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Efecto de la Sobreexpresión y del Silenciamiento de Genes del Metabolismo de Giberelinas sobre el Desarrollo de Tabaco

Memoria de tesis doctoral presentada por

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CERTIFICA: Que D. LINA MARCELA GALLEGO GIRALDO, Ingeniera Agrónomo, ha realizado bajo mi dirección, en el Instituto de Biología Molecular y Celular de Plantas, el trabajo que con el título de "Efecto de la sobreexpresión y del silenciamiento de genes del metabolismo de giberelinas sobre el desarrollo de tabaco", presenta para optar al grado de Doctora Ingeniero Agrónomo.

Para que así conste a todos los efectos oportunos firma el presente certificado en Valencia a 20 de mayo de 2008.

Dra. Isabel López Díaz

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ÍNDICE

Índice *IX*
Abreviaturas *XI*
Resúmenes *XIII*

INTRODUCCIÓN 1

1. ESTRUCTURA Y ACTIVIDAD BIOLÓGICA DE LAS GIBERELINAS 1

2. METABOLISMO DE LAS GIBERELINAS 3

2.1. Biosíntesis 3
2.1.1. Síntesis de *ent*-kaureno a partir de geranilgeranildifosfato (GGDP) 3
2.1.2. Conversión de *ent*-kaureno a GA_{12} 4
2.1.3. Síntesis de GAs de 19 y 20 carbonos a partir de GA_{12} 4
2.2. Desactivación 5
2.3. Genes que codifican los enzimas implicados en la ruta del metabolismo de GAs 6
2.3.1. Genes que codifican diterpeno ciclasas 7
2.3.2. Genes que codifican monooxigenasas dependientes de citocromo P450 7
2.3.3. Genes que codifican dioxigenasas dependientes de 2-oxoglutarato 8

3. RUTA DE SEÑALIZACIÓN DE GAs 10

3.1. Receptor de GAs y proteínas DELLA 10
3.2. Factores reguladores de las proteínas DELLA 12

4. PROCESOS FISIOLÓGICOS CONTROLADOS POR GAs 13

4.1. Las giberelinas son hormonas inductoras del crecimiento 13
4.1.1. Crecimiento del tallo 13
4.1.2. Crecimiento de raíces 14
4.2. Germinación de semillas 14
4.3. Inducción de la floración 15
4.4. Formación de flores y frutos 17
4.5 Procesos de diferenciación celular asociados a GAs 17

5. REGULACIÓN DE LOS GENES DEL METABOLISMO DE GAs 18

5.1. Regulación por luz 18
5.2. Regulación por la temperatura 20
5.3. Regulación del metabolismo de GAs por otras hormonas 20
5.4. Homeostasis del contenido de GAs 21

6. MANIPULACIÓN GENÉTICA DE LA RUTA DEL METABOLISMO DE GAs 22

6.1. RNA interferencia como herramienta para el estudio de la función de los genes 24
6.2. El tabaco como modelo para el estudio de procesos del desarrollo en plantas tipo no roseta 25

OBJETIVOS 27

RESULTADOS 29

1. CAPÍTULO I 29
La Floración en Tabaco Necesita de Giberelinas pero no es promovida por los Niveles de GA_1 y GA_4 en el Brote Apical

2. CAPÍTULO II 43
La Homeostasis de Giberelinas en Tabaco es Regulada por Genes de su Metabolismo con Diferente Sensibilidad a Giberelinas

3. CAPÍTULO III 63
Efecto del Silenciamiento Múltiple de GA 2-oxidasas en el Desarrollo del Tabaco

DISCUSIÓN 93

1. REGULACIÓN DE LA RUTA DEL METABOLISMO DE GAS EN TABACO 93

1.1. El enzima GA 3-oxidasa no es limitante en la biosíntesis de GAs en tabaco 93
1.2. El enzima GA3ox esta codificado por al menos dos genes que tienen diferente regulación 94
1.3. La expresión de los genes *NtGA20ox1* y *NtGA3ox1* puede ser utilizada como indicador de los niveles de GAs 94
1.4. Existen al menos cinco genes de GA 2-oxidasa en tabaco que se expresan diferencialmente en la planta 95
1.5. Existen dos tipos de regulación para la familia de genes de GA 2-oxidasa en tabaco de acuerdo a la sensibilidad de estos genes frente a las variaciones en los niveles de GAs 95
1.6. La homeostasis de GAs en tabaco depende de distintos genes del metabolismo de GAs en función del grado de variación en los contenidos de las mismas. 95

2. EL PAPEL DE LAS GAS EN DISTINTOS PROCESOS DEL DESARROLLO DE TABACO 96

2.1. El silenciamiento múltiple de GA 2-oxidasas afecta el desarrollo de tabaco 96
2.2. La floración en tabaco depende de las GAs, pero no es inducida por los niveles de GAs activas, GA_1 y GA_4 en el ápice 98
2.3. El exceso de GAs puede tener un efecto inhibitorio sobre la floración en tabaco 98

CONCLUSIONES 101

BIBLIOGRAFÍA 103

ABREVIATURAS

BRs = Brasinoesteroides
$C_{1.....n}$ = carbono en posición 1….n
CCC = cycocel
CH_2OH = grupo hidroximetilo
CH_3 = grupo metilo
CHO = grupo aldehido
COOH = grupo carboxilo
CPD = *ent*-copalil difosfato
CPS = *ent*-copalil-difosfato-sintasa
d = día (s)
Fe^{+2} = ión de hierro bivalente
GA = giberelina
GA_3 = ácido giberélico
GAMTs = metiltransferasas de giberelinas
GAs = giberelinas
GAs C-19 = giberelinas de 19 carbonos
GAs C-20 = giberelinas de 20 carbonos
GA2ox = GA 2-oxidasa
GA2ox/RNAi = Plantas transgénicas de silenciamiento de GA 2-oxidasa
GA3ox = GA 3-oxidasa
GA3ox-OE = Plantas transgénicas de sobreexpresión de GA 3-oxidasa
GA3ox-OE/GA 20oxOE = Híbrido de sobreexpresión de GA 3-oxidasa y GA 20-oxidasa
GA13ox = GA 13-oxidasa
GA20ox = GA 20-oxidasa
GA20ox-OE = Plantas transgénicas de sobreexpresión de GA 20-oxidasa
GGDP = geranilgeranil bifosfato
IAA = ácido indolacético
KAO = ácido *ent*-kaurenoico oxidasa
KO = *ent*-kaureno oxidasa
KS = *ent*-kaureno sintasa
MeGAs = ésteres metilados de giberelinas
pb = pares de bases (bp, base pair)
PCB = paclobutrazol
PCR = reacción en cadena de la polimerasa
RNAi = RNA de interferencia
RT-PCR = retrotrancripción y PCR
SAM = S-adenosin-l-metionina
Ser = aminoácido serina
Thr = aminoácido treonina
2ODD = dioxigenasas dependientes de 2-oxoglutarato

RESÚMENES

RESUMEN

Las giberelinas (GAs) son hormonas vegetales que regulan diversos procesos del desarrollo y crecimiento de las plantas. Los niveles de las GAs activas (GA_1 y GA_4) están regulados por enzimas de biosíntesis como los GA 20-oxidasas (GA20ox) y GA 3-oxidasas (GA3ox) y por enzimas de catabolismo GA 2-oxidasas (GA2ox). Mediante un abordaje de genética reversa se ha estudiado el papel de algunos genes del metabolismo de GAs en la homeostasis de los contenidos de GAs y en el desarrollo de tabaco (*Nicotiana tabacum* var. *xanthi*). Plantas transgénicas que sobreexpresan un gen de GA3ox (*35S:PsGA3ox1*) presentaron pequeñas variaciones en el fenotipo así como leves incrementos en el contenido de GA_1, probablemente debido a que este enzima no es limitante. Adicionalmente se encontró un aumento de expresión de algunos genes *GA2ox*, *NtGA2ox3* y *-5,* los cuales parecen tener un papel importante en el control de la homeostasis de GAs cuando suceden pequeños incrementos en su contenido endógeno, por lo tanto serían los responsables de evitar la acumulación de GA_1 convirtiéndolo en su catabolito, GA_8, en estas plantas. Paralelamente, en el estudio del efecto de la sobreexpresión conjunta de una *GA3ox* con una *GA20ox* (*35S:PsGA3ox1 x 35S:CcGA20ox1*) se observaron pocas variaciones en el fenotipo así como en el contenido de GAs activas (GA_1 + GA_4) en comparación con su parental *GA20ox*. Estos resultados, corroboran el carácter no limitante de GA3ox en el metabolismo de GAs en tabaco.

Por otro lado, se ha estudiado el papel de la familia de genes de GA 2-oxidasas (genes del catabolismo de GAs), mediante la técnica de silenciamiento génico post-transcripcional por RNA de interferencia (RNAi). Los GA2ox de tabaco están codificados por al menos 5 genes con alto grado de redundancia en su expresión. Con el objetivo de obtener plantas transgénicas de tabaco con múltiple silenciamiento génico se elaboró una construccion tipo horquilla portadora de una secuencia altamente conservada en los 5 clones de *GA2ox,* capaz de inducir la supresión de la expresión de estos genes a la vez. Las plantas transgénicas de silenciamiento *GA2ox/RNAi* presentaron en su mayoría fenotipos similares a los fenotipos de superproducción de GAs (incrementos en la longitud de hipocotilos, entrenudos, hojas, pedúnculos de frutos, así como de la altura final de plantas). Además, en estas plantas se detectaron otros fenotipos como reducción en la longitud de la raíz, retraso en la floración y baja fertilidad que también parecen estar asociados a un exceso de GAs. Todos estos resultados en las plantas de silenciamiento *GA2ox/RNAi*, sugieren la importancia de los genes *GA2ox* en el control de los niveles de GAs que modulan diversos procesos del desarrollo de tabaco.

Por último, se ha estudiado el papel de las GAs en la inducción de la floración en plantas de tabaco. Se cuantificaron los niveles de GA_1 y GA_4 en ápices en diferentes estadios del desarrollo y se encontró una reducción progresiva de sus niveles conforme las plantas se acercaban a la floración. Este resultado excluye a estas dos GAs como inductoras de la floración en tabaco. Sin embargo, tanto por deficiencia como por exceso de GAs, la floración en tabaco se retrasa. Esto indica que es necesario que el nivel de GAs esté dentro de un intervalo óptimo (encima de un mínimo y por debajo de un máximo), para que ocurra la floración.

ABSTRACT

Gibberellins (GAs) are plant hormones that regulate a wide range of developmental and growing processes in plants. Levels of active GAs (GA_1 and GA_4) are regulated by biosynthetic enzymes such as GA-20oxidases (GA20ox) and GA 3-oxidases (GA3ox) and catabolic enzymes such as GA 2-oxidases (GA2ox). The role of some GA metabolism genes on GA homeostasis and tobacco (*Nicotiana tabacum* var. *xanthi*) development has been studied using reverse genetics approach. Transgenic plants overexpressing a *GA3ox* gen (*35S:PsGA3ox1*) showed small phenotypical variations and slight increases in GA_1 content, probably because this is a non-limiting enzyme. Additionally, some *GA2ox*, *NtGA2ox3* and *-5,* increased their expression in these plants and could be responsible for the lack of GA_1 accumulation by its conversion to GA_8 (inactive form of GA_1). Furthermore, the pattern expression of these *GA2ox* genes in response to GA levels variations, suggests that they could play an important role in GA homeostasis when a small increases in endogenous GA content occurs. Parallel to this, the simultaneous expression of a *GA3ox* and a *GA20ox* (*35S:PsGA3ox1 x 35S:CcGA20ox1*), caused small variations in the phenotype and active GA levels compared to its *GA20ox* parental. These results suggest a non limiting character of GA3ox on GA metabolism in tobacco.

On the other hand, the role of GA 2-oxidases genes (catabolism genes) was studied using post-transcriptional RNA interference silencing (RNAi). Tobacco GA2ox enzymes are encoded by at least five genes with high redundancy in expression. To obtain multiple silenced transgenic plants, a hairpin construction with a high-conserved sequence for the five *GA2ox* genes was performed to silence all those genes. Most of the transgenic silenced *GA2ox/RNAi* plants showed similar phenotypes to those caused by GA over-production (increases in length of hypocotyls, internodes, leaves, fruit peduncles and in final plant height). Additionally, other phenotypic characteristics like reduction of root length, flowering delay and low fertility were detected in these plants and could be associated to a GA excess. These results suggest the important role of *GA2ox* genes in the control of GA levels that regulate many processes of tobacco development.

Finally, the role of GAs on flowering induction has been studied in tobacco plants. When the plants approximate flowering, a progressive reduction of GA_1 and GA_4 levels was observed by its quantification at different developmental stages in the apical shoots. This result excludes these two GAs as flowering-promoting factors in tobacco. Nevertheless, flowering time in tobacco is delayed either by GAs deficiency or excess. Thus, flowering occurs only when GAs levels are within an optimum range, over a certain minimum or beyond a maximum.

RESUM

Les gibberel.lines (GA) són hormones vegetals que regulen diversos processos del desenvolupament i creixement de les plantes. Els nivells de les GA actives (GA_1 i GA_4) estan regulats per enzims de biosíntesi com els GA 20-oxidases (GA20ox) i GA 3-oxidases (GA3ox) i per enzims de catabolisme GA 2-oxidases (GA2ox). Per mitjà d'aproximació de genètica reversa s'ha estudiat el paper d'alguns gens del metabolisme de GA, en l'homeostasi dels continguts de GA i en el desenvolupament de tabac (*Nicotiana tabacum* var. *xanthi*). Plantes transgèniques que sobreexpressen un gen de GA3ox (*35S:PsGA3ox1*) van presentar xicotetes variacions en el fenotip així com lleus increments en el contingut de GA_1, probablement pel fet que aquest enzim no és limitant. Addicionalment es va trobar un augment d'expressió de *GA2ox*, *NtGA2ox3* i *-5,* els quals pareixen tindre un paper important en el control de l'homeostasi de GA quan succeïxen xicotets increments en el seu contingut endogen, per tant serien els responsables d'evitar l'acumulació de GA_1 convertint-lo en GA_8 en aquestes plantes. Paral·lelament, l'estudi de l'efecte de la sobreexpressió conjunta d'una *GA3ox* amb una *GA20ox* (*35S:PsGA3ox1 x 35S:CcGA20ox1*) on es van observar poques variacions en el fenotip així com en el contingut de GA actives (GA_1 + GA_4) en comparació amb el seu parental *GA20ox*. Aquests resultats, corroboren el caràcter no limitant de GA3ox en el metabolisme de GA en tabac.

D'altra banda, s'ha estudiat el paper de la família de gens de GA 2-oxidases (gens del catabolisme de GA), per mitjà de la tècnica de silenciament gènic post-transcripcional de RNA d'interferència (RNAi). Els GA2ox de tabac estan codificats per almenys 5 gens amb alt grau de redundància en la seua expressió. Amb l'objectiu d'obtindre plantes transgèniques de tabac amb múltiple silenciament gènic es va elaborar una construcció tipus ganxo portadora d'una seqüència altament conservada en els 5 clons de *GA2ox,* capaç d'induir la supressió d'expression d'aquests gens al mateix temps per mitjà de RNAi. Les plantes transgèniques de silenciament *GA2ox/RNAi* van presentar en la seua majoria fenotips semblants als fenotips de superproducció de GA (increments en la longitud d'hipocotil, entrenucs, fulles, peduncle de fruits, així com de l'altura final de plantes). Addicionalment, en aquestes plantes es van detectar altres fenotips com la reducció en la longitud de l'arrel, retard en la floració i baixa fertilitat que també pareixen estar associats a un excés de GA. Tots aquests resultats en les plantes de silenciament *GA2ox/RNAi*, suggerixen la importància dels gens *GA2ox* en el control dels nivells de GA que modulen diversos processos del desenvolupament de tabac.

Finalment, s'ha estudiat el paper de les GA en la inducció de la floració en plantes de tabac. Es van quantificar els nivells de GA_1 i GA_4 en àpexs en diferents estadis del desenvolupament i es va trobar una reducció progressiva dels seus nivells a mesura que les plantes s'acostaven a la floració. Aquest resultat exclou a aquestes dos GA com a inductores de la floració en tabac. No obstant això, tant per deficiència o per excés de GA, la floració en tabac es retarda. Açò indica que és necessari que el nivell de GA estiga dins d'un interval òptim (damunt d'un mínim i per davall d'un màxim), perquè ocórrega la floració

INTRODUCCIÓN

1. ESTRUCTURA Y ACTIVIDAD BIOLÓGICA DE LAS GIBERELINAS

Las giberelinas (GAs) son un conjunto de compuestos químicos naturales con actividad reguladora del crecimiento y el desarrollo de las plantas. Las GAs controlan diversos procesos del desarrollo de las plantas tales como la germinación de semillas, la elongación del tallo, la expansión de las hojas, el desarrollo de los tricomas y la inducción del desarrollo de flores y frutos (Sponsel y Hedden, 2004).

Las GAs se identificaron por primera vez en el hongo *Gibberella fujikuroi*, en 1935, como el principio activo de una enfermedad conocida como "bakanae", causante de un alargamiento excesivo del tallo y los brotes de arroz. A mediados de los años 50 se aisló, a partir del filtrado secretado por el hongo, el compuesto inductor del crecimiento del tallo que se denominó ácido giberélico (giberelina A_3 o GA_3). Posteriormente, se demostró su actividad biológica en plantas, ya que la aplicación de extractos purificados de GA_3 de *Gibberella fujikuroi* a mutantes enanos de maíz y guisante era suficiente para que éstos recuperasen su altura normal (Hedden y Phillips, 2000a). Actualmente se conocen 136 GAs diferentes (http://www.plant-hormones.info) presentes en plantas, hongos y bacterias, a las que se les ha asignado un número ($GA_{1, 2, 3, \ldots n}$) según el orden cronológico de su descubrimiento (MacMillan y Takahashi, 1968).

Químicamente las GAs son ácidos diterpenos tetracíclicos naturales, cuya estructura básica está constituida por un anillo de *ent*-giberelano (Fig. 1A). Las GAs se clasifican en dos grupos atendiendo al número de átomos de carbono presentes en el esqueleto de *ent*-giberelano: las GAs con 20 átomos de carbono, GAs C-20, y las GAs con 19 átomos de carbono, GAs C-19 (Fig. 1). Las GAs de C-20 presentan varios estados de oxidación del carbono 20 que se pueden encontrar como un grupo metilo (-CH_3), hidroximetilo (-CH_2OH), aldehído (-CHO) o carboxilo (-COOH) (Fig. 1B). Las GAs C-20, que poseen un grupo aldehído en el C-20, pueden perder ese carbono por descarboxilación oxidativa formándose una γ-lactona y dando lugar a las GAs C-19 (Fig. 1), entre las que se encuentran las GAs activas.

De las 136 giberelinas descritas actualmente, solo algunas pocas poseen actividad biológica intrínseca o *per se* en la regulación del desarrollo de las plantas, principalmente GA_1, GA_4, y GA_3, las restantes son compuestos precursores o de degradación de las GAs activas (Kobayashi *et al.*, 2000). La presencia o ausencia de un grupo β-hidroxilo en las posiciones C-2, C-3 y C-13 del *ent*-giberelano de las GAs C-19 (Fig. 1) determina la existencia o no de actividad biológica de las GAs (Talón, 2000). De esta manera la β-hidroxilación en el C-3 parece ser la clave para la actividad biológica de las GAs. GA_1 y GA_4 son las giberelinas activas que se encuentran en la mayoría de especies. Aunque de acuerdo a la especie se encuentra más abundante la una o la otra, por ejemplo, en *Arabidopsis* GA_4 es mayoritaria (revisado en Yamaguchi, 2007), mientras que GA_1 es la GA predominante, en tabaco (Jordan *et al.*, 1995; Vidal

et al., 2001) y guisante (Ross *et al.*, 1992). Por otra parte, cuando la β-hidroxilación es reemplazada por otros grupos funcionales en los C-2 y C-3, se producen otras GAs activas como GA_5 y GA_6 (Fig. 1), caracterizadas especialmente en gramíneas como *Lolium* (King y Evans, 2003).

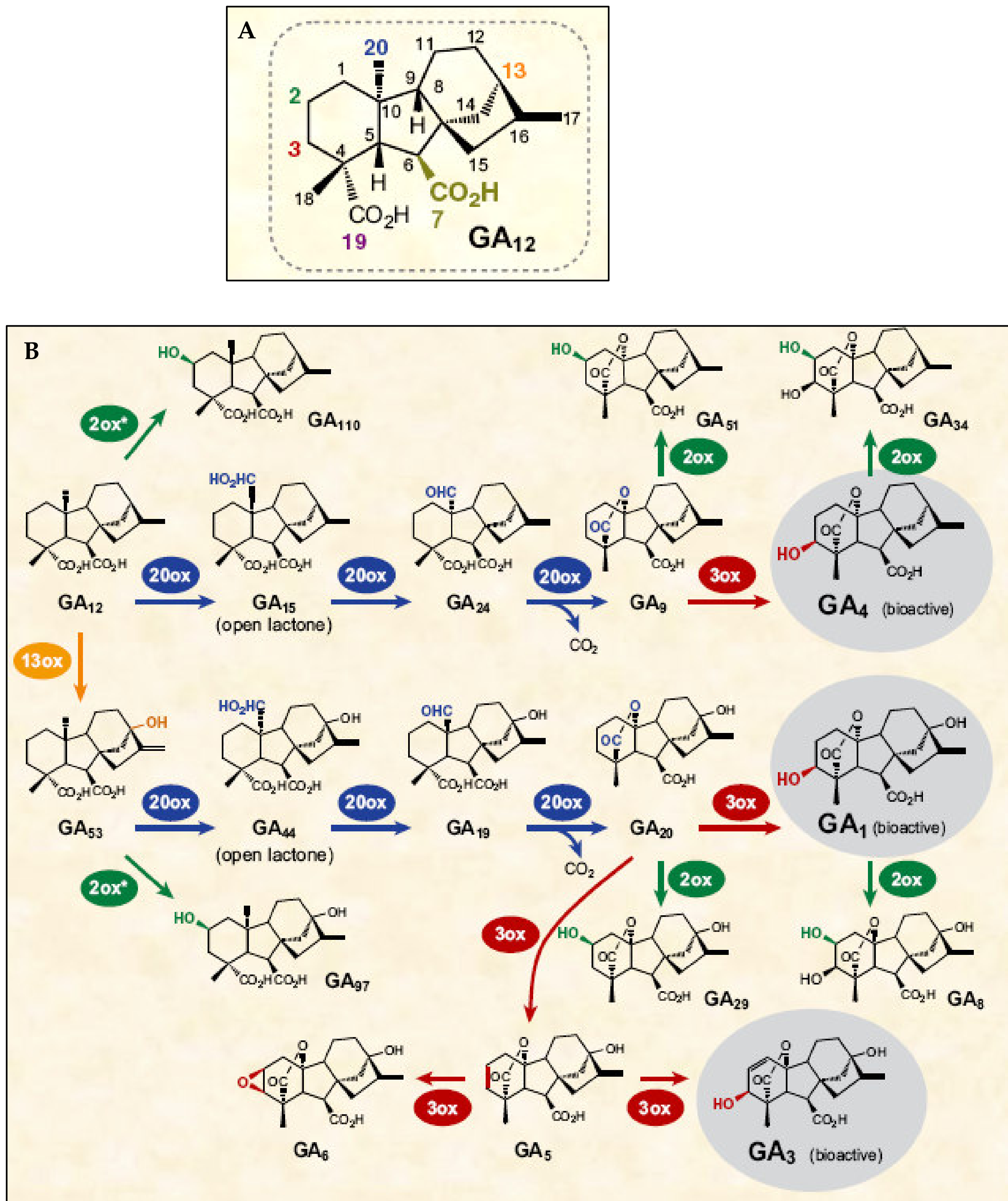

Fig. 1. Estructura química de GA_{12} en la cual se representa el anillo ***ent*-giberelano** (A) y de GAs representativas de la tercera parte de la ruta del metabolismo de GA (B)**.** Tales como: $\mathbf{GA_{12}}$, GA de C-20, $\mathbf{GA_1}$ y $\mathbf{GA_4}$, GAs activas de C-19, $\mathbf{GA_8}$ y $\mathbf{GA_{34}}$, GAs inactivas de C-19 y $\mathbf{GA_5}$ y $\mathbf{GA_6}$, otras GAs activas. Adaptado de Yamaguchi, 2007.

2. METABOLISMO DE LAS GIBERELINAS

2.1. Biosíntesis

La biosíntesis de GAs en plantas superiores se divide en tres etapas: síntesis de *ent*-kaureno a partir de geranilgeranildifosfato (GGDP), conversión de *ent*-kaureno a GA_{12} y síntesis de GAs de 19 y 20 carbonos a partir de GA_{12} (Yamaguchi, 2007).

2.1.1. Síntesis de *ent*-kaureno a partir de geranilgeranildifosfato (GGDP)

La primera parte de la ruta de biosíntesis de GAs propiamente dicha transcurre en los plastidios y está catalizada por ciclasas. Se inicia con la ciclación del GGDP, proceso que tiene lugar en dos pasos, el primero catalizado por el enzima *ent*-copalildifosfato sintasa (CPS) dando como producto el *ent*-copalil difosfato (CDP) y el segundo paso, catalizado por el enzima *ent*-kaureno sintasa (KS), donde el producto final obtenido es el *ent*-kaureno (Fig. 2) (revisado en Yamaguchi, 2007). Algunos inhibidores del crecimiento como AMO-1618 y cycocel (CCC) inhiben la actividad de la CPS, mientras que el phosphon D inhibe la actividad KS (Sponsel, 1995; Talón, 2000).

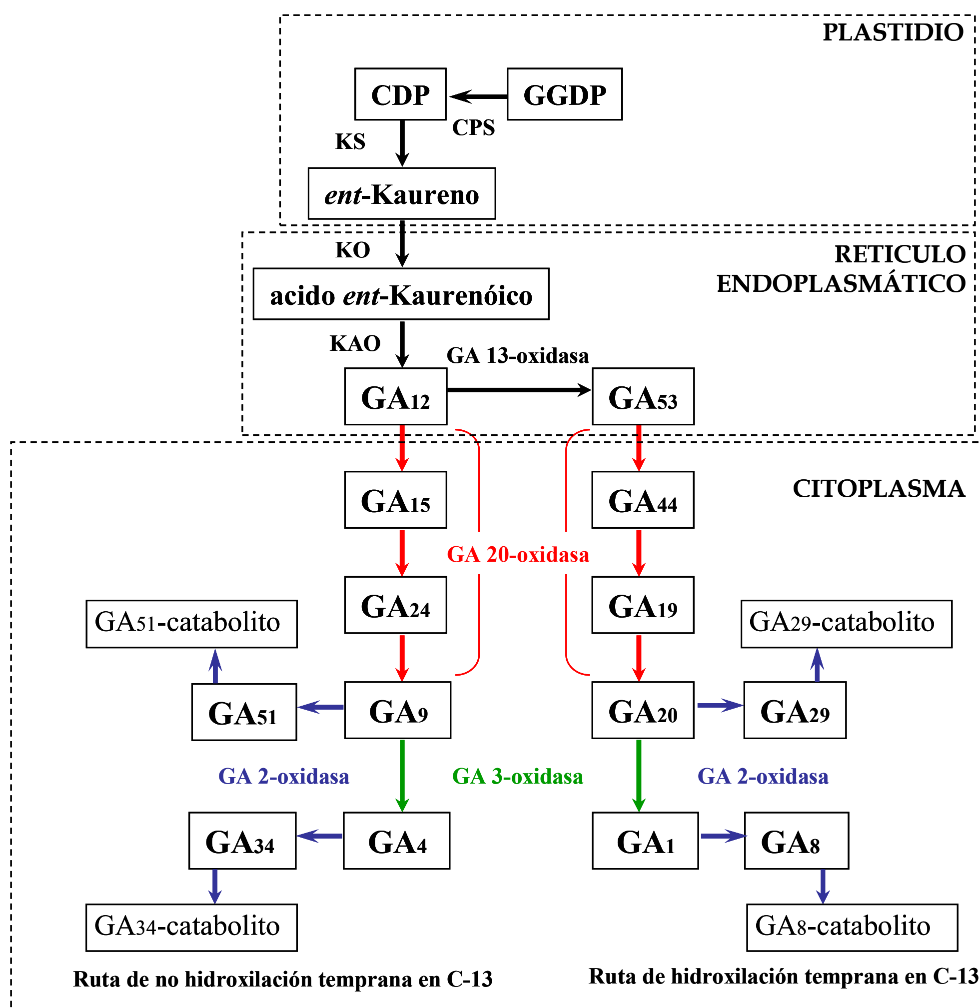

Figura 2. Esquema de la ruta del metabolismo de giberelinas (GAs). Paso de GGDP a *ent*-kaureno en el plastidio. Paso de *ent*-kaureno a GA_{12} en el retículo endoplasmático. Síntesis de GAs de C-20 y C-19 a partir de GA_{12} en el citoplasma. Ruta de la hidroxilación y no hidroxilación temprana en C-13. Síntesis de GAs activas GA_4 y GA_1. Formas inactivas tanto de las GAs activas como de sus precursores GA_9 y GA_{20}.

2.1.2. Conversión de *ent*-kaureno a GA_{12}.

La segunda etapa ocurre en la membrana del retículo endoplasmático y está catalizada por monoxigenasas del tipo citocromo P450. El *ent*-kaureno es oxidado a *ent*-kaurenol, *ent*-kaurenal y ácido *ent*-kaurenóico por la *ent*-kaureno oxidasa (KO), el cual se localiza específicamente en la cara externa del retículo endoplasmático y es el enzima que enlaza los pasos de la ruta que suceden en los plastidios, con los que ocurren en el retículo (Helliwell *et al.*, 2001a). Tanto en *Arabidopsis* (Helliwell *et al.*, 1999) como en arroz (Davidson *et al.*, 2004), se ha demostrado que KO cataliza tres oxidaciones sobre el C-19 del ent-*kaureno* (Helliwell *et al.*, 2001b). A continuación, el carbono C-7 del ácido *ent*-kaurenóico es oxidado, mediante otras tres reacciones de oxidación catalizadas por la ácido *ent*-kaurenóico oxidasa (KAO), el cual se localiza en el retículo endoplasmático, para producir GA_{12} (Fig. 2) (revisado en Yamaguchi, 2007). Los compuestos que interactúan con el citocromo P450 como el ancymidol y derivados de triazol, entre los que se encuentra el paclobutrazol (PCB), inhiben la actividad de estos enzimas (Talón, 2000).

2.1.3. Síntesis de GAs de 19 y 20 carbonos a partir de GA_{12}

La tercera parte de la ruta de biosíntesis de las GAs ocurre en el citoplasma de las células y está catalizada en su mayor parte por dioxigenasas dependientes de 2-oxoglutarato y Fe^{+2} (Yamaguchi y Kamiya, 2000). Los compuestos que mimetizan la estructura del 2-oxoglutarato, co-sustrato de las dioxigenasas, actúan como inhibidores de estos enzimas. Los principales representantes de este tipo de inhibidores son las acilciclohexanodionas, como la prohexadiona-Ca, trinexapac-ethyl y LAB 198 999 (Rademacher, 2000).

El proceso metabólico posterior a la GA_{12} puede variar según la especie, e incluso puede ser diferente entre tejidos de una misma planta. A partir de GA_{12} la biosíntesis puede tomar dos rutas descritas a continuación:

Ruta de la no hidroxilación temprana en el C-13.

El C-20 de GA_{12} se oxida dos veces dando origen a GA_{15} y GA_{24}. La posterior descarboxilación o eliminación del C-20 de GA_{24} da origen a GA_9, considerada la primera GA de C-19 de esta ruta (Fig. 1); ambos pasos están catalizados por el enzima GA 20-oxidasa (Fig. 2). La incorporación de un grupo hidroxilo en la posición 3β de GA_9 produce GA_4 (Fig. 1), compuesto con actividad biológica; éste proceso es catalizado por el enzima GA 3-oxidasa (revisado en Yamaguchi, 2007).

Ruta de la hidroxilación temprana en el C-13.

El GA_{12} es oxidado en el C-13 por el enzima monooxigenasa del tipo citocromo P450, GA 13-oxidasa (GA13ox), transformándose en GA_{53} (Figs. 1 y 2), que es el primer miembro de la ruta de síntesis de la 13-hidroxilación, ruta predominante en la mayoría de las especies. Posteriormente, el C-20 de la GA_{53} sufre dos oxidaciones consecutivas (Fig. 1) catalizadas por GA 20-oxidasas dando GA_{44} y GA_{19} (Fig. 2). A continuación se elimina el C-20 de GA_{19} sintetizándose GA_{20}, que es la primera GA de C-19 de la ruta (Fig. 2). La incorporación de un grupo hidroxilo en la posición 3β de GA_{20} produce GA_1 (Fig. 1), compuesto con actividad biológica. Este proceso es catalizado por el enzima GA 3-oxidasa (revisado en Yamaguchi, 2007). Algunos enzimas GA3ox de gramíneas pueden también catalizar la síntesis de otras GAs activas como GA_3, GA_5 y GA_6 a partir de GA_{20} (Fig. 1).

2.2. Desactivación

Las GAs de C-19, productos finales de las dos rutas de la no hidroxilación temprana en el C-13 y de la hidroxilación temprana en el C-13, se desactivan o pierden su actividad biológica de forma irreversible mediante la β-hidroxilación en el C-2, esta reacción es catalizada por GA 2-oxidasas (Fig. 1), siendo este sistema de desactivación, un aspecto importante en la regulación de los niveles de GAs activas en plantas (Ross *et al.*, 1995). De esta manera tanto GA_9, GA_4, GA_{20} y GA_1 pueden transformarse en los productos inactivos GA_{51}, GA_{34}, GA_{29} y GA_8 respectivamente (Fig. 1). Las GAs 2β-hidroxiladas pueden ser oxidadas nuevamente por GA2ox para formar los productos finales GA_{51}-catabolito, GA_{34}-catabolito, GA_{29}-catabolito y GA_8-catabolito respectivamente (Talón, 2000) (Fig. 2). Existe un tipo diferente de GA 2-oxidasas capaces de catalizar la 2β-hidroxilación en las GAs de C-20 como la GA_{12} y GA_{53}, los cuales originan los productos inactivos GA_{110} y GA_{97} respectivamente (Fig. 1). La desactivación de estos precursores, también puede reducir el flujo de la biosíntesis de GAs activas (Schömburg *et al.*, 2003).

Además de las GA 2-oxidasas, estudios sobre otros enzimas tales como las GA epoxidasas y GA metiltransferasas (GAMTs) han demostrado que ellos también pueden realizar una función catabólica dentro de la homeóstasis de los niveles de GAs durante el desarrollo de la planta. Por ejemplo, en arroz una monooxigenasa dependiente del citocromo P450, codificada por el gen *EUI (elongated uppermost internode)* cataliza la 16α,17-epoxidación de GAs, GA_4, GA_9 o GA_{12} de la ruta de la no hidroxilación temprana en C-13 (Fig. 3). Tanto la alta acumulación de GAs en el mutante *eui* como la reducción severa de los niveles de GAs en plantas transgénicas que sobreexpresan *EUI*, mostraron que la 16α,17-epoxidación repercute en la disminución de los contenidos GA_4, demostrando que EUI funciona como un enzima de inactivación de GAs (Zhu *et al.*, 2006).

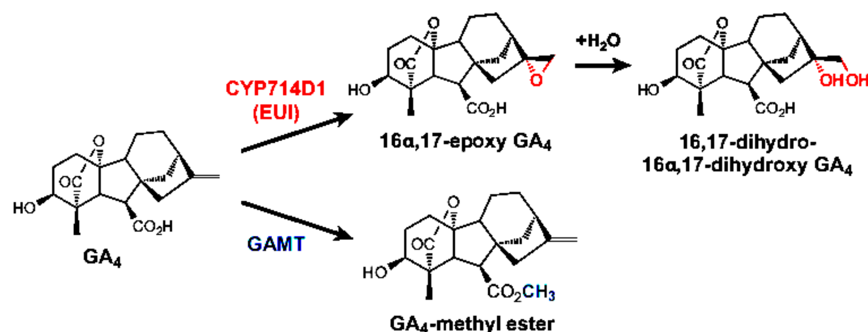

Figura 3. Otros mecanismos de desactivación de GAs. GA epoxidasas (EUI) y GA metiltransferasa (GAMT). Adaptado de Yamaguchi, 2007.

Por otra parte, los enzimas GA metiltransferasas, codificadas por los genes *GAMT1* y *GAMT2* en *Arabidopsis*, producen otras formas inactivas de GAs (Varbanova *et al.*, 2007). Estos enzimas utilizan S-adenosin-l-metionina (SAM) como un donante del grupo metilo y son capaces de metilar el grupo carboxilo del C-7 de las GAs, lo cual produce ésteres metilados de GAs (MeGAs) (Fig. 3). La sobreexpresión de estos genes en *Arabidospis*, tabaco y petunia produce enanismo. En *Arabidopsis* los genes GAMT se expresan preferentemente en las semillas y los mutantes nulos para ambos genes resultaron en plantas con semillas que presentaron menor inhibición en la germinación cuando fueron cultivas en presencia del inhibidor de biosíntesis de GAs en comparación con las semillas silvestres. Este fenotipo indica que hay un mayor contenido de GAs en las semillas mutantes GAMT, lo que indica un papel de estos genes en la desactivación de GAs en estadios específicos del desarrollo y germinación de las semillas (Varbanova *et al.*, 2007).

2.3. Genes que codifican los enzimas implicados en la ruta del metabolismo de GAs

Se han clonado todos los genes de la ruta del metabolismo de GAs en diversas especies, a excepción del gen que codifican para la GA 13-oxidasa (GA13ox), enzima que cataliza la hidroxilación del C-13 en GA_{12} para dar GA_{53} (Hedden y Phillips, 2000a). Además, se conocen mutaciones en los genes de la biosíntesis de GAs en varias especies, estos mutantes se conocen como "deficientes en GAs" y la mayor parte de ellos son enanos. Generalmente estas mutaciones son recesivas y producen bloqueos incompletos de la ruta de síntesis de GAs (revisado en Ross *et al*., 1997). Tanto en *Arabidopsis* como en arroz, los enzimas que catalizan los primeros pasos de la biosíntesis de GAs, están codificados por uno o dos genes (revisado en Yamaguchi, 2007). Mientras que los enzimas, dioxigenasas dependientes de 2-oxoglutarato, que catalizan la última parte de la biosíntesis de GA, son codificadas por familias multigénicas. Los miembros de estas familias de genes se expresan diferencialmente en los distintos órganos de la planta en función del estadio de desarrollo de la misma (revisado en Yamaguchi, 2007).

2.3.1. Genes que codifican diterpeno ciclasas

En la primera parte de la ruta, la ciclación de GGDP a *ent*-kaureno, pasando por el intermediario CDP, es catalizada por la CPS y KS.

Los genes que codifican **CPS**, se han aislado en diversas especies. En *Arabidospsis* se ha aislado un solo gen (*GA1*; Sun y Kamiya, 1994) que codifica para este enzima. En arroz se han aislado dos genes que codifican para CPS, pero solo uno de ellos (*OsCyc1*) parece participar en la biosíntesis de GAs (Prisic *et al.*, 2004) y el segundo (*OsCyc2*) está implicado en la biosíntesis de diterpenos fitoalexinas (Otomo *et al*., 2004). Además, ambos genes tienen función *syn*-copalil bifosfato sintasa (CDP), reacción implicada en la biosíntesis de fitoalexinas (Otomo *et al*., 2004 y Xu *et al*., 2004). En calabaza (*CmCPS1* y *CmCPS2;* Smith *et al*., 1998) y maíz (*An1*; Bensen *et al*., 1995, *An2;* Harris *et al,* 2005), también se han clonado dos genes que codifican este enzima. En *Arabidopsis* la expresión de CPS (*GA1*) está regulada durante el crecimiento y desarrollo de la planta. Su expresión es elevada tanto en tejidos con crecimiento activo (ápices, raíces, flores en desarrollo y semillas) como en órganos que ya no están en crecimiento (hojas expandidas). Esto puede sugerir que las hojas son sitios de biosíntesis de GAs desde donde se transportan a otros tejidos (Silvertone *et al*., 1997). En el caso de calabaza, la expresión diferencial de los genes *CPS*, a lo largo del ciclo vital sugiere que cada uno de los genes podría estar implicado en distintas etapas del desarrollo de los órganos, *CmCPS1* en los estadios tempranos y *CmCPS2* en los procesos de crecimiento posteriores (Smith *et al*., 1998).

Los genes que codifican **KS** han sido aislados en *Arabidopsis* (*GA2;* Yamaguchi *et al*., 1998b), calabaza (*CmKS*; Yamaguchi *et al.,* 1996), arroz (Margis-Pinheiro *et al*., 2005) y otras especies. En *Arabidopsis*, KS (*GA2*) se expresa de forma constitutiva en todos los órganos (Yamaguchi *et al.,* 1998b). En arroz se detecta expresión de *OsKS* en hojas y tallos (Margis-Pinheiro *et al.,* 2005). En *Arabidopsis*, el patrón de expresión de *AtKS* es similar al de *AtCPS* pero los niveles de transcritos son más elevados en el primer caso (Yamaguchi *et al*., 1998b). Este hecho sugiere que la síntesis de *ent*-kaureno estaría determinada principalmente por los niveles de expresión de *AtCPS* y por su localización. El patrón específico de expresión del gen *AtCPS* y la mayor expresión de *AtKS*, apoyan la idea de que *AtCPS* controla el lugar y la actividad de las etapas tempranas de la ruta de biosíntesis de GAs en la planta (Olszewski *et al*., 2002).

2.3.2. Genes que codifican monooxigenasas dependientes de citocromo P450

La actividad enzimática de los genes que codifican monooxigenasas dependientes de citocromo P450 (KO y KAO) es poco conocida a causa de la dificultad que supone la purificación de estos enzimas cuando se expresan en bacterias (Hedden y Phillips, 2000a). Se han clonado los genes que codifican para **KO** en *Arabidospis* (*GA3*, Helliwell *et al*., 1998), arroz

(D35, Itoh *et al.*, 2004) y otras especies. La expresión de los genes *KO* tanto en *Arabidospis* (Helliwell *et al.*, 1998) como en arroz (Itoh *et al.*, 2004) ocurre en todos los tejidos de la planta, incluidas las inflorescencias.

Los genes que codifican **KAO** se han aislado en arroz (revisado en Sakamoto *et al.*, 2004), *Arabidopsis* (Helliwell *et al.*, 2001a) y guisante (Davidson *et al.*, 2003), en esta últimas dos especies se han clonado dos genes KAO. En guisante, *PsKAO1* (gen *NA*) se expresa en tallo, brote apical, hojas, vainas y raíces (Davidson *et al.*, 2003) y *PsKAO2* en semillas.

2.3.3. Genes que codifican dioxigenasas dependientes de 2-oxoglutarato

Los enzimas que catalizan la última parte de la ruta del metabolismo de las GAs son dioxigenasas dependientes de 2-oxoglutarato (2ODD). Las GA 20-oxidasas y las GA 3-oxidasas se encargan de la síntesis de GAs y las GA 2-oxidasas de su inactivación.

La familia de **GA 20-oxidasas** (*GA20ox*) está formada por un número variable de genes según la especie. En *Arabidopsis* hay cinco genes *GA20ox* (Phillips *et al.,* 1995; Xu *et al.*, 1995), en arroz hay 4 genes (revisado en Sakamoto *et al.*, 2004) y en tabaco han sido clonados 2 genes *GA20ox* (Tanaka-Ueguchi *et al.*, 1998). Tres de los genes que codifican GA 20-oxidasas en *Arabidopsis* se expresan diferencialmente: el gen *AtGA20ox1* se expresa preferentemente en tallos y flores, el gen *AtGA20ox2* en silicuas y flores y el gen *AtGA20ox3* se expresa únicamente en silicuas (Phillips *et al.,* 1995). En algunas solanáceas como tomate, patata y tabaco, los genes que codifican GA 20-oxidasas tienen un patrón de expresión diferencial durante los diferentes estadios del desarrollo de las plantas (Rebers *et al.*, 1999, Carrera *et al*, 1999 y Tanaka-Ueguchi *et al.*, 1998). En *Nicotiana tabacum* los niveles de transcritos de *NtGA20ox1* (*Ntc12*) y *NtGA20ox2 (Ntc16*) difieren en abundancia y órgano de expresión (Tanaka-Ueguchi *et al.*, 1998).

