GOBIERNO DE CHILE
FUNDACION PARA LA
INNOVACION AGRARIA

CONTENIDO DEL INFORME TÉCNICO

PROGRAMA DE FORMACIÓN PARA LA INNOVACIÓN AGRARIA

1. Antecedentes Generales de la Propuesta

Nombre

Curso de Especialización de Postítulo en Biotecnología Agroforestal

Código

FR – 01- 1- BT- 023

Entidad Responsable Postulante Individual

Facultad de Ciencias Agronómicas, Universidad de Chile

Coordinador

Carmen Saenz Hernández

Lugar de Formación (País, Región, Ciudad, Localidad)

Santa Rosa 11315, Paradero 32, La Pintana, Santiago, Chile

Tipo o modalidad de Formación

Apoyo a la realización de actividades de formación

Fecha de realización

Del 29 de Octubre de 2001 al 15 de Enero de 2002

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Participantes: presentación de acuerdo al siguiente cuadro:

Curso: "Aspectos Moleculares de la Fijación Biológica del Nitrógeno"
Del 29 de Octubre al 2 de Noviembre

PROFESORES:

Nombre	Institución/Empresa	Cargo/Actividad
José Manuel Palacios Alberti	Universidad Politécnica de Madrid, España	Profesor de Microbiología, Investigador jpalacios@bit.etsia.upm.es
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Curso: "Aplicaciones Biotecnológicas en Patología Vegetal"
Del 26 al 30 de Noviembre del 2001

PROFESORES:

Nombre	Institución/Empresa	Cargo/Actividad
Nicola Fiore	Universidad de Chile, Facultad de Ciencias Agronómicas	Virólogo, Investigador Teléfono: 6785726
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Emilio Stefani	Istituto di Patologia Vegetale, Italia	Bacteriologo Teléfono: 39-051-2091454
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Curso: "Ingeniería Genética de Plantas"
Del 7 al 15 de Enero de 2002

PROFESORES:

Nombre	Institución/Empresa	Cargo/Actividad
Patricio Arce	Pontificia Universidad Católica de Chile	Académico parce@puc.cl
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Alejandro Venegas	Pontificia Universidad Católica de Chile	Académico avenegas@genes.bio.puc.cl
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Alejandro Marchant 42	Universidad Adventista de Chile	Académico

Problema a Resolver: detallar brevemente el problema que se pretendía resolver con la participación en la actividad de formación, a nivel local, regional y/o nacional.

Con la Actividad de Formación, se pretendía resolver los siguientes aspectos:

1. Fortalecer en Chile la Biotecnología Agroforestal, con personal altamente capacitado, que facilite el desarrollo del país en esta disciplina.

2. Aumentar el número de profesionales en Chile, capaces de elaborar y poner en práctica, proyectos productivos y de investigación en el área de la Biotecnología Agroforestal.

Objetivos de la Propuesta

Objetivo General

1. Actualizar conocimientos sobre las técnicas de biología molecular y celular empleadas en el campo de la Biotecnología Agroforestal.

Objetivos Específicos

1. Capacitar a profesionales en el área de la Biotecnología Agroforestal, de tal forma de aumentar la actual masa crítica e incentivar el desarrollo de proyectos productivos y de investigación en el tema, acordes con el desarrollo y las necesidades del país.

2. Entregar a los participantes, los conocimientos necesarios para proyectar y aplicar técnicas biotecnológicas, tanto en el ámbito industrial como en laboratorios de investigación científica.

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2. Antecedentes Generales: describir si se lograron adquirir los conocimientos y/o experiencias en la actividad en la cual se participó (no más de 2 páginas).

La Universidad de Chile, la cual ha liderado en muchas ocasiones los cambios científicos - tecnológicos del sector agrícola, y considerada como institución líder en el área de la investigación científica, se propuso realizar importantes esfuerzos para desarrollar e implementar Programas de Especialización en el área de la Biotecnología Vegetal, de tal forma de constituir un punto de referencia nacional.

En este contexto, La Facultad de Ciencias Agronómicas de la Universidad de Chile, desde el año 1998, implementó el "Curso de Especialización de Postítulo en Biotecnología Agroforestal", a través de un Convenio de Colaboración Conjunta con la Escuela de Ingenieros Agrónomos de la Universidad Politécnica de Madrid, España. Este Curso está orientado a la obtención de un certificado de asistencia y aprobación del curso, otorgado por la Universidad de Chile, luego de un año de realización de 7 cursos teóricos - prácticos, y al Título de Doctor otorgado por la Universidad Politécnica de Madrid, una vez realizada una tesis de investigación por un período de 2 años en los Laboratorios del Departamento de Biotecnología en Madrid.

Considerando los buenos resultados obtenidos en los años anteriores y la imperiosa necesidad de capacitación que actualmente requieren los profesionales jóvenes de nuestras Universidades, Institutos de Investigaciones y del Sector Privado, la Facultad decidió dictar durante al año académico 2001, dentro del marco del Curso de Especialización, los siguientes módulos docentes:

Módulo I: Aspectos Moleculares de la Fijación Biológica del Nitrógeno

Módulo II: Aspectos Biotecnológicos en Patología Vegetal

Módulo III: Ingeniería Genética de Plantas

Los mismos se llevaron a cabo Del 29 de Octubre de 2001 al 15 de Enero de 2002, en las Salas de Postgrado y en los Laboratorios de la Facultad de Ciencias Agronómicas de la Universidad de Chile, ubicada en Santa Rosa 11315, Paradero 32, La Pintana, Santiago. La actividad de formación estuvo dirigida principalmente para Ingenieros o Licenciados en Agronomía o Forestal, Biólogos, o cualquier profesional cuya área de investigación esté relacionada con el área de la Biotecnología agroforestal.

En la planificación y ejecución de estos 3 módulos, se contó con la valiosa colaboración de 16 profesores de diferentes Universidades: Universidad de Chile, Pontificia Universidad Católica de Chile, Universidad Católica de Valparaíso, Universidad Austral de Chile, Institutos de Investigación: INIA – La Platina y Empresas privadas: Semillas Pioneer Ltda. Asistieron además a los cursos un total de 80 profesionales de diferentes instituciones del país (Ver lista de participantes en la página 2), que actualmente se desempeñan en el área de la Biotecnología y la Biología Molecular.

En la clausura del "Curso de Especialización de Postítulo en Biotecnología Agroforestal", se contó con la presencia de: Sr. Mario Silva, Decano de la Facultad de Ciencias Agronómicas, Sra. Carmen Saenz, Directora de la Escuela de Postgrado de la Facultad de Ciencias Agronómicas, Sra. Marina Gambardella, Coordinadora del Curso de Postítulo y Sr. Jorge Gatica, Director de la Revista Bioplanet.

Es importante además, destacar la valiosa ayuda logística y financiera de la Fundación para la Innovación Agraria (FIA), sin la cual no hubiese sido posible la realización exitosa de los 3 módulos anteriormente mencionados.

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3. Itinerario Realizado: presentación de acuerdo al siguiente cuadro:

Fecha	Actividad	Objetivo	Lugar
Módulo I: "Aspectos Moleculares de la Fijación Biológica del Nitrógeno" Del 29 de Octubre al 2 de Noviembre del 2001			
29 Oct.	**Tema 1** Profesor: Tomás Ruiz – Argüeso Introducción: Ciclo del nitrógeno. Relevancia de la fijación biológica del nitrógeno Organismos y sistemas diazotróficos Estructura y función de la nitrogenasa Síntesis de la nitrogenasa: genes nif Métodos de determinación de la actividad nitrogenasa **Tema 2** Profesor: Tomás Ruiz – Argüeso Fijación del nitrógeno por bacterias heterótrofas Fijación asociativa Cianobacterias: fijación en vida libre y en asociación con plantas Práctico: Aislamiento de Rhizobium		Facultad de Ciencias Agronómicas, Universidad de Chile

30 Oct.	**Tema 3** Profesor. José Manuel Palacios Alberti Simbiosis Rhizobium - leguminosa: Caracteres botánicos y agronómicos de las leguminosas El grupo Rhizobium. Características fisiológicas. Especificidad. Taxonomía Presencia de Rhizobium en suelos Aislamiento y caracterización de cepas de Rhizobium **Tema 4** Profesor: José Manuel Palacios Alberti Inoculación de leguminosas Desarrollo y fisiología de los nódulos radiculares Regulación del funcionamiento del sistema simbiótico		Facultad de Ciencias Agronómicas, Universidad de Chile
31 Oct.	**Tema 5** Profesor: Tomás Ruiz – Argüeso Inoculación de leguminosas Necesidad de inoculación Producción de inoculantes Selección de cepas de Rhizobium Práctico: Inoculación de semillas		Facultad de Ciencias Agronómicas, Universidad de Chile

1 Nov	**Tema 6** Profesor: Tomás Ruiz – Argüeso Metabolismo del hidrógeno en la fijación simbiótica el nitrógeno Eficiencia energética Genes de oxidación de hidrógeno		Facultad de Ciencias Agronómicas, Universidad de Chile
2 Nov	**Tema 7** Profesor: José Manuel Palacios Alberti Otros sistemas simbióticos de fijación de nitrógeno: Actinorrizas Posibilidades de mejora de la fijación biológica de nitrógeno Caracteres de la planta y de la bacteria Extensión de la capacidad fijadora a nuevos sistemas		Facultad de Ciencias Agronómicas, Universidad de Chile
Módulo II: "Aspectos Biotecnológicos en Patología Vegetal" Del 26 al 30 de Noviembre del 2001			
26 Nov	Diagnóstico convencional y molecular de bacterias fitopatógenas: ventajas, desventajas y perspectivas. Inmunofluorescencia (IFAS). Teoría y Práctico. Metodologías avanzadas para el diagnóstico de bacterias fitopatógenas.		Laboratorio Lo Aguirre, SAG.

27 Nov	Enfermedades bacterianas objetos de la legislación fitosanitaria de la Comunidad Europea. Extracción de ADN bacteriano y PCR: Teoría y Práctico. Discusión sobre los protocolos oficiales para el diagnóstico de Ralstonia solanacearum y Erwinia amylovora.		Laboratorio Lo Aguirre, SAG.
28 Nov	Resultados PCR y metodología de extracción de agrobacterias y Xylella fastidiosa desde xilema de vid. Teoría y práctico. Partidores degenerados y su uso en PCR para el diagnóstico de virus fitopatógenos. Extracción de RNA viral y RT.Teoría y Práctico. Hibridación molecular en el diagnóstico de virus fitopatógenos. Teoría y Práctico.		Laboratorio Lo Aguirre, SAG.
29 Nov	PCR y visualización de productos de amplificación. Teoría y Práctico. Enfermedades virales objeto de la legislación fitosanitaria de la comunidad europea. Hibridación molecular para el diagnóstico de virus fitopatógenos. Teoría Y práctico.		Laboratorio Lo Aguirre, SAG.
30 Nov	Bases moleculares de la interacción hospedero/patógeno. Identificación de genes de resistencia a fitopatógenos con el uso de marcadores moleculares. Diagnóstico molecular de virus y virus - afines.		Laboratorio Lo Aguirre, SAG.

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Módulo III:
"Ingeniería Genética de Plantas", Del 7 al 15 de Enero del 2001

7 Ene	**Tema 1: Técnicas básicas utilizadas en Ingeniería Genética de Plantas** Profesor: Dr. Alejandro Venegas 1.1 Enzimas de restricción Definición y clasificación de las enzimas de restricción Aplicaciones: Fragmentación o digestión de genomas, Elaboración de mapas de restricción, Polimorfismo de fragmentos, Diagnóstico de enfermedades 1.2 Degradación enzimática. Nucleasas 1.3 Síntesis de ácidos nucleicos ADN polimerasa ARN polimerasa Ligasas, Fosfatasas y Quinasas 1.4 Extracción de ácidos nucleicos 1.5 Extracción de proteínas 1.5 Electroforesis de ácidos nucleicos y proteínas Pricipios básicos de la electroforesis Electroforesis horizpontal en geles de agarosa Electroforesis vertical en geles de poliacrilamida Visualización de las muestras Cuantificación		Facultad de Ciencias Agronómicas, Universidad de Chile

	1.6 Sondas Definición Construcción Marcaje Aplicaciones 1.7 Transferencia de ácidos nucleicos y proteínas a membrana Transferencia Southern Blot Transferencia Northern Blot Transferencia Western Blot 1.8 Secuenciación del ADN 1.9 Reacción en cadena de la polimerasa y sus aplicaciones		Facultad de Ciencias Agronómicas, Universidad de Chile

8 Ene	**Tema 2: Plantas Transgénicas** Profesor Responsable: Dra. Loreto Holuigue 2.1 Vectores de clonación utilizados en transformación genética de plantas Vectores procarióticos: Plásmidos, Cósmidos, Fágos Vectores Agrobacterium Tumefaciens, plásmido Ti vectores binarios, vectores cointegrados 2.2 Bibliotecas genómicas 2.3 Bibliotecas de DNA complementario (cDNA) 2.4 Técnicas de transformación genética de plantas. Aplicación. Ventajas y desventajas del sistema. Efectividad de Transformación. Ejemplos de transformación -mediada por Agrobacterium Tumefaciens - Electroporación - Bio - balística 2.5 Genes reporteros 2.6 Genes marcadores de selección		Facultad de Ciencias Agronómicas, Universidad de Chile

9 Ene	Profesor Responsable: Dra. Ximena Henzi 2.5 Caracterización molecular de plantas transgénicas - Detección de la presencia del gen - Número de copias - Expresión del mRNA - Expresión de la proteína 2.5 Caracterización molecular de plantas transgénicas - Análisis biológico Práctica de Laboratorio No. 1 Transformación de Plantas		Facultad de Ciencias Agronómicas, Universidad de Chile
10 Ene	Profesor Responsable: Dr. Humberto Prieto 2.6 Aplicaciones en transformación de plantas - Resistencia a patógenos: Virus, Hongos, Bacterias, Insectos, etc. Profesor Responsable: Dr. Hugo Campo 2.7 Aplicaciones de la transformación genética de plantas - Resistencia a herbicida		Facultad de Ciencias Agronómicas, Universidad de Chile
11 Ene	Profesor Responsable: Dr. Patricio Arce 2.9 Aplicaciones de la transformación genética de plantas - Cambio del sexo de las plantas		Facultad de Ciencias Agronómicas, Universidad de Chile

14 Ene	**Tema 3: Transformación de especies forestales** Profesor Responsable: Dr. Patricio Arce 3.1 Sistema de regeneración y transformación de arboles - Modificación de la lignina y la celulosa - Producción de semillas artificiales		Facultad de Ciencias Agronómicas, Universidad de Chile
15 Ene	Profesor Responsable: Dr. Patricio Arce Práctico de Laboratorio 2: Transformación de Plantas		Facultad de Ciencias Agronómicas, Universidad de Chile

Señalar las razones por las cuales algunas de las actividades programadas no se realizaron o se modificaron.

4. Resultados Obtenidos: descripción detallada de los conocimientos adquiridos. Explicar el grado de cumplimiento de los objetivos propuestos, de acuerdo a los resultados obtenidos. Incorporar en este punto fotografías relevantes que contribuyan a describir las actividades realizadas.

Con la Actividad de Formación, se lograron cumplir los objetivos iniciales propuestos en la actividad de formación, lográndose los siguientes resultados:

Entregar a los profesionales participantes (Académicos, Investigadores) en los cursos, conocimientos actualizados en las técnicas de Biología Molecular y Celular empleadas en el campo de la Biotecnología Agroforestal, con la finalidad de que puedan ser capaces de proyectar y aplicar técnicas biotecnológicas, tanto en el ámbito industrial como en laboratorios de investigación científica. Se entregaron artículos especializados en el tema, los cuales se anexan dentro del presente informe (Ver punto 9 Material Recopilado).

En los 3 módulos contamos con un total de 80 participantes, logrando en cierta medida aumentar el número de profesionales en Chile, capaces de elaborar y poner en práctica, Programas y Proyectos de Investigación y Docencia, en el área de la Biotecnología, acordes con el desarrollo y las necesidades del país. Es bueno destacar que contamos en nuestros cursos con la presencia de profesionales jóvenes que estaban buscando información en el tema con el propósito de insertarse en el área de la Biotecnología Agroforestal.

5. Aplicabilidad: explicar la situación actual del rubro en Chile (región), compararla con la tendencias y perspectivas en el país (región) visitado y explicar la posible incorporación de los conocimientos adquiridos, en el corto, mediano o largo plazo, los procesos de adaptación necesarios, las zonas potenciales y los apoyos tanto técnicos como financieros necesarios para hacer posible su incorporación en nuestro país (región).

6. Contactos Establecidos: presentación de acuerdo al siguiente cuadro:

VER LISTA DE PARTICIPANTES EN LA PAGINA 2

Institución/Empresa	Persona de Contacto	Cargo/Actividad	Fono/Fax	Dirección	E-mail

7. Detección de nuevas oportunidades y aspectos que quedan por abordar: señalar aquellas iniciativas detectadas en la actividad de formación, que significan un aporte para el rubro en el marco de los objetivos de la propuesta, como por ejemplo la posibilidad de realizar nuevos cursos, participar en ferias y establecer posibles contactos o convenios. Indicar además, en función de los resultados obtenidos, los aspectos y vacíos tecnológicos que aún quedan por abordar para la modernización del rubro.

Se destacó que es de gran importancia para Chile, que la Fundación para la Innovación Agraria (FIA), apoye constantemente este tipo de iniciativas en lo que respecta a las actividades de formación, teniendo en consideración que la Biotecnología es un área de la agricultura que necesita contar con suficiente personal altamente calificado capaz de enfrentarse día a día a los desafíos que la agricultura nacional demanda. De hecho se contó con la presencia de un alto número de participantes en dicha actividad.

Los estudiantes participantes en la actividad de formación así como algunos profesores que dictaron los módulos, ~~se~~ señalaron que debe~~mos~~ continuar con dicho Curso de Especialización en los años venideros, ya que es una buena forma de estar actualizando constantemente a todos los jóvenes que recién se incorporan a trabajar en el área de la Biotecnología y sirven además para motivar y captar a estudiantes recién egresados a incorporarse a trabajar en dicha área.

8. Resultados adicionales: capacidades adquiridas por el grupo o entidad responsable, como por ejemplo, formación de una organización, incorporación (compra) de alguna maquinaria, desarrollo de un proyecto, firma de un convenio, etc.

No tuvimos ningún resultado adicional al respecto.

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9. Material Recopilado: junto con el informe técnico se debe entregar un set de todo el material recopilado durante la actividad de formación (escrito y audiovisual) ordenado de acuerdo al cuadro que se presenta a continuación (deben señalarse aquí las fotografías incorporadas en el punto 4):

Tipo de Material	Nº Correlativo (si es necesario)	Caracterización (título)
Artículo	1	1. The bases of crown tumorigenesis.
Artículo	2	2. Generation of single-strandedd T-DNA molecules during the initial stages of T-DNA transfer from *Agrobacterium tumefaciens* to plant cell.
Artículo	3	3. Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens.*
Artículo	4	4. The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights.
Artículo	1	5. A guide to Agrobacterium binary Ti vectors.
Artículo	1	6. Optimization of *in vitro* culture conditions for Pinus *radiata* embryos and histological characterization of regenerated shoots.
Artículo	1	7. New methods of diagnosis in plant phatology – perspectives and pitfalls.
Artículo	1	8. Virus and phytoplasma detection in fruit trees.
Artículo	1	9. Biological dinitrogen fixation: Symbiotic.
Artículo	1	10. Prokaryotic nitrogen fixation.
Artículo	1	11. La simbiosis.
Apuntes	1	12. Simbiosis Rhizobium – Leguminosa.
Apuntes	1	13. Genética de la Fijación del nitrógeno.
Apuntes	1	14. Nitrogenasa: Estructura y Función.
Foto	En el disquette	15. Clausura Curso de Especialización de Postítulo en Biotecnología Agroforestal. Año Académico 2001.

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10. Aspectos Administrativos

10.1. Organización previa a la actividad de formación

a. Conformación del grupo

_____ muy dificultosa ___X_ sin problemas _____ algunas dificultades

(Indicar los motivos en caso de dificultades)

b. Apoyo de la Entidad Responsable

__X__ bueno _____ regular _____ malo

(Justificar)

Desde el comienzo del Curso de Especialización de Postítulo en Biotecnología Agroforestal, contamos en todo momento, con el apoyo de las autoridades de la Facultad de Ciencias Agronómicas de la Universidad de Chile, las mismas nos facilitaron computadores con ~~Internet con~~ libre acceso para los estudiantes del curso, los materiales docentes, audiovisuales, y de laboratorio, con los cuales fue posible la excelente realización de los módulos que se pretendían dictar durante el año académico 2001.

Se preocuparon además de la recepción y bienvenida de los profesores colaboradores tanto extranjeros como nacionales, efectuando intercambios de científicos y de colaboración con los mismos, así como estuvieron pendientes todo el tiempo del buen confort en la estadía de los profesores extranjeros en nuestro país.

c. Información recibida durante la actividad de formación

__X__ amplia y detallada _____ aceptable _____ deficiente

d. Trámites de viaje (visa, pasajes, otros)

__X__ bueno _____ regular _____ malo

e. Recomendaciones (señalar aquellas recomendaciones que puedan aportar a mejorar los aspectos administrativos antes indicados)

10.2. Organización durante la actividad (indicar con cruces)

Ítem	Bueno	Regular	Malo
Recepción en país o región de destino	X		
Transporte aeropuerto/hotel y viceversa	X		
Reserva en hoteles	X		
Cumplimiento del programa y horarios	X		

En caso de existir un ítem Malo o Regular, señalar los problemas enfrentados durante el desarrollo de la actividad de formación, la forma como fueron abordados y las sugerencias que puedan aportar a mejorar los aspectos organizacionales de las actividades de formación a futuro.

11. Conclusiones Finales

Durante la Clausura del "Curso de Especialización de Postítulo en Biotecnología Agroforestal", tuvimos la oportunidad de realizar intercambios con los profesionales, algunos profesores colaboradores de los cursos y las autoridades de la Facultad de Ciencias Agronómicas de la Universidad de Chile, resultando de dicho encuentro planteamientos muy concretos tales como la posibilidad de continuar con este tipo de actividad de formación, la cual contribuye a la actualización de los conocimientos en el área de la Biotecnología y la Biología Celular y Molecular, áreas que hoy en día están alcanzando un alto nivel de importancia en los países desarrollados y en un alto grado en Chile, debido a que las nuevas técnicas biotecnológicas están siendo puestas al servicio del desarrollo agrícola.

Se destacó que es de gran importancia para Chile, que la Fundación para la Innovación Agraria (FIA), apoye constantemente este tipo de iniciativas en lo que respecta a las actividades de formación, teniendo en consideración que la Biotecnología es un área de la agricultura que necesita contar con suficiente personal altamente calificado capaz de enfrentarse día a día a los desafíos que la agricultura nacional demanda. De hecho se contó con la presencia de un alto número de participantes en dicha actividad.

La calidad de los profesores colaboradores trascendió las expectativas de los estudiantes, se comentó el alto nivel de profesionalismo y de preparación en las exposiciones realizadas. En todos los temas expuestos se encontró un amplio dominio y actualización. Se abordaron además los principales avances que Chile está obteniendo en el campo de la Biotecnología Agroforestal. El material audiovisual utilizado (transparencias, diapositivas, CD) mostraron con suficiente claridad todos los puntos abordados.

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12. Conclusiones Individuales: anexar las conclusiones individuales de cada uno de los participantes de la actividad de formación, incluyendo el nivel de satisfacción de los objetivos personales (no más de 1 página y media por participante).

Doris Prehn Roth
Profesora Auxiliar
Facultad de Agronomía e Ingeniería Forestal
Pontificia Universidad católica de Chile

"Curso de Especialización de Postítulo en Biotecnología Agroforestal: Ingeniería Genética de Plantas"

El curso se efectuó de manera ordenada y organizada. Los profesores responsables mostraron preparación en sus temas e hicieron exposiciones claras, aptas para un alumnado heterogéneo. El apoyo tecnológico y audiovisual fue excelente, siendo la mayoría de las presentaciones tipo power-point, con apoyo de diapositivas y transparencias. Hubo facilidad para acceder a bibliografía actualizada mediante fotocopias y una sala de estudio. La infraestructura, instalaciones y acceso a casino y laboratorios fue adecuada

Los objetivos y expectativas personales puestos en el curso se referían a una actualización en los diversos temas relacionados con la ingeniería genética de plantas y se consideran totalmente cumplidos. A continuación se detalla el nivel de satisfacción de los objetivos personales por tema y profesor responsable.

Tema	**Nivel de satisfacción de los objetivos personales**
1.Técnicas básicas utilizadas en Ingeniería Genética de Plantas. Profesor: Dr. Alejandro Venegas. P. Univ. Católica de Chile	Regular
2.Plantas transgénicas. Profesora: Dra. Loreto Holuigue. P. Univ. Católica de Chile	Muy Bueno
3.Resistencia a virus y otros patógenos. Profesor: Dr. Humberto Prieto. INIA-La Platina	Bueno
4. Aplicaciones de la transformación genética de plantas. Profesor Eduardo Oyanedel. U. Católica de Valparaíso.	Regular

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5. Transformación de plantas recalcitrantes. Profesora: Dra. Ximena Henzi. Universidad Austral de Chile.	Bueno
6. Práctica de laboratorio: Transformación de plantas de papas. Profesora: Dra. Ximena Henzi u Ayudante. Universidad Austral de Chile.	Muy Bueno
7. Transformación de gramíneas y cereales. Profesor: Dr. Hugo campos. Semillas Pioneer Chile Ltda.	Muy Bueno
8. Transformación de especies forestales. Profesor: Dr. Patricio Arce. P. Universidad Católica de Chile.	Bueno
9. Práctica de laboratorio: Transformación de embriones de *Pinus radiata.* Profesor: Dr. Patricio Arce y Ayudantes.	Muy Bueno

Ana María Sabja
Académico
Universidad Austral de Chile

Curso: Ingeniería Genética de Plantas

- Temas:
-

Los temas fueron apropiados y de gran interés por la actualidad con que se expusieron.
Los expositores mostraron un buen nivel, calidad y manejo de los temas.
Las dos clases prácticas lograron reforzar los conocimientos que entregaban los expositores.

- Expectativas personales del curso

El curso me permitió ampliar algunos conocimientos respecto a transformación genética y sus aplicaciones.
En el tema del área forestal me hubiese gustado una mayor profundidad de los temas que se abordaron.

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María Teresa Barriga

El temario del curso completo me pareció muy interesante, completo y aplicable. Lamentablemente me enteré muy tarde del curso y pude atender solamente a las clases 3, 4, 5 y.

Clases entendidas: Excelente y fascinante. Tanto el contenido como las exposiciones las encontré técnicamente muy completas y en un grado de profundización muy bueno. Mi trabajo se centra en asuntos regulatorios, y la profundización biológica y agrícola del tema de biotecnología sin duda es y será un aporte y un apoyo para mi trabajo, que también involucra difusión.

Personalmente mi interés era profundizar en el tema de biotecnología con el objeto de difusión y formación. He visto cumplidos mis objetivos con creces y me encantaría poder asistir al curso completo si se repitiera el mismo o alguno parecido.

Carmen Cabrera Valenzuela
Ing. Agrónomo
Depto. Protección Agrícola
SAG

En relación a los Cursos de Especialización de Postítulo en Biotecnología Agroforestal, entregado por la Facultad de Ciencias Agronómicas de la Universidad de Chile y financiados por el FIA dentro del marco del programa de formación; me es muy grato referirme, como alumna participante, a los Módulos I y II, sobre "Ingeniería Genética de Plantas" y "Aspectos Moleculares de la Fijación Biológica del Nitrógeno".

En primer término, poder acceder con tal facilidad **en Chile** a cursos especializados, con la categoría y el nivel con que fueron entregados por la Universidad de Chile, me significa agradecer y reconocer la iniciativa de realizarlos. Sobre todo, reconocer el esfuerzo académico que significa hacer avenible los temas Biotecnológicos para profesionales, que de una u otra forma tienen distinto interés en el tema.

En lo personal y profesional destaco, la valiosa oportunidad que se me entregó para capacitarme en el tema, el poder conocer materias nuevas, reforzar o mejor comprender otras y darme cuenta con recato, de la "necesidad creada" por continuar recibiendo capacitación en otras materias vinculadas con el tema.

En mi calidad de Ingeniero Agrónomo que se desempeña en una Institución Ministerial, como es el Servicio Agrícola y Ganadero (SAG), la capacitación recibida reviste de un enorme interés para las funciones que me corresponden realizar en la Institución.

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Finalmente, me permito reiterar mi agradecimiento y reconocimiento por la iniciativa y el logro alcanzado al realizar los Cursos de Especialización de Postítulo en Biotecnología Agroforestal.

Marisol Reyes Muñoz
Ing. Agrónomo
INIA CAuquenes

El curso me pareció muy bueno. Creo que los temas estuvieron entregados en un orden bastante adecuado y con mucha claridad.

Quienes dictaron las clases fueron bastante amenos y didácticos, además el hecho de que ellos manejaban muy bien sus temas, hizo muy interesantes las clases.

En mi caso, que no trabajo directamente en el tema, fue realmente muy provechoso, me aclaró varios conceptos e ideas erradas respecto a la manipulación genética y me sirvió para enterarme de lo que se está haciendo el área.

Andrea Torres Pinto
Ingeniero Agronómo
INIA - Raihuén

Es mi interés poder colocar en práctica los conocimientos aprendidos en este curso, que me pareció muy interesante, muy actual, además de la excelencia de los expositores, la información escrita entregada y la posibilidad de tomar contacto con profesionales que desarrollan "campos" similares.

Mis más cordiales felicitaciones a los organizadores y fuentes, que nos permitieron asistir a este curso.

Erika R. Salazar Suazo
Investigador
Dpto Fruticultura

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INIA - CRI La Platina

El programa y desarrollo de curso me parecieron de muy buen nivel, así como la elección de los expositores quienes satisficieron a cabalidad la necesidad de información sobre los temas expuestos.

Se contó con buenos elementos de apoyo audiovisuales facilitando así la compresión de las charlas expuestas. Si bien considero que la incorporación de sesiones prácticas contribuyó al entendimiento de ciertos temas tratados, sugiero que a futuro el seguimiento de los resultados de los mismos esté inserto dentro del período de duración del curso, lo que se lograría dilatando el calendario el cual encontré un poco ajustado.

El apoyo logístico fue excelente traduciéndose en una buena organización y disposición a resolver los inconvenientes que siempre surgen en este tipo de eventos.

Alejandro Marchant Kemp
M.S en Ciencias
Universidad Adventista de Chile

Sean mis primeras palabras de reconocimiento a la labor que desempeña en la dirección del FIA y la vez, desear las mayores bendiciones en el presente año, junto a todos los que laboran en dicha institución.

El motivo de mi nota, es agradecer la invitación que hace el FIA a través de la REDBIO, para asistir al curso de Ingeniería Genética de Plantas, teniendo la posibilidad de recibir conocimiento de destacados investigadores en el área de la Biotecnología.

Por último, expresar mi deseo de participar en el curso antes citado, y desde ya agradecer al FIA la posibilidad que me da de asistir y beneficiarme de una temática tan importante para el desarrollo de nuestro país.

Fecha: _____17de Abril del 2002____

Nombre y Firma coordinador de la ejecución: __ Carmen Saenz Hernández_____

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MINIREVIEW

The Bases of Crown Gall Tumorigenesis

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The nine decades since Smith and Townsend demonstrated that *Agrobacterium tumefaciens* causes plant tumors (95) have been marked by a series of surprises. Among the most important of these was the report in 1958 that these tumors could be excised and propagated in vitro without exogenous plant hormones (7). Equally important were a series of reports beginning about the same time that tumors released compounds that agrobacteria could use as nutrients (24). Perhaps the most exciting discoveries, reported in the 1970s and 1980s, were that tumorigenesis required the transfer of fragments of oncogenic DNA to infected plant cells (10), that this process evolved from a conjugal transfer system (99), and that the genes that direct this process are expressed in response to host-released chemical signals (47). This DNA transfer process has become a cornerstone of plant molecular genetics. The genus *Agrobacterium* also has provided excellent models for several aspects of host-pathogen interactions, including intercellular transport of macromolecules (11), bacterial detection of host organisms (47), targeting of proteins to plant cell nuclei (3), and interbacterial chemical signaling via autoinducer-type pheromones (120),

Most of the genes required for tumorigenesis are found on large extrachromosomal elements called Ti plasmids. Indeed, transfer of Ti plasmids into certain nonpathogenic bacteria converts them into tumorigenic pathogens (43). Ti plasmids are generally referred to by the types of opines whose catabolism they direct (see below). However, this nomenclature is becoming less satisfactory as we discover that all known Ti plasmids direct the catabolism of more than one opine and that opine catabolic genes are found in a variety of combinations in different plasmids. The Ti plasmids pTiA6NC, pTi15955, pTiAch5, pTiR10, and pTiB6S3, which are widely considered to be functionally identical, are generally referred to as octopine-type Ti plasmids (or, less frequently, octopine, mannityl opine-type Ti plasmids). The DNA sequencing of these plasmids was initiated almost 20 years ago (21) and was recently completed in our three laboratories. The resulting 194,140-nucleotide sequence is a composite assembly of sequences from all of the plasmids listed above. The close similarity of these plasmids is exemplified by the sequence of a 42-kb segment of the *vir* regions of pTiA6NC and pTi15955. These sequences differ at only one base, and this polymorphism is silent at the amino acid level. We have no evidence for polymorphisms elsewhere except for a large deletion that is unique to pTiA6NC (Fig. 1). The restriction map deduced from this sequence agrees almost perfectly with the published restriction map of pTiAch5 (25). All known and suspected genes are depicted in Fig. 1, and their demonstrated or putative functions are described in Table 1. The DNA sequence of this Ti plasmid provides a useful framework to review the roles of this plasmid in the biology of plant infection and colonization.

This Ti plasmid contains 155 open reading frames (ORFs), almost all of which are likely to encode functional proteins (Fig. 1 and Table 1). The overall G+C composition of this plasmid is 55%, although a few segments are considerably richer in A's and T's, particularly in the T region (see below). Overall, the Ti plasmid exhibits a modular structure with genes of similar function or purpose grouped together. Thus, we can define five components: (i) the T region, which codes for sequences that are transferred to the plant host; (ii) the *vir* region, which directs the processing and transfer of the T-DNA; (iii) the *rep* region, which is required for replication of the Ti plasmid; (iv) the *tra* and *trb* loci, which direct the conjugal transfer of the Ti plasmid; and (v) genes that direct uptake and catabolism of opines. An exception to this clustering is the *tra* and *trb* loci, the two gene sets required for conjugal transfer, which are separated from each other by 60 kb.

TRANSFER OF TWO DNA FRAGMENTS TO HOST PLANT CELLS

During infection, *A. tumefaciens* strains carrying an octopine-type Ti plasmid transfer two fragments of DNA to the nuclei of host plants by a mechanism that requires cell-cell contact and resembles plasmid conjugation. These fragments are designated the T_L-DNA and T_R-DNA (Fig. 1, top line), and are 13 and 7.8 kb in length, respectively (4, 105). The corresponding segments of the Ti plasmid are called T regions, and each is flanked by *cis*-acting, 25-bp direct repeats, called border sequences (121, 125). The left border of the T_L-DNA is dispensable for T-DNA transfer, while the right border is essential and acts in a polar fashion, suggesting that transfer may initiate at the right border and proceed leftward (76). Inversion of the right border leads to attenuated tumorigenesis, and tumors made by such mutants contain extremely long T-DNA fragments consisting of virtually the entire Ti plasmid (76). Adjacent to the right border of T_L is another *cis*-acting site called overdrive (94), which is required for wild-type transfer efficiency and provides a binding site for the VirC1 protein (see below). A second possible overdrive sequence is located adjacent to the right border of T_R, though the role of this sequence in T-DNA transfer has not been studied.

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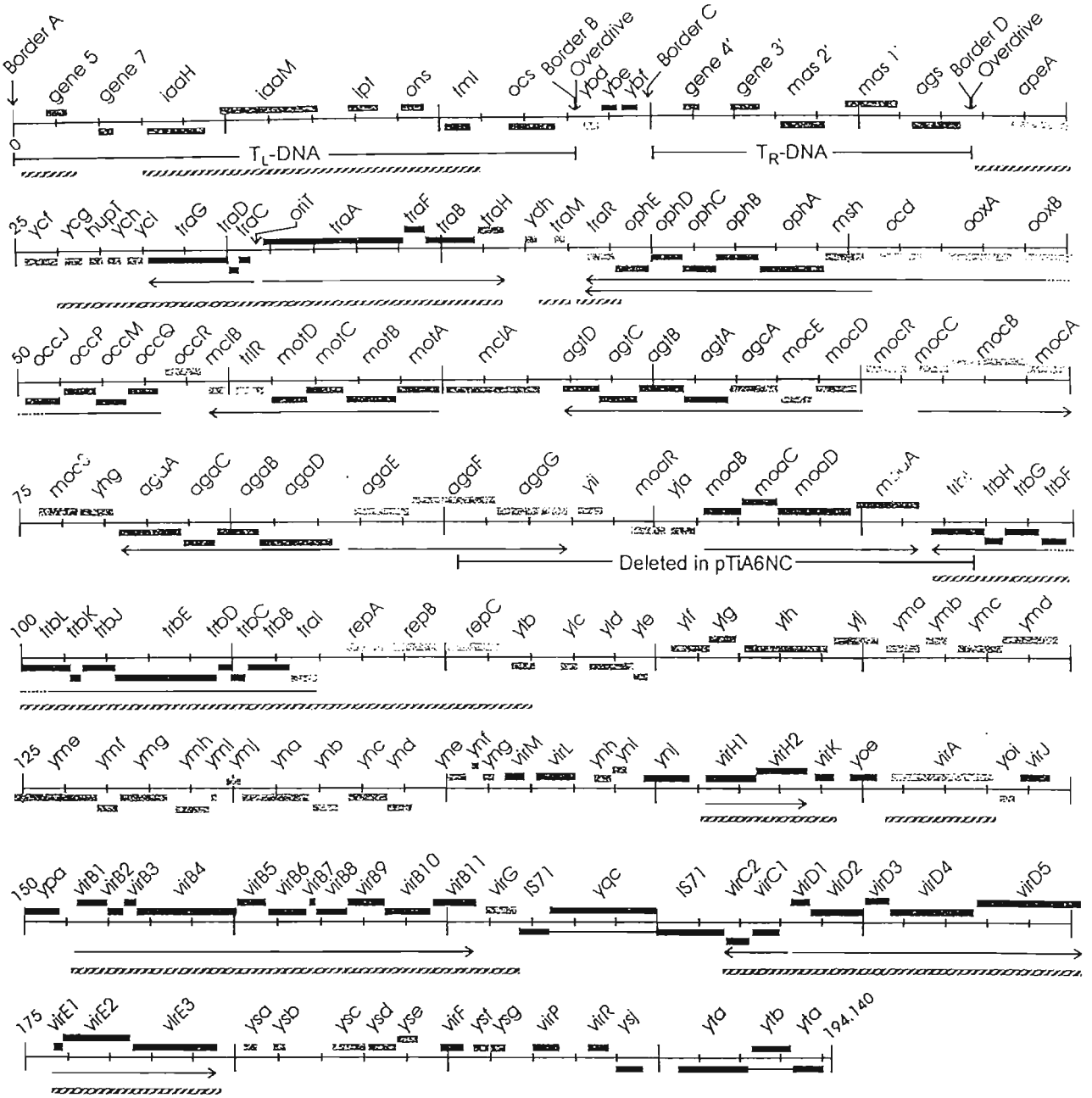

FIG. 1. Genetic map of the octopine-type Ti plasmid. Nucleotide 1 is taken as the left end of the left border of T_L. Genes transcribed from left to right are shown above the scale, while genes transcribed from right to left are shown below the scale. Known or suspected polycistronic operons are indicated with horizontal arrows. A 12.6-kb region deleted in the widely studied pTiA6NC is indicated by a horizontal bar on line 4. Regions that can form heteroduplexes with orthologous sequences of pTiC58 (27) are indicated with crosshatched bars. Green bars indicate genes that are located on the T_L region or T_R region and transferred to plant cells. Red bars indicate genes in the *vir* regulon, all of which are regulated by VirA and VirG, and many of which are required for T-strand processing and transfer. Purple bars indicate genes required for interbacterial conjugal transfer of the Ti plasmid. Light green bars indicate genes required for vegetative replication and partitioning of the Ti plasmid. Dark blue bars indicate genes encoding ATP binding cassette-type opine permeases, while light blue bars indicate opine catabolic genes. Orange bars indicate regulatory genes. Black bars indicate suspected IS elements (in this case, bars represent DNA sequence similarities rather than ORFs), while grey bars indicate ORFs of miscellaneous or unknown function. Gene names beginning with the letter "y" indicate the position of the gene, such that the second letter represents position in tens of kilobases, and the third letter indicates position in single kilobases. During the analysis of all these sequences, we found that *tml*, *mas2'*, *mas1'*, and *ags* had sequence discrepancies relative to orthologous genes from other Ti or Ri plasmids. These regions were resequenced after PCR amplification, and three errors in the original sequence (4) were detected and corrected. The resulting DNA sequence of the Ti plasmid has been deposited in the GenBank DNA sequence database (accession no. AF242881). Most Ti plasmid sequences were previously deposited in DNA sequence databases, and the original sources of these sequences are provided in the annotations of our compiled sequence.

TABLE 1. Genes encoded by the octopine-type Ti plasmid[a]

Genetic locus	Description	Reference(s)
T-DNA genes		
ags	Agropine synthase, lactonization of mannopine	24, 40
Gene *5*	Synthesis of indole-3-lactate, an auxin antagonist	57
iaaH and *iaaM*	Conversion of tryptophan to indole acetic acid (auxin)	55
ipt	Condensation of AMP and isopentenylpyrophosphate to form isopentenyl-AMP, a cytokinin	66
mas1' and *mas2'*	Mannopine synthase; condensation of glucose with glutamine or glutamate followed by reduction	24
ocs	Octopine synthase, reductive condensation of pyruvate with four basic amino acids	21
ons	Opine export from plant cells	75
tml (gene *6b*)	Auxin sensitivity	108
Borders A, B, C, D	*cis*-acting sites required for T-DNA processing, functionally equivalent to conjugal origins of transfer	125
Overdrive	*cis*-acting site for optimal T-DNA transfer; VirC1 binding site	110, 113
vir loci		
virB1-11	Type IV transport system to transfer T-DNA and Vir proteins from bacteria to host cytoplasm	106, 116
virC and *-D*	T-DNA processing. VirD1 and VirD2 nick at T-DNA borders; VirC1 binds overdrive	111, 122, 123
virE	Nuclear transport of T-DNA. VirE2 binds single-stranded DNA and has nuclear localization sites; VirE1 is a chaperone for VirE2 transport	14, 101
virF	Host range factor	73
virH1-2	P450-type oxidases; VirH2 O demethylates phenolic inducers	51
virM, *-L*, *-K*, *-J*, *-F*, *-P*, *-R*, *-D3*, *-D5*, and *-E3*	Other members of the *vir* regulon; VirP resembles phosphatases	50, 52
Interbacterial conjugation genes		
traAFB, *traCDG*	Ti plasmid conjugal DNA processing	1
trbB-J	Type IV transfer system required for Ti plasmid conjugation	1
oriT	*cis*-acting site required for conjugation	1
Vegetative replication genes		
repAB	Putative partitioning system	104
repC	Essential for vegetative replication	104
Opine uptake genes		
agaDBCA	Agropinic acid permease	70
agtABCD	Agropine permease	Unpublished data
motABCD	Mannopine permease	80
moaBCDA	Mannopinic acid permease	70
occQMPJ	Octopine permease	112
ophABCDE	Putative permease for an unknown substrate	33
Opine catabolism genes		
agaE	Conversion of agropinic acid to mannopinic acid	70
agaFG	Conversion of mannopinic acid to glutamic acid and mannose	70
agcA	Catabolic mannopine cyclase, for conversion of agropine to mannopine, related to *ags*	54
mocAB	Oxidoreductase, and dehydratase?	54
mocCD	Conversion of mannopine to glutamine and glucose	54
mocE	Kinase?	54
ocd	Ornithine cyclodeaminase for conversion of ornithine to proline	114
ooxAB	Oxidoreductase for conversion of octopine-type opines to pyruvate and corresponding basic amino acid	114
Transcriptional regulation genes		
moaR	Repressor of *agaD-A*, *agaE-G*, and *moaB-A* operons	70
mocR	Probable regulator of the *mocD-agtD* and *mocC-A* operons	54
mocS	Resembles MocR, function unknown	54
occR	LysR-type octopine-responsive regulator of the *occQ-traR* operon	115
traR and *traI*	LuxR-LuxI-type quorum sensing regulators; TraI synthesizes 3-oxooctanoylhomoserine lactone; TraR is a transcriptional activator	36, 77, 128
traM	TraR antagonist	32
trlR	TraR antagonist; TrlR resembles TraR but is truncated and may inhibit TraR by forming inactive heteromultimers	80, 127
virA and *virG*	Two-component regulators of *vir* regulon; VirA is a transmembrane histidine kinase; VirG is an OmpR-type response regulator	61
IS elements		
IS*71*L and IS*71*R	Apparent IS element, interrupted by insertion of *yqc* IS element	Unpublished data
ybe, *yoe*, and *yqc*	Resemble IS*66* of *A. tumefaciens*	Unpublished data
ybf	Resembles IS*1203* of *E. coli*	Unpublished data
ynj	Resembles IS*1313* of *A. tumefaciens*	Unpublished data
ypa	Resembles IS*869* of *A. tumefaciens*	Unpublished data
ysj	Resembles IS*492* of *Pseudomonas* sp.	Unpublished data
yta	Resembles IS*21* of *E. coli*, disrupted by IS element *ytb*	Unpublished data
ytb	Resembles IS*1111*a of *Coxiella burnetii*	Unpublished data

Continued on following page

TABLE 1—*Continued.*

Genetic locus	Description	Reference(s)
Genes with miscellaneous and unknown functions		
apeA	Exclusion of bacteriophage Ap1	1
hupT	Resembles HNS-type proteins	1
mclA and *-B*	Resembles methyl-accepting chemotaxis proteins; possible role in chemotaxis to opines. McIB is severely truncated and inhibits chemotaxis in *A. tumefaciens*	80, 127
mtsh	Resembles methionine synthase	33
yhg	Resembles oxidoreductases; possible role in opine catabolism	70
ylb	Resembles DNA invertases; possible role in plasmid maintenance	Unpublished data
yld	Resembles DNA invertases; possible role in plasmid maintenance	Unpublished data
yle	Resembles plasmid stability locus; possible role in plasmid maintenance	Unpublished data
ylf-yng	Functions unknown	Unpublished data
ysa	Resembles integration host factor; weakly induced by *vir* inducing stimuli	52
ysb	Resembles cold shock proteins	52
ysc, *ysd*, and *yse*	*ysc* and *ysd* resemble 3′ end of *traA* of Ti plasmid; *yse* resembles Ti plasmid *traF*; none is detectably expressed	29, 52

[a] Types of genes correspond to bars in Fig. 1 as follows: T-DNA genes, dark green bars; *vir* loci, red bars; interbacterial conjugation genes, purple bars; vegetative replication genes, light green bars; opine uptake genes, dark blue bars; opine catabolism genes, light blue bars; transcriptional regulation genes, orange bars; IS elements, black bars; and genes with miscellaneous and unknown functions, grey bars.

In the presence of proteins encoded by the *vir* region (see below), the DNA within the T regions undergoes several processing steps (Fig. 2). Each border is cleaved on the bottom DNA strand at a site exactly 4 nucleotides from its left end. This reaction is catalyzed by the VirD2 protein (see below), which remains covalently bound to the 5′ end of each cleaved strand. While the top strand remains in duplex form, approximately half of the bottom strands can be recovered in a single-stranded linear form, referred to as T strands (97). These T strands are thought to represent the transferred form of the T-DNA and are probably formed by displacement during rolling-circle DNA synthesis that initiates from the 3′ ends of each right border. At an early stage of transformation, T strands can be detected in plant cells (124), showing that the T-DNA is transferred in a single-stranded form. T strands are integrated into the host genome at apparently random sites by illegitimate recombination (72) and are stably transmitted to daughter plant cells upon mitotic cell division, and during meiosis and syngamy.

EXPRESSION AND FUNCTIONS OF TRANSFERRED GENES

Collectively, T_L-DNA and T_R-DNA encode 13 proteins (Fig. 1, dark green bars). The nontranscribed regions of each transferred gene possess many of the features of plant genes, including typical eukaryotic TATA and CAAT boxes, transcriptional enhancers, and poly(A) addition sites (6). No introns have been reported for any of the *A. tumefaciens* transferred genes, although at least one T-DNA gene in *Agrobacterium rhizogenes* contains an intron in its 5′ nontranslated region (71). The coding regions of the T-DNAs have a G+C content of approximately 50%. However, the intergenic regions, especially the 3′ nontranslated regions, are extremely poor in G's and C's, approximately 20 to 30%.

One group of T-DNA genes directs the production of plant growth hormones that are responsible for the proliferation of the transformed plant cells (6). The *iaaM* and *iaaH* products direct the conversion of tryptophan via indoleacetamide to indoleacetic acid (auxin). The *ipt* product condenses isopentenyl pyrophosphate and AMP (6), and host enzymes are presumed to convert the resulting isopentenyl-AMP into the cytokinin zeatin by removal of the phosphoribosyl group and hydroxylation of one methyl group of the isopentenyl moiety. Two other T-DNA genes are thought to play ancillary roles in tumorigenesis. The gene *5* product directs the synthesis of indole-3-lactate, an antagonistic auxin analogue (57), while *tml* (also designated gene *6b*) increases the sensitivity of plant cells to phytohormones by a mechanism that remains to be discovered (108). This gene can provoke tumors in certain host plants in the absence of the other oncogenes (42).

A second set of transferred genes directs the production of bacterial nutrients called opines. Octopine-type Ti plasmids direct their hosts to synthesize at least eight opines. The *ocs* gene encodes octopine synthase, which reductively condenses pyruvate with either arginine, lysine, histidine, or ornithine to produce octopine, lysopine, histopine, or octopinic acid, respectively, all of which can be detected in crown gall tumors (24). The *mas2′* product is thought to condense glutamine or glutamic acid with glucose (although this has not been experimentally demonstrated), while the *mas1′* product reduces these intermediates, forming mannopine and mannopinic acid, respectively. The *ags* product catalyzes the lactonization of mannopine to form agropine. Mannopine and agropine also can spontaneously lactamize to form agropinic acid (24). Thus, tumors induced by strains harboring octopine-type Ti plasmids can produce as many as four members of the octopine family and four members of the mannityl opine family.

Ti PLASMID-ENCODED PROTEINS REQUIRED FOR T-DNA PROCESSING AND TRANSFER

Proteins responsible for T-DNA processing and transfer are encoded by the *vir* region of the Ti plasmid. Twenty genes in this region are essential for wild-type levels of pathogenesis on most host plants and are expressed in six operons, *virA*, *-B*, *-C*, *-D*, *-E*, and *-G*. The proteins required for border cleavage are encoded by *virD1* and *virD2*, with the VirD2 protein remaining covalently bound to the 5′ end of the T-strands (98, 123). Purified VirD2 cleaves single-stranded oligonucleotides containing border sequences at the same site, creating a covalent bond between the 5′ phosphate and tyrosine 29 (86). This reaction is fully reversible, indicating that the DNA-protein phosphodiester linkage is a high-energy bond and suggesting that a reverse reaction might be important for the integration of T-DNA into the plant genome (109). VirD2 alone was not able to cleave the same sequence in double-stranded form but was able to do so in the presence of VirD1 (90). VirC1 binds to the overdrive site, which lies adjacent to the left border (111). VirC1 and VirC2 are not required for T-region process-

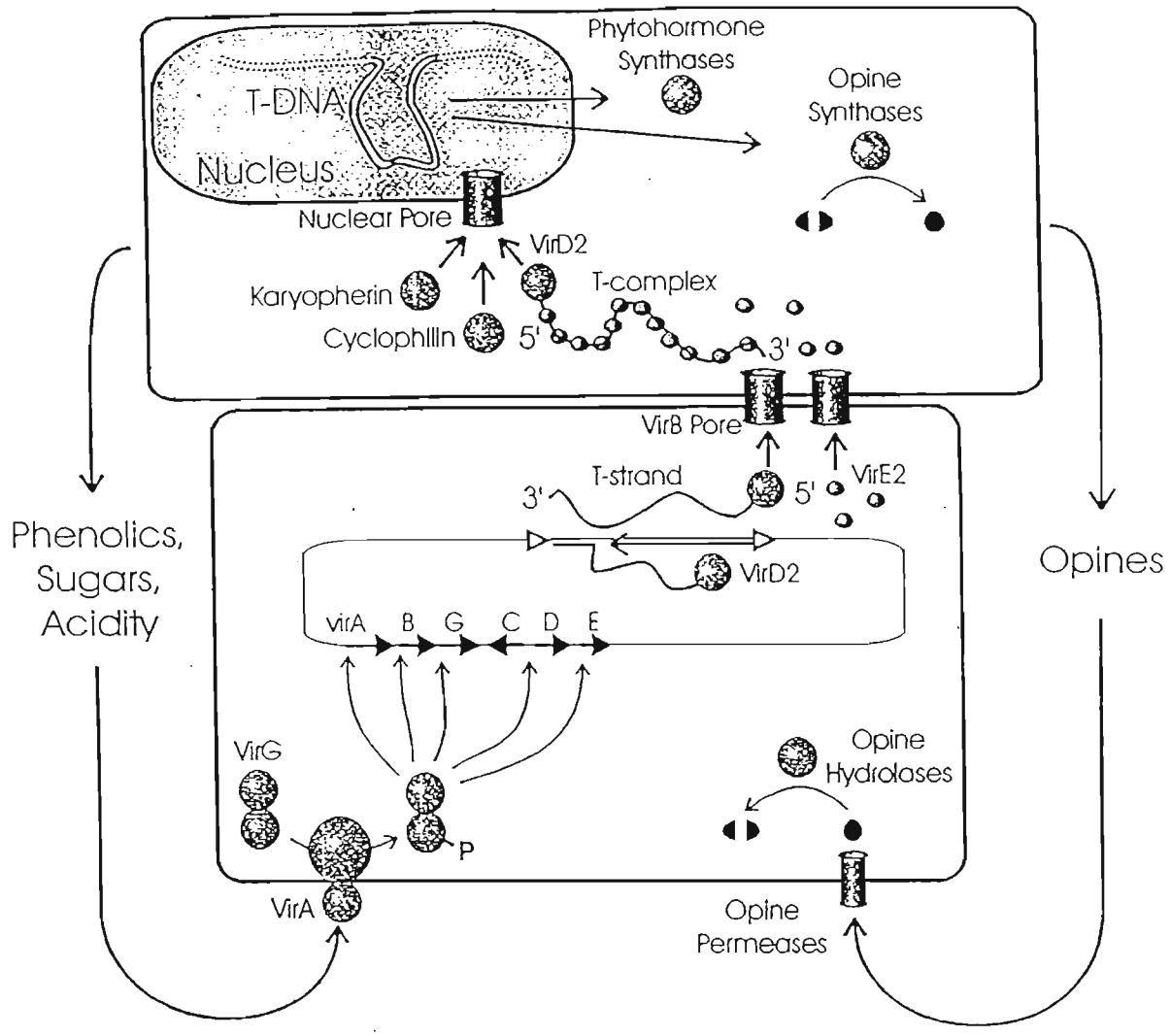

FIG. 2. Two-way exchange of chemical signals between *A. tumefaciens* and host plants. Wound-released chemical stimuli are perceived by the VirA to VirG proteins, which leads to transcription of *vir* promoters. T-DNA is processed by the VirD2 protein, and single-stranded linear T strands are formed by strand displacement. T strands and VirE2 are translocated from the bacteria via a pore encoded by the *virB* operon and form a T complex within the plant cytoplasm. T complexes are transported into the nucleoplasm via the host protein karyopherin alpha, and the T-DNA is integrated into genomic DNA. Transferred genes encode phytohormone synthases that lead to plant cell proliferation and opine synthases that provide nutrients to the colonizing bacteria. Opines are released from the plant cell, enter the bacteria via dedicated opine permeases, and are catabolized via opine-specific catabolic proteins. Opine permeases and catabolic enzymes are encoded by the Ti plasmid. For the sake of clarity, the relative orientations of *vir* genes and T-DNA have been inverted.

ing but are required for efficient T-strand transfer into most host plants, suggesting that they play a role in T-strand export.

The T-DNA transfer apparatus is encoded by the *virB* operon, which contains 11 genes (11). Each except VirB1 is essential for tumorigenesis (5). All 10 essential proteins have been localized to the inner or outer membrane, and most appear either to be integral membrane proteins or to be exported from the cytoplasm (107). Two VirB proteins, VirB4 and VirB11, are peripherally bound to the others and located primarily in the cytoplasm, although a small part of VirB4 may span the inner membrane (19). VirB4 and VirB11 have ATPase activity and are thought to provide the energy required for export of other protein subunits, for T-strand transport, or both (12, 93). VirB proteins direct the production of pili that resemble conjugative pili (31), and VirB2 is the major subunit of these pili (58). VirB2 is processed to a 7.2-kDa product that is cyclized such that the amino terminus is linked to the carboxyl terminus via an amide bond (26). Cyclization does not require any Ti plasmid-encoded proteins but does not occur in *Escherichia coli*, suggesting that this reaction requires a protein encoded elsewhere in the *A. tumefaciens* genome. VirB7 may help to anchor this pilus to the bacterial cell, as it is an outer membrane lipoprotein that forms disulfide bonds with the periplasmically localized VirB9 (2, 96). The VirB mating bridge is thought to be coupled to the T-strand complex by the VirD4 protein, which is located in the inner membrane and is absolutely required for transfer (67, 82). VirB1 possesses sequence motifs found in bacterial transglycosylases and eukaryotic lysozymes, suggesting a role in the localized digestion of the peptidoglycan (78).

The VirB apparatus delivers T strands to the plant cell cytoplasm, where additional steps are required to transport

this DNA to the nucleoplasm and to integrate it into host DNA. The carboxyl terminus of VirD2 contains a nuclear localization signal that is thought to guide nuclear targeting by interacting with the karyopherin alpha and cyclophilin proteins (3, 23, 44). The VirE2 protein appears also to play a role in nuclear import. This protein binds tightly and cooperatively to single-stranded nucleic acids, forming coiled, cylindrical filaments (14). Like VirD2, VirE2 contains nuclear localization sites that mediate transport of the T-DNA from the cytoplasm to the nucleoplasm (15).

Transgenic plants expressing VirE2 can be transformed by *virE2* mutants of *A. tumefaciens*, indicating that VirE2 is required only in plant cells (15). Similar data have been obtained for another protein, VirF, which is required for tumorigenesis on such plants as tomato and *Nicotiana glauca* (87). Mutations in either *virE2* or *virF* can be complemented extracellularly, that is, by coinfection with a helper strain possessing the *vir* region but lacking an oncogenic T-DNA (73, 84). Initially, it was thought that such complexes were formed within the bacterium, but more recent genetic evidence suggests that VirE2 and T-DNA are transferred separately and form complexes in the plant cell cytoplasm (101). Transfer of VirE2 requires VirE1, while transfer of T-DNA does not, suggesting that VirE1 acts as an export chaperone for VirE2 (22, 101). Conversely, transfer of T-DNA requires VirC1 and VirC2 while transfer of VirE2 does not require either protein (13). These studies provide the best evidence that T strands and VirE2 are transferred independently, although biochemical evidence addressing this hypothesis will await future studies. These data indicate that the *virB*-encoded transfer system, in addition to transferring T-DNA, can carry out contact-dependent translocation of at least three proteins, VirD2, VirE2, and VirF. This property of protein transport is highly reminiscent of the family of type III protein translocation systems of plant and animal pathogens, although these systems have independent ancestries (18, 37).

Many aspects of T-DNA transfer resemble interbacterial conjugal transfer of plasmid DNA (63). In both processes, transfer is initiated by single-stranded scissions at specific *cis*-acting sites. Moreover, the protein that catalyzes the scission remains bound to the 5′ end of the cleaved strand, and in both cases, DNA is transferred in a single-stranded form. The most direct evidence that the T-DNA transfer apparatus evolved from a conjugal transfer system is the extensive sequence similarities between Vir proteins and certain Tra proteins. For example, all 11 VirB proteins resemble the mating pair formation (Mpf) subset of Tra proteins encoded by the IncN plasmid pKM101 and show a lower degree of similarity to the Tra proteins of IncW, IncP, and IncF plasmids (48, 62). Similarly, the VirD1, VirD2, and VirD4 proteins resemble the donor transfer and replication (Dtr) subset of Tra proteins. In fact, the *virB* and *virD* operons together would constitute a complete set of conjugation proteins. The T-DNA border resembles the *oriT* sites of IncP plasmids, and nicking occurs at identical positions in the two transfer systems (117). The gene family of Vir and Tra proteins also includes the Ptl proteins of *Bordetella pertussis*, which direct the export of pertussis toxin; the VirB proteins of *Brucella* spp., which are required for intracellular survival; and other protein translocators of bacterial pathogens, collectively referred to as type IV export systems (18). The VirC and VirE proteins do not significantly resemble known transfer proteins, and VirC1 resembles a plasmid partitioning protein (38).

Some members of the *vir* regulon are not essential for tumorigenesis on all hosts and may be required only in specific hosts or may play other roles in pathogenesis. These include *virD5*, *-E3*, *-F*, *-H*, *-J*, *-K* *-L*, *-M*, *P*, and *-R* (50, 52). However, the lack of an apparent role in tumorigenicity could be a consequence of functional redundancy. For example, *virJ* is essential for tumorigenicity, but only in the absence of the homologous chromosomal gene *acvB* (49). The *virH* operon consists of two genes whose products resemble the family of P450 monooxygenases (51). VirH2 chemically modifies certain phenolic *vir* gene inducers by O demethylation, converting them to noninducers (51). For example, the inducer ferrulic acid is O demethylated to create the noninducer caffeic acid. This finding suggests that VirH2 acts as a regulatory governor.

UPTAKE AND CATABOLISM OF OPINES

As described above, several T-DNA-encoded genes direct the synthesis of opines, which serve the bacteria as nutrient sources. Over 40 genes are devoted to opine uptake and catabolism. These include no fewer than six ATP binding cassette-type permeases (Fig. 1, dark blue bars) and 12 opine catabolic enzymes (light blue bars), whose functions are summarized in Table 1. These opine permeases are only distantly related to each other, suggesting that they were adapted from diverse sources. An additional gene (*mclA*) could encode a protein that resembles methyl-accepting chemoreceptors. *A. tumefaciens* strains are chemotactic toward opines, and chemotaxis requires the cognate periplasmic opine binding proteins (each a component of an opine uptake system) but does not require Ti plasmid-encoded methyl-accepting chemotaxis proteins (53). It seems likely that this is another example of redundancy in which these periplasmic binding proteins can interact either with chromosomally encoded or with Ti plasmid-encoded methyl-accepting chemotaxis proteins.

Characteristically, Ti plasmids code only for the opine catabolism systems that correspond to the set of opine biosynthesis genes located in the T regions. This presents the interesting problem of how these paired gene systems arise and how they remain grouped together despite the fact that they are located in different segments of the plasmid. Sequence analysis of the mannopine-agropine catabolic loci indicated that certain of these genes resemble the cognate opine biosynthetic genes. The catabolic protein AgcA, which interconverts mannopine and agropine, resembles the agropine synthase protein Ags, which carries out the same reaction. In fact, *ags* can complement an *agcA* mutant for catabolism of agropine (40). Similarly, MocC and MocD, which together degrade mannopine, resemble Mas1′ and Mas2′, which synthesize mannopine (54). Based on these comparisons, we have suggested that the T-region genes coding for mannityl opine synthesis by the transformed plant cells arose by gene duplication from bacterial genes required for catabolism of these or closely related substrates (54). However, not all opine synthases resemble their corresponding catabolic enzymes. For example, the octopine and nopaline synthases do not resemble their cognate catabolic enzymes.

REPLICATION FUNCTIONS

A DNA fragment containing just *repA*, *repB*, and *repC* provides all functions required for stable replication in *A. tumefaciens* (104). Only *repC* is critical for vegetative replication, while *repA* or *repB* is required for stable plasmid inheritance. RepA and RepB resemble a family of plasmid partitioning systems that are thought to ensure that during cell division each daughter cell inherits at least one copy of the plasmid. All three genes resemble replication genes of other large, low-copy-number plasmids present in members of the family *Rhi-*

zobiaceae (64). Incompatibility functions also are determined by the DNA fragment containing *repABC* (56). The octopine-type Ti plasmid is incompatible with nopaline-type Ti plasmids (41), but in spite of the relatedness of their replicators, the octopine Ti plasmid is compatible with Ri plasmids (17).

INTERBACTERIAL CONJUGATION OF Ti PLASMIDS

The octopine Ti plasmid is capable of interbacterial conjugation (28) and contains a complete transfer system (Fig. 1, purple bars). On the basis of similarity to other conjugation systems, the cluster of *tra* genes probably is required for DNA transfer and replication, while the *trb* gene cluster is probably required for mating pair formation and could direct the synthesis of conjugal pili. In the closely related conjugation system of pTiC58, *traB* is not essential for transfer, although it is required for maximal efficiency, while *traH* is not required for efficient transfer (29) and is here designated a *tra* gene simply because it lies in the *tra* regulon (see below). All other *tra* and *trb* genes of pTiC58 are known or thought to be required for efficient conjugation (29, 65), with the exception of *trbK*, which is probably not required for conjugation but may mediate entry exclusion (28). The three operons of the conjugal transfer system are strongly conserved among all of the Ti plasmids analyzed to date (although one report [103] claimed otherwise, that study was based upon an incorrect DNA sequence). These genes also resemble the *tra* genes of at least one symbiosis megaplasmid, pNGR234a of *Rhizobium* sp. strain NGR234 (30). The Tra system functions independently of the T-DNA transfer system described above (16).

The *tra* genes appear to have diverse origins. TraG, TraF, and all 11 Trb proteins closely resemble IncP-type Tra proteins. In contrast, TraA, the putative nickase-helicase of this system, does not closely resemble any IncP-type Tra protein. Instead, the amino-terminal domain of TraA, which should contain *oriT* nicking activity, resembles the strand transferase of the IncQ plasmid RSF1010, while its carboxyl-terminal domain, which contains a possible helicase, resembles Tra proteins of IncN, IncW, and IncF plasmids. The *oriT* also resembles the corresponding site in RSF1010. Interestingly, the Vir system seems also to have chimeric origins, as all 11 VirB proteins resemble IncN Tra proteins, while two VirD proteins, VirD2 and VirD4, resemble IncP-type Tra proteins. The T-DNA borders resemble the *oriT* site of IncP plasmids (117). In all cases, sequence similarities between Ti plasmid Tra proteins and corresponding Vir proteins are relatively weak.

REGULATED EXPRESSION OF Ti PLASMID-ENCODED GENES

Virtually all of the genes described above are tightly regulated by proteins that also are encoded on the Ti plasmid (Fig. 1, orange bars). For example, the *vir* regulon is coordinately induced in response to host-released phenolic compounds in combination with monosaccharides and extracellular acidity in the range of pH 5.0 to 5.5 (47). This acidity may be necessary to protonate phenolic compounds, which would increase their membrane permeability. These chemical stimuli are detected by the transmembrane two-component sensor kinase VirA, which phosphorylates the response regulator VirG. Phospho-VirG positively regulates all *vir* promoters, including those of *virA* and *virG*, which results in positive autoregulation of this regulon (100, 119).

VirA contains four functional domains, designated the periplasmic, linker, kinase, and receiver domains (9), and exists as a dimer both in the presence and in the absence of inducing stimuli (85). The periplasmic domain is required for detection of a sugar binding protein called ChvE (8, 92), while the linker domain is required for detection of phenolic compounds, and the receiver plays an inhibitory role in *vir* gene expression (9). VirA can undergo autophosphorylation in vitro and transfers its phosphoryl group to Asp52 of VirG (46). The carboxyl-terminal domain of VirG binds to sequences called *vir* boxes that are found near all VirG-regulated promoters (88, 118). While there is still some controversy about whether phenolic inducers bind directly to VirA (59), genetic evidence suggests that this is so, since *virA* genes from different strains of *A. tumefaciens*, when introduced into an isogenic background, encode proteins that are stimulated by different types of inducers (60).

All opine uptake and catabolic systems are induced by their cognate substrates. For example, octopine induces transcription of a 14-kb *occQ-traR* operon via the OccR protein, a LysR-type regulator. OccR binds to its binding site, which lies directly upstream of the *occQ* promoter, in the presence or absence of octopine but undergoes a conformational change in response to octopine (115). The mannopine and agropine permeases and catabolic enzymes also are induced by the cognate opines, probably via the MocR protein, which resembles the LacI repressor of *E. coli*. Similarly, regulated expression of the *aga* and *moa* genes by the cognate opines requires the MoaR repressor, which resembles yet another family of regulators, including the galacticol repressor of *E. coli* (70). Expression of the opine catabolism gene sets also is influenced by global control systems. Transcription of the mannityl opine catabolism genes, while inducible by their cognate substrates, also is controlled by catabolite repression (39, 127), since these genes are not induced by mannopine or agropine when favored carbon sources such as glutamate or succinate also are provided. Furthermore, these catabolic genes are part of the nitrogen assimilation regulon, since catabolite repression by succinate is not observed when mannopine is provided as the sole source of nitrogen (39).

The TraR-TraI system positively controls expression of the *tra* and *trb* operons (Fig. 1, purple bars). Transcription of this regulon is controlled by a regulatory cascade that is initiated by octopine acting through OccR, which leads to expression of *traR* (33). TraR in turn is a direct positive regulator of the *tra* and *trb* genes (36). TraR is a member of the LuxR family of quorum-sensing transcriptional regulators (35), and its activity requires *N*-3-oxooctanoyl-L-homoserine lactone (126). Synthesis of this compound, called an autoinducer, is directed by the TraI protein, which utilizes 3-oxooctanoyl-acyl carrier protein and *S*-adenosylmethionine as substrates (36, 77). This compound is synthesized in the bacterial cytoplasm but diffuses across the cell envelope and acts as a bacterial pheromone, providing a mechanism for the bacteria to estimate their population densities (35). Since the Ti plasmid encodes both TraI and TraR, each conjugal donor takes a census of other donors rather than of recipients (34). Purified TraR binds one molecule of this compound per protein monomer and binds directly to dyad symmetrical DNA sequences called *tra* boxes, which are found directly upstream of the *traA*, *traC*, and *traI* promoters (34, 128). TraR stimulates transcription of *tra* promoters in vitro on supercoiled templates but is largely inactive on linear templates (128). DNA binding by TraR requires the autoinducer (68).

TraR activity is antagonized by two proteins encoded by the *traM* and *trlR* genes. Interestingly, the *traM* gene is positively regulated by TraR, thereby creating a negative autoregulatory loop (32). TraM is an antiactivator and directly interacts with the carboxyl terminus of TraR (45, 69). This interaction rapidly

inhibits TraR activity and disrupts TraR-DNA complexes (69). TrlR is very closely related to TraR in its autoinducer binding domain but lacks a DNA binding domain (80, 127) and is thought to form inactive heterodimers with TraR. The *trlR* gene is positively regulated by mannopine. Consistent with this, mannopine inhibits *tra* gene expression, while inhibition is abolished by a *trlR* mutation (80, 127).

INSERTION SEQUENCES (ISs) AND UNCHARACTERIZED ORFS

A number of possible ISs are present on the Ti plasmid (Fig. 1, black bars), although transposition of these elements has not been detected experimentally. Three of these resemble IS66, which was originally found inserted in the *iaaH* gene of a strain of an *A. tumefaciens* mutant that causes shooty teratomas rather than tumors. However, several of these IS66-like elements are considerably shorter than IS66, suggesting that they may be defective remnants of the original element. The other possible IS-like elements resemble a wide variety of IS elements in many eubacteria.

While most of the genes in the Ti plasmid have been ascribed functions, a contiguous 24-kb region (coordinates 112 to 136) contains 25 ORFs that have no known function (Fig. 1, grey bars). Most of the ORFs in this region are at least 100 codons in length and have moderately strong translation initiation motifs, and many of these ORFs appear to be translationally coupled to adjacent ORFs. All of these considerations suggest that these ORFs are expressed genes, but the functions of their products are at present unknown. Approximately half of these genes resemble genes identified in genome sequencing projects, though none of these homologous genes has been characterized genetically or biochemically.

RELATION OF THE OCTOPINE-TYPE Ti PLASMIDS TO OTHER PLASMIDS OF THE *RHIZOBIACEAE*

The modular structure of this Ti plasmid is entirely in keeping with the model presented by Otten and colleagues in which these elements evolve by IS-mediated intramolecular rearrangements and by recombination with other plasmids (83). Signs of such recombination events are scattered over the length of this plasmid. For example, *trlR* may well have arisen by a recombination event that fused the mannityl opine catabolism region with its attendant mannopine-regulated *traR* allele to the region just upstream of the octopine catabolism locus (80, 127). Similarly, the structural and regulatory association of the functional *traR* allele with the *occ* operon arose from a fortuitous recombination event that fused *traR* to *occ*. Interestingly, such associations of *traR* with various opine catabolism operons are a consistent feature of Ti plasmids (28, 33, 80, 127).

Despite this plasticity, certain gene associations seem to be strongly conserved among these elements. Most notably, the *repABC* complex is tightly linked with the *trb* operon in all Ti and opine catabolism plasmids that have been examined to date (pTiC58, pTi-SAKURA, and pAtK84b) (64). This conservation extends to at least four plasmids present in members of the genus *Rhizobium* (64), suggesting that this linkage is strongly selected. Consistent with this interpretation, the intergenic region separating the two divergently oriented gene systems on many of these plasmids, including all Ti plasmids, contains two copies of the *tra* box sequence. Coupled with the recent observation that copy number of the nopaline-type Ti plasmid is positively enhanced by TraR in a quorum-dependent fashion (64), it is reasonable to conclude that conjugal transfer and plasmid replication are functionally linked.

CONCLUSIONS

As described above, most of the genes of the Ti plasmid play direct or indirect roles in some aspect of tumorigenesis or tumor colonization. We understand the roles of most of the T-DNA-encoded genes, although the functions of some remain mysterious. We have some insights about the processing and transfer of the T-DNA, although our understanding of the VirB-encoded pore is rudimentary, as are the steps involved in nuclear transport and integration. At least three Vir proteins are thought to be transferred from the bacterium into plant cells during infection, though the physical detection of these proteins in plant cells remains a goal for future studies. It will be interesting to identify any additional translocated proteins and to elucidate their functions. VirA and VirG remain important paradigms for host detection, and the multidomain structure of VirA remains fertile ground for future work. Future studies will decide once and for all whether VirA binds phenolic inducers directly or through an accessory phenolic binding protein. Of the 34 known VirG-regulated genes, one-third do not seem essential for tumorigenesis (at least on certain host plants), suggesting that plant-released *vir*-inducing signals elicit multiple bacterial responses that remain to be described.

Another challenge lies in comparative analysis of the many different Ti plasmids that have been isolated, as well as other plasmids found in members of the *Rhizobiaceae*. We know that approximately 65 kb of octopine-type plasmids are conserved in the nopaline-type Ti plasmid pTiC58 (Fig. 1, crosshatched boxes), including part of the T-DNA and the *tra*, *trb*, *rep*, and *vir* regions (27), while the remaining 130 kb are not conserved. As more Ti plasmids and related plasmids are characterized (102), it will be possible to refine our insights about the evolution of these genetic elements.

The use of *A. tumefaciens* to create transgenic plants has become routine for many dicots as well as for some monocots, and yet new insights about fundamental aspects of *Agrobacterium*-plant interactions will lead to improved technologies in plant transformation. Future work will lead, for example, to further expansion of the organism's host range, to new approaches to transferring extremely long fragments of DNA, and to new approaches to using T-DNA to disrupt plant genes.

It is striking that such a large portion of the Ti plasmid is devoted to opine uptake and catabolism, and few of these systems have been studied in any depth. Studies of opine chemotaxis, uptake, and catabolism will continue. In addition, one challenge for the next 10 years will be to apply these insights about opines to agriculture. Several reports have already appeared showing that bacteria that utilize a particular opine enjoy a competitive advantage in colonizing transgenic plants that produce the same opine (81, 89). We suspect that this technology may revolutionize efforts to foster beneficial plant-microbe associations.

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Generation of single-stranded T-DNA molecules during the initial stages of T-DNA transfer from *Agrobacterium tumefaciens* to plant cells

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Activation of the T-DNA transfer process of Agrobacterium *by the plant signal molecule acetosyringone generates a single-stranded, unipolar, linear T-DNA molecule (T-strand)—a potential conjugative intermediate in the transfer of the T-DNA to plant cells. Acetosyringone induction also leads to S_1 nuclease-sensitive sites at the Ti plasmid T-DNA borders, and other molecular changes associated with the Ti plasmid T-DNA sequences, which may correspond to specific steps of T-strand synthesis.*

DURING the genetic transformation of plant cells by the soil pathogen *Agrobacterium tumefaciens* (reviewed in ref. 1), a specific segment of DNA, the T-DNA, is recognized in and mobilized from the large (>200 kilobase pairs; kbp) Ti plasmid of the bacterium, transferred across the cell walls of the bacterium and plant cell, and integrated as an unaltered fragment into the plant nuclear genome. Analysis of the transfer process has focused on what defines the T-DNA; the genetic requirements for transfer other than the T-DNA; and the mechanism of transfer.

In the Ti plasmid the T-DNA is bounded by essentially identical 25-base pair (bp) direct repeats[2-5]. These sequences define the T-DNA, for any DNA, and only DNA, located between T-DNA borders is efficiently transferred and integrated[6-9]. The T-DNA transfer process is directed by the products of the Ti plasmid virulence (*vir*) and chromosomal virulence (*chv*) loci (reviewed in refs 10, 11). Whereas *chv* expression is constitutive, *vir* expression is tightly regulated[12,13], and its activation initiates the transfer process. This activation is mediated by specific phenolic compounds present in the exudates of wounded and actively metabolizing plant cells. One such compound is acetosyringone (AS; 4-acetyl-2,6-dimethoxyphenol)[14].

Genetic analyses of the 25-bp sequences have indicated that they are polar in function[15,16]. While deletion of the left border repeat has no significant effect on pathogenicity[17], deletion of the right repeat totally abolishes it[15,16,18]. Furthermore, when the orientation of the right border is reversed with regard to its natural orientation on the Ti plasmid, the efficient transfer and/or integration of the T-DNA sequences is greatly attenuated. These results indicate that T-DNA transfer may occur in a rightward to leftward fashion, determined by the orientation of the 25-bp border repeats, and suggest that transfer might be via a conjugative mechanism[15].

