GOBIERNO DE CHILE
FUNDACIÓN PARA LA
INNOVACIÓN AGRARIA

PROGRAMA DE CAPTURA Y DIFUSIÓN TECNOLÓGICA

INFORME TÉCNICO Y DE DIFUSIÓN

FIA-CD-V-2005-1- A - 157

2006

GOBIERNO DE CHILE
FUNDACIÓN PARA LA
INNOVACIÓN AGRARIA

07 MAR. 2006
1064

CONTENIDO DEL INFORME TÉCNICO

Fecha de entrega del Informe

24 Febrero 2006

Nombre del coordinador de la ejecución

M.Teresa Pino Q.

Firma del Coordinador de la Ejecución

1. ANTECEDENTES GENERALES DE LA PROPUESTA

Nombre de la propuesta

Consultoría sobre nuevas herramientas biotecnológicas usadas para inducir resistencia a frío y otros estreses abióticos en la agricultura.

Código

FIA-CD-V-2005-1- A - 157

Entidad responsable

Instituto de Investigaciones Agropecuarias La Platina

Coordinador(a)

María Teresa Pino

Tipo de Iniciativa(s)

☐ **Gira** ☐ **Beca** ☐ **Evento** ☒ **Consultores** ☐ **Documentos**

Fecha de realización (inicio y término)

22 Enero 2006 y 27 Enero 2006

2. RESUMEN DE LA PROPUESTA

Resumir en no más de una página la justificación, actividades globales, resultados e impactos alcanzados con la propuesta completa. Cuando exista más de una iniciativa, cada una de ellas debe ser resumida en forma específica. Estos resúmenes deben sintetizar los aspectos principales de la propuesta y cada una de sus iniciativas en forma general.

CONSULTORES

La Consultoría de Dr Tony H.H. Chen se enmarca en la necesidad de consolidar un área de estudio y mejoramiento en la resistencia a estreses abióticos como heladas, salinidad y sequía. Tan sólo las heladas pueden causar importantes pérdidas en producción y daños que van desde manchas superficiales en hojas, brotes, flores y frutos hasta la muerte total de la planta. En esta última temporada, las bajas temperaturas colocaron en jaque la producción de frutales en la zona central de Chile. En damasco, el daño superó el 70% mientras que en vides afectaron variedades tempranas de uva de mesa como "Superior" y "Flame"; Ante fenómenos climáticos cómo heladas granizadas y sequía a los agricultores no les quedan más que resignación ya que prácticamente nada se puede hacer. El uso de métodos de detección de heladas es engorroso, de alto costo y difíciles de aplicar en grandes superficies. Además, los expertos en el tema sostienen que la mayoría de los métodos de control de heladas son poco efectivos y deben necesariamente ser acompañados con variedades tolerantes al frío.

El desarrollo de variedades resistente a heladas, se ha descrito como el camino más acertado desde el punto de vista económico, sin embargo esto no es tan fácil ya que la resistencia a las bajas temperaturas es controlada por varios genes, lo cual dificulta la tarea de los mejoradores. Algunos logros interesantes se han obtenido en esta materia a través de mejoramiento tradicional y uso de marcadores moleculares; sin embargo, dada las características de como la resistencia al frío es trasmitida, el mejoramiento convencional ha sido un trabajo de largo aliento, que ha requerido mucho tiempo y con pocos logros ya que no se han logrado ganar más allá de 2ºC en resistencia. Hoy gracias al uso de la biotecnología y las nuevas herramientas disponibles, han aparecidos otras alternativas como el uso de genes Cod A y genes CBF (factores que regulan la transcripción de genes responsable de la aclimatación al frío y de la resistencia a heladas), los cuales prometen un gran impacto en la agricultura. De hecho, estudios en Arabidopsis, papas, tomate y otras especies han mostrado mejoras importantes en la resistencia a bajas temperaturas. El objetivo de la consultoría del Dr.Chen fue conocer las nuevas herramientas biotecnológicas que hoy se están usando para inducir resistencia a heladas y otros estreses abióticos en hortalizas y frutales con la meta final de evaluar su implementación en Chile. Para cumplir con este objetivo Dr Chen estuvo en INIA Platina conociendo la instalaciones y facilidades con que cuenta INIA La Platina con el fin de evaluar la aplicabilidad de la investigación que el lleva a cabo en los EEUU. Asimismo, se discutió con él los principales estreses abióticos que afectan la fruticultura y hortalizas en Chile, en particular en vides y carozos. Por otra parte el consultor expuso una charla técnica titulada ***Mejoramiento de la tolerancia a bajas temperaturas y otros estreses en tomate mediante ingeniería genética de la biosíntesis de glycebetaina,*** la cual se uso como base para abordar el tema. Asimismo, Dr Chen dio una recomendación técnica en cuanto a cómo aplicar esta tecnología en vides y se comprometió tanto con el Centro de Estudios Avanzados en Zonas Aridas (CEAZA) e INIA a dar su apoyo y orientación en cuanto a cómo usar y aplicar esta tecnología en vides.

3. ALCANCES Y LOGROS DE LA PROPUESTA GLOBAL

Problema a resolver, justificación y objetivos planteado inicialmente en la propuesta

Los problemas abióticos como heladas, sequía y sales son factores limitantes en la producción Hortofrutícola en diferentes áreas de Chile; salinidad y sequía en el norte, bajas temperaturas e incidencia de heladas en la zona centro sur. Aun cuando en Chile se están haciendo algunos esfuerzos estudiando tolerancia a heladas en tomate a través de QTL y búsqueda de genes, sin embargo falta mucho por hacer en particular en frutales. El mejoramiento genético para el desarrollo de variedades resistente a heladas, se ha descrito como el camino más acertado desde el punto de vista económico para combatir los efectos de las heladas, sin embargo el mejoramiento convencional es un trabajo de largo aliento, complejo y a través del cual no se ha logrado mejorar la resistencia a heladas significativamente. Esto se debe en parte la resistencia a las bajas temperaturas es controlada por varios genes, lo cual dificulta la tarea de los mejoradores. Debido al desarrollo de la biotecnología se han logrado grandes avances en el entendimiento de este fenómeno: tan sólo en la última década se han identificado varios genes asociados a tolerancia a frío cuya expresión ectopica ha resultado en el incremento en la resistencia al frío en varias especies. Entre estos se encuentran los genes Cod A y genes CBF (factores que regulan la transcripción de genes responsable de la aclimatación al frío y de la resistencia a heladas), los cuales prometen un gran impacto en la agricultura. De hecho, estudios en Arabidopsis, papas, tomate y otras especies han mostrado mejoras importantes en la resistencia a bajas temperaturas. Dado, que el efecto CodA genes y CBF genes constituye un tema de actualidad en el tema de estrés al frío; Nosotros buscamos evaluar la factibilidad de incorporar esta tecnología al programa de mejoramiento en frutales que actualmente desarrollamos.

Resumiendo, se planteó una consultoría para dar a conocer las nuevas herramientas biotecnológicas que actualmente están siendo usadas para inducir resistencia a frío y otros estreses abióticos en la agricultura, con la meta final de evaluar su utilización en un programa de mejoramiento genético en Chile.

Objetivos alcanzados tras la realización de la propuesta

A través de esta consultoría de Dr Chen se dio a conocer los resultados obtenidos en E.U.A. y Japón referente al uso genes CodA y los factores de transcripción FT de *Arabidopsis* (AtCBFs) para mejorar la tolerancia a heladas en el área hortofrutícola. Asimismo, se analizó la aplicabilidad de esta tecnología en vides y papas. Dr Chen sobre la base de la implementación en laboratorio que cuenta tanto el INIA como las universidades ratificó la aplicabilidad de estas técnicas en Chile y se recomendó iniciar un estudio de este tipo en solanaceas como papa y tomate y en frutales como vides y carozos en particular aprovechando la plataforma de transformación en vides que ha desarrollado INIA. Además, para aplicar esta tecnología Dr Chen orientó las líneas de investigación a seguir en cultivos como papas Chilenas y en frutales como vides y ofreció los genes con las cuales él trabaja para ser evaluados enestas especies.

Resultados e impactos esperados inicialmente en la propuesta

Los resultados e impactos esperados se cumplieron completamente. Primero, a través de las reuniones de trabajo y charla técnica investigadores de INIA y otras Instituciones como el CEAZA, Universidad de Talca, Universidad de Chile fueron capacitados, en total asistieron 31 personas. Segundo, Dr Chen entregó una recomendación técnica y orientó la aplicación de la biotecnología en especies como solanaceas y vides. Tercero, Dr Chen se comprometió a entregar tanto los genes como dar las directrices para aplicar esta tecnología en vides. Asimismo, INIA buscará fondos de financiamiento para iniciar un convenio con Dr.Chen para consolidar esta investigación y aplicación en papas y frutales.

Resultados obtenidos

Descripción detallada de los conocimientos y/o tecnologías adquiridos y/o entregados. Explicar el grado de cumplimiento de los objetivos propuestos, de acuerdo a los resultados obtenidos. Para consultorías es necesario anexar el informe final del consultor.

Primero, a través de las reuniones de trabajo y charla técnica investigadores de INIA y otras Instituciones como el CEAZA, Universidad de Talca, Universidad de Chile entre otros fueron capacitados, en total asistieron personas.

Segundo, Dr Chen entregó una recomendación técnica y orientó la aplicación de la biotecnología en especies de como solanáceas y vides.

Tercero, Dr Chen se comprometió a entregar tanto los genes como dar las directrices para aplicar esta tecnología en vides. Asimismo, INIA buscará fondos de financiamiento para iniciar un convenio con Dr.Chen para consolidar esta investigación y aplicación en papas y frutales.

Resultados adicionales

Describir los resultados obtenidos que no estaban contemplados inicialmente como por ejemplo: formación de una organización, incorporación de alguna tecnología, desarrollo de un proyecto, firma de un convenio, entre otros posibles.

Además, parte de los resultados que Dr Chen a obtenido con con relación a FT (CBF) de Arabidopsis en Papas fueron expuestos en El seminario de estrés abiótico realizado en la Universidad de Talca- a través de una ponencia presentada por la coordinadora de esta consultoría M.Teresa –Pino –se adjunta publicación.

Aplicabilidad

Explicar la situación actual del sector y/o temática en Chile (región), compararla con las tendencias y perspectivas presentadas en las actividades de la propuesta y explicar la posible incorporación de los conocimientos y/o tecnologías, 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).

El mejoramiento genético para tolerancia heladas y otros estreses abióticos en especies hortofrutícolas están recién comenzando en Chile; En la actualidad la PUCV lleva algunos años evaluando la herencia a resistencia a frío en tomate a través mejoramiento convencional apoyado con Marcadores moleculares: en particular están buscando QTL asociados. Por otra parte, la universidad de Talca desarrolla un proyecto de investigación en Lycopersicum chilensis que busca genes asociados a resistencia a estreses, PUC está desarrollando un proyecto en cítricos para buscar resistencia a sales, INIA y CEAZA están comenzando un proyecto para buscar resistencia a sales en vides en la IV región. Además, otras instituciones están buscando resistencia a heladas en especie forestales como el Eucalipto. La situación actual de este tema en Chile Dr Chen la resumió como lo que sucedía 20 años atrás en los EEUU; con la ventaja que a nivel mundial existen muchos resultados y técnicas que hace 20 anos atrás no existían y podrían ser aplicadas en Chile. Dr Chen visualiza un gran potencial para aplicar esta tecnología en el país dada la diversidad agroclimática que existe en Chile; lo cual hace que diferentes estreses abióticos (sales, sequía y temperaturas extremas) afecten diferentes áreas donde se desarrolla agricultura y donde estas tecnologías tendrían aplicación.

Detección de nuevas oportunidades y aspectos que quedan por abordar

Señalar aquellas iniciativas que surgen como vías para realizar un aporte futuro para el rubro y/o temática en el marco de los objetivos iniciales de la propuesta, como por ejemplo la posibilidad de realizar nuevas actividades.

Indicar además, en función de los resultados obtenidos, los aspectos y vacíos tecnológicos que aún quedan por abordar para ampliar el desarrollo del rubro y/o temática.

Producto de esta consultoría surge la importancia de generar y continuar con proyectos de investigación y desarrollo en el tema; proyectos que deberían estar bien conducidos con objetivos y metas claras a seguir ya que la resistencia a estreses y en particular a bajas temperaturas, es uno de los problemas fisiológicos más complejos que existen en plantas.

Las debilidades de la investigación en esta área y su aplicación se basan fundamentalmente en que este tema es muy nuevo en Chile y se desconocen muchos aspectos. Otra debilidad es la baja cantidad de proyectos y trabajos que se desarrollan en el tema; se requieren buscar más recursos o fondos para consolidar esta línea de trabajo en el país.

Sin embargo, el desarrollo de un seminario sobre estrés abiótico en la Universidad de Talca, la presente consultaría desarrollada por el INIA, los proyectos que están operando y otras iniciativas marca un inicio en el tema que no se debería abandonar.

4. ASPECTOS RELACIONADOS CON LA EJECUCIÓN DE LA PROPUESTA

Programa Actividades Realizadas

Nº	Fecha	Actividad	Iniciativa
1	22 Enero 2006	Viaje desde Oregon (EEUU) a Chile	
2	23 Enero 2006	Espera en Atlanta EEUU debido a problemas climáticos	
3	24 Enero 2006	Llegada a Chile mañana. Reconocimiento de las facilidades de laboratorio e implementación para estudios en las técnicas que el desarrolla. Discusión de los principales problemas de estrés abióticos que enfrenta Chile.	Dada a la plataforma de transformación genética que ha desarrollado INIA, aplicar esta tecnología en dichas especies. En una primera etapa concentrar el trabajo en vides, y solanaceas
4	25 Enero 2006	Charla técnica Almuerzo de Dr. Chen, U.de talca y U. De la Serena Taller de trabajo entre Dr Chen, CEAZA e INIA para analizar su aplicabilidad en vides	Intención para cooperar y orientar el estudio de tolerancia a sales en vides IV región.
5	26 Enero 2006	Trabajo de Dr Chen con la coordinadora del proyecto para delinear líneas de investigación en solanáceas y vides.	Estrategia como desarrollar la investigación
6	27 Enero 2006	Viaje a la V región Viaje Santiago a EEUU	Realizar una próxima visita a la IV región, para visitar el proyecto conocer las condiciones agroclimáticas de la IV Región y a la zona sur de Chile-zona papera

Detallar las actividades realizadas en cada una de las Iniciativas, señalar y discutir las diferencias con la propuesta original, y rescatar lo más importante de cada una de ellas. Por ejemplo, en el caso de Giras discutir las actividades de cada visita; Becas, analizar las exposiciones más interesantes; Consultores, detallar el itinerario y comentarios del consultor; Eventos, resumir y analizar cada una de las exposiciones; y Documentos, analizar brevemente los contenidos de cada sección.

GIRAS

BECAS

CONSULTORES

En esta propuesta hubo dos cambios. Primero aun cuando Dr Chen partió el 22 de Enero desde Pórtland (Oregon) perdió la combinación con el vuelo Atlanta a Santiago de Chile debido a problemas climátcos. Debió pasar una noche en Atlanta y llegó a Chile el 24 de enero en la mañana- esto causó que los gastos se redujeran debido a su retraso de un día. Segundo, debido a que la reunión del FIA fue suspendida el día 27 de Enero se llevó a Dr Chen a una reconocimento de la zona de vides de la V región.

EVENTOS

DOCUMENTOS

Contactos Establecidos

Presentar los antecedentes de los contactos establecidos durante el desarrollo de la propuesta (profesionales, investigadores, empresas, etc.), de acuerdo al siguiente cuadro:

Institución Empresa Organización	Persona de Contacto	Cargo	Fono/Fax	Dirección	E-mail
CEAZA	Andrés Zurita	investigador	56-51-204378/ 56-51-334741		azurita@userena.cl
U.Talca	Simón Ruiz	investigador	56-71-200268		sruiz@utalca.cl

Material elaborado y/o recopilado

Entregar un listado del material elaborado, recibido y/o entregado en el marco de la propuesta. Se debe entregar adjunto al informe un set de todo el material escrito y audiovisual, ordenado de acuerdo al cuadro que se presenta a continuación.

También se deben adjuntar fotografías correspondientes a la actividad desarrollada. El material se debe adjuntar en forma impresa y en un medio electrónico (disquet o disco compacto).

Elaborado

Tipo de material	Nombre o identificación	Preparado por	Cantidad
Presentación de Dr Chen	Charla- 25 de Enero 2006	Dr. Chen	1
Publicación sobre CodA y Tomate	Artículo#1 en tomate	Park J, Chen T.H.H et al	1
Publicación sobre AtCBF1 en Paps	Articulo# 2 en papa	Pino M.T., Chen T.H.H et al.	1

Recopilado

Tipo de Material	Nº Correlativo (si es necesario)	Caracterización (título)
Artículo	1	Park, E, Z. Jeknić, A. Sakamoto, J. DeNoma, N. Murata, and T. H.H. Chen. 2003. Genetic engineering of cold-tolerant tomato via glycinebetaine biosynthesis. Cryobiology and Cryotechnology. 49:77-85.
Articulo	2	Pino, M.T., J. S. Skinner, Z. Jeknić, E.J. Park, P.M. Hayes, and T.H.H. Chen. 2005 Ectopic Overexpression of AtCBF1 in Potato Enhances Freezing Tolerance. In: T.H.H. Chen, M. Uemura, and S. Fujikawa (Eds.), Cold Hardiness in Plants: Molecular Genetics, Cell Biology, and Physiology. CABI International, Oxon, UK. Page 103- 123.
Diapositiva- impresión de la presentación	3	Mejoramiento de la tolerancia a bajas temperaturas y otros estreses en tomate mediante ingeniería genética de la biosíntesis de glycebetaina

Programa de difusión de la actividad

En esta sección se deben describir las actividades de difusión de la actividad, adjuntando el material preparado y/o distribuido para tal efecto.

