FBT01-1-001

INFORME DE DIFUSIÓN
PROGRAMA FORMACION PARA LA PARTICIPACION

1 Nombre de la propuesta :

"Formación de profesionales en técnicas de genómica funcional"

1.1 Modalidad

Pasantías de formación

1.2 Lugar donde se llevo a cabo la formación

Francia. Perpignan (estadía 1)
Bélgica. Gent (estadía 2)

1.3 Rubro / Area temática de la actividad de formación

Actividades de formación en Biotecnología Silvoagropecuaria

1.4 Fecha en la que se efectúo la actividad de formación:

20 de Octubre 2001- 01 de Marzo 2002 (estadía1)
15 de Octubre 2001- 01 de Abril 2002 (estadía2)

1.5 Postulante

Paula Andrea Salinas Salvo (estadía1)
María Francisca Blanco Herrera (estadía 2)

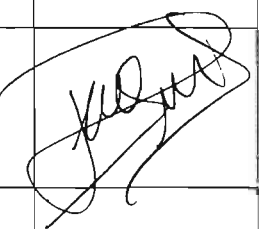
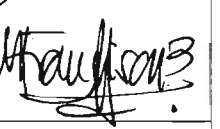
1.6 Entidad Responsable

Pontificia Universidad Católica de Chile

1.7 Coordinador

Dra María Loreto Holuigue Barros

1.8 Identificación de los participantes de la propuesta

NOMBRE	RUT	TELEFONO FAX E-MAIL	DIRECCION POSTAL	ACTIVIDAD PRINCIPAL	FIRMA
Paula Andrea Salinas Salvo	13.011.788-0	2385749 2225515 psalinsa@genes.bio.puc.cl	Av Grecia 2510. Depto 406. Ñuñoa	Estudiante de Post grado	
María Francisca Blanco Herrera	13.443.370-1	7331255 2225515 mfblanco@puc.cl	Camino Santa Sara Condominio Tunquelen parc.19. Batuco	Tesista de pregrado	

2. ACTIVIDADES DE TRASFERENCIA

2.1. Resumen actividades de transferencia PROPUESTAS

FECHA	ACTIVIDAD	OBJETIVO	LUGAR	Nº y TIPO BENEFICIARIOS
Marzo 2002	Seminarios departamentales.	Presentación oral de las técnicas de genómica aprendidas por ambas alumnas.	Departamento de Genética Molecular y Microbiología de la Pontificia Universidad Católica de Chile.	25 personas. Estudiantes de pre y post grado del área ciencia, doctores en ciencias.
Mayo 2002	Reunión anual del grupo de plantas de la Pontificia Universidad Católica de Chile y Universidad de Chile.	Presentación oral de las técnicas de genómica aprendidas y los resultados aplicados a las líneas de investigación de ambas alumnas.	Salón de Seminarios de la Facultad de Ciencias de la Universidad de Chile.	60 personas Estudiantes de pre y post grado del área ciencia, doctores en ciencias.
Octubre 2002	Reunión Anual de la Sociedad de Bioquímica de Chile	Presentación oral de las técnicas de genómica aprendidas y los resultados aplicados a las líneas de investigación de ambas alumnas.	No establecido aún.	500 personas Estudiantes de pre y post grado del área ciencia, doctores en ciencias.

2.1. Resumen actividades de trasferencia REALIZADAS

FECHA	ACTIVIDAD	OBJETIVO	LUGAR	Nº y TIPO BENEFICIARIOS
Marzo 2002	Seminarios departamentales.	Presentación oral de las técnicas de genómica aprendidas por ambas alumnas.	Departamento de Genética Molecular y Microbiología de la Pontificia Universidad Católica de Chile.	25 personas. Estudiantes de pre y post grado del área ciencia, doctores en ciencias.

2.2. Detalle por actividad de transferencia REALIZADAS

Fecha:28 de febrero - 2002

Lugar (Ciudad e Institución)

Universitè Perpignan, Perpignan, Francia.

Exposición oral en ingles al laboratorio del Dr. Manuel Echeverría . Se discutió acerca de los resultados obtenidos, las técnicas aplicadas tanto del *footprinting in vivo* como de la expresión transciente de proteínas de fusión construidad tambien en la estadía en su laboratorio.

Fecha:4 de Abril - 2002

Lugar (Ciudad e Institución)

Santiago. Pontificia Universidad Católica de Chile

Exposición oral al grupo de la Dra. Loreto Holuigue de los resultados obtenidos en el laboratorio de Francia. Descripción detallada de la técnica de *Footprinting in vivo* y planificación del desarrollo de esta técnica en nuestro laboratorio.

Fecha:11 de Abril - 2002

Lugar (Ciudad e Institución)

Santiago. Pontificia Universidad Católica de Chile

Exposición oral utilizando transparencias de los resultados obtenidos en Francia y detalla de la técnica de footprinting in vivo en el departamento de Genética molecular y Microbiología a el grupo de biología molecular de plantas dirigidos por los doctores Xavier Jordana, Patricio Arce y Loreto Holuigue.

Fecha:29 de Marzo - 2002

Lugar (Ciudad e Institución)

Universidad de Gent. Bélgica

Exposición en inglés del montaje de la técnica de AFLP-TP en *Oryza sativa* bajo el contexto de la interacción entre etileno e hipoxia generada por tratamientos de submersión y discusión de los resultados preliminares en el laboratorio de Genética Molecular de la Dra Dominique Van der Straeten.

Fecha:18 de Abril - 2002

Lugar (Ciudad e Institución)

Santiago. Pontificia Universidad Católica de Chile

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Actividad (en este punto explicar con detalle la actividad realizada y mencionar la información entregada)

Exposición oral de la técnica de AFLP-TP (amplified fragment length polymorfism based transcript profiling) y de los resultados preliminares obtenidos en el contexto del estudio de la via de señalización temprana del Acido Salicílico en *Arabidopsis thaliana.* Departamento de Genética molecular y Microbiología a el grupo de biología molecular de plantas dirigidos por los doctores Xavier Jordana, Patricio Arce y Loreto Holuigue

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FUNDACIÓN PARA LA
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2.2. Especificar el grado de éxito de las actividades propuestas, dando razones de los problemas presentados y sugerencias para mejorar.

La técnica de genómica funcional, AFLP-TP presentada en las actividades de difusión fue de gran éxito entre la concurrencia. Es una técnica relativamente nueva (1996) en la cual se obtiene información acerca de la expresión genómica completa, seguido del análisis de todos los genes involucrados en un proceso biológico particular o expresado bajo ciertas condiciones. La idea principal es implementar la técnica en nuestro laboratorio y mostrándo luego los resultados, expandirla a otros sistemas biológicos y a otros temas.

El público asistente a las actividades de difusión se mostró muy interesado y la discusión sostenida a continuación fue enriquecedora y en su mayoría, inquietudes respecto de la técnica, aplicabilidad, protocolos, material y análisis.

Por otro lado, el análisis de las secuencias reguladoras de los genes que permite la expresión génica diferencial es otro punto esencial en el estudio de los procesos biológicos. En este sentido la técnica de foortprinting in vivo permite realizar un análisis real in vivo de la unión de proteínas a las secuencias promotoras. En las presentaciones realizadas se analizaron los resultados y la realización del experimentos en si, por lo que discutió acerca de la elección de la región promotora en estudio, de la importancia de cada paso de la técnica y cuan realizable es este tipo de experimentos en Chile. Tambien se discutió el diseño de oligonucleótidos nuevos que pudieran dar un resultado favorable para el estudio del promotor de la glicosil transferasa y el análisis de los promotores de otros genes de expresión temprana en la vía del ácido salicilico.

La audiencia pudo apreciar la importancia y utilidad de esta técnica enfocadas a sus propios temas de estudio, es asi como se hablo de la posibilidad de realizar este tipo de estudios en sus modelos vegetales de trabajo. Considerando el interes de la audiencia pienso que el exito de la presentación fue bastante alto.

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2.3. Listado de documentos o materiales mostrados en las actividades y entregados a los asistentes (escrito y/o visual). (Se debe adjuntar una copia del material)

Tipo de material	Nombre o identificación	Idioma	Cantidad
Presentación Power Point	Estudio de la Via de Señalización Temprana de SA utilizando **AFLP-TP** en *Arabidopsis thaliana*	Inglés-español	1
Presentación Power Point	Análisis de **Foot printing** *in vivo* del promotor de Glicosil transferasa en *Arabidopsis thaliana*	Inglés-españo	1

3. ASPECTOS ADMINISTRATIVOS

Indicar los problemas administrativos que surgieron en la preparación y realización de las actividades de difusión.

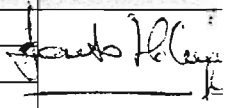
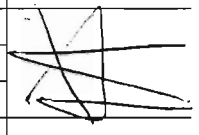
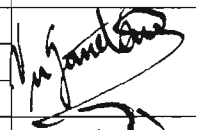
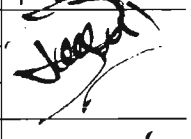
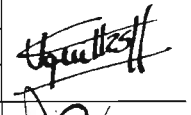
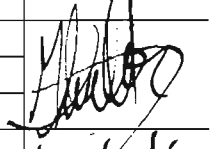
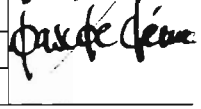
No surgió ningún problema administrativo para la realización de las actividades

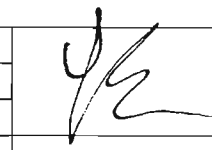
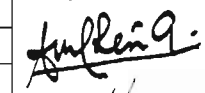
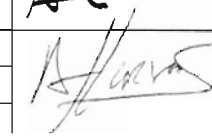
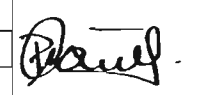
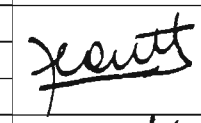
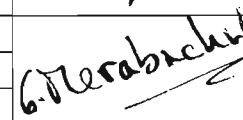
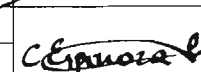
Fecha: _22/Mayo/ 2002_

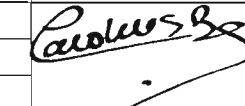
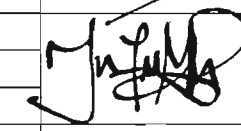
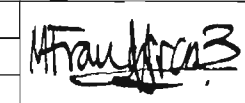
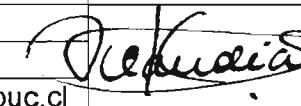
Firma responsable de la ejecución: _____

GOBIERNO DE CHILE
FUNDACIÓN PARA LA INNOVACIÓN AGRARIA

ASISTENTES A ACTIVIDAD DE DIFUSIÓN

Nombre	Actividad Principal	Institución o Empresa	Teléfono Fax e-mail	Firma
Mª Loreto Holuigue Barros	Profesor asistente	Pontificia Universidad Católica de Chile	6862895 2225515 lholuig@genes.bio.puc.cl	
Xavier Jordana de Buen	Profesor asistente	Pontificia Universidad Católica de Chile	6862668 2225515 xjordana@genes.bio.puc.cl	
Patricio Arce	Profesor adjunto	Pontificia Universidad Católica de Chile	6862897 2225515 parce@genes.bio.puc.cl	
Virginia Garretón	Doctor	Pontificia Universidad Católica de Chile	6862901 2225515 vbgarret@genes.bio.puc.cl	
Paula Salinas	Tesista doctorado	Pontificia Universidad Católica de Chile	6862901 2225515 psalinsa@genes.bio.puc.c	
Carolina Uquillas	Tesista doctorado	Pontificia Universidad Católica de Chile	6862894 2225515 cuquilla@genes.bio.puc.c	
Luis González	Post doctorante	Pontificia Universidad Católica de Chile	6862894 2225515 lgonzale@genes.bio.puc.cl	
Mauricio Quiroz	Coinvestigador	Pontificia Universidad Católica de Chile	6862894 2225515 mjquiroz@hotmail.com	
Pascale Clement	Post doctorante	Pontificia Universidad Católica de Chile	6862894 2225515 pclement@genes.bio.puc.cl	

Ingrid Letelier	Alumno doctorado	Pontificia Universidad Católica de Chile	6862894 2225515 letelie@genes.bio.puc.cl	
Gabriel León	Alumno doctorado	Pontificia Universidad Católica de Chile	6862894 2225515 gleon@genes.bio.puc.cl	
Alvaro Elorza	Tesista doctorado	Pontificia Universidad Católica de Chile	6862894 2225515 aelorza@genes.bio.puc.cl	
Isabel Gómez	Tecnólogo médico	Pontificia Universidad Católica de Chile	6862894 2225515 mgomez@genes.bio.puc.cl	
Paola Cañón	Tesista pregrado	Pontificia Universidad Católica de Chile	6862897 2225515 pcanong@puc.cl	
Felipe Aquea	Licenciado en Biología	Pontificia Universidad Católica de Chile	6862897 2225515 jaquea@puc.cl	
Nicole Ehrenfeld	Tesista doctorado	Pontificia Universidad Católica de Chile	6862894 2225515 kehrenfe@puc.cl	
Geneviev Merabachvili	Alumno doctorado	Pontificia Universidad Católica de Chile	6862894 2225515 gmerabac@genes.bio.puc.cl	
Hannetz Roschzttardtz	Alumno doctorado	Pontificia Universidad Católica de Chile	6862901 2225515 Hannetz_r@hotmail.coml	
Carmen Espinoza	Alumno doctorado	Pontificia Universidad Católica de Chile	6862897 2225515 cespinc@hotmail.com	

Consuelo Medina	Coinvestigador	Pontificia Universidad Católica de Chile	6862579 2225515 Parce2@genes.bio.puc.cl	
Carolina Serrano	Tesista doctorado	Pontificia Universidad Católica de Chile	6862579 2225515 clserran@puc.cl	
Tomás Matus	Biologo	Pontificia Universidad Católica de Chile	6862579 2225515 jtmatus@puc.cl	
Francisca Blanco	Tesista pregrado Bioquímica	Pontificia Universidad Católica de Chile	6862901 2225515 mfblanco@puc.cl	
Claudia Stange	Tesista doctorado	Pontificia Universidad Católica de Chile	6862579 2225515 cstange@genes.bio.puc.cl	

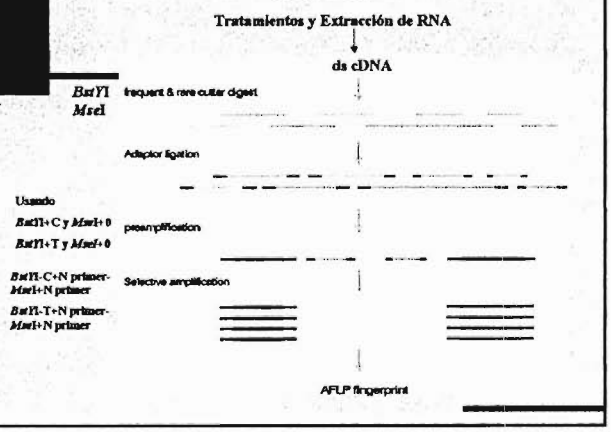
Estudio de la Vía de Señalización Temprana de SA usando AFLP- Transcript Profiling en *Arabidopsis thaliana*

Mª Francisca Blanco

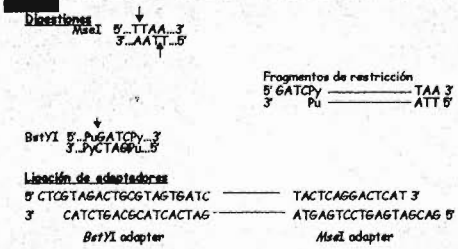
Estrategia Experimental

- Plantas de Arabidopsis thaliana WT de 15 días de edad crecidas en medio MS sólido.
- Tratamiento:Planta completa fue puesta a flotar solución de SA 0.5 mM por los siguientes tiempos: 15min, 30min, 1hr, 2.5hr y 5hr. Los respectivos controles por los mismos tiempos en solución MS.
- Preparación de RNA (método Trizol)
- AFLP-TP.Amplified Fragment Lenght Polymorphism- based transcript profiling.