Here we use hybridization techniques to directly identify and characterize novel structures associated with the T-DNA and its border sequences in *Agrobacterium* following induction of *vir* gene expression with AS. A variation of the Southern blotting transfer procedure is used to distinguish between single-stranded (ss) and double-stranded (ds) DNA molecules present in total

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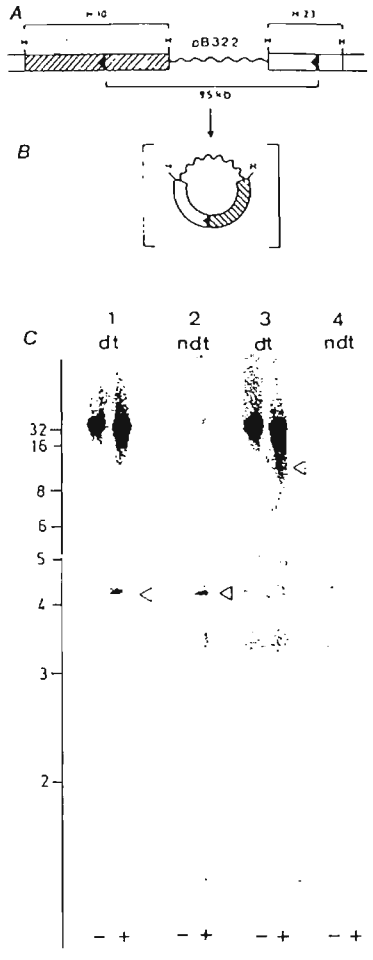

Fig. 1 Hybridization analysis of total DNA prepared from AS-induced *Agrobacterium*. *A*, T-DNA region of the pGV3850 Ti plasmid[6]. The interior portion of the T-DNA of pTiC58 has been replaced with PBR322 (wavy line). *Hin*dIII fragments 10 (hatched) and 23 (white) from pTiC58 carrying the right and left 25-bp T-DNA border sequences respectively (black arrows); H, *Hin*dIII restriction site. *B*, Structure of the 9.5-kbp ds T-DNA circle molecule isolated after transformation of *E. coli* with undigested total DNA from AS-induced pGV3850 *Agrobacteria*. *C*, Southern blot hybridization analyses of *Agrobacterium* DNA. Total DNA prepared from uninduced (−) and AS-induced (+) 3850 *agrobacteria* was analysed by hybridization against nick-translated ^{32}P-labelled pBR322 probe. Lane 1, denatured transfer (dt) of untreated DNA; lane 2, non-denatured transfer (ndt) of untreated DNA; lane 3, denatured transfer of total DNA digested with S_1 nuclease; lane 4, non-denatured transfer of S_1-treated DNA. Scaling on left is in kbp. Arrows indicate novel signals observed in the AS-induced lanes. Note that all of these novel signals are also observed, albeit at a lower level, with DNA prepared from *Agrobacterium* co-cultivated with *Nicotiana tabacum* protoplast cells (data not shown).

Methods. An overnight culture of 3850 *Agrobacteria* grown in YEB liquid medium was resuspended in MSSP medium[14] at 0.05 A_{600} units ml^{-1} and grown at 28 °C with high aeration. After 5 h of preincubation growth, acetosyringone (Janssen) was added to half of the MSSP culture at 100 μM, a concentration that is non-limiting for induction of the Ti-plasmid *vir* genes[14]. The uninduced and AS-induced cultures were grown for another 12–18 h and a parallel culture of the *virB*::*lac* strain A348(pSM30) (refs 10, 14) was used to monitor *vir* induction; under these conditions the cultures undergo two to three doublings, and >100-fold increases in β-galactosidase activity are observed in A348(pSM30). The bacteria were collected by centrifugation and total DNA prepared as described previously[33]. Briefly, the pellet from 5 ml of cells is lysed in 200 μl TE (50 mM Tris, 20 mM Na_2-EDTA, *p*H 8.0), 100 μl 5% sodium sarkosyl and 100 μl pronase (10 mg ml^{-1}) for 45 min at 37 °C. The lysate is then vortexed for 15 s (light-shear), extracted twice with phenol and twice with chloroform, and the DNA recovered by EtOH precipitation. *E. coli* was transformed with uninduced and AS-induced DNA as described previously[19], and the AS-induced preparation was determined to give transformants carrying the ds T-DNA circle molecule diagrammed in *B*. Aliquots (1 μg) of total *Agrobacterium* DNA (either untreated or S_1-digested) were then electrophoresed in 0.9% TBE agarose gels containing 0.5 μg ml^{-1} EtBr, transferred to nitrocellulose in 10×SSC, and analysed by Southern blot filter hybridization. Two different transfer condtions have been used, denatured and non-denatured. For denatured transfer, the agarose gel is soaked in denaturing solution for 60 min followed by neutralizing solution for 60 min before capillary blotting. For non-denatured transfer, the gel is soaked in H_2O for 10 min, then 10×SSC for 10 min, before blotting. For the S_1 nuclease digestions, 1 μg total DNA in 200 μl S_1 digestion buffer was incubated with 50 U S_1 nuclease (Boehringer Mannheim) for 30 min at 20 °C. The reaction is terminated by adding 20 μl 10×S_1 termination buffer, followed by phenol extraction and EtOH precipitation. Unless otherwise specified, all buffers and conditions used here and in Figs 2–5 are according to Maniatis *et al.*[34].

DNA prepared from these cells. Evidence is presented for (1) free ss T-DNA molecules (T-strands) whose polarity corresponds to that of the T-DNA borders; (2) ss endonuclease-sensitive structures associated with the Ti plasmid T-DNA borders (S_1 border sites); and (3) molecular alterations associated with the internal sequences of the Ti plasmid T-region (T-region structures). We discuss the potential role of each of these AS-induced T-DNA homologous molecules in the transfer of the T-DNA to the plant cell.

Free T-DNAs in AS-induced cells

For these studies we used *Agrobacterium* carrying Ti plasmid pGV3850 (ref. 6). pGV3850 has been derived from the nopaline C58 Ti plasmid, and the structure of its minimal T-DNA region is shown in Fig. 1*A*. Strain 3850 has been used previously to isolate and identify a 9.5 kbp ds T-DNA circle molecule (Fig. 1*B*) following transformation of *Escherichia coli* with total DNA prepared from plant[19] or AS-induced *Agrobacteria*[14]; this T-DNA circle has been proposed as a candidate for the T-DNA molecule that is transferred to the plant cell. Our initial experiments aimed to identify the presence of this molecule directly in *Agrobacterium*.

Total DNA was prepared from AS-induced cells and found to produce ds T-DNA circles in *E. coli*; this AS-induced DNA, along with total DNA prepared from uninduced cells, was fractionated by agarose gel electrophoresis and transferred to a nitrocellulose filter following gel denaturation (normal Southern transfer procedure, see below). Two T-DNA homologous signals are observed in Fig. 1*C*, lane 1. Since the DNA is undigested, the upper signal represents Ti-linked T-DNA sequences, while the lower signal represents free T-DNA sequences. This novel signal is specific to the AS-induced DNA, and thus corresponds to a Ti-independent T-DNA molecule whose synthesis is the result of the AS-induced activation of the pGV3850 *vir* loci.

The free T-DNA signal does not migrate as a 9.5-kbp ds DNA molecule (supercoiled, relaxed-circular or linear), but instead migrates at a size corresponding to a ds linear molecule of 4.4 kbp. Thus, by hybridization we find no evidence for ds T-DNA circles in AS-induced bacteria. This result is not totally unexpected because the frequency of recovery of these molecules in *E. coli* is low. We obtain on average 50 ds circle transformants per μg AS-induced DNA (at a transformation efficiency of 10^7 transformants per μg supercoiled pBR322 plasmid DNA). Since 1 μg of total *Agrobacterium* DNA contains ~1.7 ng of pGV3850 T-DNA[19], at most only one in every 100 AS-induced cells harbours a ds T-DNA circle. That these molecules are not detected by hybridization indicates that the transformation results are representative of their actual concentration in the AS-induced DNA. We discuss below a model for how ds circle molecules might be generated at a low frequency as a result of AS induction (Fig. 6).

Free T-DNAs are single stranded

Since the size of the AS-induced free T-DNA molecule is about half that of the T-region of pGV3850 (Fig. 1*A*), it may be a single-strand copy of the pGV3850 T-DNA. The following experiments demonstrate that the free T-DNA molecule has properties characteristic of ss DNA. Evidence is also given for other novel T-DNA-homologous molecules present in the AS-induced bacteria; in contrast to the free T-DNA, these structures

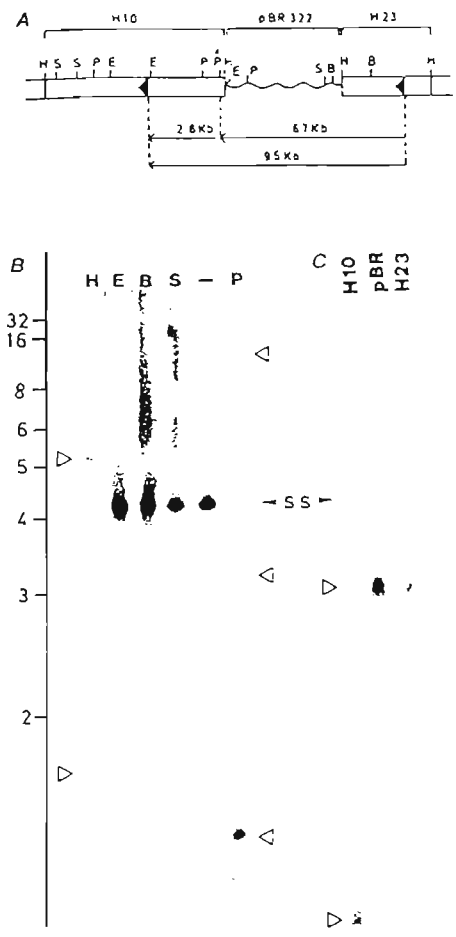

Fig. 2 Restriction endonuclease treatment of AS-induced DNA. *A*, Restriction map of the T-DNA region of pGV3850. Black arrows, T-DNA border sequences; the 9.5-kb arrow designates the ss T-DNA molecule, and the 2.8-kb and 6.7-kb arrows correspond to the ss fragments that would be produced by *Pst*I cleavage of the ss T-DNA molecule at the *Pst*I* site. *B*, Aliquots (1 μg) of AS-induced DNA were digested to completion with five restriction endonucleases, fractionated on 0.9% agarose, transferred under non-denaturing conditions to nitrocellulose, and analysed by hybridization against *Hin*dIII fragment 10/pBR322 probe. H, *Hin*dIII; E. *Eco*RI; B, *Bam*HI; S, *Sal*I; P, *Pst*I; −, undigested; ss, free T-DNA molecule; open arrows, unexpected fragments produced by *Hin*dIII or *Pst*I digestion of the AS-induced DNA. *C*, Three equivalent non-denatured transfers of *Pst*I-digested AS-induced DNA were hybridized against *Hin*dIII fragment 10 probe (H10); pBR322 probe (pBR); and *Hin*dIII fragment 23 probe (H23). Scaling is different from that in *B*, such that the signals marked with triangles correspond to ss DNA molecules of ~6.6 and 2.8 kb, respectively. Note that different preparations of AS-induced DNA were used in the experiments of *B* and *C*. The ~9.2-kbp signal observed in the *Pst*I digest in *B* is not observed in *C*; this difference may reflect the detection of different levels of AS-induced events in the two preparations.

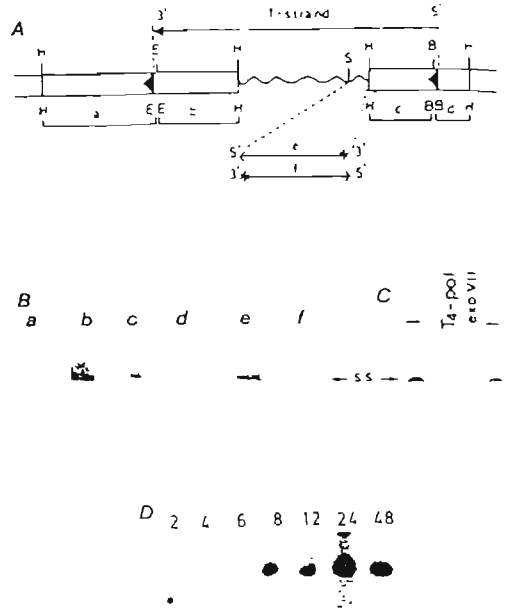

Fig. 3 Molecular characterization of ss T-DNA molecules. *A*, Six probes used to analyse the sequence content and strandedness of the ss T-DNA molecule. *B*, *Bcl*I; *E*, *Eco*RI; *H*, *Hin*dIII. The *Eco*RI site of probes *a* and *b* falls 55 bases inside the left T-DNA border, and the *Bcl*I site of probes *c* and *d* falls 42 bases inside the right border.

Methods. Fragments *a*, *b*, *c* and *d* were gel-purified twice and labelled by nick translation. Probes *e* and *f* are 3'-end-labelled probes where the position of the label is marked by an asterisk, and correspond to the upper and lower strands of the pGV3850 T-DNA, respectively. For probe *e*, pBR322 was digested with *Hin*dIII, 3'-end-labelled at the *Hin*dIII site by Klenow fill-in, recut with *Sal*I, and the resultant 622-bp *Hin*dIII/*Sal*I fragment was gel-purified. For probe *f*, the order of the restriction digests was reversed to give a 622-bp *Sal*I/*Hin*dIII fragment, 3'-end-labelled at the *Sal*I site. The polarity of the ss T-DNA molecule, or T-strand, is shown at the top of the figure. *B*, Sequence content and strandedness of the ss T-DNA. Six equivalent strips of a non-denatured transfer of undigested AS-induced DNA hybridized against the six probes shown in *A*. ss, Single-stranded T-DNA signal. *C*, Exonuclease sensitivity of the ss T-DNA. The non-denatured transfer of untreated (−), T4 polymerase-digested (T4-pol), and exonuclease VII-digested (ExoVII) AS-induced DNA was hybridized against nick-translated pBR322 probe. For exonuclease digestion, 1-μg aliquots of AS-induced DNA were incubated with 2.5 U T4 polymerase (Anglian Biotechnology Ltd) or 1.1 U exonuclease VII (Gibco-BRL), respectively, for 30 min at 37 °C. Each reaction was carried out in 15 μl in the enzyme buffer recommended by the manufacturer. Note that under identical conditions these treatments have no effect on M13 ss circular phage DNA; also, the ss T-DNA is fully degraded after 90 min digestion with T4 polymerase (data not shown). *D*, Kinetics of synthesis of the ss T-DNA molecule. A 50-ml culture of pGV3850 *Agrobacteria* pregrown for 5 h was then induced with AS at 100 μM. At 2, 4, 6, 8, 12, 24 and 48 h after the start of induction, 5-ml aliquots were removed, frozen at −70 °C, and total DNA was then prepared. These DNA samples were fractionated on agarose and transferred to nitrocellulose without gel denaturation, and the amount of ss T-DNA molecules present in the AS-induced cells for each time point was assessed by hybridization against nick-translated pBR322 probe. Note that by EtBR staining the amount of DNA in the 24-h lane was about double that for the other lanes.

are still linked to the Ti plasmid, as they are only detected after enzymatic digestion.

Transfer assay for ss T-DNAs. DNA binds to nitrocellulose only if it is single-stranded[20] or associated with protein[21]. In the Southern blotting technique, ds DNA fragments fractionated in an agarose gel must be denatured before being transferred to nitrocellulose[20]. Thus, ss (and protein-associated) DNA molecules can be easily distinguished from ds molecules by comparing their transfer to nitrocellulose from agarose gels treated with or without NaOH before transfer. Figure 1*C*, lanes 1 and 2, show the denatured and non-denatured transfers of uninduced and AS-induced total DNA. Of the two T-DNA-homologous signals present in the AS-induced DNA sample, only the lower signal, which represents the free T-DNA molecule, transfers without gel denaturation (Fig. 1*C*, lane 2). This free T-DNA must be ss DNA because the AS-induced total DNA has been fully deproteinated during its preparation (Fig. 1

legend) and RNase digestion of the DNA before hybridization analysis does not affect the ss signal (data not shown).

Nuclease sensitivity of ss T-DNAs. S_1 nuclease is a ss-specific endonuclease[22]. In Fig. 1*C*, the denatured (lane 3) and non-denatured (lane 4) transfers of S_1 nuclease-digested samples of uninduced and AS-induced total DNA demonstrate that the free T-DNA molecule is fully degraded by S_1 nuclease. Interestingly, S_1 treatment also appears to release the T-region from the AS-induced T1 plasmid. A novel T-DNA homologous signal is observed in the S_1-treated AS-induced sample in the denatured transfer (Fig. 1*C*, lane 3), and this signal corresponds to a ds molecule, which migrates as a linear fragment of 9.5 kbp, the precise size of the pGV3850 T-DNA from its left to right T-DNA borders (Fig. 1*A*). Thus, AS induction also results in a Ti plasmid whose T-DNA borders are sensitive to cleavage by S_1 nuclease (see Figs 4 and 5).

The activity of type II restriction endonucleases is limited primarily to duplex DNA. Aliquots of AS-induced DNA were digested to completion with *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I and *Sal*I (each of these enzymes cleaves within the T-DNA region of pGV3850; Fig. 2*A*), and hybridized to the *Hin*dIII fragment 10/pBR probe following non-denatured transfer (Fig. 2*B*). Because the ss signal is present in all the digests (except for *Pst*I), the free T-DNA is generally resistant to restriction digestion.

The *Hin*dIII and *Pst*I results are more complex. The ss signal is weak in the *Hin*dIII lane and absent in the *Pst*I lane; also, several novel signals are observed in these lanes. Several restriction enzymes have been shown to restrict ss DNA[23], and the sensitivity of the free T-DNA to *Hin*dIII and *Pst*I may be an example of this effect. Interestingly, fragments both smaller and larger than the ss T-DNA molecule are produced by *Hin*dIII and *Pst*I digestion (Fig. 2*B*). While the smaller fragments can be cleavage products of the ss T-DNA, the larger fragments must be derived from a large molecule, presumably the AS-induced Ti plasmid. Thus, AS induction also results in a Ti plasmid molecule whose T-region carries ss sequences (see Fig. 5).

Characterization of ss T-DNAs

ss T-DNAs are full-length and unipolar. Six identical strips of a non-denatured transfer of undigested AS-induced DNA were hybridized separately with the six probes labelled *a* to *f* in Fig. 3*A*. Probes *a* and *d* correspond to pGV3850 sequences just outside the left and right T-DNA border repeats; probes *b* and *c* correspond to T-region sequences just within these repeats; and probes *e* and *f* correspond to the upper and lower strands of the pGV3850 T-DNA region, respectively. Figure 3*b* shows that the ss T-DNA molecule hybridizes only to probes *b*, *c* and *e*. Thus, it is composed only of sequences located internal to the T-DNA borders, and which correspond to the lower strand of the pGV3850 T-region. We designate this unipolar ss T-DNA molecule as the T-strand.

T-strands are linear. The T-strand is sensitive to both exonuclease VII (3′→5′ and 5′→3′ activities specific for ss DNA[24]) and T4 polymerase (3′→5′ exonuclease that accepts both ss and ds DNA as substrate[25]) (Fig. 3*C*). Thus, the T-strand, as isolated, must be a linear ss DNA molecule whose 3′ terminus (and perhaps also 5′ terminus) is available to exonuclease digestion.

The *Pst*I cleavage products of the T-strand indicate that its 5′ and 3′ ends map to the right and left T-DNA borders, respectively. Two fragments of ~3.3 and 1.4 kbp (~ss lengths 6.7 and 2.8 kbp) are detected. The 6.6-kbp fragment is homologous to pBR322 and *Hin*dIII fragment 23, while the 2.8-kbp fragment is homologous only to *Hin*dIII fragment 10 (Fig. 2*C*). Cleavage of a circular T-strand molecule at any (or all) of its three *Pst*I sites would yield fragments of different sizes and sequence content from those observed. The simplest explanation for the results of Fig. 2*C* is that the T-strand is a linear ss molecule, cleaved by *Pst*I principally at its middle *Pst*I site (just within the right end of its *Hin*dIII fragment 10 sequences), to produce a 6.7-kbp 5′ and a 2.8-kbp 3′ fragment.

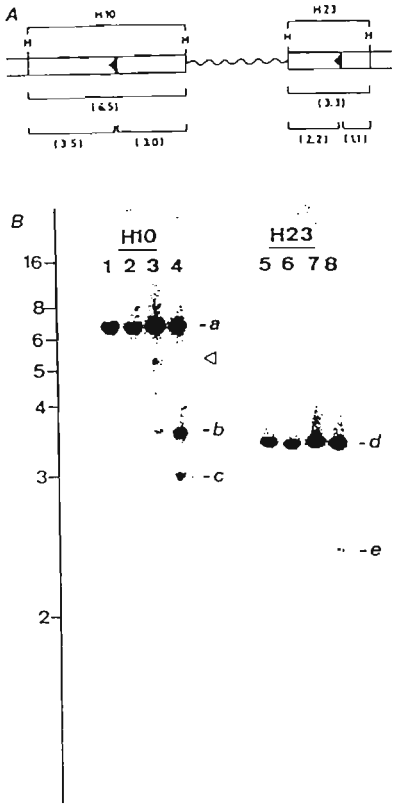

Fig. 4 Identification of AS-induced S_1-sensitive T-DNA border structures. *A*, *Hin*dIII fragments produced by ds cleavages of the pGV3850 T-DNA borders. Fragments *b* and *c* result from cleavage of the left border repeat carried by *Hin*dIII fragment 10 (*a*). Fragments *e* and *f* result from cleavage of the right T-DNA border carried by *Hin*dIII fragment 23 (*d*). Fragment sizes are in kbp. Black arrows, T-DNA border sequences; H, *Hin*dIII. *B*, Uninduced and AS-induced DNA was digested with *Hin*dIII, and half of each sample was treated further with S_1 nuclease. The four samples were analysed on two denatured transfers hybridized against nick-translated *Hin*dIII fragment 10 (lanes 1–4) or *Hin*dIII fragment 23 probe (lanes 5–8). Lanes 1, 5, uninduced DNA; lanes 2, 6, uninduced DNA plus S_1 treatment; lanes 3, 7, AS-induced DNA; lanes 4, 8, AS-induced DNA plus S_1 treatment. *a*–*f* refer to the fragments shown in *A*. The open arrow indicates a novel S_1-sensitive fragment of ~5.2 kbp. Scaling is in kbp. Note that the low level of the border cleavage fragments (*b*, *c*, *e*, *f*) seen in the absence of S_1 treatment (lane 3, and lane 7 after long exposure) may reflect mechanical breakage of the AS-induced S_1-sensitive border structures during sample preparation.

T-strand synthesis is limited. Using a probe homologus to both strands of the T-DNA, the intensity of the T-strand signal is ~5–10-fold less than the Ti-plasmid T-region signal (Fig. 1*C*, lane 1; also Fig. 5). As the T-strand corresponds to one strand of the T-region, and the copy number of the Ti plasmid is about two (and assuming no specific loss of T-strands during DNA preparation), we estimate that on average each AS-induced cell carries 0.4–0.8 T-strand molecules. As T-DNA homologous molecules are not found in the culture medium (data not shown), this low copy number is probably not due to export of the T-strand. Figure 3*D* demonstrates that the relative amount of T-strands begins to plateau within 8 h after the start of AS induction, and shows only a doubling during an additional 40 hours of induction. Thus, T-strand synthesis is a limited process.

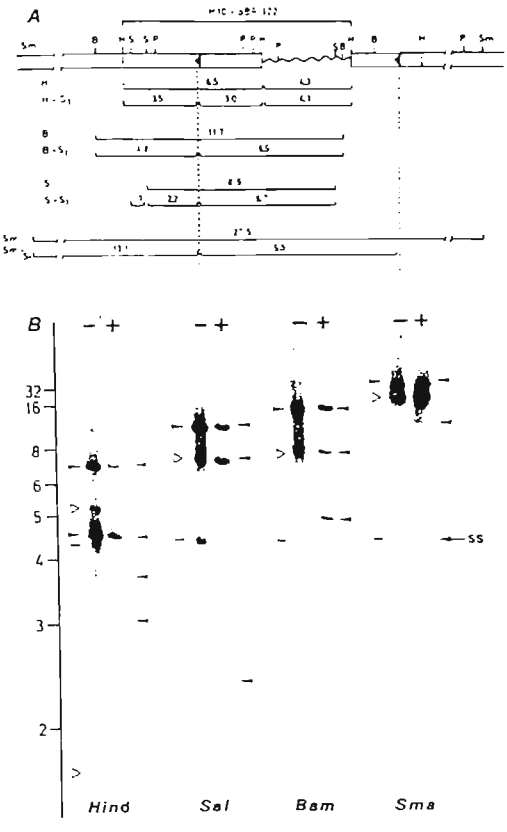

Fig. 5 Hybridization analysis of T-region intermediate structures. *A*, Restriction map of the left portion of the pGV3850 T-DNA region and border cleavage fragments predicted to arise after S_1 treatment of AS-induced DNA. Sizes are given in kbp. B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; S_1, S_1 nuclease. The black arrows and dotted lines indicate the positions of the T-DNA borders. *B*, Denatured transfers of *Hin*dIII, *Bam*HI, *Sal*I and *Sma*I digests of AS-induced DNA treated (+) and untreated (−) with S_1 nuclease were hybridized against nick-translated *Hin*dIII fragment 10/pBR322 probe (*A*). Scaling on left is in kbp. The single-stranded T-strand is indicated by ss and the bar adjacent to the digests. Solid arrows indicate the expected fragments before and after S_1 treatment and correspond in size to the fragments shown in *A*. The open arrows indicate unexpected AS-induced fragments which correspond to novel T-DNA homologous structures linked to pGV3850. Note that other unexpected signals are observed when the filter is reprobed with *Hin*dIII fragment 23 sequences, and that all of the unexpected fragments are specific to AS-induced DNA and have been observed with DNA prepared from at least two independent AS-induced bacterial cultures (data not shown).

AS-induced alterations of the Ti plasmid

Since the T-strand must be produced from the Ti-plasmid T-region, several molecular reactions involving these sequences should occur in bacterial cells that are actively carrying out different steps of T-strand synthesis, or other early steps of T-DNA transfer. To gain insight into these reactions, the novel T-DNA homologous hybridization signals that result from fragmentation of the AS-induced Ti plasmid with restriction enzymes and S_1 nuclease are characterized below. These data provide evidence for specific reactions associated with the Ti-plasmid T-DNA border repeats and with the internal sequences of the T-region.

S_1-sensitive T-DNA border sites. Figure 4 demonstrates that AS induction leads to the generation of Ti-plasmid T-DNA border structures which are sensitive to cleavage by S_1 nuclease. Uninduced and AS-induced DNA was restricted with *Hin*dIII, half of each sample was treated further with S_1 nuclease, and the fragments homologous to the left and right border regions of pGV3850 were detected by hybridization. The AS-induced + S_1 lanes contain novel signals whose sizes correspond exactly to the fragments that would be produced by ds cleavages at the left and right T-DNA border sequences of pGV3850 (Fig. 4A) and the intensities of these signals are greatly increased by S_1 digestion of the DNA. We designate these S_1-sensitive structures S_1 border sites.

The relative intensities of the signals which correspond to S_1 border cleavage fragments show that ~30% of the left T-DNA borders and ~10% of the right T-DNA borders of the AS-induced Ti plasmid population are S_1-sensitive. Thus, the generation of S_1 border sites is a frequent event. Furthermore, since a 9.5-kbp ds T-DNA fragment is produced by S_1 digestion of AS-induced DNA that is unrestricted (Fig. 1*C*, lane 3), or restricted with *Sma*I (which does not cleave within the pGV3850 T-region (Fig. 5)), S_1 border sites can simultaneously occur on both the right and left border repeats on a single Ti plasmid. We note that incubation of the uncut AS-induced DNA alone does not result in the release of the 9.5-kbp T-DNA fragment from the AS-induced Ti plasmid (data not shown); that is, the S_1 border site does not correspond to a ds staggered cleavage of the T-DNA border sequence. Other experiments have shown that the S_1-sensitive structure corresponds to an AS-induced endonucleolytic cleavage in the bottom strand of the 25-bp border sequence (K. Wang and M. Van Montagu, in preparation); S_1 is known to cleave opposite such structures[26].

Other AS-induced T-region structures. Figure 5 demonstrates that other novel Ti-plasmid-linked T-region structures are also present in the AS-induced cell population. Three different classes of T-DNA-homologous signals are observed when AS-induced DNA is digested with various restriction enzymes and S_1 nuclease (Fig. 5*B*): those that correspond to the ss T-DNA molecule (black bars, $-S_1$ lanes); those that correspond to the predicted border cleavage fragments (Fig. 5*A*) produced by S_1 digestion of the AS-induced S_1 border sites (solid arrows, $+S_1$ lanes); and those that correspond to novel unexpected fragments (open triangles) derived from pGV3850 Ti plasmids whose internal T-region sequences have been altered as a result of AS induction.

For example, the 5.2-kbp fragment present in the $-S_1$ *Hin*dIII sample (Fig. 5*B*; also Figs 2*B* and 4*B*) is S_1-sensitive, binds to nitrocellulose, specifically hybridizes to *Hin*dIII fragment 10 (and not to pBR322 or *Hin*dIII fragment 23; data not shown), and is ~1.3 kbp smaller than *Hin*dIII fragment 10 (Fig. 5*A*). Thus, this novel *Hin*dIII fragment must be partially single-stranded and derived from an AS-induced Ti plasmid whose T-region is partially single-stranded or associated with ss T-DNA sequences, as in a D-loop structure. More perplexing fragments are also detected: the ~15-kbp S_1-insensitive *Sma*I signal corresponds to a ds DNA fragment which is ~12 kbp smaller than the *Sma*I fragment that covers the T-region of the uninduced Ti plasmid; also, in the $-S_1$ *Bam*HI and *Sal*I lanes, signals are observed whose sizes correspond to the S_1 cleavage fragments of these digests (Fig. 5*A*) which are internal, but not external, to the left T-DNA border. While the present data do not allow the precise identification of the T-region structure(s) to which these unexpected fragments correspond, they illustrate the complexity of the molecular reactions associated with the Ti-plasmid T-DNA sequences which occur in AS-induced *Agrobacterium*.

Discussion

Agrobacterium tumefaciens transfers its Ti-plasmid T-DNA to plant cells; and this process is activated by the induction of the Ti-plasmid virulence genes with the plant phenolic compound AS. We show that AS induction results in the generation of several novel T-DNA homologous molecules in *Agrobacterium*: a linear ss molecule, the T-strand, which corresponds to the lower strand of the Ti-plasmid T-region; and Ti-plasmid molecules whose T-DNA border repeats are sensitive to cleavage

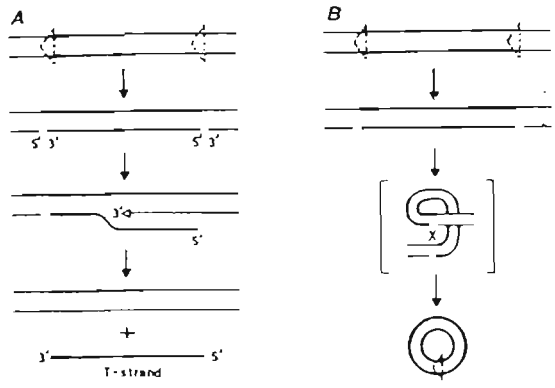

Fig. 6 Proposed reactions associated with the Ti-plasmid T-region in AS-induced cells. *A*, Model for the generation of the T-strand; *B*, model for the generation of a ds T-DNA circle molecule. Thick lines represent T-region sequences, thin lines represent adjoining Ti-plasmid sequences external to the 25-bp T-DNA border repeats, indicated by the dashed triangles. ss breaks in the bottom strand of the border repeats correspond to S_1 border sites, and the wavy line corresponds to a newly synthesized bottom strand of the T-region. This model implies that ss molecules are not generated leftward of the left T-DNA border; while we have not observed such molecules, they might not have been detected if they are heterogeneous in length.

by S_1 nuclease (S_1 border sites), and whose internal sequences have been altered (T-region structures). The specific properties of these AS-induced molecules allows the formulation of mechanistic models for the generation of the T-strand, and for its potential transfer to the plant cell.

Figure 6*A* presents a model for the generation of the T-strand molecule. First, ss endonucleolytic cleavages occur within the left and right T-DNA border sequences on the Ti plasmid. These cleavages correspond to S_1 border sites and provide free 3' OH groups from which DNA synthesis can be primed. Second, using the top strand of the T-region as template, DNA synthesis initiates at the right border cleavage site, and proceeds unidirectionally across the T-region. This synthesis displaces the bottom strand of the T-region and produces a transitory triple-stranded structure which may correspond to one of the AS-induced T-region structures that we have detected. Third, DNA synthesis terminates when it encounters the left border cleavage site, and the displaced bottom strand is released from the Ti plasmid, as the free T-strand ss molecule. Alternatively, a 5'→3' helicase activity unwinds the T-region of the nicked Ti plasmid to free the T-strand molecule and produce a Ti plasmid whose T-region is momentarily ss prior to replacement strand synthesis. Since we observe at most one T-strand molecule per AS-induced cell, T-strand production must be tightly regulated.

The sequences internal to the T-DNA of the wild-type Ti plasmid encode genes whose expression in the transformed plant cell result in the tumorous phenotype, crown gall[1], and when the orientation of the right border on the Ti plasmid is flipped by *in vitro* manipulation), phenotypically transformed plant cells are not obtained[15,16]. The model of Fig. 6*A* explains this functional polarity of the right T-DNA border; that is, the T-strand corresponds to the bottom strand of the T-region and must be generated in a right-to-left (5' to 3') direction. Thus, if the orientation of the right border is reversed in the Ti plasmid, ss molecules will be generated away from (rightward of) the T-DNA tumour genes. This model may also explain how the ds T-DNA circle molecules, recovered in *E. coli* transformed with AS-induced DNA, are generated at a low frequency in response to AS induction. Since nicked DNA stimulates recombination events[28], the S_1 border sites could promote pairing and recombination between the T-DNA border repeats of the AS-induced Ti plasmid (Fig. 6*B*).

Assuming that the T-strand is the transfer intermediate, we can speculate on the mechanism of its transfer. This mechanism might share features with known bacterial processes which mediate the transfer of specific DNA molecules between bacteria, such as phage infection or conjugation. An important distinction between these two processes is that only phage infection involves the synthesis of many copies of the molecule destined for transfer. Since the T-strand is present at about one copy per AS-induced cell, it is unlikely that it is transferred to the plant cell as an infectious phage particle.

In bacterial conjugation, one strand of a ds donor molecule is transferred as a linear ss molecule from the donor to recipient cell[29]. This process is initiated by nicking one strand of the donor molecule at a specific site (designated *ori*T, origin of transfer)[30], and the strand destined for transfer is unwound in a 5'→3' direction. Concomitant to unwinding, the unwound strand is mobilized to the recipient cell, and this transfer is accompanied by DNA synthesis on the donor molecule to replace the mobilized strand. The T-DNA homologous molecules that we have described correspond to the structures which would be predicted to occur if T-DNA transfer occurs through a conjugative mechanism. The S_1 border sites are analogous to nicked *ori*T sites, the T-strand is analogous to the linear ss DNA molecule transferred during bacterial conjugation, and the internal T-region structures are analogous to the replacement strand synthesis intermediates of the donor molecule. Furthermore, bacterial conjugation requires direct contact between donor and recipient cells[31], and the same requirement is observed for the T-DNA transfer process[32].

If the T-strand is transferred by conjugation to the plant cell, it is still not known how it ultimately finds its way into the plant cell nucleus and becomes integrated into the nuclear genome. While the deproteinized T-strand that we have described is a naked linear molecule, presumably it is transferred as a complex that carries proteins which in part mediate the post-transfer events. These proteins, as well as the proteins involved in the generation and transfer of the T-strand, are probably encoded by the plant-inducible Ti-plasmid *vir* loci[10].

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Note added in proof: The nondenatured transfer assay for single-stranded DNAs has also been recently described by Reile, H. T., Michel, B. and Ehrlich, S. D. *Proc. natn Acad. Sci. U.S.A.* 83, 2541–2545 (1986).

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LETTERS TO NATURE

Binary pulsar with a very small mass function

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Much of the interesting physics concerning neutron stars and their evolution depends for its experimental foundation on observations of the rotation rates of pulsars. To continue recent efforts of our group in this area[1–3], we began a series of pulse-arrival-time observations of ~70 pulsars in January 1985. Most of the pulsars in this study were discovered in the Princeton/NRAO pulsar survey of the preceding two years[4,5]. Soon after we began these observations it became clear that PSR2303+46 was a binary pulsar[2]; it is now evident that PSR1831–00 is also a member of a binary system, the seventh such radio pulsar known. It moves in an orbit with a period of 1.81 days, a small eccentricity, and an unusually small mass function of 0.00012 $M_\odot$ (where $M_\odot$ is the mass of the Sun). With a period $P = 0.521$ s and period derivative $\dot{P} \leqslant 10^{-17}$ s s^{-1}, PSR1831–00, like the other known binary pulsars, has a relatively weak magnetic field. We discuss the features of this system that provide clues to its evolutionary history and outline possible models for its formation.

Most of our observations were made with the 92-m transit telescope at Green Bank, West Virginia, at a frequency of 390 MHz. The data acquisition system has been described by Stokes *et al.*[5]. Briefly, a dual-channel parametric up-converter amplifies two orthogonal linear polarizations and provides a system noise temperature of 50 K at high galactic latitudes. In the direction of PSR1831-00 (galactic coordinates $l = 30.8°$, $b = 3.7°$) the system temperature is 180 K, equivalent to a flux density of ~150 Jy. In each polarization an 8-MHz pass-band is divided into 32 sub-channels, each 250 kHz wide; the signals are detected, summed in a de-disperser, and then integrated for two minutes in a signal averager synchronized to the apparent pulsar period. For each pulsar, the resulting profiles are cross-correlated with a standard profile for that pulsar. This procedure determines phase offsets which, when added to reference times near the centre of the integrations, yield effective pulse arrival times.

For PSR1831–00, one to ten arrival times were obtained in this way on each of 19 days in January, February, April, July and November 1985, and February 1986. The number of observations obtainable on a single day is limited by the small hour-angle range through which the 92-m telescope can track (~20 min at the celestial equator), and this in turn limits the accuracy with which one can measure a pulsar's apparent period on a given day. Nevertheless, for virtually all non-binary pulsars it is possible to fit data obtained over two or three days to a single barycentric period and to count pulses unambiguously

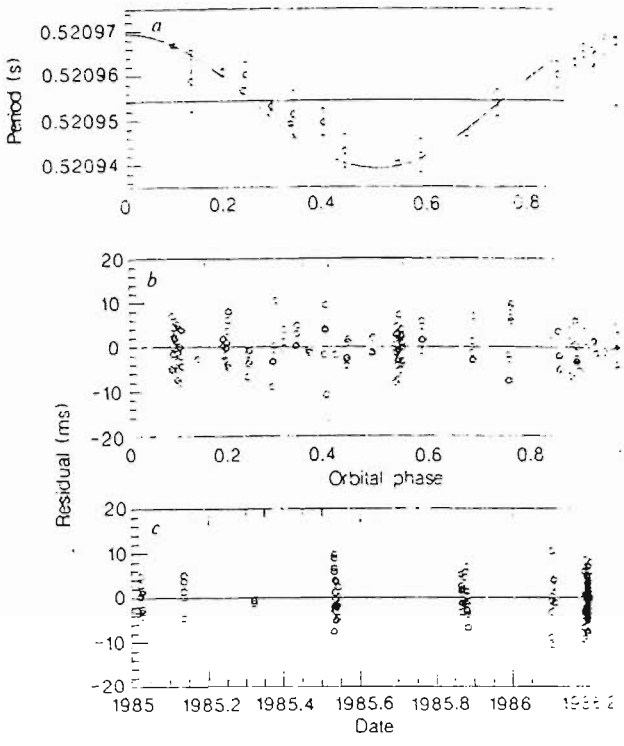

Fig. 1 Pulsar period as a function of orbital phase (*a*), and arrival-time residuals as function of both orbital phase (*b*) and date (*c*). The sinusoid superimposed on the measured periods in *a* corresponds to the orbital parameters listed in Table 1.

from day to day. The data for PSR1831–00 were not amenable to such treatment; it was rarely possible to fit data taken one or two days apart to a single period. However, the day-to-day period changes were small (a few parts in 10^5) and only marginally significant, making the binary nature of this pulsar hard to recognize.

These difficulties were compounded by the weakness of the pulsar's signal (2–5 mJy) and its variability. We did not detect the pulsar in a number of our attempts to observe it, and there is some evidence that it has become weaker in the past year. The variability may be due to refractive interstellar scintillation. Our failures to detect the pulsar show no correlation with orbital phase, so there is no evidence that eclipses are involved.

Redoubled observational efforts in February 1986 showed that the data were consistent with a nearly circular orbit having a 1.81-day period and a maximum radial velocity of ±8.7 km s^{-1}. We were still not able, however, to connect pulsar phases unambiguously between observing days. Consequently, during March 1986 we observed PSR1831–00 at 430 MHz with the Arecibo 305-m telescope. The data acquisition scheme is described in ref. 6. The pulsar is close to the southern declination limit of the Arecibo antenna and can therefore be observed for

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Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*

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We show here that Agrobacterium tumefaciens *virulence* (Vir) *gene expression is activated specifically by the plant molecules acetosyringone (AS) and α-hydroxyacetosyringone (OH-AS). These molecules induce the entire* vir *regulon in* Agrobacterium *as well as the formation of T-DNA intermediate molecules. AS and OH-AS occur specifically in exudates of wounded and metabolically active plant cells and probably allow* Agrobacterium *to recognize susceptible cells in nature.*

SOIL bacteria often form specialized interactions with plant cells. Usually a particular bacterium interacts with only a few species and/or types of plant cells[1,2]. A primary step in the formation of a bacterial/plant interaction is the detection by the bacterium of the appropriate susceptible plant cell. This recognition then triggers the activation of the bacterial genes whose products direct the development and/or maintenance of the interaction. The soil is a complex biological and chemical environment, and the signals that mediate the detection of a specific plant cell by a bacterium are unknown.

A. tumefaciens, a soil phytopathogen, genetically transforms dicotyledonous plant cells to cause the neoplastic disease crown gall[3,4]. Only cells that have been wounded are seen to be susceptible[5,6]. The bacterium transfers a specific segment of DNA, the T-DNA, from its large (>200 kilobases, kb) tumour-inducing (Ti) plasmid into the susceptible plant cell, where it becomes integrated into the nuclear genome[7,8]. In the Ti plasmid, the T-DNA is defined and bounded by identical 25-base pair (bp) direct repeats; only DNA between these T-DNA borders is transferred to the plant genome[9-12]. During co-cultivation with plant cells, independent T-DNA circles are formed in *Agrobacterium*; these molecules arise by a specific recombination between the 25-bp sequences at the ends of the T-DNA and are potential intermediates in the transfer of the T-DNA from *Agrobacterium* to the plant cell[13].

The Ti plasmid genes required for plant transformation are not contained in the T-DNA but are located in the ~40-kb *vir* region[14-16]. Genetic analysis of the *vir* region of the A6 Ti plasmid has shown that it encodes at least six separate complementation groups, *virA*, *B*, *C*, *D*, *E* and *G*, and *pinF* that are organized as a single regulon (ref. 17; S.E.S., in preparation). In the vegetative bacterium only *virA* and *virG*, the *vir* regulatory genes, are significantly expressed; however, when *Agrobacterium* is co-cultivated with plant cells the expression of *virB*, *C*, *D*, *E*, *G* and *pinF* is induced to high levels[17,18]. This activation of *vir* expression by plant cells probably initiates the steps of T-DNA transfer and integration into the plant cell genome. We have shown that *vir* induction and the production of T-DNA circles is mediated by a small deffusible factor produced by actively growing plant cells[17]. Here, we purify and establish the chemical identity of this factor, and demonstrate that its production is related to plant cell wounding and that its recognition by the bacterium is a highly specific process. This factor is likely to be the signal that allows *Agrobacterium* to recognize in nature a plant cell susceptible to transformation.

Purification of signal molecules

To purify the plant molecule(s) that specifically signals *Agrobacterium* to initiate its interaction with plant cells requires first, an efficient and quantitative bioassay for a primary event in this transformation process, specifically the induction of *vir* gene expression; and second, a starting source of the signal molecule(s). We have previously described an assay for *vir* induction in *Agrobacterium* that used gene fusions between the pTiA6 *vir* loci and the *Escherichia coli lacZ* gene[17,18]. Briefly, in a bacterium carrying a *vir*::*lac* gene fusion, the production of β-galactosidase (the *lacZ* gene product) is controlled by the *vir* locus to which *lacZ* has been fused. Thus, the state of expression of the locus can be monitored by measuring the β-galactosidase activity present in the bacterium; increased activity indicates increased *vir* expression. (The relative amount of induced activity reflects the relative amount of *vir*-inducing activity to which the cell has been exposed.) Here we use *Agrobacterium* strain A348(pSM30) (ref. 17) to detect and measure *vir*-inducing activity. This strain contains wild-type pTiA6 and pSM30, a *virB*::*lac* fusion plasmid, and gives high levels (up to ~100-fold of basal activity) of induced β-galactosidase activity[17].

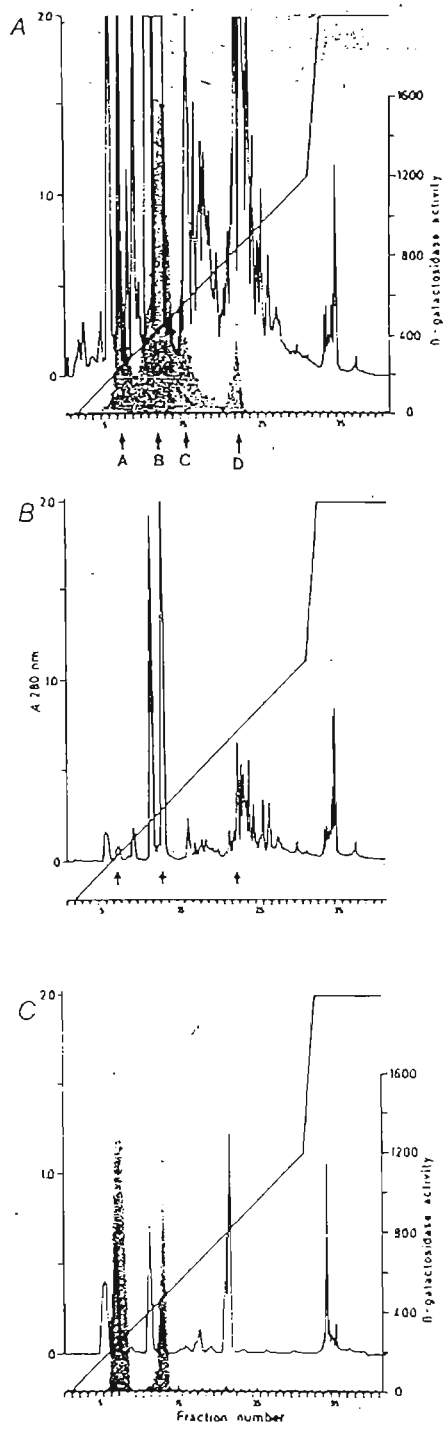


Fig. 1 RPC/FPLC fractionation of the *vir*-inducing activity in plant-cell exudates. The organic compounds present in plant cell medium conditioned with root culture (*cmr*; *A*, *B*), or with leaf disks (*cml*; *C*), were prepared by chloroform extraction and analysed by RPC/FPLC. The material analysed in *A*, *B* and *C* was prepared from 2 litres *cmr*, 40 ml *cmr* and 50 ml *cml*, respectively. Each sample was fractionated on a C-2/C-18 RPC column eluted with a linear methanol gradient. In *A* and *C*, column fractions were bioassayed for *vir*-inducing activity in *Agrobacterium*. The diagonal line represents the elution gradient and the solid line indicates the ultraviolet absorbance measured at 280 nm of the column fractions; shaded curves indicate relative specific units of β-galactosidase activity induced in the *virB*::*lacZ* tester strain A348(pM30) by the column fractions. The major peaks of *vir*-inducing activity in *A* are indicated by arrows labelled A, B, C and D. *B*, Arrows indicate peaks corresponding to peaks A, B and D of *A*.

Methods. Root culture of *N. tabacum* transformed with *Agrobacterium rhizogenes* A15834 was grown and maintained as described previously[17]. Transformed roots were used because they are easy to propagate. Every 72 h the conditioned medium (*cmr*) was removed and stored at −20 °C. Leaf disks were prepared from 6-week-old untransformed *N. tabacum* SR1 plants, and 2-g samples of 1.5-cm-diameter disks were incubated in 50 ml MS medium (Murashige and Skoog[28] salts, 3% sucrose, supplemented with 0.018% K_2HPO_4, 0.01% inositol, 0.0001% biotin, pH 5.5) in 150-mm Petri dishes. After 72 h, the conditioned medium (*cml*) was removed and stored at −20 °C. The conditioned medium (*cmr* and *cml*) was filtered through 0.22 μm nitrocellulose and the filtrate extracted twice with a 25% volume of chloroform and the pooled chloroform phase was back-extracted with 1 vol. MS medium, rotary-evaporated to dryness and resuspended in 500 μl 20% CH_3OH, 0.1% CH_3COOH for analysis by RPC/FPLC. (Note that initial solvent extraction experiments were performed to determine the solvent partitioning character of the *cmr* and *cml vir*-inducing activities. In these experiments 50 ml of the respective conditioned medium was extracted with chloroform; the interphase and chloroform phase were lyophilized and each resuspended in 2 ml MSSP (MS medium supplemented with 12.5 mm sodium phosphate, pH 5.5[17]), and the aqueous phase was blown with a stream of air to remove traces of chloroform. Each of these samples was bioassayed for *vir*-inducing activity; only the chloroform phase material contained this activity.) A Pep RPC pre-packed 5 mm × 50 mm column (HR5/5) containing 6-μm silica particles with C-2 and C-18 alkyl side chains (Pharmacia) was pre-equilibrated with 10% CH_3OH, 0.1% CH_3COOH. The column was run at a flow rate of 0.7 ml min^{-1} using a Pharmacia FPLC system equipped with the LCC500 chromatographic programmer. A single-path ultraviolet monitor was used to monitor absorbance at 280 nm. The column was eluted with a linear gradient of 10–60% $CH_3OH:H_2O$ (v/v), 0.1% CH_3COOH. We collected 40 1.4-ml (2-min) fractions; 140-μl aliquots (*A*) or the entire fractions (*C*) were lyophilized, resuspended in 1.5 ml MSSP and bioassayed for *vir*-inducing activity. Overnight cultures of strain A348(pSM30) were centrifuged and resuspended in MSSP medium. Material to be tested for *vir*-inducing activity was inoculated with bacteria at 0.1 absorbance unit ml^{-1} at 600 nm cm^{-1}. Incubations were for 10 h at 28 °C and 200 r.p.m. Specific units of β-galactosidase activity were determined as described previously[17,18] and are expressed as U per bacterial cell.

Induction of *vir* expression occurs during co-cultivation of *Agrobacterium* with plant cells, and during incubation of bacteria in plant-cell exudates. We have shown that the medium in which *Nicotiana tabacum* root culture has grown (designated *cmr* for conditioned medium roots) contains substantial amounts of a *vir*-inducing activity[17]. This activity stimulates the expression of each of the inducble pTiA6 *vir* loci, and also the formation of T-DNA circular intermediates, indicating that it triggers in *Agrobacterium* the initiation of plant cell transformation. This activity has relative molecluar mass less than 1,000; is stable to boiling, freezing, lyophilization, and high and low pH; and is partially hydrophobic, as it is retained by silica C-18 and completely elutes from this matrix with 40% CH_3OH (ref. 17). Here we purify and identify this *cmr* activity.

The above properties suggest that the *cmr vir*-inducing activity is composed of one or more small organic molecules; that it completely partitions into the organic solvent chloroform (Fig. 1) confirms this identity, and provided a basis for its purification. The *cmr vir*-inducing activity was fractionated by reverse-phase chromatography (RPC) using a high-resolution fast-performance liquid chromatography (FPLC) system. Activity was localized to two major and two minor peaks that had eluted, respectively, with 18 (peak A), 27.5 (peak B), 32 (peak C) and 42% (peak D) CH_3OH (Fig. 1*A*). Thus, the *cmr vir*-inducing activity is represented by at least four distinct compounds. When less material is loaded onto the FPLC column (Fig. 1*B*), peak B is the major component of the organic fraction of *cmr*. Because of their low abundance, the peak C and peak D activities were not analysed further. The signal molecules in peaks A and B were purified to homogeneity by further RPC fractionation and analysed by gas chromatography/mass spectroscopy (GC-MS) (Fig. 2) and ultraviolet absorption spectroscopy (Fig. 3).

Identification of *vir* inducers

The peak B compound, the major *cmr vir*-inducing molecule, is 4-acetyl-2,6-dimethoxyphenol, based on several experimental observations: (1) The GC/MS spectrum of the peak B compound

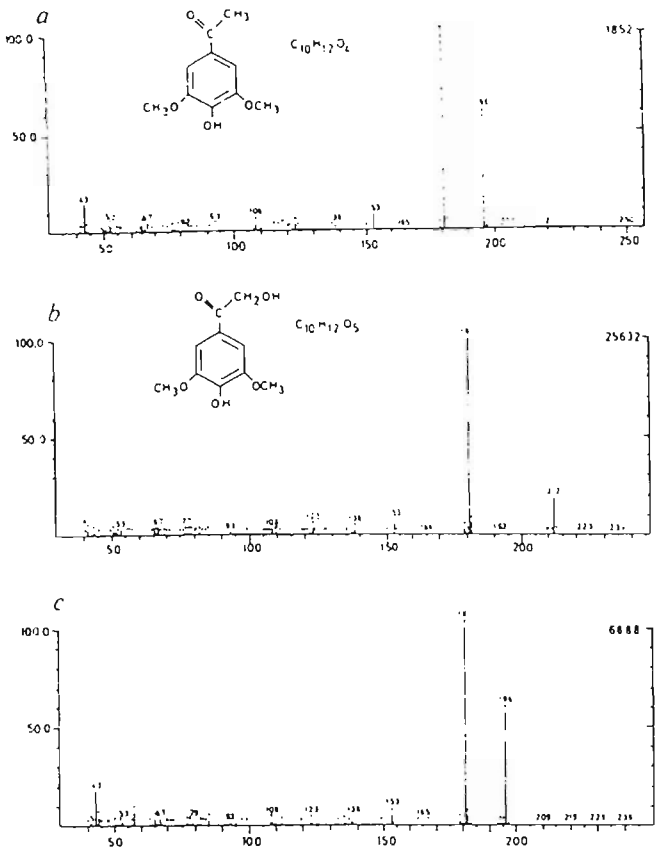


Fig. 2 Mass spectra of *vir*-inducing molecules. The mass spectra of purified peak B compound (Fig. 1), purified peak A compound (Fig. 1) and authentic acetosyringone (Janssen Chimica), are shown in *a*, *b* and *c*, respectively. Vertical axes, relative intensity of the fragment ions; horizontal axes, the mass/charge ratio of the fragment ions. The determined chemical formulae and structures of peak B and peak A (from Fig. 1) are indicated in *a* and *b*, respectively.

Methods. The biologically active compounds present in the peak A and B fractions were purified to homogeneity by RPC/FPLC; fractionation conditions were identical to those described in Fig. 1, except for the column solvent. The fractions containing peak A or peak B (from Fig. 1*A*) were pooled, lyophilized, resuspended in 5% acetonitrile, 0.1% trifluoroacetic acid (TFA) and injected onto the C-2/C-18 column equilibrated with this buffer. The column was eluted with a linear gradient of 5–30% acetonitrile, 0.1% TFA, and monitored for ultraviolet absorbance at 280 nm and for biological activity. For each sample a single peak was resolved from other minor ultraviolet-absorbing compounds, and a portion of the peak fraction was lyophilized, dissolved in 500 μl chloroform and evaporated to 40 μl under a nitrogen stream for GC/MS analysis. Separate portions of each sample were dissolved in CH_3OH for ultraviolet absorption analysis (Fig. 3) and in MSSP for analysis of biological activity. We injected 0.5 μl of sample into a 0.32 mm × 25 m Carlo Erba (HRGC) gas chromatography column SF-52, directly coupled to a Finnigan 4000 mass spectrometer. GC fractionation was with a 50–250° gradient, 5° min^{-1}. Data were collected and processed on a Nova 3 computer (Data General).

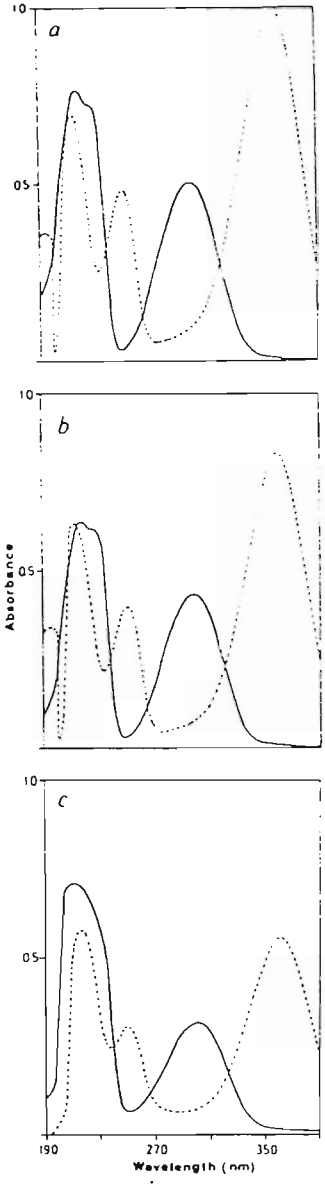


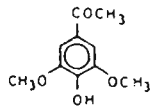
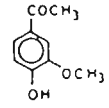
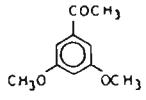
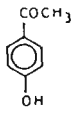
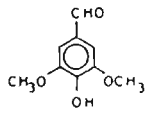
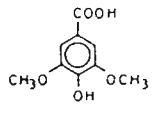
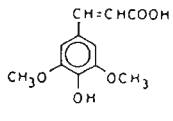
Fig. 3 Ultraviolet absorption spectroscopy of *vir*-inducing compounds purified from *cmr*. Spectra were determined in both CH_3OH (solid line) and CH_3OH/NaOH (dashed line). *a*, Spectra of peak B compound. *b*, spectra of commercially obtained acetosyringone; *c*, spectra of peak A compound.

Methods. Absorption spectra were measured in a Beckman DU-6 spectrophotometer scanning at 60 nm min^{-1}. For each compound dried material was resuspended in CH_3OH (HPLC grade, Rathburn Chemicals) to an approximate concentration of 50 μM, and scanned against CH_3OH as a blank solution. The *p*H of the sample was subsequently adjusted to basic *p*H by the addition of 1 M NaOH to 20 mM and the sample scanned against CH_3OH/20 mM NaOH. (Note that when the base-adjusted sample of each compound is readjusted to acid *p*H by adding 1 M HCl to 40 mM, the determined spectrum is equivalent to the original CH_3OH spectrum of the compound (data not shown). Thus, the base-induced redshift of each compound is fully reversible in the conditions used.)

(Fig. 2*a*) indicates a molecule of relative molecular mass (M_r) 196, chemical composition $C_{10}H_{12}O_4$ and general structure acetyl-dimethoxyphenol. (2) Comparison of the ultraviolet absorption spectrum of the peak B compound in CH_3OH with its spectrum in CH_3OH/NaOH (Fig. 3*a*) indicates that the molecule exhibits a strong absorption shift to longer wavelengths in the presence of NaOH; this base-induced redshift is diagnostic of phenolic compounds in which the phenolic hydroxyl group is *para* (but not *ortho* or *meta*) to a conjugated ring substituent, such as a ketone or allyl group[19]. (3) The *p*-hydroxyl derivative, 4-acetyl-2,6-dimethoxyphenol, commonly termed acetosyringone (AS), is commercially available and greatly stimulates *vir* expression in *Agrobacterium* (see below). AS and the purified peak *B* compound co-elute on RPC/FPLC (data not shown) and their GC/MS spectra are indistinguishable (Fig. 2). Furthermore, their respective ultraviolet spectra, both in CH_3OH and in CH_3OH/NaOH, are identical (Fig. 3*a*, *b*). (4) The quantitative *vir*-inducing activities of authentic AS and the peak *B* compound are equivalent. In the experiment described in Fig. 4, the units of induced β-galactosidase activity as a function of concentration of inducing compound were determined for each compound at several concentrations ; the respective activity/concentration curves are identical.

The peak *A* compound, the second major *cmr vir*-inducer, is 4-(2-hydroxyacetyl)-2,6-dimethoxyphenol, termed α-hydroxy acetosyringone (OH-AS), an analogue of AS. This identification is based on the following observations: (1) The GC/MS spectrum of the peak A compound (Fig. 2*c*) indicates a molecule of M_r 212 whose only significant fragmentation product has M_r 181. The GC/MS spectrum of compound A following trimethylsilyation indicates a mass increase corresponding to two trimethylsilyl groups (data not shown). Thus, compound A contains two hydroxyl groups and has general structure of either (3-propanol)dimethoxyphenol ($C_{11}H_{14}O_4$) or (2-hydroxyacetyl)dimethoxyphenol ($C_{10}H_{12}O_5$). Only the latter structure is consistent with the observation that the peak A compound is more polar than AS, as it elutes before AS on RPC/FPLC

Table 1 Biological activity of derivatives of acetosyringone

a acetosyringone		110	109	93	29
b acetovanillone		80	33	1.9	1.6
c 3,5-dimethoxy acetophenone		ND	1.0	ND	ND
d 4-hydroxy acetophenone		ND	1.1	ND	ND
e syringaldehyde		86	51	2.2	ND
f syringic acid		21	8.6	1.7	ND
g sinapinic acid		104	98	68	18

Acetysyringone (*a*) and six related compounds; acetovanillone (*b*); 3,5-dimethoxy acetophenone (*c*); 4-hydroxyacetophenone (*d*); syringaldehyde (*e*); syringic acid (*f*); and sinapinic acid (*g*), were tested for their ability to induce β-galactosidase in the *Agrobacterium virB*::*lacZ* strain A348(pSM30). All compounds were purchased from Janssen Chemica, and prepared as 0.1 M solutions in dimethyl sulphoxide. Each compound was serially diluted into MSSP (Fig. 1, legend) medium to 200, 50, 5 and 0.5 μM (*a*, *b*, *e*, *f*, *g*) or 50 μM (*c*, *d*). These solutions were inoculated with bacteria at 0.05 absorbance unit ml^{-1} at 600 nm cm^{-1} and incubated at 28 °C, 200 r.p.m. After 12 h, the bacterial β-galactosidase activity in each sample was determined. The data are expressed as activity of the bacteria incubated in the presence of a compound relative to the basal activity present in bacteria incubated without added compound. The basal activity for the pSM30 strain is 10 U. ND, not determined.

Fig. 1). (2) Comparison of the ultraviolet adsorption spectrum of the peak A compound in CH_3OH with its spectrum in CH_3OH/NaOH indicates that, similarly to AS, this molecule exhibits a strong base-induced redshift (Fig. 3*c*). Thus, compound A contains a phenolic hydroxyl group *para* to a conjugated ring substituent. Furthermore, the absorption spectra of compound A are almost identical to the equivalent spectra of AS; the respective absorption maxima occur at identical wavelengths (the largest maxima occur at 298 nm in CH_3OH and 355 nm in CH_3OH/NaOH), although their relative extinction coefficients are different. These similarities strongly indicate that the peak *A* compound is very closely related to AS. (3) The quantitative *vir*-inducing activity of the peak A compound is approximately equivalent to that of AS (Fig. 1). As described below, the structure/activity specificity of *vir*-induction indicates that only molecules with similar structure to AS induce

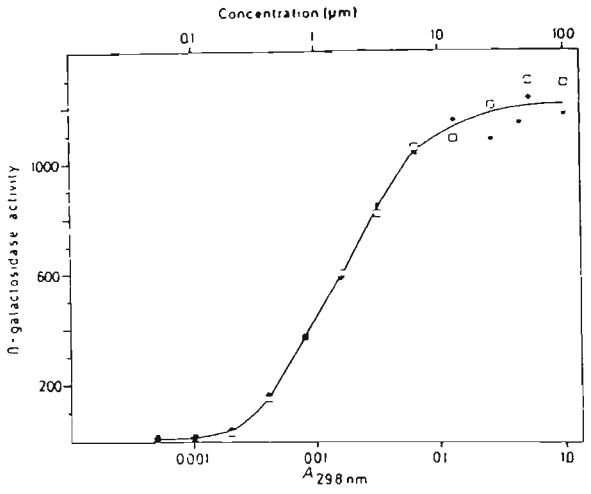


Fig. 4 Comparison of *vir*-inducing activity of purified peak *B* compound (Fig. 2) and commercially obtained acetosyringone. Vertical axis, β-galactosidase activity (U $bacterium^{-1}$) in the *Agrobacterium virB*::*lacZ* strain A348(pSM30); lower horizontal axis, relative concentration of purified peak B compound (Fig. 2) or authentic AS (Janssen Chimica) in the inducing medium, measured as absorbance at 298 nm (cm^{-1}). The extinction coefficient of AS at 298 nm is 10,300 (ref. 29); this value was used to calculate the concentration of inducer compound in the inducing medium, indicated by the upper horizontal axis.
Methods. Purified peak B compound and authentic AS were separately dissolved in MSSP to 1.0 absorbance unit ml^{-1} at 298 nm (cm^{-1}), measured against fresh MSSP. Each solution was serially diluted and the resultant samples were inoculated with bacterium at 0.09 absorbance unit ml^{-1} at 600 nm (cm^{-1}). Samples were incubated for 14 h at 28 °C, 200 r.p.m. and the β-galactosidase activity of the bacteria in each sample determined.

Thus, we have identified plant molecules that induce *vir* expression. Our next experiments seek to provide insight into the relationship between these molecules and *Agrobacterium* in nature. For the *Agrobacterium*/plant transformation system to be most efficient, its activation should be limited to the presence of susceptible plant cells. This could be achieved if activation is signalled only by molecules specific to these cells. For instance, such moleclues should first, be produced by different types of plant cells, as *Agobacterium* can transform several different cell types[20]; second, be specifically synthesized by susceptible plant cells, such as wounded cells; and third, be available to the bacterium in quantities sufficient for efficient activation. The following experiments demonstrate that AS has these properties and that activation of *Agrobacterium* is a highly specific process.

Different plant tissues produce AS/OH-AS

To determine whether AS and OH-AS are specific to roots or whether they are also produced by other plant tissues, we purified and identified the *vir*-inducing activity produced by leaf cells (Fig. 1). Medium in which *N. tabacum* leaves that have been cut into disks have been cultured (*cml*; conditioned medium leaves) contains substantial amounts of inducing activity that completely partitions into chloroform. The RPC/FPLC profile and corresponding bioassay profile of this material (Fig. 1*C*) show that the *cml* activity fractionates into two peaks that co-elute with OH-AS and AS. Subsequent purification and GC/MS and ultraviolet spectrophotometric analysis of the active molecules in these peaks confirmed that the major *vir*-inducing activity in *cml* is composed of OH-AS and AS (data not shown). Thus, these two compounds are present in the exudates of at least two different plant tissues.

AS and OH-AS are exudate-specific

In the soil, *Agrobacterium* probably detects plant cells through their exudates. To determine whether AS and OH-AS are

exudate-specific we assessed their relative concentrations within the *N. tabacum* leaf disks of Fig. 1C. These disks were extracted with chloroform and the extract was analysed by RPC/FPLC fractionation and corresponding bioassay. The relative concentrations of AS and OH-AS are <0.5% of the organic compounds present in the total leaf disks (data not shown). In comparison, the relative concentrations of these compounds in the *cml* extract is ~5% and greater (Figs 1C, 5A). Thus, AS and OH-AS probably do not leak out of damaged plant cells and are exudate-specific compounds.

Concentration of *vir* inducers in *cmr*/*cml*

Figure 4 shows the relationship between the concentration of AS (both commercial and *cmr*-purified) and induction of *virB* expression as measured in units of β-galactosidase activity. Under the conditions used, AS at ≥10 μM, and AS at 1.5 μM, induce maximal (1,200 U) and half-maximal (600 U) expression, respectively. Note that AS induction does not significantly affect cell viability (that is, induction is a non-lethal event), and that concentrations of AS >200 μM are not significantly toxic to the bacteria. Using the dose-response curve of Fig. 4, the concentration of *vir*-inducing compounds in plant-cell exudates relative to AS can be estimated. Our starting *cmr* and *cml* preparations typically induce between 250 and 500 U β-galactosidase activity in A348 (pSM30); this activity approximately corresponds to between 0.5 and 1 μM AS.

Biological activity of acetyosyringone

The primary molecular signal for the initiation in *Agobacterium* of the events of plant cell transformation should induce in the bacterium both the entire *vir* regulon and the initial steps of T-DNA transfer. We determined that AS has these activities. Several *vir*::*lac* and *pin*::*lac* *Agrobacterium* strains were incubated in 20 μM AS (both commerical and *cmr*-purified) and assayed for β-galactosidase activity. Inductions of all the previously identified inducible *vir* loci (*B*, *C*, *D*, *E*, *G*) and *pinF* was obtained, and the levels of induction were at least 20–50% higher than those stimulated during co-cultivation with plant cell cultures or incubation with *cmr*[17] (data not shown). AS also induces the production of T-DNA intermediates: when bacteria are incubated with 20 μM AS for 12–18 h, T-DNA intermediates[13] are found at a frequency two- to fivefold greater than that obtained following 48-h co-cultivation with protoplasts[17] (data not shown). We have also seen that AS induces the appearance and/or disappearance of several major proteins in *Agrobacterium* (P. Engström, P.Z., and S.E.S., in preparation). Thus, a single compound is sufficient to trigger the complete activation of *vir* and the initial events of T-DNA transfer; and AS (probably OH-AS also) is a primary signal for plant cell transformation by *Agrobacterium*.

Molecular specificity of *vir* induction

Table 1 shows the respective *vir*-inducing activities of AS (*a*) and analogues of AS (*b*–*g*). These results indicate that *Agrobacterium vir* expression is efficiently induced by molecules that conform to a structure best represented by AS itself, and suggest that *Agrobacterium* has evolved to recognize and respond to AS as a specific signal for the activation of plant cell transformation. For instance, AS without one methoxy group (*b*) has greatly attenuated *vir*-inducing activity, whereas AS without both methoxy groups (*d*) or its hydroxyl group (*c*) is inactive. The acetyl substituent of AS is important for activity. The formyl (*e*) and carboxylic acid (*f*) analogues of AS have attenuated activity, whereas the cinnamic acid analogue of AS, sinapinic acid (*g*), has approximately equivalent activity to AS. This latter result is interesting in that sinapinic acid is a precusor of lignin, an integral cell-wall constituent of all vascular plants (see below).

Wounding stimulates AS/OH-AS production

Although many dicotyledonous plant cells can be transformed by *Agrobacterium*, only cells that have been wounded or traumatized are susceptible. Therefore, we tested whether the production of AS and OH-AS is stimulated by plant cell wounding. Figure 5 shows the RPC/FPLC profiles of the organic extracts of *cml* produced by equivalent amounts of *N. tabacum* leaf disks (wounded cells; Fig. 5*a*) or intact leaves (unwounded; Fig. 5*b*). Comparison of these profiles indicates that the 'wounded' *cml* contains >10-fold more AS and OH-AS than the 'unwounded' *cml*, demonstrating that wounding stimulates the appearance of these molecules in the cell exudate. The low levels of AS and OH-AS in the 'unwounded' leaf exudate could be caused by the cut stem surfaces of the leaves.

These results do not define which cells of the wounded tissue produce AS and OH-AS. For example, damaged or dead cells could release AS, although such cells are not good targets

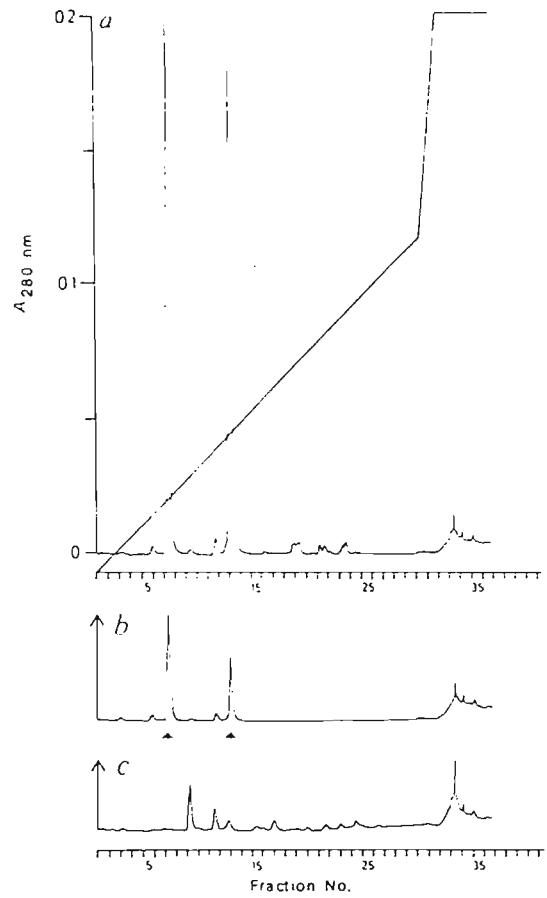


Fig. 5 Effect of wounding and inhibition of plant cell metabolism on production of AS and OH-AS by plant cells. RPC/FPLC fractionations of equivalent amounts of medium conditioned with: *a*, 1.5-cm-diameter leaf disks; *b*, intact leaves; *c*, leaf disks in the presence of cycloheximide. The vertical axes are drawn to the same scale in *a*–*c*. Arrows, elution positions of OH-AS (fraction 7), and AS (fraction 13).

Methods. Leaves (4 g) excised from 6-week-old *N. tabacum* SR1 plants were treated as described below and incubated in 50 ml MS medium in a 150-ml Petri dish. Care was taken to use visually equivalent leaves in each experiment. *a*, 5–6 1-cm^2 disks were obtained per leaf; *b*, intact leaves were used; *c*, same as *a* but with 5 p.p.m. (12.8 μM) cycloheximide (Actidion, 99%; Aldrich Chemicals) added to the medium. After a 72-h incubation, 40 ml of each respective conditioned medium was recovered, filtered through 0.22 μm nitrocellulose and 35 ml extracted with chloroform (Fig. 1), and the organic pellet was resuspended in 400 μl 10% CH_3OH, 0.1% CH_3COOH. We analysed 100 μl of the sample by RPC/FPLC using the conditions described in Fig. 1. The remaining 5 ml of conditioned medium was bioassayed for *vir*-inducing activity; the units of β-galactosidase activity induced by the conditioned media of *a*, *b* and *c* were 565, 135 and 20, respectively.

Agrobacterium infection. We tested whether active plant cell metabolism is required for the production of AS by analysing the *cml* produced by leaf disks incubated in cycloheximide. This material does not contain AS and OH-AS (Fig. 5c). These results confirm and extend our previous observation that in plant/*Agrobacterium* co-cultivations only actively growing plant cell cultures are able to stimulate efficient *vir* gene expression[17]. Our data concerning the production of AS and OH-AS in total exudates suggest that these compounds are specifically synthesized and exuded by metabolically active wounded cells.

Discussion

The molecules that we have purified signal *Agrobacterium* to activate the expression of its virulence genes, setting in motion a series of molecular events ultimately resulting in the transfer of T-DNA from the bacterium to the plant cell.

Wounded cells are known to be susceptible to *Agrobacterium* infection, perhaps because the intact cell walls of undamaged cells restrict T-DNA transfer, or because T-DNA integration is dependent on host-cell replication and wounding stimulates this replication. Because *Agrobacterium* responds to AS and OH-AS to initiate plant cell transformation, these molecules potentially represent the signal that *Agrobacterium* detects and recognizes in the soil as susceptible plant cells. The presence of AS and OH-AS specifically in the exudates of wounded but actively metabolizing plant tissues supports our hypothesis.

The activation of *Agrobacterium vir* expression by plant signal molecules probably involves at least two steps: extracellular recognition and intracellular response. The first step could depend on the signal molecule acting as a chemical attractant and/or nutritive source for the bacterium. The latter must depend on the ability of the bacterium to convey the information of the signal from outside to inside the cell and to activate *vir* expression. The mechanism of these events is unknown. Induction of the pTiA6 *vir* region is attentuated in *virA* and does not occur in *virG* mutant bacteria (S.E.S., in preparation). Also, the amino-acid sequence of the *virG* gene product is highly homologous with several positive regulatory proteins of *E. coli*[21]. Because AS can cause the induction of the complete *vir* regulon, we suggest that this compound acts to activate allosterically the *virG* protein, which then activates *vir* transcription by directly interacting with *vir* gene promoter sequences. The *virA* protein potentially functions in the initial extracellular/intracellular recognition and/or intracellular transport of the signal molecule.

AS and OH-AS have not previously been identified as natural component of plant cells, suggesting that the appearance of these molecules in nature is not widespread. Thus, these molecules potentially represent to *Agrobacterium* only those cells which are its desired targets. In addition, these or related molecules might also serve to initiate other bacterial/plant interactions in the soil. The observed resistance of most monocotyledonous plants to *Agrobacterium* could result because these plants do not produce, or only produce in low quantities, *vir* signal molecules. AS and OH-AS could be useful for obtaining *Agrobacterium* transformation of plant species previously seen to be resistant to *Agrobacterium*, and also for the analysis of the initial molecular steps of the T-DNA transfer process.

Although it is important for *Agrobacterium* to detect and respond to such molecules, it is equally important that it does not respond to closely related but functionally different molecules; we have shown that *vir* induction is most efficiently stimulated by AS and by the lignin precursor sinapinic acid. This latter compound is not present in detectable quantities in our exudate preparations; however, sinapinic acid could be present in the soil in exudates of wounded cells in the process of cell-wall rebuilding.

It is interesting to speculate on the function of AS and OH-AS in plants. These compounds are likely products of the shikimic acid biosynthetic pathway that provides the plant cell with the precursors to a broad spectrum of molecules, including the flavonoids, and lignins[22–24]. These classes of compounds are important to a plant subjected to stress or injury. For example, many flavonoid-derived phytoalexins are potent inhibitors of the growth of invading pathogens[25], whereas lignin, a major component of the cell wall, provides a physical barrier to invasion[26]. Thus, AS and OH-AS could be part of the wound response[27] of plant cells. These compounds are potentially toxic to other soil pathogens, and *Agrobacterium* has evolved to be resistant to AS and OH-AS and to use these chemicals to recognize wounded cells. Alternatively, the compounds could be products related to lignin repair in damaged cells.

We propose that there is a continual excretion of wound-related phenolics during growth, caused by abrasion from the soil. *Agrobacterium* may be attracted to plants by recognition of these compounds. However, significant levels of *vir* induction and the events of T-DNA transfer will only occur if signal molecules are present in sufficient concentrations. As the highest concentrations are found at wound sites, *A. tumefaciens* effectively infects only these sites.

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Halford and Boone County with Harrow Blood whose respective NADs were 0.19 and 0.22, implied a high degree of genetic relatedness between each pair of genotypes. Okinawa and Yunnan, both introduced from eastern Asia, lied in a close group (NAD = 0.63) and share relatively similar phenotypes. The first major bifurcation in the dendrogram divided the 20 rootstocks into two groups. One group (Lovell, Halford, Montclar, Bailey, Harrow Blood, Boone County, Tennessee Natural, and GF 305) is susceptible to root-knot nematodes, whereas the second group (BY520-9, BY520-8, SL1089, SL1090, Higama, Nemaguard, Flordaguard, Yunnan, Okinawa, and Nemared), with the exception of Rubira and Siberian C, is tolerant or resistant.

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Mutagenesis and Tissue Culture for Selecting *Phytopthora cactorum*-resistant Strawberry (*Fragaria xananassa*) Plants

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The aim of this study was to isolate *Phytopthora cactorum*-resistant strawberry plants, regenerated from gamma-irradiated explants on a shoot regeneration medium. Three gamma doses (0, 5, 10, 15 krad) were used to irradiate strawberry axillary buds taken from in vitro-grown plants. After irradiation, axillary buds were cultured on a shoot regeneration medium containing 0.75 mg BA/liter and 0.4 mg IBA/liter. Shoot regeneration occurred mainly from axillary buds irradiated with 5 and 10 krad. The highest dose (15 krad) produced few shoots. The shoot regeneration rate was highest at the 50-krad dose. All the regenerated plants were transferred in the greenhouse. The crude extract of *P. cactorum*, isolated from the strawberry field, was prepared in sterile water; 1 ml of it was put directly in the center of the crown of each of 400 regenerated plants. After 2 weeks, leaves of most of the plants wilted. Only 20 plants survived the first round of selection; they grew slowly when compared with the control and also showed some tolerance to drought. Further investigations are in progress to reconfirm the resistance of selected putative disease-resistant strawberry plants.