En la realización de estas actividades, se deberán seguir los lineamientos que establece el "Instructivo de Difusión y Publicaciones" de FIA, que le será entregado junto con el instructivo y formato para la elaboración del informe técnico.

Ver anexo: se adjunta presentation de Dr. Chen impresa

5. PARTICIPANTES DE LA PROPUESTA

GIRAS, BECAS: Ficha de Participantes

CONSULTORES: Ficha de(l) Consultor(es)

EVENTOS: Ficha de Expositores y Organizadores

DOCUMENTOS: Ficha de Autores y Editores

Nombre	**Tony Hwei-Hwang**
Apellido Paterno	**Chen**
Apellido Materno	
RUT Personal	
Dirección, Comuna y Región	**4017 ALS Bldg. Corvallis Oregon 97331-7304.**
Fono y Fax	**01-541-737-5444**
E-mail	chent@hort.oregonstate.edu
Nombre de la organización, empresa o institución donde trabaja / Nombre del predio o de la sociedad en caso de ser productor	**Biotecnología y estrés fisiológico** **Departamento de Horticultura** **Oregon State University. USA**
RUT de la organización, empresa o institución donde trabaja / RUT de la sociedad agrícola o predio en caso de ser agricultor	
Cargo o actividad que desarrolla	Investigador y profesor
Rubro, área o sector a la cual se vincula o en la que trabaja	Frutales y hortalizas

Participantes en actividades de difusión

Es necesario registrar los antecedentes de todos los asistentes que participaron en las actividades de difusión. El listado de asistentes a cualquier actividad deberá al menos contener la siguiente información:

Nombre	Andrés
Apellido Paterno	Zurita
Apellido Materno	Silva
RUT Personal	
Dirección, Comuna y Región	La Serena. Chile
Fono y Fax	56-51-204378
E-mail	azurita@userena.cl
Nombre de la organización, empresa o institución donde trabaja / Nombre del predio o de la sociedad en caso de ser productor	Universidad de la serena
RUT de la organización, empresa o institución donde trabaja / RUT de la sociedad agrícola o predio en caso de ser agricultor	
Cargo o actividad que desarrolla	Investigador del CEAZA
Rubro, área o sector a la cual se vincula o en la que trabaja	Genética Vegetal

Participantes en actividades de difusión

Es necesario registrar los antecedentes de todos los asistentes que participaron en las actividades de difusión. El listado de asistentes a cualquier actividad deberá al menos contener la siguiente información:

Nombre	Simón
Apellido Paterno	Ruiz
Apellido Materno	
RUT Personal	
Dirección, Comuna y Región	Talca. Chile
Fono y Fax	56-71-200268
E-mail	sruiz@utalca.cl
Nombre de la organización, empresa o institución donde trabaja / Nombre del predio o de la sociedad en caso de ser productor	Universidad de Talca
RUT de la organización, empresa o institución donde trabaja / RUT de la sociedad agrícola o predio en caso de ser agricultor	
Cargo o actividad que desarrolla	Investigador
Rubro, área o sector a la cual se vincula o en la que trabaja	Genética Vegetal

6. EVALUACIÓN DE LA PROPUESTA

Evaluación de la actividad para cada INICIATIVA

En esta sección se debe evaluar la actividad en cuanto a los siguientes ítems:

a) Efectividad de la convocatoria (cuando corresponda)

Asistieron 31 personas. Después de la Charla se sostuvo un almuerzo con Dr Chen. INIA, U de Talca y CEAZA y luego una posterior reunión de trabajo entre CEAZA e INIA para abordar el tema de tolerancia a salinidad en vides.

b) Grado de participación de los asistentes (interés, nivel de consultas, dudas, etc)

La charla de Dr. Chen se prolongó por prácticamente 2 horas en la cual hubo gran interés interés y un alto nivel de consultas. Las cuales prosiguieron después de la charla en el café.

c) Nivel de conocimientos adquiridos por los participantes, en función de lo esperado (se debe indicar si la actividad contaba con algún mecanismo para medir este punto y entregar una copia de los instrumentos de evaluación aplicados)

NO

d) Problemas presentados y sugerencias para mejorarlos en el futuro (incumplimiento de horarios, deserción de participantes, incumplimiento del programa, otros)

Elaborar una ficha más completa por participante y recalcar que todos completen la información ya que algunas personas no firmaron la hoja de asistencia

Aspectos relacionados con la postulación al programa de Captura y Difusión

a) Información recibida por parte de FIA para realizar la postulación

__X__ amplia y detallada ____ aceptable ____ deficiente

Justificar: Tanto la información entregada en la bases s como el apoyo entregado por el especialista del FIA son claras y detallada

b) Sistema de postulación al Programa de Formación o Promoción (según corresponda)

____ adecuado ____ aceptable ____ deficiente

Justificar:

c) Apoyo de FIA en la realización de los trámites de viaje internacionales (pasajes, seguros, otros) (sólo cuando corresponda)

__X__ bueno ____ regular ____ malo

Justificar: El FIA compró los pasajes y seguros eficientemente y los hizo llegar oportunamente al consultor.

d) Recomendaciones (señalar aquellas recomendaciones que puedan aportar a mejorar los aspectos administrativos antes indicados).- Ya que en algunos puntos se solicita la misma información, sería recomendable revisarla para evitar duplicidad de información

7. Conclusiones Finales de la Propuesta Completa

En el caso de Giras Tecnológicas, en lo posible presentar conclusiones individuales por participante.

El mejoramiento en frutales y hortalizas en resistencia a heladas y otros estreses abióticos aun cuando es muy incipiente en Chile tiene un gran potencial; primero, existen problemas abióticos en diversas áreas del país que disminuyen la producción y en temporadas como la 2006 las heladas causan importantes pérdidas; segundo, dado los avances que existen a nivel mundial en este tema, la investigación en Chile no debería comenzar de cero. El mejoramiento usando las herramientas expuestas por Dr Chen deberían concentrarse en frutales y en especies solanáceas. En frutales como vides se debería basar fundamentalmente en los siguientes puntos en orden de importancia y aplicabilidad: primero, buscar patrones y plantas resistentes a estreses abióticos en germoplasma chileno; segundo, transformar patrones con genes que ya han sido identificados a nivel mundial aprovechando la plataforma de transformación que ha desarrollado INIA: Tercero, buscar genes de resistencia en material nacional y cuarto, buscar financiamiento para desarrollar este tema.

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INIA LA PLATINA

INVITACION CHARLA

La Fundación para la Innovación Agraria en conjunto con el Instituto de Investigaciones Agropecuarias INIA, tienen el agrado de invitar a usted a una charla sobre estrés abiótico para el día 25 de Enero de 2006 a las 10:30 hrs., Sala 1, en el CRI La Platina, ubicado en Santa Rosa 11610, par. 33 La Pintana, Santiago.

*La charla titulada "**Mejoramiento de la tolerancia a bajas temperaturas y otros estreses en tomate mediante ingeniería genética de la biosíntesis de glycebetaina"** será expuesta por Dr. Tony H.H.Chen, especialista en estrés abiótico de la Universidad del Estado de Oregon. EEUU.*

Agradecemos su asistencia y la difusión de esta invitación a los posibles interesados. Favor confirmar su asistencia al teléfono 7575148 (Unidad de Mejoramiento Genético CRI La Platina) email: mtpino@inia.cl

Santiago, 18 de Enero de 2006.

GOBIERNO DE CHILE
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GOBIERNO DE CHILE
INIA LA PLATINA

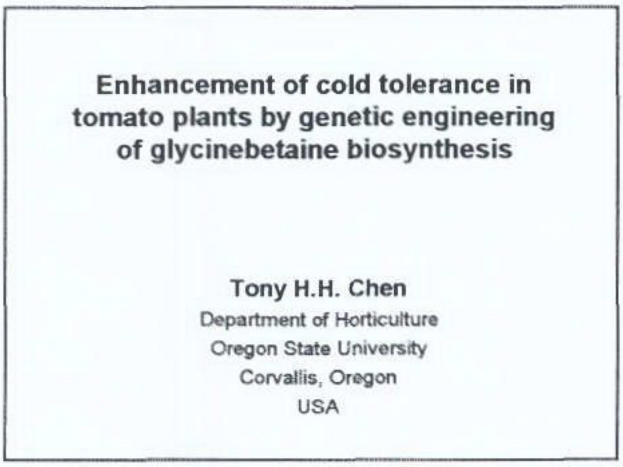
CHARLA TECNICA

"Mejoramiento de la tolerancia a bajas temperaturas y otros estreses en tomate mediante ingeniería genética de la biosíntesis de glycebetaina

Contratación de Consultores
FIA: BID-CD-V-2005-1-A-157

Dr. Tony H.H.Chen
Universidad de Oregon

25 de Enero de 2006.



Enhancement of cold tolerance in tomato plants by genetic engineering of glycinebetaine biosynthesis

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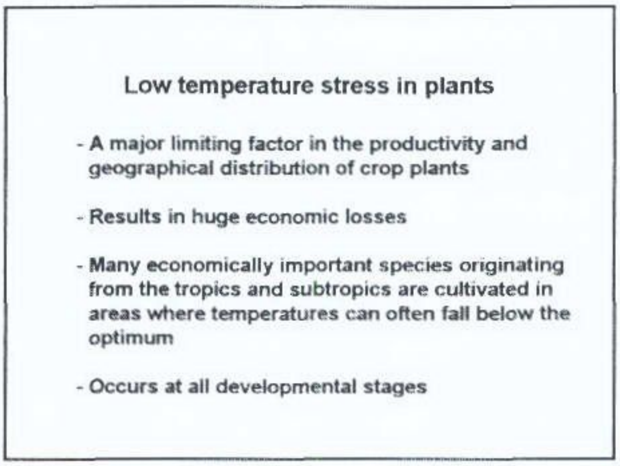
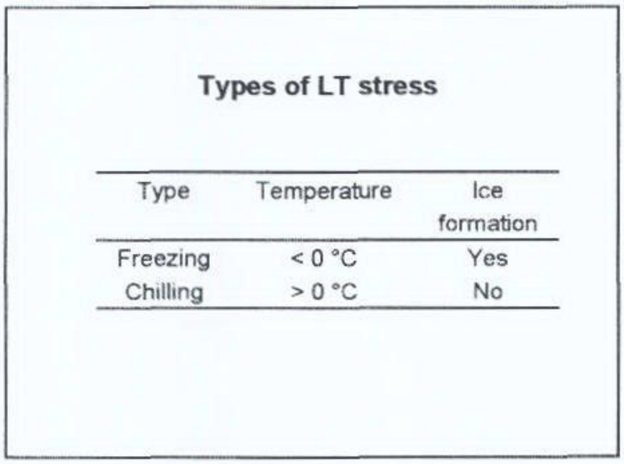


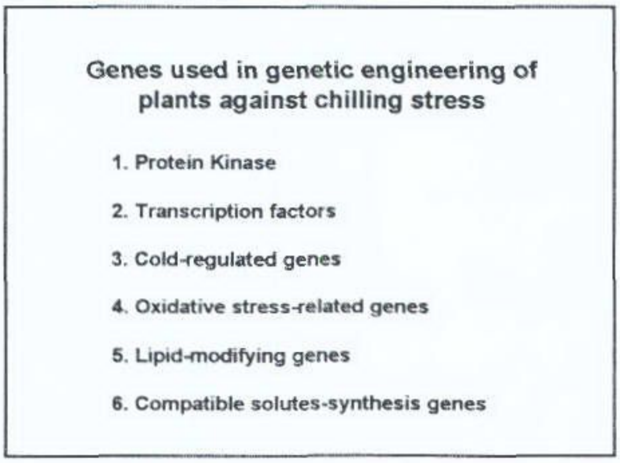
Low temperature stress in plants

- A major limiting factor in the productivity and geographical distribution of crop plants
- Results in huge economic losses
- Many economically important species originating from the tropics and subtropics are cultivated in areas where temperatures can often fall below the optimum
- Occurs at all developmental stages



Types of LT stress

Type	Temperature	Ice formation
Freezing	< 0 °C	Yes
Chilling	> 0 °C	No



Genes used in genetic engineering of plants against chilling stress

1. Protein Kinase
2. Transcription factors
3. Cold-regulated genes
4. Oxidative stress-related genes
5. Lipid-modifying genes
6. Compatible solutes-synthesis genes

Compatible solutes

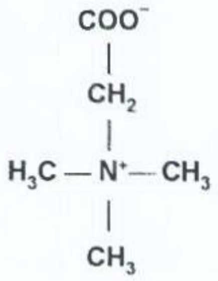
Definition

- Low molecular weight, highly soluble compounds that are non-toxic at high concentrations

Types:

1. Polyols and sugars : trehalose, raffinose, mannitol
2. Amino acids : proline
3. Betaines and related compounds: Glycinebetaine

Glycinebetaine (GB)



$$\begin{array}{c} COO^- \\ | \\ CH_2 \\ | \\ H_3C - N^+ - CH_3 \\ | \\ CH_3 \end{array}$$

Protective roles of GB on stress tolerance

1. Regulates osmotic balance
2. Stabilizes the complex proteins and membranes: PSII, Rubisco, lipid peroxidation, mitochondrial electron transport system
3. Protects transcriptional and translational machinery – a potential chaperonin

GB-accumulator:

e.g. barley, wheat, spinach

Non-accumulator:

e.g. *Arabidopsis*, potato, rice, tomato

GB accumulation in plants

- GB accumulated in natural GB-accumulators in response to abiotic stresses
- Levels of GB is correlated with the degrees of stress tolerance in natural GB-accumulators
- Exogenous application of GB improves stress tolerance of GB non-accumulators

Tomato
(*Lycopersicon esculentum* L.)

- An important horticultural crop
- A GB non-accumulator
- A chilling-sensitive species originating from the tropics

Effects of chilling temperature on developmental stages of tomato plants

- Germination
  - fail below 9 °C and reduced at 10-15°C
- Plant growth
  - Extended exposure below 6°C can kill plants
- Reproduction stage
  - Fruit set is inhibited below 13°C, primarily due to reduced pollen viability and the failure of anthers to release pollen grains
- Post-harvest stage
  - Chilling-injured fruits become blotchy and exhibit increased disease susceptibility

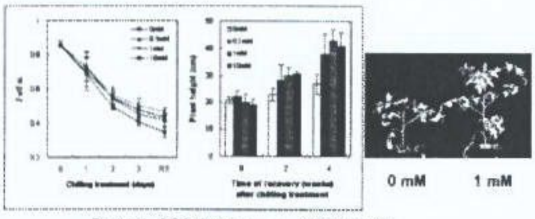
Tomato and GB

- Does not accumulate GB
- Foliar applied GB is readily taken up by tomato leaves (Mäkelä et al. 1996)
- Exogenous GB application increased salt and drought tolerance (Mäkelä et at. 1999)
- Exogenous GB application increased yield in greenhouse and field (Jokinen et al. 1999; Mäkelä et al. 1998)

⟶ *Does GB also enhance tomato chilling tolerance ?*

1. **Exogenous Application of Glycinebetaine Increases Chilling Tolerance in Tomato Plants**
2. **Genetic Engineering of Glycinebetaine Synthesis Protects Seeds, Plants and Flowers from Chilling Damage**
3. **Accumulation of Glycinebetaine in Various Subcelluar Compartments Confers Similar Levels of Enhanced Tolerance to Abiotic Stresses**

Exogenous application of GB increases chilling tolerance in tomato plants (cv. Moneymaker)



Treatment: 3°C for 3 days ⟶ 25°C for 4 weeks

→ - **Enhanced chilling tolerance in tomato plants was achieved with the application of GB (0.1 – 10.0 mM)**
- 1 mM GB is an optimal concentration

The majority of GB is localized in the cytosol

GB treatment (mM)	GB			GB in chloroplasts (%)
	Leaves		Isolated chloroplast	
	μmol g^{-1} FW	nmol mg^{-1} chlorophyll	nmol mg^{-1} chlorophyll	
0.1	0.09±0.01	7.78±0.98	0.98±0.11	21.55±3.20
1.0	1.09±0.13	89.21±1.69	1.75±0.94	3.28±0.41
10.0	10.88±1.38	900.49±102.48	3.20±3.98	0.58±0.07

→ Cytosolic GB can be translocated into subcellular compartments, likely resulting in protecting organelles from chilling stress

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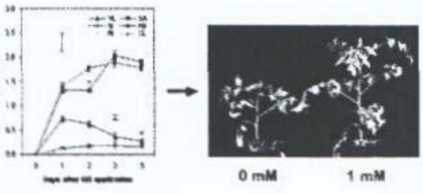
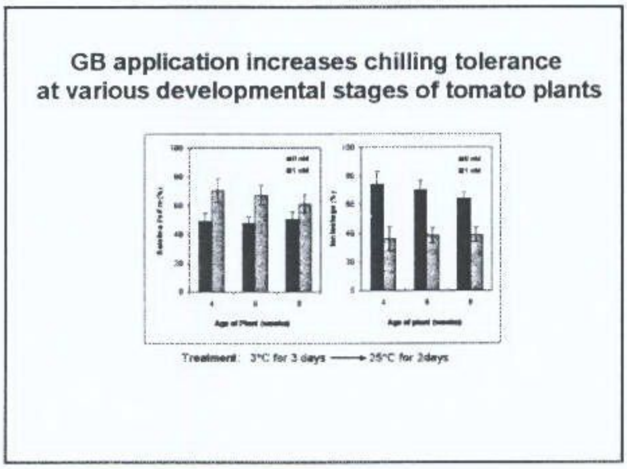
GB is translocated into meristemic tissues