Las **GA 3-oxidasas** catalizan el último paso de la biosíntesis de las GAs activas convirtiendo GA_{20} y GA_9 en GA_1 y GA_4 respectivamente. Los genes que codifican GA 3-oxidasas (*GA3ox*) han sido clonados en diversas especies. En *Arabidopsis* se han aislado cuatro genes *GA3ox* (Chiang *et al.,* 1995; Yamaguchi *et al.,* 1998a), en guisante (Martin *et al.,* 1997) y en arroz (Itoh *et al.,* 2001) dos genes y en tabaco solo se ha aislado un gen *GA3ox* (Tanaka-Ueguchi *et al.*, 1998, Itoh *et al.*, 1999).

El análisis de la función de los miembros de la familia de genes *GA3ox* en *Arabidopsis*, indica que existe una expresión diferencial de cada miembro de la familia, lo que sugiere que cada gen podría tener un papel individual en la biosíntesis de GAs durante el desarrollo de la planta. Sin embargo se ha visto solapamiento funcional, ya que se ha demostrado que los genes *AtGA3ox1* y -2 son los principales responsables de la biosíntesis de GA activa durante el desarrollo vegetativo pero también son necesarios para el desarrollo reproductivo (Mitchum *et*

al., 2006). En guisante, el gen homólogo al de *Arabidopsis* (*GA4*) corresponde al locus *LE* utilizado por Mendel en sus investigaciones sobre la herencia de los caracteres (Martin *et al.*, 1997). En arroz, el gen *OsGA3ox1* se expresa en flores y el gen *OsGA3ox2*, (mutante enano *d18*) se expresa principalmente en hojas (Itoh *et al.*, 2001). En tomate, el gen *LeGA3ox2* muestra un incremento gradual de su expresión en las flores hasta la antesis y disminuye en las etapas tempranas de la formación del fruto (Rebers *et al.*, 1999). En tabaco el gen *NtGA3ox1* (*Nty*) se expresa preferentemente en tejidos en crecimiento activo y zonas de elongación celular tales como ápices (apical y radical), tapetum y granos de polen en desarrollo (Itoh *et al.*, 1999).

Las **GA 2-oxidasas** oxidan el C-2 de sus substratos (GAs activas y sus precursores) originando compuestos 2β-hidroxilados, inactivos (Ross *et al.*, 1995). Esta característica les ha conferido el nombre de enzimas desactivadores. Se han clonado GA 2-oxidasas (*GA2ox*) en diversas especies. En *Arabidopsis* hay ocho genes (Thomas *et al.*, 1999; Yamaguchi, 2007), en arroz existen seis genes (Sakamoto *et al.*, 2004; Sakai *et al.*, 2003), en tomate hay 5 genes (Serrani *et al.*, 2007) y tanto en guisante (Martin *et al.*, 1999) como en tabaco hay dos clones de *GA2ox*. En guisante se detectan niveles elevados de transcritos del gen *PsGA2ox1* en raíz y semillas, mientras que hay niveles reducidos en el brote apical. Un patrón de expresión diferente ha sido descrito para *PsGA2ox2*, ya que los transcritos de este gen abundan en el brote apical y escasean en las semillas (Lester *et al.*, 1999). Estas diferencias de expresión entre los dos genes de *GA2ox* en guisante explican el fenotipo de tallo elongado encontrado en el mutante "*slender*" *sln* (Reid *et al.*, 1992; Ross *et al.*, 1993). En *Arabidopsis* por el contrario, los genes *AtGA2ox1* y *AtGA2ox2* presentan el mismo patrón de expresión (tallo, flores y silicuas) y esto explica probablemente la ausencia de mutantes en estos genes (Thomas *et al.*, 1999). Aunque específicamente, el gen *AtGA2ox2* se ha encontrado abundantemente en semillas y parece tener un papel importante en la germinación regulada por el fitocromo (Yamauchi *et al.*, 2007). En *Arabidopsis,* los mutantes simples para los genes *AtGA2ox7* y *AtGA2ox8* no presentan mayores alteraciones en el fenotipo, solo en el mutante doble *AtGA2ox7* y *AtGA2ox8* se detecta un fenotipo moderado (Schömburg *et al.*, 2003), lo que indica la existencia de redundancia génica en esta familia de *GA2ox*. Por otra parte, el gen *OsGA2ox1* de arroz, se expresa alrededor del ápice y su abundancia disminuye cuando las plantas se acercan al cambio de fase vegetativa a reproductiva, indicando la importancia de este gen en la regulación del contenido de GAs en el ápice durante la transición floral (Sakamoto *et al.*, 2001b).

Dentro de la familia de genes que codifican GA 2-oxidasas, se han descrito tres subfamilias génicas *GA2ox*. El primer (I) y segundo (II) grupo de *GA2ox* utilizan las GAs de C-19 como sustrato. Mientras que el tercer (III) grupo de *GA2ox* utiliza las GAs de C-20 como substrato (Lee y Zeevart, 2005). Los enzimas del grupo II catalizan una sola oxidación en el C-2 de sus substratos (monocatalíticos) y en el grupo I, algunos enzimas catalizan dos oxidaciones

consecutivas (multicatalíticos) (Thomas *et al.,* 1999) originando por ejemplo, GAs tales como GA_8-catabolito (Fig. 2). En *Arabidopsis,* los genes *AtGA2ox1*, *-2* y *-3* son del grupo I (Schömburg *et al.*, 2003) y *AtGA2ox4* y *-6* son del grupo II, la clasificación de estos últimos dos genes se determinó según la similitud de secuencia con otros enzimas del grupo II, sin embargo aun no han sido caracterizados en cuanto a su preferencia de sustrato. El gen *AtGA2ox5* no se expresa por ser probablemente un pseudogen (Hedden y Phillips, 2000a). En guisante, el mutante "*slender*" (*sln)* tiene bloqueado el paso de GA_{20} a GA_{29} y el paso de GA_{29} a GA_9-catabolito (Ross *et al.* 1995). Este bloqueo se debe a la mutación en el gen *PsGA2ox1* que codifica para un enzima con características multifuncionales capaz de catalizar dos oxidaciones consecutivas sobre el C-2 de los substratos (Martin *et al.* 1999; Lester *et al.* 1999). En otras especies como *Nerium oleander* también se han caracterizado enzimas GA2ox de los grupos I y II, unas con actividad monocatalítica (NoGA2ox1 y -2) y NoGA2ox3, enzima multicatalitica, capaz de catalizar el paso de GA_9 a GA_9-catabolito y el paso de GA_{51} a GA_{51}-catabolito (Ubeda-Tomás *et al*., 2006). En el tercer grupo de genes *GA2ox* en *Arabidopsis* existen dos miembros (*AtGA2ox7* y *-8*). Mediante genética reversa, se ha demostrado que los genes *AtGA2ox7* y *AtGA2ox8* utilizan como substrato las GAs de C-20 (Schömburg *et al*., 2003).

3. RUTA DE SEÑALIZACIÓN DE GAs

3.1. Receptor de GAs y proteínas DELLA

La cascada de señales que se desencadena tras la percepción de GAs se conoce menos que la biosíntesis. Sin embargo, tras la reciente identificación del receptor soluble de GAs, se ha podido avanzar en el conocimiento del proceso de señalización (revisado en Ueguchi-Tanaka *et al*., 2007). La caracterización del gen *GID1* (*GIBBERELLIN INSENSITIVE DRARWF 1*) de arroz y sus homólogos en *Arabidospsis*, han demostrado su afinidad estructural con las GAs activas (Ueguchi-Tanaka *et al*., 2005, Nakayima *et al*., 2006). Resultados obtenidos en levadura han postulado que tras la percepción de las GAs activas por el receptor, se forma un complejo compuesto por GAs-GID1 y las proteínas DELLA (revisado en Ueguchi-Tanaka *et al*., 2007).

Las proteínas DELLA actúan como reguladores negativos de la respuesta a GAs (Harberd, 2003). Cuando ocurre la percepción de las GAs por el receptor, se induce la fosforilación de las proteínas DELLA destinadas a la degradación por el proteosoma 26S (Hare *et al.,* 2003; Viertra, 2003). Tras la degradación estos reguladores negativos (proteínas DELLA), se activa la transcripción de los genes de respuesta a GAs involucrados en diversos procesos del desarrollo de las plantas (Fig. 4). Las proteínas DELLA fosforiladas son poliubiquitinadas por el complejo enzimático SCF-E3 ubiquitina ligasa, cuya subunidad F-box posee un dominio C-terminal que confiere la especificidad de la unión al sustrato que se debe ubiquitinar (Thomas y Sun, 2004). En *Arabidopsis* las proteínas DELLA son degradadas vía un complejo de

ubiquitinación SCF^{SLY1} cuya proteína F-box esta codificada por el gen SLY1 (McGinni *et al.*, 2003). En arroz, la proteína que forma parte del complejo SCF E3 Ubiquitin ligasa, a través del cual se inicia la degradación de las proteínas DELLA, esta codificada por el gen GID2 (Gomi *et al.*, 2004) (Fig. 4).

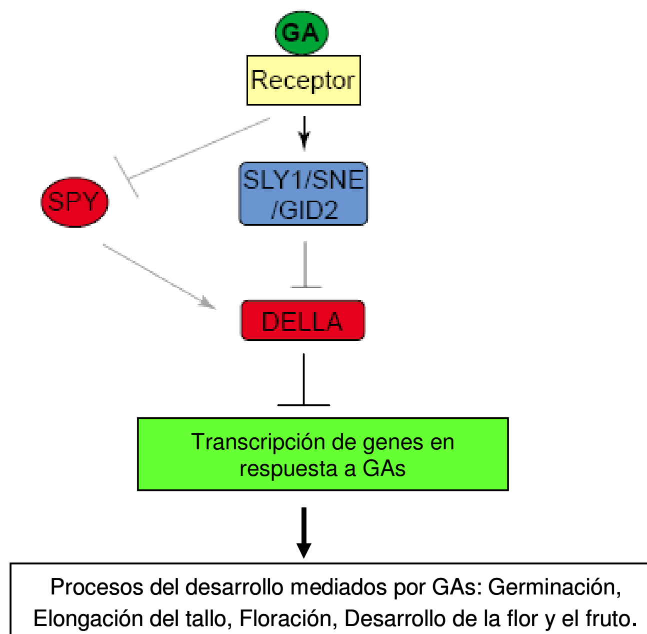

Figura 4. Modelo de la ruta de señalización de GAs. Adaptado de Fleet y Sun, 2005.

Las proteínas DELLA pertenecen a una familia de factores de transcripción, la familia GRAS (Pysh *et al.*, 1999). La estructura de las proteínas DELLA posee dos dominios. El del extremo C-terminal denominado dominio GRAS, importante para la función de la proteína y común a todas las proteínas de las misma familia, y el del extremo N-terminal denominado DELLA importante para la regulación por GAs (Thomas *et al.*, 2005). Este dominio contiene dos secuencias consenso de aminoácidos: "DELLA", que le da nombre a estas proteínas y "VHYNP" (Thomas *et al.*, 2005). En *Arabidospsis,* la familia DELLA esta compuesta por 5 miembros, GAI (Peng *et al.*, 1997), RGA (Silverstone *et al*, 1998), RGL1, RGL2 y RGL3 (Richards *et al.*, 2001). La redundancia génica entre los miembros de la familia DELLA en *Arabidopsis* hace que las mutaciones de perdida de función para un solo miembro no presenten fenotipo. Se han identificado los genes ortólogos que codifican estas proteínas en maíz (D8; Peng *et al.,* 1999), trigo (RHT; Peng *et al.,* 1999), cebada (SLN1; Chandler *et al.,* 2002), guisante (LA y CRY; Weston *et al.*, 2008) y arroz (SLR1; Ikeda *et al.,* 2001). En arroz la presencia de un solo miembro DELLA hace que el mutante de pérdida de función se comporte como si estuviese saturado de GAs.

La identificación de las proteínas DELLA como reguladores negativos ha sido posible gracias a la caracterización de mutantes que afectan el dominio DELLA. La eliminación o mutación del dominio DELLA provoca una acumulación de la proteína DELLA mutante, RGA en *Arabidospis* y SLR1 en arroz, en el núcleo de las células en presencia de GAs (Dill *et al.*, 2001).

Los mutantes DELLA de distintas especies se clasifican en dos grupos: (1) mutantes enanos e insensibles a GAs, causados por mutaciones semi-dominantes de ganancia de función (por ejemplo, por deleción o alteración de la región DELLA), y (2) mutantes altos insensibles a GAs, causados por mutaciones recesivas de perdida de función (por ejemplo, mutaciones nulas). Los mutantes *gai-1* de *Arabidopsis*, *d8* de maíz y *Rht* de trigo forman parte del primer grupo. Su fenotipo es similar al de los mutantes deficientes en GAs, enanos y con hojas color verde oscuro, fenotipo que no es revertido por la adición exógena de las mismas. Además, los niveles endógenos de GAs están elevados en estos mutantes respecto a los de las plantas silvestres (Richards *et al.,* 2001). Por el contario, los mutantes cuádruple DELLA (*gai-t6/rga-24/rgl1-1/rgl1-*2) en *Arabidopsis*, *slr1* de arroz, *la/cry^{s}* de guisante y *sln1* de cebada forman parte del segundo grupo. Estas plantas presentan el fenotipo típico de plantas tratadas exógenamente con niveles saturantes de GAs, entrenudos largos, hojas estrechas y esterilidad masculina. La concentración de GAs endógenas en estos mutantes es mas baja que en las plantas silvestres. Además son resistentes al efecto de los inhibidores de la biosíntesis de GAs, lo que indica que estas mutaciones causan una respuesta constitutiva a las mismas (Richards *et al*., 2001).

3.2 Factores reguladores de las proteínas DELLA

Existen proteínas que regulan la actividad de las proteínas DELLA. Dichas proteínas pueden ejercer una regulación positiva si disminuyen la estabilidad de las proteínas DELLA para ser degradadas, o negativa si aumentan la estabilidad frente a la degradación (Fig. 4).

Entre los reguladores negativos se encuentran el gen SPY de *Arabidopsis* (Fig. 4), una N-acetilglucosamina transferasa (OGT) (Thornton *et al*., 1999) que cataliza la modificación post-traduccional de los residuos Ser/Thr de las proteínas por adición de B-N-acetylglucosamina. La modificación post-traduccional de las proteínas DELLA por acción de este enzima podría estabilizarlas, evitando la degradación por ubiquitinación en un mecanismo de competencia por los sitios de fosforilación de la proteína (Thomas *et al*., 2005). Por ello el fenotipo de los mutantes *spy* es como el de las plantas tratadas exógenamente con niveles saturantes de GAs.

PHOR1 es una proteína que actúa como regulador positivo de las proteínas DELLA. Dicha proteína, aislada de patata, contiene un dominio u-box en el extremo animo terminal que lleva asociada, en la mayoría de los casos, la actividad E3 ubiquitina ligasa (revisado en Olszewski *et al*., 2002). Dado que dicha actividad forma parte del complejo de ubiquitinación SCF E3 ubiquitina ligasa, PHOR1 podría estar implicada en la degradación de las proteínas DELLA.

4. PROCESOS FISIOLÓGICOS CONTROLADOS POR GAs

4.1. Las giberelinas son hormonas inductoras del crecimiento

El crecimiento se entiende como el aumento irreversible del tamaño de una célula, órgano, tejido u organismo. El crecimiento se debe a dos acontecimientos a nivel celular: (1) división, en el cual una célula madura se divide en dos células separadas y (2) expansión o aumento de tamaño celular de las células hijas. En las plantas, la división celular se restringe a determinadas zonas que tienen células producidas constantemente por un meristemo. Las puntas de las raíces y tallos (ápices) tienen meristemos. Estos meristemos se forman durante el desarrollo embrionario, mientras se origina la semilla y se llaman meristemos primarios. Las raíces y los tallos crecen por meristemos que continuamente se están renovando indeterminadamente hasta que este meristemo se transforma en reproductivo. Sin embargo, por si sola la división celular no causa aumento de tamaño, necesita del incremento de los productos celulares de la división para que se origine el crecimiento. El desarrollo se debe a la suma entre el crecimiento y la diferenciación celular, en el cual una célula que quizás haya alcanzado su tamaño definitivo, se especializa para realizar una función determinada. La diversidad de tipos celulares en que se pueden especializar una célula, explica los diversos tejidos y órganos de una planta determinada (Salisbury y Ross, 1992).

Las giberelinas actúan como reguladores del crecimiento y desarrollo en las plantas, controlando procesos tales como la germinación de las semillas, la elongación del tallo, la expansión de las hojas, el crecimiento de la raíz y del tubo polínico, la formación de tricomas, el desarrollo de flores y frutos y la inducción de la floración en condiciones no inductivas de día corto (Sponsel y Hedden, 2004; Thomas *et al.,* 2005; Yamaguchi, 2007).

4.1.1. Crecimiento del tallo

Los mutantes deficientes en GAs son fenotípicamente enanos y la adición de GA exógena restaura el crecimiento normal del tallo en diversas especies (revisado en Hedden y Phillips, 2000a). Además, en general, elevados niveles de GAs están asociados con planta altas y bajos niveles con enanismo (Richards *et al*., 2001). La respuesta más evidente inducida por las GAs en la elongación del tallo es a nivel de entrenudos. Las GAs inducen la elongación mediante la división y expansión celular, siendo este último proceso el que mayoritariamente contribuye al incremento en la altura de la planta (Hooley, 1994). En primer lugar, se ha demostrado que las GAs promueven la división celular porque estimulan que las células que se encuentran en la fase G1 entren en la fase S y también a que se acorta la fase S (Liu y Loy, 1976). En segundo lugar, las GAs promueven el crecimiento celular debido a un incremento en la hidrólisis de almidón, sacarosa y fructanos, con lo que se originan moléculas de fructosa y glucosa. Estas

hexosas proporcionan energía vía respiración, la cual contribuye a la formación de la pared celular y también hacen momentáneamente más negativo el potencial hídrico de la planta. Como resultado de esta disminución del potencial, el agua penetra con mayor rapidez, provocando la expansión celular y diluyendo los azúcares (Glasziou, 1969). En tercer lugar las GAs aumentan la plasticidad de la pared celular, promoviendo la expansión de las células jóvenes derivadas del meristemo intercalar de los entrenudos (Taylor y Cosgrove, 1989). En cuarto lugar las GAs incrementan la expresión de los componentes necesarios para la elongación celular, como la tubulina o el canal de agua de la membrana del tonoplasto (Huang y Lloyd, 1999; Phillips y Huttly, 1994). Por último, las GAs podrían estar implicadas en la reorientación de los microtúbulos, y por tanto en la determinación de la polaridad de la expansión celular (Baluska *et al*., 1993), proceso en que también es necesaria la participación de las auxinas. En *Arabidopsis* el gen *AtKSS* (mutante semi-enano, *lue1*) que codifica para una proteína similar a una microtubulin kataninas p60, sugiere la implicación de las GAs en la orientación de los microtúbulos ya que el nivel de mensajeros de *AtKSS* es modulado por los niveles de GAs. El mutante *lue1*, presenta una orientación de los microtúbulos alterada y una reducción en la elongación del tallo. Además, tiene alterados los niveles de expresión del gen *AtGA20ox1* en comparación con plantas silvestres (Bouquin *et al.,* 2003).

4.1.2. Crecimiento de raíces

Las raíces de los mutantes deficientes en GAs, son cortas, es el caso del mutante *na* en guisante (Yaxley *et al*., 2001) o el mutante *ga1-3* en *Arabidopsis* (Fu y Harberd, 2003). La longitud de dichas raíces se restaura con aplicaciones exógenas de GAs, en combinación con la mutación slender (*sln*; mutante que tiene bloqueado el catabolismo de GAs) en guisante (Yaxley *et al*., 2001) o bien en combinación con mutaciones de respuesta constitutiva a GAs que afectan a las proteínas DELLA en *Arabidopsis* (Fu y Harberd, 2003). Esto último indica que las proteínas DELLA inhiben el crecimiento de la raíz y que las GAs promueven dicho crecimiento mediante la degradación de estas proteínas (Fu y Harberd, 2003). Además, en guisante se ha encontrado que el contenido de GA_1 en el ápice radical y en las raíces laterales es mayor que en las demás zonas de la raíz, indicando que el contenido de esta GA activa es importante en las zonas de crecimiento activo de este órgano (Yaxley *et al*., 2001). Recientemente, en *Arabidopsis* se ha visto que la respuesta a GAs, mediada por las proteínas DELLA, controla el crecimiento de la raíz regulando específicamente la elongación de las células de la endodermis de este tejido (Ubeda-Tómas *et al*., 2008).

4.2. Germinación de semillas

Las GAs tiene un papel importante en la estimulación de la germinación en un amplio número de especies (revisado en Thomas *et al*., 2005). Algunos mutantes deficientes en GAs de

Arabidopsis o tomate son incapaces de germinar, salvo si son tratados exógenamente con GAs. (Koornneef y Van der Veen, 1980, Koornneef *et al.*, 1990). En cereales, las GAs estimulan la germinación mediante la degradación de las proteínas DELLA (SLN1 en maíz y SLR1 en arroz) (Gubler *et al.*, 2002; Ikeda *et al.*, 2001). Esta degradación de las proteínas DELLA conlleva la activación del factor de transcripción GAMYB (factor de tipo MYB que se induce por GAs) el cual activa la expresión de α-amilasa y otros enzimas hidrolíticas o proteasas, que degradan las reservas del endospermo. De esta manera se promueve la movilización de las reservas de las semillas y se estimula la expansión del embrión (revisado en Finkelstein *et al.,* 2007).

4.3. Inducción de la floración

El éxito reproductivo de una planta depende de que la floración tenga lugar en el momento más adecuado de su desarrollo y cuando las condiciones ambientales sean favorables. Las plantas en un estadio concreto de su ciclo vital, pasan de la fase de desarrollo vegetativo a la fase reproductiva. El cambio de esta fase se denomina transición floral. La transición floral involucra el cambio de identidad del meristemo apical el cual cesa de producir hojas y en su lugar comienza a formar flores. En la fase juvenil o de desarrollo vegetativo la planta es incapaz de florecer como consecuencia de la incompetencia o la falta de capacidad de respuesta de los órganos que participan en la inducción de la floración. El estímulo floral solo es percibido cuando las plantas están en la fase adulta y como consecuencia se produce el inicio del desarrollo reproductivo. Se desconocen cuales son los mecanismos moleculares responsables de la transición de la fase juvenil a la adulta. Los tratamientos que aceleran el tiempo de floración, como determinados fotoperiodos, vernalización (exposición a bajas temperaturas) o tratamientos con giberelinas activas, suelen acelerar el cambio de fase y la adquisición de la competencia para florecer, indicando la existencia de elementos comunes en dichas respuestas (revisado en Azcón-Bieto y Talón, 2000). Existen tres grupos de plantas en función de su respuesta al fotoperiodo: plantas de día largo, que requieren días largos para florecer, plantas de día corto, que requieren días cortos y plantas independientes o neutras al fotoperiodo. En estas últimas la floración se da una vez la planta alcanza un determinado estado del desarrollo. En especies dependientes de fotoperiodo que tienen un habito de crecimiento en roseta, la floración esta asociada generalmente con la inducción de la elongación del tallo.

En la especie modelo, *Arabidopsis,* planta de día largo y de tipo roseta, se han identificado los genes que afectan la inducción de la floración mediante la caracterización de mutaciones que dan lugar a fenotipos de floración temprana o tardía, y el análisis de variación genética para el tiempo de floración inherentes a las diferentes poblaciones naturales (ecotipos) (revisado en Parcy, 2005). En *Arabidopsis*, los genes que participan en la regulación del tiempo de floración se organizan en dos rutas: en respuesta a señales ambientales (fotoperiodo y vernalización) y en respuesta a señales endógenas (ruta autónoma y GAs). Durante los últimos años se han

realizado progresos que ayudan a entender las bases moleculares de cómo se integran las cuatro rutas que promueven la transición de la floración en esta especie (Araki, 2001). Estas rutas parecen actuar finalmente de manera directa o indirecta sobre los genes responsables de la iniciación de los meristemos florales, regulando su expresión y su función (Blázquez *et al*., 1998). Como genes integradores de la señales para la transición floral, se han identificado en *Arabidopsis* los genes *LFY*, *SOC1* y *FT* (revisado en Mouradov *et al.,* 2002).

Las condiciones de fotoperiodo en las que se induce la floración, en la mayoría de plantas, dependientes de fotoperiodo, son las de día largo (Levy y Dean, 1998). Estas condiciones se denominan condiciones inductivas de la floración. La importancia de las GAs en la inducción de la floración se pone de manifiesto por la capacidad de estas hormonas para inducir este proceso en condiciones no inductivas, es decir, en día corto (Metzger, 1995), y por la falta de floración de los mutantes de *Arabidopsis, ga1-3* deficientes de GAs, cuando se cultivan en dichas condiciones (Wilson *et al*., 1992). En condiciones de día corto no inductivas, la floración está mediada por una ruta independiente del fotoperiodo y dependiente de GAs, ya que en dichas condiciones esta hormona induce la expresión del gen de identidad del meristemo floral *LEAFY* (Blázquez *et al*., 1998). También se ha encontrado que tras el incremento en los niveles de GA_4 en el ápice, se induce la expresión de este mismo gen de identidad floral en *Arabidopisis* (Eriksson *et al*., 2006), contribuyendo a la idea de que las GAs promueven tanto la iniciación de la floración como la elongación del tallo (boolting) en esta especie (Xu *et al*., 1997; Zeevaart *et al***.,** 1993). En el caso de *Lolium temulentum* (especie dependiente de fotoperiodo de día largo), se ha demostrado que GA_5 y GA_6 son las GAs inductoras de la floración (King *et al.,* 2003).

Sin embargo, el efecto positivo de las GAs en la inducción floral no puede generalizarse en todas las plantas, tanto en especies dependientes o independientes de fotoperiodo. Por ejemplo en *Silene Armeria,* el día largo promueve la floración y el incremento en el contenido de GA_1 en los brotes apicales, lo que provoca el desarrollo del tallo, sin embargo cuando este mismo efecto se pretende mimetizar con aplicaciones exógenas de GAs en condiciones no inductivas, es decir de día corto, se promueven la elongación del tallo pero las plantas no florecen (Azcón-Bieto y Talón, 2000). En especies como *Fuchsia hybrida*, *Pharbitis nil* (King *et al.,* 2000) o la vid, las GAs actúan retrasando el tiempo de floración (Boss y Thomas, 2002) y los mutantes enanos deficientes de GAs en guisante, no presentan retraso en el tiempo de floración, ni un aumento de las GAs activas previo a la transición floral (Murfet y Reid, 1987; Ross *et al*., 1992). Lo anterior indica que el papel de las GAs en la inducción del tiempo de floración no parece ser igual para las plantas dependientes o independientes de fotoperiodo.

4.4. Formación de flores y frutos

Las GAs son necesarias para el desarrollo normal de la flor. Mutantes deficientes en GAs como *ga1-3* de *Arabidopsis* (Koornneef y van der Veen, 1980) y *gid-1/2* en tomate son androestériles y muestran un desarrollo incompleto de estambres y pétalos (Nester y Zeevaart, 1988; Jacobsen y Olszewki, 1991). En dichos mutantes el desarrollo de las anteras se encuentra detenido en la fase pre-meiótica G1. En petunia, las anteras son fuente de GAs necesarias para el desarrollo de la flor y su eliminación provoca la inhibición de la pigmentación y el crecimiento de pétalos (Weiss y Haley, 1989). En *Arabidopsis* y petunia después de la antesis las GAs estimulan la germinación del polen y el crecimiento del tubo polínico (Metzger, 1995). La sobreexpresión de un gen de catabolismo de GAs (*PsGA2ox2*) en *Arabidospis,* produjo plantas transgénicas con menor crecimiento del tubo polínico, y este defecto fue parcialmente rescatado por la aplicación exógena de GAs o por combinaciones con mutaciones en la sensibilidad de GAs tales como *sly-5, gar2-1, rga-2* y *rga-2/gai-d5* (Swain *et al*., 2004). Esto indica que, tanto la biosíntesis, como la vía de señalización de GAs son importantes en el desarrollo del polen y del tubo polínico.

Las GAs también son importantes en la formación de los frutos (Gillaspy *et al.,* 1993). La aplicación exógena de GAs promueve la fructificación y afecta el desarrollo del fruto en especies cultivadas (Pharis y King, 1985). Además, la aplicación exógena de GAs a ovarios emasculados no polinizados induce el desarrollo partenocárpico del fruto (Gustafson, 1960; Alabadí *et al.,* 1996; Fos *et al.,* 2000, 2001). Por otra parte, la aplicación de un inhibidor de la síntesis de GAs (Paclobutrazol) a frutos polinizados inhibe su desarrollo, efecto revertido por la aplicación de GA_3 (Fos *et al.,* 2000, 2001).

4.5. Procesos de diferenciación celular asociados a GAs

El mantenimiento del meristemo apical del tallo y la iniciación de los primordios foliares están regulados, en parte, por GAs y por algunos genes homeóticos de tipo KNOTTED1-like homeobox (KNOX), que codifican reguladores transcripcionales (Fleet y Sun, 2005). Dos factores de transcripción de la familia KNOTTED de tabaco, *NtH15*, y arroz *OsH1* han sido estudiados en relación con las GAs. La sobreexpresión de *OsH1* en tabaco provoca enanismo y una reducción de los niveles de expresión de *GA20ox* y de GAs activas (Kusaba *et al.,* 1998). Por otra parte, el análisis de hibridación *in situ* en tabaco muestra que el gen KNOX (*NtH15*) se expresa en el meristemo apical en una zona, donde está excluida la expresión del gen *NtGA20ox1 (Ntc12)*, siendo dichos genes mutuamente excluyentes (Tanaka-Ueguchi *et al.,* 1998). En tabaco, la regulación que ejerce la proteína KNOX, *NtH15*, sobre la expresión de *NtGA20ox1,* es a través de la unión a una secuencia de 8pb, presente en la región 5´ no traducible y el primer intrón de *Ntc12* (Sakamoto *et al*., 2001a).

La interacción entre los genes *KNOX* y las GAs también afecta a la morfología de las hojas. En especies de hoja simple como tabaco o *Arabidopsis*, se ha visto que la expresión de genes KNOX se presenta en el meristemo, donde se reprime la expresión de *GA20ox*. Sin embargo las GAs se acumulan en los primordios foliares de estas especies para promover el crecimiento determinado (revisado en Sakamoto et el., 2001a). En tomate, los genes KNOX se expresan tanto en el meristemo como en los primordios foliares, donde la disminución de GA20ox promueve la disección de las hojas (Hay *et al*., 2002). Además, la represión de la expresión de *GA20ox*, causada por la sobreexpresión de los genes *KNOX*, en tabaco, tomate y *Arabidopsis* genera la formación de meristemos ectópicos en las hojas y de hojas mas lobuladas que en plantas silvestres (Hay *et al*., 2002; Sakamoto *et al*., 2001a).

Otro tipo de reguladores transcripcionales de los genes de la biosíntesis de GAs también están implicados en el mecanismo por el que esta hormona controla el crecimiento de la planta. Uno de estos reguladores transcripcionales se ha descrito en tabaco, la proteína de tipo bZIP (REPRESSION OF SHOOT GROWTH; RSG) que activa la transcripción de *KO* (*ent*-kaureno oxidasa) (Fukazawa *et al*, 2000) y está implicada en la regulación por retroalimentación de la *GA20ox* (Igarashi *et al*., 2001).

5. REGULACIÓN DE LOS GENES DEL METABOLISMO DE GAs

Las plantas perciben las variaciones en el ambiente que les rodea, y responden a estas variaciones con cambios en el hábito de crecimiento y desarrollo. La expresión de los genes del metabolismo de GAs, está regulada por factores ambientales, como las condiciones de luz, temperatura y por factores endógenos como otras hormonas o las propias GAs.

5.1. Regulación por luz

Durante el desarrollo vegetal son muchos los procesos controlados por GAs tales como la germinación de semillas, la desetiolación, la tuberización entre otros, que también se ven influenciados por la luz. En algunos casos los efectos producidos por la luz pueden ser mimetizados mediante la alteración en el contenido de GAs. La luz afecta el desarrollo de las plantas de acuerdo a la cantidad, calidad, duración y dirección de la misma. La cantidad, calidad y duración de la luz generan respuestas fotoperiódicas del crecimiento en las plantas mediante la estimulación del foto-receptor dual o fitocromo. La dirección de la luz genera respuestas fototrópicas del crecimiento mediada por el criptocromo. Las GAs participan en los cambios que ejerce la luz sobre las respuestas del crecimiento y desarrollo de las plantas.

El control ejercido por la luz durante el proceso de germinación de semillas sobre los genes del metabolismo de GAs, principalmente los que codifican *GA3ox*, está relacionado con la

percepción de cambios en la calidad de la misma. En dicha regulación están implicados los fitocromos, que inducen la producción de GAs activas mediante el aumento de la expresión de *GA3ox* (Yamaguchi *et al.*, 1998a). La expresión de *AtGA3ox1* y *-2* en semillas embebidas de *Arabidopsis* y de *LsGA3ox1* en semillas de lechuga se induce tras 1 h de tratamiento con luz roja (Yamaguchi *et al.*, 1998a, Toyomasu *et al.*, 1998). Esta inducción es revertida por un pulso de luz roja lejana.

La fotomorfogénesis se refiere al crecimiento en luz de una planta, después de la germinación donde previamente tenía un crecimiento en oscuridad (escotomorfogénesis). El crecimiento en oscuridad genera plantas etioladas. Al cambiar a condiciones de crecimiento en luz se da un proceso de desetiolación, donde suceden cambios en la morfología de la planta etiolada como la reducción del crecimiento del hipocotilo, la desaparición del gancho apical y la expansión y apertura de los cotiledones (Alabadí *et al.*, 2004). En guisante y en *Arabidopsis*, plántulas tratadas con inhibidores de GAs presentan fenotipo desetiolado cuando crecen en oscuridad y el efecto del inhibidor es revertido por la aplicación exógena de GAs (Alabadi *et al.*, 2004). Además el mutante *ga1-3* de *Arabidopsis*, deficiente en GAs, presenta fenotipo desetiolado en condiciones de oscuridad, lo que indica que los cambios en el fenotipo durante la fotomorfogénesis están relacionados con el metabolismo de GAs. En guisante se ha observado que dichos cambios están relacionados con el contenido de GAs en el brote apical de las plántulas durante las 2 primeras horas de exposición a luz, tras ser cultivadas en oscuridad. En experimentos de desetiolación en guisante a tiempos cortos se ha observado que el contenido de GA_1 disminuye y que el de su catabolito GA_8 aumenta (Gil y García-Martínez, 2000), además se ha detectado que la expresión del gen *PsGA3ox1* disminuye y que la expresión del gen *PsGA2ox2* incrementa rápidamente (en menos de 1 hora). El incremento de *PsGA2ox2* correlaciona con la rápida disminución en el contenido de GA_1 (Reid *et al.*, 2002). Tras 4 horas de exposición a la luz se detecta un aumento de los niveles de transcritos de *PsGA20ox1*, lo que sugiere que dicho aumento está relacionado con la regulación por retroalimentación de este gen en respuesta a la disminución del contenido de GA_1 (Gil y García-Martínez, 2000).

La tuberización en patata se induce en condiciones de día corto y se inhibe por la aplicación de GAs (Kamiya y García-Martínez, 1999). En condiciones de día largo, no inductoras de la tuberización, los niveles endógenos de GAs en las plantas son mayores que en condiciones de día corto (Okazawa, 1960, Railton y Wareing, 1973). La aplicación de inhibidores de la biosíntesis de GAs provoca la tuberización en condiciones de crecimiento no inductoras (Jackson y Prat, 1996), lo que sugiere que las GAs ejercen una inhibición de la tuberización en condiciones de día largo. Sin embargo, debe existir otro factor inhibidor de la tuberización ligado a las condiciones de día largo aparte de las GAs, porque mutantes severos de biosíntesis de las mismas o líneas antisentido de *GA20ox1* no tuberizan en dichas condiciones (Carrera *et*

al., 2000). El patrón de expresión diurno de los tres genes de *GA20ox* de patata depende de las condiciones del fotoperiodo. En condiciones de día corto, la sobreexpresión en antisentido de un gen *GA20ox*, disminuye el contenido de GA_{20} y GA_1 en las hojas y produce un adelanto de la tuberización (Carrera *et al*., 2000).

5.2. Regulación por la temperatura

La temperatura es uno de los factores ambientales que influye sobre el desarrollo y crecimiento de las plantas. Estudios realizados, cuando se expone a bajas temperaturas tanto en procesos de estratificación de semillas (Yamaguchi *et al*., 2004) como de vernalización en plantas (Zanewich y Rood, 1995), demuestran que parte de los cambios que se producen en el desarrollo durante estos procesos están relacionados con una alteración del metabolismo de GAs. El aumento de GA_{20} que se produce durante la vernalización en *Winter canola*, indica que podría haber un incremento de la expresión de *GA20ox*s durante este proceso (Zanewich y Roddo, 1995). *Eustoma grandiflorum* Shinn es otra especie que requiere vernalización para la inducción del crecimiento del tallo y la floración. Estudios para determinar el papel de las GAs en la vernalización revelan que los genes que codifican los enzimas de biosíntesis de GAs, CPS, GA20ox y GA3ox, así como el gen regulador de la señalización de estas hormonas, SPY, son inducidos durante este proceso, lo que sugiere que la expresión de dichos genes está regulada durante la vernalización (Mino *et al*., 2003). Por otra parte, las altas temperaturas en cítricos incrementan la elongación del tallo en plántulas, a través de variaciones en el metabolismo de GAs, ya que tanto la expresión del gen *CcGA20ox1* como el contenido de GA_1 aumentan cuando las plántulas se cultivan a 32ºC durante el día y 27ºC durante la noche respecto a las que se cultivan a 17ºC durante el día y 12ºC durante la noche (Vidal *et al*., 2003). En guisante se ha demostrado que la expresión del gen *PsGA2ox2* es regulado por la temperatura, ya que en una condición de temperatura con un coeficiente negativo DIF (baja temperatura de día/alta temperatura en la noche; 13ºC/21ºC), la abundancia de este gen aumenta y el contenido de GA_1 disminuye al igual que la elongación del tallo, lo que indica un papel importante de este gen del catabolismo de GAs en la modulación de sus contenidos en respuesta a la temperatura (Stavang *et al*., 2005).

5.3. Regulación del metabolismo de GAs por otras hormonas

La implicación de varias hormonas, como por ejemplo las GAs, el etileno, el acido absícico (ABA), los brasinoesteroides (BRs) y las auxinas (IAA) entre otras, en el control de diversos procesos del desarrollo de las plantas es frecuente aunque la forma como interaccionan o cooperan para controlar dichos procesos dista de estar claro. Entre los casos de interacción entre hormonas que se conoce que pueden influir en la regulación de la expresión de los genes de biosíntesis y catabolismo de GAs, se encuentran por ejemplo la interacción entre GAs y IAA

o entre GAs y BRs. Estas interacciones se han estudiado a través de diversos procesos del desarrollo de las plantas, tales como la germinación de semillas, la elongación del hipocotilo o la fructificación.

Las auxinas estimulan la elongación del tallo mediante la regulación de la biosíntesis de GAs. En guisante, la decapitación del ápice o la aplicación de inhibidores del transporte de auxinas, provoca una disminución de la expresión de *PsGA3ox1*. La aplicación de IAA en estas plantas recupera la expresión normal de este gen y disminuye la expresión de *PsGA2ox1* y -2 (O´Neill y Ross, 2002). Esto provoca un aumento en el contenido de GA_1 y como consecuencia la elongación del tallo (Ross *et al.*, 2000). En tabaco, el mismo tipo de estudio muestra que la aplicación de IAA en tallos decapitados regula positivamente la GA 20-oxidasa (Wolbarg y Ross, 2001). De la misma forma, en *Arabidopsis* la aplicación de auxinas controlan la expresión de algunos genes del metabolismo de GAs en plántulas (Frigerio *et al.,* 2006). Las auxinas también actúan como reguladoras de la expresión de los genes de GAs durante las primeras etapas del desarrollo del fruto. En guisante, el engrosamiento del pericarpo, esta mediado por el aumento en la expresión de *PsGA3ox1* en dicho tejido. Este aumento es promovido por el transporte de auxinas desde la semilla al pericarpo, donde se produce un aumento de GA_1 (Ozga y Reinecke, 2003). En tomate, las auxinas inducen la fructificación aumentando la biosíntesis de GAs a través de la actividad GA 20-oxidasa, GA 3-oxidasa y CPS, lo que conlleva a un mayor contenido en GA_1 (Serrani *et al.,* 2007).

Durante la germinación de las semillas los BRs regulan positivamente la expresión de *GA20ox* en *Arabidopsis* (Bouquin *et al.*, 2001), de forma que esta hormona podría actuar estimulando la biosíntesis de GAs. Aunque está no sería la única vía de interacción puesto que la aplicación de BRs rescata la germinación en mutantes *ga1-3* que tienen bloqueada la síntesis por encima de la acción de las *GA20ox* (Steber y McCourt, 2001). Durante el crecimiento del hipocotilo los BRs regulan la biosíntesis de las GAs, ya que en *Arabidopsis*, la aplicación de BRs provoca la elongación del hipocotilo durante el crecimiento en la oscuridad de mutantes de biosíntesis *ga1-3* e insensibles a GAs, *sly1-2* (Steber y McCourt, 2001). Sin embargo, la aplicación de GAs sobre el mutante de biosíntesis de BRs *det2-1* (Chory *et al.*, 1991) no restaura la elongación del hipocotilo en oscuridad, lo que indica que la acción de las GAs en este proceso del desarrollo depende de BRs.

5.4. Homeostasis del contenido de GAs

Las GAs activas ejercen a través de algunos componentes de su ruta de señalización la auto-regulación de su biosíntesis, de manera que la síntesis *de novo* y el catabolismo de GAs, están controlados por los niveles endógenos de las GAs activas en la planta (Elliott *et al.*, 2001; Israelsson *et al.*, 2004). La regulación transcripcional de los genes implicados en el metabolismo de las GAs tiene lugar mediante un mecanismo de retroalimentación negativa en el caso de los

genes biosintéticos (*GA 20-oxidasa* y *GA 3-oxidasa*), o positiva en el caso de los genes catabólicos (*GA 2-oxidasa*) (Fig. 5). En plantas deficientes en GAs de diversas especies o en plantas silvestres tratadas con inhibidores de la biosíntesis de GAs, las bajas concentraciones de GAs activas inducen la expresión de los genes *GA 20-oxidasa* y *GA 3-oxidasa*; por el contrario dicha expresión disminuye con la aplicación de GA exógena (Martin *et al.*, 1996; Phillips *et al.*, 1995; Toyomasu *et al.*, 1997). Para las *GA 2-oxidasas* también se ha demostrado que la expresión de estos genes es inducida cuando hay altos niveles de GAs endógenas (Thomas *et al.*, 1999; Elliott *et al.*, 2001).

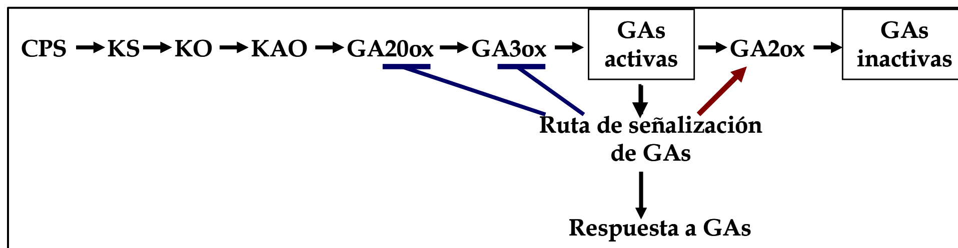

Figura 5. Esquema de la regulación por retroalimentación negativa de genes de biosíntesis (*GA 20-oxidasa*, *GA 3-oxidasa*) y retroalimentación positiva de genes de catabolismo (*GA 2-oxidasa*) de GAs. Adaptado de Fleet y Sun, (2005).

Este mecanismo por retroalimentación, está mediado por los componentes de la vía de transducción de señal de las GAs (Yamaguchi y Kamiya, 2000), ya que los mutantes afectados en la respuesta a GAs (insensibles o constitutivos) tienen alterado el control homeostático de sus niveles endógenos de GAs activas. Así mismo, se ha demostrado que este tipo de regulación transcripcional, importante en el control de los niveles de GAs, se activa en las plantas transgénicas que tienen alterada la ruta de metabolismo de GAs (Hedden y Phillips, 2000b; Yamaguchi y Kamiya, 2000).