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Monitoring Prunus Necrotic Ringspot Virus Infection by Hybridization with a CRNA Probe following in Vitro Grafting

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In vitro micrografting was tested as a technique for inoculating peach [*Prunus persica* (L.) Batsch] with prunus necrotic ringspot virus (PNRSV). Cultured 'Suncrest' shoots derived from a naturally infected tree (as indicated by ELISA testing) maintained virus in vitro, with virus concentrations in growing tips and folded leaves being several times those of fully expanded leaves. The infected shoots served as graft bases and the source of virus. Grafted tips were derived from 'Suncrest' trees that had tested negative for the virus. Leaf samples were collected from the tips following grafting and analyzed for the presence of virus by slot-blot hybridization with a digoxigenin-labeled cRNA probed derived from PNRSV RNA 3. Rates of successful grafting were 55% and 73% in three trials and PNRSV was found in all tips analyzed. Virus concentrations approximated those found in the source shoots, suggesting that this method should be useful for screening transformed peach shoots for coat protein-mediated resistance to PNRSV.

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An Evaluation of Antibiotics for the Elimination of *Agrobacterium tumefaciens* from Apple Leaf Explants in Vitro and for the Effect on Regeneration

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A range of antibiotics was evaluated for their effect on eliminating *Agrobacterium tumefaciens* supervirulent strain EHA101(pEHA101) from leaf explants of 'Royal Gala' apple (*Malus domestica* Borkh) and on regeneration. After long-term (38 days) exposure to 100-$\mu g \cdot ml^{-1}$ concentrations of either cefotaxime (cef), carbenicillin (carb), mefoxin (mef), or combinations of these antibiotics, only on carb or carb with mef was regeneration not inhibited. None of the above antibiotics or antibiotic combinations eliminated *A. tumefaciens* from leaf explants. Short-term (1-18 hours), vacuum infiltration with 500- to 1000-$\mu g \cdot ml^{-1}$ concentrations of either of the above antibiotics did not inhibit regeneration, but did not eliminate *A. tumefaciens* from leaf explants. After a 30-min vacuum infiltration with a 2000-$\mu g \cdot ml^{-1}$ concentration of either cef, carb, or mef, only cef reduced the number of leaf explants with *A. tumefaciens*.

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Essential Oils of *Boronia megastigma*—Selection of Improved Genotypes

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Boronia megastigma is cultivated or picked from natural stands in Western Australia for the production of essential oil. Boronia absolute is extracted from the highly perfumed flowers. It is currently valued at between US$4000 and US$7000 per kilogram, and world consumption for perfumery is about 1 tonne. The variation in essential oil composition within and between populations has indicated considerable variation in oil components. Some individuals have high β-ionone and low levels of pinenes. Principle components analysis indicated that the content of β-ionone and dodecyl acetate were tightly linked, as were the monoterpenes, α-pinene, β-pinene, and, to a lesser extent, limonene. Separate linkages between the desirable oil components (β-ionone and dodecyl acetate) and the undesirable components (α-pinene, β-pinene, and limonene) will facilitate selection of plants to be used in oil production.

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Effect of Irradiance Level and Iron Chelate Source on the Shoot-tip Culture of *Carica papaya* L.

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Interaction between irradiance levels (5–40 $mM \cdot m^{-2} \cdot s^{-1}$) and iron chelate sources ($FeNa_2EDTA$ and FeNaDTPA) on the establishment, growth, and proliferation of shoot tips of *Carica papaya* were tested. Reduced irradiance level (5 $mM \cdot m^{-2} \cdot s^{-1}$) enhanced the establishment of shoot tips regardless of the source of iron chelate tested. At higher irradiance levels (30 and 40 $mM \cdot m^{-2} \cdot s^{-1}$), presence of FeNaDTPA in the medium enhanced establishment of shoot tips. Continuous or alternating light/dark (16/8 h) photoperiods at high irradiance levels had no effect on the establishment or growth of the culture. At higher irradiance levels, the cultures produced smaller leaves as compared to lower irradiance levels. Low irradiance and $FeNa_2EDTA$ was preferred during the proliferation stage.

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Transformation of Grape (*Vitis vinifera* L.)

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Transgenic grapevines were regenerated from somatic embryos produced from immature zygotic embryos of two seedless grape selections and from leaves of in vitro-grown plants of 'Thompson Seedless'. Somatic embryos were bombarded with gold microparticles using the Biolistic PDS-1000/He device (Bio-Rad Labs) and then exposed to engineered *A. tumefaciens* EHA101 (E. Hood, WSU). Alternately, somatic embryos were exposed to *A. tumefaciens* without bombardment. Following cocultivation, secondary embryos multiplied on Emershad and Ramming proliferation medium under kan selection. Transgenic embryos were identified after 3 to 5 months and developed into rooted plants on woody plant medium with 1 mM N6-benzyladenine, 1.5% sucrose, and 0.3% activated charcoal. Seedless selections were transformed with plasmids pGA482GG (J. Slightom, Upjohn) and pCGN7314 (Calgene), which carry GUS and NPTII genes. 'Thompson Seedless' was transformed with pGA482GG and pGA482GG/TomRSVcp-15 (D. Gonsalves, Cornell Univ.) containing the tomato ringspot virus coat protein gene. Integration of foreign genes into grapevines was verified by growth on kan, GUS, and PCR assays, and Southern analyses.

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Somatic Embryogenesis in Muscadine Grape

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Low frequency of in vitro regeneration has hampered the adoption of genetic engineering technique for improving the quality of muscadine grape. This study is to develop a straightforward method for high-frequency regeneration of muscadine grapes in vitro. Leaves, petioles, and immature ovules of muscadine grapes were cultured on various media. Embryogenic callus, somatic embryos were formed after 9 weeks inoculated on embryo rescue (ER) medium. The somatic embryos were isolated and subcultured on fresh medium to promote enlargement and in-

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REVIEW

The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights

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This review is dedicated to Jeff Schell, one of the founders of modern 'Agrobiology', the genetic and molecular dissection of crown gall disease. Together with notable scientists at the University of Gent, Belgium, Jeff spearheaded the discovery of the Ti-plasmid. The elusive 'tumor inducing principle' was uncloaked and provided impetus for an incredibly fruitful subsequent 25 years of analyses. Scientists all over the world were caught up in unraveling the underlying mechanisms of *Agrobacterium*-mediated gene transfer to plants, and along the way uncovered a movable feast of fundamental insights. Below we summarize a sampling of *Agrobacterium's* most recently recognized accomplishments.

Introduction

The soil phytopathogen *Agrobacterium tumefaciens* has been extensively studied since 1907, when as *Bacterium tumefaciens*, it was identified as the causative agent of crown gall disease (Smith and Townsend, 1907). This disease is characterized by the tumorous growth of plant tissues in the stem, and is a significant problem in the cultivation of grape vines, stone fruit and nut trees. The first indication of the cellular or biochemical mechanisms involved in tumorigenesis coincided with the discovery of the plant growth regulator auxin. *Agrobacterium*-induced tumors were shown to be sources of auxin (Link and Eggers, 1941), and capable of growth in culture in the absence of both bacteria as well as the complement of plant growth regulators normally required to incite growth of callus from sterile plant tissues (White and Braun, 1941). Cytokinin was identified as a plant growth regulator in 1955 and shortly thereafter was strongly implicated in the growth of *Agrobacterium*-induced tumors (Braun, 1958). Braun first proposed that *Agrobacterium* was the source of a 'tumor inducing principle', possibly DNA, that permanently transformed plant cells from a state of quiescence to active cell division (Braun, 1947; Braun and Mandle, 1948). The transforming principle, however, remained elusive. With the advent of molecular techniques came the first evidence that crown gall tumors, cultured axenically, contained DNA of bacterial origin (Schilperoort *et al.*, 1967), although this conclusion was debated. Identification of the tumor-inducing (Ti) plasmid (Van Larebeke *et al.*, 1974; Van Larebeke *et al.*, 1975; Zaenen *et al.*, 1974) narrowed the search to genetic elements derived from this plasmid and ultimately resulted in the discovery of T-DNA (transferred DNA), a specific segment transferred to plant cells (Chilton *et al.*, 1977; Chilton *et al.*, 1978; Depicker *et al.*, 1978). Braun (1982) provides an interesting historical review of early work on *Agrobacterium tumefaciens*, notably studies conducted prior to the advent of molecular techniques.

Presumably, elicitation of tumors provides *Agrobacterium tumefaciens* with some advantage. This advantage derives from the production by tumors of unusual amino acid-like compounds called opines (reviewed in Dessaux *et al.*, 1993). Although opines are structurally diverse, a tumor produces only certain opines that are strictly dependent on the infecting strain, and the opines produced by a gall are specifically catabolized by that strain of *Agrobacterium* (Goldman *et al.*, 1968; Petit *et al.*, 1970). Furthermore, the ability to metabolize opines is tightly correlated with virulence; loss of virulence is always accompanied by the loss of the ability to degrade a specific opine (Petit and Tourneur, 1972). Before the identification of the Ti-plasmid, these observations were the first indication that tumorigenesis involved the transfer of genetic material from bacteria to plants. It is now known that the enzymes for catabolism of specific opines are encoded on the Ti-plasmid and complement the opine biosynthetic pathways encoded on the T-DNA. Thus, by the introduction of genetic material into plant cells, 'genetic colonization' (Schell *et al.*, 1979), *Agrobacterium tumefaciens* creates a unique habitat wherein it solely is genetically equipped to utilize the predominant carbon-nitrogen source.

Overview of *Agrobacterium*-mediated gene transfer

The tumorous transformation of plants by *Agrobacterium* results from the stimulation of plant cell division by gene products encoded by a segment of DNA (T-DNA) trans-

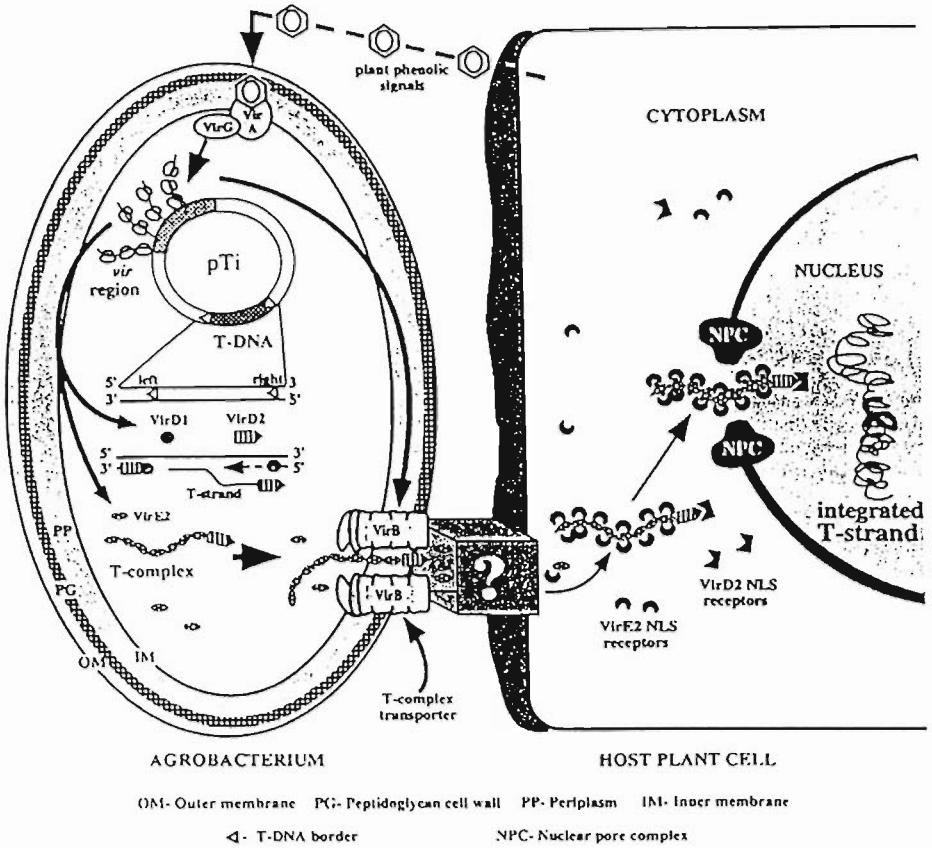

Figure 1. Basic steps in the transformation of plant cells by *Agrobacterium tumefaciens* (see text for details). Adapted from Sheng and Citovsky (1996).

ferred from the bacterium to the plant (Figure 1). The T-DNA and the virulence (*vir*) region, whose products generate the transfer intermediate (T-complex) and mediate its transport, are located on the tumor-inducing plasmid (pTi). The expression of genes in the *vir* region is induced by the exudate from wounded plants. Phenolics, such as acetosyringone, are the most potent inducers found in wound exudate, but sugars and acidic pH amplify the response. The T-complex comprises a single strand (ss) copy of the T-DNA (T-strand) with a single molecule of the Vir protein VirD2 covalently bound to the 5′ end, and coated along its length with the ssDNA binding protein, VirE2. The *vir* system of *Agrobacterium* will process and transfer any DNA between the flanking 25 bp direct repeats (right and left borders) that delimit the T-DNA; hence, the utility of *Agrobacterium* for the genetic engineering of plants. A *vir*-specific apparatus, the T-complex transporter, mediates transfer of the T-complex from the bacterium to the plant cell and is assembled from 12 membrane-associated, *vir*-specific proteins. Inside the plant cell, the T-complex is imported into the nucleus where the T-strand becomes stably integrated into a plant chromosome (reviewed in Christie, 1997; Sheng and Citovsky, 1996; Zambryski, 1997).

Fundamental insights into biological processes

Throughout its study, *Agrobacterium tumefaciens* has both spurred and benefited from advances in numerous biological processes. Table 1 highlights 12 fundamental insights derived from the analysis of the interaction between *Agrobacterium* and susceptible plant cells; selected references are listed in Table 1. To provide focus to the present review, we discuss T-DNA transfer in particular (Table 1, items 7–12). The first section focuses on analysis of the T-DNA element and attempts to place its processing and transport in a broader context. The second half of the review highlights current research on *Agrobacterium* (Table 1, item 12) and again illustrates its continued utility as a model experimental system.

Interkingdom 'conjugal' DNA transfer (Table 1, item 7)

Discovery of the T-strand directly provoked the hypothesis that the transfer of DNA from *Agrobacterium* to the plant cell is not mechanistically unique, but might be evolutionarily related to bacterial conjugation (Stachel and Zambryski, 1986a; Stachel et al., 1986b). Comparison of the synthesis of conjugal DNA transfer intermediates and

Table 1. Insights into fundamental biological processes derived from research on *Agrobacterium tumefaciens*

Insights*
1. **Communication** between microbes and plants in the soil environment: Identification of plant phenolics as *vir* inducers (Stachel *et al.*, 1985; Stachel *et al.*, 1986a).
2. VirA/VirG bacterial **two-component system** for transcriptional regulation (Stachel and Zambryski, 1986b; Winans *et al.*, 1986).
3. **Plant promoters:** Characterization of nopaline and octopine synthase promoters as two of the first plant promoters (De Greve *et al.*, 1982; Depicker *et al.*, 1982).
4. Novel pathways for **plant hormone** synthesis: T-DNA encoded genes for auxin and cytokinin biosynthesis responsible for tumor phenotype (Akiyoshi *et al.*, 1984; Barry *et al.*, 1984; Buchmann *et al.*, 1985; Inze *et al.*, 1984; Schröder *et al.*, 1984; Thomashow *et al.*, 1984).
5. **Quorum sensing** for transcriptional regulation of conjugation: Opines stimulate conjugation of Ti-plasmid when cell density rises above some threshold (Fuqua and Winans, 1994; Fuqua *et al.*, 1994; Piper *et al.*, 1993; Zhang *et al.*, 1993).
6. Biotechnology: **Vectors** for plant transformation (Bevan, 1984; Hoekema *et al.*, 1983; Zambryski *et al.*, 1983).
7. **Interkingdom 'conjugal' DNA transfer:** Identification of T-strand transfer intermediate.
8. Evolutionary conservation of *nic* **sites** (T-DNA borders as sites of initiation and termination for T-strand production) and **nicking enzymes** (VirD1 and VirD2 together produce ss nicks in T-DNA borders).
9. Trafficking of nucleic acids: VirE2 is a **single strand nucleic acid binding protein** (SSB).
10. **Plant nuclear localization signals:** VirD2 (and subsequently VirE2) reveal sequence requirements for plant cell nuclear import.
11. **Non-homologous recombination:** Analysis of T-DNA insertion into plant DNA.
12. **Type IV secretion systems:** The VirB T-complex transporter paradigm.

*Selected primary references for insights 1–6 (as these are not discussed in the text); see text for references related to insights 7–12.

the T-strand revealed extensive similarities. First, short nucleotide sequences required in *cis* are functionally polar in directing DNA transfer. Second, transfer is initiated at ss nicks in these motifs by sequence and strand-specific relaxases. Single-stranded, linear DNA is transferred from donor to recipient following its displacement from the plasmid. This evolutionary link was further supported by the discovery that transfer from donor to recipient of T-complex and conjugal DNA of several incompatibility (Inc) groups is mediated by an apparatus assembled from very similar proteins, encoded by operons conserved among several conjugation as well as protein export systems, designated type IV secretion systems (Christie, 1997; Lessl and Lanka, 1994; Winans et al., 1996).

Prior to defining the ss nicking reaction that initiates T-DNA transfer (below), whether the T-DNA is transferred in an ss or ds form was much debated (reviewed in Zambryski, 1992). The argument was put to rest by two very different strategies that assayed the nature of the T-DNA copy upon arrival in the plant cell. Yusibov *et al.* (1994) detected a PCR-amplified, T-DNA homologous segment within hours after *Agrobacterium* infection of tobacco protoplasts; if the plant cytoplasmic fraction first was first treated with an ss-specific nuclease, the T-DNA signal was lost. Secondly, a sensitive extrachromosomal recombination assay was employed (Tinland *et al.*, 1994), in which *in planta* recombination of the T-DNA was required to yield a full length copy of the reporter gene *uidA* (β-glucuronidase) from two overlapping coding fragments separated by an insertion. While recombination would produce an intact gene regardless of fragment polarity if the transfer intermediate is ds, a complete *uidA* can be obtained through recombination only from segments of opposite polarity if the transfer intermediate is ss. β-glucuronidase activity in infected protoplasts was an order of magnitude greater from the T-DNA bearing *uidA* segments of opposite polarity relative to segments of the same polarity. Thus, both studies provide strong confirmation for an ss transfer intermediate.

Nic *sites and nicking enzymes (Table 1, item 8)*

In type IV secretion systems that transfer DNA, synthesis of the transfer intermediate is initiated by strand-specific nicks in particular sequences, *oriT* for conjugation and T-DNA right border for T-strand (Stachel *et al.*, 1986b; Wang *et al.*, 1987). Four groups of transfer origins can be distinguished by sequence analysis (reviewed in Pansegrau and Lanka, 1996). The largest group is IncP, which includes the origins of transfer of all IncP plasmids, T-DNA borders, transfer origins from conjugative transposons, vegetative replication origins of plasmids from Gram-positive bacteria, and replication origins from ss bacteriophages. The origin for conjugal transfer of the entire Ti-plasmid between agrobacteria belongs to the IncQ group. Differences among groups in conserved nucleotides at the *nic* site indicate that the DNA:protein interactions required for substrate recognition may vary. The nicking reactions, however, probably proceed by a similar molecular mechanism, as all form a covalent bond between the cleaving enzyme and the nucleotide on the 5′ side of the *nic*. Thus, processing of nucleic acid inter-

mediates for transfer to a recipient probably originated with the evolution of specific sequence substrates for ss nicking.

During nicking, a relaxase breaks the phosphate bond between a specific pair of nucleotides in one strand of the *nic* site. In most systems, specificity for binding of the relaxase to the *nic* site is provided by an auxiliary protein which recruits the relaxase to the *nic* site (reviewed in Pansegrau and Lanka, 1996). In others, however, *nic* site recognition and cleavage functions are combined in a single protein (Pansegrau and Lanka, 1996). In *Agrobacterium*, VirD1 likely first recognizes and binds the T-DNA border to promote binding of VirD2 relaxase (Lessl and Lanka, 1994; Pansegrau and Lanka, 1996). The complex of supercoiled plasmid and nicking proteins for bacterial–bacterial conjugation systems, termed the relaxosome, is present throughout the cell cycle. In the absence of contact with a suitable recipient, the reaction equilibrium of the relaxosome does not favor the cleavage reaction. Once a recipient is physically contacted, a signal is transmitted to the relaxosome that alters the equilibrium to initiate cleavage, followed by synthesis of a transfer intermediate. *Agrobacterium* employs transcriptional regulation of VirD1 and VirD2 to ensure that a pTi-relaxosome (Filichkin and Gelvin, 1993) is assembled and active only in the presence of a plant susceptible to infection. Generally, homologies among relaxases parallel homologies of the transfer origins.

In vivo in *Agrobacterium*, VirD1/D2 are both required for the nicking reaction (Scheiffele *et al.*, 1995; Stachel *et al.*, 1987; Yanofsky *et al.*, 1986). *In vitro*, VirD2 alone nicks ss oligonucleotides bearing the T-DNA border sequence (Jasper *et al.*, 1994; Pansegrau *et al.*, 1993a). In the presence of an excess of cleavage products, VirD2 can also catalyze the reverse reaction, joining two pieces of ssDNA (Pansegrau *et al.*, 1993a). Notably, VirD2 catalyzes the cleavage of the IncP *oriT* in an ss oligonucleotide, but TraI, the IncP homolog of VirD2, cannot cleave a ss T-DNA right border (Pansegrau *et al.*, 1993a). The ability to recognize heterologous *nic* sites may reflect a role for VirD2 in initiating the integration of the T-strand to the 3′ side of an ss nick in plant DNA. VirD2 may have evolved to tolerate more variability in the sequences to which it will bind to facilitate T-strand ligation into non-homologous DNA. When *nic* sites are presented in supercoiled double-stranded (ds) plasmids, VirD1 is essential for VirD2 nicking. The VirD1/D2 complex is unable to cleave its cognate *nic* site presented in a relaxed ds circle or a linear double strand.

IncP relaxases have three conserved motifs at their N-termini (Ilyina and Koonin, 1992; Pansegrau *et al.*, 1994). Motif I contains a tyrosine that forms a phosphodiester bond between its aromatic hydroxyl group and the 5′ phosphoryl group of the DNA during cleavage (Pansegrau *et al.*, 1993b). This tyrosine, at position 29 in VirD2 and position 22 in TraI, cannot be altered without abolishing nicking activity (Vogel and Das, 1992). Motif III is the most highly conserved, and contains two histidines necessary for cleavage that may activate the tyrosine by co-ordinate binding of $Mg2+$ (Vogel *et al.*, 1995). Motif II may recognize a sequence 3′ to the *nic*. Critical amino acids for these functions have been identified in TraI by mutagenesis, and are predicted in VirD2 based on the presence of identical or similar residues within equivalent context (Pansegrau *et al.*, 1994). As with *nic* sequences, the similarity among the molecular reactions that cleave *nic* sites and the proteins that mediate these reactions strongly suggests that these systems share a common ancestor.

ssDNA binding proteins (Table 1, item 9)

Transfer of nucleic acids through cell membranes is essential in all living organisms (Citovsky and Zambryski, 1993). Cellular physiology of eukaryotes is dependent on the nuclear import/export of RNA. Pathogenesis, especially viral, often requires genome transport into the nucleus or from cell to cell. During transport, the nucleic acid is potentially a target for nucleolytic degradation. In addition, transported nucleic acids must be efficiently targeted to their sites of action. These functions, protection and localization, are largely provided by proteins associated with nucleic acids (Citovsky and Zambryski, 1993). Identification of the *Agrobacterium* protein VirE2 as a sequence non-specific, ss DNA binding (SSB) protein not only furthered our understanding of *Agrobacterium*-mediated genetic transformation of plants, but also provoked insights in two areas – nuclear import in plant cells and plant virus spread from cell-to-cell.

VirE2 is a *vir*-inducible, ss nucleic acid binding protein (Christie *et al.*, 1988; Citovsky *et al.*, 1988; Das, 1988; Gietl *et al.*, 1987). *In vitro*, VirE2 binds ss DNA regardless of sequence; the binding is strong and co-operative suggesting that T-strands are fully coated withVirE2 (Citovsky *et al.*, 1989; Sen *et al.*, 1989). VirE2:ssDNA complexes are resistant to 3′ or 5′ exonucleases, as well as endonucleases (Citovsky *et al.*, 1989; Sen *et al.*, 1989). VirE2:ssDNA complexes formed *in vitro* are unfolded and less than 2 nm in diameter (Citovsky *et al.*, 1989), but under some conditions this complex adopts a coiled, telephone cord-like conformation (Citovsky *et al.*, 1997).

Binding of VirE2 to T-strand was originally proposed to occur *in bacterium* prior to export (Christie *et al.*, 1988; Zupan and Zambryski, 1997). Alternatively, it has been proposed that VirE2 and the VirD2:T-strand are exported independently from the bacterium and formation of the T-complex is completed in the plant cell cytoplasm (Binns *et al.*, 1995; Gelvin, 1998; Lee *et al.*, 1999; Sundberg *et al.*, 1996). That VirE1 physically interacts with VirE2 suggests

the binding of VirE2 to the T-strand is regulated *in vivo* but does not resolve the question of where or when this occurs (Deng *et al.*, 1999; Sundberg and Ream, 1999; Sundberg *et al.*, 1996). In either case, once bound to the T-strand, VirE2 provides protection from nucleolytic degradation. If T-strand is transported as a nucleoprotein complex, a specific conformation, maintained by VirE2, may well be a prerequisite for transit through transmembrane channels that mediate transfer. Before discussing targeting functions of VirE2, we highlight how these results impacted on other research in our laboratory.

The requirement for an SSB in the transfer of nucleic acids during pathogenesis by *Agrobacterium* directly stimulated the hypothesis that a similar activity might be involved in the cell-to-cell spread of plant viruses. Movement of plant viruses from infected cells to healthy cells was long presumed to take place through plasmodesmata (Esau, 1948), natural plant intercellular connections. The estimated size exclusion limit (SEL) of plasmodesmata (0.9–1.0 nm Stokes' radius), however, was far below the size of whole virus particles (12–80 nm) or free viral genomes with irregular, folded structures and Stokes' radii of at least 10 nm. Therefore, to exploit plasmodesmata, plant viruses must increase the plasmodesmata SEL or synthesize a transfer intermediate compatible with transport through plasmodesmata (reviewed in Ghoshroy *et al.*, 1997). Thus, an ss DNA:SSB complex was predicted to serve as the transfer intermediate in movement of viral nucleic acids through plasmodesmata as well as transfer of T-strand from bacterium to plant cell.

Genetic evidence suggested that viral-encoded proteins, termed movement protein (MPs), mediated cell-to-cell movement (reviewed in Carrington *et al.*, 1996; Citovsky, 1999; Mushegian and Koonin, 1993). The first indication of the mechanism behind this activity derived from studies of P30, the MP of tobacco mosaic virus (TMV), a positive sense RNA virus. Transgenic tobacco expressing P30 allowed the diffusion of fluorescently labeled, microinjected 10 kDa dextrans from the injected cell to adjacent cells while the same tracer remained confined to the injected cell in control plants (Wolf *et al.*, 1989). Thus, the SEL of plasmodesmata in transgenic plants had been increased to approximately 3 nm (the Stoke's radius of 10 kDa dextran). Plasmodesmata with these dimensions, however, would still be unable to traffic TMV RNA or viral particles.

Using a biochemical approach, Citovsky *et al.* (1990) demonstrated that P30 binds ss nucleic acids. The binding is strong, co-operative and sequence non-specific. P30:ss nucleic acid complexes are less than 2 nm in diameter, compatible with the P30-induced increase in the SEL of plasmodesmata (Citovsky *et al.*, 1990; Citovsky *et al.*, 1992a). The *in vivo* and *in vitro* activities of P30 generate a very specific model for the spread of plant viruses from cell to cell. After infection and replication of the TMV genome, P30 sequesters a portion of the RNA for MP-mediated transport to adjacent cells while the remainder is encapsidated for spread by vectors to other plants. P30 expression during infection of a leaf is transient, temporally partitioning the replicated genomes between nucleoprotein complexes for cell-to-cell spread and viral particles for transfer to new hosts. After transport into a neighboring cell, replication, cell-to-cell movement and encapsidation are recapitulated.

Since the initial characterization of P30 as an ss nucleic acid binding protein, SSB activity has been demonstrated for many plant viral MPs (Table 2) (reviewed in Carrington *et al.*, 1996; Lazarowitz and Beachy, 1999; Mushegian and Koonin, 1993). The fact that most MPs have SSB activity suggests that transport through plasmodesmata of nucleoprotein intermediates is a common mechanism for cell-to-cell spread of many plant viruses; this simple model was provoked by our studies of VirE2 and the formation of a transferable T-complex by *Agrobacterium*.

Plant nuclear localization signals (Table 1, item 10)

Integration of the T-strand requires that the T-complex be imported into the plant cell nucleus, a tightly regulated process in which proteins larger than 40–60 kDa must possess a nuclear localization signal (NLS) to mediate their import through the nuclear pore (Goerlich, 1997; Melchior and Gerace, 1995). As the T-strand is presumed to be completely coated with proteins, the signals that target T-complex to the nucleus most likely reside in its associated proteins, VirD2 and VirE2 (Zambryski, 1992). Therefore, in the evolution of the *vir*-system to exploit a eukaryotic host, these prokaryotic proteins acquired motifs that function as NLSs. Nuclear targeting of VirD2 and VirE2 were among the first studies of nuclear import in plants.

Nuclear localization of VirD2. Sequence analysis of VirD2 revealed a sequence homologous to the bipartite type of NLS (Howard *et al.*, 1992). The nuclear localizing function of this sequence was confirmed by expressing a VirD2:-GUS fusion in tobacco protoplasts (Howard *et al.*, 1992) and by immunolocalization (Tinland *et al.*, 1992). That *Agrobacterium* was severely reduced in tumorigenicity when the two stretches of basic amino acids in the VirD2 bipartite NLS were deleted validated the biological relevance of the NLS-like sequence (Shurvinton *et al.*, 1992).

VirE2 NLSs (discussed below) are present in vast excess over the single VirD2 NLS. This raises the question of whether the NLS of VirD2 is superfluous or has a unique role in the import of T-complex. The fact that *Agrobacterium* bearing an NLS-deleted VirD2 was attenuated in virulence suggests the latter. Possibly, VirD2 ensures that the 5′ end of the T-strand enters the nucleus

Table 2. Representative plant pathogen proteins with single-strand nucleic acid binding activity*

Pathogen	Protein	Nucleic acid binding activity and function	References
Agrobacterium tumefaciens	VirE2	ssDNA; protection and nuclear import	(Christie *et al.*, 1988; Citovsky *et al.*, 1988; Citovsky *et al.*, 1989; Citovsky *et al.*, 1992; Das, 1988; Sen *et al.*, 1989)
Tobacco mosaic virus (TMV)	P30	ssRNA, ssDNA; cell-to-cell movement	(Citovsky *et al.*, 1990; Citovsky *et al.*, 1992a; Waigmann *et al.*, 1994)
Cauliflower mosaic virus (CaMV)	P1	ssRNA, ssDNA; cell-to-cell movement	(Citovsky *et al.*, 1991; Thomas and Maule, 1995)
Squash leaf curl virus	NSP (BV1, BR1) MPB (BC1, BL1)	ssDNA; nuclear shuttle, cell-to-cell movement	(Pascal *et al.*, 1994; Sanderfoot and Lazarowitz, (SqLCV) 1995; Sanderfoot *et al.*, 1996)
Cucumber mosaic virus (CMV)	3a	ssRNA, ssDNA; cell-to-cell movement	(Ding *et al.*, 1995; Li and Palukaitis, 1996; Vaquero *et al.*, 1997)
Barley stripe mosaic virus (BSMV)	βb	ssRNA, dsRNA	(Donald *et al.*, 1997)
Potato leafroll virus (PLRV)	pr17	ssRNA, ssDNA	(Tacke *et al.*, 1991)
Potato virus Y (PVY)	HC-Pro	ssRNA	(Maia and Bernardi, 1996)

*Additional movement proteins with SSB activity are described in Mushegian and Koonin (1993), Carrington *et al.* (1996), and Lazarowitz and Beachy (1999).

first, avoiding entry in an awkward 'elbow' conformation. Initiation at the 5′ end may be a common feature of the translocation of ss nucleic acids across the nuclear pore in either direction. For example, export of 75s rRNA (Balbioni Rings) from the nuclei of salivary gland cells in *Chironomus tentans* initiates at the 5′ end (Mehlin *et al.*, 1992).

VirD2 was exploited in yeast two-hybrid analysis to identify an *Arabidopsis* protein of the karyopherin-α family (AtKAPα) (Ballas and Citovsky, 1997). AtKAPα rescued a yeast mutant defective in nuclear import and mediated nuclear import of VirD2 in permeabilized yeast cells. The interaction between VirD2 and AtKAPα provides strong evidence that *Agrobacterium* has co-opted the eukaryotic process of nuclear import to assist in the efficient genetic transformation of plant cells.

Nuclear localization of VirE2. VirE2, the most abundant protein component of the T-complex, contains two bipartite NLSs. Both NLSs were required for maximum accumulation of a VirE2:GUS fusion protein in the nucleus (Citovsky *et al.*, 1992b). The T-strand from the nopaline strain of *Agrobacterium* requires 600 monomers of VirE2 to coat it completely (Zambryski, 1992). Therefore, VirE2 provides NLSs along the entire length of the T-complex, potentially facilitating uninterrupted nuclear uptake of T-complex.

VirE2-mediated nuclear import of ssDNA was assayed directly by microinjection of complexes formed *in vitro* from fluorescently labeled DNA and purified VirE2 into stamen hair cells of the flowering plant *Tradescantia virginiana* (Zupan *et al.*, 1996). Fluorescent DNA accumulated in the nucleus specifically when microinjected as a complex with VirE2. ssDNA microinjected alone remained cytoplasmic. These data suggest that T-strand is imported into the nucleus by a protein import pathway via its association with VirE2. Other data have been interpreted to suggest that VirE2 does not provide nuclear-targeting but only protects T-strand from nucleolytic degradation inside the plant cell (Rossi *et al.*, 1996); however, the entire *virE2* gene was deleted, simultaneously abolishing both protective and nuclear localizing functions.

T-complex nuclear import also reveals novel features of this process. First, accumulation of VirD2 and VirE2 GUS-fusions in the nuclei of leaf and immature root epidermal cells, but not in mature root epidermal cells, suggests nuclear import may be developmentally regulated. Cell-type specific NLS-binding proteins could result in specific gene expression by admitting different subsets of transcription factors into the nucleus (reviewed in Whiteside and Goodbourn, 1993). Secondly, plants may tolerate greater variability in the primary sequence of bipartite NLSs. While the NLS of VirD2 conforms to the consensus 'animal' bipartite sequence, the NLSs of VirE2 are less homologous. Both of these proteins are imported into plant cell nuclei but only VirD2 is imported into the nuclei of animal cells (Guralnick *et al.*, 1996). When either NLS of VirE2 was altered to conform precisely to the 'animal' bipartite signal, the protein accumulated in animal cell nuclei and mediated nuclear import of ss nucleic acid (Guralnick *et al.*, 1996).

Integration (Table 1, item 11)

In the final step of T-DNA transfer, the incoming ssDNA of the T-complex is stably integrated into a plant chromosome (Gheysen *et al.*, 1991). Integration may induce small

deletions, less than 100 bp, of plant DNA at the insertion site. In addition, integration also generates 'filler' DNA, less than 50 bp homologous to nearby plant DNA, at the T-DNA-plant junction. Integration of the 5′ end of the T-strand is relatively precise often occurring at the penultimate 5′ nucleotide bound to VirD2 (Tinland *et al.*, 1995). Some mutations in VirD2 result in large deletions at the 5′ end suggesting that VirD2 mediates the precision of integration at this end. At the 3′ end, integration usually includes most of the left border region, although larger deletions are more common at this end. Deletions of up to 1000 bp at the 3′ end of the T-strand transferred from a *virE2*⁻ strain suggest that VirE2 plays a significant role in protecting the T-strand from nucleolytic degradation (Rossi *et al.*, 1996).

The mechanics of integration remain largely unknown. After nuclear import, the T-strand is likely made ds with the concomitant displacement of VirE2. Conversion to a ds form is supported by transient expression of reporter genes in the T-DNA, extrachromosomal homologous recombination of T-DNA prior to integration, and the complex pattern of multiple T-DNAs at a single insertion site (discussed in De Buck *et al.*, 1999).

Given that the T-strand does not encode enzymes that catalyze integration, these functions must be mediated by host cell factors. As not more than 5 (usually 0–3) bp of homology have been found between either end of the T-DNA and the plant sequences at the insertion site, most models usually propose illegitimate recombination as the model for T-strand integration (De Buck *et al.*, 1999). Double-strand break repair via non-homologous end-joining (Britt, 1999) of the ds T-strand is consistent with many of the products observed as a result of T-DNA integration. The precision of T-DNA borders at the 5′ end of the T-strand suggested that VirD2 initiates integration by ligating the 5′ end to an exposed 3′-OH in plant DNA. An alternative model proposes that the T-strand remains ss, and integration initiates by docking of the 3′ end of the T-strand via 2–5 bases of microhomology (Tinland *et al.*, 1995). The latter model, however, does not explain the pattern of integration for tandem arrays of multiple T-DNAs. In either case, both 5′ and 3′ integration are likely to be assisted by plant machinery for DNA metabolism; DNA replication and repair type enzymes are prime candidates. It will be interesting to determine whether VirD2 and VirE2 directly interact with structural or enzymatic components of plant chromatin or DNA metabolism.

Type IV secretion systems (Table 1, item 12)

Systems that secrete various substrates through the bacterial envelope are currently classified into four types. Type I, typified by *Escherichia coli* hemolysin export, is sec-independent and requires 3–4 accessory proteins (Fath and Kolter, 1993). In the type II system, typified by pullulanase export in *Klebsiella oxytoca*, the substrate is exported into the periplasm by the *sec*-dependent general secretory pathway (GSP) then secreted across the outer membrane via a specialized terminal branch of the GSP (Pugsley, 1993). Type III secretion (Hobbs and Mattick, 1993), typified by Yop export in the human pathogen *Yersinia pestis* (Cornelis and Wolf-Watz, 1997), is sec-independent, translocates protein substrates from the bacterial cytoplasm into the eukaryotic host cytoplasm, and requires more than 20 accessory proteins. Type III systems also include bacterial flagellar assembly proteins which 'secrete' flagellar components to the exterior of the cell.

Type IV secretion systems comprise a growing family of multiprotein complexes that span the bacterial envelope (Salmond, 1994). Originally, the type IV family included the *virB* operon and systems for conjugal DNA transfer. Homologs of six *virB* genes were then found in the *ptl* operon of *Bordetella pertussis*, which encodes a transporter that exports pertussis toxin (reviewed in Lessl and Lanka, 1994; Winans *et al.*, 1996). Subsequently, type IV systems essential for virulence have been identified in human pathogens (discussed below), and mediate transfer of proteinaceous factors, such as CagA (Odenbreit *et al.* 2000; Stein *et al.* 2000), that subvert host defense mechanisms. The export of proteins suggests type IV secretion systems evolved from a protein exporter system. The *Agrobacterium* VirB transporter as well as those for bacterial conjugation may incidentally transfer DNA by virtue of its forming a nucleoprotein complex with proteins targeted for export, exemplified by the T-complex.

Recent advances: bacterial secretion and pathogenesis

Structure and function of the T-transporter

Over the past 5 years, much of the research on *Agrobacterium*-mediated gene transfer has focused on the *vir*-specific type IV secretion system, the T-complex transporter (T-transporter). This apparatus is assembled from 11 proteins encoded by the *virB* operon, and VirD4. The T-transporter facilitates transfer of the T-complex to plant or yeast cells and mediates conjugal transfer of the non-self transmissible, but mobilizable, plasmid RSF1010 (an IncQ plasmid) to *Agrobacterium*. It can also transfer proteins, such as VirE2 and VirF, to plant cells (reviewed in Christie, 1997; Zupan *et al.*, 1998).

While the mechanics of T-transporter function are not known, the basic architecture of its structural components is emerging. Assembly is most likely preceded by hydrolysis of the peptidoglycan layer by VirB1 (Figure 2a) (Baron *et al.*, 1997a). This activity is essential for the mobilization

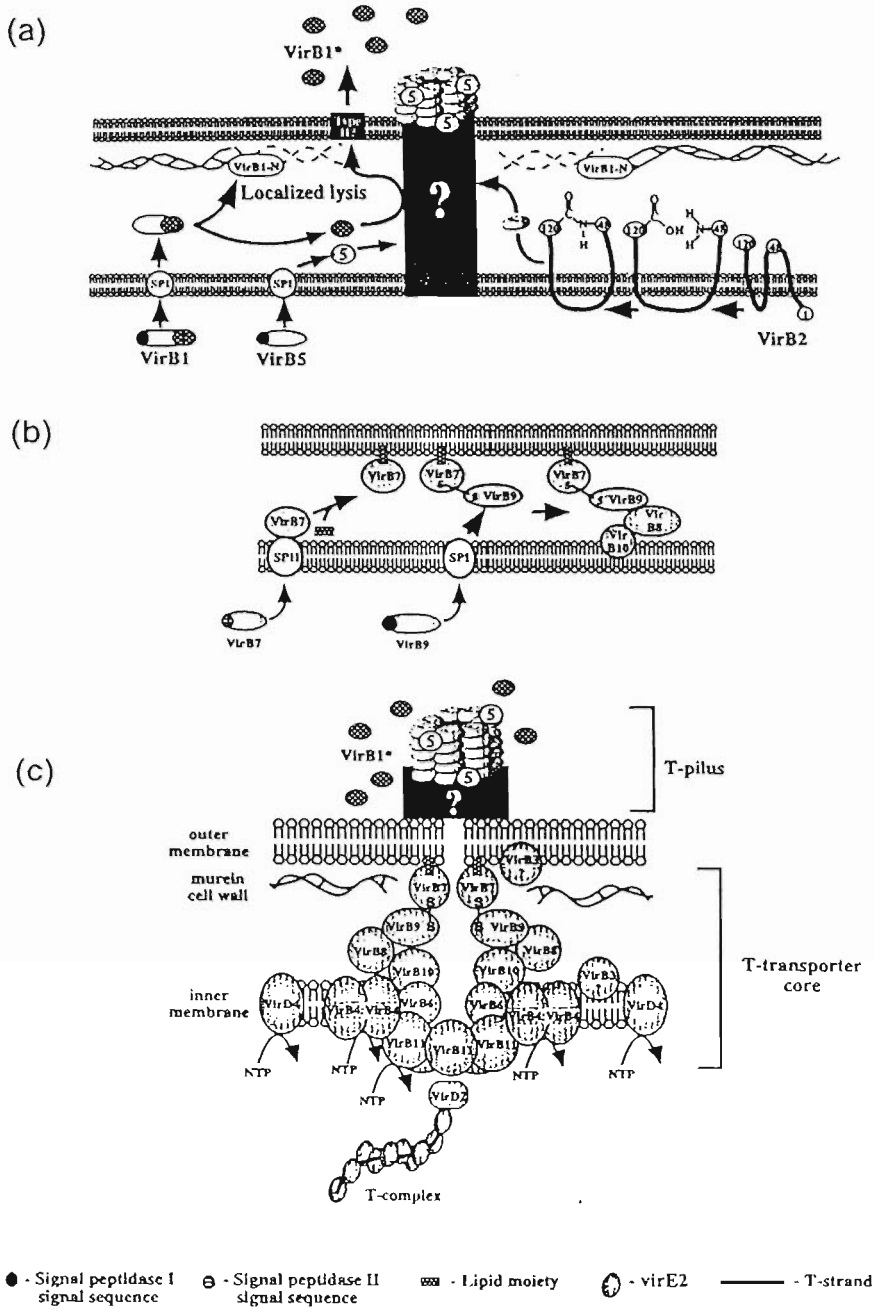

Figure 2. Assembly of the *Agrobacterium tumefaciens* T-complex transporter.

(a) Assembly of the T-pilus. VirB1 is processed into two domains following SPI mediated transport into the periplasm. The N-terminal portion (VirB1-N, white oval) may be involved indirectly in pilus formation (as well as assembly of the transporter core) by inducing localized lysis of the peptidoglycan layer. The C-terminal VirB1* (cross-hatched oval) may chaperone pilus components VirB2 and VirB5 to the exterior of the cell. VirB2 (major) and VirB5 (minor) pilus components are shown as donut-shaped and gray ovals, respectively. VirB2 processing is described in the text. VirB2 and VirB5 are mobilized to the cell surface by an unknown mechanism that likely requires additional VirB proteins. The black box indicates that it is not known how the T-pilus is anchored in the bacterial envelope.

(b) Assembly of the T-complex transporter core begins with the lipid-modification of VirB7 resulting in anchoring in the outer membrane. VirB7 forms a heteromultimer with VirB9 which promotes the stability of many other VirB proteins, possibly through interactions with VirB8 and VirB10 (Das and Xie, 2000).

(c) The T-complex transporter is suggested to comprise a pilus and channel 'core' (see text). Whether the pilus and core are coupled either physically or functionally is not known (black box).

of VirB2 and VirB5 to the cell surface to form the T-pilus, the best documented structural feature of the transporter (Figure 2a). Interaction and subcellular localization studies suggest at least some of the remainder of the VirB/VirD4

proteins assemble into a multimeric complex that spans the bacterial envelope (Figure 2b). This hypothetical 'core' of the T-transporter likely translocates substrates out of the bacterium. Energy to drive assembly and translocation is potentially provided by the three ATPases (VirB4, VirB11 and VirD4) associated with the T-transporter (Figure 2c) (reviewed in Christie, 1997; Zupan *et al.*, 1998). Increasingly, non-*vir* functions are also being identified as essential for transporter assembly. Below, we discuss each of the known components of the T-transporter.

VirB1 is bifunctional

Illustrations depicting large, multicomponent, membrane-spanning complexes in bacteria usually neglect to represent the peptidoglycan layer (Dijkstra and Keck, 1996). This omission reflects the lack of information on how penetration of this layer is achieved during complex assembly. The size limit for diffusion through naturally occurring channels in the peptidoglycan layer is only ≈50 kDa (Dijkstra and Keck, 1996), too small to allow the assembly of a multiprotein apparatus large enough to traffic nucleoprotein complexes. Work on VirB1 provided some of the first evidence that secretion systems may include dedicated factors that modify the bacterial cell wall to accommodate their assembly. Sequence comparisons revealed similarities among chicken egg white lysozyme and the N-terminus of VirB1, as well as proteins involved in conjugal DNA transfer, invasion by the human pathogens *Salmonella enterica* and *Shigella* spp. and others (Table 3) (Dijkstra and Keck, 1996; Lehnherr *et al.*, 1998; Mushegian *et al.*, 1996). Based on lysozyme homology, these proteins are predicted to possess glycosidase activity, and indeed they share homology with lytic transglycosylases across a region that forms the catalytic site (Table 3). Although VirB1 glycosidase activity has not been demonstrated directly *in vitro*, agrobacteria, carrying VirB1 mutations in two residues predicted to be critical for hydrolysis of glycosidic bonds were severely attenuated in virulence (Mushegian *et al.*, 1996).

VirB1 is processed to release its C-terminal third, VirB1*, which is then secreted (Baron *et al.*, 1997a). Approximately half of VirB1* remains loosely associated with the exterior of *Agrobacterium* cells, and the rest can be recovered from culture supernatants (Baron *et al.*, 1997a). Processing and secretion of VirB1* are not coupled, are independent of the Ti-plasmid, but do require the signal peptide (Llosa *et al.* 2000). Thus, factors that mediate processing of VirB1 and secretion of VirB1* are localized in the periplasm or outer membrane and are not subject to *vir*-regulation. VirB1 is processed to VirB1* in other Rhizobiaceae, but secretion is specific to *Agrobacterium* (Weininger, Domke and Baron, personal communication). These observations suggest that VirB1* is synthesized and secreted for a specific role, most likely at the cell surface, during T-transporter assembly or function. A bifunctional VirB1 is supported by partial complementation (tumor and T-pilus assays) of a *virB1* deletion strain with constructs expressing either the N-terminal lysozyme-homologous region or the C-terminal VirB1* (Llosa *et al.* 2000; Weininger, Domke and Baron, personal communication). Thus, each domain of VirB1 probably has its own role in tumorigenesis.

As VirB1 is the first product of the polycistronic transcript of the *virB* operon, it is likely to have an early role. Its loose exterior location suggests that VirB1* may play a non-structural role mediating pilus formation, such as chaperone activity for VirB2 during transport to the cell exterior. Alternatively, association of VirB1* with VirB9 (Baron *et al.*, 1997a) suggests that it may function in the assembly of the T-transporter 'core', perhaps before VirB1* is secreted to the exterior of the cell. Finally, association of VirB1* with the exterior of *Agrobacterium* suggests that it may be available to interact with the plant cell surface (Figure 2a).

T-pilus

The only documented structural component of the T-transporter is the T-pilus, a long, flexible, filamentous appendage observed on the surface of *vir*-induced

Table 3. Selected proteins with lysozyme or transglycosylase homology

Protein	Organism or plasmid	Function	Reference
VirB1	*A. tumefaciens*	Transporter assembly	(Baron *et al.*, 1997a; Dijkstra and Keck, 1996; Mushegian *et al.*, 1996)
VirB1	*Brucella suis*	Survival; intracellular multiplication	(O'Callaghan *et al.*, 1999)
TraN	IncP plasmid	Conjugation	(Lessl *et al.*, 1992)
TraL	IncN plasmid	Conjugation	(Mushegian *et al.*, 1996; Pohlman *et al.*, 1994)
ORF19/ORF169	IncFII R1 plasmid	Conjugation	(Graus *et al.*, 1990; Loh *et al.*, 1989)
Slt70	*E. coli*	Peptidoglycan degradation	(Holtje *et al.*, 1975; Thunnissen *et al.*, 1994)
IpgF	*Shigella* spp.	Invasion	(Allaoui *et al.*, 1993)
IagB	*Salmonella enterica*	Invasion	(Miras *et al.*, 1995)
Pm404	*Proteus mirabilis*	?	(Dijkstra and Keck, 1996)

Agrobacterium cells (Fullner *et al.*, 1996). Formation of T-pili is dependent on expression of VirB proteins and is correlated with VirB-mediated transfer of T-complex to plant cells and the transfer of IncQ plasmids between bacteria (Fullner, 1998).

The F-pilus of the F-incompatibility system of *E. coli* is the best characterized component of any type IV transporter (Firth *et al.*, 1996). This pilus mediates cell surface contact between donor and recipient, a prerequisite for conjugal DNA transfer. F-pili have been observed to shorten and 'retract.' Presumably, retraction brings the cell surfaces of the donor and recipient into close contact to facilitate transfer of F-plasmid.

The inside diameter of the F-pilus (2 nm) (Silverman, 1997) is barely compatible with the transfer of nucleoprotein complexes through the lumen. The diameter of the nucleoprotein T-complex is < 2 nm (Citovsky *et al.*, 1989). Although the interior dimensions of the *Agrobacterium* virulence pilus have not been reported, the exterior dimensions (10 nm) (Lai and Kado, 1998) are comparable to the F-pilus (8 nm) (Silverman, 1997). As the T-pilus is extremely thin and the T-strand is likely to be transferred as a nucleoprotein complex, it is difficult to imagine how the T-pilus lumen serves as a conduit for delivery of an elongated T-complex into the host without some additional features. Either a conformational change in the pilus structure itself and/or the widening of the transmembrane channel may diminish the physical constraints to egress of the T-complex. Whether the pilus serves as the conduit for T-complex transport, as well as the host cell tether, remains unresolved. If the pilus serves only as a tether, the T-complex may move directly through the VirB transmembrane channel into the plant cell by an unknown mechanism. Dürrenberger *et al.* (1991) suggest that conjugating bacteria become tightly juxtaposed along their length to form patches of intimate contact. If the tether and conduit are separated functionally, they may not be linked physically. The latter scenario provides two opportunities for interaction between the host cell surface components and the bacteria, i.e. interactions via the pilus and interactions via the channel.

Virulence pili may also have a regulatory role in T-transporter function. While attachment of *Agrobacterium* is obviously critical for the genetic transformation of plant cells, attachment is chromosomally, not Ti-plasmid, encoded (Matthysse and Wagner, 1994). Furthermore, expression of the *vir*-specific type IV transporter components can be induced by soluble plant factors in the absence of plant cell contact *per se*. T-complex, however, is not detected in *vir*-induced culture supernatants; thus, the T-transporter may not be functional without a plant host cell. Type III secretion systems require host cell contact for transporter assembly and function (Cornelis and Wolf-Watz, 1997). Perception and transduction of a contact signal by the T-pilus may trigger transporter activity, either by assembling transporter components at the site of closest proximity to the recipient cell or by inducing activity in assembled transporters specifically in the region of contact.

T-pilus assembly. Two Vir proteins have been identified in T-pili preparations. The major component, VirB2, is translated as a 12.3 kDa protein that is quickly processed to a 7.2 kDa protein associated with the inner membrane (Jones *et al.*, 1996; Lai and Kado, 1998). The VirB2 homolog, TraA, is the F-pilus subunit and is similarly processed from a 12.3 kDa propilin to a 6.2 kDa inner membrane protein (Firth *et al.*, 1996). In the presence of recipient cells, TraA is mobilized by an unknown mechanism to the surface of the donor cell where it polymerizes to form the pilus. In contrast, *Vir*-induced cultures of *Agrobacterium* assemble T-pili without host plant cells (Lai and Kado, 1998; Schmidt-Eisenlohr *et al.*, 1999a). Strains of *Agrobacterium* with deletions of *virB3*, *virB4*, *virB5*, *virB9* or *virB10* produce VirB2, but the processed form accumulates inside rather than on the exterior of the cell, and pili are not observed (Lai and Kado, 1998).

The leader peptide (47 amino acids) of VirB2 propilin is cleaved during insertion into the inner membrane where it accumulates prior to T-pilus assembly (Eisenbrandt *et al.*, 1999). Pilin subunits in other systems undergo a variety of further post-translational modifications. TraA of the F-pilus is acetylated at the C-terminus (Moore *et al.*, 1993), and the subunit of type IV adhesive pili in *Neisseria meningitidis* is glycosylated (Virji *et al.*, 1993). VirB2, as well as its IncP homolog TrbC, forms an intramolecular bond between its N- and C-termini producing a cyclic polypeptide, a rare reaction in prokaryotes (Eisenbrandt *et al.*, 1999). Prior to cyclization, TrbC undergoes two additional proteolytic reactions following leader peptide removal; the final reaction requires TraF and removes four amino acids at the C-terminus. In the absence of TraF, TrbC is not cyclized; cyclization may have specific sequence requirements necessitating prior processing by TraF. The Ti-plasmid does not encode a TraF homolog and VirB2 does not undergo proteolytic processing subsequent to removal of the signal peptide (Figure 2a).

VirB2 is cyclized in the absence of other Ti-plasmid genes but little is known about this reaction (Eisenbrandt *et al.*, 1999). Membrane topology studies predict that both the N- and C-termini of VirB2 protrude into the periplasm after insertion into the inner membrane (Eisenbrandt *et al.*, 1999). This conformation may facilitate formation of the intramolecular bond by bringing the polypeptide termini into close contact (Figure 2a). Either cyclization is autocatalytic or the putative cyclase represents a novel unidentified chromosomal factor required for virulence.

In addition to VirB2, VirB5 is found as a minor component in T-pili preparations (Schmidt-Eisenlohr *et al.*,

1999a). TraC, the IncN VirB5 homolog, also associates with an exocellular polymeric structure that may be the pilus of pKM101 (IncN) (Schmidt-Eisenlohr *et al.*, 1999b) thus suggesting that VirB5 homologs may function as auxiliary structural proteins in pili of type IV secretion systems (Figure 2a). In contrast to VirB2, cellular levels of VirB5 were strongly correlated with the abundance of other *Vir* proteins, indicating its stabilization by protein–protein interactions (Schmidt-Eisenlohr *et al.*, 1999a). Thus, additional VirB components, as well as VirB2 and VirB5, are required for T-pilus formation. Isolated pili have terminal knobs, but it is unknown whether these knobs reside at the pilus tip or base anchoring the pilus in the bacterial envelope (Schmidt-Eisenlohr *et al.*, 1999a). By analogy to pap-pili adhesins, the knob may be a distal feature of the T-pilus that mediates interaction with the plant cell.

Assembly of the T pilus also involves VirB6 (Fullner *et al.*, 1996). Based on its predicted extreme hydrophobic character, VirB6 was suggested to form a pore in the inner membrane (Christie, 1997; Das and Xie, 1998) that might function in egress of the T-complex. Although cellular levels of most VirB proteins were unaffected by in-frame deletion of *virB6*, pili were not formed (Hapfelmeier, Domke, Zambryski and Baron, personal communication). Pili were restored by *trans*-complementation with *virB6*, suggesting that VirB6 may function in pilus assembly and not directly in the translocation of the T-complex.

Assembly of a T-complex transporter 'core'

A membrane spanning conduit is generally assumed to represent the channel 'core' for T-complex export. Insights into the composition of this core, its assembly or its function require determination of the specific protein–protein interactions.

VirB7, VirB8, VirB9 and VirB10. The best documented interaction for components of the T-transporter is between the outer membrane lipoprotein VirB7 and periplasmic VirB9 (Anderson *et al.*, 1996; Baron *et al.*, 1997b; Das *et al.*, 1997; Fernandez *et al.*, 1996a; Spudich *et al.*, 1996). Strains carrying mutations in VirB7 or VirB9 that disrupt formation of the heteromultimer accumulated significantly lower cellular levels of VirB4, VirB5, VirB8, VirB10 and VirB11 (Fernandez *et al.*, 1996b). In the absence of the VirB7:VirB9 heterodimer, these proteins may be degraded. Thus, the VirB7:VirB9 heterodimer may play a critical role initiating or stabilizing transporter assembly through physical interactions (Figure 2b).

VirB10 forms high molecular weight complexes, dependent on the VirB7:VirB9 heterodimer (Beaupré *et al.*, 1997; Ward *et al.*, 1990). Two-hybrid analysis in yeast indicates VirB10 interacts physically with VirB9 as well as VirB8 (Das and Xie, 2000), although these proteins were not identified in the VirB10 complexes in *Agrobacterium* (Beaupré *et al.*, 1997; Ward *et al.*, 1990). Alteration of a single amino acid in VirB8 disrupted the two-hybrid interaction with VirB9 and caused avirulence suggesting the VirB8:VirB9 interaction is essential. Possibly, VirB8:VirB9 association in the periplasm forms links with VirB10 in the inner membrane and with VirB7 in the outer membrane (Figure 2c).

The ATPases: VirB4, VirB11 and VirD4. The T-transporter includes three proteins (VirB4, VirB11 and VirD4) with homology to ATPases. These proteins are presumed to provide the energy that drives either transporter assembly, T-complex translocation, or both. Strains carrying VirB4 mutants with defects in the Walker A nucleotide binding motif are avirulent (Christie *et al.*, 1989; Fullner *et al.*, 1994) and exert a dominant negative effect when co-expressed with wild-type VirB4 (Berger and Christie, 1993). Trans-dominance suggests VirB4 is incorporated into a multimer. VirB4 also mediated dimerization of the λ c1 repressor protein and conferred immunity to λ infection (Dang *et al.*, 1999). As the VirB4 Walker A motif mutants also conferred λ immunity, ATP binding is dispensable for dimerization. However, dimerization may be essential for ATPase activity as occurs in ATPase subunits associated with a variety of membrane transport systems (Davidson *et al.*, 1996; Nikaido *et al.*, 1997).

Co-synthesis, specifically of VirB3, VirB4, VirB7, VirB8, VirB9 and VirB10, in agrobacterial recipient cells significantly stimulates VirB-mediated conjugation of the non-self transmissible IncQ plasmid RSF1010 (Bohné *et al.*, 1998). The increase in conjugation efficiency, proposed to result from the assembly of these VirB proteins into a transmembrane structure, may provide an assay for testing hypotheses regarding the assembly and function of individual VirB proteins. For example, recipients expressing either wild-type VirB4 or Walker A box mutants exhibit the same level of enhanced conjugation efficiency. Potentially, VirB4 homomultimers are required for the assembly of a complex including additional VirB proteins, and this structure can assemble in the absence of ATP-binding by VirB4 (Dang *et al.*, 1999). In this scenario, VirB4 may act as an essential component of the scaffolding for transporter assembly. In contrast, the donor requirement for a wild-type VirB4 suggests that ATP-binding confers the T-transporter with 'one-way' activity to export DNA (Figure 2c).

The homology between VirB4 and VirB3 and the IncF proteins TraL and TraC, respectively, suggests that these *Agrobacterium* proteins have a role in pilus assembly (Jones *et al.*, 1994). The IncF proteins are essential for pilus formation but are not pilus structural components. While a connection to T-pilus assembly has not been established for either VirB4 or VirB3, they may physically interact. In a *virB4* deletion strain, cellular levels of VirB3 are reduced,

and VirB3 is localized to the inner membrane rather than both inner and outer membranes as in wild-type strains (Jones *et al.*, 1994). VirB3 specific localization, mediated by VirB4, may promote mobilization of VirB2 to the exterior of the cell.

VirB11 is a member of the PulE superfamily and is the most widespread of the VirB proteins (Krause *et al.*, 2000). In addition to the type IV secretion systems, homologs are found in fimbrial genes from *Pseudomonas aeruginosa* and pathogenic *Neisseria*, the comG operon involved in *Bacillus subtilis* competence, and the pullulunase secretion system of *Klebsiella oxytoca*. PulE homologs are cytoplasmic or weakly associated with the cytoplasmic face of the inner membrane. Two members of this family, TrbB of the IncPα plasmid RP4 and HP0525 from the *cag* pathogenicity island of *Helicobacter pylori*, form six membered ring-shaped structures *in vitro* (Krause *et al.* 2000). In the absence of ATP, TrbB formed significantly fewer rings. Other di- and monophosphate nucleotides stabilized the TrbB hexamers as well, indicating that nucleotide binding but not hydrolysis is required. Curiously, HP0525 formed rings even without NTPs. In *Agrobacterium*, mutations in the Walker A box of VirB11 are not transdominant but increase VirB11 association with the cytoplasmic face of the inner membrane (Rashkova *et al.*, 1997). If VirB11 is similar to TrbB, loss of ATP-binding may prevent this class of VirB11 mutants from assembling into hexamers at the inner membrane or from interacting with other VirB components in the transporter. TrbB/HP0525 hexameric rings have an outer diameter of about 12 nm and a central channel approximately 3 nm in diameter. Perhaps the first step in transfer of the T-complex from the bacterial cytoplasm occurs through the channel in a hexameric ring of VirB11 (Figure 2c).

VirD4 homologs are present in almost all type IV secretion systems as well as the F-system (Christie, 1997). All homologs possess a Walker A motif necessary for function and all are integral inner membrane proteins. Mutants of the homologs *traD* (F) and *traG* (RP4, IncP) still produce conjugal pili and attach to recipient cells, but conjugal DNA transfer does not occur (Balzer *et al.*, 1994; Firth *et al.*, 1996). Thus, VirD4 homologs are suggested to mediate introduction of the nucleoprotein complex into the transporter by an energy-dependent mechanism (Cabezon *et al.*, 1997).

New required chromosomal activities

Although the virulence of *Agrobacterium tumefaciens* is largely attributed to the combined activities of the *Vir* proteins, recent work suggests more and varied contributions by chromosomally encoded factors. Attachment was the first step in virulence shown to be mediated by chromosomally encoded products. These *chv* (chromosomal virulence) genes encode proteins that function in synthesis and export of polysaccharides thought to enable recognition and binding of host cell-surface factors (Matthysse and Wagner, 1994). In fact, such polysaccharides may facilitate non-specific 'sticking' to plant cells, as additional data suggest that these proteins contribute to the stability of the bacterial envelope by regulating the periplasmic osmoticum (Swart *et al.*, 1994).

Sequence analysis has identified signal peptidase I and II sites in several VirB proteins (Kuldau *et al.*, 1990; Ward *et al.*, 1988), which implies constitutive secretion pathways are involved in the assembly of the transporter. More specific activities are also indicated. After VirB1 is delivered into the periplasm by the general secretory pathway, processing of VirB1, potentially by a periplasmic protease, releases VirB1* (Baron *et al.*, 1997a). Subsequently, a type II secretion system may transport VirB1* to the exterior of the cell. This would be analogous to the secretion of elastase by *Pseudomonas aeruginosa* (McIver *et al.*, 1995). Elastase is exported into the periplasm by the general secretory pathway where an intramolecular domain, which serves as a chaperone, is cleaved autoproteolytically but remains associated with the elastase. Secretion of the elastase across the outer membrane is mediated by the Xcp apparatus, a type II secretion apparatus required for pathogenicity (McIver *et al.*, 1995).

Intramolecular cyclization, a process required for maturation of VirB2, is an uncommon protein modification; either cyclization occurs autocatalytically or it requires a function encoded on the chromosome (Eisenbrandt *et al.*, 1999). Interactions between VirB proteins and chromosomal factors important for transporter assembly and function are fertile areas for future investigation.

Type IV secretion: a rapidly growing family

The type IV secretion system family is expanding. In addition to conjugal plasmid systems, recently identified homologs occur in animal pathogens and are required for virulence. The list includes *Bordetella pertussis* (whooping cough), *Helicobacter pylori* (gastric ulcers), *Legionella pneumophila* (Legionnaire's disease) and *Rickettsia prowazekii* (epidemic typhus), reviewed in (Christie, 1997; Covacci *et al.*, 1999). As these bacteria represent members of the α, β, ε and γ subgroups of the Proteobacteria, type IV transporters have clearly evolved for a variety of purposes.

Gene clusters in *Brucella suis* (brucellosis) (O'Callaghan *et al.*, 1999) and *L. pneumophila* (Segal *et al.*, 1999) are the most recent additions to the type IV secretion system family. Notably, these organisms possess nearly complete sets of *virB* and *virD4* homologs. *L. pneumophila* contains *virB2-11* and *virD4*, but their specific roles in pathogenesis have not been demonstrated (Segal *et al.*, 1999). *B. su*

has a gene cluster, also referred to as *virB*, that appears to be a single transcriptional unit and includes homologs of all *Agrobacterium virB* genes in the same order. A twelfth gene in this cluster encodes a protein similar to a mating pair factor from *Enterobacter aerogenes* and adhesin from *Pseudomonas* sp. Strains carrying mutations in either *B. suis virB5* or *virB9* were unable to survive or multiply intracellularly in a variety of cultured animal cells (O'Callaghan *et al.*, 1999). *B. suis* strains with mutations in *virB2*, *virB4* or *virB10* were severely attenuated in rates of intracellular multiplication. Thus, the *virB* region in *Brucella suis* is essential for survival and multiplication in macrophages.

Perspectives

Originally, interest in *Agrobacterium* was sparked by the fact that it caused tumors, and research was expected to provide clues into animal tumor pathogenesis. While this goal was never realized, after nearly 100 years, it is now evident that research in 'Agrobiology' has application to animal pathogenesis. Many bacteria employ a type IV secretion system to transfer conjugal plasmids carrying resistance factors into bacterial recipients and virulence determinants, either DNA or protein, into susceptible plant or animal hosts. Assembly of the *Agrobacterium* T-complex transporter currently serves as the paradigm for type IV secretion systems. The T-transporter transports nucleoprotein complexes as well as protein alone. Of type IV transporters, the T-transporter also recognizes the greatest phylogenetic diversity of hosts, i.e. plants, bacteria and yeast. These attributes confer unparalleled flexibility in experimental design. Given the significance of type IV secretion systems in plant biotechnology and health issues, research on *Agrobacterium*-mediated gene transfer should continue to supply us with insight into a wide range of biological questions.

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technical focus

A guide to *Agrobacterium* binary Ti vectors

Agrobacterium-based plasmid vectors allow the transformation of a wide range of plant species by capitalizing on a natural bacterial system to introduce DNA into the nuclear genome of plants. It is often a complex task to consider fully all the possible plasmid vectors and *Agrobacterium* strains available, and it can thus be difficult to take full advantage of these research tools. This practical guide is a survey of the many binary Ti plasmid vectors and *Agrobacterium* strains available, and aims to help researchers to make an informed decision about the system that is best suited to their needs.

Agrobacterium tumefaciens is a soil bacterium. It is pathogenic to a range of dicot plant species, causing the formation of crown galls or tumours at or close to infection sites[1]. The proliferated tissue in the tumour provides the bacterium with unusual amino acids (opines), which are an important carbon and nitrogen source, at the expense of the host plant. Genes required to establish a tumour and to bring about opine biosynthesis are transferred from *Agrobacterium* and hence this bacterium has been called Nature's genetic engineer.

As part of this sophisticated parasitism, *Agrobacterium* transfers a discrete portion of its DNA (T-DNA) into the nuclear genome of the host plant. Most of the machinery necessary for this T-DNA transfer resides on a tumour-inducing (Ti) plasmid. This Ti plasmid includes the T-DNA itself, delimited by 25 bp imperfect repeats [known as the right and left borders (RB and LB, respectively)] that define the boundaries of the T-DNA and ~35 virulence (*vir*) genes, clustered together into a *vir* region. The combined action of the *vir* genes achieves the delivery of the T-DNA to the nucleus of the host plant cell[1,2]. The T-DNA contains the genes for inducing tumour formation and opine biosynthesis, and these genes, even though they are bacterial in origin, have evolved to function only in plant cells.

Agrobacterium-mediated transformation systems take advantage of this natural plant transformation mechanism. Removal of all the genes within the T-DNA does not impede the ability of *Agrobacterium* to transfer this DNA but does prevent the formation of tumours. Ti plasmids and their host *Agrobacterium* strains that are no longer oncogenic are termed 'disarmed'. There are two key advances that have made *Agrobacterium* transformation the method of choice. These are the development of binary Ti vectors and of a range of disarmed *Agrobacterium* strains. This article outlines the options that are currently available to the researcher and provides a basis for making the most appropriate decision.

Binary Ti vector plasmids

The two main components for successful *Agrobacterium*-mediated gene transfer, the T-DNA and the *vir* region, can reside on separate plasmids. These form the basis of modern Ti plasmid vectors, termed binary Ti vectors[3]. The *vir* gene functions are provided by the disarmed Ti plasmids resident in the *Agrobacterium* strain. The T-DNA, within which are the gene(s) to be transferred, is provided on the vector.

Most *in vitro* gene manipulation techniques use *E. coli* and consequently binary Ti vectors replicate in both *E. coli* and *Agrobacterium*. Some of the important binary Ti vector types available are listed in Table 1, which also illustrates the gradual refinements that have been implemented since the introduction of the first vectors. These refinements have increased the flexibility of the vectors, allowing a range of uses and plant species, and aiding the development of more 'user-friendly' formats to facilitate *in vitro* gene manipulation.

Antibiotic resistance genes for plasmid selection and maintenance in culture

The most commonly used plasmid marker genes are those encoding resistance to kanamycin (e.g. pBIN19 and pGreen), gentamicin (e.g. the pCGN series), tetracycline (e.g. the pJJ series) and streptomycin and/or spectinomycin (e.g. pVZP series) (Table 1). However, *Agrobacterium* strains are marked with antibiotic resistances that are either chromosome or Ti-plasmid localized (Table 2). Therefore, the binary Ti vector and bacterial antibiotic resistance markers cannot be duplicated. In addition, penicillin-based antibiotics (e.g. carbenicillin and amoxycillin) are used to kill or inhibit the further growth of *Agrobacterium* several hours or days after co-cultivation

Table 1. A selection of binary Ti vectors published in the peer-reviewed literature[a]

Vector[b]	Size (kb)	Unique restriction sites in T-DNA	LacZ	Bacterial selection on	Selectable marker at	Replication origin		Mobilization	Ref.
						Agrobacterium	*E. coli*		
pBIN19	11 777	9	Yes	Kanamycin	RB	pRK2	pRK2	Yes	14
pC22	17 500	2	No	Ampicillin, streptomycin and spectinomycin	RB	pRi	ColE1	Yes	21
pGA482	13 200	7	No	Tetracycline	RB	pRK2	ColE1	Yes	31
pPCV001	9200	6	No	Ampicillin	RB	pRK2	ColE1	Yes	6
pCGN1547	14 440	5	Yes	Gentamicin	LB	pRi	ColE1	Yes	32
pJJ1881	25 700	4	No	Tetracycline	LB	pRK2	pRK2	Yes	24
pPZP111	8909	9	Yes	Chloramphenicol	LB	pVS1	ColE1	Yes	7
pGreen0029	4632	18	Yes	Kanamycin	LB	pSa	pUC	No	5

[a]In the cases of families of binary vectors only those which confer kanamycin resistance on transgenic plants are described. Sizes are based on restriction enzyme digestion patterns or on nucleotide sequence.

[b]See Box 1 for e-mail addresses for obtaining most of the plasmids listed.

Abbreviations: LacZ, β galactosidase α subunit gene for α-lac complementation; LB, left border; RB, right border. Mobilization refers to the ability of the plasmid to be transferred from *E. coli* to *Agrobacterium* by conjugation.

Table 2. Disarmed *Agrobacterium tumefaciens* strains defined by the *Agrobacterium* chromosomal background and the Ti plasmid they harbour[a]

Agrobacterium strain[a]	Chromosomal		Ti plasmid		Opine[b]	Ref.
	Background	Marker gene[c]		Marker gene[c]		
LBA4404	TiAch5	rif	pAL4404	spec and strep	Octopine	3
GV2260	C58	rif	pGV2260 (pTiB6S3ΔT-DNA)	carb	Octopine	32
C58C1	C58	–	Cured	–	Nopaline	33
GV3100	C58	–	Cured	–	Nopaline	34
A136	C58	rif and nal	Cured	–	Nopaline	35
GV3101	C58	rif	Cured	–	Nopaline	34
GV3850	C58	rif	pGV3850 (pTiC58Δonc. genes)	carb	Nopaline	36
GV3101::pMP90	C58	rif	pMP90 (pTiC58ΔT-DNA)	gent	Nopaline	6
GV3101::pMP90RK	C58	rif	pMP90RK (pTiC58ΔT-DNA)	gent and kan	Nopaline	6
EHA101	C58	rif	pEHA101 (pTiBo542ΔT-DNA)	kan	Nopaline	37
EHA105	C58	rif	pEHA105 (pTiBo542ΔT-DNA)	–	Succinamopine	38
AGL-1	C58, RecA	rif, carb	pTiBo542ΔT-DNA	–	Succinamopine	39

[a]See Box 1 for e-mail addresses for obtaining most of the strains listed.
[b]Grouped according to the opine catabolism of the original progenitor wild-type strain and/or non-disarmed parental Ti plasmid. This generally accepted classification of *Agrobacterium* strains does not necessarily imply that their disarmed counterparts still make opines.
[c]Antibiotic resistance gene used to select for that strain of *Agrobacterium* or Ti plasmid.
Abbreviations: rif, rifampicin resistance; gent, gentamicin resistance; nal, nalidixic acid resistance; kan, kanamycin resistance gene for bacteria (*nptI* or *nptIII*); carb, carbenicillin and ampicillin resistance; spec and strep, spectinomycin and streptomycin resistance; –, no marker gene present.

with target explants. Some binary Ti vectors and *Agrobacterium* strains harbour the ampicillin resistance gene (encoding a β-lactamase; Tables 1 and 2) and therefore it might not be easy to remove such *Agrobacterium* strains from tissue culture. When such strains and plasmids are used, proprietary mixtures of a penicillin and a β-lactamase inhibitor (e.g. Augmentin™) or cephalosporins (e.g. cefotaxime) are effective.

Practical implications of plasmid replication

A broad host range replication origin (*ori*) is found in many binary Ti vectors, which permits plasmid maintenance in a wide range of Gram-negative bacteria including *E. coli* and *Agrobacterium*. Alternatively, two separate origins can be present on the plasmid, permitting its maintenance in each of the bacterial species concerned.

The low copy number in *E. coli* of the broad host range plasmids that contain a RK2 *ori*, as in pBIN19 (Table 1), can be a problem for efficient manipulation of DNA. The copy number of several binary Ti vectors has been improved by incorporating the *Col*E1 *ori* from pBR322, achieving a significant enhancement of *E. coli* plasmid yields. Binary Ti vectors such as pC22 and pCGN1547 (Table 1) have an *ori* derived from the Ri plasmid of *Agrobacterium rhizogenes*[4] and a *Col*E1 *ori* for maintenance in *E. coli*. The pGreen vectors contain both a broad host range *ori* (pSa) and a *Col*E1 *ori* derived from a pUC derivative[5]. In *Agrobacterium*, plasmids such as pC22 and pCGN1547 (Table 1), which have a Ri *ori*, are present as a single copy in *Agrobacterium*.

Size matters

There has been a progressive reduction in the sizes of binary Ti vectors (Table 1). In pPCV001 and related vectors[6], and in pMON10098 (Fig. 1), the *trans*-acting replication functions derived from the progenitor plasmid RK2 have been removed, leaving a miniature replicon binary Ti vector (Table 1), with only the replication origin (*Ori*V) remaining. For this plasmid to replicate in *Agrobacterium*, strains need to possess the RK2 replicase and *trf* genes integrated into their genome [as in strain GV3101::pMP90(RK); Table 2].

The pPZP plasmids[7] contain the pVS1 *ori* (Ref. 8), which is considerably smaller than the DNA fragments from pRK2 or pRi that are necessary for plasmid replication. As a consequence, the basic vectors containing pVS1 replication sequences are smaller than those with the pRi or pRK2 replication region (Table 1). Combining both these advances to reduce plasmid size, the pGreen vector uses the relatively small pSa replication locus, which has been subdivided into the pSa *ori* and the pSa replicase gene[5] (*rep*A). The *repA* gene is resident on a compatible plasmid (pSoup) in *Agrobacterium* and provides pGreen replication functions *in trans*.

Transfer of binary Ti vectors between E. coli *and* Agrobacterium

The binary Ti vector from *E. coli* can be transferred to *Agrobacterium* via bacterial conjugation or direct DNA transfer (electroporation or freeze–thaw techniques)[9]. For the pGreen vector, transformation of *Agrobacterium* is obligatory.

The binary Ti vector T-DNA

In older binary Ti vector designs, fragments of Ti plasmid DNA containing the RB and LB imperfect repeats were used from several different Ti plasmid types. However, the sequence for these borders varies little between Ti plasmids[10] and border sequences from all Ti plasmids function in heterologous *Agrobacterium* strains[11].

Some Ti plasmids (those from the so-called octopine-producing strains) also contain an 'overdrive' sequence immediately external and adjacent to RB (Ref. 12). This overdrive motif enhances T-DNA transfer from octopine strains, such as the commonly used LBA4404 (Table 2). More recent designs of binary Ti vectors (e.g. pBECKS$_{2000}$ and pGreen series; Tables 1 and 3) contain synthetic T-DNA borders. In pGreen the sequences are copied from pTiT37 sequences but also incorporate an octopine Ti-plasmid-derived overdrive sequence[5].