AFLP- TP

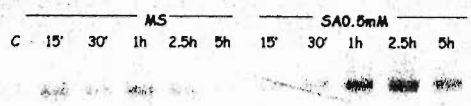


cDNA-AFLPs: principios



Chequear la activación de la vía temprana por el tratamiento de SA 0.5 mM

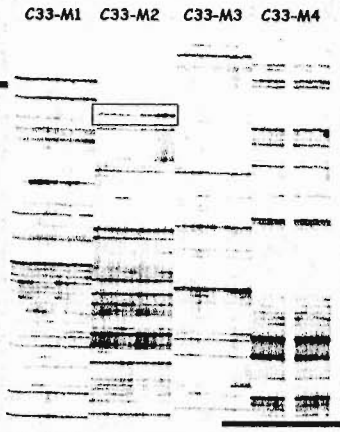
Northen utilizando sonda GST 6



Gel de cDNA-AFLP

BstC+33-Mse+1
BstC+33-Mse+2
BstC+33-Mse+3
BstC+33-Mse+4

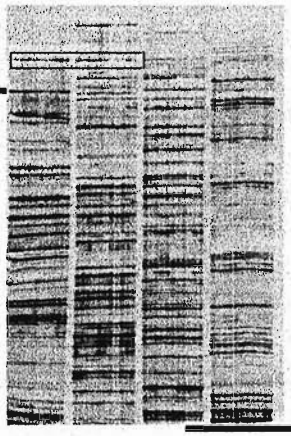
Carriles:
1.-Control (15days old)
2.-MS 15'
3.-MS 30'
4.-MS 1h
5.-MS 2.5h
6.-MS 5h
7.-SA 0.5mM 15'
8.-SA 0.5mM 30'
9.-SA 0.5mM 1h
10.-SA 0.5mM 2.5h
11.-SA 0.5mM 5h



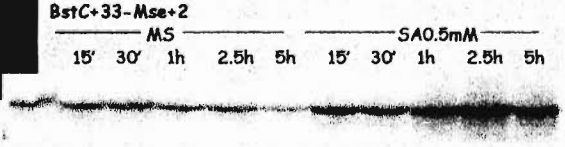
Gel de cDNA-AFLP

BstT+33-Mse+1
BstT+33-Mse+2
BstT+33-Mse+3
BstT+33-Mse+4

Carriles:
1.-Control (15days old)
2.-MS 15'
3.-MS 30'
4.-MS 1h
5.-MS 2.5h
6.-MS 5h
7.-SA 0.5mM 15'
8.-SA 0.5mM 30'
9.-SA 0.5mM 1h
10.-SA 0.5mM 2.5h
11.-SA 0.5mM 5h

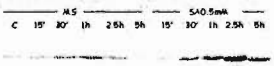


BstC+33-Mse+2



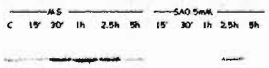
GST 25 upregulated
Plant Phisiol 1993,102: 1193-1201

BstT+33-Mse+1



12-oxophytodienoate reductase (OPR1) upregulated

BstT+33-Mse+2



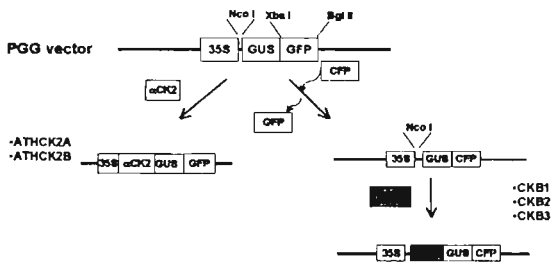
Cytochrome B6 downregulated

Resultados

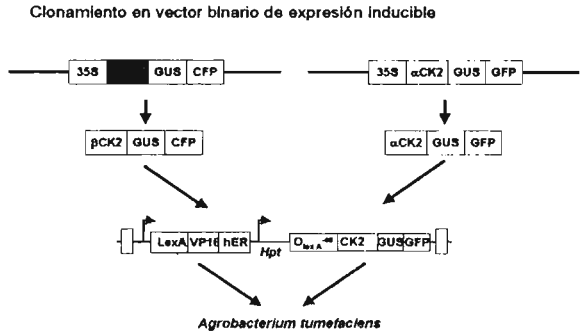
BstC24- Mse2	447	At3g10930	Unknown protein	Upregulated
BstT42- Mse1	279	At2g37710	Putative receptor-like protein kinase	Upregulated
BstT42- Mse2	190	At1g05560	UDP-Glucosyltransferase	Upregulated
BstT42- Mse4	319	At2g43820	Putative glycosyltransferase	Upregulated
BstT42- Mse4	308	At1g05560	UDP-Glucosyltransferase	Upregulated
BstC33- Mse2	339	AT2G29420	GST-25	Upregulated
BstT33- Mse1	600	Y10617	12-oxophytodienoate reductase	Upregulated
BstT33- Mse1	481	AY048242	Putative Aldehyde dehydrogenase	Upregulated
BstT33- Mse2	587	petB	Cytochrome B6	Downregulated
BstC24- Mse4	184	At2g33790	Putative proline rich protein	Downregulated
BstT42- Mse1	114	At4g16860	Disease resistance RPP5 like protein	Downregulated
BstT33- Mse2	477	At4g27230	Histone H2A- like protein	Downregulated

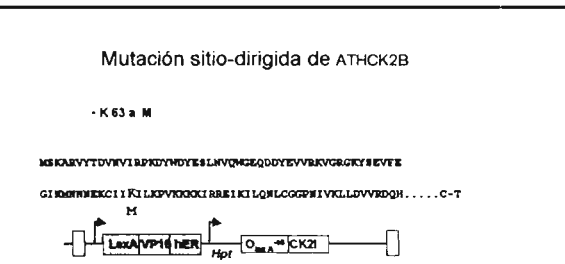
Lo que falta...

- Realizar las combinaciones restantes (nomenclatura: A C G T = 1 2 3 4) *BstY*IC+2 primers, *Mse*I+1 primers *BstY*IT+2 primers, *Mse*I+1 primers.
- Análisis computacional de los geles mediante Quantar Pro
- Definir cluster de genes tempranos
- Análisis mediante Northen blot de genes modelos (confirmar que las cinéticas de expresión son comparables con TP-AFLP)
- Análisis computacional y mediante footprinting de promotores de genes que resulten interesantes.

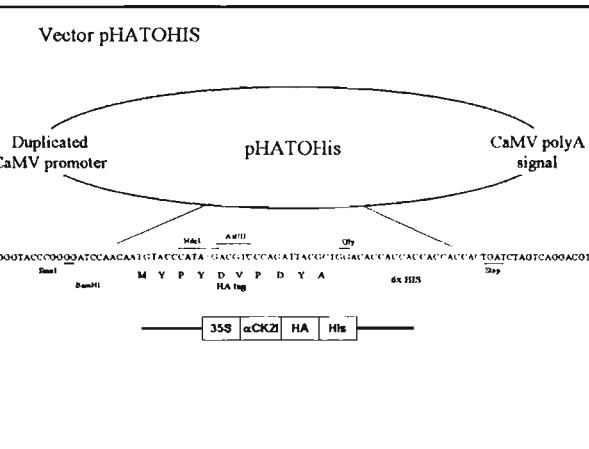


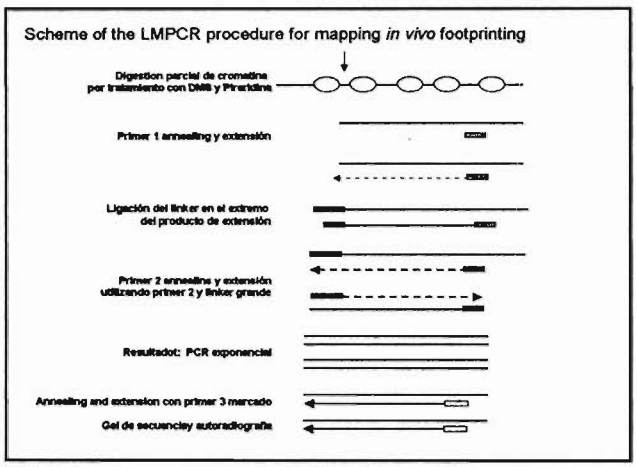
Expresion transiente en celulas de cebolla
•Actividad GUS
•Microscopio de Epifluorescencia

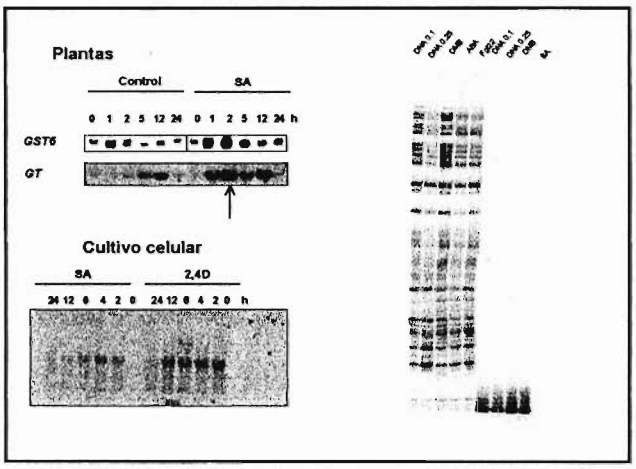


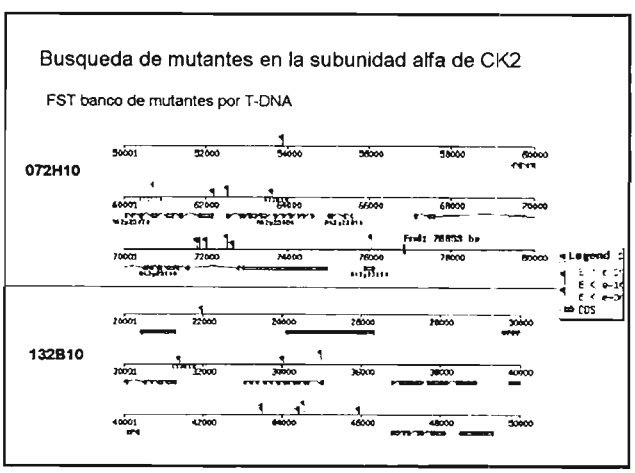


• Expresion de mutante y detección por Westerblot




Scheme of the LMPCR procedure for mapping *in vivo* footprinting
Digestion parcial de cromatina por tratamiento con DMS y Piridina
Primer 1 annealing y extensión
Ligación del linker en el extremo del producto de extensión
Primer 2 annealing y extensión utilizando primer 2 y linker grande
Resultado: PCR exponencial
Annealing and extension con primer 3 marcado
Gel de secuencia y autoradiografía

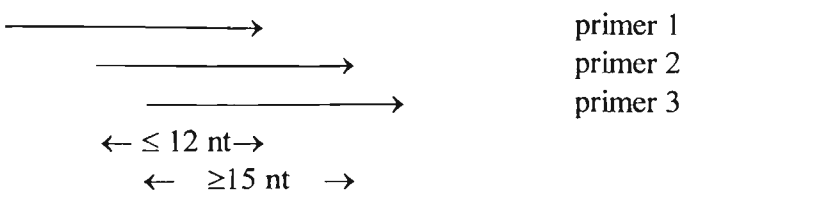

Plantas
Control
SA
0 1 2 5 12 24 0 1 2 5 12 24 h
GST6
GT
Cultivo celular
SA
2,4D
24 12 0 4 2 0 24 12 0 4 2 0 h


Busqueda de mutantes en la subunidad alfa de CK2
FST banco de mutantes por T-DNA
072H10
132B10
Legend
CDS

IN-VIVO-FOOTPRINTING

Experimental design:

@3 sequence specific primers, one linker



@Each primer should have an extending 3' end to the previous one. The overlap between primers 1 and 2 should not exceed 12 nucleotides. On the contrary primers 2 and 3 should overlap over at least 15 nucleotides. Ideally each primer should have a higher Tm than the previous one.

@The Tm of the three primers is calculated as 81.5+16.6(log M)+0.41(%GC)-500/n

n.....length of primer
M.....Molarity of salt in buffer (0.047 for 1x Taq buffer)

In general
primer 1: 17 to 32mer Tm of 54 to 59
primer 2: 21 to 43mer Tm 59 to 65
primer 3: 28 to 45mer Tm 62 to 68

In general the annealing temperatures will be chosen as follows:
"PCR 1" annealing temperature = Tm+1°C
PCR 2: annealing temperature=Tm+2°C
PCR 3: annealing temperature=Tm+4°C

The longer oligonucleotide of the linker should have a Tm that matches the Tm of the primer 2.

Tm GL = 64°C.

Gel-purification of Oligo-nucleotides

@ Precipitate 50 µg oligonucleotide with 10 µg tRNA with 3 vol EtOH/ 0.3 MnaACas final concentration (pH (5.5) o:n –20°C

@ Prepare a 15% acrylamide/bis (19:1):

	37.6 mL	40% acrylamide mix
	48 g	urea

De-ionise for 30 minutes

Add	10 mL	10 x TBE
		aqua dest.
	---	
	100 mL	

Polymerise with 500 µL 10 % APS and 56 µL TEMED
Pre-run the polymerised gel at 200 V (≈16 mA)
Load the gel, run for ≈4 h at 200 V (BBP at the bottom of the gel)
Remember: The bromphenolblue will ≈comigrate with a 17mer
Illuminate the gel above a silica gel plate (TLC F254, Merck), with UV lamp and cut out the band
Elute o/n in EB about 300 and 600 µl by slice
Elution buffer= 0,1%SDS
0.5M ammonium ac
10 mM MgAc
Precipitate with 3 vol EtOH/ NaAC 0,3M >2 h –20°
Centrifuge, wash and resuspend in 30 µL aqua
Quantify at A260

DMS treatment of naked DNA
Precipitate DNA in 10 µg aliquots, pellet and wash with 80% EtOH
Dissolve in 6 µL H2O
Add 200 µL Cacodylatbuffer (50 mM sodium cacodylate pH8, 1mM EDTA)
Add 1 µL DMS
Incubate 2' at 20°C
Add 20.6 µL 3M NaAC., pH=5.5, and 618 µL EtOH
10' in dry ice/EtOH bath

Proceed with Piperidin cleavage protocol

Piperidine cleavage of DMS treated DNA

Resuspend pelleted DNA from the in-vitro modification and the in-vivo DMS treatments (10 µg/tube)
In 250 µL of O.3 M NaAC., pH=5.5
Add 750 µL EtOH
Incubate 10' in dry ice/EtOH
Pellet, wash with 70% EtOH
In the meantime prepare 1 M Piperidine by diluting 10 M stock in H2O
Add 100 µL 1M Piperidine
Incubate 30' at 90°C

Freeze in liquid N2
Lyophilise in speedvac w vacuum trap (ca. 1h)
Dissolve in 100 µL H20
Freeze and lyophilise
Dissolve in 100 µL H20
Freeze and lyophilise
Resuspend in 250 µL 0.3 M NaAC. PH=5.5 and ADD 750 µl of EtOH and precipitate
Pellet, wash pellet in EtOH 70% and resuspend in 10 µL H20
Quantify samples by loading 0.5µL on a 2% agarose gel, to confirm equal amounts in each lane.
The piperidine cleaved DNA will migrate as a smear at around 100 to 400 bp

Annealing of linker oligonucleotides

Prepare linker solution:

250 mM Tris-HCl, pH=7.5
5 mM MgCl2
20 pmol/µL of each linker oligonucleotide (final concentration)

Incubate:

5' 94°C
30' RT

store in small aliquots at –20°C

Hybridisation & elongation of primer #1 ("PCR 1")

Add:

2 µL DNA (1 µg/µL)
1,5 µL DyNAzyme buffer (Finzyme)
MgSO4, 2mM final
0.4 µL dNTP Mix, 10 mM
1 µL primer 1, 0.15 pmol, freshly diluted
0.5 µL Vent exo-, 2 U/µL
H2O ad 15 µL

or add to _x Mastermix:

DyNAzyme buffer (Finzyme)
MgSO4, 2mM final
dNTP Mix, 10 mM
primer 1, 0.15 pmol, fr. diluted
5 µL Vent exo-, 2 U/µL
H2O ad

5' 94°C
30' (Tm+1)°C
10' 72°C or (optional) 76°C, when there is eg high GC%
Put on +4°C immediately after the program is completed

set 1:

primer	concentration	dilution

set 2:

primer	concentration	dilution

set 3:

primer	concentration	dilution

set 4:
primer concentration dilution

Mix : (17x)

Ligation of linker oligonucleotides
Add:

2.5 µL	annealed linker,kept in ice	42,5	42,5
15 µL	"PCR" 1		
6 µL	Ligase buffer (5x)	51	102
55 µL	H2O	186	93,5
1 µL	Ligase (Gibco/BRL) (~~50 U/µl~~)	25,5	17
~~30 µL total~~			

30°C 10 min.

Incubate on 16°C, add 70 µl of H2O
Precipitate with EtOH/NaAC and 10 µg tRNA, wash with 80% EtOH and resuspend in 35 µL H2O

Amplification with primer #2 and linker primer (PCR 2) Mix PCR 4

35 µL DNA
5 µL 10xDyNAzyme buffer Taq (Vgl. zu 1. PCR)
1 µL dNTP Mix, 10 mM
primer 2 : 5 pmol
large linker oligonucleotide: 5 pmol
1.25 µL DyNAzyme Taq
H20 ad 50 µL

2' 94°C
1' 94°C
1'30'' Tm+2°C } 15x
2' 72°C

Store at –20°C

Oligonucleotide labeling of primer #3

150 pmol primer 3
3 µL 10x PNK buffer
10 µL γP32 ATP 10 µCi/µL
2 µL T4 PNK
H20 ad 30 µL

Incubate 30' at 37°C
10' at 70°C
Add:
70 µL H20
10 µg tRNA
10 µL 3M NaAC. PH=5.5
300 µL EtOH
2h –20°C
pellet, wash with 80% EtOH
Suspend in 30 µL H20
Count 1 µL in 3H (Cerenkov mode), multiply cpm with 2 to get P32 cpm
Expect: 3 to 4 x10exp6 cpm/µL

Sequencing reaction with labelled primer #3 (PCR3)

Add:

15 µL of PCR2
0.8 µL dNTP Mix, 10 mM
0.5 µL 10x DyNAzyme buffer
~~1.25~~ µL DyNAzyme
~~0.45~~ µL H20
2 µL labeled primer 3

1' 94°C
2' (Tm+4)°C
3' 72°C
10 cycles, then
7' 72°C
4°C

Add 380 µL Taq Stop buffer
10 µg tRNA
1000 µL EtOH

-20°C on
pellet, wash ETOH 80%
Suspend in 10 µL loading buffer
Boil 5'
Load 2 µL

Taq Stop buffer: 8 mM Tris pH 7.5
200 mM NaAC. PH=5.5
3 mM EDTA

loading buffer:
95% formamide
0.05% bromophenolblue
0.05% xylenocyanol
20 mM EDTA

load on denaturating acrylamide gel : 6% (for longer migration 5%)
in TBE X1.
prerun 45W 30 mn
load 2 µl of samples

PROTOCOLE DE TRANSFECTION DE PROTOPLASTES

A partir de plantes d'*Arabidopsis thaliana* cultivées *in vitro* durant 4 semaines.

- Couper les feuilles des plantes et les mettre dans une boite de pétri stérile.
- Lacérer les feuilles de manière à ce qu'elles soient toutes coupées environ une fois (≈15 s).
- Recouvrir les feuilles avec la solution de digestion (≈15 ml).
- Fermer les boites avec du ruban Micropore.
- Laisser digérer toute la nuit (≈16 h).
- Séparer les protoplastes de leur matrice en pippetant et relachant délicatement le milieu (≈30 s) a l'aide d'une pipette stérile 3 ml.
- Filtrer le milieu contenant les protoplastes avec un tamis de 370 µm monté sur un autre tamis de 38 µm, dans un bécher stérile de 150 ml.
- Répartir le contenu du bécher dans des tubes stériles de 15ml.
- Centrifuger 5 minutes à 600 rpm, à température ambiante.
- Eliminer le surnageant, et resuspendre chaque culot dans 10ml de milieu W5A.
- Centrifuger 5 minutes à 600 rpm, à température ambiante.
- Resuspendre chaque culot dans 5 ml de milieu W5A, puis pooler tous les tubes dans un tube stérile de 50 ml.
- Prélever 100 µl de la solution de protoplastes et les déposer sur une cellule de Nageotte. Il faut multiplier par 800 le nombre de protoplastes comptés sur un ligne (faire la moyenne de 3 lignes) pour obtenir la concentration en protoplastes / ml.
- Répartir le contenu du tube dans des tubes stériles de 15ml.
- Centrifuger 5 minutes à 600 rpm, à température ambiante.
- Eliminer le surnageant, et resuspendre chaque culot dans un volume nécessaire de milieu MaMg pour atteindre la concentration de 10^6 protoplastes / ml. Pooler tous les tubes dans un tube stérile de 50 ml.
- Mettre ce tube dans la glace 30 minutes.
- Disposer 1 ml de protoplastes dans une cuve de plaque multipuits (3,5 cm de diamètre).
- Ajouter 50 µg d'ADN (≈ 50 µl).
- Ajouter 1 ml de solution PEG 40% délicatement, en faisant des cercles dans la solution protoplastes / ADN (≈30 s).
- Laisser à température ambiante 30 minutes.
- Ajouter délicatement 2 ml de solution W5A, attendre 5 minutes.
- Ajouter délicatement 4 ml de solution W5A, attendre 5 minutes.
- Transférer les protoplastes dans un tube 15 ml, puis compléter qsp 15 ml avec de la solution W5A.
- Centrifuger 5 minutes à 600 rpm, à température ambiante.
- Éliminer le surnageant, et resuspendre chaque culot dans 1 ml de milieu de culture.
- Laisser les tubes de protoplastes entre 24 h et 36 h, à l'obscurité.

