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Interacción de Giberelinas y Auxinas en la Fructificación del Tomate

Memoria presentada por
JUAN CARLOS SERRANI YARCE

Directores
Dr. JOSÉ LUÍS GARCÍA MARTÍNEZ
Dr. MARIANO FOS CAUSERA

Tutor
Dr. VICENTE MORENO FERRER

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D. JOSÉ LUIS GARCÍA MARTÍNEZ, Doctor Ingeniero Agrónomo, Profesor de Investigación del Consejo Superior de Investigaciones Científicas adscrito al Instituto de Biología Molecular y Celular de Plantas y D. MARIANO FOS CAUSERA, Doctor en Ciencias Biológicas, Catedrático de Escuela Universitaria del Departamento de Biología Vegetal de la Universidad Politécnica de Valencia.

CERTIFICAN: Que D. JUAN CARLOS SERRANI YARCE, Ingeniero Agrónomo, ha realizado bajo nuestra dirección, en el Instituto de Biología Molecular y Celular de Plantas, el trabajo que con el título de "Interacción de Giberelinas y Auxinas en la Fructificación del Tomate", presenta para optar al grado de Doctor Ingeniero Agrónomo.

Para que así conste a todos los efectos oportunos firman el presente certificado en Valencia a 7 de enero de 2008.

Dr. José Luís García Martínez Dr. Mariano Fos Causera

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ABREVIATURAS

2,4-D = ácido 2,4-diclorofenoxiacético
2,4,5-T = ácido 2,4,5-triclorofenoxiaético
4-Cl-IAA = ácido 4-cloroindol-3-acético
ARF8 = auxin response factor 8
$C_{1.....n}$ = carbono en posición 1….n
cv = cultivar
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)
dpa = días post antesis
EM = Mesocarpo externo
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 = 2β-hidroxilasa de giberelinas
GA3ox = 3β-hidroxilasa de giberelinas
GA13ox = 13-hidroxilasa de giberelinas
GA20ox = 20-hidroxilasa de giberelinas
GGDP = geranilgeranil bifosfato
IAA = ácido indolacético
IAA-Asp = indol-3-acetil-L-aspartato
IAOx = indol-3-acetaldoxima
IBA = ácido indol-3-butírico
IGP = fosfato indol-3-glicerol
IM = mesocarpo interno
IPA = ácido indol-3-pirúvico
KAO = ácido *ent*-kaurenoico oxidasa
KO = *ent*-kaureno oxidasa
KS = *ent*-kaureno sintasa
LAB 198999 = etil éster ácido carboxílico 3,5-dioxi-4-butiril-ciclohexano
MCPA = 2,4-metilfenoxiacético
MeGAs = ésteres metilados de giberelinas
NAA = ácido naftalenacético
NPA = ácido naftiltalámico
OxIAA = 2-oxindol-3-acético
pb = pares de bases (bp, base pair)
PCB = paclobutrazol
PCIB = ácido α-(ρ-clorofenoxi) isobutírico
Pa = células cuyo eje mayor es paralelo a la epidermis
Pe = células cuyo eje mayor es perpendicular a la epidermis
SAM = S-adenosin-l-metionina
TIBA = ácido triiodobenzóico
Tpm = triptamina
Trp=triptófano

RESÚMENES

RESUMEN

El efecto de la aplicación de giberelinas (GAs) y auxinas durante la fructificación y desarrollo del fruto, ha sido investigado en tomate (*Solanum lycopersicum* L.) cv Micro-Tom. Los resultados indican que constituye un sistema adecuado para el estudio de la regulación hormonal en tomate. Para evitar la competencia entre frutos dentro del mismo racimo, solo un fruto por racimo y hasta dos racimos por planta se utilizaron en los experimentos. Ovarios no polinizados (emasculados) se desarrollaron partenocárpicamente en respuesta a la aplicación de $GA_3 > GA_1 = GA_4 > GA_{20}$, aunque no de GA_{19}, y de diferentes auxinas tales como los ácidos indolacético (IAA), naftalenacético (NAA) y 2,4-diclorofenoxiacético (2,4-D), siendo este último el más eficiente. La morfología de los frutos inducidos por auxinas y giberelinas es diferente. En los frutos tratados con GA_3 el tejido locular se desarrolla pobremente dejando las cavidades loculares vacías, mientras que los frutos tratados con 2,4-D presentan pseudoembriones y cavidades loculares llenas. A nivel interno, el GA_3 indujo células de mayor tamaño en el mesocarpo interno, lo cual estaba correlacionado con un mayor nivel de ploidía (mayor MCV, mean C value), mientras el 2,4-D favoreció las divisiones celulares, por lo que el número de capas celulares en el pericarpo fue superior al tratamiento con GA_3 y al polinizado. Los frutos polinizados tuvieron un mayor tamaño y peso que los frutos inducidos con GA_3 aunque ambos fueron más pequeños y de menor peso que los tratados con 2,4-D. El grosor del pericarpo de los frutos inducidos con GA_3 y 2,4-D no mostró diferencias hasta 20 días después de antesis, por lo que el tener menos células en el pericarpo (frutos inducidos con GA_3) podría ser compensado teniendo un mayor tamaño celular. El uso de inhibidores de biosíntesis de GAs tales como el pablobutrazol (PCB) y LAB 198999 disminuyó la fructificación y desarrollo del fruto, efecto revertido con la aplicación de GA_3. Sin embargo, en frutos polinizados el LAB redujo el contenido de GA_1 y GA_8 pero incrementó el de GA_{53}, GA_{44}, GA_{19} y GA_{20}, lo cual puede indicar que GA_1 es la GA activa en el crecimiento del fruto de tomate. Ambos resultados sugieren que la fructificación depende de GAs. Al analizar el efecto de la polinización en los niveles de transcritos de los genes de biosíntesis de GAs, se observó que se incrementaron los niveles de *SlGA20ox1*, *-2*, y *-3*, y de *SlCPS*, pero no los de *SlGA3ox1* y *-2*. Esto, junto con la no inducción de la fructificación al

aplicar GA_{19}, sugiere que la actividad GA 20-oxidasa es limitante en el ovario. Igualmente, para ver el efecto de la polinización en los niveles de transcritos de enzimas de inactivación de GAs, se aislaron 5 clones completos de cDNA de genes que codifican GA-2-oxidasas (*SlGA2ox1*, *-2*, *-3-*, *-4* y *-5*). Si bien los niveles de transcritos de esos genes no disminuyeron tempranamente luego de la polinización (5 días post antesis, dpa), todos se redujeron, especialmente *SlGA2ox2*, luego de 10 dpa. Así, la polinización regula la fructificación activando la biosíntesis de GAs por medio del aumento de los niveles de *GA20ox*. Con el propósito de establecer la posición de los nuevos genes de catabolismo (*SlGA2ox1*, *-2*, *-3-*, *-4* y *-5*) aislados dentro de las subfamilias respectivas, se realizó un análisis filogenético de los genes de la familia *GA2ox*. Dicho análisis mostró la existencia de tres subfamilias que podrían caracterizarse por sus diferentes propiedades bioquímicas. De otra parte, la aplicación de los inhibidores PCB y LAB sobre frutos tratados con 2,4-D e IAA, redujo significativamente la fructificación, y ese efecto fue contrarrestado con la aplicación de GA_3. Al medir los contenidos de GAs en frutos inducidos con 2,4-D, se observó que estos tenían un contenido más alto de GA_1 y GA_8 que los polinizados, aunque las concentraciones de los precursores (GA_{53}, GA_{44}, GA_{19}, GA_{20}) fueron similares, mientras que el contenido de GA_{29} disminuyó en éstos. Estos últimos resultados sugieren que el efecto de las auxinas esta mediado por GAs. De la misma manera, la aplicación de GAs marcadas radiactivamente a ovarios no polinizados, en presencia o no de 2,4-D, produjo variaciones en el metabolismo de las GAs. El 2,4-D indujo el metabolismo de [^{14}C]GA_{12} a [^{14}C]GA_9 putativa, y de [^{14}C]GA_{20} principalmente a [^{14}C]GA_1, no a [^{14}C]GA_{29} como ocurrió en el control, mientras que el metabolismo de [^{14}C]GA_1 a [^{14}C]GA_8 fue menor en ovarios tratados con 2,4-D. El análisis de los transcritos de ovarios inducidos con 2,4-D, mostró que la auxina incrementó los niveles de expresión de *SCPSs*, *SlGA20ox1*, *-2* y *-3 SlGA3ox1* y *SlGA3ox1* pero no los de *SlGA3ox2*, mientras que solo los niveles de expresión de *SlGA2ox2* fueron menores en frutos tratados con 2,4-D. En conclusión, y de acuerdo a los resultados obtenidos, se sugiere que las auxinas inducen la fructificación y el crecimiento del fruto de tomate 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 de GA_1.

RESUM

L'efecte de l'aplicació de gibberel·lines (GA) i auxines durant la fructificació i desenvolupament del fruit, ha estat investigat en la tomaca (*Solanum lycopersicum* L.) cv Micro-Tom. Els resultats indiquen que constitueix un sistema convenient per a l'estudi de la regulació hormonal en tomaca. Per a prevenir la competència entre fruits dins del mateix ramell, només un fruit per ramell i fins a dos ramells per planta es van utilitzar en els experiments. Ovaris no pol·linitzats (emasculats) es van desenvolupar de forma partenocàrpica en resposta a l'aplicació de $GA_3 > GA_1 = GA_4 > GA_{20}$, encara que no de GA_{19}, i de diferents auxines com ara els àcids indolacètics (IAA), naftalènic (NAA) i 2,4-diclorofenoxiacètic (2,4-D), sent aquest últim el més eficient. La morfologia dels fruits induïts per auxines i gibberel·lines és diferent. En els fruits tractats amb GA_3 el teixit locular es desenvolupa pobrament deixant les cavitats loculars buides, mentre que els fruits tractats amb 2,4-D presenten pseudoembrions i cavitats loculars plenes. A nivell intern, el GA_3 va induir cèl·lules més grans en el mesocarpi intern, la qual cosa estava correlacionada amb un major nivell de ploïdia (major MCV, *mean C value*), mentre el 2,4-D va afavorir les divisions cel·lulars, per la qual cosa el nombre de capes cel·lulars en el pericarpi va ser superior al tractament amb GA_3 i al pol·linitzat. Els fruits pol·linitzats van tindre un major grandària i pes que els fruits induïts amb GA_3 encara que ambdós van ser més xicotets i de menor pes que els tractats amb 2,4-D. El grossor del pericarpi dels fruits induïts amb GA_3 i 2,4-D no va mostrar diferències fins a 20 dies després d'antesi, per la qual cosa el tindre menys cèl·lules en el pericarpi (fruits induïts amb GA_3) podria ser compensat tenint un major grandària cel·lular. L'ús d'inhibidors de biosíntesi de les GA com ara el pablobutrazol (PCB) i LAB 198999 va disminuir la fructificació i desenvolupament del fruit, efecte revertit amb l'aplicació de GA_3. No obstant això, en fruits pol·linitzats el LAB va reduir el contingut de GA_1 i GA_8 però va incrementar el de GA_{53}, GA_{44}, GA_{19} i GA_{20}, la qual cosa pot indicar que GA_1 és la GA activa en el creixement del fruit de tomaca. Ambdós resultats suggereixen que la fructificació depén de les GA. En analitzar l'efecte de la pol·linització en els nivells de transcrits dels gens de biosíntesi de les GA, es va observar que s'incrementaren els nivells de *SlGA20ox1*, *-2*, i *-3*, i de *SlCPS*, però no els

de *SlGA3ox1* i *-2*. Açò, juntament amb la no inducció de la fructificació en aplicar GA_{19}, suggereix que l'activitat GA 20-oxidasa és limitant en l'ovari. Igualment, per a veure l'efecte de la pol·linització en els nivells de transcrits d'enzims d'inactivació de les GA, es van aïllar 5 clons complets de cDNA de gens que codifiquen per a GA-2-oxidases (*SlGA2ox1*, *-2*, *-3-*, *-4* i *-5*). Si bé els nivells de transcrits d'aquests gens no van disminuir prompte després de la pol·linització (5 dies post antesis dpa), tots es van reduir, especialment *SlGA2ox2*, després de 10 dpa. Així, la pol·linització regula la fructificació i activa la biosíntesi de les GA mitjançant l'augment dels nivells de *GA20ox*. Una anàlisi filogenètica dels gens de la família *GA2ox* va ser realitzada, per a establir la posició dels nous gens de catabolisme (*SlGA2ox1*, *-2*, *-3-*, *-4* i *-5*) aïllats dins de les subfamílies respectives. Aquesta anàlisi va mostrar l'existència de tres subfamílies que podrien caracteritzar-se per les seues diferents propietats bioquímiques. D'una altra part, l'aplicació dels inhibidors PCB i LAB sobre fruits tractats amb 2,4-D i IAA, va reduir significativament la fructificació, i aquest efecte va ser contrarestat amb l'aplicació de GA_3. En mesurar els continguts de les GA en fruits induïts amb 2,4-D, es va observar que aquests tenien un contingut més alt de GA_1 i GA_8 que els pol·linitzats, encara que les concentracions dels precursors (GA_{53}, GA_{44}, GA_{19}, GA_{20}) van ser semblants, mentre que el contingut de GA_{29} hi va disminuir. Aquests últims resultats suggereixen que l'efecte de les auxines està mediat per les GA. De la mateixa manera, l'aplicació de les GA marcades de forma radioactiva a ovaris no pol·linitzats, en presència o no de 2,4-D, va produir variacions en el metabolisme de les GA. El 2,4-D va induir el metabolisme de $[^{14}C]GA_{12}$ a $[^{14}C]GA_9$ putativa, i de $[^{14}C]GA_{20}$ principalment a $[^{14}C]GA_1$, no a $[^{14}C]GA_{29}$ com va ocórrer en el control, mentre que el metabolisme de $[^{14}C]GA_1$ a $[^{14}C]GA_8$ va ser menor en ovaris tractats amb 2,4-D. L'anàlisi dels transcrits d'ovaris induïts amb 2,4-D, va mostrar que l'auxina va incrementar els nivells d'expressió de *SCPSs*, *SlGA20ox1, -2* i *-3 SlGA3ox1* i *SlGA3ox1* però no els de *SlGA3ox2*, mentre que només els nivells d'expressió de *SlGA2ox2* van ser menors en fruits tractats amb 2,4-D. En conclusió i d'acord amb els resultats obtinguts, se suggereix que les auxines indueixen la fructificació i el creixement del fruit de

tomaca, i augmenten la biosíntesi de les GA a través de l'activitat GA 20-oxidasa, GA 3-oxidasa i CPS, el que comporta a un major contingut de GA_1.

ABSTRACT

The effect of applied gibberellin (GA) and auxin on fruit-set and growth has been investigated in tomato (*Solanum lycopersicum* L.) cv Micro-Tom. Results indicate that this cultivar is a convenient model system to perform research on the hormonal regulation of tomato. It was found that to prevent competition between developing fruits, only one fruit per truss and two trusses should be left per plant. Unpollinated ovaries responded to $GA_3 > GA_1 = GA_4 > GA_{20}$ (but not to GA_{19}) and to different auxins [indol-3-acetic acid, naphthaleneacetic acid, and 2,4-dichlorophenoxyacetic acid (2,4-D)], 2,4-D being the most efficient. GA- and auxin-induced fruits had different internal morphology, poor locular tissue development and empty locular cavities in the case of GA_3, and pseudoembryos development and filled locular cavities in the case of 2,4-D. Also, GA_3 produced larger cells in the internal region of the mesocarp correlated with higher mean C values (MCV), while 2,4-D promoted cell division and thus produced more cell layers in the pericarp than GA_3 and pollinated treatments. Pollinated fruits had higher size and weight than GA_3-treated fruits, but both were smaller compared to 2,4-D treatment. Pericarp thickness of GA_3- and 2,4-D-induced fruits did not showed differences upon 20 dpa, reason why having fewer cells (GA_3) could be compensated by a larger cell size. Two different inhibitors of GA biosynthesis [LAB 198999 and paclobutrazol (PCB)] decreased fruit growth and fruit-set, an effect reversed by GA_3 application. LAB 198999 reduced GA_1 and GA_8 content, but increased that of their precursors GA_{53}, GA_{44}, GA_{19} and GA_{20} in pollinated fruits. This supports the hypothesis that GA_1 is the active GA for tomato fruit growth. Both results demonstrate that fruit-set on tomato depends on GAs. The effect of pollination of GA biosynthesis genes was performed analyzing transcript levels of *SlCPS*, *SlGA20ox1*, *-2* and *-3*, and *SlGA3ox1* and *-2*. Pollination increased transcript content of *SlGA20ox1*, *-2*, and *-3*, and *SlCPS*, but not of *SlGA3ox1* and *-2*. This summarized to the non-induction of fruit set by GA_{19}, suggests that GA 20-oxidase activity was limiting in unpollinated ovaries. To

investigate whether pollination also altered GA inactivation, full length cDNA clones of genes encoding enzymes catalyzing GA 2-oxidases (*SlGA2ox1*, *-2*, *-3*-, *-4* and *-5*) were isolated and characterized. Transcript levels of these genes did not decrease early after pollination (5 dpa fruits), but transcript content reduction of all of them, mainly of *SlGA2ox2*, was found later (from 10 dpa). Thus, pollination mediates fruit-set by activating GA biosynthesis mainly through up regulation of *GA20ox*. A phylogenetic reconstruction of the *GA2ox* family was necessary to establish new genes position between subfamilies. It was showed the existence of three gene subfamilies maybe distinguished by its biochemical properties. Other aspect, is that fruit development induced by the auxins indol-3-acetic acid and 2,4-dichlorophenoxyacetic acid (2,4-D) were significantly reduced by simultaneous application of inhibitors of GA biosynthesis (paclobutrazol and LAB 198999), and that this effect was reversed by applied GA_3. Parthenocarpic fruits induced by 2,4-D had higher contents of the active GA_1 and of GA_8 than pollinated fruits, while concentrations of the precursors (GA_{53}, GA_{44}, GA_{19}, GA_{20}) and GA_{29} were similar. Radioactive-labelled GAs applied to unpollinated ovaries, in presence or not of 2,4-D, showed that auxin induced the metabolism of [^{14}C]GA_{12} to putative [^{14}C]GA_9, and of [^{14}C]GA_{20} mainly to [^{14}C]GA_1, not to [^{14}C]GA_{29} as occurred in control. [^{14}C]GA_1 metabolism to [^{14}C]GA_8 was much lower in 2,4-D-treated ovaries than in control. Transcript levels of genes encoding copalyldiphosphate synthase (*SlCPS*) and *SlGA20ox1*, *-2* and *-3* and *SlGA3ox1*, but not those encoding *SlGA3ox*2, were higher in unpollinated ovaries treated with 2,4-D, while transcript levels of *SlGA2ox2* were lower in 2,4-D treated ovaries. Our results support the idea that auxins induce fruit-set and growth in tomato by enhancing GA biosynthesis through GA 20-oxidase, GA 3-oxidase and CPS, leading to higher GA_1 content.

INTRODUCCIÓN

1. EL FRUTO DE TOMATE

El tomate (*Solanum lycopersicum* L.) es uno de los frutos carnosos tipo baya más estudiados. Su gran importancia económica radica en ser una de las especies hortícolas más ampliamente cultivadas no solo en España sino a nivel mundial, gracias a su gran adaptabilidad, variabilidad, características organolépticas y usos.

1.1. Fructificación

La formación del fruto es uno de los procesos del desarrollo más complejos de las plantas. Existen diferentes etapas en ese proceso, pero la base del futuro crecimiento y desarrollo es el **cuajado**. El cuajado o fructificación se entiende como la reanudación del crecimiento del ovario de la flor, pasando de una condición estática a un rápido crecimiento y depende de una exitosa polinización y fecundación de los óvulos (Gillaspy *et al.,* 1993). En la **polinización** ocurre la dehiscencia de las anteras, lo cual permite que los granos de polen caigan en el estigma, que es receptivo desde 1 ó 2 días antes. Las anteras de las flores de tomate están unidas adyacentemente por una serie de pelos que forman un cono estaminal fusionado. Dicho cono rodea estrechamente el estilo, lo cual hace que el polen de una flor caiga sobre su mismo estigma autopolinizándose, generalmente en el momento de la antesis (Picken, 1984; Ho y Hewitt, 1986). Una vez en el estigma, los granos de polen deben germinar, y esto puede tardar entre 0,5 y 20 horas (Picken, 1984).

El gametofito femenino o saco embrionario en las angiospermas está formado por ocho núcleos haploides distribuidos en siete células organizadas con un patrón y una disposición característica. Las tres células que se sitúan en el extremo más cercano al micrópilo son la ovocélula o gameto femenino y las dos sinérgidas que flanquean a la ovocélula. Las tres células que se sitúan en el extremo más alejado del micrópilo son las antípodas. En el centro, ocupando casi todo el volumen del saco embrionario, se sitúa la célula polar que contiene dos núcleos polares que se fusionarán para formar el núcleo secundario diploide (Farabee, 2001). El gametofito masculino está compuesto por dos núcleos: un núcleo vegetativo que formará el tubo polínico al germinar en el estigma y un núcleo generativo que volverá a dividirse originando dos células espermáticas o

gametos masculinos. Una vez germina la célula vegetativa se forma el tubo polínico que crece a través del estilo y penetra en el óvulo a través del micrópilo. Mientras esto ocurre, la célula generativa que estaba dentro del grano de polen, sufre un proceso de división que da origen a las dos células espermáticas. Una vez formado el tubo polínico, las dos células espermáticas descienden por el mismo tubo y penetran en el saco embrionario a través de una de las dos sinérgidas, teniendo lugar la rotura del tubo polínico y la liberación de las 2 células espermáticas. Tras la migración de las células espermáticas se produce el proceso de la doble **fecundación**. Una célula espermática se une con la ovocélula para formar el cigoto a partir del cual se desarrollará el embrión. La otra célula espermática se une al núcleo secundario diploide para formar un núcleo triploide, el núcleo del endospermo, a partir del cual se originará el endospermo que servirá como reserva de nutrientes para el embrión (Farabee, 2001). El número de óvulos fecundados, y por consiguiente el número de semillas, dependen del número de células espermáticas que alcancen los óvulos (Ho y Hewitt, 1986) y que estarán fecundados en su mayoría antes de 30 horas tras la polinización (Chamarro, 1995).

Tanto la polinización como la fecundación dependen de factores ambientales tales como humedad y temperatura, así como de aspectos morfológicos de la flor (Picken, 1984; Ho y Hewitt, 1986). A 20 ºC, en el endospermo se desarrollan de 2 a 8 núcleos entre las primeras 48 a 96 horas luego de la fecundación, y a las 120 horas ya se ha formado un pro-embrión de 10 células (Ho y Hewitt, 1986). Si la polinización o un estímulo equivalente no se produce, el crecimiento del ovario se detiene (Nitsch, 1952) y senesce eventualmente, aunque este proceso no se observa durante el primer mes después de antesis (Alabadí *et al.,* 1996).

1.2. Desarrollo del fruto

Durante el desarrollo de la flor, el crecimiento temprano del ovario de tomate es debido fundamentalmente a un incremento en el número de células (Mapelli *et al*., 1978; Varga y Bruinsma, 1986). Tras la fecundación, el desarrollo del fruto puede dividirse en dos grandes fases: la primera, en la que el crecimiento se produce mayoritariamente por divisiones celulares, y la segunda, en la que el crecimiento tiene lugar principalmente por expansión celular (Gillaspy *et al.,* 1993; Ho y Hewitt, 1986).

El tiempo necesario para que el ovario se convierta en fruto maduro puede tardar entre 7 y 9 semanas desde la antesis (Chamarro, 1995).

Fase I. Crecimiento por divisiones celulares. Tras la fecundación suceden más divisiones celulares, que continuarán entre 7 y 10 días post antesis (dpa) (Mapelli *et al.,* 1978; Varga y Bruinsma, 1986). Estas divisiones se dan en todos los tejidos del fruto aunque mayoritariamente en el pericarpo y en el tejido placentario. Las células en división son pequeñas y ricas en sustancias citoplásmicas, además de poseer vacuolas pequeñas (Gillaspy *et al.,* 1993). La importación de asimilados por el ovario dos días después de la fecundación sufre un aumento considerable (Archbold *et al.,* 1982).

En los óvulos fecundados, el zigoto se divide transversalmente para formar un embrión bi-celular después de 48 horas. La división transversa de las dos células genera un embrión de cuatro células alineadas. La célula apical generará los cotiledones, la siguiente el hipocotilo y la raíz primaria, la siguiente la cofia y la última el suspensor (Varga y Bruinsma, 1986).

Se han investigado diversos aspectos de la distribución espacial y temporal de la actividad mitótica en los tejidos del fruto (ver Suzuka *et al.,* 1989; Daidoji *et al.,* 1992). Como resultado de los mismos, se sabe que en las etapas tempranas de la fase I, en el pericarpo la actividad mitótica es mayor en la zona exterior que en la interior, y que las células de la columela y de la placenta muestran también alta actividad mitótica. En las semillas en desarrollo, las divisiones celulares tienen lugar en las capas periféricas del tegumento más que en el embrión (Gillaspy *et al.,* 1993). Al final de la fase I sólo se detecta actividad mitótica en la capa más externa tanto del pericarpo (exocarpo), como del tejido placentario (Gillaspy *et al.,* 1993). Por el contrario, en un estudio reciente con 20 líneas de tomate, Cheniclet *et al.* (2005) observaron que la capa subepidérmica del exocarpo es la que presenta mayor actividad mitótica, y que la mayoría de las divisiones eran de tipo periclinal, en contraste con las divisiones anticlinales de la epidermis, por lo que serían las capas subepidérmicas la principal fuente de nuevas capas celulares. Estos autores también encontraron que el número inicial de capas del pericarpo en antesis variaba entre 9 y 12, y que a lo largo del crecimiento y hasta la maduración, podría incrementar su número entre 5 y 17, aunque

la mayoría de las divisiones ocurrirían antes de 10 dpa. Coombe (1976) sugirió que tanto la dirección de las divisiones (periclinales, anticlinales, o al azar) y la expansión celular, son factores que contribuyen al tamaño final del fruto.

Las semillas en desarrollo pueden controlar y mantener la tasa de divisiones celulares en los tejidos circundantes (tejido locular y placenta), ya que se ha observado que el número de óvulos fecundados determina la tasa inicial de crecimiento del ovario, (Varga y Bruinsma, 1986). También es sabido que existe una correlación entre el número de semillas en desarrollo y el crecimiento del fruto (Gustafson, 1939; Hobson y Davies, 1970; Varga y Bruinsma, 1986).

Fase II. Crecimiento por expansión celular. La fase de expansión celular suele durar hasta el inicio de la maduración. La expansión celular en algunas especies puede hacer que un fruto incremente su tamaño 100 veces o más (Coombe, 1976); en tomate, el volumen de células de la placenta, del tejido locular y del mesocarpo puede aumentar más de 10 veces, mientras que las células del exocarpo y del endocarpo, que continúan dividiéndose, se expanden menos (Gillaspy *et al.,* 1993). Conforme va engrosando la pared celular primaria, la vacuola ocupa la mayor proporción del volumen final de la célula y comienzan a acumularse almidón, ácidos orgánicos y otras sustancias (Chamarro, 1995), quedando el citoplasma reducido a una fina capa pegada a la pared celular (Ho y Hewitt, 1986). Las semillas no crecen en proporción directa al fruto pues no incrementan su tamaño de forma paralela (Gillaspy *et al.,* 1993).

Un aspecto importante en la expansión celular es la **endorreduplicación**. Se trata de un fenómeno por el cual las células duplican su contenido de DNA sin dividirse (Cookson *et al.,* 2006), lo cual se traduce en un aumento del nivel de ploidía. La endorreduplicación es con frecuencia un evento somático en muchos órganos y tejidos de plantas y suele estar asociado con actividad metabólica y expansión celular (revisión de Cheniclet *et al.,* 2005). El pericarpo se vuelve poliploide en muchas especies (Coombe, 1970), incluyendo el tomate donde los valores de ploidía varían entre 2 C y 256 C (Joubès *et al.,* 1999; Cheniclet *et al.,* 2005). Joubès *et al.* (1999) demostraron que el tejido locular también es poliploide, pero no la epidermis. Una función de la endorreduplicación podría ser la de favorecer la expansión celular, lo cual ha sido

demostrado en pericarpo de tomate donde se ha encontrado una estrecha correlación entre el tamaño celular y el nivel de ploidía (Cheniclet *et al.,* 2005). Esto ha sido observado igualmente en órganos de *Arabidopsis thaliana* tales como raíz (Sugimoto-Shirasu *et al.,* 2005) e hipocotilo (Kondorosi *et al.,* 2000). En tomate se ha observado una correlación entre el peso del fruto y el tamaño celular (Cheniclet *et al.,* 2005), por lo que la endorreduplicación podría tener un papel importante en el tamaño final del fruto al favorecer la expansión celular.

Como resumen de todo lo dicho anteriormente, el tamaño final del fruto depende del número inicial de células dentro del ovario antes de la fecundación, del número de divisiones celulares, del número de semillas en desarrollo y de la expansión celular (Varga y Bruinsma, 1986; Bohner y Bangerth, 1988; Mapelli *et al.,* 1993).

1.3. Estructura del fruto

El fruto de tomate es una **baya** compuesta básicamente por un pericarpo, tejido locular, placenta y semillas. En las variedades cultivadas, los frutos tienen dos o más carpelos que determinarán el número de lóculos (Ho y Hewitt, 1986).

Pericarpo. Durante el proceso de crecimiento, la pared del ovario se convierte en el pericarpo que posee paredes externas, radiales e internas (Varga y Bruinsma, 1986). La pared externa se compone de exocarpo, mesocarpo y endocarpo (Figs. 1 y 2). El **exocarpo** está compuesto por una epidermis que no posee estomas, y varias capas subepidérmicas de tipo colenquimático (pared gruesa). La epidermis la cubre una fina cutícula que engrosará a medida que se desarrolla el fruto (Wilson y Sterling, 1976). Desde la epidermis salen tricomas que desaparecen durante la maduración. El **mesocarpo** está compuesto por células parenquimáticas y haces vasculares (Figs. 1 y 2), aunque las células de su parte central suelen ser más alargadas que aquellas cercanas a la epidermis y a los lóculos (Ho y Hewitt, 1986). Finalmente, el **endocarpo** se compone de una capa celular que delimita las cavidades loculares (Fig. 2). También son parte del pericarpo las paredes radiales del fruto llamadas **septos** (Fig. 1), que separan las cavidades loculares, y la pared interna llamada **columela** (Ho y Hewitt, 1986; Gillaspy *et al.,* 1993). Tanto la columela como los septos están compuestos principalmente por

células parenquimáticas, teniendo la columela células menos pigmentadas y amplios espacios intercelulares.

Tejido locular, placenta y semillas. Los lóculos contienen las semillas, que están incluidas dentro del tejido locular, masa gelatinosa que en etapas tempranas del desarrollo del fruto es firme y que está constituido por células parenquimáticas (Fig. 1). El tejido locular colinda con la placenta (dado que surge a partir de ésta), y comienza a expandirse muy temprano durante el desarrollo dentro de los lóculos hasta rodear completamente las semillas. Éstas se componen del embrión, el endospermo y la testa o cubierta seminal que suele ser un tejido duro con pelos, y que es la capa más externa de la semilla derivada a partir del tegumento que rodea inicialmente al óvulo (Chamarro, 1995). El tegumento es un tejido de varias capas: una interna, una externa y una zona de tejido en el medio de estas dos. Su función principal es proteger al embrión y las sustancias de reserva (Esau, 1976).

Haces vasculares. Hay dos tipos de sistemas vasculares en el fruto, uno que se extiende desde el pedicelo a través del pericarpo (Figs. 1 y 2) y otro que pasa por los septos hacia las semillas. El sistema vascular se compone principalmente de tubos de floema y vasos de xilema con presencia de traqueidas. En el extremo proximal del fruto, los vasos que pasan a través del pericarpo están distribuidos de manera uniforme con pocas ramificaciones, mientras que en el extremo distal se ramifican considerablemente. Al aumentar la ramificación, la sección de los vasos disminuye pero la proporción de floema a xilema aumenta (Ho y Hewitt, 1986).

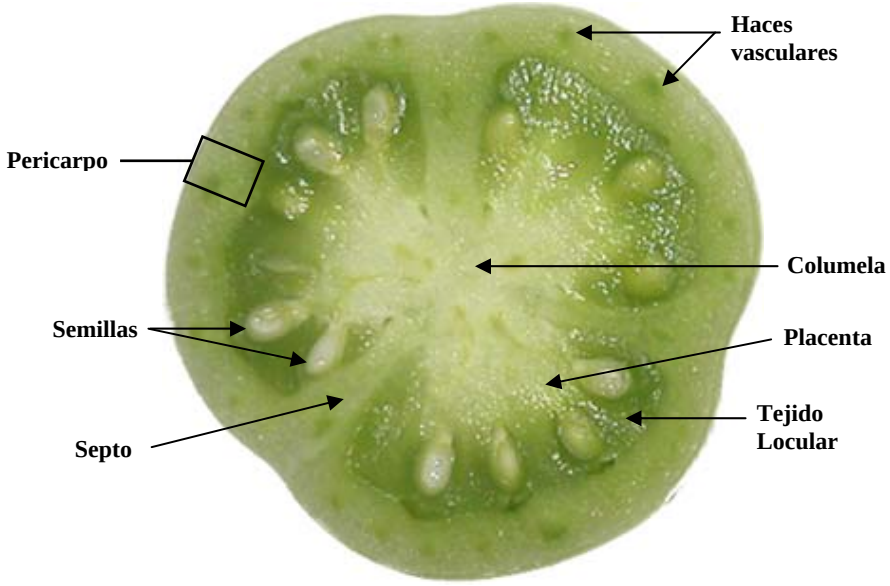

Figura 1. Corte transversal de un fruto de tomate. Se observan el pericarpo (cuya sección ampliada se observa en la Fig. 2), los septos, las semillas en contacto con la placenta, rodeadas de tejido locular, y en el eje central se encuentra la columela. Los haces vasculares se observan como puntos oscuros ubicados a lo largo del pericarpo. Fruto de 20 días post antesis (dpa).

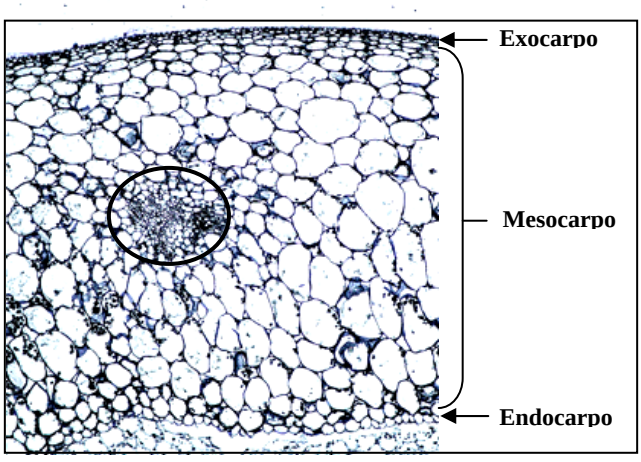

Figura 2. Pericarpo de un fruto de tomate. Corte histológico en parafina de una sección de pericarpo de fruto de 20 dpa donde se observa el mesocarpo, el exocarpo y el endocarpo. En el círculo se observa un haz vascular.

2. GIBERELINAS

2.1. Estructura química

Las giberelinas (GAs) son ácidos diterpenos tetracíclicos naturales, cuya estructura básica está constituida por un anillo de *ent*-giberelano, algunos de los cuales poseen actividad hormonal (Talón, 2000) (Fig. 3). Las GAs pueden actuar como reguladores endógenos del crecimiento controlando diversos procesos del desarrollo de las plantas como la germinación, la elongación del tallo, la expansión de las hojas, el desarrollo de los tricomas y la inducción de flores y frutos (Pharis y King, 1985; Huttly y Phillips, 1995; Sponsel, 1995; Hedden y Kamiya, 1997).

Hacia 1935 se utilizó por primera vez el término giberelina, cuando unos fitopatólogos japoneses descubrieron una sustancia producida por el hongo *Gibberella fujikuroi* que causa crecimiento excesivo de los tallos y brotes de la planta del arroz, provocando la enfermedad conocida como "bakanae". 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 aislaron en plantas compuestos de estructuras similares al ácido giberélico, y es en este momento cuando las giberelinas adquieren mayor importancia para los fisiólogos vegetales (Hedden, y Proebsting, 1999; Talón, 2000). Actualmente se conocen al menos 136 diferentes GAs (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). Las GAs se clasifican en dos grupos atendiendo al número de átomos de carbono presentes en su estructura: las GAs C-20, con 20 átomos de carbono en el esqueleto de *ent*-giberelano y las GAs C-19, con 19 átomos de carbono.

Las GAs C-20 presentan varios estados de oxidación del carbono 20 (C-20) que se puede encontrar como un grupo metilo ($-CH_3$), hidroximetilo ($-CH_2OH$), aldehído (CHO) o carboxilo (-COOH) (Sponsel, 1995). 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, entre las que se encuentran las

GAs activas biológicamente. La presencia o ausencia de grupos hidroxilo y la estequiometría de los mismos en las posiciones C-2, C-3 y C-13 del *ent*-giberelano de las GAs C-19 determina la existencia o no de actividad biológica de las GAs (Talón, 2000).

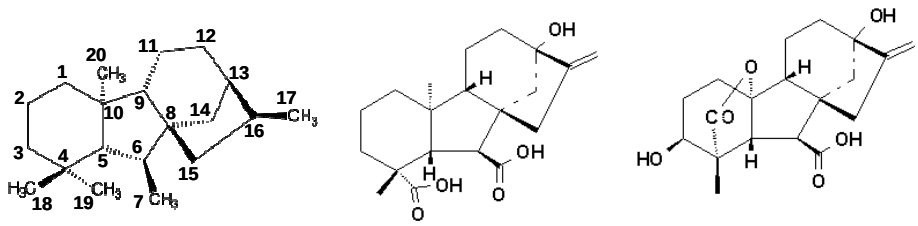

Fig. 3. Estructura química del *ent*-giberelano y de GAs representativas C-20 (GA_{52}) y C-19 (GA_1). Adaptado de http://www.plant-hormones.info/gibberellins.htm.

2.2. Actividad biológica de las giberelinas

De todas la 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, GA_3 y GA_7. Las restantes GAs son compuestos precursores o de degradación de las GAs activas (Kobayashi *et al*., 2000). Las formas activas suelen ser GAs C-19 con un grupo β-hidroxilo en el C-3.

La GA activa GA_3 se ha encontrado en un número reducido de plantas, de ahí que su función como fitohormona endógena no pueda generalizarse a todos los vegetales. En el caso de GA_4 y GA_7 es posible que su actividad se deba a la conversión en la planta a GA_1 y GA_3 respectivamente (Talón, 2000). La β-hidroxilación en el C-3 parece ser la clave para la actividad mientras que la β-hidroxilación en el C-2 provoca la pérdida de la actividad biológica. Éste constituye por lo tanto sistema de desactivación y por tanto, un punto en la ruta que es de vital importancia en la regulación de los niveles de GAs activas en plantas (Ross *et al.*, 1995). La oxidación completa del C-20 a carboxilo produce también la inactivación de las GAs (Fig. 4) (Lange *et al*., 1997). Sin embargo, es sabido que otros enzimas tales como las GA epoxidasas y GA

metiltransferasas (GAMTs) también pueden realizar una función catabólica dentro de la homeóstasis de los niveles de GAs durante la fructificación y desarrollo del fruto. Por ejemplo, en arroz el gen *EUI* cataliza la 16α,17-epoxidación de GAs de la ruta de no hidroxilación temprana en el C-13. En el mutante *eui* y en plantas transgénicas que sobreexpresan *EUI*, la 16α,17-epoxidación redujo la actividad biológica de GA_4 demostrando que EUI funciona como un enzima de inactivación de GAs (Zhu *et al*., 2006). De otra parte, en *Arabidopsis* se ha demostrado que los genes *GAMT1* y *GAMT2*, codifican enzimas que utilizan S-adenosin-l-metionina (SAM) como un donante del grupo metilo para metilar el grupo carboxilo de las GAs, lo cual produce ésteres metilados de GAs (MeGAs) (Varbanova *et al.*, 2007).

2.3. Biosíntesis

La biosíntesis de GAs en plantas superiores se puede dividir en tres etapas:

2.3.1. Síntesis de *ent*-kaureno a partir de geranilgeranildifosfato

La primera parte de la ruta de biosíntesis de GAs propiamente dicha transcurre en los proplastidios 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. 4) (MacMillan, 1997; Hedden y Phillips, 2000; Talón, 2000).

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

La segunda etapa ocurre en la membrana del retículo endoplasmático y está catalizada por monoxigenasas del tipo P-450. El *ent*-kaureno es oxidado a *ent*-kaurenol, *ent*-kaurenal y ácido *ent*-kaurenóico por la *ent*-kaureno oxidasa (KO) (Fig. 4). A continuación, el ácido *ent*-kaurenóico es oxidado a ácido *ent*-7α-hidroxikaurenóico, el cual, tras una contracción del anillo B produce GA_{12}-aldehído que es oxidado finalmente a GA_{12}. Estos tres últimos pasos de la vía son catalizados por la ácido *ent*-kaurenóico oxidasa (KAO) (ver revisión de Olszewski *et al*., 2002).

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

La tercera parte de la vía de biosíntesis de las GAs ocurre en el citoplasma de las células y está catalizada en su mayor parte por dioxigenasas dependientes de 2-cetoglutarato y Fe^{+2} (Yamaguchi y Kamiya, 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. Las dos rutas principales (Fig. 4) son:

Ruta de la hidroxilación temprana en el C-13. El GA_{12} es oxidado en el C-13 por la enzima GA 13-hidroxilasa (GA13ox, una monoxigenasa P-450) transformándose en GA_{53}, 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 catalizadas por GA 20-oxidasas (GA20ox) dando GA_{44} y GA_{19}. En una ramificación de la ruta metabólica principal, el C-20 de GA_{19} puede ser oxidado de nuevo dando lugar a GA_{17}. Esta ramificación constituye un proceso de inactivación irreversible que produce compuestos tricarboxílicos sin actividad biológica. La ruta principal de la 13-hidroxilación continúa con la eliminación del C-20 de GA_{19} sintetizándose GA_{20}, que es la primera GA C-19 de la ruta. La incorporación de un grupo hidroxilo en la posición 3β de GA_{20} produce GA_1, compuesto de elevada actividad biológica. Este proceso es catalizado por la enzima GA 3-oxidasa (GA3ox). Al final de la ruta, las GAs C-19 se desactivan de forma irreversible mediante la 2β-hidroxilación, catalizada por GA 2-oxidasas (GA2ox). Así, tanto GA_{20} como GA_1 pueden transformarse en los productos inactivos GA_{29} y GA_8 respectivamente. Las GAs 2β-hidroxiladas pueden ser oxidadas nuevamente por GA2ox para formar los productos finales GA_{29}-catabolito y GA_8-catabolito (Talón, 2000) (Fig. 4).

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, que es la primera GA C-19 de esta ruta; ambos pasos están catalizados por la GA20ox. La incorporación de un grupo hidroxilo en la posición 3β de GA_9 produce GA_4, compuesto con actividad biológica; éste proceso es catalizado por la enzima GA3ox. Al final de esta ruta, al igual que ocurría en la ruta de la 13-hidroxilación, las GAs C-19 se desactivan de forma irreversible mediante la 2β-

hidroxilación, proceso catalizado por la GA2ox. GA_9 y GA_4 se transforman en este caso, en los productos inactivos GA_{51} y GA_{34} respectivamente. Las GAs 2β-hidroxiladas pueden ser oxidadas nuevamente por las GA2ox para formar los productos finales GA_{51}-catabolito y GA_{34}-catabolito (Talón, 2000) (Fig. 4).

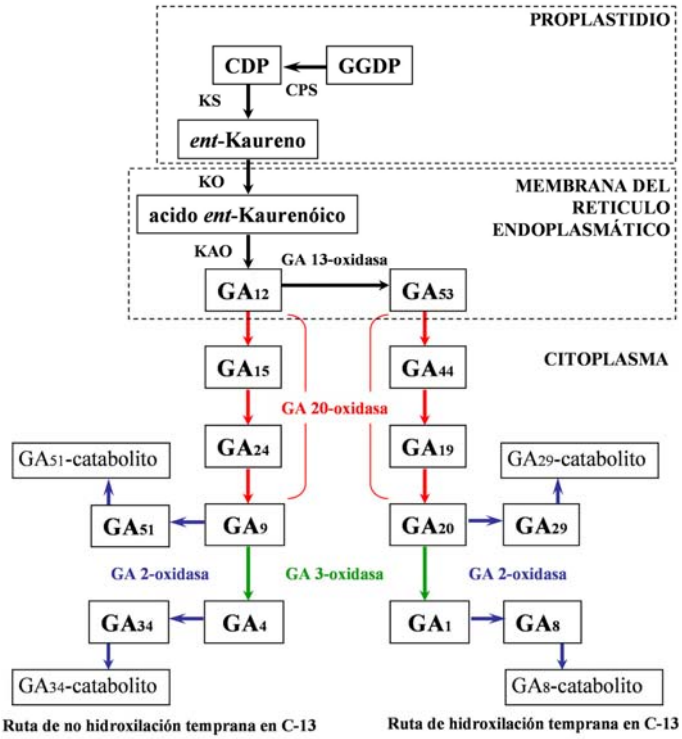

Figura 4. Esquema de la ruta de biosíntesis de giberelinas.

2.4. Genes que codifican enzimas implicados en el metabolismo de las GAs

En diversas especies se han clonado la mayoría de los genes que codifican los enzimas del metabolismo de GAs, excepto el de GA C-13 hidroxilasa. Los enzimas CPS, KS y KO están codificados en la mayoría de las especies estudiadas por genes únicos, y la expresión génica de los mismos no presenta regulación feed-back (revisado en Hedden y Phillips, 2000). De *Arabidopsis,* han sido aislados dos genes que codifican KAO, los cuales se expresan en todos los tejidos estudiados (Helliwell *et al.*, 2001). Por otro lado, los enzimas citosólicos GA20ox, GA3ox y GA2ox, codificados por familias

multigénicas, presentan un patrón específico de expresión para cada uno de los individuos de la familia multigénica, (revisado en Hedden y Phillips, 2000). La sobreexpresión de GA 20-oxidasa en plantas transgénicas de *Arabidopsis thaliana* puede producir una alteración de las concentraciones de GAs activas, lo que estaría indicando que una regulación de estos genes sería crucial en la modulación del flujo en la parte final de la ruta (Hedden y Phillips, 2000a y 2000b; Yamaguchi y Kamiya, 2000).