Selectable marker genes in the T-DNA

There is polarity of T-DNA transfer from *Agrobacterium* to the plant cell: RB precede LB (Ref. 2). Most of the early binary T vectors have their selectable marker genes i the RB (e.g. pBIN19; Fig. 1, Table 1). Th means that the selectable marker gene wi be transferred into the plant cell first. bacterium-to-plant T-DNA transfer is inte rupted then transgenic plants could l

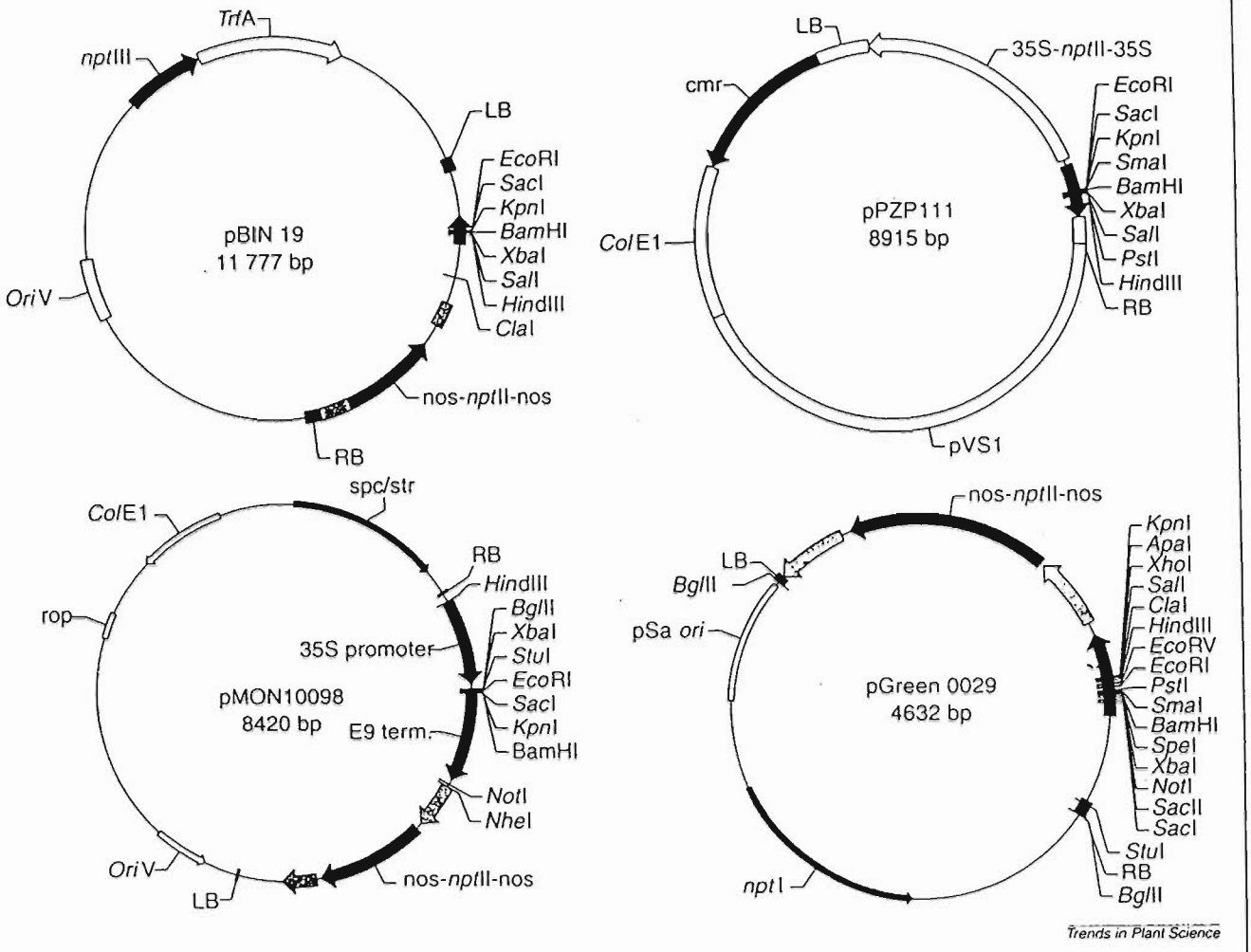

Fig. 1. Restriction maps of plasmids whose complete nucleotide sequence has been deposited in the EMBL database (Accession numbers: pBIN19, U09365; pPZP111, U10487; pGreen, AJ007829) or is available on the Internet (pMON10098 and pGreen, Box 1). *npt*I, *npt*II and *npt*III are different genes with non-homologous sequences that all encode kanamycin resistance. Abbreviations: LB, left 25 bp imperfect repeat; ori, origin of replication; RB, right 25 bp imperfect repeat.

Table 3. Binary Ti vector families whose members harbour different selectable marker genes for plant transformation[a]

	Vector series									Refs
	Bar	Ble	Dhfr	Gent	Hyg	Kan	Spec	Sul	Empty	
pMON			•	•	•	•				40,41
pBIB/pBIG					•	•				42
pGPVT	•	•	•		•	•				43
pJJ	•				•	•	•			24
pPZP				•		•			•	7
pNFHK1					•	•				29
$pBECKS_{2000}$	•				•	•				44
pGreen	•				•	•		•	•	5

[a]See Box 1 for e-mail addresses for obtaining most of the plasmids listed.
Abbreviations for resistance genes: Bar, bialaphos (or phosphinothricin, glufosinate ammonium); Ble, bleomycin; Dhfr, methotrexate; Gent, gentamicin; Hyg, hygromycin; Kan, kanamycin (or G418); Spec, spectinomycin; Sul, sulfonamides (or Asulam). Empty refers to the availability of a basic binary vector with no selectable marker genes for plant transformation.

produced with no additional sequences other than the selectable marker gene. More recently constructed binary Ti vectors contain their plant selectable marker gene nearest to the LB, to ensure that gene(s) of interest will have been transferred before the selectable marker gene (Table 1).

User support

Further important refinements include the availability of complete sequences for some binary Ti plasmids (e.g. pBIN19, pMON series, pGreen), which allows accurate restriction maps to be compiled. In addition, three binary vector systems, pGreen series, the pMON series and the pCAMBIA series, now have supporting websites (Box 1). To our knowledge, the pCAMBIA vectors have not been described in the peer-reviewed literature.

Selectable marker genes

Many improved binary Ti vectors now provide a choice of plant selectable marker genes and the promoters that initiate and drive their transcription (Table 3). This has been dictated, to some extent, by the tissue culture requirements of an expanding range of transformable plant species, including the cereals. The consequence of this requirement is that families of binary Ti vector plasmids are often available as versions of the same basic design (Table 3). The pGreen vector system (Table 3, Fig. 1), for example, permits any combination of selectable marker genes in order to explore such options as double and single selection protocols, and alternative selectable marker genes. This degree of flexibility in modern binary Ti vector design will also permit the incorporation of new or novel selectable marker genes as they appear.

A caution must also be sounded at this point about a mutation in the coding sequence of some of the kanamycin selectable marker genes (*aph3'II*, *nptII* or *neo*) in some binary Ti vectors[13]. This can prove crucial for the efficient transformation and selection of some species with kanamycin[13]. The most prominent binary Ti vector containing this defect is pBIN19 (Refs 13,14).

Agrobacterium strains

Strains of *Agrobacterium* that are useful for Ti-vector-based plant transformation are defined by their chromosomal background and resident Ti plasmid (Table 2). The C58 chromosomal background has proved to be popular for plant transformation and now harbours several kinds of wild-type and disarmed Ti plasmids, including strains that are effective at transforming cereals.

Significant modifications to the virulence of *Agrobacterium* have expanded the range of plant species that are susceptible to T-DNA transformation by improving the frequency of T-DNA transfer, most notably to the cereals. The main modification enhancing virulence has been to boost the expression of, or to introduce a change in, the activation state of the *virG* product, which activates transcription of the rest of the *vir* cluster[1,2,15]. Another modification is the enhancement of *virE1* gene expression, which encodes a single-stranded DNA binding protein that coats the transferred T-strand of the T-DNA (Refs 1,2). Expression of *VirE1*, along with that of *virG*, can be limiting in conventional *Agrobacterium* strains when large sections of DNA (>50 kb) need to be transferred using specialized binary Ti vectors called binary bacterial artificial chromosome (BiBAC) plasmids[16] (see below). In both cases, this is achieved by placing the *vir* genes on a co-resident plasmid that is compatible with the binary Ti vectors, thus boosting their expression by increasing their total copy number in *Agrobacterium*[15,16].

T-DNA integration

There is no sequence specificity for T-DNA integration into the host plant genome, although this non-homologous or illegitimate recombination might select for potentially transcribed sequences[17]. The accuracy of T-DNA integration can be a problem. Extraneous vector DNA transferred into the genome of plants has been associated with aberrant transgene expression[18]. A compilation of data on integration sites also indicates that the LB is prone to incomplete nicking and, as a consequence, that vector DNA adjacent to the LB might be transferred during transformation[19].

Variations on binary Ti plasmid designs for specific purposes

Certain experimental requirements have led to the development of specialist binary Ti vectors. Usually, these are based on the binary Ti vector families described here. The possible advantages of using such specialist plasmids should still be set against the general considerations summarized in Box 2. Given the flexibility of modern binary Ti plasmids, it might be more convenient to build new plasmids specific to the requirements of the experiment than to compromise on the requirements of the plant tissue culture system, *Agrobacterium* strain and selectable marker gene required for efficient transformation.

Binary vectors have been adapted for uses in promoter and enhancer trapping, gene activation tagging, T-DNA insertional inactivation of genes, and transposon mutagenesis[20–24]. To these ends, some binary plasmids, such as pC22 (Ref. 21; Table 1), pUCD2340 and others[25], harbour λcos sites,

Box 1. E-mail addresses and websites for obtaining plasmids and *Agrobacterium* strains

A136
gnester@u.washington.edu

AGL-1
ludwig@biology.ucsc.edu

EHA101 and EHA105
eehood@prodigene.com

GV2260, GV3101, GV3850, C58C1
inzed@gengenp.rug.ac.be

GV3101::mp90(RK) and pPCV series
koncz@mpiz-koeln.mpg.de

LBA4404 (requesting accession PC2760)
www.cbs.knaw.nl/Necb

pBECKS$_{2000}$
acm@soton.ac.uk.

pBIN19
michael.bevan@bbsrc.ac.uk

pC22
inzed@gengenp.rug.ac.be

pCAMBIA series
http://www.cambia.org.au/main/r_et_camvec.htm

pGA482
genean@vision.postech.ac.kr

pGPVT series
www.atcc.org (quote ATCC nos. 77388-77392)

pGreen series
www.pgreen.ac.uk

pJJ series
jonathan.jones@bbsrc.ac.uk

pMON
http://www.hos.ufl.edu/kleeweb

pNFHK1
mbhattac@iastate.edu

pPZP series
maliga@mbcl.rutgers.edu

Box 2. Some questions to ask when choosing a binary vector

Do I have a specialized requirement?
- Gene tagging by T-DNA insertion or transposon mutagenesis
- Promoter or enhancer trapping
- Constitutive expression or regulated expression?
- Quantitative transgene expression studies
- Transfer of large DNA sequences (>50 kb)?
- Will the T-DNA insert and flanking sequences in the plant need to be analysed?

How many genes do I want to transform into plants?
- Different single transgenes in different lines?
- Multiple transgenes in a single line (transgene stacking)?

  If yes, will this be by:

  (1) Crossing of independent lines?
  - If yes – will different marker or reporter genes in different lines help me to select hybrids?

  (2) Multiple rounds of transformation?
  - If yes – will I need more than one selectable marker gene or can I remove the marker gene in some way (for example, by co-transformation followed by segregation)?

  (3) Insertion of multiple genes into one binary Ti vector?

  If yes:
  - Will it be desirable to have marker genes at the right and left borders?
  - Will a good choice of unique restriction sites be required in the vector?
  - Can multiple transgenes be added easily, one after the other, into the vector?

Is the vector suitable for the plant transformation method?
- Does the binary Ti vector series have the required selectable marker?
- Will the preferred *Agrobacterium* strain be compatible with my choice of vector?
- Are the cultured explants affected by antibiotics used to remove *Agrobacterium*?

How many plant species do I want to transform?
- Will the binary vector have the right selectable marker gene(s) for all species?
- Will different *Agrobacterium* strains be used and be compatible with the vector?

plasmid origins and bacterial selectable marker genes within their T-DNAs. These allow T-DNAs and flanking DNA to be excised from transgenic plants.

Binary Ti plasmids are also available for promoter transcriptional or translational fusions to reporter gene coding sequences, such as versions of pBIN19 (Table 1) that harbour a promoterless GUS (*uidA*) coding sequence that allow translation fusions in all three possible reading frames [pBI101.1, pBI101.2, pBI101.3; available from Clontech (Palo Alto, CA, USA)][25,26]. The plasmids pMON10098 (Fig. 1) and pROK1 (Ref. 27), a derivative of pBIN19 (Fig. 1), contain a cauliflower mosaic virus 35S promoter linked to a polyadenylation sequence in their T-DNAs. This permits the direct cloning of a coding sequence into the T-DNA of the plasmid, with the aim of proceeding to *Agrobacterium* and plant transformation as the next step. Other binary vectors have been created that allow the inducible expression of a coding sequence in a transgenic plant. For example, a glucocorti- [illegible] is available on a pMON derivative[28] (Table 1).

A further refinement that deserves more attention is the development of binary Ti plasmids with different selectable marker genes at both the LB and the RB [e.g. the pBIN19 derivative pNFHK1 (Ref. 29)]. Transformants that have undergone simultaneous double selection show improved and more consistent transgene expression than single marker gene selections. This might be an advantage in quantitative studies on transgene expression.

BiBAC binary Ti plasmid vectors have been developed that permit the insertion of large portions of DNA (up to at least 150 kb) and their transfer to *Agrobacterium* and thence to a plant[16,29]. Such vectors have low copy number in both *E. coli* (using either a P1 or F *ori*) and *Agrobacterium* (using a Ri *ori*)[16,30].

Future developments

There are three trends in plant science research that will stimulate the further development of binary Ti plasmid [illegible] The first is the continued development of user-friendly vector systems applicable to a wide range of species. This is already happening and plant researchers will only be restrained by their imagination in adapting these vector families for specific experimental purposes.

Second, plant genomics, with its demands for high throughput assessment of gene functions, will place considerable demands on the efficiency with which *Agrobacterium* technology can be handled. For example, the increasing use of map-based cloning approaches for gene isolation requires, at some stage, the complementation of mutants in the subject species and its relatives. Therefore, the development of more flexible BiBAC vectors that can take account of a wide variety of transformation systems is important.

Finally, the increasing debate about the safety of genetically modified plants for release into the environment is already ensuring that licencing authorities in many countries have tightened or will be tightening their requirements over the types of genetically modified plants that will receive approval. In particular, the removal (or absence in the first place) of extraneous DNA sequences that are not needed for expression of the introduced trait could stimulate the development of tightly defined vectors that make analysis of such plants easier to conduct.

The innovations that defined the early advances in plant transformation using *Agrobacterium*-mediated techniques have been followed by a series of smaller but important improvements in Ti vector design. As the demands and expectations of plant transformation evolve, so have the vectors, becoming progressively more user friendly. In an ever-demanding field, no single vector, strain or marker gene will predominate. Thus, as consumers of binary Ti vector technology, it might well pay researchers to shop around.

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Table 1. A selection of binary Ti vectors published in the peer-reviewed literature[a]

Vector[b]	Size (kb)	Unique restriction sites in T-DNA	LacZ	Bacterial selection on	Selectable marker at	Replication origin		Mobilization	Ref.
						Agrobacterium	*E. coli*		
pBIN19	11 777	9	Yes	Kanamycin	RB	pRK2	pRK2	Yes	14
pC22	17 500	2	No	Ampicillin, streptomycin and spectinomycin	RB	pRi	ColE1	Yes	21
pGA482	13 200	7	No	Tetracycline	RB	pRK2	ColE1	Yes	31
pPCV001	9200	6	No	Ampicillin	RB	pRK2	ColE1	Yes	6
pCGN1547	14 440	5	Yes	Gentamicin	LB	pRi	ColE1	Yes	32
pJJ1881	25 700	4	No	Tetracycline	LB	pRK2	pRK2	Yes	24
pPZP111	8909	9	Yes	Chloramphenicol	LB	pVS1	ColE1	Yes	7
pGreen0029	4632	18	Yes	Kanamycin	LB	pSa	pUC	No	5

[a]In the cases of families of binary vectors only those which confer kanamycin resistance on transgenic plants are described. Sizes are based on restriction enzyme digestion patterns or on nucleotide sequence.

[b]See Box 1 for e-mail addresses for obtaining most of the plasmids listed.

Abbreviations: LacZ, β galactosidase α subunit gene for α-lac complementation; LB, left border; RB, right border. Mobilization refers to the ability of the plasmid to be transferred from *E. coli* to *Agrobacterium* by conjugation.

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Table 2. Disarmed *Agrobacterium tumefaciens* strains defined by the *Agrobacterium* chromosomal background and the Ti plasmid they harbour[a]

Agrobacterium strain[a]	Chromosomal		Ti plasmid		Opine[b]	Ref.
	Background	Marker gene[c]		Marker gene[c]		
LBA4404	TiAch5	rif	pAL4404	spec and strep	Octopine	3
GV2260	C58	rif	pGV2260 (pTiB6S3ΔT-DNA)	carb	Octopine	32
C58C1	C58	–	Cured	–	Nopaline	33
GV3100	C58	–	Cured	–	Nopaline	34
A136	C58	rif and nal	Cured	–	Nopaline	35
GV3101	C58	rif	Cured	–	Nopaline	34
GV3850	C58	rif	pGV3850 (pTiC58Δonc. genes)	carb	Nopaline	36
GV3101::pMP90	C58	rif	pMP90 (pTiC58ΔT-DNA)	gent	Nopaline	6
GV3101::pMP90RK	C58	rif	pMP90RK (pTiC58ΔT-DNA)	gent and kan	Nopaline	6
EHA101	C58	rif	pEHA101 (pTiBo542ΔT-DNA)	kan	Nopaline	37
EHA105	C58	rif	pEHA105 (pTiBo542ΔT-DNA)	–	Succinamopine	38
AGL-1	C58, RecA	rif, carb	pTiBo542ΔT-DNA	–	Succinamopine	39

[a]See Box 1 for e-mail addresses for obtaining most of the strains listed.
[b]Grouped according to the opine catabolism of the original progenitor wild-type strain and/or non-disarmed parental Ti plasmid. This generally accepted classification of *Agrobacterium* strains does not necessarily imply that their disarmed counterparts still make opines.
[c]Antibiotic resistance gene used to select for that strain of *Agrobacterium* or Ti plasmid.
Abbreviations: rif, rifampicin resistance; gent, gentamicin resistance; nal, nalidixic acid resistance; kan, kanamycin resistance gene for bacteria (*npt*I or *npt*III); carb, carbenicillin and ampicillin resistance; spec and strep, spectinomycin and streptomycin resistance; –, no marker gene present.

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Optimization of *in vitro* culture conditions for *Pinus radiata* embryos and histological characterization of regenerated shoots

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ABSTRACT

Different *in vitro* culture conditions were tested on *Pinus radiata* organogenic embryos. Optimum shoot induction occurred at 26.1°C, whereas the best elongation resulted at 21.4°C. Supplements of 2.5 mg/l or 5 mg/l of BAP added to the induction media produced a similar number of regenerated shoots, which differed statistically from 1.0 mg/l of BAP and 0.025 mg/l TDZ. Addition of 10 mg/l $MnSO_4$ to $LP_{1/2}$ medium significantly increased the number and quality of *in vitro* regenerated shoots. The removal the apical region of shoots cultured in LP 2.5 mg/l of BAP increased the number of *de novo* generated shoots by 23%, compared to a control group with intact shoots. Approximately 70% of the *in vitro* shoots of *P. radiata* were of wet phenotype (hyperhydrated appearance); the rest were waxy in appearance. Histological cuts did not produce any differences in phenotypes, but scanning electronic microscopy of needles gave evidence of differences in epicuticular wax deposits.
Abbreviations: LP: Quoirin and LePoivre basal medium, without plant growth regulators; LP_1: LP medium + 1 mg/l BAP; $LP_{2.5}$: LP medium+ 2.5 mg/l BAP; LP_5 : LP medium + 5 mg/l BAP; $LP_{1/2}$: LP basal medium at half strength of macroelements, 2% commercial sugar, ammonium nitrate 100 mg/l, calcium nitrate 564.5 mg/l, hydroxyquinoleine 1.25 mg/l, MS vitamins and without plant growth regulators; $LPT_{0.025}$: LP medium + 0.025 mg/l TDZ; BAP: N-6 benzylaminopurine; TDZ: Thidiazuron.

Key words: *P. radiata*; BAP, TDZ, *in vitro* culture; wet and waxy phenotype

INTRODUCTION

Pinus radiata is Chile's most important forest species for wood and cellulose production. Selection of elite phenotypes and controlled pollination are currently used for its genetic improvement. However, this is an expensive and laborious method that produces a small number of seeds with high genetic and commercial value. With the aim of increasing the number of selected individuals obtained by traditional methods, *in vitro* micropropagation from the embryos of controlled pollinated seeds has been implemented. Given the multiplying effect of the process, it is essential to ensure the conservation of the elite genotypes throughout the successive stages of propagation.

Micropropagation of *P. radiata* is based on the induction of zygotic embryos by organogenesis (1). The success of the method depends upon the number and quality of the adventitious shoots produced per embryo. Our results have shown high heterogeneity in the regenerative capacity of the embryos and in the quality of the shoots produced. A significantly high proportion of regenerated shoots show dark green and clustered needles that appear to be hyperhydrated. This phenotype, which is also difficult to acclimatize, was described as "wet" by Aitken-Christie *et al* (2). High quality shoots that can be acclimatized suc-

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cessfully were designated "waxy" (2) due to their appearance.

Some authors propose that the shoot generation ability of *in vitro* cultures of *P. radiata* has a genetic component (4, 17). It is not clear to date whether the wet and waxy phenotypes had a genetic origin, if they were determined by nutritional and environmental factors, or if they were somaclonal variants induced by *in vitro* conditions. Aitken-Christie *et al* (2), suggest that the wet phenotype corresponds to an intermediate state of hyperhydricity between the vitrified (translucid) and the waxy shoot and that there might be culture medium factors that trigger this response.

Cytokinins, micronutrients and microenvironmental conditions have been documented to play an important role in the morphogenic response of several forest species (2, 4, 5, 22, 25, 26). In our current work, we have studied the effect of different culture conditions on the number and quality of shoots produced through embryo organogenesis. Temperature effect, different BAP concentrations and manganese addition to embryo cultures were specifically tested. The induction of lateral buds through apex removal and needle pruning of regenerated shoots was also assayed.

Morphological and histological characterizations of the two observed phenotypes were carried out to examine the differences at the cellular and tissue levels.

MATERIALS AND METHODS

Plant material and culture conditions

Selected *P. radiata* seeds were provided by the Forestal Mininco, S.A. nursery. The seeds were surface sterilized by soaking them in 2% (w/v) aqueous $NaHClO_4$ for 10 minutes and in 50% (v/v) aqueous H_2O_2 for 5 minutes, followed by three rinses in sterile distilled water. The seeds were then stratified at 4°C for 24 hours and sterilized again in 50% (v/v) H_2O_2 for 5 min. After rinsing, the embryos were obtained by dissecting the seeds.

The embryos were cultured in an inverted position (2) with the cotyledons immersed in a bud induction medium (20) (LP2.5 supplemented with 3% w/v commercial sugar, 0.8% Gibco agar and 2.5 mg/l BAP). The pH was adjusted to 5.8 before autoclaving for 20 minutes at 121°C. After 3 weeks, explants were passed to elongation steps for approximately four months (2). In this stage, the explants were successively transferred every 4 weeks to glass jars (7 x 10 cm) containing 20 ml of hormone-free $LP_{1/2}$ medium. Cultures were maintained at a 16-hour photoperiod with a light intensity of 60 $\mu Em^{-2}s^{-1}$ at 22 +/- 6°C. Isolated shoots with an average height of 1.2 cm were placed on LP medium containing 0.2% activated charcoal (Merck 2186) for one additional transfer before rooting. Root initiation was induced on a water-agar medium containing 2.0 mg/l indolebutyric acid (IBA) and 0.5 mg/l napthaleneacetic acid (NAA) for 5 days. The induced shoots were then transferred onto a low sugar $LP_{1/2}$ (containing 10 g/l commercial sugar) to evaluate rooting.

Temperature assay

The effect of temperature on shoot induction and the elongation processes was studied. Sixty embryos were pre-cultured as described above for one week. The embryos were then individually cultured in 8.0 x 2.5 cm glass jars containing $LP_{2.5}$ medium and used in the following assays.

Shoot induction: 12 groups of five embryos each were transferred to an illuminated (60 $\mu Em^{-2}s^{-1}$) chamber with a controlled continuous temperature gradient at one of the following temperatures: 13.5°C; 16.3°C; 19.7°C; 22.9°C, 24.1°C; 26.1°C; 28.5°C; 30.8°C; 31.3°C; 31.9°C; 32.8°C; 34.1°C. The explants were shoot induced for three weeks, transferred to $LP_{1/2}$ and then cultured for an additional month in the temperature gradient controlled chamber. After 7 weeks of exposure to regulated temperatures, half of the meristematic tissue of each embryo was transferred to the regular growth chamber (22 +/- 6°C). Four weeks later (week 11) the shoot buds per explant at each temperature treatment were counted.

Shoot elongation: the remaining meristematic clumps were maintained in the temperature gradient controlled chamber, now adjusted to 13.5°C; 17.7°C; 21.8°C; 24.1°C; 26.1°C; 28.5°C; 30.8°C; 31.3°C; 31.9°C; 32.8°C; 34.1°C for another four weeks to identify the best shoot elongation temperature. Since the temperatures set at this stage were not the same as those of the induction step, the following changes were made: the embryos induced at 16.3°C were elongated at 17.7°C; those induced at 22.9°C were elongated at 21.8°C; and those induced at 19.7°C were equally distributed between these two temperatures. All other embryos had the same induction and elongation temperatures. At this point, one-third of the shoots were transferred to the regular growth chamber. The remaining shoots (2/3) were maintained in the temperature-controlled chamber and evaluated after 4 weeks of culture (15 weeks total) to determine the quantity of waxy shoots and the total number of shoots produced at each temperature. This assay was repeated twice. As a control, 35 embryos were cultured for 15 weeks in the regular growth chamber.

Cytokinin effect on shoot induction

Cytokinin concentrations tested on adventitious bud induction were selected from previous reports (2,10,15). Twenty-five embryos were cultured in one of the following treatments: Thidiazuron 0.025 mg/l ($LP_{T0.025}$); Benzyladenine 1 mg/l ($LP_{1.0}$), 2.5 mg/l ($LP_{2.5}$), or 5.0 mg/l ($LP_{5.0}$). After the induction period (3 weeks), the explants were cultured in hormone-free $LP_{1/2}$ for six months. The number of buds per embryo and the percentage of waxy shoots were evaluated. Statistical analysis was done applying the Mann-Whitney test ($P< 0.05$), and the Z test corrected for continuity.

Effect of additional Manganese on the elongation step

Fifteen 7-week-old induced embryos were cultured in LP medium containing 10 mg/l $MnSO_4$ (12, 21) and tested against an identical control group cultured in a normal LP (0.76 mg/l $MnSO_4$) (20). Shoot elongation steps were performed as described above for four months. At the end of this period, the following parameters were evaluated: a) number of buds per embryo, b) total number of shoots produced per treatment, c) percentage of waxy shoots in each treatment, and d) average height of the shoots produced in each treatment.

Induction of axillary shoots

From 33 to 116 isolated shoots with an average height of 1.7 cm were cultured for 2 weeks in the following multiplication media: $LP_{1/2}$: control, intact shoots, $LP_{2.5}$: intact shoots, $LP_{2.5}H$: half-pruned back needles, and $LP_{2.5}A$: apically-pruned shoots. The shoots were then transferred to the elongation steps ($LP_{1/2}$ for four months), after which each treatment was evaluated for its ability to produce axillary shoots as follows: multiplication factor (n° of final shoots / n° of initial shoots). Statistical analysis of the discrete data was carried out by the Mann-Whitney test ($P< 0.05$) to compare means.

Morphological and histological analysis of wet and waxy Pinus radiata shoots

Morphological analysis: Wet and waxy *in vitro* cultured *Pinus radiata* shoots were characterized with a Nikon SMZ-10 stereoscopic microscope and photographed. A scanning electron microscope (SEM) was used to examine the epicuticular needle surface of wet and waxy shoots according to Fowke (11). Needles were fixed in 50% v/v aqueous glutaraldehyde, dehydrated in a graded series of absolute acetone with 20% increments, freeze-dried and coated with gold/palladium using a splutter coating unit. Samples were then examined under a JEOL JSM-25-S-II scanning electron microscope for epicuticular wax development.

Histological analysis: Samples were prepared for optical microscopy according to the O'Brien and McCully modified method

(19). Needles were fixed in FAA solution (formaldehyde 5%, acetic acid 5%, ethanol 63% and water 27%), gradually dehydrated in a graded series of absolute ethanol using 20% increments, vacuum infiltrated, and gradually embedded in Paraplast. Samples were sectioned in a rotating microtome and stained with safranine (0.5% w/v) and fast green (0.5% w/v). Sections were observed and photographed using a Nikon HSX-DX optical microscope.

RESULTS

Temperature assay

The best temperature for the induction and elongation of *Pinus radiata* shoots was determined by evaluating the quantity and quality of single shoots generated per embryo. The number of shoots produced at each temperature is shown in Figure 1. The best shoot bud induction (after 11 weeks of culture) occurred at 26.1°C, obtaining 134 shoots per embryo. This temperature level also yielded the highest percentage of waxy shoots (34%, not shown). These tendencies continued after 6 months of culture. Lower temperatures caused a significant reduction in the response. Conversely, higher temperatures inhibited shoot development (absence of bars above 30.8°C in Figure 1). Shoot elongation was evaluated by assessing the quantity and quality of single shoots with an average height of 1 cm after four months of culture. The best shoot elongation temperature (evaluated after 15 weeks of culture) was obtained at 21.8°C. Elongation was also acceptable at 24.1°C; 26.1°C and 28.5°C (Fig 1). The control treatment carried out in the growth chamber yielded 31 shoots per embryo with 28% waxy shoots.

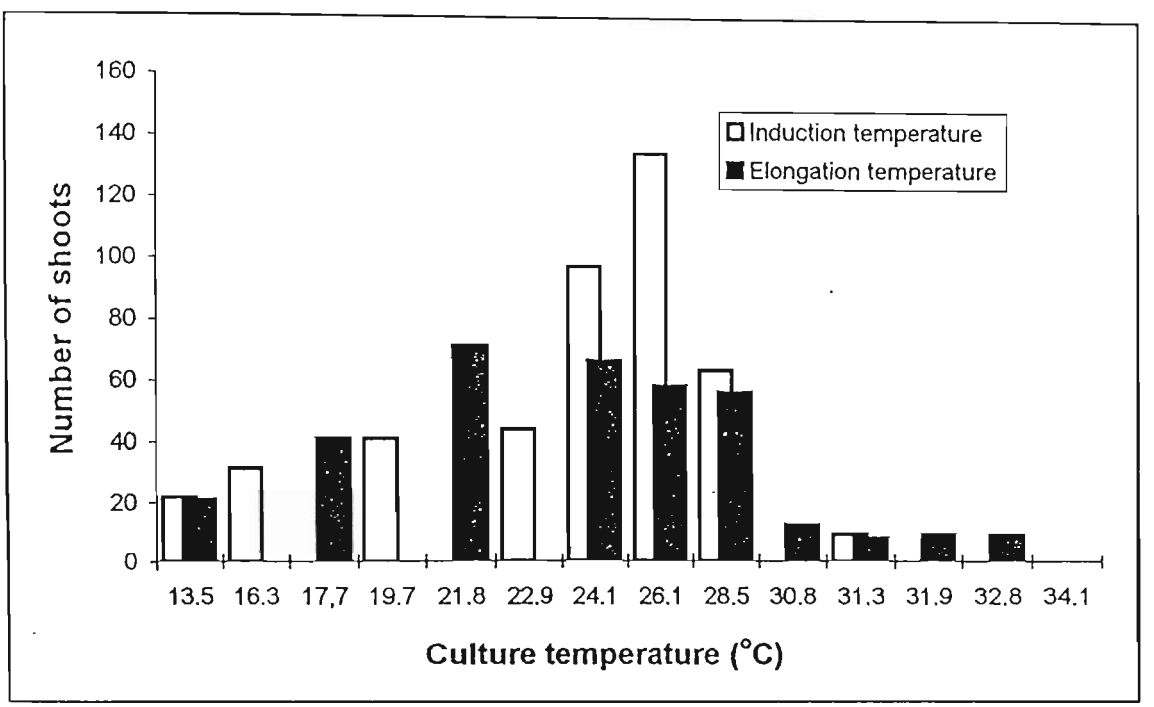

Figure 1: Effect of temperature on the induction and elongation of *P. radiata* shoots*.

Induction temperature: number of shoot buds per embryo at the end of the induction treatment (11 weeks).

Elongation temperature: number of shoots (1cm height) per embryo at the end of the elongation treatment (15 weeks).

* Absence of bars above 30.8 °C means no shoot development.

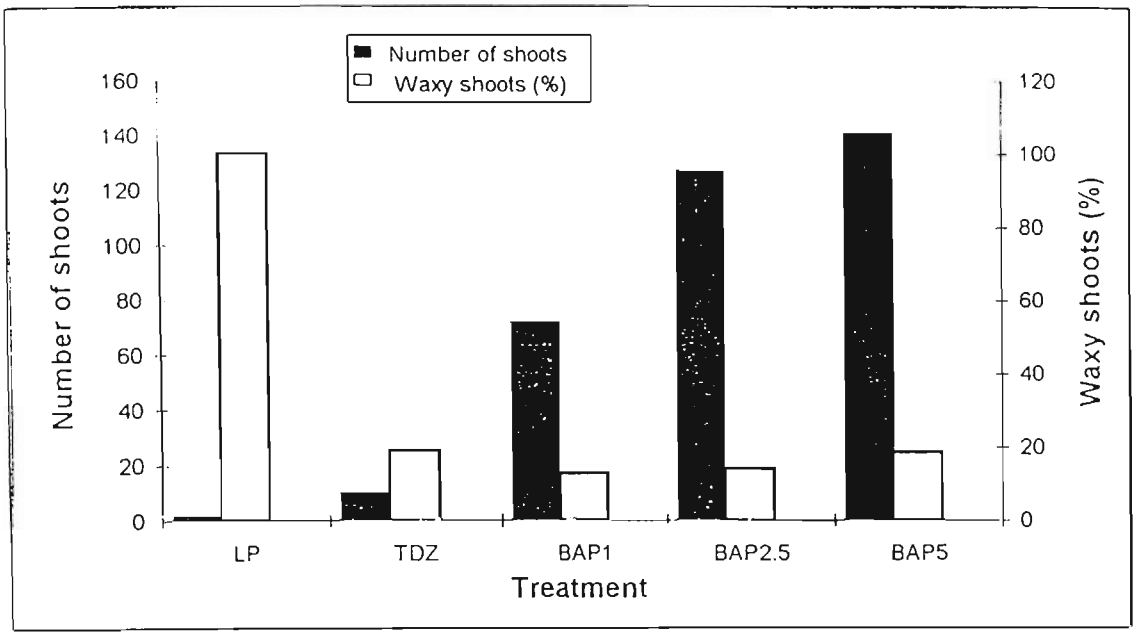

Figure 2: The number of shoots produced per embryo and the percentage of waxy phenotypes obtained from the *in vitro* culture of *P. radiata* with different treatments of BAP or TDZ.

25 embryos were cultured in each treatment and evaluated 6 months later.

Evaluation of cytokinin concentration on bud induction

BAP induced a higher average number of buds per explant than TDZ. Overall, 5 mg/l BAP proved to be the best concentration in terms of number of shoot buds per explant (140.8) (Fig 2) and total number of shoots (1,267, not shown). Although data on the difference in the number of shoot buds produced per explant between 2.5 mg/l and 5 mg/l BAP were not statistically significant, the latter generated better quality shoots (Fig 2). Regeneration of shoots per embryo with 0.025 mg/L TDZ and other previously-tested concentrations (0.001 mg/l and 0.05 mg/l) was 10 times less productive than with 5 mg/l BAP. However, shoots induced with TDZ were larger than those induced with BAP.

Effect increased $MnSO_4$ in LP medium

We observed that greenhouse-acclimatized *in vitro Pinus radiata* plants were deficient in Mn. A comparative foliar analysis of the Mn content of *in vitro* cultured shoots and *ex vitro* plants grown in the greenhouse showed a significant decrease of this microelement in the *in vitro* cultured shoots (20 ppm *in vitro*, 197 ppm *ex vitro*).

Since the Mn concentration in LP medium is 13 times lower than in other media used in *Pinus radiata in vitro* cultures (21), we tested the addition of $MnSO_4$. LP medium was supplemented with 10 mg/l of Mn (as $MnSO_4$) to compare the effect of manganese on the growth and elongation of shoots in relation to LP basal medium (0.76 mg/l $MnSO_4$). After 10 weeks, an increase of 53.8% in the number of shoots per embryo was obtained in the 10 mg/l $MnSO_4$ treatment. The percentage of waxy shoots at this concentration was also greater (80%) (Table 1).

Induction of axillary shoots

Pinus radiata shoots were induced for two weeks on LP medium supplemented with 2.5 mg/l BAP, with or without the pruning of lateral needles and apex, in order to assess large scale propagation by axillary bud multiplication of elongated shoots. A good response was obtained in

TABLE I

Effect of an increase in the concentration of Manganese in the number and quality *of P. radiata* shoots generated *in vitro*.

Treatments	Total number of shoots produced	Number of shoots produced per embryo	Shoot Quality
$LP_{1/2}$ + 0.76 mg/l $MnSO_4$	782*	52	Size: 1 cm waxy shoots: 14.4%
$LP_{1/2}$ + 10 mg/l $MnSO_4$	1037**	80	Size: 1 cm waxy shoots: 26%

*15 embryos were cultured for 4 months before their evaluation. **13 embryos were cultured for 4 months before their evaluation.

all BAP-treated shoots. An increase of 53% in the number of axillary shoots occurred when they were induced on $LP_{2.5}$, compared to the control $LP_{1/2}$ (Fig 3). $LP_{2.5}$ treatment led to the production of an average of 4 axillary buds per shoot, compared to 1.87 with $LP_{1/2}$ (Fig 3). Removal of the apical meristem ($LP_{2.5A}$) increased the effect of $LP_{2.5}$ (intact needles) by 23%.

Morphological and histological analysis of in vitro wet and waxy Pinus radiata shoots

The *in vitro* culture of *Pinus radiata* embryos produced wet and waxy phenotypes (Figs 4A-B). However, the predominant wet phenotype (70%) was not suitable for greenhouse acclimatization (62%

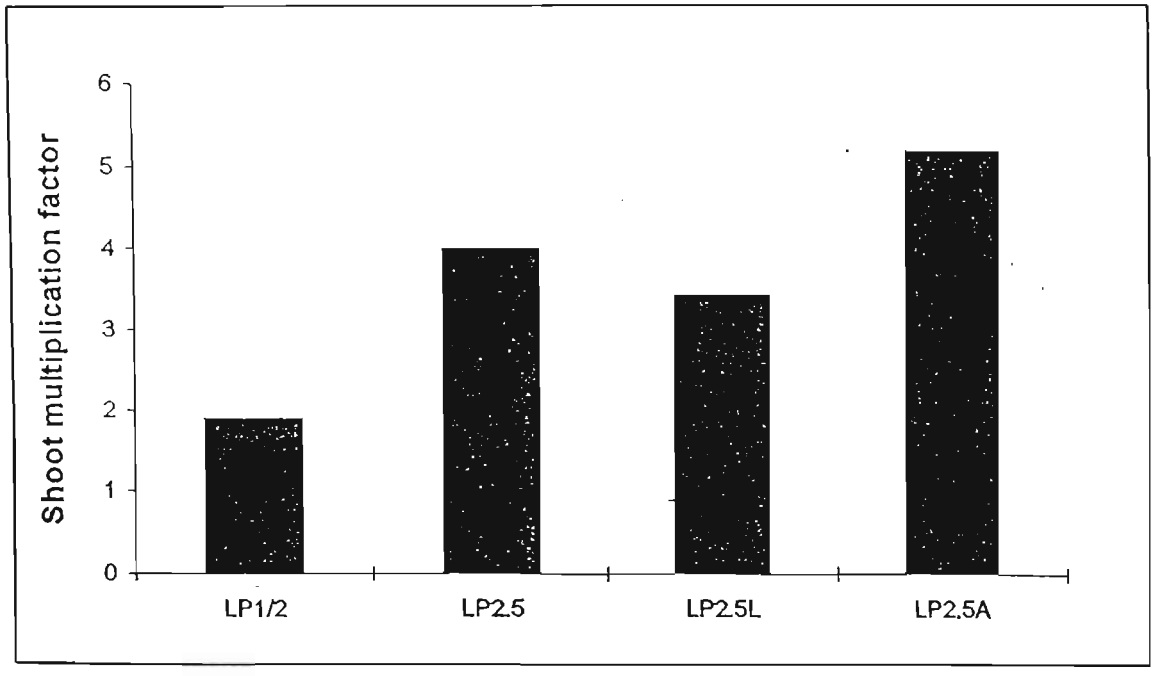

Figure 3: Effect of cutting on shoots and the addition of BAP in LP medium on the induction of lateral buds in *P. radiata* shoots grown *in vitro*.

The shoot multiplication factor was calculated as follows: Number of final shoots produced/ Number of initial shoots.

mortality) due to its high susceptibility to infection and dehydration. Conversely, the waxy phenotype showed a 67% *in vitro* rooting rate and 2% mortality during greenhouse acclimatization.

Histological sections showed similar tissue organization in both phenotypes. Epidermis structure, resin channels, and vascular conducts all showed normal appearance (Fig 4 C-D). Needle surfaces of

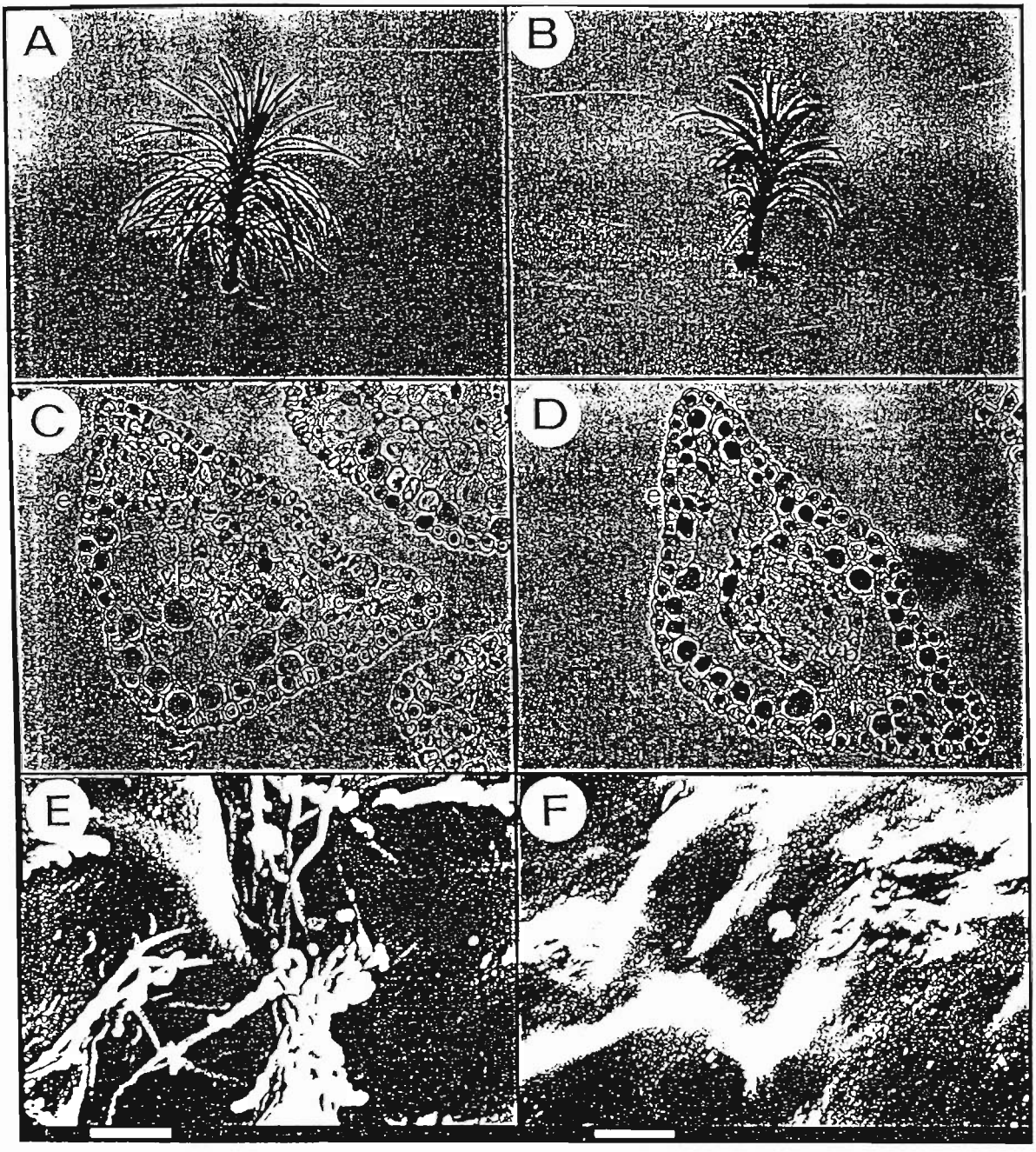

Figure 4: Morphological and histological characteristic of waxy and wet phenotypes. A-B. Appearance of waxy and wet phenotypes respectively. C-D. Histological cuts of *P. radiata* needles of waxy and wet phenotypes respectively grown *in vitro*. e: epidermis, rc: resinous channels, vb: vascular bundle. E-F: Scanning electron microscopy of the epidermal surface of *P. radiata* needles of waxy and wet phenotypes grown *in vitro*. E: Detail of globular and tubular waxes that are present in aged needles of waxy phenotype (20,000x). F: Needle epidermis of the wet phenotype with a marked reduction in globular waxes and an absence of tubular waxes (20,000x).

wet and waxy shoots are shown in Figures 4E-F. Tubular epicuticular wax deposits were abundant on the epidermis of waxy needles (Fig 4E), but were absent in wet needles (Fig 4F). Globular epicuticular wax was less abundant on wet needles than on waxy needles.

DISCUSSION

The protocol for *in vitro* regeneration of *Pinus radiata* was optimized by changing both environmental components and the culture medium. By means of a detailed study, we determined the best temperature for induction and shoot elongation processes. The optimum temperature for shoot induction based on the number and quality of shoots was 26.1°C (Fig 1), which is similar to temperatures used by Aitken-Christie *et al* (1) (28°C daytime and 24°C nighttime). Optimum shoot elongation, however, was achieved at a lower temperature (21.8°C), with favorable results at temperatures up to 28.5°C. Previous information given by other authors has shown that the elongation is optimal at temperatures of 24°C and 20°C (day and night, respectively) (1,2). Temperatures above 28.5°C considerably affect the shoot elongation process, producing clear signs of dehydration, necrosis and browning of the lower needles.

The stimulating effect of cytokinins on the induction of shoots on *P. radiata* embryos, as described by other authors (5, 22), was also tested in our work. A concentration of 2.5 mg/l of BAP was sufficient to obtain a high number of good quality shoots per embryo. Previous experiments conducted in our laboratory using concentrations of 0.001 mg/l to 0.05 mg/l TDZ did not induce more than 15 shoots per embryo (not shown). However, even when TDZ did not show a significant effect on the number of shoots (Fig 2), those induced were significantly larger and more vigorous than the BAP-induced shoots. Our results differ from other reports (14), which have demonstrated that TDZ stimulates shoot proliferation in various legumes (10) and woody species (14). Through shoot induction with BAP, a high percentage of wet shoots were obtained in all the treatments. This phenotype is not desirable due to its high mortality (62% in our case) during acclimatization in the greenhouse (18). It is possible that the high cytokinin concentration (BAP 5 mg/l) used for shoot induction increases the endogenous concentration of this growth regulator, influencing the number of hyperhydrated shoots (8). We did notice however, that in one of the analyzed families, induction with 1 and 2.5 mg/l BAP also yielded a high percentage of wet shoots, which suggests a partial genetic component for this response. In this sense, the evaluation of the response obtained with different families of *Pinus radiata* showed a high variability in the percentage of wet and waxy shoots produced (not shown). Bergmann and Stomp (4) previously described a genotypic effect on the rooting of *P. radiata*. We, however, have not found genetic differences between wet and waxy phenotypes in preliminary RAPDS analysis (23).

Adding 10 mg/l of manganese ($MnSO_4$) to LP medium showed a significant stimulating effect on the number and quality of generated shoots (Table 1). The concentration of this microelement in the original LP medium (0.76 mg/l) apparently did not cover the *P. radiata* requirements. Important lignin synthesis enzymes, such as peroxidase, require manganese as a cofactor, which could justify adding it in higher concentrations to *in vitro* cultures of woody species (12, 21).

While evaluating the induction of axillary shoots over a 6-month period, we observed that BAP alone in the culture medium increased the number of new shoots per explant by 53% over that of the control group. An additional 23% increase in new shoots was achieved by removing the shoot apex. It is likely that a decrease in the endogenous level of auxins, due to the loss of the apical meristem in conjunction with an increase of the cytokinins level in the culture medium, triggered axillary bud development in *in vitro* cultured shoots. Das *et al* (7) also determined that the regeneration of *Vigna mungo* shoots was observed only when explants were derived from the

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New methods of diagnosis in plant pathology — perspectives and pitfalls[1]

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As a spin-off from fundamental molecular biological research, there has been a remarkable increase in new methods for diagnosis (i.e. detection and identification) in recent years. Because of their origin, these new methods all have in common that they use structural elements of the target organisms such as nucleic acids, lipids, fatty acids, proteins, polyamines and polysaccharides as a basis. These structural elements are either used as templates for development of so-called probes for detection (and identification) or they are placed into man-made patterns and used for identification/classification. The article presents the advantages and perspectives of the new methods compared with conventional ones. It may be noted that, in many studies, the specificity and reproducibility of the new methods has not been adequately treated or has even been only assumed. These features, which are closely linked with experimental and sampling error, lead to the principal pitfalls of the new methods, which are also reviewed.

Introduction

As a spin-off from fundamental molecular biological research, there has been a remarkable increase in new methods for diagnosis, i.e. detection and identification, of microorganisms, viruses and viroids. Due to their origin, these methods have in common that they use structural elements of the target organisms as a basis, such as nucleic acids, polyamines, proteins, lipids, fatty acids and (lipo)polysaccharides. These structural elements may be either used for, or function as, templates for the development of so-called probes for detection (and identification), or else may be placed into man-made patterns and used for identification/classification.

The new methods appear to be fast replacing conventional methods. Fig. 1 illustrates this for four phytopathological disciplines over the past 10 years. The largest changes took place where conventional methods are very laborious and the organism cannot be cultured (especially in virology and mycoplasmatology). The least change was observed for disciplines where morphology still plays an important role (entomology). The question is whether the new methods can and should completely replace the conventional ones which are based on and use an accumulation of data on organisms over many years. Or are they no more than a welcome addition to already existing methods? In other words, do these new methods, as is sometimes claimed, provide us with 'final' or 'ultimate' answers, making data from conventional methods superfluous?

The purpose of this article is to present in a non-exhaustive way a short description of new methods used in plant pathology, their perspectives (theoretical and practical) and also some of their pitfalls. Since the advantages of the new methods have so often been stressed in the literature, the pitfalls will here receive a little more attention. These pitfalls should not be understood as fatal drawbacks but as a stimulation for optimal use of the new methods and for integration of data generated with them into the existing body of evidence on organisms,

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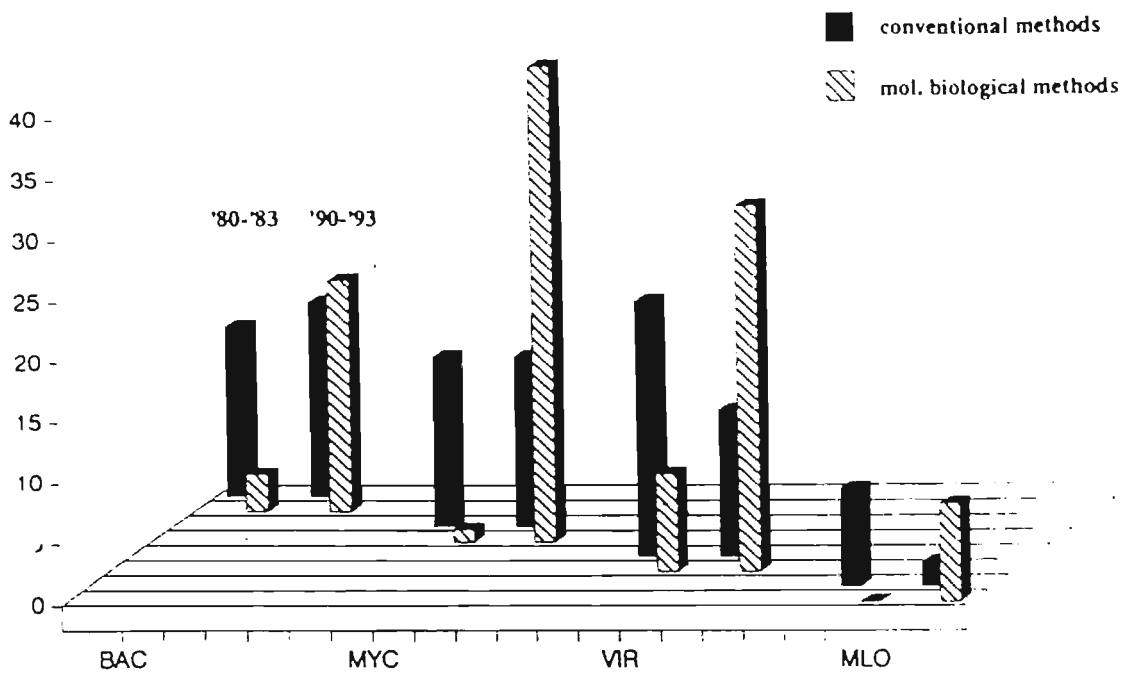

Fig. 1. Increase in the use of molecular biological detection and identification methods for different disciplines (bacteria, fungi, viruses, MLOs), in articles published in *Phytopathology* over the years 1990/1993 as compared with 1980/1983. Black columns, articles based on conventional methods; shaded columns, articles based on molecular biological methods.
Utilisation intensifiée des méthodes moléculaires de détection et d'identification pour les différentes disciplines biologiques (bactéries, champignons, virus, MLO), en fonction du nombre d'articles publiés dans *Phytopathology* en 1990/1993 et en 1980/1983. Colonnes noires, articles basés sur des méthodes classiques; colonnes hachurées, articles basés sur des méthodes moléculaires.

viruses and viroids. The literature cited is very far from exhaustive (more information becomes available every day) and is used as illustrative background material.

Examples of new methods used for detection (and identification)

Proteins

Methods using monoclonal antibodies (Schots, 1990; Hagler *et al.*, 1993) or monospecific polyclonal antibodies (Niepold & Huber, 1988; Errampalli & Fletcher, 1993) have the advantage of using antisera which are potentially more specific than ordinary polyclonal sera. However, cross reactions with non-target organisms may still occur. Sometimes antibodies are used in combination with nucleic acid methods mentioned below and/or with magnetic capture, using magnetic beads. In the latter case, antigens (e.g. bacteria, viruses) are first trapped from a solution by antibodies coated on magnetic beads and the beads trapped by a magnet. Subsequently PCR can be performed on the concentrated antigens (Wetzel *et al.*, 1992; Kapperud *et al.*, 1993). Another example of immuno-PCR is a method where antibodies directed to RNA-DNA hybrids may be used for sensitive detection of PCR products. An RNA probe is hybridized to a DNA PCR product on a blot, whereafter immuno-enzymatic assay with an anti RNA-DNA hybrid antibody takes place (Blais & Phillippe, 1993).

Relatively new is the use of two-dimensional (2D) electrophoresis of proteins in the study of insects (Osakabe & Sakagami, 1993) and the use of serology for the detection and identification of nematodes (Lawler & Harmey, 1993; Lawler *et al.*, 1993).

Nucleic acids

Nucleic acid sequences are used as probes and primers in the following methods. They include:

(1) random sequences (chromosomal, plasmid, mitochondrial, ribosomal);
(2) internal transcribed spacers;
(3) genes encoding a toxin or enzyme;
(4) variable region rRNA.

Dot slot-filter hybridization

These hybridization methods use short single-stranded nucleic acid molecules specific for the target organism ('probes'). The nucleic acid of the target organism is fixed on a nitrocellulose or nylon membrane (filter). Hybridization methods have proved not to be very sensitive for detection, i.e. a high copy number of target nucleic acid must be present for a sufficient signal. The signal can be achieved by radioactive or nonradioactive (e.g. biotin-streptavidin) labelling (Rasmussen & Reeves, 1992). Usually sensitivity is equal to or less than ELISA. This may be enhanced by application of enrichment culture (Manulis, 1992). Useful applications are: (1) detection of organisms in diseased tissue or on agar plates (colony-blotting) and (2) verification of identity of PCR products (see below) and in RFLP studies (see below). In the latter case, target nucleic acids are transferred from a gel to a membrane in order to be analysed ('blotting').

In situ hybridization using rRNA-targeted oligonucleotide probes

In this method, short (20–30-meric) oligonucleotide probes are used against 16S or 23S rRNA. These oligonucleotide probes can be used for *in situ* hybridization, because they are able to diffuse through the cell wall of microorganisms which are present in thin tissue sections or in plant or soil extracts fixed on a microscopic slide. For Gram-positive bacteria, a lyzozyme step is sometimes necessary to enhance penetration of the probe into the cell (Hahn *et al.*, 1993). Sensitivity may also depend on the metabolic activity of the cells (Hahn *et al.*, 1992). When probes have been labelled with a fluorescent dye or a gold label, the microorganisms can be vizualized by incident light (fluorescence) microscopy (Amann *et al.*, 1990; Poulsen *et al.*, 1993). Technical sensitivity can be enhanced by using confocal laser scanning microscopy (Lizard *et al.*, 1994).

Polymerase chain reaction

With this method, target nucleic acid is artificially multiplied by repeated cycles of: (1) denaturation (melting) of nucleic acid, (2) annealing of short (specific) oligomer strands of nucleic acids ('primers'), and (3) extension of nucleic acid strands in the presence of free nucleotides and a thermostable nucleic acid polymerase (usually Taq-polymerase, isolated from the hot-spring thermophilic bacterium *Thermus aquaticus*).

Theoretically the sensitivity of PCR is very high, since one copy of target DNA in a sample can be detected. In practice, however, sensitivity is usually lower. To enhance specificity, 'nested' PCR may be performed: a first primer pair is used to multiply a larger fragment of the target, then a second primer pair is used to recognize and multiply a smaller part of the amplified sequence (White *et al.*, 1992; Yourno, 1992; Henson & French, 1993; Kapperud *et al.*, 1993). To verify identy of PCR products, hybridization with probes via blotting is often applied. When specific restriction sites are present in the product, restriction enzyme analysis (REA) can equally well be performed (see also under RFLP) (Jones & Dunkle, 1993; Powers & Harris, 1993).

Table 1. Comparison of new methods of identification/classification by pattern analysis
Comparaison des nouvelles méthodes d'identification et de classification basées sur l'analyse des profils

Method	Automation	Databank	Cost level	User-friendly
Fatty acid analysis	+	+	+ +	+ + +
Protein electrophoresis				
— whole cell	+	(+)	+	+ -
— isozymes	(+)	-	+	- +
RFLP analysis	-	-	+ +	+
RAPD analysis	+	(+)	+ +	+ +
rRNA sequencing	+	+	+ + -	-

PCR is also performed in combination with immunomagnetic capture (Kapperud *et al.*, 1993; Grant *et al.*, 1993) and fluorescent labels (Cano *et al.*, 1993) in order to increase sensitivity and/or specificity. In some tests, an ELISA-plate set-up is achieved (White *et al.*, 1992; Galindo *et al.*, 1993).

Examples of new fingerprinting methods used for identification and classification

Table 1 presents some new methods for identification and classification, together with some of their characteristics. Their principles are presented below.

Fatty acid profiling

Patterns are generated by automated gas-chromatographic analysis of whole-cell fatty acids. Microbial ID (Newark, US) has developed a highly standardized extraction and analysis procedure and software for identification. The databank of the system is at present one of the largest in the world and includes libraries for aerobic and anaerobic bacteria, clinically important bacteria, actinomycetes, mycobacteria and yeasts. A library of fungi is in preparation. Moreover, the system includes library-generating software, allowing inclusion of one's own (unknown) strains, and statistical software to perform taxonomic research.

Cells are grown for 24 or 48 h on a standard medium and 40 mg wet weight of cells is used for analysis. Fatty acid analysis (FAA) has high discriminating power (below species level) as a result of the high degree of standardization achieved and the stability of fatty acid patterns in microorganisms. There is good congruence with RNA/DNA : DNA hybridization data (Lechevalier & Lechevalier, 1988; Sasser, 1990; Janse, 1991; Welch, 1991).

Protein profiling

Whole-cell protein SDS-polyacrylamide electrophoresis

Profiles are obtained by extracting proteins from 40–50 mg wet weight of cells, denaturing and negatively charging the proteins and finally running electrophoresis in a polyacrylamide gel. After electrophoresis, proteins are stained and patterns compared. Comparison is visual or by computer-based analysis. In the latter case, gels are scanned and banding patterns digitized into peak patterns. Databases can then be constructed by the laboratory. Due to greater difficulty in inter-laboratory standardization than for FAA, no standard libraries are available with the gel scan and analysis software GELCOMPAR (AppliedMaths, Kortrijk, BE). GELCOMPAR

allows sophisticated data handling, library generation and statistical analysis for identification and taxonomic purposes. Whole-cell protein electrophoresis discriminates at a low taxonomic level (below species level) and there is a good correlation with RNA/DNA-DNA hybridization data (Kersters & De Ley, 1980, Vauterin & Vauterin, 1992; Vauterin *et al.*, 1992).

Isozyme analysis

Fingerprints produced by this method are based on the presence of multiple molecular forms (isozymes) of certain enzymes. These forms have similar properties, but are slightly different in amino-acid sequence and therefore in electrophoretic mobility. Different electrophoretic techniques, such as starch gel, polyacrylamide gel electrophoresis and/or isoelectric focussing, may be used to separate isozymes in their native form. Detection is by treatment with an appropriate colour-forming substrate for a particular enzyme. The technique is relatively cheap, numerous genetic loci may be compared (more than 100 enzymes can be used) and discriminates at species and (less frequently) below species level. The method is relatively time-consuming and standardization is not easy. Isozyme patterns may or may not be congruent to RFLP patterns; see below (Bonde *et al.*, 1993; Damai *et al.*, 1993; Oudman, 1992).

Restriction fragment-length polymorphism (RFLP) analysis

Patterns obtained by this method are based on mutations in the DNA sequence that change the recognition sequence of a restriction enzyme. Total DNA can be analysed in an agarose gel and fragments vizualized in an agarose gel after staining with ethidium bromide. However, fragments are usually (too) numerous and overlap so that scanning with a densitometer is difficult. Because of this problem, probes are used which render visible only the fragments which hybridize to the probe. To obtain a pattern, DNA is blotted after agarose gel electrophoresis on a filter (so-called Southern blotting). RFLP analysis discriminates at low taxonomic level, often strain level (Grimont & Grimont, 1991; Nicholson *et al.*, 1993; Vrain, 1993; Darasse *et al.*, 1994).

Random amplified polymorphic DNA (RAPD) analysis

With the RAPD method, patterns are obtained by PCR amplification of genomic DNA with arbitrary, short (about 10 bp), randomly chosen primers at a permissive annealing temperature of 36–45°C. The pattern of amplification products discriminates at low taxonomic level, often strain level. No prior sequence information about the target, probe, blotting and hybridization and restriction sites are necessary, making it a very fast method (Welsh & McClelland, 1990; Caetano-Anolles *et al.*, 1991; Cenis, 1993; Cenis *et al.*, 1993; Ouellet & Seifert, 1993).

Ribosomal DNA RFLP analysis (ribotyping)

Patterns are based on DNA restriction fragments containing all or part of the genes coding for 16 and 23S rRNA. Because genes coding for rRNA are very stable, a single probe can be used to subtype, for example, all eubacteria. Ribosomal operons are multiple, allowing discrimination at and below species level (Stull *et al.*, 1988; Grimont & Grimont, 1991). As in RFLP analysis, DNA is electrophoresed and transferred to a filter, and labelled rRNA used for probing. Labelling is by ^{32}P or by immuno-detected acetylaminefluorene.

Advantages of the new methods

The advantages of the new methods when compared with conventional ones are the following.

(1) They are rapid, sensitive and usually cost-effective.
(2) Thanks to (1), new methods may be suitable for integration into large-scale certification/inspection schemes for plants and plant products.
(3) Commercially available, standardized test kits can be produced.
(4) Non-culturable organisms, such as mycoplasma-like organisms (MLOs) (Lee *et al.*, 1993; Namba *et al.*, 1993; Vega *et al.*, 1993) or museum specimens, such as butterflies and dried fungi (Balat & Zakharov, 1992; Wingfield & Wingfield, 1993), can be analysed. Genetically modified organisms (GMOs) may be traced in the environment more easily (Kluepfel, 1993).
(5) New methods are apparently less sensitive to mutation or variation. But, for variation in a single uredinospore culture, see Doudrick *et al.* (1993) and for sensitivity of RAPD analysis to effects of cell/colony age, see Coutinho *et al.* (1993).
(6) Discrimination is possible at a low taxonomic level, even at the strain level.

Pitfalls of the new methods

In diagnosis of plant pathogenic organisms (and to a certain extent also of viruses, viroids and insects), Koch's postulates play a very important role. These postulates can be exemplified, in the diagnosis of a bacterial disease using conventional methods, by the following steps: (1) assessment of symptoms; (2) isolation of pathogenic bacteria; (3) pure culture of isolated bacteria; (4) identification of pure culture; (5) pathogenicity test; (6) reisolation from inoculated plants; (7) reidentification of pure culture; (8) diagnosis report. For many of the new methods, Koch's postulates are under pressure for several reasons. Firstly, the specificity of a test is assumed, but is often still far from certain. The number of strains or varieties of target and non-target organisms which have been tested to prove specificity is often dramatically low. However, it is precisely this specificity which is critical, since many new methods do not retain the organism in a living state, such that it can be reintroduced into a host and checked for pathogenicity. Secondly, reproducibility is often also assumed, but only tested to a very limited extent. This is especially important in analysis of patterns obtained by PAGE, RFLP, RAPD analysis where many parameters demand scrupulous standardization. These pitfalls and others will be presented below, together with some examples from the literature to show their relevance. The first six especially concern detection, the other five classification/identification.

Specificity and reproducibility unknown or only tested to a limited extent

Probes and primers should be tested with an extensive range of related and unrelated pathogenic and saprophytic organisms and known negative extracts of different regions/habitats. For example, Prosen *et al.* (1993) devised a PCR-based amplification of the phaseolotoxin gene region of the DNA of the toxin-producing bacterium *Pseudomonas syringae* pv. *phaseolicola*. They mentioned that *P.s. glycinea* also produces a phaseolotoxin-like substance. The primer pair was said to be specific for the detection of *P.s. phaseolicola* even though *P.s. glycinea* was not apparently tested for possible cross reaction. Although these authors tested 15 strains of the target organism, 15 strains of the related *P.s. syringae*, 11 other non-related pathogenic species (one strain each) and 32 saprophytes, their conclusion that they detected the pathogen in a commercial seed lot that failed to yield the pathogen by conventional plating methods is not necessarily valid. There are at least two other conclusions possible: (1) a cross reaction with a non-target organism took place, or (2) only nucleic acid of the target organism was detected. Statements that RAPD and PCR primers *generally* did not react with other species (e.g. Ouellet & Seifert, 1993) are not very informative and may make additional research necessary.

The reproducibility of RAPD analysis can be enhanced by annealing at lower stringency and

introducing a 1 min ramp time between annealing and extension (Lawrence *et al.*, 1993). In many cases, researchers generate their own patterns only once or a few times, let alone make any interlaboratory comparisons (Rademaker *et al.*, 1992).

Results may be influenced by change in environmental and experimental conditions and biochemicals used (experimental error)

Cenis (1993) found many non-reproducible bands in RAPD-PCR and asked for cautious standardization, given the enormous variety of thermal cycler and reagent sources. Relatively small changes in temperature and salt regimes may considerably change specificity of probes (Mirza *et al.*, 1993). In an excellent article, Sneath (1989) discusses the influence of experimental error (and sampling error) in relation to analysis of RNA sequence data in taxonomy.

Impossibility of discriminating between viable cells in a sample and non-viable cells, or traces of target nucleic acid

This pitfall is important in international trade, especially in the case of quarantine pests. What measures should be taken if one does not know whether the target cells detected are viable or not? What is the significance of a few copies of target DNA in a sample? In most cases, these two questions have not, or only poorly, been investigated. Josephson *et al.* (1993) report on the possibility of false positives with respect to viable cells in environmental samples analysed with PCR. Free DNA degraded in a period of 3 weeks. Smalla *et al.* (1993) found traces of free *Pseudomonas* DNA after a period of 5 months in soil.

PCR products are not necessarily derived from target DNA — verification is necessary

Due to the risk of cross-reacting PCR products (bands) from non target-organisms in ethidium bromide gel (ethidium bromide being a non-discriminative stain for nucleic acid), the identity of PCR products should be verified. This can be done by blotting products on a filter and hybridizing with a specific probe, or by restriction-enzyme analysis. In the latter case, the target sequence should contain a specific restriction site for an enzyme used (Marques *et al.*, 1993).

High concentrations of non-target organisms may give weak false positive PCR results (Blais & Phillippe, 1993).

Contamination in the PCR test may lead to false positives

The PCR test is very liable to the problem of contamination. Only one or a few copies of target DNA present in rooms where PCR is performed may already deteriorate results. For possible solutions of this problem, using for example ultraviolet irradiation of reagents, treatment of reagents or PCR reactions with exonucleases or substitution of dUTP for dTTP, see Henson & French (1993) or White *et al.* (1992).

A negative PCR test may have many hidden causes

(1) The target sequence may be absent from the sample, or may have disappeared, e.g. by the influence of nucleases (Gibson & McKee, 1993).
(2) Products in the sample or media, such as humic acids, proteins, fats, detergents, lysozyme, NaOH, alcohols and nucleases (Rossen *et al.*, 1992; Henson & French, 1993; Tebbe & Vahjen, 1993) may cause inhibition. For rapid but still laborious protocols for extraction of DNA from soil, see Smalla *et al.* (1993).
(3) The primer pair used is too specific, missing part of the population of the target.

(4) Experimental conditions are not suitable for optimal binding of primers. Wylie *et al.* (1993) stated that a rapid extraction procedure without phenol could be used to test lupin seed for the presence of cucumber mosaic cucumovirus when seed infection was more than 0.5%; at lower infection percentages the phenol procedure should be used. For official routine testing, this presents a dilemma: how do you know beforehand the infection percentage before you start a test?

Changing probes/primers/enzymes/methods/chemicals may yield different (conflicting) patterns or no patterns at all

RFLP of nuclear rDNA of *Pythium* spp. yielded species-specific patterns, while RFLP of mitochondrial DNA did not show much interspecific variation (Chen, 1992). The amount of DNA loaded may already be of importance (Nicholson *et al.*, 1993). *Listeria monocytogenes* and *L. innocua* were similar on the basis of the V2 region of 16S rRNA, but different on the basis of the V9 region (Czajka *et al.*, 1993).

An insufficient number of strains is tested, so that important conclusions are poorly supported

In a study where only a few isolates per pathotype of the fungus *Magnaporthe (Pyricularia) grisea* were sampled over a large geographic area, it was found that there was a close correlation between fingerprint and pathotype (Levy *et al.*, 1991). Subsequent work on a microgeographic scale (two fields) showed much more variation to be present in the pathotypes and much less correlation between pathotype and fingerprint (Xia *et al.*, 1993). Culture collections may be especially biased in this respect, since in many cases only readily identifiable strains are included. Deviating or intermediate strains are often not included.

In a study on RAPDs of *Fusarium oxysporum* f.sp. *pisi*, Grajal-Martin *et al.* (1993) found that only strains of race 2 showed little variability and were therefore identifiable. For races 1, 5 and 6, no generalized race-specific patterns were found. Race 2 was said to have a conserved region, but the term 'conserved' in fact told nothing, since most of the strains were from one area near Washington (US).

Only a small part of the structural elements of an organism is used in new tests: sampling error

Many polymorphisms were found between two races of *Meloidogyne hapla* and three populations of *M. arenaria* with a similar esterase pattern. In contrast, the banding patterns of the four races of *M. incognita* were very similar (Cenis, 1993). Sometimes isolates of different *formae speciales* of *F. oxysporum* appeared to be more similar with mitochondrial (mt)DNA RFLP analysis than were certain isolates of the same *forma specialis*. However, the genetically identical *formae speciales* are still highly host-specific (Kim *et al.*, 1992).

In a study using mtDNA and RFLP, two typical isolates of *Ophiostoma novo-ulmi* had mtDNAs similar to *O. ulmi* and clustered with the latter species. It was suggested that, because of the finding, rare ingression (whatever that may be) of *O. ulmi* mtDNA into *O. novo-ulmi* took place. The possible sampling error or limitations of the technique used remained unquestioned (Bates *et al.*, 1993).

Ouellet & Seifert (1993) found no difference between strains of *Fusarium graminearum* with RAPD primer pairs although they could be differentiated into vegetative compatibility groups. Moreover several primer sets were needed to allow identification at species level.

Seventy arbitrary primer pairs for race 2 and 4 of *Cochliobolus carbonum* did not differentiate (Jones & Dunkle, 1993). The authors accordingly suggested that the races were closely related. On the other hand, race 3 was very variable and hardly identifiable. The conclusion from these

findings could also be that the method is not very suitable for discriminating between pathogenic races.

In a study on 13 isolates from five different hosts of *Phytophthora porri*, De Cock *et al.* (1992) stated that it could not be concluded from the results whether differences in restriction patterns of mtDNA arose from differences in host or geographic origin. Unfortunately, the authors ended with the almost rhetorical conclusion that 'restriction pattern analysis has provided stable characters correlated with the host of the isolates and some other characters'.

Answers from automated identification systems using standard libraries are as good as these libraries and as present-day taxonomy

The more extensive the library, the more likely it is that an unknown organism with a high similarity to an organism included in the library really is the latter organism. This is especially so when the unknown was isolated from a niche where the organism is known to occur (e.g. diseased plant) and selection has already taken place. However, when the unknown originates from a complex habitat where any organism may be expected, even a high similarity can only be an indication. Verification using other methods is necessary to prove identity.

Points of reference usually determine choice of patterns

Many points of reference may be chosen for pattern analysis by the new methods: pathogenicity, host range or virulence; biological activity; geographic origin; difference in ecological niche; morphological difference. The choice is arbitary, and indeed the points of reference have often been determined by conventional methods. The existing groups (points of reference) are often only partly correlated with the patterns found by molecular methods. Sometimes even different species may show very similar patterns. A supporting set of patterns, often called 'largely' or 'generally supporting' in publications, may be just an accident and should be thoroughly evaluated before it is used to prove anything, in view of the risk of circular argument (Forster & Coffey, 1993).

In an RFLP study of *Frankia* strains, no correlation was found with geographic origin. However, site diversity was found and very low diversity on acid soils. Such patterns may really reflect some other point of reference (Jamann *et al.*, 1992). In a study of 38 strains of *Colletotrichum gloeosporioides*, isolates from mango grouped easily, while isolates from other hosts showed some possible grouping only within geographic localities. No RFLP was found that could be used to identify isolates from avocado, banana or papaya on a worldwide basis (Hodson *et al.*, 1993).

Conclusions

It is sometimes suggested that molecular methods, being genetic, are of a higher and therefore better order than conventional methods, which are phenotypic. However, there is no such contradiction. After cracking organisms and isolating their nucleic acids, these nucleic acids no longer function. They can be immobilized in gels, cut into pieces, etc., and their analysis is a purely phenotypic one.

Living organisms are able to switch genes on and off, etc. One should avoid a short-sighted, reductionistic view of molecular phenotypes, which will inevitably lead to problems in detection, identification and classification. So modern methods should be seen as a welcome addition to already existing ones. They should be carefully checked for specificity and reproducibility and their limitations should be realized. This is especially true for disciplines where saprophytic organisms play a role. In the case of virology and of non-culturable MLOs, lower stringency is perhaps required.

The great advantage of modern methods, especially those based on nucleic acids, is that they are or can be made specific at a very low taxonomic level, even at the strain level.

Nouvelles méthodes de diagnostic en phytopathologie: perspectives et pièges

L'un des résultats de la recherche fondamentale en biologie moléculaire a été le développement remarquable des nouvelles méthodes de diagnostic (détection et identification) au cours de ces dernières années. En raison de leurs origines, ces nouvelles méthodes ont pour caractéristique commune d'utiliser comme base des éléments structuraux des organismes étudiés, tels que les acides nucléiques, les lipides, les acides gras, les protéines, les polyamines et les polysaccharides. Ces éléments structuraux sont, soit utilisés comme matrice pour développer des soi-disant sondes de détection (et d'identification), soit disposés en catégories artificielles facilitant l'identification et la classification. L'article présente les avantages et perspectives des nouvelles méthodes par rapport aux méthodes conventionnelles. Il faut noter que, dans de nombreuses études, la spécificité et la reproductibilité des nouvelles méthodes ne sont traitées qu'insuffisamment, voire encore simplement supposées exister. Ces caractéristiques principales, fortement influencées par les erreurs expérimentales et d'échantillonnage, risquent de piéger l'expérimentateur dans des interprétations fausses, qui sont également passées en revue.

Новые методы диагностики патогенов растений. Перспективы и трудности

Значительное развитие за последние годы новых диагностических методов (в частности, выявления и идентификации) явилось одним из сопутствующих результатов фундаментальных исследований в области молекулярной биологии. По своему происхождению все эти новые методы имеют общую черту : использование в качестве основы таких структурных элементов организмов-мишеней, как нуклеиновые кислоты, жиры, жирные кислоты, белки, полиамины и полисахариды. Эти структурные элементы используются либо как матрицы для создания так называемых зондов для детектирования (и идентификации) или же применяются для идентификации и классификации патогенов в условиях внесения в искусственно созданные структуры. В настоящем сообщении рассматриваются достоинства и перспективы применения новых методов по сравнению с общепринятыми. Следует отметить, что во многих исследованиях вопрос о специфичности и воспроизводимости результатов новых методов либо недостаточно освещен, либо только кратко упоминается. Эти параметры, в значительной степени зависящие от погрешностей, возникающих при проведении эксперимента и при отборе проб, могут приводить к заблуждениям при интерпретации результатов новых методов диагностики, что также является предметом обсуждения.

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own, these molecules are non-fluorescent, but once hybridized to the target PCR product, a conformational change occurs restoring fluorescence. The reactions can be continuously monitored in specifically designed thermocyclers, such as TaqMan (Roche Molecular Systems Inc). It is possible to detect multiple targets in the same solution because molecular beacons can be made in many different colours utilizing a broad range of fluorophores. This technology has already been used in a multiplex PCR assay for the detection of four pathogenic retroviruses.[24]

Methods for obtaining virus sequence

Knowledge of viral nucleic acid is crucial for designing molecular assays and may be obtained in a number of ways.

(1) Purify the virus and clone. Isolation procedures have to be optimized for each virus, or even each strain of a virus and important factors for success are (a) choice of host plant species, and (b) extraction medium.[25] Many fruit tree viruses have been isolated from cultures transferred to herbaceous hosts, including *Apple chlorotic leaf spot virus*, *Plum pox virus*, *Apple stem grooving virus* and *Apple stem pitting virus*. This minimizes the presence of interfering substances in the initial extract.[26] Viral RNA templates are isolated from the purified virion preparations by removing the coat protein from the viral genome using a variety of physical and chemical agents such as heat and sodium dodecyl sulphate (SDS). Once obtained, the viral template is used to generate cDNA with first strand synthesis generally being driven by a reverse transcriptase and appropriate primer(s).

(2) dsRNA. Many viruses of woody plants cannot be purified by current methods, even from a herbaceous propagation host. Isolation of dsRNA, a stable replicative intermediate, from infected cells is a useful method for obtaining starting material for virus characterization if encapsidated ssRNA is difficult to obtain. For some disorders dsRNA may be the only virus or disease-specific molecule that can be detected. Morris and Dodds[27] developed a method for the isolation and analysis of dsRNA from virus-infected plants and fungi, based on the chromatographic adsorption and release of dsRNA from cellulose powder such Whatman CF11. In the presence of 16% buffered ethanol, dsRNA, but not other nucleic acids, is bound to cellulose in a column. After washing the column with buffered ethanol, the dsRNA can be eluted with ethanol-free buffer. This procedure has been adapted by many; perhaps the simplest to follow is that developed by Valverde,[28] which produces dsRNA free from detectable host DNA and RNA using two cycles of chromatography. Controls are essential to avoid confusion with the dsRNA genomes of cryptic viruses or non-viral dsRNAs that may be detected in plants.