SOLUTIONS

- **Milieu de culture (500 ml):**

Milieu Murashige et Skoog : 2,2g
Mannitol 0,4M : 36,4g
Glucose 0,4M : 36g
MES 0,1% : 0,5g
Cefotaxime 100mg/l : 50mg
Ajuster à pH 5,8 avec KOH, steriliser par filtration.

- **Milieu de digestion (500 ml):**

Mannitol 0,5M : 45,5g
Milieu Murashige et Skoog : 2,2g
Cellulase 1,2% : 6g
Macérozyme 0,8% : 4g
Ajuster à pH 5,8 avec KOH.
Centrifuger 5 minutes à 10000 rpm.
stériliser par filtration.

- **Milieu MaMg (500 ml):**

Mgcl2 15mM : 0,7g
MES 0,1% : 0,5g
Mannitol 0,4M : 36,4g
Ajuster à pH 5,6 avec KOH, stériliser par autoclave.

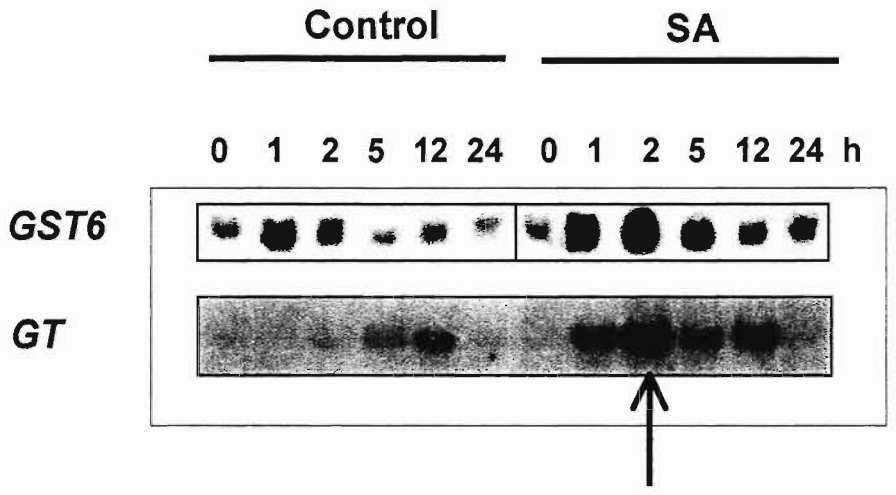
- **Solution de PEG 40% (50 ml):**

PEG 4000 40% : 20g
$Ca(NO3)_2$ 0,1M : 1,18g
Mannitol 0,4M : 3,64g
MES 0,1% : 0,05g
Homogénéiser en chauffant.
Ajuster à pH 6 avec KOH, stériliser par autoclave.

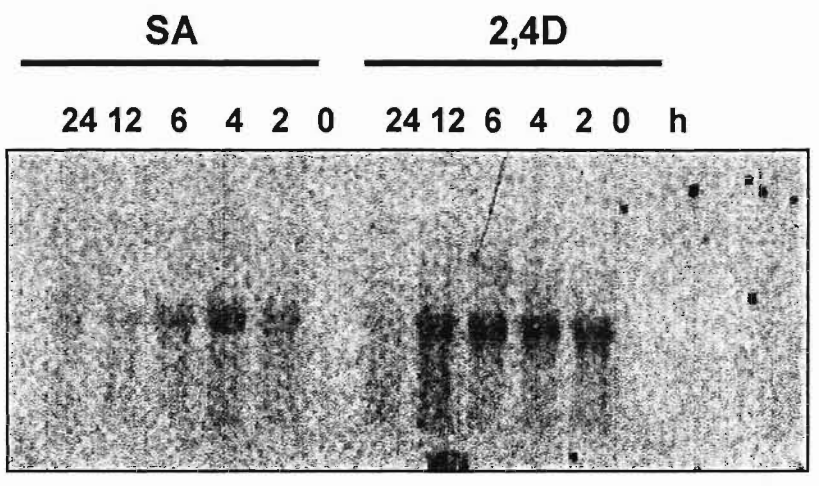
- **Milieu W5A (2000 ml):**

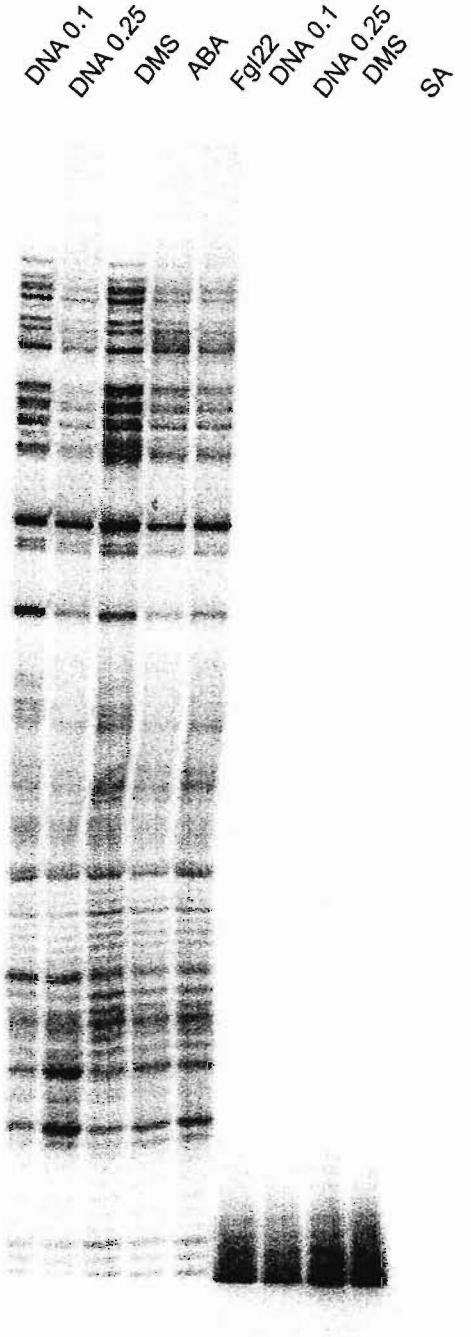
Mannitol 0,6M : 218,4g
Mgcl2 1mM : 0,2g
Glucose 5mM : 1,8g
Nacl 154mM : 18g
CaCl2 1,2mM : 0,36g
KCl 5mM : 0,76g
MES 0,1% : 2g
Ajuster à pH 5,8 avec KOH, stériliser par autoclave.

Plantas



Cultivo celular





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Multilevel regulation of histone gene expression during the cell cycle in tobacco cells

Jean Philippe Reichheld+, Claude Gigot and Nicole Chaubet-Gigot*

Institut de Biologie Moléculaire des Plantes du CNRS, 12 rue du Général Zimmer, 67084 Strasbourg cedex, France

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ABSTRACT

The respective involvement of transcriptional and post-transcriptional mechanisms in coupling H3 and H4 histone gene expression to the S phase of the cell cycle has been studied in synchronized tobacco cells. Induction of histone gene expression at the G1/S transition is shown to be essentially directed by an increase in the transcription rate in response to cellular signals occurring at the initiation step of DNA replication. Histone gene induction thus precedes the burst of DNA synthesis. However, when the elongation step of DNA replication is ineffective or artificially arrested, feedback mechanisms apparently act at the translation level to avoid overproduction of histone proteins from their mRNAs. At the end of S phase, post-transcriptional mechanisms ensure a rapid degradation of histone mRNAs. Transcription factors are bound to the *cis*-elements of histone promoters throughout the cell cycle, thus suggesting a post-translational modification of some of them to trigger promoter activation at the G1/S transition. Based on these results, a model is proposed for histone gene transcriptional induction in connection with the components of the cell cycle machinery.

INTRODUCTION

The genes encoding histones, the major proteinaceous constituents of the eukaryotic chromatin, have been extensively used as models in animals and yeast for understanding the molecular mechanisms of cell cycle-regulated gene expression (reviewed in 1–3). More recently, similar studies based on both structural and functional approaches developed on plant histone genes have led to the identification of *cis*-elements responsible for the specific activity of histone gene promoters in plant meristems. Interestingly, the sequences of the *cis*-elements are conserved among the plant histone gene classes and in various plant species, while they are rather class- and species-specific in the animal kingdom. The set of plant histone-specific *cis*-elements consists of a highly conserved plant histone-specific octameric sequence CGCGGATC (OCT), followed 10–30 bp downstream by a hexamer CCGTCC and 8–10 bp further downstream by a nonameric element of consensus sequence CC/AATC-CAACG (NON) (4). Additionally, a CCAAT box-like motif and one or two copies of a degenerate octamer differing by 1 nt from the highly conserved sequence are often present in the region surrounding the above-cited elements. Some promoters are characterized by the presence of a particular form of the octamer, the highly conserved sequence being associated in a reverse orientation with an additional non-specific motif, the hexamer ACGTCA (HEX) (5).

In all the plant histone gene promoters, the full complement of *cis*-elements were found closely associated within a rather short region (120–200 bp) whose chromatin structure displayed increased accessibility to nucleases in cycling cells, suggesting that structural modifications are induced upon transcriptional activation (6). Deletion studies demonstrated that these small promoter regions were sufficient to drive meristem- and S phase-specific GUS expression in transgenic plants (7,8) and cell suspensions (9,10). *In vivo* footprinting experiments revealed a bimodular organization of the promoters with a proximal cell division-specific set of interactions occuring over the nonameric and the CCGTCC elements, whereas the other elements displayed more or less constitutive DNA–protein interactions, irrespective of the proliferative state of the cells (11). By *in vitro* footprinting experiments, the nonamer was shown to bind multiple and as yet unidentified wheat and tobacco nuclear proteins (12,13). The ACGTCA hexamer was shown to bind the leucine zipper transcription factors HBP-1a and b (14), which have been well characterized but whose function in histone gene regulation is not yet clear. Functional studies of the activity of mutagenized histone promoters in transgenic plants have shown that all the motifs described above behaved as positive regulatory elements (8,15,16), some of them, the nonamer and the hexamer CCGTCC, proving essential for meristem-specific expression (16).

The respective importance of these different motifs in the regulation of gene expression during the cell cycle is at the moment less well documented. Indeed, until recently, the study of cell cycle-regulated gene expression in plants suffered from the lack of a suitable cell synchronization system. Recently, the highly synchronizable tobacco BY2 (TBY2) cell suspension described in Nagata *et al.* (17) has been used to study histone gene expression during the different phases of the cell cycle (18,19). As expected for genes encoding proteins whose function consists of packaging the newly synthesized DNA into chromatin, maximal mRNA accumulation was found in S phase. From expression studies of a histone promoter–reporter gene construct,

*To whom correspondence should be addressed. Tel: +33 03 88 41 72 97; Fax: +33 03 88 61 44 42; Email: nicole.chaubet@ibmp-ulp.u-strasbg.fr

+Present address: Laboratory of Genetics, University of Ghent, Ledeganckstraat 35, 9000 Ghent, Belgium

it appeared that this S phase-specific expression resulted mainly from transcriptional regulation (10). However, it has been suggested that post-transcriptional mechanisms might also play a role in regulating histone gene expression at some particular stage of the cell cycle (19,20).

In order to establish an overall picture of the mechanisms coupling histone gene expression to DNA replication in plants, we investigated H3 and H4 histone gene expression at different levels: transcription, mRNA accumulation and protein synthesis in BY2 cells throughout the cell cycle and in the presence of various inhibitors of DNA synthesis. We show that induction of histone gene expression at the G1/S transition occurs essentially at the transcriptional level and is coupled with the initiation of DNA replication, but independent of the rate of DNA elongation. In contrast, synthesis of the histone proteins is strictly coupled to the DNA synthesis rate by the means of post-transcriptional, possibly translational, regulatory mechanisms. At the end of the S phase, both transcriptional and post-transcriptional mechanisms act in concert to induce a rapid decay of histone transcripts. Interestingly, *in vivo* analysis of DNA–protein interactions at the level of a H3 histone gene promoter at various stages of the cell cycle failed to reveal significant changes upon cyclic gene transcriptional activation, thus suggesting that gene activation at the G1/S transition results from structural or chemical modification of a factor(s) bound to the promoter throughout the cell cycle, rather than from the establishment of new interactions with S phase-specific factors.

MATERIALS AND METHODS

DDBJ/EMBL/GenBank database accession no. Y14195

Plant material

A tobacco BY2 (*Nicotiana tabacum* L. cv. Bright Yellow 2) cell suspension was maintained by weekly subculture and synchronized by a 24 h subculture of stationary phase cells in a medium containing aphidicolin, followed by extensive washes, as previously described (17). DNA synthesis and mitotic index were measured as described (19). Cell viability was estimated by fluorescence microscopy analysis after fluorodiacetate (FDA, 50 µg/ml) staining. Tobacco leaves were collected from 7-week-old greenhouse grown plants (cv. Petite Havana SR1).

Nucleic acid analysis

Isolation and blotting of total RNAs was performed as previously described (19). Hybridization was under standard high stringency conditions with random primed ^{32}P-labelled H3 or H4 histone coding regions from *Arabidopsis thaliana* (21). For run-on transcription, nuclei were prepared from 50 ml cell suspension according to Cox and Goldberg (22) and purified on 76% Percoll/2 M sucrose gradients. 10^7 nuclei were incubated for 10 min at 30°C in 400 µl buffer [25 mM Tris–HCl, pH 8.5, 6.5 mM $MgCl_2$, 25% glycerol, 0.1 M $(NH_4)_2SO_4$] in the presence of 0.5 mM ATP, GTP and CTP, 80 U RNasin and 120 µCi [α-^{32}P]UTP, then successively treated with DNase (200 U) and proteinase K. The transcripts were purified by phenol extraction and hybridized to Southern blots of restricted plasmids (10^6 c.p.m./cm^2). Hybridization was performed for 36 h at 48°C in 10% formamide, 6× SSC, 5% Denhardt's, 1% SDS, 50 µg/ml *Escherichoia coli* tRNA. Washes were in 2× SSC, 0.1% SDS for 45 min at 65°C. Radioactive signals were quantified using a PhosphorImager (Molecular Dynamics).

Protein analysis

Protein *in vivo* labelling was performed by a 2 h incubation at 27°C of 5 ml cell suspension with 50 µCi [^{35}S]methionine. Total proteins were extracted by grinding frozen cells and sonicating in buffer (50 mM Tris–HCl, pH 7.5, 1.5 M NaCl, 1 mM EDTA, 10 µM leupeptin and pepstatin, 1 mM aprotinin, 1 mM PMSF and 50 µM DTT). After centrifugation, the supernatant was dialysed against the same buffer with 150 mM NaCl and the protein content measured according to Bradford (23). Proteins were separated on 16% polyacrylamide gels, transferred to Immobilon-P membranes (Millipore) and incubated with anti-human H3 histone antibodies. After washing, blots were incubated with peroxidase-conjugated secondary antibodies and revealed by chemiluminescence (Amersham). For determination of histone synthesis rate, 100 µg extracted total proteins were immunoprecipitated with antibodies for 15 h at 4°C in buffer (10 mM Na phosphate, pH 7, 150 mM NaCl, 0.1% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS, 2% protein A–Sepharose and 200 µg/ml BSA). The precipitated proteins were analysed by 16% PAGE.

In vivo footprinting by the LMPCR method

The method described in detail for plant material (24) was used with some modifications. Fifty millilitres of cell suspension or 5×10^6 leaf nuclei isolated according to Curie *et al.* (25) were treated for 2 min at 20°C with 0.5% DMS. After extensive washes in ice-cold buffer, DNA was extracted according to Zimmermann and Goldberg (26) and purified on RPC5 columns as described (27). As a control, naked genomic DNA was treated with DMS and the reaction stopped by precipitation. DMS-treated DNAs were then cleaved with 1 M piperidine and recovered by lyophilization and ethanol precipitation. Two micrograms were used for first strand synthesis in 15 µl buffer [20 mM Tris–HCl, pH 8.9, 40 mM NaCl, 5 mM $Mg(SO_4)_2$, 0.01% gelatin and Triton X-100, 0.2 mM dNTPs] with 10 nM first primer and 1 U Vent (exo$^-$) DNA polymerase (Biolabs). After a 5 min denaturation step at 94°C, annealing was performed for 30 min at T_m + 1°C, followed by extension for 10 min at 76°C. The linker was prepared as in Brignon *et al.* (24). For linker ligation to the extended DNA fragments, 25 µl buffer (44 mM Tris–HCl, pH 7.5, 12 mM $MgCl_2$, 30 mM DTT, 75 µg/ml BSA, 1.5 mM ATP, 2 µM linker, 2.5 U T4 DNA ligase) were added and the mix incubated for 20 h at 17°C. DNA was precipitated and then amplified in 16 cycles (1 min at 94°C, 2 min at T_m + 5°C, 3 min at 76°C) in the Vent buffer with 1 U Vent polymerase, 2 mM dNTPs and 100 nM each second primer and long oligomer of the linker. One third of the reaction products were extended in 10 cycles with the end-labelled third primer. After extraction and precipitation, the amplified products were analyzed on standard 6% polyacrylamide gels and autoradiographed. The primers are shown in Figure 4a.

The promoter sequence of the BY2 H3 histone gene was established by genomic sequencing combined with LMPCR on genomic DNA cleaved according to Maxam and Gilbert (28). Specific primers have been chosen in the partial 5′-region of a H3 histone gene previously isolated from a tobacco genomic library (unpublished data). The resulting sequence was confirmed by sequencing the complementary strand.