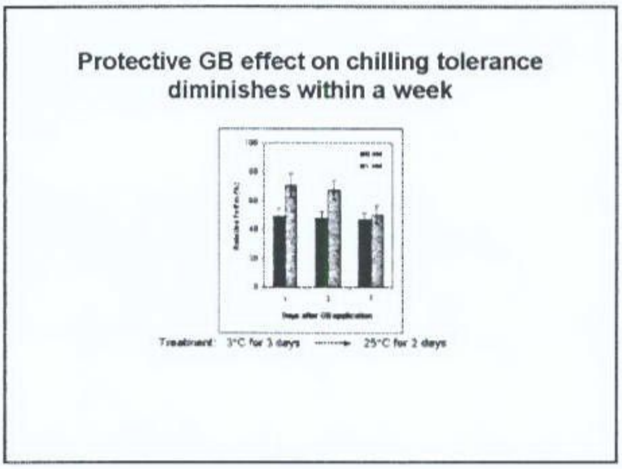
Treatment: 20 mM GB

→ High level of GB in meristemic tissues might further increase chilling tolerance



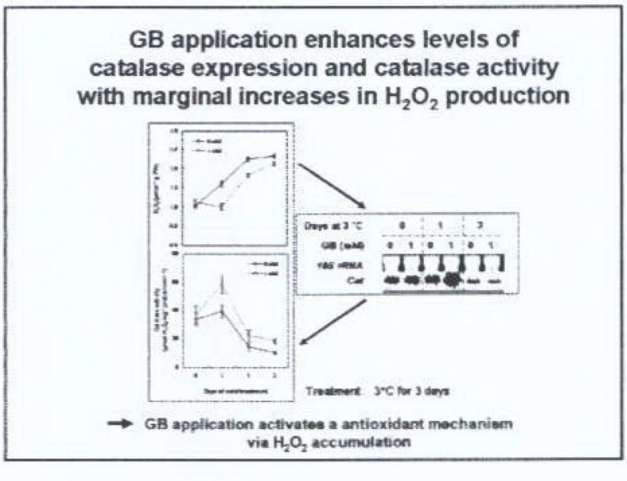
GB application increases chilling tolerance at various developmental stages of tomato plants

Treatment: 3°C for 3 days ⟶ 25°C for 2days



Protective GB effect on chilling tolerance diminishes within a week

Treatment: 3°C for 3 days ⟶ 25°C for 2 days



GB application enhances levels of catalase expression and catalase activity with marginal increases in H_2O_2 production

Treatment: 3°C for 3 days

→ GB application activates a antioxidant mechanism via H_2O_2 accumulation

Conclusions

1. GB application enhances chilling tolerance at different developmental stages of tomato plants
2. GB application activates an antioxidant response mechanism — marginal increase in H_2O_2 accumulation
3. Beneficial effect of GB on chilling tolerance diminishes within a week, suggesting that *genetic engineering of a GB biosynthetic pathway into tomato* may enhance chilling tolerance without frequent application of GB

1. **Exogenous Application of Glycinebetaine Increases Chilling Tolerance in Tomato Plants**
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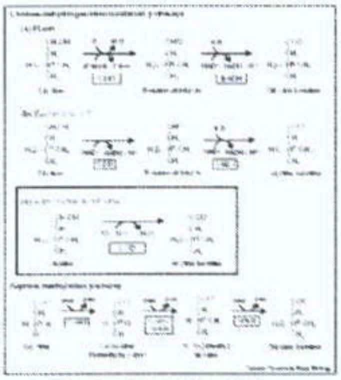
Biosynthesis of GB



CMO - choline monooxygenase
BADH - betaine aldehyde dehydrogenase

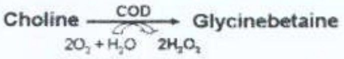
CDH - choline dehydrogenase
BADH - betaine aldehyde dehydrogenase

COD – choline oxidase

GSMT - glycine sarcosine methyltransferase
SDMT – sarcosine dimethylglycine methyltransferase
SAM – S-adenosylmethionine
SAH – S-adenosylhomocysteine

Current opinion in plant biology (2002) 5, 250-257

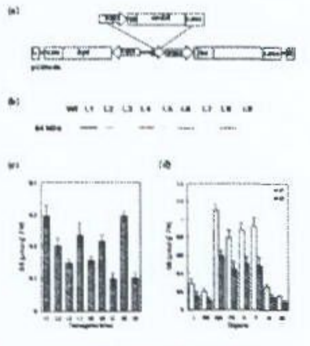
Genes encoding choline oxidase (COD)



$$\text{Choline} \xrightarrow[2O_2 + H_2O \quad 2H_2O_2]{\text{COD}} \text{Glycinebetaine}$$

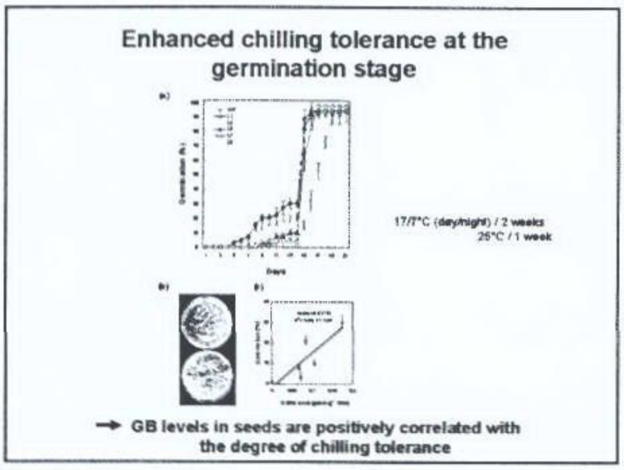
- *codA* from *Arthrobacter globiformis*
- *cox* from *Arthrobacter pascens*

Generation of chloroplast-targeted *codA* transgenic tomato plants



The majority of GB is localized in the chloroplasts

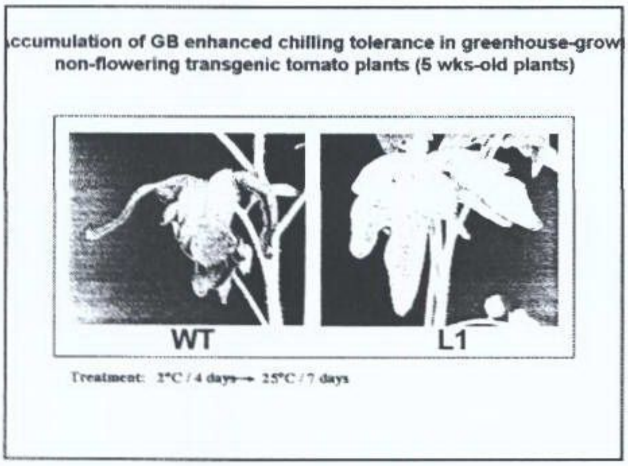
Transgenic line	GB in leaves (nmol·mg⁻¹ chlorophyll)	GB in isolated chloroplasts (nmol·mg⁻¹ chlorophyll)	% Intact chloroplasts	% GB in chloroplasts
L1	23.5±2.8	9.32±1.8	57.9±5.1	68.6±6.3
L3	15.3±1.9	6.89±1.2	58.7±5.8	76.7±7.7
L5	14.7±2.1	7.09±1.1	55.9±4.8	86.3±6.9
L7	11.3±1.8	4.28±0.6	63.2±5.7	60.0±5.6
L9	10.8±1.5	3.58±0.5	55.2±6.7	60.0±7.5



Enhanced chilling tolerance at the germination stage

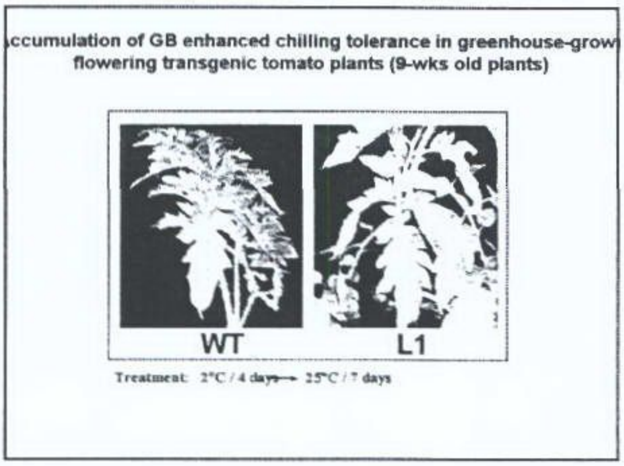
17/7°C (day/night) / 2 weeks
25°C / 1 week

→ GB levels in seeds are positively correlated with the degree of chilling tolerance



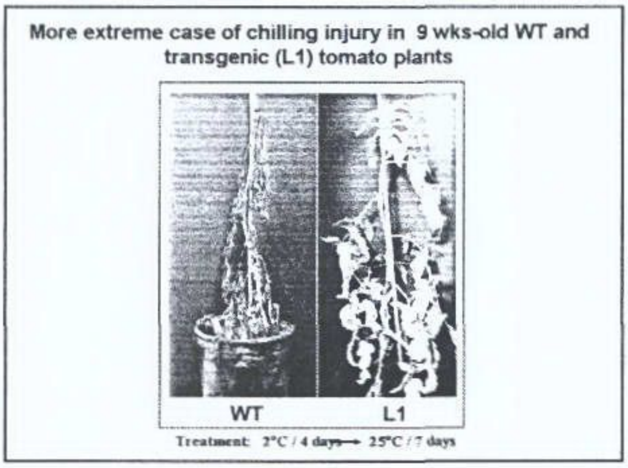
ccumulation of GB enhanced chilling tolerance in greenhouse-grow non-flowering transgenic tomato plants (5 wks-old plants)

Treatment: 2°C / 4 days→ 25°C / 7 days



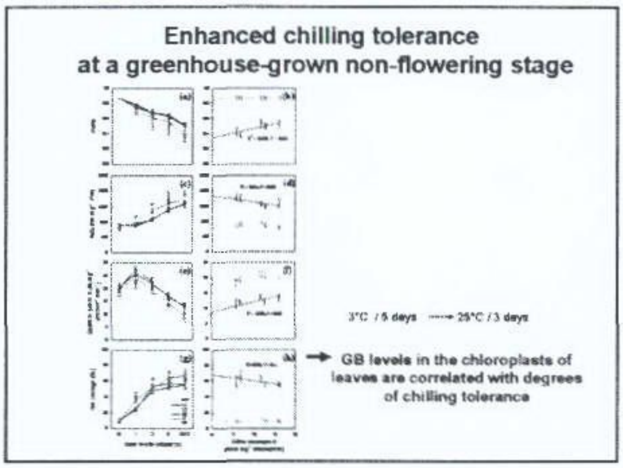
ccumulation of GB enhanced chilling tolerance in greenhouse-grow flowering transgenic tomato plants (9-wks old plants)

Treatment: 2°C / 4 days→ 25°C / 7 days



More extreme case of chilling injury in 9 wks-old WT and transgenic (L1) tomato plants

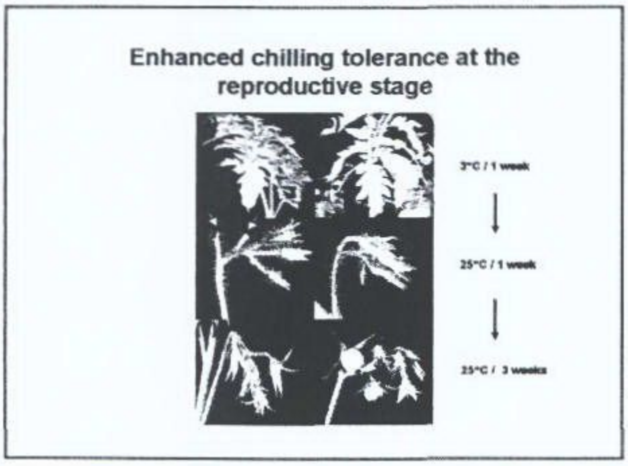
Treatment: 2°C / 4 days → 25°C / 7 days



Enhanced chilling tolerance at a greenhouse-grown non-flowering stage

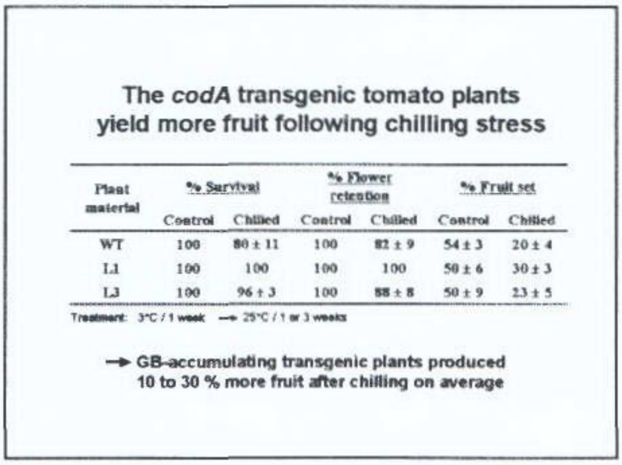
3°C / 5 days → 25°C / 3 days

→ GB levels in the chloroplasts of leaves are correlated with degrees of chilling tolerance



Enhanced chilling tolerance at the reproductive stage

3°C / 1 week
↓
25°C / 1 week
↓
25°C / 3 weeks



The *codA* transgenic tomato plants yield more fruit following chilling stress

Plant material	% Survival		% Flower retention		% Fruit set	
	Control	Chilled	Control	Chilled	Control	Chilled
WT	100	80 ± 11	100	82 ± 9	54 ± 3	20 ± 4
L1	100	100	100	100	50 ± 6	30 ± 3
L3	100	96 ± 3	100	88 ± 8	50 ± 9	23 ± 5

Treatment: 3°C / 1 week → 25°C / 1 or 3 weeks

→ GB-accumulating transgenic plants produced 10 to 30 % more fruit after chilling on average

Conclusions

1. Tomato plants transformed with a chloroplast-targeted *codA* construct accumulates GB and exhibits enhanced chilling tolerance during their entire life cycle
2. GB accumulation by COD induces marginal increase of H_2O_2, resulting in retention of high catalase activity during chilling stress
3. GB levels in the chloroplasts are positively correlated with the degree of transgenic plant chilling tolerance

Endogenous levels of GB in chloroplast-targeted *codA* transgenic plants are very low compared to natural GB-accumulators

Genes	Host	GB accumulation	References
COD/ codA	*Arabidopsis*	~1.2 μmol/g FW	Alia et al. (1998); Hayashi et al (1997); Sakamoto et al. (2000)
	Brassica juncea	~1.0 μmol/g FW	Prasad and Pardha Saradhi (2004)
	Tomato	~ 0.3 μmol/g FW	Park et al. (2004)
	Rice	~1.0 μmol/g FW	Sakamoto et al. (1998)

- Spinach: 30 ~ 40 μmol/g FW under stress conditions

Questions

- GB accumulation in the *codA* transgenic plants is low compared to natural GB-accumulators

↓

1) Would a further increase of GB in transgenic tomato plants confer higher degrees of chilling tolerance ?

2) Would targeting COD to the cytosol provide protection like the chloroplast-targeted form ?