En tabaco se ha descrito que los genes *NtGA20ox1* (*Ntc12*) y *NtGA3ox1* (*Nty*), reducen su expresión cuando las plantas son tratadas exógenamente con GA_3, y que por el contrario sus transcritos aumentan cuando se aplica un inhibidor de síntesis de GAs. Sin embargo, la regulación por retroalimentación no es igual para todos los genes de biosíntesis, puesto que para el gen *NtGA20ox2* (*Ntc16*) sólo se detectó inducción de su expresión cuando se trató con el inhibidor de biosíntesis de GAs (Tanaka-Ueguchi *et al.*, 1998, Itoh *et al.*, 1999).

No se tiene información de que exista una regulación por retroalimentación causada por GAs sobre los genes que codifican enzimas implicados en la primera parte de la ruta de biosíntesis de las mismas, CPS, KS y KO (ciclasas o monooxigenasas dependientes de P450).

6. MANIPULACIÓN GÉNETICA DE LA RUTA DEL METABOLISMO DE GAs

La mayoría de los procesos fisiológicos controlados por GAs son de gran importancia en especies cultivadas, tales como el crecimiento del tallo, la floración y la fructificación. En

agricultura, la aplicación de GAs o inhibidores de la biosíntesis de GAs es una práctica extendida (Hedden and Phillips, 2000b). Las variedades semi-enanas de trigo y arroz de la "Revolución verde" que mostraron un mayor rendimiento y menores problemas de encamado bajo condiciones de elevada fertilización (Sakamoto *et al.*, 2004), son mutantes que están afectados en la transducción de señal (proteínas DELLA) o en la ruta de biosíntesis de GAs (Peng *et al.*, 1999). Posteriormente, la clonación de los genes de la ruta de biosíntesis y respuesta a GAs ha permitido su manipulación genética, con la que ha sido posible obtener plantas transgénicas con contenidos o percepción a GAs alterados.

Actualmente, se han descrito varios casos de plantas transgénicas donde se sobreexpresan los genes del metabolismo de GAs, o se produce el silenciamiento de los mismos mediante la sobreexpresión del RNA mensajero de estos genes en antisentido. En *Arabidopsis*, Fleet *et al.* (2003) observaron que líneas transgénicas de sobreexpresión de los genes ***CPS, KS* y *CPS/KS*** (genes que participan en las primeras partes de la ruta de biosíntesis de GAs), no mostraban un fenotipo de exceso de GAs, lo cual podía deberse a una limitación en el flujo metabólico de los enzimas posteriores en la ruta de biosíntesis y/o un incremento en el catabolismo de GAs.

Por otra parte en numerosas especies vegetales se ha demostrado que la sobreexpresión de los genes que codifican **GA20ox** implica un incremento de la concentración de GAs activas, lo que indica que estos genes tienen un papel muy importante en la regulación de los últimos pasos de la ruta de biosíntesis de GAs (Yamaguchi y Kamiya, 2000). En concreto, la sobreexpresión de genes que codifican GA 20-oxidasas en *Arabidopsis* produce plantas con dormición reducida en las semillas, hojas más grandes y de color claro, floración temprana y mayor altura (Coles *et al.*, 1999; Huang *et al.*, 1998). En tabaco, la sobreexpresión de genes de *GA20ox* produce plantas que tienen mayores contenidos de GAs activas y fenotipos de exceso de GAs tales como: hipocotilos y entrenudos alargados, aumento del tamaño final de la planta (Vidal *et al.*, 2001), mayor producción de biomasa y mayor producción de lignina por la estimulación de la formación del xilema (Biemelt *et al.*, 2004). En patata, la sobreexpresión de *GA20ox*, produce plantas altas con retraso en la tuberización y un crecimiento vegetativo prolongado en día corto (Carrera *et al.*, 2000). En álamo, la sobreexpresión de la *GA20ox* incrementa además de la velocidad de crecimiento, el número y longitud de las fibras xilemáticas, carácter de gran importancia en la producción de papel (Eriksson *et al.*, 2000). Otras especies donde se ha sobreexpresado *GA20ox,* son arroz (Oikawa *et al.*, 2004) o cítricos (Fagoagoa *et al.*, 2007) y en todos los casos las plantas exhibieron fenotipos de exceso de GAs.

El silenciamiento de la expresión de GA 20-oxidasa mediante la sobreexpresión en antisentido de *GA20ox* en *Arabidopsis*, dio como resultado plantas de altura y contenidos de GAs

reducidos (Coles *et al*., 1999). En patata, la expresión de *StGA20ox* en forma antisentido acelera el proceso de tuberización e incrementa el rendimiento de tubérculos (Carrera *et al*., 2000).

La sobreexpresión de genes que codifican **GA3ox**, enzima que cataliza el último paso de la síntesis de GAs activas, se ha descrito en álamo donde las plantas transgénicas no exhibieron grandes cambios ni en la altura, ni en el contenido de GAs (Israelsson *et al*., 2004). Por el contrario, en *Arabidospis* la sobreexpresión de una *GA3ox* de calabaza produjo plantas con fenotipo de exceso de GAs (Radi *et al*., 2006).

La sobreexpresión de genes de ***GA2ox*** responsables de la inactivación de GAs, provoca fenotipos severos de deficiencia de GAs. En tabaco, la sobreexpresión de una *GA2ox* de adelfa causa una reducción en la elongación de entrenudos, floración tardía y presencia de hojas pequeñas y oscuras (*NoGA2ox3*; Ubeda-Tomás *et al*., 2006). Fenotipos similares se han encontrados en otras especies donde se han sobreexpresado constitutivamente una *GA2ox*, tales como: *Arabidopsis (AtGA2ox7* y *AtGA2ox8*; Schömburg *et al*., 2003), arroz (*OsGA2ox1*; Sakamoto *et al*., 2004), álamo (*PtaGA2ox1*; Busov *et al*., 2003), *Nicotiana silvestres* (*SoGA2ox3*; Lee y Zeevaart, 2005) y patata (*StGA2ox1*; Kloosterman *et al*., 2007) entre otros. La sobreexpresión de *PsGA2ox2* en *Arabidopsis*, produjo aborto de las semillas y un menor crecimiento del tubo polínico, indicando el papel de las GAs en el normal crecimiento del tubo polínico (Swain *et al*., 2004).

Por otra parte, el silenciamiento del gen *StGA2ox1* en patata generó plantas transgénicas sin mayores cambios en la parte aérea de la planta, solo se observó fenotipo en los tubérculos, debido probablemente a la mayor abundancia de este gen es este tejido. El silenciamiento de este gen produjo estolones alargados que favorecen una mayor tuberización (Kloosterman *et al.*, 2007).

De la variedad de fenotipos obtenidos en las plantas transgénicas donde se ha sobreexpresado o silenciado alguno de los genes de GAs, se deduce que la manipulación genética de la ruta es una herramienta muy útil tanto para obtener características agronómicas deseables, como para el estudio del papel de los genes del metabolismo de GAs en el desarrollo de las plantas.

6.1. RNA interferencia como herramienta para el estudio de la función de los genes

El objetivo de proyectos actuales de genómica, es la identificación de la función biológica de todos los genes de genoma de algunas especies, sobretodo en las especies donde su genoma ha sido secuenciado en su totalidad. Para determinar la función de un gen se ha recurrido a diversas vías; una de ellas es inferir la función de un gen determinado por su homología de secuencia con otros genes de función conocida en otras especies, otra vía implica técnicas tales como las mutaciones de pérdida de función inducidas por inserciones de T-DNA o por

elementos transponibles (Page y Grossniklaus, 2002) y la sobreexpresión de genes en antisentido. Sin embargo dichas aproximaciones son limitadas a un número reducido de especies (especies con genoma reducido), en adición a los problemas de la redundancia génica causada por las familias multigénicas y a la poliploidía (Travella *et al*., 2006).

Se ha demostrado que la inyección de pequeñas moléculas de RNAs de doble cadena (entre 21-28 nucleótidos) en nemátodos, pueden inducir la degradación del RNA mensajero de secuencia homóloga a los RNAs de doble cadena introducidos, y este proceso se conoce como **RNA de interferencia** (Fire *et al*., 1998). Este proceso facilita el silenciamiento génico post-transcripcional (PGTS) y recientemente ha sido usado para el estudio de la función de alrededor de 4000 genes en el cromosoma I y III de *Caenorhabditis elegans* (Fraser *et al*., 2000; Gonczy *et al*., 2000). El silenciamiento génico inducido por pequeños RNAs de doble cadena ha revelado su importancia no solo como herramienta en el estudio de la función de los genes, sino además, en la regulación del desarrollo y la protección del genoma frente a ácidos nucleicos extraños tales como virus y viroides, tanto en plantas como animales (revisado en Agrawal *et al*., 2003).

El RNA de interferencia permite el silenciamiento de uno, varios o todos los miembros de una familia multigénica y se presenta como una herramienta poderosa en el estudio de la función de los genes (Meins *et al*., 2005). Los RNAs de doble cadena son detectados por la planta hospedera como un RNA aberrante y se activa la maquinaria de silenciamiento génico endógeno, el cual consiste en el reconocimiento y degradación de los RNA mensajeros diana con secuencias homólogas y la amplificación de la señal de silenciamiento, mediante los complejos enzimáticos Dicer y posteriormente RISC (revisado en Brodersen y Voinnet, 2006). Esta herramienta ha sido eficiente en el silenciamiento génico en varias especies, tales como petunia, *Arabidopsis,* cebada, arroz, *Coffe arabica*, entre otras (Stam *et al*., 1997; Chuang y Meyerowitz, 2000; Zentella *et al.,* 2002; Miki *et al*., 2005; Ogita *et al*., 2003). Las construcciones de secuencias homólogas invertidas, separadas por un intrón (tipo horquilla), han demostrado supresión eficiente en el silenciamiento post-transcripcional de los genes homólogos a dichas secuencias (Wesley *et al*., 2001).

Durante este trabajo se ha utilizado la tecnología de silenciamiento por RNA de interferencia, así como la sobreexpresión de genes, con el fin de manipular y estudiar la regulación de la ruta del metabolismo de GAs en tabaco.

6.2. El tabaco como modelo para el estudio de procesos del desarrollo en plantas tipo no roseta

Nicotiana tabacum var *xanthi* es una planta herbácea perenne, de la familia de las solanáceas, de cuyas hojas se produce la mayor parte del tabaco consumido hoy en el mundo. Es oriunda de América tropical, y está estrechamente emparentada con otras plantas cultivadas

comercialmente, como el tomate (*Solanum lycopersicum*) y la papata (*Solanum tuberosum*). Su tallo es erecto, de sección circular, el cual puede alcanzar 120 cm de altura y sus hojas son alternas, lanceoladas de color verde pálido. Esta especie de *Nicotiana* es de día neutro, lo que indica que la inducción de la floración no depende del fotoperiodo, además de no presentar la formación de una roseta de hojas basales contrario a lo que sucede en *Arabidopsis*. La planta de tabaco florece tras aparecer una flor terminal, cuando las plantas tienen aproximadamente 3 meses de edad. Las flores son rosadas, con un pequeño cáliz de 1 a 2 cm y una corola pubescente, de cinco lóbulos aovados, de hasta 4 cm de longitud. El ovario es glabro; la planta es hermafrodita, produciendo flores de ambos sexos. La inflorescencia es de tipo panícula terminal con varias ramificaciones generalmente compuestas. El fruto es una cápsula parda, ovoide, de 15 a 20 mm de largo. Las semillas son esféricas, de color pardo y 0.5 mm de diámetro (Jordan *et al.*, 1995).

Tanto tabaco como tomate, sirven como organismos modelo para tratar de dilucidar cuestiones biológicas básicas. Por ejemplo, la genómica de las solanáceas, en la cual se intenta responder al interrogante de cómo puede un mismo conjunto común de genes o proteínas dar origen a organismos morfológica y ecológicamente tan diferenciados entre sí como son las especies de esta familia. Adicionalmente, *Nicotiana tabacum* se ha empleado en diversos estudios de la biología del desarrollo como planta modelo gracias a su facilidad de transformación y a un ciclo de vida relativamente corto (alrededor de 5 meses). En el estudio de procesos del desarrollo como la inducción de la floración, donde una gran parte del conocimiento se ha derivado de plantas dependientes de fotoperiodo y de tipo roseta como *Arabidopsis*, el tabaco se presenta como una planta adecuada para estudiar este proceso ya que representa a un gran número de especies de día neutro y de hábito de crecimiento tipo no roseta. Los conocimientos adquiridos en tabaco pueden ser extrapolables a plantas que comparten características de su desarrollo, las cuales son difícilmente transformables o no se pueden transformar, como es el caso del guisante. Otros procesos del desarrollo controlados por GAs o la regulación de su propio metabolismo pueden presentar características propias en algunas especies. Por ejemplo, en *Arabidopsis*, la superproducción del enzima de GAs, CPS, no induce alteraciones fenotipicas mientras que en tabaco altera la arquitectura de la inflorescencia (Alapont, 2003). Por tanto el estudio comparado de procesos del desarrollo en varias especies modelo ayuda a comprender que características son generales y cuales están delimitadas a especies concretas.

En este trabajo se estudio como las giberelinas regulan diversos procesos del desarrollo en tabaco, tales como la elongación del tallo y la inducción de la floración, además de estudiar como la propia ruta del metabolismo de la hormona esta regulada y responde a distintas manipulaciones.

OBJETIVOS

La regulación del metabolismo de giberelinas (GAs) en la planta es un proceso dinámico, en el que intervienen muchos enzimas, además de estar afectado por factores tanto endógenos como ambientales. Con el objetivo de estudiar la regulación del metabolismo de GAs en tabaco y de cómo los cambios en este metabolismo influyen en procesos del desarrollo dependientes de GAs, se han planteado los siguientes objetivos específicos:

1. Estudiar el papel que tienen los niveles de GAs activas en la transición floral en tabaco. Para ello se cuantificarán los niveles de GAs activas y la expresión de los genes del metabolismo de las mismas en el ápice de tabaco en diferentes estadíos del desarrollo y se estudiará el efecto que tiene el disminuir o el aumentar los contenidos de GAs sobre el tiempo de floración.

2. Estudiar el efecto de la manipulación de genes de biosíntesis (GA 3-oxidasa y GA 20-oxidasa) en el desarrollo de tabaco. Para ello se determinará el efecto de la sobreexpresión de un gen de GA 3-oxidasa y de la sobreexpresión conjunta de un gen de GA 3-oxidasa y GA 20-oxidasa, sobre el metabolismo de GAs y sobre los caracteres fenotípicos controlados por las GAs. Igualmente, se determinará el grado de correlación entre el contenido de GAs y las variaciones en los niveles de expresión de los genes del metabolismo de GAs en respuesta a dichas manipulaciones genéticas.

3. Estudiar el papel de las GA 2-oxidasas en el desarrollo de tabaco. Para ello se realizara un estudio funcional de los genes de GA 2-oxidasa mediante el silenciamiento génico post-transcripcional. Este abordaje permitirá determinar cuales procesos del desarrollo dependientes de GAs pueden ser afectados por el silenciamiento de dichos genes. Igualmente, se determinará como se regula la expresión de estos genes en respuesta a GAs.

RESULTADOS

CAPÍTULO I

La Floración en Tabaco Necesita de Giberelinas pero no es Promovida por los Niveles de GA_1 y GA_4 en el Brote Apical

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Flowering in Tobacco Needs Gibberellins but is not Promoted by the Levels of Active GA_1 and GA_4 in the Apical Shoot

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Flowering of *Nicotiana tabacum* cv Xhanti depends on gibberellins because gibberellin-deficient plants, due to over-expression of a gibberellin 2-oxidase gene (*35S:NoGA2ox3*) or to treatment with the gibberellin biosynthesis inhibitor paclobutrazol, flowered later than wild type. These plants also showed inhibition of the expression of molecular markers related to floral transition (*NtMADS-4* and *NtMADS-11*). To investigate further the role of gibberellin in flowering, we quantified its content in tobacco plants during development. We found a progressive reduction in the levels of GA_1 and GA_4 in the apical shoot during vegetative growth, reaching very low levels at floral transition and beyond. This excludes these two gibberellins as flowering-promoting factors in the apex. The evolution of active gibberellin content in apical shoots agrees with the expression patterns of gibberellin metabolism genes: two encoding gibberellin 20-oxidases (*NtGA20ox1* = *Ntc12*, *NtGA20ox2* = *Ntc16*), one encoding a gibberellin 3-oxidase (*NtGA3ox1* = *Nty*) and one encoding a gibberellin 2-oxidase (*NtGA2ox1*), suggesting that active gibberellins are locally synthesized. In young apical leaves, GA_1 and GA_4 content and the expression of gibberellin metabolism genes were rather constant. Our results support that floral transition in tobacco, in contrast to that in Arabidopsis, is not regulated by the levels of GA_1 and GA_4 in apical shoots, although reaching a threshold in gibberellin levels may be necessary to allow meristem competence for flowering.

Keywords: Flowering — Gibberellins — Tobacco.

Abbreviations: GA2ox, gibberellin 2-oxidase; GA3ox, gibberellin 3-oxidase; GA20ox, gibberellin 20-oxidase; PCB, paclobutrazol; RT–PCR, reverse transcription–PCR.

Introduction

Floral transition, during the life cycle of plants, is precisely determined by environmental and endogenous signals. Flowering involves changes in the identity of the primordia arising at the flanks of the shoot apical meristem, from leaves to flowers. Gibberellins have been suggested as intermediate messengers for the transition to flowering induced by environmental or endogenous signals (Parcy 2005).

In *Arabidopsis thaliana*, an integrated network of genetic pathways has been proposed to regulate the induction of floral meristem identity genes and floral transition (Araki 2001). The two main pathways mediating environmental responses are the photoperiod and vernalization pathways. The other two are the autonomous pathway and the gibberellin pathway, which acts upon flowering in non-inductive short-day conditions. A large number of genes have been identified, including those regulating the integration of these pathways (*LFY*, *SOC1* and *FT*, the so-called floral pathway integrators; Mouradov et al. 2002). The mRNA of *FT* has been recently identified as a component of the long sought 'florigen' (a graft-transmissible floral signal that moves from leaf to shoot apex) and induces flowering in response to photoperiod induction (Huang et al. 2005).

The involvement of gibberellins in Arabidopsis flowering is supported by physiological and genetic evidence. Mutants in which gibberellin levels are severely reduced, such as *ga1-3*, are unable to flower in short days (Wilson et al. 1992), and the exogenous application of gibberellins, in these conditions, accelerates flowering in wild-type Arabidopsis (Langridge 1957). Furthermore, gibberellins activate molecular targets such as the floral meristem identity gene *LFY* (Blázquez et al. 1998), and the levels of GA_4 increase dramatically in the shoot apex shortly before floral initiation (Eriksson et al. 2006), indicating that in Arabidopsis GA_4 is the active gibberellin in the regulation of both shoot elongation and flower initiation during short-day conditions. Tobacco (*Nicotiana tabacum*) cv Xhanti is a day-neutral plant, and flowering time is not substantially influenced by photoperiod, although the number of nodes produced (i.e. the amount of vegetative growth before floral initiation) can be affected by temperature, light intensity, photoperiod, nutrition and other factors. *Nicotiana tabacum* plants, when growing under a specific set of environmental conditions, produce

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a uniform number of nodes before the meristem ends growth by forming a terminal flower (McDaniel 1996). Many genes homologous to those known in Arabidopsis regulating floral transition and flower development have been cloned and investigated in tobacco, and shown to have characteristics similar to or distinct from those in Arabidopsis. For instance, in tobacco, two genes homologous to the Arabidopsis *LFY* have been identified (*NFL1* and *NFL2*; Kelly et al. 1995). These genes show divergences in their functional roles with Arabidopsis *LFY* since the ectopic expression of *35S:NFL1* is less effective in promoting precocious flowering than *35S:AtLFY* in both tobacco and Arabidopsis (Ahearn et al. 2001).

Several findings have suggested that an increase in gibberellin biosynthesis contributes to the promotion of flowering by long photoperiods in different species. On one hand, gibberellins accumulate when plants are induced to flower by transferring them to inductive conditions (Arabidopsis whole plants, Xu et al. 1997; Arabidopsis petioles, Gocal et al. 2001; *Spinacea oleracea*, Zeevaart et al. 1993; *Lolium temulentum*, King and Evans 2003), and, on the other, exogenous application of gibberellins to certain species can be as efficient as single long-day treatments to promote flowering (Pharis et al. 1987, Gocal et al. 2001). These results cannot be extrapolated to all plant species. For instance, gibberellins do not seem to be a major flowering signal in *Sinapis alba* (Corbesier et al. 2004). Furthermore, in *Fuchsia* (King et al. 2000) and grapevine (Boss and Thomas 2002), gibberellins actually inhibit flowering. Therefore, although the role of gibberellins in the regulation of stem elongation is believed to be true in most species, this is not the case for flowering.

In higher plants, active gibberellins are synthesized from the common intermediate GA_{12} by two parallel pathways that differ in the presence or absence of an early step of C-13 hydroxylation. Both pathways progress by successive oxidations at C-20, catalyzed by gibberellin 20-oxidases (GA20oxs), to produce GA_{20} or GA_9. These intermediates are converted into the active GA_1 and GA_4, respectively, by gibberellin 3-oxidases (GA3oxs). The content of bioactive gibberellins (GA_1 and GA_4) and their precursors (GA_{20} and GA_9) can be regulated by gibberellin 2-oxidases (GA2oxs) that, by catalyzing their 2β-hydroxylation, generate inactive forms (Hedden and Phillips 2000; Fig. 1). Both active GA_1 and GA_4 are present in tobacco, but their relative abundance, and that of some of their precursors, indicates that the early 13 hydroxylation pathway is the prevalent one in this species (Jordan et al. 1995, Vidal et al. 2001).

Manipulation of the gibberellin pathway in tobacco results in conspicuous differences in plant stature and gibberellin levels. Thus, transgenic plants overexpressing a GA20ox gene (*35S:CcGA20ox1*), coding for a limiting gibberellin biosynthetic enzyme, have a higher content of active gibberellins and elongated phenotype but essentially unaltered flowering (Vidal et al. 2001; Fig. 1). In contrast, transgenic plants overexpressing a GA2ox gene (*35S:NoGA2ox3*), which reduces the level of active gibberellins and induces dwarfism, flowered later than the wild type (Ubeda-Tomás et al. 2006; Fig. 1). This phenotype agrees with results obtained by constitutive overexpression of other GA2oxs in species such as rice (*OsGA2ox1*; Sakamoto et al. 2001b), Arabidopsis (*AtGA2ox7* and *AtGA2ox8*; Schömburg et al. 2003) and *Nicotiana sylvestris* (*SoGA2ox3*; Lee and Zeevaart 2005). Delay of flowering in transgenic *35S:NoGA2ox3* tobacco plants was proportional to the intensity of the dwarf phenotype and therefore to the gibberellin deficiency (Ubeda-Tomás et al. 2006). Extremely dwarf tobacco plants rarely flower and, when they do, the development of flower buds is very frequently aborted and they do not produce seeds. Flowering of these dwarf plants is induced by exogenous GA_3 application. In contrast, the phenotype of semi-dwarf plants was similar to that of control plants during early vegetative growth until approximately the eighth internode, and subsequently the developing stem of these plants showed a gibberellin-deficient phenotype with shorter internodes (Ubeda-Tomás et al. 2006). Flower development is slightly affected in these plants, but seeds are viable. When treated exogenously with GA_3, the semi-dwarf phenotype (height and flowering time) reverted to wild type (Ubeda-Tomás et al. 2006), indicating that gibberellin levels might be important for floral initiation.

To investigate the role of gibberellins in the control of flowering in tobacco, we have quantified the levels of active gibberellins and the expression of gibberellin metabolism genes in apical shoots and young apical leaves during development. We found that the content of GA_1 and GA_4 in apical shoots and the expression pattern of gibberellin metabolism genes did not correlate with flower promotion in tobacco but rather with the ontogenic variation of stem growth.

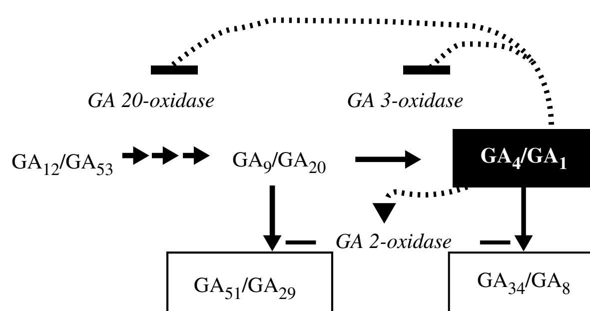

Fig. 1 Last steps of gibberellin metabolism showing feedback (dotted line with bar) and feedforward (dotted lines with arrow) regulation. Active gibberellins are in filled squares and inactive metabolic products in open squares.

Results

Effect of gibberellin content modification on tobacco flowering

Developmental stages and flowering molecular markers. As a first step to study the relationship between gibberellins and flowering, we identified the transition from vegetative to reproductive development under our experimental conditions and tested for suitable molecular markers for these phases. Freshly cut apices of plants at different ages were analyzed under a microscope. The stage of phase transition was established by counting the number of leaves ($\geq$3 cm) when floral initiation (change in the shape of the meristem and in the type of primordia) was apparent. In our conditions, floral transition took place in the apex of plants bearing 20–25 leaves longer than 3 cm (stages 2 and 3) although the flowering node (determined after the plant has completed growth) was about no. 38 (Table 1). The evolution of leaf and internode sizes during development is probably due to growth rate changes in the apex. Fig. 2A displays the lengths of leaves and internodes of 83-day-old wild-type plants. The sizes of both organs increased in parallel during the initial vegetative stages, reaching maximum sizes around node no. 10–12. Then, the internode lengths decreased progressively before floral transition (to about half of the largest length), but the sizes of leaves remained constant (Fig. 2A).

Two MADS-box genes involved in floral transition, *NtMADS-4* and *NtMADS-11*, have been described previously in tobacco (Jang et al. 2002). Since these genes were shown to be expressed during reproductive development, by RNA blot analyses (Jang et al. 2002), we tested their expression by semi-quantitative reverse transcription–PCR (RT–PCR) to determine if we could use them as markers of floral transition in our experiments. We found that both genes were detected only in apical shoots of plants at stage 2 of development (Fig. 2B) and subsequently. In young apical leaves, the expression of *NtMADS-11* was delayed even further, transcripts being detected in plants at stage 3 (Fig. 2B). These results confirm the relationship between the expression of these genes and floral transition in tobacco and their convenience as developmental markers.

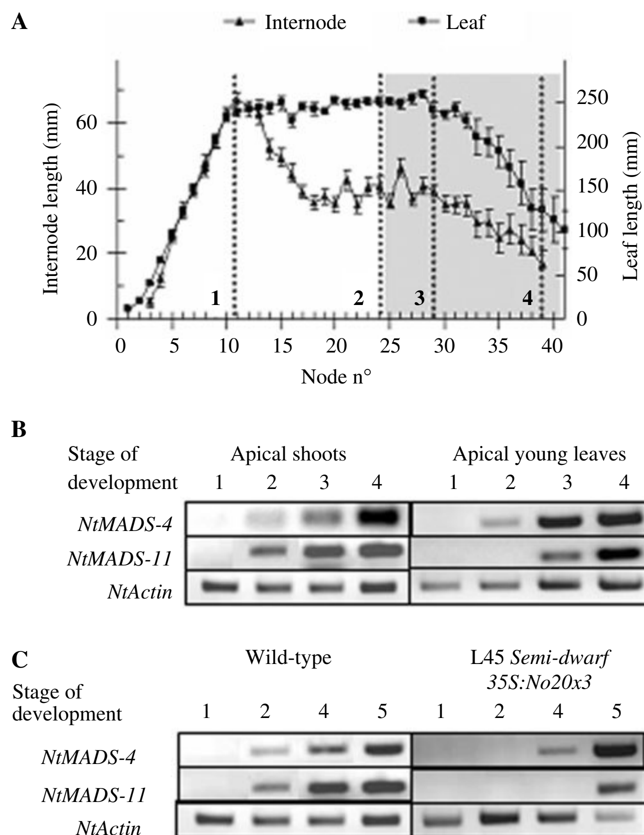

Fig. 2 Developmental stages and expression of flowering molecular markers. (A) Length of internodes and leaves of wild-type *Nicotiana tabacum* at day 83. The values are the average of 8–10 plants ±SE. Dotted lines indicate the position at which the apical shoots were cut when taking the samples (node of first leaf $\leq$0.5 cm) at the different developmental stages for gibberellin quantification and RT–PCR analysis. Leaves and internodes in the gray area were developed after floral transition. (B) Expression of *NtMADS-4* and *NtMADS-11* by semi-quantitative RT–PCR, in apical shoots and young apical leaves from wild-type plants at different stages of development (1 = 8 leaves, 2 = 20 leaves, 3 = 25 leaves and 4 = 35 leaves $\geq$3 cm). *NtActin* was amplified as an internal control. (C) Expression of *NtMADS-4* and *NtMADS-11* by semi-quantitative RT–PCR, in apical shoots from wild-type and L45 semi-dwarf transgenic plants at different stages of development (1 = 8 leaves, 2 = 20 leaves, 4 = 35 leaves and 5 = 45 leaves $\geq$3 cm). *NtActin* was amplified as an internal control

Table 1 Phenotype of wild-type tobacco plants treated with GA_3 and paclobutrazol (PCB)

	Untreated	GA_3	PCB	PCB + GA_3
Stem length (cm)	137.4 ± 3.8	240.6 ± 5.9	20 ± 1.2	158 ± 4.3
Flowering time (days to anthesis)	94.5 ± 3.4	100.7 ± 4.5	>200	145 ± 6.2
Flowering time (no. of nodes)	38.4 ± 0.4	46.8 ± 1.0	>61	45 ± 2.5

Treatments were performed by irrigation of 15-day-old plants during 2 months once per week with nutrient solution containing GA_3 (10^{-5} M), PCB (10^{-5} M) and PCB (10^{-5} M) + GA_3 (10^{-5} M). Results are the average of 9–10 plants per treatment ±SE.

Flowering in semi-dwarf transgenic plants overexpressing a GA2ox. Semi-dwarf transgenic plants (*35S:NoGA2ox3*, line L45) overexpressing a GA2ox, which reduces the active gibberellin content, have about 37% more vegetative nodes than wild-type plants and therefore show flowering delay (Ubeda-Tomás et al. 2006). We compared the expression of the molecular markers *NtMADS-4* and *NtMADS-11* in apical shoots of L45 and wild-type plants during development (Fig. 2C) to see if molecular events associated with floral transition were also altered in transgenic plants. We only detected *NtMADS-4* transcripts in semi-dwarf transgenic plants at or after stage 4 (35 leaves $\geq$3 cm), when flower development was completed in the wild type but not in L45. Furthermore, *NtMADS-11* could be detected only at stage 5 (45 leaves $\geq$3 cm). Therefore, the late flowering phenotype of L45 plants correlated with late expression of the genetic markers *NtMADS-4* and *NtMADS-11*.

Flowering in tobacco plants treated with GA_3 or paclobutrazol. Treatment of wild-type tobacco plants with the gibberellin biosynthesis inhibitor paclobutrazol (PCB) reduced stem height and inhibited flowering (Table 1). None of the nine plants treated with 10^{-5} M PCB flowered after producing 60 vegetative leaves in 200 d of growth. Untreated plants flowered at about node 38 after 95 d of culture. However, simultaneous application of 10^{-5} M GA_3 and PCB reduced the inhibition of flowering, indicating that flowering delay was due to the lack of gibberellins (Table 1). On the other hand, the application of GA_3 alone (Table 1), which stimulated stem elongation (plants treated with GA_3 were 75% taller), produced a slight delay on the flowering node (flowering node on GA_3-treated plants was 46.8 ± 1.0 vs. 38.4 ± 0.4 on the wild type).

The inhibitory effect of PCB on flowering was associated with a delay in the expression of markers *NtMADS-4* and *NtMADS-11*, detected by the absence of transcripts in young apical leaves of these plants (Fig. 3). The application of GA_3 partially reversed the effect of PCB on *NtMADS-4* but not on *NtMADS-11*. Interestingly, application of GA_3 alone reduced the expression of both flowering markers (Fig. 3), in agreement with the slight delay produced on the flowering node.

Evolution of gibberellin content in apical shoots and young apical leaves of tobacco during development

In order to study the relationship between gibberellins and flowering, we quantified the active gibberellins, GA_1 and GA_4, and the immediate precursor of GA_1, GA_{20}, in wild-type and transgenic *35S:NoGA2ox3* plants during development. The developmental stage at sampling is expressed by the number of leaves $\geq$3 cm. The content of GA_1 and GA_4 (Fig. 4A, B) was very high in apical shoots at stage 1 (plants with eight leaves), decreased considerably at floral transition (stages 2 and 3) and remained low afterwards. GA_4 was less abundant than GA_1 but its pattern of variation was similar. The content of GA_{20} in apical shoots was low compared with active gibberellins during vegetative development, decreased slightly during flower transition and increased afterwards (Fig. 4C). Active gibberellins were less abundant in young apical leaves than in apical shoots, and their levels evolved differently during development (Fig. 4D, F). The content of GA_1, the predominant active gibberellin, remained constant and did not show the strong decline that took place in the apices during flowering induction (Fig. 4D), whereas GA_4 levels decreased during the reproductive stages (Fig. 4E). In contrast to apical shoots, GA_{20} in leaves was the most abundant gibberellin and its level presented a maximum in early flower transition (Fig. 4F).

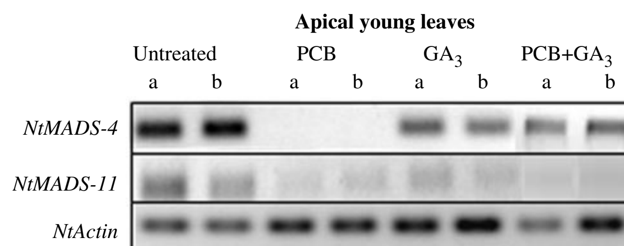

Fig. 3 Effect of GA_3 and paclobutrazol on the expression of flowering molecular markers *NtMADS-4* and *NtMADS-11* by semi-quantitative RT–PCR in tobacco wild-type plants. Plants were untreated or treated with GA_3 (10^{-5} M), PCB (10^{-5}) or both, by irrigation until the plants had 20 leaves ($\geq$3 cm). Samples were young apical leaves of three plants, and two samples (a and b) were analyzed per treatment. *NtActin* was amplified as an internal control.

We also quantified gibberellin levels in the semi-dwarf transgenic line L45, which has a late flowering phenotype as described before (Ubeda-Tomás et al. 2006, and Fig. 2C). In apical shoots, levels of GA_1, GA_4 and GA_{20} were lower in L45 than in wild-type plants, as expected (Fig. 4A–C). The same occurred for the gibberellin content in young apical leaves (Fig. 4D–F). In this case, however, the differences in content were greater for GA_1 and GA_{20}. At the vegetative stage, the contents of GA_1 and GA_4 were similar between wild-type and transgenic plants. After this stage, the contents of active gibberellins in transgenic plants decreased more than in the wild type, but followed a similar pattern before and after flowering (Fig. 4). The gibberellin-deficient phenotype in transgenic plants (*35S:NoGA2ox3*) was displayed only at late vegetative phases and it was never detectable before the stage of eight leaves (Ubeda-Tomás et al. 2006, this work). Therefore, the differences in contents of gibberellins (mostly GA_1 and GA_{20}) between transgenic and wild-type plants correlated with the late dwarf phenotype.

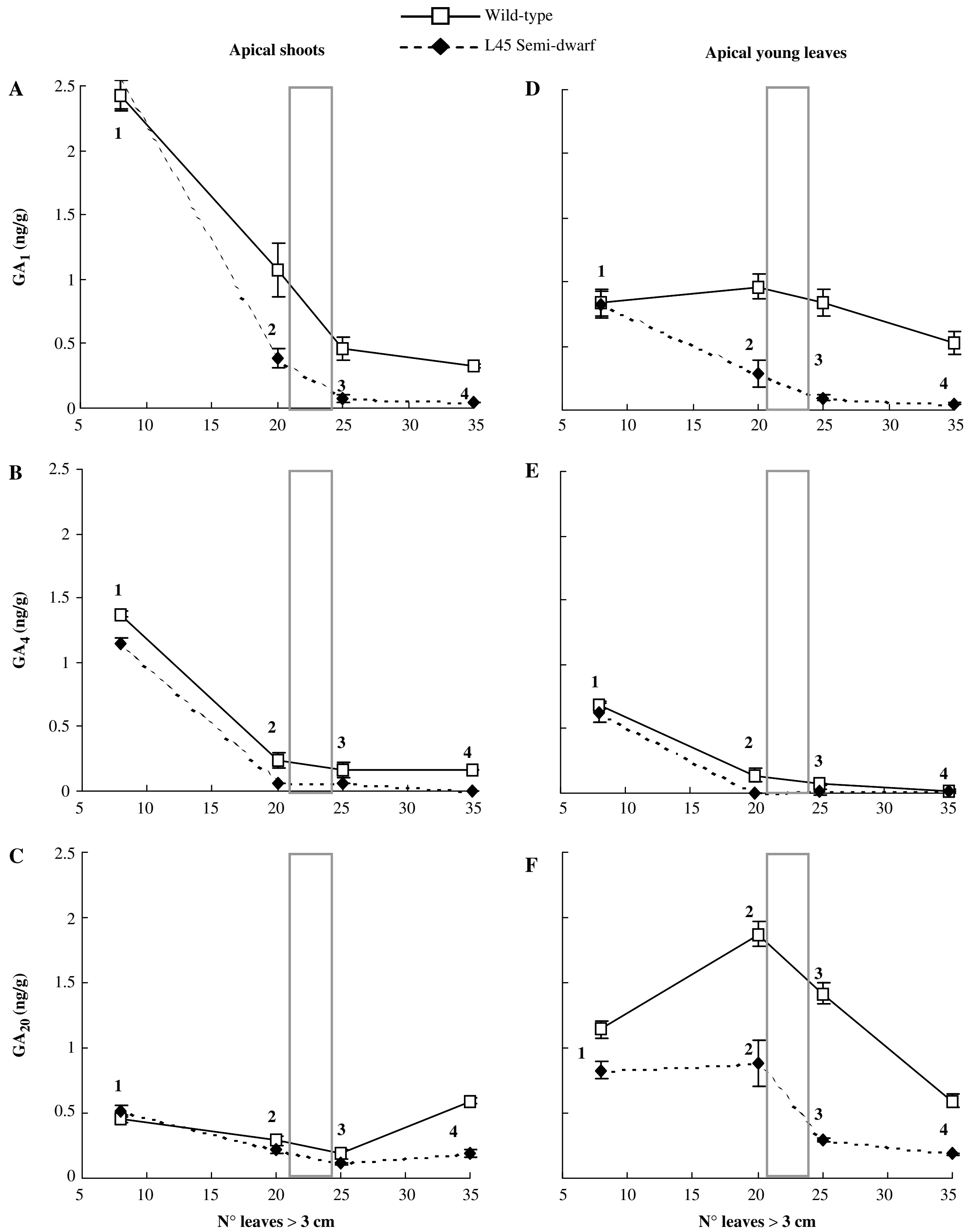

Fig. 4 GA_1, GA_4 and GA_{20} (ng g^{-1}) content in apical shoots (A, B and C) and young apical leaves (D, E and F) from wild-type and L45 semi-dwarf transgenic tobacco plants at different stages of development, classified by the number of leaves ≥3 cm (1–4). The dotted area indicates floral transition in the wild type. Values are averages of five biological replicates ±SE.

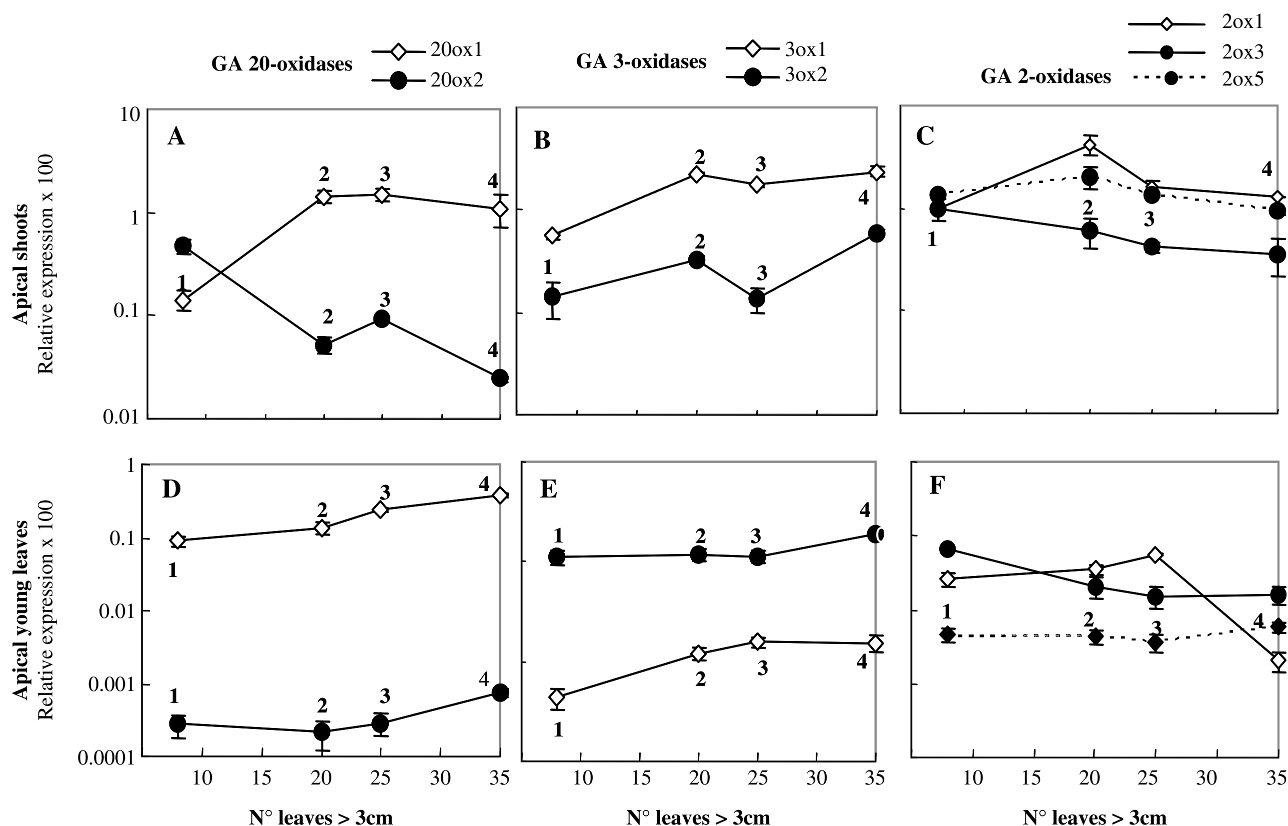

Fig. 5 Expression of genes involved in the metabolism of gibberellins in apical shoots (A, B and C) and apical young leaves (D, E and F) from wild-type tobacco during development (stages 1–4). Real time RT–PCR analysis of GA20oxs (*Nt20ox1* and *Nt20ox2*), GA3oxs (*NtGA3ox1* and *NtGA3ox2*) and GA2oxs (*NtGA2ox1*, *NtGA2ox3* and *NtGA2ox5*). The mRNA levels are relative to *NtActin* mRNA. Results are the average of two independent samples ±SE. Relative expression of mRNA was multiplied by 100. Note that the scale is logarithmic.

Expression pattern of gibberellin metabolism genes during development.

The GenBank database contained sequences encoding two GA20oxs (*NtGA20ox1*, *Ntc12*; and *NtGA20ox2*, *Ntc16*), one GA3ox (*NtGA3ox1*, *Nty*) and two GA2oxs (*NtGA2ox1* and *NtGA2ox2*, 97% identical) of *N. tabacum* at the time of starting this work. We completed the 3′ untranslated region of the *NtGA3ox1* (*Nty*) and isolated a new cDNA *GA3ox* clone (*NtGA3ox2*, 98% identical to the *N. sylvestris* gene *NsGA3ox1*) and two new cDNA clones for GA2oxs (*NtGA2ox3* and *NtGA2ox5*). The functions of all these genes have been inferred from their high homology to previously identified gibberellin clones, except for the product of *Nty* whose enzymatic activity had been confirmed in vitro (Itoh et al. 1999).

Relative levels of mRNAs from genes encoding GA20oxs (*NtGA20ox1* and *NtGA20ox2*), GA3oxs (*NtGA3ox1* and *NtGA3ox2*) and GA2oxs (*NtGA2ox1*, *NtGA2ox3* and *NtGA2ox5*) were quantified by real time RT–PCR, to see whether the changes in gibberellin content correlated with the expression of gibberellin metabolism genes. Some of these genes showed significant changes of expression in apical shoots during transition from vegetative to reproductive development (Fig. 5A–C). For instance, *NtGA20ox1* and *NtGA3ox1* transcript levels increased 10- and 4.5-fold, respectively, between stages 1 and 2, while *NtGA20ox2* showed the opposite pattern, with a reduction of expression of about 9.5-fold. *NtGA2ox1* transcripts increased four times between stages 1 and 2. The other *GA3ox* (*NtGA3ox2*) and *GA2ox* (*NtGA2ox3* and *NtGA2ox5*) genes showed minor variations of expression during plant development.