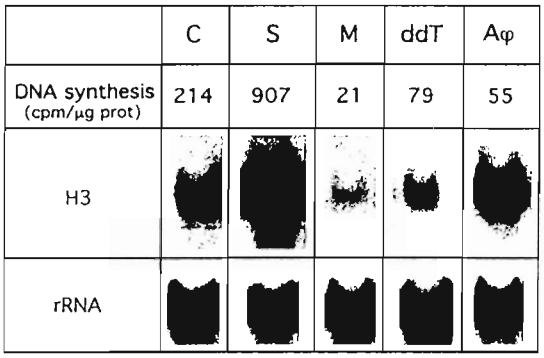

Figure 1. Effect of different DNA synthesis inhibitors on the histone mRNA level. Stationary phase cells were subcultured for 24 h in standard medium (C) or in the presence of the indicated DNA synthesis inhibitors. The DNA synthesis rate was measured at the end of the treatment by [^{3}H]dTTP pulse labelling. The histone mRNA level (H3) was determined at the end of the treatment and in released mid S phase cells (S) by blot hybridization of total RNAs to an *Arabidopsis* H3 histone coding region. Similar patterns were found using a H4 probe. The blot was reprobed to a rDNA probe as a loading control (rRNA). Aφ, aphidicolin 20 μg/ml; ddT, dideoxythymidine triphosphate 0.5 mM; M, mimosine 2 mM.

RESULTS

Induction of histone gene expression is coupled with initiation of DNA replication

In order to clarify the relationships between histone gene induction and the onset of DNA replication, we studied the effect of DNA synthesis inhibitors on H3 and H4 histone gene expression. Mimosine has been shown to block DNA synthesis by inhibiting deoxyribonucleotide metabolism (29), whereas aphidicolin and dideoxythymidine triphosphate (ddTTP) affect the activity of specific DNA polymerases (30,31). Stationary phase BY2 cells were diluted into fresh medium in the presence of either inhibitor. After 24 h, the DNA synthesis rate and the steady-state amounts of histone mRNA were estimated (Fig. 1). In order to optimize the inhibition conditions, a range of concentrations were first tested for each inhibitor and the effect on cell viability determined. In no case did the inhibitor treatment affect cell viability by >5% and no mitotic figures were observed (data not shown). Concentrations of mimosine which almost completely blocked DNA synthesis also reduced the histone mRNA amount to a very low level (Fig. 1). As expected from their similar cellular targets, mimosine had an effect similar to that previously observed with hydroxyurea (19). Although both aphidicolin and ddTTP significantly inhibited DNA synthesis, they did not have the same effect on histone mRNA accumulation. Whereas aphidicolin treatment resulted in a high mRNA level similar to that reached in S phase (Fig. 1; 19), ddTTP significantly inhibited the accumulation of histone transcripts as compared with control cells.

Our previous results suggested that hydroxyurea and aphidicolin blocked progression of the cell cycle before and after the induction of histone transcript accumulation respectively (19). Interestingly, the results obtained after ddTTP treatment suggest that ddTTP blocks progression of the cell cycle prior to aphidicolin. These results need to be linked to work on properties of DNA polymerases in plants (31,32). While aphidicolin has been shown to inhibit DNA polymerase α, the enzyme which initiates DNA replication in animal and yeast cells, it appears to have no influence on the corresponding plant enzyme, at least *in vitro*. Therefore, plant cells treated with aphidicolin are likely to be arrested at a step later than the initiation of DNA replication, probably at the elongation step, since aphidicolin is able to inhibit the plant counterpart of animal DNA polymerase δ (32). In contrast, ddTTP, being able to inhibit the plant counterpart of DNA polymerase α, is likely to block the cells just at or prior to the initiation step of DNA replication. This assumption is in agreement with the experimental results presented above and suggests that induction of histone gene expression temporally occurs in parallel with the initiation of DNA replication.

Histone gene expression is regulated at the transcriptional, post-transcriptional and possibly translational levels

The accumulation of H3 and H4 histone mRNAs under conditions of low DNA synthesis in aphidicolin-treated cells was rather surprising and might result from either sustained transcription or increased transcript stability or from a combination of both mechanisms. It also raised the question whether these mRNAs were translated, thus possibly leading to an overproduction of histones in the absence of newly synthesized DNA with which to associate. Such accumulation of histone mRNAs in the absence of DNA synthesis has also been observed when aphidicolin is re-added in the middle of S phase (19). In order to dissect the mechanisms involved in the regulation of histone gene expression during the cell cycle and under conditions of perturbed DNA synthesis, we compared the mRNA steady-state levels with the transcription rate of the corresponding genes and with the synthesis rate of the histone proteins in aphidicolin-synchronized cells (Fig. 2). In one sample, aphidicolin was re-added at mid S phase to a concentration previously shown to totally block DNA synthesis (19). The gene corresponding to the *A.thaliana* translation elongation factor (EF-1α), which is known to be constitutively expressed throughout the cell cycle (33,34), was used as a reference for quantification of the blots. In stationary phase cells, H3 and H4 histone mRNAs as well as transcription rates were undetectable (Fig. 2b and c). After the 24 h aphidicolin treatment, in the absence of any significant DNA synthesis, histone mRNAs accumulated to a high level, which could be connected to an active transcription rate (Fig. 2b and c, lane C0). Histone transcript level as well as transcription rate peaked 1 h after the release from aphidicolin, thus showing that the histone mRNAs accumulated as a result of transcriptional activation. In the second part of S phase, however, the histone mRNA level and transcription rate decreased with different kinetics (Fig. 2b and c, lanes C3), the transcription rate being low (30% of the maximum) 3 h after release from the aphidicolin block while the mRNA level was still at a high level (70%). One hour later, at the end of S phase (lane C4), the mRNA level and the transcription rate were again roughly equivalent. These results suggest that a modification of mRNA stability occurs at the end of S phase between 3 and 4 h culture, leading to a rapid and selective degradation of the histone mRNAs. During the following phases of the cell cycle, histone transcriptional rate and transcript amount stabilized at a basal level which might correspond to the replication-independent activity already demonstrated for plant histone promoters (35) or to the expression of some replication-independent histone variants (36,37).

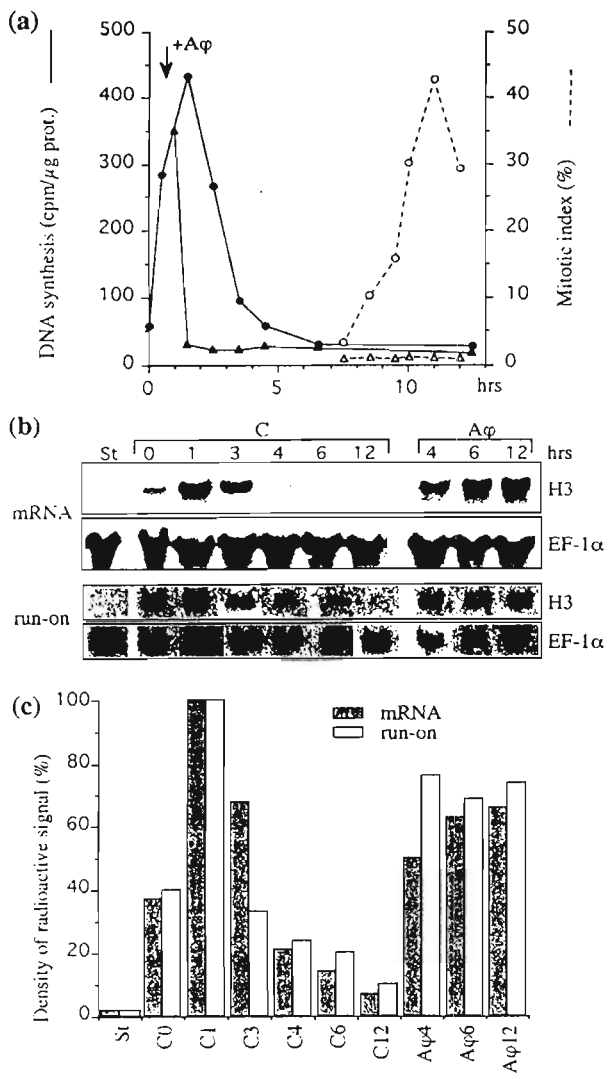

Figure 2. Histone gene transcription and mRNA abundance in the unperturbed and arrested cell cycle. Cells were released from a 24 h aphidicolin treatment. S phase was allowed to proceed without perturbation in control cells (circles, C) or was interrupted in mid S phase (arrow) by addition of 20 μg/ml aphidicolin (triangles, Aφ). A typical experiment is shown. (**a**) DNA synthesis rate determined from 30 min [^{3}H]dTTP pulses (filled symbols) and mitotic index (open symbols). (**b**) mRNA level (mRNA) and transcription rate (run-on) of H3 histone (H3) and translation elongation factor 1α (EF-1α). Similar patterns were found using the H3 or H4 histone probes or a probe specific to the 5′-UTR of the BY2 H3 gene. (**c**) PhosphorImager quantitated signals for H3 mRNA level (white bars) and transcription rate (shaded bars).

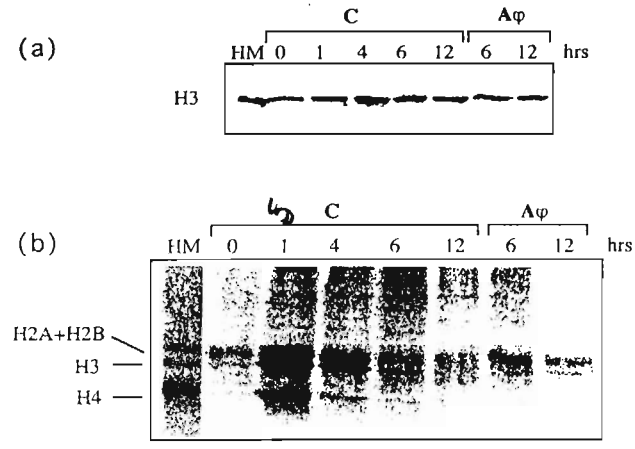

Figure 3. Histone protein amount and synthesis rate in the unperturbed and arrested cell cycle. Cells were collected from the same experiment as in Figure 2 and incubated for 2 h with [^{35}S]methionine. The indicated times correspond to the start of the treatment. (**a**) Western blot of total BY2 proteins revealed with an anti-human H3 antibody. HM, maize histones. (**b**) Immunoprecipitation of labelled proteins with an anti-human H3 antibody. The position of the various histones is indicated. HM, Coomassie staining of maize histones.

As revealed by immunoblotting with an antibody raised against the human H3 protein, the total amount of histone H3 increased significantly during the 5 h after release from the aphidicolin block, corresponding to doubling of the genome (DNA and associated proteins), and remained constant during the subsequent phases of the cycle (Fig. 3a). This variation in the total histone amount resulted from their rate of synthesis, as revealed by [^{35}S]methionine labelling and immunoprecipitation with the same anti-human H3 antibody (Fig. 3b). Surprisingly, although the antibody only revealed H3 histones on the immunoblot of total proteins (Fig. 3a), it was able to precipitate all classes of major histones (Fig. 3b). It might be that at the ionic strength used for the immunoprecipitation experiments, the various histones still interact with each other and therefore co-precipitate with H3. Advantage could be taken of this particular fact to verify that all the core histones had the same pattern of synthesis during the cell cycle. After the 24 h aphidicolin treatment, histone synthesis was very low (lane C0), thus appearing unrelated to transcript amount. After release from the aphidicolin block, histone synthesis was high during S phase (lane C1) and then decreased to a basal level (lanes C4, C6, C12), thus mimicking the mRNA level.

Upon addition of aphidicolin at mid S phase, DNA synthesis quickly declined within 1 h to the basal level reached in the G2 phase of control cells (Fig. 2a, triangles) and no mitotic figures could be observed. However, both the histone mRNA amount and the transcription level remained very high (65–75% of the level in S phase) for at least 11 h (Fig. 2b and c, lanes Aφ4–Aφ12). This result clearly shows that the maintainance of high steady-state levels of histone mRNA in the presence of aphidicolin is due to sustained gene transcription and not to an increase in mRNA stability. Under these conditions, although the histone mRNA amount was high, the amount of histone protein did not significantly increase as compared with control cells (Fig. 3a, compare lanes C6 and C12 with Aφ6 and Aφ12 respectively) and the translation rate remained at a basal level (Fig. 3b). These results thus strongly suggest the existence of a post-transcriptional, possibly translational, control blocking the translation of histone mRNAs when DNA synthesis is interrupted.

Protein–DNA interactions in the promoter region of a histone gene remain essentially unchanged throughout the cell cycle

As shown above, induction of H3 and H4 histone gene expression at the G1/S transition is mostly due to transcriptional activation. In order to understand the molecular mechanisms involved in this transcriptional regulation, we studied the protein–DNA interactions

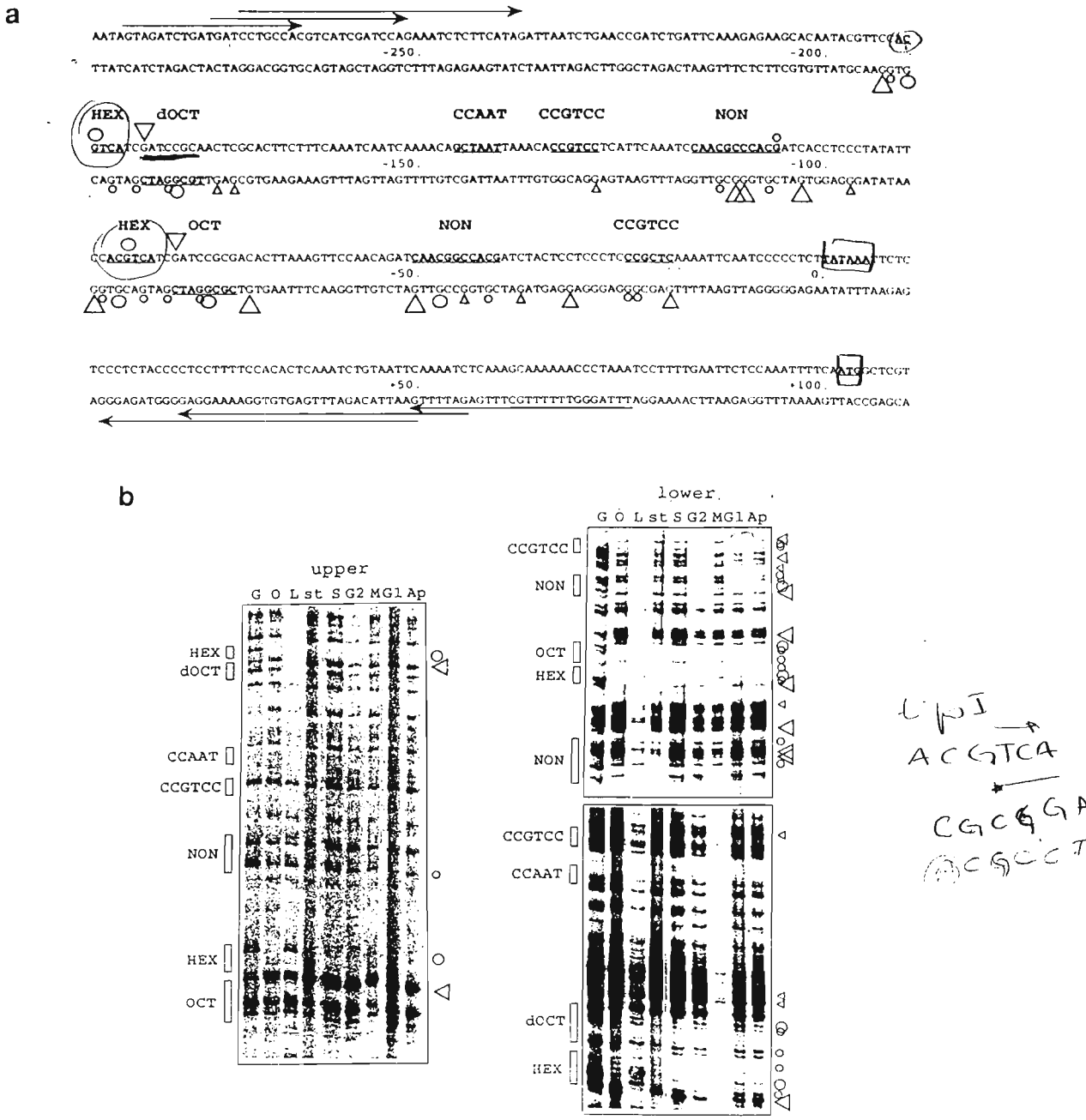

Figure 4. Protein–DNA interactions on a BY2 histone promoter in the unperturbed and arrested cell cycle. Cells collected from the same experiment as in Figure 2 were treated with DMS and protein–DNA interactions were revealed by the LMPCR method. (**a**) Histone promoter sequence and schematic representation of the footprints. Nucleotides are numbered relative to the TATA box. Remarkable motifs are underlined and their abbreviation indicated above: HEX, hexamer; OCT, octamer; dOCT, degenerate octamer; NON, nonamer. The two sets of overlapping primers used for the LMPCR are indicated with arrows. Residues protected from DMS are indicated with circles, those hypersensitive to DMS with triangles of various sizes according to the signal intensity. (**b**) *In vivo* footprinting of the upper and lower strands of the BY2 histone promoter. Lane G, naked DNA; lane 0, stationary cells subcultured for 24 h in the presence of aphidicolin; lane L, fully expanded tobacco leaves; lane st, stationary phase cells; lane S, S phase cells; lane G2, G2 phase; lane M, anaphase; lane G1, G1 phase; lane Aφ, aphidicolin-arrested cell cycle (12 h after addition of aphidicolin at mid S phase). Positions of the motifs of interest are indicated. The symbols represent the footprints in S phase cells. They are reported on the promoter sequence in (a).

in the promoter region of a histone H3 gene from BY2 at different phases of the cell cycle using the DMS/LMPCR *in vivo* footprinting method (38) adapted to plant material (24). All the consensus motifs previously identified in plant histone gene promoters were found within 200 bp upstream from the typical TATA box of the H3 promoter (Fig. 4a). Two copies of the so-called type I element composed of the hexamer ACGTCA motif paired with a reverse-oriented octamer CGCGGATC (5) were found at −87 and −191 from the TATA box, the more distal one having a degenerate octamer instead of the highly conserved

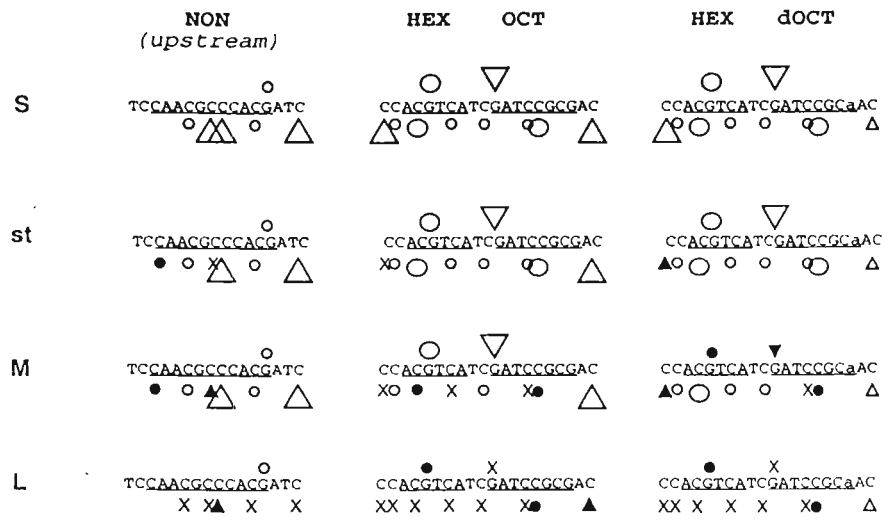

Figure 5. Schematic representation of the changes in the protein–DNA interactions. Only the sequence of the upper strand is shown. Signals occuring over the upper and lower strands are indicated respectively above and below the sequence. The symbols are the same as in Figure 4. Solid symbols indicate changes in signal intensity relative to S phase cells. A cross means a loss of signal. The downstream nonamer and CCGTCC are not represented. They display decreased signals only in leaves.

octameric motif. Two sequences, CAACgcCCACG (–116) and CAACggCCACG (–50), had significant homologies with the nonamer (CC/AATC-CANCG) element. As in other plant histone promoters, these two sequences were closely linked with a CCGTCC (or CCGCTC) sequence respectively located 11 bp upstream or 16 bp downstream of the nonamer element. A CCAAT box-like sequence was also found just upstream from the CCGTCC sequence (–145).