2.4.1. Ciclasas y monooxigenasas

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. Estos enzimas están localizados principalmente en los protoplastidios de los tejidos meristemáticos de los brotes pero no en los cloroplastos maduros (Talón, 2000). 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).

En la bibliografía se ha descrito el aislamiento de los genes que codifican CPS y KS en distintas especies. Así, se han aislado genes codificantes de CPS en *Arabidopsis* (*GA1*; Sun y Kamiya, 1994), maíz (*An1*; Bensen *et al.*, 1995), guisante (*LS*; Ait-Alí *et al.*, 1997), tomate (Imai *et al.*, 1996) y calabaza (*CmCPS1* y *CmCPS2*; Smith *et al.*, 1998), y genes codificantes de KS en *Arabidopsis* (*GA2;* Yamaguchi *et al.*, 1998b) y calabaza (*CmKS*; Yamaguchi *et al.*, 1996).

En *Arabidopsis* se han encontrado niveles altos de transcritos de *AtCPS* en tejidos de rápido crecimiento, por ejemplo, en los brotes apicales, en los ápices radiculares y en anteras y frutos en desarrollo. 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 CPS y su localización. El patrón específico de expresión del gen *AtCPS* y la mayor expresión de *AtKS*, apoyan la idea que AtCPS podría actuar como un 'guardián', controlando el lugar y la actividad de las etapas tempranas de la ruta de biosíntesis de GAs (Olszewski *et al*., 2002). Sin

embargo, Fleet *et al*. (2003) observaron que líneas transgénicas de sobreexpresión de *CPS*, *KS* y *CPS/KS* no mostraban un fenotipo de exceso de GAs, lo cual podía deberse a 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.

2.4.2. Dioxigenasas

Estos enzimas catalizan la última parte de la ruta del metabolismo de las GAs. Las GA 20-oxidasas y las GA 3-oxidasas se encargan de la activación de GAs y las GA 2-oxidasas de su inactivación.

Los genes que codifican GA 20-oxidasas pertenecen a familias multigénicas. Sólo una GA 20-oxidasa, la *CmGA20ox,* presenta una actividad catalítica atípica y viene de semillas de calabaza (Lange *et al*., 1994). La peculiaridad de este enzima reside en que tras catalizar las dos oxidaciones consecutivas en el C-20 de los substratos, no se produce la descarboxilación, y en su lugar se producen los ácidos tricarboxílicos de GA_{17} o GA_{25} (en el caso de la ruta de la no 13-hidroxilación), compuestos desactivados irreversiblemente. Gracias a la clonación de la GA 20-oxidasa de calabaza, se pudieron encontrar enzimas homólogos en otras especies tales como *Arabidopsis thaliana* (Phillips *et al.,* 1995; Xu *et al.,* 1995), espinaca (Wu *et al.,* 1996), guisante y judía (García-Martínez *et al.,* 1997), *Marah macrocarpus* (MacMillan *et al,* 1997), arroz (Toyomasu *et al.,* 1997), lechuga (Toyomasu *et al.,* 1998), tomate (Rebers *et al.,* 1999), y patata (Carrera *et al.,* 1999). Tres de los genes que codifican GA 20-oxidasas en *Arabidopsis thaliana* se expresan diferencialmente: el gen *AtGA20ox1* se expresa preferentemente en tallo y flores, el gen *AtGA20ox2* en silicuas y flores y el gen *AtGA20ox3* se expresa únicamente en silicuas (Phillips *et al.,* 1995).

Los diferentes genes que codifican GA 20-oxidasas en *Solanum lycopersicum* tienen un patrón de expresión diferencial durante el desarrollo de las yemas florales. Los niveles de transcritos de *LeGA20ox1* y de *LeGA20ox2* aumentan en las etapas previas a la antesis, mientras que se reducen en las flores abiertas. Sin embargo, tras la antesis aumentan los niveles de los transcritos de los genes *LeGA20ox1* y *LeGA20ox3* (Rebers

et al., 1999). Por tanto, las GA 20-oxidasas de tomate se expresan de forma diferencial en las distintas etapas del desarrollo de las yemas florales (Rebers *et al.,* 1999).

Las GA 3-oxidasas catalizan el último paso de la biosíntesis de las GAs activas convirtiendo GA_{20} y GA_9 a GA_1 y GA_4 respectivamente. Además, estos enzimas presentan, al igual que las GA 20-oxidasas, una preferencia de substrato; así, en *Arabidopsis* las GA 3-oxidasas transforman GA_9 a GA_4 como la GA activa predominante. En cuanto a las GA 3-oxidasas, también están codificadas por pequeñas familias multigénicas. Se han aislado GA 3-oxidasas de *Arabidopsis thaliana* (Chiang *et al.,* 1995; Yamaguchi *et al.,* 1998a), guisante (Martin *et al.,* 1997; Lester *et al.,* 1997), tomate (Yang *et al.,* 1998), lechuga (Toyomasu *et al.,* 1998), tabaco (Tanaka-Ueguchi *et al.,* 1998), arroz (Itoh *et al.,* 2001) y espinaca (Lee y Zeevaart, 2002) entre otras. En guisante, el gen homólogo a *GA4* corresponde al locus *LE* utilizado por Mendel en sus investigaciones sobre la herencia de los caracteres (Martin *et al.,* 1997; Lester *et al.,* 1997). En tomate, el gen *LeGA3ox2* muestra un incremento gradual en los niveles de trascritos hasta la antesis y su expresión es diferencial en diferentes tejidos, al igual que *LeGA3ox1* (Rebers *et al.,* 1999).

Las GA 2-oxidasas presentan poca especificidad de substrato dado que utilizan las GAs activas así como sus precursores. Las GA 2-oxidasas oxidan el C-2 de sus substratos originando compuestos 2β-hidroxilados, inactivos (Ross *et al.,* 1995). Esta característica les ha conferido el nombre de enzimas desactivadores. Recientemente se ha planteado la existencia de dos grupos de genes de GA 2-oxidasa. El primero utiliza las GAs C-20 como substrato y el segundo las GAs C-19. Es así, como en *Arabidopsis* se ha demostrado que los genes *AtGA2ox7* y *AtGA2ox8* utilizan como substrato las GAs C-20 (no GAs C-19 como en el caso de *AtGA2ox1, -2 y -3*), y su sobreexpresión en plantas transgénicas produce fenotipos enanos (Schomburg *et al*., 2003). Los genes *AtGA2ox4 y -5* no han sido caracterizados en cuanto a su preferencia de sustrato, mientras que el gen *AtGA2ox6* no se expresa por ser probablemente un pseudogen (Hedden y Phillips, 2000). Dentro del grupo de GA 2-oxidasas que utilizan como substrato las GAs C-19 se pueden encontar dos tipos: el primero, que cataliza una sola oxidación en el C-2 de sus substratos (monocatalíticos), y el segundo, que cataliza dos

oxidaciones consecutivas (multicatalíticos) (Thomas *et al.,* 1999). En esta segunda reacción se abre la γ-lactona y se forma una cetona y un doble enlace entre el C-10 y el átomo de carbono adyacente (C-1, C-5 ó C–9).

Ross *et al.* (1995) plantearon que dos enzimas distintas estaban implicadas en el fenotipo de la mutación "*slender*" (*sln)* de guisante dado que observaron que ésta tenía bloqueado el paso de GA_{20} a GA_{29} y el paso de GA_{29} a GA_{29}-catabolito. Sin embargo, Martin *et al*. (1999) y Lester *et al*. (1999) determinaron que se trataba de un solo enzima (PsGA2ox1) que se encontraba en gran concentración en las semillas, y que tenía características multifuncionales (multicatalíticas), y de esa forma, era capaz de catalizar dos oxidaciones consecutivas sobre el C-2 de los substratos.

Existen clonadas GA 2-oxidasas de diversas especies tales como calabaza (Lange *et al.,* 1997), *Arabidopsis thaliana* (Thomas *et al.,* 1999), guisante (Martin *et al.,* 1999), arroz (Sakamoto *et al.*, 2001; Sakai *et al.,* 2003), espinaca (Lee y Zeevaart, 2002 y 2005), lechuga (Nakaminami *et al.,* 2003), *Vigna angularis* (Park *et al.,* 2005) y adelfa (Ubeda-Tomás *et al.,* 2006), entre otras, pero ninguna en tomate. 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 el gen *PsGA2ox2*, ya que los transcritos de este gen abundan en el brote apical y escasean en las semillas (Lester *et al.,* 1999). En *Arabidopsis* por el contrario, los genes *AtGA2ox1* y *AtGA2ox2* presentan el mismo patrón de expresión y el gen *AtGA2ox3* no se detecta mediante análisis northern (Thomas *et al.,* 1999).

2.5. Regulación de la ruta de biosíntesis de giberelinas por retroalimentación

Las GAs activas ejercen a través de su modo de acción un mecanismo de regulación sobre la biosíntesis de GAs, de manera que la síntesis *de novo* y el catabolismo de éstas, 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 (*GA20oxidasa* y *GA3oxidasa*), o positiva en el caso de los genes catabólicos (*GA2oxidasa*) (Fig. 5). Este mecanismo está inducido por la respuesta a las GAs activas (Yamaguchi y Kamiya, 2000), proporcionando de este modo a las plantas un control homeostático de sus niveles endógenos de GAs activas. Estos resultados, ponen de manifiesto la regulación transcripcional de la mayoría de los genes del metabolismo de GAs, mediante un mecanismo de retroalimentación que originan los niveles endógenos de GAs activas (Fig. 5) (Phillips *et al.,* 1995; Yamaguchi y Kamiya, 2000).

Para controlar los niveles de GAs activas en las plantas, este tipo de regulación transcripcional de las dioxigenasas es importante, pues la sobreexpresión de estos genes produce una alteración en los niveles de GAs activas en las plantas transgénicas (Hedden y Phillips, 2000; Yamaguchi y Kamiya, 2000). En plantas deficientes en GA (*gib-1* de tomate) o en plantas silvestres tratadas con inhibidores de la biosíntesis de GAs, las bajas concentraciones de GAs son la razón del aumento de los niveles de transcritos del gen *GA20oxidasa*; dichos niveles disminuyen con la aplicación de GA exógena (Martin *et al.,* 1996, Phillips *et al*., 1995; Toyomasu *et al.,* 1997).

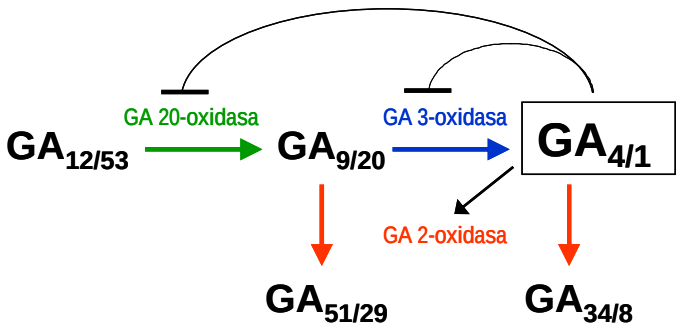

Figura 5. Esquema de la regulación por retroalimentación negativa de genes de biosíntesis (GA 20-oxidasa, GA 3-oxidasa) y retroalimentación positiva de genes de catabolismo (GA 2-oxidasa) de GAs.

3. AUXINAS

3.1. Estructura química

Las auxinas se definen como sustancias orgánicas que promueven la elongación celular cuando se aplican en concentraciones bajas a tejidos vegetales (Cohen *et al.,* 2003). El ácido indolacético (IAA) es la auxina más estudiada y la de mayor presencia natural en plantas. Los diferentes tipos de auxinas se encuentran en plantas como ácidos libres o formas conjugadas (Fig. 6).

La actividad de cada auxina es diferente. Por ejemplo, se ha visto que el derivado halogenado 4-Cl-IAA es más activo que el IAA en el coleóptilo de avena (Nigovic *et al.,* 1996) y además puede estimular la síntesis de giberelinas, aunque también se sabe que el IAA afecta dicha síntesis en tabaco y guisante (Normanly *et al*., 2004). Tanto el IAA como sus precursores, pueden sufrir conversiones a ácido indol-3-láctico, indol-3-etanol y ácido indol-3-butírico (IBA). Éste último ha sido usado comercialmente más que el IAA debido a su eficiencia en promover el desarrollo de raíces adventicias, lo cual puede ser debido a su mayor estabilidad frente al catabolismo *in vivo* (Bartel *et al.,* 2001). Algunos estudios revelan que el IBA es capaz de convertirse en IAA y éste a su vez en IBA, pero no es del todo claro si el IBA es una auxina *per se* o un precursor del IAA (Normanly *et al*., 2004). En muchos tejidos la mayoría de auxinas se encuentran conjugadas con compuestos tales como glucosa, *myo*-inositol y aminoácidos (Fig. 6). Los dos tipos más comunes de conjugados son los derivados tipo éster y los derivados tipo amida (Normanly *et al*., 2004). Gran parte del efecto biológico de estas dos clases de conjugados se asocia con la capacidad de liberar el IAA en un tejido por hidrólisis. Igualmente estos conjugados pueden llegar a tener una actividad auxínica específica.

3.2. Biosíntesis del IAA

Existen dos rutas principales para la formación del IAA en plantas. Una parte del triptófano y la otra es independiente de éste. Mediante la ruta del triptófano, que es la más común, éste sufre una desaminación para convertirse en ácido indol-3-pirúvico (IPA), seguida de una descarboxilación que produce indol-3-acetaldehido, que luego es oxidado a IAA (Fig. 6A).

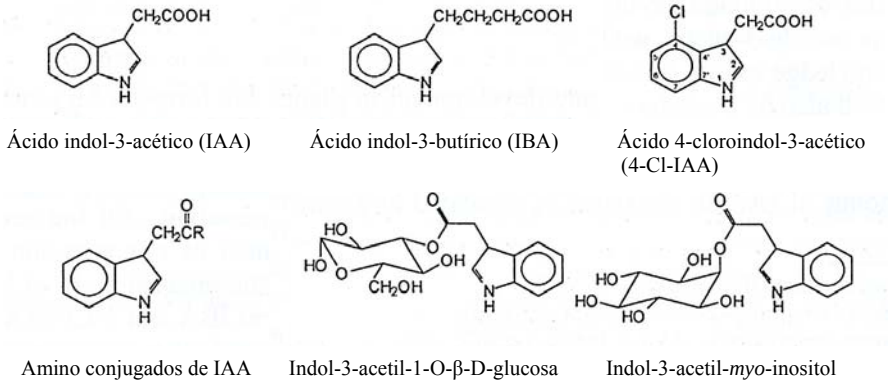

Figura 6. Estructura química del ácido indolacético, auxinas derivadas (IBA, 4-Cl-IAA) y algunos de sus conjugados. Adaptado de Normanly *et al*. (2004).

Existen rutas alternativas, aunque no tan frecuentes, de obtención de IAA a partir del triptófano. Por ejemplo, se puede obtener indol-3-acetaldehído a partir de triptamina sin pasar por IPA, ó, a partir del mismo triptófano se puede obtener indol-3-acetaldoxima, que una vez convertida en indol-3-acetonitrilo, puede producir IAA (Fig. 7A) (Srivastava, 2002).

La otra ruta (que no utiliza el triptófano) parte del corismato, que es convertido en antranilato gracias a la antranilato sintasa y luego convertido en 5-fosforibosilantranilato por la acción de la fosforibosil antranilato transferasa. Éste es convertido en un derivado de deoxiribulosa por medio de la fosforibosil antranilato isomerasa. Luego, y gracias a la IGP sintasa, se forma fosfato indol-3-glicerol a partir del cuál, tienen lugar las conversiones subsecuentes a indol por la acción de la triptófano sintasaα y a triptófano por la acción de la sintasaβ. El IAA puede ser producido bien a partir de indol o de indol-3-glicerol fosfato (Fig. 7B) (Srivastava, 2002).

Se cree que los lugares más importantes de síntesis del IAA pueden ser los tejidos jóvenes en general (yemas y hojas jóvenes, frutos jóvenes y semillas inmaduras), dado que los niveles endógenos en estos tejidos son más altos que en los demás. Dentro del las células parece haber dos sitios de biosíntesis, dado que el triptófano es sintetizado en los plastidios, aunque también se ha encontrado éste a nivel citoplásmico

(Srivastava, 2002). Si bien no han sido clonados los enzimas de biosíntesis del IAA a partir del triptófano, en la última década se han encontrado genes que codifican nitrilasas que están asociados con síntesis de IAA *in vitro*. Igualmente, se han identificado genes del metabolismo de triptófano a indol-3-acetaldoxima (IAOx) en *Arabidopsis*, que codifican enzimas que pertenecen a la familia de monoxigenasas del citocromo P450 (Normanly *et al*., 2004). También las proteínas YUCCA (flavina monooxigenasas) son capaces de convertir triptamina (Tpm) a N-hidroxil triptamina *in vitro* (Cohen *et al.,* 2003). Los defectos en el desarrollo que presentan los mutantes *yuc1yuc4* y *yuc1yuc2yuc6* pueden ser recuperados con la expresión del gen iaaM, que es un gen bacteriano de biosíntesis de auxinas y que codifica una triptófano-2-monoxigenasa que cataliza la conversión de triptófano en indol-3-acetamida (Cheng *et al*., 2006).

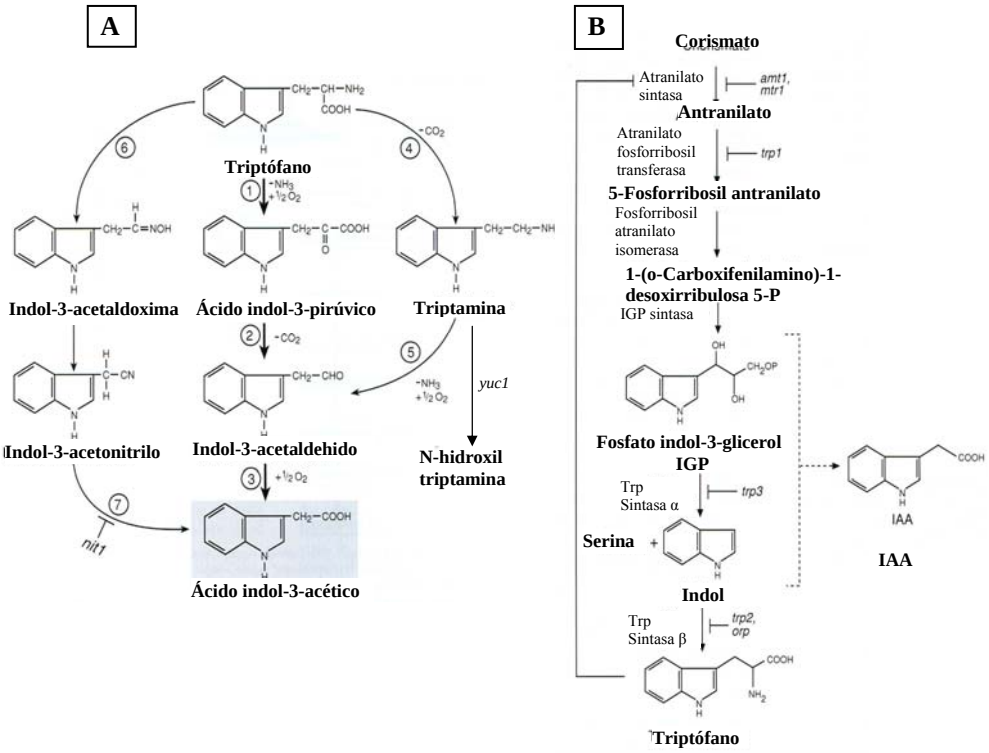

Figura 7. Rutas de biosíntesis de IAA. **A**. Ruta a partir del triptófano. **B**. Ruta a partir del corismato. En ambas rutas se señalan los sitios de acción de diversas mutaciones tales como *trp1, trp2, trp3, amt1*, *nit1* y *yuc1* en *Arabidopsis*; *orp* en maíz; *mtr1* en *Lemma gibba*; Adaptado de Srivastava (2002).

3.3. Regulación de los niveles de IAA (homeostasis)

Los niveles de esta hormona están regulados en los tejidos de la planta de una manera precisa por una serie de mecanismos (Srivastava, 2002; Bandurski *et al.,* 1995) (Fig. 8):

- Síntesis *de novo*, bien desde el triptófano o desde precursores indólicos de éste.
- Hidrólisis de los conjugados amida- ó éster-IAA
- Transporte de un lugar de la planta a otro.
- Conversión de IBA a IAA y de IAA en IBA.
- Catabolismo oxidativo.
- Síntesis de conjugados.

Figura 8. Interacciones metabólicas y de señalización en la producción de IAA. TRP: triptófano; IBA: ácido indol-3-butírico. Adaptado de Normanly *et al*. (2004)

3.4. Catabolismo del IAA

El catabolismo oxidativo del IAA es una modificación química del núcleo indol que da lugar a la pérdida de actividad auxínica. Estudios sobre el catabolismo del IAA, indican que esta hormona se encuentra ubícuamente en la planta, que diversos enzimas tipo peroxidasa catalizan la descarboxilación oxidativa del IAA, y que puede no haber una ruta predominante de catabolismo (Normanly *et al.,* 2004). Hay dos rutas:

Ruta del ácido oxindol-3-acético.El primer producto producido en esta ruta es el ácido 2-oxindol-3-acético (OxIAA), que es metabolizado por hidroxilación en la posición 7 y con la adición de glucosa forma 7-OH-OxIAA-glucósido, cuyo metabolismo posterior no es claro (Bandurski *et al.,* 1995).

Oxidación de IAA-Asp. En plántulas de *Vicia*, se demostró que el indol-3-acetil-L-aspartato (IAA-Asp) es oxidado a di-Ox-IAA-aspartato (Bandurski *et al.*, 1995). Luego el producto es glicosilado a 3-(0-β-glucosil). El IAA-Asp también puede ser oxidado por una peroxidasa formando 2-OH-Ox-IAAsp.

3.5. Inhibidores del transporte de IAA

Diversos compuestos sintéticos son capaces de inhibir la acción del IAA. Entre ellos se encuentran el ácido triiodobenzóico (TIBA) y el ácido naftiltalámico (NPA), que inhiben el trasporte polar (basipétalo) del IAA. También está el ácido α-(ρ-clorofenoxi) isobutírico (PCIB) u otros compuestos llamados antiauxinas para los cuales se ha sugerido una acción de interacción con el receptor de IAA (Svirastava, 2002).

3.6. Auxinas sintéticas

Se han encontrado diversos compuestos con actividad auxínica. Los más importantes son los ácidos diclorofenoxiacético (2,4-D), 2,4,5-triclorofenoxiaético (2,4,5-T), naftalenacético (NAA) y 2,4-metilfenoxiacético (MCPA) y derivados del ácido benzóico (Fig. 9). La mayoría de estos compuestos son más estables en los tejidos que el IAA y son metabolizados más lentamente, por lo que su acción a bajas concentraciones puede ser más eficiente que el IAA (Svirastava, 2002).

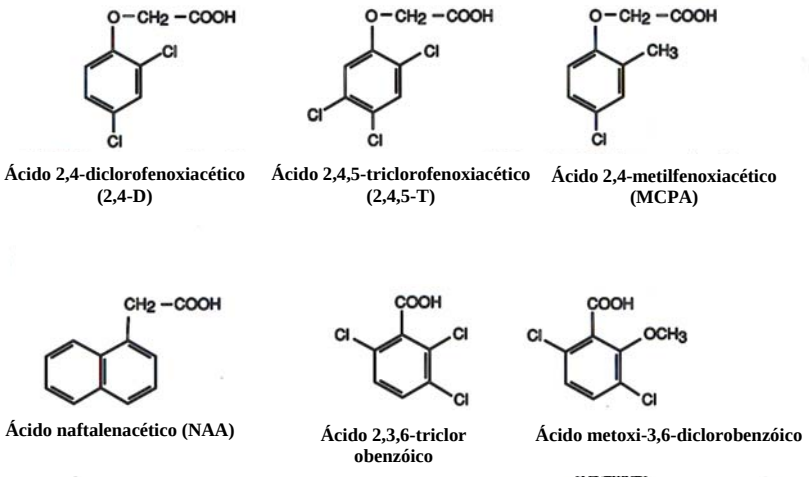

Figura 9. Diversas auxinas sintéticas.

4. REGULACIÓN HORMONAL DEL CRECIMIENTO Y DESARROLLO DEL FRUTO EN TOMATE

Hemos visto en el capítulo de fructificación cómo se desarrolla un fruto con semillas a partir de un ovario polinizado. Sin embargo, el fruto también puede desarrollarse a partir de un ovario no fecundado y por consiguiente en ausencia de semillas por medio de un fenómeno llamado **partenocarpia**, término introducido por Noll (1902). En tomate, la partenocarpia puede ser natural, como en el caso genotipos que posean los genes *pat*, *pat-2, pat-3/pat-4* (Philouze, 1983; George *et al.,* 1984) o bien inducida con hormonas aplicadas a ovarios, lo cual ha sido objeto de revisiones recientes (Gorquet *et al.,* 2005; Srivastava y Handa, 2005). La partenocarpia natural puede ser facultativa u obligada dependiendo de las condiciones ambientales (Ho y Hewitt, 1986).

Sin descartar la participación de otras hormonas en el desarrollo partenocárpico del fruto, varios estudios en líneas partenocárpicas de tomate han demostrado que las hormonas endógenas más relevantes en la partenocarpia son las **auxinas** (Mapelli *et al.,* 1978; Bohner y Bangerth, 1988) y **giberelinas** (GAs) (Mapelli *et al.,* 1978; Bohner y Bangerth, 1988; Fos *et al.,* 2000, 2001). Por otra parte, la aplicación exógena de auxinas (Gustafson, 1936; Asahira *et al.,* 1967; Nitsch, 1970; Sjut y Bangerth, 1981; Bünger-Kibler y Bangerth, 1982/83; Chareonboonsit *et al.,* 1985; revisión de Abad y Monteiro, 1989; Koshioka *et al.,* 1994; Alabadí *et al.,* 1996) y GAs (Gustafson, 1936, 1960; Nitsch, 1952; Sjut y Bangerth, 1981; Bünger-Kliber y Bangerth, 1982/83; Alabadí *et al.,* 1996, Alabadí y Carbonell, 1998; Fos *et al*., 2000, 2001) induce la fructificación de ovarios no polinizados en tomate, lo cual sugiere un papel importante de estas hormonas en la fructificación y desarrollo del fruto.

4.1. Las giberelinas y la fructificación del tomate

Diversos estudios has demostrado la importancia de las GAs en la fructificación del tomate, lo cual se basa en las siguientes evidencias:

En primer lugar, la aplicación de GAs a ovarios emasculados no polinizados induce el desarrollo partenocárpico del fruto (Gustafson, 1936, 1960; Nitsch, 1952; Sjut y Bangerth, 1981; Bünger-Kibler y Bangerth, 1982/83; Alabadí *et al.,* 1996; Alabadí y Carbonell, 1998; Fos *et al.,* 2000, 2001). En segundo lugar, 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). En tercer lugar, se han identificado GAs de las rutas de 13-hidroxilación y de no hidroxilación temprana tanto en ovarios (Fos *et al.,* 2000), como en frutos polinizados en desarrollo e inducidos con auxinas (Bohner *et al.,* 1988; Koshioka *et al.,* 1994). Cuarto, se ha encontrado que genes que codifican enzimas de la ruta de biosíntesis de GAs, se expresan durante el desarrollo de la flor y del fruto (Rebers *et al.,* 1999). Finalmente, plantas transgénicas de tomate que tenían silenciado el gen *SlDELLA* (que codifica proteínas cuya degradación es inducida por GAs) produjeron partenocarpia facultativa (Martí *et al*., 2007).

Bünger-Kibler y Bangerth (1982/83) observaron que el tratamiento con GA_3 hace que la tasa de divisiones celulares decrezca, por lo cual esa aparente inhibición de la división celular por parte de GA_3 podría explicarse como un efecto inhibitorio de la mitosis para estimular la endorreduplicación y la expansión celular. Algunos trabajos sugieren un rol de las GAs en la división celular (ver revisión de Varga y Bruinsma, 1986), aunque publicaciones más recientes confieren un papel más claro de las GAs en la expansión celular (Srivastava y Handa, 2005). Como se dijo anteriormente, Bünger-Kibler y Bangerth (1982/83) han descrito que en frutos tratados con GA_3 la división celular decrece en el tiempo, mientras lo contrario ocurre al aplicar auxinas en un periodo corto de tiempo. El tamaño celular es mayor en los frutos inducidos con GA_3, por lo que se podría sugerir un mecanismo de compensación en el cual hay menos células pero de mayor tamaño. Estos mismos autores y Asahira *et al.* (1968) observaron que los frutos inducidos con GA_3 son más pequeños que los frutos polinizados y que los frutos tratados con auxinas.

Los niveles de GAs en el fruto varían a lo largo del desarrollo, al igual que varían en frutos con semillas y sin semillas (Mapelli *et al*., 1978). Se cree que el nivel incrementado de GAs en el ovario antes de la fecundación se relaciona con el desarrollo

partenocárpico. Así, el contenido endógeno de sustancias tipo GA en ovarios de líneas partenocárpicas de tomate (*pat*) es mayor que en líneas normales (Mapelli *et al*., 1978; Mapelli y Lombardi, 1982); en el caso de *pat-2* aumenta el contenido de GA_{20} (Fos *et al.,* 2000), mientras en *pat-3/pat-4* es GA_1 (Fos *et al.,* 2001). En frutos partenocárpicos *pat*, el primer pico de actividad de GAs ocurre a los 7 dpa y el segundo a los 27 dpa, mientras que en frutos con semillas ocurren a los 20 y 35 dpa respectivamente (Mapelli *et al.,* 1978). Es de notar, que estos dos picos de GAs se manifiestan uno en fase de divisiones celulares (Fase I) y otro en fase de expansiones celulares (Fase II). En frutos con semillas, ambos picos se encuentran en la fase de expansiones celulares (Mapelli *et al.,* 1978). El primer pico de GAs en frutos partenocárpicos, podría estar asociado a un incremento hormonal necesario para el desarrollo del ovario en ausencia de la polinización.

4.2. Las auxinas y la fructificación del tomate

Diversos estudios han demostrado la importancia de las auxinas en la fructificación y desarrollo del fruto de tomate. En primer lugar, la aplicación exógena de auxinas al ovario induce la formación de frutos partenocárpicos (Gustafson, 1936; Asahira *et al.,* 1967; Nitsch, 1970; Sjut y Bangerth, 1981; Bünger-Kibler y Bangerth, 1982/83; Chareonboonsit *et al.,* 1985; revisión de Abad y Monteiro, 1989; Koshioka *et al.,* 1994; Alabadí *et al.,* 1996).; Segundo, existe un incremento de sustancias auxínicas e IAA después de la antesis (Mapelli *et al.,* 1978; Sjut y Bangerth, 1981). En tercer lugar, líneas transgénicas que mostraban regulación negativa de *SlIAA9* (un gen *Aux/IAA*), tienen la capacidad de desarrollarse partenocárpicamente (Wang *et al.,* 2005). Cuarto, un análisis del transcriptoma de tejidos en crecimiento de tomate, mostró expresión de genes de auxinas relacionados con su síntesis (amidohidrolasas del IAA), transporte (*PIN7*) y respuesta (*Aux/IAA*) (Lemaire-Chamley *et al.,* 2005). Por último, en *Arabidopsis thaliana* se ha demostrado que el factor transcripcional ARF8 (AUXIN RESPONSE FACTOR 8), el cual interactúa con proteínas Aux/IAA, inhibe el desarrollo del fruto hasta que la fecundación tiene lugar (Goetz *et al*., 2006). Igualmente se ha demostrado que la mutación del gen *ARF8* (*Atarf8-4)* produce partenocarpia en *Arabidopsis* y tomate (Goetz *et al*., 2007).

Se ha sugerido que el crecimiento del fruto no es causado directamente por la producción de auxinas en las semillas (Varga y Bruinsma, 1976), sino que la auxina estimula el desarrollo del embrión, lo que generaría una actividad de sumidero por parte de las semillas, de lo cual podrían aprovecharse los tejidos circundantes (Varga y Bruinsma, 1986). La señalización por auxinas sin embargo, parece ser un prerrequisito para el desarrollo del tejido locular y para la expansión celular del mismo de acuerdo a los resultados obtenidos por Lemaire-Chamley *et al.* (2005). Esto parece estar de acuerdo con la teoría que plantea que las auxinas producidas por las semillas o por los tejidos circundantes a ellas, sirven para acelerar el crecimiento del fruto por expansión celular (Gillaspy *et al.,* 1993).

Se ha sugerido un papel importante de las auxinas en la división celular. La aplicación de auxinas al ovario, o el bloqueo de la salida de éstas, produce una acumulación suficiente para activar la fructificación y la división celular (Gillaspy *et al.,* 1993). También se ha observado que uno de los dos grandes picos de producción de auxinas se encuentra en la fase de divisiones celulares (Mapelli *et al.,* 1978). Por otro lado, un análisis de transcritos de DR12, que es un factor de respuesta a auxinas (ARF), muestra su acumulación en frutos y tiene un papel importante en la división celular (Jones *et al.,* 2002). Bohner y Bangerth (1988) observaron, en experimentos con líneas isogénicas de tomate, que el decrecimiento en la tasa de divisiones celulares está asociado con una rápida disminución del contenido de IAA. Además, no encontraron correlación entre la concentración de IAA y la expansión celular, aunque se han encontrado correlaciones positivas entre nivel de auxinas y expansión celular en diversos tejidos de otras especies (ver revisión de Cleland, 2004). Por último, se ha observado que los frutos inducidos con IAA tienen una actividad de división celular mucho mayor que los frutos polinizados y que los tratados con GA_3 (Bünger-Kibler y Bangerth, 1982/83).

Por otra parte, en frutos partenocárpicos inducidos con auxinas se ha descrito la formación de pseudoembriones (que son estructuras similares al embrión, desarrolladas a partir de las células internas del tegumento). Si estos frutos eran tratados con uniconazol (un inhibidor de la biosíntesis de giberelinas), la formación de

pseudoembriones era inhibida. Dicho efecto fue contrarrestado con la aplicación de GA_3, lo cual sugería que el desarrollo de pseudoembriones estaba relacionado con el crecimiento partenocárpico del fruto (Kataoka *et al.,* 2003)

En general, el cuajado del fruto de tomate es inducido más eficientemente por auxinas que por giberelinas (revisión de Abad y Monteiro, 1989; Koshioka *et al.,* 1994), siendo los primeros de mayor tamaño que los segundos (Sjut y Bangerth, 1982/83; Alabadí *et al.,* 1996). En cuanto a morfología celular, los frutos inducidos por auxinas tienen más células y de menor tamaño que los tratados con GA_3, lo que sugiere que las auxinas inducen las divisiones celulares y las GAs la expansión celular. Esto último ha sido confirmado por Bohner y Bangerth (1988) en líneas isogénicas de tomate.

La partenocarpia natural puede estar relacionada con la capacidad de las líneas sin semillas de crear un umbral adecuado de concentración de reguladores del crecimiento en la antesis, antes de la polinización (Mapelli *et al.,* 1978). Como se describió en el apartado 4.3, las GAs poseen picos de acumulación que difieren en frutos partenocárpicos y no partenocárpicos. Algo similar ocurre con los picos de auxinas, cuyo patrón también es bi-modal. En frutos con semillas, el primer pico se observa a los 10 dpa y coincide con la iniciación de las expansiones celulares (fase II) (Mapelli *et al.,* 1978; Gillaspy *et al.,* 1993; Srivastava y Handa, 2005). El segundo pico de acumulación de auxinas ocurre tarde en el desarrollo del fruto (35 dpa) y coincide con el final del desarrollo del embrión. Por el contario, en frutos partenocárpicos el primer pico de auxinas coincide en el tiempo con el primer pico en frutos normales (10 dpa), aunque es mucho menor. El segundo pico no es detectable, lo cual tiene una clara explicación por el hecho de que las semillas son la principal fuente de auxinas y los frutos partenocárpicos no las tienen (Gillaspy *et al.,* 1993).

4.3. Hormonas y endorreduplicación

Se ha planteado que los frutos inducidos con GA_3, los cuales tienen un alto porcentaje de células que han sufrido expansión, pueden tener un nivel de endopoliploidía mayor (Bünger-Kibler y Bangerth, 1982/83), ya que se observó un incremento en el contenido de DNA en células parenquimáticas en expansión. Esto

mismo no se ha podido demostrar en frutos inducidos con auxina, dado que sus células no mostraron incremento en tamaño como en el caso de frutos tratados con GA_3. Igualmente, el incremento de DNA en frutos de líneas partenocárpicas como *pat* es mayor que en frutos con semillas, y es exponencial al final de las divisiones celulares y cuando comienza la expansión celular, la cual es favorecida por un alto nivel de hormonas en los frutos partenocárpicos (Mapelli *et al.,* 1978).

4.4. Interacción de giberelinas y auxinas

De acuerdo con lo expuesto anteriormente, las auxinas y GAs están implicadas en la fructificación y desarrollo del fruto de tomate; de esta manera, se hace necesario estudiar las posibles interacciones entre ellas. En otras especies, las auxinas y GAs parecen interactuar en diversos procesos dependientes de GAs, ya que se ha observado que las primeras pueden alterar el metabolismo o modo de acción de las GAs. Por ejemplo, en guisante y tabaco, el IAA transportado de la yema apical induce la síntesis de GA_1 y el alargamiento del entrenudo, ya que regula positivamente la expresión de genes de biosíntesis de GAs y negativamente la expresión del gen *GA2ox* en el caso de guisante (O´Neill y Ross, 2002; Ross *et al.,* 2002). De la misma forma, las auxinas controlan la expresión de enzimas relacionados con el metabolismo de GAs en plántulas de *Arabidopsis* (Frigerio *et al.,* 2006). Se ha descrito que el 4-Cl-IAA, al parecer sintetizado en óvulos fertilizados de guisante, aumenta los niveles de transcritos que codifican los enzimas de biosíntesis de GAs en el pericarpo (Ngo *et al.,* 2002; Ozga *et al.,* 2003). La interacción o 'cross-talk' entre auxinas y GAs se ha demostrado en *Arabidopsis* ya que la degradación de RGA (regulador transcripcional putativo que reprime la señalización de GAs) es dependiente de IAA (Fu y Harberd, 2003). Este mecanismo de control de la elongación de la raíz depende de GAs a través de la degradación de las proteínas DELLA, que son represores del modo de acción de las GAs (Dill *et al.,* 2001). Igualmente en *Arabidopsis*, al analizar por medio de un microarray la expresión de genes en respuesta a la aplicación exógena de GA_4, se observó la regulación positiva de diversos genes que codificaban proteínas de transporte de auxinas (Ogawa *et al*., 2003).

En tomate, varios trabajos han descrito que la aplicación de GAs altera el contenido de auxinas: primero, cuando se aplican GAs a flores no polinizadas se observa un incremento del nivel de auxinas en el ovario (Sastry y Muir, 1963), y segundo, se han observado niveles incrementados de IAA de frutos tratados con GA_3 respecto a frutos no tratados (Bünger-Kibler y Bangerth, 1982/83). También se ha observado el efecto contrario, es decir, un incremento significativo en el nivel endógeno de GAs cuando se estimula el crecimiento partenocárpico del fruto con auxinas (Sjut y Bangerth, 1978). El incremento de GAs en la fase de expansión celular ocurre cuando se da el máximo crecimiento, que coincide con el decrecimiento del nivel de auxinas. Esto es consistente con el modelo en el cual las auxinas estimulan la biosíntesis y posterior acumulación de GAs, lo cual es requerido para expansiones posteriores (Gillaspy *et al.*, 1993).

Estos resultados, sugieren que la acción cooperativa o secuencial de GAs y auxinas es parte de una cadena de transducción de señales que conlleva a la fructificación.

5. EL CULTIVAR MICRO-TOM

Micro-Tom es un cultivar de tomate que presenta un fenotipo enano (Fig. 10). Este cultivar se caracteriza por tener entrenudos cortos, frutos de pequeño tamaño y una apariencia compacta, aunque el tamaño de las hojas es grande comparado con el resto de la planta. Otro aspecto es que ocupa poco espacio y crece rápidamente comparado con cultivares no enanos. Algunos autores sugieren que el cultivar Micro-Tom constituye un sistema adecuado para investigar la regulación hormonal del cuajado y desarrollo del fruto (Meissner *et al*., 1997; Eyal y Levy, 2002; Dan *et al*., 2006) debido a su pequeño tamaño, rápido crecimiento y facilidad para la transformación genética. Este cultivar fue desarrollado como planta ornamental a partir de cruce entre los cultivares Florida Basket y Ohio 4013-3 (Scott y Harbaugh, 1989). Del primero provienen las características de cultivar enano y la resistencia a *Fusarium*, mientras que el segundo le confirió el tamaño de hoja pequeño y las características del fruto.

Figura 10. Planta de Micro-Tom.

Las hojas de Micro-Tom son de color verde oscuro y esta característica se mantiene a lo largo del desarrollo (Scott y Harbaugh, 1989). El número de días desde la siembra hasta el desarrollo del fruto rojo varía entre 75 a 98 días (Scott y Harbaugh, 1989), aunque los frutos rojos aparecen después de 45 dpa y la planta se determina luego del cuarto racimo floral, aunque otros racimos pueden salir de brotes axilares (datos no publicados). El fenotipo de Micro-Tom es debido a la presencia de varias mutaciones puntuales en los genes: *Dwarf (D)* (que codifica una 6-deoxocatasterona dehidrogenasa, de la ruta de biosíntesis de brasinosteroides, que conduce a un mis-splicing); *Self-pruning (SP)* (que controla el fenotipo determinado/indeterminado), y del gen *Internode Length Reduction* (*Ilr*) (probablemente similar a *Miniature, Mnt*, aún no caracterizado) (Martí *et al.,* 2006). El fenotipo enano de Micro-Tom no es el resultado de deficiencias de GAs; éste cultivar tiene diversos genes *Cf* (*Cladosporium fulvum*) de resistencia (Martí *et al.,* 2006).

Si bien, los frutos polinizados de Micro-Tom son normales, se ha sugerido ser precavidos en el uso de este cultivar debido a sus mutaciones. Particularmente, no se sabe si los ovarios de Micro-Tom responden a tratamientos hormonales y, en particular, a auxinas y GAs en relación con la fructificación.

OBJETIVOS

La fructificación y desarrollo del fruto son procesos complejos en los que intervienen muchos factores, entre ellos, la regulación hormonal. Con objeto de estudiar la implicación de las hormonas giberelinas y auxinas en la fructificación y desarrollo del fruto de tomate, y su posible interacción a lo largo del crecimiento del mismo, se han planteado los siguientes objetivos:

1) Caracterizar la fructificación inducida por auxinas y giberelinas (GAs) en el cultivar de tomate Micro-Tom. Para ello se estudiará el efecto de la aplicación de diferentes GAs y auxinas sobre la fructificación y desarrollo del fruto en esta variedad y su efecto a nivel histológico y de ploidía en el pericarpo y tejido locular.

2) Determinar si las GAs son necesarias para la fructificación y desarrollo del fruto. Para ello se utilizarán inhibidores de la ruta de biosíntesis de GAs y se estudiará el efecto de la polinización sobre los niveles de transcritos de genes de las rutas de biosíntesis y catabolismo de GAs. Previamente, será necesario aislar y caracterizar la actividad de clones completos de cDNA de genes del catabolismo de GAs (*GA2ox*) de los que no se conoce ninguno hasta el momento en tomate.

3) Determinar si el efecto de las auxinas está mediado por GAs. Para abordar este punto se utilizarán inhibidores de la ruta de biosíntesis de GAs en frutos inducidos por auxinas, y se estudiará el efecto de las auxinas sobre los niveles de transcritos de genes de las rutas de biosíntesis y catabolismo de GAs. Igualmente, se estudiará el efecto del 2,4-D sobre el metabolismo *in vivo* de GAs.

RESULTADOS

CAPÍTULO I

Efecto de las Giberelinas y Auxinas en la Inducción del Crecimiento Partenocárpico del cv Micro- Tom de Tomate

Juan C. Serrani, Mariano Fos, Alejandro Atarés y José L. García-Martínez
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ORIGINAL ARTICLES

Effect of Gibberellin and Auxin on Parthenocarpic Fruit Growth Induction in the cv Micro-Tom of Tomato

Juan C. Serrani · Mariano Fos · Alejandro Atarés · José L. García-Martínez

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Abstract The effect of applied gibberellin (GA) and auxin on fruit-set and growth has been investigated in tomato (*Solanum lycopersicum* L.) cv Micro-Tom. It was found that to prevent competition between developing fruits only one fruit per truss should be left on the plant. Unpollinated ovaries responded to GA_3 and to different auxins [indol-3-acetic acid, naphthaleneacetic acid, and 2,4-dichlorophenoxyacetic acid (2,4-D)], 2,4-D being the most efficient. GA_3- and 2,4-D-induced fruits had different internal morphology, with poor locular tissue development in the case of GA, and pseudoembryos development in the case of 2,4-D. Also, GA_3 produced larger cells in the internal region of the mesocarp (IM) associated with higher mean C values, whereas 2,4-D produced more cell layers in the pericarp than pollinated fruits. The smaller size of GA_3-compared with 2,4-D-induced fruits was due to them having fewer cells, only partially compensated by the larger size of IM cells. Simultaneous application of GA_3 and 2,4-D produced parthenocarpic fruits similar to pollinated fruits, but for the absence of seeds, suggesting that both kinds of hormones are involved in the induction of fruit development upon pollination. It is concluded that Micro-Tom constitutes a convenient model system, compared to tall cultivars, to investigate the hormonal regulation of fruit development in tomato.