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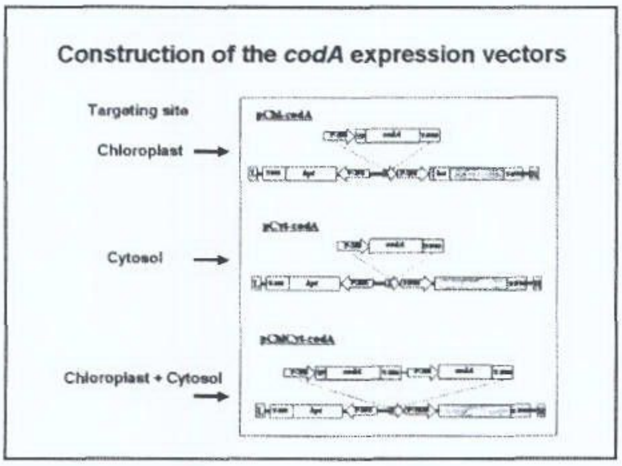


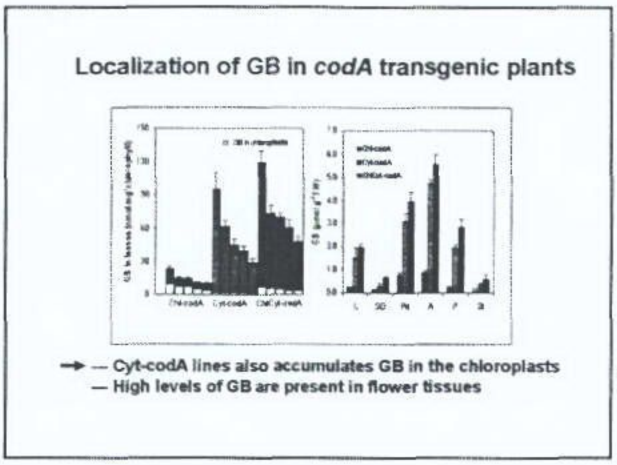
Construction of the *codA* expression vectors

Targeting site

Chloroplast →

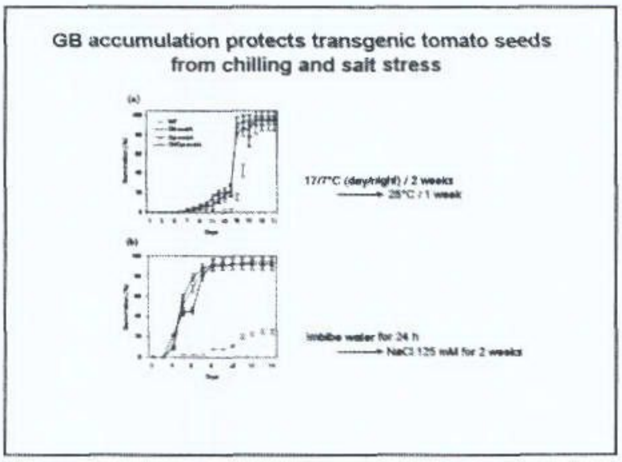
Cytosol →

Chloroplast + Cytosol →



Localization of GB in *codA* transgenic plants

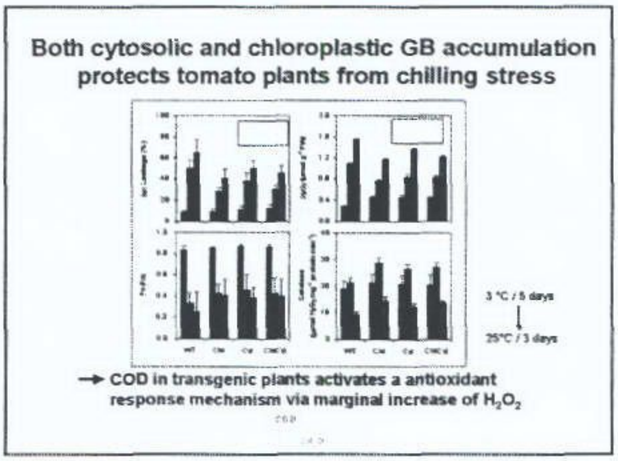
→ — Cyt-codA lines also accumulates GB in the chloroplasts
— High levels of GB are present in flower tissues



GB accumulation protects transgenic tomato seeds from chilling and salt stress

17/7°C (day/night) / 2 weeks
→ 25°C / 1 week

Imbibe water for 24 h
→ NaCl 125 mM for 2 weeks

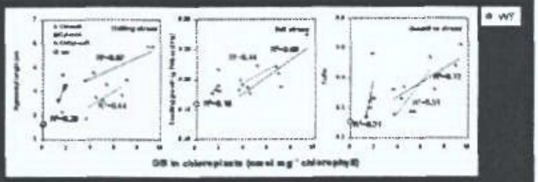


Both cytosolic and chloroplastic GB accumulation protects tomato plants from chilling stress

3 °C / 5 days
↓
25°C / 3 days

→ COD in transgenic plants activates a antioxidant response mechanism via marginal increase of H_2O_2

GB accumulation in transgenic plants enhances tolerance to various stresses



3°C / 1 week
→ 25°C / 1 week

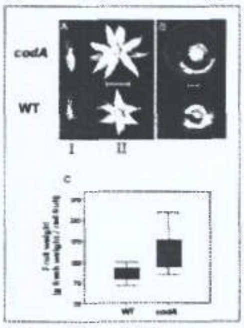
Imbibe water for 24 h
→ 125 mM NaCl / 2 week

5 µM MV / 16h

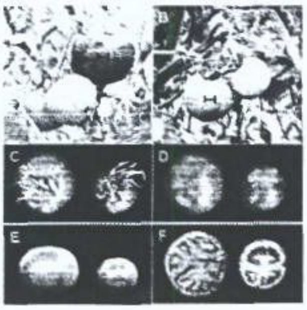
→ GB accumulation in chloroplasts of transgenic plants is related to enhanced stress tolerance in a dose-dependent manner

1. All three *codA* transgenic types exhibits similar degrees of tolerance to abiotic stresses, even though each type accumulated different amounts of GB
2. Cytosolic GB in the Cyt-codA lines was transported, while limited, to subcelluar compartments, including the chloroplasts
3. Levels of GB in the chloroplasts of the Chl-codA lines is correlated with enhanced stress tolerance in a dose-dependent manner

Transformation with the *codA* gene resulted in enlarged flowers and fruits



Flower Removal Experiment



低温生物工学会誌〔Cryobiology and Cryotechnology〕, Vol. 49, No. 2, 77～85, 2003

[Review]

Genetic Engineering of Cold-Tolerant Tomato via Glycinebetaine Biosynthesis

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Tomato (*Lycopersicon esculentum* Mill.) plants, which normally do not accumulate glycinebetaine (GB), are susceptible to chilling stress. Exposure to temperatures below 10℃ causes various symptoms of injury, and greatly decreases fruit set (i.e., the number of fruits per plants) in most cultivars. Conventional breeding efforts have failed to produce cultivars with satisfactory cold tolerance. Therefore, we have transformed tomato (cv. Moneymaker) with the *cod*A gene of *Arthrobacter globiformis*, which encodes choline oxidase to catalyze the conversion of choline to GB. These transgenic plants express the *cod*A gene and synthesize choline oxidase, while accumulating up to 0.23 μmol of GB per gram fresh weight in their leaves. During various phases of growth and development, from seed germination to fruit production, the GB-accumulating plants are more tolerant of chilling stress than their wild-type counterparts. Moreover, at the reproductive stage, our transgenic tomato plants yield, on average, 30% more fruit after an episode of chilling stress. We demonstrate here that introducing the biosynthetic pathway of GB into tomato via metabolic engineering is an effective strategy for improving fruit production under cold-stress conditions.

(Received for publication October 14, 2003)

INTRODUCTION

Many crop species that originated in tropical and subtropical regions are susceptible to chilling injury when temperatures fall below about 15℃[1,2]. This stress can delay growth and development, reduce productivity, and, in extreme cases, kill plants[1]. For chilling-susceptible species, the reproductive stage, which includes the formation of floral organs, fruit, and seed[1], is most sensitive. Although such fruit and vegetable crops can be grown in northern climates in greenhouses, the energy required to maintain minimum temperatures increases the costs of production.

Cold tolerance is controlled by many genes[illegible]. Traditional approaches to crop breeding have made limited progress in enhancing tolerance to low temperatures. In view of the economic incentives for developing cold-tolerant crop plants, considerable

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[Key words : Chilling injury, Choline oxidase, Glycinebetaine (GB), Tomato ; 冷温障害, コリン酸化酵素, グリシンベタイン, トマト]

attention has recently been paid to the possible benefits gained from genetic engineering. Already, a number of genes have been used to produce transgenic plants with increased chilling tolerance[3,4].

Plants have evolved various mechanisms that allow them to adapt to unfavorable environments. One such means involves the accumulation of glycinebetaine (GB), a so-called "compatible solute," in response to both high salinity and low temperatures[5-8]. Several taxonomically distant plants have been identified as natural accumulators of GB, e.g., spinach and wheat, whereas others, such as *Arabidopsis*, potato, rice, and tomato, are considered non-accumulators[8]. Evidence exists that indicates GB might play an important role in tolerance to abiotic stress. For example, its accumulation is induced under adverse conditions, with GB levels being correlated with the extent of enhanced tolerance[9]. Likewise, exogenous application of GB improves the growth and survival of a wide variety of plants under many types of stress[5,10-16]. GB is also effective in stabilizing the quaternary structures of enzymes and complex proteins, as well as maintaining the highly ordered state of membranes at high salt concentrations or extreme temperatures *in vitro*[17]. Finally, introducing the biosynthetic pathway to GB into non-accumulator species increases their tolerance to various kinds of abiotic stress[17].

Here, we report that exogenous applications of glycinebetaine increase chilling tolerance in tomato (*Lycopersicon esculentum* Mill.) plants. Moreover, we demonstrate that GB-accumulating transgenic tomatoes, which are produced through the expression of a bacterial gene for choline oxidase, exhibit enhanced tolerance to chilling stress at several developmental stages.

TOMATO CHILLING INJURY

The tomato is a very popular vegetable in many countries. Because these plants originated in the Tropics, most cultivars suffer chilling injury when subjected to temperatures of <10℃[18,19,20]. Extended exposure below 6 ℃ can kill plants; temperatures below 13℃ inhibit fruit set[21]. Cold stress delays the onset and lowers the rate of seed germination, resulting in poor performance[21,22]. At later stages, low temperatures cause reduced growth and development, interfering with the formation of flowers and fruit as well as substantially decreasing yields[22]. These negative effects on fruit set are due mainly to reduced pollen viability and the failure of anthers to release pollen grains when nighttime temperatures are low[23]. Pollen development in the tomato is particularly sensitive to such environmental disruptions[24]. For example, if their immature flowers are repeatedly exposed to night temperatures below 10℃, infertile pollen grains are produced[24]. Two periods of pollen development, at about 11 and 6 d before anthesis, are particularly sensitive.

Post-harvest storage of tomato fruit at low temperatures results in slow and abnormal ripening, blotchy color development, greater susceptibility to disease, and increased rates of respiration and ethylene production[25]. Whereas green-mature fruit should not be stored for more than 2 weeks at 13℃, light-red fruit can be held up to 1 week at 10℃ without exhibiting chilling injury. Longer storage times for ripe fruit (i.e., at <10℃) lead to lower quality, as measured by a loss in color, firmness, and shelf life.

Chilling tomato plants for 3 d at 3 ℃ (± 1 ℃) under a PPDF of 250 μmol $m^{-2}s^{-1}$ was shown to reduce the chlorophyll fluorescence ratio, F_v:F_m, and stomatal conductance[25]. Photosynthetic CO_2 uptake was also decreased by 43%[25]. Thylakoid membranes that were isolated from previously chilled leaves produced more reactive oxygen species (ROS) than those from non-chilled plants, and the rate of production increased with time spent in the light[25]. The entire chain electron transport was measured in thylakoids isolated from control and chilled leaves. Treatment at 25℃ reduced such transport, a rate that continued to decline as the assay temperature was decreased from 25 to 5 ℃[25]. Nevertheless, the rate of electron transport in thylakoids from chilled leaves was less responsive to changes in temperature than that from control leaves, so that the transport rate in leaves chilled below 20℃ was actually greater than in control samples held at the same temperature[25].

Moreover, a chilling treatment that induced changes in Chl fluorescence, stomatal conductance, photosynthesis, and growth, did not, on a percentage basis, significantly affect the lipid profile or distribution of total fatty acids[25]. If extensive lipid peroxidation had occurred in the thylakoid membranes at that time, the level of polyunsaturated fatty acids should have declined, but this was not observed. Furthermore, the expected accumulation of thiobarbituric acid reactive species in the chilled thylakoid membranes did not occur either[25]. The lack of evidence for a build-up in peroxidative by-products indirectly suggests that free radical scavengers were at sufficient levels to maintain membrane integrity[25].

The roles of lipid- and water-soluble antioxidants in tomato leaves following chilling stress was manifested by an associated decline in the level of α-tocopherol, as well as reduced / oxidized ascorbate and glutathione ratios[26]. Activity of superoxide dismutase also decreased, while that of ascorbate peroxidase, monodehydroascorbate reductase, and glutathione reductase did not change in response to low-temperature treatments.

A major contributing factor to injury by chilling is the oxidative stress that is so induced[27]. In cold-sensitive plants, exposure to low temperatures under illumination often leads to the peroxidation of membrane lipids and the depletion of antioxidant compounds, thereby indicating an increase in the levels of reactive oxygen species. Furthermore, an increase in H_2O_2 levels in maize[27] and tomato[28] seedlings during low-temperature exposure provides direct evidence for an increase in reactive oxygen species.

Oxidative stress apparently plays a dual role in low-temperature responses of chilling-sensitive plants, as both a source of injury and a signal to increase antioxidant defenses[27, 29]. For example, H_2O_2 content was nearly doubled when tomato seedlings were chilled[28] (E.J. Park, unpublished). This sensitivity in tomatoes also varied diurnally[29], being highest when chilling occurred at the end of a dark period. Those seedlings then became more resistant to chilling injury upon exposure to the light, so that they showed higher levels of H_2O_2 than seedlings at the end of the dark period. Exogenous application of H_2O_2 also increased chilling tolerance, but with little evidence of diurnal variation in sensitivity. All these results support the theory that H_2O_2 confers increased chilling tolerance to tomato seedlings, similar to that reported with maize[27].

Catalase is the primary H_2O_2 scavenger in plant cells[9]. Because this enzyme serves as a major protectant against the accumulation and toxicity of H_2O_2, changes in catalase activities have been evaluated in tomato plants during low-temperature stress. Under light, catalase activity is reduced in response to chilling for a number of plant species, including tomato[28, 30]. Researchers believe that this decrease is primarily due to the failure of repair synthesis and suppressed translation[30], as well as reduced expression of the catalase gene[28].

To test the hypothesis that modifying the H_2O_2-scavenging mechanism in plants can lead to changes in oxidative-stress tolerance, tomatoes were transformed with an antisense catalase gene construct[29]. Those that expressed the antisense gene showed a 2- to 8-fold reduction in total catalase activity, and a 2-fold increase in levels of H_2O_2 in leaf extracts. These transgenic plants were more sensitive to exogenous application of H_2O_2 and also became more sensitive to chilling stress. Their reduced catalase activity likely led to a failure to scavenge the ROS that accumulated during such stress. In addition, when transgenic tomato plants that expressed an *Arabidopsis* CBF1 gene were exposed to chilling stress, they had increased levels of catalase activity and catalase transcript, and also showed greater tolerance to low temperatures compared with wild-type (WT) plants[2].

EXOGENOUS GLYCINEBETAINE APPLICATION INCREASES CHILLING TOLERANCE IN TOMATO PLANTS

GB protects cells from salt stress by maintaining osmotic balance with the environment[31], and by stabilizing the quaternary structure of complex proteins[17, 32-34]. In photosynthetic systems, GB stabilizes the oxygenevolving photosystem II (PSII) complex[17, 35, 36] at high concentrations of NaCl. Because low-temperature stress is considered a form of dehydration[37], compatible osmolytes may be regarded as water substitutes. These osmolytes maintain a hydrophilic environment for all types of cellular macromolecules, such as proteins and membranes, thereby reducing the sensitivity of molecular structures to this lack of water.

Even though tomato plants do not normally accumulate GB[9], exogenous GB is taken up readily when applied to their leaves. Being translocated rapidly to other parts of the plant[38], it increases tolerance to both salt and drought[14] and improves yields in the greenhouse and field[12, 13].

We have examined the effect of exogenous GB applications on the chilling tolerance of *in vitro*-grown seedlings as well as those produced in the greenhouse. Although various test amounts of GB (1 to 100 μM) significantly increased the tolerance of *in-vitro* seedlings (Fig. 1), concentrations higher than 10000 μM appeared to be toxic. We also applied GB to the foliage of seven-week-old WT plants. Control plants, sprayed with water only, suffered extensive chilling injury, whereas those treated with 50 mM GB showed little damage (Fig. 2). However, for all plants, higher concentrations (100 and 150 mM) were detrimental (data not shown). Thus, exogenously applied GB appears to be effective in protecting tomato plants against chilling. Our results agree with those from previous tomato experiments, in which foliar GB applications enhanced tolerance to abiotic stresses[13, 14].

Based on these observations, we postulated that the introduction of a GB biosynthetic pathway into tomato plants might increase tolerance to chilling stress, thereby eliminating the need for exogenously applied GB. Such advances, gained via metabolic engineering of GB, have already been extensively reviewed[3, 4, 15, 39, 40].

TRANSGENIC TOMATO PLANTS EXPRESSING A BACTERIAL *codA* GENE ACCUMULATE GB AND INCREASE CHILLING TOLERANCE

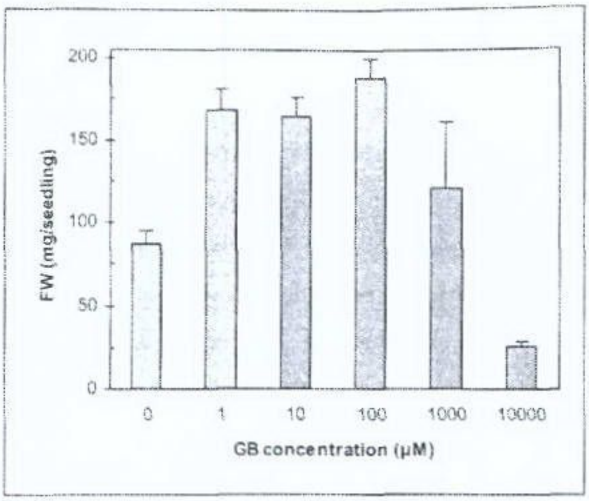


Fig. 1. Exogenously applied GB increased chilling tolerance of *in vitro*-grown tomato seedlings. Seeds (WT) were germinated on presoaked filter paper in Petri dishes containing various concentrations of GB (1 to 10,000 μM) for 5 d. They were transferred to an MS medium (50 ml) in Magenta GA-7 vessels supplemented with the same amount of GB used for germination, then grown until the seedlings were approximately 2 cm tall. The seedlings were chilled at 3 ℃ (±1 ℃) for 7 d under a 16-h photoperiod, then transferred to a warm growth chamber (25℃, ±2 ℃). Fresh weights (FW, mg / seedling) were measured 7 d later.