The two type I elements displayed very similar footprints, suggesting that they interact with the same or very similar factors (Fig. 4a and b). The same holds true for the two copies of the CCGTCC hexamer. The two nonameric motifs, which differ by only one base difference, showed the same global reactivity towards DMS, but the relative intensities of the signals on the different G residues of the *cis*-element were slightly different, suggesting that the strength of the interactions are probably affected by the nucleoprotein environment of the complexes. Surprisingly, when studying the protein–DNA interactions at various steps of the cell cycle, no major modifications were found between S phase, when histone genes are transcribed, and the remainder of the cycle, when histone genes are not transcribed. Such results indicate that there is no transcription factor binding specifically to the promoter during S phase. It is thus likely that transcriptional induction of histone genes at the G1/S transition is mediated by a post-translational modification of a factor(s) already bound to the promoter. Otherwise, it is noteworthy that interactions seemed somewhat loosened during anaphase (Fig. 4b). A similar observation has been reported in a study of human mitotic chromosomes by LMPCR, leading to the assumption that transcription complexes should be reformed *de novo* after each cellular division (39).

Protein–DNA interactions in cells arrested by the DNA synthesis blocking agent aphidicolin either before or during S phase (Fig. 4b, lanes 0 and Aφ) were identical to those observed in S phase cells, thus confirming the results obtained from run-on experiments showing that, although DNA synthesis is blocked, histone gene transcription nevertheless proceeds in aphidicolin-arrested cells. Intriguingly, in stationary phase cells which do not express histone genes, the signals revealed over the *cis*-elements display only minor differences as compared with cells undergoing the cell cycle (Fig. 5). The reason for this is unknown, but it can be hypothesized that stationary BY2 cells have not really exited the cell cycle and that the full complement of transacting factors are maintained bound to the histone promoter to ensure a rapid response to a potential re-entry into S phase. In contrast, only a few weak protections against DMS attack were detected in the non-cycling cells of fully expanded leaves, thus showing the quasi-absence of specific protein–DNA interactions on histone promoters in differentiated cells.

DISCUSSION

The data presented in this article obtained with a highly synchronizable BY2 tobacco cell suspension confirm previous observations showing that plant histone mRNAs specifically accumulate during the S phase of the cell cycle, as in animals and yeast (8,9,19). Comparison between the H3 and H4 histone mRNA amounts, protein synthesis and transcription rate of the corresponding genes during the cell cycle showed that accumulation of histone mRNAs and proteins in S phase is essentially due to transcriptional induction. This mechanism of regulation is quite different from that acting in animal systems, where accumulation of histone mRNAs in S phase is only partly due to an increase in transcription rate and relies essentially on increased processing and stability of the transcripts (1–3). This difference in the mechanism of regulation is likely to be related to the absence in the 3′-regions of plant histone genes of the palindromic and purine-rich motifs which are responsible for maturation of the non-polyadenylated animal histone messengers (1). However, by comparing the transcription rate and the mRNA amount, we provide evidence for the existence of post-transcriptional mechanisms acting on plant histone mRNAs at the end of S phase, leading to a rapid decay of the transcripts. This result confirms previous studies based on mRNA turnover measurements in the presence of

transcription inhibitors (19,20). As such a rapid decay was not observed for reporter mRNAs transcribed from a construct containing a plant histone promoter but no histone terminator (9), it can be assumed that the targets for selective destabilization at the end of S phase are located in the histone 3′- and/or coding region.

Considering the role of histone proteins in packaging DNA into chromatin, the synthesis of histones obviously needs to be tightly linked to DNA synthesis. Whereas in animals and yeast an arrest of DNA synthesis by aphidicolin or other blocking agents results in a dramatic drop in histone mRNA levels mediated by both transcriptional arrest and increased mRNA turnover, our results indicate that in plants, neither transcription of histone genes nor the transcript amount decrease upon a block in DNA synthesis. It has been previously postulated that the arrest of plant histone gene transcription is only dependent on signals connected to the natural completion of chromosome replication (19). However, upon interruption of DNA replication some rescue mechanisms avoiding overproduction of histones relative to DNA are apparently acting at the translational level, since histone proteins are no longer synthesized from their mRNAs. One hypothesis accounting for this result could be autoregulation of histone synthesis by free histones present in the cytoplasm, preventing histone mRNA translation. Such a mechanism has already been proposed in animal systems to explain the degradation of histone mRNAs upon a replication block (1,3).

The mechanisms which couple the induction of histone gene transcription to the onset of DNA replication remain still largely unknown in any kingdom. The treatment of freshly subcultured BY2 cells with various inhibitors of DNA synthesis acting on different cellular targets allowed us to identify the timing of histone gene induction with respect to the onset of DNA replication. Hydroxyurea and mimosine inhibit synthesis of deoxynucleotides, the precursors necessary for DNA synthesis (29,40). The arrest in the cell cycle produced by these two inhibitors occurs before the induction of histone gene transcription. In contrast, aphidicolin, which was shown *in vitro* to inhibit the plant DNA polymerase responsible for elongation of DNA chains (31), arrested the cycle after induction of histone genes. Under these particular conditions, it was apparent that the induction of histone gene transcription somewhat preceded the burst in DNA synthesis. Another inhibitor, ddTTP, which was shown to inhibit the plant DNA polymerase involved in initiation of DNA replication (31,32), did not allow the transcription rate of histone genes to reach a high level, thus preventing the mRNAs accumulating to the same level as in aphidicolin-treated cells. It can thus be concluded that induction of histone gene transcription at the G1/S transition takes place between two very close chronological steps, defined by the respective arrests caused by ddTTP and aphidicolin, and, hence, is temporally linked to the initiation of DNA replication. This time of induction is likely to be the same for most living organisms. However, such correlations could not be established before, because in mammals and yeast, aphidicolin inhibits both DNA polymerases and thus does not allow discrimination between the initiation and elongation steps of DNA replication (32).

We investigated the evolution of DNA–protein interactions by LMPCR *in vivo* footprinting during the cyclic activation of histone gene transcription at the G1/S transition and in arrested cells. Protein binding was detected on three different motifs corresponding to *cis*-elements previously described for their conservation in plant histone promoters (4,11). These *cis*-elements, namely the nonamer CC/AATC-CAACG, the CCGTCC sequence and the type I element composed of the widely distributed hexamer ACGTCA paired with a reverse copy of the plant histone-specific octamer CGCGGATC, were repeated twice in the promoter as slightly degenerate forms. The *in vivo* protein footprints over the hexamer motifs in the two type I elements are quite similar to each other and to those previously observed in a maize H4 histone promoter (11), suggesting that identical proteins exist in maize and tobacco. These *in vivo* footprints resemble the footprints of the bZIP factors HBP-1a and b observed *in vitro* over the same type I hexamer in a wheat H3 histone promoter (14). The protein footprints over the octamer motifs seem somewhat less conserved between tobacco and maize, as well as between type I and type II promoters (11,16), which may indicate some fluctuations in the octamer binding protein family or some dependence of binding on the nucleoprotein environment. Globally, protein–DNA interactions did not vary greatly during progression through the cell cycle, thus indicating a constitutive interaction of transacting factors with the different *cis*-elements, regardless of the transcriptional activity of the gene. We therefore postulate that cyclic activation of histone gene transcription at the G1/S transition results from modified protein–protein and protein–DNA interactions over one or several motifs. As there is increasing evidence for cyclin/cdk (cyclin-dependent kinase) complex mediation of cell cycle regulation by phosphorylation of a variety of substrates, it can be proposed that the post-translational modification occuring on the histone promoter binding factor(s) at the G1/S transition might consist of a transient phosphorylation of a prebound factor by a specific cyclin/cdk complex. In keeping with this hypothesis, the nonameric element has been shown to bind nuclear proteins from BY2 cells *in vitro* and binding was dependent on phosphorylation (13). The nonamer might thus be one of the targets for a cell cycle-dependent phosphorylation mechanism. Its involvement in conferring cell division-specific expression on a reporter gene in transgenic plants has been shown by functional studies of the *cis*-elements of plant histone promoters (16). Our hypothesis of gene induction mediated by phosphorylation is further reinforced by recent results showing that the crucial cell cycle-specific phosphorylation regulators cdc2 and cyclin A contribute to the transcriptional complex interacting with human histone promoters (41,42). A completely different pattern of *in vivo* footprints was found in the differentiated cells of fully expanded leaves, where almost no interactions were found. This fact suggests that the transacting factors of the histone promoters are synthesized and/or bind to the promoters at the entry of cells into the cell cycle, at the transition G0/G1. Surprisingly, the footprints observed in stationary phase BY2 cells were only slightly weaker than those in G1, S and G2 cells, suggesting that these non-dividing cells have not really exited the cell cycle.

Taken together, the results presented in this paper suggest a two-step mechanism for histone gene induction in tobacco cells and allow the proposal of a regulatory model taking into account the timing of induction with respect to DNA replication. We postulate that transacting factors bind to the histone promoters at entry into the cell cycle, possibly resulting from *de novo* synthesis or post-translational modifications mediated by the cycle regulators present at this stage of the cell cycle, potentially cyclin D/cdk complexes, whose existence has recently been demonstrated in plants (43). Then, phosphorylation of a factor(s) already bound to the promoter by a cyclin/cdk complex specific for the G1/S

transition could modify the structure of the transcriptional complex and trigger histone gene transcription. Two tobacco type A cyclins recently identified are good candidates for belonging to this kinase complex, the first being induced slightly before and the second in parallel with the histone genes, at least at the transcript level (34).

Since histone gene induction is concomitant with the initiation of DNA replication and since the cyclin A/cdk complex has been proposed to play a direct role in initiation of DNA synthesis by phosphorylating initiator proteins (44), one therefore may postulate that transcriptional induction of histone and possibly a set of other genes at the G1/S transition and initiation of DNA replication may be physically interrelated by means of common factors, such as cyclins and cdks, or by a common multifunctional transcription–replication complex. This hypothesis is supported by data reporting co-localization of replication and transcription complexes, called 'factories' in early S phase cells (45). More precise experiments also revealed the existence of replication origins in the regions surrounding several genes transcribed in early S phase, among which is a *Physarum* histone gene (46,47).

To check this model of a multifunctional complex, it will be crucial in the future to isolate the various transacting factors constituting the histone transcriptional complex and to investigate their potential involvement in the DNA replication process, and vice versa.

ACKNOWLEDGEMENTS

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Department of Plant Genetics, VIB – RUG
Marnik Vuylsteke, updated January, 2002

AFLP-BASED TRANSCRIPT PROFILING

The AFLP-based transcript profiling protocol (AFLP-TP) is an improved version of the cDNA-AFLP method described by Bachem et al. (Plant J. 9: 745-753, 1996). AFLP-TP is a fragment-based, genome-wide expression technique allowing the analysis of "all" genes involved in a particular biological process or expressed under certain conditions. In the approach, unique transcript tags derived from the 3' end region of expressed genes are PCR amplified and displayed on acrylamide gels. Selective amplification of subsets of transcript tags allows to fractionate the initial pool of tags and to detect low abundant messengers. Because it is PCR based and because of the fractionation, the sensitivity is significantly higher compared to other techniques including microarrays. Furthermore, as it is a fragment-based technique, it allows to discriminate between homologous sequences provided that distinct restriction fragments are obtained from the homologues.

The first step in the AFLP-TP approach is the conversion of mRNA into ds cDNA using a biotinylated oligo-dT primer. We start with about 2 ug total RNA for cDNA synthesis.

The cDNAs are digested with two restriction enzymes in a two-step reaction. After digestion with the first enzyme, the 3' most regions are captured on dynabeads. Digestion with the second enzyme releases the restriction fragments or transcript tags. The enzymes have been chosen such that the majority of the cDNAs is cut. The combination used in the protocol (*BstY*I and *Mse*I) cuts around 80% of all messengers (based on in silico analysis using available full-length cDNA sequences). Around 10% of the fragments is however too small or too large to be displayed on acrylamide gels. Almost 70% of the fragments are *BstY*I/*Mse*I fragments, the other 10% are *Mse*I/*BstY*I fragments. To screen all of them, the two series of analyses have to be done, with as first cutting enzyme *BstY*I and *Mse*I, respectively.

*BstY*I recognizes the sequence PuGATCPy and after digestion and adapter ligation, a pre-amplification is done using a *BstY*I-primer with a T or C as 3' nucleotide. In this way, the total pool of fragments is subdivided in two. In a subsequent amplification, selective primers are used. Increasing the number of selective nucleotides reduces the number of fragments amplified but results in amplification of fragments that are derived from low abundant messengers. Usually, primers with 1 or 2 selective nucleotides are used. In several plant species, a total of 3 selective nucleotides (e.g. *BstY*I+1 and *Mse*I+2 primers) results in profiles that are not too dense, while the sensitivity is already high enough to detect low abundant messengers.

After generating profiles, differentially expressed genes can readily be detected and isolated from the gels for further characterization. Depending on the density of the gel pattern, good quality sequence can be obtained for 50% to 80% of the isolated fragments by direct sequencing.

1. Total RNA isolation

Total RNA is isolated using the "Trizol"-method.

To check the yield/quality of the isolated RNA, a sample (1/10 of the total) is analyzed on an agarose gel. The RNA should appear as a faint smear from approximately 10 kb down, with trace rRNA bands. Spectrophotometric determination of the concentration (A_{260}=1 corresponds to 40 µg/ml) and purity (A_{260}/A_{280}) can be done.

2. Double stranded cDNA synthesis

- *First strand cDNA synthesis :*

Add together	20 µl total RNA (~2.0 µg)
	1 µl oligo-dT_{25}-**bio** (700 ng/µl)
	4 µl H_2O (DEPC!!!!)
Then add	8 µl 5x First Strand buffer
	4 µl 0.1 M DTT
	2 µl 10 mM dNTPs
	+ 1 µl Superscript II (200 U/µl)
	40 µl

Incubate 2 hr at 42°C

DEPC-water : 100 ml water + 100 µl DEPC (diethylpyrocarbonate),
Let stand overnight at room temperature and autoclave

- *Second strand synthesis :*

Add together	40 µl first strand reaction mixture
	16 µl 10x Second Strand buffer (E. Coli ligase buffer)
	3 µl 10 mM dNTPs
	6 µl 0.1 M DTT
	1.5 µl E.coli ligase (10 U/µl) (or 15 units)
	5.0 µl E.coli polymerase I (10 U/µl) (or 50 units) (DNA polymerase I)
	1.6 µl RNAse-H (1 U/µl) (or 1.6 units) (Ribonuclease H)
	H_2O to 160 µl

Incubate 1 hr at 12°C and 1 hr at 22°C in a thermoblock

Purify the cDNA using a Qiaquick spin column (qiaquick PCR purification kit, qiagen).
Check the quality and yield of cDNA by agarose gel electrophoresis (1 % agarose / 0.5x TAE).

5x First Strand buffer:

250 mM Tris.HCl pH 8.3; 15 mM $MgCl_2$; 375 mM KCl

10x Second Strand buffer:

188 mM Tris.HCl pH 7.0; 46 mM $MgCl_2$; 906 mM KCl

1500 µM NAD^+; 100 mM $(NH_4)_2SO_4$

500 ng of the double stranded cDNA preparation is used for AFLP.

3. AFLP template preparation (using *BstY*I and *Mse*I)

First digestion :

Mix: 20 µl cDNA (500 ng)
10 Units *BstY*I
4 µl 10x RL-buffer
H_2O to 40 µl

Incubate 2 hrs at 60°C

10x RL-buffer: 100 mM Tris-HAc (pH 7.5), 100 mM MgAc, 500 mM KAc, 50 mM DTT, 50 ng/µl BSA (optional).

Immobilization of 3'-terminal cDNA fragments on dynabeads :

The biotinylated 3'-end fragments are separated from the non-biotinylated fragments by binding to streptavidin beads (Dynal).

- Per sample, wash 10 µl dynabeads once in 100 µl 2x STEX and resuspend it in 40 µl 2x STEX (the same volume as the restricted cDNA) (washing dynabeads for 7-8 samples maximally in one tube).
- Add the beads (40ul) to the restricted cDNA (40ul), to give a final volume of 80 µl.
- Incubate the mixture at room temperature for 30 minutes with gentle agitation (1000 rpm).
- Collect the beads with the magnet, wash once with 100 µl 1x STEX and transfer to a fresh tube.
- Wash **4** additional times with 100 µl 1x STEX
- Resuspend the beads in 30 µl $T_{10}E_{0.1}$ and transfer again to a fresh tube.

2x STEX: 2 M NaCl, 20 mM Tris-HCl (pH 8.0), 2 mM, 0.2 % Triton X-100

Second digestion :

Add to the 30 µl beads-suspension:
10 Units enzyme *Mse*I
4 µl 10x RL-buffer
H_2O to 40 µl

Incubate 2 hr at 37 °C with gentle agitation (1000 rpm) to ensure that beads are resuspended occasionally

Collect the beads using the magnet and transfer the supernatant, containing the liberated template fragments, to a clean tube for the subsequent adapter ligation reaction.