J. C. Serrani · A. Atarés · J. L. García-Martínez (✉)
Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC), Universidad Politécnica de Valencia, Avda de los Naranjos s/n, 46022 Valencia, Spain
e-mail: jlgarcim@ibmcp.upv.es

M. Fos
Departamento de Biología Vegetal, Universidad Politécnica de Valencia, 46022 Valencia, Spain

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Introduction

Fruit development occurs normally after fruit-set (defined as the changeover from the static condition of the flower ovary to the rapidly growing condition of the young fruit) following ovary fertilization. In the case of tomato (*Solanum lycopersicum* L.), one of the most studied fleshy fruits, fruit growth takes place in two consecutive phases: an active division phase, lasting about 7–10 days postanthesis, and a cell expansion phase (Gillaspy and others 1993). During the growth process the ovary wall develops into a pericarp composed of exocarp, mesocarp, and endocarp, while the placental parenchyma, supported by the columella, grows by division and expansion, enclosing the developing seeds and filling the locular cavities with a jelly-like homogenous tissue (locular tissue) (Ho and Hewitt 1986; Gillaspy and others 1993).

Fruit-set can also be induced by application of plant growth substances to unpollinated ovaries; the role of hormones on tomato fruit growth has been the subject of recent reviews (Gorquet and others 2005; Srivastava and Handa 2005). Application experiments of gibberellins (GAs) to unpollinated ovaries (Sjut and Bangerth 1982/83; Alabadí and Carbonell 1998; Fos and others 2000, 2001) and of inhibitors of GA biosynthesis to pollinated ovaries (Fos and others 2000, 2001) suggest that fruit-set in tomato depends on GAs. Overexpression of genes of IAA biosynthesis (Pandolfini and others 2002) and auxin application (review of Abad and Monteiro 1989; Koshioka and others 1994) also induce fruit-set and growth,

generally more efficiently that GAs (Asahira and others 1967; Bünger-Kibler and Bangerth 1982/83; Sjut and Bangerth 1982/83; Alabadí and others 1996; Alabadí and Carbonell 1998). Transcriptome analysis of expanding locular cells from pollinated fruits showed preferential expression of genes involved in synthesis, transport, and response to auxins in this tissue (Lemaire-Chamley and others 2005). Transgenic lines displaying downregulation of *SlIAA9*, an *Aux/IAA* gene, display parthenocarpic fruit development capability (Wang and others 2005). These observations, together with the increase in the content of auxin-like substances (Mapelli and others 1978) and IAA (Sjut and Bangerth 1981) after anthesis, indicate that these hormones are also involved in tomato fruit-set and development. There is some information indicating that GA and auxin application induces different histologic development of tissue ovaries. For instance, Asahira and others (1968) reported that although auxin induced cell enlargement, GA-treated fruits had poor cell division and enlargement. In contrast, Bünger-Kibler and Bangerth (1982/83) found that IAA application resulted in fruits with smaller cells, whereas GA_3-induced fruits had considerably less but larger cells. Also, development of pseudoembryos (embryo-like or embryoid structures, originated from division of the innermost integument cells and formed in the ovule cavity) with different morphology in auxin- and GA-induced fruits has been described (Kataoka and others 2003).

The tomato cultivar Micro-Tom (Scott and Harbaugh 1989) has been proposed as a convenient model system to perform research on the hormonal regulation of berry fruit development because of its small size, rapid growth, and easy transformation (Meissner and others 1997; Eyal and Levy 2002; Dan and others 2006). It has been shown that the phenotype of this cultivar is the result of point mutations in the genes *Dwarf* (*D*) (encoding 6-deoxocatasterone dehydrogenase, of the brassinosteroid biosynthesis pathway, which leads to mis-splicing), *Self-Pruning* (*SP*) (which controls the determinate/indeterminate phenotype), and *Internode length reduction* (*Ilr*) (probably similar to *Miniature, Mnt*, still uncharacterized) (Martí and others 2006). The dwarf phenotype of Micro-Tom is not the result of GA deficiency (Martí and others 2006). The Micro-Tom cultivar also carries several *Cf* (*Cladosporium fulvum*) resistance genes (Martí and others 2006). Although pollinated ovaries of Micro-Tom develop into normal fruits, it has been suggested this cultivar should be used with caution due to the above-mentioned mutations. In particular, it is not known whether Micro-Tom ovaries respond to hormones shown to be capable of inducing fruit-set in wild-type cultivars.

In this work we have carried out a comparative characterization, at histologic levels, of the response of unpollinated Micro-Tom ovaries to GAs and auxins, and have shown that this cultivar constitutes a good and advantageous system, compared to tall cultivars, to investigate hormonal regulation of fruit development. Parthenocarpic fruits induced by GAs and auxin show different morphologic and histologic development. It is suggested that both kinds of hormones are necessary for normal fruit development after pollination.

Materials and Methods

Plant Material and Growth Conditions

Tomato plants (*Solanum lycopersicum* L.) cv Micro-Tom (seeds obtained originally from Dr A. Levy) were used in the experiments. Plants (one per pot) were grown in 1-L pots with a mixture of peat:vermiculite (1:1), cultured in a greenhouse under 24°C (day)/20°C (night) conditions, and irrigated daily with Hoagland's solution. Natural light was supplemented with Osram lamps (Powerstar HQI-BT, 400W) to get a 16-h light photoperiod.

Only one flower per truss and the first two trusses were left per plant for the experiments, unless otherwise stated. All nonselected flowers were removed 2 days before anthesis.

Plant Hormone Applications

Application of gibberellic acid (GA_3) (Duchefa, Haarlem, The Netherlands), indol-3-acetic acid (IAA) (Duchefa), naphthaleneacetic acid (NAA) (Duchefa), and 2,4-dichlorophenoxyacetic acid (2,4-D) (Duchefa) was carried out to emasculated ovaries the day equivalent to anthesis (day 0) in 10 µl of 5% ethanol and 0.1% Tween 80 (Sigma-Aldrich, St. Louis, MO, USA) solution, and their weight determined 20 days later (cell expansion stage). Control ovaries were treated with the same volume of solvent solution. Flower emasculation was carried out two days before anthesis (day -2) to prevent self-pollination. Due to the low fruit weight variability of the experimental system, only eight ovaries (from four plants) were used per treatment.

Histology and Determination of Cellular Parameters

Ovary and fruit tissue sections were fixed in 4% paraformaldehyde-0.1 M sodium phosphate buffer pH 7.2 by applying a vacuum for 3 min and then for 8 h at 4°C. After dehydration in ethanol (15, 30, 50, 70, 85, and 100% series), the samples were embedded in paraffin [immersed in

Histo-clear (National Diagnostics, Atlanta, GA, USA)/ethanol series (2:1, 1:1, 1:2), followed by Histo-clear/paraffin series (2:1, 1:1, 1:2), and finally paraffin (Paraplast Plus, Sigma-Aldrich].

Sections (8 μm thick) were cut with a microtome (model HM 330, Microm, Walldorf, Germany) and stained with toluidine blue. Sections of 0, 5, and 10 days postanthesis (dpa) were viewed on a microscope (eclipse E600, ACT-1 software, Nikon, Tokyo, Japan) and photographed with a spot digital camera (DMX1200F, Nikon). Tissue fragments from 20, 30, and 45 dpa were observed on a stereomicroscope (SMZ200, Nikon) because of their large size, and images were acquired with a digital camera (Colorview 12 CCD, Olympus, Tokyo, Japan). All measurements were performed in six to eight independent sections, using analySIS® (Soft Imaging System, Münster, Germany) software.

For cell size and shape determinations, squares between 0.5 and 1.5 mm^2 (depending on fruit age) were delimited on sections of external mesocarp (EM), located between the central ring of vascular bundles and epidermis, and on sections of internal mesocarp (IM), between the ring of vessels and endocarp, and their surface area and the numbers of cells inside the squares calculated. Mean cell area was estimated as the ratio square cell area/cell number.

Number of cell layers was estimated by counting the number of cells along a line across the pericarp perpendicular to the epidermis and endocarp (avoiding vascular vessels).

Cell sections were circular (C) or more or less elliptical, and in the latter case their major axis was perpendicular or parallel to the epidermis and endocarp. For cell shape classification we determined the lengths of the major axes perpendicular (A) and parallel (B) to the epidermis in 50–60 cells per fruit region (EM and IM) and section. When $A > B$ the cells were elliptical perpendicular to the epidermis (Pe), and when $A < B$ they were elliptical parallel to the epidermis (Pa). Cell eccentricity ($0 \le e \le 1$) is a parameter that determines how much a cell section deviates from being circular (e = 0 for a perfect circle):

$$e^2 = (1 - m^2/M^2),$$

where m = minor axis and M = maximum axis. For classification purposes, cells with $e \le 0.5$ were considered circular, and those with $e > 0.5$ elliptical. For $e = 0.5$, $m = 0.866M$. This means that we considered a cell as circular when $0.866M \le m \le M$, and elliptical when $m < 0.866M$. Therefore, we established that cells with $0.866A \le B \le 1.134A$ were C, cells with $B < 0.866A$ were Pe, and cells with $B > 1.134A$ were Pa.

Ploidy Determination

Ploidy was determined in entire 0- and 5-dpa ovaries and in exocarp (peel removed with a razor blade including several layers of cells in addition to the actual epidermis), mesocarp, and locular gel of pollinated and hormone-induced fruits from 10 dpa. Two hundred to 400 mg of fresh tissue was sliced with a razor blade into thin strips smaller than 0.5 mm in a glass Petri dish containing 0.4 ml of nuclei isolation buffer (high-resolution DNA kit, solution A: nuclei isolation, Partec GmbH, Münster, Germany). The nuclei extract was mixed with 1 ml of staining buffer (high-resolution DNA kit, solution B: DAPI staining; Partec GmbH, Münster, Germany), shaken for 1 min, and filtered through 100-μm nylon mesh (Nyblot). The filtrates (more than 5000 nuclei per extract) were analyzed using a Partec PA-II flow cytometer (Partec GmbH). Data were plotted on a semilogarithmic scale. Calibration of C values was performed using young leaf extract.

Peak areas were used to determine nuclei numbers with different ploidy. Ploidy distribution represented nuclei percentages corresponding to all the peaks. Mean C values (MCV) correspond to the sum of the number of nuclei of each ploidy level multiplied by its endoreduplication cycle, divided by the total number of nuclei (Barow and Meister 2003).

Results

Effect of Number and Position of Ovaries per Truss on Fruit Growth

With the aim of establishing a system without competition between growing fruits in Micro-Tom, we induced fruit-set by GA_3 application to different numbers of unpollinated ovaries (1-3) in the first three trusses. GA_3 was chosen because there was previous evidence that GAs are necessary for fruit-set in tomato (Fos and others 2001). All the GA_3-treated ovaries set and developed to mature fruits (Figure 1). The mean weight of the total GA_3-induced fruits was similar in the three trusses, indicating that there was no competition between trusses. However, despite GA_3 being applied simultaneously to the three ovaries of a truss (synchronized treatment), the weight of fruits within a truss depended on fruit position, the fruit coming from the older flower being larger than the fruit coming from the youngest one (Figure 1). In the case of trusses where only one or two unpollinated ovaries were treated with GA_3, some of the untreated ovaries of the same truss also developed parthenocarpically (Figure 1). This means that GA_3 can be transported acropetally and alter the growth of other ovaries within the truss. As a result, only one ovary/

fruit per truss and a maximum of three trusses per plant were used in the experiments.

Response of Unpollinated Ovaries to Auxins and Gibberellin

Three different kinds of auxins were applied to unpollinated ovaries for fruit growth-inducing activity in Micro-Tom: IAA, NAA, and 2,4-D. 2,4-D was active at all the doses assayed (20-2000 ng per ovary), but NAA only at 200 and 2000 ng per ovary, and IAA at 2000 ng per ovary (Figure 2). Unpollinated ovaries treated with solvent solution (control) did not grow (< 5 mg at day 20 compared to about 1 mg at day 0) but did not abscise. Due to the much higher activity of 2,4-D, this auxin was used in all the subsequent experiments. This kind of auxin has also been used as a substitute for IAA by many authors in diverse application experiments.

Weight comparisons of 20-day-old parthenocarpic fruits induced by GA_3 and 2,4-D showed that the maximum response to GA_3 was attained with 2000 ng per ovary, whereas the maximum to 2,4-D was obtained with a dose about ten times lower (200 ng per ovary), and fruits of similar size to pollinated fruits even with just 20 ng per ovary (Figure 3A). Parthenocarpic fruits larger than pollinated fruits could be obtained with high doses of 2,4-D, although the weight of GA_3-induced fruits was always lower than pollinated fruits (Figure 3A).

Although fruits were different sizes, the external morphology of GA_3- and 2,4-D-induced fruits was quite similar to pollinated fruits at optimal doses. Sometimes fruits induced with 2,4-D were elongated at the stylar end (results not presented). At higher doses of 2,4-D (> 200 ng per ovary), the fruits were more flattened (results not shown) and the external surface had a small number of protuberances produced by mesocarp cell proliferation but not connected to the vascular system (Figure 3C). In contrast, the internal morphology of GA_3 and 2,4-D fruits was quite different. Jelly tissue development was almost absent in GA_3-induced fruits at all doses, so these fruits had empty locular cavities (Figure 3B). A similar effect, though less severe, could be observed at supraoptimal doses of 2,4-D (Figure 3B). Also, 2,4-D produced several morphologic effects absent in GA_3-induced fruits: (1) development of pseudoembryos, which remained engulfed into the locular tissues at advanced fruit growth stages (Figure 4C, F). In GA_3-induced ovaries some ovules had limited growth, but most of them degenerated (Figure 4B, E); (2) an increase in the number of vascular bundles (Figure 5C); and (3) development of tracheids connecting transversally the

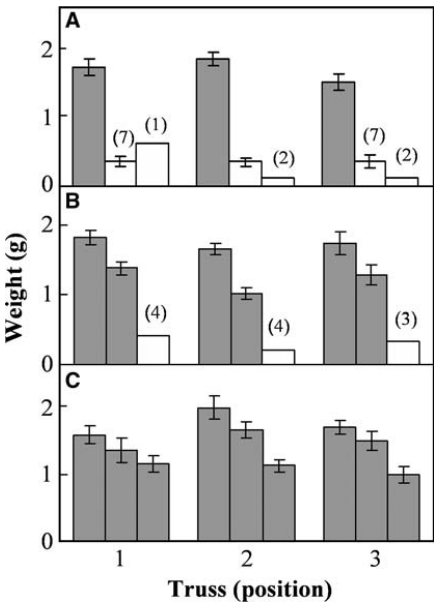

Fig. 1 Effect of number of unpollinatated tomato ovaries per truss treated with GA_3 (2000 ng per ovary) on parthenocarpic growth. **A** One ovary per truss. **B** Two ovaries per truss. **C** Three ovaries per truss. In all cases plants with the first three trusses and three unpollinated ovaries per truss were used, and both treated and untreated ovaries were left on the truss. Fruits were collected 20 days after treatment. Values are means of eight replicates ± SE. All GA_3-treated ovaries developed. In the case of untreated ovaries, figures on histograms indicate the number of ovaries grown (when < 8). At each truss, left bar corresponds to the lower ovary, middle bar to middle ovary, and right bar to upper ovary. Gray bars correspond to GA_3-treated ovaries and white bars to nontreated ovaries. Truss position: 1, lower; 2, medium; 3, upper

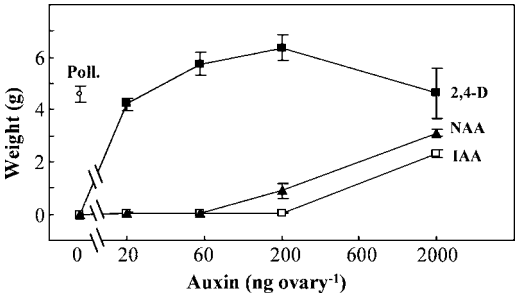

Fig. 2 Dose response of unpollinated tomato ovaries to 2, 4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), and naphthaleneacetic acid (NAA) treatment. Fruits were collected 20 days after hormone application, and values are means of eight fruits ± SE. Values of pollinated ovaries are also included as control. Poll., pollinated

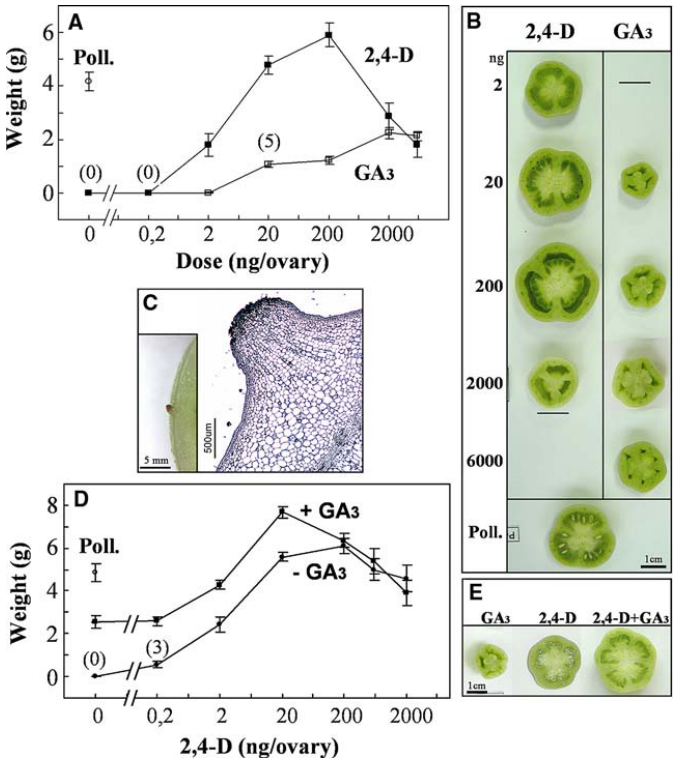

Fig. 3 Response of unpollinated ovaries to GA_3 and 2,4-D treatments. **A** Dose response to GA_3 and 2,4-D (0-6000 ng per ovary). **B** Pictures of transverse sections of representative pollinated, GA_3-, and 2,4-D-induced fruits with different amounts of hormones. **C** Representative protuberance on the epidermis of 2,4-D-induced fruits. **D** Effect of GA_3 (2000 ng per ovary) on the response to 2,4-D. **E** Pictures of transverse sections of representative fruits induced by single and joint application of GA_3 (2000 ng) and 2,4-D (20 ng). Fruits were collected 20 days after treatment. Values are means of eight fruits ± SE. Figures in brackets indicate the numbers of fruits developed when less than eight. Values of pollinated ovaries are also included as control. Poll., pollinated

longitudinal vascular bundles in the cambium region of the pericarp (Figure 5C, D).

The application of GA_3 (2000 ng per ovary) simultaneously with up to 20 ng of 2,4-D produced an additive growth effect. At supraoptimal doses of 2,4-D (200 ng and more), GA_3 could not revert the negative effect of 2,4-D (Figure 3D). Interestingly, at low doses of 2,4-D (2 and 20 ng), simultaneous addition of GA_3 produced fruits of similar size and shape (including development of locular tissue) to pollinated fruits (Figure 3D, E).

Histology of Pollinated and GA_3- and Auxin-Induced Fruits

The different pericarp parameters measured during fruit development are depicted in Figure 6A, which shows a transverse section of a representative 20-day-old pollinated fruit. Because gradient differences between epidermis and endocarp were apparent, two different regions were considered for cell size and shape determinations: external mesocarp (EM), located between the central ring of vascular bundles and epidermis, and internal mesocarp (IM), between the ring of vascular vessels and endocarp, as described in Materials and Methods. Representative micrographs of transverse pericarp sections from GA_3-induced (2000 ng per ovary) and 2,4-D-induced (200 ng per ovary) 20-day-old fruits are presented in Figure 5B and C. Figure 5D shows a mesocarp region containing more bundles of tracheids in 2,4-D-treated fruits (as mentioned before).

Fruits induced by GA_3 and 2,4-D had thicker pericarp than pollinated fruits ($P = 0.008$) throughout development, more with 2,4-D than with GA_3 ($P = 0.001$) (Figure 6A). The number of pericarp cell layers increased between day 0 and day 10 (cell division period) and remained relatively constant afterward (expansion period). Some increase in the number of layers occurred in pollinated and GA_3-induced fruits after day 20. The number of layers produced in 2,4-D-induced fruits was higher ($P = 0.006$) and in GA_3-induced fruits the number was lower ($P = 0.0015$) than in pollinated fruits (Figure 6B). The combined application of GA_3 and 2,4-D had an intermediate effect on pericarp thickness and number of layers compared to separate hormone application (data not presented). No apparent differences in mean cell size (estimated as transversal

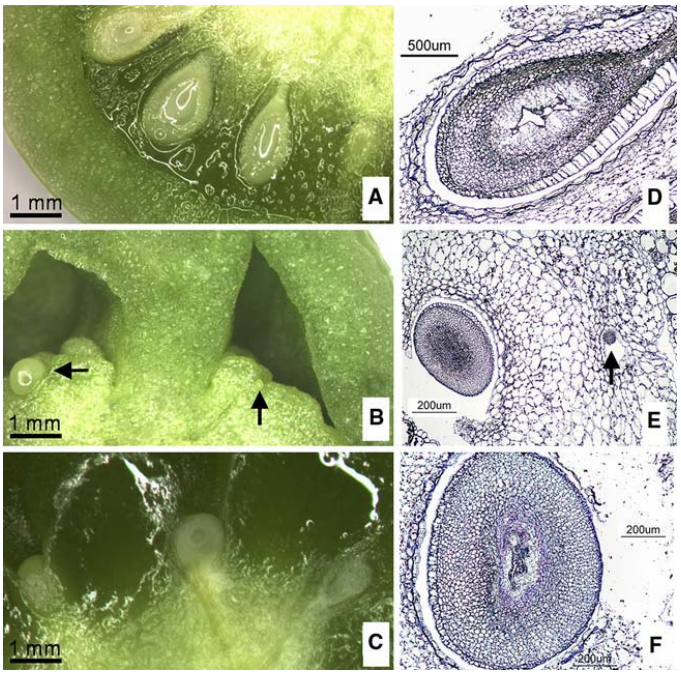

Fig. 4 Images of developing seeds in pollinated fruits (**A, D**), poor developing and degenerated ovules in GA_3-induced fruits (**B, E**), and pseudoembryos in 2,4-D-induced fruits (**C, F**). Cross-sections (D, E, F) were obtained from paraffin-embedded tissues. Fruits were collected 20 days after pollination or hormone treatment. Arrows in C indicate ovule position in GA_3-treated ovaries

section areas) between treatments were found in the EM region of the mesocarp at different developmental stages (Figure 6C). In contrast, significantly larger cells were present in the IM region of GA_3-induced fruits compared to pollinated and 2,4-D-induced fruits (P = 0.007) (Figure 6D).

At day 0 similar percentages (about 33%) of circular (C) and elliptic cells with their major axis oriented parallel (Pa) and perpendicular (Pe) to the epidermis were found in transverse sections of the entire pericarp (Figure 7). These percentages changed dramatically during fruit development, mainly during cell expansion, though differently in the external (EM) and internal mesocarp (IM). Compared with day 0, at day 5 (cell division stage) changes in cell shape were found in GA_3-induced ovaries (with higher % of Pe cells in both EM and IM; P = 0.004) and in pollinated ovaries (with an increase of Pa cells in EM; P = 0.008). In 20-day-old fruits (cell expansion stage) no apparent differences in cell orientation (estimated as % of cell number) between pollinated and GA_3- and 2,4-D-induced fruits were found. In this case most of the cells in EM were Pa (60–70%), whereas in IM they were Pe (more than 80%) (Figure 7). However, in IM the Pe cells were more elliptical in GA_3-induced fruits (e = 0.78 ± 0.03) than in pollinated (e = 0.68 ± 0.04; P = 0.0001) and 2,4-D-induced fruits (e = 0.70 ± 0.04; P = 0.002). No significant differences of eccentricity were found between fruits in EM cells.

Ploidy of Pollinated and GA_3- and Auxin-Induced Fruits

Ploidy of cells from day 0 ovaries was essentially similar to that of very young leaves, which were used as a reference pattern. This ploidy level increased progressively during fruit development in all fruit tissues (exocarp, mesocarp, and locular gel plus placenta), where cells with C values up to 256 were found in 45-day-old fruits (data not presented). The distribution of C values showed some variation between tissues, but no large differences between pollinated and parthenocarpic fruits were found, particularly for exocarp and locular plus placenta (see Figure 8A as an example for tissues of 20-day-old fruits).

The time course of mean C values (MCV) showed a continuous increase in the exocarp, mesocarp, and locular gel plus placenta during fruit growth. No significant differences of MCV were found between pollinated and GA_3- and 2,4-D-induced fruits in the exocarp (Figure 8B). In contrast, MCVs of mesocarp cells in GA_3-induced fruits were higher than those in pollinated (P = 0.0003) and 2,4-D-induced fruits (P = 0.002) from 5 days after anthesis

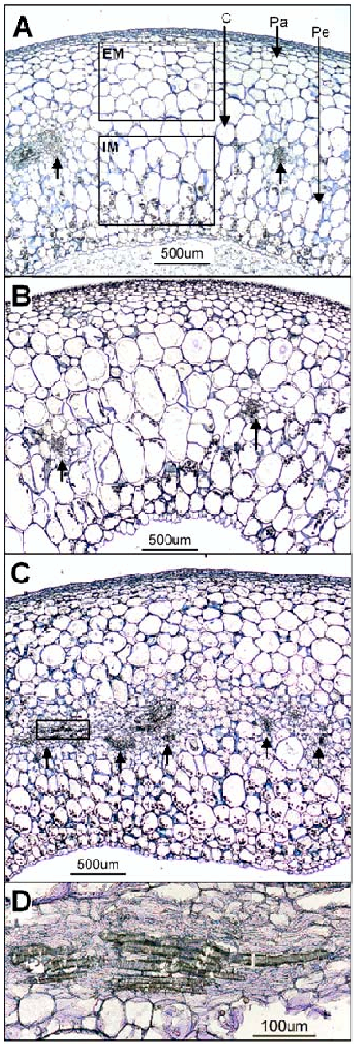

Fig. 5 Effect of GA_3, 2,4-D, and pollination on pericarp histology. Representative pictures of transverse pericarp sections of 20-day-old fruits. **A** Pollinated. **B** Parthenocarpic fruit induced with GA_3 (2000 ng per ovary). **C** Parthenocarpic fruit induced with 2,4-D (200 ng per ovary). **D** Enlarged square from C showing representative tracheid bundles developed in 2,4-D-induced fruits

(Figure 8B and inset). In locular gel plus placenta, MCVs of GA_3-induced fruits were also higher than those of 2,4-D-induced fruits ($P = 0.001$) from 15 days after anthesis, but smaller than pollinated fruits ($P = 0.001$) (Figure 8B).

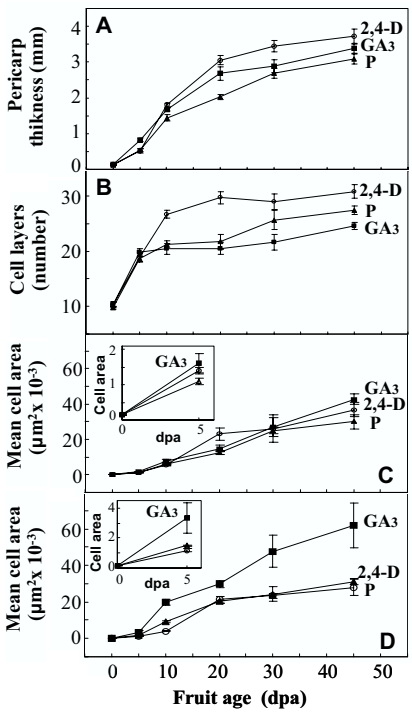

Fig. 6 Effect of GA_3, 2,4-D, and pollination on pericarp development. **A** Pericarp thickness. **B** Number of cell layers. **C** Mean cell area of external mesocarp. **D** Mean cell area of internal mesocarp. Values are means of eight sections, one section per fruit, and eight fruits. The parameters determined are indicated in the transversal pericarp section of pollinated ovary (Figure 5A). EM, external mesocarp; IM, internal mesocarp; C, circular cell; Pa, elliptic cell parallel to the surface of the pericarp; Pe, elliptic cell perpendicular to the epidermis. Arrows indicate the position of longitudinal vascular bundles

Discussion

Micro-Tom as a Model System to Investigate the Hormonal Regulation of Fruit-Set and Growth

We have found that unpollinated ovaries of tomato cv Micro-Tom grow parthenocarpically in response to GA and auxin application (Figures 2, 3). This means that in spite of carrying the mutations d, sp, and ilr/mnt that produce a very dwarf habit and small fruits (Martí and others 2006), the response of this cultivar to GAs and auxins was similar to that observed previously in diverse wild-type cultivars of tomato (Asahira and others 1967; Sjut and Bangerth 1982/83; Koshioka and others 1994; Alabadí and others 1996; Alabadí and Carbonell 1998; Fos and others 2000, 2001).

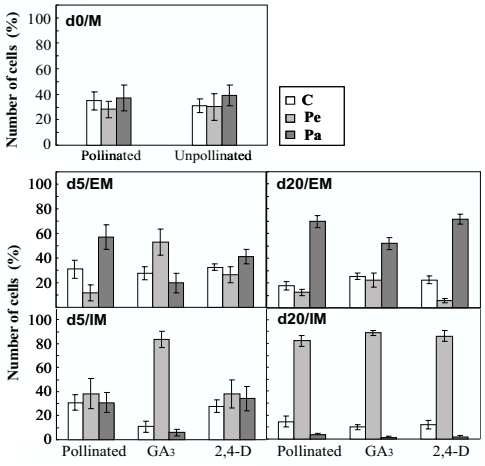

Fig. 7 Effect of GA_3 (2000 ng per ovary), 2,4-D (200 ng per ovary), and pollination on mesocarp cell shape. White bars correspond to circular cells (C), light-gray bars to elliptic cells perpendicular to the epidermis (Pe), and dark-gray bars to elliptic cells parallel to the epidermis (Pa). See Materials and Methods for definitions of C, Pa, and Pe cells. Determinations were carried out at day 0 (equivalent to anthesis), at day 5 (representative cell division stage), and at day 20 (representative cell expansion stage). External (EM) and internal (IM) mesocarp regions were analyzed separately at days 5 and 20. Values are means of 50-60 cells per region and fruit, from six fruits ± SE

Therefore, our results support the proposed idea that Micro-Tom may be a good system to investigate the hormonal regulation of berry fruit development (Meissner and others 1997). Moreover, compared to tall cultivars, Micro-Tom has the advantage of having a reduced stature, which allows many plants to be cultivated in a relatively small space and thus a large number of experimental replicates and a shorter fruit development cycle (less than 3 months from seeding compared to more than 4 months in tall cultivars). Interestingly, although Micro-Tom is a brassinosteroid mutant (Martí and others 2006), pollinated fruits grow normally and parthenocarpic fruit-set could not be induced nor enhanced by brassinolide application (data not presented), indicating that brassinosteroids are not involved in fruit-set and growth in tomato. However, the observation that brassinosteroid synthesis seems to be localized in developing tomato fruit, associated with the locular jelly and seeds (Montoya and others 2005), may deserve further investigation. It is common that people use several ovaries per truss in hormone application experiments (Alabadí and others 1996; Fos and others 2000; Kataoka and others 2003). We have shown that to prevent growth competition between fruits in Micro-Tom, only one ovary per truss should be used because hormone transport between ovaries of a truss occurs, at least in the case of applied GAs (Figure 1). The reduced number of fruits that have to be left per plant to prevent competition may be a disadvantage compared to tall cultivars showing weaker or no competition between fruits.

Gibberellins and Auxins Have Different Effects at Morphologic and Histologic Levels

Parthenocarpic fruits induced by optimal doses of GA_3 and 2,4-D had external shapes quite similar to pollinated fruits. However, at high doses of 2,4-D some malformations were apparent (similar to those described for overexpression of IAA-biosynthesis genes; Pandolfini and others 2002) and small epidermal protuberances were produced that were never found in pollinated and GA_3-induced fruits

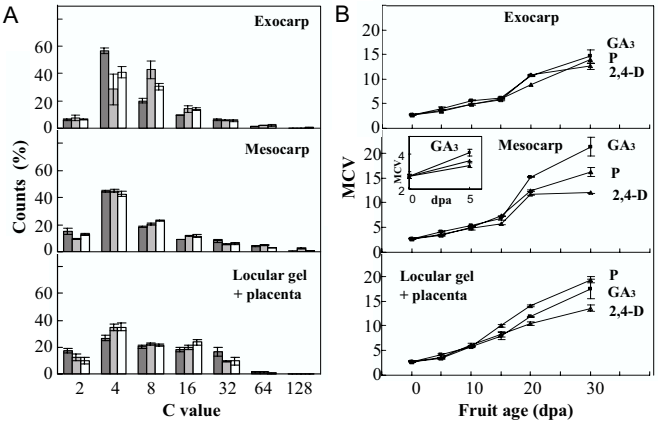

Fig. 8 Effect of GA_3 (2000 ng per ovary), 2,4-D (200 ng per ovary), and pollination on fruit cell endoreduplication. **A** Percentages of nuclei with different C values in exocarp, mesocarp, and locular and placenta tissues from 20-day-old fruits. **B** Time course of mean C values (MCV) in exocarp, mesocarp, and locular and placenta tissues. Dark bars, pollinated fruits; gray bars, GA_3-treated ovaries; white bars, 2,4-D-treated ovaries

(Figure 3C). In contrast, GA_3- and 2,4-D-induced fruits were quite different internally in that locular tissue of GA_3-induced fruits barely developed, resulting in almost empty locular cavities, whereas 2,4-D-induced fruits had filled locules similar to those of pollinated fruits (except at very high doses of hormone) (Figure 3B). Puffiness following GA_3 and 2-hydroxymethyl-4-chlorophenoxyacetic acid (another synthetic auxin) application has been described previously (Asahira and others 1968); in the latter case, however, it was probably the result of an overoptimum amount of applied auxin, as occurred with 2,4-D (Figure 3B), although no dose effect was investigated. Another interesting difference between GA_3- and 2,4-D-induced fruits was that most of the unfertilized ovules degenerated in the former case, whereas they developed to some extent in the case of auxin application (due to proliferation of the outer integuments and the formation of pseudoembryos from division of the innermost integument cells in the internal ovule cavity) (Figure 4). Induction of pseudoembryos by auxin application has been reported earlier (Asahira and others 1967; Kataoka and others 2003) but their possible relation to parthenocarpic fruit growth is unclear.

Both GA_3- and 2,4-D-induced fruits had thicker pericarp compared with that of pollinated fruits (Figure 6A). However, at the cellular level this was the result of different processes: increase of cell divisions (cell layers) in the case of 2,4-D (Figure 6B), and increase of cell size in the IM in the case of GA_3 (Figure 6D). In addition, 2,4-D (at doses of 20 ng or more per ovary) stimulated vascular development, characterized by an increase in the number of vascular bundles (see arrows in Figure 5C), and multiple transversal bundles of tracheids apparently connecting the former ones (Figure 5D), which were never present in pollinated or GA-induced fruits. This probably facilitates the arrival of nutrients which, together with the higher number of cells, produces the greater growth of auxin-induced fruits. Interestingly, the addition of a small quantity of 2,4-D (2-20 ng) to the optimal dose of GA_3 (2000 ng) produced fruits essentially identical in size and shape to pollinated ones, including normal development of locular tissue and vascular bundles (Figure 3D, E). These observations suggest that there is an interaction between these hormones during tomato fruit development.

Gibberellins and Auxins Have a Different Effect at the Cellular Level

In both pollinated and parthenocarpic grown fruits most of the cells had an elliptical shape, although their orientation was not homogeneous throughout the mesocarp. For instance, most of the cells in the EM were parallel to the epidermis (Pa), whereas in the IM they were perpendicular (Pe) (Figure 7). Interestingly, although there was no qualitative difference in the distribution (percentages) of Pa and Pe cells between pollinated and 2,4-D- and GA-induced fruits, absolute eccentricity values of IM cells in GA_3-treated fruits were significantly higher than those of pollinated and 2,4-D-induced fruits. This effect of GA on polar cell elongation is common in stem-elongating tissues (Cowling and Harberd 1999). It is in contrast, however, to the effect on mesocarp cell expansion induced by GA_3 in other species like pea (Vercher and Carbonell 1991), where multidirectional expansion was observed. The increase of Pe cells in the IM may be relevant to the mode of action of GAs on fruit-set in tomato because this effect could be detected in ovaries 5 days after treatment but not in pollinated or 2,4-D-induced fruits of the same age.

Ploidy in different fruit tissues (exocarp, mesocarp, and locular gel plus placenta) increased progressively during growth due to endoreduplication up to 256C. This agrees with ploidy data reported from different tomato cultivars (Bergervoet and others 1996; Joubès and others 1999; Cheniclet and others 2005). Mature IM cells of fruits induced with GA_3 were larger (Figure 6D) and had higher MCV (Figure 8B) than 2,4-D-induced and pollinated fruits. In the case of 2,4-D lower ploidy compared with pollinated fruits, it was not associated with reduced mesocarp cell size. This suggests that GAs may regulate the size of mesocarp cells by altering ploidy levels. This is in agreement with the strong positive correlation between mean cell size and ploidy observed in 20 tomato lines displaying a large fruit weight range (Cheniclet and others 2005). GA_3-induced fruits also had fewer mesocarp cells (Figure 6B). This raises the possibility that the increase of cell size in GA_3-induced fruits is not direct but due to compensation induced by reduction of cell divisions (a similar effect to that described in relation to leaf morphogenesis; Tsukaya 2006), and that this compensation is mediated by ploidy level alteration. Thus, the smaller size of GA_3- compared to 2,4-D-induced fruits (about 3 times at optimal doses; Figure 3A) would be due to reduced cell divisions in the former, only partially compensated by cell size increase. The effect of GA_3 on ploidy is rapid because it was observed as early as 5 days after hormone application, associated with an increase of IM cells. The importance of ovary cell number at anthesis to final fruit size has been previously reported in pollinated *Lycopersicon pimpinellifollium* fruits using near-isogenic mutants (Bohner and Bangerth 1988). Also, Bertin and others (2003) suggested that the large variation of fruit size between competing fruits from two isogenic lines bearing cells of similar size was due to genes controlling cell division. According to these authors, the fruit size difference disappears in the absence of fruit competition, probably as a result of hormonal imbalance within the truss.

Conclusions

Parthenocarpic fruit-set and growth in response to GA and auxin application in the dwarf cultivar Micro-Tom were similar to those described previously in diverse tall cultivars. Therefore, the results presented show that Micro-Tom, due to its small size and reduced reproductive cycle, is a convenient cultivar of tomato for use as a model in research on the hormonal regulation of fruit-set and growth. It was also found that GAs and auxins seem to interact to regulate fruit growth through cell division and expansion.

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References

Abad M, Monteiro AA (1989) The use of auxins for the production of greenhouse tomatoes in mild-winter conditions: a review. Sci Hort 38:167–192

Alabadí D, Carbonell J (1998) Expression of ornithine decarboxylase is transiently increased by pollination, 2,4-dichlorophenoxyacetic acid, and gibberellic acid in tomato ovaries. Plant Physiol 118:323–328

Alabadí D, Agüero MS, Pérez-Amador MA, Carbonell J (1996) Arginase, arginine decarboxylase, ornithine decarboxylase, and polyamines in tomato ovaries. Changes in pollinated ovaries and parthenocarpic fruits induced by auxin and gibberellin. Plant Physiol 112:1237–1244

Asahira T, Takeda Y, Nishio T, Hirabayashi M, Tsukamoto Y (1967) Studies on fruit development in tomato. I. Ovule development and content of diffusible auxin in synthetic auxin- and gibberellin-induced parthenocarpic tomato fruits in relation to their development. Mem Res Inst Food Sci Kyoto Univ 28:47–74

Asahira T, Takagi H, Takeda Y, Tsukamoto Y (1968) Studies on fruit development on tomato. II. Cytokinin activity in extracts from pollinated, auxin- and gibberellin-induced parthenocarpic tomato fruits and its effect on the histology of the fruit. Mem Res Inst Food Sci Kyoto Univ 29:24–54

Barow M, Meister A (2003) Endopolyploidy in seed plants is differently correlated to systematics, organ, life strategy and genome size. Plant Cell Environ 26:571–584

Bergervoet JHW, Verhoeven HA, Gilissen LJW, Bino RJ (1996) High amounts of nuclear DNA in tomato (*Lycopersicon esculentum* Mill.) pericarp. Plant Sci 116:141–145

Bertin N, Borel C, Brunel B, Cheniclet C, Causse M (2003) Do genetic make-up and growth manipulation affect tomato fruit size by cell number, or cell size and DNA endoreduplication? Ann Bot 92:415–424

Bohner J, Bangerth F (1988) Cell number, cell size and hormone levels in semi-isogenic mutants of *Lycopersicon pimpinellifolium* differing in fruit size. Physiol Plant 72:316–320

Bünger-Kibler S, Bangerth F (1982/83) Relationship between cell number, cell size and fruit size of seeded fruits of tomato (*Lycopersicon esculentum* Mill.), and those induced parthenocarpically by the application of plant growth regulators. Plant Growth Regul 1:143–154

Cheniclet C, Rong WY, Causse M, Frangne N, Bolling L, Carde JP, Renaudin JP (2005) Cell expansion and endoreduplication show a large genetic variability in pericarp and contribute strongly to tomato fruit growth. Plant Physiol 139:1984–1994

Cowling RJ, Harberd NP (1999) Gibberellins control *Arabidopsis* hypocotyl growth via regulation of cellular elongation. J Exp Bot 50:1351–1357

Dan Y, Yan H, Munyikwa T, Dong J, Zhang Y, Armstrong CL (2006) MicroTom, a high-throughput model transformation system for functional genomics. Plant Cell Rep 25:432–441

Eyal E, Levy AA (2002) Tomato mutants as tools for functional genomics. Curr Opin Plant Biol 5:112–117

Fos M, Nuez F, García-Martínez JL (2000) The gene *pat-2*, which induces natural parthenocarpy, alters the gibberellin content in unpollinated tomato ovaries. Plant Physiol 122:471–479

Fos M, Proaño K, Nuez F, García-Martínez JL (2001) Role of gibberellins in parthenocarpic fruit development induced by the genetic system *pat-3/pat-4* in tomato. Physiol Plant 111:545–550

Gillaspy G, Ben-David H, Gruissem W (1993) Fruits: a developmental perspective. Plant Cell 5:1439–1451

Gorquet B, Van Heusden AW, Lindhout P (2005) Parthenocarpic fruit development in tomato. Plant Biol 7:131–139

Ho L, Hewitt J (1986) Fruit development. In: Atherton JG, Rudish J (eds), The tomato crop. New York: Chapman and Hall, pp 201–239

Joubès J, Phan T-H, Just D. Rothan C. Bergounioux C, Raymond P, Chevalier C (1999) Molecular and biochemical characterization of the involvement of cyclin-dependent kinase A during the early development of tomato fruit. Plant Physiol 121:857–869

Kataoka K, Uemachi A, Yazawa S (2003) Fruit growth and pseudoembryo development affected by uniconazole, an inhibitor of gibberellins, in *pat-2* and auxin-induced parthenocarpic tomato fruits. Sci Hort 98:9–16

Koshioka M, Nishijima T, Yamazaki H, Liu Y, Nonaka M, Mander LN (1994) Analysis of gibberellins in growing fruits of *Lycopersicon esculentum* after pollination or treatment with 4-chlorophenoxyacetic acid. J Hort Sci 69:171–179

Lemaire-Chamley M, Petit J, García V, Just D, Baldet P, Germain V, Fagard M, Mouassite M, Cheniclet C, Rothan C (2005) Changes in transcriptional profiles are associated with early fruit tissue specialization in tomato. Plant Physiol 139:750–769

Mapelli SC, Frova C, Torti G, Soressi G (1978) Relationship between set, development and activities of growth regulators in tomato fruits. Plant Cell Physiol 19:1281–1288

Martí E, Gisbert C, Bishop GJ, Dixon MS, García-Martínez JL (2006) Genetic and physiological characterization of tomato cv. Micro-Tom. J Exp Bot 57:2037–2047

Meissner R, Jacobson Y, Melamed S, Levyatuv S, Shalev G, Ashri A, Elkind Y, Levy AA (1997) A new model system for tomato genetics. Plant J 12:1465–1472

Montoya T, Nomura T, Yokota T, Farrar K, Harrison K, Jones JGD, Kaneta T, Kamiya Y, Szekeres M, Bishop G (2005) Patterns of *Dwarf* expression and brassinosteroid accumulation in tomato reveal the importance of brassinosteroid synthesis during fruit development. Plant J 42:262–269

Pandolfini T, Rotino GL, Camerini S, Defez R, Spena A (2002) Optimisation of transgene action at the post-transcriptional level: high quality parthenocarpic fruits in industrial tomatoes. BMC Biotechnol 2:1–11

Scott JW, Harbaugh BK (1989) Micro-Tom. - A miniature dwarf tomato. Florida Agric Exp Sta Cir 370:1–6

Sjut V, Bangerth F (1981) Effect of pollination or treatment with growth regulators on levels of extractable hormones in tomato ovaries and young fruits. Physiol Plant 53:76–78

Sjut V, Bangerth F (1982/1983) Induced parthenocarpy – a way of changing the levels of endogenous hormones in tomato fruits

(*Lycopersicon esculentum* Mill.). 1 Extractable hormones. Plant Growth Regul 1:243–251

Srivastava A, Handa AK (2005) Hormonal regulation of tomato fruit development: a molecular perspective. J Plant Growth Regul 24:67–82

Tsukaya H (2006) Mechanism of leaf-shape determination. Ann Rev Plant Biol 57:477–496

Vercher Y, Carbonell J (1991) Changes in the structure of ovary tissues and in the ultrastructure of mesocarp cells during ovary senescence or fruit development induced by plant growth substances in *Pisum sativum*. Physiol Plant 81:518–526

Wang H, Jones B, Li Z, Frasse P, Delalande C, Regad F, Chaabouni S, Latché A, Pech J-C, Bouzayen M (2005) The tomato *Aux/IAA* transcription factor *IAA9* is involved in fruit development and leaf morphogenesis. Plant Cell 17:2676–2692

CAPÍTULO II

Regulación de la Fructificación y el Crecimiento del Fruto de Tomate por Giberelinas

Juan Carlos Serrani, Rafael Sanjuán, Omar Ruiz-Rivero, Mariano Fos y José Luis García-Martínez
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Gibberellin Regulation of Fruit Set and Growth in Tomato[1][W]

Juan Carlos Serrani, Rafael Sanjuán, Omar Ruiz-Rivero, Mariano Fos, and José Luis García-Martínez*

Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-Consejo Superior de Investigaciones Científicas (J.C.S., R.S., O.R.-R., J.L.G.-M.), and Departamento de Biología Vegetal (M.F.), Universidad Politécnica de Valencia, 46022 Valencia, Spain

The role of gibberellins (GAs) in tomato (*Solanum lycopersicum*) fruit development was investigated. Two different inhibitors of GA biosynthesis (LAB 198999 and paclobutrazol) decreased fruit growth and fruit set, an effect reversed by GA_3 application. LAB 198999 reduced GA_1 and GA_8 content, but increased that of their precursors GA_{53}, GA_{44}, GA_{19}, and GA_{20} in pollinated fruits. This supports the hypothesis that GA_1 is the active GA for tomato fruit growth. Unpollinated ovaries developed parthenocarpically in response to $GA_3 > GA_1 = GA_4 > GA_{20}$, but not to GA_{19}, suggesting that GA 20-oxidase activity was limiting in unpollinated ovaries. This was confirmed by analyzing the effect of pollination on transcript levels of *SlCPS*, *SlGA20ox1*, *-2*, and *-3*, and *SlGA3ox1* and *-2*, encoding enzymes of GA biosynthesis. Pollination increased transcript content of *SlGA20ox1*, *-2*, and *-3*, and *SlCPS*, but not of *SlGA3ox1* and *-2*. To investigate whether pollination also altered GA inactivation, full-length cDNA clones of genes encoding enzymes catalyzing GA 2-oxidases (*SlGA2ox1*, *-2*, *-3*, *-4*, and *-5*) were isolated and characterized. Transcript levels of these genes did not decrease early after pollination (5-d-old fruits), but transcript content reduction of all of them, mainly of *SlGA2ox2*, was found later (from 10 d after anthesis). We conclude that pollination mediates fruit set by activating GA biosynthesis mainly through up-regulation of *GA20ox*. Finally, the phylogenetic reconstruction of the *GA2ox* family clearly showed the existence of three gene subfamilies, and the phylogenetic position of *SlGA2ox1*, *-2*, *-3*, *-4*, and *-5* was established.