In young apical leaves, the expression of gibberellin genes was much lower than in apical shoots and remained rather constant during plant development (Fig. 5D–F).

Discussion

Gibberellin content and flowering induction in tobacco

The treatment of wild-type plants with the gibberellin biosynthesis inhibitor PCB produced dwarfism and delay in

flowering (Table 1). This PCB effect mimics the phenotype of semi-dwarf *35S:NoGA2ox3* transgenic plants (Ubeda-Tomás et al. 2006). The flowering delay in these plants is associated with the down-regulation of flowering molecular markers. (Figs. 2C, 3). In tobacco wild-type plants, floral transition seems to correlate with a rise in the expression of the molecular markers *NtMADS-4* and *NtMADS-11* (Jang et al. 2002; Fig. 2B) but this rise is not detected in semi-dwarf *35S:NoGA2ox3* and PCB-treated plants, in contrast to the wild type (Figs. 2C, 3). The application of GA_3, via irrigation, rescued the late flowering phenotype in PCB-treated and semi-dwarf transgenic plants (Table 1; Ubeda-Tomás et al. 2006) and induced the expression of the flowering molecular markers *NtMADS-4* and *NtMADS-11* (Fig. 3). This suggests that flowering delay in these plants was due to the gibberellin deficiency. The flowering delay of PCB-treated and transgenic plants did not stop meristem activity (because the apex continued producing vegetative leaves), but just induced a much longer vegetative phase before floral transition took place.

On the other hand, in the tall transgenic *35S:CcGA20ox1* plants with a higher gibberellin content, flowering time is not modified (Vidal et al. 2001). This suggests that an increase in the active gibberellin content cannot promote early flowering in tobacco. Interestingly, GA_3 application to wild-type plants which stimulated stem elongation had a negative effect on the expression of *NtMADS-4* and *NtMADS-11* (Fig. 3) and a slight delay in the flowering node (Table 1). The inhibition of floral transition caused by GA_3 application was probably due to an excess in the content of active gibberellins. This excess would be achieved by GA_3 application and not by ectopic expression of *35S:CcGA20ox1*, since GA_3 is very poorly metabolized, because it is resistant to GA2ox inactivation compared with active GA_1 and GA_4, and therefore remained in the plant for longer (Nakayama et al. 1990). The occurrence of a gibberellin inhibitory effect on flowering has been reported previously in other plant species. For instance, the application of GA_3 had an inhibitory effect on *Fuchsia hybrida* and *Pharbitis nil* (King et al. 2000), resembling the case of tobacco. Also in grapevine (*Vitis vinifera*) gibberellin may have an inhibitory effect on flowering because a dwarf gibberellin-insensitive mutant flowers earlier than the wild type (Boss and Thomas 2002). This is in contrast to the gibberellin-insensitive *gai* mutant of Arabidopsis, which is delayed in flowering (Wilson et al. 1992). Therefore, it is possible that in tobacco flowering is only allowed, or favored, when gibberellin levels are between a minimum (which is not reached in dwarf transgenic and PCB-treated plants) and a maximum (which was overcome with continuous application of GA_3 at 10^{-5} M). Interestingly, the flowering delay due to gibberellin excess seems to be smaller than that due to gibberellin shortage (Table 1).

To investigate further the role of gibberellins in flowering, we determined the gibberellin content in apical shoots and young apical leaves of tobacco during development. The levels of active GA_1 and GA_4 in apical shoots decreased during the vegetative phase, reaching very low levels at floral transition and afterwards (Fig. 4A, B). This would not be expected if any of these two gibberellins were flowering-promoting factors. Our data on the progressive reduction in the content of active GA_1 and GA_4 in the apices of tobacco plants correlated with the reduction of internode lengths before floral transition (Fig. 2A). Interestingly, in contrast to internodes, the size and active gibberellin content in leaves remained constant before and after floral transition (Figs. 2A, 4D, E). The decline of GA_1 and GA_4 in apical shoots before floral transition is probably related to the decrease in stem growth and does not seem to have a role in inducing the reproductive phase, since semi-dwarf late flowering plants (L45) displayed a similar decline. However, we cannot exclude that the levels of GA_1 and GA_4 in tissues other than apices and apical leaves (for instance old leaves or roots) may have relevance in floral transition signaling. In summary, we can conclude that floral transition in tobacco was associated with a significant reduction in stem growth rate and a significant reduction in active gibberellin in apical shoots but not in leaves.

Although GA_1 and GA_4 have been established in many species as the active gibberellins for stem elongation, their roles in floral induction have not been demonstrated so clearly. In Arabidopsis, GA_4 is the active gibberellin, regulating stem elongation as well as floral initiation, under short-day conditions (Eriksson et al. 2006). However, in the long-day monocot *Lolium temulentum*, the florigenic gibberellins are GA_5 and GA_6, which have little effect on stem elongation. In contrast, GA_1 and GA_4 cause stem elongation and seem to be inactive in floral induction (King and Evans 2003). Our results in tobacco do not show any peak of GA_1 and GA_4 before floral induction, in contrast to Arabidopsis, which has been proposed as a model for dicot flowering (Eriksson et al. 2006). Therefore, we cannot exclude the possibility that specific gibberellins, other than GA_1 and GA_4, are responsible for flowering induction in tobacco.

The role of active gibberellins in the control of flowering is not universally accepted. The evidence for the involvement of gibberellin biosynthesis in the regulation of floral induction comes mainly from photoperiod-responsive rosette plants, where stem elongation (bolting) coincides with flowering. In other cases such as pea (*Pisum sativum*), mutations causing gibberellin deficiency have minimal effect on the node of flower initiation (Murfet and Reid 1987).

Tobacco, like pea, is not a rosette plant and flowering is not associated with stem elongation. In fact, the internode length of tobacco plants diminishes when approaching floral transition (Fig. 2A), and a similar ontogenic variation in internode length is found in pea, correlating with a GA_1 decline in young apical tissues (Ross et al. 1992). Therefore, floral transition in tobacco, as well as in pea, might not be induced by an increase of active gibberellins in the apical shoot. However, gibberellins are necessary for flowering in tobacco because dwarf transgenic and PCB-treated plants show an extended vegetative phase and late flowering. This requirement for gibberellins may imply only a minimum level, or gibberellin threshold, that would be important for the meristem to reach a stage of competence for flowering. In fact, there is evidence supporting a role for gibberellin in cell differentiation. Sakamoto et al. (2001a) found that the expression of a GA20ox gene (*Ntc12*) of tobacco is excluded from the apical meristem by direct repression of a KNOX homeodomain protein, suggesting a role for gibberellins in determination of cell fate in tobacco. In rice, the ring-shaped expression around the shoot apex of the GA2ox (*OsGA2ox1*) is drastically reduced after the phase transition from vegetative to reproductive growth (Sakamoto et al. 2001b), which may indicate a role for gibberellins in cell differentiation. Therefore, the presence of a certain amount of gibberellin within some meristematic cells may be necessary for acquiring competence for flowering. In dwarf transgenic and PCB-treated plants, this amount of gibberellin would not be reached, competence for flowering would not be achieved, and plants would continue through vegetative growth. On the other hand, the application of high doses of GA_3 (10^{-5} M) may alter the normal pattern of gibberellin distribution within the meristem and this may also interfere with flowering (Table 1).

However, once the meristem becomes competent for flowering, floral transition will take place depending on signals different from the levels of GA_1 or GA_4. In fact, an increase in gibberellin levels, within certain limits, should not accelerate flowering because gibberellins only affect the meristem competence and not the floral transition. This may explain why flowering time is not affected in tall transgenic plants (*35S:CcGA20ox1*) with high gibberellin content (Vidal et al. 2001) and why the application of moderate doses of GA_3 (10^{-6} M) does not have any effect on flowering (unpublished results).

Expression of gibberellin metabolism genes in apical shoots and leaves

We found major changes of expression in some of the gibberellin metabolism genes, in the apical shoots of tobacco, between vegetative and reproductive growth (Fig. 5A–C). Many gibberellin genes, coding for the dioxygenases GA20oxs, GA3oxs and GA2oxs, can be positively or negatively regulated by the levels of active gibberellins (Fig. 1; Hedden and Phillips 2000). The changes of expression found between stages 1 and 2, in *NtGA20ox1* (*NTc12*) and *NtGA3ox1* (*Nty*) (Fig. 5A, B), could be explained by negative feedback regulation, which has been previously described for these genes (Tanaka-Ueguchi et al. 1998, Itoh et al. 1999). In contrast, *NtGA20ox2* (*NTc16*), which shows little feedback regulation (Tanaka-Ueguchi et al. 1998), had a continuous reduction of expression in the apical shoot, matching the decline of active gibberellins. The expression pattern of the GA2ox gene *NtGA2ox1* (which increased about four times between stages 1 and 2) also fits the evolution of active gibberellins. These results may suggest that both enzymes (encoded by *NtGA20ox2* and *NtGA2ox1*) could contribute to the reduction of active gibberellin, before floral transition, and are probably regulated by ontogenic signals. The transcripts levels for the other three genes had relatively milder variations.

The patterns of expression of gibberellin biosynthesis genes in leaves diverged considerably from those in apical shoots (Fig. 5). In general they showed very small changes in expression, in agreement with the low variation in the content of active gibberellin, mainly GA_1. Most of the genes were expressed at lower levels in leaves than in apical shoots (Fig. 5). However it is interesting to note that the active gibberellin content in leaves was not much lower than in apical shoots (see Fig. 4, stage 3). This result could be explained by a reduction in the expression of gibberellin biosynthesis genes compensated by a reduction in the expression of gibberellin catabolic genes in leaves. This may indicate a poorer requirement for gibberellin content regulation in the leaves (with low variation in their levels) than in the apical shoots. In contrast to Arabidopsis (Eriksson et al. 2006), there is a reasonable correlation between active gibberellin content and the expression pattern of gibberellin genes in both tissues, apical shoots and apical leaves, which indicates that the active gibberellins are locally synthesized and not imported from other tissues.

Interestingly, GA_{20}, the precursor of active GA_1 (Fig. 1), was the most abundant gibberellin in leaves and showed a maximum during flower transition. This variation in the content did not correlate with the expression pattern of any of the gibberellin metabolism genes investigated. Because of the great number of members in the gibberellin dioxygenase families in Arabidopsis, we cannot exclude the existence of as yet unidentified genes in tobacco that could be responsible for that pattern. The rather different pattern of GA_{20} and its product GA_1 suggests that leaves could be a source of gibberellin precursors that can be exported to other organs.

In summary, the results presented in this work show that floral transition in tobacco depends on gibberellins but, in contrast to Arabidopsis, is not regulated by the levels of GA_1 and GA_4 in apical shoots. Also, in contrast to Arabidopsis (Eriksson et al. 2006), the active gibberellin content in apical shoots correlated roughly with the expression of gibberellin metabolism genes, suggesting that active gibberellins are not imported from other organs but are locally synthesized.

Materials and Methods

Plant material and growth conditions

Nicotiana tabacum cv Xanthi (photoperiod independent) and transgenic tobacco (L45 overexpressing *35S:NoGA2ox3*) plants were used as experimental material. The plants were cultured in pots containing vermiculite:peat (1 : 1), watered with nutrient solution and grown under long-day conditions (16 h light and 8 h of darkness at 25°C).

The stage of plant development was established according to the total number of plant leaves ≥3 cm in length. We established five stages of development named: stage 1, plants with eight leaves ≥3 cm; stage 2, plants with 20 leaves ≥3 cm; stage 3, plants with 25 leaves ≥3 cm; stage 4, plants with 35 leaves ≥3 cm; and stage 5, plants with 45 leaves ≥3 cm. Floral transition in the wild type takes place between stages 2 and 3.

The time of floral transition was determined by studying the morphological changes of the meristem from shoot apices of plants at different developmental stages, using freshly cut apices observed under a stereomicroscope (model SMZ88, Nikon). Flowering time was established by the number of days to the first anthesis and by the number of nodes on the primary shoot formed below the terminal flower.

Apical shoot samples consisted of the entire apical portion of the shoot containing leaves ≤0.5 cm long. Young leaf samples consisted of apical leaves 0.5–3 cm in length. Samples were frozen immediately in liquid N_2 before storage at −80°C, until analysis.

Gibberellin quantification

Gibberellins were quantified in apical shoots and apical young leaves of five biological replicates from plants at different stage of development. Each sample consisted of material from five plants. Aliquots of 10 mg of lyophilized material were homogenized using a mortar and pestle and extracted using an MM301 Vibration Mill for 3 min (Retsch GmbH and Co., Haan, Germany) in 1 ml of 80% MeOH + 0.02% diethyl dithiocarbamate as antioxidant containing gibberellin internal standards ([$^2H_2$]GA_1, [$^2H_2$]GA_8, [$^2H_2$]GA_{19}, [$^2H_2$]GA_{20}, [$^2H_2$]GA_{44}, [$^2H_2$]GA_{53}, [$^2H_2$]GA_{19}, [$^2H_2$]GA_4, [$^2H_2$]GA_9, [$^2H_2$] GA_{24}, [$^2H_2$]GA_{34}) (purchased from Profesor Lewis Mander, Australia). After centrifugation (13,000 r.p.m. for 3 min), the supernatant was evaporated to dryness under vacuum. The residue was dissolved in 500 µl of 1% acetic acid at pH 2.5–3. This solution was loaded onto a pre-equilibrated Isolute C8-EC cartridge (International Sorbent Technology, UK). The cartridges were washed with 2 ml of 5% MeOH–1% acetic acid, eluted with 2 ml of 80% MeOH and evaporated to dryness. The residue was dissolved in 200 µl of 2-propanol and methylated with trimethylsilyl-diazomethane in hexane (Aldrich). The gibberellins were separated by HPLC and the fractions were dried, trimethylsilylated in 20 µl of pyridine and BSFA + 1% TMCS, and analyzed by GC–MS in selected reaction monitoring mode using a JEOL JMS MStation (JEOL, Tokyo, Japan). as described previously by Moritz and Olsen (1995).

RNA isolation and reverse transcription

Total RNA was isolated from apical shoots and young apical leaves using an RNeasy Plant Mini Kit and treated with RNase-free DNase set (Qiagen), according to the manufacturer's instructions. RNA concentration was measured using a Nanodrop ND-1000 Spectrophotometer (Wilmington, DE, USA). A 1.5 µg aliquot of total RNA was used for cDNA synthesis using a Taq Man® Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) with oligo(dT) primers, at final volume of 20 µl.

PCR primer design and real-time RT–PCR analysis

Primers were designed using Primer Express™ v2.0 software (Applied Biosystems). The primer concentration for real-time PCR was optimized for each primer gene pair, and the best primer concentration was determined by calculating PCR efficiency and C_T values (SYBR® Green PCR Master Mix and RT-PCR Protocol Applied Biosystems).

Gene transcription of *Nt20ox1* (*Ntc12*, AB012856.1), *Nt20ox2* (*NTc16*, AB016084), *Nt3ox1* (*Nty*, AB032198), *NtGA2ox1* (AB125232.1), *NtGA3ox2*, *NtGA2ox3* and *NtGA2ox5* (cloned in our laboratory, EF471116, EF471117 and EF471118) was analyzed by quantitative real-time PCR. *NtActin* (U60489) was amplified as a loading control. The primer pairs displayed in Supplementary Table S1 were used for PCR amplification.

The analyses were carried out using two biological replicates of apical shoots and apical young leaves at different stages of development. Each sample consisted of material from five plants. All PCRs were performed in triplicate and a template-free control was included for gene amplification. The entire experiment was carried out twice at different times of the year, with similar results. Only results from one of the experiment are shown in this work.

PCRs were performed in an optical 96-well plate with an ABI PRISM® 7000 Sequence detection System (Applied Biosystems), using SYBR® Green to monitor double-stranded DNA synthesis. The reactions contained 12.5 µl of SYBR® Green Master Mix reagent (Applied Biosystems), the optimal concentration of primers for each gene, RNase-free water and 1 µl of cDNA solution in a final volume of 25 µl. The thermal profile conditions for all PCRs were: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 1 min. A melting curve program was used to confirm the presence of only one amplicon according to the T_m expected for each gene amplification. Data were analyzed by the SDS 2.0 software (Applied Biosystems). To compare data from different PCR runs, C_T values for all genes were normalized to the C_T value of *NtActin*. PCR efficiency (E) was estimated with the LinRegPCR: Analysis of Real-Time PCR Data v7.5 program (Ramakers et al. 2003) for each gene. Relative expression of mRNA was calculated by the ratio = ($E^{Ct\ control}/E^{Ct\ target}$), as described by Pfaffl (2001).

Semi-quantitative RT–PCR

Transcription of *NtMADS-4* and *NtMADS-11* genes (Jang et al. 2002) was analyzed by semi-quantitative RT–PCR. The primer pairs used for PCR amplification were: *NtMADS-4* (forward 5′-CGAGTTCCACTTCGATTGTCAT-3′ and reverse 5′-TGCGGATTAGCCACAGCAA-3′) and *NtMADS-11* (forward 5′-ACAAGCTCTCGAAGCAGGTGAA-3′ and reverse 5′- CAG TGTTCTGTTGTTGTTGCCG-3′). *NtActin* (U60489; primer

pairs was the same as used in real-time PCR) was amplified as a loading control. The appropriate number of PCR cycles (PCR in exponential phase) was determined for each gene by trial runs.

Application of GA_3 and paclobutrazol

Separate and joint applications of GA_3 (Duchefa Biochemie, Haalen, The Netherlands) and PCB (Duchefa Biochemie) were performed by irrigation of plants with 10^{-5} M solutions once per week during 2 months. Treatments (10 plants per treatment) started when the plants were 15 d old.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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SUPPLEMENTAL DATA

Table S1. Primers pairs used in real time RT-PCR.

Gene	Forward	Reverse
NtGA20ox1	5'-TGTAGCACGAGAACTTCC-3'	5'-ACGGCATGCTTCACCAACA-3'
NtGA20ox2	5'-TGAGGTTCCTTCTTCACAGCA-3'	5'- CTCCCCTAAAAGCTCCATTACC-3'
NtGA3ox1	5'-CGGCTTTGTCCCCTCTA-3'	5'-CTTATCGAGTTTAGCCAACTTGCA-3'
NtGA3ox2	5'-TGCGCTCCTCGTATTGGATT-3'	5'AAAGGTAACTACTAGTCAAATTAGCCAACT-3'
NtGA2ox1	5'- CGTTCAATTTCTTTGCCCAC-3'	5'-GCAGTTGTCTTTGGAGAAGTGC-3'
NtGA2ox3	5'-AAGTCCCACCTTGTTACTTG-3'	5'-GCTATCTATAGGAAATCCAATG-3'
NtGA2ox5	5'-ATCCATCGCTATTCCCGGA-3'	5'-GCTATCTTTTCCAGCGCCAA-3'
NtActin	5'-CATTGGCGCTGAGAGATTCC-3'	5'-GCAGCTTCCATTCCGATCA-3'

CAPÍTULO II

La Homeóstasis de Giberelinas en Tabaco es Regulada por Genes de su Metabolismo con Diferente Sensibilidad a Giberelinas

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Gibberellin Homeostasis in Tobacco is Regulated by Gibberellin Metabolism Genes with Different Gibberellin Sensitivity

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Gibberellins are phytohormones that regulate growth and development of plants. Gibberellin homeostasis is maintained by feedback regulation of gibberellin metabolism genes. To understand this regulation, we manipulated the gibberellin pathway in tobacco and studied its effects on the morphological phenotype, gibberellin levels and the expression of endogenous gibberellin metabolism genes. The overexpression of a gibberellin 3-oxidase (biosynthesis gene) in tobacco (3ox-OE) induced slight variations in phenotype and active GA_1 levels, but we also found an increase in GA_8 levels (GA_1 inactivation product) and a conspicuous induction of gibberellin 2-oxidases (catabolism genes; *NtGA2ox3* and *-5*), suggesting an important role for these particular genes in the control of gibberellin homeostasis. The effect of simultaneous overexpression of two biosynthesis genes, a gibberellin 3-oxidase and a gibberellin 20-oxidase (20ox/3ox-OE), on phenotype and gibberellin content suggests that gibberellin 3-oxidases are non-limiting enzymes in tobacco, even in a 20ox-OE background. Moreover, the expression analysis of gibberellin metabolism genes in transgenic plants (3ox-OE, 20ox-OE and hybrid 3ox/20ox-OE), and in response to application of different GA_1 concentrations, showed genes with different gibberellin sensitivity. Gibberellin biosynthesis genes (*NtGA20ox1* and *NtGA3ox1*) are negatively feedback regulated mainly by high gibberellin levels. In contrast, gibberellin catabolism genes which are subject to positive feedback regulation are sensitive to high (*NtGA2ox1*) or to low (*NtGA2ox3* and *-5*) gibberellin concentrations. These two last *GA2ox* genes seem to play a predominant role in gibberellin homeostasis under mild gibberellin variations, but not under large gibberellin changes, where the biosynthesis genes *GA20ox* and *GA3ox* may be more important.

Keywords: Feed-back — Gibberellin — Homeostasis — Over-expression — Regulation.

Abbreviations: GA2ox, gibberellin 2-oxidase; GA3ox, gibberellin 3-oxidase; GA20ox, gibberellin 20-oxidase; 3ox-OE, gibberellin 3-oxidase overexpressor; 20ox-OE, gibberellin 20-oxidase overexpressor; RT–PCR, reverse transcription–PCR.

Introduction

Bioactive gibberellins are important natural regulators of growth and development throughout the life cycle of higher plants, including seed germination, stem elongation and flowering (Hedden 1999). The gibberellin metabolic pathway has been elucidated and the majority of the genes encoding the enzymes have been cloned and characterized (reviewed in Hedden and Phillips 2000a). The early steps in gibberellin biosynthesis are catalyzed by enzymes [i.e. ent-copalyl diphosphate synthase (CPS), ent-kaurene synthase (KS), ent-kaurene oxidase (KO) and ent-kaurenoic acid (KAO)] mostly encoded by single-copy genes producing GA_{12}. In the final stages of gibberellin biosynthesis, GA_{12} or its C-13 hydroxylated form GA_{53} are converted into active GA_1 and GA_4 by two parallel pathways that differ in the presence or absence of the early step of C-13 hydroxylation (Fig. 1). Both pathways progress by successive oxidations at C-20, catalyzed by gibberellin 20-oxidases (GA20oxs), to produce GA_{20} or GA_9. These intermediates are converted into the active GA_1 and GA_4, respectively, by gibberellin 3-oxidases (GA3oxs). Both active GA_1 and GA_4 are present in most species, but their relative abundance differs. In tobacco, the predominance of GA_1, and of some of their precursors, indicates that the early C-13 hydroxylation pathway is the prevalent one (Jordan et al. 1995, Vidal et al. 2001).

The content of bioactive gibberellins (GA_1 and GA_4) and their precursors (GA_{20} and GA_9) can be regulated by gibberellin 2-oxidases (GA2oxs) that generate inactive forms by catalyzing their 2β-hydroxylation (Fig. 1). In addition to GA2ox-inactivating genes, the importance of GA20ox and GA3ox in regulating gibberellin content

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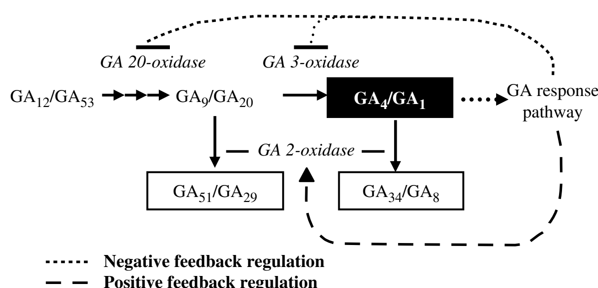


Fig. 1 Gibberellin metabolic pathway showing gibberellin 20-, 3- and 2-oxidation steps. Active gibberellins are indicated by a filled square and inactive gibberellin products by open squares. Positive and negative regulations are indicated by dotted lines.

has also been demonstrated (Hedden and Philips 2000a). These three enzymes are 2-oxoglutarate-dependent dioxygenases encoded by small multigene families with some degree of functional redundancy. Differential and tissue-specific regulation of gibberellin gene family members is frequently found, presumably to maintain appropriate levels of gibberellins in different organs during development (Mitchum et al. 2006). In tobacco, two *GA20ox*, two *GA3ox* and three *GA2ox* genes are known to be differentially and tissue specifically expressed (Itoh et al. 1999, Sakamoto et al. 2001. Gallego-Giraldo et al. 2007). Moreover, environmental stimuli such as light and temperature specifically regulate the expression of some gibberellin genes (Kamiya and García-Martínez 1999, Ait-Ali et al. 1999, Stavang et al. 2007). In addition to developmental and environmental regulation of gibberellin biosynthesis, there are homeostatic mechanisms that control gibberellin levels. Gibberellin homeostasis is maintained by negative feedback regulation of *GA20ox* and *GA3ox* biosynthesis genes (Fig. 1). Transcript levels of some of these gene family members are reduced by elevated gibberellin levels or gibberellin treatment, and increased by gibberellin deficiency conditions (Hedden and Phillips 2000a). Positive induction of GA2oxs by elevated levels of gibberellins may also contribute to gibberellin homeostasis, and has been demonstrated in several species such as *Arabidopsis* (Thomas et al. 1999) and pea (Elliott et al. 2001).

Gibberellin promotes plant growth by relieving the restraint of a family of nuclear growth-repressing DELLA proteins via the 26S proteasome pathway (Harberd 2003). When gibberellin signaling is altered by mutations in DELLA proteins that act as negative regulators, the expression of *GA20ox* and *GA3ox* is altered (Sun and Gubler 2004), indicating that feedback regulation operates through the gibberellin signal transduction pathway. However, whether the feedback regulation of *GA2ox* gene expression takes place through DELLA and the signal transduction pathway has not been reported, to our knowledge.

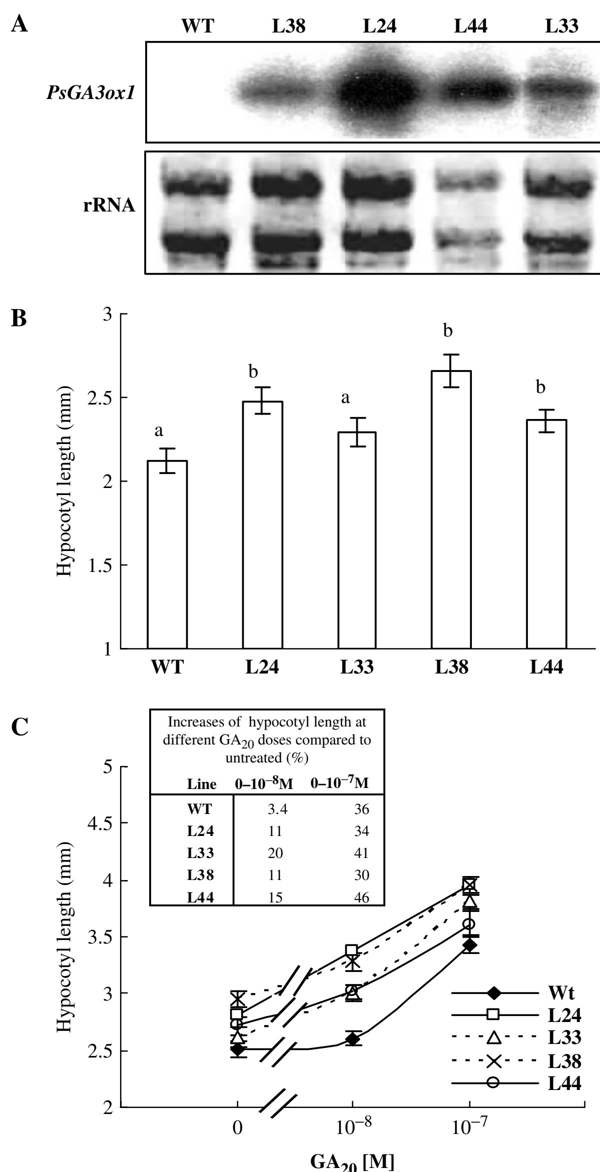
Overexpression of gibberellin metabolism genes in transgenic plants is informative because increased flux through the pathway could identify rate-limiting steps in gibberellin biosynthesis (Hedden and Phillips 2000b). For instance, the overexpression of genes controlling the early steps of gibberellin biosynthesis in *Arabidopsis* (*AtCPS* and *AtKS*) confers no phenotypic alterations or increase in active gibberellins (Fleet et al. 2003), suggesting a critical limiting step later in the pathway. However, overexpression of GA20oxs produces elongated phenotypes associated with gibberellin overproduction in *Arabidopsis* (Huang et al. 1998, Coles et al. 1999), potato (Carrera et al. 2000), hybrid aspen (Eriksson et al. 2000), rice (Oikawa et al. 2004) and tobacco plants (Vidal et al. 2001, Biemelt et al. 2004), indicating the limiting activity of this enzyme in the gibberellin pathway. Overproduction of GA3ox, which catalyzes the last step in gibberellin biosynthesis, has generated conflicting results. In hybrid aspen, overexpression of a GA3ox from *Arabidopsis* resulted in no major changes in morphology and only small changes in GA_1 and GA_4 levels (Israelsson et al. 2004), leading the authors to conclude that 20-oxidation rather than 3-oxidation is the limiting step for gibberellin biosynthesis. In contrast, overexpression of a pumpkin GA3ox in *Arabidopsis* resulted in a gibberellin overdose phenotype with increased levels of endogenous GA_4 (Radi et al. 2006), suggesting that GA3ox catalyzes a rate-limiting step in *Arabidopsis*.

In order to understand the contribution of GA3ox, relative to the GA20ox, we have investigated the effect of overexpressing a *GA3ox* gene alone and in combination with *GA20ox* in tobacco plants. In addition to studying their effect on active gibberellin levels and morphological phenotype, we also examined the effect on the expression of endogenous gibberellin metabolism genes. To expand our understanding of how gibberellin homeostasis is maintained in plants, we also studied the expression pattern of gibberellin metabolism genes in response to different GA_1 concentrations and its correlation with the hypocotyl elongation response. Our data indicate that gibberellin metabolism genes respond to different gibberellin levels: some of them are sensitive to low while others are responding to high gibberellin concentration. Our results suggest that feedback regulation of different *GA2ox* genes shows differences that may be related to different roles in the regulation of gibberellin homeostasis.

Results

Effect of PsGA3ox1 overexpression on tobacco phenotype

A total of four homozygous transgenic *35S:PsGA3ox1* overexpressor (3ox-OE) lines with diverse levels of *PsGA3ox1* expression (L24, L33, L38 and L44) were isolated (Fig. 2A). All the transgenic 3ox-OE lines had a longer



Increases of hypocotyl length at different GA_{20} doses compared to untreated (%)

Line	0–10^{-8}M	0–10^{-7}M
WT	3.4	36
L24	11	34
L33	20	41
L38	11	30
L44	15	46

Fig. 2 Phenotypic characterization of wild-type (Wt) and homozygous transgenic tobacco plants overexpressing *35S:PsGA3ox1* (3ox-OE). (A) Northern analysis of *PsGA3ox1* expression in wild-type and 3ox-OE transgenic lines (L24, L33, L38 and L44). rRNA transfer into the membrane was used as the loading control. (B) Hypocotyl length of 7-day-old wild-type and homozygous 3ox-OE transgenic lines (L24, L33, L38 and L44) grown in MS medium. Results are means ± SE ($n = 30$). Values with a different letter above the bars were significantly different ($P < 0.05$). (C) Response sensitivity of wild-type and 3ox-OE lines to GA_{20}. Seedlings were cultured in MS medium without or with different doses (10^{-8}, 10^{-7} and 10^{-6} M) of GA_{20}. Results are the means of 50 seedlings ± SE. Increases of hypocotyl lengths between 0 and 10^{-8} M GA_{20} or 0 and 10^{-7} M GA_{20} are represented in the inset of C.

hypocotyl, although only three of them (L24, L38 and L44) were significantly different from control plants (9–20% longer) (Fig. 2B). Final plant height of the four lines was significantly longer (about 7%) compared with wild-type plants (Table 1). The width and length of leaves were also significantly greater in transgenic 3ox-OE plants compared with the wild type, except for the leaf width of L24 (Table 1). Other phenotypical characters such as percentage germination, flowering node, stem diameter and fruit weight were not affected in tobacco transgenic 3ox-OE plants (Table 1). We verified that in planta PsGA3ox1 was active in converting the substrate GA_{20} into its product GA_1 (Fig. 1). With that purpose, the in vivo activity of PsGA3ox1 was determined by growing seedlings of these transgenic 3ox-OE plants in a MS medium containing different GA_{20} doses. At low concentration of GA_{20} (10^{-8} M), there was an increase in hypocotyl length of 11–20% in the transgenic 3ox-OE lines, while wild-type hypocotyl increased only 3.4% (Fig. 2C and inset). These results suggest that GA_{20} was converted more efficiently into active gibberellin in transgenic 3ox-OE that in wild-type plants, presumably due to *PsGA3ox1* overexpression.

Gibberellin levels in homozygous transgenic 3ox-OE plants

Gibberellin levels were quantified in the apical portion of transgenic 3ox-OE and wild-type plants at the stage of 18 developed leaves. Compared with control plants, GA_{20} concentration was dramatically reduced in all transgenic 3ox-OE lines (Fig. 3A). Since GA_{20} is a substrate of GA3ox, the decrease of GA_{20} in transgenic 3ox-OE provides further evidence of effective *PsGA3ox1* activity in vivo. In contrast, the GA_1 level showed mild but significant increases in some transgenic 3ox-OE lines (L24 and L38; Fig. 3B). Interestingly, the level of GA_8 in all transgenic 3ox-OE lines was higher than in wild-type plants (Fig. 3C), suggesting the production of more GA_1, because GA_8 is a catabolic product of GA_1 (Fig. 1). No significant differences of GA_4 content were found between wild-type and transgenic 3ox-OE plants (Fig. 3D).

Expression analysis of gibberellin metabolism genes in transgenic 3ox-OE plants

To investigate if transcriptional regulation of gibberellin metabolism had been modified in transgenic 3ox-OE plants, we quantified mRNA expression of endogenous genes encoding GA20oxs (*NtGA20ox1* and *-2*), GA3oxs (*NtGA3ox1* and *-2*) and GA2oxs (*NtGA2ox1*, *-3* and *-5*). In wild-type seedlings (hypocotyls plus cotyledons), the transcript abundance of gibberellin metabolism genes was different: *NtGA20ox2* had undetectable expression (data not shown), while *NtGA3ox1*, *NtGA20ox1* and *NtGA2ox1* had intermediate levels, and *NtGA2ox3*, *NtGA2ox5* and *NtGA3ox2* were the most abundantly expressed genes

Table 1 Phenotypic characterization of wild type (Wt) and homozygous transgenic lines overexpressing *35S:PsGA3ox1* (L24, L33, L38 and L44)

	Percentage germination	Stem height (cm)	No. of vegetative nodes	Width of leaf No. 30 (cm)	Length of leaf No. 30 (cm)	Stem diameter (cm)	Fruit weight (mg)
Experiment I							
Wt	51	151 ± 3a	43.5 ± 3.4a	12.7 ± 0.2a	21.0 ± 0.9a	1.45 ± 0.03a	210 ± 6a
L33	48	160 ± 2b	47.8 ± 2.6a,b	14.8 ± 0.4b	26.2 ± 0.6b	1.5 ± 0.05a	200 ± 3a
L38	52	165 ± 2b,c	50.4 ± 3.8a,b	15.2 ± 0.8b	27.0 ± 0.63b	1.44 ± 0.06a	190 ± 5a
L44	38	162 ± 3c	47.6 ± 3.2c	14.7 ± 0.2b	27.2 ± 0.6b	1.44 ± 0.06a	210 ± 8a
Experiment II							
Wt	–	180 ± 4a	49.2 ± 0.8a	13.5 ± 0.3a	23 ± 0.5a	1.42 ± 0.05a	–
L24	–	190 ± 6b	50.9 ± 2.0a	14.0 ± 0.2a	28 ± 0.6b,c	1.46 ± 0.06b	–
L38	–	197 ± 9b	50.7 ± 1.3a	15.5 ± 0.3b	26.5 ± 0.3b	1.48 ± 0.05bc	–

The percentage germination is the result of a representative experiment out of two independent experiment with similar results and was determined after 100 h, when approximately 50% of wild-type seeds had germinated in MS medium. At least 100 seeds were cultured for each line. For others phenotypical characters, two independent experiments were carried out at different periods of the year. All plants were cultured in a greenhouse at 28°C under long day conditions. Results are the means of 12 plants per line ± SE. For fruit weight, at least 40 fruits were taken. In each column, values with different letter were significantly different ($P < 0.05$).

(Fig. 4A, B, C in Wt). In the transgenic 3ox-OE plants, the expression of *NtGA20ox1*, *NtGA3ox1* and *NtGA3ox2* (gibberellin biosynthesis genes) was similar to that in wild-type plants (Fig. 4A, B). In contrast, transcript levels of *NtGA2ox3* and *NtGA2ox5* (gibberellin catabolism genes) showed a large increase (between 10- and 60-fold) in transgenic 3ox-OE (Fig. 4C). These results suggest that gibberellin catabolism genes were induced in transgenic 3ox-OE compared with wild-type plants, consistent with the increase in GA_8 levels found in these plants (Fig. 3C). The three *NtGA2ox* genes studied in this work have not been tested in vitro for GA2ox activities, but their functions have been inferred from their high homology to previously identified genes whose activity have been experimentaly confirmed (Supplementary data S4). The comparison of the sequences suggests that NtGA2ox1, -3 and -5 are most probably active enzymes of group I (Lee and Zeevaart 2005) that catalyze C19- gibberellins (GA_{20}, GA_1, GA_9 and GA_4) as substrates. However, we cannot predict if they are multicatalytic (Serrani et al. 2007) or if they have substrate preferences.

Generation and characterization of hybrid transgenic plants overexpressing a GA3ox (PsGA3ox1) and a GA20ox (CcGA20ox1)

In order to investigate the effect of simultaneous *GA3ox* and *GA20ox* overexpression in tobacco, we generated hybrid plants by crossing the transgenic 3ox-OE (lines L38 and L44) generated in this work and 20ox-OE (line L5), previously isolated in our laboratory (Vidal et al. 2001). We also crossed the wild type with transgenic 20ox-OE (line L5) to obtain parental hemizygous plants with a single copy of the transgene *35S:CcGA20ox1* (Wt×L5). A hybrid of crosses 3ox/20ox-OE (L38×L5 and L44× L5) carrying one copy of each transgene (*35S:PsGA3ox1* and *35S:CcGA20ox1*) exhibited a slender phenotype like the 20ox-OE parental L5 line (Fig. 5A). Compared with 3ox-OE parents, hybrids showed an elongated hypocotyl and faster germination (Fig. 5B, C). Compared with 20ox-OE parental seeds, hybrid seeds germinated faster than Wt×L5 seed (Fig 5B) and L5 seeds (data not shown). After 100 h of culture, hybrid seeds germinated between 10% (for L44×L5) and 25% (for L38×L5) more than hemizygous Wt×L5 seeds (Fig. 5B). The hypocotyl length (Fig. 5C) and final height (Fig. 5D) of 3ox/20ox-OE hybrids were also significantly greater than those of Wt×L5 plants. The number of vegetative nodes was higher in hybrid 3ox/20ox-OE compared with hemizygous Wt×L5 or wild-type plants (Fig. 5D). This may explain the slight increase in plant height since the internode length was not affected.

Gibberellin levels in hybrid transgenic 3ox/20ox-OE plants

Gibberellin levels were quantified in the apical portion of hybrid L38×L5 (3ox/20ox-OE) plants and the parental lines L38 (3ox-OE) and L5 (20ox-OE), at the stage of 18 developed leaves. Compared with parental 3ox-OE (L38), GA_1, GA_{20} and GA_8 contents were reduced in hybrid L38×L5 plants, while the GA_4 level was higher in the hybrid than in the 3ox-OE parental plant (Table 2). The reduction of GA_1, GA_{20} and GA_8 can be explained by the decrease in the early 13-hydroxylation pathway caused by the *GA20ox* overexpression, as described by Vidal et al. (2001). The other 20ox-OE parent (L5) contained gibberellin levels similar to those of hybrid plants (Table 2). However, the total active gibberellin content (GA_1 plus GA_4) in hybrid plants was slightly but significantly higher

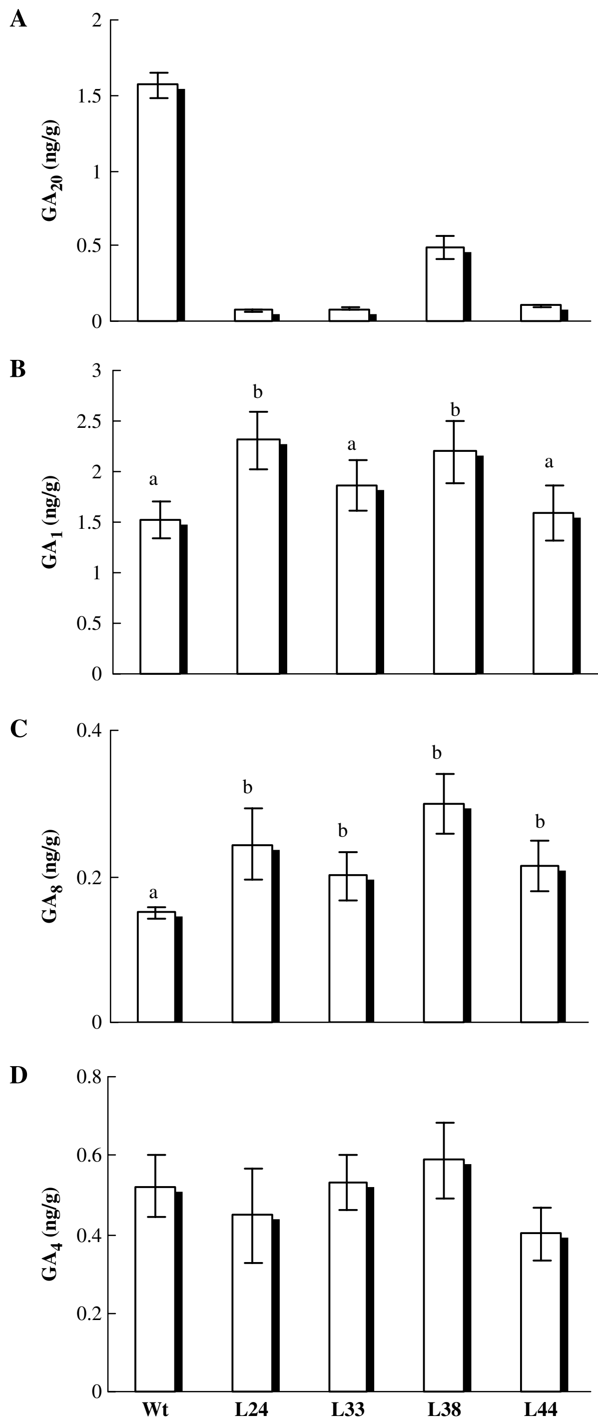


Fig. 3 Content (ng g^{-1} FW) of GA_{20} (A), GA_1 (B), GA_8 (C) and GA_4 (D) in apical shoots of wild-type (Wt) and transgenic 3ox-OE plants. Results are means of three replicates ± SE (five plants per replicate). Values with a different letter above the bars were significantly different ($P<0.05$).