Adapter preparation :

All adapters (*BstY*I- and *Mse*I-adapter) are non-phosphorylated oligonucleotides

For the *BstY*I-adapter, mix oligo F and R such that an adapter is obtained at a concentration of 5 pmol/µl.

*BstY*I-F: 5' – CTCGTAGACTGCGTAGT – 3'
*BstY*I-R: 5' – GATCACTACGCAGTCTAC – 3'

*BstY*I adapter : 5 pmol/µl

*BstY*I-R (100 µM)	: 2.5 µl
*BstY*I-F (100 µM)	: 2.5 µl
H_2O	: 45 µl
	50 µl

For *Mse*I, mix oligo F and R such that an adapter is obtained at a concentration of 50 pmol/µl.

*Mse*I-F: 5' – GACGATGAGTCCTGAG – 3'
*Mse*I-R: 5' – TACTCAGGACTCAT – 3'

MseI adapter : 50 pmol/µl

*Mse*I-R (100 µM)	: 25.0 µl
*Mse*I-F (100 µM)	: 25.0 µl
	50.0 µl

Adapter ligation :

Add to the supernatant (containing the template fragments; 40 µl) a mix of 10 µl containing:

1 µl *BstY*I adapter (= 5 pmol)
1 µl *Mse*I adapter (= 50 pmol)
1 µl 10 mM ATP (Pharmacia)
2 µl 5 x RL-buffer
1 µl T4 DNA ligase (5 U/µl) (or 1 unit) (Pharmacia)
10 Units *BstY*I
H_2O to 10 µl

Incubate 3 hrs at 37°C

4. Pre-amplification with non-selective primers

Dilute the adapter ligation 2-fold with $T_{10}E_{0.1}$ and use 5 µl as a template in the pre-amplification. *BstY*I-T+0 and *BstY*I-C+0 are combined with the *Mse*I+0 primer

Primers:

*BstY*IT+0: 5' – GACTGCGTAGTGATCT – 3'
*BstY*IC+0: 5' – GACTGCGTAGTGATCC – 3'
*Mse*I+0: 5' – GATGAGTCCTGAGTAA – 3'

The reaction contains:

5.0 µl 2-fold diluted adapter ligation
1.5 µl *BstY*I+0 primer (50 ng/µl = 75 ng)
1.5 µl *Mse*I+0 primer (50 ng/µl = 75 ng)
2.0 µl 5 mM dNTPs (--> 0.2 mM final concentration of each dNTP)
0.2 µl Taq polymerase (AmpliTaq, Perkin-Elmer, 5 units/µl) (or 1 unit)
5.0 µl 10x PCR-buffer
H_2O to 50 µl

10x PCR-buffer : 100 mM Tris.HCl pH 8.3, 25 mM $MgCl_2$, 500 mM KCl

It is advisable to work with mixes of reagents as much as possible to avoid differences in amplification between different reactions; the following procedure is suggested:

Primer mix:

1.5 µl *BstY*I+0-primer
1.5 µl *Mse*I+0-primer
2.0 µl 5 mM dNTPs
20.0 µl H_2O

Taq mix:

0.2 µl Taq polymerase (5 units/µl)
5.0 µl 10 x PCR-buffer
14.8 µl H_2O

Each reaction contains 5 µl template, 25 µl of primer mix and 20 µl of Taq mix

PCR amplification:	**No hot start!!!**
	30 sec. 94°C
25 cycles	60 sec. 56°C
	60 sec. 72°C

Check the pre-amplification by running 10 µl of the reaction mixture on an agarose gel.

5. Selective amplification-reaction using ^{33}P labeled primer

Primer labeling :

For one single AFLP reaction, 5 ng of labeled primer is needed.

Reaction Mix for ^{33}P:

0.1 µl of a 50 ng/µl primer stock-solution
0.1 µl ^{33}P-γ-ATP (≈2000 Ci/mmol ≈ 50 pmol; Amersham)
0.05 µl 10x T4-buffer
0.2 units T4-kinase
H_2O to 0.5 µl

10x T4-buffer: 250 mM Tris.HCl pH 7.5, 100 mM $MgCl_2$, 50 mM DTT,
5 mM spermidine (3HCl-form; Sigma)

0.5 µl final volume yields labeled primer at a concentration of 10 ng/µl

Incubate 45 min at 37°C and, subsequently, 10 min at 80°C (inactivation of the kinase).

The actual reaction volumes depend on the number of AFLP amplifications that need to be performed with the selective primer.

PCR reaction :

For each amplification, 5 µl of a **600-fold dilution** of the pre-amplification mixture is used as template.

A single PCR reaction contains:

5.0 µl pre-amplification mix (diluted 600-fold in $T_{10}E_{0.1}$)
0.5 µl labeled *BstY*I+N-primer (10 ng/µl --> 5 ng)
6 µl unlabeled *Mse*I+N-primer (5 ng/µl --> 30 ng)
0.8 µl 5 mM dNTPs
2.0 µl 10x PCR-buffer
0.12 µl AmpliTaq-"Gold" polymerase (5 U/µl) (0.6 units)
H_2O to 20 µl

$T_{10}E_{0.1}$-buffer : 10 mMTris-HCl (pH8.0), 0.1 mM EDTA
10x PCR-buffer : 100 mM Tris-HCl (pH 8.3), 25 mM $MgCl_2$, 500 mM KCl

Again, work with mixes. The AFLP-reactions may be done as follows, in case one particular labelled primer is used in combination with many different *Mse*I+N primers:

5.0 µl template DNA
5.0 µl *Mse*I-primer (dilute stock to 6 ng/µl with H_2O --> 30 ng)
10.0 µl mix; amounts for 100 µl or 10 reactions
- 5.0 µl labeled *BstY*I-primer (10 ng/µl --> 50 ng)
- 8.0 µl 5 mM dNTPs
- 20.0 µl 10x PCR-buffer
- 6 units AmpliTaq-Gold polymerase (1.2 µl of 5 U/µl)
- 65.8 µl H_2O

10x PCR – buffer : 100 mM Tris-HCl (pH 8.3), 25 mM $MgCl_2$, 500 mM KCl

PCR amplification :

10 min 94°C **(HOT START!!!!)**

13 cycles : 30 sec 94°C
30 sec 65°C ↓ 0.7 °C/cycle
60 sec 72°C

23 cycles: 30 sec 94°C
30 sec 56°C
60 sec 72°C

Gel analysis :

After PCR amplification, the samples are analyzed on 5% polyacrylamide gels using the Sequigel system (Bio-Rad). Gel electrophoresis is done as in conventional AFLP analysis.
A labeled 10 bp sizing standard (Sequamark, Research Genetics) is included as size marker.
After running, the gel is vacuum-dried on a whatmann paper and exposed on a Biomax MR autoradiogram (Kodak) or scanned in a phosphor-imager.

6. Selective amplification- reaction using IRD-700 labeled primers (LI-COR)

PCR- reaction

For each amplification, 5 µl of a **600-fold dilution** of the pre-amplification mixture is used as a template.

A single PCR-reaction contains :

5.0 µl pre-amplification mix (600x diluted in $T_{10}E_{0.1}$)
0.8 µl IRD-700 labeled *BstY*I+N primer (6 ng/µl, diluted in $T_{10}E_{0.1}$)
3 µl unlabeled *Mse*I+N primer (10 ng/µl, diluted in H_2O)
0.8 µl dNTP's (5 mM)
2 µl 10x PCR-buffer II (Perkin Elmer)
2.4 µl $MgCl_2$ (25 mM, Perkin Elmer)
0.2 µl Ampli TAQ DNA polymerase (5 U/µl, Perkin Elmer)
H_2O to 20 µl

PCR amplification :

13 cycles	94°C - 10 sec	
	63°C - 30 sec	↓ 0.7 °C/cycle
	72°C - 60 sec	
25 cycles	94 °C - 10 sec	
	54 °C - 30 sec	
	72 °C - 60 sec	↑ 1 sec/cycle
72 °C - 2 min		

Gel Analysis :
After PCR amplification, the samples are analyzed on a 6.5 % polyacrylamide gel (Kbplus gel matrix) using the LI-COR. The 50-700 bp sizing standard from LI-COR is used as size marker.

TP-BUFFERS

10 x RL-buffer :

100 mM Tris-Hac (pH 7.5)
100 mM $MgAc_2$
500 mM KAc
50 mM DTT

10 ml
1 ml Tris-Hac 1M (pH7.5)
1 ml $MgAc_2$ 1 M
1.25 ml KAc 4M
0.077 g DTT
Add H_2O to 10 ml

2xSTEX- buffer :

2 M NaCl
20 mM Tris-HCl (pH 8.0)
2 mM EDTA (0.5 M)
0.2 % TritonX-100

100 ml
40 ml NaCl 5M
2 ml Tris-HCl 1M (pH 8.0)
400 µl EDTA (0.5 M)
2 ml TritonX-100 (10 %)
Add H_2O to 100 ml

10 x PCR-buffer :

100 mM Tris-HCl (pH 8.3)
25 mM $MgCl_2$
500 mM KCl

10 ml
1 ml Tris-HCl 1 M (pH 8.3)
250 µl $MgCl_2$ 1 M
2.5 ml KCl (2 M)
Add H_2O to 10 ml

$T_{10}E_{0.1}$:

10 mM Tris-HCl (pH 8.0)
0.1 mM EDTA (pH 8.0)

100 ml
1 ml Tris-HCl 1 M (pH 8.0)
20 µl EDTA 0.5 M
Add H_2O to 100 ml

Formamide loading dye (^{33}P) :

2 ml EDTA 0.5 M (pH 8.0)
98 ml Formamide
0.06 g bromophenol blue

10 x T4-buffer :

250 mM Tris-HCl (pH 7.5)
100 mM $MgCl_2$
50 mM DTT
5 mM spermidine (3 HCl.form)
0.06 xylenecyanol

10 ml
2.5 ml 1M Tris-HCl (pH 7.5)
1 ml $MgCl_2$ 1 M
0.077 g DTT
0.013 g spermidine
Add H_2O to 10 ml

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TECHNICAL ADVANCE

Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: Analysis of gene expression during potato tuber development

Christian W.B. Bachem[1,3,*], Rutger S. van der Hoeven[1], Steef M. de Bruijn[2], Dick Vreugdenhil[2], Marc Zabeau[3] and Richard G.F. Visser[1]

[1]Department of Plant Breeding, and [2]Department of Plant Physiology, Graduate School of Experimental Sciences, Wageningen Agricultural University, P.O. Box 386, 6700 AJ Wageningen, The Netherlands and

[3]Keygene N.V., Agro Business Park 90, P.O. Box 216, 6700 AE Wageningen, The Netherlands

Summary

Using a highly synchronous *in vitro* tuberization system, in combination with an amplified restriction fragment polymorphism (AFLP[†])-derived technique for RNA fingerprinting (cDNA-AFLP[‡]), transcriptional changes at and around the time point of potato tuberization have been analyzed. The targeted expression analysis of a specific transcript coding for the major potato storage protein, patatin and a second transcript, coding for ADP-glucose pyrophosphorylase, a key gene in the starch biosynthetic pathway is described. This paper confirms that kinetics of expression revealed by cDNA-AFLP analysis are comparable to those found in Northern analysis. Furthermore, this paper reports the isolation and analysis of two tuber-specific transcript-derived fragments (TDFs) coding for the lipoxygenase enzyme, which are differentially induced around the time point of tuber formation. Analysis of the two *lox* TDFs demonstrates that it is possible to dissect the expression modalities of individual transcripts, not independently detectable by Northern analysis. Finally, it is shown that using cDNA-AFLP, rapid and simple verification of band identity may be achieved. The results indicate that cDNA-AFLP is a broadly applicable technology for identifying developmentally regulated genes.

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*For correspondence (fax +31 317 483457; e-mail Christian.Bachem@users.pv.wau.nl).

†AFLP is a trademark filed by Keygene N.V.

‡A detailed protocol of the cDNA-AFLP protocol is available on the WWW page http://www.spg.wau.nl/pv/staff/aflp.htm.

Introduction

Methods for isolating differentially expressed genes, by the use of differential cDNA library screening, have been work-intensive and likely to yield clones derived from abundantly expressed genes. More recently, techniques for RNA fingerprinting have been developed allowing the detection of DNA fragments derived from RNA using cDNA synthesis and subsequent PCR amplification (McClelland *et al.*, 1995). In the differential display method (Liang and Pardee, 1992), cDNA is subjected to PCR amplification using a poly-dT primer (3') and arbitrary oligonucleotides for the 5'-primer. In plants, this technique tends to give a preponderance of 3'-untranslated sequences in the resulting DNA fragments, which make the data difficult to characterize further by database searches (Heidstra, personal communication). In an alternative protocol (Welsh *et al.*, 1992) arbitrary primers are used both for cDNA synthesis and PCR amplification. Both variants of RNA fingerprinting require a low annealing temperature during thermo-cycling in order to achieve visualizable products. As a consequence, the quantity of individual amplification products, is not only a function of initial concentration of that cDNA species, but also is dependent on the quality of a particular match between primer and template (McClelland *et al.*, 1995). As a result, abundant cDNAs with poor matches to the primers used, are likely to outperform rarer species with perfect matches, during the course of PCR amplification.

Both RNA and DNA fingerprinting methods rely on the selective amplification of a subset of DNA molecules from a more complex pool. Recently, a novel method for achieving this aim in genomic DNA has been developed and has been described as amplified restriction fragment length polymorphism (AFLP) (Vos *et al.*, 1995). The system is based on the use of highly stringent PCR conditions, facilitated by adding double-stranded adaptors on the ends of restriction fragments which serve as primer sites during amplification. Selective fragment amplification is achieved by adding one or more bases on to the PCR primers which will only then be successfully extended if the complementary sequence is present in the fragment flanking the restriction site, thereby reducing the number of visualized bands.

In this paper we present a reliable and robust method

of RNA fingerprinting, based on AFLP, which allows the detailed characterization of gene expression in a wide range of biological processes. We have applied this technique to the analysis of tuber formation in potato. Investigation of possible regulatory pathways directing potato tuber organogenesis has been difficult to execute in a whole-plant system (Jackson *et al.*, 1993). In order to avoid these problems, we have used a synchronized *in vitro* tuberization system in which a detailed physiological analysis of the tuberization process has been carried out (Hendriks *et al.*, 1991). All parameters examined in our *in vitro* tuberization system and in others using a number of different potato varieties, indicate that they are largely comparable to *in vivo* grown material (Peterson *et al.*, 1985). Such a synchronous developmental system is a prerequisite for a detailed dissection of differential gene expression.

A broad range of physiological, biochemical and molecular genetic studies have been carried out on developing potato tubers. Genes coding for major storage proteins (patatin gene family) have been extensively studied (Peterson *et al.*, 1985). Also considerable progress has been made in isolation and characterization of genes associated with starch synthesis (Preiss, 1991). One of these, ADP-glucose pyrophosphorylase, has been shown to play a central role in the regulation of starch synthesis (Müller-Röber *et al.*, 1992; Preiss, 1991). However, little is known about the regulation of the process of tuber organogenesis. Early investigations using grafting experiments indicated the presence of a hormone-like factor produced in the leaves which induces tuber formation (Gregory, 1956). More recently, a compound termed tuberonic acid, which is chemically related to jasmonic acid, has been shown to have strong tuber-inducing activity (Koda *et al.*, 1991). The enzyme responsible for synthesis of jasmonates and related compounds in plants is lipoxygenase. It is also known that potato tubers produce large amounts of this enzyme (Pinskey *et al.*, 1971). Two potato *lox* genes have been described from root (Feltkamp *et al.*, 1994) and from tuber (Casey, 1995). Although potato *lox* genes have been shown to be induced in potato tubers after wounding (Geerts *et al.*, 1994) no involvement of *lox* genes in tuberization has yet been demonstrated.

In this paper we analyse the expression of two genes known to be expressed during tuberization, namely, patatin and ADP-glucose pyrophosphorylase and demonstrate that the transcript-derived fragments (TDFs) generated by cDNA-AFLP reveal an expression pattern correlated closely to data obtained using traditional methods. Furthermore, the expression analysis of two highly homologous and novel *lox* genes is described, which are differentially expressed during tuberization.

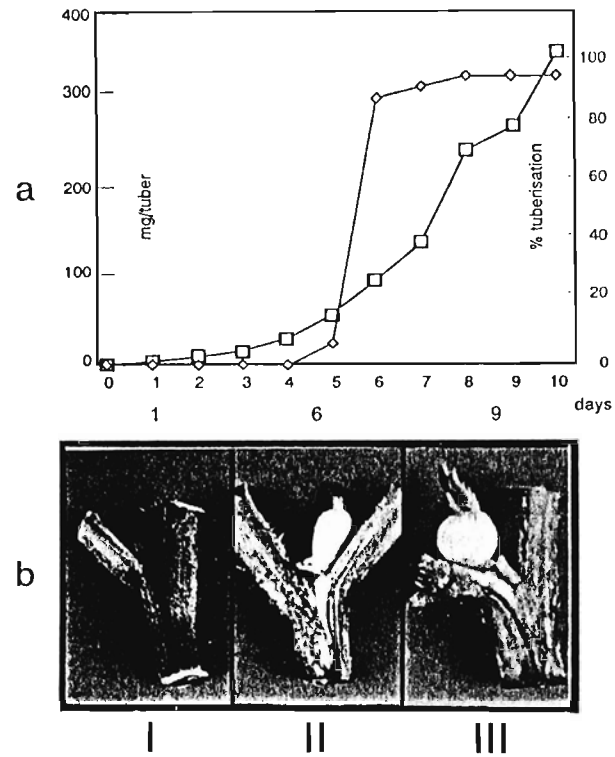

Figure 1. Tuberization profile of the April 1994 harvest.
(a) Tuber mass (square symbols) and tuberization percentage (diamond symbols) plotted as a function of the harvest time points in days.
(b) Tuberizing explants showing the stages of growth of the axillary bud on days 0, 6, and 10 on panels I, II and III, respectively.

Results

Tissue culture system and synchronicity of tuberization

Two separate series of tuberizing nodal cuttings were harvested and used for the isolation of RNA for template production (January/February 1994 and April 1994). Both harvests gave the same banding patterns in cDNA-AFLP analysis and thus, the April harvest was used for further analysis. Figure 1a shows the tuberization profile of this harvest along with the fresh weight development. Figure 1b shows three examples of the development of the axillary buds from the nodal cuttings during tuber induction. The day 0 sample represents the axillary bud before the start of the *in vitro* culture (Figure 1b, panel I). During the first few days a slight elongation of buds was observed, followed by swelling from day 5 onwards (Figure 1b, panel II; day 6), resulting in microtubers (Figure 1b, panel III; day 10). As can be seen on the graphs (Figure 1a), tuberization was highly synchronous, increasing from 5 to 90% between day 5 and day 6.