Fruit set has been defined as the changeover from the static condition of the flower ovary to the rapidly growing condition of the young fruit following ovary fertilization. In the case of tomato (*Solanum lycopersicum*), one of the most studied fleshy fruits, fruit growth takes place after fruit set in two consecutive phases: an active division, lasting about 7 to 10 DPA, and a cell expansion phase (Gillaspy et al., 1993). During the growth process the ovary wall develops into a pericarp composed of exocarp, mesocarp, and endocarp, while the placental parenchyma, supported by the columella, grows by division and expansion, enclosing the developing seeds and filling the locular cavities with a jelly-like homogenous tissue (locular tissue; Ho and Hewitt, 1986; Gillaspy et al., 1993).

GAs constitute a group of plant hormones that control developmental processes such as germination, shoot elongation, tuber formation, flowering, and fruit set, and growth in diverse species (Hedden and Kamiya, 1997; Olszewski et al., 2002). The metabolism of GA has been deeply investigated and is quite well understood (Sponsel and Hedden, 2004). In summary, *ent*-kaurene, synthesized from geranylgeranyl diphosphate by the action of two cyclases, is metabolized by the action of P450-dependent monoxygenases to GA_{12} and/or GA_{53}, which in turn are metabolized by GA 20-oxidases and GA 3-oxidases, acting consecutively, to active GAs through two parallel pathways: the non-13-hydroxylation (leading to GA_4) and the early 13-hydroxylation one (leading to GA_1 and GA_3 in some cases; Supplemental Fig. S1). Active GAs and their precursors can be irreversibly inactivated by GA 2-oxidases introducing a hydroxyl at the 2β position (Sponsel and Hedden, 2004). The existence of genes encoding GA deactivating enzymes catalyzing 16α,17-epoxidation in rice (*Oryza sativa*; Zhu et al., 2006) and formation of GA methyl esters in Arabidopsis (*Arabidopsis thaliana*; Varbanova et al., 2007) has been reported, although the importance of these reactions for GA homeostasis in other species is unknown. Most of the genes encoding all those enzymes have been cloned in many plant species (Hedden and Kamiya, 1997; Hedden and Phillips, 2000; Sponsel and Hedden, 2004) and their expression is regulated by endogenous and environmental factors (Yamaguchi and Kamiya, 2000; García-Martínez and Gil, 2002). GA 20-oxidases, GA 3-oxidases, and GA 2-oxidases are 2-oxoglutarate-dependent dioxygenases that have been found to be encoded by small gene families (e.g. in the case of Arabidopsis 5 *GA20ox*, 4 *GA3ox*, and 7 *GA2ox*), whose expression is temporarily and developmentally regulated (Hedden and Phillips, 2000). The *GA2ox* family is particularly complex since it is composed of two classes differing in their substrate specificity, C_{19}-GAs and C_{20}-GAs, respectively (Schomburg et al., 2003). In

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* Corresponding author; e-mail jlgarcim@ibmcp.upv.es.

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addition, some GA2ox enzymes using C_{19}-GAs as substrates have multicatalytic activity, converting the GAs successively to 2β-hydroxylated metabolites and to GA catabolites (Supplemental Fig. S1; Thomas et al., 1999; Ubeda-Tomás et al., 2006).

Analysis of GAs has shown that seeded fruits of tomato contain mainly GAs from the early 13-hydroxylation biosynthetic pathway (Bohner et al., 1988; Fos et al., 2000) and that pollination induces an increase of GA content in the ovary (Mapelli et al., 1978; Koshioka et al., 1994), suggesting that these hormones are involved in fruit set and growth of tomato. This hypothesis is supported by results of GA application experiments to unpollinated ovaries (Sjut and Bangerth, 1982, 1983; Alabadí and Carbonell, 1998; Fos et al., 2000, 2001) and of inhibitors of GA biosynthesis to pollinated ovaries (Fos et al., 2000, 2001). There is, however, no demonstration on the nature of the active GA, nor on the possible changes in GA metabolism affected by pollination in relation to fruit set and early fruit growth in tomato.

The tomato 'Micro-Tom' (Scott and Harbaugh, 1989) has been proposed as a convenient model system to carry out research on the hormonal regulation of berry fruit development due to its small size, rapid growth, and easy transformation (Meissner et al., 1997; Eyal and Levy, 2002; Dan et al., 2006). The phenotype of this cultivar is the result of mutations in the genes *Dwarf* (encoding 6-deoxocatasterone dehydrogenase, of the brassinosteroid biosynthesis pathway), *Self-Pruning* (which controls the determinate/indeterminate phenotype), and *Internode length reduction* (probably similar to *Miniature* still uncharacterized; Martí et al., 2006). The dwarf phenotype of 'Micro-Tom' is not the result of GA deficiency (Martí et al., 2006). It has been found that pollinated ovaries of 'Micro-Tom' develop into normal fruits and that unpollinated ovaries respond to GA_3 and auxin (but not to brassinosteroid) application (Serrani et al., 2007), showing that 'Micro-Tom' constitutes a good experimental system to investigate the role of hormones in fruit development.

In this work, using the tomato 'Micro-Tom', we have shown by application of different GAs and inhibitors of GA biosynthesis that tomato fruit set after pollination depends on GAs, and that GA_1 is the active form to induce fruit development. Pollination increased the expression of genes encoding GA20ox, but not of those encoding GA3ox, supporting the hypothesis that GA 20-oxidase activity is limiting in unpollinated ovaries. Five members of the *SlGA2ox* family have also been isolated to investigate the effect of pollination on expression of genes of GA catabolism. No decrease in transcript levels was found for any of these genes early after pollination (at day 5 after anthesis), indicating that fruit set may not be induced by regulation of GA inactivation. Phylogenetic analysis of genes encoding GA2ox indicates the existence of three subfamilies denoted I, II, and III, the new five *SlGA2ox* being clustered within groups I and II, constituted by enzymes using C_{19}-GAs as substrates.

RESULTS

Effect of Inhibitors of GA Biosynthesis on Growth of Pollinated Fruits

To investigate whether the development of pollinated fruits depends on GAs, two different kinds of inhibitors of GA biosynthesis were used: LAB 198999, an acylcyclohexanedione derivative which inhibits 2-oxoglutarate-dependent dioxygenases (Santes and García-Martínez, 1995), was applied to pollinated ovaries, and paclobutrazol, an inhibitor of P450-dependent monooxygenases (Hedden and Graebe, 1985), to the roots in the nutrient solution. In the case of LAB 198999, direct application to the ovaries was carried out 2 d after pollination, after removing stamen and petals, to facilitate absorption. This inhibitor was applied at that time because earlier application might prevent pollen germination or fertilization. It was shown previously that removal of those organs 2 d after pollination did not reduce the number of seeds per fruit nor the final fruit weight (Fig. 1A). Paclobutrazol was applied to the roots because direct treatment of pollinated ovaries the day equivalent to anthesis or later was not efficient. Paclobutrazol application was started when flowers on which the effect of the inhibitor was going to be determined were about 7 d before anthesis (estimated by flower bud size) to ascertain that it was transported in time to the pollinated ovary.

LAB 198999 application (0.3–10 mM) reduced the weight of the fruit, an effect which was reversed by exogenous GA_3. At the highest doses of inhibitor (3 and 10 mM) fruit set was also reduced, but could not be recovered by GA_3 (Fig. 1B), probably due to nonspecific toxic effect of the inhibitor (necrotic spots appeared on the surface of the ovary) at those doses. In the case of paclobutrazol application, both fruit set and final fruit size decreased proportionally to the dose of inhibitor and at 10^{-2} M fruit set was 0% (Fig. 1C). This inhibition was fully reverted with GA_3 application (Fig. 1C). Vegetative growth of plants treated with LAB 198999 was not affected (due probably to direct ovary application) and in the case of paclobutrazol the apical shoot length was only slightly reduced (due probably to application after flowering time, when most vegetative growth had already occurred). Interestingly, both kinds of inhibitors did not prevent the development of seeds in developed fruits (data not presented).

Effect of Inhibitors of GA Biosynthesis on GA Content of Pollinated Fruits

To assess the effect of modification of endogenous GA content in relation to early fruit development, GAs from the early 13-hydroxylation pathway were quantified in 10-d-old pollinated ovaries control or treated with 1 mM LAB 198999 (dose of inhibitor at which the effects are fully reverted by applied GA_3; Fig. 1B). At that time, the weight of LAB 198999 treated ovaries was about half of control (Table I). This weight reduction was associated with significantly lower concentration

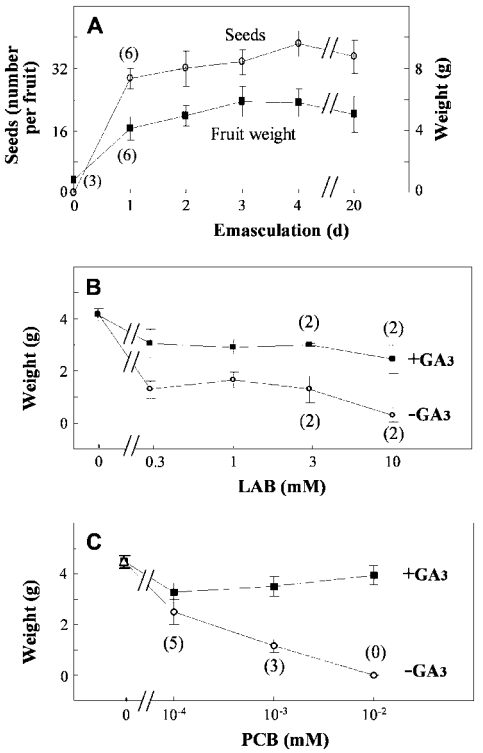

Figure 1. Fruit set and growth inhibition of pollinated ovaries with inhibitors of GA biosynthesis and its reversal by GA_3 application. A, Effect of time of emasculation and removal of petals, anthers, and style on number of seeds and fruit growth of pollinated ovaries (at day 0). B, Effect of different doses of LAB 198999. C, Effect of different doses of paclobutrazol (PCB). Pollination was carried out at day 0. LAB 198999 was applied directly to the ovary in 10 μL solution, 2 d after anthesis, after emasculation and petal removal. Paclobutrazol was applied to the roots in the nutrient solution, every 2 d, from 7 d before anthesis to 15 d after anthesis. GA_3 (2,000 ng) was applied to the ovary in 10 μL solution at anthesis. Fruits were collected 20 d after treatment. Values are data from eight fruits ±SE. One-hundred percent of fruits developed in all treatments, except those marked with figures in parentheses (number of fruits developed over eight treated).

(about half) of GA_1 (the active GA), of its metabolite GA_8 (about one tenth), and of GA_{29} (a metabolite of GA_{20}, more than half; Table I). In contrast, LAB 198999 produced accumulation of all precursors of GA_1 (GA_{53}, GA_{44}, GA_{19}, and GA_{20}; Table I). These results strongly support that fruit development in tomato depends on GAs, and specifically on GA_1.

Response of Unpollinated Ovaries to Application of Different Kinds of GAs

Diverse GAs from the early 13-hydroxylation pathway (GA_1, GA_3, GA_{19}, and GA_{20}) and GA_4 (from the non-13-hydroxylation pathway) were tested for their activity to induce fruit set and growth of unpollinated ovaries. As in many other systems, GA_3 was the most active followed by GA_1 and GA_4 (equally active), and GA_{20}. Interestingly, GA_{19} (the immediate metabolic precursor of GA_{20}) was completely inactive (Fig. 2). These results suggested that GA 20-oxidase activity is limiting in unpollinated ovaries.

Effect of Pollination on Transcript Levels of Genes Encoding Enzymes of GA Biosynthesis

To test the last hypothesis we compared in unpollinated and pollinated ovaries transcript levels of *SlCPS*, *SlGA20ox1*, *-2*, and *-3* and *SlGA3ox1* and *-2*, genes previously cloned by Rebers et al. (1999) that encode three kinds of GA biosynthesis enzymes. The expression of those genes in diverse tomato organs is given in Figure 3. All the genes were expressed in aerial vegetative (leaves and internodes) and reproductive (flowers and their diverse parts) tissues. In roots we could only detect transcripts of *SlGA20ox3* and *SlGA3ox1*. Transcripts of *SlCPS*, *GA20ox3*, and *SlGA3ox1* and *-2* were detected in ovaries of flowers at anthesis, and transcripts of all the analyzed genes, except of *SlGA3ox2* also in pollinated 20-d-old fruits.

Expression of *SlCPS* was detected in unpollinated ovaries before anthesis (day 3) but decreased later on (from day 0 to 20 DPA; Fig. 4). In contrast, in entire (E) pollinated ovaries *SlCPS* transcript levels did not decrease and remained similar or higher than unpollinated ovaries before anthesis. Transcripts were present both in pericarp and developing seeds, more in the latter than in the former (Fig. 4).

Almost undetectable expression of *SlGA20ox1* was found in unpollinated ovaries (between −3 and 20 DPA). In the case of *SlGA20ox2*, high expression was detected before anthesis (day 3), but dropped to undetected or very low in unpollinated ovaries between day 0 and day 20 (Fig. 4). Interestingly, transcript levels of both *SlGA20ox1* and *-2* were very high in entire pollinated ovaries (5–20 DPA; at least 10-fold those of unpollinated ovaries). Transcript content could also be analyzed separately in pericarp and seeds of 10- and 20-d-old fruits. Transcripts were equally distributed in the pericarp and seeds at day 10, but were much more concentrated in seeds at day 20 (Fig. 4). *SlGA20ox3* transcripts could be clearly detected and their levels did not vary in unpollinated ovaries (from day 3 to day 20). Interestingly, they increased also (about twice) in pollinated ovaries, particularly in developing seeds at day 20 (Fig. 4).

SlGA3ox1 transcript content was high in unpollinated ovaries before anthesis (day 3) and decreased from anthesis until day 20. Similar levels were found in unpollinated and pollinated ovaries until day 20 (Fig. 4). At day 10 and day 20 transcripts were concentrated in developing seeds (Fig. 4). In contrast, transcripts of *SlGA3ox2*, detected in ovaries before anthesis, were at very low levels or not detected in unpollinated ovaries

Table I. *Effect of LAB 198999 on weight and endogenous GA content (ng g fresh weight^{-1}) of pollinated fruits*

Fruits were collected 10 d after pollination (8 and a half days after 1 mM LAB 198999 application). Fruit weight data are means of 26 (−LAB) and 31 (+LAB) fruits, and GA data from three biological replicates (aliquots of about 5 g each) ±SE.

	Weight	GA_1	GA_8	GA_{19}	GA_{20}	GA_{29}	GA_{44}	GA_{53}
	g fruit^{-1}							
−LAB	1.04 ± 0.06	2.7 ± 0.8	31.4 ± 0.3	8.7 ± 0.4	23.5 ± 0.6	18.5 ± 2.6	2.7 ± 0.1	<0.1
+LAB	0.47 ± 0.04	1.2 ± 0.0	3.3 ± 0.9	30.5 ± 0.9	50.5 ± 6.1	7.0 ± 1.2	3.4 ± 0.2	3.1 ± 1.6

after anthesis. In day 10 and day 20 pollinated ovaries *SlGA3ox2* transcripts were barely detected and present mainly in the seeds (Fig. 4).

Cloning and Characterization of Genes Encoding Enzymes of GA Inactivation in Tomato

At the time of starting this work no GA 2-oxidase had been cloned in tomato, to our knowledge. Therefore, to know whether pollination increased active GA content by also altering GA inactivation, we isolated genes encoding GA2ox. Using reverse transcription (RT)-PCR and degenerated primers, followed by 5′ and 3′ RACE only one full-length cDNA clone could be isolated (*SlGA2ox1*, EF441351; see ''Materials and Methods''). This cDNA was 1,281 bp long (including 88 and 143 bp in the 5′and 3′ untranslated regions, respectively) and encoded a protein of 349 amino acids.

Using BLAST search of EST databases we identified 18 sequences with high similarity to *SlGA2ox1* and *GA2ox* from other species, which corresponded apparently to four additional different incomplete genes (gene 2, AW930043, BI935635, AW222239, BE434782, BE433301, BE435345; gene 3, AW030357, AI777086, BI921857, AW031637; gene 4, BI208568, AW931003, AW030225; and gene 5, AI899222, AI487548, AI488712, AW650238, AW650160). Full-length clones of these genes were isolated by 5′ and 3′ RACE, amplified, and named accordingly *SlGA2ox2* (EF441352; 322 amino acids long), *SlGA2ox3* (EF441353; 344 amino acids long), *SlGA2ox4* (EF441354; 341 amino acids long), and *SlGA2ox5* (EF441355; 346 amino acids long). Recently, the sequence of a clone similar to our *SlGA2ox2* (EF017805) was also submitted to GeneBank.

A phylogenetic analysis was carried out with the sequences of all published GA dioxygenase genes from tomato and those of the Arabidopsis genome, including the five putative GA2ox genes isolated in this work, previously published sequences of tomato GA20ox (three genes) and GA3ox (two genes), plus all sequences encoding GA dioxygenases (five *GA20ox*, four *GA3ox*, and seven *GA2ox*) in Arabidopis. Four groups corresponding to GA20ox, GA3ox, GA2ox using C_{19}-GAs as substrate, and GA2ox using C_{20}-GAs as substrate were found. The five *SlGA2ox* genes from tomato clustered with the group of GA2ox of Arabidopsis using C_{19}-GAs as substrate, suggesting that all of them encode this kind of enzymes (Supplemental Fig. S2).

After subcloning the five *SlGA2ox* genes in the expression vector pET45b, the activity of the expressed proteins was analyzed using [^{14}C]GA_1, [^{14}C]GA_4, [^{14}C]GA_9, [^{14}C]GA_{12}, [^{14}C]GA_{20}, and [^{14}C]GA_{53} as substrates. Separation of radioactive metabolites by HPLC showed that extracts from *SlGA2ox1* metabolized [^{14}C]GA_1 and [^{14}C]GA_4 to compounds with the same retention times as [^{14}C]GA_8 and [^{14}C]GA_{34}, respectively; those from *SlGA2ox3* metabolized [^{14}C]GA_1, [^{14}C]GA_4, and [^{14}C]GA_9 to compounds with the same retention times as [^{14}C]GA_8, [^{14}C]GA_{34}, and [^{14}C]GA_{51}, respectively, and those from *SlGA2ox4* metabolized [^{14}C]GA_1, [^{14}C]GA_4, [^{14}C]GA_9, and [^{14}C]GA_{20} to compounds with the same retention time as [^{14}C]GA_8, [^{14}C]GA_{34}, [^{14}C]GA_{51}, and [^{14}C]GA_{29} (Supplemental Fig. S3). Activity of *SlGA2ox5* extracts was very poor and only small peaks corresponding to putative [^{14}C]GA_{34} and [^{14}C]GA_{51} were found using [^{14}C]GA_4 and [^{14}C]GA_9 substrates, respectively (Supplemental Fig. S3). [^{14}C]GA_{12} and [^{14}C]GA_{53} were not metabolized in any case (Supplemental Fig. S3), confirming that *SlGA2ox1*, *-3*, *-4*, and *-5* encoded C_{19} GA 2-oxidases. Expressed extracts from *SlGA2ox2* did not metabolize any of the six labeled GAs used as substrates (data not presented), suggesting that the corresponding protein was probably inactive.

Phylogenetic Analysis of GA 2-Oxidases

To better locate the new *SlGA2ox* genes within the large *GA2ox* family, a phylogenetic analysis was performed with all of the full-length *GA2ox* genes found

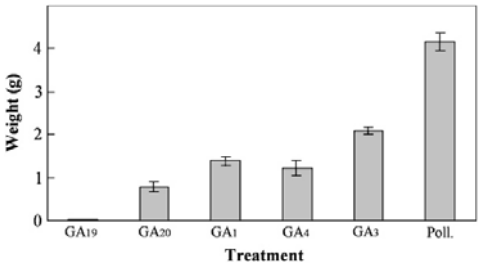

Figure 2. Response of unpollinated tomato ovaries to GA_1, GA_3, GA_4, GA_{19}, and GA_{20} (2,000 ng per ovary) application. Fruits were collected 20 d after treatment and values are means of eight fruits ±SE. Values of pollinated ovaries are also included as control. Poll., Pollinated.

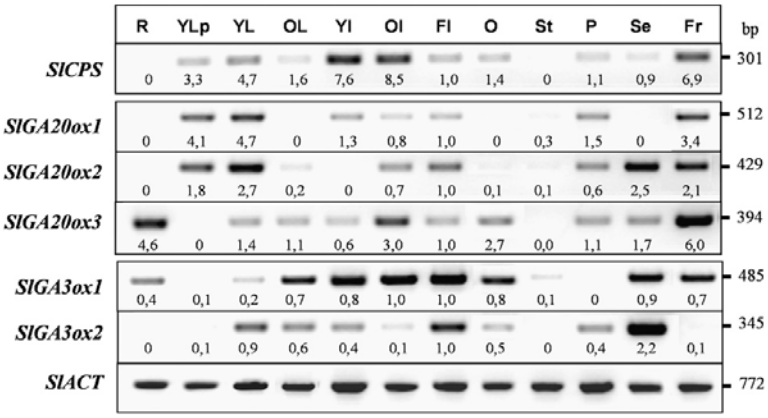

Figure 3. Distribution of transcript levels of *SlCPS*, *SlGA20ox1*, *-2*, and *-3*, and *SlGA3ox1* and *-2* in different organs of tomato. Semiquantitative transcript analysis was carried out by RT-PCR, as described in "Materials and Methods," using total RNA from roots (R), young leaves before flowering (YLp), young and old leaves from plants at flowering (YL, OL), young and old internodes (YI, OI), flowers (Fl), ovary at anthesis (O), stamens (St), sepals (Se), petals (Pe), and 20-d-old fruit (Fr). For each gene, figures below the blots mean normalized values of gene expression versus that of *Actin* (used as an internal control; flower expression set at 1.0). Data come from a representative experiment out of two biological replicates with similar results.

in the databases, using the outgroup sequence AtGA20ox1 to position the root of the tree. The analysis showed the existence of three large subfamilies of GA2ox (Fig. 5): groups I and II correspond to GA2ox using C_{19}-GAs as substrate (the occurrence of these two groups was pointed out earlier by Elliott et al., 2001), and group III corresponds to GA2ox using C_{20}-GAs as substrate. According to this phylogenetic tree, OsGA2ox5 and -6, and NsGA2ox1, for which catalytic properties have not been reported yet, would use C_{20}-GAs as substrates. These subfamilies are similar to those described by Lee and Zeevaart (2005) in a previous analysis carried out with a selected number of sequences (20 versus 44 in this work). The topology of the root tree indicates that groups I and II are more closely related to each other than to group III. In other words, these data suggest that group III diverged from all other GA 2-dioxygenase genes before the split between groups I and II. Both monocot and dicot genes are present in each of the three groups, indicating that the gene duplication events that gave rise to these three subfamilies occurred before the split between monocots and dicots.

Interestingly, the seven GA2ox reported in the literature as having multicatalytic activity were located in group I (underlined in Fig. 5; see also appropriate references in Fig. 5 legend). Certainly, not all GA2ox present in this group have been shown to be multicatalytic. Absence of annotation of this biochemical property in enzymes of group I may be due to: (1) the catalytic properties have not been investigated in these enzymes; (2) catabolite formation may have not been

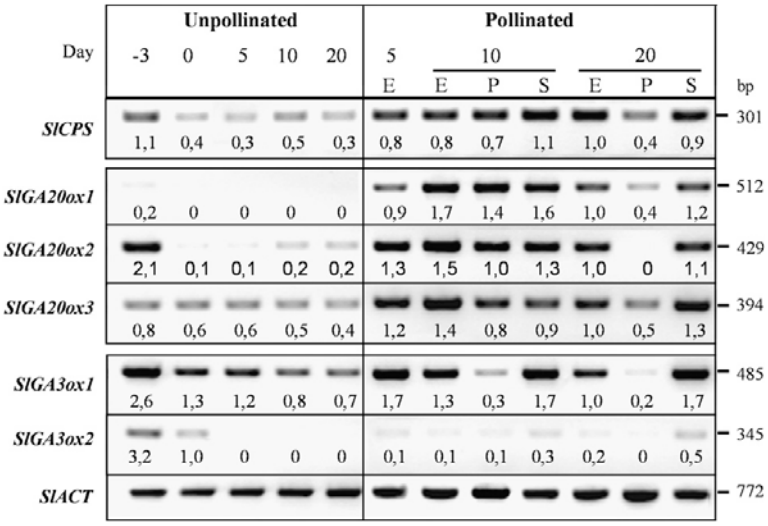

Figure 4. Effect of pollination on transcript levels of *SlCPS*, *SlGA20ox1*, *-2*, and *-3*, and *SlGA3ox1* and *-2* genes. Semiquantitative transcript analysis was carried out by RT-PCR, as described in "Materials and Methods," using total RNA from unpollinated (days 0, 5, 10, and 20) and pollinated (day 5, 10, and 20) ovaries. E, Entire ovary; P, pericarp; S, seeds. For each gene, figures below the blots mean normalized values of gene expression versus that of *Actin* (used as an internal control; expression of entire 20-d-old pollinated fruits set at 1.0 for all the genes but for *SlGA3ox2*, where expression of day 0 unpollinated ovaries was used as reference). Data come from a representative experiment out of two biological replicates with similar results.

detected since it depends strongly on enzyme concentration and is adversely affected by dilution (Martin et al., 1999).

Amino acid sequence comparison of all GA2ox enzymes used to construct the phylogenetic tree of Figure 5 is given in Supplemental Figure S4. Interestingly, groups I and II differ in at least two specific amino acids at conserved regions that might be related to their possible different catalytic properties. For instance, within the sequence (N/T/S)GDXG(W/R/E/D/H)X(L/V/I)E(Y/H)(L/I)L (located between positions 90 and 100 of AtGA2ox1) the W present in all the sequences of group I (except in SlGA2ox2 that has an R) is substituted by a D/E in all the sequences of group II (except in VaGA2oxB3 that has an H). Also, within the sequence (Y/F)XX(F/L)(T/K)(W/R)X(E/D/Q)(Y/F)K (located between positions 294 and 303 of AtGA2ox1), the E present in all the sequences of group I (at position 296 of AtGA2ox1) is substituted by diverse nonacidic amino acids in all the enzymes of group II. According to these predictions (see Fig. 5; Supplemental Fig. S4) of the five genes isolated in this work, SlGA2ox1 and -3 would be monocatalytic (confirmed in this work) and SlGA2ox2, -4, and -5 would be multicatalytic (a prediction that we were unable to confirm; possible reasons for the absence of this kind of activity are given in the ''Discussion'').

Effect of Pollination of Transcript Levels of Genes Encoding Enzymes of GA Inactivation in Tomato

Distribution of *SlGA2ox1* to *-5* transcripts in diverse tomato organs is presented in Figure 6. *SlGA2ox1* was expressed only in ovaries at anthesis and developing pollinated fruits. The other four genes were expressed to different extents in leaves (young and old), internodes (young and adult), and flowers at anthesis. In the roots we could only detect transcripts of *SlGA2ox3*, *-4*, and *-5*. In flowers at anthesis, *SlGA2ox2* transcripts were present in all the organs (ovary, stamens, petals, and sepals), *SlGA2ox3* mainly in petals and sepals, *SlGA2ox4* in ovary, petals, and sepals, and those of *SlGA2ox5* only in ovaries. Developing 20-d-old fruits contained transcripts of all *GA2ox* genes, except *SlGA2ox3*.

The effect of pollination on expression of *SlGA2ox1* to *-5* is shown in Figure 7. In unpollinated ovaries transcripts of all genes were present before or at the time of anthesis (day 3 and day 0). In unpollinated ovaries expression of all *SlGA2ox* remained high later on, except for *SlGA2ox3* whose transcripts were at very low levels or undetected between day 0 and day 20 (in agreement with results presented in Fig. 6). In 5-d-old pollinated ovaries (a time at which fruit set and some growth had occurred already) transcript levels of the five *SlGA2ox* genes were similar to those of unpollinated ovaries. In contrast, in 10- and 20-d- old pollinated ovaries transcript levels of all *SlGA2ox* were lower than in unpollinated ovaries, particularly in the case of *SlGA2ox2* and -3 (in the latter case transcripts were barely detected). An exception was *SlGA2ox1* at day 10 where transcript levels were not reduced. Pericarp and seeds could be separated in 10- and 20-d-old fruits and therefore *GA2ox* transcript content were also analyzed in both organs at those times. *SlGA2ox1* was always highly expressed in the pericarp and in seeds at day 10. In contrast, *SlGA2ox4* and *-5* were expressed mostly in the developing seeds and therefore they may not contribute to GA homeostasis in the pericarp.

DISCUSSION

Fruit set and fruit growth of pollinated 'Micro-Tom' ovaries was reduced significantly, on a dose-effect response, by application of paclobutrazol, an inhibitor of GA biosynthesis that inhibits P450-dependent dioxygenases. The effect of paclobutrazol was fully counteracted by applied GA_3 (Fig. 1C). LAB 198999, another inhibitor of GA biosynthesis that inhibits 2-oxoglutarate-dependent dioxygenases, also reduced fruit set and fruit growth, but the former effect could not be reverted by GA application (Fig. 1B), probably due to nonspecific toxic effect. These results support the hypothesis that tomato fruit development depends on GAs, as suggested previously (Fos et al., 2000, 2001).

The reduction of fruit growth (about 50%) by LAB 198999 was associated with a reduction of GA_1 content to about 50% whereas GA_8 content was reduced to 10% (Table I). At the same time, in LAB 198999 treated fruits there was accumulation of GA_{53}, GA_{44}, GA_{19}, and GA_{20} (Table I). Since the early 13-hydroxylation is the main GA metabolic pathway in tomato (Bohner et al., 1988; Koshioka et al., 1994; Fos et al., 2000, 2001) this means: (1) that GA_1 is the main active GA in tomato fruit development; (2) that the precursors of GA_1 are not active per se but only after conversion to this active hormone. GA_1 has been shown to be the active GA in shoot growth of many species such as pea (*Pisum sativum*; Ingram et al., 1984), lettuce (*Lactuca sativa*; Waycott et al., 1991), rice (Fujioka et al., 1988), spinach (*Spinacia oleracea*; Zeevaart et al., 1993), and *Salix* (Olsen et al., 1995). In contrast, GA_4 is the main active hormone in others species like cucumber (*Cucumis sativus*; Nakayama et al., 1991) and Arabidopsis (Cowling et al., 1998). Application of GA_4 is certainly capable of inducing tomato fruit development also (Fig. 2), but this hormone may have a minor physiological role because the non-13-hydroxylation pathway seems to be minor in this species. GA_{20} and GA_1 were almost equally active to induce parthenocarpic fruit growth in tomato, while GA_{19} was completely inactive (Fig. 2). This suggests that unpollinated ovaries are capable of metabolizing GA_{20} but not GA_{19} to GA_1 and, therefore, that the activity of GA 20-oxidase (that metabolizes GA_{19} to GA_{20}) but not that of GA 3-oxidase (that metabolizes GA_{20} to GA_1) is limiting in unpollinated tomato ovaries. Interestingly, in *pat-2*, a facultative parthenocarpic mutant of tomato, parthenocarpy is

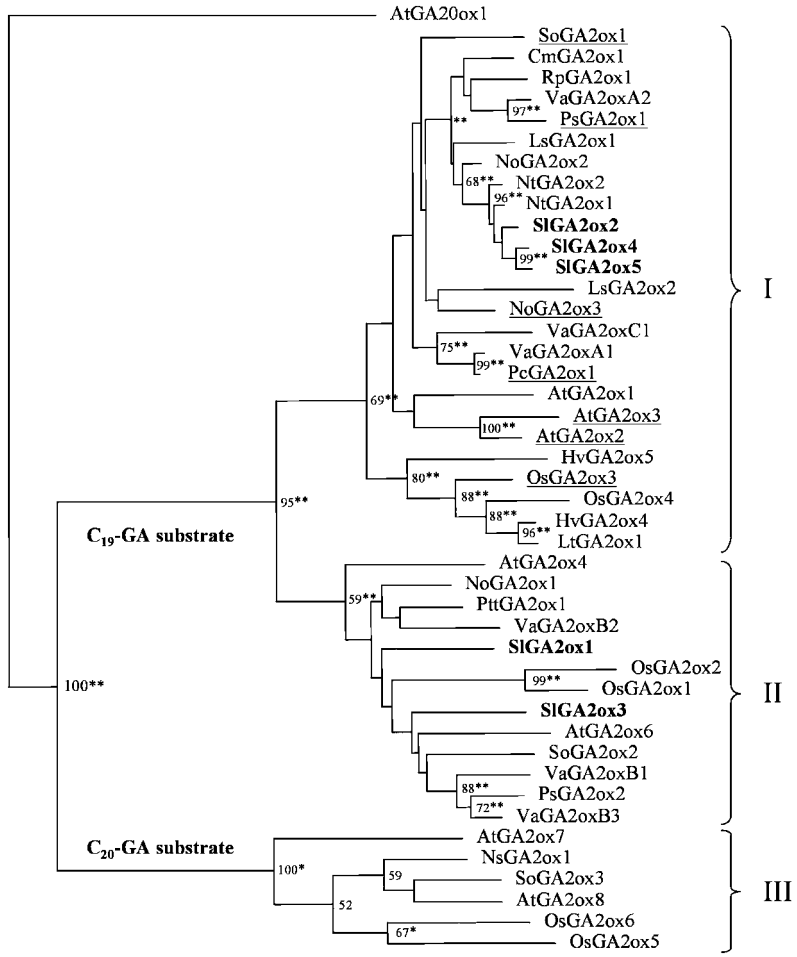

Figure 5. Maximum-likelihood phylogenetic tree based on comparison of GA2ox protein sequences from different species. The tree was rooted using AtGA20ox1 as outgroup and branch lengths are proportional to the estimated sequence divergence. Bootstrap values above 50% are shown, whereas asterisks indicate statistical significance according to the weighted least-squares likelihood ration test (**, $P < 0.01$; *, $P < 0.05$). The three *GA2ox* subfamilies I, II, and II are indicated, and genes that have been shown to codify for multicatalytic enzymes are underlined. The five genes characterized in this study are shown in bold type. Accession numbers corresponding to the sequences in the tree are the following: AtGA20ox1, X83379; AtGA2ox1, AJ132435; AtGA2ox2, AJ132436 (Thomas et al., 1999); AtGA2ox3, AJ322437 (Thomas et al., 1999); AtGA2ox4, NM103695; AtGA2ox6, NM100121; AtGA2ox7, AC079284; AtGA2ox8, AL021960; CmGA2ox1, AJ315663; HvGA2ox4, AY551432; HvGA2ox5, AY551433; Ls2ox1, AB031206; Ls2ox2, AB031207; LtGA2ox1, DQ324114; NoGA2ox1, AY594291; NoGA2ox2, AY594292; NoGA2ox3, AY588978 (Ubeda-Tomás et al., 2006); NsGA2ox1, Ay242858; NtGA2ox1, AB125232; NtGA2ox2, AB125233; OsGA2ox1, AB059416; OsGA2ox2, AB092484; OsGA2ox3, AB092485 (Sakai et al., 2003); OsGA2ox4, AC132485; OsGA2ox5, BAC10398; OsGA2ox6, AL662958; PcGA2ox1, AJ132438 (Thomas et al., 1999); PsGA2ox1, AF056935 (Martin et al., 1999); PsGA2ox2, AF100954; PttGA2ox1, AY392094; RpGA2ox1, DQ641499; SlGA2ox1, EF441351; SlGA2ox2, EF441352; SlGA2ox3, EF441353; SlGA2ox4, EF441354; SlGA2ox5, EF441355; SoGA2ox1, AF506281 (Lee and Zeevaart, 2002); SoGA2ox2, AF506282; SoGA2ox3, AY935713; VaGA2oxA1, AB181372; VaGA2oxA2, AB181373; VaGA2oxB1, AB181374; VaGA2oxB2, AB181375; VaGA2oxB3, AB181376; VaGA2oxC1, AB181377.

associated with a dramatic increase of GA_{20} and more GA_1 and GA_8 contents (Fos et al., 2000), due probably to enhanced activity of GA 20-oxidase.

The above-mentioned hypothesis was supported by results of comparing the effect of pollination on transcript levels of diverse *SlGA20ox* and *SlGA3ox* genes of tomato previously isolated by Rebers et al. (1999). *SlGA3ox2* transcripts were almost undetected in unpollinated and pollinated ovaries, whereas *SlGA3ox1* transcripts were present in unpollinated ovaries at day 0 and remained essentially constant in both unpollinated and pollinated ovaries at least until day 20 (Fig. 4). This supports the idea that GA 3-oxidase activity (encoded from *SlGA3ox1*) is present in ovaries before pollination and that pollination does not alter that activity. In contrast, *SlGA20ox1* and *-2* transcripts were at very low levels or undetected at day 0 and in 5- to 20-d-old unpollinated ovaries, but at high levels in 5- to 20-d-old pollinated ovaries. Transcript levels of *SlGA20ox3*, which were present in unpollinated ovaries, also increased upon pollination (Fig. 4). This suggests that GA 20-oxidase activity increases upon pollination, as indicated by previous GA application experiments (Fig. 2). However, we cannot decide, based on our data, whether the three *SlGA20ox* are or not equally important for fruit development regulation because transcripts of all of them were similarly distributed in the pericarp and seeds, at least until day 10 (Fig. 4). In any case, our results do not support a role for GA 3-oxidase activity for fruit development and are in contrast with the suggestions of Bohner et al. (1988) and Koshioka et al. (1994) based on endogenous GA content analyses that 3β-hydroxylation of GA_{20} is a rate-limiting step in GA_1 biosynthesis after pollination in tomato.

Since transcript levels of *SlCPS* were higher in pollinated than in unpollinated ovaries, activity of earlier biosynthetic enzymes (e.g. copalyldiphosphate synthase [CPS]) might also contribute to the increase of GA content after pollination. CPS (formerly *ent*-kaurene synthetase A) activity is certainly present in extracts of tomato fruits (Bensen and Zeevaart, 1990). Arabidopsis CPS transcripts occur in actively growing tissues, particularly in developing flowers and seeds (Silverstone et al., 1997), and expression of *PsCPS* (locus *LS*) seems to play an important role on the regulation of GA biosynthesis in relation to seed development in pea (Ait-Ali et al., 1997). In contrast, overexpression of *AtCPS* in Arabidopsis, although increasing *ent*-kaurene production did not result in increase of active GAs (Fleet et al., 2003). Rebers et al. (1999) found that the expression of all the GA biosynthetic genes analyzed in this work (*SlCPS*, *SlGA20ox*, and *SlGA3ox*) change during flower bud development in tomato, with different patterns of mRNA accumulation, indicating a complex regulatory mechanism for controlling GA biosynthesis during flower development. However, no comparison of transcript levels in unpollinated and pollinated tomato ovaries was carried out. GA metabolism during fruit set and growth has also been investigated in pea. In this case, the increase of GA content upon pollination (Rodrigo et al., 1997) is also associated with an increase of *PsGA20ox1* expression (Van Huizen et al., 1997). But in contrast to tomato, the presence of seeds seems also to up-regulate the expression of a *GA3ox* (*PsGA3ox1*; Ozga et al., 2003).

GA levels are a result of GA biosynthesis and inactivation (Hedden and Phillips, 2000). Therefore, modification of active GA levels may be due to simultaneous transcription alteration of genes encoding GA biosynthesis (e.g. GA20ox and/or GA3ox) and GA inactivating enzymes (GA2ox, GA epoxidases, and GA methyltransferases [GAMTs]). For instance, GA_1

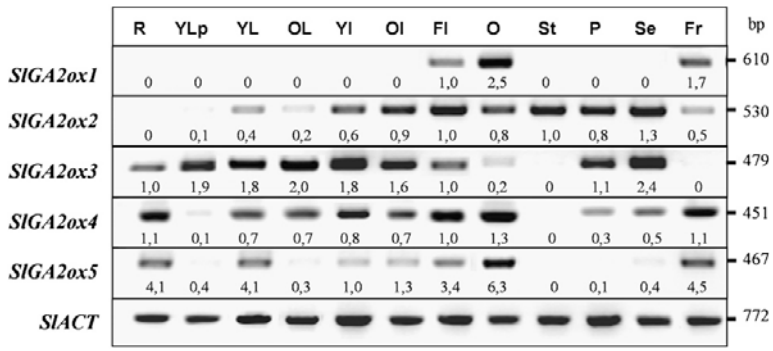

Figure 6. Distribution of transcript levels of *SlGA2ox1*, *-2*, *-3*, *-4*, and *-5* in different organs of tomato. Semiquantitative transcript analysis was carried out by RT-PCR, as described in "Materials and Methods," using total RNA from roots (R), young leaves before flowering (YLp), young and old leaves from flowering plants (YL, OL), young and old internodes (YI, OI), flowers (Fl), ovary at anthesis (O), stamens (St), sepals (Se), petals (Pe), and 20-d-old fruit (Fr). For each gene, figures below the blots mean normalized values of gene expression versus that of *Actin* (used as an internal control; flower expression set at 1.0 for all the genes, except for *SlGA2ox5*, where expression of YI was used as reference). Data come from a representative experiment out of two biological replicates with similar results.

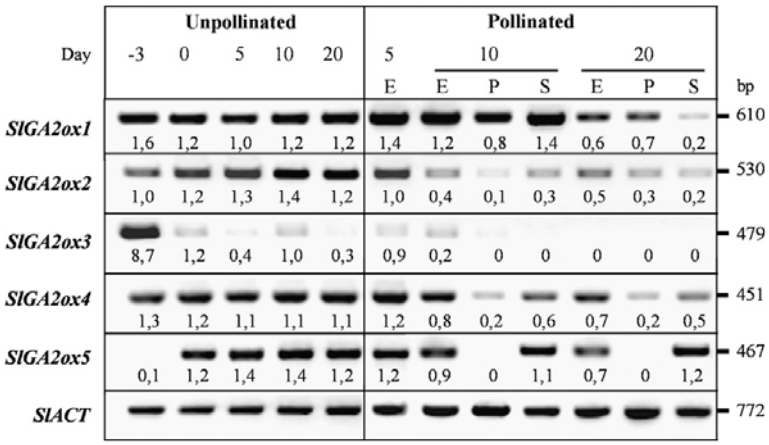

Figure 7. Effect of pollination on transcript levels of *SlGA2ox1*, *-2*, *-3*, *-4*, and *-5* genes. Semiquantitative transcript analysis was carried out by RT-PCR, as described in "Materials and Methods," using total RNA from unpollinated (days 0, 5, 10, and 20) and pollinated (days 5, 10, and 20) ovaries. E, Entire ovary; P, pericarp; S, seeds. For each gene, figures below the blots mean normalized values of gene expression versus that of *Actin* (used as an internal control; expression of unpollinated day 5 and 10 ovaries set at 1.0 for *SlGA2ox1*, *-3*, and *-4*, of pollinated day 5 ovaries for *SlGA2ox2*, and seeds from day 10 pollinated ovaries for *SlGA2ox5*). Data come from a representative experiment out of two biological replicates with similar results.

content decrease in the shoot during deetiolation in pea is due to down-regulation of *PsGA3ox1*, which controls the conversion of GA_{20} to GA_1, and by up-regulation of *PsGA2ox2*, encoding a GA2ox that converts GA_1 to inactive GA_8 (Symons and Reid, 2003). Developing siliques of null mutants of *GAMT1* and *GAMT2* have higher GA_1 and GA_4 contents and their seeds are more resistant to ancymidol, suggesting that they also contain more active GAs (Varbanova et al., 2007). Since GA2ox are generally considered the main GA inactivating enzymes, to know whether the increase of GA_1 upon pollination in tomato ovary is not only due to enhanced GA biosynthesis (through increase of *GA20ox* transcript levels, and may be *SlCPS*, as shown before), but also to reduction of catabolic activity, five cDNA clones encoding putative GA 2-oxidases from tomato (*SlGA2ox1* to *-5*) were isolated. *SlGA2ox1*, *-3*, and *-4* and *-5* to a lesser extent were shown to encode active C_{19} GA2ox using different kinds of GAs as substrates (Supplemental Fig. S3). Expressed *SlGA2ox2* extracts did not show activity with any of the six GAs used as substrate, suggesting that the corresponding protein was inactive in spite of carrying the purported amino acids binding Fe^{2+} and 2-oxoglutarae, and essentially all the amino acids conserved in GA2ox (Supplemental Fig. S4). A reason for SlGA2ox2 inactivity might be the presence of a mutation leading to the change of W (conserved in all GA2ox from group I) by an R at position 92 (Supplemental Fig. S4). This observation points out the possible importance this W residue has for GA2ox activity. Additionally, SlGA2ox2, -4, and -5 have a D at a site (position 44 of SlGA2ox2) where most GA2ox have a conserved G (Supplemental Fig. S4), which might also affect their activity.