This simple technique is not applicable to all viral groups. There has been no report of dsRNA in plants infected with negative sense RNA viruses such as rhabdoviruses, and indeed in our hands none has been observed for the cytorhabdovirus, *Strawberry crinkle virus*. This technique has been of particular value for characterizing recalcitrant viruses of fruit crops, and in some cases has resulted in the unexpected cloning of a non-target virus; eg Jelkmann[29] cloned the previously unknown *Cherry virus A* from dsRNA extracted from plant tissue infected with *Little cherry virus*.

To generate cDNA for cloning and sequence analysis it is necessary to melt the dsRNA to ssRNA. This can be done by boiling in water and quick cooling on ice, or by chemical treatments with methylmercuric hydroxide, glyoxal and dimethyl sulphoxide or formamide and formaldehyde.[30]

(3) Conserved sequence in related viruses. Degenerate oligonucleotides designed to conserved sequences or sequence motifs, from reverse translated viral amino acids sequences, may be used to amplify sequences of related viruses. In this way all members of a genus[31] or even several genera[32] of viruses, may be detected.

(4) cDNA library construction using subtractive hybridization. Subtractive cloning is a powerful technique that allows the isolation of genes expressed or present in one cell population but not in another. It may be used, for example, to obtain pathogen genes from diseased plants. Although there are several different methods,[33] the basic theory behind subtraction is simple. Complementary nucleic acids from two samples (healthy and infected material) are mixed together, with healthy in excess, denatured and allowed to anneal. Duplexes formed between the healthy and infected material are then removed, as is unhybridized healthy material, leaving a population enriched for sequences unique to the infected material. When complex tissues are used, multiple rounds of subtraction must be performed to remove completely sequences that occur in both sets of plants. Reiterative subtraction requires the infected material to be regenerated or amplified after subtraction using PCR, amplification of a cDNA library or by in vitro transcription. As with many molecular biology techniques, commercial kits are available. These generally make use of: (a) immobilization (in which the infected material is hybridized to healthy material immobilized on to a solid phase, leaving unbound infected sequences not represented in healthy material); or (b) PCR-based methodology (in which infected-infected hybrids can be selected and amplified exponentially, through the incorporation of specific primer binding sites).

Assays for phytoplasmas

Phytoplasmas in fruit trees present different problems from those caused by viruses. They are insect-transmitted and are more likely to spread between trees within orchards and between orchards. They multiply only in phloem tissue and may be irregularly distributed throughout the tree. They also occur in low concentrations and may be subject to seasonal fluctuations.[34] Therefore, any test to detect phytoplasmas in fruit trees must be sensitive, must utilize samples from several different parts of the tree, and must take into account the time of year most likely to give a correct result. The most suitable tissue samples are leaf petioles and midribs. Bark samples

cation. Alternatively, cDNA synthesis can be carried out in the same tube, speeding up the time for the test and reducing the risk of amplicon contamination; this requires the design of cDNA synthesis reaction mixes that are compatible with the PCR reaction mix. Reverse transcription (RT)-PCR is an extremely sensitive method for detecting and quantifying specific RNAs; it is about 100 times more sensitive than dot blot hybridization for *Cherry mottle leaf virus* testing[16] and about 1,000 times more sensitive than ELISA for *Plum pox virus* assays.[17]

Problems with the technique arise with extracting target sequence, in this case total RNA. There is a need to purify or treat the material to remove DNA polymerase inhibitors such as the polysaccharides and phenolic compounds found in fruit plants. Detection of *Cherry virus A* in cherry leaf and wood samples was facilitated by using RNA purified by chromatography on a Qiagen column, to remove inhibitors.[18] Nucleic acid purification may not be needed; simply diluting crude extracts allows cDNA synthesis and subsequent PCR detection of *Cherry leaf roll virus*[19] and *Apple stem grooving virus*.[20] The ability to detect viruses in crude plant extracts simplifies the time-consuming sample preparation, giving a speedier assay.

IC/RT-PCR. Immuncocapture PCR combines antibody binding and PCR into an extremely sensitive and simplified test. Viral RNA from immunocaptured particles is used as the template for reverse transcription followed by PCR amplification (Figure 5). This technique was first used for fruit tree viruses as a means of overcoming the problems of PCR inhibitors found in woody tissues, and the apparent viral concentration achieved has proved useful for the detection of viruses that are present at extremely low concentrations. This concentration effect may explain why IC/RT-PCR was found to be 250 times more sensitive than direct PCR for the detection of *Plum pox virus*.[21] The drawback of this technique is that immunocapture requires antigen-specific antibody (although not as highly specific as for ELISA) as well as nucleic acid information. An alternative is plate capture (PC/RT-PCR) in which virus is bound to the plate without the need for antibody treatment; this enables the simultaneous detection of more than one virus (multiplex PCR). This technique has been used to detect both *Apple stem grooving virus* and *Cherry mottle leaf virus*.[22] However, multiplex PCR often requires extensive optimization because primer-dimer formations and the occurrence of non-specific products may interfere with the amplification of specific products. Other important factors limiting the sensitivity of multiplex PCR are the relative concentration of each primer set and the copy number of each target sequence, which are of course much harder to determine. An alternative is to use degenerate, broad-spectrum primers to amplify at the genus level or above in one round of PCR, and to follow this with a second, nested PCR (using primers internal to the original set) for further characterization, should that level of specificity be required.

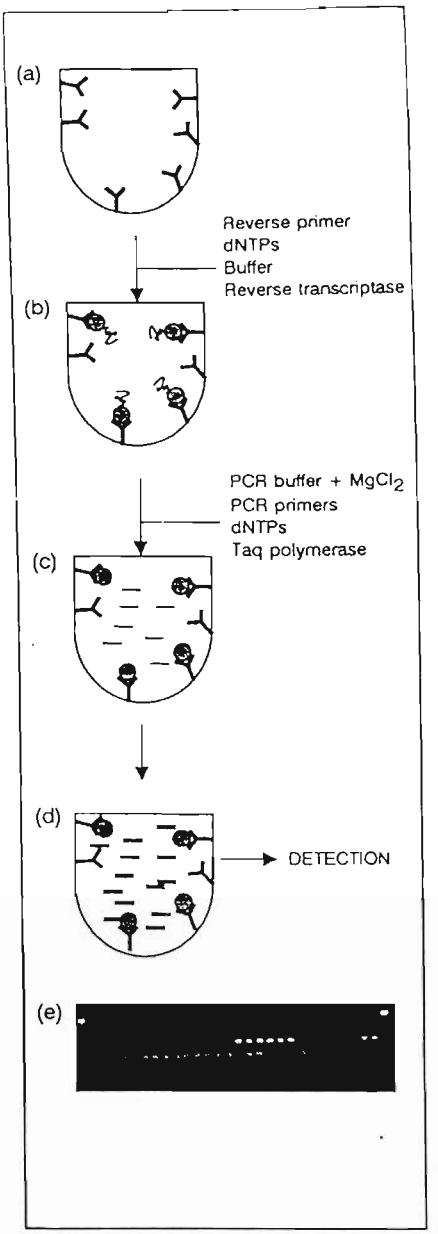

Figure 5. Immunocapture RT-PCR. (a) Wells are coated with specific antibody before the addition of plant extract. (b) After immunocapture of virus particles, reverse transcription reagents are added. (c) First strand cDNA is synthesized and PCR reagents are added. (d) PCR exponentially amplifies a specific piece of the viral genome. (e) Amplified fragments are detected by agarose gel electrophoresis (positive samples are the clear bands above the hazy primer-dimer bands).

Diagnostic PCR assays require numerous negative controls because it is possible for a single copy of contaminating target sequence to produce a positive PCR result. The main benefit of PCR is the ability to detect the virus at any time of the year, and budwood received can be tested before planting. Apart from sample preparation, the other drawback of PCR-based assays is the means for detecting the amplification product. Traditionally this has been achieved by agarose gel electrophoresis using ethidium bromide for visualization of DNA. However, detection methods are available that are more rapid and sensitive than gel electrophoresis and have the potential for automation. PCR ELISA and PCR ELOSA allow direct labelling of PCR products by using a labelled nucleotide or a labelled primer (oligonucleotide) respectively during PCR amplification. Subsequent semi-quantitative detection occurs by a hybridization-based microtitre plate assay (ELISA). This technology has already been incorporated into a rapid detection method for the measurement of telomerase activity in tissue or cell extracts (Boehringer Mannheim). An alternative system uses molecular beacons to provide quantitative results, and has the added benefit of tests being conducted in closed reaction tubes. Molecular beacons are oligonucleotide probes that can report the presence of specific nucleic acids in homogeneous solutions.[23] On their

proteins can be cloned and expressed at high levels. However, problems encountered include: cell toxicity, protein instability, improper processing or post-translational modification, and inefficient translation. A major difficulty is that polypeptide gene products expressed in *E.coli* may accumulate as insoluble aggregates that lack functional activity. Also, the biological activity and immunogenicity of the recombinant protein may differ from natural protein, and antisera raised to recombinant virus proteins expressed in *E.coli* have not been reliable for use in ELISA to detect fruit tree viruses.[12]

Alternative expression hosts include yeasts and vertebrate cells. Yeasts such as *Saccharomyces cerevisiae* and *Pichia pastoris* are among the simplest eukaryotic organisms. These two species are not the only ones employed, but their use has been facilitated by 'off-the-shelf' availability and they have all the advantages of *E.coli* (high levels of expression, easy scale-up, rapid growth) combined with some of the post-translational modifications found in mammalian cells. Recombinant proteins produced in yeast often possess the antigenicity and functional activity of native proteins, but there are many cases, generally unpublished, in which despite optimizing all of the parameters, only low levels of expression are achieved.

Many eukaryotic post-translational modifications such as phosphorylation, glycosylation, precursor processing and targeting, are carried out in insect cells. Baculoviruses, which produce large amounts of structural, functional and antigenically authentic protein, are commonly used for recombinant protein expression in insect cells. This expression system is most popular for the expression of viral glycoprotein genes; glycoproteins are components of the outer shell of many animal virion particles[13] and are therefore the obvious target for antigens in immunodiagnostic tests. Glycoproteins are not restricted to animal viruses and are found in both the plant rhabdovirus and tospovirus genera.

All recombinant protein expression is rather unpredictable and researchers interested in generating high protein yields often carry out expression in one system, for example yeast, in parallel with equivalent studies in another expression host such as a bacterium, or baculovirus.

Molecular assays

A good diagnostic test for the detection of plant viruses combines sensitivity with speed and the ability to process large numbers. The most widely used technique is ELISA, which is sensitive, easy to carry out and has a high throughput. However, ELISA may not always be applicable. Specific antisera are unavailable for many fruit viruses or are unable to detect low virus titres, especially in dormant wood, reducing their use to specific times of the year. The following techniques rely on knowledge of the viral nucleic acid and identification of sequences that are unique or characteristic for a virus or strain.

(1) Hybridization. Hybridization refers to the formation of double-stranded molecules between the nucleic acid under test and a probe (nucleic acid of complementary sense labelled with a radioactive or chemical marker). Virus nucleic acids are immobilized on to an inert support, the most popular being nitrocellulose and nylon. Most plant viruses have ssRNA genomes and will bind to the membrane without the need for the removal of secondary structure . (treatment with heat or helix destabilizing agents such as formamide). Purification of nucleic acid is not necessary and the most commonly used procedure for testing large numbers of samples is dot blot hybridization, in which a small amount of sap is extracted from the plant under test and a spot is applied to the membrane. Membranes are baked or UV cross-linked to bind the nucleic acid before incubating for about two hours in a prehybridization solution to block non-specific sites. During this time it is usual to label the probe; the most common is cDNA made to part of the RNA genome by reverse transcription, either directly from the viral RNA or from a cDNA clone. There are many ways to incorporate a label and both uniform and end labelling have been used successfully for dot blot analysis, but the method used is dependent to some extent on the type of label. Hybridization of the probe to the test nucleic acid usually takes place overnight and excess probe is washed off before detection, and quantification if required. RNA probes have been shown to be more sensitive than ELISA during field indexing trials for plum pox virus (PPV).[14] When appropriate information is available it is possible, by using highly strain-specific regions of the viral genome, to generate diagnostic probes that discriminate between different strains of a virus. However, with the exception of tests for viroid detection, this technique has been largely superseded by PCR-based detection assays.

(2) PCR techniques. The invention of the Polymerase Chain Reaction (PCR) by Mullis *et al*[15] has revolutionized molecular biology, and the sensitivity, speed and versatility of PCR have been successfully applied to the detection and identification of many plant pathogens. PCR is conducted in a few hours, it permits adjustment of sensitivity and specificity depending on the choice of primers, and it can be used to identify hard-to-culture pathogens such as phytoplasmas. PCR is an enzymatic procedure that exponentially amplifies a specific segment of DNA, the essential step being:

(1) melting of target DNA;
(2) annealing of two synthetic oligonucelotide primers designed to flank the region of interest; and
(3) primer extension by a thermostable DNA polymerase (*Taq* polymerase).

RT-PCR. In the above format, PCR is only appropriate for the detection of plant viruses with DNA genomes. However, many plant viruses have RNA genomes, and for amplification by PCR of single or double-stranded viral RNA, a modification of the procedure is required. RNA is reverse transcribed into cDNA before amplification, and this can be carried out as a two-step procedure: cDNA is synthesized in one reaction and then a small aliquot is used in a subsequent PCR amplifi-

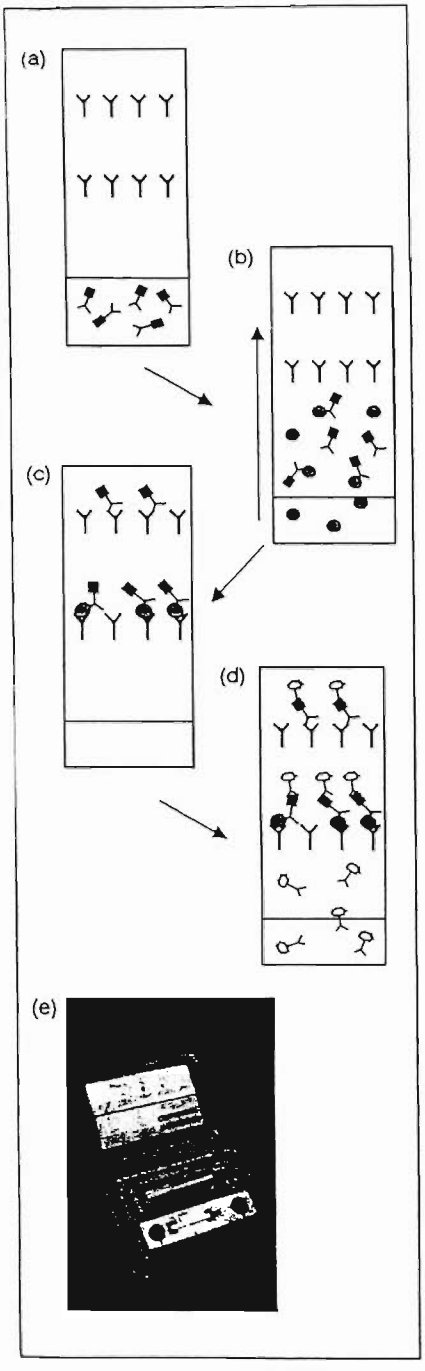

Figure 4. Dipstick lateral flow device. (a) A membrane striped with control (upper band) and antivirus antibodies (lower band) is placed in a well containing activated antivirus antibodies. (b) After addition of a virus-containing sample, material flows in the direction of the arrow; any virus present (spheres) binds to the activated antivirus antibodies on the way. (c) As the sample reaches the other end of the membrane, the activated antibody-virus complex becomes attached to the antivirus antibody stripe, while unbound activated antibody becomes attached to the control antibody stripe. (d) The addition of gold-labelled antibody to the well produces a visible colour reaction in both stripes to denote a positive reaction. (e) A kit for virus detection in the field — a result can be obtained in 10 minutes.

of lateral flow devices for detecting plant viruses that operate on a principle similar to that of a pregnancy test. In the dipstick assays, developed at Horticulture Research International,[10] strips of nitrocellulose membrane are striped with (a) a narrow zone of antibody to the virus under test, and (b) a stripe of control antibody (Figure 4). A test is conducted by grinding plant tissue, adding extract to a vial, placing the dipstick in the vial to soak up the extract, and transferring the dipstick to a second vial to soak up labelled antibody. A positive reaction appears as a coloured stripe on the dipstick.

This test can be performed in the field and the result is obtained within about 10 minutes. The assay is close to the ideal for speed, simplicity, user-friendliness and reliability. It is slightly less sensitive than a standard ELISA and will doubtless be developed for many pathogens and crops in the future. It is ideal for farmers, nurserymen and advisers assessing disease situations in the field. Kits are available for several important viruses: plum pox, tomato spotted wilt, turnip mosaic, as well as for *Xanthomonas campestris* (from HortiTech Diagnostic and Crop Protection Services, Selby, North Yorkshire, UK). A test more closely resembling the pregnancy test has also been developed for *Plum pox virus*.[11]

The main obstacle to providing serological assays is obtaining high quality antiserum. This in turn depends upon the quality of the immunogen (ie the virus preparation used to incite the immune response in the animal that produces the antiserum). For many viruses it is difficult or impossible to make the pure preparations necessary for immunization. For these, there are other strategies for laboratory detection, either by using a molecular route to obtaining the immunogen as detailed below, or by employing a technique that detects the virus nucleic acid.

Molecular routes to obtaining antisera

The primary function of the coat protein is to protect viral genomic nucleic acids by encapsidation, and antisera raised to 'purified' viruses react to this protein. Where purification of the virus cannot be achieved but sequence data of the genome are known, or at least of the coat protein gene, then expression and purification of a recombinant coat protein can be used to generate virus-specific antisera.

The use of recombinant proteins has increased in recent years, as has the wealth of techniques and products employed for their amplification and purification. A key factor has been the construction of fusion protein vectors, in which special affinity tags are added to the protein sequence of interest. The use of these affinity tags simplifies subsequent detection and purification (by affinity chromatography) of the recombinant fusion proteins. The two most commonly used tags are glutathione S-transferase (GST tag) and 6 histidine residues (6xHis tag) both of which can be used in any expression system. The small 6xHis tag has an advantage in being poorly immunogenic so that the fusion protein can be used directly as an immunogen in antibody production, therefore avoiding tag cleavage.

A number of heterologous expression systems are available and the choice of host (phage, bacteria, yeast, plants, filamentous fungi, insect or mammalian cells grown in culture) depends on the specific requirements and applications for the recombinant protein. In general, expression of plant viral coat proteins is carried out in the bacterium *Escherichia coli*, which offers many advantages. It has been widely used for the expression of recombinant proteins; there are many references, there is a wide choice of cloning vectors it is easy to manipulate and control gene expression; and it has rapid growth with high yields. With careful choice of host strains, vectors and growth conditions, most recombinant

allowing the object to be seen (Figure 2).

There are many variations of this procedure, pioneered by Derrick.[3] Coating the grids with antiserum is similar to coating a microtitre plate for enzyme-linked immunosorbent assay (ELISA) and, not surprisingly, the sensitivity of immunosorbent electron microscopy is of the same order as ELISA and can detect about 1 ng/ml^{-1} of virus in a plant extract.[4] It is difficult to find low numbers of virus particles, particularly if they are isometric and small, and many plant viruses fall into this category. However, visibility can be improved dramatically by 'decorating' the trapped particles. An 'overcoat' of antibodies is attached to the virus coat protein, effectively increasing its size and making the particles easier to see. This can make it feasible to scan several grid squares thoroughly for the presence of only one or two virus particles in a few minutes. It also allows viruses with similar particle morphology to be distinguished in a mixture (Figure 2).

IEM methods cannot be used on large numbers of samples as preparation, although easy for three or four samples at a time, is fiddly and does not lend itself to 'mass production'. Only small amounts of sample and unpurified antiserum are required, eg 100 samples can be trapped and decorated with just 15 µl crude antiserum. IEM techniques are therefore ideal for quick tests on small numbers of samples to detect and/or identify a virus, especially when antiserum is in limited supply.

Serology: ELISA

Since the advent of ELISA for use with plant viruses,[5] some form of ELISA has become the preferred method for the assessment of virus infection in plants. The assays are quick (1–2 days, depending on the format) and can cope with many samples (100-plus a day with one operator and many more if the process is semi-automated). The tests are easy to do and no special training is required; the results are reproducible and sensitivity is high (end point of about 1ng/ml^{-1} of extract).

The standard ELISA is a double-antibody sandwich (DAS, Figure 3). Assays are usually conducted in a 96 well microtitre plate. The polystyrene surface is coated with antibodies to the virus under test. The test extract is then incubated in the plate, so that the virus is trapped by the specific antibodies. Trapped virus is detected by incubation with a second solution of antibodies, which have been conjugated to an enzyme (commonly horseradish peroxidase or alkaline phosphatase). An appropriate enzyme substrate is then added, which changes colour in the presence of the antibody-enzyme conjugate. The reaction is quantitative and the degree of colour change is proportional to the amount of antigen (virus) present.

There are many variations of the ELISA format, mainly to simplify the test or to make it more sensitive. In the triple-antibody sandwich (TAS) the test is conducted as for DAS, but the second antibody is not conjugated and is produced in a different animal from the trapping antibody. The second antibody is then detected with a species-specific conjugated antibody, eg anti-mouse if the second antibody is raised in a mouse. Such conjugates can be obtained commercially and obviate the need for making a special enzyme conjugate for each virus under test. Another way around the need for virus-specific enzyme-antibody conjugates is FAB ELISA.[6] In this test, the plate is coated with antibodies that have had the 'tail' of the molecule removed by digestion with pepsin (a very simple procedure). The trapped virus is then detected with whole antibody molecules using a Protein A-enzyme conjugate. Protein A attaches specifically to the part of the antibody molecule that is removed from the coating antibodies; thus it reacts only with antibodies specifically attached to trapped virus particles, and not with the coating antibodies.

Sensitivity can be increased by use of a fluorogenic substrate,[7] but this requires an expensive fluorimeter to assess the results, and is not widely used. Several other methods of amplifying the signal from antigen–antibody reactions are possible,[8] but all suffer from the problem of raised background reactions as well as increased specific signal.

Cellulose nitrate membranes have been used as the solid phase in assays for many viruses. Plant extracts can be spotted directly on to the membrane, instead of trapping the antigen with specific antibody, and the assay is then completed by soaking the membrane in antibody-conjugate solution, followed by a substrate that produces an insoluble, coloured product. This has been little used for fruit tree viruses, possibly because of problems with non-specific reactions; these can be overcome, but may require the use of toxic chemicals, eg chloroform,[9] which is undesirable in a routine procedure.

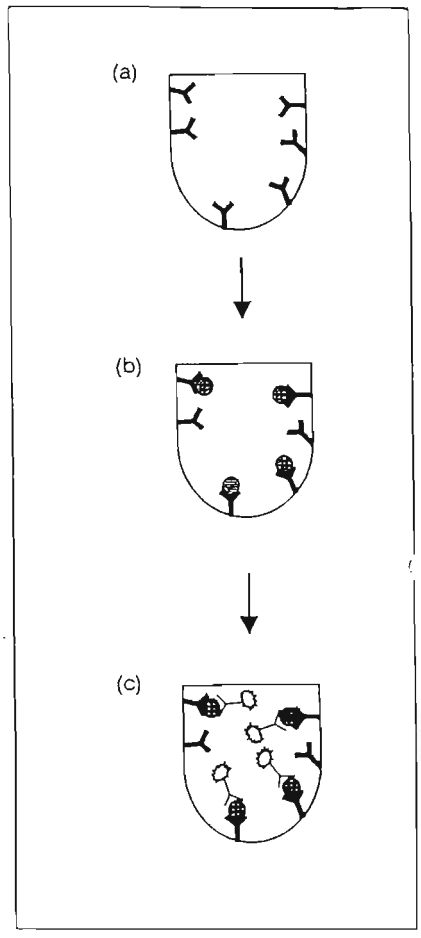

Figure 3. Enzyme-linked immunosorbent assay. (a) Wells in an ELISA plate are coated with specific antibody. (b) Plant extract is added and virus is trapped by the antibodies. (c) Detection is by incubation with a second round of antibodies (enzyme-linked), which produce a colour in the presence of the enzyme substrate.

Serology: lateral flow devices

A recent innovation is the adaptation

before the trees are finally assessed. In the case of Virginia Crab, the trees are cut down, the bark is stripped and the wood examined for symptoms of pitting, grooving and necrosis at and above the graft union.

Four replicate trees are needed for each field test, plus negative and positive controls. Field assays are generally recognized as being the most sensitive, and experience shows that they can be used to detect a wide range of virus strains. They are the baseline against which any new assay is judged. These tests score very well on sensitivity but extremely badly on speed. The numbers of trees that can be tested depend upon the agronomy facilities available, such as the land and skilled personnel to do the grafting; these are readily available and cheap in some countries but not in others, eg the UK. For apple, at least 12 virus, virus-like and phytoplasma diseases have to be tested for, and these require six indicator cultivars. Two of these indicators have to be grown as fruiting trees, as symptoms only show in the fruit. As four replicates are used of each indicator, plus positive and negative controls, approximately 36 trees are required to conduct a full range of tests on one apple tree to be reasonably sure that it is free from viruses and phytoplasmas. Fruiting trees are kept for three cropping years before accepting negative results; this is necessary to avoid the possibility of missing symptoms in years when the climate is unsuitable for symptom production. Long-term planning is therefore required to conduct the complete range of tests for viruses of apple trees.

Bioassays using trees in the glasshouse

A variation of the field bioassay can be conducted in the glasshouse.[1] Small grade rootstocks are planted in forestry pots and double-budded in the same way as for the standard field test. However the trees are grown under controlled temperature in the glasshouse, and results can be achieved within a few months for many temperate fruit tree viruses. This system was devised in the USA, but although it is in routine use there, it has not been widely used elsewhere. Although results are obtained quickly, temperature-controlled glasshouses are expensive and the technique requires more attention to husbandry than growing trees in the field.

A similar assay has been in use in many countries for several decades to detect stone fruit viruses. Seedlings of the homozygous peach, GF 305, are grafted at two or three months with two or more buds from the plant under test. The peach is cut back to a bud above the inoculum two weeks later and symptoms develop in the following 1–5 months. This peach selection is sensitive to about 50 virus and virus-like disorders of stone fruit.[2]

Laboratory assays

Apart from the direct use of the electron microscope to visualize virus particles, laboratory tests for viruses can be divided into those that detect the protein component of plant viruses (ie serology), and those that detect the nucleic acid or some part of it. The main advantages of laboratory tests are their speed and specificity. Specificity can also be a disadvantage as variant strains may remain undetected. Reagents have to be tailored for the pathogen under test, and reagents do not exist for all pathogens; much research on fruit tree viruses is directed towards obtaining and refining reagents and assays.

Electron microscopy and immunosorbent electron microscopy (IEM)

Some viruses can be seen in the electron microscope in preparations that are simple to make and take only one or two minutes to prepare. This is possible with extracts that contain quite high numbers of particles, which have a distinct appearance, but unfortunately, this is not the case with most viruses infecting temperate fruit trees. However, good results can be obtained with the use of antiserum, providing the virus is easy to distinguish from plant organelles and debris. Electron microscope grids (metal discs that are punctured with holes and covered with an electron-transparent support such as a fine film of carbon) are coated with antiserum to the virus being sought. This is then rinsed and floated on a crude plant extract. After washing, the preparation is then 'stained' with a solution containing an appropriate heavy metal salt. This accumulates around objects adhering to the grid and forms an electron-opaque layer,

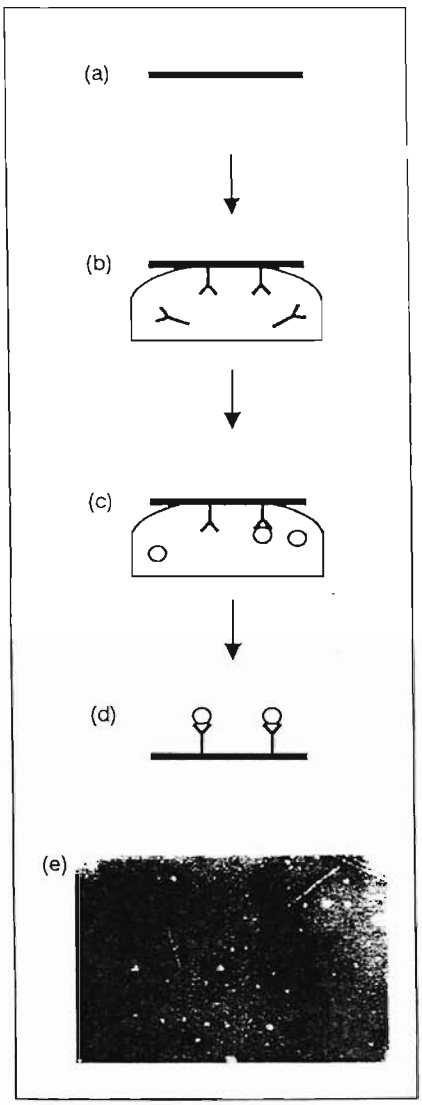

Figure 2. Immunosorbent electron microscopy. A carbon-coated electron microscope grid (a) is floated on a solution containing antibodies, which bind to the carbon layer (b). The antibody-coated grid is then floated on plant extract (c) and selective virus particles are adsorbed (d). The grid is then stained and viewed with an electron microscope. (e) Electron micrograph of hop mosaic and hop latent virus particles that have been adsorbed with mixed antisera and then selectively decorated with antiserum to hop mosaic virus (heavily coated particles).

hosts are not known, an assay may be feasible using plants related to the crop host that are particularly sensitive to the virus under test. Grafting is usually the only practical means of experimental transmission to woody plants, and grafts are only satisfactory between related species. For fruit trees, this therefore entails growing related tree species as indicators of infection.

Table 1. Methods of virus detection (the more stars the better).

Type	Sensitivity	Speed	Numbers	Total
Graft	•••		•	4
Herb	•	••	••	5
EM	•	•••	•	5
EM/IMM	••	•••	•	6
ELISA	••	•••	•••	8
Hybr	••	•••	•••	8
PCR	•••	•••	••	8
PCR/IMM	•••	•••	••	8

Herbaceous bioassays

Herbaceous plants are normally inoculated mechanically. Material is taken from the plant under test, added to extraction buffer and pulverized with a pestle and mortar. The buffer can contain chemicals to prevent oxidation, degradation of the virus or precipitation of virus particles by the tannins that occur in woody plants; it also dilutes inhibitors of infection. The resulting soup is applied to leaves of the sensitive indicator plant (species of *Nicotiana*, *Cucurbita* and *Chenopodium* are susceptible to a very wide range of viruses and are frequently-used indicators) with a finger or spatula. Infection takes place through wounds that are encouraged by the inclusion of an abrasive powder, such as Celite or Carborundum, and excess inoculum is washed off immediately after application to avoid inhibition or cytotoxic effects.

Sap inoculation into herbaceous hosts is not a very sensitive means of assay, particularly for fruit viruses. This is partly due to substances in fruit plant extracts that inactivate, degrade or precipitate viruses. Also, virus concentrations fluctuate in different tissues and through the growing season, so that there may only be a small window of opportunity in which tests are reasonably successful. Symptoms take from a few days to two or three weeks to appear, but the test plants have to be grown beforehand, and this may take up to six weeks. Symptoms may appear at the points of entry into the plant (local lesions) and/or they may be systemic, but they are not necessarily diagnostic as many different viruses can induce similar symptoms. Further tests are usually required precisely to identify the causal virus. The corollary of this is that, as the tests are not specific, viruses previously not known to infect the plant under test may be found. Several replicates of each test are recommended and the plants need to be grown within extremes of temperature. This can therefore require expensive glasshouse or screenhouse facilities and the space available is the main constraint on the numbers of tests that can be done. For example, to carry out the number of tests that can be done in one enzyme-linked immunosorbent assay (ELISA) plate in 1–2 days will require approximately 3 m^2 of glasshouse bench space for 2–3 weeks. The main attributes of these assays are given a rough score in Table 1 for comparison with other methods.

Bioassays using trees in the field

Different cultivars and species can vary greatly in their sensitivity to virus infection. For example, *Apple stem grooving virus* is latent in many commercial apple cultivars but will severely affect the graft union between apple rootstocks or commercial varieties and Virginia Crab. Necrosis at the union and deep grooves in the bark of Virginia Crab-apple are used as a sensitive diagnostic test for *Apple stem grooving virus*. Virginia Crab is also sensitive to another virus, which is usually latent in apple cultivars, *Apple stem pitting virus*. As the name suggests, this causes pitting in the wood of Virginia Crab. This crab-apple can therefore be used to test for two viruses affecting apples.

Tests are normally carried out in field plots that have been planted with virus-free rootstocks in the winter. These are grown during the year, and at the end of the summer are 'double-budded' (Figure 1). Each rootstock receives two buds. The upper one is the sensitive 'indicator', eg Virginia Crab, and the lower bud is from the tree under test. The upper bud is allowed to grow for two years

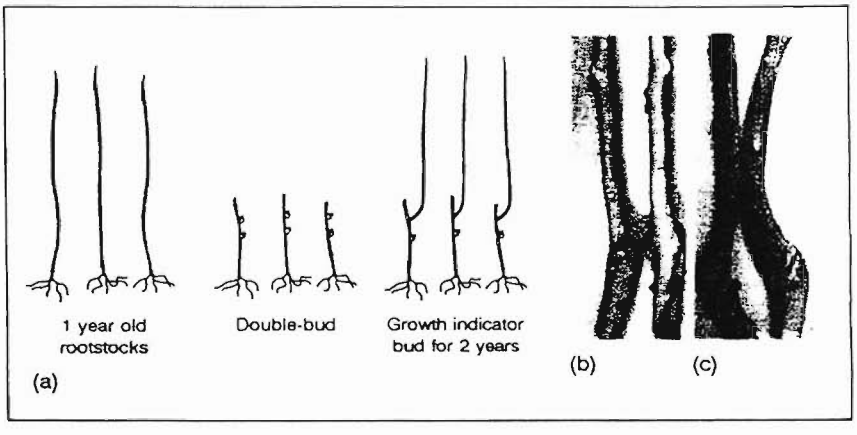

Figure 1. (a) Procedure for double-budding rootstocks with material under test (lower bud) and an indicator variety (upper bud). Plants are grown for 2–3 years in the field before the final symptom assessment. (b) Rootstock, graft union and healthy cv. Virginia crab-apple virus indicator. (c) Virginia crab indicator showing pitting in the wood characteristic of *Apple stem pitting virus* and necrosis at the graft union symptomatic of *Apple stem grooving virus*, two years after grafting.

Virus and phytoplasma detection in fruit trees

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Efficient detection methods are vital for the control of viruses and phytoplasmas in fruit and other perennial crops. This paper reviews the diagnostic tests currently available for detecting such pathogens. Biological tests are generally slow and results may be difficult to interpret. Serological assays are reliable and easy to conduct and some are very rapid. An abundance of molecular techniques offers higher sensitivity and facilitates the introduction of laboratory tests for diseases that currently have to be assayed biologically.

Perennial fruit plants, particularly trees, are a big investment, take several years to reach cropping potential, and can be in the ground for decades. It is therefore vital to start with the best quality disease-free plants that are available. This is particularly important for virus diseases, because once infected, a plant cannot be freed from infection. Many of the virus diseases affecting fruit crops spread slowly or not at all and if healthy material is planted, it will stay free from infection for many years. It is therefore quite feasible to propagate virus-free plants in the open, to release them to growers, and for their plantations to remain free from infection for most or all of their lifespan. The keys to doing this successfully are:

(1) satisfactory monitoring, regulation and control of the plant material to ensure that it does not become mixed with material of uncertain health and origin;
(2) really good, unequivocal tests for the pathogens.

This paper reports on the means available to test for the presence of viruses in temperate fruit trees. Some diseases can only be assayed by biological methods when the pathogen is unknown, although assumed to be viral. However, an increasing number of pathogens are being detected by molecular methods and a molecular approach can provide the possiblility of developing assays for viruses that other methods could not detect. The pros and cons of biological, serological and nucleic acid tests are explained with reference to temperate fruit tree viruses. These methods are in use for most crops and the technology, both biological and molecular, is equally applicable to the production of healthy planting material of any perennial crop. Information is included on phytoplasmas, as these have much in common with viruses and, about 30 years ago, they were found to be the cause of many diseases previously thought to be of viral origin.

Bioassays

The basis of bioassays for plant viruses is the availability of plants that are sensitive to infection and display characteristic symptoms in response to specific viruses. Some viruses that infect perennial fruit plants can also infect fast-growing herbaceous plants such as species of *Nicotiana* and *Chenopodium* . For such viruses it may be possible to conduct an assay using these plants; if such

Chapter 14

♦

Biological Dinitrogen Fixation: Symbiotic

Peter H. Graham

♦

An' I am blest, becos me feet 'ave trod
A land 'oo's fields reflect the smile o' God
C.J. Dennis

A mutualistic symbiosis is an association between two organisms from which each derives benefit. It is usually a long-term relationship and, in the case of symbiotic dinitrogen (N_2) fixation, often involves development of a special structure to house the microbial partner. Each N_2-fixing symbiotic association involves an N_2-fixing prokaryotic organism, the microsymbiont (e.g., *Rhizobium*, *Klebsiella*, *Nostoc*, or *Frankia*) and a eukaryotic, usually photosynthetic, host (e.g., leguminous or non-leguminous plant, water fern, or liverwort). These symbioses contribute more than 100 million metric tons of combined nitrogen per year to the global nitrogen economy and account for more than 65% of the nitrogen used in agriculture. Rates of N_2 fixation vary with host, microsymbiont, and environment but in temperate clover pastures may reach 600 kg N fixed ha^{-1}. Grain legumes fix 100 to 200 kg N ha^{-1} growth $cycle^{-1}$ and supply 40 to 85% of the plant's nitrogen needs through symbiosis. The direct availability of the fixed nitrogen permits the host to grow in soils that are nitrogen deficient and, at the same time, reduces losses by denitrification, volatilization, and leaching, thus improving the sustainability of an agricultural system. Dinitrogen fixation is likely to become even more important in the future as population increases in many developing countries necessitate sharply increased crop production, while pollution, energy, and cost concerns limit significant increases in the use of fertilizer nitrogen.

Box 14–1

The Importance of Symbiotic Dinitrogen Fixation. Symbiotic N_2 fixation is the single greatest contributor to the global nitrogen economy. Principal contributors include:

- leguminous plants and their associated rhizobia,
- actinorhizal plants in symbiosis with *Frankia*,
- the water fern *Azolla* and its microsymbiont *Anabaena*, and
- lichen symbioses involving cyanobacteria.

The Symbiosis Between Legumes and Rhizobia

Legumes have been used in crop rotations since the time of the Romans. Theophrastus (370–285 B.C.) stated " . . . beans are not a burdensome crop to the ground, they even seem to manure it. . . . wherefore the people of Macedonia and Thessaly turn over the ground when it is in flower." However, it was not until detailed nitrogen balance studies became possible that leguminous plants were shown to accumulate nitrogen from sources other than soil and fertilizer. In 1886 Hellriegel and Wilfarth demonstrated that the ability of legumes to convert dinitrogen from the atmosphere into compounds that could be used by the plant was associated with the presence of swellings or **nodules** on the legume root. They related this association to the presence of particular bacteria within the nodule. Later in this chapter we discuss the different types of root-nodule bacteria, but for the moment can refer to them collectively as **rhizobia.** It was then a series of short, but important, steps to the isolation of rhizobia from nodules by Beijerinck in 1888 and to the completion of Koch's postulates by the demonstration of their ability to reinfect the legume and to fix dinitrogen in symbiosis (Chapter 1).

Groupings of Rhizobia and Their Separation into Species

Early studies showed that each rhizobial strain or isolate had a finite host range, nodulating certain legumes but not others. This led to the concept of cross-inoculation, with legumes grouped according to the different rhizobia with which they formed nodules. Thus rhizobia isolated from species of *Medicago* (e.g., alfalfa) would also nodulate *Melilotus*, and vice versa, though rhizobia isolated from these hosts would not nodulate *Trifolium* spp. (clovers). More than 20 different cross-inoculation groups were identified, with the bacteria from the clover, medic, bean, lupine, pea, and soybean groups named as separate species within the single genus *Rhizobium* (e.g., *R. trifolii* for clover). Host specificity is still important in the identification of rhizobia, but more recently other traits have assumed greater significance in their classification. There were a number of reasons for this:

- Initial studies involved mainly legumes of agricultural importance. Study of less traditional legumes blurred cross-inoculation boundaries. For example, the bacterial strain NGR234, originally isolated from *Lablab purpureus*, the hyacinth

bean, nodulates with 34 different species of legume and with a nonlegume, *Parasponia andersonii* (Stanley and Cervantes, 1991). Some nonlegumes, such as rice and wheat, can be induced to form nodule-like structures with rhizobia if pretreated with plant enzymes or hormones. Even now, though, less than 15% of the roughly 19,700 species of legumes have been evaluated for nodulation.

- Many anomalous results have been reported. One study gave more than 500 examples where strains were either promiscuous (i.e., they nodulated legumes from other cross-inoculation groups) or failed to nodulate legumes from their own group.
- Nodulation genes of some rhizobia are plasmid-borne. Strains losing the plasmid as the result of exposure to high temperature also lose the ability to form nodules and for many years could not be identified. In one soil, noninfective rhizobia lacking the symbiotic plasmid outnumbered those capable of nodule formation by 40 to 1.
- Taxonomic methods were developed to compare strains on the basis of many different traits. Computer-based numerical classification, along with taxonomic methods based on differences in cell DNA or RNA, often resulted in groupings at odds with those based on host range.

Some of the traits now used in the classification of rhizobia are listed in Table 14–1. The original genus *Rhizobium* is now divided into 4 genera and 16 species, as shown in Table 14–2. Most of these changes have occurred since 1985.

Similarities between the rhizobia and other organisms have been identified. Thus the fast-growing species *Rhizobium tropici* shows a close affinity to species of *Agrobacterium*, a plant-pathogenic bacterium causing crown gall and hairy root diseases, while some strains of the slower-growing *Bradyrhizobium* produce bacteriochlorophyll and are more closely related to the photoautotroph, *Rhodopseudomonas palustris*.

Table 14–1 **Characteristics for the phenotypic and phylogenetic characterization of rhizobia.**

Phenotypic traits*
Range of substrates used as sources of energy (e.g., sugars, sugar alcohols, and complex carbohydrates)
Range of substrates used as sources of nitrogen (e.g., amino acids, urea, and nitrate)
Resistance to specific antibiotics
Electrophoretic mobility of different cell enzymes
Tolerance to different stresses (e.g., salt, temperature, and pH)
Phylogenetic traits† (refer to Chapters 8)
Pattern of banding of DNA restriction fragments (RFLPs)
Degree of hybridization with specific DNA probes
16S rRNA sequence analysis

*Phenotypic traits can be observed in culture.
†Phylogenetic traits are related to cell DNA or RNA composition.

Table 14–2 **Genera and species of the root-nodule bacteria of legumes. Genera in the square brackets refer to host legumes nodulated by each species of root-nodule bacteria. Common names are included for well-known legume genera. In several examples in this list, different species of root-nodule bacteria nodulate the same legume.***

Rhizobium†
R. leguminosarum (with three biovars: trifolii [*Trifolium*, clovers], viciae [*Pisum*, peas; *Vicia*, field beans; *Lathyrus*; and *Lens*, lentil], and phaseoli [*Phaseolus*, bean]
R. loti [*Lotus*, trefoil]
R. tropici [*Phaseolus*, bean; *Leucaena*, Ipil-Ipil, and *Macroptilium*]
R. etli [*Phaseolus*]
R. galegae [*Galega*, *Leucaena*]
R. huakuii [*Astragalus*, milkvetch]
R. ciceri [*Cicer*, chickpea]
R. mediterraneum [*Cicer*, chickpea]
Sinorhizobium
S. meliloti [*Melilotus*, sweetclover; *Medicago*, alfalfa; and *Trigonella*, fenugreek]
S. fredii [*Glycine*, soybean]
S. saheli [*Sesbania*]
S. teranga [*Sesbania*, *Acacia*, wattle]
Bradyrhizobium
B. japonicum [*Glycine*, soybean]
B. elkanii [*Glycine*]
B. huanmingense [*Glycine*]
Azorhizobium
A. caulinodans [*Sesbania*]

*Other genus and species names exist in the literature. Some predate the present names. Others (e.g., Photobacterium) have not been accepted as valid.
†Strains of *Rhizobium* and *Bradyrhizobium* that do not belong in any named species are usually identified by the host from which they were isolated, e.g., *Rhizobium* spp. (*Acacia*) or *Bradyrhizobium* spp. (*Lupinus*).

The Infection Process

Nodule Initiation and Development

The process of nodule formation is outlined here. Greater detail can be obtained by reading Hirsch (1992).

Infection. Mechanisms by which rhizobia infect their hosts and induce root- or stem-nodule formation include:

- penetration of root hairs and formation of infection threads as found in plants such as clovers and beans,
- entry via wounds or sites of lateral root emergence, as found in peanut and the pasture legume *Stylosanthes*, and
- penetration of root primordia, as found on the stem of plants such as *Sesbania*.

Root-hair infection has been studied for many years, using small-seeded legumes that were inoculated with rhizobia, embedded in agar, and grown between glass slides. Such

Fåhreus slides permitted observation of the different steps of the infection process under the microscope. Infection begins with rhizobial attachment to immature, emerging root hairs of a compatible host. Deformation and curling of the root hair follows (Fig. 14–1), with the root hair surface at the point of infection hydrolyzed to permit penetration of the rhizobia. Rhizobia then move down the root hair toward the root cortex.

Rhizobia never gain free intracellular access to their host. During infection, and as they move down the root hair, they become enclosed within a plant-derived

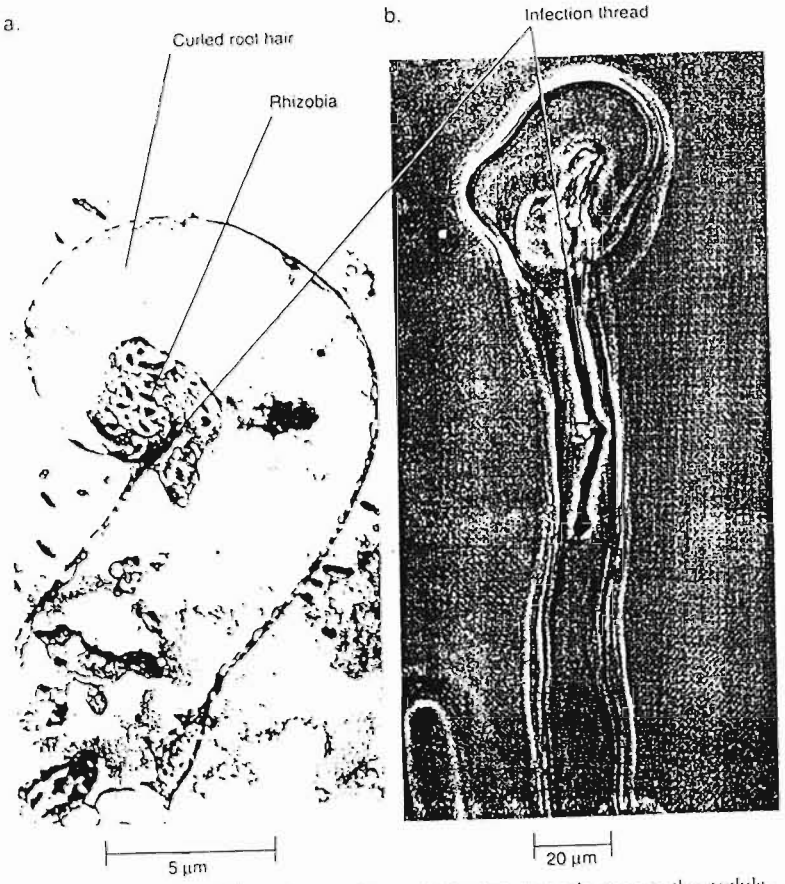

Figure 14–1 Root hair deformation, curling, and infection: an early stage in the nodulation of clover by rhizobia. (a) The initiation of infection thread formation showing the plant-derived gelatinous matrix surrounding 10 to 20 cells of rhizobia. *From Sahlman and Fåhreus (1963). Used with permission.* (b) Movement of the infection thread down the root hair toward the root cortex. *From Fåhreus (1957). Used with permission.*

infection thread. They remain surrounded by this material until released into modified cells of the root cortex, where again they are enclosed within a plant-derived **peribacteroid membrane.** These membranes protect the bacteria from the defense responses of the host.

The demonstration that the nodulation genes of many rhizobia are carried on plasmids led to more detailed molecular studies of infection. Two different groups of genes are required for infection:

- *Common* nodulation genes (*nod* A,B,C) are found in all rhizobia. A fourth gene (*nod* D) is sometimes included in this group but is unique in being the only *nod* gene expressed in the absence of a suitable host.
- *Host-specific* nodulation genes (*nod* E, F, G, H, I, J, L, M, P, Q in the case of *S. meliloti*) differ with type of rhizobia and define the host range.

Because *nod* D is the only *nod* gene expressed in the absence of the host, studies to determine the host factor(s) needed to trigger expression of other *nod*-genes soon followed. *Flavonoids,* complex phenolic compounds exuded from the legume root, were implicated and hypothesized to interact with the product of *nod* D prior to the expression of the other genes. Considerable specificity has been shown in this interaction. For example, luteolin is the principal flavonoid involved in *nod*-gene expression in *S. meliloti,* whereas naringenin and genistein are required for *B. japonicum.*

Characterization of the different nodulation genes led to the detection and characterization of a series of substances, termed lipo-oligosaccharides or *nod factors,* which are responsible for root hair deformation and curling and the division of cortical cells in the root at concentrations lower than $10^{-9}M$. Composition of these nod factors varies with microsymbiont but in each case includes a backbone of β 1, 4-linked, N-acetylglucosamine units specified by the common *nod* genes. The chemical composition of the major lipo-oligosaccharide produced by *S. meliloti* is shown in Figure 14–2. The *nod* genes in *Bradyrhizobium* are not located on plasmids, but are otherwise analogous to those found in *Rhizobium.* Even in *Rhizobium,* not all of the genes contributing to nodule formation and function are found on plasmids. Figure 14–3 shows the effect of mutation in the chromosomal genes required for bacterial **lipopolysaccharide** production on nodule morphology and nitrogenase activity.

Nodule development and function. As the infection thread penetrates the root cortex and the rhizobia it contains are released into host cells, cell division and enlargement of these cells results in the formation of a visible nodule. Root nodules differ in appearance and structure, a trait determined by the host legume. Determinate nodules, such as those which occur on soybean and *Phaseolus,* are round and have no pronounced meristematic region. In contrast, the indeterminate nodules of peas, medics, and clovers are elongated with a pronounced meristematic region, and increase in length over the growing season.

Examination of an alfalfa nodule under the microscope reveals four distinct zones, as shown in Figure 14–4:

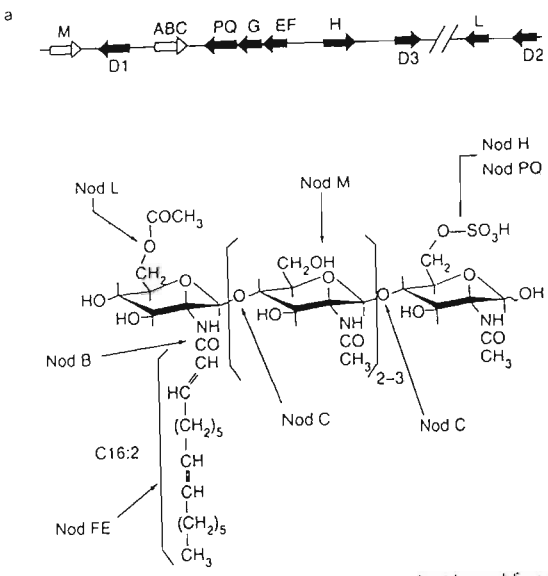

Figure 14–2 Structure of the major lipo-oligosaccharide nod factor produced by *S. meliloti* and the role of different *nod* genes in its biosynthesis. (a) A simplified genetic map of the *nod* gene region showing structural and regulatory genes and their organization. (b) The lipo-oligosaccharide molecule in this species has a backbone of β 1,4-linked glucosamine residues, and carries N-acylated, N-acetylated, and O-sulfated side chains. Site of action of the nod gene products (enzymes) are shown. As an example of *nod* gene function, the products of *nod* PQ exhibit homology with the enzymes ATP sulfurylase and APS kinase. *From Denarie and Cullimore (1993). Used with permission.*

- Meristematic region in which host cells undergo active division but show little infection by rhizobia (M).
- A region in which many plant cells are infected but in which the bacteria have not undergone changes in size and shape (TI). Din trogen fixation is limited.
- Region of active N_2 fixation (ES), often red or pink in color due to the presence of **leghemoglobin.** Host cells will contain many rhizobia, which may be misshapen. Such bacteria are referred to as **bacteroids.**
- Region of nodule senescence in which the symbiosis is breaking down (LS). Bacteroids may undergo lysis, and the degradation of leghemoglobin results in a green or brown coloration.

Nodules with a large pink or red region usually are active in N_2 fixation and are said to be *effective*. If the nodule is white or greenish brown, either the symbiosis is ineffective or the nodule is undergoing breakdown and is said to be senescing.

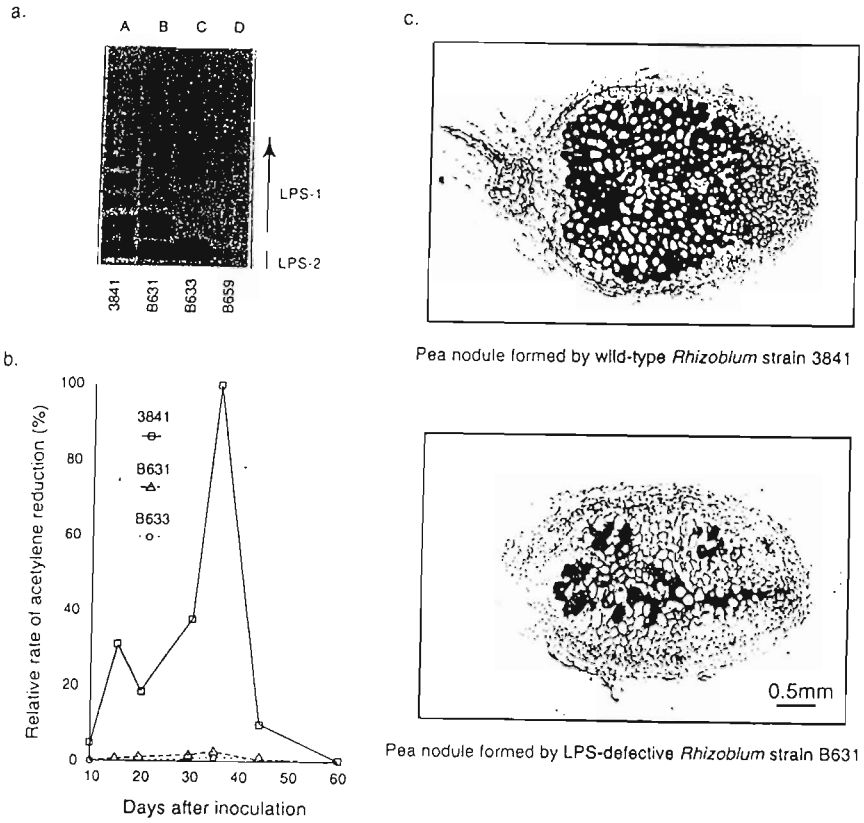

Figure 14–3 Influence of mutations in lipopolysaccharide (LPS) production on nodulation and nitrogenase activity (an index of N_2 fixation potential) in pea seedlings. (a) Periodate silver stained polyacrylamide gels showing differences in the lipopolysaccharide of strain 3841 (wild type) and of three mutants derived from it. (b) Seasonal profiles of nitrogenase activity achieved by the wild type strain and by two LPS mutants. (c) Differences in the morphology of pea nodules produced by strain 3841 and B631. Nodules produced by such mutant strains contain few infected cells. *From Perotto et al. (1994). Used with permission.*

In indeterminate nodules like those in alfalfa, bacteroids produce ammonia (see Fig. 13–2), which is exported to the host cell and there converted via glutamine, glutamate, and aspartate to asparagine. Asparagine is then exported to the shoot. The overall reaction for this series of changes is:

2 ammonia + 3ATP + oxaloacetate →

L-asparagine + AMP + 2ADP + $2P_i$ + NAD + PP_i

Determinate nodules export a very different end product. Glutamate and aspartate are produced but are then used to synthesize purines such as xanthine. These

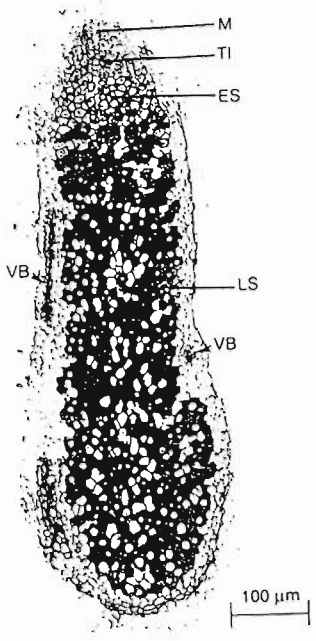

Figure 14–4 The internal organization of an indeterminate root nodule: M, meristematic tissue; TI, region with infection thread penetration; ES, region of early symbiotic activity; LS, region of late symbiotic activity; VB, vascular bundle. The nodule is attached to the root at the bottom of the photo. *Photo courtesy C. P. Vance.*

are converted in neighboring noninfected cells to the ureides, allantoin and allantoic acid. Because the level of these substances in nodules and xylem sap is usually correlated with recent N_2 fixation, ureide analysis of bleeding sap is often used to estimate N_2 fixation.

Relatively few infections result in root-nodule formation. Successful infections may be visible as early as five to six days after inoculation with rhizobia, with active N_2 fixation beginning eight to fifteen days thereafter. During this period a number of proteins are produced in the root hair or nodule that are not found in host or bacteria alone. Expression of these substances, called **nodulins**, may be both time-and-tissue dependent. "Early" nodulins have been recovered from infected root hairs less than six hours after inoculation. "Late" nodulins, which are more often related to nodule function and N_2 fixation, include leghemoglobin and the nodule enzymes nitrogenase, uricase, and glutamine synthetase

Host-Rhizobium Specificity

Specificity can occur at each stage of the nodulation process. Thus, only 25 to 30% of legumes in the subfamily Caesalpinioideae ever form nodules, while infectiveness subgroups have been identified within several cross-inoculation groups. One such subgroup includes *Trifolium tembense*, a pasture species com-

Box 14–2

Events Leading to Nodulation and N_2 Fixation in Legumes.

- Attachment of rhizobia to the root begins within 1 minute of inoculation.
- Number of attached rhizobia increases with time up to several hours.
- Root hair curling begins within 5 hours.
- Infection threads become visible within 3 days of inoculation.
- Nodules become visible within 5 to 12 days.
- N_2 fixation is often evident in 15-day-old plants.

Table 14–3 **Levels of host-*Rhizobium* specificity affecting nodule formation in legumes.**

Legume species that are never nodulated (e.g., *Cassia bicapsularis*)
Cross inoculation group specificity
Infectiveness subgroups
Nonnodulating plant genotypes, e.g.,
the rj_1 gene of soybean
the nod 125 mutant of *Phaseolus vulgaris*
Nodulation preference

mon in Kenya and Tanzania. This species nodulates with rhizobia from *T. rueppellianum* and *T. usambarense* but either fails to nodulate or is ineffective with rhizobia from other African and European clovers. Similarly, the pea variety Afghanistan will nodulate with pea rhizobia from the center of origin of this crop in the Middle East, but not with most of the rhizobia from pea varieties in Europe. These and additional examples of specificity listed in Table 14–3 have often been a problem in the introduction of new plant germplasm.

Host-rhizobial interactions also influence levels of N_2 fixation. Thus, peanuts and cowpeas are nodulated by and fix dinitrogen with many different soil bradyrhizobia, whereas *Centrosema* and *Desmodium* species often nodulate with these strains but fix little dinitrogen. A consequence is that when cowpeas or peanuts are introduced into a new area, they are often well nodulated and grow vigorously, even in the absence of inoculation, whereas *Centrosema* and *Desmodium* species may have many nodules but grow poorly.

Box 14–3

Infectiveness and Effectiveness.

- *Infectiveness* is the ability of a rhizobial strain to form nodules with a particular legume.
- *Effectiveness* is the ability of those nodules to fix dinitrogen.

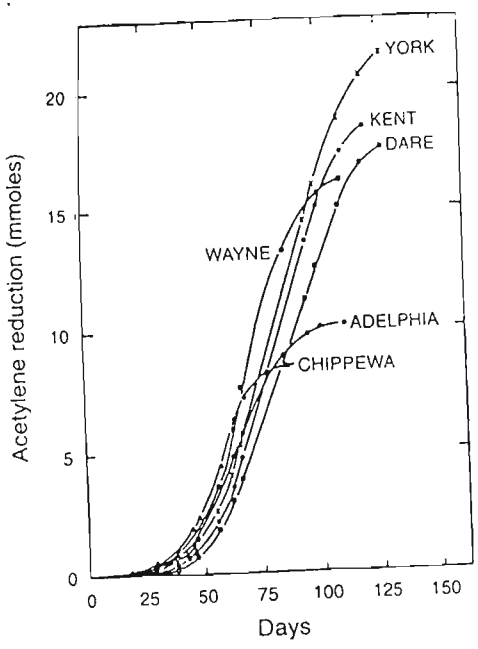

Figure 14–5 Influence of soybean maturity group on potential N_2 fixation, as estimated with the acetylene reduction method. The varieties shown are of increasing vegetative period, with Chippewa early to flower and York and Kent having much longer vegetative periods. Once podfill begins, available energy for N_2 fixation can be insufficient for continued nodule activity. *From Hardy et al. (1973). Used with permission*

Even different varieties of the same species can vary in their ability to fix dinitrogen with the same rhizobial strain. This often relates to differences in maturity, with early-flowering varieties more likely to be limited in their N_2-fixing ability (Fig. 14–5). Genetic differences in earliness of nodulation, nodule mass, nodule senescence, and enzyme function have also been reported, and attempts to improve current levels of N_2 fixation through plant breeding are under way in a number of laboratories. Perhaps the simplest approach to improve N_2 fixation of new varieties is to inoculate plants with rhizobia, and to grow and select the varieties under conditions of nitrogen deficiency. The growth and yield of the plant genotypes being tested will then depend heavily on their ability to fix dinitrogen.

Influence of Environmental Factors

Environmental factors influence all aspects of nodulation and symbiotic N_2 fixation, in some cases reducing rhizobial survival in soil and in others affecting nodulation or even growth of the host. Critical factors include acidity, temperature, mineral nutrition, and salinity and alkalinity.

Acidity

In Latin America alone there are more than 800 million ha of Oxisols and Ultisols, the pH of which is usually less than 5.0. In this region, as in other regions of the world

Box 14–4

The Symbiotic Environment. Environmental factors influence all aspects of the symbiosis between legumes and rhizobia, including:

- survival of the rhizobia on the seed and in soil,
- infection and nodule formation, and
- N_2 fixation.

where soils are acid (Fig. 14–6), N_2 fixation may be markedly reduced. This may be because of the direct effect of H^+ concentration, the presence of toxic levels of aluminum and manganese, or deficiencies of calcium, phosphorus, and molybdenum.

Soil acidity per se can limit rhizobial growth and persistence in soil. Fast-growing rhizobia are generally considered more sensitive to acidity than bradyrhizobia, but strains of *R. tropici* and *R. loti* may also be acid tolerant. In contrast, most isolates of *S. meliloti* are particularly acid sensitive. In one study an average of 89,000 *S. meliloti* cells g^{-1} were reported in soils of near neutral pH, but only 37 cells g^{-1} in soils of pH less than 6.0. Surprisingly, not all of the strains recovered from acid soils are acid tolerant, suggesting that microsites of more favorable pH can occur.

Failure to nodulate is also common in acid soils, in part because of lowered numbers of rhizobia, but also because pH limits rhizobial attachment to infectible root hairs. Although it is common in the United States to lime acid soils, the large areas involved and the cost and availability of limestone preclude this approach in many other countries. Alternative practices include the use of acid-tolerant inoculant strains and host cultivars and the pelleting (coating) of inoculated seed with a layer of ground rock phosphate or limestone. In Australia, the use of relatively acid-tolerant *S. meliloti* strains such as WSM419, together with *Medicago* species collected from acid soils in Sardinia, has permitted extension of the area sown to annual medics by some 350,000 ha since 1985 (Ewing and Howieson, 1989). Similarly, an acid-tolerant *R. tropici* strain, CIAT899, is now the strain recommended for inoculating *Phaseolus vulgaris* in the acid soils of Brazil.

Plant species vary in tolerance to aluminum and manganese, but are generally more affected by these ions than are the rhizobia. Thus, some rhizobia tolerate 100 μM aluminum and 300 μM manganese, but reduced root growth of alfalfa (*Medicago sativa*) occurs at only 8 μM aluminum, and nodulation in cowpea is inhibited at 25 μM aluminum.

Temperature

Rhizobia are mesophiles and most do not grow below 10°C or above 37°C. Exceptions are rhizobia associated with certain Arctic legumes, and bradyrhizobia collected from the hot, dry Sahel savannah of Africa. Maintaining favorable temperatures during the shipment and storage of inoculant, and seed inoculation and planting, is particularly critical. Exposure to high temperatures at these times can lead to the loss of the symbiotic plasmid in *Rhizobium* or reduce cell numbers below the levels needed for good nodulation.

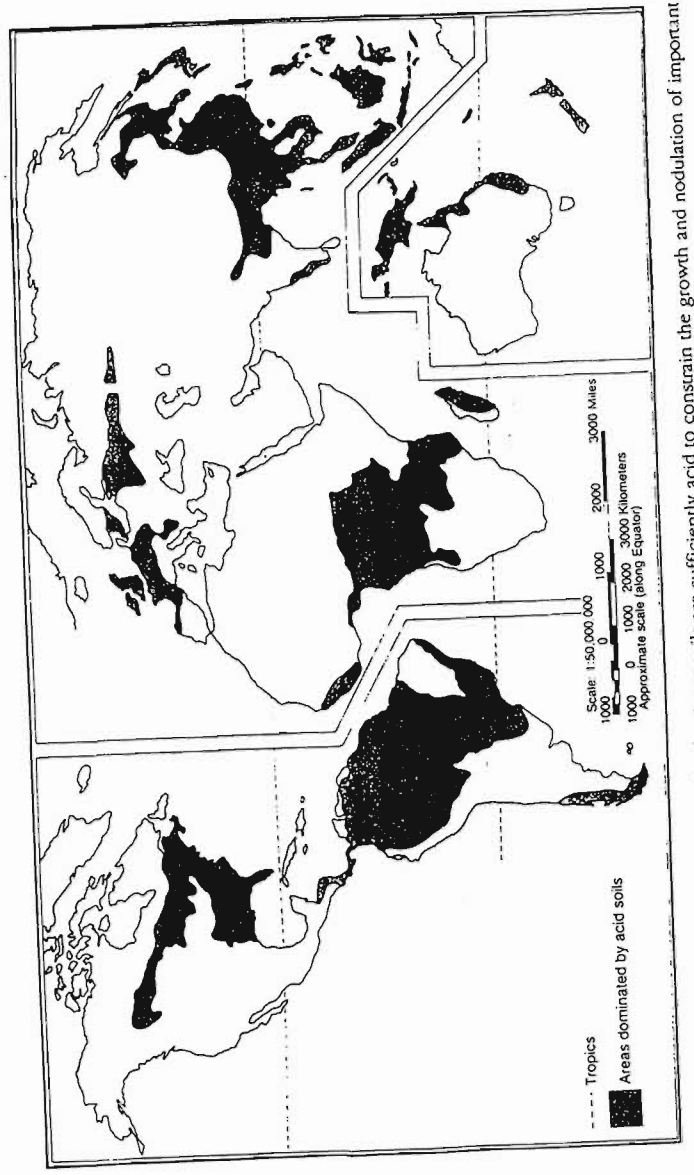

Figure 14–6 Regions of the world where the dominant soils are sufficiently acid to constrain the growth and nodulation of important leguminous crops. *From van Wambeke (1976).*

Temperature also influences nodule growth, N_2 fixation, and the time period for which nodules are active. The optimum temperature for many legumes is around 25°C; exposure to temperatures of greater than 40°C, even for short periods, can cause irreparable loss of nodule function.

Mineral Nutrition

Although well-nourished plants generally nodulate and fix dinitrogen better than those that are nutrient limited, several elements have specific functions in nodulation and symbiotic N_2 fixation (Table 14–4). Adequate levels of these elements are essential for effective N_2 fixation (see also Table 13–2), and failure to supply them results in the generalized yellow chlorosis typical of nitrogen deficiency. Several of these elements warrant specific mention.

Phosphorus. Leguminous plants dependent on N_2 fixation commonly require more phosphorus than similar plants supplied fertilizer nitrogen. Nodules are an important phosphorus sink and commonly have the highest concentration of that element in the plant. The high energy cost of N_2 fixation with its need for large amounts of ATP leads to the elevated phosphorus requirement. In the case of the legume nodule, this is compounded by the energy cost of building and maintaining functioning nodules. Bacterial strains and host cultivars differ in their phosphorus-use efficiencies, but cultivar variation has been studied mainly in host plants fertilized with nitrogen. Field-grown legumes form tripartite symbioses with both *Rhizobium* or *Bradyrhizobium* and arbuscular mycorrhizal fungi. This has an added energy cost to the host, but the benefit from additional phosphorus uptake for N_2 fixation can be considerable.

Molybdenum. The principal function of molybdenum in the legume is as a component of the nitrogenase enzyme complex. As such, the requirement for molybdenum is satisfied by supplying as little as 100 to 500 g ha^{-1} of this element. How to supply this small amount can be a problem, especially under acid soil conditions where adsorption reduces the availability of soil molybdenum to the plant. Molybdenum salts have sometimes been incorporated with the inoculant. While the effect varies with the molybdenum salt added, this can drastically reduce survival of rhizobia in the inoculant and its addition is not recommended.

Iron. Iron is a component of leghemoglobin, which functions in the regulation of oxygen supply to bacteroids. It is also a component of both the Fe and FeMo proteins

Table 14–4 **Elements having specific functions in the nodulation or N_2 fixation of legumes.**

Molybdenum	FeMoCo protein of nitrogenase
Phosphorus	Energy transformations in the nodule
Iron	Fe and FeMo proteins of nitrogenase
	Leghemoglobin
	FeS centers of nitrogenase
	Unspecified function in nodule development
Calcium	Attachment of rhizobia to root hairs
	Cell wall integrity in *Rhizobium*
Sulfur	FeS centers of nitrogenase
Cobalt	Nodule coenzyme function
Nickel	Hydrogenase function

of the nitrogenase complex and is essential for early nodule development. Plants which are iron-deficient develop many nodule initials but few functioning nodules. Both host and bacterial strain can differ in efficiency of iron utilization. In the case of the bacteria, some strains can produce iron-sequestering siderophores and so compete more effectively for iron in the rhizosphere.

Salinity and Alkalinity

The effects of saline or alkaline conditions are likely to be greater on the host or symbiosis than on the rhizobia. Alkaline soil conditions limit the availability of iron, zinc, manganese, and boron in the soil, thereby reducing plant growth and N_2 fixation. Foliar fertilization with micronutrients is often an effective remedy. Legumes as a group are also markedly sensitive to salt, with some species affected by concentrations as low as 80 mM. In contrast, strains of rhizobia from *Medicago* and *Acacia* often tolerate 500 mM salt. Cells of *Rhizobium* exposed to high salt concentrations often accumulate osmoregulants such as glutamic acid, trehalose, glycine betaine, and proline, which help to maintain turgor in the cell and limit the damage caused by salts.

Legume Inoculation

When a new legume species is introduced into a region, soils are unlikely to contain appropriate rhizobia, and inoculation is usually needed for adequate nodulation and N_2 fixation. Yield increases following this initial inoculation are often of the order of 50%, with clear differences evident between inoculated and noninoculated plants, as seen in Figure 14–7. Inoculation in subsequent years is usually not necessary. In fact, where a legume has an extensive history of cultivation in a region, most soils contain abundant rhizobia, and even noninoculated plants are heavily nodulated. If inoculation is practiced in such an area, the inoculant strain usually produces only a small fraction of the nodules formed, and a yield response is unlikely.

Figure 14–7 Response of soybeans to inoculation in Florida. The right-hand plot in the foreground received inoculation, while the adjacent plot did not. *Photo courtesy of W. Scudder and D.H. Hubbell. Used with permission.*

Box 14–5

Inoculation in the American Midwest. In the American Midwest, agricultural soils usually contain 1,000 to 10,000 soybean rhizobia g^{-1}. These indigenous rhizobia limit nodule formation by inoculant strains, which often produce less than 20% of the nodules formed. Because the indigenous or naturalized populations often fix less dinitrogen than the inoculant strain, the full benefits of the symbiosis may not be realized. Soybeans grown in this region derive only 30 to 40% of their nitrogen needs from N_2 fixation.

Need for Inoculation

A simple, three-treatment experiment will establish the need for inoculation. The treatments are:

- noninoculated control plots,
- plots inoculated with a strain of *Rhizobium* or *Bradyrhizobium* effective on the host legume, and
- plots inoculated with the same strain, but also supplied fertilizer nitrogen.

Extensive nodulation of the noninoculated plants means that the soil already contains indigenous rhizobia able to nodulate this host. The contrast between the noninoculated plants and those supplied with nitrogen will then be a measure of the effectiveness of the native rhizobia. If the noninoculated plants are green and vigorous, inoculation is probably not necessary. Absence of nodulation in the noninoculated plants, coupled with heavy nodulation of plants receiving inoculation, indicates that inoculation is needed. The differences in plant growth among the three treatments are indicators of the efficiency of N_2 fixation by the inoculant strain. If excellent plant growth is achieved in all three treatments then either the native rhizobia are highly effective and inoculation is not necessary or the site was higher in available nitrogen than was anticipated. Poor growth in all treatments would imply that a factor other than nitrogen was limiting plant growth.

Strain Selection and Testing

If inoculation is required, the strain or strains employed must meet the following criteria:

- form highly effective nodules with all commonly used varieties of the legume species for which it is recommended,
- be competitive in nodule formation and persistent in the soil,
- tolerate extremes of acidity, temperature, and other soil conditions,
- grow well in simple, inexpensive culture media,
- be genetically stable, and
- survive well on the seed prior to seed germination.

Inoculant-quality rhizobia should be selected after screening at several levels. The initial step is usually a growth chamber or greenhouse study of numerous strains obtained from other collections or from the field. Marked variation in nodulation and N_2 fixation usually is evident. Poorer strains are discarded, and the remaining strains may be tested with different varieties of the legume to eliminate any possibility of host-strain interaction or may be further tested under field conditions. Ideally such field trials are conducted at sites that vary in numbers of indigenous rhizobia; they should be followed up in subsequent growing seasons to ensure that the inoculant strains persist in the soil. Finally, environmental and cultural factors that could influence strain performance in the field need to be considered.

Inoculants and Inoculation

The number of rhizobia per seed necessary to ensure good nodulation varies with seed size and environmental conditions. In countries where inoculant quality is regulated by law, the usual standard is from 1,000 rhizobia per seed for small-seeded legumes such as clover to 100,000 rhizobia per seed for bean and soybean. In the early 1900s, soil from previously planted fields was the principal, but far from ideal, inoculant. The supply was limited, moving soil was cumbersome, and the possibility of transferring root pathogens or nematodes was a major concern. The inoculant industry now manufactures pure cultures of rhizobia for seed and soil inoculation. Inoculants range from simple tube cultures sufficient for small quantities of exotic seed to large-scale, fermenter-grown cultures mixed with peat or other carrier material and used in the commercial inoculation of large areas of crops such as soybean, bean, peanut, and clover.

The large-scale production of inoculants is a simple process designed to supply a minimum of 10^8 to 10^9 highly effective rhizobia per gram of product. Inoculants from many countries do not meet this standard. Factors contributing to this failure include:

- inadequate testing of the inoculant strain,
- mutation in the inoculant strain after repeated subculture or storage at high temperature,
- inappropriate culture media,
- contamination of the rhizobial culture,
- carrier materials that will not support suitable populations of rhizobia, and
- poor storage and transport conditions.

Characteristics of a good inoculant carrier are shown in Table 14–5. The most common carrier is peat, but compost, sterile bagasse (derived from the milling of sugarcane), coal, polyacrylamide, vegetable oils, and soil have all been used successfully. No listing of physical or chemical properties can fully explain why some peats make suitable inoculant carriers and others do not.

Four procedures are commonly used in legume inoculation. For additional detail, refer to Somasegaran and Hoben (1994).

Table 14–5 **Qualities of a good inoculant carrier material.**

High water-holding capacity
Nontoxic to rhizobia
Readily available, inexpensive, and easily processed
Sterilizable by autoclaving (pressurized steam) or radiation
Good adherence to seed
Good buffering capacity

- *Seed inoculation:* The inoculant is mixed with milk or some other slightly adhesive material (called the *sticker*), and the seed is uniformly covered with this suspension. The seed is dried in the shade and sown the same day.
- *Seed pelleting:* The sticker is a stronger adhesive, such as gum arabic or methyl cellulose, and the seed, once inoculated as above, is rolled in finely ground limestone or rock phosphate. Pelleting combats unfavorable soil conditions such as low pH and allows aerial sowing. Preinoculation of seed for subsequent sale is not recommended because rhizobial numbers on the seed can decline dramatically during storage.
- *Soil inoculation with a granular peat or liquid:* The inoculant is banded below the seed and makes contact with the emerging radicle. Soil inoculation is most useful for seed that has been treated with fungicide or for conditions where higher than normal inoculation rates are desirable.
- *Inoculation in the planter seed box:* The inoculant is mixed directly with seeds in a planter box attached to a tractor. Inoculant and seed tend to separate, providing uneven coverage. This simple method is usually an "insurance measure" when soils are already likely to contain rhizobia.

Strain Competitiveness and Persistence

Even without inoculation, it is common for a newly introduced legume to have a few nodules. These arise from seed- or dust-borne rhizobia or from native legumes having compatible rhizobia. When these nodules rot, they can release more than 10^{10} rhizobia g^{-1}, ensuring a buildup in the soil to levels of 10^3 to 10^4 rhizobia g^{-1} of soil. Unfortunately, many of these organisms are not particularly effective, and do little to benefit subsequent plantings. Worse, they can limit the ability of inoculant rhizobia to form nodules and become established in the soil.

Attempts to overcome this problem using strains selected for superior competitive ability, heavier than normal inoculation rates, and improved carrier and delivery systems have all had limited success. Accentuating this problem, rhizobia are not very mobile in soil, and as the root elongates, they may be left behind. One solution is host cultivars that nodulate preferentially with the inoculant strain or exclude indigenous rhizobia. Several soybean cultivars that can restrict nodulation by the indigenous strains but nodulate normally with specific inoculant strains have been identified (Table 14–6). Differences in the response of the wild *Phaseolus vulgaris* accession G21117 to inoculation with strain CIAT632 and CIAT 899 are shown in Figure 14–8.

Table 14–6 **Differences in the nodule occupancy of three genotypes of soybean differing in ability to restrict nodulation by strain USDA123. Data are from a 2-year field evaluation.**

Genotype	% of nodules occupied by strain		
	USDA123	USDA122 or USDA138	Other
Williams (nonrestrictive)	76	20	4
PI371607 (restrictive)	3	89	8
PI377578 (restrictive)	5	92	3

From Keyser and Li (1992). Used with permission.