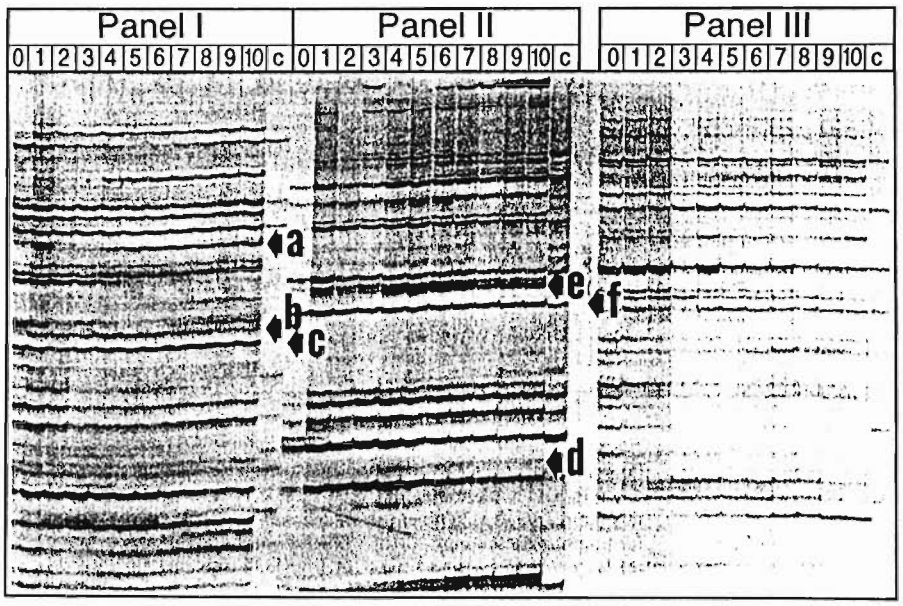

Figure 2. Example of a cDNA-AFLP gel autoradiogram.
The three primer combinations used are shown in panels I, II and III. Each panel represents 11 lanes corresponding to amplified template from day 0–10, respectively. The 12th lane labelled c is a control template derived from a mixture of RNA samples isolated from mature field-grown tubers and leaf. Arrows label bands described in the text.

Choice of parameters for cDNA-AFLP analysis of gene transcription during tuberization

The cDNA-AFLP technique uses the standard AFLP protocol (Vos *et al.*, 1995) on a cDNA template. In genomic AFLP, two restriction enzymes are generally used for the preparation of PCR template. The choice of enzymes is determined on one hand, by the complexity of the genome under investigation and on the other hand by such factors as the methylation status of the DNA. For cDNA template, the choice of restriction enzyme pair is determined by the frequency of cleavage. Ideally, the restriction enzymes should cut every cDNA species once (rare cutter) and subsequently cut the cDNA on one or both sides adjacent (frequent cutter) to yield fragments of between 100 and 1000 bp in size. We used restriction enzymes which, in a review of all available potato cDNAs, came closest to these preconditions (*Ase*I and *Taq*I). Around 45% of all analysed cDNAs had an *Ase*I site and around 87% of these had additional *Taq*I sites, thus, a little less than half of all genes expressing in a given tissue could potentially be visualized using this enzyme combination. In genomic AFLP with plant DNA, three selective bases on the end of each primer are required to give a storable banding pattern. The lower complexity of the cDNA allows the use of two selective bases for each primer giving a total of 256 possible primer combinations. The preparation of several templates made with different restriction enzyme combinations would potentially allow the visualization of virtually every transcript as a TDF using a finite and practically manageable number of cDNA-AFLP reactions.

Parameters for identifying TDFs with potential relevance for the tuberization process were chosen on the basis of their differential expression pattern. We chose TDFs for further study which appeared strongly just before or during tuberization, that is, between days 3 and 6 in our *in vitro* tuberization system (Figure 2, arrows a, d and e). TDFs which appeared directly on day 1 were avoided as the likelihood of association with trauma or other tissue culture effects was assumed to be high. In addition to day 0 template, control templates were also run routinely in the form of a 1:1 mixture of template derived from potato leaf RNA and mature field-grown tuber RNA (Figure 2, lane c). Bands appearing in this control were regarded as not specific for tuberization, but were not necessarily excluded from further investigation. Templates were also made from various plant tissues and these templates were then used to analyze tissue-specific expression of individual TDFs rather than in routine screening.

General characteristics of cDNA-AFLP expression fingerprints

Figure 2 shows a typical cDNA-AFLP gel produced by amplification of template derived from tuberizing axillary products harvested during the day 0 to day 10 period. Three different primer combinations are shown using two selective bases at each restriction enzyme site (Figure 2, panels I–III). The largest visible products (Figure 2, panel II) are around 1000 bp in size and the lower end of the gel represents approximately 100 bp. In this size window an average of 40 bands can be scored for each primer combination. In Figure 2, various expression patterns can be detected across the 10 day period: (i) steady increase (arrow a), (ii) steady decrease (arrow b), (iii) constitutive (arrow c), and (iv) transient expression (arrow d). The contrasting banding patterns of the last lane (Figure 2,

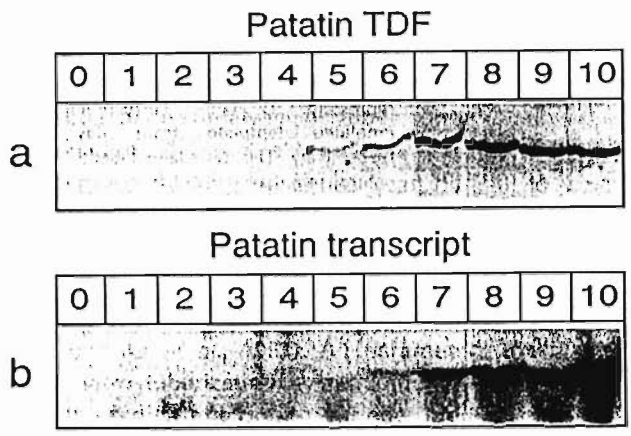

Figure 3. Comparison of patatin gene expression with cDNA-AFLP and Northern analysis.
(a) Expression profile of the 570 bp patatin TDF in template derived from day 0 to day 10 plant material.
(b) Northern analysis of total RNA from day 0 to day 10 plant material probed with patatin cDNA fragment.

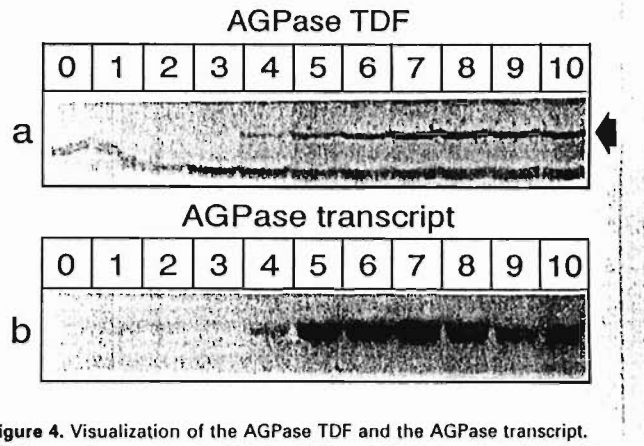

Figure 4. Visualization of the AGPase TDF and the AGPase transcript.
(a) Expression profile of a 391 bp AGPase TDF in template derived from day 0 to day 10 plant material. (The small dots seen around the bands in this figure, are the alignment reference points on the autoradiogram used for fragment isolation.)
(b) Northern analysis of total RNA from day 0 to day 10 plant material probed with AGPase cDNA fragment.

lane c) in each panel represent different bands derived from adding a 1:1 mixture of two templates from different organs (leaf and tuber).

Owing primarily to the stringent conditions that may be chosen in cDNA-AFLP, resolution of the banding pattern is high and the background remains at an acceptable level (Figure 2). Furthermore, quite radical changes in the intensity of individual bands over the 10 day period (Figure 2, arrow e), do not seem to affect the patterns of other bands in the same lane (Figure 2, arrow f). This leads to the conclusion that the PCR remains largely proportional and independent of the concentration of individual substrates in the reaction.

Using an optimized combination of standard protocols for isolating, reamplifying and cloning individual TDFs, has allowed the identification of a number of transcripts which are related to tuberization events. Sequence analysis showed that in all cases appropriate terminal sequences were found corresponding to the two selective base extensions used in the PCR indicating the stringency and fidelity of the system (see example shown in Figure 6).

Analysis of gene expression using cDNA-AFLP and Northern analysis

To examine the accuracy of the cDNA-AFLP process, control genes were chosen, the expression pattern of which was well described and documented. In addition to DNA sequence information, the presence of suitable *Ase*I and *Taq*I sites in the coding region of the gene was a prerequisite. The patatin B2 gene (STPATB2, EMBL Data Library accession number X13178; Stiekema *et al.*, 1988) was found to fulfil these criteria. The TDF was predicted to yield a fragment of 570 bp when selective base extensions AA (*Ase*I-end) and AC (*Taq*I-end) were used on the 3′ ends of the primers. The autoradiograph (Figure 3a) shows a band of the expected size and transcription pattern when compared with the Northern analysis (Figure 3b). The identity of this TDF was further verified by isolation, cloning and DNA sequence analysis. Alignment of the sequences from the patatin TDF and STPATB2 showed an almost perfect match (99% over the 500 bp sequenced).

The results demonstrate clearly that the onset of patatin expression begins concomitant with tuber formation (day 5; see Figure 1). The lack of a further increase of amplification product after day 8 in the cDNA-AFLP (transcript continues to increase in the Northern), indicates that the transcription of this mRNA, specifically targeted using this primer pair, does not increase beyond day 8.

A second gene, known to be induced during tuberization was also found to be differentially expressed during tuberization. The gene coding for the large subunit of the ADP-glucose pyrophosphorylase (AGPase; Müller-Röber *et al.*, 1992) is induced as part of the starch synthesis machinery and can be seen clearly as a transcript from day 4 in our *in vitro* tuberization system (Figure 4b). The corresponding TDF of 391 bp shows a very similar expression pattern over the full range of developmental stages analyzed (Figure 4a). The sequence analysis shows a 97% homology to the *Solanum tuberosum* cDNA (STGLL, EMBL Data Library accession number X61187; Nakata *et al.*, 1991).

From the above comparison of Northern and cDNA-AFLP analysis it can be concluded that the cDNA-AFLP method gives highly reliable results over a broad range of template concentrations. The quantitative response in the cDNA-AFLP system seems to be broadly proportional to the input DNA. Moreover, transcripts with known sequences can be targeted for analysis and used for individual gene expression analysis or for controlling the system.

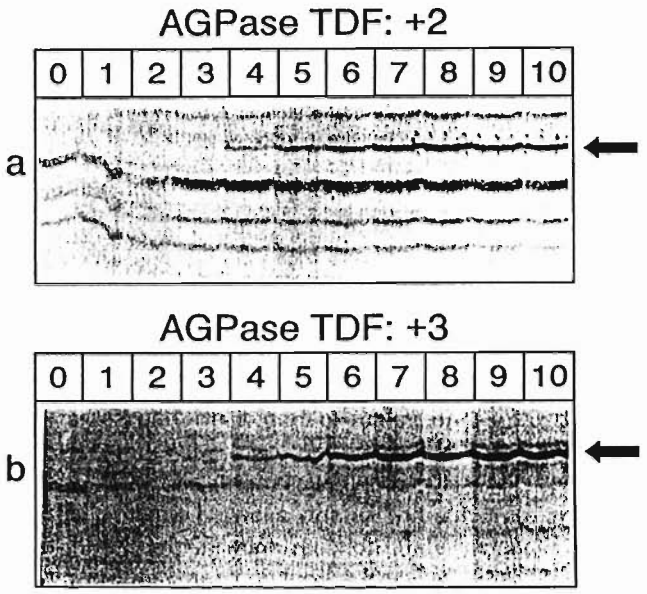

Figure 5. Verification of band identity using cDNA-AFLP.
(a) AGPase TDF identified using +2 bp selective primer extensions and coamplification of other bands.
(b) Amplification of the AGPase TDF using +3 bp selective primer extensions.