Transcripts of the five *SlGA2ox* genes were detected in different tissues (Fig. 6), suggesting that their expression is developmentally regulated. All of them were expressed in unpollinated ovaries before and/or at the time of anthesis and also up to day 20 in unpollinated ovaries, at more or less extent. However, no decrease of expression was observed in any of the *SlGA2ox* genes in pollinated ovaries 5 d after anthesis, a time at which fruit set has already been established, as shown by the observation that a significant growth had occurred. This means that the effect of pollination on early fruit development may not be mediated by an effect on GA inactivation through GA2ox. However, we cannot discard a possible effect of GA2ox on later growth of tomato fruit (because transcripts of all *SlGA2ox* genes were lower in pollinated than in pollinated ovaries at day 10 and/or day 20), nor a possible role of other GA catabolic enzymes (e.g. GA epoxidases and GAMT) in GA homeostasis during fruit set and growth.

The phylogenetic analysis of GA2ox, using all the sequences available in data bank and AtGA20ox1 as outgroup (Fig. 5), indicates that a first split occurred between enzymes using C_{20}-GAs as substrate (group III) and those using C_{19}-GAs, and that divergence between groups I and II occurred more recently. The five *SlGA2ox* genes isolated in this work were distributed between groups I and II, and therefore, according to this prediction, should differ in their catalytic properties. While SlGA2ox1 and -3 presented monocatalytic activity, as expected, no multicatalytic activity

could be demonstrated for SlGA2ox2, -4, and -5 (expressed *SlGA2ox2* was completely inactive). Therefore, our results do not support the proposed hypothesis. However, since the three translated sequences of SlGA2ox2, -4, and -5 present changes in specific conserved amino acids that might affect activity, and it has been reported that detection of GA catabolites may be difficult and dependent on enzyme concentration (Martin et al., 1999), it may not be possible to completely discard that hypothesis before carrying out more biochemical work to substantiate it. Monocot and dicot genes are both present in each of the three groups, indicating that gene subfamilies I, II, and III were originated from gene duplications early in evolution. Finally, additional gene duplications occurred within each of the groups I and II as indicated by the presence of several duplicates of Arabidopsis and other species in those groups, whereas no further duplication seem to have occurred within the more ancestral group III (Fig. 5). Altogether, the data support the general hypothesis that acquisition of evolutionarily novel functions among GA dioxygenases is associated with gene duplication events, as previously shown for other gene families (Sanjuan and Marin, 2001).

The results of experiments of GA and inhibitors of GA biosynthesis application presented here, as well as of GA quantification analysis support the hypothesis that fruit set and early growth in tomato depend on GAs and that GA_1 is the active hormone involved in these processes. Pollination increases the content of GAs in the ovary by increasing GA biosynthesis (through up-regulating *GA20ox* and *SlCPS*, but not *GA3ox* expression), not by reducing GA catabolic inactivation through GA2ox.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Plants of tomato (*Solanum lycopersicum*) 'Micro-Tom' (seeds obtained originally from Dr. A. Levy) were used in the experiments. Plants (one per pot) were grown in 1 L pots with a mixture of peat:vermiculite (1:1), cultured in a greenhouse under 24°C (day)/20°C (night) conditions, and irrigated daily with Hoagland solution. Natural light was supplemented with Osram lamps (Powerstar HQI-BT, 400W) to get a 16 h light photoperiod.

Only one flower per truss, and the first two trusses were left per plant to prevent interaction between fruits at the same truss (Serrani et al., 2007).

Plant Hormone Applications

Application of GAs (GA_1, GA_4, GA_{19}, and GA_{20}, obtained from Prof. L. Mander, Australian National University, Canberra, Australia) and GA_3 (Duchefa) was carried out to unpollinated ovaries in 10 μL of 5% ethanol, 0.1% Tween 80 solution. Flower emasculation was carried out 2 d before anthesis to prevent self-pollination. LAB 198999 (3,5-dioxo-4-butyryl-cyclohexane carboxylic acid ethyl ester; BASF) was applied in 10 μL of 5% ethanol, 0.1% Tween solution to pollinated ovaries, at different times after pollination, after removal of petals and stamens. Equal volumes of solvent solution was applied to control ovaries. Paclobutrazol (Duchefa) was applied to the roots in the nutrient solution.

Quantification of GAs

GAs were quantified following the protocol described in Fos et al. (2000). In summary, aliquots (about 3–5 g fresh weight) of frozen material were extracted with 80% methanol and, after removing the organic phase, the water fraction was partitioned against ethyl acetate and purified by QAE-Sephadex chromatography and C_{18} cartridges. The GAs where then separated by reverse-phase HPLC chromatography (4-μm C_{18} column, 15 cm long, 3.9 mm i.d.; NovaPak, Millipore) and appropriate fractions grouped for gas chromatography-single ion monitoring analysis after methylation and trimethylsililation. [17,17-^{2}H]GA_1, [17,17-^{2}H]GA_8, [17,17-^{2}H]GA_{19}, [17,17-^{2}H]GA_{20}, [17,17-^{2}H]GA_{29}, [17,17-^{2}H]GA_{44}, and [17,17-^{2}H]GA_{53} (purchased from Prof. L. Mander) were added to the extracts as internal standards for quantification and [^{3}H]GA_{20} and [^{3}H]GA_9 to monitor the separation of GAs after HPLC using a 10% to 100% methanol gradient. Quantification was carried out by gas chromatography-single ion monitoring using a gas chromatograph (model 5890, Hewlett-Packard) coupled to a mass-selective detector (model 5971A, Hewlett-Packard). The concentrations of GAs in the extracts were determined using the calibration curves methodology.

Isolation of cDNA Clones of *GA2ox* from Tomato

Total RNA was isolated from 20-d-old pollinated fruits using a phenol-chloroform method (Bartels and Thompson, 1983). Clones of *SlGA2ox* were isolated by RT-PCR using degenerated oligonucleotides. Two micrograms of total RNA were reverse transcribed with a first-strand cDNA synthesis kit (Amersham Biosciences) in 33 μL total volume reaction. PCR was performed taking 1 μL aliquot of cDNA solution in a 50 μL total volume reaction containing 0.2 mM of each dNTP, 2 mM $MgCl_2$, 1× reaction buffer, 1 unit of NETZYME DNA Polymerase (Fermentas Gmbh), and 1 μM of degenerated primers A [5′-(GA)TXGGXTT(CT)GGXGA(AG)(CA)(CA)(AT)-3′] and B [5′-X(GC)CX(GC)(AC)(AG)AA(AG)TAXATCAT-3′]. Thermocycling conditions for amplification consisted of initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C/30 s, 45°C/60 s, and 72°C/60 s, and finally 10 min extension at 72°C. The products of an amplified band of about 250 bp, separated on 1% agarose gel electrophoresis, were purified (CONCERT Rapid Gel Extraction system, GIBCO-BRL), cloned in the pGEM-T Easy Vector (Promega), and sequenced. Six of these clones (out of 11 sequenced) were identical and homologous to *GA2ox* previously cloned from diverse species. Sequences of the 5′ and 3′ regions were obtained by RACE (RACE cDNA amplification kit, CLONTECH) using appropriate primers (Supplemental Table S1) and the following conditions for amplification: 95°C/5 min followed by five cycles of 94°C/30 s and 72°C/2.5 min, five cycles of 94°C/30 s and 70°C/2.5 min, and 30 cycles of 94°C/30 s and 68°C/2.5 min, and finally 10 min extension at 72°C. A full-length cDNA clone, named *SlGA2ox1*, was obtained by RT-PCR using appropriate primers (Supplemental Table S1) and the following thermocycling conditions: 94°C/2 min, followed by 40 cycles of 94°C/1 min, 57°C/2 min, and 72°C/3 min, and 10 min extension at 72°C, cloned in the pGEM-T Easy Vector and sequenced.

Additional *GA2ox* clones of tomato were identified by searching for tomato sequences homologous to *GA2ox* from diverse species (including Arabidopsis [*Arabidopsis thaliana*] and *SlGA2ox1*, previously cloned) in GenBank EST databases. Four groups coming from 18 ESTs corresponding to genes different to *SlGA2ox1* were identified. Using this sequence information 5′ and 3′ regions were obtained by RACE, when necessary, as described before. Full-length cDNA clones (named *SlGA2ox2*, *SlGA2ox3*, *SlGA2ox4*, and *SlGA2ox5*) were amplified by RT-PCR using RNA from pollinated fruits (*SlGA2ox2*, *-4*, and *-5*) and mature leaves (*SlGA2ox4*), the primers given in Supplemental Table S1, and the thermocycling conditions described previously for *SlGA2ox1* (but using as annealing temperatures of 50°C for *SlGA2ox3* and *-4*, and 54°C for *SlGA2ox2* and *-5*). Amplified products were cloned in pGEM-T Easy Vector and sequenced.

Heterologous Expression of cDNA Clones and Determination of Enzyme Activities

Coding cDNA sequences of *SlGA2ox1*, *-2*, *-3*, *-4*, and *-5* were amplified by PCR, cloned using a Zero Blunt TOPO Cloning kit (Invitrogen), and inserted as a translational fusion into the pET45b prokariote expression vector (Novagen) using *Bam*HI-*Hin*dIII (*SlGA2ox1*, *-3*, and *-4*) and *Not*I-*Xho*I (*SlGA2ox2* and *-5*) sites. Recombinant clones were sequenced and expressed in BL21 (pLysS) D3 *Escherichia coli* cells (Novagen) following the manufacturer's instructions. Activity of expressed proteins from at least two sequenced PCR independent clones of each gene was determined enzymatically using appropriate cofactors [17-^{14}C]GA_1, [17-^{14}C]GA_4, [17-^{14}C]GA_9, [17-^{14}C]GA_{12}, [17-^{14}C]GA_{20}, and [17-^{14}C]GA_{53} (333 Bq, 100–150 pmol; purchased from Dr. L. Mander, Australian National University, Canberra) as

substrates, and 93 μL aliquots of cell lysates in a total 100 μL reaction volume as described elsewhere (García-Martínez et al., 1997). Metabolic products were separated by HPLC, detected using an on-line radioactive monitor (Radioflow Detector LB 508, Berthold Technologies), and identified by their retention times compared to pure GAs.

Semiquantitative RT-PCR

Total RNA was isolated from different tomato organs: roots, young and old leaves, young and old internodes, flowers, and separated flower organs at anthesis. Unpollinated and pollinated ovaries at 0, 5, 10, and 20 DPA were also collected and pericarp and seeds of 10- and 20-d-old pollinated ovaries separated for RNA extraction. RNA was treated with DNAse, according to manufacturer's protocol using an RNAeasy Plant mini kit (Quiagen). Then, 2 μg of total RNA were reverse transcribed with a first-strand cDNA synthesis kit (Amersham Biosciences) in 33 μL total volume reaction. PCRs were performed taking 1 μL aliquots of cDNA solution in a 50 μL total volume reaction containing 0.2 mM of each dNTP, 2 mM $MgCl_2$, 1× reaction buffer, 1 unit of NETZYME DNA Polymerase (Fermentas Gmbh), and 1 μM of the appropriate pair of primers (Supplemental Table S2). PCR conditions for amplification of *SlCPS*, *SlGA20ox1*, *-2*, and *-3* and *SlGA3ox1* and *-2* consisted of initial denaturation at 94°C for 2 min, followed by 32 cycles of 94°C/30 s, 57°C/60 s, and 72°C/60 s, and finally 10 min extension at 72°C. For amplification of *SlGA2ox1*, *-2*, *-3*, *-4*, and *-5*, 31 cycles were used with annealing temperatures of 60°C (*SlGA2ox1*) or 62°C (*SlGA2ox2*, *-3*, *-4*, and *-5*), and for *SlCPS* 33 cycles and 61°C. In the case of *Actin* annealing temperature of 60°C and 24 cycles were used. In all cases, the number of cycles was chosen to give amplified products within the linear synthesis reaction. Fifteen microliter aliquots of PCR products were separated on 1% 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 Actin as internal control, by comparing expression ratios to that of the specific tissues indicated in the figure legends (set to 1.0).

The analyses were carried out in duplicate using biologically independent material with similar results. Only data from one representative replicate are given under "Results."

Phylogenetic Analyses

Nucleotide sequences were translated into protein sequences using GeneDoc software (available at http://www.psc.edu/biomed/genedoc) and aligned with *MUSCLE* algorithm (freely available at http://www.drive5.com/muscle) using default parameters. Sequences were highly divergent, which led us to pursue the phylogenetic reconstruction using amino acid rather than nucleotide sequences. The best model of protein evolution was selected based on the Akaike criterion with the ProtTest on-line server (http://darwin.uvigo.es/software/prottest_server.html). The Jones-Taylor-Thornton evolutionary model (Jones et al., 1992) with evolution rates varying according to a Gamma distribution plus a class of invariant sites was judged optima in both phylogenetic analyses. In the 44 *GA2ox* dataset, the inferred parameters were $\alpha = 1.20$ for the shape of the Gamma and $P = 0.04$ for the fraction of invariant sites, whereas for the 26-sequence dataset containing only tomato and Arabidopsis *GA2ox*, *GA3ox*, and *GA20ox* genes, the estimated values were $\alpha = 1.36$ and $P = 0.01$. A maximum-likelihood tree was obtained with the *proml* implementation of the *PHYLIP* package version 3.66 (freely available at http://evolution.genetics.washington.edu/phylip.html) using the Hidden Markov model method of inferring different rates of evolution at different amino acid positions (Felsenstein and Churchill, 1996), with six discrete classes for the rates and prior probabilities chosen according to the above estimated parameters. To identify ancestral and derivate clusters in the 44 *GA2ox* dataset analysis, the outgroup AtGA20ox1 was used to root the tree, whereas in the other analysis, the tree was left unrooted.

To assess the statistical significance of each internal branch, 1,000 bootstrap pseudoreplicates of the protein alignments were generated using the *seqboot* implementation of the *PHYLIP* package version 3.66. The maximum-likelihood procedure was repeated for 100 of the pseudoreplicates (doing more pseudoreplicates would be computationally too intensive) and a consensus tree was obtained using the *consense* implementation of the same package, setting all parameters at their default values. The branch lengths of the tree were then estimated using the same maximum-likelihood method. A node is judged statistically significant if it is supported by a high bootstrap proportion, though the appropriate threshold value depends on many factors (Hillis and Bull, 1993). To have an additional criterion for clade selection, we performed a weighted least-squares likelihood ratio test (Sanjuan and Wrobel, 2005) on each node using the *WeightLESS* implementation (freely available at http://www.iopan.gda.pl/~wrobel). To do that, we used the 1,000 pseudoreplicates to estimate the involved parameters, the distance matrix derived from the above Jones-Taylor-Thornton plus Gamma plus invariant class evolutionary model, and the above consensus tree.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers EF441351 to EF441355.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Scheme of GA metabolic pathways.

Supplemental Figure S2. Maximum-likelihood phylogenetic tree based on comparison of *GA20ox*, *GA3ox*, and *GA2ox* protein sequences from Arabidopsis and tomato.

Supplemental Figure S3. HPLC radioactivity traces of products of [^{14}C]GA_{12}, [^{14}C]GA_9, [^{14}C]GA_4, [^{14}C]GA_{53}, [^{14}C]GA_{20}, and [^{14}C]GA_1, incubated with heterologous expression products of *SlGA2ox1*, *-3*, *-4*, and *-5* after 2 h incubation at 30°C.

Supplemental Figure S4. Alignment of amino acid sequences corresponding to GA 2-oxidases from groups I, II, and III used to construct the phylogenetic tree of Figure 5.

Supplemental Table S1. Primer sequences used to amplify full-length cDNA clones of *SlGA2ox1*, *-2*, *-3*, *-4*, and *-5*.

Supplemental Table S2. Primer sequences used for semiquantitative RT-PCR analysis of diverse GA metabolism genes of tomato.

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LITERATURE CITED

Ait-Ali T, Swain SM, Reid JB, Sun TP, Kamiya Y (1997) The *LS* locus of pea encodes the gibberellin biosynthesis enzyme *ent*-kaurene synthase A. Plant J **11:** 443–454

Alabadí D, Carbonell J (1998) Expression of ornithine decarboxylase is transiently increased by pollination, 2,4-dichlorophenoxyacetic acid, and gibberellic acid in tomato ovaries. Plant Physiol **118:** 323–328

Bartels D, Thompson RD (1983) The characterization of cDNA clones coding for wheat storage proteins. Nucleic Acids Res **11:** 2961–2977

Bensen RJ, Zeevaart JAD (1990) Comparison of *ent*-kaurene synthetase A and B activities in cell-free extracts from young tomato fruits of wild-type and *gib-1*, *gib-2*, and *gib-3* tomato plants. J Plant Growth Regul **9:** 237–242

Bohner J, Hedden P, Bora-Haber E, Bangerth F (1988) Identification and quantitation of gibberellins in frutis of *Lycopersicon esculentum*, and their relationship to fruit size in *L. esculentum* and *L. pimpinellifolium*. Physiol Plant **73:** 348–353

Cowling RJ, Kamiya Y, Seto H, Harberd NP (1998) Gibberellin dose-response regulation of *GA4* gene transcript levels in Arabidopsis. Plant Physiol **117:** 1195–1203

Dan Y, Yan H, Munyikwa T, Dong J, Zhang Y, Armstrong CL (2006) MicroTom, a high-throughput model transformation system for functional genomics. Plant Cell Rep **25:** 432–441

Elliott RC, Ross JJ, Smith JJ, Lester DR, Reid JB (2001) Feed-forward regulation of gibberellin deactivation in pea. J Plant Growth Regul **20:** 87–94

Eyal E, Levy AA (2002) Tomato mutants as tools for functional genomics. Curr Opin Plant Biol **5:** 112–117

Felsenstein J, Churchill GA (1996) A hidden Markov model approach to variation among sites in rate of evolution. Mol Biol Evol **13:** 93–104

Fleet CM, Yamaguchi S, Hanada A, Kawaide H, David CJ, Kamiya Y, Sun TP (2003) Overexpression of *AtCPS* and *AtKS* in Arabidopsis confers increased *ent*-kaurene production but no increase in bioactive gibberellins. Plant Physiol **132:** 830–839

Fos M, Nuez F, García-Martínez JL (2000) The gene *pat-2*, which induces natural parthenocarpy, alters the gibberellin content in unpollinated tomato ovaries. Plant Physiol **122:** 471–479

Fos M, Proaño K, Nuez F, García-Martínez JL (2001) Role of gibberellins in parthenocarpic fruit development induced by the genetic system *pat-3*/*pat-4* in tomato. Physiol Plant **111:** 545–550

Fujioka S, Yamane H, Spray CR, Gaskin P, MacMillan J, Phinney BO, Takahashi N (1988) Qualitative and quantitative analyses of gibberellins in vegetative shoots on normal, *dwarf-1*, *dwarf-2*, *dwarf-3*, and *dwarf-5* seedlings of *Zea mays* L. Plant Physiol **88:** 1367–1372

García-Martínez JL, Gil J (2002) Light regulation of gibberellin biosynthesis and mode of action. J Plant Growth Regul **20:** 354–368

García-Martínez JL, López-Díaz I, Sánchez-Beltrán MJ, Phillips AL, Ward DA, Gaskin P, Hedden P (1997) Isolation and transcript analysis of gibberellin 20-oxidase genes in pea and bean in relation to fruit development. Plant Mol Biol **33:** 1073–1084

Gillaspy G, Ben-David H, Gruissem W (1993) Fruits: a developmental perspective. Plant Cell **5:** 1439–1451

Hedden P, Graebe JE (1985) Inhibition of gibberellin biosynthesis by paclobutrazol in cell-free homogenates of *Cucurbita maxima* endosperm an *Malus pumila* embryos. J Plant Growth Regul **4:** 111–122

Hedden P, Kamiya Y (1997) Gibberellin biosynthesis: enzymes, genes and their regulation. Annu Rev Plant Physiol Plant Mol Biol **48:** 431–460

Hedden P, Phillips A (2000) Gibberellin metabolism: new insights revealed by the genes. Trends Pharmacol Sci **5:** 523–530

Hillis DM, Bull JJ (1993) An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst Biol **42:** 182–192

Ho L, Hewitt J (1986) Fruit development. *In* JG Atherton, J Rudish, eds, The Tomato Crop. Chapman and Hall, New York, pp 201–239

Ingram TJ, Reid JB, Murfet IC, Gaskin P, Willis CL, MacMillan J (1984) Internode length in *Pisum*: the *Le* gene controls the 3β-hydroxylation of gibberellin A_{20} to gibberellin A_1. Planta **160:** 455–463

Jones DT, Taylor WR, Thornton JM (1992) The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci **8:** 275–282

Koshioka M, Nishijima T, Yamazaki H, Liu Y, Nonaka M, Mander LN (1994) Analysis of gibberellins in growing fruits of *Lycopersicon esculentum* after pollination or treatment with 4-chlorophenoxyacetic acid. J Hortic Sci **69:** 171–179

Lee DJ, Zeevaart JAD (2002) Differential regulation of RNA levels of gibberellin dioxygenases by photoperiod in spinach. Plant Physiol **130:** 2085–2094

Lee DJ, Zeevaart JAD (2005) Molecular cloning of *GA-Oxidase3* from spinach and its ectopic expression in *Nicotiana sylvestris*. Plant Physiol **138:** 243–254

Mapelli SC, Frova C, Torti G, Soressi G (1978) Relationship between set, development and activities of growth regulators in tomato fruits. Plant Cell Physiol **19:** 1281–1288

Martí E, Gisbert C, Bishop GJ, Dixon MS, García-Martínez JL (2006) Genetic and physiological characterization of tomato cv. Micro-Tom. J Exp Bot **57:** 2037–2047

Martin DN, Proebsting WM, Hedden P (1999) The *SLENDER* gene of pea encodes a gibberellin 2-oxidase. Plant Physiol **121:** 775–781

Meissner R, Jacobson Y, Melamed S, Levyatuv S, Shalev G, Ashri A, Elkind Y, Levy AA (1997) A new model system for tomato genetics. Plant J **12:** 1465–1472

Nakayama M, Yamane H, Murofushi N, Takahashi N, Mander L, Seto H (1991) Gibberellin biosynthetic pathway and the physiologically active gibberellin in the shoot of *Cucumis sativus* L. J Plant Growth Regul **10:** 115–119

Olsen JE, Junttila O, Moritz T (1995) A localised decrease of GA_1 in shoot tips of *Salix pentandra* seedlings precedes cessation of shoot elongation under short photoperiod. Physiol Plant **95:** 627–632

Olszewski N, Sun TP, Gubler F (2002) Gibberellin signalling, biosynthesis, catabolism, and response pathways. Plant Cell (Suppl) **14:** S61–S80

Ozga JA, Yu J, Reinecke DM (2003) Pollination-, development-, and auxin-specific regulation of gibberellin 3β-hydroxylase gene expression in pea fruit and seeds. Plant Physiol **131:** 1137–1146

Rebers M, Kaneta T, Kawaide H, Yamaguchi S, Yang Y-Y, Imai R, Sekimoto H, Kamiya Y (1999) Regulation of gibberellin biosynthesis genes during flower and early fruit development of tomato. Plant J **17:** 241–250

Rodrigo MJ, Garcia-Martínez JL, Santes CM, Gaskin P, Hedden P (1997) The role of gibberellins A_1 and A_3 in fruit growth of *Pisum sativum* L. and the identification of gibberellins A_4 and A_7 in young seeds. Planta **201:** 446–455

Sakai M, Sakamoto T, Saito T, Matsuoka M, Tanaka H, Kobayashi M (2003) Expression of novel rice gibberellin 2-oxidase gene is under homeostatic regulation by biologically active gibberellins. J Plant Res **116:** 161–164

Sanjuan R, Marin I (2001) Tracing the origin of the compensasome: evolutionary history of DEAH helicase and MYST acetyltransferase gene families. Mol Biol Evol **18:** 330–343

Sanjuan R, Wrobel B (2005) Weighted least-squares likelihood ratio test for branch testing in phylogenies reconstructed from distance methods. Syst Biol **54:** 218–229

Santes CM, García-Martínez JL (1995) Effect of the growth retardant 3,5-dioxo-4-butyryl-cyclohexane carboxylic acid ethyl ester, an acylcyclohexanedione compound, on fruit growth and gibberellin content of pollinated and unpollinated ovaries in pea. Plant Physiol **108:** 517–523

Schomburg FM, Bizzell CM, Lee DJ, Zeevaart JAD, Amasino RM (2003) Overexpression of a novel class of gibberellin 2-oxidases decreases gibberellin levels and creates dwarf plants. Plant Cell **15:** 151–163

Scott JW, Harbaugh BK (1989) Micro-Tom—a miniature dwarf tomato. Florida Agr Expt Sta Cir **370:** 1–6

Serrani JC, Fos M, Atarés A, García-Martínez JL (2007) Effect of gibberellin and auxin on parthenocarpic fruit growth induction in the cv Micro-Tom of tomato. J Plant Growth Regul (in press)

Silverstone AL, Chang CW, Krol E, Sun TP (1997) Developmental regulation of the gibberellin biosynthetic gene *GA1* in *Arabidopsis thaliana*. Plant J **12:** 9–19

Sjut V, Bangerth F (1982) Induced parthenocarpy—a way of changing the levels of endogenous hormones in tomato fruits (*Lycopersicon esculentum* Mill.). 1. Extractable hormones. Plant Growth Regul **1:** 243–251

Sponsel V, Hedden V (2004) Gibberellin biosynthesis and inactivation. *In* P Davies, ed, Plant Hormones: Biosynthesis, Signal Transduction, Action! Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 63–94

Symons GM, Reid JB (2003) Interactions between light and plant hormones during de-etiolation. J Plant Growth Regul **22:** 3–14

Thomas SG, Phillips AL, Hedden P (1999) Molecular cloning and functional expression of gibberellin 2-oxidases, multifunctional enzymes involved in gibberellin deactivation. Proc Natl Acad Sci USA **96:** 4698–4703

Ubeda-Tomás S, García-Martínez JL, López-Díaz I (2006) Molecular, biochemical and physiological characterization of gibberellin biosynthesis and catabolism genes from *Nerium oleander*. J Plant Growth Regul **25:** 52–68

Van Huizen R, Ozga JA, Reinecke DM (1997) Seed and hormonal regulation of gibberellin 20-oxidase expression in pea pericarp. Plant Physiol **115:** 123–128

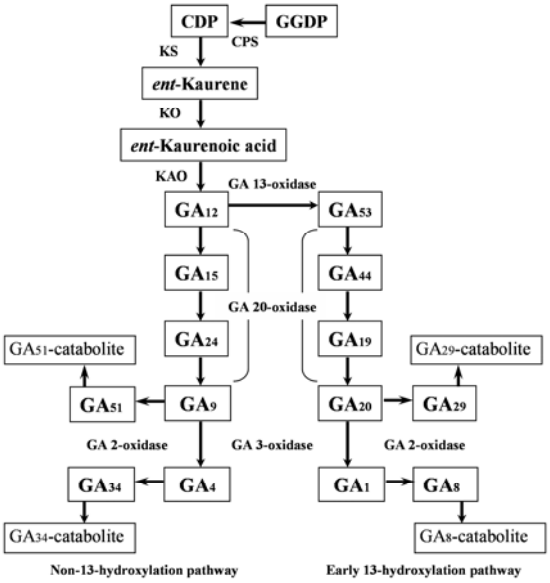
Varbanova M, Yamaguchi S, Yang Y, McKelvey K, Hanada A, Borochov R, Yu F, Jikumaru Y, Ross Y, Cortes D, et al (2007) Methylation of gibberellins by Arabidopsis GAMT1 and GAMT2. Plant Cell **19:** 32–45

Waycott W, Smith VA, Gaskin P, MacMillan J, Taiz L (1991) The endogenous gibberellins of dwarf mutants of lettuce. Plant Physiol **95:** 1169–1173

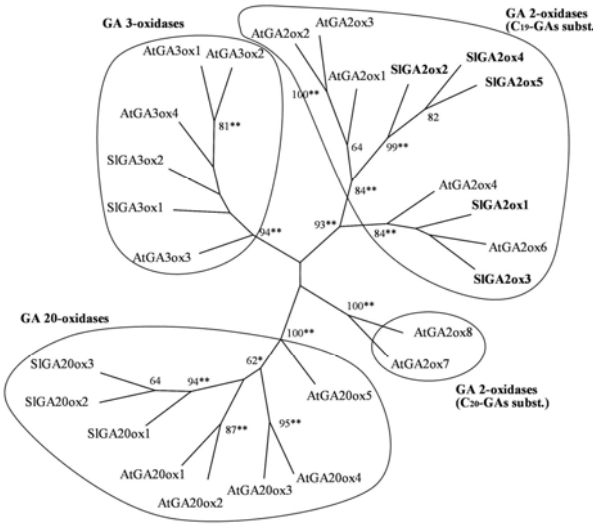
Yamaguchi S, Kamiya Y (2000) Gibberellin biosynthesis: its regulation by endogenous and environmental signals. Plant Cell Physiol **41:** 251–257

Zeevaart JAD, Gage DA, Talon M (1993) Gibberellin A_1 is required for stem elongation in spinach. Proc Natl Acad Sci USA **90:** 7401–7405

Zhu Y, Nomura T, Xu Y, Zhang Y, Peng Y, Mao B, Hanada A, Zhou H, Wang R, Li P, et al (2006) *ELONGATED UPPERMOST INTERNODE* encodes a cytochrome P450 monooxygenase that epoxidizes gibberellins in a novel deactivation reaction in rice. Plant Cell **18:** 442–456

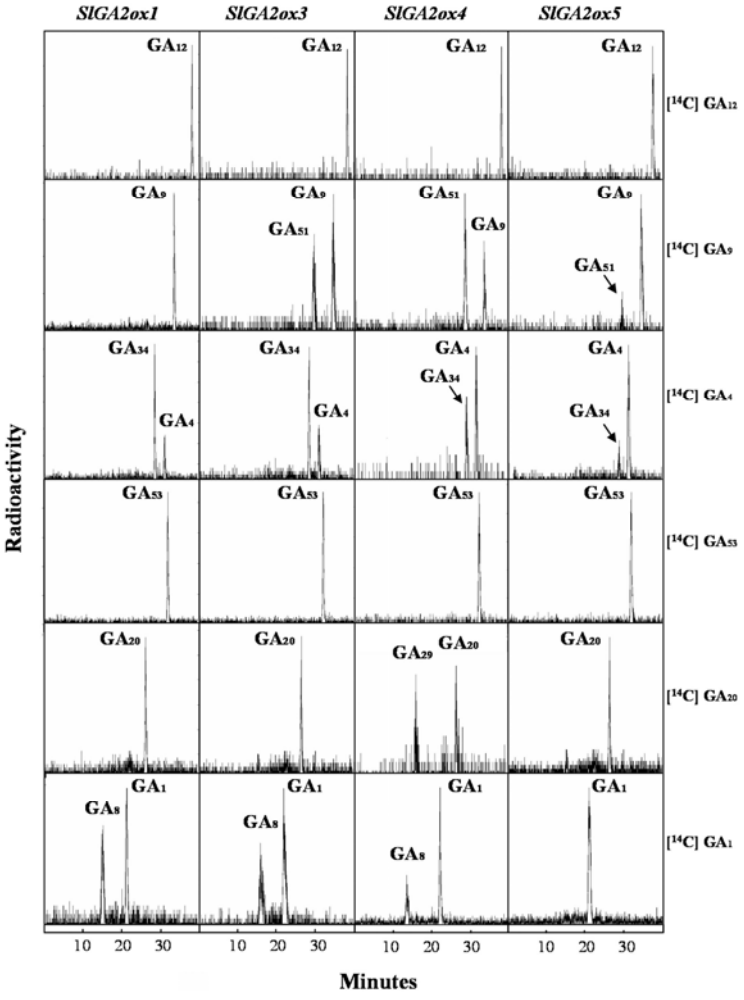


Supplementary Figure 1. Scheme of GA metabolic pathways.

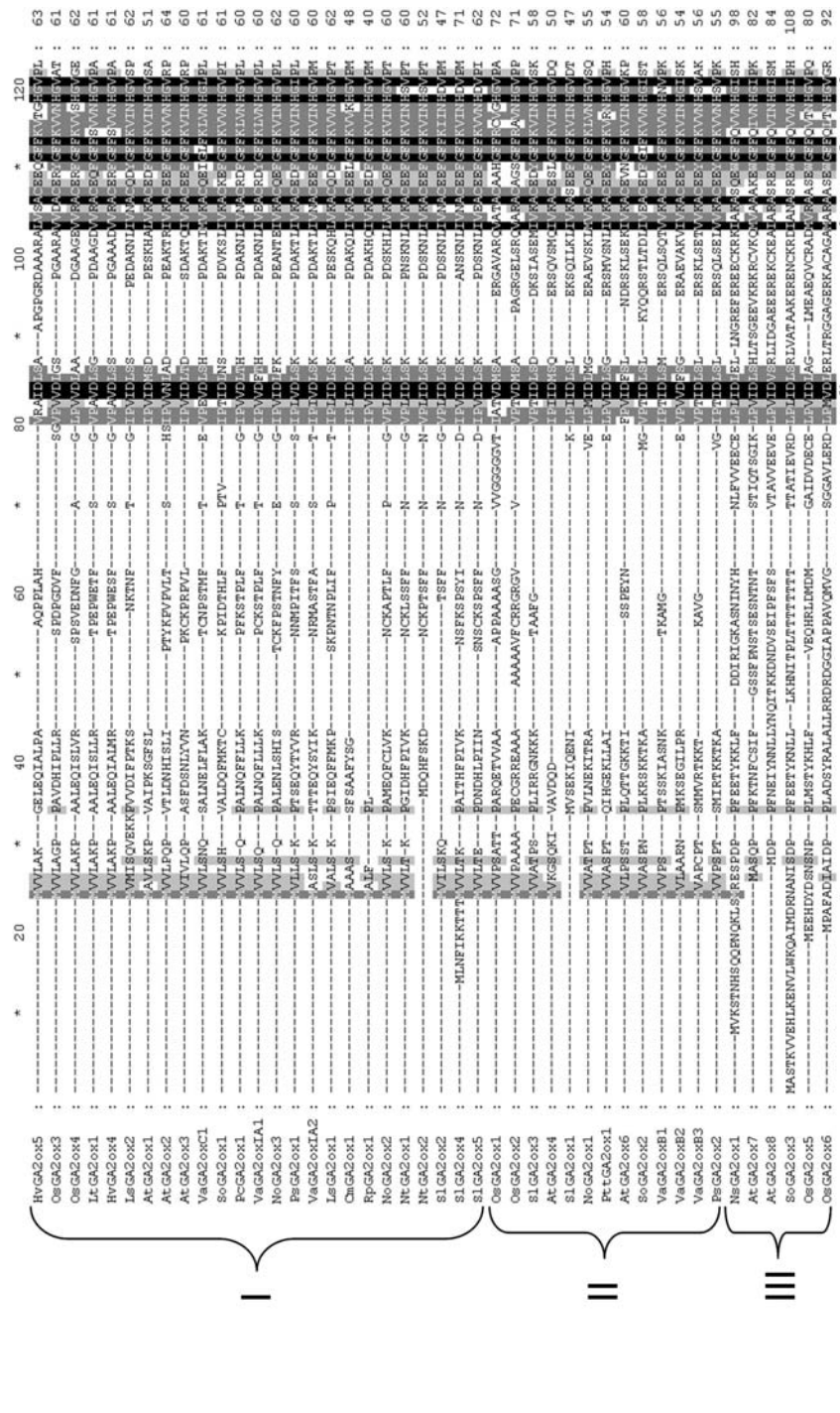


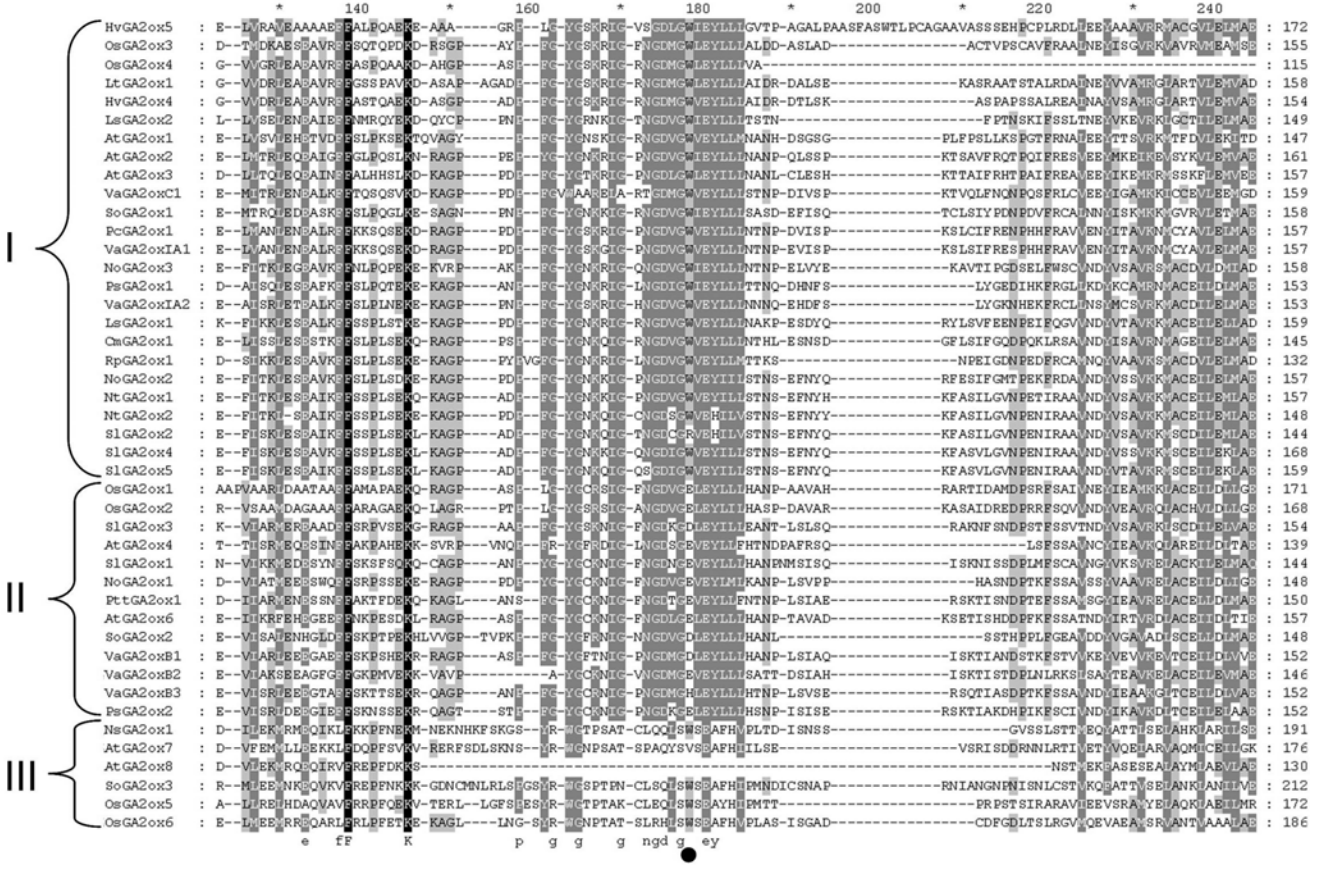
Supplementary Fig. 2. Maximum likelihood phylogenetic tree based on comparison of *GA20ox*, *GA3ox* and *GA2ox* protein sequences from Arabidopsis and tomato. Branch lengths are proportional to sequence divergence. Bootstrap values above 50% are shown, whereas asterisks indicate statistical significance according to the weighted least-squares likelihood ration test (**, $P < 0.01$; *, $P < 0.05$). The five genes characterized in this study are shown in bold type. Accession numbers corresponding to the sequences in the tree are the following: AtGA20ox1, X83379; AtGA20ox2, X83380; AtGA20ox3, X83381; AtGA20ox4, NM104778; AtGA20ox5, DQ056484; AtGA3ox1, L37126; AtGA3ox2, AF070937; AtGA3ox3, NM118289; AtGA3ox4, NM106682; AtGA2ox1, AJ132435; AtGA2ox2, AJ132436; AtGA2ox3, AJ322437; AtGA2ox4, NM103695; AtGA2ox6, NM100121; AtGA2ox7, AC079284; AtGA2ox8, AL021960; SlGA20ox1, AF049898; SlGA20ox2, AF049899; SlGA20ox3, AF049900; SlGA3ox1, AB010991; SlGA3ox2, AB010992; SlGA2ox1, EF441351; SlGA2ox2, EF441352; SlGA2ox3, EF441353; SlGA2ox4, EF441354; SlGA2ox5, EF441355.

Supplementary Fig. 3. HPLC radioactivity traces of products of [^{14}C]GA_{12}, [^{14}C]GA_9, [^{14}C]GA_4, [^{14}C]GA_{53}, [^{14}C]GA_{20}, and [^{14}C]GA_1, incubated with heterologous expression products of *SlGA2ox1*, *-3*, *-4* and *-5* after 2 h incubation at 30ºC.



Supplementary Fig. 4. Alignment of amino acid sequences corresponding to GA 2-oxidases from groups I, II and III used to construct the phylogenetic tree of Fig. 5. ○, Fe^{2+} binding residues; ↑, 2-cetoglutarate binding residues; ●, amino acids conserved in groups I and II.