Expression of the codA gene in transgenic tomato plants

We transformed tomato (cv. Moneymaker) with a 35S::*codA* expression cassette via *Agrobacterium*-mediated transformation[41]. The coding region of the *codA* gene was preceded by the transit peptide sequence of a small subunit of the tobacco Rubisco gene to target the choline oxidase into chloroplasts. A total of 45 independent primary transgenic tomato lines (T0) were generated, all of which possessed the hygromycin resistance gene (*hpt*), as confirmed by PCR. Staining for β-glucuronidase (GUS) activity revealed that 28 out of the 45 lines expressed this gene. Western-blotting analysis also confirmed that all 28 GUS-positive lines accumulated a 64-kDa protein that corresponded to choline oxidase; no such protein was detected in the WT plants. Furthermore, 19 independent homozygous T2 and T3 transgenic lines were obtained by self-pollination, and were used for GB analysis. Extraction and analysis of GB by HPLC was performed according to the method of

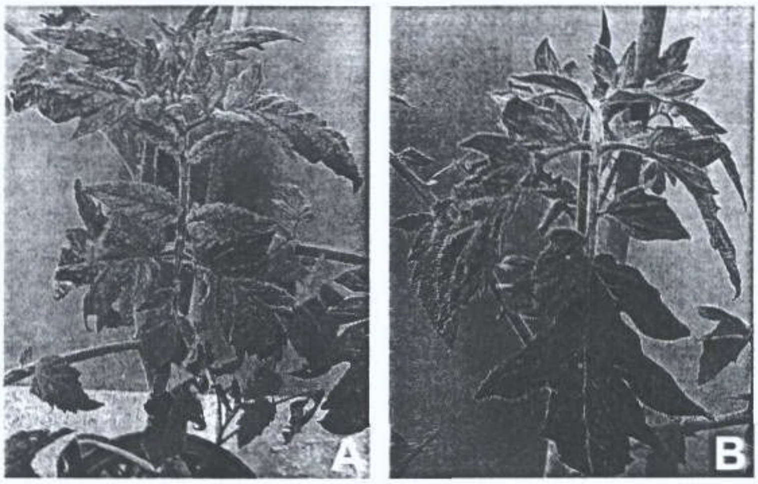


Fig. 2. Exogenously applied GB increased chilling tolerance of WT tomato plants. Seven-week-old greenhouse-grown plants were sprayed with either water (A) or a 50-mM GB solution (B). Tween-20 (0.005% v / v) was included in all solutions as a wetting agent. One day after the foliar application, plants were transferred to a cold room (3 ℃, ±1 ℃) and chilled for 5 d. Following this stress period, plants were returned to the greenhouse (25℃, ±2 ℃), and the extent of chilling injury was visually scored 7 d later. The red arrows (A) indicate chilling injury in control plants sprayed with water only.

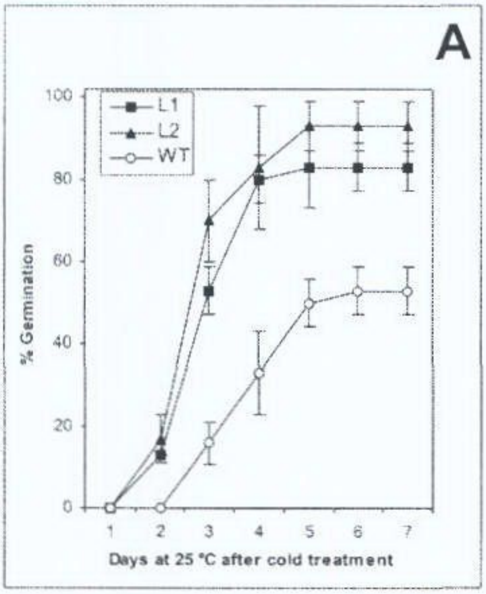
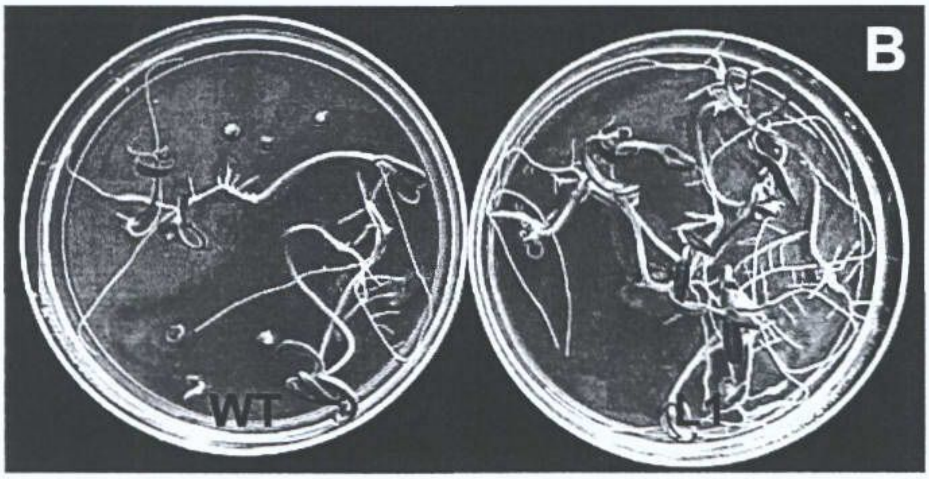


Fig. 3. Comparison of chilling tolerance between WT and transgenic tomato lines at germination stage. Imbibed seeds were chilled at 3 ℃ (±1 ℃) in the light for 2 weeks, then transferred to a warm growth chamber (25℃, ±2 ℃). Evidence of germination was recorded daily for 7 d. (A) Percent germination of WT and two independent transgenic lines (L1 and L2) after cold treatment. Values represent mean % germination of 3 independent experiments. Bars indicate SE of the mean. (B) Seed germination at Day 5 after chilling treatment.



Naidu[42], with two exceptions: a strong anion-exchange resin AG 1-X8 (Bio-Rad, Hercules, CA), as described by Bessieres et al.[43], and refractive indices were used to estimate amounts of GB. The level of GB ranged from 0.068 to 0.230 μmol/g fresh weight (FW); no GB was detected in WT plants. For further study, we then selected two homozygous transgenic lines, L1 and L2, with GB contents of 0.22 and 0.18 μmol/g FW, respectively.

The amount of GB measured in transgenic tomato plants (0.07-0.23 μmol/g FW) was lower than that reported in other plant species expressing the same *codA* construct[3], and was much lower than those in natural GB-accumulators, such as spinach (30-40 μmol/g FW)[9], when exposed to stress conditions. For future practical applications, it may be necessary to generate plants with the capacity to synthesize higher levels of GB by enhancing the availability of choline, the GB precursor[44].

In transgenic *Arabidopsis* plants expressing the *codA* gene flanked with chloroplast transit peptide, choline oxidase is efficiently targeted into the chloroplasts[45], and most GB is localized there[46]. It is likely that, in our transgenic tomato plants, most of the GB was also localized in the chloroplasts. That, however, remains to be examined. In transgenic rice plants that are transformed with the chloroplast-targeted *codA* gene, the photosynthetic machinery is more efficiently protected against salt and cold than are plants expressing the non-targeted *codA* gene, despite the fact that the latter accumulates five times more GB[47]. The localization of GB in the chloroplasts might also have been responsible for protecting against low temperatures in our transgenic tomato plants. However, targeting choline oxidase to cell compartments other than chloroplasts should be considered because targeting to mitochondria and the cytosol can also improve the tolerance of the photosynthetic machinery to salt and chilling stress[48, 49].

Transformation with the codA gene protects seed germination against chilling stress

Seeds were imbibed and then incubated at 3 ℃ (±1 ℃) in a cold room for two weeks prior to germination in a warm growth chamber at 25℃ (±2 ℃). One week after the end of the chilling treatment, only 53% of the WT seeds had germinated, whereas the success rates were 93% and 83% for seeds from the L2 and L1 transgenic lines, respectively (Fig. 3A). In addition, those two lines had produced rooted seedlings that were larger and more vigorous than those arising from WT seeds (Fig. 3B). Without the chilling treatment, seeds from the transgenic lines germinated approximately 1 d earlier than the WT. These results demonstrate that transformation with the *codA* gene significantly increased chilling tolerance at the seed germination stage, and resulted in more robust tomato seedlings.

Accumulation of GB enhances chilling tolerance of young seedlings

Tomato seedlings that had been grown *in vitro* were held for two weeks at 3 ℃ (±1 ℃), in either the light or dark. Chilling is known to be more damaging under illumination[1]. Although neither the WT nor the L1 and L2 transgenic plants grew at all in the light, after their transfer to the warm growth chamber (25℃, ±2 ℃), the

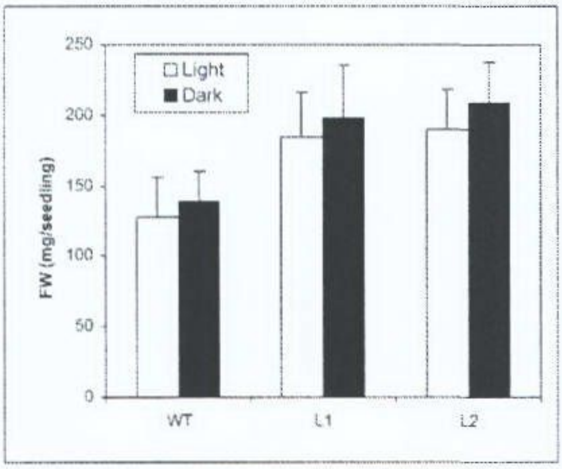


Fig. 4. Effects of low temperature on *in vitro*-grown tomato plants. WT, L1, and L2 seeds were germinated and grown at 25℃ (±2℃) until seedlings reached ≈ 2 cm in height. They were then transferred to a cold room (3 ℃, ±1 ℃) under either light (open bar) or darkness (hatched bars). Seedling heights were measured 7 d later. Values represent means of heights ±SE for 3 independent experiments, each involving 5 seedlings (n=15).

transgenic seedlings resumed normal growth (100% survival). In contrast, a number of WT seedlings suffered extensive damage, as evidenced by severely necrotic lesions and moribund shoot tips. The rest survived but grew poorly in those warm conditions. When chilled in darkness, transgenic seedlings continued to grow even at the low temperature (3 ℃, ±1 ℃), but the WT plants did not grow at all. When the plants were returned to the warm chamber, the seedlings of the two transgenic lines grew more vigorously than the WT (Fig. 4), with longer shoots and better-developed root systems (data not shown).

Accumulation of GB enhances chilling tolerance in greenhouse-grown non-flowering transgenic tomato plants

Five-week-old greenhouse-grown plants were chilled in a cold room (3 ℃, ±1 ℃) for 4 d. Both the transgenic and the WT plants wilted soon after being transferred to that environment. Upon their return to the greenhouse (25℃, ±2 ℃), the transgenics recovered more quickly, with significantly less extensive chilling injury ($P < 0.01$). About 20 to 25% of the total leaf area on the transgenic plants was damaged, compared with about 55% injury on the WT plants. Young leaves of the L1 and L2 plants recovered and resumed normal growth, while those from the WT plants developed necrotic lesions.

Improved setting of fruit in codA-transgenic tomato plants after chilling stress

Flowering WT and L1 transgenic plants were chilled at 3 ℃ (±1 ℃) for 7 d, then returned to the greenhouse (25℃, ±2 ℃). After this transfer, 20% of the WT plants died within 3-4 d, whereas 100% of the transgenics survived (Table 1). During the first week back in the greenhouse, the WT leaves became severely necrotic and the plants retained only about 82% of their flowers (Table 1). Their fruit set was greatly delayed compared with that of the L1 plants. Some WT inflorescences retained all of their flowers but none had set fruit three weeks after the stress treatment (Table 1). In contrast, the L1 transgenic plants showed much less chilling injury, and all their flowers were retained (Table 1). Although this stress reduced the number of fruit in both WT and L1 plants, fruit set was higher ($P \le 0.052$) in the latter (Table 1). For these experiments, the tomato plants were raised in a greenhouse without manual pollination. Under those conditions, the frequency of fruit set in both WT and L1 plants was about 50%. However, the GB-accumulating transgenic tomato plants had, on average, about 30% more fruit than the WT after an episode of chilling stress.

CONCLUSIONS

Tomato plants that express the *codA* gene exhibit significantly increased tolerance to chilling at various stages of development, from seed germination to fruit formation. Our findings are agriculturally important because the incidence of cold stress under field conditions is unpredictable, and plants can be exposed to low temperatures at any time during their life cycle. Therefore, enhanced tolerance to chilling at multiple stages of growth and development should greatly reduce

Table. 1. *cod*A-transgenic tomato plants showed enhanced chilling tolerance during reproductive phase. Nine-week-old WT and L1 transgenic plants, at the stage when two or three flowers of the first inflorescence had reached anthesis, were incubated at 3 ℃ (±1 ℃) under 16-h photoperiod for 7 d. Plants were then returned to greenhouse (25℃, ±2 ℃) for three weeks. Flower retention and fruit set were quantified one and three weeks after cold treatment.

Plant Material	% Survival		Flower retention (%)		Fruit set (%)	
	Control	Chilled	Control	Chilled	Control	Chilled
WT	100	80.1 ± 11.2	100	81.5 ± 9.2	51.7 ± 2.9	19.7 ± 3.7
L1	100	100	100	100	54.2 ± 6.3	29.9 ± 3.2

Mean ± SE (Standard error)

the negative impact of low temperatures, thereby stabilizing tomato production in locations where plants are currently prone to such stress. Here, both *in-vivo* expression of the *codA* gene and foliar GB applications rendered tomato plants more tolerant to low-temperature stress.

Engineering tolerance to abiotic stress is a promising approach to the improvement of crop plants[3,4]. The ability of the *codA* gene to improve cold tolerance at the flowering and fruiting stages is very important because many food crops are grown for their fruits. Furthermore, it is the reproductive phase, including flowering and fruiting, that is generally most sensitive to environmental stresses.

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8

Ectopic Overexpression of *AtCBF1* in Potato Enhances Freezing Tolerance

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Introduction

The incidence of frost has a significant impact on worldwide agricultural production, causing considerable crop-productivity loss and limiting the geographical distribution of many important crop species. While it is grown in a wide range of climates, cultivated potato (*Solanum tuberosum*) is a frost-sensitive species. In most potato production areas, frost can significantly reduce yield, and particularly hard frosts can even result in the complete destruction of an entire plantation. All *S. tuberosum* cultivars are frost-sensitive and lack the ability to cold acclimate. In contrast, many other wild tuber-bearing *Solanum* species (e.g. *S. acaule*, *S. commersonii*) can survive temperatures of about −5°C pre-acclimation, and tolerate temperatures as low as −10°C following cold acclimation (Chen and Li, 1980; Costa and Li, 1993).

An initial research approach to solve the frost injury problem of potato has involved the attempt to genetically transfer frost-tolerant genes from frost-hardy wild species to the frost-sensitive cultivated varieties. Such attempts to transfer frost-tolerant genes to cultivated potato by traditional breeding have proved to be time-consuming, and have not resulted in a significant increase in frost tolerance of cultivated potato (Cardi *et al.*, 1993; Estrada *et al.*, 1993; Iovene *et al.*, 2004). This slow progress in frost-tolerance improvement in potato and other crop plant species is mainly due to the fact that frost tolerance and cold acclimation are complex genetic traits that involve multiple genes at distinct chromosomal locations, and to the lack of knowledge of the key genes involved in the process.

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Recently, new biotechnology-based approaches have shown promise for both the direct improvement of plant cold tolerance, and as a means of evaluating the roles of individual genes important to the process of cold acclimation. This has principally involved a concerted effort conducted in the model plant system *Arabidopsis* to understand the key genes involved in the cold signal transduction mechanism and the resultant genes that are regulated by low temperature. Cold acclimation involves the action of a large suite of cold-regulated (*cor*) genes (Fowler and Thomashow, 2002). The identification of CBF transcription factors (also called DREB1s) and their role in the coordinate regulation of *cor* genes has significantly advanced our understanding of the genes and the ways by which plants adapt to low temperature (Stockinger *et al.*, 1997; Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Thomashow, 2001; Fowler and Thomashow, 2002). The *Arabidopsis CBF* genes and their homologues from other species have been employed as a new approach towards improving cold tolerance in crop plants such as *Brassica napus* (Jaglo *et al.*, 2001), tomato (Hsieh *et al.*, 2002a,b) and tobacco (Kasuga *et al.*, 2004).

Wild *Solanum* species differ in frost-tolerance level and cold-acclimation potential, representing a rich genetic source for improvement of these traits in cultivated potato. The advances made in *Arabidopsis* in understanding the function of the genetic components of cold tolerance have led us to study whether an endogenous CBF pathway is involved in controlling the frost-tolerance and cold-acclimation potential of wild *Solanum* species. We are investigating this by: (i) determining if characterized *CBF*s from *Arabidopsis* affect frost-tolerance traits in wild and domesticated potato; (ii) isolating and characterizing the endogenous *CBF*s of potato; and (iii) determining the role they play in potato freezing-tolerance and cold-acclimation. The latest results on ectopic expression of *Arabidopsis CBF1* (*AtCBF1*), its effect on enhancing freezing tolerance in wild and domesticated potato species, and that ectopic *AtCBF1* overexpression in *Solanum* species mimics many of the multiple biochemical and structural changes that are observed in wild-type (WT) plants during cold acclimation will be reported in this chapter.