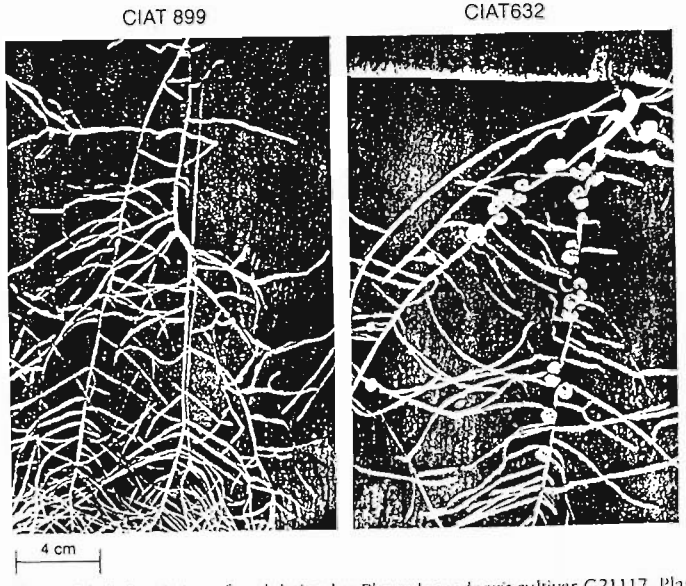

Figure 14–8 Restriction of nodulation by *Phaseolus vulgaris* cultivar G21117. Plants on the left were inoculated with *R. tropici* strain CIAT899 and those on the right with *R. etli* strain CIAT632. Both strains produce normal nodules and are effective on most cultivars of *P. vulgaris*. However, restricted nodulation occurs when CIAT899, but not CIAT632, is used to inoculate G21117. *From Kipe-Nolt et al. (1992). Used with permission.*

Other Important Symbiotic Dinitrogen-Fixing Associations

Frankia and the Actinorhizal Symbiosis

Frankia is an actinomycete that forms actinorhizal, N_2-fixing nodules with a range of angiosperms (Table 14–7). The host species are not typical crop plants, but several are important in agroforestry, the ecology and nitrogen economy of marginal soils, mine spoil reclamation, or the stabilization of sand dunes. Rates of N_2 fixation are highly variable but can be equivalent to those achieved by leguminous symbioses.

Table 14–7 **Families and genera of actinorhizal plants**

Family	Genera with nodules
Betulaceae	*Alnus* (alder)*
Casuarinaceae	*Allocasuarina, Casuarina* (Australian pine), *Ceuthostoma,* and *Gymnostoma*
Coriariaceae	*Coriaria*
Datiscaceae	*Datisca*
Elaeagnaceae	*Elaeagnus, Hippophae* (sea buckthorn), and *Shepherdia*
Myricaceae	*Comptonia* and *Myrica* (myrtle)
Rhamnaceae	*Ceanothus* (snowbrush), *Colletia, Discaria, Kentrothamnus, Retanilla, Talguenea,* and *Trevoa*
Rosaceae	*Cercocarpus* (mountain mahogany), *Chamaebatia, Cowania, Dryas,* and *Purshia*

*Common names for some important examples are included in parentheses.

Isolation of *Frankia* from nodules was not achieved until 1978, and still requires very specific methodologies. However, numerous isolates are now available, and information on morphological, genetic, and specificity differences is beginning to accumulate. Particular points of interest include:

- *Frankia* is a Gram-positive, filamentous organism characterized by multilocular sporangia and N_2-fixing vesicles *in vitro*.
- Nodule formation results from root hair infection or intercellular invasion. Nodules are perennial, modified lateral roots with lobes up to 5 cm in length.
- Host specificity exists, but needs further definition. Three to four host-specificity groups have been suggested, though species of *Myrica* and *Gymnostoma* appear to be promiscuous and nodulate with strains from all groups.
- Few *Frankia* isolates produce spores within the nodule. Although this trait is regulated by the organism, nodules in which spore formation occurs seem to have a higher energy cost for N_2 fixation and so contribute less to the host.
- Vesicle production occurs under conditions of nitrogen limitation, with the type of vesicle produced dependent on the host plant. Vesicles are borne as terminal swellings or on short hyphal branches; at maturity, they show a pronounced lipid envelope that protects the nitrogenase from oxygen.

Additional information on the biology and symbiotic specificity of *Frankia* is provided by Benson and Silvester (1993).

Azolla/Anabaena Symbiosis

The aquatic fern *Azolla* is a common green manure used in Vietnam and China for rice production. *Azolla* maintained in slow-flowing creeks or overwintered in protected beds is introduced into paddies between plantings of rice and is then either incorporated before rice seedlings are transplanted or left to be shaded out as the rice canopy develops. The low C/N ratio of the fern facilitates rapid nitrogen mineralization after incorporation, with yields in the subsequent rice crop increased by as much as 1,000 kg ha $^{-1}$. In this case N_2 fixation is because of the heterocystous cyanobacterium

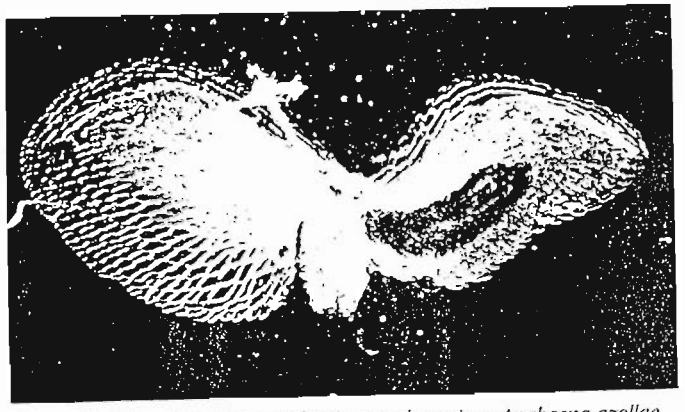

Figure 14–9 Location of the symbiotic cyanobacterium *Anabaena azollae* within the leaf (frond) of the water fern *Azolla*. The frond has been cleared, with the *Anabaena* filaments visible as the darkened region to the center-right of the frond. *Photo courtesy T.A. Lumpkin. Used with permission.*

Anabaena azollae growing within cavities in the dorsal leaf lobe (Fig. 14–9). Under favorable conditions, rates of N_2 fixation can reach 2 kg ha^{-1} day^{-1}.

Four aspects of this symbiosis warrant particular comment:

- *Azolla* can be cured of its microsymbiont, but no one has succeeded in reintroducing *Anabaena*.
- Dinitrogen fixation in *Anabaena* occurs predominantly in specialized cells called **heterocysts.** Under free-living conditions, only 6 to 10% of the cells in the filament are heterocysts, but in the mature *Azolla* frond, this frequency rises to 20 to 30%.
- Gram-positive bacteria that do not fix dinitrogen, identified as species of *Arthrobacter*, have also been found in the leaf cavity and are thought to play a role in this symbiosis. However, no definite function is known for these organisms.
- During heterocyst formation by free-living *Anabaena*, a small piece of DNA is deleted, leaving the *Nif* HDK genes all regulated by a single promoter. In contrast, all cells of the *Azolla* microsymbiont have this arrangement.

Although the yield benefits from the use of *Azolla* can be appreciable, they are offset to some extent by labor costs for storage, propagation, and field distribution of the fern, by the need in some areas to tie up scarce land while the inoculum is multiplied, and by insect and disease problems. Nonetheless, the *Azolla/Anabaena* symbiosis continues to be important in Asia, with some estimates of the area sown to *Azolla* in China being as high as 1.5 million ha annually. Additional information on this symbiosis is provided by Lumpkin and Plucknett (1980) and Peters and Meeks (1989).

Summary

This chapter highlights the potential of N_2 fixation in legumes, and problems associated with utilizing this symbiosis. It also introduces other N_2-fixing symbioses, some of which have potential for use in agriculture. As with the legumes, their greater exploitation will require a multidisciplinary and ecological approach.

Symbiotic N_2 fixation currently accounts for more than 65% of the nitrogen used in agriculture. As the world's population increases, this contribution must increase. Problems in the availability of fertilizer nitrogen and groundwater pollution resulting from excessive fertilization will limit the degree to which fertilizer nitrogen usage can be increased. Although inoculant production in many regions of the world leaves much to be desired, the inoculant technology reviewed in this chapter is within the reach of most countries and needs only to be properly and consistently applied. However, other bottlenecks need to be remedied if symbiotic N_2 fixation is to assume a more important role in the agriculture of the twenty-first century. Areas of research that need to be resolved include:

- improving the ability of different varieties to fix dinitrogen,
- overcoming the problem of low nodule occupancy by inoculant strains,
- improving the persistence of inoculant strains in soil,
- enhancing the tolerance of both host and microsymbiont to environmental stresses, and
- understanding better the contribution of N_2 fixation to the nitrogen economy of both modern and traditional farming systems.

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Study Questions

1. Competition for energy between developing pods and nodules illustrates the influence of energy supply on nodule function. What experimental treatments can you suggest to study the importance of energy supply in nodulation and N_2 fixation?

2. Dinitrogen-fixing symbioses adopt different strategies to protect nitrogenase from the inhibitory effects of oxygen. Give three examples discussed in this chapter.

3. You join the Peace Corps in Nepal and are assigned to the development of an inoculant industry for that country. Describe the steps you would need to take to achieve such a development.

4. In Latin America and Africa, N_2-fixing crops such as bean and cowpea are often grown in association with corn. How might this affect N_2 fixation?

5. Develop a method to screen strains of *Rhizobium* for differences in pH tolerance.

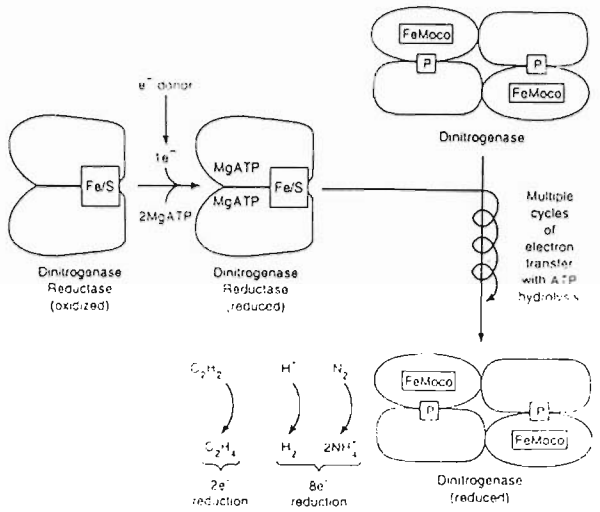
6. Legumes abound in most situations. Find one such plant and describe its nodulation. How many nodules does it have, how are they distributed, and what type of nodule are they? Describe the internal appearance of the nodule. How would you determine whether the nodules were formed by a *Rhizobium* or *Bradyrhizobium* strain?

PROKARYOTIC NITROGEN FIXATION

10

A Model System for the Analysis of a Biological Process

Edited by: Eric W. Triplett



horizon scientific press

27

UPTAKE HYDROGENASES IN ROOT NODULE BACTERIA

T. Ruiz-Argüeso, J. Imperial, and J. M. Palacios

Abstract

Uptake hydrogenases in diazotrophic root nodule bacteria (rhizobia, *Frankia*) ca recycle the H_2 generated as a by-product of the nitrogenase reaction and have potential to improve the energy efficiency of the symbiosis. This is especially relevan in view of the fact that uptake hydrogenases are uncommon in many of the rhizobi used as legume inoculants. The Hup (Hydrogen uptake) system has been studied i depth only in two species of root nodule bacteria, *Rhizobium leguminosarum* bv *viciae* and *Bradyrhizobium japonicum*. In these organisms, a multigenic (18-24 genes cluster responsible for synthesis of an active hydrogenase has been isolated. Th current status of research on characterization of the functions of their gene product and the regulation of their expression is reviewed. This information, together wit available gene transfer technology, opens the door to biotechnological exploitatio of the Hup system for the design and generation of more energy efficient rhizobia inoculants.

Introduction

Uptake hydrogenases catalyze the oxidation of molecular hydrogen to protons, an are present in many aerobic bacteria, where electrons are fed to the respiratory chair oxygen acts as the final acceptor, and energy can be obtained through oxidativ phosporylation. The "knallgas" bacteria, where H_2 is used as the only energy sourc for growth (1, 2) represent an extreme example of this potential. Uptake hydrogenase can be important for nitrogen-fixing organisms because of the concomitan unavoidable reduction of protons to hydrogen catalyzed by nitrogenase (see Chapte 3 in this volume). The hydrogen generated can be recycled by uptake hydrogenase from the same organism, therefore increasing the energy efficiency of nitroge fixation (3, 4). This hydrogen recycling is especially relevant for the diazotrophi symbioses between plants and root nodule bacteria because it has the potential t increase the energy efficiency of the symbiosis and to improve plant productivit Thus, it is not surprising that it has been the subject of a good number of reviews i

the past (5-10). This chapter will focus on recent advances in our understanding of the only two systems studied in depth, those of *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* bv. *viciae*, and on the biotechnological applications of these systems. Other aspects, such as the occurrence and physiology of hydrogenase systems in rhizobia, have not progressed at the same pace and are well covered by the above reviews.

Hydrogenases and Hydrogen Recycling in Legume Nodules

Uptake hydrogenase in rhizobia is a dimeric ($\alpha\beta$), membrane-bound [NiFe] enzyme that has only been isolated and characterized from *B. japonicum*, either as free-living cells or as soybean bacteroids (see 5, 7, 8 for reviews). Both primary structure (see below) and properties of membrane-bound, [NiFe] hydrogenases and their ancillary systems are extremely well conserved in widely diverging bacteria (11-13). For this reason, many results obtained with specific systems (e.g. the well-known *Escherichia coli*) have often been extrapolated to lesser known systems. Recently, the crystal structure of the [NiFe] hydrogenase from *Desulfovibrio gigas* has been solved (14-16), and it is expected that the key structural features will be conserved in other systems. Noteworthy among them are: a bimetallic [Ni-Fe] active site in the large subunit, with biologically uncommon CO and CN ligands to the iron, and three iron-sulfur clusters in the small subunit (14, 15).

Rhizobia expressing hydrogenase activity (Hup$^+$, from Hydrogen uptake) are able to reoxidize the H_2 generated by nitrogenase and produce root nodules which evolve little or no H_2. Given the potential of these uptake hydrogenase systems to improve the energy efficiency of diazotrophic symbioses, it is somewhat surprising that the ability to recycle hydrogen is not widely distributed in rhizobia (see 8, for a review, and also below). In many rhizobia of the genera *Rhizobium*, *Sinorhizobium*, and *Mesorhizobium*, the Hup$^+$ phenotype is a rare trait. In some cases, the genetic determinants for the *h*ydrogen *up*take (*hup*) system seem to be present, but conditions appropriate for their expression have not been found (see 10). Despite repeated screenings (see 8), reliable hydrogenase activity measurements and the presence of *hup* genes have only been documented for fast-growing rhizobia in the following cases: i) some strains of *R. leguminosarum* bv. *viciae*; ii) among the bean rhizobia, those *R. tropici* strains belonging to subgroup IIb (17, 18); and iii) uncharacterized strains nodulating *Astragalus* (19). Strains capable of efficiently recycling H_2 have not been found in fast-growing rhizobia nodulating important legume crops such as alfalfa and clovers (20) or chickpeas (21). Among the slow-growing rhizobia (*Bradyrhizobium spp.*), the Hup$^+$ phenotype is not uncommon in lupine and soybean strains and it is quite common among strains of the cowpea miscellanea. Finally, most *Azorhizobium* strains are Hup$^+$. The reasons for this patchy distribution are not clear, but they may be related to: i) possible specific roles of the Hup system in the biology of some rhizobia in the soil (e.g. chemolithotrophy in *B. japonicum*: 5, 22); and ii) plasmid encoding of the Hup system in many rhizobia (see below). As a result, most strains commonly used as legume inoculants are Hup$^-$. As we discuss

below, this opens possibilities for biotechnological exploitation of the known Hup systems.

Very little is known about hydrogenases in other root nodule symbiotic systems. No studies have been carried out with the non-legume *Parasponia*-rhizobial symbiosis. Studies with the actinorhizal symbioses also lag behind, despite the fact that all the tested *Frankia* isolates nodulating *Alnus* (23) or *Casuarina* (24) exhibited a Hup$^+$ phenotype.

Genetic Determinants for Hydrogenase Synthesis

The synthesis of hydrogenase enzyme requires the concerted action of a large numbe. of proteins. In *R. leguminosarum* and *B. japonicum* the whole set of genetic determinants involved in this process is clustered in DNA regions of over 15 kb (Figure 1), which have been identified in the symbiotic plasmid of *R. leguminosarum* (25) and in the chromosome of *B. japonicum* (26). Most of hydrogen oxidation genes from rhizobia have been designated *hup* genes, and a specific set of genes has been designated as *hyp* after their homology to a previously described hydrogenase pleiotropic gene operon from *E. coli*. The *R. leguminosarum* cluster includes 18 genes (*hupSLCDEFGHIJKhypABFCDEX*) closely linked and transcribed in the same direction (Figure 1). The *B. japonicum* cluster of hydrogen oxidation determinants contains 24 genes (*hupNOPUVSLCDFGHIJKhypABFCDEhoxXAhupT*).

A general model explaining the role of each gene product on the process of hydrogenase synthesis is not yet available. Gene products required for hydrogenase synthesis include the hydrogenase structural subunits (HupSL), along with a large array of proteins whose known or proposed functions are summarized in Table 1. Potential functions ascribed to these gene products in hydrogenase synthesis or activity are electron transport (HupC), subunit proteolytic processing (HupD), hydrogenase scaffolding (HupK), redox modifications (HupI), and processing of nickel (products of *hyp* gene cluster). Some of these functions have been first described in other bacteria, mainly in *E. coli*, and have been generalized on the basis of the wide conservation of hydrogenase systems among different bacteria (see 11-13, for reviews). Genes involved in the regulation of *hup* expression are present in the *B. japonicum* cluster, namely *hupUV*, *hoxA* and *hupT* genes (see Regulation section below). A more detailed description of the specific role of most of Hup and Hyp proteins in rhizobia has been thoroughly covered recently (10). New information available on the possible roles of HupE, HypX (HoxX) and HypB in hydrogenase synthesis is reviewed below.

hupE (27) is the only *R. leguminosarum hup* gene not present in the *B. japonicum* hydrogenase cluster. It encodes a hydrophobic protein of about 19 kDa, including a leader peptide and six potential transmembrane domains. Genes homologous to *hupE*, although infrequent in hydrogenase systems, have been identified in connection with hydrogen oxidation genes of *Rhodobacter sphaeroides* (EMBL accession Y14197), *Rhodocyclus gelatinosus* (EMBL accession X52522), *Synechocystis* sp. (28), and *Aquifex aeolicus* (29). More interestingly, proteins displaying high homology to *R. leguminosarum* HupE have been identified in urease gene clusters

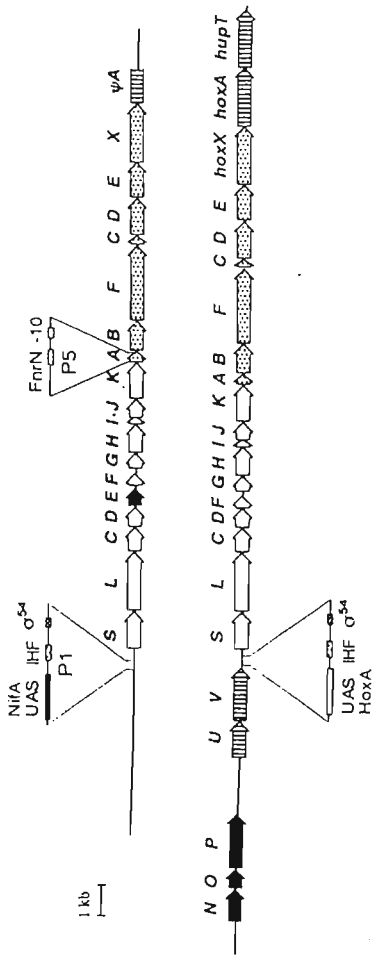


Figure 1. Genetic organization of the *hup* regions of *R. leguminosarum* bv. *viciae* and *B. japonicum*. Arrows indicate gene size and direction of transcription. *hyp* genes are indicated by a light grey fill. Regulatory genes are indicated by vertical stripes. Dark-filled genes are putative Ni transport genes unique to either *R. leguminosarum* or *B. japonicum*. Relevant features of P1 and P5 promoters from *R. leguminosarum* and of the main structural promoter from *B. japonicum* are indicated in the enlarged regions (see text).

Table 1. *hup* Gene Functions in *Rhizobium leguminosarum* bv. *viciae* and *Bradyrhizobium japonicum*

Gene				Function[c]
R. leguminosarum	*B. japonicum*	Size[a]	Identity (%)[b]	
	hupN	40		(?) Ni transport
	hupO	19		(?) Ni transport
	hupP	63.5		(?) Ni transport
	hupU	35.5		Regulation (sensor H_2ase small subunit)
	hupV	52		Regulation (sensor H_2ase large subunit)
hupS	*hupS*	34.5	89	Hydrogenase small subunit
hupL	*hupL*	66	89	Hydrogenase large subunit
hupC	*hupC*	28	67	Cytochrome *b*
hupD	*hupD*	22	65	HupL-processing specific peptidase
hupE		19		(?) Ni transport
hupF	*hupF*	10.5	45	
hupG	*hupG*	16	48	
hupH	*hupH*	30.5	51	
hupI	*hupI*	8	70	Rubredoxin
hupJ	*hupJ*	18.5	45	
hupK	*hupK*	39	32	(?) Scaffolding protein
hypA	*hypA*	12.5	49	
hypB	*hypB*	32.5	56	Ni binding
hypF	*hypF*	80.5	51	Hydrogenase processing
hypC	*hypC*	8	63	Hydrogenase processing
hypD	*hypD*	43.5	73	Hydrogenase processing
hypE	*hypE*	36.5	77	Hydrogenase processing
hypX	*hoxX*	62	55	Hydrogenase processing
hoxA[d]	*hoxA*	53	57[e]	Regulation (activator)
	hupT	50		Regulation (repressor)

[a] Gene product size (kDa) for *R. leguminosarum* (or *B. japonicum* where appropriate)
[b] % identical amino acids in sequences from both organisms
[c] Question marks (?) denote proposed functions
[d] The *R. leguminosarum* ψ*hoxA* lacks the C-terminal half
[e] % identity of the first 136 aas conserved in ψ*hoxA*

from *Bordetella bronchiseptica* (30) and *Alcaligenes eutrophus* (EMBL accession Y13732). Urease is a soluble nickel-containing enzyme, and the presence of a homologous membrane protein in both hydrogenase and urease systems strongly suggests that HupE could be involved in Ni supply to the cell (30). It has to be noted, however, that the putative activity of *R. leguminosarum* HupE as Ni transporter is not sufficient to ensure adequate levels of this element for hydrogenase synthesis, since hydrogenase activity in pea bacteroids is limited by nickel level of the rooting solution, even at high concentrations (over 100 μM, see Nickel Uptake below).

hypX is the distal gene in the *R. leguminosarum* gene cluster (Figure 1). It has been shown to belong to the *hyp* operon along with *hypBFCDE* genes (31). In *B. japonicum* a gene homologous to *hypX*, designated *hoxX*, precedes the gene encoding the regulatory protein HoxA. The actual function of HypX is not clear. Initial sequence comparisons of *B. japonicum* HoxX suggested that it could act as sensory histidine kinase in connection with HoxA (32). However, the analysis of HypX-deficient mutants from *R. leguminosarum* (31) and *B. japonicum* (33) revealed that such mutants expressed lower levels of hydrogenase activity but accumulated unprocessed forms of the enzyme, suggesting a role for HypX/HoxX at post-translational rather than at transcriptional level, specifically at the stage of Ni-dependent processing of

the enzyme. Further sequence comparison of *R. leguminosarum* HypX and homologous proteins (31) revealed the presence of sequence motifs characteristic of two different types of enzymes: i) enzymes using N_{10}-formyltetrahydrofolate as C1 donor, and ii) enzymes using XCO-SCoA as substrate. Based on these homologies it has been proposed that HypX/HoxX could be a bifunctional enzyme involved in the incorporation of one-carbon groups into the active site of hydrogenase (see above) through an unknown mechanism.

Several ancillary proteins required for hydrogenase synthesis are involved in nickel metabolism. Among these, HypB may have a central role in nickel provision for the enzyme. HypB proteins from *R. leguminosarum* and *B. japonicum* are able to bind nickel *in vitro*, likely through histidine-rich stretches present in both proteins (34, 35). Further studies have shown that HypB from *B. japonicum* is associated to the ability of storing nickel in the cell for hydrogenase synthesis (36). The mechanism for nickel storage and transfer to hydrogenase has not been elucidated yet. This mechanism may require energy supply, since studies carried out in *E. coli* and *B. japonicum* have demonstrated that HypB has GTPase activity (35, 37). The available data suggest that HypB may have an important role in nickel sequestration/storage in the bacteroid, under conditions where the microsymbiont must compete with plant enzymes for available nickel (36).

Other Functions Required for Hydrogenase Activity

Nickel Uptake

The incorporation of nickel into the active center of hydrogenase is an essential step for the synthesis of the enzyme. The environments in which rhizobial hydrogenase is expressed (legume nodules, agricultural soils, and laboratory conditions) usually contain low amounts of nickel, and bacteria oxidizing H_2 in these environments should possess an efficient uptake system for this element. A gene cluster (*hupNOP*) involved in nickel metabolism has been identified in *B. japonicum* (38). A *B. japonicum* HupNOP$^-$ mutant showed an 80% reduction in hydrogenase activity at low nickel concentrations (0.5 μM), and this defect could be complemented by high nickel (50 μM). However, since this mutant maintained the same K_m for nickel transport (62 μM) as the wild type strain, it was concluded that HupN was not a nickel transporter, but that it was rather involved in some nickel-insertion/metabolism steps for proper assembly of hydrogenase (39). Although no direct role of HupN on nickel transport has been demonstrated, it is interesting to note that this protein shares a high degree of homology with proteins described in *A. eutrophus* (HoxN, 40) and *Helicobacter pylori* (NixA, 41). HoxN and NixA have been demonstrated to mediate nickel transport when expressed in *E. coli* cells (41, 42). A protein homologous to this family of nickel transporters has also been reported in the urease gene cluster of *Bacillus* sp TB90 (UreH, 43). These data suggest that HupN might be indeed a nickel transporter, acting in concert with HupO and HupP through an unknown mechanism. Since the K_m value described for *B. japonicum* cells is relatively high (62 μM) and HoxN and NixA have been described as high-affinity

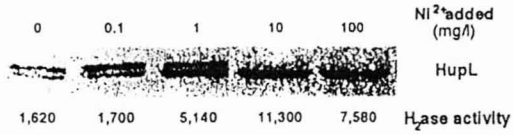


Figure 2. Effect of nickel on hydrogenase processing and activity in *R. leguminosarum* bv. *viciae* bacteroids. Ni^{2+} was added to pea plant nutrient solution. Processed and unprocessed forms of HupL were visualized by immunoblotting using antisera raised against *B. japonicum* HupL protein. Bacteroid hydrogenase activity is expressed as nmoles H_2 oxidized per hour per mg protein (after ref. 44).

(K_m 0.34 μM and 0.011 μM, respectively) low-capacity nickel transporters, it is possible that the Ni uptake mediated by HupN in *B. japonicum* could be masked by a second system also functional in this bacterium.

In *R. leguminosarum* studies on nickel transport for hydrogenase synthesis have been hampered by the symbiosis-specific expression of *hup* system. It has been clearly established that nickel availability limits hydrogenase expression at the level of processing of the enzyme subunits (Figure 2; 44). In fact, full processing of the subunits was only observed at nickel concentrations in the phytotoxic range (over 1 mM) indicating that the provision of nickel to the bacteroid is a limiting factor for this process. It is not clear, however, whether this limitation is due to the bacterial or the plant component of the symbiosis. Recent results of nickel transport experiments with intact pea symbiosomes indicate that the peribacteroid membrane is not a specific barrier for Ni transport into the bacteroid (Báscones *et al.*, unpublished).

Hydrogenase Translocation

An additional aspect important for hydrogenase synthesis is the translocation of the enzyme to the periplasmic side of the membrane. This process is likely mediated by HupS signal peptide. The features of signal peptides present at the N-terminus of HupS-like subunits of different hydrogenases are rather unusual (45). Although they keep the overall structure of regular signal peptides, they are much longer (27-50 residues) than those recognized by the general secretory Sec system (18-26 residues) and present a twin-arginine motif (R-R-x-F-x-K). This motif is conserved in the N-terminus domain of all [NiFe] hydrogenase small subunits, including those from *R. leguminosarum* and *B. japonicum*. Analysis of mutant signal peptides has demonstrated that arginine residues within this motif are essential for enzyme export (45). A novel translocation system for proteins containing this type of signal peptide was later identified in maize thylakoid membranes (46). *E. coli* genes (*tat* genes, for twin arginine translocation) encoding a homologous system have been identified, and shown to mediate hydrogenase translocation to the periplasm (47). The current model for *E. coli* Tat system includes the participation of a putative membrane channel protein (TatC) along with other proteins (TatA, TatB, TatD) which should act as branches at the membrane targeting/reception stage of the secretion pathway (48). *tat*-like genes involved in hydrogenase metabolism have also been identified in

A. chroococcum (49). Preliminary experiments carried out in our laboratory demonstrated the presence of *tatC* also in *R. leguminosarum* (Meloni *et al.*, unpublished).

Regulation of Hydrogenase Gene Expression

Analysis of the hydrogenase system in root nodule bacteria has revealed the existence of regulatory mechanisms controlling Hup expression in free-living and symbiotic cells. Hydrogenase synthesis has been observed in two different physiological situations. First, free living cultures of *B. japonicum* induce hydrogenase activity in response to microaerobic conditions in the presence of hydrogen and traces of nickel. This expression in vegetative cells has not been observed in *R. leguminosarum*. Second, hydrogenase activity is also induced in bacteroids within legume nodules, where hydrogen generated by nitrogenase is a substrate for the enzyme. Both types of induction respond to very different regulatory mechanisms.

Hydrogenase expression in vegetative cells of *B. japonicum* is controlled by a complex mechanism responding to the simultaneous presence of three environmental signals: hydrogen, low oxygen tensions and traces of nickel (22). Although a complete model for this regulation is not available, it is known that under those conditions *hupSL* expression requires σ^{54}, IHF (50) and HoxA (51). HoxA is a DNA-binding transcriptional activator of the NtrC family (32), and homologous proteins have also been described in hydrogenase systems from *A. eutrophus* (52) and *R. capsulatus* (HupR1, 53). In addition to HoxA, three other proteins affect expression of *hup* genes in vegetative cells: HupU, HupV, and HupT (Figure 1). HupUV complex constitutes a pseudohydrogenase apparently able to respond to adequate levels of oxygen, hydrogen and nickel (54). A recent study performed with *A. eutrophus* HupUV complex has demonstrated that this pseudohydrogenase contains an active center much like that of standard hydrogenase (55).

HupT protein was previously described as repressor of Hup activity in *R. capsulatus* (56) and in *A. hydrogenophilus* (HoxJ*, 57). This protein has the features of a histidine kinase, including the ability to autophosphorylate. A HupT homologue acting as repressor of *hup* expression has been recently described in *B. japonicum* (van Soom *et al.*, submitted). Thus, the model for regulation of hydrogenase in this bacterium could be similar to that proposed for *A. eutrophus* (57). In this model, HupUV sensor complex transduces the presence of environmental conditions adequate for hydrogenase synthesis into activation of HoxA, probably by modifying HupT (HoxJ*) repressor activity. Active HoxA would then promote transcription of hydrogenase structural genes and downstream genes leading to hydrogenase synthesis. Such regulatory model does not apply to *R. leguminosarum*. In this bacterium *hupUV* and *hupT* genes have not been found, and only a non-functional pseudogene (*hoxA*) has been identified downstream from *hypX* (31). Consistent with this, no induction of *hup* genes has ever been observed in vegetative cells of *R. leguminosarum* (58).

Symbiotic expression of *hup* genes has been studied in detail in *R. leguminosarum*. In this system, two major promoters have been identified (Figure 1): i) A -12/-24 promoter (designated P1) controls symbiotic expression of the operon containing hydrogenase structural genes and several additional genes located downstream. "*In situ*" hybridization experiments demonstrated that *hupSL* are co-expressed both temporally and spatially with nitrogenase structural genes in pea nodules (59), suggesting the presence of a common regulator for both *nif* and *hup* systems. Furthermore, data obtained in experiments of heterologous expression in *Klebsiella pneumoniae*, *E. coli* and *R. etli* led to the conclusion that in *R. leguminosarum* transcription of hydrogenase structural genes is controlled by a σ^{54} type promoter activated by NifA with the concurrence of IHF (60); ii) A second promoter (designated P5) has been identified upstream of *hypB* within the coding sequence of *hypA*. P5 is an Fnr-type promoter containing a consensus anaerobox sequence that controls the expression of the *hypBFCDEX* genes. It has been demonstrated that FnrN, a transcriptional activator homologous to *E. coli* Fnr, activates P5 in bacteroids and in vegetative cells exposed to microacrobic conditions (61). Furthermore, two functional copies of the corresponding gene (*fnrN1* and *fnrN2*) have been identified in *R. leguminosarum* UPM791 (62). A double mutant affected in both copies induced ineffective nodules lacking both hydrogenase and nitrogenase activities. In addition to *hypBFCDEX* genes, *R. leguminosarum* FnrN also controls the microaerobic induction of a *fixNOQP* operon, involved in the synthesis of a cbb_3-type, high affinity terminal oxidase essential for bacteroid respiration (62). These results provide additional evidence for the close relationship between hydrogenase and nitrogenase systems in *R. leguminosarum*.

In the case of *B. japonicum* the regulation of symbiotic expression of Hup activity has not been fully clarified. It has been shown that HoxA is not essential for transcription of hydrogenase structural genes in soybean nodules (33, 51). Additionally, it has been proposed that nitrogenase and hydrogenase are co-regulated through $FixK_2$ (33), although the possibility that NifA still affects HupSL expression in the nodule has not been discarded.

Another factor affecting hydrogenase expression in *R. leguminosarum* is the legume host. Some legume hosts have been shown to be more permissive than others for expression of hydrogenase. Host-plant mediated control of the Hup phenotype has been reported for the symbionts of pea, soybean, cowpea and common bean (10). The molecular basis for this effect is not understood yet. In the case of *R. leguminosarum* bv. *viciae*, it has been demonstrated that strain UPM791 induces hydrogenase activity in pea bacteroids but is unable to express this activity in lentils (63). A hydrogenase expression analysis with different hosts nodulated by this strain, including several *Vicia* and *Lathyrus* species and lentil varieties demonstrated that the lack of bacteroid hydrogenase activity is specific to lentils. Preliminary data arising from experiments carried out with reporter gene fusions suggest that the blocking of Hup expression is exerted at the level of transcription of *hup* structural genes (Toffanin *et al.*, unpublished).

Table 2. Strategies Followed to Generate New Hup⁺ Strains of Root Nodule Bacteria

Strategy	Source of *hup* system	Recipient Hup⁻ species	Refs
Cosmid-borne *hup* genes	*B. japonicum*	*S. meliloti*,	(83)
	B. japonicum	*R. leguminosarum* bv. *viciae*,	
	R. leguminosarum	*R. leguminosarum* bv. *trifolii*, *B. japonicum*	(75)
	R. leguminosarum	*M. ciceri*	(81)
	R.leguminosarum	*R. etli*	(76)
	B. japonicum	*M. loti*	(77)
Plasmid-borne *hup* genes stabilized with *par*	*B. japonicum*	*Sinorhizobium* sp.	(78)
Native *sym-hup* plasmids	*R. leguminosarum*	*R. leguminosarum* bv. *viciae*	(74)
	R. leguminosarum	*S.meliloti*	(79)
			(80)
	R. leguminosarum	*M. ciceri*	(81)
Chromosome-integrated cosmid DNA	*B. japonicum*	*M. ciceri*	(84)
Chromosome-integrated *hup* cluster by site-specific recombination	*B. japonicum*	*M. ciceri*	(82)
Chromosome-integrated *hup* cluster by use of minitransposons	*R. leguminosarum*	*R. leguminosarum* bv. *viciae*, *R. leguminosarum* bv. *trifolii*, *R. etli*, *M. loti*, *M. ciceri*, *S. meliloti*, *B. japonicum*	(*)

* Báscones *et al.*, unpublished

Biotechnological Applications

It has been estimated that the energy losses associated to the hydrogen production by legume nodules account for 40 to 60% of the energy available for nitrogen fixation (64). These energy loses constitute the main source of inefficiency of the rhizobia-legume symbiosis, and those root nodule bacteria strains that express hydrogenase activity are expected to carry out a more efficient fixation of N_2 by reutilizing the H_2 produced by the nitrogenase (65). The ability to oxidize H_2 is generally considered a beneficial property of N_2-fixing organisms, whether in the symbiotic or in the free living state, and several mechanisms by which the presence of a hydrogenase might increase the overall efficiency of nitrogen fixation have been postulated (66, 67). These include: i) provision of an additional source of energy and reductant to the N_2-fixing system; ii) protection of nitrogenase from O_2 damage; and iii) prevention of H_2 inhibition of nitrogenase-catalyzed N_2 reduction. The direct or indirect experimental evidence accumulated in support of these potential benefits of the hydrogenase system has been discussed in detail in previous reviews on this topic (5, 8, 9).

Although convincing evidence from *in vitro* experiments has shown that the hydrogenase system has a potential to improve N_2 fixation, the actual contribution of H_2 recycling ability to increase productivity of nodulated legumes is still a conflicting issue. By comparing groups of Hup⁺ and Hup⁻ *B. japonicum* strains or

wild-type strains and nearly isogenic (except for the presence of the Hup system) Hup⁻ mutants, Evans and coworkers provided strong evidence for a positive effect of the *hup* system in productivity of soybeans (6, 68). Particularly significant was an experiment with soybean plants grown to maturity in large concrete tiles in which significant increases (11%) in seed yield and total nitrogen content were detected in soybean plants inoculated with a *B. japonicum* Hup⁺ revertant strain as compared with plants inoculated with the corresponding Hup⁻ mutant (69). Positive effects on legume productivity associated to the presence of hydrogenase activity have also been observed in bean (*Phaseolus vulgaris*) (70, 71). However, in one particular case, a negative effect of the hup system on productivity of soybeans was reported by using a different Hup⁻ *B. japonicum* mutant as control (72). No significant increases in productivity were found associated to the *hup* system in experiments with *R. leguminosarum* Hup⁺ strains (73, 74).Variations in experimental conditions such as use of rhizobial strains that do not efficiently recycle H_2 or where the H_2 oxidation is not coupled to ATP generation in the host legume, the harvesting of plants before maturity, or use of insufficient number of replicates for statistical significance,, particularly in field experiments, and overall the use of inadequate Hup⁻ control strains are to be blamed for the differences observed (6, 8). The need to compare Hup⁺ and Hup⁻ strains that are isogenic except for the H_2 oxidation characteristic has repeatedly been emphasized (6). However, since we know now that the H_2 oxidation capacity is coded by a multigenic system, Hup⁻ mutants deleted of the entire *hup* cluster should be more appropriate controls than Hup⁻ mutants affected in a single hydrogenase gene. Alternatively, the effect of *hup* on legume productivity can be examined by comparing Hup⁻ wild-type strains of root nodule bacteria and genetically engineered, derivative Hup⁺ strains that have received the entire *hup* cluster. The construction of these strains is a first step in a more general biotechnological objective aimed to extend the Hup phenotype to rhizobia that nodulate important legume crops and lack the *hup* system.

Transfer of *hup* genes to heterologous backgrounds is now facilitated by the progress made in identification and characterization of genetic determinants for hydrogenase synthesis and in the regulation of their expression (see above). Several strategies have been used to transfer the *hup* system from Hup⁺ strains of *B. japonicum* and *R. leguminosarum* bv. *viciae* into Hup⁻ recipient strains of different rhizobia (Table 2). The transfer of the *hup* system in a plasmid or cosmid basis frequently faces problems of instability of the Hup character in root nodules in the absence of selective pressure for maintenance. The instability of pLAFR1-derived cosmids containing the *hup* system in nodules appears to be partly host-dependent, and it is higher in *S. meliloti* and *B. japonicum* (10^{-2} to 10^{-4}) (75) than in *R. etli* and *M. loti* (10^{-1}) (76, 77). Maintenance rate of the Hup phenotype can be stabilized by addition of the par genes to a cosmid containing the *hup* system (78). When native *hup*-containing symbiotic plasmids from *R. leguminosarum* have been used, the presence of non-*hup* DNA information imposes an extra metabolic load to the recipient cell which can mask the effect of the *hup* system on the symbiotic behavior (74, 79-81). Integration of the plasmid-borne *hup* cluster into the chromosome appears to be the strategy of choice, although its large size (over 15 kb) restricts its usefulness for *in vitro* recombination (82).

The strategy we have found to be more rapid and flexible for integration of the *hup* system into the chromosome of recipient strains is the use of mini-transposons based on the delivery system originally designed by de Lorenzo *et al.* (85) and later on modified by Wilson *et al.* (86). Genes to be integrated are cloned between the inverted repeats of Tn*5* adjacent to a marker antibiotic resistance gene. Since the rest of the transposition system is located outside these repeats, the procedure results in a stable integration. Using adequate suicide vectors one can eventually transfer any DNA fragment into the chromosome. This system allowed us to stably integrate the entire 18 kb *hup* cluster from *R. leguminosarum* bv. *viciae* strain UPM791 into the chromosome of Hup⁻ strains of *B. japonicum*, *M. ciceri*, *M. loti*, *R. leguminosarum* bv. *viciae* and bv. *trifolii*, *R. etli* and *S. meliloti* (Báscones *et al.*, unpublished). Hydrogenase expression analysis of these newly generated Hup⁺ strains in symbiosis with their corresponding legume hosts showed a broad range of expression levels depending on the bacterial species and even on the strains. From the *hup*-minitransposons containing vectors, it is straightforward to remove the antibiotic resistance gene in order to construct strains with the *hup* system stably integrated into the chromosome for use in field trials or for commercial release.

Stable integration of the *hup* system into the chromosome still does not ensure acquisition of the H_2 recycling capacity by the recipient Hup⁻ strains. Efficient expression of hydrogenase genes and hydrogenase activity in symbiosis with the corresponding host legumes are also required. As indicated above, the bacterial genetic background drastically affects the levels of heterologous expression of the *R. leguminosarum hup* system. The molecular basis for this bacterial host control in *hup* expression is not known at present, although differences in regulation of the *hup* genes by NifA and FnrN, the two major activators of *R. leguminosarum hup* system (see above) might account for the observed differences. Based on what we know from the symbiotic regulation of *hup* gene expression, two other factors may also control expression of the *hup* system in the newly-generated Hup⁺ strains. First, nickel availability to the host plant severely limits the expression of the *R. leguminosarum* hydrogenase genes in the *Pisum sativum* symbiosis (see above), and likely in other symbioses such as the *M. loti-Lotus corniculatus* system (Brito *et al.*, submitted). Second, the host legume can have an effect on hydrogenase expression (see above). In summary, although the tools for introducing the *hup* system in any strain of rhizobia in a stable form are now available. Much research is still needed to achieve an efficient expression of the *hup* system in the different legume hosts and to evaluate their effect on legume productivity before succeeding in obtaining a commercially attractive, superior inoculant strain.

Concluding Remarks

Important advances have been made in recent years on the study of genetic determinants of the *hup* system in the two model systems, *B. japonicum* and *R. leguminosarum* bv. *viciae*, as well as on their regulation of expression. This information will allow for the generation of well-defined Hup⁻ strains. In addition, these new constructs allow testing of the hypothesized positive effect of the system on the legume-rhizobial symbioses. Further studies are also needed on gene product functions and on the mechanism of active hydrogenase assembly, and on the yet uncharacterized rhizobial *hup* systems as well as that from *Frankia*. Finally, construction and test of new Hup⁺ strains will allow further characterization of the plant and bacterial factors (Ni, energy coupling, plant host effect, ...) that limit hydrogenase expression in symbiosis.

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diferenciados de Rhizobium: unos de crecimiento rápido y productores de ácido, y otros de crecimiento lento que alcalinizan el medio de cultivo. En general, los de crecimiento lento se corresponden con leguminosas tropicales frente a los de regiones templadas que tienen crecimiento rápido.

El Rhizobium vive normalmente en el suelo en forma saprófita utilizando nitrógeno combinado. Normalmente es aerobio y la temperatura óptima, tanto para su crecimiento en laboratorio como para la infección y la formación del nódulo, es de 30 °C, aunque soporta temperaturas desde 3 °C hasta 33 °C. Tiene necesidad de ciertos nutrientes para su desarrollo, siendo importantes el Ca, Mo, Co, vitamina B12, etc. Tiene gran sensibilidad al pH, prefiriendo la neutralidad. Es Gram negativa y móvil y carece de formas de resistencia.

3.- La Simbiosis

La asociación entre las leguminosas y el Rhizobium es de gran importancia tanto desde el punto de vista agronómico como económico. La fijación anual del nitrógeno por vía simbiótica se ha calculado en 175×10^6 Tm, frente a 30×10^6 Tm que es la producción anual de abonos nitrogenados (aunque esta cifra aumenta rapidamente).

Esta asociación tiene un caracter benéfico muy claro para la planta, que utiliza el nitrógeno fijado por la bacteria para sus necesidades simbióticas. A cambio la bacteria recibe azúcares y otros nutrientes que necesita. De esta forma, la planta se independiza del nitrógeno de la alimentación de las plantas no simbióticas (gramíneas etc.), y los rizobios no tienen que competir con demás microorganismos del suelo para obtener alimentos.

La leguminosa y la bacteria podrían proliferar de manera en suelos pobres en nitrógeno y materia orgánica donde, por separado, llevarían una vida efímera.

La fijación del nitrógeno se realiza en formaciones especiales de la raíz que se llaman NODULOS: La bacteria penetra en la planta por los pelos radiculares por medio de un mecanismo que todavía no se conoce bien e introduce en el tejido radicular para formar el nódulo. En este proceso coopera la planta. Dentro del nódulo la bacteria cambia de forma y transforma su equipo enzimático produciendo nitrogenasa, que es el enzima responsable de la fijación. Un nódulo efectivo tiene un color rosáceo producido por un pigmento (Leghemoglobina), cuyo papel parece ser el de proteger a la nitrogenasa frente al oxígeno que la inactiva.

La simbiosis Rhizobium-Leguminosa puede ser aprovechada por la agricultura con gran rendimiento, introduciendo la bacteria en los cultivos de leguminosas, lo que supone una evidente ventaja pues se ahorra la utilización de abonos nitrogenados y se controlan mejor las hierbas, obteniéndose altas producciones.

4.- Los Inoculantes

Los inoculantes son preparados industriales que contienen un altísimo número de bacterias seleccionadas por su especificidad para nodular con una planta determinada, así como por su capacidad para fijar nitrógeno. Por lo tanto, se trata de introducir la bacteria que no

teresa allí donde no la haya o donde exista una población menos efectiva.

Cuando se siembran leguminosas sin usar inoculantes las plantas pueden ser invadidas por los rizobios silvestres del suelo que pueden formar nódulos pero ser poco o nada eficientes. Siempre que se siembren leguminosas, deben ser inoculadas. De esta manera se evita el riesgo de un fracaso completo, debido a que no exista la bacteria en el suelo, o un relativo fracaso si la bacteria no posee la mayor eficiencia posible. Hay que señalar también que la bacteria apropiada que formó nódulos excelentes durante cierto período, puede desaparecer del suelo de un año para otro y no dar nódulos, por lo tanto, en la nueva siembra de la misma planta.

Por estos motivos es necesario que las cepas de rizobio que se usen en los inoculantes comerciales reunan las siguientes características:

- Alta eficiencia para la fijación simbiótica de nitrógeno en condiciones de campo.
- Capacidad de sobrevivencia sobre la semilla inoculada.
- Capacidad de sobrevivencia en el suelo.
- Capacidad competitiva para colonizar la raíz y para formar nódulos en presencia de razas autóctonas de menor eficiencia.

Existen diferentes formas de fabricar inoculantes. A continuación se describe la producción de inoculantes con turba como soporte, ya que es la más utilizada en el mundo y por otra parte este tipo de inoculantes es el único que se fabrica en España hasta el momento.

En el proceso de fabricación de inoculantes, se distinguen 6 etapas (ver diagrama):

a) Preparación de la turba: Después de extraída la tur-

ba se deja drenar, se extiende y se seca primero al a
re y luego en hornos a 60 °C. Una vez seca, se muele
malla 200 y se almacena.

b) Preparación del medio que contiene los rizobios: S
prepara a partir de un cultivo puro de una cepa previ
mente seleccionada por sus características simbiótica
y por su facilidad de multiplicación en medio líquido
Dicha bacteria se multiplica en fermentadores con air
ción en un medio de cultivo apropiado hasta alcanzar
una población de aproximadamente 10^{10} bacterias/ml.
rante la fermentación se controla el pH, pureza y con
centración.

c) Impregnación: Consiste en la inoculación de la tur
con el cultivo puro de rizobios procedente del fermen
dor.

d) Conservación: El inoculante se conserva hasta su d
tribución a 4 °C para maximizar su vida útil.

e) Control de calidad: La producción de inoculantes se
realiza bajo un severo control de calidad. Además del
control del caldo de impregnación se realizan controle
del inoculante producido, con lo cual se asegura que e
inoculante contiene una concentración suficiente de ri
zobios vivos por gramo.

f) Distribución: Las condiciones de transporte y conse
vación del inoculante durante la distribución son de s
ma importancia, ya que influyen decisivamente en la ca
lidad del inoculante. Cualquier falta en la distribuci
o en el uso del inoculante hace que éste llegue en mal
condiciones al agricultor con el consiguiente perjuici
económico. Por ello es importante que tanto el distrit

. ...so el agricultor observen detenidamente las instrucciones de uso que acompañan al inoculante.

3.- Técnicas de inoculación

La inoculación consiste en recubrir las semillas con un cultivo de la cepa apropiada de Rhizobium.

La siguiente información sobre dosis de adhesivos, agua e inoculante, depende del tamaño de la semilla a inocular. Podemos distinguir tres tamaños básicos:

Semillas pequeñas: > 200.000 semillas/Kg. Ej.: Trébol blanco, Lotus

Semillas medianas: 200.000 - 20.000 semillas/Kg. Ej.: Alfalfa, Trébol subterráneo.

Semillas grandes: < 20.000 semillas/Kg. Ej.: Soja, Habas, Garbanzo.

a) INOCULACION SIMPLE:

La forma más simple de inoculación es mezclar el inoculante con agua azucarada en proporciones determinadas; después agregar esta mezcla a la semilla revolviendo lo mejor posible hasta obtener un recubrimiento uniforme.

El azúcar actúa como adhesivo; debido a su viscosidad, hace que el inoculante se pegue a la superficie de la semilla.

Como se puede ver en el siguiente cuadro, cuanto menor es el tamaño de la semilla mayor es la cantidad de agua que necesita:

Tamaño semilla	Kilos semilla	Gramos inoculante	Litros agua azucarada (1)
Pequeñas	25	250	3
Medianas	25	250	2
Grandes	100	500	1

(1) En todos los casos se utiliza el azúcar a razón de 100 gr/l. de agua.

Recomendaciones especiales: es muy importante tomar las siguientes precauciones a la hora de inocular:

- Las bolsas de inoculante deben conservarse en un lugar lo más frío posible, pero sin congelarlas, hasta el momento de su empleo. Se recomienda conservarlas en nevera a 4 °C.
- Deben ser utilizados antes de la fecha de vencimiento que da el fabricante.
- No utilizar agua con alto contenido en sales. El agua destilada o de lluvia son ideales. El agua de la red de abastecimiento debe hervirse durante 10 minutos para desalojar el cloro.
- La operación de la inoculación se realizará a la sombra. La semilla inoculada no debe ser expuesta a la luz directa del sol.
- Se procederá a la siembra inmediatamente después de la inoculación, asegurándose que la tierra esté bien preparada y con el grado de humedad apropiado para una rápida y uniforme germinación de la semilla. Si a pesar

se todo se siembra en suelo seco, regar inmediatamente después de la siembra.

- No se puede usar el inoculante con semillas que han sido tratadas con fungicidas, insecticidas y otras drogas sin un asesoramiento previo.
- No debe ponerse el inoculante en contacto con recipientes o instrumentos sucios de petróleo, jabón, desinfectantes, etc.
- No debe usarse cualquier fertilizante junto a la semilla inoculada, porque la acidez provocada por algunos pueden impedir la nodulación.
- No utilizar más de 30 Kg de nitrógeno por hectárea.

9 - PILDORIZACION DE LA SEMILLA

Con esta técnica se trata de adherir lo mejor posible el inoculante a las semillas mediante un adhesivo y un polvo de recubrimiento, de modo que la semilla quede cubierta de una capa dura que contiene a la bacteria y la protege.

Este método se inició en 1.948 en Australia, debido a las dificultades que tenían para implantar praderas en suelos ácidos y para lograr buena nodulación en campos abonados con superfosfato de calcio que daña a la bacteria.

La semilla inoculada y revestida, almacenada a baja temperatura, conserva vivo un número suficiente de rizobios al menos durante cuatro semanas, pero conviene sembrar de inmediato. Si se almacena por más de tres semanas, hay que sembrarlas en suelos con un contenido óptimo de humedad.

El peleteo de la semilla complica y encarece el proc de inoculación, pero puede ser necesario en ciertas cunstancias, sobre todo en siembras de pratenses ye se obtienen los siguientes ventajes:

- Protección de la bacteria del sol y sequedad
- Protección de la bacteria de los fertilizantes.
- Protección de la bacteria de suelos ácidos

Para formar un pelet hay que inocular la semilla de misma forma que en la inoculación simple, pero utilido un adhesivo más fuerte que el azúcar y después hque recubir la semilla inoculada con un polvo finam molido.

Hay muchos tipos de adherentes: Goma arábiga, Carbotil-celulosa, Metil-etil-celulosa, Dextrina, Carbop Methocel, Colex "A", etc.

El mejor es la goma arábiga comercial en polvo. Se en solución acuosa al 40%. Cuesta mucho el disolver puede requerirse un ligero calentamiento. Debe ser más pura posible (ya que las impurezas pueden perjucar al rizobio), y no contener conservantes.

También da buenos resultados la metil-etil-celulosa LOFAS "S"), utilizada al 5% en solución de agua. Ur vez preparada, debe dejarse reposar una noche para espese.

El material de recubrimiento debe estar muy finamen molido, hasta la consistencia de polvo (el 100% debe pasar malla 300). Se utiliza Carbonato Cálcico o Fosto de Roca, según el tipo de rizobio de que se trat

a) Se usa carbonato cálcico para los rizobios de crmiento rápido y formadores de ácido como son los

nosas de zonas templadas.

b) Se usa fosfato de roca para recubrir las semillas de especies tropicales (Soja), ya que las bacterias que las nodulan son de crecimiento lento y formadoras de álcali.

Una vez que la semilla está inoculada, se mezcla con el polvo de recubrimiento y se revuelve hasta que todas las semillas quedan cubiertas por una capa.

Después es conveniente dejar orear los palets durante unas horas para que se endurezca la cubierta y no queden pegadas unas semillas a otras.

También en este caso varían las dosis requeridas de acuerdo a la talla de la semilla. Cuanto más pequeña es la semilla, más material se necesita para el recubrimiento.

Tamaño de semilla	Semilla	Adhesivo (1)	Polvo de recubrimiento (2)
pequeña	25 Kg	3 l.	60 %
mediana	25 Kg	2 l.	40 %
grande	100 Kg	1 l.	25 %

1) Solución acuosa de goma arábiga al 40% ó Celofas "A" al 5%.

2) Expresado en porcentaje del peso de la semilla al tratar.

Sin embargo, estas cantidades son orientativas y se recomienda ajustar las proporciones para cada caso en particular, según el tipo de semilla y las condiciones de siembra.

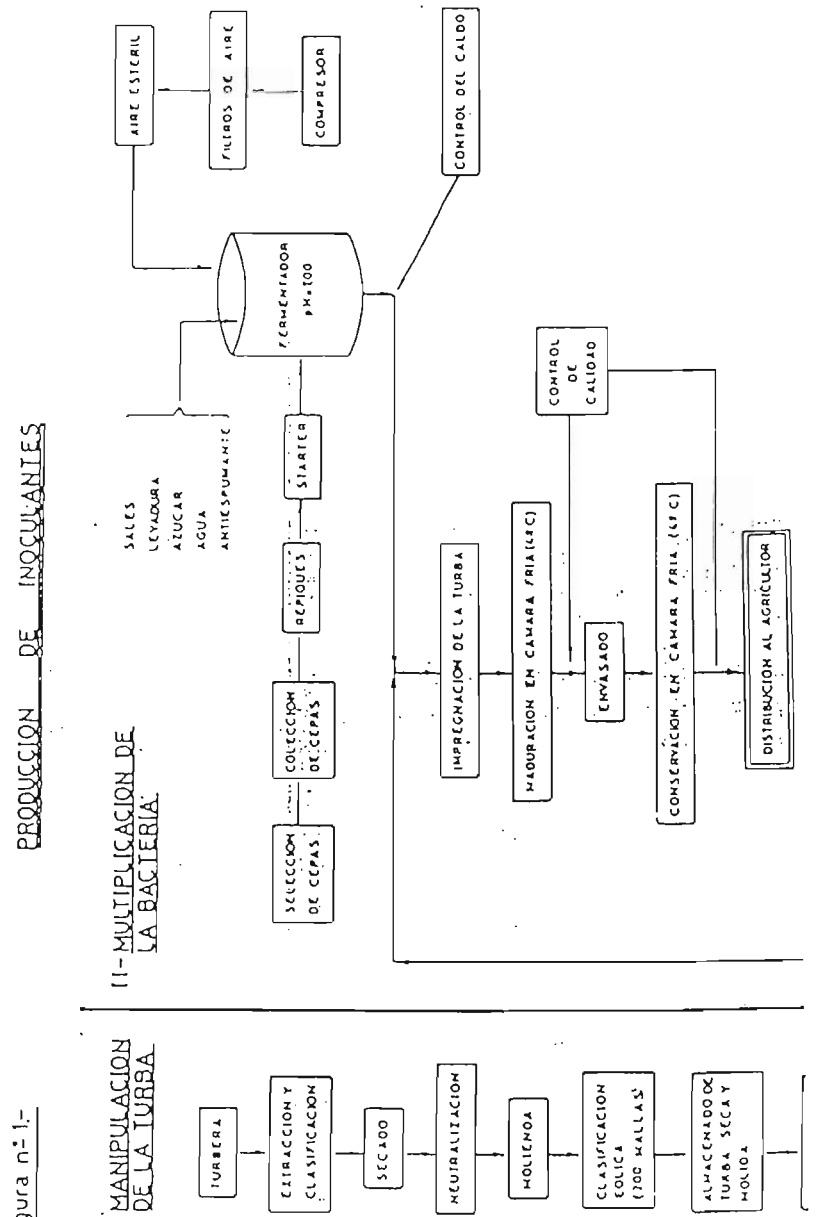

Figura nº 1:-

CRECIMIENTO DE PLANTAS EN CONDICIONES BACTERIOLOGICAMENTE CONTROLADAS.

Las semillas se esterilizaron superficialmente siguiendo esencialmente la técnica de Vincent (1970).

Las semillas se sumergen 1 minuto en etanol del 96% y 3 minutos en una solución de cloruro mercúrico (0,2%) acidificada con ClH concentrado (5ml/l) a la que se añade 0,1 ml/l de Tween 80. Posteriormente se lavan 9 veces con agua estéril y se germinan en placas de agar al agua (1%) a 20ºC.

Las semillas germinadas (raicilla de 0,5-2 cm) se sembraron en unidades de cultivo tipo Leonard (Leonard, 1943) modificadas en nuestro laboratorio (Fig. 2). Esencialmente las unidades Leonard constan de un tiesto (7 x 14 cm Ø y 13 cm de altura) que contiene como soporte vermiculita lavada, y una lata (10 cm Ø x 12 cm altura) con 500 ml. de solución nutritiva para plantas carente de nitrógeno combinado (Leonard 1943). Una torcida hecha de gasa permite el ascenso de la solución nutritiva desde la lata hasta la vermiculita. El conjunto del tiesto y la lata ensamblados y cubiertos en papel de aluminio, se esterilizó en autoclave durante 2 h 30 min a 1 atm de sobrepresión. En cada unidad Leonard se sembraron 4 semillas germinadas que se inocularon con 10 ml de un cultivo de Rhizobium crecido en medio YM a 28ºC durante 3 días (10^9 bacterias/ml). La superficie de cada unidad se recubrió con arena parafinada estéril (Vincent, 1970) para evitar la contaminación con otras cepas de Rhizobium.

Las plantas se crecieron en una cámara climática provista de una iluminación de 30 000 lux (16 h./día) a nivel de las plantas y un ciclo de temperatura noche/día de 15/25ºC. Durante el crecimiento, las plantas se regaron con solución nutritiva Leonard libre de nitrógeno. Esta solución contenía por litro: ClK 0,0745 g; PO_4HK_2 0,174 g.; SO_4Mg7H_2O 0,246 g.; SO_4Ca2H_2O 0,344 g.; citrato férrico 1,7 mg. y y 0,5 ml solución stock de microelementos. La solución de microelementos contenía (mg/l): SO_4Cu5H_2O, 78; SO_4Zn7H_2O, 220; SO_4Mn7H_2O, 2030; $Mo_7O_{24}(NH_4)_64H_2O$, 10; BO_3H_3, 1430.

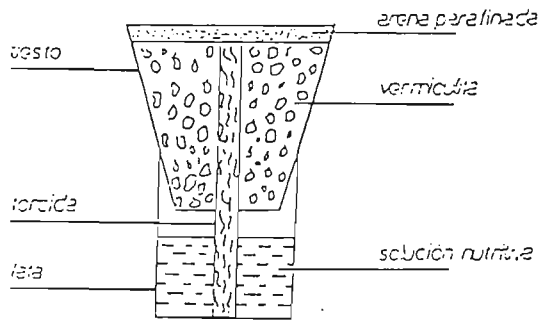

Figura 2. Esquema de una unidad de cultivo "tipo Leonard" utilizada para el crecimiento de plantas bajo condiciones bacteriológicamente controladas.

MEDIO : YEAST MANNITOL BROTH (g/litro)

Manitol, 10g; Extracto de levadura 0,4 g; ClNa 0,1g; SO_4Mg, 0,4g; PO_4HK_2, 0,5g; Se ajusta pH a 6,8. Se distribuye en matraces de 200-300 ml a razón de 50 ml por matraz. Se esteriliza a 120ª 20' en autoclave.

8. DETERMINACION DE ACTIVIDADES ENZIMATICAS EN NODULOS.

8.1. Actividad nitrogenasa por reducción de acetileno.

La actividad nitrogenasa en nódulos se estimó midiendo la velocidad de reducción de acetileno por cromatografía de gases esencialmente según lo descrito por Schwinghamer et al (1970).

Los ensayos se realizaron en viales de 33 ml conteniendo de 0,1 a 0,2 g de nódulos procedentes de las plantas de una unidad Leonard unidos a pequeños segmentos de raiz. Los viales se cerraron con tapones de goma y se reemplazó un 10% del volumen de aire por C_2H_2 y, después de 15 y 30 min de incubación a 20ºC, se tomaron dos muestras de 0,5 ml de la atmósfera del vial. El contenido de etileno (C_2H_4) de las muestras se leyó en un cromatógrafo Konic modelo Cromatix KNK 2000 equipado con un detector de ionización de llama y una columna (0,3 x 186 cm) rellena de Porapak R (malla de 80-100). Como gas portador se usó N_2, a un flujo de 18 ml/min. Las temperaturas de la columna, del inyector y del detector fueron 50, 100, y 150°C respectivamente. La velocidad de reducción de acetileno se expresó en µmoles de C_2H_4 por gramo de peso fresco de nódulo y por hora.

8.2. Producción de hidrogeno.

La velocidad de producción de hidrogeno por nódulos se cuantificó por cromatografía de gases siguiendo esencialmente el método descrito por Hanus et al (1980). Las muestras de nódulos se incubaron en las mismas condiciones que las descritas para la determinación de la velocidad de reducción de acetileno con la excepción de que no se incluye C_2H_2 en la atmósfera de los viales. A los 15 y 30 min de incubación se toman alícuotas de 0,5 ml de gas y se inyectan en un cromatógrafo de gases Konic, modelo KNK 2000, equipado con un detector de conductividad térmica y una columna molecular Sieve 5A (malla 40-60) de 0,64 x 186 cm. Las temperaturas de la columna, el inyector y el detector fueron respectivamente 100, 150 y 150°C. La velocidad de producción de hidrógeno se expresó en µmoles de H_2 por gramo de peso fresco de nódulos y por hora.

Simbiosis *Rhizobium*-leguminosa

1.- Leguminosas: Caracteres botánicos y agronómicos

2.- Rhizobium: Biología. aislamiento y caracterización.

3.- Formación y funcionamiento de los nódulos radiculares de las leguminosas

4.- Otras simbiosis: actinorrizas.

1.- Leguminosas

Botánica:

Angiospermas del orden Rosales

Familia muy diversificada (20.000 especies en 750 géneros)

Hojas : alternas, compuestas, estipuladas
Flores : hermafroditas, corola-cáliz 5 p.
Fruto : vaina (LEGUMBRE)

Nódulos radiculares.

Subfamilias :

Mimosoideas: 2,900 sp, 65 géneros (Mimosa, Inga, Acacia).
Nodulación: 90%

Caesalpinoideas: 1.800 sp., 18 géneros (Cercis, Cassia, Ceratonia)
Nodulación: 25%

Papilionoideas: 14.000 sp., 500 generos (Pisum, Phaseolus, Medicago, Genista)
Nodulación: 97%

Importancia:

Aspectos históricos: Mesopotamia (6.000 a. C.), China (5.000), Méjico (4.000)
Centros de origen:

Usos actuales: **Alimentación** (humana y animal)
Otros : Mejora de suelos, madera, extracción de compuestos, ornamental

Limitaciones agronómicas: Requerimientos de alta iluminación
Mala adaptación a condiciones extremas
Menor productividad potencial
Factores antinutritivos

Table 10-2 N_2 Fixation in Legumes[86,77,53,74,93]

Legume	Fixation (kg N ha^{-1} $year^{-1}$)	
	Minimum/Maximum	Average
Clover	45–670	250
Pea	50–500	150
Alfalfa	90–340	250
Lupin	140–200	150
Soybean	60–300	100
Peanut	50–150	100
Lentils	50–150	80
Broadbean	100–300	200
Sesbania rostrata	600–800	700
N_2-fixing trees	80–500	150

2.- Grupo *Rhizobium*

Biología:

Bacterias G(-), α-Proteobacterias, aeróbicas, mótiles.
Hábitat natural: Suelo

Metabolismo: amplio rango de compuestos como fuente de C (C4 y C6)
Rutas metabólicas centrales: Entner-Doudoroff y Pentosas P

Inducen nódulos radiculares en leguminosas.

Cultivo: Medios ricos: TY (triptona-extracto de levadura)
YMB (extracto de levadura-manitol)
Medio minimo: sales min. + glutamato + manitol + vitaminas

Clasificación:

Caracteres:
Rango de hospedadores
Caracteres fisiológicos: veloc. crecimiento, acid/basif. del medio, empleo de fuentes de C
Caracteres genéticos: 16S, "DNA fingerprints"

Géneros:
Rhizobium: Pisum, Phaseolus, Vicia
Bradyrhizobium: Glycine, Lupinus
Mesorhizobium: Lotus, Cicer
Sinorhizobium: Medicago, Glycine
Azorhizobium: Sesbania.

Aislamiento y cuantificación en suelo:

- uso de "plantas trampa" y técnicas de "numero más probable".
- Medios selectivos directos: poco eficaces (Rojo Congo)

FILOGENIA BACTERIANA

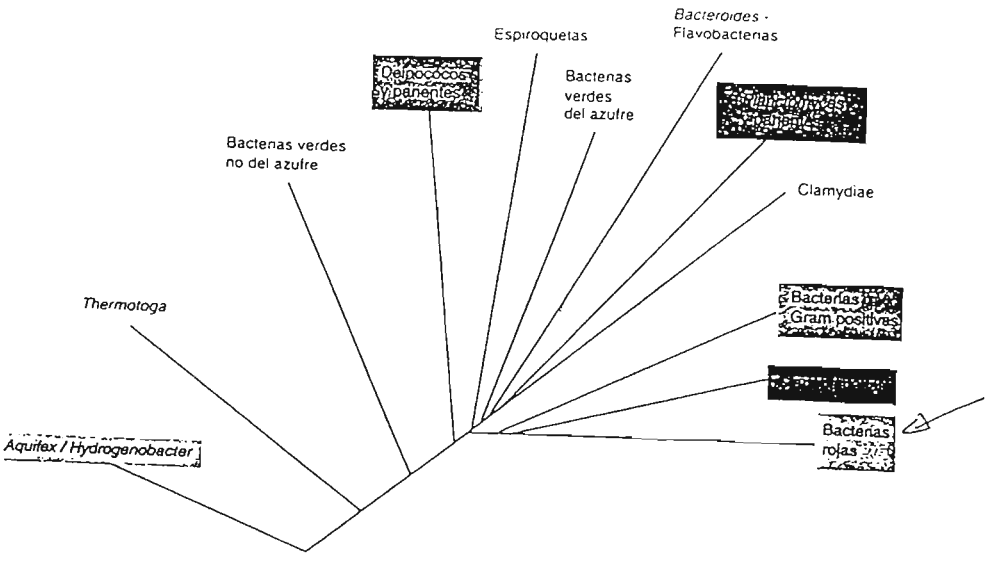

Figura 16.1 Árbol filogenético detallado de los principales linajes (reinos) de bacterias basados en la comparación de secuencias de RNA ribosómico 16S. Por razones estadísticas, la posición relativa de las ramas en este árbol difieren ligeramente de las que se muestran en el árbol universal (véase Figura 15.12), pero la longitud de las ramas es proporcional a las distancias evolutivas correctas calculadas entre dos grupos cualesquiera. Las bacterias rojas también reciben el nombre de Proteobacteria. Para una discusión sobre *Aquifex* e *Hydrogenobacter*, véase la Sección 17.3.

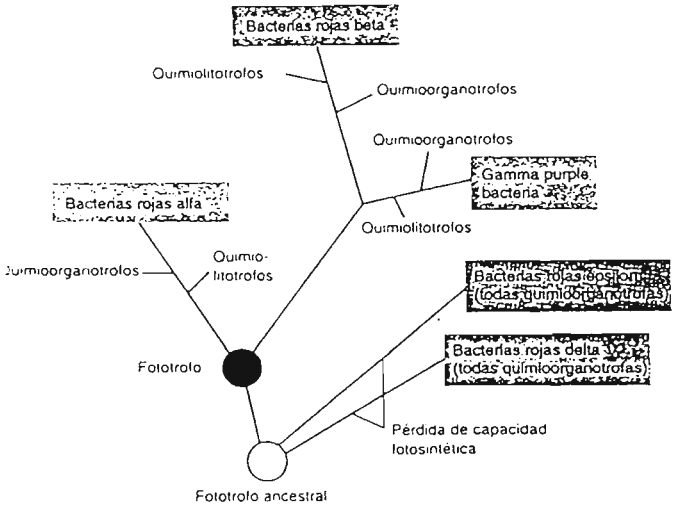

TABLA 16.1 Bacterias (fototróficas anoxigénicas) rojas y verdes

Grupo	Género
Grupo Alpha	*Rhodospirillum*[a], *Rhodopseudomonas*[a], *Rhodobacter*[a], *Rhodomicrobium*[a], *Rhodovulum*[a], *Rhodopila*[a], *Rhizobium*, *Nitrobacter*, *Agrobacterium*, *Aquaspirillum*, *Hyphomicrobium*, *Acetobacter*, *Gluconobacter*, *Beijerinckia*, *Paracoccus*, *Pseudomonas* (some species)
Grupo Beta	*Rhodocyclus*[a], *Rhodoferax*[a], *Rubrivivax*[a], *Spirillum*, *Nitrosomonas*, *Sphaerotilus*, *Thiobacillus*, *Alcaligenes*, *Pseudomonas*, *Bordetella*, *Neisseria*, *Zymomonas*
Grupo Gamma	*Chromatium*[a], *Thiospirillum*[a], y otras bacterias púrpuras del azufre[a], *Beggiatoa*, *Leucothrix*, *Escherichia* y otras bacterias entéricas, *Legionella*, *Azotobacter*, especies fluorescentes de *Pseudomonas Vibrio*
Grupo Delta	*Myxococcus*, *Bdellovibrio*, *Desulfovibrio* y otras bacterias reductoras de sulfato *Desulfuromonas*
Grupo Epsilon	*Thiovulum*, *Wolinella*, *Campylobacter*, *Helicobacter*

[a]Grupos representativos de organismos fototróficos.