I
II
III
HvGA2ox5 : E--LVRAVEAAAAEEGALPQAERE-AAA-----GRI--LC-YGSKRIG-VSGDLGWIEYLLLGVTP-AGALPAASFASWTLPCAGAAVASSSEHPCPLRDLIEELAAAVRKYACGVLELMAE : 172
OsGA2ox3 : D--TVDKAESSAVREESQTQPDKD-RSGP----AYI--FG-YGSKRIG-FNGDMGWLEYLLLALDD-ASLAD----------ACTVPSCAVFRAAINELISGVRKVAVRVMEAVSE : 155
OsGA2ox4 : G--VVGRIEAEAVREEASPQAAKD-AHGP----ASI--FG-YGSKRIG-RNGDMGWLEYLLLVA-- : 115
LtGA2ox1 : G--VVDRIEAEAVREEGSSPAVKD-ASAP--AGADI--FG-YGSKRIG-RNGDMGWLEYLLLAIDR-DALSE----------KASRAATSTALRDAINEVVAVRGYARTVLEYVAD : 158
HvGA2ox4 : G--VVDRIEAEAVREEGASTQAEKD-ASGP----ADI--FG-YGSKRIG-RNGDMGWVEYLLLAIDR-DTLSK----------ASPAPSSALREAINAVVSAVRGYARTVLEYVAE : 154
LsGA2ox2 : L--LVSEIENEAIEEENMRQYEKD-QYCP----PNI--FG-YGRNKIG-TNGDVGWIEYLLLTSTN----------------FPTNSKIFSSLTNEVKEVRKHGCTLLELMAE : 149
AtGA2ox1 : E--LVSVIEHETVDEESLPKSEKTQVAGY------I--FG-YGNSKIG-RNGDVGWVEYLLLNANH-DSGSG----------PLFPSLLKSPGTFRNAIEELTTSVRKYTFDVLEKITD : 147
AtGA2ox2 : E--LVTRIEQEAIGEEGLPQSLKN-RAGP----PEI--FG-YGNKRIG-FNGDVGWIEYLLLNANP-QLSSP----------KTSAVFRQTPQIFRESIEELMKEHKEVSYKVLEYVAE : 161
AtGA2ox3 : D--LVTQIEQEAINEEALHHSLKD-KAGP----PDI--FG-YGTKRIG-FNGDLGWLEYLLLNANL-CLESH----------KTTAIFRHTPAIFREAIEELIKEYKRVSSKFLEYVEE : 157
VaGA2oxC1 : E--MITRIENEALREETQSQSVKD-KAGP----PDI--FG-VAAREIA-RTGDMGWVEYLLLSTNP-DIVSP----------KTVQLFNQNPQSFRLCIEELIGAYKKVCCEVLEEMGD : 159
SoGA2ox1 : E--MTRQIEDEASKEESLPQGLKE-SAGN----PNI--FG-YGNKKIG-RNGDVGWIEYLLLSASD-EFISQ----------TCLSIYPDNPDVFRCAINNVISKVKKVGVRVLETMAE : 158
PcGA2ox1 : E--LVANIENEALREEKKSQSEKD-RAGP----PDI--FG-YGSKRIG-FNGDVGWVEYLLLNTNP-DVISP----------KSLCIFRENPHHFRAVIENVITAVRNVCYAVLELMAE : 157
VaGA2oxIA1 : E--LVANIENEALREEKKSQSEKD-RAGP----PDI--FG-YGSKGIG-FNGDVGWVEYLLLNTNP-EVISP----------KSLSIFRESPHHFRAVIENVITAVRNVCYAVLELMAE : 157
NoGA2ox3 : E--FITKIEGEAVREENLPQPEKE-KVRP----AKI--FG-YGNKRIG-QNGDVGWIEYLLLNTNP-ELVYE----------KAVTIPGDSELFWSCINDVVSAVRSVACDVLDYIAD : 158
PsGA2ox1 : D--AISQIESEAFKEESLPQTEKE-KAGP----ANI--FG-YGNKRIG-LNGDIGWIEYLLLTTNQ-DHNFS----------LYGEDIHKFRGLIKDVKCAVRNVACEKLDEMAE : 153
VaGA2oxIA2 : E--AISRIETEALREESLPLNEKE-KAGP----PNI--FG-YGSKRIG-HNGDVGWVEYLLLNNNQ-EHDFS----------LYGKNHEKFRCLINSVMCSVRKVACDKLELMAE : 153
LsGA2ox1 : K--FIKKIESEALKEESSPLSTKE-KAGP----PDI--FG-YGNKRIG-RNGDVGWVEYLLLNAKP-ESDYQ----------RYLSVFEENPEIFQGVINDVVTAVKKVACEKLELMAD : 159
CmGA2ox1 : E--LVSSIESESTKEESLPLSEKQ-RAGP----PSI--FG-YGNKQIG-RNGDVGWVEYLLLNTHL-ESNSD----------GFLSIFGQDPQKLRSAVNDVISAVRNVAGEKLELMAE : 145
RpGA2ox1 : D--SIKKIESEAVREESLPLSEKE-KAGP----PYIVGFG-YGNKRIG-LNGDVGWVEYLLLTTKS----------------NPEIGDNPEDFRCAVNQVVAAVKSVACDVLELMAD : 132
NoGA2ox2 : E--FITKIESEAVREESLPLSDKE-KAGP----PDI--FG-YGNKKIG-FNGDIGWVEYIILSTNS-EFNYQ----------RFESIFGMTPEKFRDAINDVVSSVKKVACEKLELMAE : 157
NtGA2ox1 : E--FITKIESEAIKEESSPLSEKQ-KAGP----ADI--FG-YGNKKIG-FNGDVGWVEYILLSTNS-EFNYH----------KFASILGVNPETIRAAINDVVSAVKKVACEKLELMAE : 157
NtGA2ox2 : E--FITKI-SEAIKEESSPLSEKE-KAGP----PDI--FG-YGNKQIG-CNGDSGWVEHILVSTNS-EFNYY----------KFASILGVNPENIRAAINDVVSAVKKVACEKLELMAE : 148
SlGA2ox2 : E--FISKIESEAIKEESSPLSEKL-KAGP----ADI--FG-YGNKQIG-TNGDCRWEHILVSTNS-EFNYQ----------KFASILGVNPENIRAAINDVVSAVKKVSCDKLELMAE : 144
SlGA2ox4 : E--FISKIESEAVREESSPLSEKL-KAGP----ADI--FG-YGNKKIG-QNGDIGWVEYILLSTNS-EFNYQ----------KFASVLGVNPENIRAAINDVVSSVKKVSCEKLEKMAE : 168
SlGA2ox5 : E--FISKIESEAIKEESSPLSEKL-KAGP----ADI--FG-YGNKQIG-QSGDIGWVEYILLSTNS-EFNYQ----------KFASVLGVNPENIRAAINDVVTAVKRVSCEKLEKMAE : 159
OsGA2ox1 : AAPVAARIDAATAAEGAMAPAEKQ-RAGP----ASI--LC-YGCRSIG-FNGDVGELEYLLLHANP-AAVAH----------RARTIDAMDPSRFSAIVNEYIEAVKKVACEKLDLLGE : 171
OsGA2ox2 : R--VSAAIDAGAAAEGARAGAEKQ-LAGR----PTI--LC-YGSRSIG-ANGDVGELEYLLIHASP-DAVAR----------KASAIDREDPRRFSQVINDVVEAVRQVACHVLDLLGE : 168
SlGA2ox3 : K--VIARIERDAADEESRPVSEKG-RAGP----AAI--FG-YGSKNIG-FNGDKGDLEYILIEANT-LSLSQ----------RAKNFSNDPSTFSSVTNDVVSAVRKVSCDKLELVAE : 154
AtGA2ox4 : T--TISRIEQSSINEGAKPAHEKR-SVRP---VNQI--FR-YGFRDIG-LNGDSGELEYFLLFHTNDPAFRSQ----------LSFSSAINCYIEAVKQVARELDLTAE : 139
SlGA2ox1 : N--VIKKIEDSYNEESKSFSQKQ-CAGP----ANI--YG-YGCKNIG-FNGDNGEVEYLLLHANPNMSISQ----------ISKNISSDPLMFSCAINGVKSVREVACKLELMAE : 144
NoGA2ox1 : D--VIATIEESWQEESRPSSEKE-RAGP----PDI--YG-YGCKNIG-FNGDVGEVEYLMIKANP-LSVPP----------HASNDPTKFSSAISSVVAAVREVACELDLHGE : 148
PttGA2ox1 : D--IIARIENSSSNEGAKTFDEKQ-KAGL----ANS--FG-YGCKNIG-FNGDTGEVEYLLFNTNP-LSIAE----------RSKTISNDPTEFSSAISGVIEAVREVACELLDLMAE : 150
AtGA2ox6 : E--IIKRFEHEGEEENKPESDKL-RAGP----ASI--FG-YGCKNIG-FNGDLGELEYLLLHANP-TAVAD----------KSETISHDDPFKFSSATNDVIRTVRDVACEIDLTIE : 157
SoGA2ox2 : E--VISAIENHGLDEESKPTPEKHLVVGP--TVPKI--FG-YGFRNIG-NNGDVGDLEYLLLHANL----------------------SSTHPPLFGEAIDDVVGAVADVSCELLDLMAE : 148
VaGA2oxB1 : E--VIARIEEGAEEESKPSHEKR-RAGP----ASI--FG-YGFTNIG-FNGDMGDLEYLLLHANP-LSIAQ----------ISKTIANDSTKFSTVVKEVVEVREVCSELLDLVVE : 152
VaGA2oxB2 : E--VIAKSEEAGFGEEGKPMVEKK-VAVP----------A-YGCKNIG-VNGDMGELEYLLLSATT-DSIAH----------ISKTISTDPLNLRKSISAVTEAVRDVACEILELMAE : 146
VaGA2oxB3 : E--VISRIEEEGTAEESKTTSEKR-QAGP----ANI--FG-YGCRNIG-FNGDMGHLEYLLLHTNP-LSVSE----------RSQTIASDPTKFSSAINDVIEAAKGHTCELLDLVAE : 152
PsGA2ox2 : E--VISRIDEEGIEEESKNSSEKR-QAGT----STI--FG-YGCKNIG-FNGDKGELEYLLLHSNP-ISISE----------RSKTIAKDHPIKFSCIVNDVIKAVRDHTCELLDLAAE : 152
NsGA2ox1 : D--IIEKIRMEQIKLEKKPFNEKM-NEKNHKFSKGS--RR-YGTPSAT-CLQQISWSSAFHVPLTD-ISNSS----------GVSSLSTTVEQVATTVSEVAHKVARLISE : 191
AtGA2ox7 : D--VFEMILLEEKKLEQDPFSVRV-RERFSDLSKNS--RR-YGNPSAT-SPAQYSVSSAFHILSE----------VSRISDDRNNLRTIVETVVQEIARVAQMICELLGK : 176
AtGA2ox8 : D--VIEKIRQEQIRVERFPDFKKS---NSTVEKGASESEAVAYMVAEVLAE : 130
SoGA2ox3 : R--MIEEINKEQVKVERFPNKKR-GDNCMNLRLSFGSIR-YGSPTFN-CLSQISWSSAFHIFMNDICSNAP----------RNIANGNFNISNLCSTVKQEATTVSEVANKIANLVSE : 212
OsGA2ox5 : A--LVREIHDAQVAVERRPFQEKV-TERL--LGFSIESIR-YGTPTAK-CLEQISWSSAYHIPMTT----------------PRPSTSIRARAVHEEVSRAVYEVAQKIAELLMR : 172
OsGA2ox6 : E--LVEEIRREQARLERLPFETKE-KAGL----LNG-SIR-YGNPTAT-SLRHISWSSAFHIPLAS-ISGAD----------CDFGDLTSLRGVVQEVAEAVSRVANTLAAAAAE : 186
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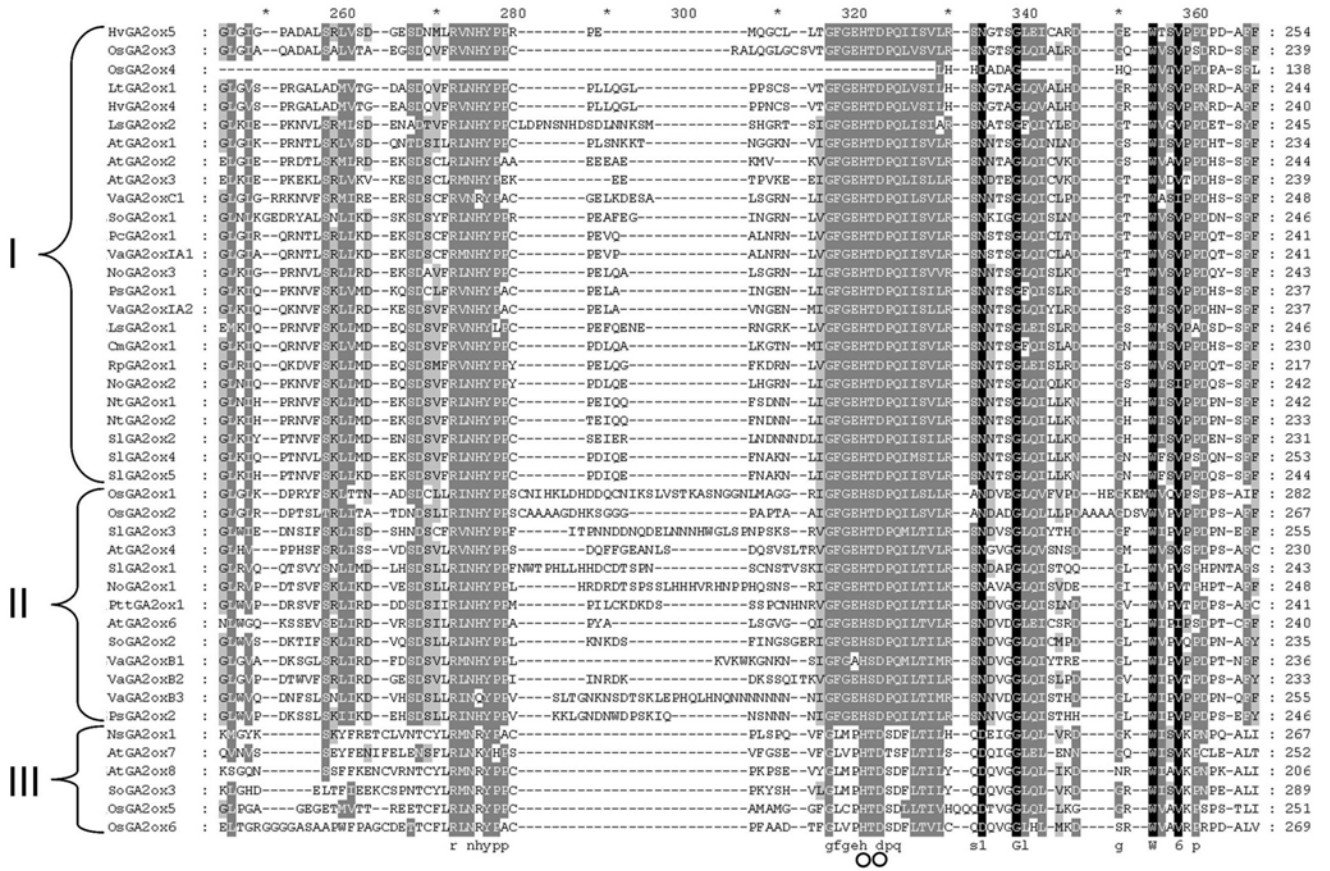
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III
HvGA2ox5
OsGA2ox3
OsGA2ox4
LtGA2ox1
HvGA2ox4
LsGA2ox2
AtGA2ox1
AtGA2ox2
AtGA2ox3
VaGA2oxC1
SoGA2ox1
PcGA2ox1
VaGA2oxIA1
NoGA2ox3
PsGA2ox1
VaGA2oxIA2
LsGA2ox1
CmGA2ox1
RpGA2ox1
NoGA2ox2
NtGA2ox1
NtGA2ox2
SlGA2ox2
SlGA2ox4
SlGA2ox5
OsGA2ox1
OsGA2ox2
SlGA2ox3
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AtGA2ox6
SoGA2ox2
VaGA2oxB1
VaGA2oxB2
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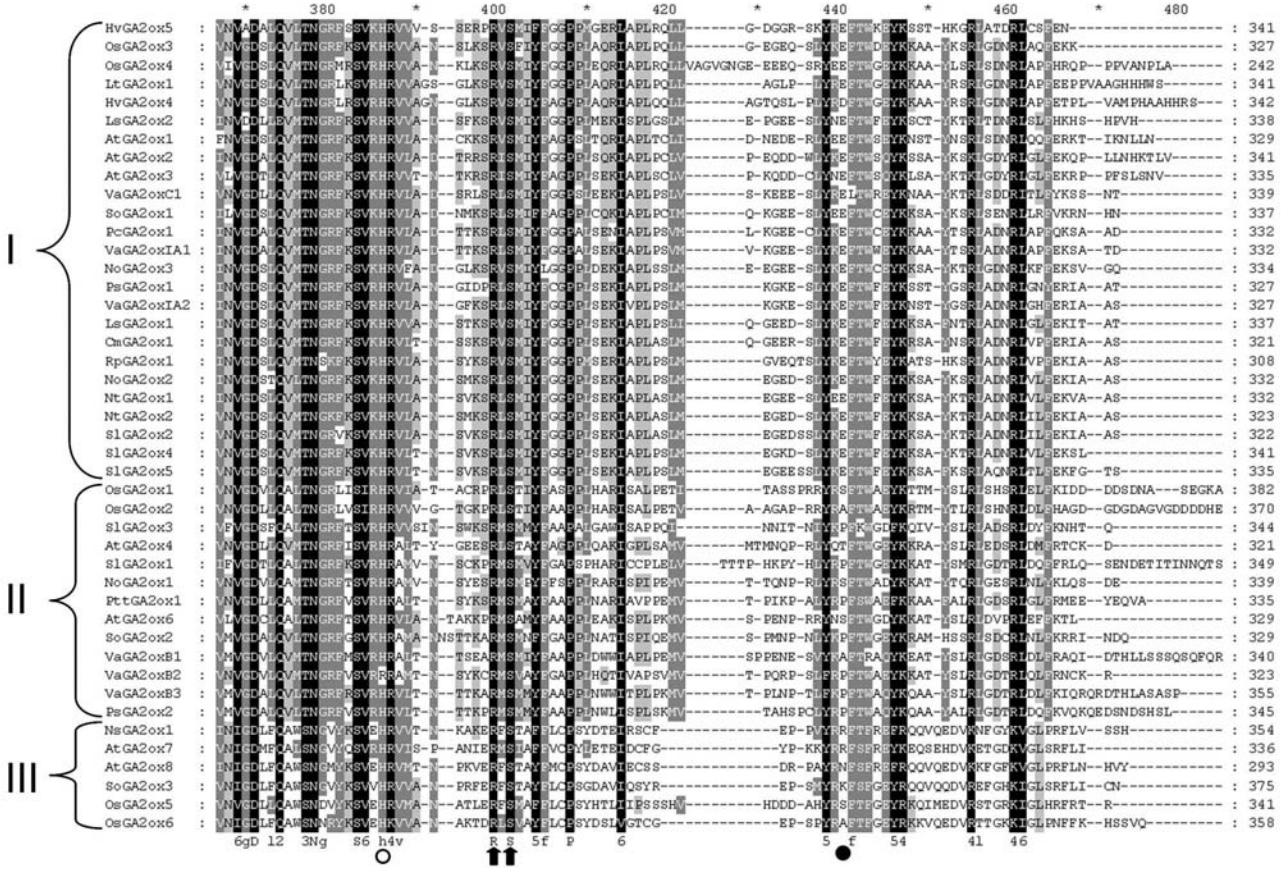
I
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HvGA2ox5
OsGA2ox3
OsGA2ox4
LtGA2ox1
HvGA2ox4
LsGA2ox2
AtGA2ox1
AtGA2ox2
AtGA2ox3
VaGA2oxC1
SoGA2ox1
PcGA2ox1
VaGA2oxIA1
NoGA2ox3
PsGA2ox1
VaGA2oxIA2
LsGA2ox1
CmGA2ox1
RpGA2ox1
NoGA2ox2
NtGA2ox1
NtGA2ox2
SlGA2ox2
SlGA2ox4
SlGA2ox5
OsGA2ox1
OsGA2ox2
SlGA2ox3
AtGA2ox4
SlGA2ox1
NoGA2ox1
PttGA2ox1
AtGA2ox6
SoGA2ox2
VaGA2oxB1
VaGA2oxB2
VaGA2oxB3
PsGA2ox2
NsGA2ox1
AtGA2ox7
AtGA2ox8
SoGA2ox3
OsGA2ox5
OsGA2ox6

Supplementary Table I. Primer sequences used to amplify full-length cDNA clones of *SlGA2ox1*, *-2*, *-3*, *-4* and *-5*.

Gene	Sense primer	Antisense primer
SlGA2ox1	5'- CCTCAACTTCCAACATGGTTTCTG -3'	*Not* I-d$(T)_{18}$
SlGA2ox2	5'- CACTTACCAAAATCAACCATGGTG -3'	5'- CCCACAATGAGCATCTTGACAACC -3'
SlGA2ox3	5'- CATTCGATTAATTATGGTAGTAGC -3'	*Not* I-d$(T)_{18}$
SlGA2ox4	5'- ACAAACAACAATTTCTACCAAAGT -3'	*Not* I-d$(T)_{18}$
SlGA2ox5	5'-CACCAGCAACAGTTGTAACAAGA-3'	5'- GATCCAAACATGGTATATTTGCGGAGG -3'

Supplementary Table II. Primer sequences used for semiquantitative RT-PCR analysis of diverse GA metabolism genes of tomato. *SlCPS* (AB015675), *SlGA20ox1* (AF049898), *SlGA20ox2* (AF049899), *SlGA20ox3* (AF049900), *SlGA3ox1* (AB010991), *SlGA3ox2* (AB010992), *SlGA2ox1, -2, -3, -4* and *-5*, and *SlACT (Actin*) (AB199316).

Gene	**Sense**	**Antisense**
SlCPS	5'-GGAAAATTGGCTACTGACGGTAGG-3'	5'-GGCATCCAATTCGGAAGCA-3'
SlGA20ox1	5'-GGAGCTCGCCTTAGGAACG-3'	5'-GTAGAAGCTAAGAGAACGTGTACACG-3'
SlGA20ox2	5'-CAACGTCTCAGGACTACAAGTTTTC-3'	5'-AGGCTAAGGTCTTGATCTACATTGG-3'
SlGA20ox3	5'-ACACCATCACTCCAAATTTCAAC-3'	5'-CCATGAGGTTCCATTTCTATGTC-3'
SlGA3ox1	5'-GTGAAACCAAAGAAGGATGTG-3'	5'-GCATCAGTAAATCCATTTAAAGGGA-3'
SlGA3ox2	5'-GTAACGGTTCCTCTCCTTCGC-3'	5'-ACCTACTTGGACGCCACTTTG-3'
SlGA2ox1	5'-ACCCCACATCTTCTCCATCAT G-3'	5'-ACATGTTTCATCAAGGGTTCGAT-3'
SlGA2ox2	5'-GCCATGCTCAGAGATTGAACGATTG-3'	5'-CCCACAATGAGCATCTTGACAACC-3'
SlGA2ox3	5'-GCTAACAATCCTTCGATCAAATGACG-3'	5'-GCATAATGCATACACCTCCAAGGCC-3'
SlGA2ox4	5'-GTCGATTTTAAGATCCAACAACACTTCCGGT-3'	5'-CATCATTTTCAACATAACGAGTCCTTCC-3'
SlGA2ox5	5'-ATATCGGTATTAAGATCCAACAACACATCC-3'	5'-GATCCAAACATGGTATATTTGCGGAGG-3'
SlACT	5'-ATGTATGTTGCCATCCAGGCTG-3'	5'-CCTTGCTCATCCTATCAGCAGCAATACC-3'

CAPÍTULO III

La Fructificación Inducida por Auxinas en Tomate Depende de Giberelinas

Juan Carlos Serrani, Mariano Fos, Omar Ruíz Rivero y José Luis García-Martínez

Enviado

Auxin-induced fruit-set in tomato is gibberellin dependent

Juan Carlos Serrani[1], Mariano Fos[2] and José Luis García-Martínez[1,*]

[1]Instituto de Biología Molecular y Celular de Plantas (Universidad Politécnica de Valencia-CSIC), and [2]Departamento de Biología Vegetal, Universidad Politécnica de Valencia, 46022-Valencia, Spain

*For correspondence (fax +34 963877859; e-mail jlgarcim@ibmcp.upv.es)

Corresponding author: Dr José Luis García-Martínez, IBMCP, Universidad Politécnica de Valencia-CSIC, Avda de los Naranjos s/n, 46022-Valencia, Spain

Phone: + 34-963877865; Fax: +34-963877859; e-mail: jlgarcim@ibmcp.upv.es

Running title: Auxin regulation of GA metabolism in tomato fruit

Key words: Auxin, Fruit-set, GA metabolism, Gibberellin, Micro-Tom, Tomato

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Summary: 250

Introduction: 962

Results: 1544

Discussion: 1355

Experimental procedures: 1041

Acknowledgments: 48

References: 1292

Summary

Tomato (*Solanum lycopersicum* L.) fruit-set and growth depend on gibberellins (GA). Auxins, another kind of hormones, can also induce parthenocarpic fruit growth in tomato, although their possible interaction with GA is unknown. We showed that fruit development induced by the auxins indol-3-acetic acid and 2,4-dichlorophenoxyacetic acid (2,4-D) were significantly reduced by simultaneous application of inhibitors of GA biosynthesis (Paclobutrazol and LAB 198999), and that this effect was reversed by applied GA_3. This suggested that the effect of auxin was mediated by GA. Parthenocarpic fruits induced by 2,4-D had higher contents of the active GA_1 (about five times) and of GA_8 (a GA_1 metabolite) than pollinated fruits, and similar concentrations of the precursors GA_{53}, GA_{44}, GA_{19}, and GA_{20}, while GA_{29} content was reduced in 2,4-D induced fruits. Radioactive-labelled GAs applied to unpollinated ovaries showed that 2,4-D induced the metabolism of [^{14}C]GA_{12} to putative [^{14}C]GA_9, and of [^{14}C]GA_{20} mainly to [^{14}C]GA_1, not to [^{14}C]GA_{29} as occurred in control. [^{14}C]GA_1 metabolism to [^{14}C]GA_8 was much lower in 2,4-D-treated ovaries than in control. Transcript levels of genes encoding copalyldiphosphate synthase (SlCPS), SlGA20ox1, -2 and -3 and SlGA3ox1, but not those encoding SlGA3ox2, were higher in unpollinated ovaries treated with 2,4-D. Transcript levels of *SlGA2ox2* (out of the five *SlGA2ox* genes known to encode this kind of GA inactivating enzymes) were lower in 2,4-D treated ovaries. Our results support the idea that auxins induce fruit-set and growth in tomato, at least partially, by enhancing GA biosynthesis (GA 20-oxidase and maybe CPS), and decreasing GA inactivation (GA2ox) activities, leading to higher GA_1 content.

Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most studied fleshy fruits due to their great commercial interest. In this species fruit development occurs normally after fruit-set (changeover from the static condition of the flower ovary to the rapidly growing condition of the young fruit) induced by fertilization, in two consecutive phases: an active division, lasting about 7-10 d post-anthesis, and a cell expansion phase (Gillaspy *et al*., 1993). The ovary wall develops during fruit growth into a pericarp composed of exocarp, mesocarp and endocarp, while the placental parenchyma, supported by the columella, grows by division and expansion, enclosing the developing seeds and filling the locular cavities with a jelly-like homogenous tissue (locular tissue) (Gillaspy *et al*., 1993; Ho and Hewitt, 1986).

Parthenocarpic fruit-set and growth can be induced by application of diverse plant growth substances to unpollinated ovaries, mainly auxins and gibberellins (GAs) (García-Martínez and Hedden, 1997; Gorquet *et al.,* 2005; Srivastava and Handa, 2005). GA metabolism in plants takes place from geranylgeranyl diphosphate, which is converted to *ent*-kaurene by the action of two consecutive cyclases (copalyldiphosphate synthase, CPS, and *ent*-kaurene synthase, KS), followed by the action of P450 monoxygenases (*ent*-kaurene oxidase, KO, and *ent*-kaurenoic acid oxidase, KAO), and of three kinds of Fe^{2+}- and 2-oxoglutarate-dependent dioxygenases (the biosynthetic enzymes GA 20-oxidases and GA 3-oxidases, and the inactivating enzymes GA 2-oxidases), which are encoded by small multigenic families (Sponsel and Hedden, 2004) (Supplementary Fig. 1). GA biosynthesis may occur through two parallel pathways: the non-13-hydroxylation, leading to GA_4 as the active GA, and the early-13-hydroxylation pathway, leading to the active GA_1 (Supplementary Fig. 1). The latter is the main metabolic pathway in tomato (Fos *et al.,* 2000). Fruit-set in tomato depends on gibberellins (GAs), as shown by application of GA biosynthesis inhibitors to pollinated ovaries (Fos *et al.,* 2000, 2001; Serrani *et al.,* 2007b), and of GAs to unpollinated ovaries (Alabadí and Carbonell, 1998; Fos *et al.,* 2000, 2001; Serrani *et al.,* 2007a; Sjut and Bangerth, 1982/83), and the active form is GA_1 (Serrani *et al.,* 2007b). It has been found that the increase of GA content in the ovary upon pollination is associated with upregulation of *SlGA20ox1, -2* and *-3* genes, which encode GA 20-oxidase biosynthetic

enzymes, but not of those encoding SlGA3ox, nor with downregulation of genes encoding SlGA2ox (inactivating enzymes) (Serrani *et al.,* 2007b). Expression increase of *SlGA20ox1* 24 h after pollination has also been reported (Olimpieri *et al.,* 2007).

Auxin application (review of Abad and Monteiro, 1989; Koshioka *et al.,* 1994; Serrani *et al.,* 2007a) and overexpression of genes of indole-3-acetic acid (IAA) biosynthesis (Pandolfini *et al.,* 2002), induce fruit-set and growth in tomato, generally more efficiently that GAs. Moreover, transcriptome analysis of expanding locular cells from pollinated fruits show preferential expression of genes involved in synthesis, transport and response to auxins in this tissue (Lemaire-Chamley *et al.,* 2005), and transgenic lines displaying downregulation of *SlIAA9*, an *Aux/IAA* gene, present parthenocarpic fruit development capability (Wang *et al.,* 2005). Interestingly, mutations in the *AUXIN RESPONSE FACTOR8* gene induces parthenocarpic development in Arabidopsis, indicating that ARF8 acts as an inhibitor in the absence of fertilization (Goetz *at al*., 2006). All these observations, together with the increase of auxin-like substances (Mapelli *et al.,* 1978) and IAA (Sjut and Bangerth, 1981) content early after anthesis indicate that these hormones are also involved in tomato fruit-set and development. However, GA and auxin application induce different morphological and histological development of fruit tissues. For instance, while parthenocarpic growth induced by auxin is associated with more cell divisions in the mesocarp, GA-induced fruits have much larger mesocarp cells (Serrani *et al.,* 2007a). Also, the presence of pseudoembryos with unknown function in auxin- but not in GA-induced fruits has been reported (Kataoka *et al.,* 2003; Serrani *et al.,* 2007a).

Auxins have been shown to interact with GAs in diverse physiological GA-dependent processes by altering GA metabolism or mode of action. In the case of elongating internodes of pea and tobacco, IAA transported from the apical shoot induces the synthesis of GA_1, the active hormone stimulating internode elongation, by upregulating in both cases the expression of GA biosynthetic genes, and downregulating the expression of a *GA2ox* gene in the case of pea (O´Neil and Ross, 2002; Ross *et al.,* 2002). Auxins have also been shown to control the expression of genes encoding enzymes involved in GA metabolism in Arabidopsis seedlings (Frigerio *et al.,* 2006). Fruit-set and growth in pea depend on GAs, whose content in the pericarp increases upon pollination (Rodrigo *et al.,* 1997). 4-Cl-IAA, presumably synthesized in fertilized

pea ovules, enhances transcript levels encoding GA20ox (Ngo *et al.,* 2002) and GA3ox (Ozga *et al.,* 2003) in the pericarp. On the other hand, Arabidopsis root elongation is controlled by GAs through degradation of DELLA proteins (repressors of the mode of action of GAs; Sun and Gubler, 2004). In this case, existence of cross-talk between auxin and GAs has been demonstrated by showing that RGA degradation is IAA dependent (Fu and Harberd, 2003).

In this work we have investigated the interaction between auxin and GA content during tomato fruit-set using the dwarf cv Micro-Tom, a BR-deficient mutant (Meissner *et al.,* 1997; Scott and Harbaugh, 1989). The phenotype of this cultivar is the result of several point mutations (*D, SP* and *Ilr*), but not of GA deficiency (Martí *et al.,* 2006), and has been shown to constitute a good experimental system to investigate the hormone regulation of fruit-set and growth in tomato (Serrani *et al.,* 2007a, b). We have found, using inhibitors of GA biosynthesis, that induction of fruit-set induced by auxins is mediated by GAs. Auxin alters GA metabolism and increases active GA_1 level in unpollinated ovaries through upregulation of genes encoding enzymes of GA biosynthesis (*CPS* and *GA20ox*) and downregulating at least one gene encoding a GA2ox enzyme.

Results

Inhibitors of GA biosynthesis reduce auxin-induced parthenocarpic fruit-set and growth

To investigate whether the development of auxin-induced fruits depends on GAs, unpollinated tomato ovaries were treated with 2,4-dichlorophenoxyacetic acid (2,4-D) the day equivalent to anthesis, in the absence or presence of two different kinds of inhibitors of GA biosynthesis: LAB 198999, an acylcyclohexanedione derivative which inhibits 2-oxoglutarate-dependent dioxygenases (Santes and García-Martínez, 1995), and Paclobutrazol (PCB), an inhibitor of P450-dependent monooxygenases (Hedden and Graebe, 1985). LAB 198999 was applied directly to ovaries and PCB to the roots. Inhibitors were also applied to unpollinated and pollinated ovaries not treated with 2,4-D, as controls.

In pollinated ovaries, fruit-set was totally inhibited by PCB application, but in the case of LAB 198999 only final fruit weight was reduced (Figs 1A, B). This agrees

with previous results showing that GAs are necessary for fruit-set and growth in tomato (Fos *et al.,* 2000; Serrani *et al.,* 2007b). In unpollinated ovaries treated with two different doses of 2,4-D (6 and 20 ng), PCB reduced fruit-set and weight, an effect which was reversed by exogenous GA_3 (Fig. 1A). In the case of LAB 198999, both fruit-set and final fruit size decreased with 6 ng 2,4-D, and only fruit weight with 20 ng 2,4-D. This inhibition was fully reversed with GA_3 application (Fig. 1B). As shown in Fig. 1C, 2,4-D-induced fruits treated with PCB were similar to fruits non-treated with PCB, but smaller, and the locular cavities were filled with locular gel. The application of GA_3 reversed the effect of PCB on fruit size (Fig. 1C).

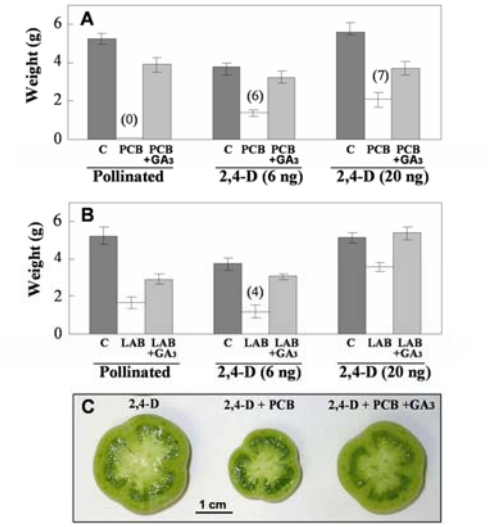

Figure 1. Inhibition by PCB (A) and LAB 198999 (B) of fruit-set and growth of pollinated and parthenocarpic fruits induced by 2,4-D (6 and 20 ng), and reversion by GA_3. (C) Photography of representative parthenocarpic fruits induced by 2,4-D (6 ng), alone or plus PCB without or with GA_3. Fruits were collected 20 d after anthesis or hormone treatment. Values are means of eight fruits ± SE, except when otherwise specified. Values in brackets indicate the number of fruits set when less than eight. PCB was applied at 10^{-5} M to the pots, and LAB 198999 (0.1 M) and GA_3 (2000 ng) to the ovaries.

We wanted to know whether the results obtained with 2,4-D, a synthetic auxin, were also valid for indole-3-acetic acid (IAA), an endogenous auxin in tomato (Kojima *et al.,* 2002; Varga and Bruinsma, 1976). IAA was less efficient than 2,4-D: poorer locular tissue development occurred in the first case (compare Figs 1 and 2). We used a dose of PCB (the most efficient GA biosynthesis inhibitor; Fig. 1) that did not affect the response of unpollinated ovaries to GA_3 (Fig. 2A). PCB application reduced both fruit-set and fruit weight of IAA-induced ovaries, effects which were reversed by GA_3

application (Figs 2A, B). Interestingly, IAA plus GA_3 application had a synergistic effect on fruit growth (Figs 2A and B).

Auxins have been reported to induce formation of pseudoembryos (embryo-like or embryoid structures, originated from division of the innermost integument cells and formed in the ovule cavity) (Kataoka *et al.,* 2003; Serrani *et al.,* 2007a). Application of GA biosynthesis inhibitors to auxin-induced ovaries did not affect pseudoembryo development (data not presented).

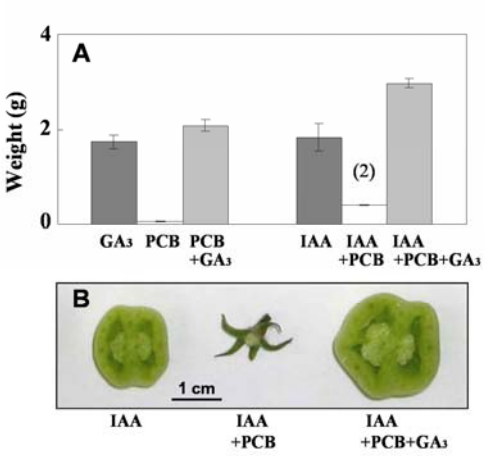

Figure 2. (A) Effect of PCB on parthenocarpic fruit induction by GA_3 and IAA and reversion by GA_3. (B) Photography of representative parthenocarpic fruits induced by IAA, alone or plus PCB without or with GA_3. Fruits were collected 20 d after hormone application. Values are means of eight fruits ± SE, except when otherwise specified. Values in brackets indicate the number of fruits set when less than eight. PCB was applied at 10^{-5} M to the pots, and LAB 198999 (0.1 M), GA_3 (2 µg) and IAA (2 µg) to the ovaries.

Parthenocarpic fruits induced by2,4-D contain high GA levels

The concentration of GA_{53}, GA_{44}, GA_{19}, GA_{20}, GA_{29}, GA_1 and GA_8, GAs from the early-13-hydroxylation pathway (Supplementary Fig. 1) (the main GA metabolic pathway in tomato; Fos *et al.,* 2000), was quantified in 10-d-old pollinated, and unpollinated ovaries treated with 2,4-D. Unpollinated ovaries untreated with 2,4-D were not analyzed because they barely grow, compared with their weight at d0, and started to degenerate at d10.

The content of GA_1, the active GA during fruit-set in tomato (Serrani *et al.,* 2007b), was about five times higher in 2,4-D-induced than in pollinated fruits (Table 1). GA_8 content was also higher (about 30%) in 2,4-D-induced fruits (Table 1). In contrast, the concentration of all precursors of GA_1 (GA_{53}, GA_{44}, GA_{19} and GA_{20}) were similar in pollinated and 2,4-D-induced fruits (Table 1). Only the content of GA_{29}, a metabolite of GA_{20}, was lower (about 70%) in 2,4-D-induced fruits (Table 1).

In order to examine whether the high content of GA_1 and its precursors in 2,4-D-induced fruits was a result of growth induced by 2,4-D, GA concentration of parthenocarpic fruits induced by GA_3 application was also determined (Table 1). As expected, GA_3 content in these fruits was relatively high (almost 61 ng g^{-1}). GA_1 was also present in these fruits at 3.7 ng g^{-1}, higher concentration than in pollinated, but lower than in 2,4-D-induced fruits. This GA_1 may come mainly from the applied solution (where it was present at about 3% of applied GA_3). In contrast, the concentration of all the GA_1 precursors and GA_{29}, was much smaller in GA_3-induced than in pollinated and 2,4-D-induced fruits (Table 1).

	Weight (g $fruit^{-1}$)	GA_{53}	GA_{44}	GA_{19}	GA_{20}	GA_{29}	GA_1	GA_8	GA_3
Pollinated	1.26	0.6	2.3	8.7	26.6	9.7	1.1	22.9	-
	± 0.06	± 0.1	± 0.4	± 1.1	± 1.9	± 0.6	± 0.3	± 2.4	
Unpollinated	1.51	0.4	2.3	10.0	22.2	5.7	5.3	31.5	-
+ 2,4-D	± 0.07	-	± 0.3	± 0.9	± 0.2	± 0.6	± 0.4	± 2.1	
Unpollinated	0.55	0.2	0.7	1.8	14.1	0.3	3.7	13.8	60.9
+ GA_3	± 0.03	± 0.05	± 0.1	± 0.5	± 1.5	± 0.05	± 0.2	± 1.2	± 3.5

Table 1. Endogenous GA content (ng g fresh $weight^{-1}$) of pollinated and unpollinated fruits induced by 2,4-D or GA_3 application**.** Fruits were collected 10 d after pollination and 2,4-D (200 ng) or GA_3 (2000 ng) application. Weight data are means of eight fruits, and GA data from three biological replicates (aliquots of about 5 g each) ± SE.

Effect of 2,4-D on in vivo metabolism of GAs in unpollinated ovaries

[17-^{14}C]GA_{12} and [17-^{14}C]GA_{53}, substrates of GA 20-oxidases, [17-^{14}C]GA_{20}, substrate of GA 3-oxidase, and [17-^{14}C]GA_{20} and [17-^{14}C]GA_1 (both from the early-13-hydroxylation pathway), purported substrates of GA 2-oxidases (see Supplementary Fig. 1), were applied to unpollinated ovaries from emasculated flowers to know the possible effect of 2,4-D on in vivo GA metabolism. GA_{12} and GA_{53} were not metabolized in untreated ovaries whereas GA_{12} was converted to a compound with the same retention time as GA_9 (Fig. 3) in 2,4-D treated ovaries. GA_{20} was metabolized to a compound with the same retention time as GA_1 in untreated and 2,4-D-treated ovaries, but this metabolite was much more abundant in the latter case (Fig. 3). Interestingly, a

compound with the same retention time as GA_{29} (a GA2ox metabolite of GA_{20}) was present in untreated but not in 2,4-D-treated ovaries (Fig. 3). Finally, GA_1 was metabolized to a compound with the same retention time as GA_8 in untreated and treated ovaries, but much less in the latter case (Fig. 3).

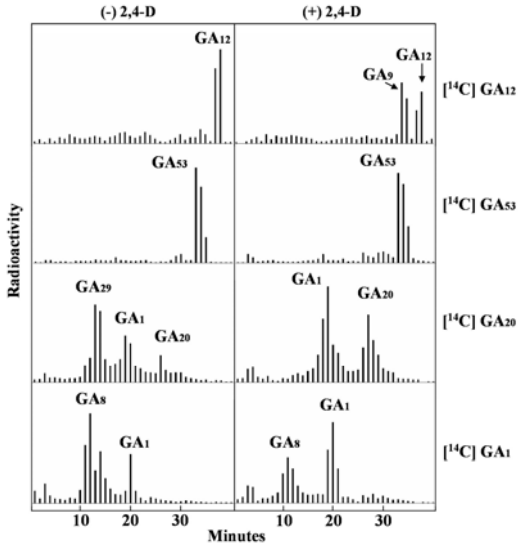

Figure 3. Radioactive HPLC traces of metabolites of [17-^{14}C]GA_{12}, [17-^{14}C]GA_{53}, [17-^{14}C]GA_{20} and [17-^{14}C]GA_1 applied to unpollinated ovaries. The GAs, without or with 2,4-D (200 ng), were applied at d0 and ovaries collected 2 days after application. See more details in Experimental Procedures.

These results show that the high content of GA_1 and its precursors in parthenocarpic 2,4-D-induced ovaries is a consequence of an alteration of GA metabolism, mainly of an increase of GA biosynthesis (GA20ox activity) and reduction of GA inactivation (GA2ox activity).

Effect of 2,4-D on transcript levels of genes encoding enzymes of GA biosynthesis

To investigate whether the altered in vivo GA metabolism in 2,4-D-induced fruits was the result of a modification of transcript activity of GA metabolism genes, we compared transcript levels of *SlCPS*, *SlGA20ox1*, *-2* and *-3*, and *SlGA3ox1* and *-2*, which encode three kinds of GA biosynthesis enzymes (Rebers *et al.,* 1999), in unpollinated ovaries untreated or treated with 2,4-D, by semiquantitative RT-PCR.

Expression of *SlCPS* was detected in non-treated ovaries before anthesis (d-3) but it was very low later on (from 0 to 20 day post anthesis, dpa). In contrast, *SlCPS* transcript levels in 2,4-D treated fruits did not decrease after anthesis and therefore were

much higher than in non-treated ovaries. *SlCPS* transcripts were present both in pericarp and locular gel of 10- and 20-d-old 2,4-D-induced fruits (Fig. 4).

In unpollinated and non-treated ovaries, expression of *SlGA20ox1* (between d-3 and 20 dpa) was not detected (Fig. 4). In the case of *SlGA20ox2*, high expression was found before anthesis (d-3), but dropped to undetected or very low expression between d0 and d20 in non-treated ovaries (Fig. 4). *SlGA20ox3* transcript levels of non-treated ovaries were relatively low and constant between d-3 and d20 (Fig. 4). In contrast, *SlGA20ox1*, *-2* and *-3* transcript levels were high in the entire fruit as well as in the pericarp and locular gel of 2,4-D-induced ovaries 5 and 10 d after hormone application (Fig. 4). At 20 d after 2,4-D application *SlGA20ox3* transcript contents were also high in entire fruit, pericarp and locular gel, but only in the locular gel in the case of *SlGA20ox1* and *-2* (Fig. 4). *SlGA3ox1* transcripts were at relatively high level in unpollinated ovaries before anthesis (d-3), and decreased progressively from the day equivalent to anthesis until d20 (Fig. 4). At 10 and 20 dpa, the expression of *SlGA3ox1* was higher in 2,4-D-induced than in non-induced ovaries, transcripts being concentrated mostly in the locular gel (Fig. 4). In the case of *SlGA3ox2* transcripts were detected in ovaries before anthesis, but were at very low level or not detected in unpollinated ovaries both untreated and treated with 2,4-D (Fig. 4).

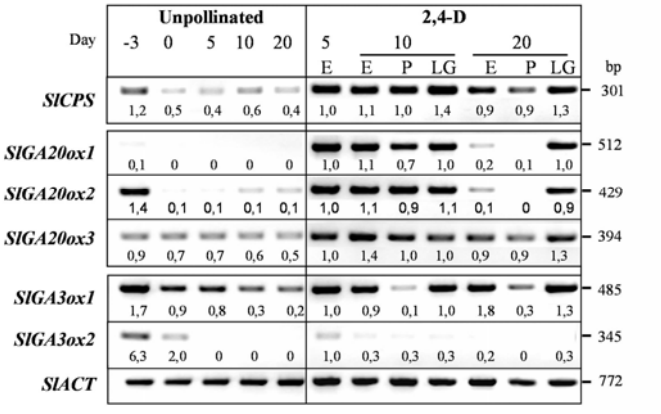

Figure 4. Effect of 2,4-D application to unpollinated ovaries on transcript levels of *SlCPS*, *SlGA20ox1, -2* and *-3,* and *SlGA3ox1* and *-2* genes. Semiquantitative transcript analysis was carried out by RT-PCR, as described in Experimental procedures, using total RNA from unpollinated d0, d5, d10 and d20 ovaries untreated or treated with 2,4-D (200 ng). For each gene, the number below the blots mean normalized values of gene expression versus that of *Actin* (internal control), using entire 5-d-old 2,4-D-induced fruits as reference (set 1.0). E, entire ovaries; P, pericarp; LG, locular gel, including unfertilized ovules. Data come from a representative experiment out of two biological replicates with similar results.

Effect of 2,4-D on transcript levels of genes encoding enzymes of GA inactivation

To determine whether the high GA content in 2,4-D-induced ovaries was also due to reduced GA-inactivation activity, transcript levels of *SlGA2ox1*, *-2*, *-3*, *-4* and -5, genes encoding GA 2-oxidases (considered as the main GA catabolic enzymes) in tomato (Serrani *et al.*, 2007b) were estimated by semiquantitative RT-PCR.

SlGA2ox1, -2, -4 and *-5* transcript content in unpollinated non-treated ovaries was high and constant between d-3 and d20, with the exception of *SlGA2ox5,* which could not be detected at d-3 (Fig. 5). In contrast, *SlGA2ox3* transcript levels were very high at d-3, slightly detectable between d0 and d10 and almost undetected at d20 (Fig. 5). In the case of unpollinated ovaries treated with 2,4-D, expression of *SlGA2ox1* at 5 d and 10 d after treatment was similar to that of non-treated ovaries of similar age, but decreased at 20 d in entire fruit and in the pericarp (while remaining high in the locular gel) (Fig. 5). Expression of *SlGA2ox2* decreased between 5 and 20 d after 2,4-D application (at least 4-fold compared with those non-treated). In the case of *SlGA2ox3*, expression at d5 was similar to that of untreated ovaries, but essentially undetectable at 10 and 20 d (Fig. 5). *SlGA2ox4* transcript contents in 2,4-D induced fruits 5- and 10-d-old were similar to those of untreated ovaries of similar age, but decreased at d20 (Fig. 5). In the case of *SlGA2ox5* transcript level did not decrease at d5, but was much lower at d10 and d20 (Fig. 5). As occurred with *SlGA2ox1*, transcript levels of *SlGA2ox4* and *SlGA2ox5* remained high in the locular gel.