Frost has a Significant Impact on Potato Production

S. tuberosum, or cultivated potato, is widely grown around the world and represents the fourth most important food crop after rice, maize and wheat. In many of these regions, the risk of frost damage is prevalent and frequently results in reduced potato yield and tuber quality. In most temperate zones, frost risks for potato are mainly encountered in the early spring and late autumn. In contrast, in the Andean Highland of South America, for example, frost damage can occur any time during the growing season and seriously reduce potato production (Chen and Li, 1980; Barrientos *et al.*, 1994; Vega and Bamberg, 1995). Frost damage is also a serious problem for the highly profitable early potato crop in the Mediterranean, which is planted from late autumn to early spring, when the potential for frost damage is highest and can cause significant loss in production (Iovene *et al.*, 2004).

Frost injury of plants and tissues primarily results from the severe cellular dehydration that occurs upon formation of extracellular ice and the damage associated with it. Extracellular ice formation can lead to altered membrane properties, cellular membrane damage, protein denaturation and the generation of reactive oxygen species (ROS) that damage various cellular components (Palta and Li, 1980; Toivio-Kinnican *et al.*, 1981; Steponkus, 1984; Guy, 1990; McKersie, 1991; Thomashow, 1999). ROS accumulation is due to a failure in the electron transfer reaction, and has been connected to decreased photosynthetic efficiency, photosystem II damage and subsequent increased damage upon exposure to high intensity light (Steffen and Palta, 1989; McKersie, *et al.*, 1991; O'Kane *et al.*, 1996; McKersie *et al.*, 2000). Differences also exist in the capacity of wild versus domesticated potato to deal with freezing stress-related photosynthetic damage. While only a temporary reduction in photosynthetic capacity is observed in the wild potato *S. commersonii* following freezing stress, highly reduced and irreversible photosynthetic capacity occurs in *S. tuberosum* (Seppanen and Coleman, 2003).

Multiple Changes Occur in Potato Plants During Cold Acclimation

Multiple biochemical and morphological alterations in potato are associated with the increase in freezing tolerance that accompanies cold acclimation. These include changes in the level and composition of carbohydrates, proteins, nucleic acids, amino acids, growth regulators, phospholipids and fatty acids (Li, 1984). The process of cold acclimation is associated with both the synthesis of cryoprotective polypeptides (Artus *et al.*, 1996; Steponkus *et al.*, 1998) and the accumulation of compatible solutes with cryoprotective properties (Gilmour *et al.*, 2000; Taji *et al.*, 2002), which include free amino acids (e.g. proline), quaternary ammonium compounds (e.g. glycine betaine) and carbohydrates (e.g. sucrose). These substances are believed to play an important role by increasing the internal osmotic pressure and preventing the loss of water from the cell during freezing-induced dehydration (Nanjo *et al.*, 1999; Thomashow, 1999).

The commonly observed accumulation of free proline in the leaves, shoots and roots of angiosperms during cold stress is considered one of the most widespread stress-induced responses (Chu *et al.*, 1974). Cold acclimation-induced changes in proline content have been reported in multiple species including perennial rye grass, barley, lucerne, winter rape, winter wheat and annual bluegrass (Draper, 1972; Chu *et al.*, 1974; Paquin, 1977; Stefl *et al.*, 1978; Dionne, 2001b). In potato, an increase in leaf proline content of three- to tenfold was observed during cold hardening without changes in water status (Van Swaaij *et al.*, 1985). Also, the exogenous application of proline increased potato frost tolerance (Van Swaaij *et al.*, 1985). While some studies suggest that the increase in proline content is related more to drought stress than cold stress, this association has not been demonstrated in potato (Levy, 1983). Later studies in *Arabidopsis* have shown similar results, where photoperiod and cold acclimation were highly associated with proline-content accumulation and an enhancement in freezing tolerance (Wanner and Junttila, 1999). Similar to cold-acclimated plants, transgenic *Arabidopsis* plants ectopically expressing *CBF* genes have increased proline content and an improved freezing-tolerance capacity (Liu *et al.*, 1998; Gilmour *et al.*, 2000).

Accumulation of sugars is another commonly observed biochemical change associated with cold acclimation. Sugars act as effective cryoprotectants *in vitro* (Carpenter and Crowe, 1988) and impart a protective benefit to membranes, cells and plants during cold stress (Sanitarius, 1973; Strauss and Hausser, 1986; Livingston and Henson, 1998; Vijn and Smeekens, 1999; Taji *et al.*, 2002). Detailed descriptions of alterations in sugar content during cold acclimation have been reported for many plant species (Gilmour *et al.*, 2000; Hincha *et al.*, 2000; Dionne *et al.*, 2001a). A comparison of multiple *Solanum* species found that both free sugars and starch increase during cold acclimation, and *S. commersonii* in particular, one of the most cold-tolerant *Solanum* species, showed the highest increase in sugar content following cold acclimation (Chen and Li, 1980). However, this study also demonstrated that sugar accumulation alone cannot be the basis for the differences between *Solanum* species in cold-acclimation potential. *S. tuberosum*, which cannot cold acclimate, was also found to accumulate sugars when grown under low temperatures (Chen and Li, 1980).

Previous research has shown that a specific subset of proteins is synthesized during cold acclimation (Guy, 1990). Members of the dehydrin protein family accumulate to high levels in response to low temperature, abscisic acid (ABA) application and drought stress (Close, 1996, 1997). COR, late embryogenesis abundant (LEA) and similar soluble proteins also accumulate in cold-acclimated plants and are probably critical to the freezing-tolerance mechanism (Thomashow, 1998; Iba, 2002). An early study in tuber-bearing *Solanum* species found a high correlation between protein synthesis and freezing tolerance where the *Solanum* species capable of cold acclimation exhibited an increase in soluble proteins (Chen and Li, 1980). In *S. commersonii*, cold acclimation induces the synthesis of many new polypeptides (Tseng and Li, 1987, 1990; Ryu and Li, 1994), and similar results were reported for *S. commersonii* cell cultures in response to cold acclimation and ABA application (Lee *et al.*, 1992).

It is well documented that the membrane lipid composition changes during cold acclimation in a wide range of plants. In fact, changes in membrane fluidity and lipid composition are thought to be associated with freezing tolerance (Palta and Li, 1980; Williams *et al.*, 1988; Palta *et al.*, 1993; Steponkus *et al.*, 1993; Welti *et al.*, 2002). In a comparison between freezing-tolerant *S. commersonii* and freezing-sensitive *S. tuberosum*, of the membrane lipid changes that occur during cold acclimation, common changes noted include decreased palmitic acid and cerebroside, while increases occurred for the unsaturated to saturated fatty acid ratio, free sterols and sitosterol (Palta *et al.*, 1993). *S commersonii*-specific lipid changes included decreased linolenic acid content and the sterol to phospholipid ratio, while increases occurred for phosphatidylethanolamine, linoleic acid and the acylated steryl glycoside to steryl glycoside ratio. The results indicated that the changes in lipid composition are associated with increased freezing tolerance during cold acclimation.

The transition to low temperature is known to alter leaf growth patterns and leaf cell ultrastructure in some plant species, suggesting that these structural and morphological alterations may play an important role in freezing tolerance (Kaku, 1973; Palta and Li, 1979; Ristic and Ashworth, 1993). Changes in leaf structure and cell-wall thickness, such as formation of double to triple palisade layers, occur in frost-tolerant potato species when grown at low temperature, but are not observed in freezing-sensitive potatoes (Chen *et al.*, 1977; Palta and Li, 1979; Estrada, 1982). Similar results have been reported in other species as well, such as changes in cell ultrastructure and increased thickness of mesophyll cells leading to leaf cell enlargement in *Arabidopsis* (Ristic and Ashworth, 1993) and winter oilseed rape (Stefanowsna *et al.*, 1999, 2002).

The expression of many genes is affected during the development of freezing tolerance in cold-hardy wild potato, and has been studied most extensively in the cold-hardy wild potatoes *S. commersonii* and *S. sogarandinum*. Many of these genes encode homologues to proteins proposed to play roles in the stress-tolerance responses of other plant systems. Examples include potato homologues to osmotin, cyclophilin – an RNA-binding glycine-rich protein, dehydrins, SAM decarboxylase, glucosyl transferase and cell-cycle protein CD48, among others (Zhu *et al.*, 1993; Rorat *et al.*, 1997; Meza-Zepeda *et al.*, 1998; Rorat *et al.*, 1998; Baudo *et al.*, 1999). In addition to cold, the expression of many of these genes also responds to exogenous ABA application and other abiotic stresses, such as salt and drought. Together, these results suggest that many of these genes may be employed in multiple stress response pathways.

Attempts to Transfer Frost-hardiness Genes from Wild Potato Species to Cultivated Potato

Temperatures below −3°C are typically lethal to *S. tuberosum* cultivars ($2n = 4\times = 48$), which are incapable of cold acclimation. In contrast, wild potato species are able to survive this temperature range, and can survive even colder temperatures after cold acclimation (Chen and Li, 1980; Costa and Li, 1993). Wild potato species, such as *S. acaule*, *S. commersonii*, *S. boliviense*, *S. chomatophium*, *S. multidissectum*, *S. megistacrolobum* and *S. sanctae-rosae*, can survive temperatures from −4°C to −6°C prior to acclimation. *S. commersonii* ($2n = 2\times = 24$), endemic to Argentina, Paraguay and Uruguay, is one of the most cold-hardy potato species, tolerating temperatures as low as −10°C following cold acclimation (Chen and Li, 1980).

Attempts to transfer frost-hardiness genes by traditional breeding from wild genotypes to cultivated potato have been challenging and met with minimal success to date. Potato breeders have also employed somatic fusion, embryo rescue and bridging-cross strategies to overcome the natural barriers associated with interspecific crossing between wild and cultivated potato species. Unfortunately, linkage drag typically limits the use of wild potato species, with many exotic genes which impart undesirable traits (e.g. high alkaloid content, long stolons) being co-transferred with cold-hardiness genes. Under these potato breeding schemes, time-consuming backcrosses, evaluations and phenotypic selections are needed to restore the desired phenotype of an improved cultivar (Cardi *et al.*, 1993; Estrada *et al.*, 1993; Pavek and Corsini, 2001; Iovene *et al.*, 2004).

Inheritance studies on F1 and backcross progenies of *S. commersonii* × *S. cardiophylum* showed that freezing-tolerance and cold-acclimation traits are under independent genetic control and determined by a small number of genes

(Stone *et al.*, 1993). Characterization of somatic hybrids between frost-tolerant *S. commersonii* and frost-sensitive *S. tuberosum* has been carried out without any notable increase in cold hardiness, with most of the results showing a minor increase in freezing tolerance after cold acclimation, but not prior to (Cardi *et al.*, 1993; Nyman and Waara, 1997; Palta *et al.*, 1997). For example, the offspring of *S. commersonii* × *S. tuberosum* somatic hybrids, while freezing-sensitive like the *S. tuberosum* parent prior to cold acclimation, increase slightly in freezing tolerance following cold acclimation (Chen *et al.*, 1996). Similar results were reported in a later study characterizing the freezing tolerance of selfed and backcrossed progenies derived from *S. tuberosum* × *S. commersonii* somatic hybrids (Chen *et al.*, 1999). Aneuploid hybrids derived from 5X × 4X crosses of *S. commersonii* × *S. tuberosum* had lethal freezing temperatures (LT_{50}) that did not differ from those of cultivated potato prior to acclimation, while the LT_{50} of the hybrids following cold acclimation were distributed between the wild and cultivated parental values, with an observed maximum 3°C gain in freezing tolerance (Iovene *et al.*, 2004).

Transformation of Agronomic Crops with *Arabidopsis CBF* Genes Enhances Freezing Tolerance

The CBF (C-repeat-binding factor) transcription factors (also termed DREB1, dehydration-responsive element-binding factor) play an important role in abiotic stress response in *Arabidopsis*, including freezing tolerance and cold acclimation (Gilmour *et al.*, 1998; Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998; Medina *et al.*, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000). A more detailed description of the role(s) of CBFs in plant cold acclimation can be found in several chapters in this volume (chapters 1–4).

CBF genes have been isolated from a wide range of plant species, indicating that the CBF response pathway is probably conserved among most plant species. Constitutive overexpression of *CBF1*, *CBF3* and *CBF4* in *Arabidopsis* induces both the expression of multiple *cor* genes and increase in freezing tolerance without a low-temperature stimulus (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998; Gilmour *et al.*, 2000; Haake *et al.*, 2002). Overexpression of *CBF3* in *Arabidopsis* has been shown to induce the expression of *cor* genes and results in a concomitant increase in freezing, salt and drought tolerance under non-stressed conditions (Liu *et al.*, 1998; Kasuga *et al.*, 1999; Gilmour *et al.*, 2000; Maruyama *et al.*, 2004). Many of the biochemical changes associated with the cold acclimation process are also observed in *CBF3*-overexpressing plants, including an increase in the total soluble sugars glucose, fructose, sucrose and raffinose to levels similar to those of cold-acclimated *Arabidopsis* plants (Gilmour *et al.*, 2000).

Ectopic overexpression of the *Arabidopsis CBFs* has also been conducted in a number of crop plants and resulted in the successful improvement of stress-tolerance characteristics, including cold tolerance (Table 8.1). Due to its rapid and direct mode, in contrast to traditional breeding, genetic engineering with the *Arabidopsis CBF* genes, or *CBF* homologues from other species, has been suggested as an effective and general means of improving stress tolerance in crop plants (Holmberg and Bulow, 1998; Bajaj *et al.*, 1999; Zhang *et al.*, 2000). However, constitutive overexpression of the *Arabidopsis CBF* genes is often associated with undesirable phenotypic alterations of the transgenic plants. For example, while *Arabidopsis* plants constitutively overexpressing *CBF3* have enhanced cold tolerance, both severe growth retardation and a delay in flowering are also observed in unstressed conditions (Liu *et al.*, 1998; Gilmour *et al.*, 2000). Similar growth retardation results have also been observed in transgenic tobacco and tomato plants overexpressing *CBF1 and CBF3* (Hsieh *et al.*, 2002a,b; Kasuga *et al.*, 2004), side effects that are undesirable in crop plants. To address this problem, the stress-inducible *rd29A* promoter has been used to control *CBF* transgene expression. Studies in *Arabidopsis* and tobacco have shown that use of the *rd29A* promoter alleviates these negative effects on plant growth in transgenic plants for the most part (Kasuga *et al.*, 1999, 2004). Together, these results suggest that it is possible to use *CBF* genes to improve freezing tolerance in crops plants while minimizing negative pleiotropic effects.

Table 8.1. Examples of *Arabidopsis CBF*s imparting enhanced cold tolerance to transgenic plants.

Arabidopsis CBF	Promoter[a]	Transgenic species	Phenotypic expression	References
CBF1	35S	*Arabidopsis*	Increased freezing tolerance	Jaglo-Ottosen *et al.* (1998)
CBF1	rd29A, 35S	*Arabidopsis*	Increased cold, drought and salinity tolerance	Kasuga *et al.* (1999)
CBF3	35S	*Arabidopsis*	Increased freezing tolerance	Liu *et al.* (1998), Gilmour *et al.* (2000)
CBF1, CBF2, CBF3	35S	Canola	Increased freezing tolerance	Jaglo *et al.* (2001)
CBF1	35S	Strawberry	Increased freezing tolerance	Owens *et al.* (2002)
CBF1	35S	Tomato	Enhanced water deficit, oxidative and chilling stress tolerance	Hsieh *et al.* (2002a,b)
CBF3	rd29A, 35S	Tobacco	Increased drought and cold stress tolerance	Kasuga *et al.* (2004)
CBF4	35S	*Arabidopsis*	Increased freezing tolerance	Haake *et al.* (2002)

[a] 35S: a constitutive CaMV 35S promoter; rd29A: a stress-inducible *Arabidopsis rd29A* promoter.

As a first step in dissecting the role of a CBF cold response pathway in potato, we transformed two species that contrast in cold tolerance, S. *commersonii* and *S. tuberosum*, with a 35S promoter-driven *AtCBF1* construct, and determined the effect on freezing-tolerance and cold-acclimation potential. We assessed whether *AtCBF1* overexpression leads to improvement of frost-tolerance and/or cold-acclimation potential in each species, if the level of improvement differed, and if any of the phenotypic changes observed in WT plants during cold acclimation were imparted.

Transformation of *S. commersonii* and *S. tuberosum* with *35S::AtCBF1*

We generated transgenic *S. tuberosum* and *S. commersonii* lines overexpressing *AtCBF1* under control of the constitutive CaMV 35S promoter. Explants of leaf and stem from *S. commersonii* PI 243503 (clone 13) and *S. tuberosum* (cv. Umatilla) plants were transformed via *Agrobacterium*-mediated transformation. Putative transgenic-potato shoots were selected *in vitro* based on Kanamycin resistance (50 mg/l), and polymerase chain reaction (PCR) was used to confirm the presence of the transgene in the potato genome for each line.