GENEROS Y ESPECIES DEL GRUPO *Rhizobium*

Género	Especie	Hospedador
Bradyrhizobium	B. japonicum	*(Glycine max)*
	B. elkanii	(")
	B. liaoningense	(")
	(Lupinus)	
	(Arachis)	
	(Vigna)	
	(Macroptilium)	
	(P. lunatus)	
Photorhizobium	BTAi	*(Aeschynomene)*
Azorhizobium	A. caulinodans	*(Sesbania)*
Sinorhizobium	S. meliloti	*(Medicago sativa)*
	S. medicae	*(M. polymorpha)*
	S. fredii	*(G. max)*
	S.sahelii, terangae	*(Acacia, Prosopis, Leucaena)*
Mesorhizobium	M. loti	*(Lotus)*
	M. huakui	*(Astragalus)*
	M. ciceri	*(Cicer)*
	M. mediterranei	*(Cicer)*
	M. tiansanense	*(G. max)*
	M. plurifarium	*(Acacia, Prosopis, Leucaena)*
Rhizobium	R. leguminosarum	
	bv. viciae	*(Pisum, Lens, Vicia, Lathyrus)*
	bv. trifolii	*(Trifolium)*
	bv. phaseoli	*(Phaseolus)*
	R. etli	*(Phaseolus vulgaris, Leucaena)*
	R. tropici	*(P. vulgaris, Leucaena)*
	R. gallicum	
	R. giardini	
	R. mongolense	
	R. hainanense	*(P. vulgaris)*
	R. galegae	*(Galega officinalis)*
	R. huauthense	*(Sebania herbacea)*
Allorhizobium	A. neptunica	*(Neptunia natans)*

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Methylotrophic *Methylobacterium* Bacteria Nodulate and Fix Nitrogen in Symbiosis with Legumes

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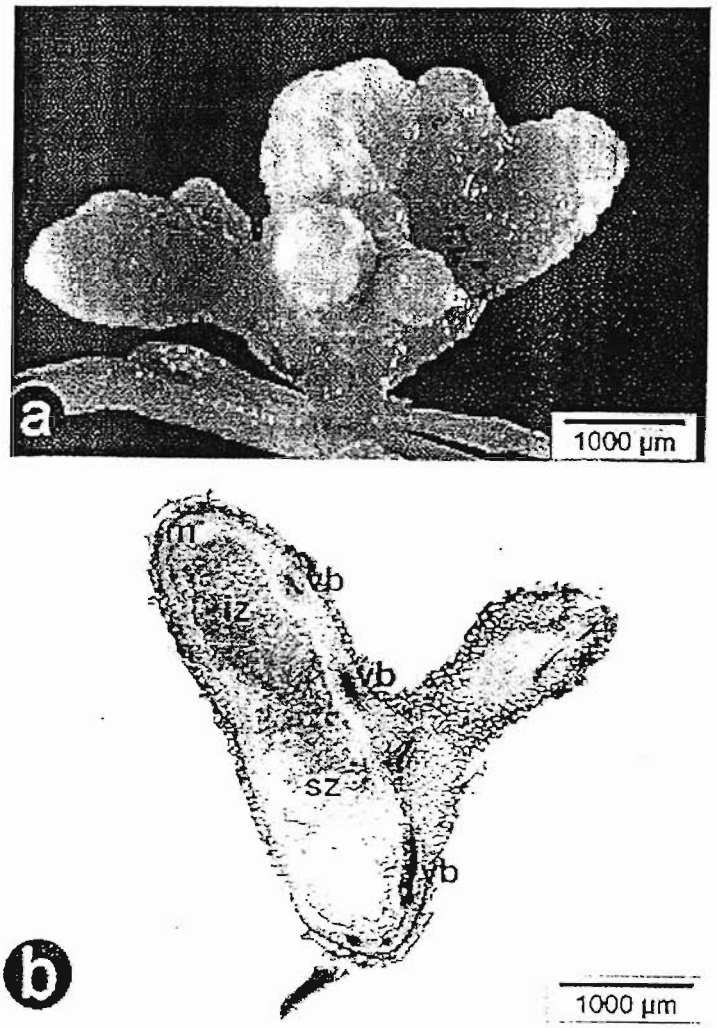
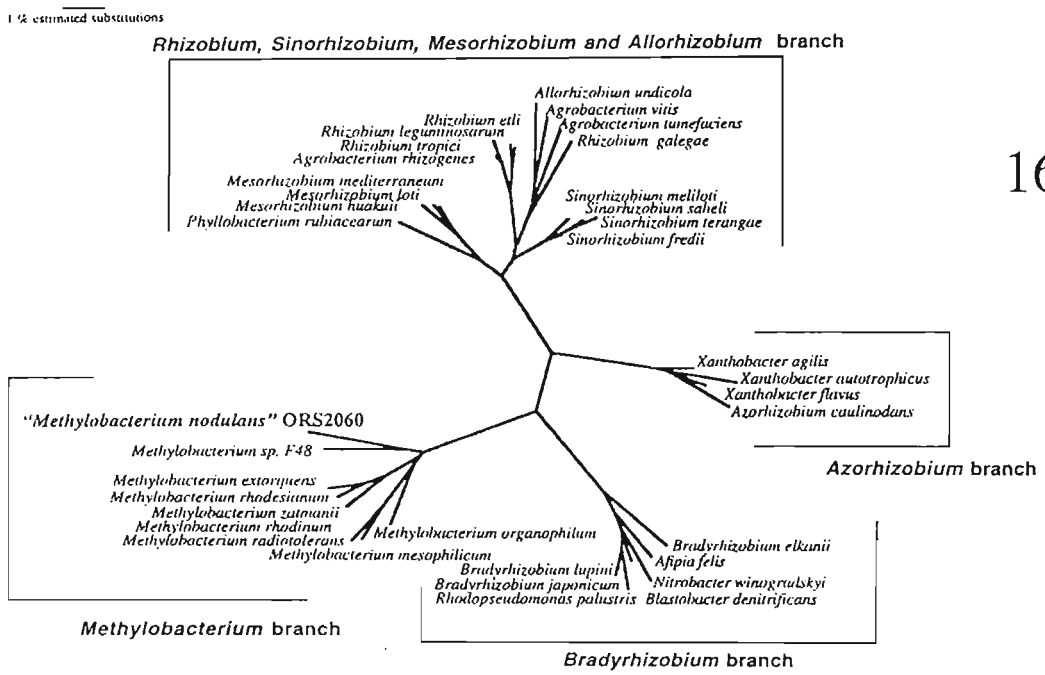


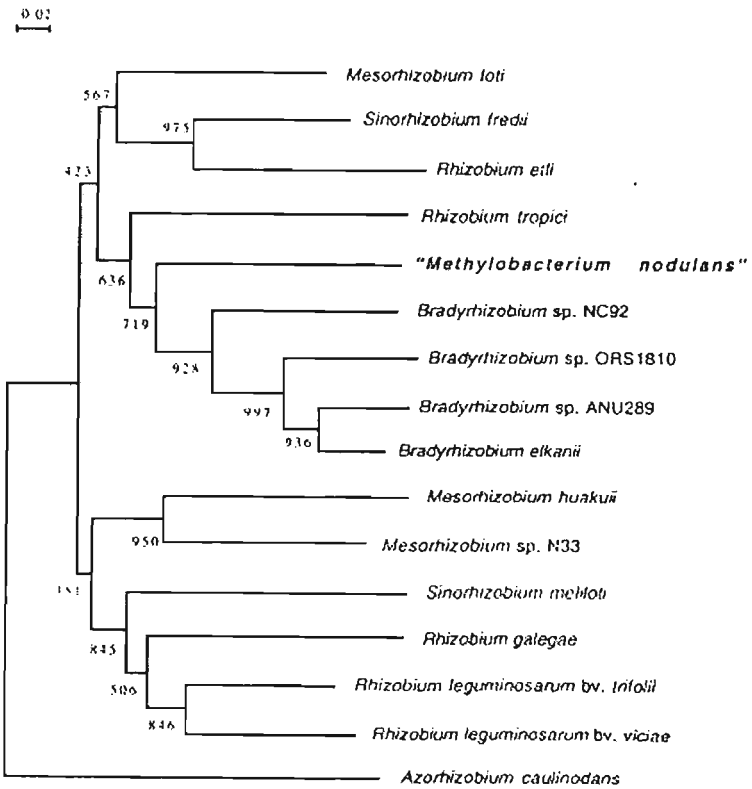
Nodulos de "*Methylobacterium nodulans*" en *Crotalaria perrottetii*
(Sy et al, 2001)

Arbol filogenético de fijadores simbióticos de nitrógeno

(Sy et al, 2001. J- Bact. 183:214-220)



16S

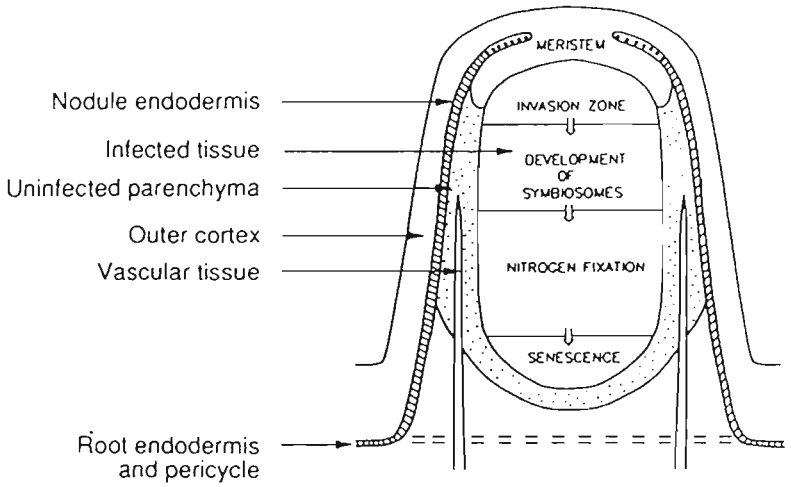


NodA

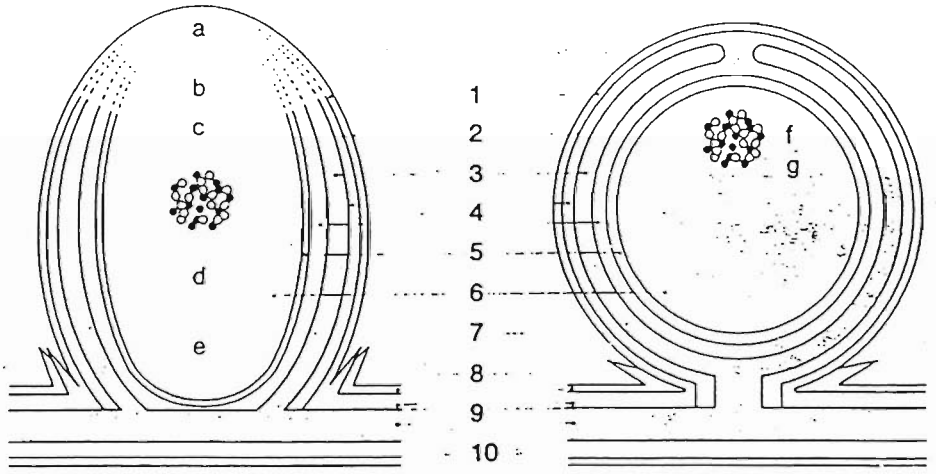
3.- Nódulos radiculares

El nódulo resulta de una proliferación de células vegetales, parte de las cuales se infecta de células de *Rhizobium*

Estructura general del nódulo



Nódulos determinados e indeterminados



Membrana peribacteroidal

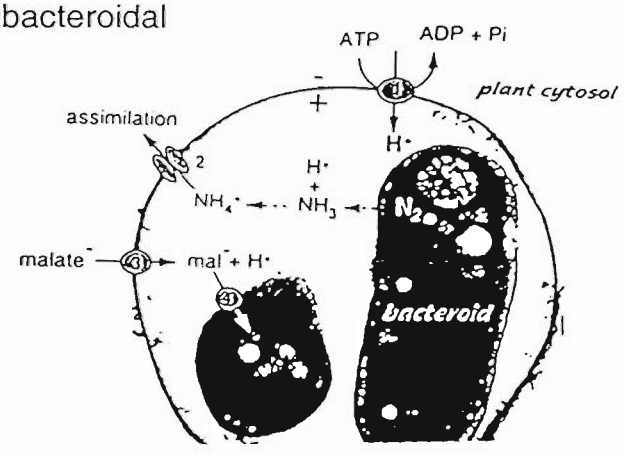
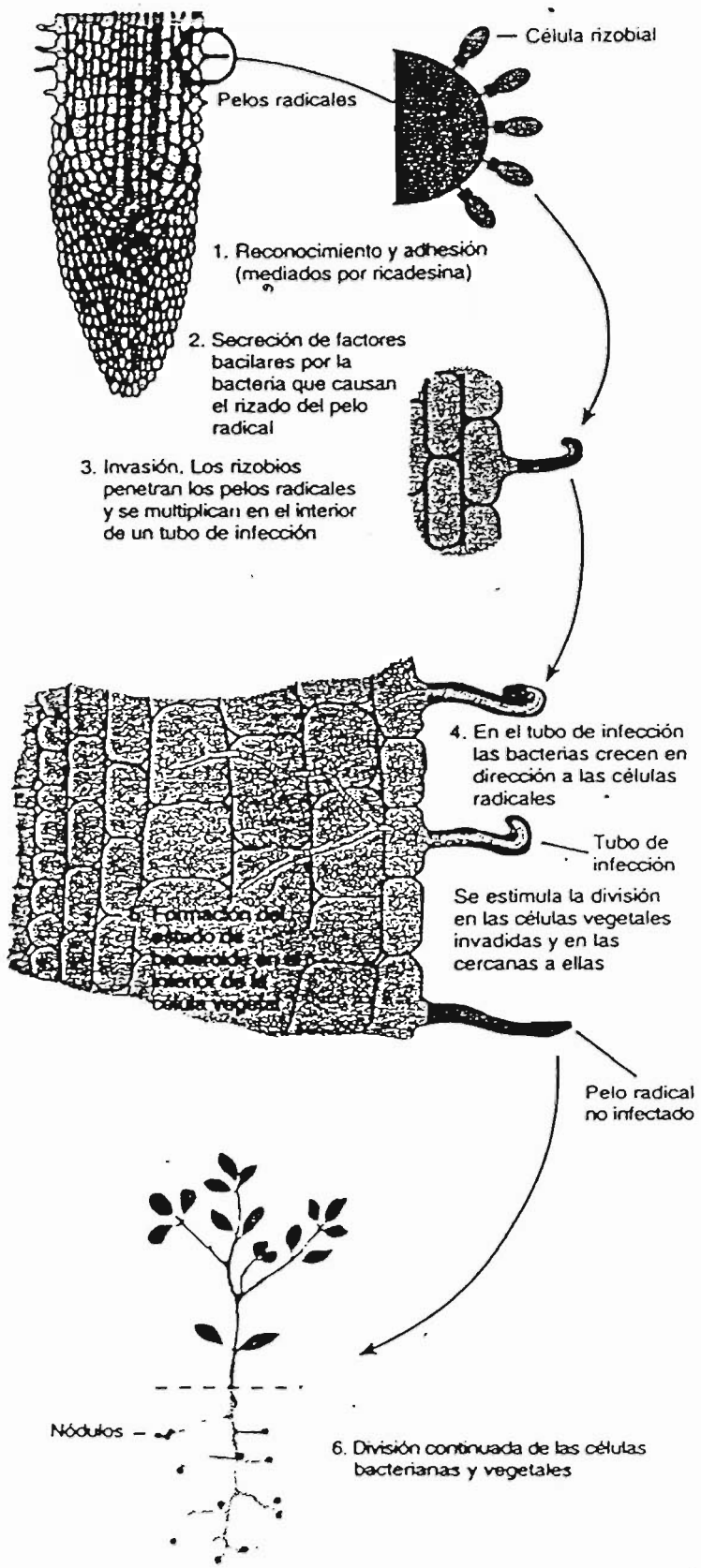


Fig. 3. Structure and function of a typical symbiosome. The photograph shows two bacteroids surrounded by the peribacteroid membrane (PBM). The major metabolic exchanges between the plant cytosol and the bacteroids are shown schematically. 1, Proton-pumping ATPase which energises the PBM and acidifies the peribacteroid space (PBS); 2, PBM monovalent cation channel which catalyses ammonium efflux from the symbiosome; 3, PBM dicarboxylate carrier which catalyses malate uptake into the PBS; 4, bacteroid dicarboxylate carrier which catalyses malate uptake from the PBS into the bacteroid.

Proceso general de formación del nódulo

- quimiotaxis
- adhesión
- reconocimiento
- progreso de hilo infectivo
- liberación de simbiosomas
- modificación en bacteroides
- fijación de nitrógeno
- senescencia



Quimiotaxis

Proceso general en bacterias para detección de compuestos atrayentes.

Mediado por quimiorreceptores de membrana ligados a cadena de transducción de señal al motor flagelar

Rhizobium es atraído por distintos compuestos presentes en exudados radiculares de leguminosas

Chemotactic response of *R. leguminosarum* UPM791 and mcpA::Tn5 (AL8) and pSym-cured (128C53.5) derivatives towards different low molecular weight compounds

	Strains		
Amino acids(1mM)	UPM791	AL8	128C53.5
Homoserine	26.0	25.6	20.4
Alanine	8.7	14.3	9.1
Arginine	10.9	12.1	2.2
Aspartic acid	13.0	12.1	0.0
Asparaguine	39.0	34.5	29.5
Cysteine	-16.8	-3.6	-16.3
Glutamic acid	39.1	36.8	25
Histidine	21.7	32.3	13.6
Glycine	10.9	30.0	15.9
Leucine	-2.2	-1.1	-5
Serine	15.2	16.6	13.6
Threonine	19.6	16.6	18.2
Tryptophan	0.0	-1.1	-10.0
Valine	26.1	18.8	15.9
Organic Acids (1mM)			
Citric	34.8	23.3	22.7
Fumaric	15.2	16.6	6.8
Malic	13	16.6	9.1
Succinic	2.2	3.6	-2.3
Gluconic	23.9	18.8	13.6
Sugars			
Fructose	-4.4	1.4	0
Glucose	0	1.4	0
Maltose	21.7	21.1	18.2
Raffinose	8.7	1.4	-6.8
Ribose	4.4	1.4	4.6
Sucrose	10.9	3.1	0
Xilose	13.0	14.3	13.6

Values represent the Chemotaxis Coeficient (C.C.) $=\frac{(d2 - d1)x100}{d1}$ x100. and are the average of three replicates.

Table 3.2. Substances detected in plant root exudates

Kind of compound	Exudate components	Plants most studied
Sugars	Glucose, fructose, sucrose, maltose, galactose, rhamnose, ribose, xylose, arabinose, raffinose, oligosaccharide	*Triticum aestivum, Hordeum vulgare, Phaseolus vulgaris, Pinus* spp
Amino compounds	Asparagine, α-alanine, glutamine, aspartic acid, leucine/isoleucine, serine, -aminobutyric acid, glycine, cystine/cysteine, methionine, phenylalanine, tryosine, threonine, lysine, proline, tryptophane, β-alanine, arginine, homoserine, cystathionine	*Triticum aestivum, Zea mays, Avena sativa, Pisum sativum, Phalaris* spp., *Trifolium* spp., *Oryza sativa, Gossypium barbadense, Lycopersicon esculentum, Pinus* spp., *Robinia pseudo-acacia, Boutelova gracilis*
Organic acids	Tartaric, oxalic, citric, malic, acetic, propionic, butyric, succinic, fumaric, glycolic, valeric, malonic	*Triticum aestivum, Zea mays, Phaseolus vulgaris, Lycopersicon esculentum, Brassica* spp., *Pinus* spp., *Robinia pseudo-acacia*
Fatty acids and sterols	Palmitic, stearic, oleic, linoleic, linolenic acids; cholesterol, campesterol, stigmasterol, sitosterol	*Phaseolus vulgaris, Arachis hypogaea*
Growth factors	Biotin, thiamine, niacin, pantothenate, choline, inositol, pyridoxine, *p*-amino benzoic acid, n-methyl nicotinic acid	*Triticum aestivum, Phalaris* spp., *Phaseolus vulgaris, Pisum sativum, Trifolium* spp., *Medicago* spp., *Gossypium barbadense*
Nucleotides, flavonones and enzymes	Flavonone, adenine, guanine, uridine/cytidine, phosphatase, invertase, amylase, protease, polygalacturonase	*Triticum aestivum, Zea mays, Pisum sativum, Trifolium* spp.
Miscellaneous compounds	Auxins, scopoletin, fluorescent substances, hydrocyanic acid, glycosides, saponin (glucosides), organic phosphorus compounds, nematode cyst or egg-hatching factors, nematode attractants, fungal mycelium growth stimulants, mycelium-growth inhibitors, zoospore attractants, spore and sclerotium germination stimulants and inhibitors, bacterial stimulants and inhibitors, parasitic weed germination stimulators	*Avena sativa, Medicago* spp., *Trifolium* spp., *Pisum sativum, Lycopersicon esculentum, Lactuca* spp., *Fragaria vesca, Musa paradisiaca, Zea mays*

EXUDADOS RADICULARES

Table 3.4. Amino acids (μg g^{-1} dry wt. root) in culture medium of 14-day-old pea seedlings. Average of 5 analyses (Boulter et al. 1966)

Acids	Culture solution	Quartz sand
Cysteic acid	Trace	Trace
Aspartic acid	280	740
Threonine	71	222
Serine*	68	232
Homoserine	190	1412
Glutamic acid	307	654
Proline	Trace	Trace
Glycine	41	160
Alanine	39	190
Valine	22	78
Cystine	14	N.I.
Isoleucine	13	43
Leucine	21	76
Tyrosine	19	54
Phenylalanine	17	67
γ-Aminobutyric acid	Trace	Trace
Ornithine	45	192
Lysine	29	123
Histidine	35	117
Arginine	61	219
Ammonia	1312	1540

* May contain asparagine and glutamine

Table 3.3. Sugars in exudates of barley and wheat (Vančura 1964)

Compound	% of reducing sugars	
	Barley	Wheat
Oligosaccharides	27.8	26.7
Maltose	5.4	3.1
Galactose	13.6	4.0
Glucose	9.5	16.8
Arabinose + fructose	19.0	17.7
Xylose	15.0	15.9
Ribose	1.3	0.9
Rhamnose	6.8	14.9
Deoxyribose	0.8	–
Deoxysugar	0.8	–

Adhesión bacteria-pelo radical

Adhesinas bacterianas

- EPS, CPS, LPS
- Rhicadhesina

Adhesinas de planta:

lectinas: PSL (*Pisum*)
Trifoliina A (*Trifolium*)

Reconocimiento

Dialogo mediado por flavonoides (planta) y factores nod (bacteria)

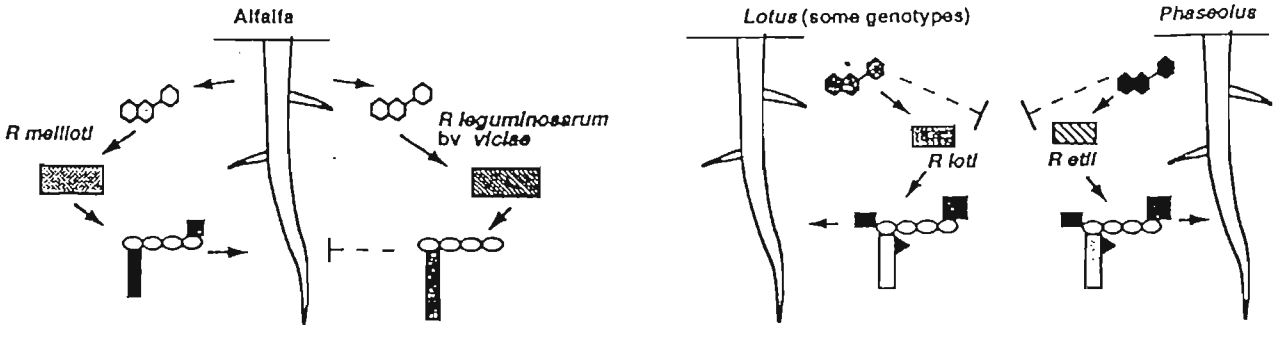
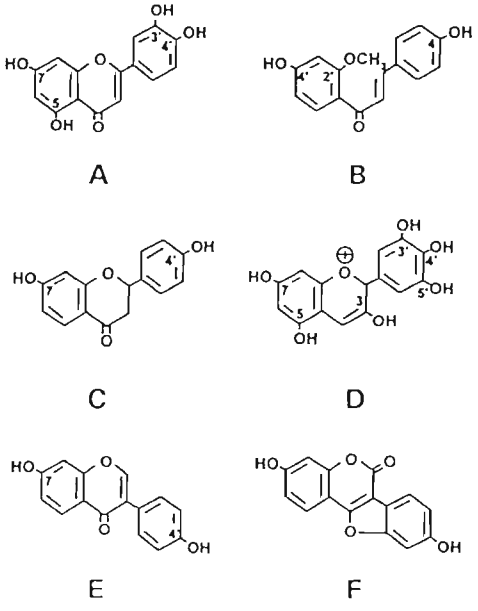
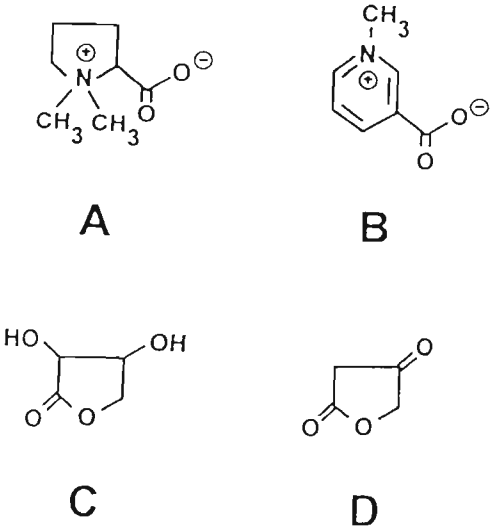


Figure 1. Schematic of Symbiotic Signal Exchange and Host Specificity.

Las raíces de las leguminosas excretan compuestos (iso)flavonoides que inducen transcripción de genes de nodulación en *Rhizobium*

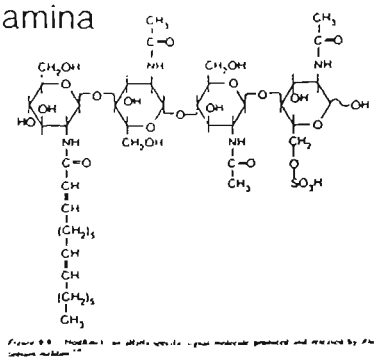


biaceas p. y



Estructura y función de factores nod

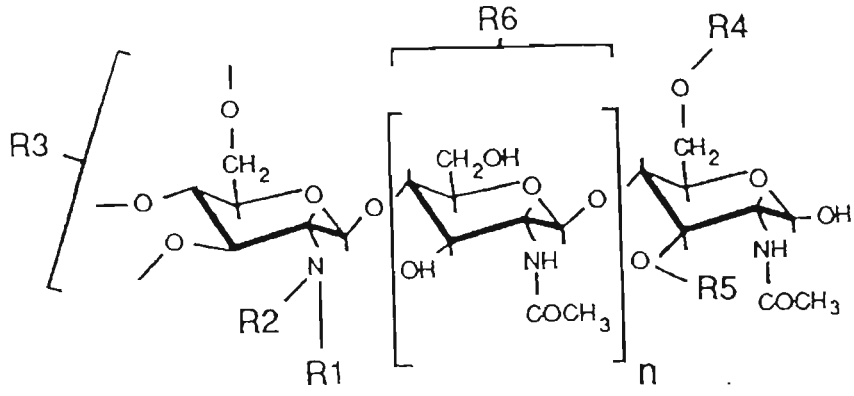
Los factores nod son oligómeros de N-acetil glucosamina "adornados" (Lipoquitooligosacaridos, LCO).



Fotocopia fig. 9-9

Los "adornos" de LCOs son esenciales para la especificidad del reconocimiento

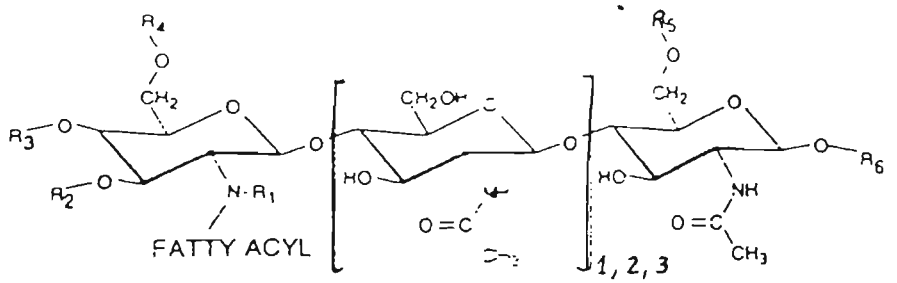
Rhizobiaceas, p. 396



LCOs inducen cambios importantes en las raíces de las leguminosas compatibles

Tejido	Cambios
Epidermis	Formación de nuevos pelos radiculares Deformacion, ramificación, hinchamiento Formación de "cayados de pastor" Estimulación de correintes citoplásmicas Alcalinización Depolarización de membrana Modulación de flujos de protones y calcio Oscilaciones periócadicas en niveles de calcio Inducción de nodulinas tempranas
Cortex	Hinchazón celular (etileno) Crecimiento apical de células del cortex externo Formación de hilos de preinfección Formación de primordios nodulares Formación de nódulos completos (Medicago) Inducción local de genes del ciclo celular Inducción de nodulinas tempranas
Periciclo	Inducción de enod40
Raíz completa	Produccion de flavonoides adicionales inducción de hidrolasa de factoores nod

DIVERSIDAD DE FACTORES NOD

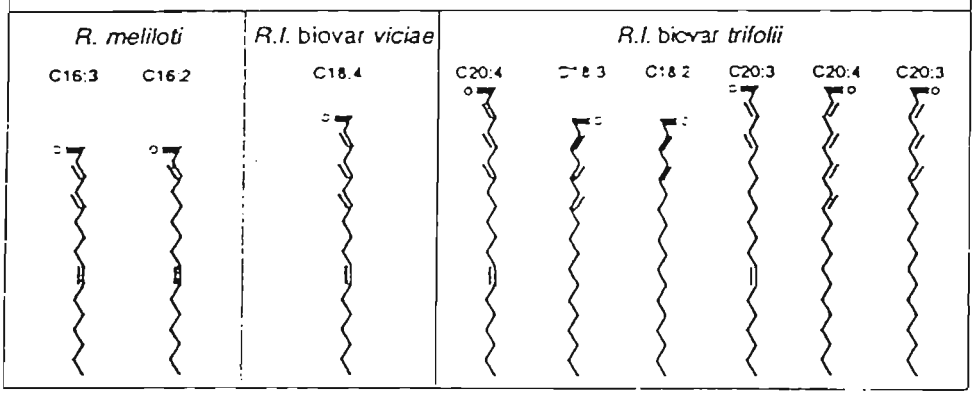


RHIZOBIA ASSOCIATED WITH INDETERMINATE NODULATING PLANTS

R4 = ACETYL

R5 = SULFATYL (*R.meliloti*)
ACETYL (*R. leguminosarum* bv. *viciae* strain TOM)

FATTY ACYL = common, (ω-1) hydroxylated (*R. meliloti*)
or highly unsaturated



RHIZOBIA ASSOCIATED WITH DETERMINATE NODULATING PLANTS

R1 = METHYL (NGR234, *B. elkanii*, *R. tropici*, *R. loti*, *R. etli*, *A. caulinodans*)

R2 = CARBAMYL (NGR234)

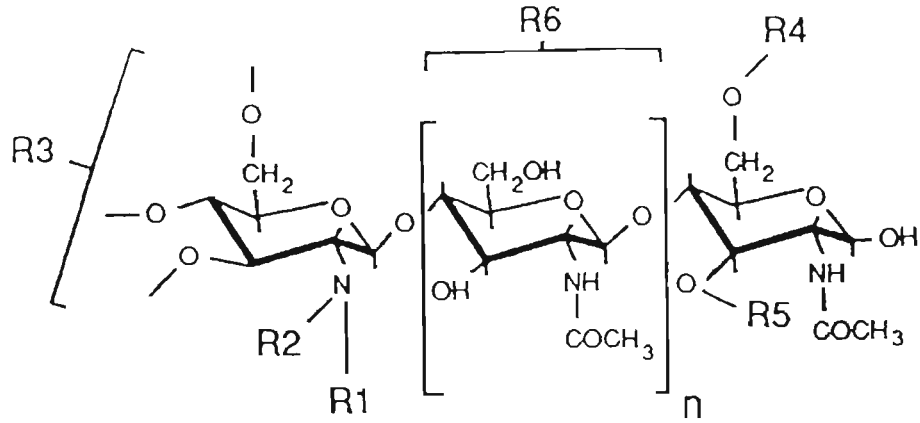
R3 = CARBAMYL (NGR234, *B. elkanii*, *R. loti*, *R. etli*)

R4 = ACETYL (*B. japonicum*, *B. elkanii*)
CARBAMYL (*A. caulinodans*)

R5 = 2–O–METHYL FUCOSYL (*B. japonicum*, *B. elkanii*, NGR234, *R. fredii*)
2–O–METHYL –3–O–ACETYL FUCOSYL (NGR234)
2–O–METHYL –4–O–SULFATYL FUCOSYL (NGR234)
3–O–ACETYL FUCOSYL (*R. loti*, *R. etli*)
FUCOSYL (*B. elkanii*, *R. fredii*)
SULFATYL (*R. tropici*)

R6 = GLYCERYL (*B. elkanii*)
MANNOSYL (*R. tropici*)

FATTY ACYL = COMMON



Host plant[b]	Rhizobial species	Nod factor substituents[c]						n[d]	Ref.
		R1	R2[e]	R3	R4	R5	R6[f,g,h]		
Medicago	*S. meliloti*	H	C16:2.C16:3 C18:C26(w-1)OH	Ac(C-6).H	S	H		1,2,3	1,2,3,4
Vicia	*R.l.* bv. *viciae*	H	C18:1.C18:4	Ac(C-6)	H	H		2,3	5
Pisum cv. Afghanistan	*R.l.* bv. *viciae* TOM	H	C18:1.C18:4	Ac(C-6)	Ac	H		2,3	6
Trifolium	*R.l.* bv. *trifolii*	H	C18:1.C18:3 C20:3.C20:4	Ac(C-6)	H	H		1,2,3	7,8,9
Astragalus	*R. huakii*	H	C18:4	H	S	H		3	27
Galega	*R. galegae*	H	C18:2;C18:3	Cb(C-6)	H	H	Ac(C-3)	2	27
Lotus	*M. loti*	Me	C18:1	Cb(C-4)	AcFuc	H		3	10
		H	C18:1.C18	Cb(C-3)	Fuc AcFuc	H	Fuc	3	26
Phaseolus	*R. etli*	Me	C18:1	Cb(C-4).H	AcFuc	H		3	11,12
	R. tropici	Me	C18:1	H	S.H	H		3	13,14
	R.l. bv. *phaseoli*	Me	C18:1	H	H	H		3	15
Acacia	*R.* sp. GRH2	Me.H	C18:1	H	S.H	H		2,3,4	16
	S. teranga	Me	C16:0.C18:0.C18:1	Cb	S.H	H		3	17
Lablab	*R.* sp. NGR234	Me	C18:1	Cb(C-6 and C-3 or C-4) H	MeFuc AcMeFuc MeSFuc	H		3	18,19
Glycine	*S. fredii*	H	C18:1	H	MeFuc.Fuc	H	glc	1,2,3	20,21
	B. japonicum	H	C18:1	H	MeFuc	H		3	22,23
	B. elkanii	H.Me	C18:1	Ac(C-6).H,Cb	MeFuc.Fuc	H		2,3	23
Sesbania	*A. caulinodans*	Me	C18:1	Cb(C-6)	Fuc.H	D-Ara.H		2,3	24,25,28
	S. saheli	Me	C18:1;C16:0	Cb(C-3, C-4 or C-6)	Fuc.H	D-Ara.H		2,3	29
	S. teranga bv.*sesbaniae*	Me	C18:1;C16:0	Cb(C-3, C-4 or C-6)	Fuc.H	D-Ara.H		2,3	29

Table 2. Structure of Nod factors from various rhizobial species[a]

This table is based on Table 1 of Dénarié *et al.*,(1996) copyright © Annual Reviews Inc and is published here by kind permission of Annual Reviews Inc and J. Dénarié, F. Debellé and J.C. Promé. b. Plant genera from which rhizobial strains were isolated. c. In *B. elkanii*, C-1 of the terminal reducing glucosaminyl residue can be occasionally substituted with glycerol. d. The bold numbers indicate the number of glucosamine residues of the most abundant Nod factors. e. Selected fatty acyl substiuents. f. In *M. loti* Fuc is linked α (1-3) to the glucosamine adjacent to the acylated glucosamine. g. In *R. galegae* the acetyl group is linked to the 3-C of the glucosamine adjacent to the reducing glucosamine. h. One of the minor Nod-factor components made by *S. fredii* contained a glucose residue instead of glucosamine.

Reference: 1, Lerouge *et al.*,(1990); 2, Roche *et al.*,(1991b); 3, Schultze *et al.*,(1992), 4, Demont *et al.*,(1993); 5, Spaink *et al.*,(1991); 6, Firmin *et al.*,(1993); 7, Spaink *et al.*,(1995a); 8; Orgambide *et al.*,(1995); 9, Van der Drift *et al.*,(1996); 10, Lopez-Lara *et al.*,(1995a); 11, Cárdenas *et al.*,(1995); 12, Poupot *et al.*,(1995a); 13, Poupot *et al.*,(1993); 14, Folch-Mallol *et al.*,(1996); 15, K.E. Wilson, R. Carlson, J. Firmin and J.A. Downie (unpublished); 16, Lopez-Lara *et al.*, (1995b); 17, Lorquin et al., (1997), 18, 19, Price *et al.*, (1992, 1996); 20 ,21, Bec-Ferté *et al.*,(1994, 1996); 22, Sanjuan *et al.*,(1992); 23, Carlson *et al.*,(1993), 24, 25, Mergaert *et al.*,(1993, 1996); 26, Olsthoorn *et al.*,(1997); 27, Yang *et al.*,(1997); 28, Mergaert *et al.*,(1997); 29, Lorquin *et al.*, (1997).

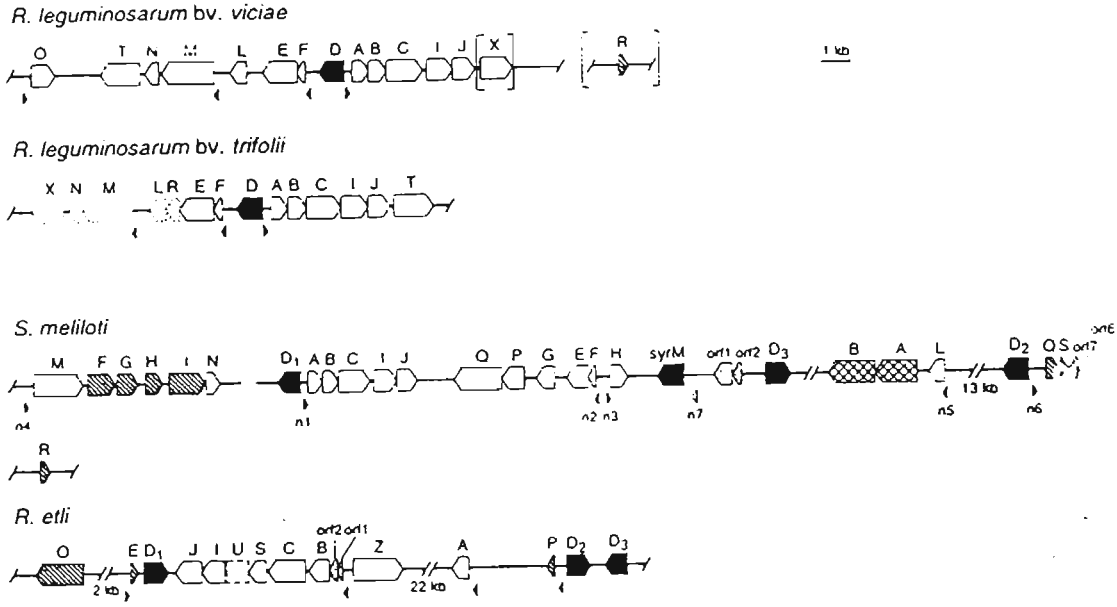
Síntesis de factores de nodulación

- Son determinantes clave para la especificada del reconocimiento planta bacteria.

- Están sintetizados por la accion coordinada de más de 20 genes nod.

-Existen genes nod "comunes" implicados en la síntesis del esqueleto carbonado básico y genes "específicos responsables de los "adornos de los LCOs.

- Se conocen las funciones de la mayoría de los genes nod en distintos Rhizobium



Regulación de la expresión de genes nod

- NodD es el regulador central que interacciona con los flavonoides específicos de la leguminosa correspondient, convirtiéndose en activador transcripcional de los demás genes nod.

- Los genes nod están organizados en operones con promotorescon "cajas nod" que interaccionan con NodD activado

- Se han descrito sistemas adicionales de regulación presentes en algunas especies (NodV/NodW)

- se han descrito represoresimplicados en la existencia de niveles óptimos de expresion de los genes nod

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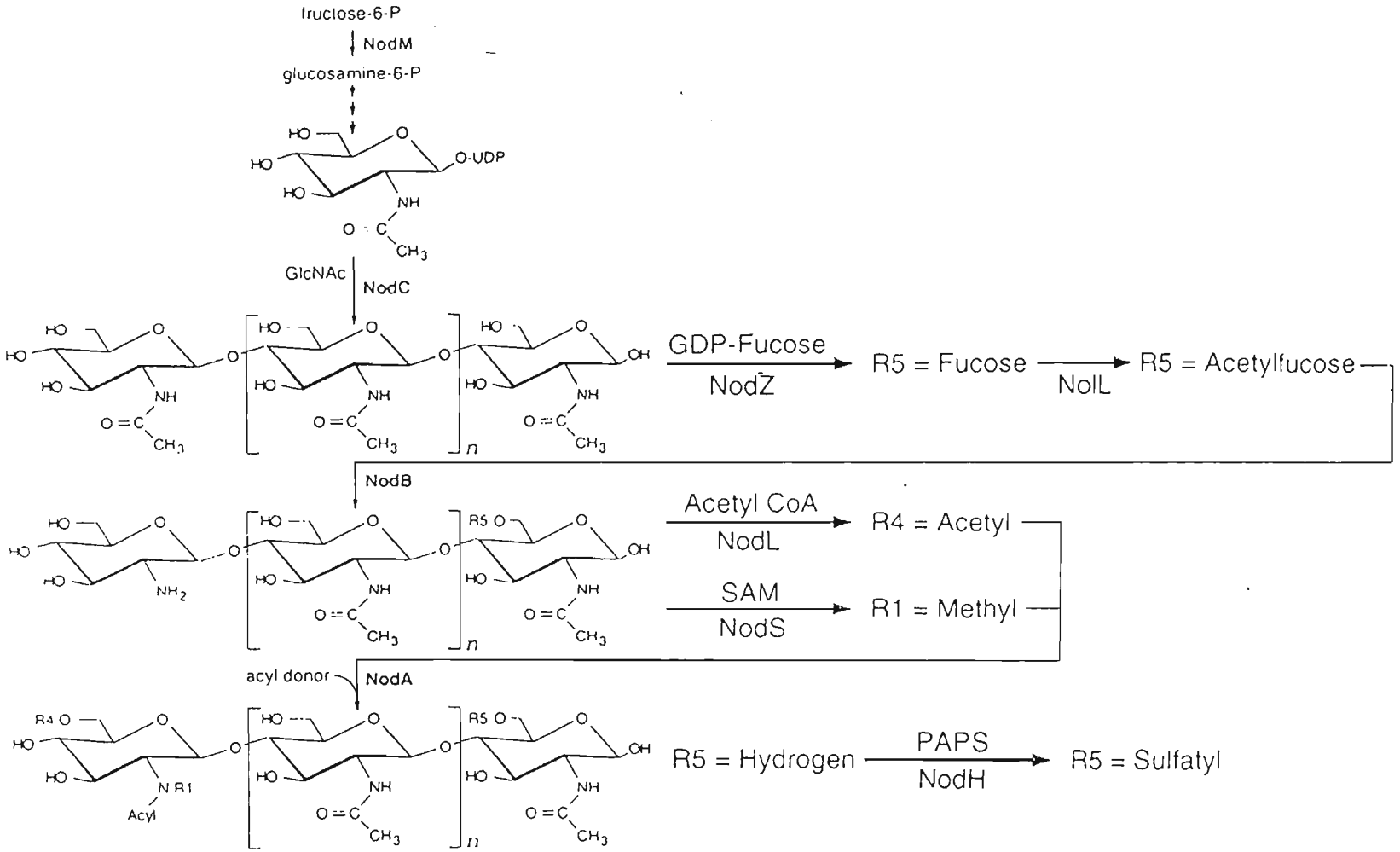


FIGURE 3. Model for the biosynthesis of LCOs. In the absence of strain-specific modifying enzymes, the groups R1, R4, and R5 represent hydrogen groups. The functions of the NodB from *R. meliloti* (John et al., 1993), NodZ from *B. japonicum* (Quinto et al., 1996), NodL from *R. leguminosarum* (Bloemberg et al. 1995b), NodS from *A. caulinodans* (Geelen et al., 1995), and NodH from *R. meliloti* (Schultze et al., 1995) proteins have been demonstrated by *in vitro* analysis of enzymatic activity of purified proteins. The functions of NodM from *R. meliloti* and *R. leguminosarum* (Baev et al., 1991; Marie et al., 1992), NodC from *R. fredii*, *A. caulinodans*, *R. leguminosarum* (Deiannino et al., 1995; Geremia et al., 1994; Kamst et al., 1995; Spaink et al., 1994b), NolL from *R. loti* (Scott et al., 1995), and NodA from *R. meliloti* and *R. leguminosarum* (Atkinson et al., 1994; Röhrig et al., 1994; Spaink et al., 1994b) have been inferred from indirect evidence.

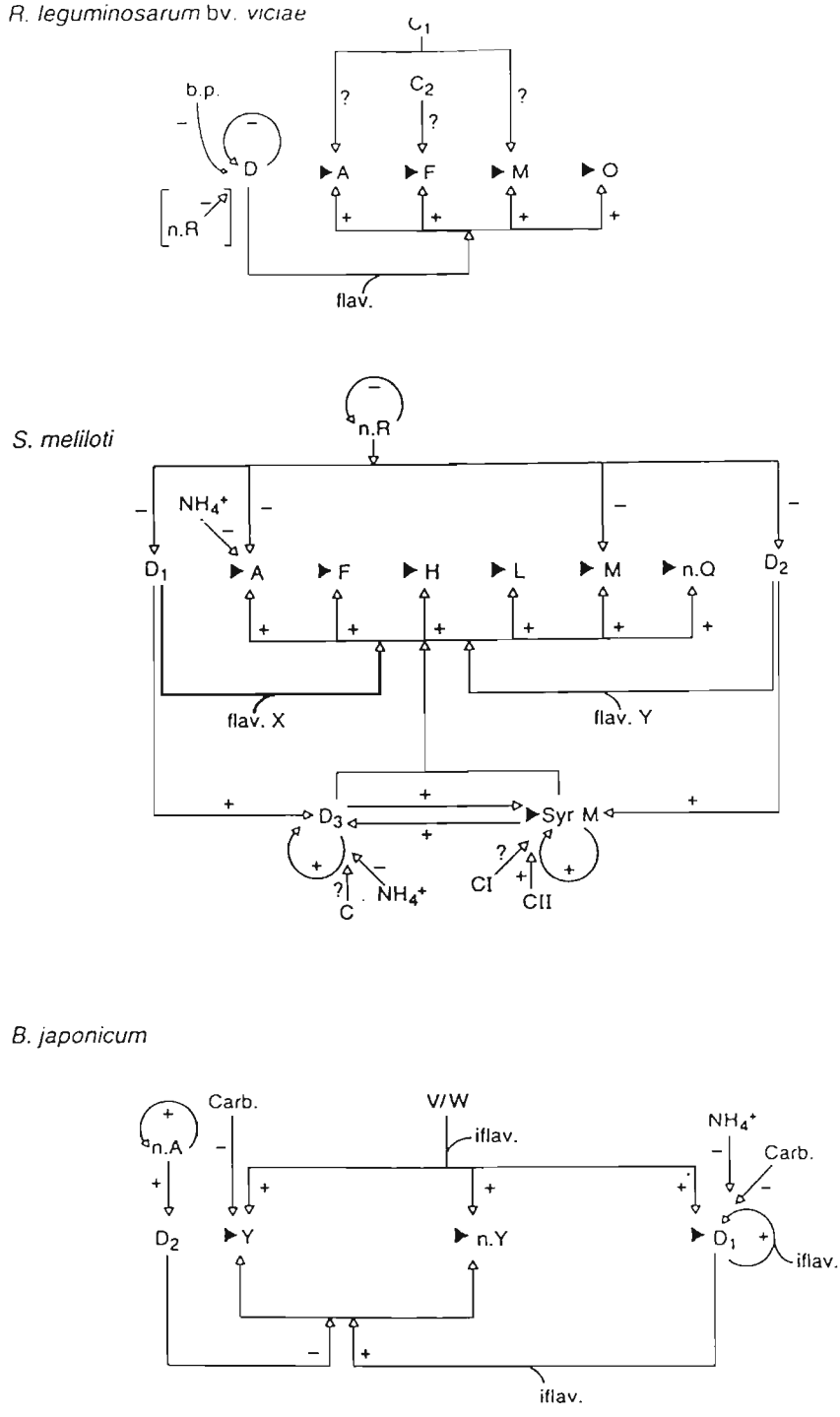


Figure 6. Summary of the regulation of nodulation gene expression in *R. leguminosarum* bv. *viciae*, *S. meliloti* and *B. japonicum*. Stimulation (+) and inhibition (-) of transcription are indicated. The involvement of flavonoids and isoflavonoids is depicted with flav. and iflav., respectively. Nodulation genes and operons are indicated by the letter of the first *nod* gene (*e.g.* A: *nodA* indicating the *nodABCIJ* operon, D: *nodD*) or by n. followed by a letter for *nol* genes (*e.g.* n.R: *nolR*). *Nod* boxes are indicated by a black arrowhead. b.p.: bacteroid-specific protein which binds *in vitro*. C, C1, C2, CI, CII: Proteins of unknown nature which bind specifically to *nod* box DNA *in vitro*. Environmental factors affecting nodulation gene transcription are also indicated. Carb.: carbohydrates. For more details, see text. Relevant literature references are as follows: *R. leguminosarum* bv. *viciae*: Hong *et al.*, 1987; Schlaman *et al.*, 1992a; Spaink *et al.*, 1987a; Zaat *et al.*, 1987; *S. meliloti:* Barnett *et al.*, 1996; Cren *et al.*, 1995; Dusha *et al.*, 1989; Fisher *et al.*, 1988; Kondorosi *et al.*, 1989, 1991a,b; Maillet *et al.*, 1990; Mulligan and Long,1989; *B. japonicum*: Dockendorff *et al.*, 1994a; Garcia *et al.*, 1996; Göttfert *et al.*, 1990; Sanjuan *et al.*, 1994; Stacey *et al.*, 1994.

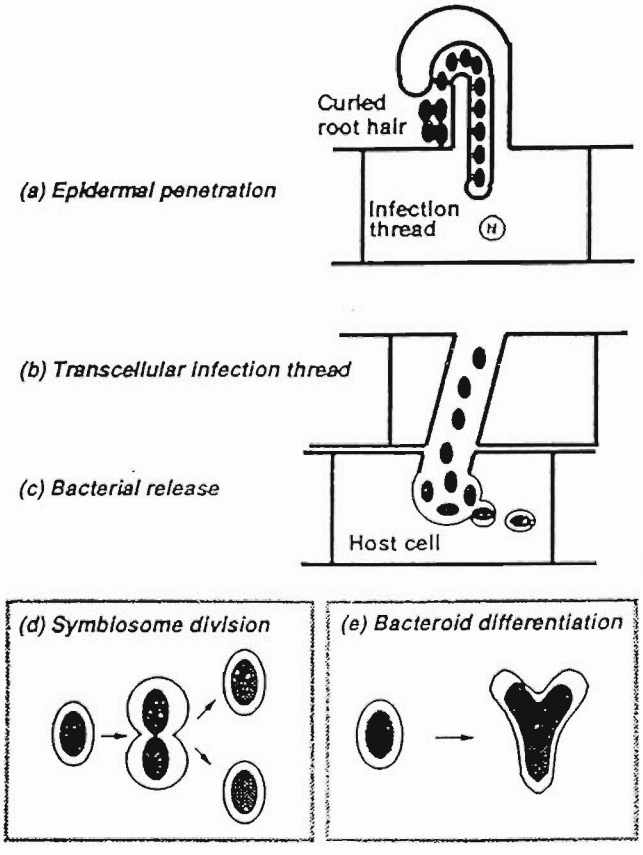
Progreso del hilo infectivo

Hilo de infección: canal de entrada originado por la planta en respuesta a factores nod.

El hilo contiene una matriz con diferentes glicoproteínas implicadas en reconocimiento.

Las células de *Rhizobium* invaden el canal de infección atravesando epidermis hasta cortex.

Los componentes de las capas celulares externas de Rhizobium (EPS, LPS) son críticas para el progreso del canal, inhibiendo respuestas de defensa de la planta.



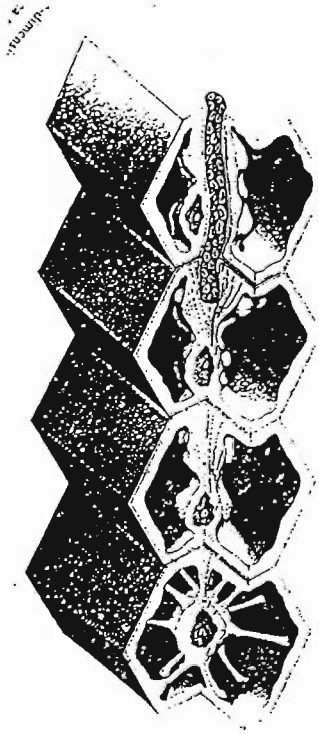


Figure 5. Stages in host cell invasion *Pisum sativum* by *Rhizobium*. (a) Epidermal penetration. Infective rhizobia cause characteristic curling and deformation on emerging and growing root hairs. Bacteria invade the plant through a newly formed tunnel, the infection thread, created by the inversion of apical growth at the tip of the root hair cell. Inward growth of the infection thread is coordinated by the plant cytoskeleton in association with the nucleus (N). (Alternative forms of epidermal penetration include "crack entry" at the point of emergence of lateral roots, e.g. *Arachis*, or dissolution of radial cell walls of the epidermis, *e.g. Mimosa*.) (b) By continuing growth of transcellular infection threads, bacterial invasion spreads from cell to cell through the root cortex and into the invasion zone in the post-meristematic zone of the incipient nodule. [Alternative forms of tissue invasion include colonisation of intercellular spaces, *e.g. Arachis*, or bacterial release (as symbiosomes) into the cytoplasm of host cells that divide mitotically to generate the central infected tissue, *e.g. Phaseolus*.] (c) Endocytosis. Individual bacteria are released through the plasmalemma from an unwalled infection droplet, which arises as an extrusion from the infection thread. Rhizobia engulfed by plasma membrane are termed bacteroids. They occupy a discrete cytoplasmic compartment, termed the symbiosome, which is bounded by a plant-derived symbiosomal (or peribacteroid) membrane. (Alternatively, bacteria may be released from the unwalled tip of an invasion structure which penetrates a host cell but does not exit from the other face (the infection peg), *e.g. Phaseolus, Glycine max*. In woody legumes, *e.g. Andira*, and in *Parasponia*, membrane-enclosed symbiosomes do not exist, but nitrogen-fixing bacteria develop within tubular intrusions termed 'fixation threads'.) (d) Symbiosome division. As the enclosed bacteria divide inside the host cell, the bounding symbiosomal membrane divides concomitantly. (Alternatively, as in determinate nodules, *e.g. Phaseolus*, this synchrony breaks down in the later stages of development and bacteroids are eventually packaged in clusters of 6-12.) (e) Bacteroid differentiation. When bacteroids have ceased division, they develop the capacity for biological nitrogen fixation. This is accompanied by substantial enlargement and morphological differentiation of bacteroids in host cells of indeterminate nodules. (Alternatively, there is no morphological

Liberación de bacterias en el cortex

El hilo infectivo progresa hasta las células del cortex, donde se produce un "goteo" de bacterias que quedan envueltas en membrana peribacteroidal, originando el SIMBIOSOMA

Se producen divisiones coordinadas de las células bacterianas manteniéndose envueltas por la membrana peribacteroidal.

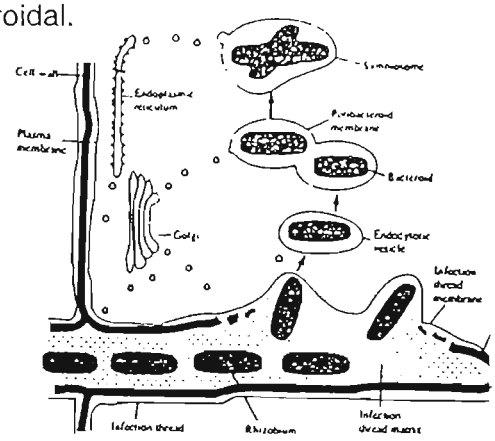


Fig. 1 fotocopias

Modificación en bacteroides

Las bacterias de los simbiosomas sufren modificaciones estructurales complejas, transformándose en **bacteroides.**

Las bacterias se adaptan al ambiente endofítico mediante:

- cambios en LPS
- variaciones en EPS específicos (glucanos cíclicos)
- modificación de cadenas de transporte (alta afinidad)

- síntesis de nitrogenasa.

Las bases moleculares de estos procesos no están bien establecidas

Senescencia del nódulo

Originada por autolisis del simbiosoma.

Relacionada con auto-oxidación de leghemoglobina y aparición de formas activas de oxígeno.

El simbiosoma queda convertido en vesícula lítica, con destrucción de bacteroides.

En leg. herbáceas con nódulos indeterminados coincide con llenado de vainas ¿Relocalización de nitrógeno?

Bacterias no diferenciadas sobreviven en los restos del nódulo e incrementan la población del suelo.

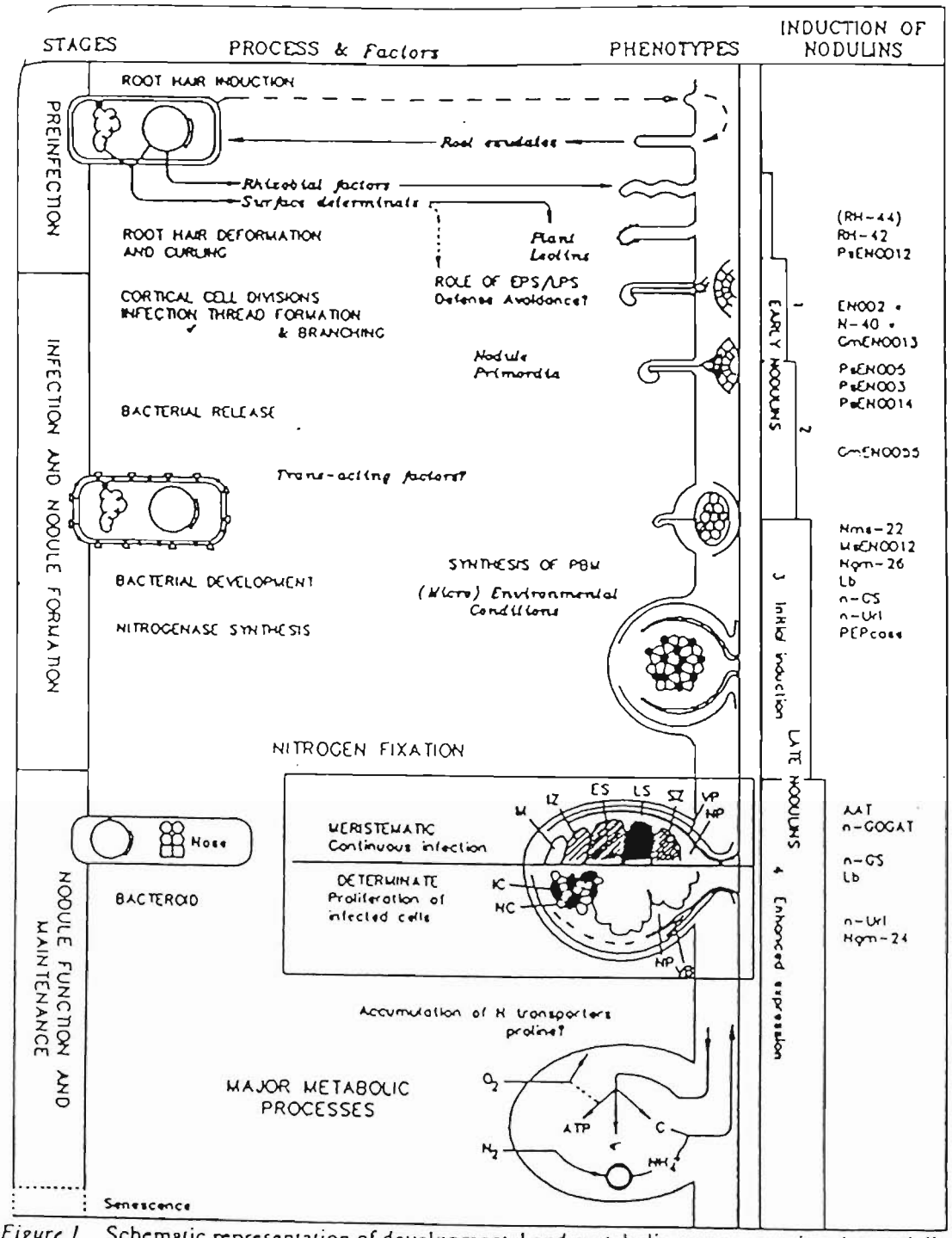


Figure 1 Schematic representation of developmental and metabolic events associated to nodulin expression. Distinct stages, processes, and factors *(left* and *center)* are shown in relation to nodule developmental phenotypes. The figure also depicts the sequence of expression of nodulins and nodule-enhanced activities *(right)* in an arbitrary time scale. At least four steps in nodulin induction are indicated (1–4), with representative examples of several species (* = ubiquitous nodulin type). Nodule types are represented in the middle (MERISTEMATIC: M = meristem; IZ = infection, ES = early symbiotic, LS = late symbiotic and SZ = senescing zones; VP = vascular bundle; NP = nodule parenchyma. DETERMINATE: IC = infected and NC = uninfected cell; VB = vascular bundle; NP = nodule parenchyma/inner cortex.) Major metabolic processes *(bottom)* are described as linked pathways of carbon, nitrogen, and oxygen metabolism, in which several nodulins participate.

TABLE 3
Some Early Nodulin (*ENOD*) Genes and Their Purported Functions

Name	Description	Location	Author
RH-42	42-kDa acidic protein	Found in root hairs exclusively	Gloudemans et al., 1989
RH-44	44-kDa protein	Elevated in root hairs post-inoculation	Gloudemans et al., 1989
LTP	Homology to a lipid transfer protein	In all tissues tested except nodules; up-regulated in root hairs postinoculation	Krause et al., 1994
ENOD40	No long ORFs except in *GmENOD40a*; may function as a small peptide or as an RNA	Generally found in dividing cells or cells competent to divide; in nodule apex and pericycle of mature nodules; also in stems, leaves, and flowers	(Soybean) Kouchi and Hata, 1993; (Pea) Matvienko et al., 1994; (Medics) Asad et al., 1994; Crespi et al., 1994; (Vetch) Vijn et al., 1995; (Bean) Papadopoulos et al., 1996
ENOD12	Proline rich; assumed to be a cell wall protein	Root hairs/epidermal cells; nodule primordium; invasion zone of mature nodules; also expression in stems	(Pea) Scheres et al., 1990a; (*Medicago truncatula*) Pichon et al., 1992; (Alfalfa) Allison et al., 1993
MtENOD11	Similar to *MtENOD12*	Similar expression pattern as *MtENOD12*	D. G. Barker, personal communication; Cook et al., 1995
Mtrip1	Peroxidase	Root epidermis	Cook et al., 1995
PsENOD5	Related to arabinogalactans in plasma membrane	Nodule primordium; zones 3 and 4 of mature nodules; in infected cells only	Scheres et al., 1990b
PsENOD3	Possibly a metal-binding protein	Early symbiotic zone of mature nodules	Scheres et al., 1990b
PsENOD14	Has a cysteine cluster; may be a metal-binding protein	Same expression pattern as *PsENOD3*	Scheres et al., 1990b
MsENOD10	Proline-rich protein; presumed cell wall protein	Nodule primordium; zone 2 of mature nodules	Löbler and Hirsch, 1993
PsENOD7	Appears to be transported across membranes	Detected 8 d after inoculation; in zone 2 of nodule; appears to be nodule specific	Kozik et al., 1996
ENOD2	Proline rich; presumed cell wall protein	Inner cortex (nodule parenchyma); may be nodule specific	(Soybean) Franssen et al., 1987; (Vetch) Moerman et al., 1987; (Alfalfa) Dickstein et al., 1988; (Bean) Sánchez et al., 1988; (*Sesbania*) Strittmatter et al., 1989; (Pea) van de Wiel et al., 1990a; (Lupine) Szczyglowski and Legocki, 1990; (Cowpea) Trese and Pueppke, 1991
GmENOD13	50% similar to ENOD2	Similar expression pattern	Franssen et al., 1987
MSENOD8	Lipase like	Nodule specific	Dickstein et al., 1993; R. Dickstein, personal communication
MtPrP4	62-kDa proline-rich protein	Meristem of mature nodule; nodule specific	Wilson et al. 1994

Note: The genes are listed in their approximate order of appearance after inoculation with *Rhizobium*.

IV.- NODULINAS TARDÍAS: Función y mantenimiento del nódulo.

Son inducidas justo antes del arranque de la fijación de nitrógeno. Se incluyen las nodulinas "verdaderas" específicas del nódulo y las que son comunes a otros órganos pero que se incrementan en el nódulo. Sus principales funciones son:

- Transporte de metabolitos por la MPB.
- Creación de condiciones fisiológicas para nitrogenasa y fijación de nitrógeno.
- Asimilación de nitrógeno fijado, metabolismo del carbono, biogénesis de amidas y ureidos.

IV.1.- Enzimas de asimilación de amonio:

- Glutamín sintetasa.
- Glutamato sintasa.

IV.2.- Enzimas del metabolismo del carbono.

- Fosfoenol piruvato carboxilasa.
- Sacarosa sintasa.

IV.3.- Transportadores de nitrógeno en el nódulo.

- Aspartato aminotransferasa
- Uricasa.

IV.4.- Otras nodulinas tardías metabólicas.

- Enzimas de ureidos: pirolina-5-carboxilato reductosa.
- Colina quinasa.
- Xantina deshidrogenasa, malato deshidrogenesa, etc.
- Leghemoglobina.

V.5.- Nodulinas de la Membrana Peribacteroidal.

La MPB se forma por un proceso de endocitosis derivando de la membrana plasmática, y juega un papel regulador central en el metabolismo de nódulo como intercambiador de metabolitos entre simbiontes. Se han detectado en soja glicoproteínas y glicolípidos:

- Ngm-24: no parece proteína transportadora transmembrana.
- Ngm-26: proteína transportadora transmembrana.
- Ngm-23: estructura similar a proteínas unidas a metales.

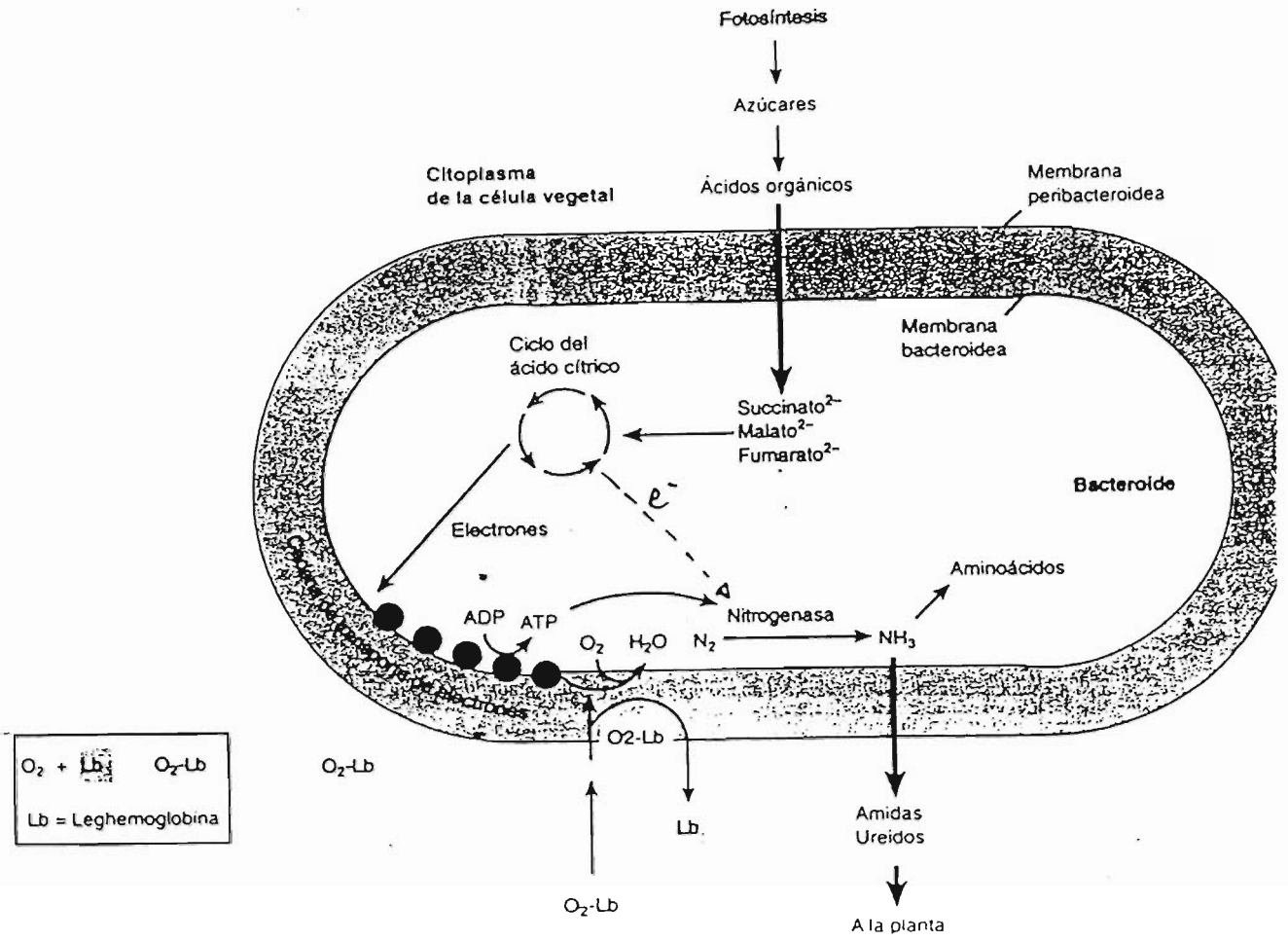
Existen otras proteínas específicas en MPB y fluido peribacteroidal (FPB) con función transportadora.

V.- NODULINAS HOMÓLOGAS Y FAMILIAS DE GENES.

V.1.- Nodulinas estructurales:

Las principales nodulinas tempranas son proteínas estructurales de la pared celular, ricas en prolinas: ENOD 2, Gm ENOD 13, Nms-30, Ps ENOD 5, 12, 3 Y 14. Todas tienen un péptido señal en el extremo N-terminal que las caracteriza.

Funcionamiento del nódulo radicular



Metabolismo nodular

- Oxígeno

- Necesario para la respiración de Rhizobium, pero

- Se requiere ambiente microaeróbico (nmolar O2) para protección de la n itrogenasa

Aparecen diversas adaptaciones:

- Barrera física a la difusión de O2 a la zona de fijación activa

- Protección respiratoria

- Oxidasas terminales de Rhizobium de alta afinidad

- **Leghemoglobina** suministraniveles bajos pero constantes de O2

- Carbono

- Los bacteroides reciben de la planta ácidos dicarboxílicos (Adc) como fuente principal de carbono

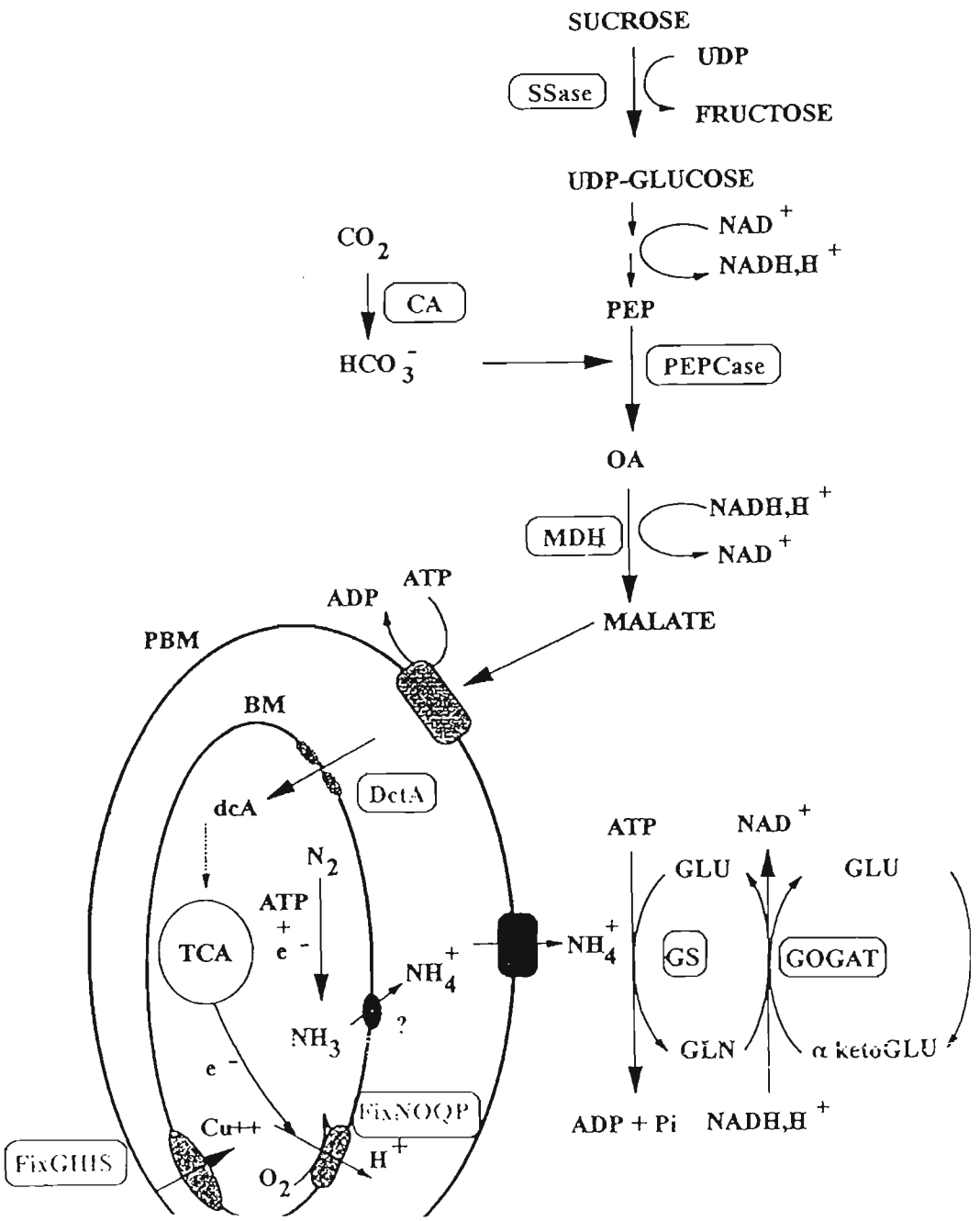
- El transporte de Adc (en bacteroide y MPB)es esencial para el funcionamiento del nódulo

- Nitrógeno

- La síntesis del sistema de fijación de nitrógeno está desacolplada del sistema de control NtrBC.

- La asimilación de amonio está reprimida en bacteroides

- Los bacteroides excretan nitrógeno fijado (NH4) que es asimilado por las célul as vegetales medianteel sistema GS/GOGAT



Regulación de la expresion de los genes de fijación de nitrogeno (nif/fix) en *Rhizobium*

El nivel de oxígeno es la señal clave para la inducción de los genes implicados en la fijación de nitrógeno

genes nif: homólogos a los descritos en *K. pneumoniae* (síntesis de N2asa

genes fix: sin correspondencia en *K. pneumoniae* (transp. de electrones)

- Componentes de los sistemas de regulación

NifA:	Regulador general de los genes nif
sigma-54:	factor sigma alternativo de promotores simbióticos.
FixK/FnrN:	activadores transcripcionales de genes inducidos por microaerobiosis

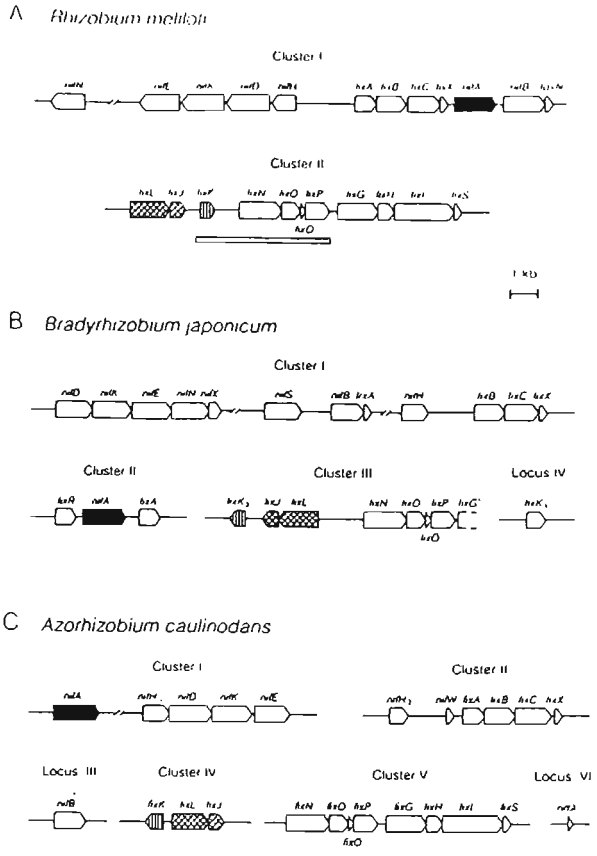
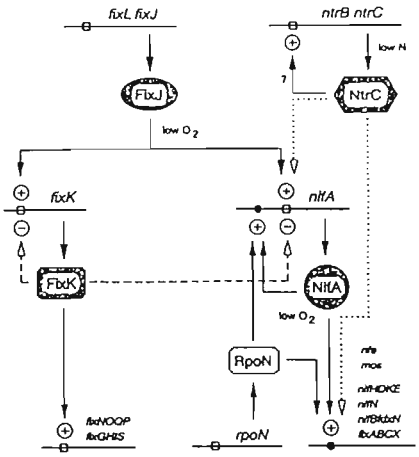


FIG. 1. Organization of *nif* and *fix* gene clusters in *R. meliloti* (A), *B. japonicum* (B), and *A. caulinodans* (C). Several known ORFs associated with some of the *nif* or *fix* genes are not shown here. In *R. meliloti* both clusters of *nif* and *fix* genes are located on the megaplasmid pSym-a, whereas in *B. japonicum* and *A. caulinodans* all of the depicted genes are present on the chromosome. Homologous regulatory genes are marked by identical patterns. The hatched bar below cluster II of *R. meliloti* refers to a region which is duplicated on the megaplasmid. For details and references, see the text and Table 1

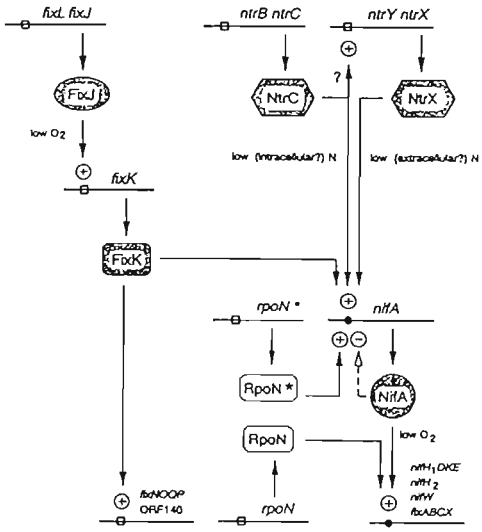
TABLE 1. *nif* and *fix* genes identified in *R. meliloti*, *B. japonicum*, or *A. caulinodans* and their known or proposed functions[a]

Gene	Product and/or (proposed) function	Reference(s)[b]		
		Rm	Bj	Ac
nif genes[c]				
nifH	Fe protein of nitrogenase	381	137	285
nifD	α subunit of MoFe protein of nitrogenase	75, 326	196	94, 108, 284
nifK	β subunit of MoFe protein of nitrogenase	75, 326	379	94, 108, 284
nifE	Involved in FeMo cofactor biosynthesis	249	6	285
nifN	Involved in FeMo cofactor biosynthesis	4, 5	6	
nifB	Involved in FeMo cofactor biosynthesis	57	136, 287	108
nifS	Cysteine desulfurase (421); activation of sulfur for metallocluster synthesis?		116	
nifW	Unknown function; required for full activity of FeMo protein			18, 200
nifX	Unknown function		163	
nifA	Positive regulator of *nif*, *fix*, and additional genes	58, 403	377	278, 304
fix genes				
fixABCX	Unknown function; required for nitrogenase activity; FixX shows similarity to ferredoxins	115	153, 154	18
fixNOQP	Microaerobically induced, membrane-bound cytochrome oxidase	42	299	242
fixGHIS	Redox process-coupled cation pump?	194	299	244
fixLJ	Oxygen-responsive two-component regulatory system involved in positive control of *fixK* (Rm, Bj, Ac) and *nifA* (Rm)	82	13	198
fixK/*fixK*$_2$	Positive regulator of *fixNOQP* (Rm, Bj, Ac), *nifA* (Ac), *rpoN*$_1$, and "nitrate respiration" (Bj); negative regulator of *nifA* and *fixK* (Rm)	28	14	199
Rm *fixK'*	Reiterated, functional copy of *fixK*	28		
Bj *fixK*$_1$	*fixK* homolog of unknown function; not essential for nitrogen fixation		15	
fixR	Unknown function; not essential for nitrogen fixation		377	

A *Rhizobium meliloti*



C *Azorhizobium caulinodans*



B *Bradyrhizobium japonicum*

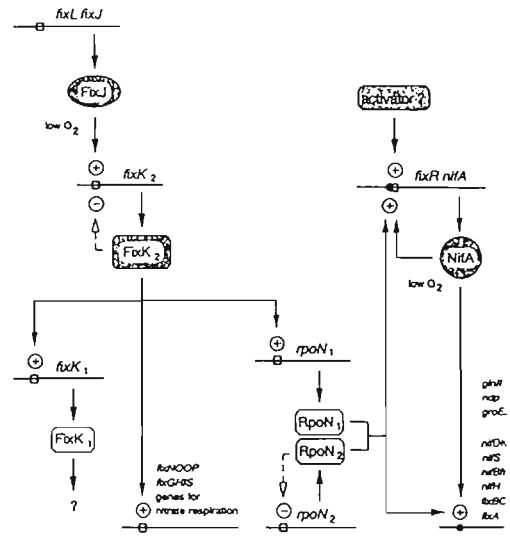
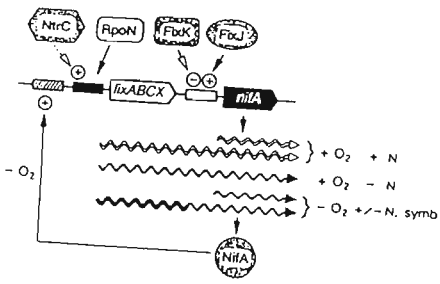
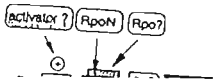


FIG. 2. Comparative models of *nif* and *fix* gene regulation
meliloti (A), *B. japonicum* (B), and *A. caulinodans* (C). Homolc
regulatory proteins are symbolized identically in panels A, B, ar
Solid arrows and open arrowheads with dashed lines indicate po
and negative regulation, respectively. Dotted lines with open ar
heads denote NtrC-mediated activation of the *R. meliloti nifH*
nifBfdxN, and *fixABCX* promoters which is observed in free-l
aerobically growing cells under nitrogen-limiting conditions but
is of no relevance for symbiotic nitrogen fixation. Solid circles inc
σ^{54}-dependent −24/−12-type promoters, whereas open boxes inc
other promoters. Key references describing substantial compone
the models presented include 28, 39, 82, 103, 319, 373, 396, and 4(
R. meliloti; 13–15, 127, 217, 376, and 377 for *B. japonicum*; anc
199, 278, 295, 296, 304, and 362 for *A. caulinodans*. For addi
references and further details, see the text.