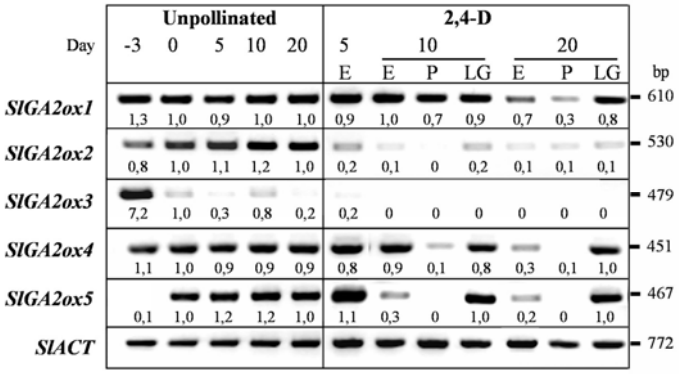

Figure 5. Effect of 2,4-D application to unpollinated ovaries on transcript levels of *SlGA2ox1, -2, -3, -4* and -5 genes. Semiquantitative transcript analysis was carried out by RT-PCR, as described in Experimental Procedures, using total RNA from unpollinated d0, d5, d10 and d20 ovaries untreated or treated with 2,4-D (200 ng). For each gene, the gene expression versus that of *Actin* (internal control), using unpollinated d0 ovaries as reference (set 1.0).E, entire ovaries; P, pericarp; LG, locular gel, including unfertilized ovules. Data come from a representative experiment out of two biological replicates with similar results.

Discussion

The results presented in this work show that parthenocarpic growth in tomato induced by auxin application is mediated by GAs. This conclusion was supported by the observation that fruit-set and growth of unpollinated ovaries induced by 2,4-D or IAA was negated or significantly reduced in the presence of inhibitors of GA biosynthesis, and the effect of these inhibitors was reversed by GA_3 application (Figs 1 and 2). Parthenocarpic fruit-set and growth in tomato can be induced by GA or auxin treatment to unpollinated ovaries (Serrani *et al.,* 2007a), and experiments of GA biosynthesis inhibitors application to pollinated ovaries support the hypothesis that tomato fruit set and growth depend on GAs synthesized after pollination and fertilization (Fos *et al.,* 2000; Serrani *et al.,* 2007b). The absence of inhibitors of auxin biosynthesis has not allowed to carry out experiments with this kind of hormones similar to those performed with GA to get direct evidence on their role in tomato fruit-set.

The application of 2,4-D induced parthenocarpic fruit-set and growth associated with high content of GA_1 (the active GA in tomato fruit-set; Serrani *et al.,* 2007b) in the ovary, as well as of its precursors and GA_8 (a GA2ox metabolite) (Table 1). The levels of GA_1 precursors were similar in 2,4-D-induced and in pollinated fruits, and those of GA_1 and GA_8 were even higher in the former (Table 1). This is in agreement with the idea that the effect of 2,4-D on tomato fruit-set is mediated by GAs through enhancement of GA biosynthesis. It could be argued that the high GA content in 2,4-D-induced fruits might be the consequence of growth induced by auxin, rather than a direct auxin effect. To test this hypothesis we induced parthenocarpic growth by applying GA_3 (a GA found at very low levels or undetected in tomato ovaries and fruit; Fos *et al.,* 2000; 2001) and quantified GAs in the fruits. GA_1 was found in GA_3-treated ovaries at a concentration about 6% the amount of GA_3. Since the GA_3 used to induce fruit-set was not absolutely pure (it contained GA_1 at about 3.3% the amount of GA_3, as determined by GC-SIM), GA_1 found in the fruit may come probably as a contaminant from the GA_3 solution. However, it is difficult to explain why the GA_1/GA_3 ratio in the fruit was higher than in the applied solution taking into account the higher metabolic stability of GA_3 (Santes and García-Martínez, 1995). A possibility to explain this apparent contradiction is that GA_3 was better transported out of the ovary than GA_1,

leading to the increase of that ratio in the fruit. In contrast, all the GA_1 precursors analyzed (GA_{53}, GA_{44}, GA_{19} and GA_{20}) were at much lower levels than in pollinated and unpollinated 2,4-D-induced fruits (Table 1). This means that fruit growth in GA_3-induced fruits was not associated to higher GA metabolism biosynthesis. The lower GA_{44}, GA_{19} and GA_{20} contents in GA_3 treated ovaries might be also a consequence of negative feed-back regulation of GA20ox, which is operative at least for *SlGA20ox1* (E. Martí and J.L. García-Martínez, unpublished results). But this possibility was not supported by the absence of accumulation of GA_{53} (a substrate of GA20ox) (Table 1). Lack of positive feed-back regulation of GA2ox was also found in GA_3-induced fruits because much lower content of GA_{29} (a GA2ox metabolite of GA_{20}) was found in GA_3-induced fruits (Table 1). This low GA_{29} content further supports the idea that GA metabolism was not stimulated in GA_3-induced fruits.

In vivo metabolism analysis showed that 2,4-D altered several aspects of GA metabolism in unpollinated ovaries. It was quite clear that 2,4-D induced the conversion of GA_{12} to putative GA_9, probably due to higher GA20ox activity (Fig. 3). Curiously, GA_{53} metabolism was not induced by 2,4-D in spite that the early-13-hydroxylation-pathway is considered to be the main one in tomato (Fos *et al*., 2000). This means that the activity of the three known SlGA20ox using GA_{12} and GA_{53} as substrates should be determined, and the possible importance of the non-13-hydroxylation pathway furher investigated. The use of GA_{20} and GA_1 as substrates of in vivo metabolism also showed that 2,4-D decreased GA2ox activity in both cases (Fig. 3).

The increase of GA content in 2,4-D-induced growing fruits was probably the result of its effect on higher transcription of genes encoding CPS and GA20ox1, -2 and -3 (GA biosynthetic enzymes), but not GA3ox (Fig. 4). In Arabidopsis, *AtCPS* transcripts are present in young developing tissues, probably the main sites of GA biosynthesis (Silverstone *et al.,* 1997). CPS (formerly *ent*-kaurene synthase A) activity has been shown to be present in extracts of tomato fruits (Bensen and Zeevaart, 1990), and the higher transcript levels of *SlCPS* suggests that early biosynthetic enzymes may contribute to the increase of GA content. 2,4-D application seems to prevent downregulation of that gene in unpollinated ovaries, whose transcript level are relatively high before anthesis. Only the expression of *SlGA2ox2* (out of the five genes encoding GA2ox analyzed) showed early downregulation in 2,4-D-induced fruits (Fig. 5).

Nevertheless, the possible effect of 2,4-D on GA inactivation through GA2ox is not clear because it has been found that the protein encoded by *SlGA2ox2* can be inactive (Serrani *et al.,* 2007b). Indeed, our results do not discard the possibility that 2,4-D may increase GA_1 content by also downregulating other kinds of GA inactivating genes, shown to regulate GA homeostasis through 16α,17-epoxidation (Zhu *et al.,* 2006) and methylation (Varbanova *et al.,* 2007). Interestingly, genes encoding SlCPS and SlGA20ox1, -2 and -3 were also found to be upregulated in young pollinated ovaries associated with GA content increase (Serrani *et al.,* 2007b). In pollinated ovaries, as in 2,4-D-induced fruits, no upregulation of gene encoding SlGA3ox2 was observed either (Serrani *et al.,* 2007b), but those of SlGA3ox1 in 2,4-D induced fruits. and maybe that contributes to a higer GA_1 content in 2,4-D treated fruits. These results suggest that pollination and/or fertilization alters GA metabolism through auxin, probably synthesized in the fertilized seeds (Varga and Bruinsma, 1986). But in contrast to 2,4-D-induced fruits (where *SlGA2ox2* expression was downregulated) no early *GA2ox* downregulation was observed in pollinated ones (see Fig. 5 and Serrani *et al.,* 2007b) (see proposed scheme on auxin mode of action in Fig. 6). Our results in tomato are comparable to the promotion of GA biosynthesis in pea by 4-Cl-IAA, because application of this auxin to deseeded pods increases transcription of both *GA20ox* (Ngo *et al.,* 2002) and *GA3ox* (Ozga *et al.,* 2003) genes in the pea pericarp. Downregulation of *PsGA2ox1* and upregulation of *PsGA2ox2* by 4-Cl-IAA in pea pericarp has been reported recently (Ozga *et al*., 2007). Regulation of GA metabolism by auxins has also been found in vegetative tissues. For instance, internode elongation depends on GAs, and it has been shown that the level of GA_1 (the active form) is regulated by IAA transported basipetally from the apical shoot through upregulation of *GA20ox* in tobacco (Wolbang and Ross, 2001), of *GA20ox* and *GA3ox* in pea (O´Neill and Ross, 2002), and of *GA3ox* in barley (Wolbang *et al.,* 2004). In the case of pea and barley downregulation of *GA2ox* genes was also detected. Application of a synthetic auxin (naphthaleneacetic acid) to Arabidopsis seedlings regulates differentially the expression of several *GA20ox* and *GA2ox* too (Frigerio *et al.,* 2006). All these results show that the mechanism regulating GA metabolism by auxin may vary with the species (and maybe tissue), enhancing the expression of different genes encoding enzymes of GA

biosynthesis in some cases, and/or downregulating the expression of GA inactivation genes in other cases.

In summary, we have shown that the effect of auxin on parthenocarpic tomato fruit-set is mediated by GAs. Auxins, probably synthesized in the seeds following pollination and ovule fertilization, increase active GA content in the fruit by upregulating genes encoding enzymes of GA biosynthesis (CPS, GA20ox, GA3ox) and downregulating at least one gene encoding GA inactivating enzymes (GA2ox), and thus inducing fruit-set. Auxins have probably other additional effects on tomato fruit development, independent of GAs (Fig. 6), because fruits induced by GA have very poor locular tissue development, in contrast to those induced by pollination and auxins (Serrani *et al*., 2007a). This hypothesis is also supported by the observation that simultaneous application of GA_3 and IAA had a synergistic effect on fruit growth (Fig. 2).

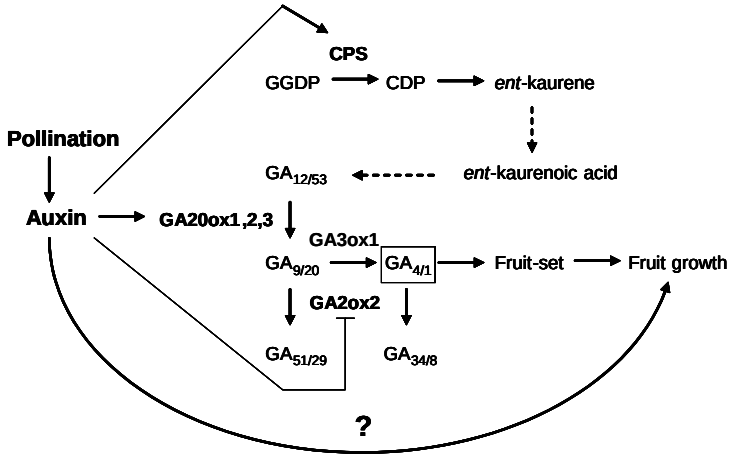

Figure 6. Proposed interaction of auxin and GAs during tomato fruit-set and growth.

Experimental procedures

Plant material and growth conditions

Plants of tomato (*Solanum lycopersicum* L.) cv Micro-Tom (seeds obtained originally from Dr A Levy) were used in the experiments. Plants (one per pot) were grown in 1 L pots with a mixture of peat:vermiculite (1:1), cultured in a greenhouse

under 24ºC (day)/ 20ºC (night) conditions, and irrigated daily with Hoagland´s solution. Natural light was supplemented with Osram lamps (Powerstar HQI-BT, 400W) to get a 16 h light photoperiod.

Only one flower per truss and the first two trusses were left per plant for the experiments, as described previously (Serrani *et al*., 2007a), unless otherwise stated. All non-selected flowers were removed 2 d before anthesis.

Plant hormone applications

Application of 2,4-D (Duchefa, Haarlem, The Netherlands), IAA (Duchefa), and GA_3 (gift of Dr Michel Beale, Rothamsted Research, UK) was carried out to unpollinated ovaries the day equivalent to anthesis, in 10 µl of 5% ethanol, 0.1% Tween 80 solution. GC-SIM analysis of the GA_3 used in the experiments showed that it also contained some GA_1 (3.3% the amount of GA_3). Flower emasculation was carried out 2 d before anthesis to prevent self-pollination. 0.1 M LAB 198999 (3,5-dioxo-4-butyryl-cyclohexane carboxylic acid ethyl ester) (BASF, Limbergerhof, Germany) was applied in 5% ethanol, 0.1% Tween solution, 10 µl per ovary the day equivalent to anthesis. In case of pollinated ovaries, LAB 198999 was applied 2 d after anthesis, after removal of petals and stamens, to ascertain that pollination was not affected by the inhibitor solution. PCB (Duchefa) was applied to the roots in the nutrient solution at 10^{-5} M every two days, starting when flowers on which the effect of the inhibitor was going to be determined were about 7 d before anthesis (estimated by flower bud size) so it would transported in time to the ovary. Equal volume of solvent solution was applied to control ovaries (in the case of LAB 198999) and to the culture medium (case of PCB).

Quantification of gibberellins

GAs were quantified following the protocol described in Fos *et al*. (2000). Briefly, aliquots (about 5 g fresh weight) of frozen material were extracted with 80% methanol and, after removing the organic phase, the water fraction was partitioned against ethyl acetate and purified by QAE-Sephadex chromatography and C_{18} cartridges. The GAs where then separated by reverse phase HPLC chromatography (4-µm C_{18} column, 15 cm long, 3.9 mm i.d.; NovaPak, Millipore, Milford, MA), and appropriate fractions grouped for GC-SIM analysis after methylation and

trimethylsililation. [17,17-^{2}H]GA_1, [17,17-^{2}H]GA_3, [17,17-^{2}H]GA_8, [17,17-^{2}H]GA_{19}, [17,17-^{2}H]GA_{20}, [17,17-^{2}H]GA_{29}, [17,17-^{2}H]GA_{44} and [17,17-^{2}H]GA_{53} (purchased from Prof. L Mander, Australian National University, Canberra) were added to the extracts as internal standards for quantification, and [^{3}H]GA_{20} and [^{3}H]GA_9 (purchased from Prof. L. Mander) to monitor the separation of GAs after HPLC using a 10 to 100% methanol gradient containing 50 µl acetic acid per litre and taking 1 ml fractions. Quantification was carried out by GC-SIM using a gas chromatograph (model 5890, Hewlett-Packard, Palo Alto, CA) coupled to a mass-selective detector (model 5971A, Hewlett-Packard). The concentrations of GAs in the extracts were determined using the calibration curves methodology.

In vivo metabolism GA metabolism

Unpollinated ovaries from emasculated flowers were treated the day equivalent to anthesis (d0) with [17-^{14}C]GA_1, [17-^{14}C]GA_{20}, [17-^{14}C]GA_{53} and [17-^{14}C]GA_{12} solutions (purchased from Prof. L. Mander; 34-55 µCi µmol^{-1}) (10 000 dpm ovary^{-1} in 10 µl of 10% methanol, two replicates of 12 ovaries per treatment) without or with 2,4-D (200 ng ovary^{-1}). Fruits (ovaries) were harvested 48 h after treatment, frozen in N_2 and kept at -80 ºC until analysis. Ground ovaries (12 per replicate and treatment) were extracted overnight at 4 ºC in 80% methanol with agitation, centrifuged at 13000 rpm and reextracted twice for 20 min in 100% methanol. The joined supernatants were taken to dryness, the residue dissolved in 10% methanol and the metabolic products separated by HPLC, as described before for quantification of GAs. Metabolites were detected using an on-line radioactive monitor (Radioflow Detector LB 508, Berthold Technologies) and identified by their retention times compared to pure GAs. Only data from one replicate, out of two with similar results, are given in Results.

Semiquantitative RT-PCR

Total RNA was isolated from ovaries about three days before anthesis (d-3), and from unpollinated ovaries at 0, 5, 10 and 20 dpa, either untreated or treated with 2,4-D solution. Entire fruits were taken 5 dpa, and pericarp and locular gel plus placenta tissues (including unfertilized ovules) were separated in the case of 10- and 20-d-old fruits. RNA was treated with DNAse, according to manufacturer's protocol using an

RNAeasy Plant Mini Kit (Quiagen, Courtaboeuf Cedex, France). Then, 2 µg of total RNA were reverse transcribed with a First-strand cDNA Synthesis Kit (Amersham Biosciences, Buckinghamshire, UK) in 33 µl total volume reaction. PCRs were performed taking 1 µl aliquots 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), and 1 µM of the appropriate pair of primers (Supplementary Table 1). PCR conditions for amplification of *SlCPS*, *SlGA20ox1, -2* and *-3* and *SlGA3ox1* and *-2* consisted of initial denaturation at 94ºC for 2 min, followed by 32 cycles of 94ºC/ 30 sec, 57ºC/ 60 sec and 72ºC/ 60 sec, and finally 10 min extension at 72ºC. For amplification of *SlGA2ox1, -2, -3, 4* and *-5*, 31 cycles were used with annealing temperatures of 60ºC (*SlGA2ox1*) or 62ºC (*SlGA2ox2*, *-3*, *-4* and *-5*), and for *SlCPS* 33 cycles and 61ºC. In the case of *Actin* annealing temperature of 60ºC and 24 cycles were used. In all cases, the number of cycles was chosen to give amplified products within the linear synthesis reaction. 15 µl aliquots of PCR products were separated on 1% agarose gel electrophoresis. The spots were stained with ethidium bromide, visualized under UV using a GeneGenius Bio Imaging System (Syngene, Frederick, MD, USA), captured with the GeneSnap program (Syngene) and quantified with the GeneTools software (Syngene). Expression was normalized using Actin as internal control, by comparing expression ratios to that of the tissues indicated in the Figure legends (set to 1.0).

The analyses were carried out in duplicate using biologically independent material, with similar results. Only data from one representative replicate are given under Results.

Acknowledgments

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References

Abad, M., Monteiro, A.A. (1989) The use of auxins for the production of greenhouse tomatoes in mild-winter conditions: a review. *Scientia Hortic*. **38**, 167-192.

Alabadí, D., Carbonell, J. (1998) Expression of ornithine decarboxylase is transiently increased by pollination, 2,4-dichlorophenoxyacetic acid, and gibberellic acid in tomato ovaries. *Plant Physiol.* **118**, 323-328.

Bensen, R.J., Zeevaart, J.A.D. (1990) Comparison of *ent*-kaurene synthetase A and B activities in cell-free extracts from young tomato fruits of wild-type and *gib-1*, *gib-2*, and *gib-3* tomato plants. *J Plant Growth Regul.* **9**, 237-242.

Fos. M., Nuez, F., García-Martínez, J.L. (2000) The Gene *pat-2*, Which Induces Natural Parthenocarpy, Alters the Gibberellin Content in Unpollinated Tomato Ovaries. *Plant Physiol*. **122**, 471-479.

Fos. M., Proaño, K., Nuez, F., García-Martínez, J.L. (2001) Role of gibberellins in parthenocarpic fruit development induced by the genetic system *pat-3/pat-4* in tomato. *Physiol Plant.* **111**, 545-550.

Frigerio, M., Alabadí, D., Pérez-Gómez, J., García-Cárcel, L., Phillips, A.L., Hedden, P., Blázquez, M.A. (2006) Transcriptional Regulation of Gibberellin Metabolism Genes by Auxin Signalling in *Arabidopsis*. *Plant Physiol.* **142**, 533-563.

Fu, X., Harberd, N.P. (2003) Auxin promotes arabidopsis root growth by modulating gibberellin response. *Nature*, **421**, 740-743.

García-Martínez, J.L., Hedden, P. (1997) Gibberellins and fruit development. In: *Phytochemistry of Fruit and Vegetables* (Tomás-Barberán, F. A., Robins, R.J., eds). Oxford: Clarendon Press, pp 263-286.

García-Martínez, J.L., Santes, C.M., Croker, S., Hedden, P. (1991) Identification, quantitation and distribution of gibberellins in fruit of *Pisum sativum* cv. Alaska during pod development. *Planta* **184**, 53-60.

Gillaspy, G., Ben-David, H., Gruissem, W. (1993) Fruits: A Developmental Perspective. *Plant Cell* **5**, 1439-1451.

Goetz, M., Vivian-Smith, A., Johnson, S.D., Koltunova, A.M. (2006) *AUXIN RESPONSE FACTOR8* Is a Negative Regulator of Fruit Initiation in *Arabidopsis*. *Plant Cell* **18**, 1873-1886.

Gorquet, B., Van Heusden, A.W., Lindhout, P. (2005) Parthenocarpic fruit development in tomato. *Plant Biol***. 7**, 131-139.

Hedden, P., Graebe, J.E. (1985) Inhibition of gibberellin biosynthesis by Paclobutrazol in cell-free homogenates of *Cucurbita maxima* endosperm an *Malus pumila* embryos. *J Plant Growth Regul***. 4**, 111-122.

Ho, L., Hewitt, J. (1986) Fruit development. In: *The Tomato Crop* (Atherton, J.G., Rudish, J., eds). New York: Chapman and Hall, pp. 201-239.

Kataoka, K., Uemachi, A., Yazawa, S. (2003) Fruit growth and pseudoembryo development affected by uniconazol, an inhibitor of gibberellin, in *pat-2* and auxin-induced parthenocarpic tomato fruits. *Scientia Hortic***. 98**, 9-16.

Kojima, K., Ohtake, E., Yu, Z. (2002) Distribution and transport of IAA in tomato plants. *Plant Growth Regul***. 41**, 99-104.

Koshioka, M., Nishijima, T., Yamazaki, H., Liu, Y., Nonaka, M., Mander, L.N. (1994) Analysis of gibberellins in growing fruits of *Lycopersicon esculentum* after pollination or treatment with 4-chlorophenoxyacetic acid. *J Hort Sci.* **69**, 171-179.

Lemaire-Chamley, M., Petit, J., García, V., Just, D., Baldet, P., Germain, V., Fagard, M., Mouassite, M., Cheniclet, C., Rothan, C. (2005) Changes in Transcriptional Profiles are Associated with Early Fruit Tissue Specialization in Tomato. *Plant Physiol***. 139**, 750-769.

Mapelli, S.C., Frova, C., Torti, G., Soressi, G. (1978) Relationship between set, development and activities of growth regulators in tomato fruits. *Plant Cell Physiol***. 19**, 1281-1288.

Martí, E., Gisbert, C., Bishop, G.J., Dixon, M.S., García-Martínez, J.L. (2006) Genetic and physiological characterization of tomato cv. Micro-Tom. *J Exp Bot***. 57**, 2037-2047.

Meissner, R., Jacobson, Y., Melamed, S., Levyatuv, S., Shalev, G., Ashri, A., Elkind, Y., Levy, A.A. (1997). A new model system for tomato genetics. *Plant J.* **12**, 1465-1472.

Ngo, P., Ozga, J.A., Reinecke, D.M. (2002) Specificity of auxin regulation of gibberellin 20-oxidase gene expression in pea pericarp. *Plant Mol Biol.* **49**, 439-446.

Olimpieri, I., Siligato, F., Caccia, R., Mariotti, L., Ceccarelli, N., Soressi, G.P., Mazzucato, A. (2007) Tomato fruit set driven by pollination or by the *parthenocarpic fruit* allele is mediated by transcriptionally regulated gibberellin biosynthesis. *Planta* DOI 10.1007/s00425-007-0533-z

O´Neill, D.P., Ross, J.J. (2002) Auxin Regulation of the Gibberellin Pathway in Pea. *Plant Physiol.* **130**, 1974-1982.

Ozga, J.A., Ayele, B.T., Reinecke, D.M. (2007) Hormonal interactions in fruit development. Abstracts of the 19th Meeting of the International Plant Growth Substances Assotiation, Puerto Vallarta, México. Abstract nº PS0103.

Ozga, J.A., Yu, J., Reinecke, D.M. (2003) Pollination-, Development-, and Auxin-Specific Regulation of Gibberellin 3ß-Hydroxylase Gene Expression in Pea Fruit and Seeds. *Plant Physiol*. **131**, 1137-1146.

Pandolfini, T., Rotino, G.L., Camerini, S., Defez, R., Spena, A. (2002) Optimisation of transgene action at the post-transcriptional level: high quality parthenocarpic fruits in industrial tomatoes. *BMC Biotechnol.* **2**, 1-11.

Rebers, M., Kaneta, T., Kawaide, H., Yamaguchi, S., Yang, Y-Y., Imai, R., Sekimoto, H., Kamiya, Y. (1999) Regulation of gibberellin biosynthesis genes during flower and early fruit development of tomato. *Plant J*. **17**, 241-250.

Rodrigo, M.J., Garcia-Martínez, J.L., Santes, C.M., Gaskin, P., Hedden, P. (1997) The role of gibberellins A_1 and A_3 in fruit growth of *Pisum sativum* L. and the identification of gibberellins A_4 and A_7 in young seeds. *Planta* **201**, 446-455.

Ross, J.J., O´Neill, D.P., Wolbang, C.M., Symons, G.M., Reid, J.B. (2002) Auxin-gibberellin interactions and their role in plant growth. *J Plant Growth Regul.* **20**, 346-353.

Santes, C.M., García-Martínez, J.L. (1995) Effect of the growth retardant 3,5-dioxo-4-butyryl-cyclohexane carboxylic acid ethyl ester, an acylcyclohexanedione compound, on fruit growth and gibberellin content of pollinated and unpollinated ovaries in pea. *Plant Physiol***. 108**, 517-523.

Scott, J.W., Harbaugh, B.K. (1989) Micro-Tom. - a miniature dwarf tomato. *Florida Agr Exp Sta Cir*. **370**, 1-6.

Serrani, J.C., Fos, M., Atarés, A., García-Martínez, J.L. (2007a) Effect of gibberellin and auxin on parthenocarpic fruit growth induction in the cv Micro-Tom of tomato. *J Plant Growth Regul.* **26**, 211-221.

Serrani, J.C., Sanjuan, R., Ruiz-Rivero, O., Fos, M., García-Martínez, J.L. (2007b) Gibberellin Regulation of Fruit-Set and Growth in Tomato. *Plant Physiol.* **145**, 246-257.

Silverstone, A.L., Chang, C.W., Krol, E., Sun, T.P. (1997) Developmental regulation of the gibberellin biosynthetic gene *GA1* in *Arabidopsis thaliana. Plant J*. **12**, 9-19.

Sjut, V., Bangerth, F. (1981) Effect of pollination or treatment with growth regulators on levels of extractable hormones in tomato ovaries and young fruits. *Physiol Plant*. **53**, 76-78.

Sjut, V., Bangerth, F. (1982/1983) Induced parthenocarpy – a way of changing the levels of endogenous hormones in tomato fruits (*Lycopersicon esculentum* Mill.). 1. Extractable hormones. *Plant Growth Regul.* **1**, 243-251.

Sponsel, V., Hedden, V. (2004) Gibberellin biosynthesis and inactivation. In: Plant Hormones: *Biosynthesis, Signal Transduction, Action* (Davies, P., ed.). Dordrecht: Kluwer Acad. Pub. pp. 63-94.

Srivastava, A., Handa. A.K. (2005) Hormonal regulation of tomato fruit development: a molecular perspective. *J Plant Growth Regul.* **24**, 67-82.

Sun, T-P., Gubler, F. (2004) Molecular mechanism of gibberellin signalling in plants. *Ann Rev Plant Biol.* **55**, 197-223.

Van Huizen, R., Ozga, J.A., Reinecke, D.M. (1997) Seed and Hormonal Regulation of Gibberellin 20-Oxidase Expression in Pea Pericarp. *Plant Physiol.* **115**, 123-128.

Varbanova, M., Yamaguchi, S., Yang, Y., McKelvey, K., Hanada, A., Borochov, R., Yu, F., Jikumaru, Y., Ross, Y., Cortes, D., Ma, C.J., Noe,l J.P., Mander, L,, Shulaev, V., Kamiya, Y., Rodermel, S., Weiss, D., Pichersky, E. (2007) Methylation of Gibberellins by *Arabidopsis* GAMT1 and GAMT2. *Plant Cell.* **19**, 32-45.

Varga, A., Bruinsma, J. (1976) Roles of seeds and auxins in tomato fruit growth. *Z. Pflanzenphysiol.* **80**, 95-104.

Wang, H., Jones, B., Li, Z., Frasse, P., Delalande, C., Regad, F., Chaabouni, S., Latché, A., Pech, J-C., Bouzayen, M. (2005) The Tomato *Aux/IAA* Transcription Factor *IAA9* is Involved in Fruit Development and Leaf Morphogenesis. *Plant Cell* **17**, 2676-2692.

Wolbang, C.M., Chandler, P.M., Smith, J.J., Ross, J.J. (2004) Auxin from the Developing Inflorescence is Required for the Biosynthesis of Active Gibberellins in Barley Stems. *Plant Physiol.* **134**, 769-776.

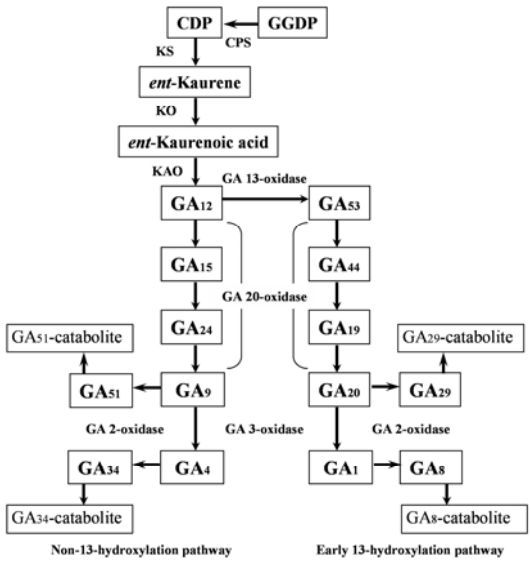
Wolbang, C.M., Ross, J.J. (2001) Auxin promotes gibberellin biosynthesis in decapitated tobacco plants. *Planta* **214**, 153-157.

Zhu, Y., Nomura, T., Xu, Y., Zhang, Y., Peng, Y., Mao, B., Hanada, A., Zhou, H., Wang, R., Li, P., Zhu, X., Mander, L.N., Kamiya, Y., Yamaguchi, S., He, Z. (2006) *ELONGATED UPPERMOST INTERNODE* Encodes a Cytochrome P450 Monooxygenase that Epoxidizes Gibberellins in a Novel Deactivation Reaction in Rice. *Plant Cell* **18**, 442-456.

Supplementary Table 1. Primer sequences used for semiquantitative RT-PCR analysis of diverse GA metabolism genes of tomato. *SlCPS* (AB015675), *SlGA20ox1* (AF049898), *SlGA20ox2* (AF049899), *SlGA20ox3* (AF049900), *SlGA3ox1* (AB010991), *SlGA3ox2* (AB010992), *SlGA2ox1*(EF441351), *SlGA2ox2* (EF441352), *SlGA2ox3* (EF441353), *SlGA2ox4* (EF441354), *SlGA2ox5* (EF441355), and *SlACT (*Actin) (AB199316).

Gene	Sense	Antisense
SlCPS	5'-GGAAAATTGGCTACTGACGGTAGG-3'	5'-GGCATCCAATTCGGAAGCA-3'
SlGA20ox1	5'-GGAGCTCGCCTTAGGAACG-3'	5'-GTAGAAGCTAAGAGAACGTGTACACG-3'
SlGA20ox2	5'-CAACGTCTCAGGACTACAAGTTTTC-3'	5'-AGGCTAAGGTCTTGATCTACATTGG-3'
SlGA20ox3	5'-ACACCATCACTCCAAATTTCAAC-3'	5'-CCATGAGGTTCCATTTCTATGTC-3'
SlGA3ox1	5'-GTGAAACCAAAGAAGGATGTG-3'	5'-GCATCAGTAAATCCATTTAAAGGGA-3'
SlGA3ox2	5'-GTAACGGTTCCTCTCCTTCGC-3'	5'-ACCTACTTGGACGCCACTTTG-3'
SlGA2ox1	5'-ACCCCACATCTTCTCCATCAT G-3'	5'-ACATGTTTCATCAAGGGTTCGAT-3'
SlGA2ox2	5'-GCCATGCTCAGAGATTGAACGATTG-3'	5'-CCCACAATGAGCATCTTGACAACC-3'
SlGA2ox3	5'-GCTAACAATCCTTCGATCAAATGACG-3'	5'-GCATAATGCATACACCTCCAAGGCC-3'
SlGA2ox4	5'-GTCGATTTTAAGATCCAACAACACTTCCGGT-3'	5'-CATCATTTTCAACATAACGAGTCCTTCC-3'
SlGA2ox5	5'-ATATCGGTATTAAGATCCAACAACACATCC-3'	5'-GATCCAAACATGGTATATTTGCGGAGG-3'
SlACT	5'-ATGTATGTTGCCATCCAGGCTG-3'	5'-CCTTGCTCATCCTATCAGCAGCAATACC-3'

Supplementary Figure 1. Scheme of GA metabolism including the non-13-hydroxylation and the early-13-hydroxylation pathways. CPS, copalyldiphosphate synthase; KS, *ent*-kaurene synthase; KO, *ent*-kaurene oxidase; KAO, *ent*-kaurenoic acid oxidase.



DISCUSIÓN

Efecto de las giberelinas y auxinas en la inducción del crecimiento partenocárpico del cv Micro-Tom de tomate.

Los resultados muestran que ovarios no polinizados del cultivar Micro-Tom fructifican partenocárpicamente en respuesta a la aplicación de GAs y auxinas, efecto que se había observado previamente en otros cultivares de tomate (Asahira *et al.,* 1967; Sjut y Bangerth, 1982/83; Koshioka *et al.,* 1994; Alabadí *et al.,* 1996; Alabadí y Carbonell, 1998; Fos *et al.,* 2000, 2001). Estos resultados, sumados a las características del cultivar, tales como su ciclo de desarrollo corto, necesidad de espacio reducido y su tamaño pequeño, apoyan la idea que Micro-Tom es un sistema ideal para el estudio de la regulación hormonal del cuajado y desarrollo del fruto propuesta por Meissner *et al.* (1997). Para evitar competencia entre los frutos, se han utilizado hasta dos racimos por planta y un solo ovario por racimo en los tratamientos hormonales, lo cual puede ser una desventaja respecto a cultivares altos.

Los frutos partenocárpicos inducidos por GA_3 y 2,4-D tienen una forma exterior similar a los frutos polinizados, aunque su tamaño difiere. Los frutos inducidos por 2,4-D son de mayor tamaño que los polinizados y los tratados con GA_3 son más pequeños. Otra diferencia, es que la inducción por GA_3 produce un escaso desarrollo del tejido locular quedando las cavidades loculares vacías, mientras que el 2,4-D (a dosis óptimas) induce la formación de un tejido locular similar al de los frutos polinizados. También se ha de apreciar que la mayoría de los óvulos no fertilizados degeneraron en el caso de GA_3, mientras que la aplicación de la auxina favoreció la formación de pseudoembriones lo cual también ha sido reportado por Asahira *et al.* (1967) y Kataoka *et al.* (2003), aunque no está clara su relación con la partenocarpia.

Los frutos inducidos con 2,4-D y GA_3 muestran pericarpos más gruesos que los polinizados, aunque hay más diferencias a nivel celular entre los tratamientos hormonales: en el caso de 2,4-D es notorio un mayor número de capas celulares por un incremento de divisiones celulares, aunque un menor tamaño celular en el mesocarpo interno y un mayor desarrollo de los haces vasculares, y en particular, mayor desarrollo de las traqueidas. Ese mayor desarrollo vascular podría estar favoreciendo la entrada de

nutrientes, lo cual, junto con un mayor número de células puede ser la causa del mayor tamaño de fruto. En el caso de frutos inducidos con GA_3 se observa un mayor tamaño celular generado por una mayor expansión celular. El tamaño de las células de los frutos polinizados es similar al de frutos inducidos por 2,4-D, aunque el número de capas celulares es menor. Se ha observado que la aplicación conjunta de ambas hormonas en dosis adecuadas, puede hacer que la morfología del fruto sea similar a la de un polinizado (datos no publicados), lo cual sugiere una interacción entre ellas durante el crecimiento y desarrollo del fruto.

La orientación espacial de las células en el pericarpo no varía sustancialmente en los tratamientos, y la forma es elíptica en la mayoría. Por ejemplo, en una etapa típicamente de expansión celular (20 dpa), en el mesocarpo externo (EM) hay más células con su eje mayor paralelo (Pa) a la epidermis, mientras que en el interno (IM) hay más células con su eje mayor perpendicular a la epidermis (Pe). En una etapa típica de divisiones celulares (5 dpa), el GA_3 indujo también frutos con células mayoritariamente perpendiculares mientras los otros tratamientos no lo hicieron. Básicamente, se observan diferencias entre los porcentajes de células Pa y Pe en las etapas del desarrollo pero no entre tratamientos, excepto para GA_3 en 5 dpa, aunque al medir la excentricidad de las células de IM, ésta fue mayor para frutos inducidos por GA_3 (a mayor excentricidad, menos circular es la célula). El efecto de elongación en las células de frutos tratados con GA_3 se observa igualmente en tejidos caulinares de elongación donde se aprecia una mayor excentricidad de las células (Cowling y Harberd, 1999), aunque en especies como guisante la expansión es multidireccional (Vercher y Carbonell, 1991). Esta expansión de las células Pe del IM puede ser un modo interesante de acción de las giberelinas en la fructificación del tomate, ya que es más temprana que en frutos polinizados o inducidos por 2,4-D.

La ploidía medida en exocarpo, mesocarpo y tejido locular más placenta aumentó progresivamente durante el crecimiento de 2 a 256 C debido al mecanismo de endorreduplicación ya descrito en otros trabajos con tomate (Bergervoet *et al.,* 1996; Joubès *et al.,* 1999; Cheniclet *et al.,* 2005). Tanto el tamaño celular como el MCV (mean C value) de células del IM de frutos inducidos con GA_3 fue mayor que en el caso

de frutos tratados con 2,4-D y polinizados, mientras que una menor ploidía en el caso de 2,4-D comparada con los polinizados, no está asociada a un tamaño menor de las células del mesocarpo. Por lo tanto, las GAs pueden estar regulando el tamaño de las células del mesocarpo al alterar los niveles de ploidía, lo cual está de acuerdo con la correlación positiva entre tamaño celular y ploidía encontrada por Cheniclet *et al.* (2005). Es interesante hacer notar que las GAs inducen frutos con células de mayor tamaño en el mesocarpo, aunque su número es inferior, lo cual sugiere la presencia de un mecanismo de compensación inducido por la reducción de las divisiones celulares (descrito por Tsukaya, 2006) y que a su vez es mediado por la alteración de los niveles de ploidía. De esta manera, el tamaño pequeño de los frutos inducidos por GA_3 comparados con los de 2,4-D (tres veces menor) puede ser determinado por pocas divisiones celulares, aunque compensado por expansión celular. Bohner y Bangerth (1988) observaron que el número de células del ovario en la antesis es determinante para el tamaño final del fruto, el cual puede ser variable cuando hay competencia entre frutos. Pero si ésta desaparece, la diferencia entre frutos pierde variabilidad como resultado del desequilibrio hormonal en el racimo.

Regulación de la fructificación y el crecimiento del fruto de tomate por giberelinas.

La fructificación y el crecimiento de ovarios de tomate Micro-Tom se redujeron significativamente con la aplicación de paclobutrazol (PCB) y LAB 198999, inhibidores de la biosíntesis de GAs, efecto que fue totalmente revertido con la aplicación de GA_3 en el caso del PCB y parcialmente en el caso de LAB. Estos resultados sugieren que el crecimiento del fruto depende de GAs, y están de acuerdo con los resultados de Fos *et al.* (2000). La reducción del peso del fruto que causó el LAB estaba asociada con la disminución de un 50% en el contenido de GA_1 comparado con GA_8 (10%), mientras que los contenidos de GA_{53}, GA_{44}, GA_{19} y GA_{20} se acumularon. Ya que la vía de 13-hidroxilación temprana en la ruta metabólica de GAs es la principal en tomate (Bohner *et al.,* 1988; Koshioka *et al.,* 1994; Fos *et al.,* 2000, 2001) esto significa: (1) que GA_1 es la GA más activa en el desarrollo del fruto de tomate; (2) que los precursores de GA_1 no son activos *per se* y deben ser convertidos a

la forma activa para actuar. En otras especies tales como guisante (*Pisum sativum*; Ingram *et al.,* 1984), lechuga (*Lactuca sativa*; Waycott *et al.,* 1991), arroz (*Oriza sativa*; Fujioka *et al.,* 1988) y espinaca (*Spinacia oleracea*; Zeevaart *et al.,* 1993), es también GA_1 la forma activa, mientras en *Arabidopsis thaliana* (Cowling *et al.,* 1998) es GA_4 la forma activa, que si bien es capaz de inducir la fructificación en tomate (Serrani *et al*., 2007a) puede que tenga un menor papel fisiológico en esta especie.

Tanto GA_{20} como GA_1 indujeron la fructificación de manera similar. Sin embargo, GA_{19} no la indujo por lo que los ovarios no polinizados (emasculados) son capaces de metabolizar GA_{20} a GA_1 pero no GA_{19}, lo que sugiere que la actividad GA 20-oxidasa (que metaboliza GA_{19} a GA_{20}) es limitante en estos ovarios. Fos *et al.* (2000) observaron que en el mutante *pat-2*, que tiene partenocarpia facultativa, hay un incremento importante de GA_{20} y de la suma de GA_1 y GA_8, posiblemente debida al incremento de la actividad GA 20-oxidasa. Para comprobar si la actividad GA 20-oxidasa es limitante en ovarios de tomate, se observaron los niveles de transcritos en frutos polinizados y no polinizados de los genes *SlGA20ox* y *SlGA3ox* de la ruta de biosíntesis de GAs. En día 0, los transcritos de *SlGA3ox2* se detectaron pobremente respecto a los de *SlGA3ox1* y fueron prácticamente indetectables en etapas posteriores (d5, d10 y d20). Los transcritos de *SlGA3ox1* estuvieron presentes en ovarios no polinizados y polinizados, y permanecieron relativamente constantes hasta el día 20. Esto sugiere que la actividad GA 3-oxidasa está presente en los ovarios antes de la polinización y ésta no altera su actividad. En oposición a esto, los transcritos de *SlGA20ox1* y *-2* fueron casi indetectables a d0, d5, d10 y d20 en ovarios no polinizados aunque presentes en polinizados, por lo que la actividad GA 20-oxidasa se incrementa debido la polinización. Los resultados sugieren la importancia de la actividad GA 20-oxidasa como limitante en ovarios de tomate, aunque no la actividad GA 3-oxidasa, lo cual contrasta con los resultados de Bohner *et al.* (1988) y Koshioka *et al.* (1994) que sugieren la 3β-hidroxilación de GA_{20} como el paso limitante en la biosíntesis de GA_1 en tomate.

Los niveles de transcritos de *SlCPS* fueron mayores en ovarios polinizados que en no polinizados, por lo que la actividad de enzimas tempranos de la biosíntesis

podrían contribuir también al incremento del contenido de GA después de la polinización. Rebers *et al.* (1999) observaron que la expresión de todos los genes de biosíntesis de GAs (*SlCPS*, *SlGA20ox* y *SlGA3ox*) cambia a lo largo del desarrollo del primordio floral en tomate, con diferentes patrones de acumulación de mRNA, lo cual indica un mecanismo complejo de regulación que controla la biosíntesis de GAs durante el desarrollo de la flor en tomate. Es sabido que los niveles de GA son el resultado de la biosíntesis y la inactivación de GAs (Hedden y Phillips, 2000). Por ello, la modificación de los niveles de giberelina activa se pueden deber a una alteración simultánea de la transcripción de los genes que codifican la biosíntesis de GAs (GA20ox y/o GA3ox) y los enzimas de inactivación (GA2o*x*). Por ejemplo, en guisante el contenido de GA_1 disminuye en el brote durante la desetiolación, lo cual es debido a la regulación negativa de *PsGA3ox1,* que controla la conversión de GA_{20} a GA_1, y también debido a la regulación positiva de *PsGA3ox2* que codifica una GA2ox que convierte GA_1 a GA_8.

Al comenzar este trabajo, ningún clon completo de genes de inactivación de GAs (*GA2ox*) se había descrito en tomate. Con objeto de saber si el incremento en el contenido de GA_1 tras la polinización en ovarios de tomate se debía no solo a un incremento en la biosíntesis de GAs (gracias al incremento de los niveles de transcritos de *GA20ox* y quizá *SlCPS*), sino también a una reducción en la actividad catabólica, se aislaron cinco clones completos de cDNA que codifican GA 2-oxidasas putativas de tomate (*SlGA2ox1* a la *-5*) y se determinó la actividad de los productos de expresión con diferentes sustratos de GAs. La única que no mostró actividad con ellos fue la correspondiente al clon *SlGA2ox2*, lo que sugiere que la proteína era inactiva a pesar de tener los aminoácidos que unen Fe^{2+} y 2-oxoglutarato y las regiones conservadas típicas de las GA 2-oxidasas. La causa de esta inactividad puede radicar en una mutación que conlleva un cambio de una W por una R en la posición 92, que es conservada en las demás GA2ox que utilizan GAs C-20 como substrato. Quizás el residuo W tiene relevancia en la actividad de las GA2ox.

Los niveles de transcritos de los cinco genes aislados de *SlGA2ox* fueron analizados en diferentes tejidos y se observó que su expresión está regulada por el desarrollo. Todos se expresaron en ovarios no polinizados en el momento de antesis y

hasta el día 20; sin embargo, no hay una disminución en la expresión en ovarios polinizados 5 días después de antesis, cuando el desarrollo ya está en curso. Esto significa que el efecto de la polinización en el desarrollo temprano del fruto no parece estar mediado por un efecto de inactivación de GAs a través de GA2ox. En etapas más tardías del crecimiento (d10 y d20), el efecto de la polinización fue el de reducir los niveles de transcritos de todos los genes *SlGA2ox* (excepto *SlGA2ox1* en d 10).