We identified 13 and 19 PCR-positive clones from *S. commersonii* and *S. tuberosum*, respectively. Northern blot analysis was used to determine which PCR-positive lines were expressing the transgene at a detectable level, and ten *S. commersonii* and three *S. tuberosum* lines expressing the *AtCBF1* transgene were identified. A much higher frequency (10/13) of the *S. commersonii* transgenics expressed detectable *AtCBF1*-transgene levels compared to *S. tuberosum*, where only 3 of 19 lines had detectable levels. Among the *S. commersonii* lines, most accumulated high levels of *AtCBF1* transcript. In contrast, *AtCBF1* transcript levels in the transgenic *S. tuberosum* lines were much lower than the level of the *S. commersonii* lines. The difference between the two species in recovery efficiency of *AtCBF1* transgene-expressing lines is currently unknown, but suggests that *S. commersonii* is better able to tolerate any negative side effects of increased *AtCBF1* expression than *S. tuberosum* cv. Umatilla; extending this to additional cultivars will determine if this observation is particular to the cv. Umatilla genotype.

Ectopic Overexpression of *AtCBF1* in *S. commersonii* Results in Altered Plant Morphology

Similar to previous studies evaluating *AtCBF* overexpression in foreign species (Jaglo *et al.*, 2001; Hsieh *et al.*, 2002a), the majority of the transgenic *S. commersonii* lines displayed visible phenotypic abnormalities relative to WT plants. Varying degrees of growth retardation were noted among the transgenic lines when grown in tissue culture. Following transplantation to soil and growth under greenhouse conditions, many of the lines recovered and grew to a similar height as those of WT plants (Fig. 8.1). This result suggests that some of the initial growth alterations may have been due to the effects of the tissue-culture growth conditions. Nevertheless, several independent transgenic lines continued to show severe growth retardation, even after a long growth period in the greenhouse, confirming that ectopic *AtCBF1* expression can also yield stable pleiotropic effects in *S. commersonii*; phenotypic alterations in flowers, leaf colour and leaf shape were also observed (data not shown). Significant phenotypic alterations or negative pleiotropic growth effects among the three transgenic *35S::AtCBF1 S. tuberosum* lines were not observed (data not shown). This may be due to the low *AtCBF1*-expression levels in these transgenic *S. tuberosum* lines, relative to the substantially higher levels seen in *S. commersonii*.

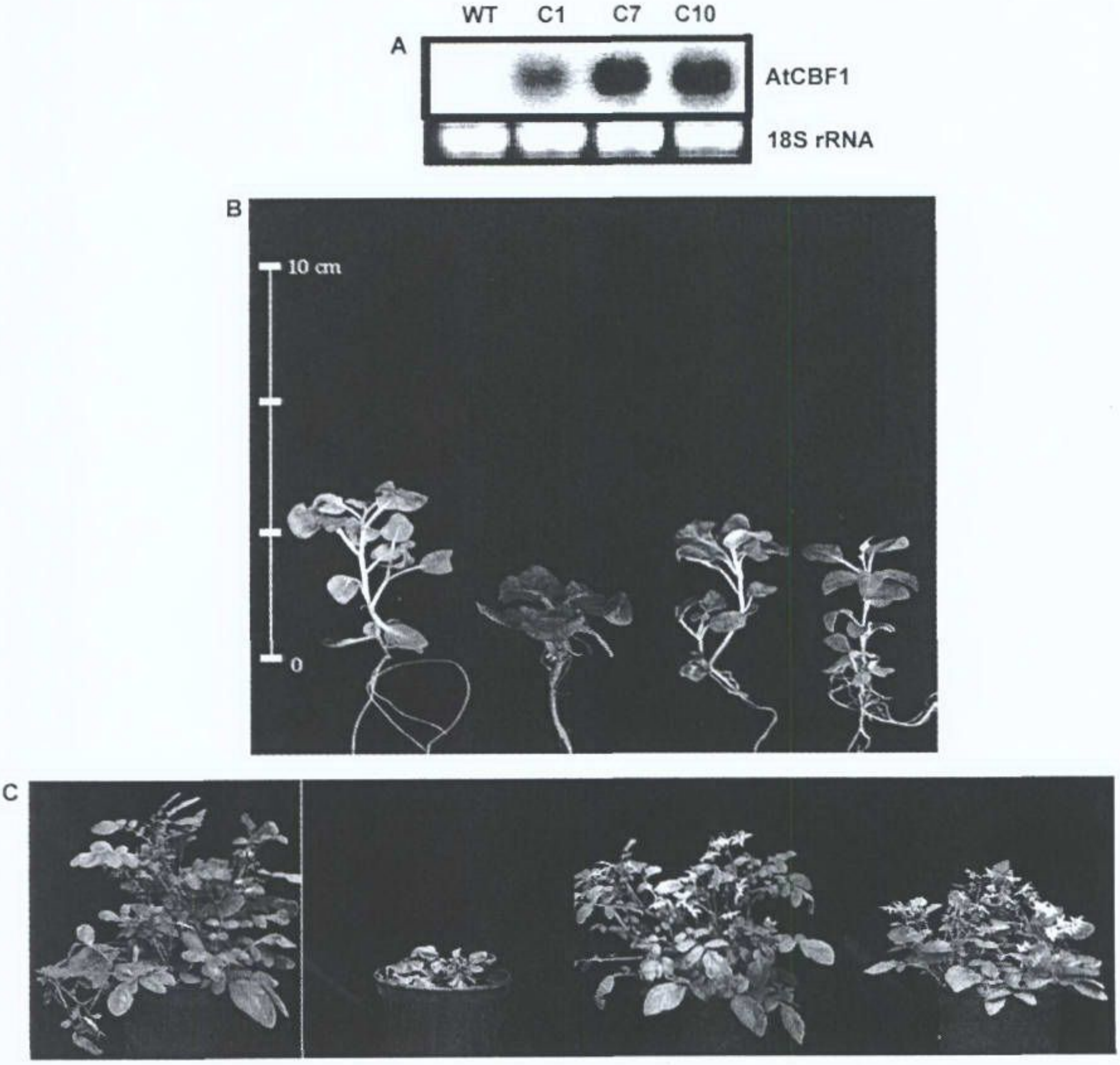

Fig. 8.1. Examples of phenotypic variations in wild-type (WT) and *AtCBF1*-overexpressing transgenic *S. commersonii*. (A) Northern blot analysis. (B) WT and *AtCBF1*-transgenic plants grown in tissue culture. (C) Twelve-week-old WT and *AtCBF1*-transgenic plants grown under greenhouse conditions. From left to right, WT and transgenic lines C1, C7 and C10.

Ectopic Overexpression of *AtCBF1* Enhances Freezing Tolerance

WT and transgenic *S. tuberosum* and *S. commersonii* plants were grown at either 25°C or cold acclimated for 2 weeks at 2°C, and freezing tolerance evaluated using a controlled freezing test (Jaglo-Ottosen *et al.*, 1998; Gilmour *et al.*, 2000). Freezing tolerance (expressed as LT_{50}) for WT *S. commersonii* plants was −6°C and −10°C, prior to and after 2 weeks of cold acclimation, respectively (Fig. 8.2). In contrast, WT plants of *S. tuberosum* had an LT_{50} of −3°C under both conditions, verifying that *S. tuberosum* is more sensitive to cold and fails to undergo cold acclimation (data not shown).

Under warm (non-acclimating) conditions, transgenic *S. commersonii* lines were from 2°C to 4°C cold hardier than WT plants (Fig. 8.2). Following 2 weeks of cold acclimation at 2°C, these transgenic lines were also hardier than the cold-acclimated WT plants. These results indicate that the constitutive overexpression of *AtCBF1* in *S. commersonii* increased both freezing-tolerance and cold-acclimation potential (Fig. 8.2). The results also suggest that an endogenous *CBF* pathway is involved in this process in *S. commersonii*, and that the constitutive *AtCBF1* activity is artificially stimulating and enhancing the pathway. We also determined LT_{50} values in WT and two of the transgenic lines cold acclimated at 2°C from 0 to 21 days, and found that both WT and transgenic plants displayed a linear increase in freezing tolerance that correlated with the length of cold acclimation at 2°C ($r^2 = 0.85$, $P \leq 0.0001$, from regression analysis). A similar exper-

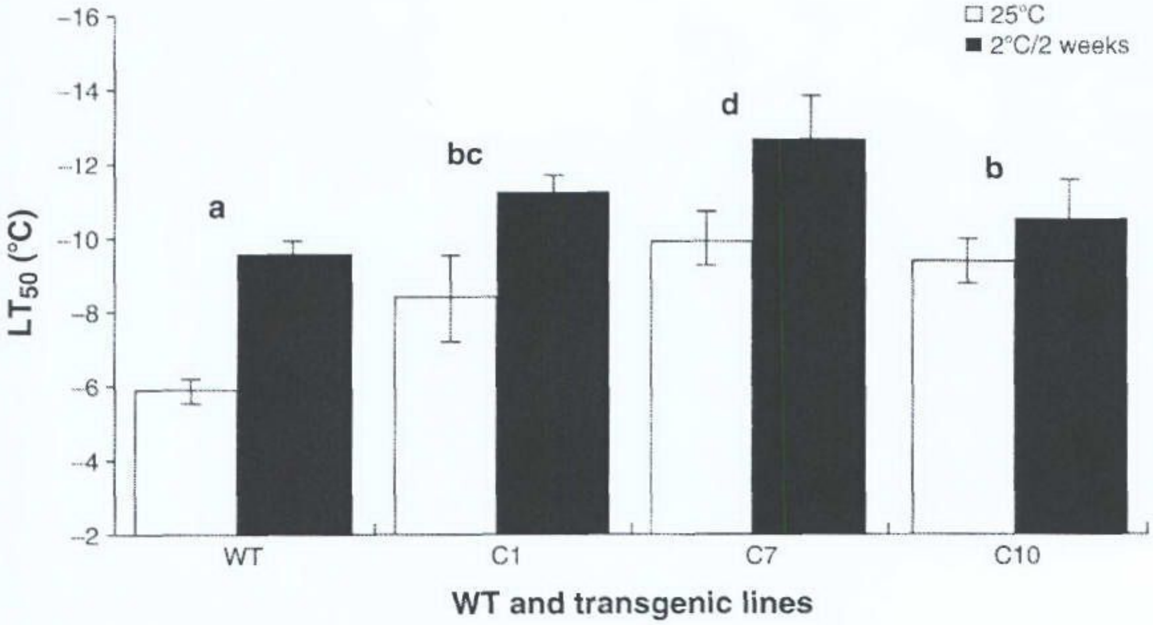

Fig. 8.2. Effect of *AtCBF1* overexpression on freezing tolerance of *S. commersonii*. LT_{50} (−°C) of wild-type (WT) and *35S::AtCBF1* transgenic lines (C1, C7, C10) growing at 25°C and after 2 weeks' cold acclimation at 2°C. Different letters indicate significant differences among lines ($P \leq 0.0001$) according to Duncan's multiple range test.

iment was conducted using WT and transgenic *S. tuberosum* plants, and transgenic lines were about 2°C cold hardier than WT plants both before and after cold acclimation (data not shown). This indicated that overexpression of *AtCBF1* increased freezing tolerance, but did not impart a cold-acclimation response to *S. tuberosum* plants. This also suggests that the freezing tolerance property of *S. tuberosum* may be responsive to enhanced levels of CBF activity.

Overexpression of *AtCBF1* in *S. commersonii* Results in Anatomical and Ultrastructural Changes in Leaves

Light microscopy cross-sectional analysis of transgenic *S. commersonii* leaves revealed both increased leaf thickness and an altered pattern of palisade and sponge cell distribution (Fig. 8.3). Transgenic plants showed significantly thickened leaves, enlarged intercellular spaces and elongated palisade cells when grown under both warm- and cold-acclimating conditions. Similar alterations were observed in WT leaves following cold acclimation, suggesting that the post-acclimation changes are related to an endogenous *CBF* activity and that constitutive *AtCBF1* expression under warm conditions in *S. commersonii* mimics the cold-acclimation effect (Fig. 8.3). In contrast, a comparative cross-sectional analysis of WT and transgenic *S. tuberosum* leaves did not show significant variations in the leaf traits mentioned above. While leaves of some *S. tuberosum* transgenic lines were thicker than WT leaves, the differences were not significant between the unacclimated and cold-acclimated plants (Fig. 8.4).

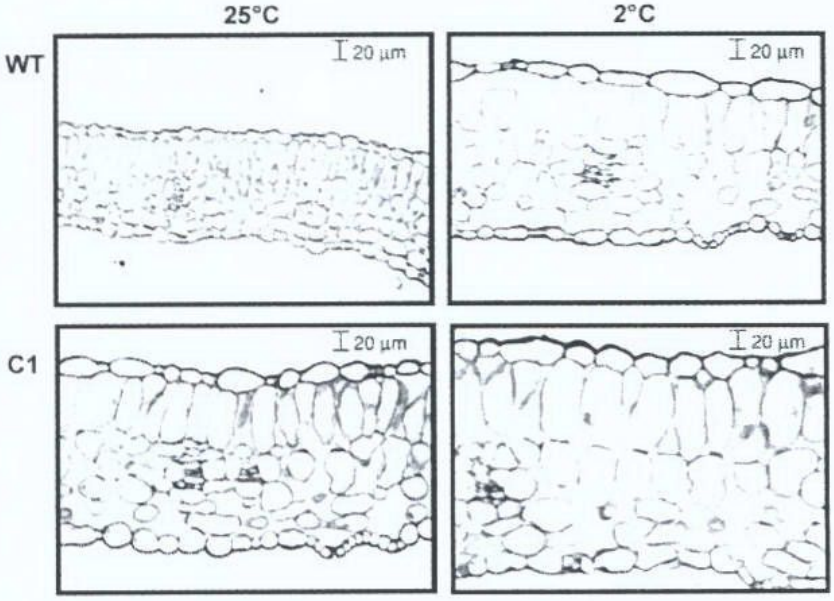

Fig. 8.3. Leaf cross sections from wild-type (WT) and *S. commersonii* transgenic-line C1 plants. Cross sections were prepared from leaves of WT and *AtCBF1*-overexpressing line C1 grown at 25°C and after 2 weeks of cold acclimation at 2°C. Light micrographs were taken at 400×.

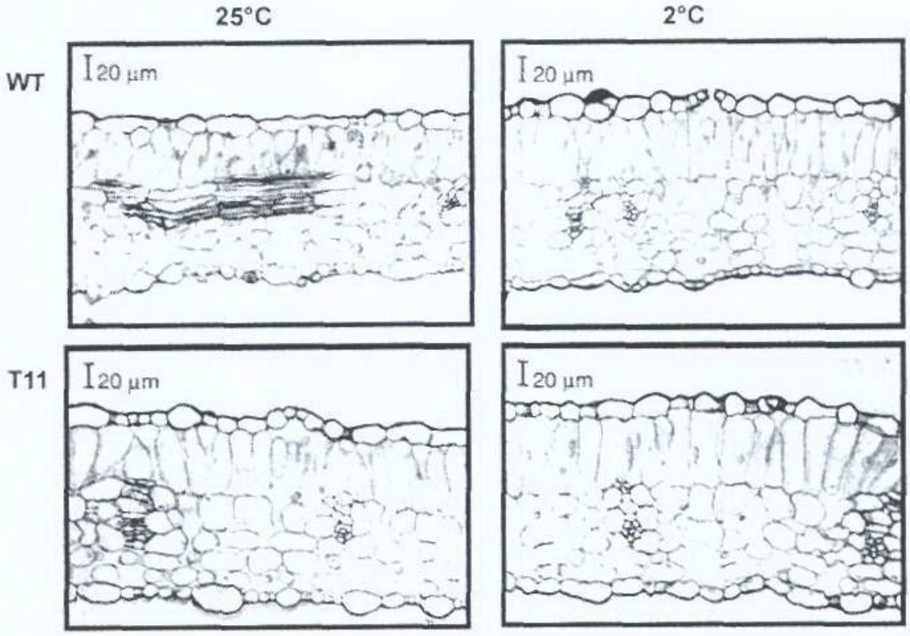

Fig. 8.4. Leaf cross sections from wild-type (WT) and *S. tuberosum* transgenic-line T11 plants. Cross sections were prepared from leaves of WT and *AtCBF1*-overexpressing line T11 grown at 25°C and after 2 weeks of cold acclimation at 2°C. Light micrographs were taken at 200×.

Transmission electron microscopy (TEM) conducted on the palisade and spongy mesophyll cells of transgenic lines revealed that, relative to WT, transgenics had an increase in chloroplast number, and decreases in the oil body number and both the size and number of starch grains (Fig. 8.5). Scanning EM analysis of the S. *commersonii* adaxial (top) and abaxial (bottom) leaf surfaces under non-acclimating conditions showed an increase in stomata per cm^2 and altered stomata morphology. This was especially true for the adaxial leaf surface, where an approximately threefold increase in stomata had occurred (data not shown).