A *Rhizobium meliloti*



B *Bradyrhizobium japonicum*



C *Azorhizobium caulinodans*

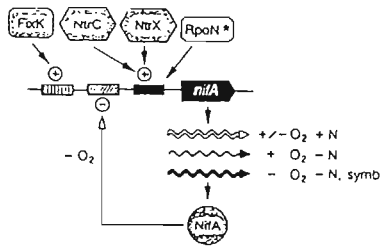


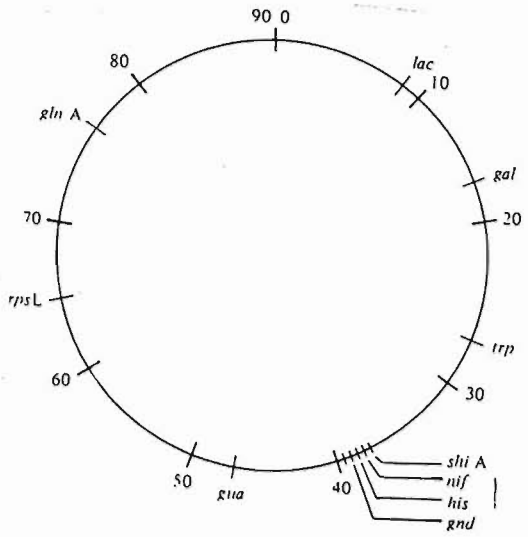
FIG. 7. Transcriptional organization and regulation o
genes in *R. meliloti* (A), *B. japonicum* (B), and *A. caulinoda*
schemes are not drawn to scale. Solid wavy lines of diffe
nesses symbolize the size and relative abundance of (*fixA*
transcripts present under the growth conditions described o
whereas open wavy lines refer to transcripts that are absen
conditions indicated. Growth conditions are as follows: aero
microaerobic or anaerobic (−O_2), nitrogen rich (+N), nit
ited (−N), and symbiotic (symb.); +/− means that the
oxygen or nitrogen conditions are not relevant. Solid and op
arrowheads refer to the positive and negative control, respe
dotted line with the open arrowhead denotes the symbiotic
vant activation of the *R. meliloti fixABCX* promoter by N
free-living conditions (see the legend to Fig. 2). Expres
meliloti nifA is additionally modulated by ammonia repres
unknown mechanism involving FixL (not shown in panc
Similarly, expression of *A. caulinodans nifA* is additionally
by *nifA* via a mechanism that is not yet understood in detail

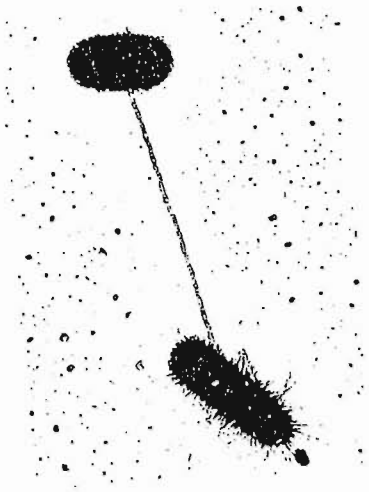
Genética FN (K. Pneumoniae)

Chile 2005

Complejicidad genética del sistema de fijación de dinitrógeno

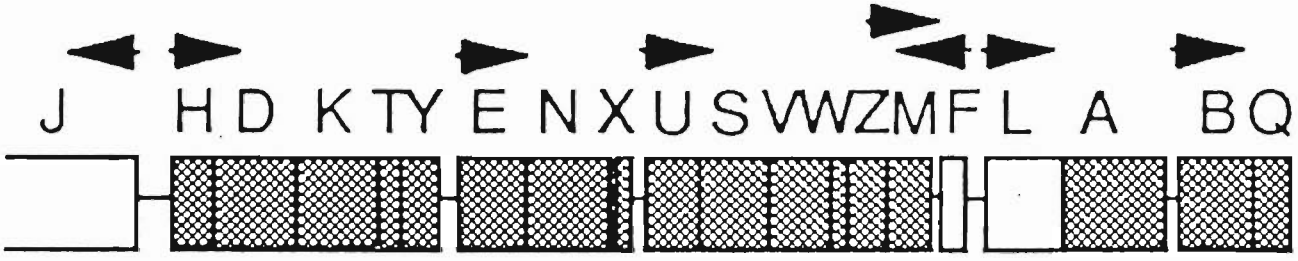
Klebsiella pneumoniae





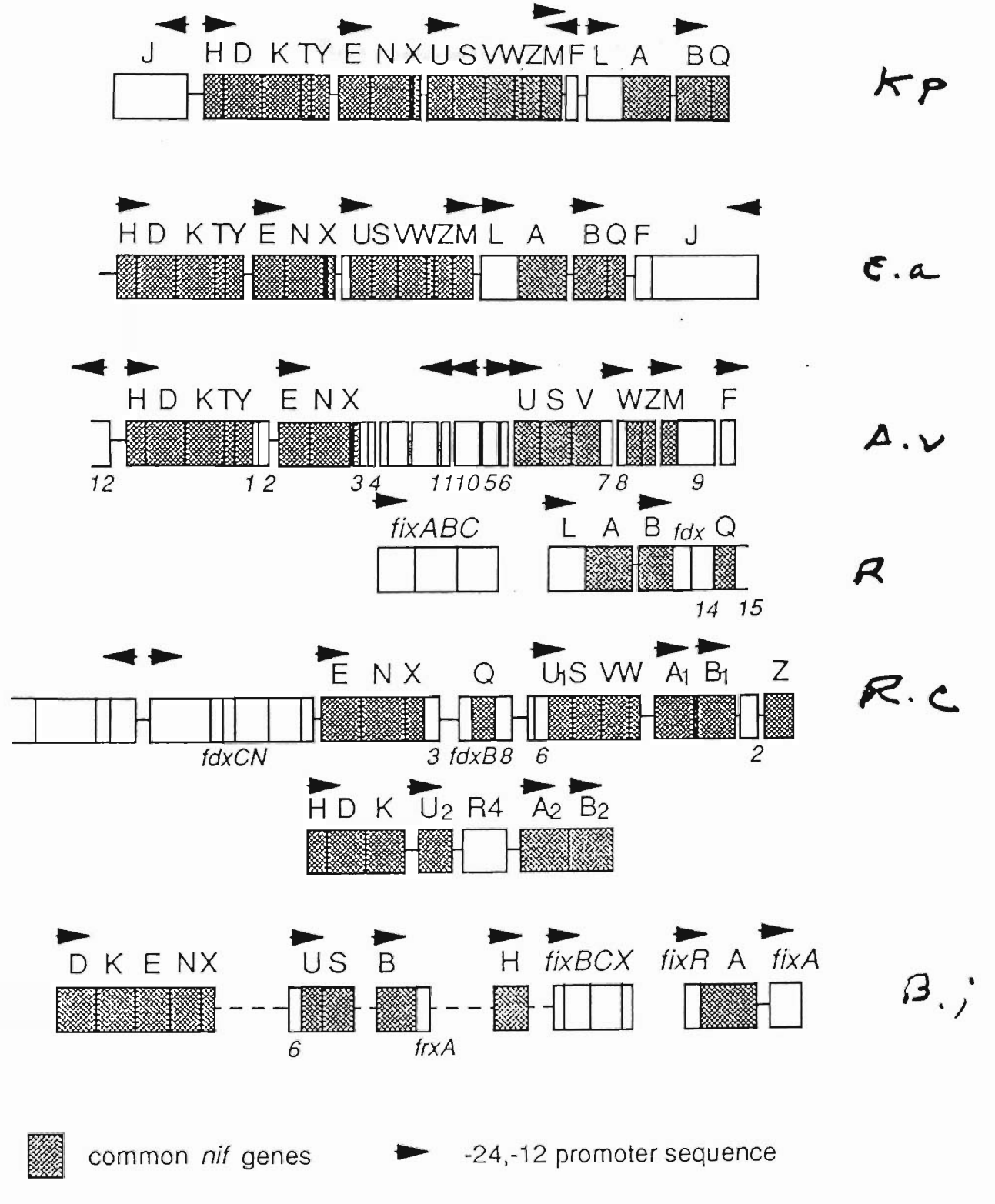
Eschericioa coli

Genes *nif*



Nitrogenasa funcional

AGRUPACIONES nif EN DISTINTAS BACTERIAS

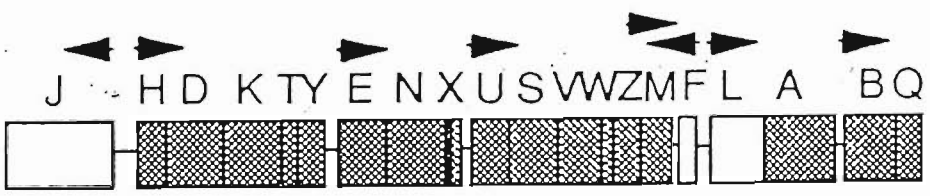


Arnold et al J. Mol Biol 203, 715-738 (1988)

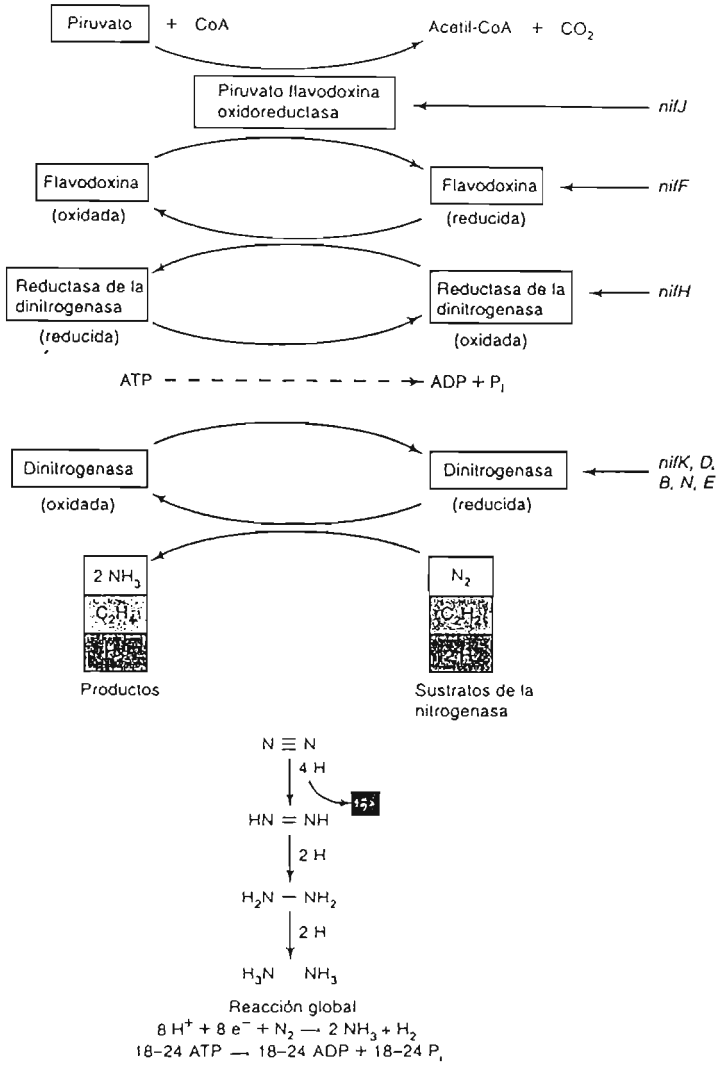
Table 2

Comparison of the molecular masses of nif *gene products determined by SDS/polyacrylamide gel electrophoresis and deduced from the nucleotide sequences*

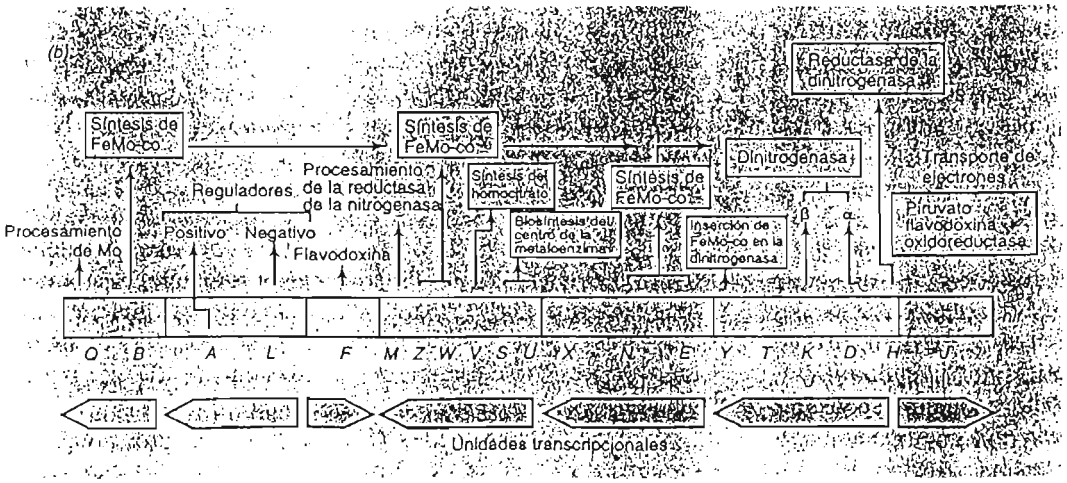
Gene	Molecular masses in kD: Determined from SDS/PAGE	Molecular masses in kD: Deduced from DNA sequence	Proposed function
J	120[1,2,3,4], 122[5]	128·038[6a] 114·354[6a]	Electron transport: pyruvate-flavodoxin oxidoreductase[4,5]
H	35/39[1b], 35[2,3]	31·902[7c]	Component II[8,9]
D	60[1], 56[2,3]	54·139[6] 54·156[10]	α-Subunit of component I[8,9]
K	60[1,2,3]	58·406[6] 57·751[11]	β-Subunit of component I[8,9]
orf4		8·321[6]	?
Y	24[1]	24·691[6]	Maturation of component I ?[12]
E	40[1], 46[3]	50·153[6]	Synthesis of FeMoco[9]
N	50[1,3]	50·567[6]	Synthesis of FeMoco[9]
X	18[1]	18·229[6]	?
U	25/32[1b], 22[2], 28[3]	29·494[6,13c]	Maturation of component I ?[12]
S	45[1], 42[3]	43·175[6] 43·259[13]	Maturation of component I[12] or component II[9]
V	42[1], 38[3]	41·078[6] 41·194[13]	Synthesis of FeMoco: homocitrate synthase[14]
W[d]		10·179[6]	?
Z	15–17[15]	16·659[6,15c]	?
M	28[1], 27[3], 31·5[15]	30·613[6,15c]	Processing of component II[12,30]
F	10[1,2], 22[3,16], 19[17]	19·112[6,18c]	Electron transport: flavodoxin[16]
L	50[1], 52[3], 45[19]	55·213[6] 55·311[22c]	*nif*-specific repression[20,21]
A	60[1], 66[3], 57[19]	58·632[6,23c] 53·319[25]	*nif*-specific activation[8,24]
B	49[26]	50·855[6] 51·032[28c]	Synthesis of FeMoco[27]
Q		19·665[6] 19·683[28c]	Processing of Mo[29]

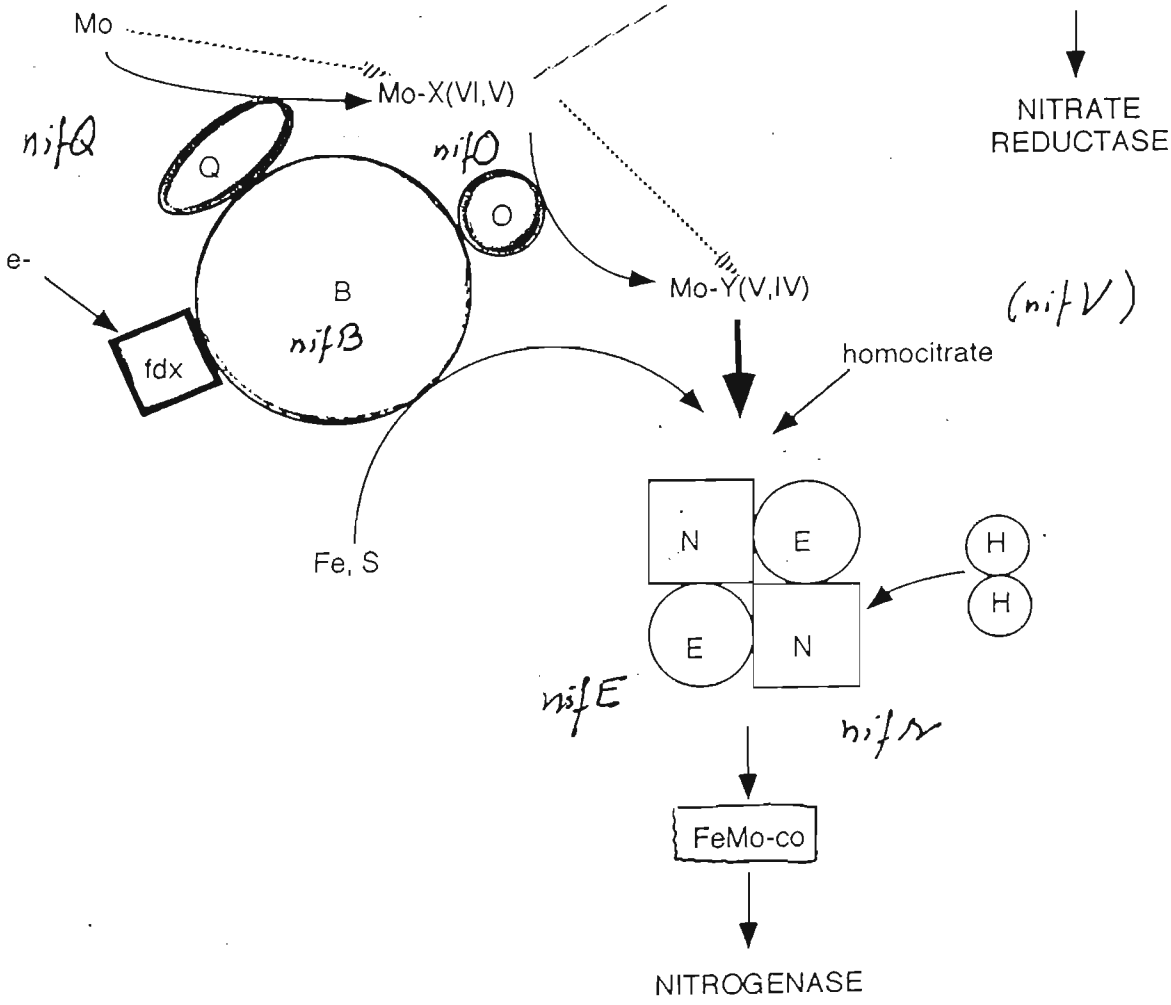


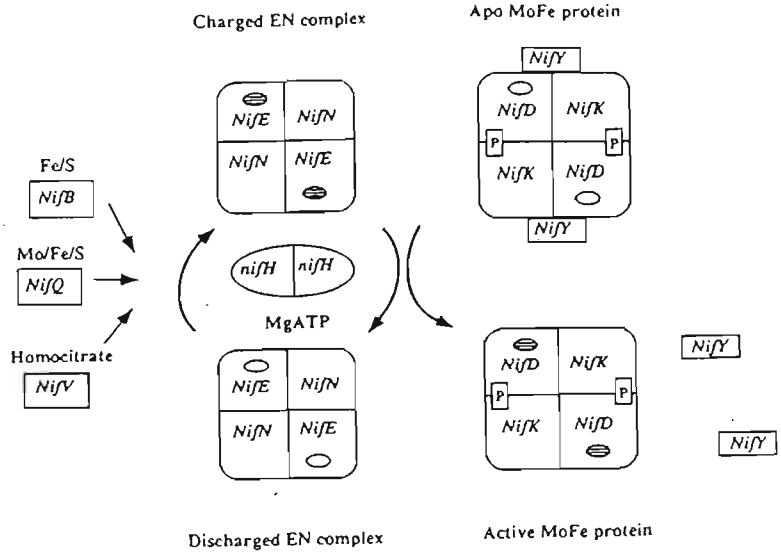
(a)


Piruvato + CoA
Acetil-CoA + CO_2
Piruvato flavodoxina oxidoreductasa
nifJ
Flavodoxina (oxidada)
Flavodoxina (reducida)
nifF
Reductasa de la dinitrogenasa (reducida)
Reductasa de la dinitrogenasa (oxidada)
nifH
ATP
ADP + P_i
Dinitrogenasa (oxidada)
Dinitrogenasa (reducida)
nifK, D, B, N, E
2 NH_3
C_2H_4
N_2
C_2H_2
Productos
Sustratos de la nitrogenasa
N ≡ N
4 H
HN = NH
2 H
$H_2N - NH_2$
2 H
H_3N NH_3
Reacción global
$8 H^+ + 8 e^- + N_2 \rightarrow 2 NH_3 + H_2$
18–24 ATP → 18–24 ADP + 18–24 P_i

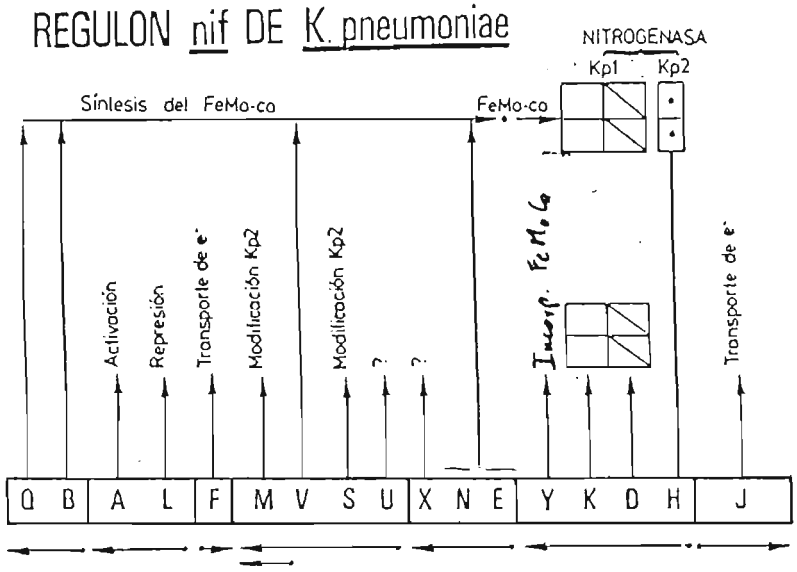
(b)


Síntesis de FeMo-co
Síntesis de FeMo-co
Reductasa de la dinitrogenasa
Dinitrogenasa
Transporte de electrones
Procesamiento de la reductasa de la nitrogenasa
Síntesis del homocitrato
Síntesis de FeMo-co
Reguladores
Procesamiento de Mo
Positivo
Negativo
Flavodoxina
Biosíntesis del centro de la metaloenzima
Inserción de FeMo-co en la dinitrogenasa
Piruvato flavodoxina oxidoreductasa
β
α
O B A L F M Z W V S U X N E Y T K D H J
Unidades transcripcionales

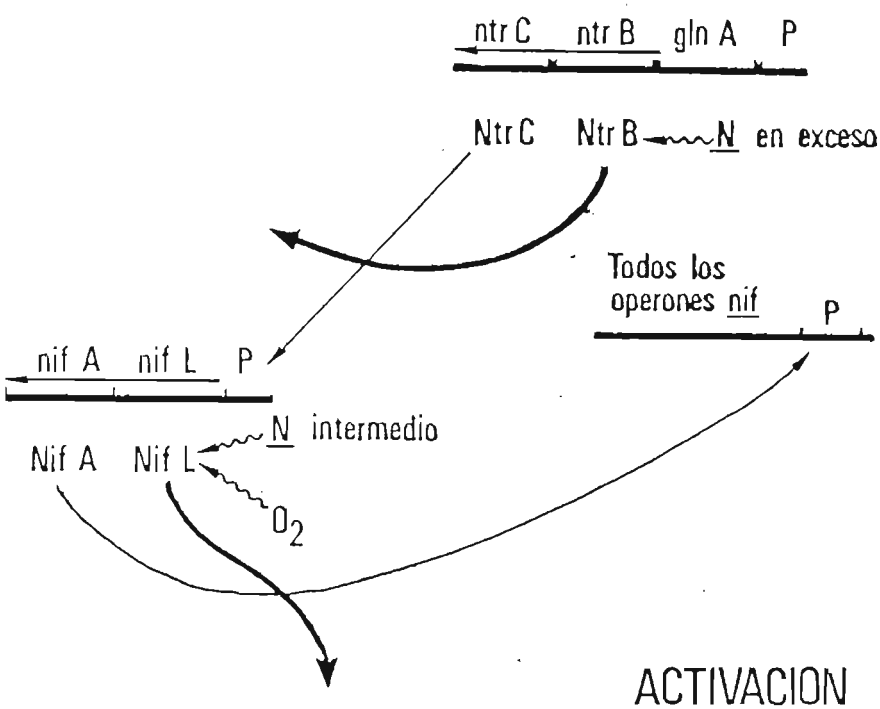

Mo
Mo-X(VI,V)
nifQ
Q
nifO
O
e-
B
nifB
fdx
Mo-Y(V,IV)
(nifV)
NITRATE
REDUCTASE
homocitrate
Fe, S
N
E
H
H
E
N
nifE
nifN
FeMo-co
NITROGENASE


Charged EN complex
Apo MoFe protein
NifY
NifD
NifK
NifK
NifD
NifY
NifE
NifN
NifN
NifE
Fe/S
NifB
Mo/Fe/S
NifQ
Homocitrate
NifV
nifH
nifH
MgATP
NifE
NifN
NifN
NifE
NifD
NifK
NifK
NifD
NifY
NifY
Discharged EN complex
Active MoFe protein
P = P cluster
= Unoccupied FeMo-co site

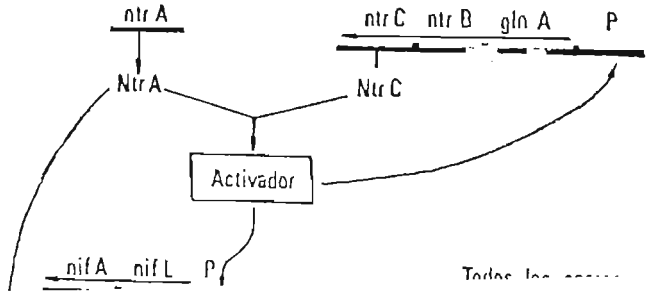
REGULON nif DE K. pneumoniae



REPRESION DE LOS GENES nif



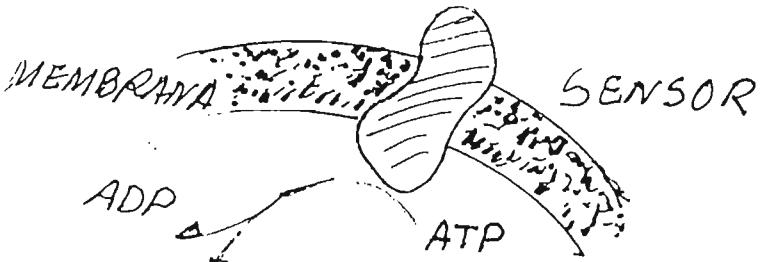
ACTIVACION DE LOS GENES nif

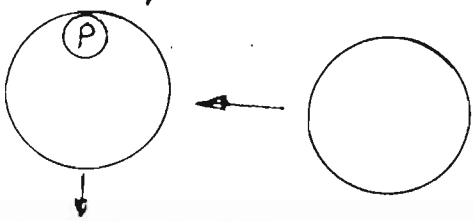


SISTEMA DE DOS COMPONENTES

Estímulo ambiental

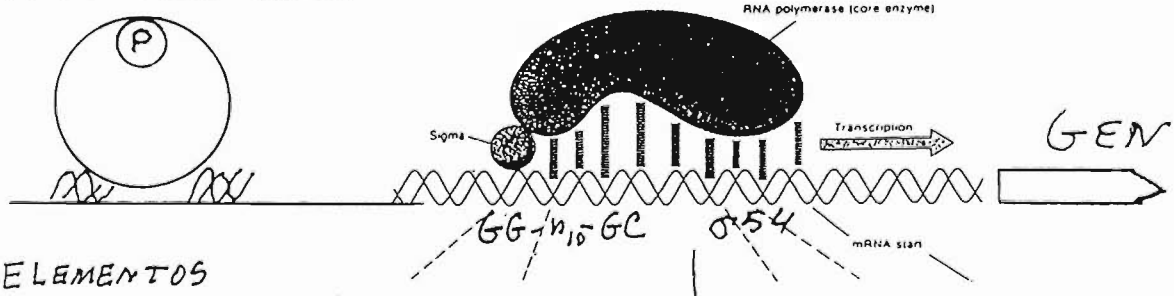
NH_4^+
O_2
A. dicarbox.
Acetosiringona





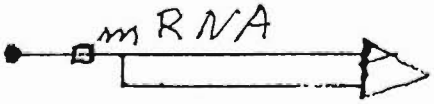
TRANSDUCCION DE LA SEÑAL

ACTIVADOR DE TRANSCRIPCION



ELEMENTOS REGULADORES

TRANSCRIPCION



TRADUCCIÓN

PROTEINA

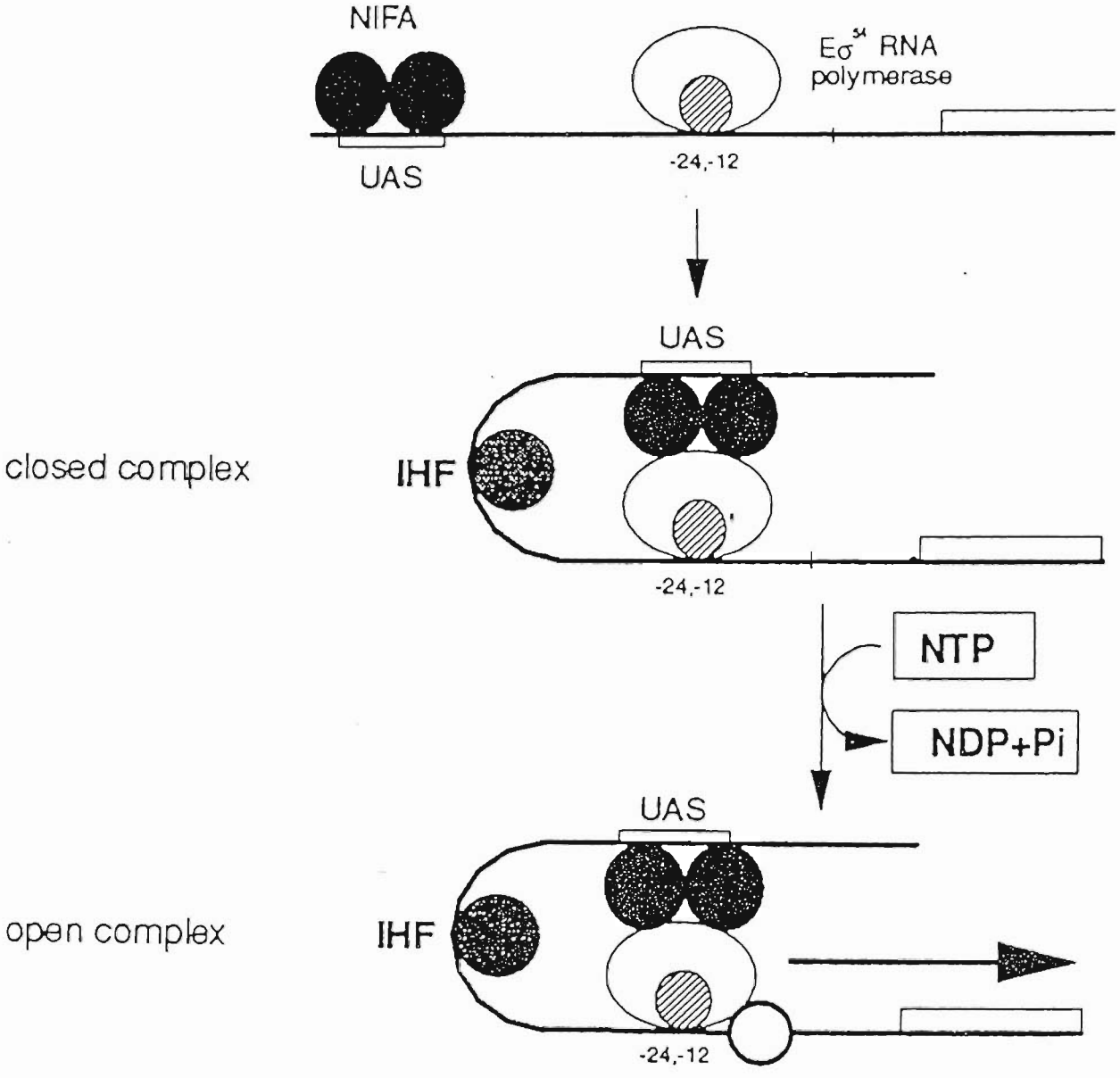


Figure 1 Pathway of transcriptional activation by NIFA

Nitrogenase
(Estructura y función)

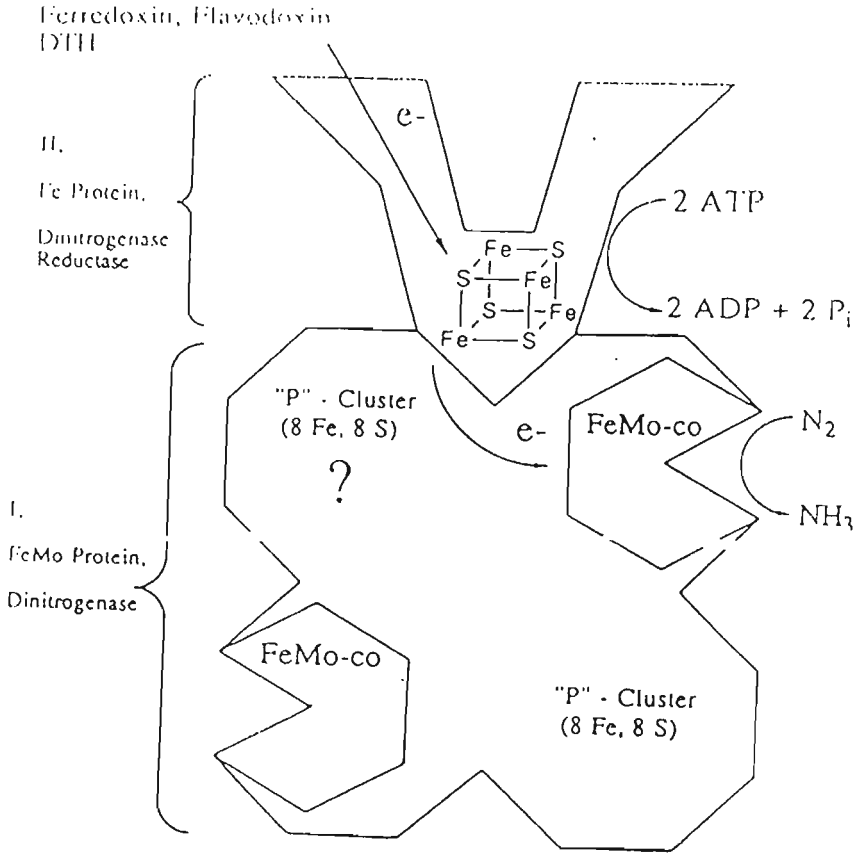


Table 18-4. Reactions Catalyzed by Nitrogenase

	Reaction	Ref.*
1.	$N_2 + 6H^+ + 6e^- \rightarrow 2NH_3$	88
2	$HCN + 6H^+ + 6e^- \rightarrow CH_4 + NH_3$	98
3	$HCN + 4H^+ + 4e^- \rightarrow CH_3NH_2$	98
4	$CH_3NC + 6H^+ + 6e^- \rightarrow CH_4 + CH_3NH_2$	133
	$CH_3NC + 4H^+ + 4e^- \rightarrow CH_3NHCH_3$	19
5	$HN_3 + 6H^+ + 6e^- \rightarrow N_2H_4 + NH_3$	132
6.	$N_3^- + 3H^+ + 2e^- \rightarrow N_2 + NH_3$	132
7.	$N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O$	88,90
8.	$C_2H_2 + 2H^+ + 2e^- \rightarrow C_2H_4$	97,38
9.	$3CH{=}CHCH_3 + 6H^+ + 2e^- \rightarrow \overline{CH_3CH_2CH_3} + 2CH_3CH{=}CH_2$	43
10.	$2H^+ + 2e^- \rightarrow H_2$	88
11.	$\overline{CH_3{-}N{=}N} + 6H^+ + 6e^- \rightarrow CH_3NH_2 + NH_3$	105
12.	$\overline{CH_3{-}N{=}N} + 8H^+ + 8e^- \rightarrow CH_4 + 2NH_3$	105
13.	$NO_2^- + 7H^+ + 6e^- \rightarrow NH_3 + 2H_2O$	157
14.	$N{\equiv}CNH_2 + 6H^+ + 6e^- \rightarrow CH_3NH_2 + NH_3$	1,3
15.	$N{\equiv}CNH_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2NH_3$	113
16.	$C_2H_4 + 2H^+ + 2e^- \rightarrow C_2H_6$	6
17.	$N_2H_4 + 2H^+ + 2e^- \rightarrow 2NH_3$	15
Catalytic activities associated with individual nitrogenase proteins:		
		Ref.
Av1	$H_2 \rightarrow 2H^+ + 2e^-$	171
Av2	$S_2O_4^{2-} \rightarrow S_2O_3^{2-} + SO_2^{2-}$	104
Stoichiometric reaction of the Fe protein with O_2:		
Kp2	$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2$	153

*The references are the most recent on the alternative substrates.

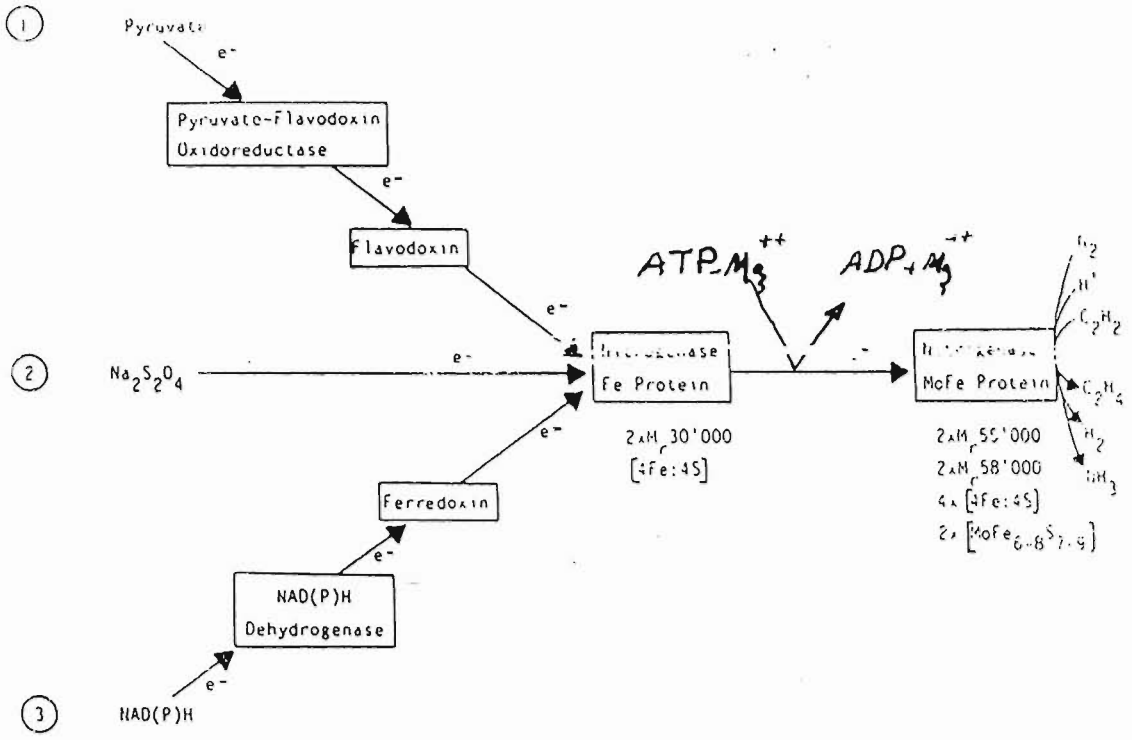
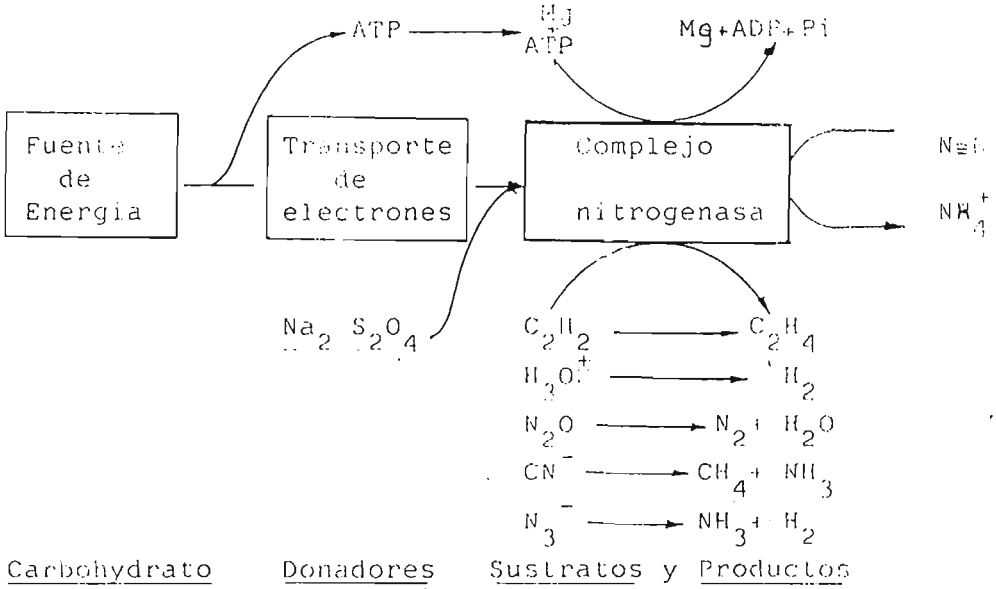


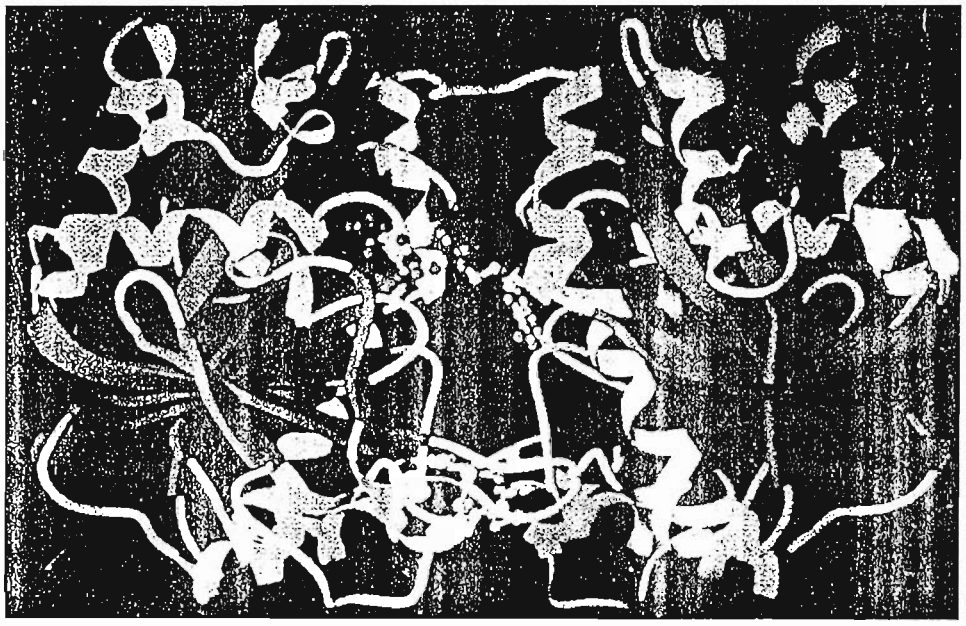
Fig. 2. Composition and function of nitrogenase, and routes of electron transport to nitrogenase. (1) In the facultative anaerobe, Klebsiella pneumoniae; (2) in vitro, i.e. in an anaerobic cell-free system with an artificial electron donor; (3) in aerobic diazotrophs, e.g. Rhizobium. The transfer of an electron from the Fe protein to the MoFe protein consumes two molecules of MgATP (see text).

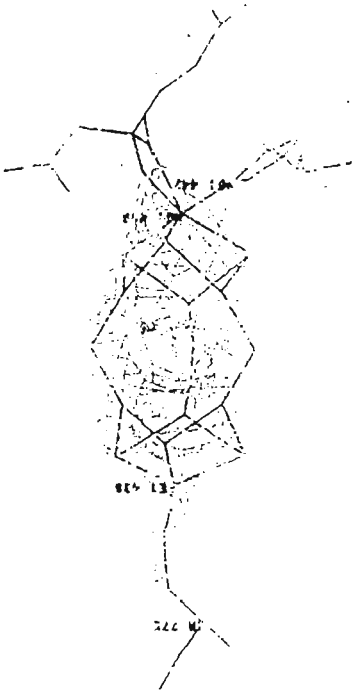


Carbohydrato	Donadores fisiológicos o no fisiológicos	Sustratos y Productos de la reacción

Characteristic	MoFe protein	Fe protein
Other names	Component 1 or dinitrogenase	Component 2 or dinitrogenase reductase
Molecular Weight	240 KD	120 KD
Conserved Cys	$C_{62}, C_{88}, C_{154}, C_{153}, C_{275}$ / C_{70} C_{95} C_{153}	$C_{39}, C_{86}, C_{98}, C_{133}$ C_{185} .....
Fe	30-32, $\bar{x}$ 30	8
S^{2-}	24-34, $\bar{x}=32$	8
Mo	2	0
Subunits	α_2 60.000	γ_2 60.000
	β_2 50.000	
Metal clusters	2 P-clusters: $[Fe_8 S_8]$ ligands: $C_{\beta 95}$ $C_{\alpha 153}$ $C_{\beta 111}$ $C_{\beta 153}$ $C_{\beta 70}$ $C_{\alpha 154}$ $C_{\alpha 62}$	1 × $[Fe_4 S_4]$ Symmetrically bridged
	2 M-clusters FeMo.co: [1 Mo: 7 Fe: 8 S: 1 homocitrate]	
	1 Mo : 3 Fe : 3 S / 4 Fe : 3 S | 3 non-protein ligands	
	& environment (H_{442}, H_{195} Q_{191} C_{275})	
Cristalographic structure	Kim and Rees Science 257, 1677-1681 (1992)	Georgiadis et al 1992 Science 257, 1653-1659

Estructura de la Subunidad II
"Fe protein"





Walker's motif
GxGxxGKS

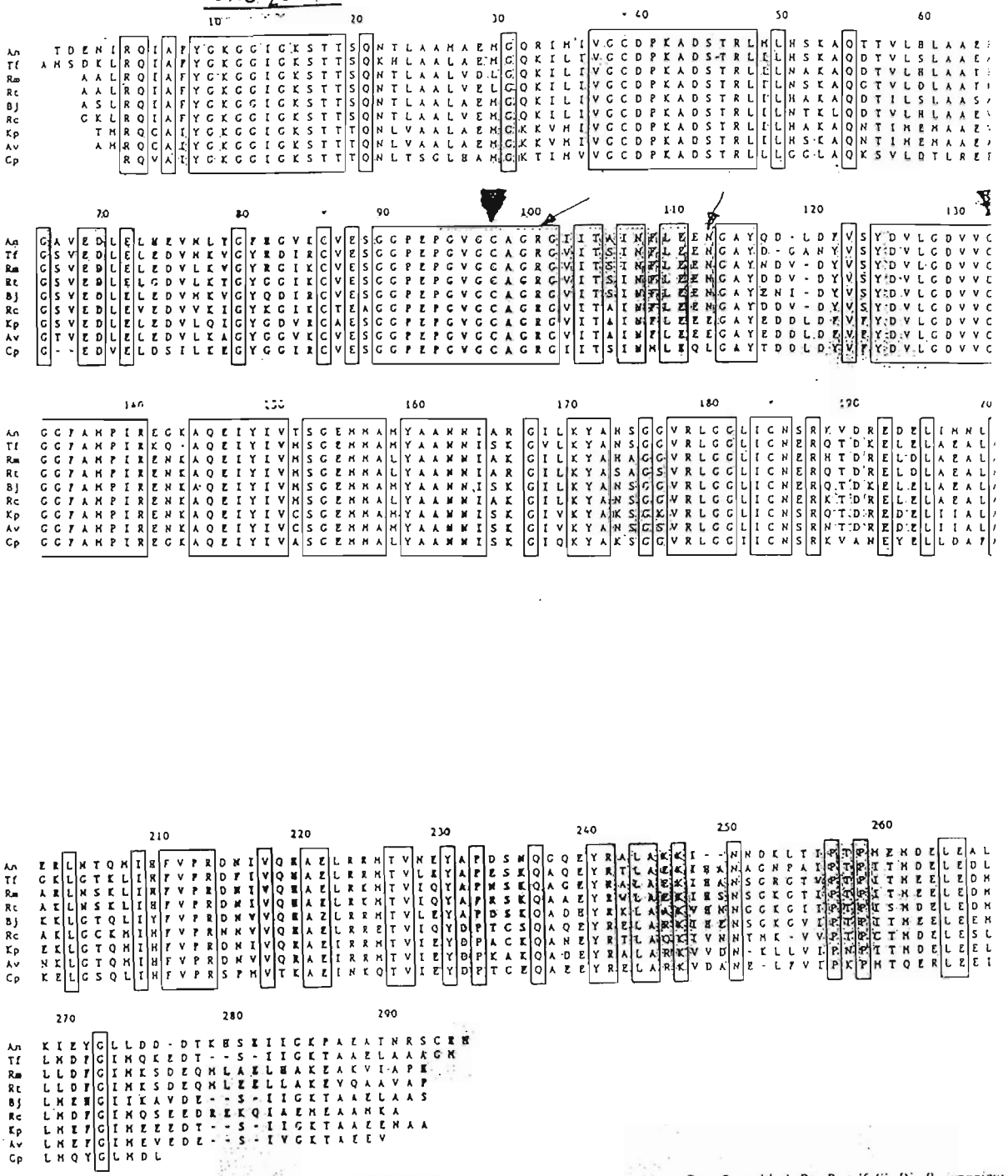


Figure 20-2. Alignment of Fe protein primary sequences. An, *Anabaena* 7120; Tf, *T. ferrooxidans;* Rm, *R. meliloti;* Rt, *R. trifolii*, Bj, *B. japonicu* Rc, *R. capsulatus;* Kp, *K. pneumoniae;* Av, *A. vinelandii;* Cp, *C. pasteurianum*. Numbering is according to the *A. vinelandii* sequence beginning fro the initiating methionine (which is not included in the figure). Residues that are conserved in all Fe proteins shown in the figure are boxed. The fi conserved Cys residues are indicated by arrowheads.

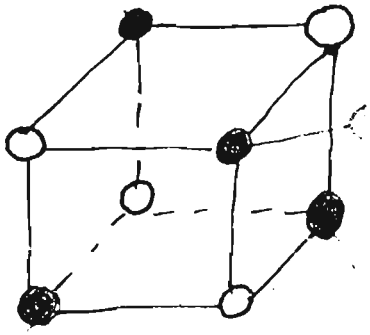
Fe

[4Fe-4S] - cluster of Ferredoxin

Fd reduced

$2\,Fe^{2+} + 2\,Fe^{3+}$

Fd oxidized



MW: 600

2x[4Fe-4

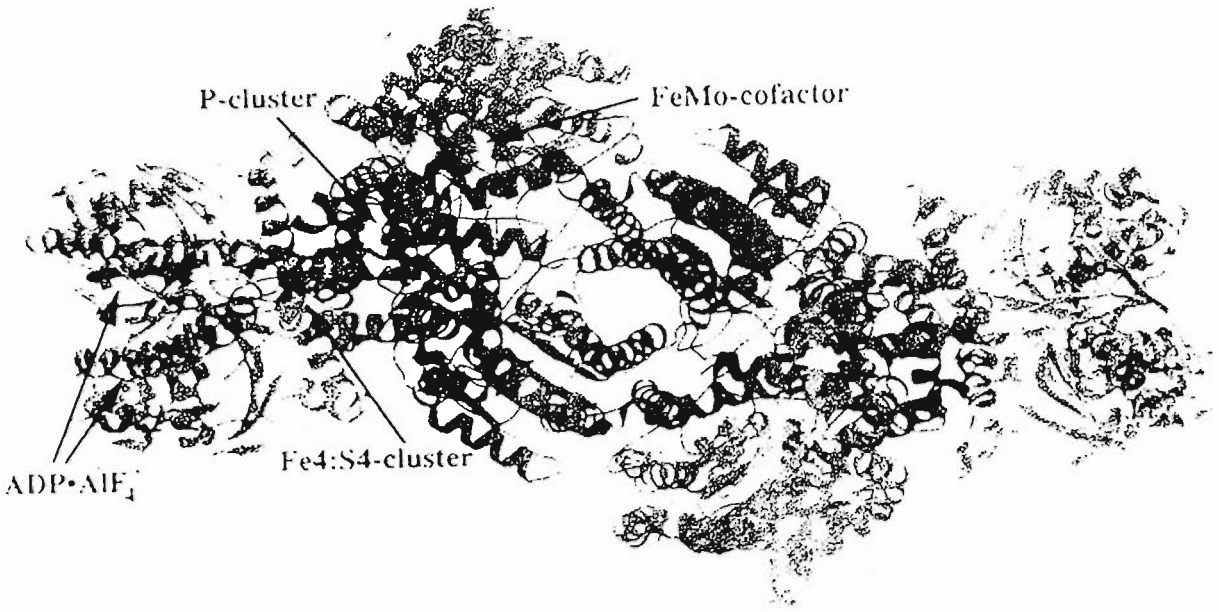
{ 8 Fe/m
{ 8 sulfid

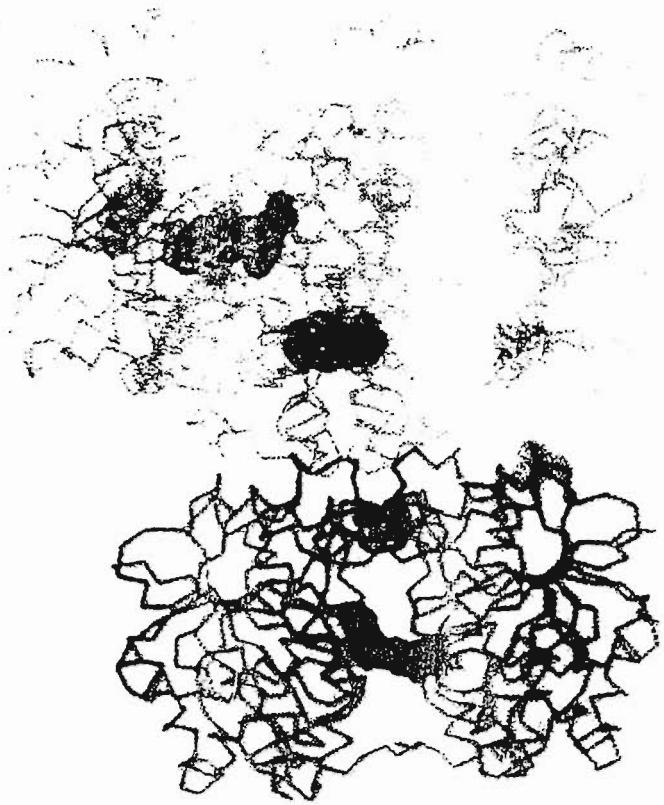
(after acidi
of Fd produ
"labile s

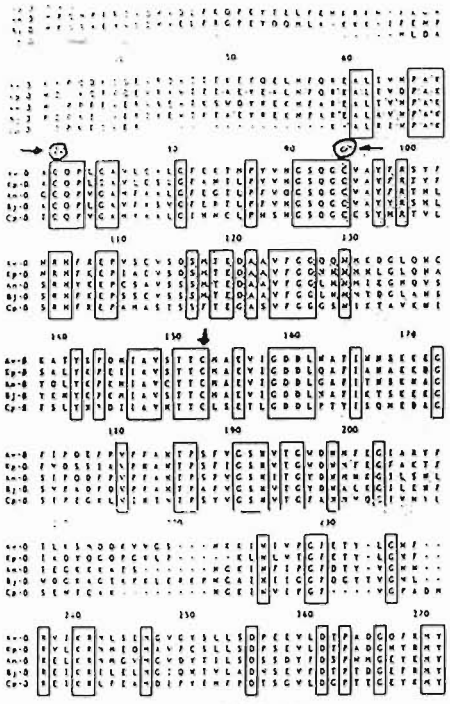
Cubanelike iron-sulfur cluster

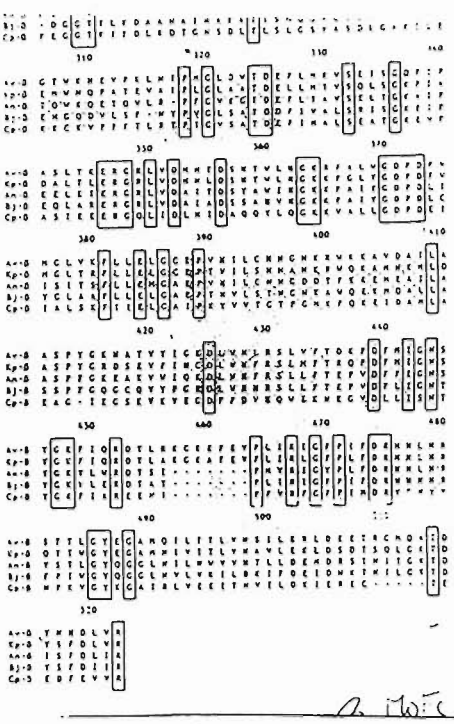
"Other Enzymes with Iron-Sulfur Cente

Hydrogenases
Formate dehydrogenase
Sulfite reductase
Pyruvate: ferredoxin oxidored
CO_2 reductase
Nitrogenase
CO dehydrogenase
Cl oxidase
Trimethylamine dehydrogen
Dihydroorotate dehydrogen
Glutamate synthetase
" "

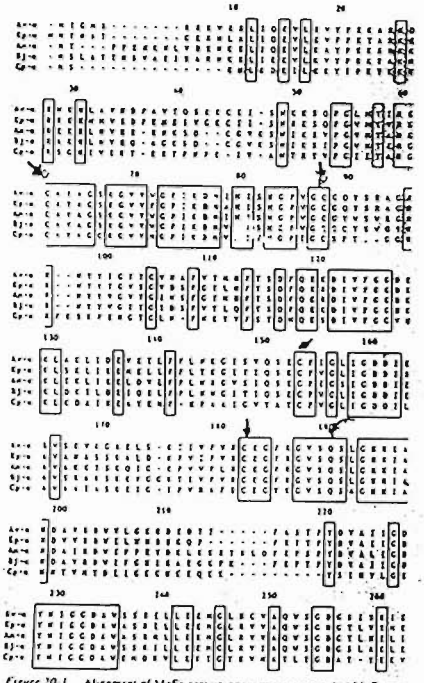

P-cluster
FeMo-cofactor
ADP•AlF4
Fe4:S4-cluster

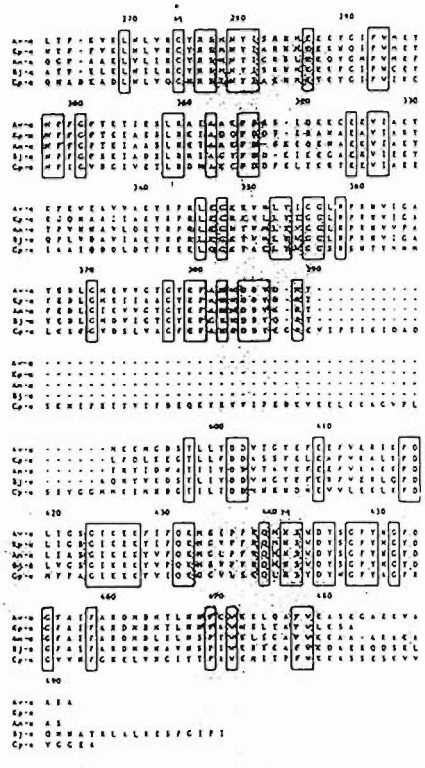






B





α

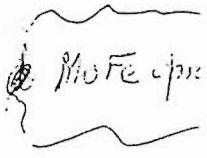


Figure 20.1 Alignment of MoFe protein primary sequences. (A) MoFe protein α-subunit alignments; (B) MoFe protein β-subunit alignments. Av, *A. vinelandii*; Kp, *K. pneumoniae*; An, *Anabaena* 7120; Bj, *B. japonicum*; Cp, *C. pasteurianum*. Numbering is according to the *A. vinelandii* sequence. Residues conserved in all sequences shown are boxed.

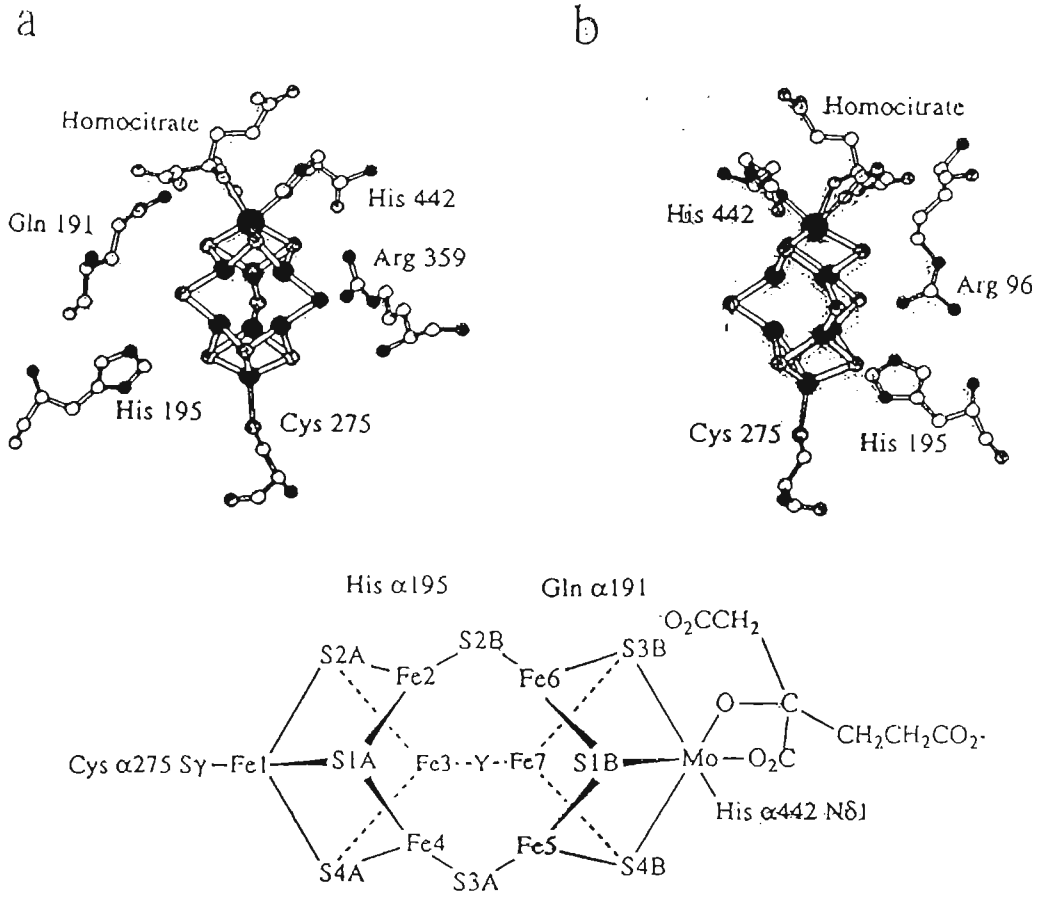


Figure 1. Structure of the FeMo-cofactor.

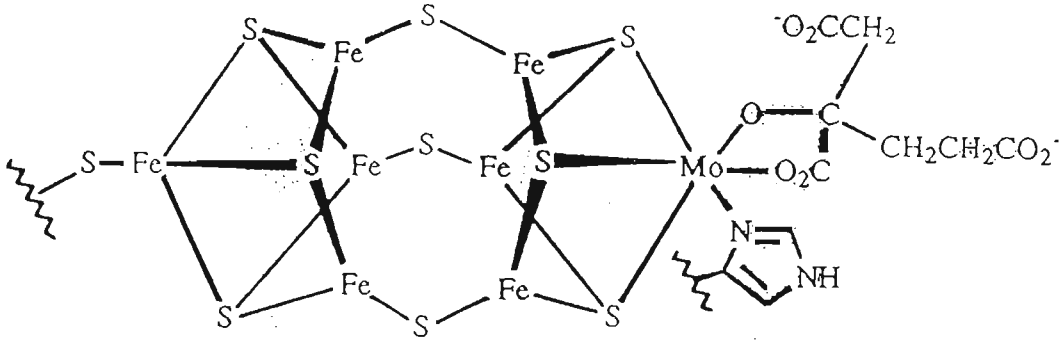


Figure 1: outline structure of FeMoco

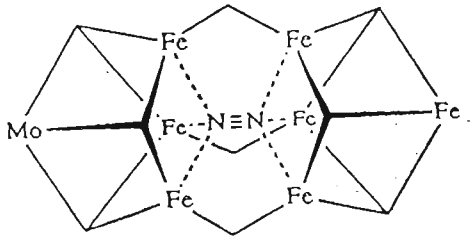


Figure 3. Hypothetical model for N_2 binding to FeMo-cofactor.

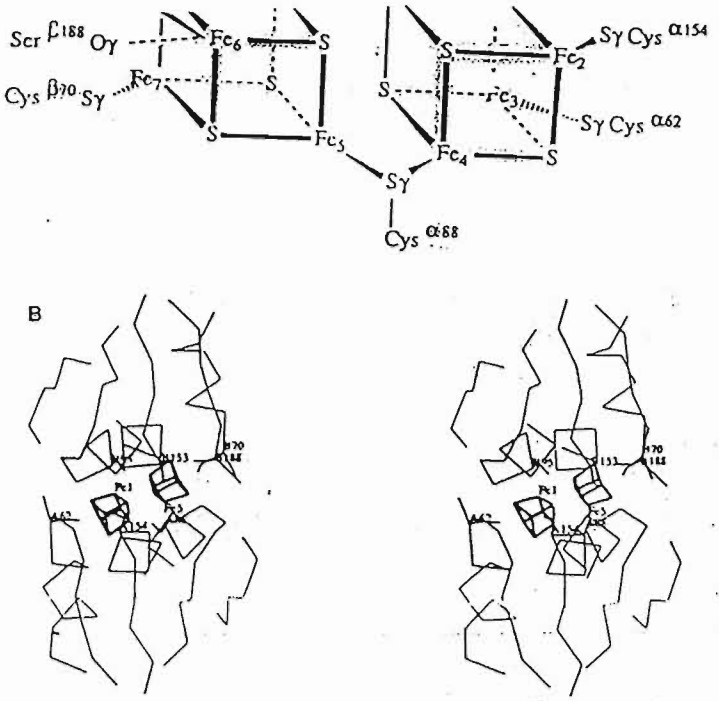


Fig. 4. (A) Schematic representation of the P-cluster model. (B) Stereoview of the P-cluster and surrounding protein model. The view is approximately along the direction of a twofold rotation axis approximately relating the α and β subunits.

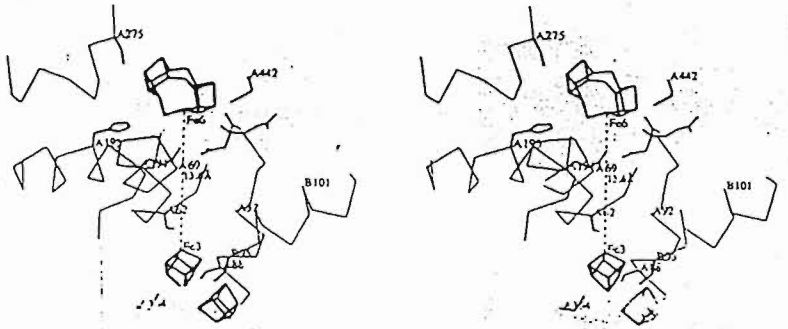
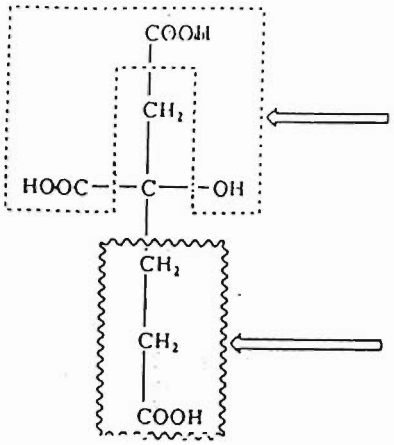


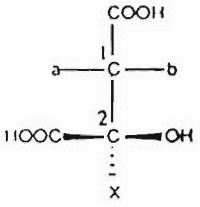
Fig. 5. Stereoview of the MoFe-protein structure in the vicinity of the FeMo-cofactor and P-cluster, including the Cα trace of the polypeptide chain and side chains of selected residues.



Essential for activity
(H^+, C_2H_2, N_2 reduction)

Essential for effective
N_2 reduction

Model for the Minimum Organic Moiety Required for the Synthesis of FeMo-co



x = $-CH_2CH_2COOH$ ('R'-homocitrate)

Fluorine substitution at 'a' (*erythro*-fluorohomocitrate) results in proton, cyanide, C_2H_2, and N_2 reduction.

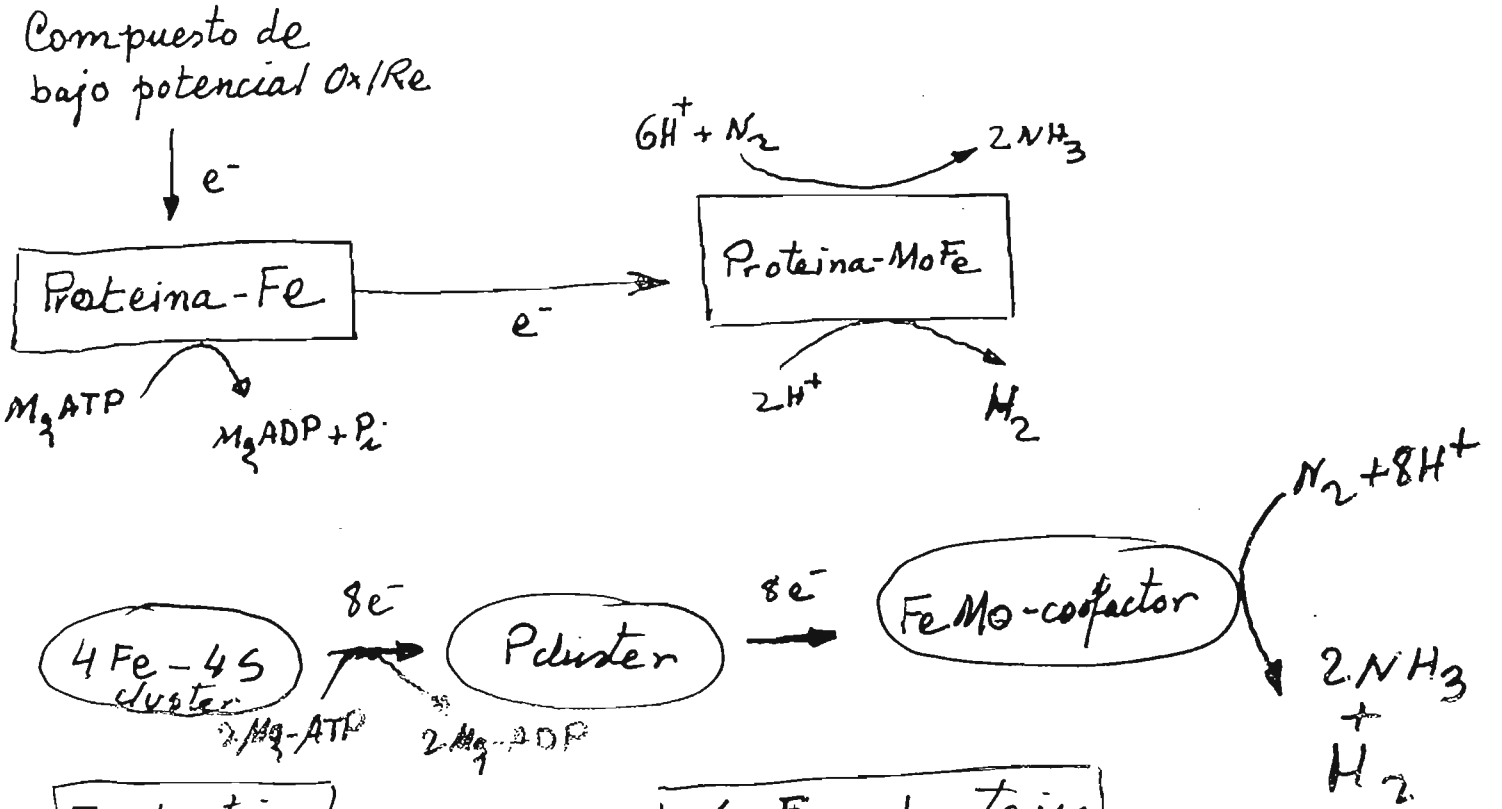
Fluorine substitution at 'b' (*threo*-fluorohomocitrate) results in proton, cyanide, and C_2H_2 reduction; but no N_2 reduction.

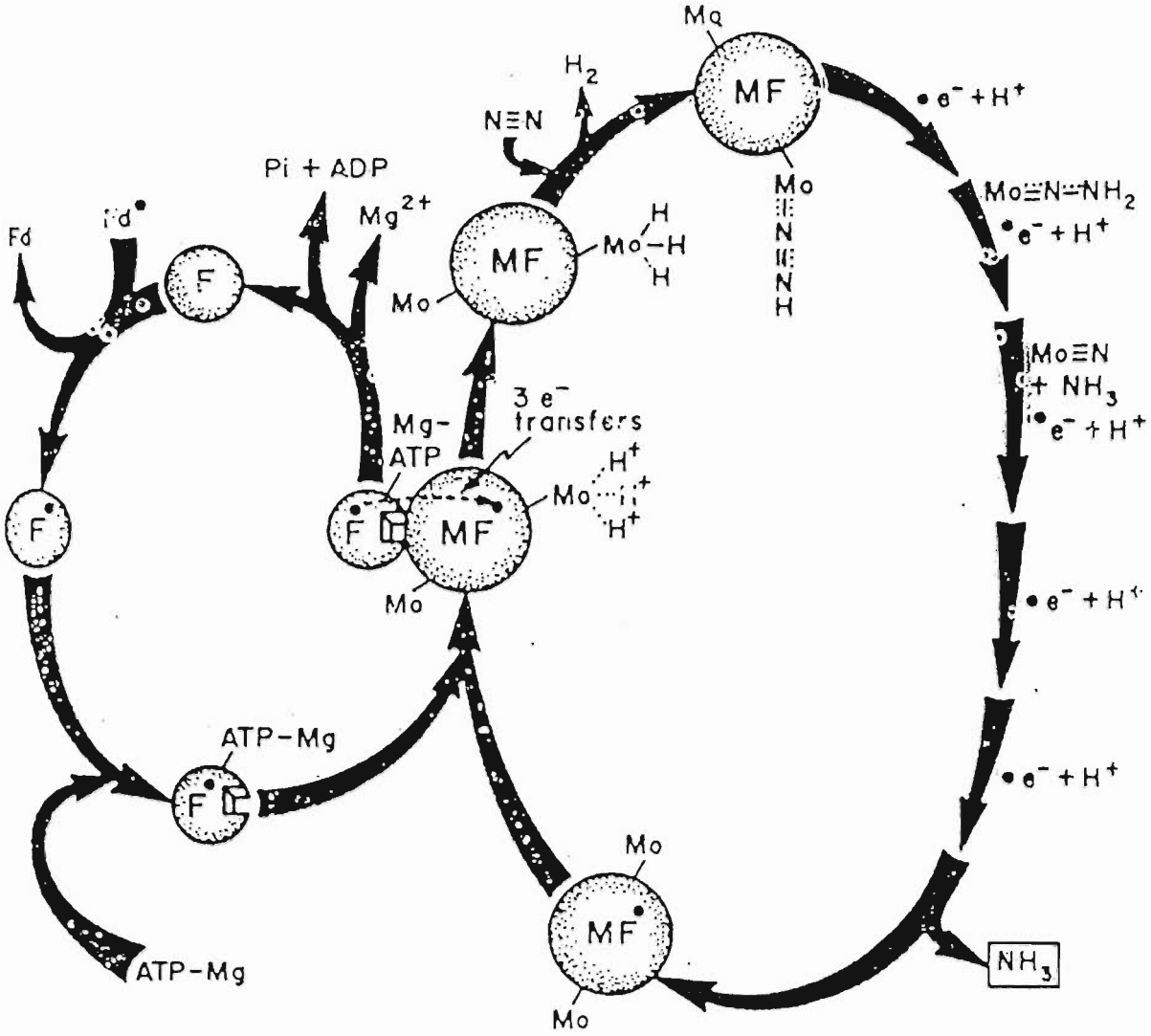
Interchanging the hydrogen atom at 'a' with the hydroxy group at the C-2 carbon results in proton and cyanide reduction without CO (COS) inhibition.

$$N_2 + 8H^+ + 8e^- + 16\,MgATP \longrightarrow 2NH_3 + H_2 + 16\,MgATP + 16\,P_i$$

Ecuación global de la Reducción de N_2

MECANISMO





$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + 16ADP + 16P_i + H_2.$$

N_2·asa 2
(VANADIO-NITROGENASA).

- Purificación de N_2·asa de *Azotobacter vinelandii* crecido con V.

  Hipótesis: Mo (contaminante) } → La N_2·asa 1 resultaba más
  V (estabilizante) } EFICIENTE en el empleo de trazos de Mo.

- Mutantes de *A. vinelandii* con lesiones en *nif HDK* → RESPONDEN a la adición de V.

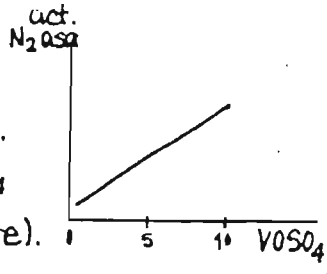
  Hipótesis: N_2·asa alternativa que se sintetiza en ausencia de Mo y presencia de V.

- En *Azotobacter chroococcum*:

  mutantes / deleción *nif HDK*
  \ mutación: tolerantes a W

  → Añadiendo 2.5 mM W crecen y fijan N_2.
  → Sin W: responden a la adición de $NiSO_4$

  (sólo con ↑[W] se añadía bastante V contaminante).

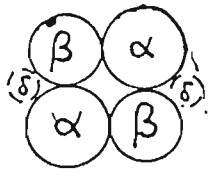


- El V copurifica con la N_2·asa activa.
- El V está en el componente I (dinitrogenasa)
- Componente II (nit. reductasa) = Componente II de N_2·asa 1.

Componente I
Pm = 210.000
2V : 23 Fe : 20 S

Componente II
Pm = 62.500
4 Fe : 4S

Subunidades: α (Pm 50.000)
β (Pm 55.000)
δ (Pm 14.000)?



Reduce etileno a acetato

N_2·asa 3

(NITROGENASA SIN Mo ni V)

En *Azotobacter vinelandii*:

cepa CA 11.6 → deleción en niF HDK (N_2 asa 1)
→ mutación: W-tolerantes

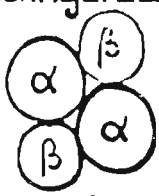
⇩

PURIFICACION de una N_2·asa sin Mo y sin V.

Estructura:

Dinitrogenasa (Componente I)

Configuración A

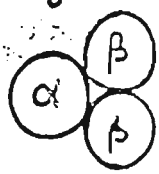


$\alpha_2\beta_2$

Pm = 216.000

24 Fe : 18 S

configuración B



$\alpha_1\beta_2$

Pm = 158.000

11 Fe : 9 S

Nitrogenasa reductasa (Componente II)

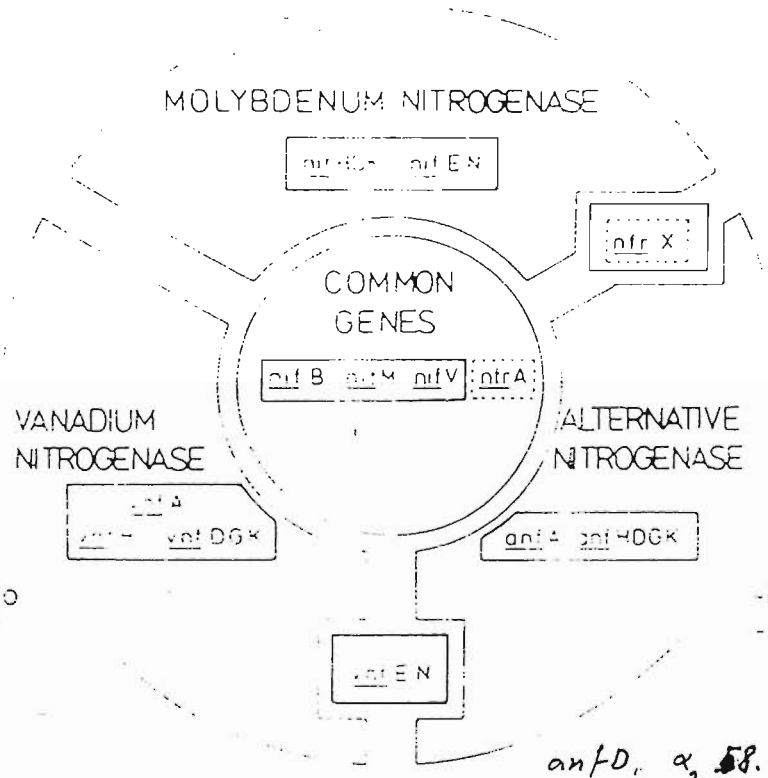
γ_2

Pm ≈ 65.000

4 Fe : 4 S

Características:

- Desvia muchos e^- para reducir H^+
- Bajos niveles de reducción de C_2H_2.
- Reducción $C_2H_2 \rightarrow C_2H_6$ (etano)
- 22.3 ATP / 2 e^- transferidos a N_2



nifD α_2 50.000
nifK β_2 61.000
nifH δ_2 30.000
+ Mo

vnfD α_2 50.000
vnfK β_2 55.000
vnfG δ_2 14.000

δ_2 62.000 = nvfH

anfD α_2 58.000
anfK β_2 51.000
anfH δ_2 30.000
anfG δ_2 15.000

Fig. 2. Genetic interrelationships between nitrogenase systems in Azo

[illegible] in A. vinelandii [illegible] the central circle. Genes required [illegible] and shown within the boxes placed between adjoining arcs. Expression [illegible] only when the metal availability corresponds to that sho[illegible] In A. chroococcum MCIMB 8003 only the Mo and V nitrogenases