El análisis filogenético de GA2ox con todas las secuencias disponibles en bases de datos, mostró que hay una primera división entre enzimas que utilizan GAs C-20 como substrato (grupo III) y aquellas que usan GAs C-19. Estás últimas se dividen en dos grupos (I y II) cuya divergencia parece ser más reciente. Los genes aislados en este trabajo se ubican en los grupos I (*SlGA2ox1* y *-3*) y II (*SlGA2ox2*, *-4*, *-5*). Algunos de los genes de otras especies cuya actividad enzimática ha sido investigada, y que presentan actividad monocatalítica se ubican en el grupo I. Por el contrario, los que presentan actividad multicatalítica pertenecen al grupo II; la hipótesis es que ambos grupos difieren en sus características catalíticas. Como se esperaba, SlGA2ox1 y -3 presentaron actividad monocatalítica, pero SlGA2ox4 y -5 no presentaron actividad multicatalítica (SlGA2ox2 fue inactivo). Nuestros resultados por lo tanto, parecen no confirmar esa hipótesis. Sin embargo, las secuencias de *SlGA2ox2*, *-4*, y *-5* presentan cambios en aminoácidos conservados específicos que pueden afectar su actividad, teniendo en cuenta que se ha reportado que es difícil encontrar catabolitos de GAs y depende de la concentración de enzima (Martin *et al.,* 1999). Quizá un análisis bioquímico más detallado podría dilucidar estos aspectos. Tanto dico- como monocotiledóneas están presentes en los tres grupos de GA2ox, lo que indica que las subfamilias génicas I, II y III se originaron por duplicaciones génicas y esto sucedió tempranamente en la evolución. Los resultados coinciden con trabajos (Sanjuan y Marin, 2001) que sugieren que la adquisición de nuevas funciones evolutivas entre dioxigenasas de GAs está asociada con eventos de duplicación génica.

La fructificación inducida por auxinas en tomate depende de giberelinas.

El desarrollo partenocárpico del fruto puede ser inducido por tratamiento con auxinas o giberelinas a ovarios no polinizados (Serrani *et al.,* 2007a) y la aplicación de inhibidores de biosíntesis de GAs en frutos polinizados apoyan la hipótesis de que la fructificación y el desarrollo del fruto de tomate depende de GAs sintetizadas tras la polinización y fecundación (Fos *et al.,* 2000; Serrani *et al.,* 2007b). Sin embargo, la ausencia de inhibidores de la síntesis de auxinas no ha permitido realizar experimentos que aporten evidencias sobre su papel específico en la fructificación. Cuando se aplicaron inhibidores de biosíntesis de GAs a frutos inducidos por 2,4-D o IAA, su crecimiento fue prácticamente nulo o muy reducido, lo cual sugiere que el crecimiento y desarrollo partenocárpico inducido por auxinas está mediado por GAs. En trabajos previos observamos que la aplicación de 2,4-D indujo el desarrollo partenocárpico del fruto, y en este trabajo hemos demostrado que el efecto del 2,4-D está asociado con un alto contenido de GA_1 (la GA activa en fructificación del tomate) en el ovario, tanto como sus precursores y GA_8. Los niveles de los precursores de la GA_1 fueron similares en frutos polinizados y en frutos tratados con 2,4-D, y los niveles de GA_1 y GA_8 fueron incluso mayores en frutos tratados con 2,4-D.

Los resultados anteriores sugieren que el efecto del 2,4-D en la fructificación está mediado por GAs (aumentando la biosíntesis de GAs). Sin embargo, es posible que el alto contenido de GA en frutos inducidos por 2,4-D sea la consecuencia del crecimiento inducido por la auxina más que el efecto directo de la misma. Para comprobar esta hipótesis, se indujo el crecimiento partenocárpico con GA_3 [una GA que se encuentran en niveles muy bajos o casi indetectables en ovarios de tomate (Fos *et al.* 2000; 2001)] y se cuantificó el contenido de GAs en el fruto. GA_1 se encontró en ovarios tratados con GA_3 en una concentración de cerca del 6% de GA_3. Puesto que el GA_3 aplicado no es absolutamente puro (contiene un 3.3% de GA_1 respecto a GA_3), el GA_1 encontrado en el fruto podría venir como un contaminante en la solución inicial, aunque es difícil de explicar por qué el cociente GA_1/GA_3 en el fruto es mayor que en la solución aplicada. Una posible explicación puede ser que el GA_3 es transportado más eficientemente fuera del ovario que el GA_1 lo cual llevaría a incrementar dicho cociente.

Por el contrario, los precursores de GA_1 analizados (GA_{53}, GA_{44}, GA_{19} y GA_{20}) en frutos tratados con GA_3, estaban a un nivel mucho más bajo que en frutos polinizados y no polinizados inducidos con 2,4-D. Estos resultados sugieren que el crecimiento de frutos inducidos con GA_3 no está asociado a una mayor biosíntesis de GAs.

El incremento del contenido de GAs en frutos inducidos con 2,4-D, fue probablemente el resultado de un aumento en la transcripción de genes que codifican CPS, GA20ox1, -2 y -3 y GA3ox1 (enzimas biosintéticos). Actividad de CPS está presente en extractos de frutos de tomate (Bensen y Zeevaart, 1990), y los altos niveles de transcripción de *SlCPS* sugieren que los enzimas tempranos en la ruta de biosíntesis podrían contribuir al incremento de GAs. Los transcritos de este gen son relativamente altos antes de la antesis y, al parecer, la aplicación de 2,4-D podría prevenir su regulación negativa. Tan solo la expresión de *SlGA2ox2* (dentro de las GA2ox) fue regulada negativamente en frutos tratados con 2,4-D. Sin embargo, dado que la proteína codificada por *SlGA2ox2* es probablemente inactiva (Serrani *et al.,* 2007b), no está claro el posible efecto del 2,4-D sobre la inactivación de GAs a través de GA2ox. Es posible por lo tanto, que haya otros genes que codifiquen GA2ox todavía no aislados que inactiven GAs C-20.

En frutos inducidos con 2,4-D el gen *SlGA2ox2* es regulado negativamente, mientras que en frutos polinizados (Serrani *et al.,* 2007b) no se observó dicha regulación temprana. Esto sugiere que la polinización y/o fecundación puede alterar el metabolismo de GAs a través de auxinas, que son sintetizadas en las semillas (Varga y Bruinsma, 1986). Este tipo de regulación también ha sido encontrado en tejidos vegetativos. Por ejemplo, se ha observado que el contenido de GA_1 es regulado por el IAA transportado basipétalamente desde el brote apical a través de una mecanismo de regulación positiva de *GA20ox* en tabaco (Wolbang y Ross, 2001) y *GA20ox* y *GA3ox* en guisante (O´Neill y Ross, 2002). También la aplicación de ácido naftalenacético (una auxina sintética) a plántulas de Arabidopsis, regula diferencialmente la expresión de varios genes de *GA20ox* y *GA2ox* (Frigerio *et al.,* 2006). Todos estos resultados apoyan la hipótesis de que existe un mecanismo de regulación del metabolismo de GAs por auxinas que parece ser diferente en cada especie y posiblemente en cada tejido, y que

aumenta la expresión de diferentes enzimas que codifican GAs, ó bien, regula negativamente los que inactivan a las GAs. Las auxinas tienen probablemente otros efectos independientes de GAs en el desarrollo del fruto de tomate. Los frutos inducidos por éstas, tienen muy poco tejido locular y son de menor tamaño en comparación con frutos polinizados o tratados con auxinas y la aplicación conjunta, por ejemplo de GA_3 e IAA, tiene un efecto sinérgico en el desarrollo.

CONCLUSIONES

1. El crecimiento y desarrollo partenocárpico del fruto en respuesta a la aplicación de giberelinas y auxinas en el cultivar enano de tomate Micro-Tom es similar al descrito previamente en otros cultivares altos. Los resultados obtenidos con Micro-Tom, permiten proponer a este cultivar como un modelo conveniente para estudiar la regulación hormonal de la fructificación, dado su corto ciclo reproductivo y su pequeño tamaño. La aplicación de GAs y auxinas induce la fructificación, aunque su efecto sobre la morfología del fruto es diferente. Las GAs favorecen la expansión celular mientras que las auxinas favorecen las divisiones celulares. Por otro lado, el tamaño celular en el mesocarpo de frutos tratados con GAs depende del nivel de ploidía.

2. Los resultados obtenidos de experimentos de aplicación de inhibidores de biosíntesis de GAs, así como los de cuantificación de GAs, apoyan la hipótesis que tanto la fructificación como el desarrollo temprano del fruto dependen de GAs, siendo GA_1 la hormona activa involucrada en estos procesos. Nuestros resultados sugieren que en el ovario la actividad GA 20-oxidasa es limitante para la inducción de fructificación por GAs. La polinización incrementa los niveles de GAs en el ovario, incrementando su biosíntesis (a través de la regulación positiva de *GA20ox* y *SlCPS*, pero no de *GA3ox*), pero no reduciendo la inactivación catabólica de GAs por medio de GA2ox.

3. El efecto de las auxinas en la fructificación y desarrollo del fruto está mediado por GAs. Las auxinas, posiblemente sintetizadas en las semillas tras la polinización y la fecundación de los óvulos, incrementan el contenido de GAs en el fruto, aumentando los niveles de transcritos de genes de biosíntesis de GAs (CPS, GA20ox y GA3ox1) y el contenido de GA_1, y así, induciendo la fructificación.

BIBLIOGRAFÍA

Abad M, Monteiro AA. 1989. The use of auxins for the production of greenhouse tomatoes in mild-winter conditions: a review. Scientia Hortic. 38: 167-192.

Ait-Ali T, Swain SM, Reid JB, Sun TP, Kamiya Y. 1997. The LS locus of pea encodes the gibberellin biosynthesis enzyme *ent*-kaurene synthase A. Plant J. 11: 443-454.

Alabadí D, Agüero MS, Pérez-Amador MA, Carbonell J. 1996. Arginase, arginine decarboxylase, ornithine decarboxylase, and polyamines in tomato ovaries. Changes in pollinated ovaries and parthenocarpic fruits induced by auxin and gibberellin. Plant Physiol. 112: 1237-1244.

Alabadí D, Carbonell J. 1998. Expression of ornithine decarboxylase is transiently increased by pollination, 2,4-dichlorophenoxyacetic acid, and gibberellic acid in tomato ovaries. Plant Physiol. 118: 323-328.

Archbold DD, Dennis FG, Flore JA. 1982. Accumulation of ^{14}C-labelled material from foliar applied ^{14}C-sucrose by tomato ovaries during fruit set and initial development. J. Amer. Soc. Hort. Sci. 107: 19-23.

Asahira T, Takeda Y, Nishio T, Hirabayashi M, Tsukamoto Y. 1967. Studies on fruit development in tomato. I. Ovule development and content of diffusible auxin in synthetic auxin- and gibberellin-induced parthenocarpic tomato fruits in relation to their development. Mem. Res. Inst. Food Sci. Kyoto Univ. 28: 47-74.

Asahira T, Takagi H, Takeda Y, Tsukamoto Y. 1968. Studies on fruit development on tomato. II. Cytokinin activity in extracts from pollinated, auxin- and gibberellin- induced parthenocarpic tomato fruits and its effect on the histology of the fruit. Mem. Res. Inst. Food Sci. Kyoto Univ. 29: 24-54.

Bandurski R, Cohen J, Slovin J, Reinecke D. 1995. Auxin biosynthesis and metabolism. In: Plant Hormones: Physiology, Biochemistry, and Molecular Biology, Davies P (ed). Kluwer Acad. Pub. Dordretch, Boston, London, pp. 39-65.

Barow M, Meister A. 2003. Endopolyploidy in seed plants is differently correlated to systematics, organ, life strategy and genome size. Plant Cell Environ. 26: 571-584.

Bartel B, Le Clere S, Magidin M, Zolman BK. 2001. Inputs to the active indole-3-acetic acid pool: *de novo* synthesis, conjugate hydrolysis and indole-3-butyric acid β-oxidation. J. Plant Growth Regul. 20: 198-216

Bartels D, Thompson RD. 1983. The characterization of cDNA clones coding for wheat storage proteins. Nucl. Acid Res. 11: 2961-2977.

Bensen RJ, Zeevaart JAD. 1990. Comparison of ent-kaurene synthetase A and B activities in cell-free extracts from young tomato fruits of wild-type and *gib-1*, *gib-2*, and *gib-3* tomato plants. J. Plant Growth Regul. 9: 237-242.

Bensen RJ, Johal GS, Crane VC, Tossberg JT, Schnable PS, Meeley RB, Briggs SP. 1995. Cloning and characterization of the maize *An1* gene. Plant Cell 7: 75-84.

Bergervoet JHW, Verhoeven HA, Gilissen LJW, Bino RJ. 1996. High amounts of nuclear DNA in tomato (*Lycopersicon esculentum* Mill.) pericarp. Plant Sci. 116: 141-145.

Bertin N, Borel C, Brunel B, Cheniclet C, Causse M. 2003. Do genetic make-up and growth manipulation affect tomato fruit size by cell number, or cell size and DNA endoreduplication? Ann. Bot. 92: 415-424.

Bohner J, Hedden P, Bora-Haber E, Bangerth F. 1988. Identification and quantitation of gibberellins in frutis of *Lycopersicon esculentum*, and their relationship to fruit size in *L. esculentum* and *L. pimpinellifolium*. Physiol. Plant. 73: 348-353.

Bohner J, Bangerth F. 1988. Cell number, cell size and hormone levels in semi-isogenic mutants of *Lycopersicon pimpinellifolium* differing in fruit size. Physiol. Plant. 72: 316-320.

Bünger-Kibler S, Bangerth F. 1982/83. Relationship between cell number, cell size and fruit size of seeded fruits of tomato (*Lycopersicon esculentum* Mill.), and those induced parthenocarpically by the application of plant growth regulators. Plant Growth Regul. 1: 143-154.

Carrera E, Jackson SD, Prat S. 1999. Cloning and expression of three *GA 20-oxidase* cDNAs from potato. Plant Physiol. 119(2): 765-774.

Cheniclet C, Rong WY, Causse M, Frangne N, Bolling L, Carde JP, Renaudin JP. 2005. Cell expansion and endoreduplication show a large genetic variability in pericarp and contribute strongly to tomato fruit growth. Plant Physiol. 139: 1984-1994.

Chamarro, J. 1995. Anatomía y Fisiología de la Planta. En: Nuez F (ed). El cultivo del tomate. Ediciones Mundi Prensa. Madrid, Barcelona, México, pp. 44-91.

Chareonboonsit S, Splittstoesser W, George W. 1985. The effects of pollination methods and auxin application upon parthenocarpic fruit set and development. Sci. Hort. 27: 1-8.

Cheng Y, Dai X, Zhao Y. 2006. Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in *Arabidopsis*. Gen. and Dev. 20:1790-1799.

Chiang HH, Hwang I, Goodman HM. 1995. Isolation of the *Arabidopsis GA4* locus. Plant Cell 7: 195-201.

Cleland R. 2004. Auxin and cell elongation. In: Plant Hormones: Biosynthesis, Signal Transduction, Action! Davies J. (ed). Kluwer Acad. Pub. Dordretch, The Netherlands, pp. 204-220.

Cohen JD, Slovin JP, Hendrickson A. 2003. Two genetically discrete pathways convert tryptophan to auxin: more redundancy in auxin biosynthesis. Trends in Plant Science 8: 197-199.

Cookson SJ, Radziejwoski A, Granier C. 2006. Cell and leaf plasticity in *Arabidopsis*: what is the role of endoreduplication? P. Cell Env. 29: 1273-1283.

Coombe B. 1976. The development of fleshy fruits. Annu. Rev. Plant Physiol. 27: 507-528.

Cowling RJ, Kamiya Y, Seto H, Harberd NP. 1998. Gibberellin dose-response regulation of *GA4* gene transcript levels in *Arabidopsis*. Plant Physiol. 117: 1195-1203.

Cowling RJ, Harberd NP. 1999. Gibberellins control *Arabidopsis* hypocotyl growth via regulation of cellular elongation. J. Exp. Bot. 50: 1351-1357.

Daidoji H, Takasaki Y, Nakane PK. 1992. Proliferating cell nuclear antigen (PCNA/cyclin) in plant proliferating cells. Immunohistochemical and quantitative analysis using autoantibody and murine monoclonal antibodies to PCNA. Cell Biochem. Funct. 10: 123-132.

Dan Y, Yan H, Munyikwa T, Dong J, Zhang Y, Armstrong CL. 2006. MicroTom, a high-throughput model transformation system for functional genomics. Plant Cell Rep. 25: 432-441.

Dill A, Jung HS, Sun T. 2001. The DELLA motif is essential for gibberellin-induced degradation of RGA. Proc. Natl. Acad. Sci. 98 (24): 14162–14167.

Elliott R, Ross JJ, Smith JJ, Lester D, Reid J. 2001. Feed-forward regulation of gibberellin deactivation in pea. J. Plant Growth. Regul. 20(1): 87-94.

Esau, K. 1976. Anatomía Vegetal. Ediciones Omega, Barcelona.

Eyal E, Levy AA. 2002. Tomato mutants as tools for functional genomics. Curr. Opin. Plant Biol. 5: 112-117.

Farabee MJ. 2001. Flowering Plant Reproduction: Fertilization and Fruits. The Online Biology Book. Estrella Mountain Community College, Arizona.

Felsenstein J, Churchill GA. 1996. A hidden Markov model approach to variation among sites in rate of evolution. Mol. Biol. Evol. 13: 93-104.

Fleet CM, Yamaguchi S, Hanada A, Kawaide H, David CJ, Kamiya Y, Sun TP. 2003. Overexpression of *AtCPS* and *AtKS* in *Arabidopsis* confers increased *ent*-kaurene production but no increase in bioactive gibberellins. Plant Physiol. 132: 830-839.

Fos M, Nuez F, García-Martínez JL. 2000. The gene *pat-2*, which induces natural parthenocarpy, alters the gibberellin content in unpollinated tomato ovaries. Plant Physiol. 122: 471-479.

Fos M, Proaño K, Nuez F, García-Martínez JL. 2001. Role of gibberellins in parthenocarpic fruit development induced by the genetic system *pat-3*/*pat-4* in tomato. Physiol. Plant. 111: 545-550.

Frigerio M., Alabadí D, Pérez-Gómez J, García-Cárcel L, Phillips A.L, Hedden P, Blázquez M.A. 2006. Transcriptional regulation of gibberellin metabolism genes by auxin signalling in *Arabidopsis*. Plant Physiol. 142: 533-563.

Fu X, Harberd N.P. 2003. Auxin promotes *Arabidopsis* root growth by modulating gibberellin response. Nature 421: 740-743.

Fujioka S, Yamane H, Spray CR, Gaskin P, MacMillan J, Phinney BO, Takahashi N. 1988. Qualitative and quantitative analyses of gibberellins in vegetative shoots on normal, *dwarf-1*, *dwarf-2*, *dwarf-3*, and *dwarf-5* seedlings of *Zea mays* L. Plant Physiol. 88: 1367-1372.

García-Martínez J.L, Santes C.M, Croker S, Hedden P. 1991. Identification, quantitation and distribution of gibberellins in fruit of *Pisum sativum* cv. Alaska during pod development. Planta 184: 53-60.

Garcia-Martinez JL, Hedden P. 1997. Gibberellins and fruit development. In: Phytochemistry of Fruit and Vegetables. Tomás-Barberán FA and Robins RJ, (eds). Clarendon Press: Oxford, pp. 263–286.

García-Martínez JL, López-Díaz I, Sánchez-Beltrán MJ, Phillips AL, Ward DA, Gaskin P, Hedden P. 1997. Isolation and transcript analysis of gibberellin 20-oxidase genes in pea and bean in relation to fruit development. Plant Mol. Biol. 33: 1073-1084.

García-Martínez JL, Gil J. 2002. Light regulation of gibberellin biosynthesis and mode of action. J. Plant Growth Regul. 20: 354-368.

George WL, Scott JW, Splittstoesser WE. 1984. Parthenocarpy in tomato. Hort. Rev. 6: 65-84.

Gillaspy G, Ben-David H, Gruissem W. 1993. Fruits: a developmental perspective. Plant Cell 5: 1439-1451.

Goetz M, Vivian-Smith A., Johnson S.D, Koltunow A.M. 2006. *AUXIN RESPONSE FACTOR8* is a negative regulator of fruit initiation in *Arabidopsis*. Plant Cell 18: 1873-1886.

Goetz M, Hooper L, Johnson S, Macedo JC, Koltunow A. 2007. Expression of aberrant forms of AUXIN RESPONSE FACTOR8 stimulates parthenocarpy in arabidopsis and tomato. P. Physiol. DOI:10.1104/pp.107.104174.

Gorquet B, Van Heusden AW, Lindhout P. 2005. Parthenocarpic fruit development in tomato. Plant Biol. 7: 131-139.

Gustafson FG. 1936. Inducement of fruit development by growth-promoting chemicals. Proc. Natl. Acad. Sci. 22: 628-636.

Gustafson FG. 1939. Auxin distribution in fruits and its significance in fruit development. Am. J. Bot. 26: 189–194.

Gustafson FG. 1960. Influence of gibberellic acid on setting and development of fruits in tomato. Plant Physiol. 35: 521-523.

Hackett D, Thimann K. 1952. The nature of the auxin-induced water uptake by potato tissue. Am. J. Bot. 39: 553-560.

Hayward HE. 1938. Solanaceae-*Lycopersicon esculentum*. In: The structure of economic plants. Cramer L (ed). McMillan, New York. Reprinted edition (1967), pp. 550 -579.

Hedden P, Graebe JE. 1985. Inhibition of gibberellin biosynthesis by Paclobutrazol in cell-free homogenates of *Cucurbita maxima* endosperm and *Malus pumila* embryos. Plant Growth Regul. 4: 11-122.

Hedden P, Kamiya Y. 1997. Gibberellin biosynthesis: enzymes, genes and their regulation. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48: 431-460.

Hedden P, Phillips A. 2000a. Gibberellin metabolism: new insights revealed by the genes. TIPS 5: 523-530.

Hedden P, Phillips A. 2000b. Manipulation of hormone biosynthetic genes in transgenic plants. Curr. Opin. Biotech. 11(2): 130-137.

Hedden P, Proebsting WM. 1999. Genetic analysis of gibberellin biosynthesis. Plant Physiol. 119: 365-370.

Helliwell CA, Sullivan JA, Mould RM, Gray JC, Peacock J, Dennis ES. 2001. A plastid envelope location of *Arabidopsis ent*-kaurene oxidase links the plastid and endoplasmic reticulum steps of the gibberellin biosynthesis pathway. Plant J. 28: 201-208.

Hillis DM, Bull JJ. 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst. Biol. 42:182-192.

Ho L, Hewitt J. 1986. Fruit development. In: The Tomato Crop. Atherton JG and Rudish J (eds). New York: Chapman and Hall, pp. 201-239.

Hobson G, Davies J. 1970. The tomato. In: The Biochemistry of Fruits and Their Products. Vol. II. Hulme AC (ed). London, Academic Press, pp. 437-482.

Huttly AK, Phillips AL. 1995. Gibberellin-regulated plant genes. Physiol. Plant. 95: 310-317.

Imai R, Yang Y, Ait-alí T, Kawaide H, Kamiya Y. 1996. Cloning and ligth-regulated expresion of two cDNAs for *ent*-kaurene synthase A from tomato. Plant Cell Physiol. Suppl. 37, S14 (Absstr.539).

Ingram TJ, Reid JB, Murfet IC, Gaskin P, Willis CL, MacMillan J. 1984. Internode length in *Pisum*. The *Le* gene controls the 3ß-hydroxylation of gibberellin A_{20} to gibberellin A1. Planta 160: 455-463.

Israelsson M, Mellerowicz E, Chono M, Gullberg J, Moritz T. 2004. Cloning and overproduction of gibberellin 3-oxidase in hybrid aspen trees. Effects on gibberellin homeostasis and development. Plant Physiol. 135(1): 221–230.

Itoh H, Ueguchi-Tanaka M, Sentoku N, Kitano H, Matsuoka M, Kobayashi M. 2001. Cloning and functional analysis of two gibberellin 3β-hydroxylase genes that are differently expressed during the growth of rice. Proc. Natl. Acad. Sci. 98: 8909–8914.

Jones B, Frasse P, Olmos E, Zegzouti H, Li ZG, Latché A, Pech JC, Bouzayen M. 2002. Down-regulation of an ARF-like gene in the tomato results in a pleiotropic phenotype including dark-green and blotchy ripening fruit. P. Journal 32: 603-614.

Jones DT, Taylor WR, Thornton JM. 1992. The rapid generation of mutation data matrices from protein sequences. CABIOS 8: 275-282.

Joubès J, Phan T-H, Just D. Rothan C. Bergounioux C, Raymond P, Chevalier C. 1999. Molecular and biochemical characterization of the involvement of cyclin-dependent kinase A during the early development of tomato fruit. Plant Physiol. 121: 857-869.

Kataoka K, Uemachi A, Yazawa S. 2003. Fruit growth and pseudoembryo development affected by uniconazole, an inhibitor of gibberellins, in *pat-2* and auxin-induced parthenocarpic tomato fruits. Scientia Hortic. 98: 9-16.

Kobayashi M, MacMillan J, Phinney B, Gaskin P, Spray CR, Hedden P. 2000. Gibberellin Biosynthesis: metabolic evidence for three steps in the early 13-hydroxylation pathway of rice. Phytochemistry 55(4): 317-321.

Kojima K, Ohtake E, Yu Z. 2002. Distribution and transport of IAA in tomato plants. Plant Growth Regul. 41: 99-104.

Kondorosi E, Roudier F, Gendreau E. 2000. Plant cell-size control: growing by ploidy? Curr. Opin. Plnat Biol. 3: 488-492.

Koshioka M, Nishijima T, Yamazaki H, Liu Y, Nonaka M, Mander LN. 1994. Analysis of gibberellins in growing fruits of *Lycopersicon esculentum* after pollination or treatment with 4-chlorophenoxyacetic acid. J. Hort. Sci. 69: 171-179.

Lange T, Robatzek S, Frisse A. 1997. Cloning and expression of a gibberellin 2β,3β-hydroxylase cDNA from pumpkin endosperm. Plant Cell 9: 1459-1467.

Lange T. 1998. Molecular biology of gibberellin synthesis. Planta 204: 409–419.

Lee DJ, Zeevaart JAD. 2002. Differential regulation of RNA levels of gibberellin dioxygenases by photoperiod in spinach. Plant Physiol. 130:2085–2094.

Lee DJ, Zeevaart JAD. 2005. Molecular cloning of *GA 2-Oxidase3* from spinach and its ectopic expression in *Nicotiana sylvestris*. Plant Physiol. 138: 243-254.

Lemaire-Chamley M, Petit J, García V, Just D, Baldet P, Germain V, Fagard M, Mouassite M, Cheniclet C, Rothan C. 2005. Changes in transcriptional profiles are associated with early fruit tissue specialization in tomato. Plant Physiol. 139: 750-769.

Lester DR, Ross JJ, Davies PJ, Reid JB. 1997. Mendel's stem length gene (*Le*) encodes a gibberellin 3β- hydroxylase. Plant Cell 9: 1435-1443.

Lester DR, Ross JJ, Smith JJ, Elliot RC, Reid JB. 1999. Gibberellin 2-oxidation and the *SLN* gene of *Pisum sativum*. Plant J. 19(1): 65-73.

MacMillan J, Takahashi N. 1968. Proposed procedure for the allocation of trivial names to the gibberellins. Nature 217: 170-171.

MacMillan J. 1997. Biosynthesis of the gibberellin plant hormones. Nat. Prod. Rep. 14: 221-244.

MacMillan J, Ward DA, Phillips AL, Sanchez-Beltran MJ, Gaskin P, Lange T, Hedden P. 1997. Gibberellin biosynthesis from gibberellin A_{12}-Aldehyde in endosperm and embryos of *Marah macrocarpus*. Plant Physiol. 113: 1369-1377.

Mapelli SC, Frova , Torti G, Soressi G. 1978. Relationship between set, development and activities of growth regulators in tomato fruits. Plant Cell Physiol. 19: 1281-1288.

Mapelli SC, Lombardi L. 1982. A comparative auxin and cytokinin study in normal and *to-2* mutant tomato plants. P. Cell Physiol. 23: 751-757.

Martí E, Gisbert C, Bishop GJ, Dixon MS, García-Martínez JL. 2006. Genetic and physiological characterization of tomato cv. Micro-Tom. J. Exp. Bot. 57: 2037-2047.

Martí C, Orzáez D, Ellul P, Moreno V, Carbonell J, Granell A. 2007. Silencing of DELLA induces facultative parthenocarpy in tomato fruits. Plant J. 52(5): 865-876.

Martin DN, Proebsting WM, Hedden P. 1991. The *SLENDER* gene of pea encodes a gibberellin 2-oxidase. Plant Physiol. 121: 775-781.

Martin DN, Proebsting WM, Hedden P. 1997. Mendel's dwarfing gene: cDNAs from the *Le* alleles and function of the expressed proteins. Proc. Natl. Acad. Sci. 94: 8907-8911.

Martin DN, Proebsting WM, Parks TD, Dougherty WG, Lange T, Lewis MJ, Gaskin P, Hedden P. 1996. Feed-back regulation of gibberellin biosynthesis and gene expression in *Pisum sativum* L. Planta 200: 159-166.

Meissner R, Jacobson Y, Melamed S, Levyatuv S, Shalev G, Ashri A, Elkind Y, Levy AA. 1997. A new model system for tomato genetics. Plant J. 12: 1465-1472.

Montoya T, Nomura T, Yokota T, Farrar K, Harrison K, Jones JGD, Kaneta T, Kamiya Y, Szekeres M, Bishop G. 2005. Patterns of *Dwarf* expression and brassinosteroid accumulation in tomato reveal the importance of brassinosteroid synthesis during fruit development. Plant J. 42: 262-269.

Nakaminami K, Sawada Y, Suzuki M, Kenmoku H, Kawaide H, Mitsuhashi W, Sassa T, Inoue Y, Kamiya Y, Toyomasu T. 2003. Deactivation of gibberellin by 2-oxidation during germination of photoblastic lettuce seeds. Biosci. Biotechnol. Biochem. 67(7):1551-1558.

Nakayama M, Yamane H, Murofushi N, Takahashi N, Mander L, Seto H. 1991. Gibberellin biosynthetic pathway and the physiologically active gibberellin in the shoot of *Cucumis sativus* L. J. Plant Growth Regul. 10: 15-119.

Nigovic B, Kojic-Prodic B, Antolic S, Tomic S, Puntarec V, Cohen JD. 1996. Structural studies on monohalogenated derivates of the phytohormone indole-3-acetic acid (auxin). Acta Cryst. B52: 332-343.

Nitsch JP. 1952. Plant hormones in the development of fruits. Quart. Rev. Biol. 27: 33-47.

Nitsch JP. 1970. Hormonal factors in growth and development. In: The biochemistry of fruits and their products. Hulme A (ed). Academic Press, London. Vol 1, pp. 427-472.

Ngo P, Ozga JA, Reinecke DM. 2002. Specificity of auxin regulation of gibberellin 20-oxidase gene expression in pea pericarp. Plant Mol. Biol. 49: 439-446.

Noll F. 1902. Fruchtbildung ohne vorausgegangene bestaubung (parthenokarpie) bei der gurke. Sitzungsb. Niederrhein. Bes. Nat. Heilk. Bonn. 149-162.

Normanly J, Slovin JP, Cohen JD. 2004. Auxin biosynthesis and metabolism. In: Plant Hormones: Biosynthesis, Signal Transduction, Action! Davies J (ed). Kluwer Acad. Pub. Dordretch, The Netherlands, pp. 36-62.

Ogawa M, Hanada A, Yamauchi Y, Kuwahara A, Kamiya Y, Yamaguchi S. 2003. Gibberellin biosynthesis and response during *Arabidopsis* seed germination.. Plant Cell. 15(7):1591-604.

Olimpieri I, Siligato F, Caccia R, Mariotti L, Ceccarelli N, Soressi GP, Mazzucato A. 2007. Tomato fruit set driven by pollination or by the *parthenocarpic fruit* allele is mediated by transcriptionally regulated gibberellin biosynthesis. Planta, DOI 10.1007/s00425-007-0533-z.

Olsen JE, Junttila O, Moritz T. 1995. A localised decrease of GA_1 in shoot tips of *Salix pentandra* seedlings precedes cessation of shoot elongation under short photoperiod. Physiol. Plant. 95: 627-632.

Olszewski N, Sun TP, Gubler F. 2002. Gibberellin signalling, biosynthesis, catabolism, and response pathways. Plant Cell 14: S61-S80.

O´Neill DP, Ross JJ. 2002. Auxin regulation of the gibberellin pathway in pea. Plant Physiol. 130: 1974-1982.

Ozga JA, Yu J, Reinecke DM. 2003. Pollination-, development-, and auxin-specific regulation of gibberellin 3ß-hydroxylase gene expression in pea fruit and seeds. Plant Physiol. 131: 1137-1146.

Pandolfini T, Rotino GL, Camerini S, Defez R, Spena A. 2002. Optimisation of transgene action at the post-transcriptional level: high quality parthenocarpic fruits in industrial tomatoes. BMC Biotechnology 2: 1-11.

Park SH, Nakajima M, Sakane M, Xu ZJ, Tomioka K, Yamaguchi I. 2005. Gibberellin 2-oxidases from seedlings of adzuki bean (*Vigna angularis*) show high gibberellin-binding activity in the presence of 2-oxoglutarate and Co[2+]. Biosci. Biotechnol. Biochem. 69(8): 1498-1507.

Pharis RP, King RW. 1985. Gibberellins and reproductive development in seed plants. Annu. Rev. Plant Physiol. 36: 517-568.

Phillips AL, Ward DA, Uknes S, Appleford NEJ, Lange T, Huttly AK, Gaskin P, Graebe JE, Hedden P. 1995. Isolation and expression of three gibberellin 20-oxidase cDNA clones from *Arabidopsis*. Plant Physiol. 108: 1049-1057.

Philouze J. 1983. Prthenocarpie naturelle chez la tomate. I. Revue bibliographique. Agronomie. 3: 311-320.

Picken AJF. 1984. A review of pollination and fruit set in the tomato. J. Hort. Sci. 59 (1): 1-13.

Rayle D, Cleyland R. 1992. The acid growth theory of auxin-induced cell elongation is alive and well. Plant Physiol. 99: 1271-1274.

Rebers M, Kaneta T, Kawaide H, Yamaguchi S, Yang Y-Y, Imai R, Sekimoto H, Kamiya Y. 1999. Regulation of gibberellin biosynthesis genes during flower and early fruit development of tomato. Plant J. 17: 241-250.

Rodrigo MJ, Garcia-Martínez JL, Santes CM, Gaskin P, Hedden P. 1997. The role of gibberellins A_1 and A_3 in fruit growth of *Pisum sativum* L. and the identification of gibberellins A_4 and A_7 in young seeds. Planta 201: 446-455.

Ross JJ, Reid JB, Swain SM, Hasan O, Poole AT, Hedden P, Willis C. 1995. Genetic regulation of gibberellin deactivation in *Pisum*. Plant J. 7(3): 513-523.

Ross JJ, O´Neill DP, Wolbang CM, Symons GM, Reid JB. 2002. Auxin-gibberellin interactions and their role in plant growth. J. Plant Growth Regul. 20: 346-353.

Sakai M, Sakamoto T, Saito T, Matsuoka M, Tanaka H, Kobayashi M. 2003. Expression of novel rice gibberellin 2-oxidase gene is under homeostatic regulation by biologically active gibberellins. J. Plant Res. 116: 161-164.

Sakamoto T, Kobayashi M, Itoh H, Tagiri A, Kayano T, Tanaka H, Iwahori S, Marsuoka M. 2001. Expression of a gibberellin 2-oxidase gene around the shoot apex is related to phase transition in rice. Plant Physiol. 125: 1508–1516.

Sanjuan R, Marin I. 2001. Tracing the origin of the compensasome: evolutionary history of DEAH helicase and MYST acetyltransferase gene families. Mol. Biol. Evol. 18: 330-343.

Sanjuan R, Wrobel B. 2005. Weighted least-squares likelihood ratio test for branch testing in phylogenies reconstructed from distance methods. Syst. Biol. 54:218-229.

Santes CM, García-Martínez JL. 1995. Effect of the growth retardant 3,5-dioxo-4-butyryl-cyclohexane carboxylic acid ethyl ester, an acylcyclohexanedione compound, on fruit growth and gibberellin content of pollinated and unpollinated ovaries in pea. Plant Physiol. 108: 517-523.

Sastry KKS, Muir RM. 1963. Gibberellin: Effect on diffusible auxin in fruit development. Science 140: 494-95.

Schomburg FM, Bizzell CM, Lee DJ, Zeevaart JAD, Amasino RM. 2003. Overexpression of a novel class of gibberellin 2-oxidases decreases gibberellin levels and creates dwarf plants. Plant Cell 15: 151-163.

Scott JW, Harbaugh BK. 1989. Micro-Tom. - A miniature dwarf tomato. Florida Agr Expt. Sta. Cir. 370: 1-6.

Serrani JC, Fos M, Atarés A, García-Martínez JL. 2007a. Effect of gibberellin and auxin on parthenocarpic fruit growth induction in the cv Micro-Tom of tomato. J. Plant Growth Regul. 26: 211-221.

Serrani JC, Sanjuan R, Ruiz-Rivero O, Fos M, García-Martínez JL. 2007b. Gibberellin regulation of fruit-set and growth in tomato. Plant Physiol. 145: 246-257.

Sjut V, Bangerth F. 1978. Endogenous hormones in artificially induced parthenocarpic fruits. Acta Hort. (ISHS) 80: 163-168.

Sjut V, Bangerth F. 1981. Effect of pollination or treatment with growth regulators on levels of extractable hormones in tomato ovaries and young fruits. Physiol. Plant. 53: 76-78.

Sjut V, Bangerth F. 1982/1983. Induced parthenocarpy – a way of changing the levels of endogenous hormones in tomato fruits (*Lycopersicon esculentum* Mill.). 1 Extractable hormones. Plant Growth Regul. 1: 243-251.

Smith MW, Yamaguchi S, Ait-Ali T, Kamiya Y. 1998. The first step of gibberellin biosynthesis in pumpkin is catalyzed by at least two copalyl diphosphate synthases encoded by differentially regulated genes. Plant Physiol. 118: 1411-1419.

Sponsel VM. 1995. Gibberellin biosynthesis and metabolism. In: Plant Hormones. Physiology, Biochemistry and Molecular Biology. Davies PJ (ed). Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 66-97.

Sponsel V, Hedden V. 2004. Gibberellin biosynthesis and inactivation. In: Davies P (ed). Plant Hormones: Biosynthesis, Signal Transduction, Action! Kluwer Acad Pub, Dordrecht, The Netherlands, pp. 63-94.

Srivastava LM. 2002. Auxins. In: Plant Growth and Development: hormones and environment. Academic Press, New York, USA, pp. 155-169.

Srivastava A, Handa AK. 2005. Hormonal regulation of tomato fruit development: a molecular perspective. J. Plant Growth Regul. 24: 67-82.

Sugimoto-Shirasu K, Roberts G, Stacey N, McCann M, Naxwell A, Roberts K. 2005. RHL1 is an essential component of the plant DNA topoisimerase VI complex and is required for ploidy-dependant cell growth. Proc. Natl. Acad. Sci. 102 (51): 18736-18741.

Sun TP, Kamiya Y. 1994. The *Arabidopsis* GA1 locus encodes the cyclase ent-kaurene synthetase A of gibberellin biosynthesis. Plant Cell 6: 1509 1518.

Symons GM, Reid JB. 2003. Interactions between light and plant hormones during de-etiolation. J. Plant Growth Regul. 22: 3-14.

Sun TP, Gubler F. 2004. Molecular mechanism of gibberellin signalling in plants. Ann. Rev. Plant Biol. 55: 197-223.

Suzuka I, Daidoji H, Matsuoka M, Kadowaki K, Takasaki Y, Nakane PK, Moriuchi T. 1989. Gene for proliferating-cell nuclear antigen (DNA polymerase delta auxiliary protein) is present in both mammalian and higher plant genomes. Proc. Natl. Acad. Sci. 9:3189-3193.

Talón M. 2000. Giberelinas. En: Fundamentos de Fisiología Vegetal. Azcón-Bieto J y Talón M (eds). Interamericana Mc Graw Hill, España, pp. 325-341.

Tanaka-Ueguchi M, Itoh H, Oyama N, Koshioka M, Matsuoka M. 1998. Over-expression of a tobacco homeobox gene, *NTH15*, decreases the expression of a gibberellin biosynthetic gene encoding GA 20-oxidase. Plant J. 15: 391-400.

Thomas SG, Phillips AL, Hedden P. 1999. Molecular cloning and functional expression of gibberellin 2-oxidases, multifunctional enzymes involved in gibberellin deactivation. Pro Natl. Acad. Sci. 96: 4698-4703.

Toyomasu T, Kawaide H, Sekimoto H, von Numers C, Phillips AL, Hedden P, Kamiya Y. 1997. Cloning and characterization of a cDNA encoding gibberellin 20-oxidase from rice (*Oryza sativa* L.) seedlings. Physiol. Plant. 99: 111-118.

Toyomasu T, Kawaide H, Mitsuhashi W, Inoue Y, y Kamiya Y. 1998. Phytochrome regulates gibberellin biosynthesis during germination of photoblastic lettuce seeds. Plant Physiol. 118: 1517–1523.

Tsukaya H, 2006. Mechanism of leaf-shape determination. Ann. Rev. Plant Biol. 57: 477-496.

Ubeda-Tomás S. 2003. Aislamiento de genes de la síntesis e inactivación de giberelinas en adelfa y estudio de sus aplicaciones biotecnológicas. Tesis Doctoral. Universidad Politécnica de Valencia-IBMCP-CSIC.

Ubeda-Tomás S, García-Martínez JL, López-Díaz I. 2006. Molecular, biochemical and physiological characterization of gibberellin biosynthesis and catabolism genes from *Nerium oleander*. J. Plant Growth Regul. 25: 52-68.

Van Huizen R, Ozga JA, Reinecke DM. 1997. Seed and hormonal regulation of gibberellin 20-oxidase expression in pea pericarp. Plant Physiol. 115: 123-128.

Varbanova M, Yamaguchi S, Yang Y, McKelvey K, Hanada A, Borochov R, Yu F, Jikumaru Y, Ross Y, Cortes D, Ma CJ, Noel JP, Mander L, Shulaev V, Kamiya Y, Rodermel S, Weiss D, Pichersky E. 2007. Methylation of gibberellins by *Arabidopsis* GAMT1 and GAMT2. Plant Cell 19: 32-45.

Varga A, Bruinsma J. 1976. Roles of seeds and auxins in tomato fruit growth. Z. Pflanzenphysiol. 80: 95-104.

Varga A, Bruinsma J. 1986. Tomato. In: CRC Handbook of Fruit Set and Development, Monselise SP (ed). Boca Raton FL. CRC Press, pp. 461-480.

Vercher Y, Carbonell J. 1991. Changes in the structure of ovary tissues and in the ultrastructure of mesocarp cells during ovary senescence or fruit development induced by plant growth substances in *Pisum sativum*. Physiol. Plant. 81: 518-526.

Wang H, Jones B, Li Z, Frasse P, Delalande C, Regad F, Chaabouni S, Latché A, Pech J-C, Bouzayen M. 2005. The tomato *Aux/IAA* transcription factor *IAA9* is involved in fruit development and leaf morphogenesis. Plant Cell 17: 2676-2692.

Waycott W, Smith VA, Gaskin P, MacMillan J, Taiz L. 1991. The endogenous gibberellins of dwarf mutants of lettuce. Plant Physiol. 95: 1169-1173.

Wilson LA, Sterling C. 1976. Studies on the cuticle of tomato fruit. I. Fine structure of the cuticle. Z. PflanzenPhysiol. 77: 359-371.

Wolbang CM, Chandler PM, Smith JJ, Ross JJ. 2004. Auxin from the developing inflorescence is required for the biosynthesis of active gibberellins in barley stems. Plant Physiol. 134: 769-776.

Wolbang CM, Ross JJ. 2001. Auxin promotes gibberellin biosynthesis in decapitated tobacco plants. Planta 214: 153-157.

Yamaguchi S, Saito T, Abe H, Yamane H, Murofushi N, Kamiya Y. 1996. Molecular cloning and characterization of a cDNA encoding the gibberellin biosynthetic enzyme *ent*-kaurene synthase B from pumpkin (*Cucurbita maxima* L.). Plant J. 10(2): 203-213.

Yamaguchi S, Smith MW, Brown RGS, Kamiya Y, Sun TP. 1998a. Phytochrome regulation and differential expression of gibberellin 3β-hydroxylase genes in germinating *Arabidopsis* seed. Plant Cell 10: 2115–2126.

Yamaguchi S, Sun TP, Kawaide H, Kamiya Y. 1998b. The GA2 locus of *Arabidopsis* thaliana encodes *ent*-kaurene synthase of gibberellin biosynthesis. Plant Physiol. 116: 1271–1278.

Yamaguchi S, Kamiya Y. 2000. Gibberellin Biosynthesis: Its regulation by endogenous and environmental signals. Plant Cell Physiol. 41: 251-257.

Yang YY, Rebers M, Toyomasu T, Kawaide H, Kaneta T, Kamiya Y. 1998. Cloning of two cDNAs encoding gibberellin 3 beta-hydroxylase (Accession no. AB010991, AB010992) of tomato (*Solanum lycopersicum* L.) seedlings (PGR8–200). Plant Physiol. 118: 1534.

Zeevaart JAD, Gage DA, Talon M. 1993. Gibberellin A_1 is required for stem elongation in spinach. Proc Natl. Acad. Sci. 90: 7401-7405.

Zhu Y, Nomura T, Xu Y, Zhang Y, Peng Y, Mao B, Hanada A, Zhou H, Wang R, Li P, Zhu X, Mander LN, Kamiya Y, Yamaguchi S, He Z. 2006. *ELONGATED UPPERMOST INTERNODE* encodes a cytochrome P450 monooxygenase that epoxidizes gibberellins in a novel deactivation reaction in rice. Plant Cell 18: 442-456.