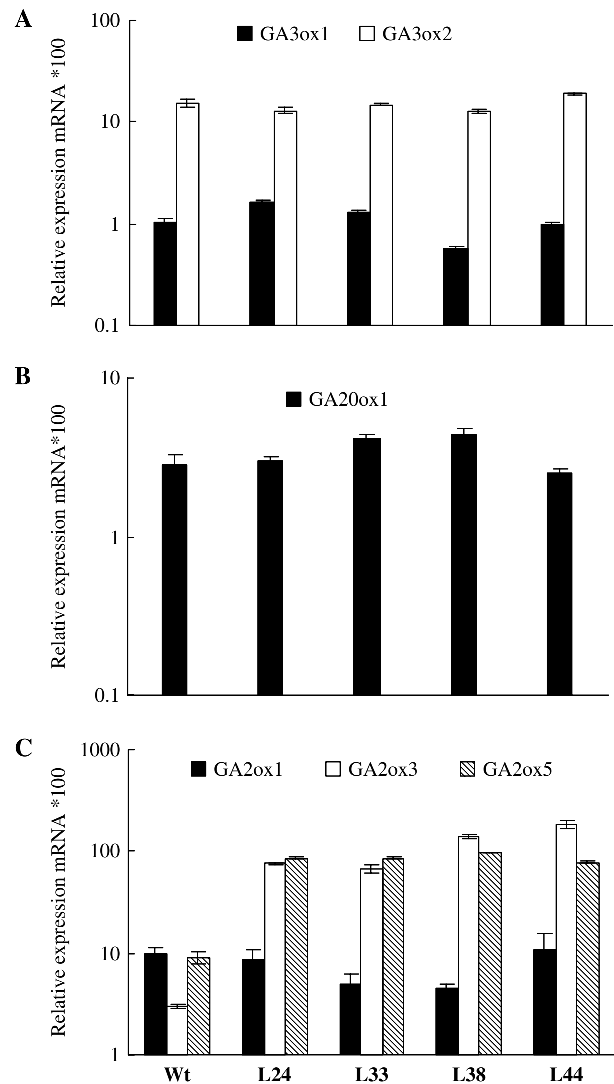


Fig. 4 Transcript levels of gibberellin metabolism genes in 7-day-old seedlings from transgenic 3ox-OE lines and the wild type quantified by real-time RT–PCR. (A) *NtGA3ox1* and *-2;* (B) *NtGA20ox1*; (C) *NtGA2ox1, -3* and *-5*. Relative expression of mRNA was normalized relative to the *NtActin* gene. Results are means of two biological replicates ± SE. Each PCR was run three times. Note that the scale is logarithmic.

than in the tall parental L5 plants (Fig. 6). As also shown in Fig. 6, a good correlation was found between total active gibberellin content and hypocotyl length for the wild type, the hybrids and their parents.

Transcriptional regulation of gibberellin metabolism genes in hybrid transgenic 3ox/20ox-OE plants

Analysis of gibberellin metabolism genes (*NtGA3ox1* and *-2*, *NtGA20ox1*, and *NtGA2ox1*, *-3* and *-5*) was

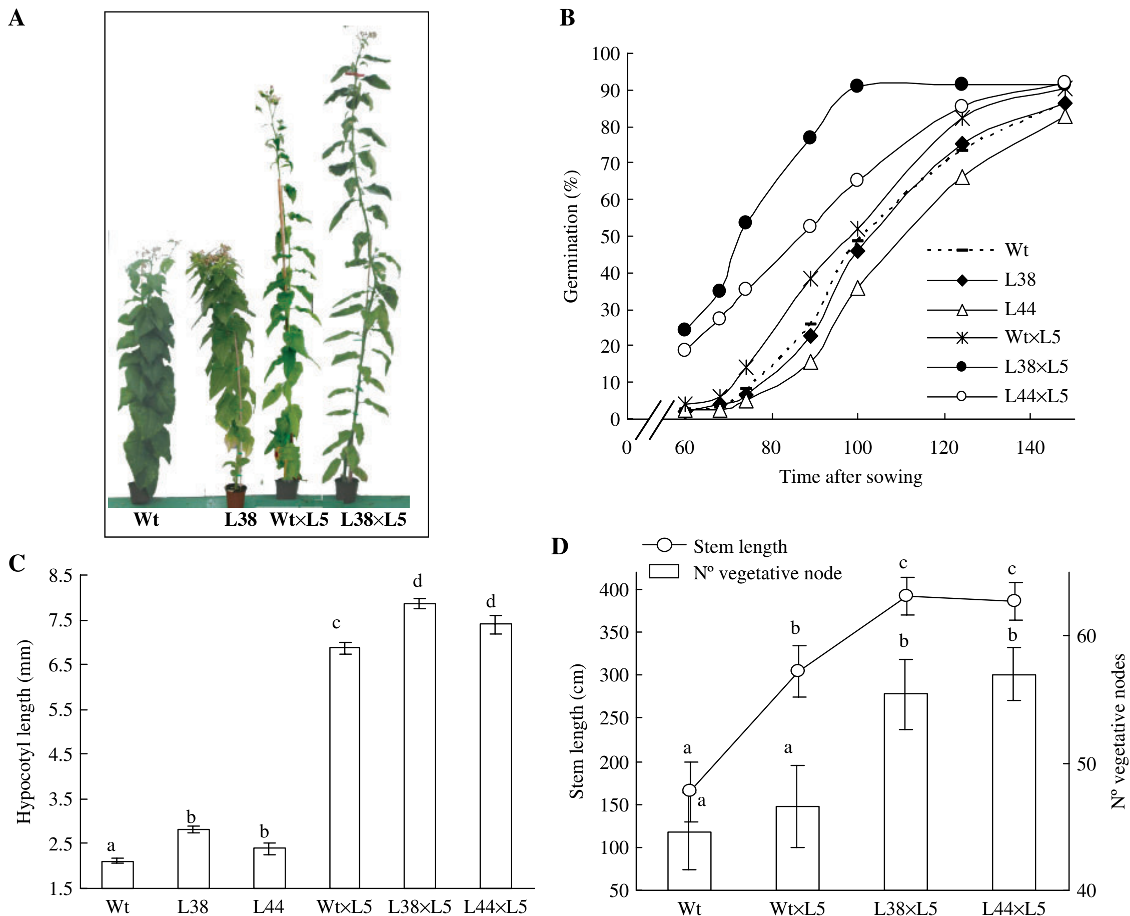


Fig. 5 Phenotypic characterization of hybrid transgenic plants overexpressing *35S:PsGA3ox1* (3ox-OE) and *35S:CcGA20ox1* (20ox-OE) (L38×L5 and L44×L5). (A) Pictures of representative plants from the wild type, transgenic parental 3ox-OE (L38), hemizygous control Wt×L5 and hybrid 3ox/20ox-OE (L38×L5). (B) Percentage of germination in transgenic hybrid 3ox/20ox-OE (L38×L5 and L44×L5), parental 3ox-OE (L38 and L44), hemizygous control Wt×L5 and wild type plants at different times after sowing. One hundred seeds were cultured in MS medium from each material. (C) Hypocotyl length of 7-day-old seedlings of transgenic hybrids 3ox/20ox-OE (L38×L5 and L44×L5), parental 3ox-OE (L38 and L44), hemizygous control Wt×L5 and the wild type. Results are the means of 40 seedlings ± SE. Values with a different letter above the bars were significantly different ($P<0.05$). (D) Stem length and number of vegetative nodes in transgenic hybrid 3ox/20ox-OE (L38×L5 and L44×L5), hemizygous control Wt×L5 and wild type plants. Results are the means of five plants ± SE. Values with a different letter above the bars were significantly different ($P<0.05$).

Table 2 Gibberellin content in the apical portion of the wild type (Wt), the transgenic *35S:PsGA3ox1* line (L38), the transgenic *35S:CcGA20ox1* line (L5) and the hybrid *35S:PsGA3ox1* × *35S:CcGA20ox1* (L38×L5)

	Gibberellin content (ng g^{-1} FW)			
	GA_{20}	GA_1	GA_8	GA_4
Wt	1.57 ± 0.08c	1.53 ± 0.2b	0.14 ± 0.007b	0.52 ± 0.07a
L38	0.49 ± 0.01b	2.20 ± 0.32c	0.30 ± 0.04c	0.59 ± 0.1a
L5	0.15 ± 0.01a	0.14 ± 0.02a	0.07 ± 0.02a	5.28 ± 0.68b
L38×L5	0.14 ± 0.01a	0.20 ± 0.02a	0.05 ± 0.02a	5.98 ± 0.5b

Results are means of three biological replicates ± SE, five plants per replicate. In each column, values with a different letter were significantly different ($P<0.05$).

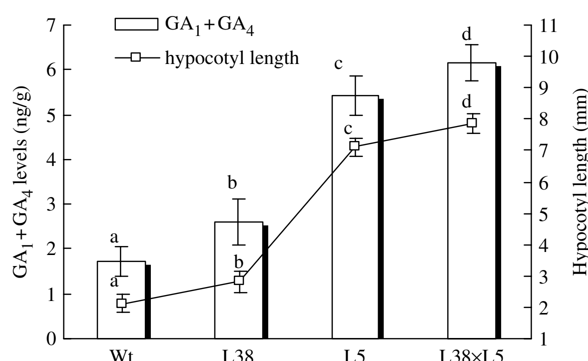


Fig. 6 Total active gibberellin content ($GA_1 + GA_4$) and hypocotyl length of the transgenic hybrid 3ox/20ox-OE line (L38×L5), parental transgenic 3ox-OE (L38) and 20ox-OE (L5), and wild-type plants. Values with a different letter above the bars were significantly different ($P<0.05$).

performed in seedlings (hypocotyls plus cotyledons) of hybrids 3ox/20ox-OE (L38×L5 and L44×L5), parental 3ox-OE (L38 and L44) and 20ox-OE (L5) plants and wild-type plants (Fig. 7). Transcript levels of *NtGA3ox1* and *NtGA20ox1* were reduced in all slender plants (Fig.7A), as expected due to the negative feedback regulation of these genes (Tanaka-Ueguchi et al. 1998, Itoh et al. 1999). Moreover, the decrease of *NtGA20ox1* expression was higher in the hybrids (3ox/20ox-OE) than in slender parental plants (L5 and Wt×L5), consistent with the increase in total active gibberellin content in the hybrid (Fig. 6). *NtGA3ox2*, which was the most highly expressed of all gibberellin genes studied, did not show negative feedback regulation. Moreover, we even found a slight induction of *NtGA3ox2* in slender 20ox-OE plants (Fig. 7B). *NtGA2ox3* and *-5* were induced in the same proportion in parental 3ox-OE and in slender transgenic 20ox-OE (hybrids 3ox/20ox-OE and 20ox-OE) plants (Fig. 7C). In contrast, the transcript level of *NtGA2ox1* was similar in the wild type and in all transgenic plants (Fig. 7C), suggesting that *NtGA2ox1* did not display any positive feedback regulation.

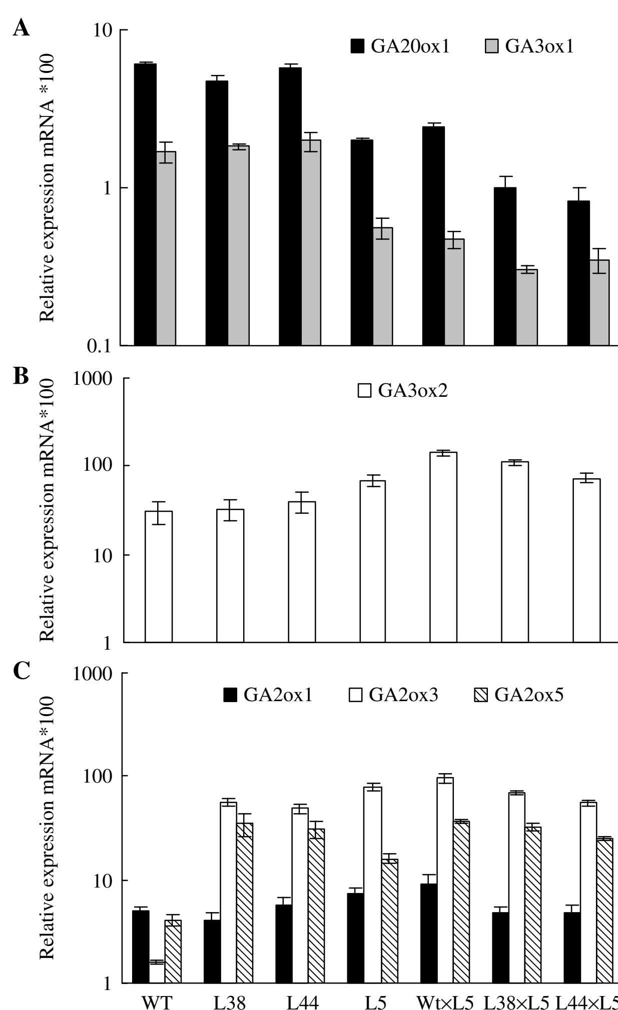


Fig. 7 Expression levels of gibberellin metabolism genes in hybrid transgenic 3ox/20ox-OE lines (L38×L5 and L44×L5), transgenic 3ox-OE lines (L38 and L44), the transgenic 20ox-OE line (L5), hemizygous control Wt×L5 and the wild type. Total RNA was extracted from 7-day-old seedlings. (A) *NtGA3ox1* and *NtGA20ox1*; (B) *NtGA3ox2*; (C) *NtGA2ox1*, *-3* and *-5* transcript levels were quantified by real-time RT–PCR. Relative expression of mRNA was normalized with respect to the *NtActin* gene. Results are means of two biological replicates ±SE. Each PCR was run three times. Note that the scale is logarithmic.

Effect of different GA_1 concentrations on the regulation of gibberellin metabolism genes

As described before, *NtGA20ox1* was repressed in transgenic 20ox-OE but not in 3ox-OE plants (Fig. 7A). In contrast, we detected similar induction of two *GA2ox* catabolism genes in transgenic 3ox-OE and in 20ox-OE plants (Fig. 7C). These results suggested that the regulation of some of the gibberellin metabolism genes is sensitive to low gibberellin levels, while others are responding only at high gibberellin levels. To test this hypothesis, we studied the expression of *GA20ox*, *GA3ox* and *GA2ox* genes in tobacco seedlings in response to different GA_1 doses (Fig. 8A). The expression level of *NtGA20ox1* and *NtGA3ox1* showed a slight variation between 0 and 10^{-7} M GA_1 and decreased strongly for 10^{-6} M GA_1. For *GA2ox*, we found two different patterns of gene expression in response to GA_1 dose. *NtGA2ox1* was only induced at high GA_1 dose (10^{-6} M), while *NtGA2ox3* and *-5* showed almost maximum induction (~6 times) at the lowest GA_1 dose (10^{-8} M; Fig. 8A). *NtGA20ox1, NtGA3ox1* and *NtGA2ox1* expression patterns showed a correlation with hypocotyl growth response to different GA_1 concentrations

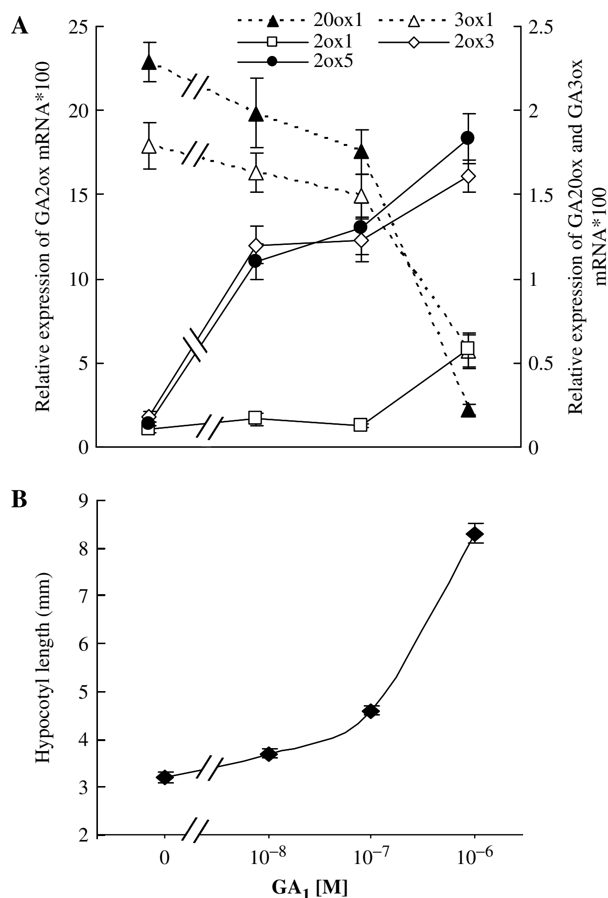


Fig. 8 Effect of different doses of GA_1 on transcript levels of selected gibberellin metabolism genes (A) and hypocotyl length (B). Total RNA was extracted from 7-day-old seedlings. Transcript levels of *NtGA20ox1*, *NtGA3ox1*, *NtGA2ox1*, *-3* and *-5* were quantified by real-time RT–PCR. The relative expression of mRNA was normalized with the *NtActin* gene. Results are the average of six value ± SE from two biological replicates. Each PCR was run three times.

(Fig. 8B), although in opposite directions: inverse in the case of *NtGA20ox1 and NtGA3ox1* and direct in the case of *NtGA2ox1*. In contrast, the *NtGA2ox3* and *-5* expression pattern did not display any correlation with hypocotyl growth (Fig. 8 and Supplementary data S2).

Discussion

Effect of PsGA3ox1 overexpression on tobacco phenotype

Overexpression of *35S:PsGA3ox1* produced mild modifications of tobacco phenotype. Hypocotyl length, stem height, and leaf width and length increased in transgenic 3ox-OE plants compared with wild-type plants (Fig. 2B and Table 1). These characters depend on gibberellin levels and therefore we expected variations in plant gibberellin levels. The main effect of GA3ox overproduction on tobacco gibberellin metabolism was a strong reduction in GA_{20} concentration in all transgenic lines (Fig. 3A), consistent with a high level of conversion of the substrate GA_{20} into the active GA_1 product by *35S:PsGA3ox1*. The reduction of the GA_{20} level, however, was not associated with a large increase of GA_1 level (Fig. 3B), in agreement with the slight changes of tobacco phenotype (Table 1). Similar results on phenotype, GA_{20} and active gibberellin levels were found in transgenic 3ox-OE plants of hybrid aspen (Israelsson et al. 2004).

In tobacco, the slight increase in active GA_1 level in transgenic 3ox-OE may be due to several reasons. First, it may be due to to the low availability of GA3ox substrate as a consequence of limited GA_{20} oxidation, and, secondly, it may be a result of the catabolism of GA_1 to inactive forms such as GA_8, by the action of GA2oxs (Fig. 1). The latter possibility is supported by the observation that GA_8 levels increased in all transgenic 3ox-OE plants compared with wild-type plants (Fig. 3C). Furthermore, two of the three *GA2ox* genes analyzed (*NtGA2ox3* and *-5*) were induced in all 3ox-OE transgenic lines (Fig. 4C), suggesting an important role for these genes in the control of gibberellin homeostasis. Interestingly, in hybrid aspen 3ox-OE transgenic plants, induction of *GA2ox* expression was not detected (Israelsson et al. 2004). This apparent discrepancy between tobacco and hybrid aspen may be a species-specific difference, or due to the fact that the genes studied in aspen were not involved in gibberellin homeostasis, like the *NtGA2ox1* gene of tobacco that remains almost unaffected in transgenic plants (Fig. 4C).

Since GA3oxs cannot enhance the flux toward active gibberellin levels, we obtained hybrid plants that incorporated the overexpression of a GA20ox (*35S:CcGA20ox1*; Vidal et al. 2001) by crossing transgenic 3ox-OE lines (L38 and L44) with a transgenic 20ox-OE line (L5). The ectopic activity of *CcGA20ox1* in these hybrid plants should provide a higher amount of substrate (GA_9) for the GA3ox. In fact, it has been demonstrated that transgenic 20ox-OE (L5) plants had higher GA_9 levels (Vidal et al. 2001). Hybrid tobacco plants (3ox/20ox-OE) displayed a slender phenotype like the 20ox-OE parental line (Fig. 5A). However, their final stem length was longer due to a greater number of vegetative internodes and not to longer internodes (Fig. 5D). This led to a flowering delay of hybrid 3ox/20ox-OE plants compared with parental plants. This observation is consistent with a previous report where an excess of active gibberellin in tobacco apical shoots was claimed to have an inhibitory effect on floral transition (Gallego-Giraldo et al. 2007).

Phenotypic differences described in hybrid 3ox/20ox-OE compared with parental 20ox-OE plants must be due to the additional effect of GA3ox overexpression

on gibberellin biosynthesis. In fact, phenotypic alterations in hybrid 3ox/20ox-OE plants correlated with a slight increase in total gibberellin active levels, $GA_1 + GA_4$, compared with the parental L5 plants (Fig. 6). Furthermore, the slight increase of active gibberellin levels in hybrid plants correlated with a further repression of endogenous *NtGA20ox1* and *NtGA3ox1* expression in hybrids (3ox/20ox-OE) compared with 20ox-OE parental plants (L5, Wt×L5; Fig. 7A). This is consistent with the negative feedback regulation of these genes reported previously (Tanaka-Ueguchi et al. 1998, Itoh et al. 1999). In contrast, expression of *GA2ox* was relatively unaffected by overexpression of a GA3ox in the 20ox-OE background (Fig. 7C). These results suggest that the expression of GA2ox genes (*NtGA2ox3* and *-5*) had reached already saturated levels in transgenic 3ox-OE plants and therefore could not be further induced in hybrid lines. Taken together, these results indicate that GA3oxs in tobacco are non-limiting enzymes in gibberellin biosynthesis, in contrast to *Arabidopsis* (Radi et al. 2006), even in the presence of the 20ox-OE background.

Regulation of gibberellin metabolism genes

Feedback regulation of genes encoding dioxygenases of gibberellin metabolism has been described previously in many species (Hedden and Phillips 2000a), including tobacco *NtGA20ox1* (Tanaka-Ueguchi et al. 1998) and *NtGA3ox1* (Itoh et al. 1999). We have confirmed that negative feedback regulation of *NtGA20ox1* and *NtGA3ox1* also occurs in slender transgenic plants, as expected by their higher active gibberellin levels. Our results also showed that the expression of a second GA3ox (*NtGA3ox2*) was unaltered in these plants (Fig. 7) as well, and in response to gibberellin application (data not shown), indicating that this gene is not under gibberellin feedback regulation. GA3oxs without feedback regulation have been described previously in *Arabidopsis* (*AtGA3ox2*, *-3* and *-4*; Yamaguchi et al. 1998, Matsushita et al. 2007). Since the existence of tissue-specific differences in transcript levels of *NtGA3ox2* (Gallego-Giraldo et al. 2007) have been reported, the regulation of this gene may depend on developmental factors. On the other hand, we found evidence for feedback regulation of GA2ox gene expression in tobacco, as was found in *Arabidopsis* (Thomas et al. 1999) and pea (Elliott et al. 2001). Expression of *NtGA2ox3* and *-5*, but not of *NtGA2ox1*, was induced in all transgenic gibberellin-overproducing plants (Fig. 7C). All these results of gene expression in transgenic plants suggested different gibberellin sensitivity for genes under feedback regulation. On one hand, *NtGA2ox3* and *-5* seem to be sensitive to low gibberellin variations, since they are induced in transgenic 3ox-OE plants which contain mild increases in gibberellin levels. On the other hand, genes such as *NtGA20ox1* and *NtGA3ox1* seem to be sensitive to high gibberellin levels, because their expression was altered only in slender transgenic plants with substantial gibberellin increases (20ox-OE and hybrid 3ox/20ox-OE). Interestingly, in citrus, it has also been described that *GA20ox1* was negatively feedback regulated only in response to large changes in the active gibberellin content (Vidal et al. 2003). The two different gene expression patterns found in response to gibberellin (one very sensitive to GA_1 for *NtGA2ox3* and *-5* and another mainly sensitive to high GA_1 concentrations for *NtGA20ox1*, *NtGA3ox1* and *NtGA2ox1*; Fig. 8) support the existence of different gibberellin sensitivities. These results are consistent with those found in transgenic plants, except for *NtGA2ox1*. This gene, whose expression was unaltered in transgenic plants, was induced by the application of 10^{-6} M GA_1, probably because the gibberellin content in transgenic plants did not reach such a high level. Therefore, although it is already known that different members of gibberellin gene families are regulated preferentially by specific environmental (Stavang et al. 2007) or developmental (Mitchum et al. 2006) cues, our data suggest that homeostatic conditions (the severity of alterations in gibberellin levels) can also trigger differentially the induction of gibberellin metabolism genes.

Interestingly, genes responding to high GA_1 concentrations displayed a correlation (direct for *NtGA2ox1* and inverse for *NtGA20ox1* and *NtGA3ox1*) between transcript levels and hypocotyl elongation induced by gibberellin (see Fig. 8 and Supplementary data S2). This relationship, between hypocotyl length (a gibberellin-dependent character) and transcript levels, was also found for *NtGA20ox1* and *NtGA3ox1* in transgenic plants with different gibberellin contents (Supplementary data S3). It has been demonstrated that negative feedback regulation of *GA20ox* and *GA3ox* genes by active gibberellin occurs via the gibberellin response pathway, because their transcription and the gibberellin content were impaired in different gibberellin response mutants (reviewed in Olszewski et al. 2002). Accordingly, in *Arabidopsis*, it was found that *AtGA3ox1* is feedback regulated by active gibberellin in a dose-dependent manner that closely mirrors stimulation of hypocotyl elongation, suggesting that similar gibberellin signal transduction processes control both *AtGA3ox1* regulation and hypocotyl elongation (Cowling et al. 1998). Therefore, we could expect correlation between gene expression and hypocotyl growth in response to gibberellin, for tobacco genes regulated through the gibberellin signal transduction pathway, as occurs in *AtGA3ox1*. The relationship between gene expression and gibberellin growth response (hypocotyl elongation) for *NtGA20ox1* and *NtGA3ox1* in tobacco supported the contention that feedback regulation of these gibberellin biosynthesis genes operates through the gibberellin response pathway.

In contrast, transcript levels of genes sensitive to low GA_1 concentrations (*NtGA2ox3* and *-5*) did not correlate with hypocotyl elongation (Fig. 8 and Supplementary data S2). While the expression of *NtGA20ox1* and *NtGA3ox1* seems to be proportional to the gibberellin content, *NtGA2ox3* and *-5* become activated to almost maximum levels with minimal increases in the gibberellin content and remain constant afterward, indicating that positive feedback regulation of these two genes may not be mediated by the same gibberellin signaling components involved in the feedback regulation of the other gibberellin biosynthesis genes. Another possibility, to explain the differences found in the expression pattern of these particular genes, could be an autonomous regulation directly mediated by the gibberellin molecule independently of DELLA signaling. For instance, in methionine biosynthesis, negative feedback regulation of the *CGS1* gene occurs through alteration of the mRNA stability in response to methionine application (Lambei et al. 2003). Because it has been demonstrated that DELLA-dependent gibberellin signaling has a central role in all gibberellin-related processes (Jianga and Fu 2007), it has been assumed that feedback regulation of *GA2ox* catabolism genes also proceeds through this signaling transduction pathway (Olszewski et al. 2002), although no experimental evidence has been published so far supporting this hypothesis. Whether or not the regulation of *NtGA2ox3* and *-5* depends on DELLA, it does not mean that feedback regulation of other *GA2ox* catabolism genes, such as *NtGA2ox1*, does not occur by this signal transduction pathway, in a similar way to the biosynthesis genes. Transcript levels of *NtGA2ox1* (and also *NtGA2ox2*; data not shown) correlated with hypocotyl growth in response to different GA_1 applications in tobacco, and the involvement of DELLA in feedback regulation of *AtGA2ox8* in *Arabidopsis* is supported by unpublished results (Gallego-Bartolomé, Alabadí and Blázquez, personal communication).

In summary, our data reveal novel features, not previously reported, of the mechanism of feedback regulation of gibberellin metabolism genes. Gibberellin metabolism genes respond to gibberellin with different sensitivity. Two of the gene members of the *GA2ox* family are positive feedback regulated by small increases in gibberellin levels and seem to play a predominant role in gibberellin homeostasis under mild variations. In the case of large variations in gibberellin levels, gibberellin homeostasis is mediated by biosynthesis genes *GA20ox* and *GA3ox* and perhaps by other *GA2ox* catabolism genes.

Materials and Methods

Plant material and growth conditions

Wild-type and transgenic tobacco plants of *Nicotiana tabacum* cv Xanthi (overexpressing *35S:CcGA20ox1*; line L5; Vidal et al. 2001) were used as experimental material. Plants were cultured in a growth chamber at 26°C and in a greenhouse at 28°C under long day conditions (16 h light, and 8 h darkness). Plants were cultured in pots containing vermiculite: peat (1 : 1) and watered with nutrient solution.

Isolation of transgenic plants overexpressing 35S:PsGA3ox1 (3ox-OE)

Sterilized seeds of tobacco were germinated and cultured in MS medium (Murashige and Skoog medium including vitamins; Duchefa, The Netherlands), 20 g l^{-1} sucrose and 6 g l^{-1} agar gel. Leaf sections from 3-week-old plants were transformed by immersion in bacterial liquid LB medium containing *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al. 1983) carrying the binary vector system pBIN-JIT-*2×35S:PsGA3ox1* (*PsGA3ox1* accession No. AF001219; Martin et al. 1997). Regeneration of tobacco explants was performed as described by Vidal et al. (2001). Non-infected leaf sections were cultured and considered as a transformation control. Through three consecutive transformation processes a total of 115 putative independent transgenic T_1 plants were obtained. Eighteen putative plants (T_1) were selected according to *PsGA3ox1* transcript level analysis performed by Northern blot (data not shown). For isolation of homozygous lines, T_1 plants were grown and self-pollinated to obtain the T_2 generation. T_2 seedlings were tested by kanamycin (150 mg l^{-1}) segregation. T_2 plants showing a 3 : 1 segregation ratio (kanamycin-resistant Km^R/kanamycin-sensitive Km^S) were selected to obtain the T_3 generation. Homozygous lines were detected by 100% Km^R segregation.

Northern blots

For Northern analysis, total RNA was isolated by using the Trizol method (Invitrogen, Carlsbad, CA, USA) from young leaves of wild-type and transgenic *PsGA3ox1*-overexpressing plants. Northern blot was prepared by electrophoresis in a formaldehyde–agarose gel, including 20 μg of total RNA. The gel was transferred by capillarity into a nylon filter (Hibond-N, Amersham Bioscience, Buckinghamshire, UK) and cross-linked with a UV Stratalinker 800 (Stratagene, La Jolla, CA, USA). Full-length *PsGA3ox1* cDNA was random-primed ^{32}P-labeled using the Kit Ready to Go DNA (Amersham Biosciences). The probe was used to hybridize the filter at 42°C in a solution containing 0.25 M KH_2PO_4 buffer, 7% SDS, 1 M EDTA, 5 M NaCl, 50% formamide, PEG 6000 and 0.1 mg ml^{-1} denatured salmon sperm. The filter was washed for 10 min at 50°C in 3× SSC, 0.5% (w/v) SDS and 1× SSC, 0.1% (w/v) SDS, and then at 55°C in 0.5× SSC, 0.1% (w/v) SDS. The primers pair used to obtain the full-length *PsGA3ox1* probe were: *PsGA3ox1S1*, 5′-TAGGTCGACCACTA TGCCTTCACTCTCCGAAGCC-3′; and *PsGA3ox1A1*, 5′-GAG CCCGGGTTAGCCCACTTGGACACTATTTTTGTTAG-3′.

Hypocotyl length measurements and hormone applications

One hundred seeds from each line were germinated and cultured in MS medium in a growth chamber at 26°C under long day conditions. For GA_{20} and GA_1 treatments, different gibberellin concentrations were applied in 50 μl of 100% ethanol added to Petri dishes with 100 ml of medium. A 50 μl aliquot of 100% ethanol was added to control plates. Seven days later, the seedlings were scanned and measured using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Quantification of gibberellins

Gibberellins were quantified in the apical portions of plants consisting of shoot apices and six apical young leaves (0.5–3 cm in length) using three biological replicates. Each sample consisted of material from five plants. The material was frozen immediately in liquid N_2 before storage at –80°C, until analysis. Aliquots of 10 mg of lyophilized material were homogenized with liquid N_2 and extracted using a MM301 Vibration Mill for 3 min (Retsch GmbH and Co., Haan, Germany) in 1 ml of 80% MeOH + 0.02% diethyl dithiocarbamate as antioxidant containing gibberellin internal standards ([$^2H_2$]GA_1, [$^2H_2$]GA_8, [$^2H_2$]GA_{19}, [$^2H_2$]GA_{20}, [$^2H_2$]GA_{44}, [$^2H_2$]GA_{53}, [$^2H_2$]GA_{19}, [$^2H_2$]GA_4, [$^2H_2$]GA_9, [$^2H_2$]GA_{24} and [$^2H_2$]GA_{34}) (purchased from Professor Lewis Mander, The Australian National University). After centrifugation (13 000 r.p.m. for 3 min), the supernatant was evaporated to dryness under vacuum. The residue was dissolved in 500 µl of 1% acetic acid at pH 2.5–3. This solution was loaded onto a pre-equilibrated Isolute C8-EC cartridge (International Sorbent Technology, Hengoed, UK), washed with 2 ml of 5% MeOH, 1% acetic acid, eluted with 2 ml of 80% MeOH and evaporated to dryness. The residue was dissolved in 200 µl of 2-propanol and methylated with trimethylsilyl-diazomethane in hexane (Sigma Aldrich, St Louis, MO, USA). The gibberellins were separated by HPLC and the fractions were dried, trimethylsilylated in 20 µl of pyridine and bis-trimethylsilyl formamide (BSFA) + 1% trimethylchlorosilane (TMCS), and analyzed by gas chromatography–mass spectroscopy in selected reaction monitoring mode using a JEOL JMS MStation (JEOL, Tokyo, Japan) as described by Peng et al. (1999).

RNA isolation and reverse transcription

Total RNA was isolated from 7-day-old seedlings without roots (hypocotyls and cotyledons) using an RNeasy Plant Mini Kit and treated with an RNase-free DNase set (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. RNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (Wilmington, DE, USA). A 1 µg aliquot of total RNA was used for cDNA synthesis using a Taq Man® Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) with random hexamers, at a final volume of 40 µl. A 1 µl aliquot of cDNA solution was used in all PCRs.

Quantitative real-time RT–PCR analysis

Transcription levels of NtGA20ox1 (Ntc12, AB012856.1), NtGA20ox2 (NTc16, AB016084), NtGA3ox1 (Nty, AB032198), NtGA3ox2 (EF471116), NtGA2ox1 (AB125232.1), NtGA2ox3 (EF471117) and NtGA2ox5 (EF471118) were analyzed by quantitative real-time PCR. NtActin (U60489) was amplified as a loading control. The primer pairs used for PCR amplification (Supplementary Table S1) were designed using Primer Express™ v2.0 software (Applied Biosystems) and their concentrations were optimized according to the manufacturer's instructions (SYBR® Green PCR Master Mix and RT-PCR Protocol, Applied Biosystems). PCRs were performed in an ABI PRISM® 7000 Sequence detection System using SYBR Green to monitor double-stranded DNA synthesis (Applied Biosystems). PCR conditions and data analysis were performed as described in Gallego-Giraldo et al. (2007). The analyses were carried out using two biological replicates. Each replicate consisted of material from 30 seedlings. All PCRs were carried out using three technical replicates.

Statistical methods

Statistical treatment of the data was by analysis of variance using Fisher's LSD procedure for multiple comparison tests (Statgraphics Plus program, version 5.1 for Windows, Statistical Graphics, Rockville, MD, USA).

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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Supplemental Data

Table S1. Primers pair used in a Real-time RT-PCR analysis.

Gene	Forward	Reverse
NtGA20ox1	5'-TGTAGCACGAGAACTTCC-3'	5'-ACGGCATGCTTCACCAACA-3'
NtGA20ox2	5'-TGAGGTTCCTTCTTCACAGCA-3'	5'- CTCCCCTAAAAGCTCCATTACC-3'
NtGA3ox1	5'-CGGCTTTGTCCCCTCTA-3'	5'-CTTATCGAGTTTAGCCAACTTGCA-3'
NtGA3ox2	5'-TGCGCTCCTCGTATTGGATT-3'	5'AAAGGTAACTACTAGTCAAATTAGCCAACT-3'
NtGA2ox1	5'- CGTTCAATTTCTTTGCCCAC-3'	5'-GCAGTTGTCTTTGGAGAAGTGC-3'
NtGA2ox3	5'-AAGTCCCACCTTGTTACTTG-3'	5'-GCTATCTATAGGAAATCCAATG-3'
NtGA2ox5	5'-ATCCATCGCTATTCCCGGA-3'	5'-GCTATCTTTTCCAGCGCCAA-3'
NtActin	5'-CATTGGCGCTGAGAGATTCC-3'	5'-GCAGCTTCCATTCCGATCA-3'

Figure S2.

Correlation between transcript levels of selected GA metabolism genes and hypocotyl length in response to GA_1 applications in tobacco wild-type. Relative gene expression of *NtGA20ox1* (A), *NtGA2ox1* (B), *NtGA2ox3* and *-5* (C) are represented in addition to hypocotyl length at different GA_1 doses. Note for *NtGA20ox1* (A), the axis of mRNA levels was drawn reversed to better visualize correlation with hypocotyl length. The date used to perform this correlation were those of figure 8.

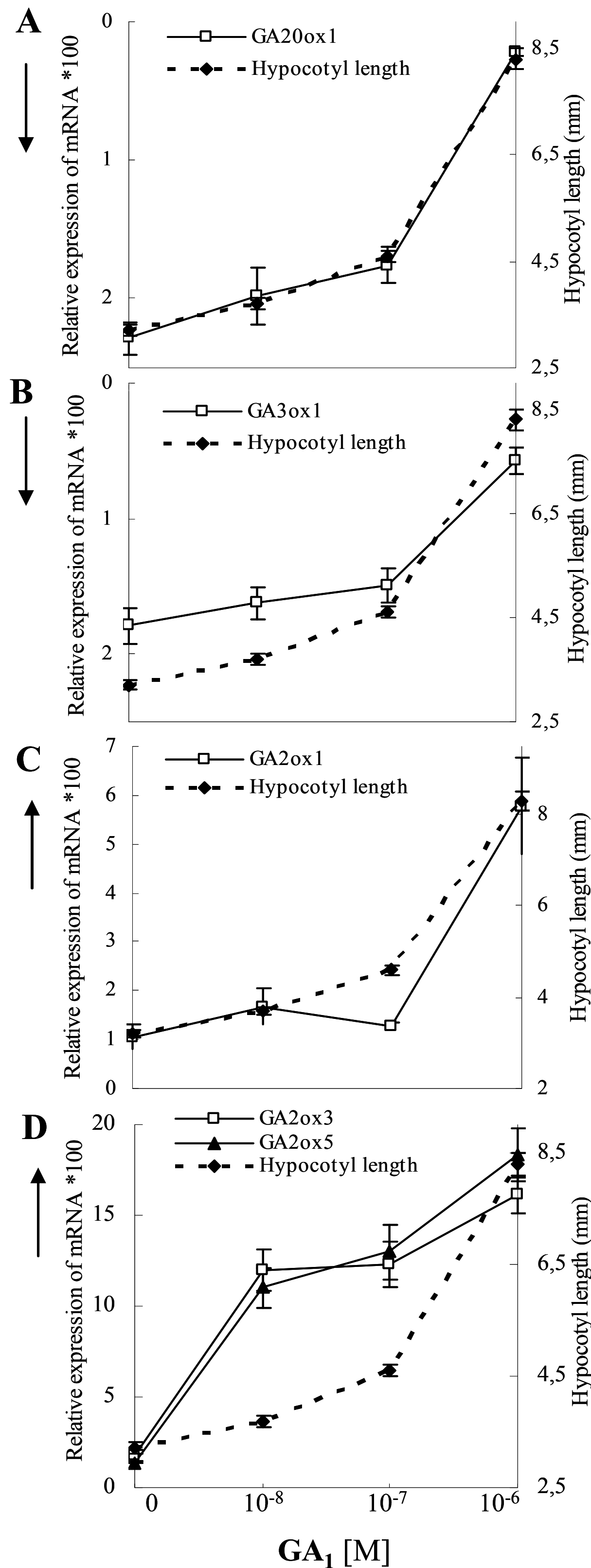


Figure S3.

Correlation between transcript levels of selected GA metabolism genes and hypocotyl elongation in transgenic tobacco plants (3ox-OE, 20ox-OE and 3ox/20ox-OE). Relative abundance of mRNA of *NtGA20ox1* (A) and *NtGA3ox1* (B) were represented in addition to hypocotyls length of diverse transgenic plants. The axis scales were adjusted to facilitate comparisons. Note that axis of mRNAs levels were drawn reversed. The date used to perform this correlation were those of figure 7A and 5C.

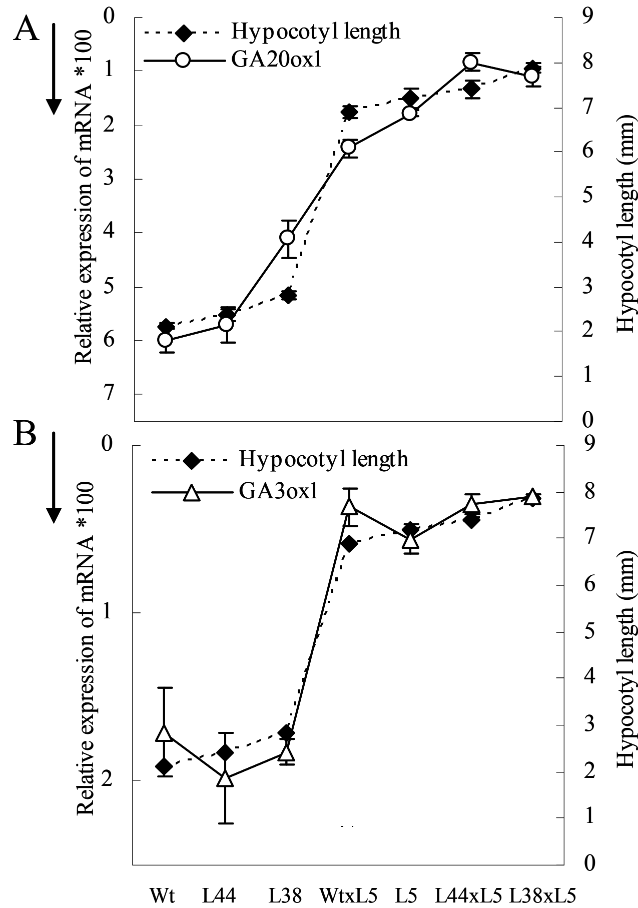
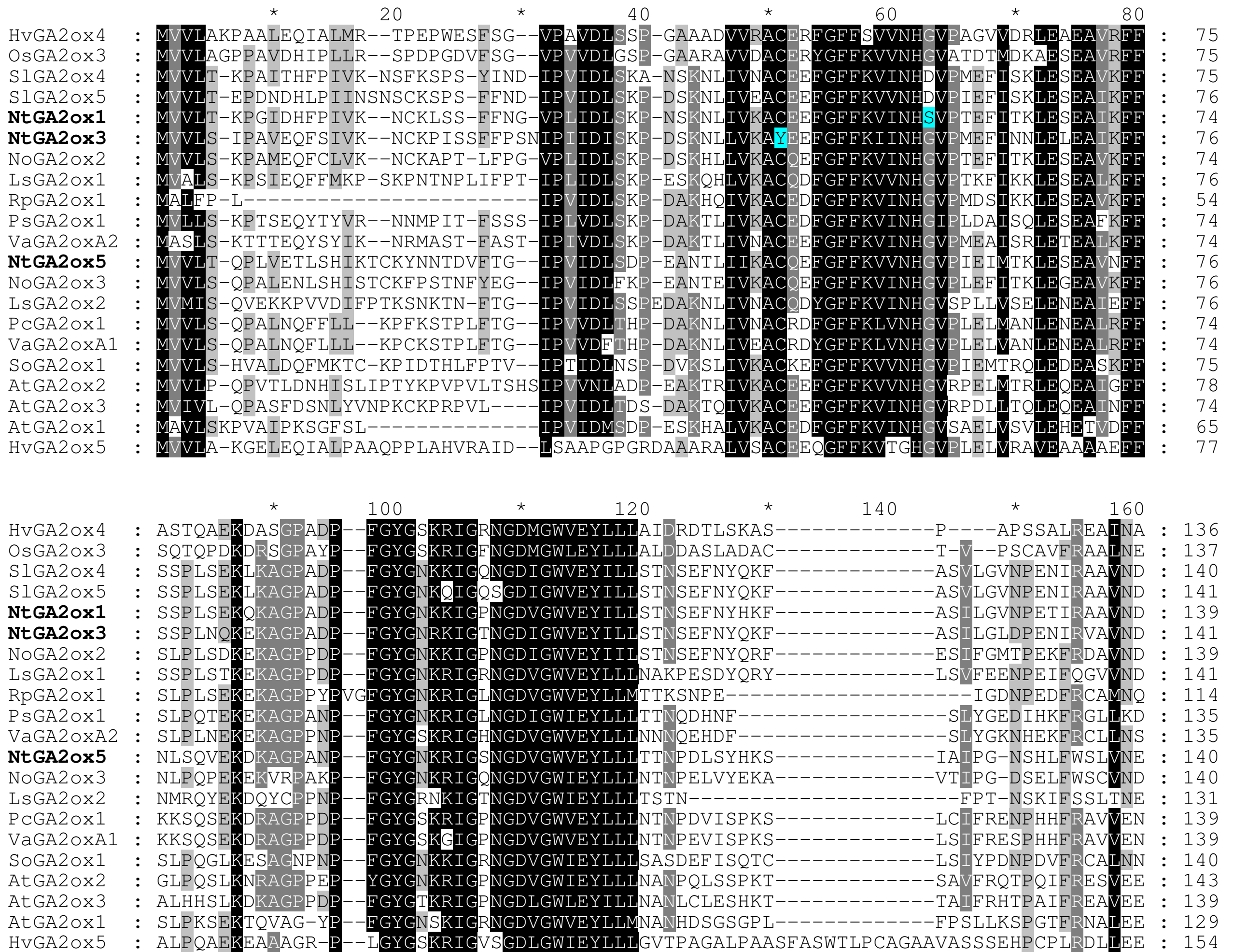


Figure S4.