Biochemical Changes Associated with Cold Acclimation are Observed in *AtCBF1*-Transgenic *S. commersonii*

In *Arabidopsis*, proline and sugar levels increase during cold acclimation (Wanner and Junttila, 1999). An increase in proline and sugar levels is also observed under non-acclimating conditions for *Arabidopsis* plants ectopically overexpressing *CBF3* (Gilmour *et al*., 2000). We observed similar results in WT and *35S::AtCBF1* transgenic *S. commersonii* (Fig. 8.6). Both proline and sugar content increase in WT *S. commersonii* following 2 weeks of cold acclimation. In unacclimated transgenic *S. commersonii*, proline content increased more than twofold relative to WT plants. Following 2 weeks of cold acclimation, the proline content of transgenic *S. commersonii* increased above the pre-acclimation levels. Similar results were

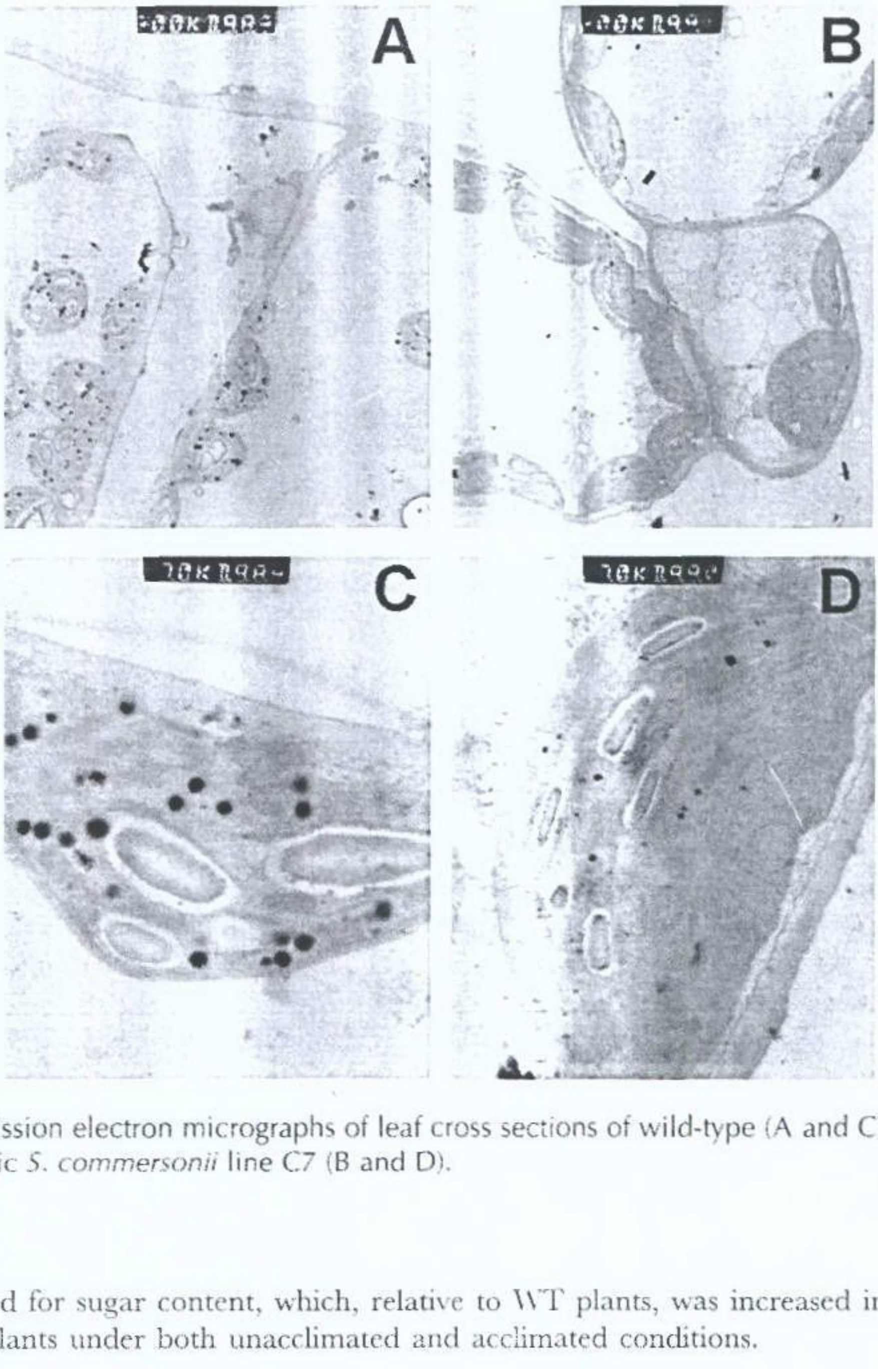

Fig. 8.5. Transmission electron micrographs of leaf cross sections of wild-type (A and C) and *AtCBF1*-transgenic *S. commersonii* line C7 (B and D).

observed for sugar content, which, relative to WT plants, was increased in transgenic plants under both unacclimated and acclimated conditions.

S. commersonii Plants Overexpressing *AtCBF1* Showed Higher Photosynthetic Capacity

We noticed that leaves of *S. commersonii AtCBF1*-overexpressing plants were generally greener than WT leaves when grown side by side, and that *AtCBF1*-overexpressing lines tend to have a higher chloroplast number per cell (Fig. 8.5). We thus compared total chlorophyll content of WT plants with one of the transgenic lines (C7) under both non-acclimated and acclimated conditions (Fig. 8.7B). While total chlorophyll (Chl *a* + *b*) content was indeed higher in the unacclimated leaves

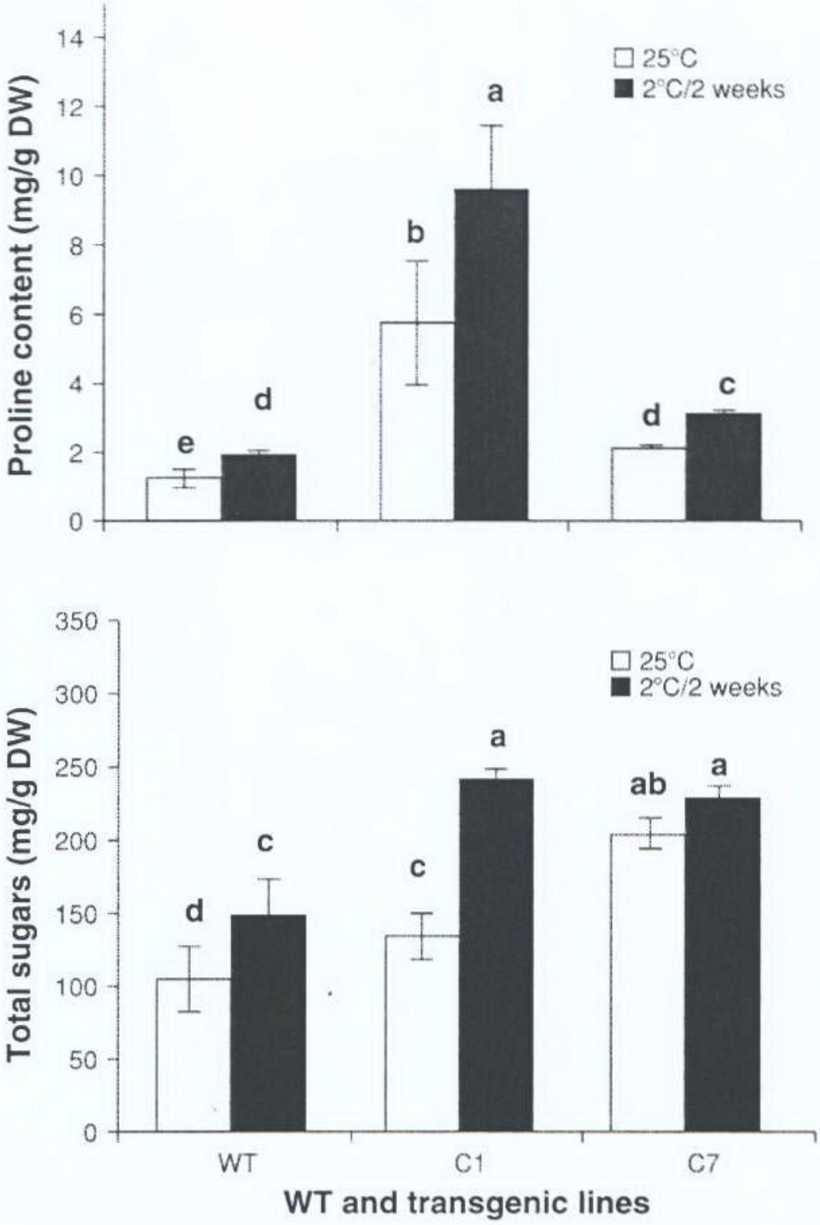

Fig. 8.6. Effect of *AtCBF1* overexpression on proline and total sugar content of *S. commersonii*. Proline and sugar content were determined from leaf tissue of wild-type (WT) and *AtCBF1*-overexpressing lines C1 and C7 grown at 25°C and after 2 weeks' cold acclimation at 2°C. Different letters indicate significant differences among lines and treatments ($P \leq 0.05$) according to Duncan's multiple range test.

of the *AtCBF1*-transgenic line relative to WT, the process of cold acclimation did not lead to a further alteration of the total chlorophyll content in either background.

Based on our observations, including the altered leaf anatomy and ultrastructural changes, we suspected that photosynthetic activities were also altered in *AtCBF1*-overexpressing lines. We compared a variety of photosynthetic parameters between WT and *AtCBF1*-overexpressing plants under both non-acclimated and acclimated conditions (Fig. 8.7). Transpiration rates were comparable between

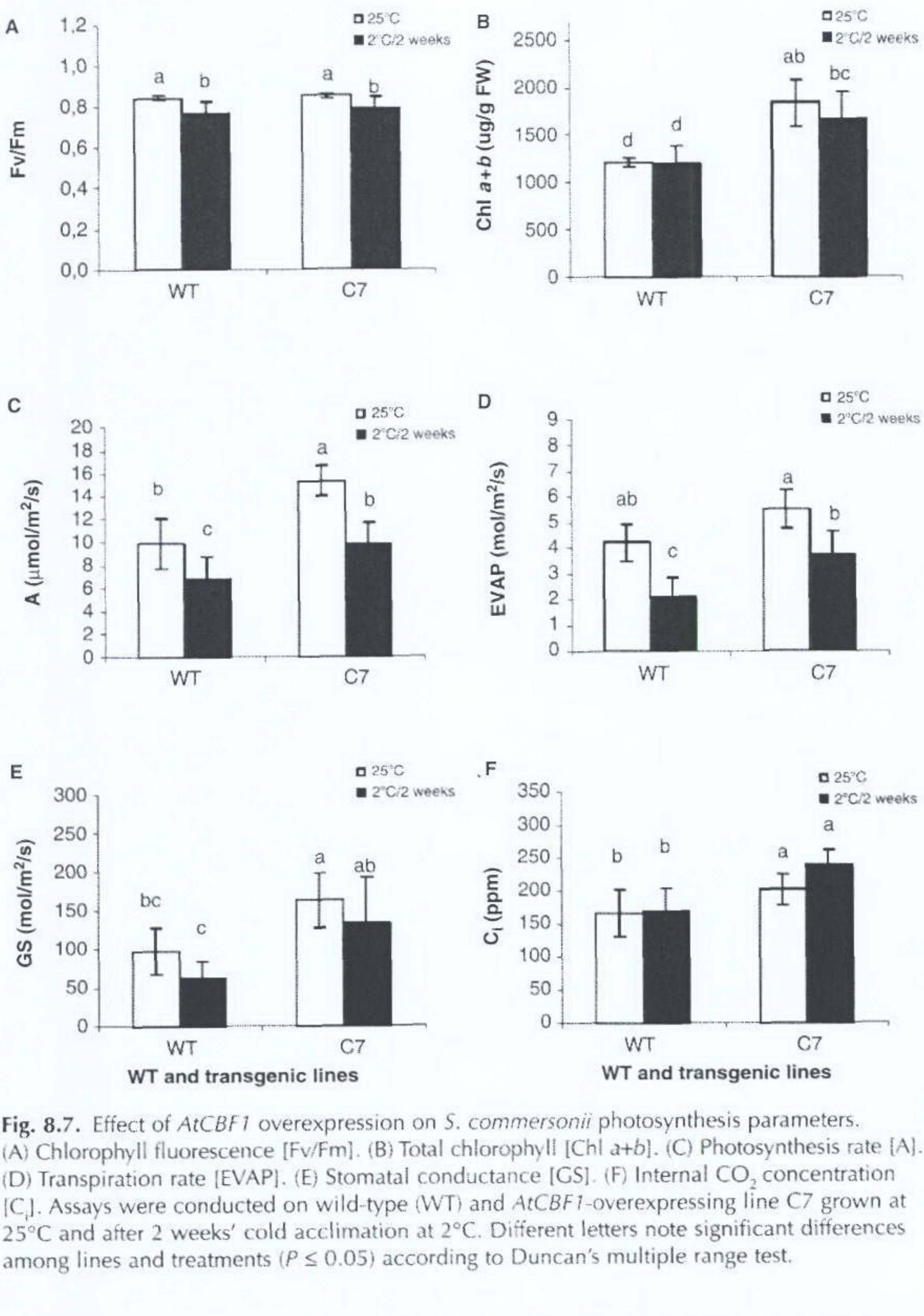

Fig. 8.7. Effect of *AtCBF1* overexpression on *S. commersonii* photosynthesis parameters. (A) Chlorophyll fluorescence [Fv/Fm]. (B) Total chlorophyll [Chl *a*+*b*]. (C) Photosynthesis rate [A]. (D) Transpiration rate [EVAP]. (E) Stomatal conductance [GS]. (F) Internal CO_2 concentration [C_i]. Assays were conducted on wild-type (WT) and *AtCBF1*-overexpressing line C7 grown at 25°C and after 2 weeks' cold acclimation at 2°C. Different letters note significant differences among lines and treatments ($P \leq 0.05$) according to Duncan's multiple range test.

WT and transgenic plants (Fig. 8.7D), and while both WT and *AtCBF1*-overexpressing lines are slightly photo-inhibited following cold acclimation, based on the decreased Fv/Fm ratio (Fig. 8.7A), the effect is not significantly different. Increases were found for photosynthetic rate (Fig. 8.7C), stomatal conductance (Fig. 8.7E) and internal CO_2 concentration (Fig. 8.7F) of transgenic plants relative to WT. These results suggest that *AtCBF1* overexpression results in an enhanced photosynthetic capacity of the transgenic plants.

Conclusions

Ectopic overexpression of *AtCBF1* significantly increased the freezing-tolerance and cold-acclimation potential of *S. commersonii*. In contrast, in *S. tuberosum*, while *AtCBF1* overexpression increased freezing tolerance, it failed to impart a cold-acclimation response to the plant. These results suggest that an endogenous *CBF* pathway is present in both plant systems and that enables the ectopic *AtCBF1* transgene expression to influence the plants' freezing-tolerance capacity. A *CBF*-based cold-acclimation pathway also appears to be present in *S. commersonii* and is affected by *AtCBF1*. This acclimation pathway appears to be disrupted in *S. tuberosum*, at some point past the *CBF* signal, as introduction of the constitutive presence of an active *CBF* failed to impart an acclimation capacity on this species.

Cold acclimation caused marked alterations in the leaf anatomy of *S. commersonii*. These included thickened leaves with smaller epidermal cells, disorganized palisade and spongy mesophyll cell layers, and increased intercellular spaces. Similar changes were not observed in *S. tuberosum* plants, which failed to cold-acclimate, suggesting that the *S. commersonii* changes are related to the acclimation process. Interestingly, we also observed these same anatomical changes in unacclimated *S. commersonii* plants overexpressing *AtCBF1*. This suggests that the cold-induced structural changes are an associated component of acclimation-based freezing-tolerance enhancement, and may be a product of the induction of endogenous *CBF* activity during the acclimation process. In addition to effects on freezing tolerance, *AtCBF1* overexpression also alters traits related to photosynthesis in *S. commersonii*. These included an increased chloroplast number and alterations of chloroplast structure and chlorophyll content, which collectively lead to an alteration of photosynthetic properties. Taken together, our results suggest that constitutive overexpression of *AtCBF1* in potato can enhance freezing tolerance. However, distinct morphological and physiological alterations are associated with this enhancement, which could have a negative contribution to the plant relative to desirable crop characteristics. We are currently investigating whether use of a stress-inducible promoter, as well as other *CBF* genes, can retain the beneficial gains in freezing tolerance, without the associated negative phenotypic alterations.

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Recomendación Tecnica a INIA La Platina

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En base a los antecedentes, Dr Tony H.H. Chen evaluó la situación actual de este tema en Chile como lo que sucedía 20 años atrás en los EEUU; con la ventaja que a nivel mundial existen muchos resultados y técnicas que hace 20 años atrás no existían y podrían ser aplicadas en Chile. Sin necesariamente comenzar de cero. Enfatizó el hecho de aprovechar la tecnología y avances que ya existen en esta área a nivel mundial.

Asimismo, y sobre la base de la implementación en laboratorio que cuenta tanto el INIA como las universidades ratificó la aplicabilidad de estas técnicas en Chile, en particular aprovechando la plataforma de transformación en vides que ha desarrollado INIA. Asimismo, destacó la necesidad, de abordar temas aun en desarrollo en Chile como los microarrays u otras técnicas.

En relación a los estreses y especies; estreses como sales y heladas deberían tener prioridad sin descartar otros estreses como sequía. En relación a las especies. Dr. Chen recomendó iniciar un estudio de este tipo en solanaceas y en frutales como vides y carozos aprovechando la plataforma de transformación que ha desarrollado INIA. En solanaceas, recomendó seguir con el tema de CBF en papas Chilenas y en otras solanaceas que sean de interés para Chile

En vides y resistencia salinidad, recomendó buscar primero resistencia en patrones chilenos (germoplasma) y paralelamente evaluar la transformación de patrones comerciales con genes que causan resistencia a salinidad. Entre los genes a evaluar indicó que existen varios circulando; entre los cuales están los TF de Arabidopsis y CodA; los cuales indicó estaría dispuestos a prestarlos con motivos de investigación y después de la búsqueda de un proyecto de investigación.