Verification of band identity using cDNA-AFLP

One of the more difficult procedures in RNA fingerprinting has been to verify that the band isolated and analyzed further is the same as the one labelled and visualized in the original amplification. Several methods have been suggested to achieve this verification. All, however, are indirect and involve several complicated manipulations (McClelland *et al.*, 1995). In cDNA-AFLP it is possible to almost totally eliminate non-target bands from the PCR by increasing the length of the selective extensions. This results in the target TDF being preferentially amplified, retaining the expected expression profile and at the predicted size. In practice, a cDNA-derived template, amplified with three selective bases at each restriction site, is sufficient to eliminate virtually all other bands from being co-amplified. When the sequence of the target band is unknown, this requires an additional 16 reactions when the initial identification was executed using two selective bases/end. Figure 5a shows an example of the AGPase TDF amplified with two selective bases and Figure 5b shows an example with the three selective bases on each end. The disappearance of other bands in the 3+3 selective base cDNA-AFLP is clearly to be seen and additionally the expression profile is maintained. The procedure of band identity verification can thus easily and rapidly be executed using cDNA-AFLP whether the sequence of the TDF is known or not. Simultaneously, further supportive data can be obtained regarding the expression of the targeted gene in the system under investigation.

```
Root-LOX                TTATACCATATTTGAGGAGGATAAACACTACAACAACGA
Tuber-LOX   TCATGATGTGATTATACCTTATTTGAGGAGGATAAACACTACAATAACGA

Root-LOX    AAACATATGCCTCGAGAACTTTGCTCTTCTTGCAAGATAATGGATCTTTG
Tuber-LOX   AAGCATATGCCTCGAGAACTTTGCTCTTCTTGCAAGATAATGGATCTTTG
TDF   531             [c]CGAGAACTTTGCTCTTCTTGCAAGATAaTGGATCTcTG
                       TaqI

Root-LOX    AAGCCACTAGCAATTGAATTGAGTTTGCCACATCCAGATGGAGATCAATT
Tuber-LOX   AAGCCACTAGCAATTGAATTGAGTTTtCCACATCCAGATGGAGATCAATT
TDF   531   AAGCCACTAGCAATTGAATTGAGTTTGCCACATCCAGATGGAGATCAATT

Root-LOX    TGGTGTTAtTAGTAAAGTGTATACTCCAAGTGATCAAGGTGTTGAGAGCT
Tuber-LOX   TGGTGTTACTAGCAAAGTGTATACTCCAAGTGATCAAGGTGTTGAGAGCT
TDF   531   TGGTGTTACTAGCAAAGTGTATACTCCAAGTGATCAAGGcGTTGAGAGCT

Root-LOX    CTATCTGGCAATTGGCCAAAGCTTATGTTGCGGTGAATGACTCTGGTGTT
Tuber-LOX   CTATCTGGCAATTGGCCAAAGCTTATGTTGCGGTGAATGACgtTGGTGTT
TDF   531   CTATCTGGCAATTGGCCAAAGCTTATGTTGCGGTGAATGACTCTGGTGTT

Root-LOX    CATCAACTAATTAGTCATTGGTTGAATACACATGCGGTGATTGAGCCATT
Tuber-LOX   CATCAACTAATTAGTCATTGGTTGAATACtCATGCaGTGATaGAGCCATT
TDF   531   CATCAACTAATTAGTCATTGGTTGAATACACATGCGGTGATTGAGCCATT
TDF   536                                   [c]cGAGCCATT
                                             [TaqI]

Root-LOX    TGTGATTGCAACAAACAGGCAACTAAGTGTGCTTCACCCTATTCATAAGC
Tuber-LOX   TGTGATTGCAACAAACAGGCAACTAAGTGTGCTTCACCCTATTCATAAGC
TDF   531   TGTGATTGCAACAAACAGGCAACTAAGTGTGCTTCACCCTATTCATAAGC
TDF   536   TGTGATTGCAACAAACAGGCAACTAAGTGTGCTTCACCCTATTCATAAGC

Root-LOX    TTCTATATCCTCATTTCCGGGACACAATGAATATTAAT
Tuber-LOX   TTCTATATCCTCATTTCCGGGACACAATGAATATTAAT
TDF   531   TTCTATATCCTCATTTCCGGGACACAATGAATATTA[oo]
TDF   536   TTCTATATCCTCATTTCCGGGACACAATGAATATTA[oo]
                                        AseI
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Figure 6. Alignment of sequences from two *lox* genes (Root-LOX; Feltkamp *et al.*, 1994 and Tuber-LOX; Casey, 1995) and sequences from *lox* TDF531 and TDF536.
Both the Root-LOX and the Tuber-LOX start from base number 1500. Restriction sites are shown below the sequences. Lower case letters in the sequences indicate mismatches. The second *Taq*I site is unique for TDF536 and is thus, represented in brackets. Square brackets surrounding sequences at the beginning and ends of the TDFs, derive from the adaptors ligated on the cDNA fragments. Restriction sites are thus inferred from restriction enzyme digestion rather than sequence data.

Differential, temporal and spatial expression of two lox *alleles during tuberization*

During further scanning of the 10 day template with different primer combinations, two TDFs were isolated (designated TDF531; 327 bp and TDF536; 98 bp), purely on the basis of their differential expression during tuber formation. Sequence analysis revealed both TDF531 and TDF536 to be highly homologous to two *lox* sequences coding for root and tuber *S. tuberosum* lipoxygenase enzymes (Figure 6). Interestingly, the two *lox* TDFs not only show a differential expression pattern during tuber formation but also have profiles distinct from one another. On the autoradiogram TDF531 is present from day 0 to 2 at low levels and increases steadily to a peak at day 10 (Figure 7a). TDF536, however, appears only on day 6 and increases to day 10 (Figure 7b).

Sequence analysis revealed a single base change in TDF536 (Figure 6), introducing an additional *Taq*I site and yielding a fragment of 98 bp as opposed to the 327 bp band in the case of TDF531. Also, other minor sequence differences can be observed in TDF531 compared with the known potato *lox* genes (lower case in Figure 6). These results suggest that at least two different *lox* genes are being expressed during tuber formation. One (TDF531) is induced prior and during tuberization while the other comes to expression well after tuber formation has begun.

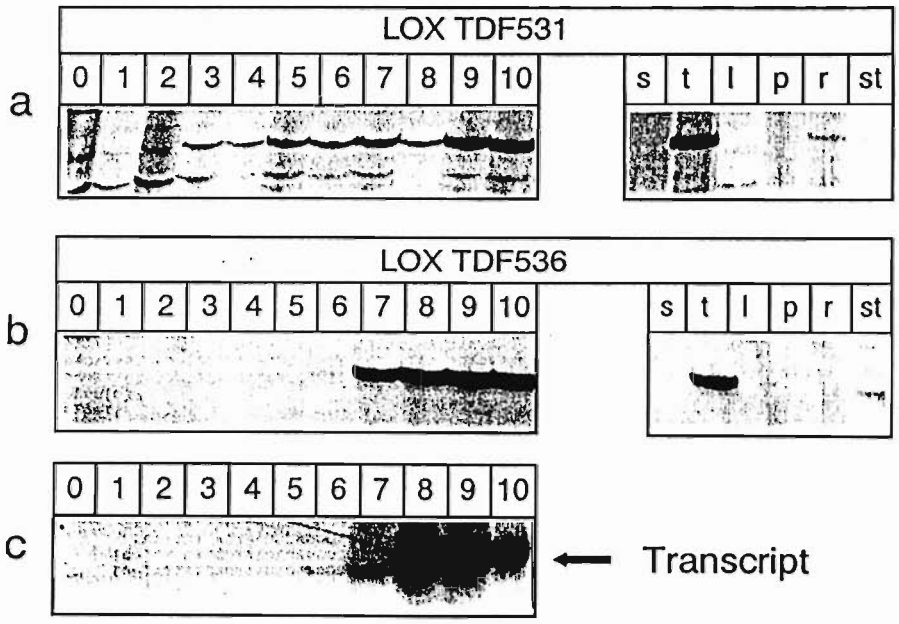

Figure 7. Analysis of *lox* TDFs and transcript in tuberizing explants.
(a) *lox* TDF531 in template derived from day 0 to day 10 plant material and in other potato tissues: s, stolons; t, 15 day tubers; l, leaf; p, petioles; r, roots; st, stems.
(b) *lox* TDF536 in template derived from day 0 to day 10 plant material and in other potato tissues (as in a).
(c) Northern analysis of *lox* transcript in total RNA isolated from day 0 to day 10 plant material. The probe was the 327 bp TDF531.

In order to establish the expression throughout the plant, cDNA-AFLP template was produced from a series of different potato tissues. The resulting banding pattern shows that both TDF531 and TDF536 are highly tuber-specific, expressed strongly in 15-day-old tubers (Figure 7a and b; lane t), not in stolons, leaves or petioles (Figure 7a and b, lanes s, l and p) and only at very low levels in stems (Figure 7a and b, lane st). Interestingly, TDF531 also appears weakly in root (Figure 7a; lane r) where TDF536 does not.

The expression of *lox* genes was also analyzed on a Northern level (Figure 7c). Here, transcript could be clearly detected at day 6, increasing to day 10. Longer exposure of the blot also revealed a low level of signal from day 4 (data not shown). Expression from the TDF536 found in the cDNA-AFLP analysis agrees well with the picture obtained in the Northern analysis (Figure 7b and c). Expression from the second gene represented by TDF531 seems to be undetectable in the initial stages of development (days 1–5) in the Northern analysis (Figure 7a and c). It can, also, not be excluded that expression of further alleles contributes to the profile obtained in the Figure 7c.

Although *lox* genes have been shown to be expressed in potato tuber tissues the specificity of the cDNA-AFLP system offers a unique opportunity to examine the expression of two such highly homologous alleles.

Discussion

Using a novel method for the visualization of gene expression we have identified a number of transcripts which show differential expression during tuber organogenesis. Previous studies have shown the induction of patatin and AGPase in an *in vitro* tuberization system (Hendriks *et al.*, 1991; Visser *et al.*, 1994). We show here that the cDNA-AFLP method faithfully reproduces these results. We further report on the expression profiles of two tuber-specific *lox* transcripts which are differentially expressed during tuberization and also show contrasting expression patterns around the point of tuberization.

In an *in vitro* tuberization system, nodal explants are stimulated to produce tubers from axillary buds by short-day conditions, low nitrogen and high sucrose levels. Several investigations have shown that these conditions produce tubers that closely mimic those of *in vivo* grown tubers on physiological, biochemical and genetic levels (Hannapel, 1991; Paiva *et al.*, 1982; Visser *et al.*, 1994). The advantages of a synchronized tuberization system for the dissection of molecular processes involved in tuber formation has been long recognized (Bourque *et al.*, 1987) and this is especially the case for gene expression studies. In the system described here, where tuberization was close to complete within a 24 h period, an ideal starting material was achieved for this type of analysis.

To visualize changes of gene expression a system of RNA fingerprinting was chosen. This has the advantage over differential cDNA library screening and other traditional techniques, in that all developmental stages or tissue types under investigation can be concurrently compared (Liang and Pardee, 1992; Welsh *et al.*, 1992). In our system the analysis across the 10 day period of the tuberization shows clear trends in gene expression emerging where each sample becomes a form of control for the neighbouring sample. It also becomes feasible to isolate genes on the basis of a band appearing only over 2–3 days during tuber formation.

The specific advantages of cDNA-AFLP over methods

described to date (reviewed in McClelland *et al.*, 1995) arise primarily from the stringency achieved by addition of universal anchors at each end of the cDNA-derived restriction fragments used as a substrate for the PCR. In RNA differential display, band intensities are the result of an interaction between the quality of primer matches and the quantity of template as a whole and of individual substrate. As a consequence there is no useful information content in a comparison between band intensities in the fingerprint and the initial concentration of input mRNA. In addition, there is a strong selection against rare mRNA species since a bad primer match on an abundant cDNA will far outperform a perfect match on a rare cDNA in the PCR under the low-stringency conditions (Liang and Pardee, 1992; Welsh *et al.*, 1992). In cDNA-AFLP primarily perfect matches between primer and substrate are tolerated in the amplification reaction. Rare instances of mismatch priming were observed in cases where transcript levels are extremely high such as with patatin. In our experiments around 70 TDFs have been sequenced and all show the expected terminal sequences. The fidelity of the method allows a much greater confidence in the initial data and permits limited conclusions to be drawn on the quantitative relationships of individual bands. Furthermore, the use of additional base extensions produces an almost unequivocal verification of band identity which is very difficult to achieve using the techniques described to date. cDNA-AFLP band verification was used to analyze the ADP-glucose pyrophosphorylase TDF. Here, other bands detected with the +2 base extensions were eliminated when the appropriate +3 extensions were used.

The use of a pre-amplification step after the production of the primary template (see Experimental procedures) allows, for practical purposes, virtually unlimited amounts of secondary template to be generated. This feature, combined with the relatively low requirement for starting material (100–200 mg tuber material) makes cDNA-AFLP the system of choice when saturation screening is to be carried out on small tissue samples. The possibility also exists to link cDNA-AFLP to genomic AFLP for rapid and precise mapping of genetic loci under investigation.

Using transcript-directed cDNA-AFLP we were able to target the reaction towards two specific transcripts; namely, patatin and AGPase. The expression profile in the cDNA-AFLP data gives a picture very comparable to the Northern data presented here and in other papers (Park *et al.*, 1985; Visser *et al.*, 1994). Very high abundance of a particular transcript such as is the case with patatin can result in the occurrence of characteristic artefacts. In the patatin TDF on days 6–10 an elevated background can also be noted (Figure 3a) which may be associated with the elevated level of this fragment in the PCR. It should be noted that when using primers with similar sequence extensions used for visualizing the patatin TDF (one base exchange at either end) a faint TDF presumably derived from the patatin cDNA appeared at the same mobility and expression pattern as described above. These TDFs are likely, therefore, to be the result of mismatch primed PCR. In the cDNA-AFLP of the patatin TDF, a weak band is just visible on day 5 not seen in the Northern (Figure 3b). This presumably is derived from the 5% of explants bearing tubers at this time point rather than an indication of patatin expression in axillary products prior to tuber formation. Visualization of this band on day 5 also indicates the sensitivity of cDNA-AFLP.

The expression of *lox* genes in plants is developmentally regulated during plant growth (Siedow, 1991). *lox* gene expression may also be induced by a series of other factors including hormonal treatment (jasmonic acid and ABA; Vick and Zimmermann, 1987), pathogen attack (Bostock *et al.*, 1992) and wounding (Geerts *et al.*, 1994). We present data here, showing that *lox* gene transcripts are differentially induced: the first transcript at a very early stage prior to tuberization and then a second transcript being expressed during organogenesis and maintaining expression at least until day 15 in our system. Furthermore, although TDF531 and TDF536 are highly homologous to both *lox* genes from roots and tubers, only the TDF531 was also found in root tissue. As the primer combination used in the amplification of TDF531 will also co-amplify both the known root-*lox* and tuber-lox fragments, the expression patterns seen in Figure 7a should be regarded as a general expression picture of several *lox* genes. The dramatic induction of the TDF536 just after the start of tuberization, however, provokes the speculation that the expression of this allele is directly linked to the tuber development process. Although the physiological role of any specific plant lipoxygenase isozyme is still unknown (Saravitz and Siedow, 1995; Siedow, 1991), the high expression of *lox* genes in tubers supports a suggested function of jasmonates in the possible regulation of tuber formation in potatoes (Koda *et al.*, 1991). Further work with antisense *lox* genes is necessary to elucidate this and other biological roles of lipoxygenase and its metabolites in developing tubers.

Experimental procedures

Tissue culture and plant material

Synchronization and growth conditions of the potato nodal cuttings (*Solanum tuberosum* cv. Bintje) were as described (Visser *et al.*, 1994). Material for template production was harvested daily and immediately frozen in liquid N_2. Samples of axillary buds were harvested and taken for the time point 0. Ten further harvests were routinely done subsequently. Tuberization was scored visually and tuber formation was defined as the onset of radial growth.

mRNA isolation, cDNA synthesis and template preparation

Total RNA was isolated from about 500 mg of fresh plant material. Total RNA concentration was determined spectrophotometrically

and then adjusted to 0.2 μg μl^{-1}. This RNA was used directly in the Northern analysis. For cDNA-AFLP, poly(A)$^+$ RNA was extracted from 2 μg of total RNA using poly-d[T]$_{25}$V oligonucleotides coupled to paramagnetic beads (Dynal A.S. Oslo, Norway). First and second strand cDNA synthesis was carried out according to standard protocols (Sambrook *et al.*, 1989). The resulting double-stranded cDNA was phenol-extracted, ethanol-precipitated and taken up in a final volume of 20 μl H_2O. Half of this volume was checked on gel and if the expected smear between 100 and 3000 bp was observed then the rest of the cDNA was subjected to the standard AFLP template production (Vos *et al.*, 1995). Restriction enzymes used for the template production were *Ase*I and *Taq*I. Fifteen cycles of non-radioactive pre-amplification using primers without extensions were carried out on a small aliquot (1/10th volume) of the primary template (94°C denaturation, 30 sec; 56°C annealing; 30 sec; 72°C polymerization, 1 min). The products of the amplification (termed secondary template) were also checked on a 1.5% agarose gel. Expected sizes were predominantly between 100 and 1000 bp. All amplification reactions were carried out on a PE-9600 thermocycler using Taq DNA-polymerase both from Perkin Elmer Corp. (Norwalk, CT, USA). The final template was then diluted 10-fold with H_2O.

The adaptors which were ligated to the restriction fragments and those used in the pre-amplification and active PCR are given below:

*Taq*I adaptor: 5'-GACGATGAGTCCTGAC
TACTCAGGACTGGC- 5'

*Taq*I pre-amplification primer: 5'-GACGATGAGTCCTGACCGA

*Taq*I amplification primer: 5'-GATGAGTCCTGACCGANN

*Ase*I adaptor: 5'-CTCGTAGACTGCGTACC
CTGACGCATGGAT- 5'

*Ase*I pre-amplification primer: 5'-CTCGTAGACTGCGTACCTAAT

*Ase*I amplification primer: 5'-GACTGCGTACCTAATNN

For PCR all the 16 different combinations of two base extensions (denoted as NN above) were available, giving in total 16^2 primer combinations. All oligonucleotides were obtained from Eurogentech (Eurogentech SA, Seraing, Belgium).

Radioactive cDNA-AFLP reactions and PAGE analysis of products

Radioactive labelling of the *Ase*I primer was carried out as described (Vos *et al.*, 1995). Thermocycling was done essentially as described above, however, with 35 cycles and including an 11 cycle touchdown (annealing temperature was reduced from 65° to 56°C in 0.7°C steps for 11 cycles and subsequently maintained at 56°C for 23 cycles). Samples were then boiled after the addition of dye and 50% formamide and separated on a 5% polyacrylamide sequencing type gel (GIBCO BRL Life Technologies Inc., Gaithersberg, MD, USA). All gels were run at standard conditions such that the 70 bp marker was 3 cm from the bottom of the gel giving a good resolution between 70–800 bp. Gels were then dried directly on to Whatman 3M paper on a slab gel dryer. Labelled DNA fragments were visualized by autoradiography. Gels and films were positionally marked prior to development. The two base selective extensions were chosen empirically to yield around 40 radioactively labelled fragments per lane.

Isolation and cloning of TDFs

Bands, identified on the autoradiograph as interesting, were lined up with markings on the film and the dehydrated gel and were excised. Excised fragments were monitored for activity. The gel fragments bonded to Whatman paper were inserted into an 1% agarose gel for support and electroeluted directly on to DE31 paper (Schleicher and Schuell, Dassel, Germany). DNA was then recovered according to the manufacturer's recommendations. DNA fragments could then be reamplified using the same PCR conditions and primers as used in the initial PCR, however, 15 cycles generally yielded sufficient product for cloning. Cloning was achieved using unpurified PCR product and the vector pGEMT (Promega, Madison, WI, USA). Conditions were used as recommended by the manufacturer.

Other molecular techniques

Basic DNA manipulations and molecular techniques were employed as described (Sambrook *et al.*, 1989). All enzymes were from Pharmacia (Uppsala, Sweden) with the exception of *Ase*I which was from NE-Biolabs Inc. (New Brunswick, NE, USA).

Sequence analysis

Sequence determination was carried out using an automated sequencer. All sequences were analyzed for homology to data banks using the WWW site of the NCBI (http://www.ncbi.nlm.nih.gov/Recipon/bs_seq.html) running the blast programs (Altschul *et al.*, 1990). The databases 'nr' and 'dbest' were used in all cases; Blastn, Blastx and tBlastx (for dbest) were all used routinely. Other analyses and manipulations were carried out using the PC-Gene programs (Intelligenetics, Mountain View, CA, USA).

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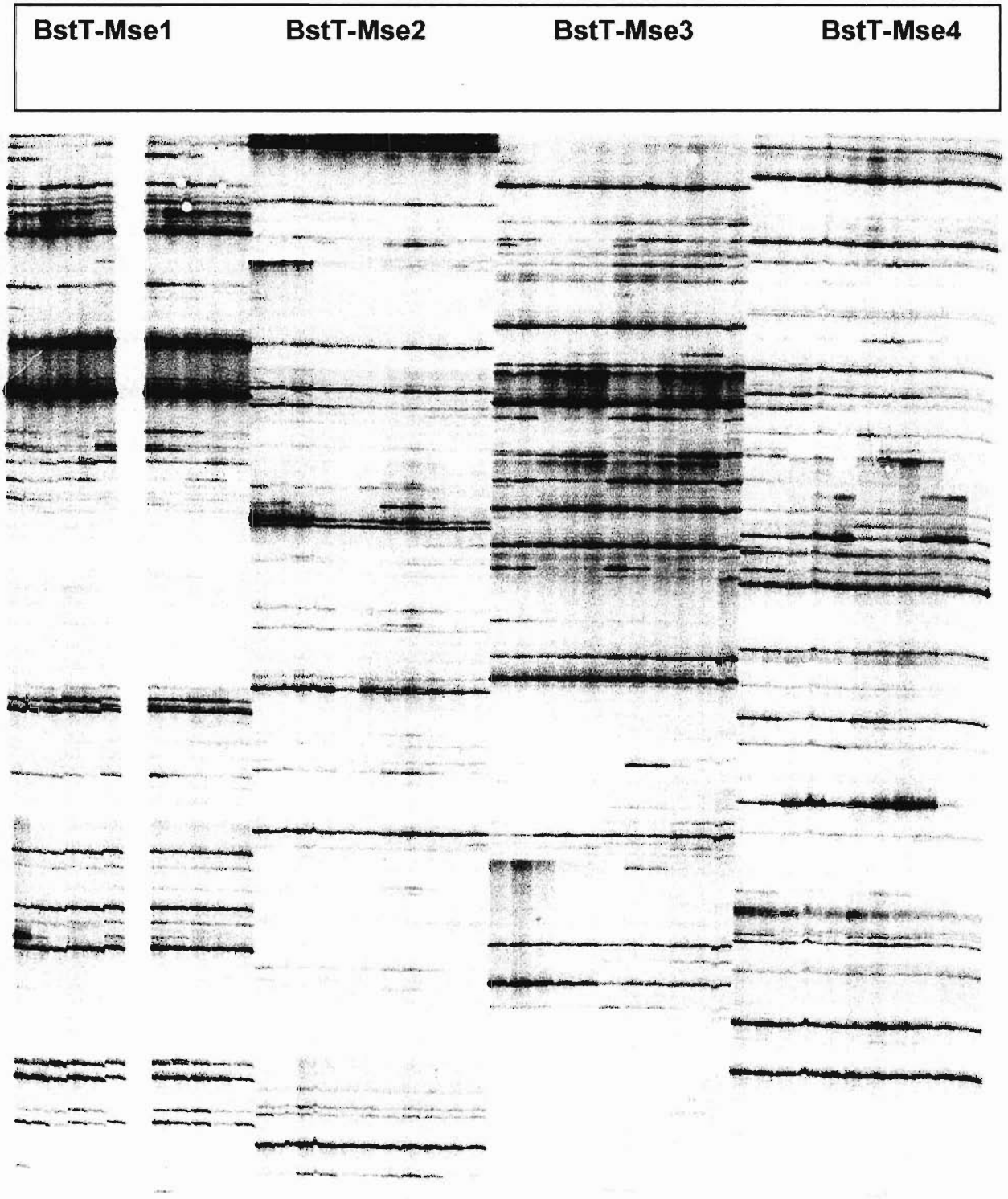
EMBL Data Library accession numbers X95666 (*S. tuberosum* mRNA homologous to *lox* gene, clone pCB531) and X95667 (*S. tuberosum* mRNA homologous to *lox* gene, clone pCB536).


BstT-Mse1
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