Alignment of amino acid sequences corresponding to 18 GA 2-oxidases whose activities have been confirmed experimentally plus the sequences of *NtGA2ox1, -3* and *-5*. Alignment was performed with ClustalW2 (**http://www.ebi.ac.uk/Tools/clustalw2/index.html**). The three tobacco genes characterized in this study are shown in bold type and positions in their sequences differing from the consensus conserved amino acids are labeled in blue color.



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                       *       180          *        200          *        220          *        240
HvGA2ox4    : YVSAMRGLARTVLEMVAEGLGV--SPRGALADMVTGEASDQVFRVNHYPPC--------PLL---QGLPPNCSVTGFGEH : 203
OsGA2ox3    : YISGVRKVAVRVMEAMSEGLGI--AQADALSALVTAEGSDQVFRVNHYPPC--------RAL---QGL--GCSVTGFGEH : 202
SlGA2ox4    : YVSSVKKMSCEILEKLAEGLKI--QPTNVLSKLLMDEKSDSVFRLNHYPPC--------PDI--QEFNAKN--LIGFGEH : 206
SlGA2ox5    : YVTAVKRMSCEILEKLAEGLKI--HPTNVFSKLLKDEKSDSVFRLNHYPPC--------PDI--QEFNAKN--LIGFGEH : 207
NtGA2ox1    : YVSAVKKMACEILEMLAEGLNI--HPRNVFSKLLMDEKSDSVFRLNHYPPC--------PEI--QQFSDNN--LIGFGEH : 205
NtGA2ox3    : YVSAMKKMACEILEMLAEGLKI--YPKNVFSKLLMDEQSDSVFRINHYPPC--------PEV--QEFNGRN--LIGFGAH : 207
NoGA2ox2    : YVSSVKKMACEILELMAEGLNI--QPKNVFSKLLMDEQSDSVFRLNHYPPY--------PDL--QELHGRN--LIGFGEH : 205
LsGA2ox1    : YVTAVKKMACEILELLADEMKL--QPRNVFSKLLMDEQSDSVFRVNHYLPC--------PEF--QENERNGRKLVGFGEH : 209
RpGA2ox1    : YVAAVKSMACDVLELMADGLRI--QQKDVFSKLLMDEQSDSMFRVNHYPPY--------PEL--QGFKDRN--LIGFGEH : 180
PsGA2ox1    : YKCAMRNMACEILDLMAEGLKI--QPKNVFSKLVMDKQSDCLFRVNHYPAC--------PEL---AINGEN--LIGFGEH : 200
VaGA2oxA2   : YMCSVRKMACDILELMAEGLKI--QQKNVFSKLLRDKESDSVFRVNHYPAC--------PEL---AVNGEN--MIGFGEH : 200
NtGA2ox5    : YVSAVRNLACLALEKIAEGLRI--EPKNVLSKMLRDEKSDSCFRLNHYPPF--------PELL-QTLSGRN--LIGFGEH : 207
NoGA2ox3    : YVSAVRSMACDVLDMIADGLKI--GPRNVLSRLLMDEKSDAVFRLNHYPPC--------PEL--QALSGRN--LIGFGEH : 206
LsGA2ox2    : YVKEVRKLGCTILELMAEGLKI--EPKNVLSRMLSDENADTVFRLNHYPPCLDPNSNHDSDLN-NKSMSHGRTSIGFGEH : 208
PcGA2ox1    : YITAVKNMCYAVLELMAEGLGI-RQR-NTLSRLLKDEKSDSCFRLNHYPPC--------PEVQ--ALN-RN--LVGFGEH : 204
VaGA2oxA1   : YITAVKNMCYAVLELMAEGLGI-AQR-NTLSRLLKDEKSDSCFRMNHYPPC--------PEVP--ALN-RN--LVGFGEH : 204
SoGA2ox1    : YISKMKKMGVRVLETMAEGLNLKGEDRYALSNLIKDSKSDSYFRLNHYPPR--------PEAFE-GINGRN--LVGFGEH : 209
AtGA2ox2    : YMKEIKEVSYKVLEMVAEELGI--EPRDTLSKMLRDEKSDSCLRLNHYPAA--------EE------EAEKMVKVGFGEH : 207
AtGA2ox3    : YIKEMKRMSSKFLEMVEEELKI--EPKEKLSRLVKVKESDSCLRMNHYPEK--------EE------TPVKE-EIGFGEH : 202
AtGA2ox1    : YTTSVRKMTFDVLEKITDGLGI--KPRNTLSKLVSDQNTDSILRLNHYPPC--------PLSNK-KTNGGK-NVIGFGEH : 197
HvGA2ox5    : YAAAVRRMACGVLELMAEGLGI--GPADALSRLVSDGESDNMLRVNHYPPR--------PEM-------QGCLLTGFGEH : 217

                       *       260          *        280          *        300          *        320
HvGA2ox4    : TDPQLVSILHSNGTAGLQVALHDGRWVSVPPNRDAFFVNVGDSLQVLTNGRLRSVRHRVVAGNGLKSRVSMIYFAGPPLA : 283
OsGA2ox3    : TDPQLVSVLRSNGTSGLQIALRDGQWVSVPSDRDSFFVNVGDSLQVLTNGRFKSVKHRVVA-NSLKSRVSFIYFGGPPLA : 281
SlGA2ox4    : TDPQIMSILRSNNTSGLQILLKNGNWFSVPSDQNSFFVNVGDSLQVMTNGRFKSVKHRVLT-NSVKSRLSMIYFGGPPLS : 285
SlGA2ox5    : TDPQIISVLRSNNTSGLQILLKNGNWFSVPPDQSSFFVNVGDSLQVMTNGRFKSVKHRVLT-NSVKSRLSMIYFGGPPLS : 286
NtGA2ox1    : TDPQIISVLRSNNTSGLQILLKNGHWISVPPDPNSFFINVGDSLQVMTNGRFKSVRHRVLA-NSVKSRLSMIYFGGPPLS : 284
NtGA2ox3    : TDPQIISLLRSNNTSGLQISLEDGHWISVPPDQNSFFINVGDSLQVMTNGRFKSVKHRVLA-NSLKSRLSMIYFGGPPLS : 286
NoGA2ox2    : TDPQIISVLRSNNTSGLQIQLKDGSWISIPPDQSSFFINVGDSTQVLTNGRFKSVKHRVLA-NSMKSRLSMIYFGGPPLS : 284
LsGA2ox1    : TDPQIISVLRSNNTSGLEISLRDGSWMSVPADSDSFFINVGDSLQVMTNGRFKSVRHRVVA-NSTKSRVSMIYFGGPPLS : 288
RpGA2ox1    : TDPQIISVLRSNNTSGLEISLRDGSWVSVPPDQTSFFINVGDSLQVMTNSKFKSVRHRVLA-NSYKSRVSMIYFGGPPMS : 259
PsGA2ox1    : TDPQIISILRSNNTSGFQISLRDGSWISVPPDHSSFFINVGDSLQVMTNGRFKSVRHRVLA-NGIDPRLSMIYFCGPPLS : 279
VaGA2oxA2   : TDPQIISLLRSNNTSGLQIYLRDGSWISVPPDHNSFFINVGDSLQVMTNGRFRSVRHRVLA-NGFKSRLSMIYFGGPPLS : 279
NtGA2ox5    : TDPQVISVVRSNNTSGLQISLKDGTWLSVPPDPYSFFINVDDSLQVMSNGRFRSVRHRVLA-DSMKSRVSMIYFGGPPLS : 286
NoGA2ox3    : TDPQIISVVRSNNTSGLQISLKDGTWVSVPPDQYSFFINVGDSLQVMTNGRFKSVKHRVFA-DGLKSRVSMIYLGGPPLD : 285
LsGA2ox2    : TDPQLISIARSNATSGFQIYLEDGTWVGVPPDETSYFINVDDLLEVMTNGRFRSVRHRVVA-DSFKSRVSMIYFGGPPLM : 287
PcGA2ox1    : TDPQIISVLRSNSTSGLQICLTDGTWVSVPPDQTSFFINVGDALQVMTNGRFKSVKHRVLA-DTTKSRLSMIYFGGPALS : 283
VaGA2oxA1   : TDPQIISVLRSNSTSGLQICLADGTWVSVPPDQTSFFINVGDALQVMTNGRFKSVKHRVLA-DTTKSRLSMIYFGGPALS : 283
SoGA2ox1    : TDPQIISVLRSNKIGGLQISLNDGTWVSVPPDDNSFFILVGDSLQVMTNGRFKSVKHRVLA-DNMKSRLSMIFFAGPPLC : 288
AtGA2ox2    : TDPQIISVLRSNNTAGLQICVKDGSWVAVPPDHSSFFINVGDALQVMTNGRFKSVKHRVLA-DTRRSRISMIYFGGPPLS : 286
AtGA2ox3    : TDPQLISLLRSNDTEGLQICVKDGTWVDVTPDHSSFFVLVGDTLQVMTNGRFKSVKHRVVT-NTKRSRISMIYFAGPPLS : 281
AtGA2ox1    : TDPQIISVLRSNNTSGLQINLNDGSWISVPPDHTSFFFNVGDSLQVMTNGRFKSVRHRVLA-NCKKSRVSMIYFAGPSLT : 276
HvGA2ox5    : TDPQIISVLRSNGTSGLEICARDGEWTSVPPDPDAFFVNVADALQVLTNGRFSSVKHRVVV-SSERPRVSMIFFGGPPMG : 296

                       *       340          *        360          *
HvGA2ox4    : QRIAPLQQLLAGTQSLPLYRDFTWGEYKKAAYRSRLGDNRLAPFETPLVAMPHAAHHRS : 342
OsGA2ox3    : QRIAPLPQLL-GEGEQSLYKEFTWDEYKKAAYKSRLGDNRLAQFEKK------------ : 327
SlGA2ox4    : EKIAPLPSLMEGK-DS-LYKEFTWFEYKKKSAYKTRLADNRLVLFEKSL---------- : 331
SlGA2ox5    : EKIAPLPSLMEGE-ESSLYKEFTWFEYKKKSAFKSRLAQNRLTLFEKFGTS-------- : 335
NtGA2ox1    : EKIAPLASLMEGE-ES-LYEEFTWFEYKKKSAYKTRLADNRLVLFEKVAAS-------- : 332
NtGA2ox3    : EKIAPLASLIKGD-QDSLYKEFTWFEYKKKSAYNSRLADNRLVLFEKVL---------- : 333
NoGA2ox2    : EKIAPLPSLMEGE-DS-LYKEFTWFEYKKKSAYKSRLADNRLVLFEKIAAS-------- : 332
LsGA2ox1    : EKIAPLPSLIQGE-EDSLYKEFTWFEYKKKSAFNTRLADNRLGLFEKITAT-------- : 337
RpGA2ox1    : ERIAPLPSLMGVE-QTSLYKEFTWYEYKKATSHKSRLADNRLVPFEKIAAS-------- : 308
PsGA2ox1    : EKIAPLPSLMKGK-ES-LYKEFTWFEYKSSTYGSRLADNRLGNYERIAAT-------- : 327
VaGA2oxA2   : EKIVPLPSLMKGK-ES-LYKEFTWFEYKNSTYGSRLADNRLGHFERIAAS-------- : 327
NtGA2ox5    : EKIAPLSCLMEEG-EESLYNEFTWCEYKKSAYKTRLGDNRLALFEKKPQTKPATSAAQ- : 343
NoGA2ox3    : EKIAPLSSLMEEG-EESLYKEFTWCEYKKSAYKTRLGDNRLKFFEK--------SVGQ- : 334
LsGA2ox2    : EKISPLGSLMEPG-EESLYNEFTWFEYKSCTYKTRLTDNRLSLFHKHS------HPVH- : 338
PcGA2ox1    : ENIAPLPSVMLKG-EECLYKEFTWCEYKKAAYTSRLADNRLAPFQKSAAD--------- : 332
VaGA2oxA1   : EKIAPLPSVMVKG-EESLYKEFTWWEYKKAAYTSRLADNRLAPFEKSATD--------- : 332
SoGA2ox1    : QKIAPLPCIMQKG-EESLYEEFTWCEYKKSAYKSRLSENRLLRFVKRNHN--------- : 337
AtGA2ox2    : QKIAPLPCLVPEQ-DDWLYKEFTWSQYKSSAYKSKLGDYRLGLFEKQPLLNHKTLV--- : 341
AtGA2ox3    : EKIAPLSCLVPKQ-DDCLYNEFTWSQYKLSAYKTKLGDYRLGLFEKRPPFSLSNV---- : 335
AtGA2ox1    : QRIAPLTCLIDNE-DERLYEEFTWSEYKNSTYNSRLSDNRLQQFERKTIKNLLN----- : 329
HvGA2ox5    : ERLAPLRQLLGDG-GRSKYREFTWKEYKSSTHKGRLATDRLCSFEN------------- : 341
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CAPÍTULO III

Efecto del Silenciamiento Múltiple de GA 2-oxidasas en el Desarrollo del Tabaco

Lina Gallego-Giraldo, José L. García-Martínez e Isabel López-Díaz.

En preparación

EFFECT OF MULTIPLE GA 2-OXIDASES SILENCING IN TOBACCO DEVELOPMENT

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Running title: Multiple silencing of GA 2-oxidases in tobacco

Subject area
(1) Growth and development.
Nº of tables 4.
Nº of black and white figures 6
Nº of colour figures 2
Supplemental data: 4; 1 Table and 3 Figures

Abbreviations

2ODDs, 2-oxoglutarate-dependent dioxygenases; GA2ox, gibberellin 2-oxidase; GA3ox, gibberellin 3-oxidase; GA20ox, gibberellin 20-oxidase; PCB, Paclobutrazol; RT–PCR, reverse transcription–PCR; RNAi, RNA interference.

ABSTRACT

Gibberellins (GA) are phytohormones that regulate plant growth and development. The inactivating enzymes gibberellin 2-oxidases play an important role in the regulation of gibberellin levels and are encoded by small multigene families with some degree of functional redundancy. To understand the role of gibberellin 2-oxidases in tobacco plant development, we induced multiple silencing by the introduction of a hairpin RNAi construct carrying a sequence homologous to five *GA2ox* genes (*NtGA2ox1* to *-5*). Although we detected overlapping expression of the target *NtGA2oxs* in most tissues, we were able to obtain transgenic (*GA2ox/RNAi*) plants with substantial phenotypic alterations associated to the silencing of several *NtGA2oxs* in different tissues. The degrees of silencing of the five target genes correlated with their level of expression in wild type and not with the similarity between the target sequence and the RNAi trigger sequence (ranging from 80 to 100%). The typical characteristics of GA over-production phenotypes (enhanced length of hypocotyls, internodes, leaves, fruit peduncles, as well of the final plant height) were found in *GA2ox/RNAi* plants. The increase in GA levels in these plants was supported by the feedback repression of GA biosynthesis genes (*NtGA20ox1* and *NtGA3ox1)* and by phenotypic reversion with a gibberellin inhibitor application (paclobutrazol). Additionally transgenic *GA2ox/RNAi* plants displayed reduction of root length, flowering delay and male sterility. Our results suggest that *GA2ox* genes are important in the regulation of GA levels controlling various aspects of plant growth and development in tobacco plants.

INTRODUCTION

Gibberellins (GAs) are phytohormones that control plant growth and diverse developmental processes, including seed germination, stem elongation, leaf expansion, and flower and fruit development (reviewed by Davis, 1995; Sponsel and Hedden, 2004).

All the genes encoding enzymes of the GA metabolic pathway have been isolated and characterized, except for GA 13-hydroxylase (GA13ox), in a large number of species (reviewed by Olszewski et al., 2002). In plants, three different classes of enzymes are required for the biosynthesis of bioactive GA from geranylgeranyl diphosphate (GGDP): cyclases, cytochrome P450 monooxygenases (P450s) and 2-oxoglutarate-dependent dioxygenases (2ODDs). Two cyclases, *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene (KS) are involved in the conversion of GGDP to *ent*-kaurene. Then, *ent*-kaurene is converted to GA_{12} by two P450s, *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO). All these enzymes are encoded by one or two genes (reviewed by Yamaguchi, 2007). In the final stages of GA biosynthesis, GA_{12} is converted to bioactive GA_4 by the action of 2ODDs enzymes in the non-hydroxylation pathway. GA_{12} is also substrate for a GA13ox for the production of GA_{53}, which is a precursor of bioactive GA_1 in the early-13-hydroxylating pathway. Both GA_{12} and GA_{53} are converted into GA_4 and GA_1 through oxidations on C-20 and C-3 by GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox). Thus, GA20ox is responsible of producing C-19-GAs from C-20-GAs. The introduction of a 3β-hydroxyl group converts inactive precursors (GA_9 and GA_{20}) into bioactive GAs (Fig. 1). The 2ODDs enzymes are encoded by small multigene families with some degree of functional redundancy (reviewed by Hedden and Phillips, 2000).

The amount of bioactive GAs is determined by both the rate of GA biosynthesis and GA inactivation. The best-characterized deactivation reaction is 2β-hydroxylation catalyzed by a class of 2ODDs, GA 2-oxidases (GA2ox). GA2ox use bioactive GAs (GA_1 and GA_4) and their precursors as substrates to generate inactive forms (Yamaguchi, 2007). A second group of GA2ox enzymes can inactivate C-20-GAs like GA_{12} and GA_{53} (Schömburg et al., 2003) and also reduce the flux to active GAs. Other GA deactivation mechanisms have been described in rice and consist of 16α, 17-epoxidation of bioactive GA_4 (Zhu et al., 2006) and methylation of GA by gibberellins methyltransferases (GAMT1 and GAMT2) in *Arabidopsis* seeds (Varbanova et al., 2007).

GA homeostasis is regulated by a feedback mechanism where bioactive GA, regulate the expression of genes coding for 2ODDs enzymes (Fig. 1) (review in Olszewski et al., 2002). With a few exceptions, most *GA20ox* and *GA3ox* genes are negatively feedback regulated by GA application (review in Hedden and Phillips, 2000). In contrast, the expression of *GA2ox* genes are positively feedback regulated after GA application (Thomas et al., 1999; Elliot et al., 2001). Moreover, it has been described that some of the *GA20ox* and *GA3ox* genes are negatively feedback regulated in a GA dose-dependant manner at least during hypocotyl growth (Cowling et al., 1998 and Gallego-Giraldo et al., 2008). Therefore, quantification of their mRNA levels can be used to estimate GA levels in this tissue.

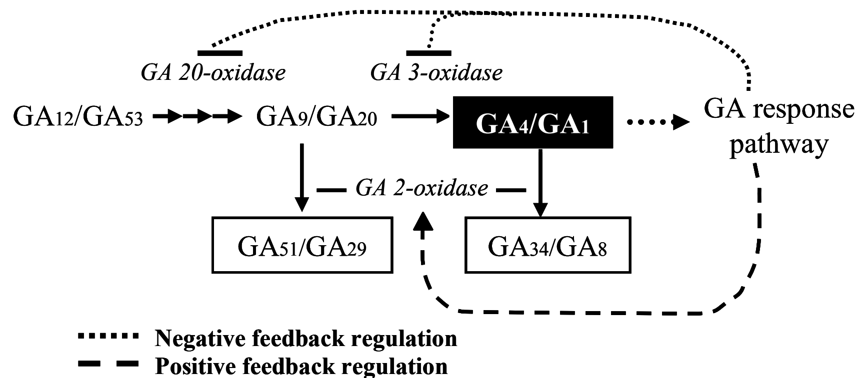

Figure 1. GA metabolic pathway showing GA 20-, 3- and 2-oxidation steps. Active GA are indicated by a solid black square and inactive GA products by open squares. Positive and negative regulations are indicated by dotted lines.

It is well known that GA levels modulate stem elongation during plant development. GA deactivation appears to be a complex and tightly regulated process which controls GA levels. Therefore, to understand *GA2ox* regulation is important to know the basis of this process. For instance, environmental factors such as light (Yamauchi et al. 2007, Reid et al., 2002) and temperature (Stavang et al., 2007) that influence growth elongation specifically regulate the expression of some *GA2ox* genes. Expression of *GA2ox* genes can also be regulated by developmental signals. In rice it has been reported that specific regulation of *OsGA2ox1* occurs during floral induction. After phase transition to the reproductive stage, expression level of this gene was drastically reduced. This drives the authors to speculate that *OsGA2ox1* plays an important role in the control of GA levels during floral transition (Sakamoto et al., 2001). However little is known about the different roles of each gene member of *GA2ox* families. There are a few genetical evidences revealing the specific function of some of these genes. In particular, a null mutation from pea (*sln)* in the *PsGA2ox1* gene produces a GA overdose (slender) phenotype, (Martin et al., 1999; Lester et al., 1999). The clear phenotype found in this mutant is due to a very specific expression pattern reported for *PsGA2ox1* and *PsGA2ox2*. *PsGA2ox1* is highly expressed in maturing seeds, where catalyzed the conversion of GA_{20} to GA_{29} and *PsGA2ox2* is preferentially expressed in shoots (Lester et al., 1999; Martin et al., 1999). The elongated shoot phenotype of this mutant is due to enhanced elongation of the first internodes only and appears to arise from transport of accumulated GA_{20} from the seed into the young shoot after germination (Reid et al., 1992; Ross et al., 1993). This indicates the importance of *PsGA2ox1* in the control of GA levels during early plant growth. In contrast single mutants of *GA2ox* genes from *Arabidopsis* have a slight phenotype due to functional redundancy among *GA2ox* genes (Schömburg et al., 2003). Only a double mutant of *AtGA2ox* genes (*AtGA2ox7* and -*8*) produced a moderate phenotype. The redundancy between *GA2ox* could explain why in some species for instance in rice, the mutations in a single *GA2ox* gene has not been isolated (Sakamoto et al., 2004).

With the aim to study the role of GA 2-oxidases in tobacco development, we induce the multiple silencing of several *GA2ox* genes by a RNA interference technique. Although we detected overlapping expression of several *NtGA2ox* genes, we were able to obtain diverse phenotypes in transgenic silenced plants. These phenotypes correlated with the degree of *GA2ox* silencing in several tissues. Our results

suggest that *GA2ox* genes are important in the regulation of GA levels controlling plant growth, flowering transition and male fertility in tobacco plants.

RESULTS

NtGA2ox gene family

Tobacco GA 2-oxidase family has been poorly studied. There are five sequences, *NtGA2ox1* to *-5,* already deposited in the database. The function of these five genes has been inferred from their high homology to previously identified *GA2ox* clones, whose enzymatic activity had been confirmed *in vitro* (Supplemental data S2). Deduced amino acid sequence showed the presence of conserved residues involved in Fe^{2+} binding (HTD located between position 248-250 and H at the position 305) and the 2-oxoglytarate binding domain (RLS located between position 218-220), suggesting that all of them encode 2-oxoglutarate-depending dioxygenases. Moreover, the five *NtGA2ox* genes clustered with the group of *GA2ox* that use C19-GAs (GA_{20}, GA_1, GA_9 and GA_4) as substrate, within the group I, that according to Serrani et al., (2007) include the enzymes having multicatalytic activity (Supplemental data S3). Among themselves *NtGA2ox1, -2* and *-4* are the most similar and *NtGA2ox5* the least (Supplemental data S3).

Expression pattern and feed-back regulation of tobacco GA 2-oxidase genes

In order to characterize the tobacco *GA2ox*, the expression of the five *GA2ox* genes in different tissues of wild type tobacco plants was analyzed by semi-quantitative RT-PCR. Expression of all *NtGA2ox* was detected both in vegetative and reproductive tissues, although transcript levels were different for each gene (Fig. 2).

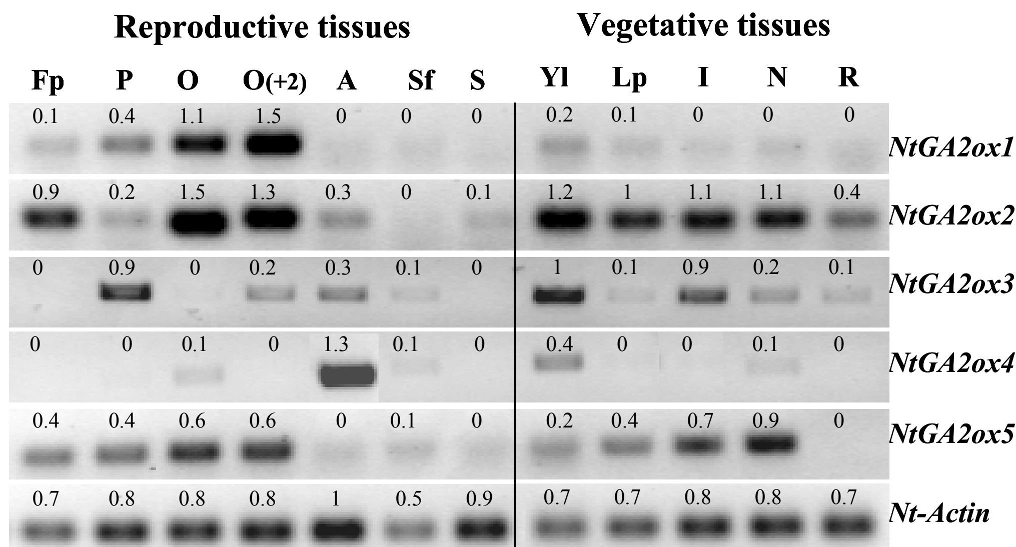

Figure 2. Distribution of transcript level of *NtGA2ox* in different tissues of tobacco wild-type plants. Transcript analysis was carried out by semi-quantitative RT-PCR, using total RNA from reproductive and vegetative tissues. The reproductive tissues were taken from flowers at 2-d-before anthesis. Flower peduncle (Fp), Petals (P), Ovary (O), Ovary at 2-d-after anthesis (O+2), Anther (A), Stamen filament (Sf) and Style (S). The vegetative tissues were taken from 40-d-old plants. Young leaves ≤ 3cm (Yl), Leaves peduncle (Lp), Internodes (I), Nodes (N) and Roots (R). For each gene, number over the amplified band are normalized respect to the *NtActin* gene (used as an internal control; anther expression set as 1.0). Results come from a representative experiment out of two biological replicates with similar results.

Within reproductive tissues, *NtGA2ox1, -2* and *-5* transcripts were the most abundant in ovaries, and were also detected in flower peduncles and petals. *NtGA2ox3, -1* and *-5* were predominantly expressed in petals and *NtGA2ox4* and *-3* in anthers. Stamen filaments and styles had very little expression of any of the five genes. On the other hand, in vegetative tissues, the expression of *NtGA2ox2* was quite high in all the organs studied (leaves, stems and roots) while *NtGA2ox1* and *NtGA2ox4* were only slightly detected in young leaves. *NtGA2ox5* was predominantly expressed in the stem (internodes and nodes) and *NtGA2ox3* in young leaves and stems.

Some of the *NtGA2ox* genes (*NtGA2ox1*, *-3* and *-5*; Gallego-Giraldo et al., 2008) are known to be positively feedback regulated by active GA levels (Fig. 1). To determine whether all of them were regulated in the same way, we compared their expression pattern in response to changes in GA concentrations. Relative mRNA abundance of *NtGA2ox* was measured by real-time RT-PCR in the aerial part of 7-d-old seedlings (hypocotyls plus cotyledons), cultured in a medium supplemented with different doses of GA_1 and Paclobutrazol (a biosynthesis inhibitor, PCB). Transcript levels of the five *GA2ox* decreased gradually with the increase of PCB doses (Fig. 3). This result is expected for a feedback repression caused by the reduction of endogenous GA content. When seedlings were grown with GA_1, *GA2ox* expression was induced although two different patterns were found. *NtGA2ox1*, *-2* and *-4* mRNA levels increased proportionally to GA_1 dose with a maximum at 10^{-6}M following a pattern parallel to hypocotyl growth response. In contrast, *NtGA2ox3* and *-5* had a major induction at a much lower GA_1 dose (10^{-8} M, Fig. 3). The variation of mRNA abundance of *NtGA2ox* genes confirms that they are all positively feedback regulated by increases of active GA contents.

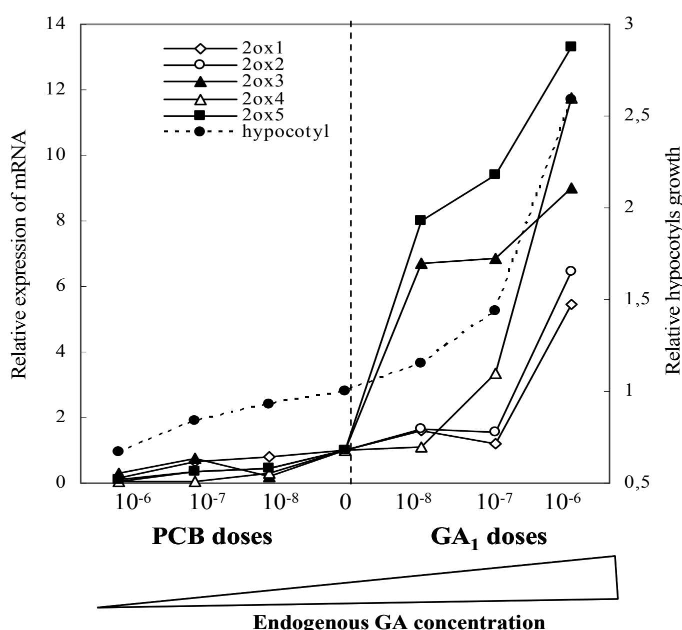

Figure 3. Feedback regulation of *NtGA2ox* genes in response to GA content modifications.
Variations of relative abundance of *NtGA2ox* mRNA and hypocotyl growth from 7-d-old seedlings cultured in a MS medium without (0) or with different doses (10^{-8} M, 10^{-7} M and 10^{-6} M) of GA_1 and PCB. Values of relative abundance of *GA2ox* mRNA were calculated from data of transcript levels of *GA2ox* quantified by real time RT-PCR. In this analysis the relative expression of mRNA was normalized relative to the *NtActin* gene and values are means of two biological replicates ± SE. Variations values of hypocotyls length were performed from data of results means of 50 seedlings ± SE. All data are expressed relative to the values in the medium without any addition.

Isolation of homozygous tobacco *GA2ox*/RNAi lines

The overlapping expression pattern between the five *NtGA2ox* genes (Fig. 2) suggests the possibility of functional redundancy between members of this family. For this reason to investigated the function of the *NtGA2ox* family we carried out an RNAi-based approach using a highly conserved region of 280-bp in the *GA2ox* family sequences with the aim of producing multiple silencing. The sequence region used to prepare the RNAi construct was perfectly conserved in the *NtGA2ox4* gene and had only a few mismatches in the others *NtGA2ox* sequences (Table 1). This similarity should be sufficient to allow the generation of hypothetical RNA fragments of at least 21-nt long complementary to all five *GA2ox* sequences (Table 1). Therefore, the RNAi construct should target indistinctly all members of the *NtGA2ox* family and inhibit their mRNA expression.

Table 1. Comparison of each *NtGA2ox* sequences with the *GA2ox*/RNAi fragment used for silencing plants. The second column indicates the hypothetical RNA fragments of at least 21-nt long complementary to all five *GA2ox* sequences that could activated a silencing phenomenon.

	Nº of mismatches between the *GA2ox*/RNAi fragment and each *NtGA2ox* sequences	Nº of continuous fragments non overlapping of at least 21-nt long identical to *GA2ox*/RNAi fragment
NtGA2ox1	14	8
NtGA2ox2	22	7
NtGA2ox3	34	3
NtGA2ox4	0	12
NtGA2ox5	56	2

The selected region was cloned in both sense and antisense orientations in the vector pHannibal (Helliwell and Waterhouse, 2003) as described in Material and Methods. The hairpin *GA2ox*/RNAi construct expressed under the control of the 35S promoter was used to obtain transgenic tobacco *GA2ox*/RNAi lines. The main phenotypes detected in 30 independent T1 plants were the increase elongation of stems, inflorescences, and flowers in addition to low fertility (fruits with reduced number of seeds). To determine whether the *GA2ox/*RNAi construct had any effect on the expression of *NtGA2ox* genes we studied individual *GA2ox* transcript levels in different tissues (leaves, anthers, filament, ovaries and style) by semi-quantitative RT-PCR analysis (data not shown). The lines showing the most extreme phenotype and a clear reduction in the abundance of *GA2ox* transcripts were selected to be studied in the next generations.

Five independent homozygous transgenic lines, segregating the kanamycin marker as a single locus, were obtained (i4.2, i9.1, i44, i46 and i51) and further characterized. We tested if the *GA2ox*/RNAi transgene was able to maintain the silencing signal in homozygous lines by detecting the accumulation of short RNA fragments (sRNA 21-25 nt long), which are characteristic of the RNAi induced post-transcriptional gene silencing (review in Meins et al., 2005). Northern blot analysis from seedlings (hypocotyls plus cotyledons) of homozygous transgenic tobacco *GA2ox*/RNAi plants confirmed the

presence of sRNA in all the *GA2ox*/RNAi lines (Fig. 4). This result indicated that the transgene was stable and active at T3 generation.

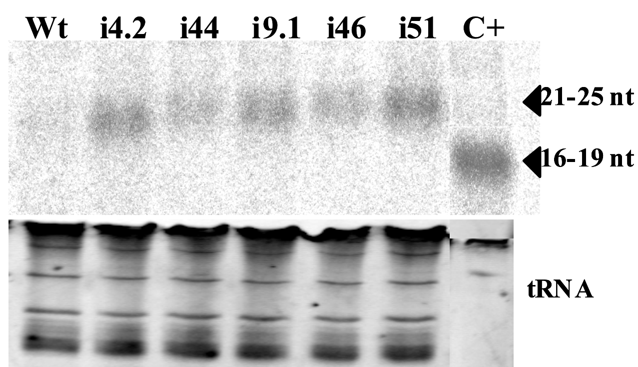

Figure 4. Detection of small RNA (sRNA 21-25 nt long) in *GA2ox/*RNAi transgenic homocigous lines. Total RNA was isolated from 7-d-old seedlings (hypocotyl plus cotyledons) of wild-type and transgenic lines (i4.2, i44, i9.1, i46 and i51) cultured in a MS medium. Total RNA was separated in a 15% denaturing polyacrilamide gel, blotted onto a Hybond-N filter and hybridized with radioactively labeled riboprobe containing the sequence used for the *GA2ox*/RNAi construct. sRNA (16-19 nt long) obtained by digestion of the *GA2ox*/RNAi sequence with RNase III enzyme was used as a positive hybridization control (C+) and as a molecular size marker.

Inhibition of *GA2ox* expression in transgenic tobacco plants by RNA interference leads to increased stem elongation, flowering delay, low fertility and variation in flower and inflorescence length

Vegetative characterization

Time of germination did not show significant difference between transgenic *GA2ox*/RNAi and wild-type plants (Table 2). In contrast, hypocotyl and internode length had higher values in transgenic *GA2ox*/RNAi than in control plants (Table 2). Leaves were bigger (Table 2 and Fig. 6B) and stems thicker (Table 2). Interestingly, root length was shorter in transgenic *GA2ox*/RNAi plants compared to wild type (Table 2). The increase in stem height (Fig. 5A) was also due to a higher number of vegetative nodes (Fig. 5B) in addition to increased internode length (Table 2). These results indicate that flowering delay occurred in all five transgenic *GA2ox*/RNAi compared to wild type plants.

Reproductive characterization

Transgenic *GA2ox*/RNAi flowers exhibited an elongated phenotype. The perianth, pistil and stamens were longer compared to wild type and frequently, styles and stamens override the petals in transgenic flowers (Fig. 6A). In fact, significant length increments were detected in ovaries, styles, filaments, anthers and petals (Table 3). In addition, we observed reduced fertility in all transgenic plants compared to wild type. This phenotype of infertility was revealed by the production of smaller fruits (Fig. 6E) with less weight and less number of seeds (Table 3). To investigate the basis of infertility, pollen viability of *GA2ox*/RNAi anthers was examined. Viable pollen grains were red darken by the aceto-carmine test, contrary to unviable pollen (Fig. 6C). Compared to wild type, low viability of pollen was found in all transgenic lines (Table 3). We also tested the possible ovules infertility by pollinating

emasculated flowers of some transgenic lines with wild type pollen. Fruit weigh was mostly restored in all cases (Table 3), indicating that transgenic ovules were fertile and that the reduced fertility of transgenic *GA2ox/RNAi* plants was mainly due to male sterility.

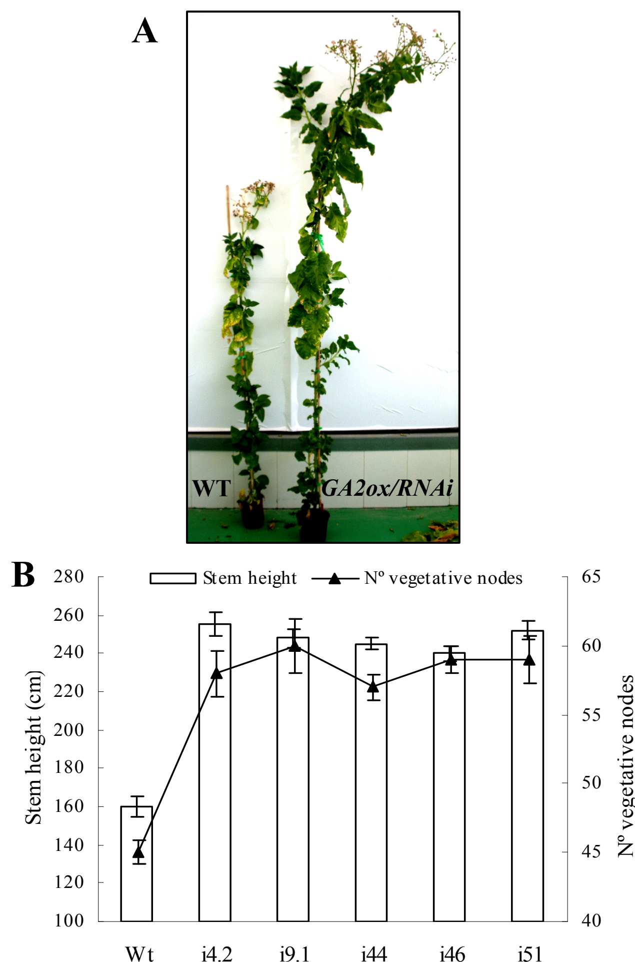

Figure 5. Flowering delay in transgenic *GA2ox*/RNAi plants. (A) Pictures of representative plants from wild type and transgenic *GA2ox/RNAi* plant. (B) Stem length and number of vegetative nodes in transgenic *GA2ox*/RNAi and wild type. Results are the average of 5 plants ± SE.

Table 2. Phenotypic characterization of wild type (Wt) and homozygous transgenic *GA2ox/*RNAi lines (i4.2, i9.1, i44, i46 and i51).

Lines	Percentage of germination	Hypocotyl length (mm)	Root length (mm)	Average of internodes length (cm)	Width of leaf Nº 30 (cm)	Length of leaf Nº 30 (cm)	Stem diameter (cm)
Wt	50^a	$3,3 \pm 0,1^a$	$14,6 \pm 0,1^a$	3.55 ± 0.6^a	13.2 ± 0.3^a	21.9 ± 0.5^a	1.5 ± 0.02^a
i4,2	48^a	$4,6 \pm 0,2^b$	$13,7 \pm 0,1^a$	4.39 ± 0.8^b	14.2 ± 0.4^b	24.5 ± 0.4^b	1.65 ± 0.04^b
i9,1	51^a	$4,7 \pm 0,2^b$	$13,6 \pm 0,1^a$	4.13 ± 0.5^b	15.3 ± 0.3^b	25.2 ± 0.3^b	1.7 ± 0.02^b
i44	52^a	$4,4 \pm 0,1^b$	$13,5 \pm 0,2^a$	4.29 ± 0.6^b	15.6 ± 0.4^b	24.5 ± 0.4^b	1.59 ± 0.03^b
i46	49^a	$4,5 \pm 0,1^b$	$13,4 \pm 0,2^a$	4.06 ± 0.2^b	14.9 ± 0.6^b	26.1 ± 0.4^b	1.63 ± 0.05^b
i51	50^a	$4,5 \pm 0,1^b$	$13,6 \pm 0,1^a$	4.27 ± 0.4^b	14.8 ± 0.5^b	25.3 ± 0.7^b	1.62 ± 0.06^b

Percentages of germination is the result of a representative experiment out of three independent experiment with similar results and were determinate after 100 hours, when approximated 50% of wild type seeds had germinated in MS medium. At least 100 seeds were cultured by line. For hypocotyl and root length, seedlings were culture in a MS medium for 7 days and at least 30 seedlings were taken for the measurements. For characters measure in adult plants, all plants were cultured in a greenhouse at 28ºC under long day (LD) conditions and these results are means of 6 plants per line ± SE. For each column, values with different letter were significantly different ($P < 0.05$).

Table 3. Reproductive organs characterization of wild type (Wt) and homozygous transgenic *GA2ox/*RNAi lines (i4.2, i9.1, i44, i46 and i51).

Lines	Style length (mm)	Stamen filament length (mm)	Petals length (mm)	Ovary length (mm)	Anther length (mm)	Fruit weight (mg)	Nº seeds by fruit	Fruit weight of pollinated flowers with wt pollen (mg)	Percentage of pollen viability
Wt	$32 \pm 0,8^{a}$	$40 \pm 0,6^{a}$	$38 \pm 0,5^{a}$	$8 \pm 0,05^{a}$	$3 \pm 0,02^{a}$	230 ± 3^{a}	2200^{a}	$246 \pm 3,2^{a}$	95 ± 2^{a}
i4,2	$38 \pm 0,5^{b}$	$48 \pm 0,4^{b}$	$47 \pm 0,5^{b}$	$14 \pm 0,05^{b}$	$4 \pm 0,05^{b}$	150 ± 4^{b}	1230^{b}	$210 \pm 5,3^{b}$	$70 \pm 1,5^{b}$
i9,1	$41 \pm 0,7^{b}$	$53 \pm 0,5^{b}$	$51 \pm 0,4^{b}$	$15 \pm 0,04^{b}$	$4,2 \pm 0,04^{b}$	113 ± 5^{b}	800^{c}	---	$72 \pm 1,4^{b}$
i44	$39 \pm 0,5^{b}$	$47 \pm 0,3^{b}$	$45 \pm 0,5^{b}$	$12 \pm 0,05^{b}$	$3,9 \pm 0,05^{b}$	160 ± 6^{b}	1150^{c}	---	$78 \pm 1,3^{b}$
i46	$39 \pm 0,6^{b}$	$48 \pm 0,5^{b}$	$45 \pm 0,4^{b}$	$13 \pm 0,02^{b}$	$3,9 \pm 0,04^{b}$	155 ± 3^{b}	1173^{b}	---	$72 \pm 1,2^{b}$
i51	$40 \pm 0,3^{b}$	$47 \pm 0,3^{b}$	$46 \pm 0,5^{b}$	$12 \pm 0,05^{b}$	$4 \pm 0,04^{b}$	157 ± 4^{b}	1050^{c}	$222 \pm 3,1^{b}$	$74 \pm 1,4^{b}$

For style, stamen, petals, ovary and anther length the results are means of at least 40 flowers taken 2-d-before anthesis. The percentage of pollen viability was determined by aceto-carmine test; values are mean of three measurements. For fruit weight at least 35 fruits were taken. For each column, values with different letter were significantly different ($P < 0.05$).

Table 4. Inflorescence characterization of wild type (Wt) and homozygous transgenic *GA2ox/*RNAi lines (i4.2, i9.1, i44, i46 and i51).

Lines	Internodes length I4 (cm)	Internodes length I5 (cm)	Branch length BA (cm)	Branch length BB (cm)	Branch length BC (cm)	Branches length BD (cm)	Branch length BE (cm)	Peduncle length PA (cm)	Peduncle length PC (cm)	Peduncle length PE (cm)
Wt	$2,5 \pm 0,5^{a}$	$2,7 \pm 0,2^{a}$	$7 \pm 1,5^{a}$	$8,6 \pm 0,7^{a}$	$8,7 \pm 1,3^{a}$	$8 \pm 1,2^{a}$	$8,3 \pm 0,8^{a}$	$1,1 \pm 0,03^{a}$	$1,2 \pm 0,06^{a}$	$1,4 \pm 0,05^{a}$
i4,2	$4,3 \pm 0,5^{b}$	$5,7 \pm 0,3^{b}$	$11 \pm 1,6^{b}$	$14 \pm 1,3^{b}$	$12 \pm 1,6^{b}$	$15 \pm 1,8^{b}$	$17 \pm 1,6^{b}$	$1,6 \pm 0,04^{b}$	$1,7 \pm 0,05^{b}$	$1,8 \pm 0,05^{b}$
i9,1	$4,7 \pm 0,3^{b}$	$5,6 \pm 0,2^{b}$	$13 \pm 1,7^{b}$	15 ± 2^{b}	$13 \pm 1,5^{b}$	$17 \pm 1,5^{b}$	$18 \pm 1,3^{b}$	$1,6 \pm 0,05^{b}$	$1,8 \pm 0,04^{b}$	$1,9 \pm 0,05^{b}$
i44	$4,5 \pm 0,5^{b}$	$5,4 \pm 0,3^{b}$	$12 \pm 1,6^{b}$	$13 \pm 1,7^{b}$	$14 \pm 1,4^{b}$	$15 \pm 1,4^{b}$	$18 \pm 1,3^{b}$	$1,5 \pm 0,05^{b}$	$1,7 \pm 0,05^{b}$	$1,7 \pm 0,05^{b}$
i46	$3,9 \pm 0,4^{b}$	$5,2 \pm 0,4^{b}$	$13 \pm 0,8^{b}$	$15 \pm 1,2^{b}$	$14 \pm 1,2^{b}$	$16 \pm 1,2^{b}$	$19 \pm 1,7^{b}$	$1,6 \pm 0,04^{b}$	$1,8 \pm 0,06^{b}$	$1,7 \pm 0,04^{b}$
i51	$4,6 \pm 0,5^{b}$	$5,1 \pm 0,3^{b}$	$14 \pm 1,2^{b}$	$15 \pm 1,3^{b}$	$13 \pm 1,4^{b}$	$15 \pm 1,3^{b}$	$18 \pm 1,5^{b}$	$1,5 \pm 0,04^{b}$	$1,7 \pm 0,04^{b}$	$1,7 \pm 0,06^{b}$

The tobacco inflorescence diagram in Fig. 6G indicates the parameters taken for the measurement. Results are means of 6 inflorescences per line ± SE at the same stage of development. Peduncle length is the average length of at lest 5 peduncle per branch ± SE. For each column, values with different letter were significantly different ($P < 0.05$).

Inflorescence characterization

A typical tobacco inflorescence is a determinate panicle type. This inflorescence architecture has a terminal flower and underneath there are several floral branches with panicle appearance. Transgenic *GA2ox*/RNAi inflorescences had elongated phenotype compared to wild type (Fig. 6F). We found length increments in some internodes of the principal stem of the inflorescence, in floral branches and in flower peduncles (Table 4; see the inflorescence diagram of Fig. 6G for the parameters measured). Additionally, the appearance of floral branches in transgenic *GA2ox*/RNAi plants changed from panicle to raceme, due to a higher production of flowers (aprox. 25 to 30) compared to wild-type (which had about 8-12) (Fig. 6F). Flower production in transgenic *GA2ox*/RNAi was extended for about one more month compared to control plants. This suggests that the activity of the meristem of *GA2ox*/RNAi floral branches may last longer than in wild-type.

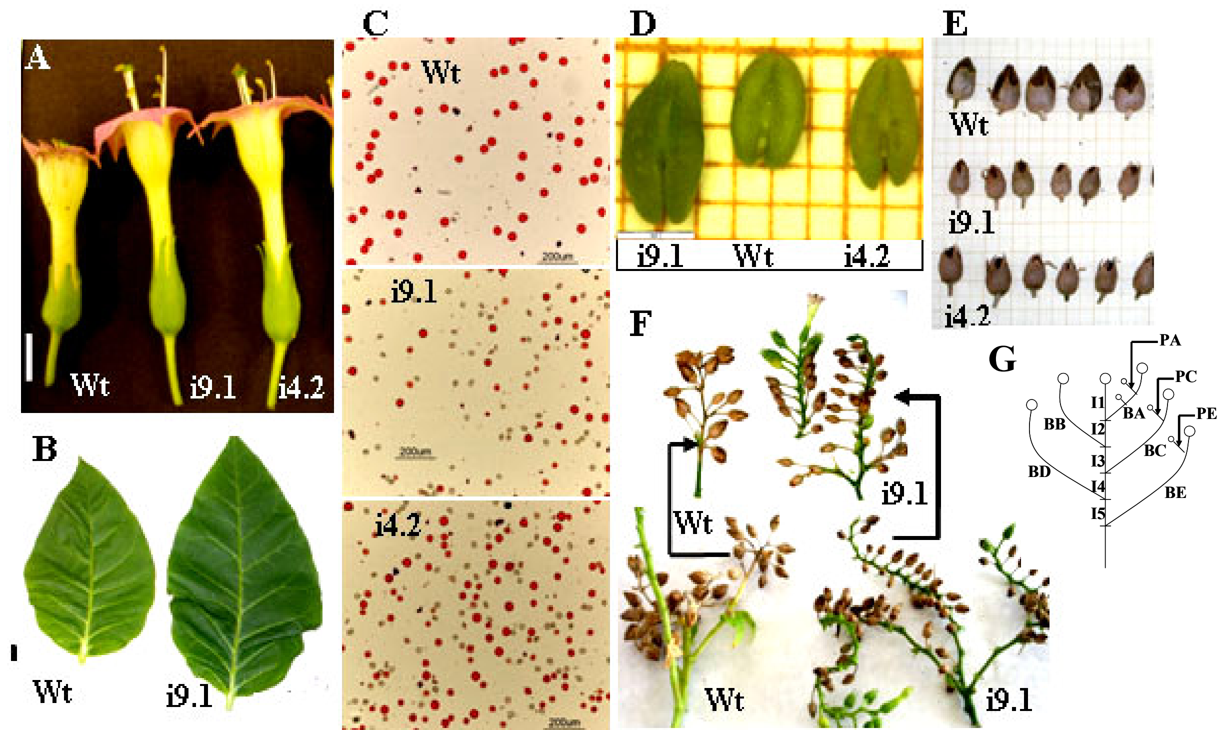

Figure 6. Phenotype of transgenic *GA2ox*/RNAi plants.
(A) Representative flowers 2-d-afther anthesis from wild type and transgenic (i9.1 and i4.2) lines. White bar represent 1 cm. (B) Representative leaves from wild type and transgenic line i9.1 at internode nº 10. These leaves were taken when their growth was completed from 40-d-old plants. Black bar represent 1 cm. (C) Tests of pollen viability by aceto-carmine staining from wild type and transgenic (i9.1 and i4.2) lines. Pollen samples were taken from anthers 2-d-afther anthesis. (D) Representative anthers from wild type and transgenic (i9.1 and i4.2) lines at 2-d-before anthesis. (E) Representative fruits from wild type and transgenic (i9.1 and i4.2) lines. (F) Picture of representative inflorescences from wild type and transgenic i9.1 line. In detail the floral branches from wild-type and transgenic line. (G) Diagram of a typical tobacco inflorescence where the parameters measured corresponding to data present in Table 4 are indicated.

Multiple silencing of *NtGA2ox* in different tissues of transgenic plants

To test how the phenotypes previously characterized in transgenic *GA2ox*/RNAi plants correlated with the reduction in the mRNA levels of the *GA2ox* genes, we quantified by real time RT-PCR their expression in tissues where phenotypic alterations had been detected. Tissues examined were seedlings (hypocotyls plus cotyledons), apical young leaves, anthers and ovaries (both 2-d-before anthesis) from transgenic (i9.1 and i4.2) and wild-type plants. In addition, we quantified the expression of GA biosynthesis genes (*NtGA20ox1* and *NtGA3ox1)* since it had been described that their transcript levels change according to the endogenous active GA content (Gallego-Giraldo et al., 2008). When the GA content increases, the mRNA abundance of these genes is reduced, due to negative feedback regulation (Fig. 1), and therefore we could use the expression of these genes as an indication of the active GA level in these tissues.

In all tissues examined we detected silencing of at least two *NtGA2ox*, although we did not find total transcript reduction for any *NtGA2ox* (Fig. 7). In transgenic *GA2ox*/RNAi seedlings a significant reduction of *NtGA2ox1* and *-2* expressions (around 57%) were detected compared to wild type (Fig. 7A).

In transgenic young leaves expression was reduced in *NtGA2ox2* (66%) and in *NtGA2ox3*, *-2* and *-4* (around 45%) (Fig. 7B). In transgenic ovaries, mRNA levels were reduced for *NtGA2ox1* (about 78%), and *NtGA2ox2* and -5 (56% and 55% respectively) (Fig. 7C). In anthers *NtGA2ox4* was the most silenced gene (81%) followed by *NtGA2ox3* (44%) (Fig. 7D). These results demonstrated the efficiency of the *GA2ox*/RNAi construct to induce degradation of its target genes in different plant tissues.

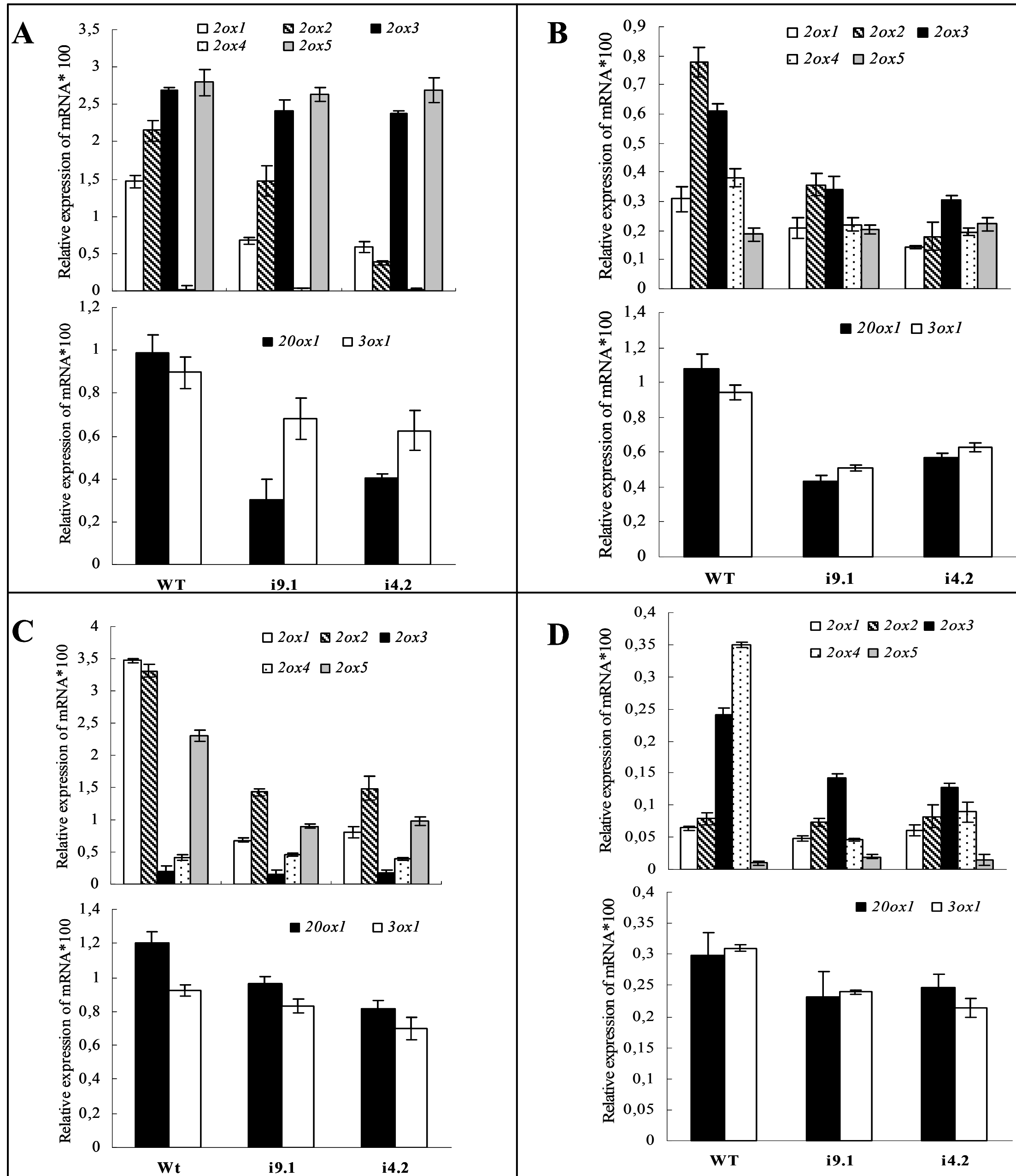

Figure 7. Multiple silencing of *NtGA2ox* in different tissues from transgenic *GA2ox*/RNAi plants. Expression levels of *NtGA2ox*, *NtGA20ox1* and *NtGA3ox1* was quantified by real time RT-PCR in wild type and transgenic i9.1 and i4.2 lines. Total RNA was extracted from (A) 7-d-old seedlings, (B) young leaves, (C) ovaries and (D) anthers, both 2-d-before anthesis. Relative expression of mRNA was normalized to the *NtActin* gene. Results are means of two biological replicates ± SE. Each PCR was run three times.

A decrease of mRNA abundance of selected biosynthesis genes (*NtGA20ox1* and *NtGA3ox1*) was detected in all tissues of transgenic *GA2ox*/RNAi lines (Fig. 7). This reduction due to negative feedback regulation indicates that an increment of active GA content occurred in these tissues. The GA content increase may be higher in seedlings and young leaves than in ovaries and anthers because *NtGA20ox1* and *NtGA3ox1* were more repressed in the former tissues (Fig. 7A to D).

Effect of Paclobutrazol treatment in transgenic *GA2ox*/RNAi plants

To test further that the phenotype of the silencing *GA2ox/*RNAi plants was caused by an excess in GA content, we tried to recover the wild-type phenotype of our *GA2ox*/RNAi plants by Paclobutrazol (PCB) application. *GA2ox*/RNAi seedlings cultured in a medium supplemented with different PCB doses, restored wild type hypocotyls (Fig. 8A) and root (Fig. 8B) lengths.

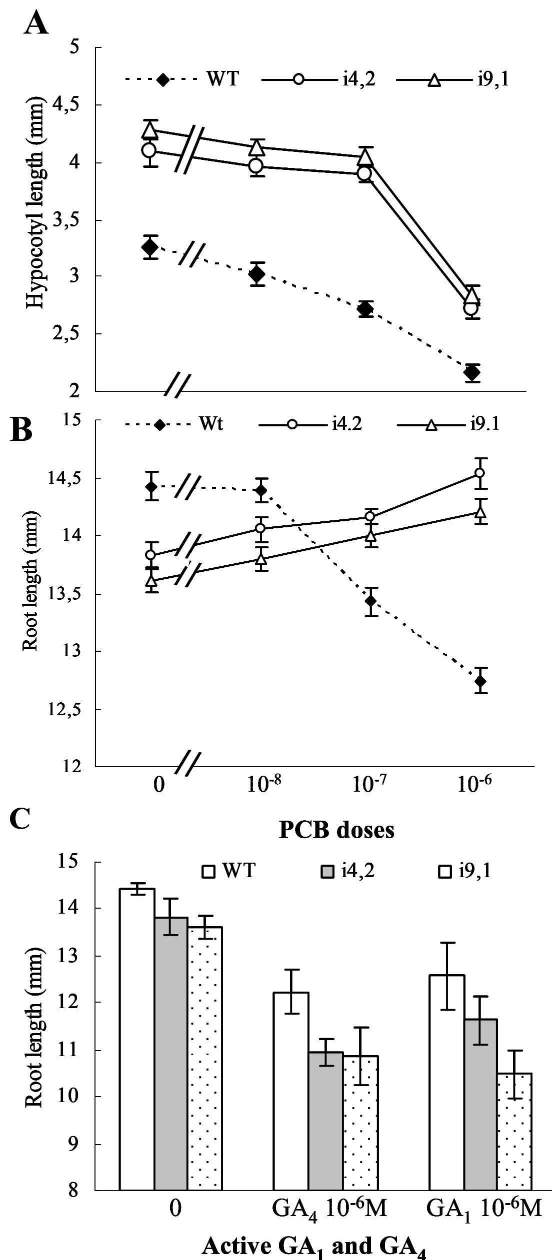

Figure 8. Effect of active GAs and paclobutrazol (PCB) treatments on *GA2ox*/RNAi phenotype. Hypocotyl (A) and root (B) length were measured in wild type and transgenic *GA2ox*/RNAi of 7-d-old seedlings cultured without or with different doses (10^{-8}M, 10^{-7}M and 10^{-6}M) of PCB. Root length (C) of wild type and transgenic *GA2ox*/RNAi of 7-d-old seedlings cultured without or with GA_1 and GA_4 at 10^{-6} M. Results are the mean of 50 seedlings ± SE.

Moreover, compared to wild type, transgenic seedlings exhibited PCB resistance since the major reduction in the *GA2ox*/RNAi hypocotyls length was only significant at high PCB doses (10^{-6} M, Fig. 8A). On the other hand, the shorter root phenotype of transgenic plants could also be attributed to an excess of GAs since PCB treatments were able to restore root length in transgenic *GA2ox*/RNAi plants (Fig. 8B) and the application of active GA_1 and GA_4 to the medium induced shortening of roots in wild type and transgenic seedlings (Fig 8C). The effect of PCB application at 10^{-6}M in transgenic lines was reverted by the simultaneous application of GA_3 (10^{-6}M), demonstrating that this effect was not toxic but was due to inhibition of GA biosynthesis (data not shown). Altogether, these results suggest that the phenotypes of *GA2ox*/RNAi plants are due to an increment in GA content.

DISCUSSION

NtGA2ox gene family

A comparison of *NtGA2ox* sequences with their closest relatives encoding 18 enzymes whose activities have been confirmed (see Supplemental data S2) revealed that the amino acids most likely to be important for GA2ox activity, are very well conserved in our genes. A phylogenetic analysis performed with the same sequences used for amino acid alignment, showed that between the five *NtGA2ox,* the gene most different is *NtGA2ox5* (Supplemental data S3). The enzyme encoded by this gene is very closely related to NoGA2ox3 (able to convert GA_1, GA_{20} and GA_9 to GA catabolites; Ubeda-Tomas et al., 2006). The other enzymes NtGA2ox1, -2, -3 and -4 are close to SlGA2ox4, shown to metabolize GA_1 to GA_8 (Serrani et al., 2007), and to NoGA2ox2 which catabolize more efficiently GA_{20} than GA_1 (Ubeda-Tomas et al., 2006). Therefore we conclude that NtGA2ox1 to -5 are most likely active enzymes that catalyze C19-GAs although it is not possible to know whether they are or not multicatalytic enzymes, and their substrate preferences.

The expression pattern of the *NtGA2ox* genes demonstrates that none of them are specific for a single tissue (Fig 2). These results shown the expression overlapping for at least two *GA2ox* genes in each of the tissues analyzed. In addition to this possible functional redundancy, our results reveal two kinds of transcript regulations within *NtGA2ox* gene family. Two of them are regulated by low increases in GA content (*NtGA2ox3* and *-5*), whereas the others (*NtGA2ox1*, *-2* and *-4*) are sensitive to large changes in GA level (Fig 3).

Multiple silencing of *NtGA2ox* genes in transgenic plants

Based on an RNAi approach and using a single hairpin construct bearing a fragment of 280 nt homologous to the *NtGA2ox* family, we were able to suppress the expression of the five *NtGA2ox* in transgenic tobacco plants. Similar results were found using a similar approach in other species like *Arabidopsis* (Kaur et al., 2005), wheat (Travella et al., 2006) and rice (Miki et al., 2005), indicating the usefulness of this approach to study gene function in gene families. In our case, the similarities between the RNAi-trigger sequence and the five *NtGA2ox* target genes varied from 100% (for *NtGA2ox4*) to 80%

(for *NtGA2ox5*; Table 1). Silencing degree of *NtGA2ox* transcripts was partial but significant in several organs (Fig. 7). Additionally, we showed that the *GA2ox/*RNAi phenotype and the presence of sRNA were maintained over at least three generations (Fig. 4, 5 and 6; Table 2, 3 and 4). These results demonstrate the efficacy of *GA2ox/*RNAi construct to degrade its target genes.

Several organs of transgenic *GA2ox/*RNAi plants, where phenotypic alterations had been detected (leaves, ovaries, anthers and seedlings) displayed reduction of mRNA abundance in more than one of the five *GA2ox* genes (Fig. 7). For instance, in young leaves, all *GA2ox* genes except *NtGA2ox5,* the less abundant in this organ, showed a certain degree of silencing (Fig. 7B). Interestingly, the reduction of mRNAs levels of *NtGA2ox1* to *-4* in transgenic plants was proportional to their relative abundance in wild type leaves (Fig. 7B and Supplemental data S4A). This was also true for ovaries and anthers (Supplemental data S4B and C). In ovaries, *NtGA2ox5,* together with *NtGA2ox1 and -2,* are the three most expressed genes and they also were significantly silenced (Fig. 7C). In anthers, the most abundant are *NtGA2ox4* and *-3* and also they were the most silenced genes (Fig. 7D). In the case of transgenic seedlings their elongated phenotype correlated with a reduction in mRNA abundance of two GA 2-oxidases (*NtGA2ox*1 and *-2*) compared to wild type (Fig. 7A). *NtGA2ox3* to *-5* the most abundant genes, did not vary their expression in transgenic seedlings compared to wild type (Fig. 7A). However, we can not discard a partial silencing of these genes in transgenic lines. It has been reported that *NtGA2ox*3 and -5 could have an important role in GA homeostasis in tobacco seedlings, because their transcript levels are induced in response to small variations in GA levels (Gallego-Giraldo et al., 2008; Fig 3). Thus, in transgenic *GA2ox/*RNAi plants the transcript levels of these genes should be highly induced compared to wild type in response to an increase in GA content. This increment of GA levels is supported by the repression of GA indicator genes *NtGA20ox1* and *NtGA3ox1* quantified in transgenic seedlings (Fig. 7A).

All together, these results indicate that the five *GA2ox* genes can be partially silenced by the *GA2ox/*RNAi construct and that the degree of silencing depends on their level of expression in each organ. It is well known that suppression efficacy depends on the level of homology between trigger and target sequence (Meyer, P, 1996). Additionally Miki et al. (2005) found that the suppression efficacy could be also affected by the concentration of the target mRNA. For instance, in young leaves the most abundant *GA2ox* genes, *NtGA2ox2* and *-3*, with 92 and 88% identity to *GA2ox/*RNAi sequence (Table 1) were silenced efficiently. In contrast, *NtGA2ox4* that had low expression level was less silenced, although its sequence was identical to the trigger sequence (Fig. 7B and Table 1). Our results suggest that the expression level of the target mRNA may be more important than the homology between trigger and target sequence if a minimum of homology is fulfilled between them.

Following this last idea, we expect that the most expressed *GA2ox* genes in other tobacco tissues (Fig. 2) should also be silenced by the RNAi construct in transgenic lines. This could explain the elongated phenotype found in other tissues like stem (node and internodes), petals and flower peduncles (Fig. 5, 6A; Table 2, 3 and 4).

***GA2ox/RNAi* phenotype and GA content**

The phenotype of transgenic *GA2ox/*RNAi plants included enhanced length of hypocotyls, internodes, leaves, fruit peduncles, as well of the final plant height (Table 2, 4 and Fig. 5), consistent with a presumed increase in GA content. The *GA2ox/*RNAi phenotype is similar to the GA-overproduction phenotype caused by overexpression of a GA 20-oxidase in tobacco (Vidal et al., 2001; Biemelt et al., 2004) and other species, like *Arabidopsis* (Huang et al., 1998; Coles et al., 1999). In addition to the GA overdose phenotype found in transgenic *GA2ox/*RNAi lines, others observations suggest that GA levels are increased in these plants. First, transgenic plants had reduced expression of several *GA2ox* genes in all tested tissues (Fig. 7). Second, genes known to be molecular markers of GA level (*NtGA20ox1* and *NtGA3ox1*; Gallego-Giraldo et al., 2008) showed a decrease of their mRNA abundance in transgenic *GA2ox*/RNAi lines (Fig. 7). Moreover, the elongated hypocotyls of *GA2ox/*RNAi plants could be restored to normal by the application of Paclobutrazol (PCB), further supporting that the phenotype is probably due to enhanced GA levels (Fig. 8A).

Interestingly, transgenic *GA2ox/*RNAi lines showed a slight but significant reduction in root length (Table 2). In fact, during regeneration of transgenic explants at T0 generation, it was necessary to apply PCB, to induce root regeneration (see Materials and Methods). Roots of some GA deficient mutants in *Arabidopsis* (Fu and Harberd, 2003) and pea (*na*; Yaxley et al., 2001) are shorter than wild type but are restored to normal growth by GA application, indicating that GAs are important for root elongation. However, our results from applications of high doses of GA in wild type roots (Fig. 8C), suggested that an excess of active GAs may inhibit root elongation. Similar phenotype was detected in tobacco GA overproducer plants (*35S:CcGA20ox*; data not shown) and in transgenic *Citrus* overexpressing a GA 20-oxidase, which rooted poorly (Fagoaga et al., 2007). Moreover, root growth inhibition in transgenic *GA2ox/RNAi* plants was reverted by reducing the GA content with PCB applications (Fig. 8B). Therefore, the small reduction in root length in transgenic *GA2ox/*RNAi plants is likely the consequence of GA increase in this organ.

Transgenic *GA2ox/*RNAi tobacco lines showed a significant delay in flowering (Fig. 5), measured as the number of vegetative nodes produced before the first flower. GAs are flowering promotion factors in *Arabidopsis* under short day conditions because an increment in GA_4 in the shoot apex regulated *LEAFY* transcription and floral initiation (Eriksson et al., 2006). In contrast, it has been reported that floral transition in tobacco might not be induced by an increase of active GAs in the apical shoot before floral transition (Gallego-Giraldo et al., 2007). However GAs are necessary for flowering because dwarf transgenic tobacco plants overexpressing a *35S:No2ox3* (Ubeda-Tomas et al., 2006) and PCB treated plants (Gallego-Giraldo et al., 2007) show an extended vegetative phase and late flowering. Moreover, an excess of GA can also induce a slight delay in flowering by GA_3 applications at saturated doses (Gallego-Giraldo et al., 2007) or in GA-overproducer transgenic hybrids (*35S:GAPs3ox1* x *35S:GACc20ox1*; Gallego-Giraldo et al., 2008). In the case of transgenic *GA2ox/*RNAi plants the inhibition of flowering induction was more pronounced than in the lasts two cases. This new evidence further support the

hypothesis suggested by Gallego-Giraldo et al., (2007), where GAs are not flowering promotion factors in tobacco. Also support that GA excess has a negative effect in the flowering time in tobacco and that flowering induction may imply a range of optimal GA concentrations. Moreover, our result indicates that the *GA2ox* could play an important role in the maintenance of adequate GA levels to flower induction.

In transgenic *GA2ox/*RNAi plants, fertility was significantly reduced since the number of seeds was 50% the number produced by wild type fruits (Table 3 and Fig. 6E). This defect in fertility is due to partial androesterility. Both the presence of less pollen viable (Fig. 6C and Table 3) and in some cases less pollen abundance in anthers of some *GA2ox/*RNAi lines (data not shown from anthers sections), support the existence of male sterility in these lines. A large body of evidence suggests that GAs are required for anther development. GA biosynthesis mutants of various species are male sterile including *ga1* in *Arabidopsis* (Koornneef and van der Veen, 1980) and *gib-1* and *gib-2* in tomato (Jacobsen and Olszewski, 1991; Nester and Zeevart, 1998, respectively). Moreover, constitutive GA response mutants like *spy* in *Arabidopsis* leads to partial male sterility (Jacobsen et al., 1998). Additionally, *Arabidopsis* flowers treated with GA_3 also exhibited reduced fertility (Jacobsen and Olszewski, 1993). Therefore, the partial male sterile phenotype found in *GA2ox/*RNAi transgenic lines may be due to an excess of GA levels in the anthers, caused by the silencing of at least two *GA2ox* genes (*NtGA2ox4* and *-3*). This GA excess is also supported by the repression of indicator genes (*NtGA20ox1* and *NtGA3ox1*) in transgenic anthers (Fig. 7D). Our results suggest the relevance of some *NtGA2ox* genes in the control of GA levels in tobacco anthers.

The inflorescence of *GA2ox/*RNAi plants displayed an elongated and complex phenotype. Their elongated internodes and fruit peduncles are characteristics shared by GA over-producer tobacco plants (Vidal et al., 2001) and therefore suggest an increment in GA levels in *GA2ox/*RNAi inflorescences. However other characters as floral branches length and the production of more flowers and fruits with less weight (Fig. 6E and 6F) were not detected in GA over-producer tobacco plants (Vidal et al., 2001; Biemelt et al., 2004) or in other species with GA overdose (*Arabidopsis*, Huang et al., 1998; Coles et al., 1999 and rice, Oikawa et al., 2004). These phenotypes could be related to the reduced fertility of *GA2ox/*RNAi plants, since it is known that *Arabidopsis* sterile mutants can produce higher number of flowers (Hensel et al., 1994). Our results suggest a role for *GA2ox* genes in the hormonal control of tobacco inflorescences growth and development.

In summary, we have obtained a range of GA overproduction phenotypes in transgenic tobacco plants by multiple silencing of *NtGA2ox* genes. These results reveal the important role of GA 2-oxidases in the regulation of GA levels in different organs.

MATERIALS AND METHODS

Accession numbers

Accession numbers for the sequences used in this study are as follows: *NtGA2ox1* (AB125232.1), *NtGA2ox2* (AB125233.1), *NtGA2ox3* (EF471117) *NtGA2ox4* (EF48000), *NtGA2ox5* (EF471118), *NtGA20ox1* (AB012856.1), *NtGA3ox1* (AB032198) and *NtActin* (U60489).

Generation of the *35S:GA2ox*/RNAi construct

The pHannibal vector (Wesley et al., 2001) was used to generate the hairpin *GA2ox* (pHan*GA2ox*) construct. Primer pairs with enzyme sites at the 5´ and 3´end, were used to amplify a conserved region of 280-bp from tobacco cDNA. This conserved region was amplified in sense (5´- 3´) and antisense (3´- 5´) orientation by primer pairs (*GA2ox-XhoI* 5´-CGC TCG AGG ATT TCT GTC CCA CCT GAT-3´; *GA2ox-EcoI* 5´-GTA GAA TTC TTG TAC AAG CTT TCT TCC CC-3´) and (*GA2ox-XbaI* 5´-CAT CTA GAT GGA TTT CTG TCC CAC CTG A-3; *GA2ox-ClaI* 5´-GTA TCG ATT GTA CAA GCT TTC TTC CCC-3´) respectively. These two PCR products (sense and antisense orientation) were cloned into pGem-Easy vector System (Promega) to confirm theirs sequences. Then, they were digested with *XhoI-EcoRI* and *XbaI-ClaI* for sense and antisense fragment respectively and ligated into the pHannibal vector to obtain pHan*GA2ox*. The *NotI* fragment from pHannibal vector carrying pHan*GA2ox* construct was subcloned into *NotI*-digested pGREENII binary vector.

Isolation of transgenic *GA2ox*/RNAi and growth plant conditions

Sterilized seeds of tobacco *Nicotiana tabacum* cv Xanthi were germinated and cultured in MS medium (Murashige and Skoog medium including vitamins; Duchefa, The Netherlands), 20 g · l^{-1} sucrose and 6 g · l^{-1} agar gel. Leaf sections from plants 3 weeks old, were transformed by immersion in bacterial liquid LB medium containing *Agrobacterium tumefaciens* strain LBA4404 (Hockema et al., 1983) carrying the binary vector system pGREENII (*35S:GA2ox/*RNAi*)*. Regeneration of tobacco explants were performed as described by Vidal et al. (2001). To promote root growth paclobutrazol (an inhibitor of GA biosynthesis) was add to the regeneration medium at $5x10^{-7}$ M. Non-infected leaf sections were cultured and considered as a transformation control. For isolation of homozygous lines, T1 plants were grown and self-pollinated to obtain the T2 generation. T2 seedlings were tested by kanamycin (150 mg · l^{-1}) segregation. T2 plants showing 3:1 segregation ratio (kanamycin resistant Km^R / kanamycin sensitive Km^S) were selected to obtain the T3 generation. Homozygous lines were identified by 100% Km^R segregation.

Transgenic *GA2ox*/RNAi and wild type tobacco plants were cultured in a growth chamber at 26ºC and in a greenhouse at 28ºC under long day (LD) conditions (16 h light, and 8 h darkness). Plants were cultured in pots containing vermiculite: peat (1:1) and watered with nutrient solution.

Northern blots for small-RNA (sRNA) detection

For northern analysis total RNA was isolated using Trizol (Invitrogen) according to the manufacture's protocol from seedling 7-days-old (hypocotyl plus cotyledons) of wild-type and transgenic *GA2ox*/RNAi plants. 30 µg of total RNA was separated by electrophoresis in a 15% denaturing polyacrylamide gel. A synthesized sRNA of 16-19 nt long from the *GA2ox*/RNAi fragment was included into the gel as a molecular size marker and a hybridization control. These sRNA were *in-vitro* transcript in two orientations (- and + polarity) from pGem-Easy vector carrying *GA2ox*/RNAi with the SP6/T7 Transcription kit (Roche) then, these transcribed fragments were annealing and digested with RNase III enzyme (Epicentre). After electrophoresis, the nucleic acids were electroblotted into nylon filter (Hibond-N, Amersham Bioscience, UK) and cross-linked with a UV Stratalinker 800 (Stratagen, USA). For sRNA detection, a radioactive RNA probe was used. This riboprobe was obtained from the fragment *GA2ox*/RNAi previously cloned into pGem-Easy vector (Promega) and transcribed in sense orientations in presence of [α-^{32}P-UTP] with the SP6/T7 Transcription kit (Roche). The electroblot was hybridized with this riboprobe under non stringent condition at 35ºC in a solution containing 50% formamide, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 1% SDS, 5x SSPE (1X SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4 and 1 mM EDTA [pH 7.7]) and 0.1 mg · ml^{-1} denatured salmon sperm. The filter was washed twice for 10 min at room temperature in 2x SSC, 0.1% (w/v) SDS and then at 50ºC in 1 x SSC, 0.1% (w/v) SDS. Hybridization signals were detected using a bioimage analyzer (Fuji Bas 1500).

Hypocotyl length measurements and Hormone applications

One hundred seeds from each line were germinated and cultured in MS medium in a growth chamber at 26ºC under LD conditions. For GA_1, GA_4 and PCB treatments, different concentrations were applied in 50 µl of 100% ethanol added to Petri dishes with 100 ml of medium. 50 µl of 100% ethanol was added to control plates. Seven days later the seedlings were scanned and measured using the ImageJ software (National Institutes of Health, USA).

RNA isolation

For semi-quantitative transcript analysis total RNA was isolated from reproductive and vegetative tissues from tobacco, using Trizol (Invitrogen) according to the manufacturer's protocol. For real-time PCR total RNA was isolated from 7-d-old seedlings without roots (hypocotyls and cotyledons) using RNeasy Plant Mini Kit (Quiagen). Total RNA to semi-quantitative or real-time PCR was treated with RNase-Free DNAse (Quiagen), according to the manufacturer's instructions. RNA concentration was measured using a Nanodrop ND-1000 Spectrophotometer (Wilmington, Delaware USA).

Semi quantitative RT-PCR analysis

2 μg of total RNA was used for cDNA synthesis using Taq Man® Reverse Transcription Kit (Applied Biosystems) with random hexamers, at a final volume of 25 μl. PCRs amplifications were performed taking 1 μl of cDNA solution in a 50 μl total volume reaction containing 0.2 mM of each dNTP, 2 mM $MgCl_2$, 1x reaction buffer, 1 U of NETZYME® DNA Polymerase (Fermentas Gmbh, Germany), 3% DMSO and 1 μM of the appropriate pair of primers (Supplementary Table S1). PCR conditions for amplification of *NtGA2ox1, -2, -3* and *-5* consisted of initial denaturation at 95ºC for 2 min, followed by 32 cycles of 95ºC/ 45 sec, 61ºC/ 45 sec and 72ºC/ 60 sec, and finally 10 min extension at 72ºC. For amplification of *NtGA2ox4* 34 cycles were used with annealing temperatures of 67ºC. In the case of *NtActin* annealing temperature of 60ºC and 27 cycles were used. 15 μl aliquots of PCR products were separated on 1.4 % agarose gel electrophoresis. The spots were stained with ethidium bromide, visualized under UV using a GeneGenius Bio Imaging System (Syngene), captured with the GeneSnap program (Syngene) and quantified with the GeneTools software (Syngene). Expression was normalized using *NtActin* as internal control, by comparing expression ratios to that of the specific tissues indicated in the Figure legends (set to 1.0).

Quantitative real-time RT-PCR analysis

1 μg of total RNA was used for cDNA synthesis using Taq Man® Reverse Transcription Kit (Applied Biosystems) with random hexamers, at final volume of 40 μl. 1 μl of cDNA solution was used in all PCR reactions. The primer pairs used for PCR amplification (Supplementary Table S1) were designed using Primer Express™ v2.0 software (Applied Biosystems). PCR's were performed in an ABI PRISM® 7000 Sequence detection System using SYBR Green to monitor double-stranded DNA synthesis (Applied Biosystems). PCR conditions and data analysis were performed as described before in Gallego-Giraldo et al., 2007). The analyses were carried out using two biological replicates. Each replicate consisted of material from 30 seedlings. All PCRs reactions were carry out by three technical replicates.

Statistical methods

Statistical treatments of the data were made by analysis of variance using the Fisher´s LSD procedure to multiple comparison tests. (Statgraphics Plus program, version 5.1 for windows, Statistical Graphics, Rockville, MD)

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Supplemental Data

Table S1. Primers pair used in a Semi-quantitative and Real-time RT-PCR analysis.

	Semi-quantitative RT-PCR	
Gene	**Forward 5´ to 3´ direction**	**Reverse 5´ to 3´ direction**
NtGA2ox1	-GTTTTCATCTTTTCATTACGTTGTCAAAC-	-GATAATTTGCAGTTTTTGACTATGGGG-
NtGA2ox2	-CGTTCAATTTCTTTGCCCAC-	-GCAGTTGTCTTTGGAGAAGTGC-
NtGA2ox3	-TGTCTTGTCAATACCAGCAGTTGAACAA-	-AATATTTTCTGGGTCAAGACCCAAAATG-
NtGA2ox4	-AGCCATTGAGCACTTGTCCAAAGCGA-	-TTCCCCTCAATGGCGGTCCTCCA-
NtGA2ox5	-ATCCATCGCTATTCCCGGA-	-GCTATCTTTTCCAGCGCCAA-
NtActin	-CATTGGCGCTGAGAGATTCC-	-GCAGCTTCCATTCCGATCA-
	Real time RT-PCR	
Gene	**Forward 5´ to 3´ direction**	**Reverse 5´ to 3´ direction**
NtGA2ox1	-TGGAAGGATTAGTTGTACAGATGTTCC-	-CGAGAGAAAGGAAATAAAGAAGAAAATC-
NtGA2ox2	-ATCGTTCAATTTCTTTGCCCACATA-	- CTTTGGAGAAGTGCTGATCCATGTT-
NtGA2ox3	-AAGTCCCACCTTGTTACTTG-	-GCTATCTATAGGAAATCCAATG-
NtGA2ox4	-CAAGAAATACGGCAAATACTTGTCTTAG-	-AATGGCTGGTTTGGACAAGCAT-
NtGA2ox5	-ATCCATCGCTATTCCCGGA-	-GCTATCTTTTCCAGCGCCAA-
NtGA20ox1	-TGTAGCACGAGAACTTCC-	-ACGGCATGCTTCACCAACA-
NtGA3ox1	-CGGCTTTGTCCCCTCTA-	-CTTATCGAGTTTAGCCAACTTGCA-
NtActin	-CATTGGCGCTGAGAGATTCC-	-GCAGCTTCCATTCCGATCA-

Supplemetal data S2. Alignment of amino acid sequences corresponding to 18 GA 2-oxidases (group I) enzymes whose activities have been confirmed experimentally and the sequences of *NtGA2ox1* to *-5*. Alignment was performed with Clustal W2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Conserved amino acids are indicated. ○ Fe^{2+} binding residues, ↑ 2-cetoglutarate binding residues and ● amino acids conserved in GA2ox enzymes group I.

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                    *        20         *        40         *        60         *        80         *       100
NtGA2ox2   : ---MD-----QHFSKD----NCKPTS---FF---NNVPLIDLSKP-DSKNL-IVKACEEFGFFKVINHSVPTEFITKL-SEAIKFFSSPLSEKEKAGPPD :  79
NtGA2ox4   : MVVLS-KPAIEHLSKAN---NCKPTS---FF---NGVPLIDLSKP-DSKNL-IVKACEEFGFFKVVNHSVPTEFITKLESEAINFFSSPLSQKEKAGPPD :  88
NtGA2ox1   : MVVLT-KPGIDHFPIVK---NCKLSS---FFNG---VPLIDLSKP-NSKNL-IVKACEEFGFFKVINHSVPTEFITKLESEAIKFFSSPLSEKQKAGPAD :  88
SlGA2ox4   : MVVLT-KPAITHFPIVK--NSFKSPS---YIND---IPVIDLSKA-NSKNL-IVNACEEFGFFKVINHDVPMEFISKLESEAVKFFSSPLSEKLKAGPAD :  89
SlGA2ox5   : MVVLT-EPDNDHLPIINS-NSCKSPS---FFND---IPVIDLSKP-DSKNL-IVEACEEFGFFKVVNHDVPIEFISKLESEAIKFFSSPLSEKLKAGPAD :  90
NtGA2ox3   : MVVLS-IPAVEQFSIVK---NCKPISS--FFPSN--IPLIDISKP-DSKNL-IVKAYEEFGFFKIINHGVPMEFINNLELEAIKFFSSPINQKEKAGPAD :  90
NoGA2ox2   : MVVLS-KPAMEQFCIVK---NCKAPT---LFPG---VPLIDLSKP-DSKHL-IVKACQEFGFFKVINHGVPTEFITKLESEAVKFFSLPLSDKEKAGPPD :  88
LsGA2ox1   : MVALS-KPSIEQFFMKP----SKPNTNPLIFPT---IPLIDLSKP-ESKQH-IVKACQDFGFFKVVNHGVPTKFIKKLESEAIKFFSSPLSTKEKAGPPD :  90
FpGA2ox1   : ---------MALFP-L-------------------IPVIDLSKP-DAKHQ-IVKACEDFGFFKVINHGVPMDSIKKLESEAVKFFSLPLSEKEKAGPPY :  68
PsGA2ox1   : MVLLS-KPTSEQYTYVR---NNMPIT---FSSS---IPIVDLSKP-DAKTL-IVKACEDFGFFKVINHGIPLDAISQLESEAFKFFSLPQTEKEKAGPAN :  88
VaGA2oxA2  : MASLS-KTTTEQYSYIK---NPMAST---FAST---IPIVDLSKP-DAKTL-IVNACEEFGFFKVINHGVPMEAISRLETEAIKFFSLPINEKEKAGPPN :  88
NtGA2ox5   : MVVLT-QPIVETLSHIK---TCKYNNTDVFTG----IPVIDLSDP-EANTL-IIKACQEFGFFKVVNHGVPIEIMTKLESEAVNFFNLSQVEKDKAGPAN :  90
NoGA2ox3   : MVVLS-QPALENLSHIS---TCKFPSTNFYEG----IPVIDIFKP-EANTE-IVKACQEFGFFKVINHGVPLEFITKLEGEAVKFFNLPQPEKEKVRPAK :  90
LsGA2ox2   : MVMIS-QVEKKPVVDIF---PTKSNKTN-FTG----IPVIDLSSPEDAKNL-IVNACQDYGFFKVINHGVSPLIVSELENEAIEFFNMRQYEKDQYCPPN :  90
PcGA2ox1   : MVVLS-QPAINQFFLL------KPFKSTPLFTG---IPVVDLTHP-DAKNL-IVNACRDFGFFKIVNHGVPLEIMANLENEAIRFFKKSQSEKDRAGPPD :  88
VaGA2oxA1  : MVVLS-QPAINQFLLL------KPCKSTPLFTG---IPVVDFTHP-DAKNL-IVEACRDYGFFKIVNHGVPLEIVANLENEAIRFFKKSQSEKDRAGPPD :  88
SoGA2ox1   : MVVLS-HVALDQFMKTC-----KPIDTHLFPTV---IPTIDINSP-DVKSL-IVKACKEFGFFKVVNHGVPIEMTRQLEDEASKFFSLPQGLKESAGNPN :  89
AtGA2ox2   : MVVLP-QPVTLDNHISLIP-TYKPVPVLTSHS----IPVVNIADP-EAKTR-IVKACEEFGFFKVVNHGVRPEIMTRLEQEAIGFFGLPQSLKNRAGPPE :  92
AtGA2ox3   : MVIVL-QPASFDSNLYVNP-KCKPRPVL--------IPVIDLTDS-DAKTQ-IVKACEEFGFFKVINHGVRPDLLTQLEQEAINFFALHHSLKDKAGPPD :  88
AtGA2ox1   : MAVLSKPVAIPKSGFSL-------------------IPVIDMSDP-ESKHA-IVKACEDFGFFKVINHGVSAEIVSVLEHETVDFFSLPKSEKTQVAG-Y :  78
HvGA2ox4   : MVVLAKPAALEQIALMR-----TPEPWESFSG----VPAVDLSSP--GAAADVVRACERFGFFSVVNHGVPAGVVDRLEAEAVRFFASTQAEKDASGPAD :  89
OsGA2ox3   : MVVLAGPPAVDHIPLLR-----SPDPGDVFSG----VPVVDLGSP--GAARAVVDACERYGFFKVVNHGVATDTMDKAESEAVRFFSQTQPDKDRSGPAY :  89
HvGA2ox5   : MVVLA-KGELEQIALPA-----AQPPLAHVRAID--LSAAPGPGRD-AAARAIVSACEEQGFFKVTGHGVPLEIVRAVEAAAAEFFALPQAEKEAAAGR- :  90
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                     *       120         *       140         *       160         *       180         *       200
NtGA2ox2   : P--FGYGNKQIGQNGDSGWVEHIIVSTNSEFNYYKF-------------ASILGV-NPENIRAAVNDYVSAVKKMACEILEMIAEGLKI--HPRNVFSKL : 161
NtGA2ox4   : P--FGYGNKQIGPNGDLGWVEYIFVATDSEISYHKF-------------SSIFGV-NPETIRTAVNDYASAVKKMACEILEMIEEGLKI--HPRNVFSKL : 170
NtGA2ox1   : P--FGYGNKKIGPNGDVGWVEYILLSTNSEFNYHKF-------------ASILGV-NPETIRAAVNDYVSAVKKMACEILEMIAEGINI--HPRNVFSKL : 170
SlGA2ox4   : P--FGYGNKKIGQNGDIGWVEYILLSTNSEFNYQKF-------------ASVLGV-NPENIRAAVNDYVSSVKKMSCEILEKIAEGLKI--QPTNVLSKL : 171
SlGA2ox5   : P--FGYGNKQIGQSGDIGWVEYILLSTNSEFNYQKF-------------ASVLGV-NPENIRAAVNDYVTAVKRMSCEILEKIAEGLKI--HPTNVFSKL : 172
NtGA2ox3   : P--FGYGNRKIGTNGDIGWVEYILLSTNSEFNYQKF-------------ASILGL-DPENIRVAVNDYVSAMKKMACEILEMIAEGLKI--YPKNVFSKL : 172
NoGA2ox2   : P--FGYGNKKIGPNGDIGWVEYIILSTNSEFNYQRF-------------ESIFGM-TPEKFRDAVNDYVSSVKKMACEILEIMAEGINI--QPKNVFSKL : 170
LsGA2ox1   : P--FGYGNKRIGRNGDVGWVEYLLINAKPESDYQRY-------------LSVFEE-NPEIFQGVVNDYVTAVKKMACEILELIADEMKI--QPRNVFSKL : 172
FpGA2ox1   : PVGFGYGNKRIGLNGDVGWVEYLIMTTKSNPE--------------------IGD-NPEDFRCAMNQYVAAVKSMACDVLEIMADGLRI--QQKDVFSKL : 145
PsGA2ox1   : P--FGYGNKRIGLNGDIGWIEYLLLTTNQDHNF-----------------SLYGE-DIHKFRGLIKDYKCAMRNMACEIIDIMAEGLKI--QPKNVFSKL : 166
VaGA2oxA2  : P--FGYGSKRIGHNGDVGWVEYLLINNNQEHDF-----------------SLYGK-NHEKFRCLINSYMCSVRKMACDILEIMAEGLKI--QQKNVFSKL : 166
NtGA2ox5   : P--FGYGNKRIGSNGDVGWVEYLLLTTNPDLSYHKS-------------IAIPG--NSHLFWSIVNEYVSAVRNIACIALEKIAEGLRI--EPKNVLSKM : 171
NoGA2ox3   : P--FGYGNKRIGQNGDVGWIEYLLINTNPELVYEKA-------------VTIPG--DSELFWSCVNDYVSAVRSMACDVIDMIADGLKI--GPRNVLSRL : 171
LsGA2ox2   : P--FGYGFNKIGTNGDVGWIEYLLLTSTN---------------------FPT--NSKIFSSLTNEYVKEVRKIGCTILEIMAEGLKI--EPKNVLSRM : 162
PcGA2ox1   : P--FGYGSKRIGPNGDVGWVEYLLINTNPDVISPKS-------------LCIFRE-NPHHFRAVVENYITAVKNMCYAVLEIMAEGLGI-RQR-NTLSRL : 170
VaGA2oxA1  : P--FGYGSKGIGPNGDVGWVEYLLINTNPEVISPKS-------------LSIFRE-SPHHFRAVVENYITAVKNMCYAVLEIMAEGLGI-AQR-NTLSRL : 170
SoGA2ox1   : P--FGYGNKKIGRNGDVGWIEYLLLSASDEFISQTC-------------LSIYPD-NPDVFRCAINNYISKMKKMGVRVLETMAEGINIKGEDRYALSNL : 173
AtGA2ox2   : P--YGYGNKRIGPNGDVGWIEYLLLNANPQLSSPKT-------------SAVFRQ-TPQIFRESVEEYMKEIKEVSYKVLEMVAEELGI--EPRDTLSKM : 174
AtGA2ox3   : P--FGYGTKRIGPNGDLGWLEYILLNANLCLESHKT-------------TAIFRH-TPAIFREAVEEYIKEMKRMSSKFLEMVEEELKI--EPKEKLSRL : 170
AtGA2ox1   : P--FGYGNSKIGRNGDVGWVEYLIMNANHDSGSGPL------------FPSILKS--PGTFRNALEEYTTSVRKMTFDVLEKITDGLGI--KPRNTLSKL : 160
HvGA2ox4   : P--FGYGSKRIGRNGDMGWVEYLLLAIDRDTLSKAS--------------P----APSSALREAINAYVSAMRGIARTVLEMVAEGLGV--SPRGALADM : 167
OsGA2ox3   : P--FGYGSKRIGFNGDMGWLEYLLLALDDASLADAC--------------T-V--PSCAVFRAAINEYISGVRKVAVRVMEAMSEGLGI--ACADALSAL : 168
HvGA2ox5   : P--LGYGSKRIGVSGDLGWIEYLLLGVTPAGALPAASFASWTLPCAGA-AVASSSEHPCPLRDLIEEYAAAVRRMACGVLEIMAEGLGI--GPADALSRL : 185
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                            *        220         *        240         *        260         *        280         *        300
NtGA2ox2   : LMDEKSDSVFRINHYPPC---------TEI---QQFNDNN--LIGFGEHTDPQIISVLRSNNTSGLQILLKNGHWISVPPDFNSFFVNVGDSLQVMTNGKF : 248
NtGA2ox4   : LMDAKSDSVFRINHYPPC---------PEI---QQFSGHN--LIGFGEHTDPQIISVLRSNNTCGLQMSLKNGHWISVPPDFNSFFINVGDSLQVMTNGRF : 257
NtGA2ox1   : LMDEKSDSVFRINHYPPC---------PEI---QQFSDNN--LIGFGEHTDPQIISVLRSNNTSGLQILLKNGHWISVPPDFNSFFINVGDSLQVMTNGRF : 257
SlGA2ox4   : LMDEKSDSVFRINHYPPC---------PDI---QEFNAKN--LIGFGEHTDPQIMSILRSNNTSGLQILLKNGNWFSVPSDQNSFFVNVGDSLQVMTNGRF : 258
SlGA2ox5   : LKDEKSDSVFRINHYPPC---------PDI---QEFNAKN--LIGFGEHTDPQIISVLRSNNTSGLQILLKNGNWFSVPPDQSSFFVNVGDSLQVMTNGRF : 259
NtGA2ox3   : LMDEQSDSVFRINHYPPC---------PEV---QEFNGRN--LIGFGAHTDPQIISLLRSNNTSGLQISLEDGHWISVPPDQNSFFINVGDSLQVMTNGRF : 259
NoGA2ox2   : LMDEQSDSVFRINHYPPY---------PDL---QELHGRN--LIGFGEHTDPQIISVLRSNNTSGLQIQLKDGSWISIPPDQSSFFINVGDSTQVLTNGRF : 257
LsGA2ox1   : LMDEQSDSVFRVNHYLPC---------PEF---QENERNGRKIVGFGEHTDPQIISVLRSNNTSGLEISLRDGSWMSVPADSDSFFINVGDSLQVMTNGRF : 261
RpGA2ox1   : LMDEQSDSMFRVNHYPPY---------PEI---QGFKDRN--IVGFGEHTDPQIISVLRSNNTSGLEISLRDGSWVSVPPDQTSFFINVGDSLQVMTNSKF : 232
PsGA2ox1   : VMDKQSDCLFRVNHYPAC---------PEL----AINGEN--LIGFGEHTDPQIISILRSNNTSGFQISLRDGSWISVPPDHSSFFINVGDSLQVMTNGRF : 252
VaGA2oxA2  : LRDKESDSVFRVNHYPAC---------PEL----AVNGEN--MIGFGEHTDPQIISLLRSNNTSGLQIYLRDGSWISVPPDHNSFFINVGDSLQVMTNGRF : 252
NtGA2ox5   : LRDEKSDSCFRINHYPPF---------PELL--QTLSGRN--LIGFGEHTDPQVISVVRSNNTSGLQISLKDGTWLSVPPDPYSFFINVDDSLQVMSNGRF : 259
NoGA2ox3   : LRDEKSDAVFRINHYPPC---------PEL---QALSGRN--LIGFGEHTDPQIISVVRSNNTSGLQISLKDGTWVSVPPDQYSFFINVGDSLQVMTNGRF : 258
LsGA2ox2   : LSDENADTVFRINHYPPCLDFNSNHDSDIN-NKSMSHGR-TSIGFGEHTDPQLISIARSNATSGFQIYLEDGTWVGVPPDETSYFINVDDLLEVMTNGRF : 260
PcGA2ox1   : LKDEKSDSCFRINHYPPC---------PEVQ---AIN-RN--IVGFGEHTDPQIISVLRSNSTSGLQICLTDGTWVSVPPDQTSFFINVGDALQVMTNGRF : 256
VaGA2oxA1  : LKDEKSDSCFRMNHYPPC---------PEVP---AIN-RN--IVGFGEHTDPQIISVLRSNSTSGLQICLADGTWVSVPPDQTSFFINVGDALQVMTNGRF : 256
SoGA2ox1   : IKDSKSDSYFRINHYPPR---------PEAFE--GINGRN--IVGFGEHTDPQIISVLRSNKIGGLQISINDGTWVSVPPDDNSFFILVGDSLQVMTNGRF : 261
AtGA2ox2   : LRDEKSDSCLRINHYPAA---------EE-------EAEKMVKVGFGEHTDPQIISVLRSNNTAGLQICVKDGSWVAVPPDHSSFFINVGDALQVMTNGRF : 259
AtGA2ox3   : VKVKESDSCLRMNHYPEK---------EE-------TEVKE-EIGFGEHTDPQLISLLRSNDTEGLQICVKDGTWVDVTPDHSSFFVLVGDTLQVMTNGRF : 254
AtGA2ox1   : VSDQNTDSILRINHYPPC---------PLSNK--KTNGGK-NVIGFGEHTDPQIISVLRSNNTSGLQINLNDGSWISVPPDHTSFFFNVGDSLQVMTNGRF : 249
HvGA2ox4   : VTGEASDQVFRVNHYPPC---------PLL----QGLPENCSVTGFGEHTDPQIVSILHSNGTAGLQVALHDGRWVSVPPNRDAFFVNVGDSLQVLTNGRL : 255
OsGA2ox3   : VTAEGSDQVFRVNHYPPC---------RAL----QGL--GCSVTGFGEHTDPQIVSVLRSNGTSGLQIALRDGQWVSVPSDRDSFFVNVGDSLQVLTNGRF : 254
HvGA2ox5   : VSDGESDNMLRVNHYPPR---------PEM---------QGCLITGFGEHTDPQIISVLRSNGTSGLEICARDGEWTSVPPDPDAFFVNVADALQVLTNGRF : 269
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                          *        320         *        340         *        360         *        380         *
NtGA2ox2   : KSVKHRVLA-NSM-KSRLSMIYFGGPPLSEKIAPLASIM--EGE-D-SLYKEFTWFEYKKSAYKTRLADNRLILFEKIAAS---------- : 323
NtGA2ox4   : KSVRHRVLA-NSV-KSRLSTIYFGGPPLRGKIAPLASIM--EGE-E-SLYKEFKWFEYKKSAYKTRLADNRLVLFEKIASK---------- : 332
NtGA2ox1   : KSVRHRVLA-NSV-KSRLSMIYFGGPPLSEKIAPLASIM--EGE-ES-LYEEFTWFEYKKSAYKTRLADNRLVLFEKVAAS---------- : 332
SlGA2ox4   : KSVKHRVLT-NSV-KSRLSMIYFGGPPLSEKIAPLPSIM--EGK-DS-LYKEFTWFEYKKSAYKTRLADNRLVLFEKSL------------ : 331
SlGA2ox5   : KSVKHRVLT-NSV-KSRLSMIYFGGPPLSEKIAPLPSIM--EGE-ESSLYKEFTWFEYKKSAFKSRLAQNRLTLFEKFGTS---------- : 335
NtGA2ox3   : KSVKHRVLA-NSL-KSRLSMIYFGGPPLSEKIAPLASLI--KGD-QDSLYKEFTWFEYKKSAYNSRLADNRLVLFEKVL------------ : 333
NoGA2ox2   : KSVKHRVLA-NSM-KSRLSMIYFGGPPLSEKIAPLPSIM--EGE-DS-LYKEFTWFEYKKSAYKSRLADNRLVLFEKIAAS---------- : 332
LsGA2ox1   : KSVKHRVVA-NST-KSRVSMIYFGGPPLSEKIAPLPSLI--QGE-EDSLYKEFTWFEYKKSAFNTRLADNRLGLFEKITAT---------- : 337
RpGA2ox1   : KSVRHRVLA-NSY-KSRVSMIYFGGPPMSERIAPLPSIM--GVE-QTSLYKEFTWYEYKATSHKSRLADNRLVPFEKIAAS---------- : 308
PsGA2ox1   : KSVRHRVLA-NGI-DPRLSMIYFCGPPLSEKIAPLPSIM--KGK-ES-LYKEFTWFEYKSSTYGSRLADNRLGNYERIAAT---------- : 327
VaGA2oxA2  : RSVRHRVLA-NGF-KSRLSMIYFGGPPLSEKIVPLPSIM--KGK-ES-LYKEFTWFEYKNSTYGSRLADNRLGHFERIAAS---------- : 327
NtGA2ox5   : RSVRHRVLA--DSMKSRVSMIYFGGPPLSEKIAPLSCIM-EEG--EESLYNEFTWCEYKKSAYKTRLGDNRLALFEKKPQTKPATSAAQ- : 343
NoGA2ox3   : RSVKHRVFA--DGLKSRVSMIYLGGPPLDEKIAPLSSIM-EEG--EESLYKEFTWCEYKKSAYKTRLGDNRLKFFEK--------SVGQ- : 334
LsGA2ox2   : RSVRHRVVA--DSFKSRVSMIYFGGPPIMEKISPLGSIM-EPG--EESLYNEFTWFEYKSCTYKTRLTDNRLSLFHKHS------HFVH- : 338
PcGA2ox1   : KSVKHRVLA--DTTKSRLSMIYFGGPALSENIAPLPSVM-LKG--EECLYKEFTWCEYKKAAYTSRLADNRLAPFQKSAAD---------- : 332
VaGA2oxA1  : KSVKHRVLA--DTTKSRLSMIYFGGPALSEKIAPLPSVM-VKG--EESLYKEFTWWEYKKAAYTSRLADNRLAPFEKSATD---------- : 332
SoGA2ox1   : KSVKHRVLA--DNMKSRLSMIFFAGPPLCQKIAPLPCIM-QKG--EESLYEEFTWCEYKKSAYKSRLSENRLLRFVKRNHN---------- : 337
AtGA2ox2   : KSVKHRVLA--DTRRSRISMIYFGGPPLSQKIAPLPCIV-PEQ--DDWLYKEFTWSQYKSSAYKSKLGDYRLGLFEKQPLINHKTIV--- : 341
AtGA2ox3   : KSVKHRVVT--NTKRSRISMIYFAGPPLSEKIAPLSCIV-PKQ--DDCLYNEFTWSQYKLSAYKTKLGDYRLGLFEKRPPFSLSNV---- : 335
AtGA2ox1   : KSVRHRVLA--NCKKSRVSMIYFAGPSLTQRIAPLTCLI--DNE-DERLYEEFTWSEYKNSTYNSRLSDNRLQQFERKTIKNLIN----- : 329
HvGA2ox4   : RSVRHRVVAG-NGLKSRVSMIYFAGPPLAQRIAPLQQLIA--GTQSLPLYRDFTWGEYKKAAYRSRLGDNRLAPFETPIVAMPHAAHHRS : 342
OsGA2ox3   : KSVKHRVVA--NSLKSRVSFIYFGGPPLAQRIAPLPQLL---GEGEQSLYKEFTWDEYKKAAYKSRLGDNRLAQFEKK------------ : 327
HvGA2ox5   : SSVKHRVVV--SSERPRVSMIFFGGPPMGERLAPLRQLLG--DG-GRSKYREFTWKEYKSSTHKGRLATDRLCSFEN------------- : 341
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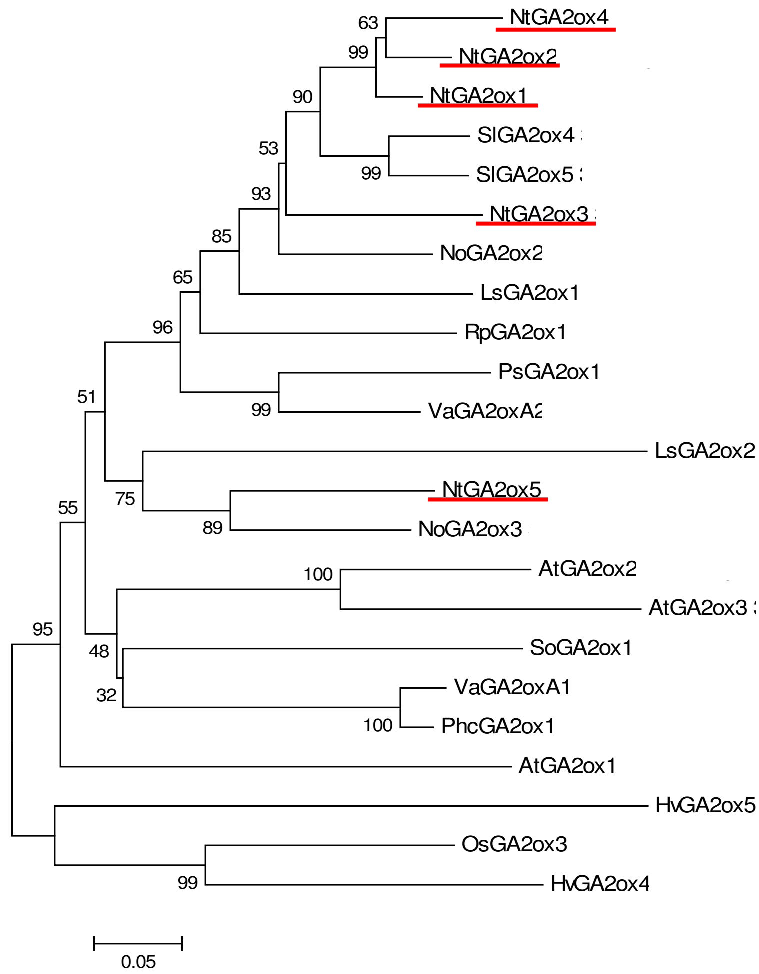
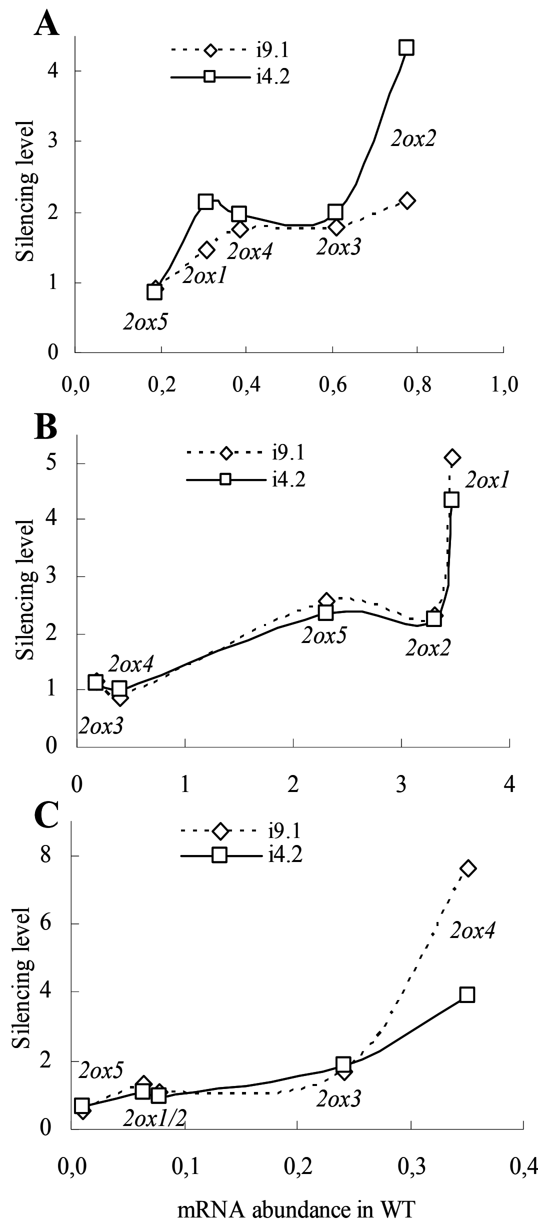
Supplemental data S3. Phylogenetic tree, based in amino acid sequences from 18 group I GA 2-oxidases enzymes whose activities have been confirmed experimentally and the sequences of *NtGA2ox1* to *-5*. This unrooted phylogenetic tree was performed with the MEGA3 program.



Supplemental data S4. Correlation of silencing level and the mRNA abundance of target genes. Silencing levels are plotted against mRNA abundance of the target gene in wild type (WT) in different organs; A. leaves, B. Ovaries and C. anthers. Silencing levels were estimated as the ratio of mRNA level between wild type and transgenic lines (i9.1 and i4.2) for each gene *NtGA2ox1* to *-5*. The data form performed these figures comes from data of the Figure 7.



DISCUSIÓN

1. Regulación de la ruta del metabolismo de GAs en tabaco

A lo largo de este trabajo se ha estudiado el papel de los genes de biosíntesis GA 3-oxidasa y GA 20-oxidasa y de catabolismo GA 2-oxidasa en la regulación de los niveles de GAs en tabaco, lo cual nos ha permitido conocer aspectos importantes de la regulación de esta hormona en la planta, tales como:

1.1. *El enzima GA 3-oxidasa no es limitante en la biosíntesis de GAs en tabaco.*

Las plantas transgénicas que sobreexpresan un gen *GA3ox* bajo el promotor constitutivo 35S (*35S:PsGA3ox1; GA3ox-OE*), presentaron pocas alteraciones fenotípicas respecto a las plantas silvestres. El ligero incremento en el contenido de la GA activa GA_1, correlaciona con las leves alteraciones fenotipicas encontradas en el hipocotilo y en la planta adulta, donde se ven pequeños aumentos en la longitud. El mayor efecto de la sobreexpresión de este gen sobre el metabolismo de GAs fue la significativa disminución en los niveles del precursor inmediato de GA_1, GA_{20}, en cuatro líneas homocigotas analizadas. Sin embargo, esta fuerte disminución en GA_{20} no produjo grandes incrementos en el contenido de GA_1 (Capitulo II). Resultados similares en cuanto a la alteración en el fenotipo y en los contenidos de GA_{20} y GA_1, fueron encontrados en álamo donde también se sobreexpresó un gen *GA3ox* (Israelsson *et al*., 2004). En todas las líneas transgénicas *GA3ox-OE* de tabaco, a diferencia de las plantas transgéncias de álamo, se detectó un aumento significativo en el contenido de GA_8 (forma inactiva de GA_1) y en la expresión de dos genes de GA 2-oxidasa (*NtGA2ox3* y *NtGA2ox5*). Estos resultados, explicarían la falta de acumulación de GA_1 causada por la actividad GA3ox en las plantas transgénicas de sobreexpresión de *GA3ox*, así como la escasa alteración del fenotipo (Capitulo II). Además, atribuyen un papel importante a estos dos genes de catabolismo en la regulación de la homeostasis de los niveles de GAs en tabaco.

El escaso efecto de la sobreexpresión de *GA3ox* sobre el fenotipo y el contenido de GAs activas en tabaco, podría deberse en parte a que los sustratos de este enzima (GA_{20} o GA_9) también fuesen limitantes y a que la sobreexpresión de este gen por si solo no fuese suficiente para incrementar el flujo en el contenido de GAs activas. Para estudiar esta hipótesis, se buscó incrementar el sustrato de este enzima en las plantas transgénicas *GA3ox-OE*. Se obtuvieron híbridos transgénicos provenientes de los cruces entre las líneas de *GA3ox-OE* con una línea de sobreexpresión de *GA 20-oxidasa* (*35S:CcGA20ox1*; *GA20ox-OE*). La línea *GA20ox-OE* previamente aislada en nuestro laboratorio, presentaba fenotipo de sobreproducción de GAs, así como un incremento en el contenido de GA_4 y GA_9 (Vidal *et al*., 2001) siendo este último el sustrato extra para la GA3ox. De esta manera, el efecto adicional que la sobreexpresión de un gen *GA3ox* tuviese sobre la sobreexpresión de una *GA20ox* revelaría el carácter limitante o no del enzima GA3ox en ciertos procesos del desarrollo, así como sobre la regulación del metabolismo de GAs. Con respecto

al parental *GA20ox-OE*, los híbridos transgénicos *GA3ox-OE/GA20ox-OE* mostraron leves variaciones en el fenotipo, así como un pequeño incremento en el contenido de GAs activas (GA_1 + GA_4). Estos resultados, refuerzan la idea de que la GA3ox no es un enzima limitante en el metabolismo de GAs en tabaco, incluso cuando se aumentan los niveles de su sustrato (Capitulo II).

1.2. *El enzima GA3ox en tabaco esta codificado por al menos dos genes que tienen diferente regulación.*

Existe un segundo gen *NtGA3ox2* clonado durante este trabajo, además del gen *NtGA3ox1*, descrito previamente por Itoh *et al*. (1999). De estos dos genes *GA3ox* de tabaco, *NtGA3ox1* es regulado por los contenidos de GAs mediante retroalimentación negativa, mientras que *NtGA3ox2* no parece estarlo (Capitulo II). La falta de regulación por retroalimentación mediada por GAs en genes del metabolismo de GAs también se ha descrito en la familia de genes *GA3ox* de *Arabidopsis*. Tres de ellos (*AtGA3ox2*, *-3* and *-4*) no presentan regulación por retroalimentación (Yamaguchi *et al*., 1998; Matsushita *et al*., 2007). Sin embargo, otros factores endógenos pueden estar regulando la expresión de estos genes. En el caso del gen *NtGA3ox2,* su nivel de expresión aumenta después de la inducción floral en el ápice y la abundancia de transcritos en este tejido es superior que en las hojas apicales de plantas de tabaco (Capitulo I). Esto podría indicar que este gen estaría regulado por factores endógenos del desarrollo.

1.3. *La expresión de los genes NtGA20ox1 y NtGA3ox1 puede ser utilizada como indicador de los niveles de GAs.*

Previamente, se había descrito que los genes *NtGA20ox1* y *NtGA3ox1* son regulados por el mecanismo de retroalimentación negativa mediado por GAs en tabaco (Tanaka-Ueguchi *et al*., 1998, Itoh *et al*., 1999). Nuestros resultados demuestran que existe una estrecha correlación entre la expresión génica de *NtGA20ox1* y *NtGA3ox1* y el contenido de GAs. Dicha correlación aparece tanto en los ápices de plantas silvestres (Capitulo I), como en los brotes apicales de las plantas transgénicas de tabaco (Capitulo II). Además en plantulas, también se encontró una correlación entre la expresión de los genes *NtGA20ox1* y *NtGA3ox1* y diferentes concentraciones crecientes de GA_1 aplicadas al medio. A su vez, el crecimiento del hipocotilo, variable dependiente de los niveles de GAs, tambien correlacionó con la expresión de estos dos genes (Capitulo II). En base a esto, proponemos que los niveles de expresión de los genes *NtGA20ox1* y *NtGA3ox1* pueden ser usados como un buen indicador de los contenidos endógenos de GAs en la planta al menos en algunos tejidos, siendo esta una herramienta importante en los estudios del metabolismo de GAs en tabaco, ya que la cuantificación de esta hormona es muy laboriosa.

1.4. *Existen al menos cinco genes de GA 2-oxidasa en tabaco que se expresan diferencialmente en la planta.*

En este trabajo se han clonado tres genes de *GA 2-oxidasa,* que se unen a los dos genes *GA2ox* depositados previamente en la base de datos. El análisis de expresión por RT-PCR semi-cuantitativo de los cinco genes *NtGA2ox* en plantas silvestres de tabaco, muestra que se expresan tanto en tejidos vegetativos como reproductivos. Además, no se encontró expresión específica de ningún gen *NtGA2ox* en un solo tejido (Capitulo III). Esto indica que existe un solapamiento de expresión dentro de esta familia de genes. En la expresión de todos los tejidos vegetativos, el gen *NtGA2ox2* fue el más abundante. La expresión de los genes *NtGA2ox3* y *-4* fue abundante en hojas jóvenes, mientras que la de *NtGA2ox5* lo fue en el tallo. En los tejidos reproductivos, los genes *NtGA2ox1, -2* y *-5* son los predominantes en ovarios, *NtGA2ox3* en pétalos y *NtGA2ox4* y *-3* en anteras.

1.5. *Existen dos tipos de regulación para la familia de genes de GA 2-oxidasa en tabaco de acuerdo a la sensibilidad de estos genes frente a las variaciones en los niveles de GAs.*

Todos los miembros de la familia génica *NtGA2ox* aislados son regulados por retroalimentación positiva mediada por GAs (Capitulo III). Este tipo de regulación ha sido descrita previamente en algunos genes *GA2ox* en *Arabidopsis* (Thomas *et al*., 1999) y en guisante (Elliott *et al.*, 2001). Sin embargo, nuestros resultados indican que la regulación por retroalimentación positiva es mucho más fina de lo descrito previamente, dado que hemos encontrado un grupo de genes en los cuales su expresión es altamente inducida cuando se producen pequeños cambios en los contenidos de GAs (el caso de los genes *NtGA2ox3* y *NtGA2ox5*). En contraposición, hay un segundo grupo de genes (en el caso de *NtGA2ox1, -2 y 4*) en los cuales solo se detecta la inducción de su expresión cuando se producen grandes cambios en los niveles de GAs (Capitulo II y III). Los resultados anteriores, sugieren que existen dos tipos de regulación por retroalimentación positiva para los genes *GA2ox* en tabaco, que difieren en la sensibilidad para responder a pequeños o a grandes cambios en las variaciones de los niveles de GAs.

1.6. *La homeostasis de GAs en tabaco depende de distintos genes del metabolismo de GAs en función del grado de variación en los contenidos de las mismas.*

Esta idea se basa en nuestros resultados de la expresión de los genes del metabolismo de GAs en las plantas transgénicas estudiadas. En las plantas de sobreexpresión de *GA3ox*, que presentaban leves variaciones en los niveles de GAs, los genes *NtGA20ox1* y *NtGA3ox1* no alteraron su expresión. Sin embargo, en las plantas tanto de sobreexpresión de *GA20ox* como en los híbridos *GA3ox-OE/GA20ox-OE*, los cuales tienen aumentos significativos en los niveles de GAs, la

expresión de estos mismos dos genes está claramente alterada (Capitulo II). En conjunto, los resultados del patrón de expresión de los genes de síntesis *NtGA20ox1* y *NtGA3ox1,* en respuesta a pequeñas o grandes variaciones en los niveles de GAs en las plantas transgénicas, sugiere que estos genes tendrían un papel clave en la homeostasis de GAs cuando hay grandes variaciones en el contenido de las mismas. Además, se sugiere que los genes de catabolismo *NtGA2ox3* y *NtGA2ox5* serían los más importantes en la modulación fina de los contenidos de GAs, ya que son los únicos que varían su expresión cuando hay ligeras variaciones en el metabolismo de las GAs activas, como por ejemplo en las plantas de sobreexpresión de *GA3ox* (Capitulo II).

2. El papel de las GAs en distintos procesos del desarrollo de tabaco

A partir de nuestros experimentos se ha obtenido una información relevante del papel de los genes de *GA 2-oxidasa* en diversos procesos del desarrollo de tabaco, así como del papel de las GAs en la transición floral.

2.1. *El silenciamiento múltiple de GA 2-oxidasas afecta el desarrollo de tabaco.*

El análisis de expresión de cinco genes *NtGA2ox* en plantas silvestres de tabaco indica que existe redundancia génica. También se ha descrito en *Arabidopsis,* redundancia génica en la familia *GA2ox*, donde mutaciones de perdida de función para un solo gen *GA2ox* presentan muy poco fenotipo, y solo en mutantes dobles de *GA2ox* se detecta un fenotipo moderado (Schömburg *et al.*, 2003). En arroz, se ha interpretado que la redundancia génica de los genes *GA2ox* ha dificultado el aislamiento de mutaciones para estos genes (Sakamoto *et al.*, 2004). Cuando la expresión de un gen *GA2ox* presenta poco solapamiento funcional con otros genes de la misma familia, las mutaciones tienen un efecto sobre el fenotipo muy claro. Tal es el caso del mutante *sln* de guisante, causado por una mutación en el gen *PsGA2ox1,* el cual tiene un papel importante en el control de los niveles de GAs en estadíos tempranos del desarrollo (Martin *et al.*, 1999; Lester *et al.*, 1999). Dado que en nuestro caso los genes *GA2ox* solapan su expresión en diversos tejidos y probablemente una mutación puntual en un solo gen, podría no producir mayores efectos en el fenotipo de las plantas, nos planteamos obtener un mutante en tabaco que tuviese silenciado todos los genes *GA2ox* a la vez.

El silenciamiento génico post-transcripcional múltiple mediado por el RNA de interferencia (RNAi), ha sido abordado con éxito previamente en *Arabidopsis* (Kaur *et al.*, 2006) y arroz (Miki *et al.*, 2005), indicando que el uso de esta estrategia es adecuada para el estudio de la función de los miembros de una familia génica. De esta manera, se diseñó una construcción tipo horquilla portadora de una secuencia homóloga a los cinco genes *GA2ox* en tabaco, potencialmente capaz de silenciar la expresión de todos.

Las plantas transgénicas de silenciamiento (*GA2ox/RNAi*) en tabaco presentaron considerables alteraciones fenotípicas en diferentes órganos, relacionadas con el silenciamiento parcial de varios genes *GA2ox*. Estos resultados indican que la construcción empleada para el silenciamiento fue capaz de silenciar los cinco miembros de esta familia génica. Es interesante destacar, que el grado de silenciamiento de los *GA2ox* se correlacionó con la abundancia de expresión de cada uno de ellos en diferentes tejidos. De esta manera, los genes más abundantes son los más silenciados y no los más homólogos a la construcción inductora de silenciamiento. Esto quiere decir que dentro de un mínimo de homología de secuencia, en nuestro caso entre el 80-100%, la eficacia del silenciamiento está ligada en mayor medida a la abundancia del mensajero que se quiere silenciar, que al grado de homología entre la secuencia inductora del silenciamiento y su mensajero diana (Capitulo III).

El efecto del silenciamiento múltiple de los genes *GA2ox* sobre el metabolismo de GAs, ha sido estudiado a través de su efecto en la expresión de los genes indicadores *NtGA20ox1* y *NtGA3ox1*. Dicha información se utilizó para tener una estimación de los contenidos endógenos de GAs en estas plantas de silenciamiento. Se encontró una disminución de la expresión de los genes *NtGA20ox1* y *NtGA3ox1* en diferentes tejidos de las plantas transgénicas *GA2ox/RNAi*, lo cual sugiere que el contenido de GAs activas en estas plantas estaría aumentado. La mayoría de los fenotipos detectados en las plantas *GA2ox/RNAi* son similares a los fenotipos de superproducción de GAs. El aumento de longitud en distintos órganos, como hipocotilo, entrenudo, hoja, pedúnculo de frutos, así como de la altura final que observamos en nuestras plantas, también se ha detectado en plantas que superproducen GAs por sobreexpresión del enzima de síntesis GA 20-oxidasa en tabaco (Vidal *et al*., 2001; Biemelt *et al*., 2004). Sin embargo, otras alteraciones fenotipicas detectadas en nuestras plantas *GA2ox/RNAi*, como son la inhibición en el crecimiento de la raíz, el retraso en la floración y la baja fertilidad no fueron detectadas en plantas superproductoras de GAs. Nuestros resultados indican que estas alteraciones fenotipicas adicionales también podrían ser consecuencia de un exceso de GAs. La inhibición del crecimiento de la raíz fue revertida disminuyendo la síntesis de GAs con aplicaciones de PCB. El retraso en la floración también se observó en plantas tratadas con aplicaciones saturantes de GA_3 o en los híbridos *GA3ox-OE/GA20ox-OE* de sobreexpresión conjunta de dos genes de síntesis. Igualmente la baja fertilidad ha sido previamente descrita en mutantes de respuesta constitutiva a GAs en *Arabidopsis* (*spy*; Jacobsen *et al*., 1998). Todos estos resultados en las plantas de silenciamiento *GA2ox/RNAi*, señalan la importancia de los genes de *GA2ox* en el control de los niveles de GAs que modulan diversos procesos del desarrollo de tabaco como son el crecimiento de distintos tejidos, el desarrollo de la antera y el tiempo de floración (Capitulo III).

2.2. *La floración en tabaco depende de las GAs, pero no es inducida por los niveles de GAs activas, GA_1 y GA_4 en el ápice.*

El papel de las GAs en el control del tiempo de floración no está concluyentemente demostrado, y la mayoría de las pruebas a favor del papel de esta hormona en la regulación de la inducción floral viene de experimentos en plantas tipo roseta, dependientes de fotoperiodo, donde la elongación del tallo coincide con la floración. La importancia de las GAs en la inducción de la floración se pone de manifiesto por la falta de floración de los mutantes de *Arabidopsis, ga1-3* deficientes de GAs, cuando se cultivan en condiciones no inductivas, es decir de día corto (Wilson *et al.*, 1992). En *Arabidopsis*, se ha demostrado que GA_4 es la GA activa que regula tanto la elongación del tallo, como la inducción del tiempo de floración en condiciones de día corto. Debido a que el contenido de GA_4 en el ápice muestra un aumento que precede a la transición floral y a la inducción el gen de identidad meristemática LEAFY (Eriksson *et al.*, 2006).

Las plantas de tabaco (*Nicotiana tabacum* var. xanthi) son independientes del fotoperiodo. Tanto tabaco como guisante no son plantas tipo rosetas y su floración no está asociada con la elongación del tallo (Ross *et al.*, 1992). La determinación de los niveles de GAs activas en los ápices de plantas silvestres de tabaco antes, durante y después de la transición floral, mostraron una disminución gradual en los contenidos de GA_1 y GA_4 en los ápices conforme las plantas se acercaban a la transición floral. Adicionalmente, la disminución en el contenido de GAs activas en el ápice correlacionó con la disminución gradual del crecimiento del tallo durante todo el desarrollo vegetativo. Estos resultados excluyen que los contenidos de GA_1 y GA_4 en el ápice, sean la señal inductora de la floración en tabaco (Capitulo I). Sin embargo, las GAs son necesarias para la floración en esta especie, puesto que plantas deficientes en GAs, bien inducidas por la sobreexpresión de un gen de catabolismo, (*35S:GANo2ox3*; Ubeda-Tomas *et al.*, 2006) o por tratamiento con un inhibidor de la biosíntesis de GAs (Paclobutrazol; PCB), tienen un periodo mayor de su fase vegetativa y florecen tardíamente.

2.3. *El exceso de GAs puede tener un efecto inhibitorio sobre la floración en tabaco.*

Por otra parte, se ha encontrado que el tiempo de floración en tabaco también puede ser afectado en plantas que sobreexpresan conjuntamente una GA 3-oxidasa y una GA 20-oxidasa, (híbridos *GA3ox-OE/GA20ox-OE*), en plantas tratadas con dosis saturantes de GA_3, y más aún en plantas transgénicas de silenciamiento múltiple de *GA2ox*. Estos resultados sugieren que también un exceso de GAs puede inhibir la transición floral (Capitulo I, II y III).

Nuestros resultados indican que los contenidos de GAs en el ápice no son la señal inductora de la transición floral en tabaco. Sin embargo, los niveles de GAs deben estar dentro de un intervalo óptimo (encima de un mínimo y por debajo de un máximo), para que el meristemo sea competente para florecer y se diferencie tras la percepción de la señal inductora de la transición floral.

CONCLUSIONES

1. Las GAs activas (GA_1 y GA_4) en el ápice no son la señal inductora de la floración en tabaco ya que sus niveles disminuyen al acercarse la transición floral. Sin embargo los contenidos de GAs activas deben estar dentro de un intervalo óptimo para que ocurra la floración (por encima de un mínimo y por debajo de un máximo), ya que tanto el déficit como el exceso de GAs retrasa la floración en tabaco.

2. El enzima GA 3-oxidasa no es limitante en el metabolismo de GAs en tabaco ya que la sobreexpresión de una GA 3-oxidasa (sola o en conjunto con una GA 20-oxidasa) no produce incrementos importantes en el contenido de GAs activas, ni en el fenotipo de tabaco. Adicionalmente, este enzima esta codificado por al menos dos genes en tabaco, de los cuales solo uno de ellos es regulado por retroalimentación negativa mediada por GAs.

3. La expresión de los genes *NtGA20ox1* y *NtGA3ox1* puede ser utilizada como un indicador de los niveles endógenos de GAs en tabaco. Existe una estrecha correlación entre los niveles de expresión de estos genes y los contenidos de GAs activas, así como con el crecimiento del hipocotilo. Además estos genes de biosíntesis *NtGA20ox1* y *NtGA3ox1* tienen un papel clave en la homeostasis de GAs cuando hay grandes cambios en sus contenidos.

4. Existen al menos cinco genes de GA 2-oxidasa en tabaco que se expresan diferencialmente en la planta y que son regulados por retroalimentación positiva mediada por GAs. Dicha regulación es modulada bien por, pequeños cambios (el caso de los genes *NtGA2ox3* y *NtGA2ox5*) o por grandes cambios en los contenidos de GAs (para los genes *NtGA2ox1, -2 y 4*). Consecuentemente, los genes *NtGA2ox3* y *-5* tienen un papel importante en la homeostasis de GAs cuando hay ligeras variaciones en el metabolismo de las mismas.

5. Los genes de catabolismo GA 2-oxidasa tienen un papel importante en diversos procesos del desarrollo en tabaco, dado que el silenciamiento múltiple de estos genes, ha dado lugar a plantas transgénicas con diversas alteraciones fenotipicas, las cuales correlacionan con fenotipos similares a los de superproducción de GAs, tales como: incrementos en la longitud de hipocotilo, entrenudos, hojas, pedúnculo de frutos, así como de la altura final de plantas. Además, en estas plantas se detectaron otros fenotipos como inhibición en el crecimiento de la raíz, retraso en la floración y baja fertilidad que también parecen estar asociados a un exceso de GAs.

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