Journal of Experimental Botany, Vol. 63, No. 16, pp. 5803–5813, 2012
doi:10.1093/jxb/ers229 Advance Access publication 3 September, 2012

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

The characterization of transgenic tomato overexpressing *gibberellin 20-oxidase* reveals induction of parthenocarpic fruit growth, higher yield, and alteration of the gibberellin biosynthetic pathway

Noemí García-Hurtado[1], Esther Carrera[1], Omar Ruiz-Rivero[1], Maria Pilar López-Gresa[1], Peter Hedden[2], Fan Gong[2] and José Luis García-Martínez[1,*]

[1] Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia (UPV)-Consejo Superior de Investigaciones Científicas (CSIC), Ingeniero Fausto Elío s/n, 46022 Valencia, Spain
[2] Rothamsted Research, Harpenden, Herts AL5 2JQ, UK

* To whom correspondence should be addressed. E-mail: jlgarcim@ibmcp.upv.es

Received 22 May 2012; Revised 18 July 2012; Accepted 23 July 2012

Abstract

Fruit-set and growth in tomato depend on the action of gibberellins (GAs). To evaluate the role of the GA biosynthetic enzyme GA 20-oxidase (GA20ox) in that process, the citrus gene *CcGA20ox1* was overexpressed in tomato (*Solanum lycopersicum* L.) cv Micro-Tom. The transformed plants were taller, had non-serrated leaves, and some flowers displayed a protruding stigma due to a longer style, thus preventing self-pollination, similar to GA_3-treated plants. Flowering was delayed compared with wild-type (WT) plants. Both yield and number of fruits per plant, some of them seedless, were higher in the transgenic plants. The Brix index value of fruit juice was also higher due to elevated citric acid content, but not glucose or fructose content. When emasculated, 14–30% of ovaries from transgenic flowers developed parthenocarpically, whereas no parthenocarpy was found in emasculated WT flowers. The presence of early-13-hydroxylation and non-13-hydroxylation GA pathways was demonstrated in the shoot and fruit of Micro-Tom, as well as in two tall tomato cultivars (Ailsa Craig and UC-82). The transgenic plants had altered GA profiles containing higher concentrations of GA_4, from the non-13-hydroxylation pathway, which is generally a minor active GA in tomato. The effect of GA_4 application in enhancing stem growth and parthenocarpic fruit development was proportional to dose, with the same activity as GA_1. The results support the contention that *GA20ox* overexpression diverts GA metabolism from the early-13-hydroxylation pathway to the non-13-hydroxylation pathway. This led to enhanced GA_4 synthesis and higher yield, although the increase in GA_4 content in the ovary was not sufficient to induce full parthenocarpy.

Keywords: Fruit set, gibberellin (GA), gibberellin 20-oxidase, Micro-Tom, parthenocarpy, tomato.

Introduction

The gibberellins (GAs) are plant hormones that control diverse developmental processes such as germination, shoot elongation, flowering, and fruit-set and growth (Hedden and Kamiya, 1997; Olszewski *et al*., 2002). Recent reviews (e.g. Sponsel and Hedden, 2004; Yamaguchi, 2008) have described in detail the plant GA metabolic pathways. In summary, as depicted in Supplementary Fig. S1 available at *JXB* online, GAs are synthesized from *ent*-kaurene, which is metabolized by the action of cytochrome P450-dependent monooxygenases to GA_{12} and/or GA_{53}. These precursors are converted to the active GA_4 and GA_1 by GA 20-oxidases and GA 3-oxidases, acting consecutively through two parallel pathways: the non-13-hydroxylation

pathway (leading to GA_4) and the early-13-hydroxylation pathway (leading to GA_1). GA_1, GA_4, and their precursors can be inactivated by GA 2-oxidases (Rieu *et al*., 2008*a*), the main GA-inactivating process. The majority of GA metabolism genes have been cloned (Hedden and Kamiya, 1997; Hedden and Phillips, 2000; Sponsel and Hedden, 2004; Yamaguchi, 2008), and their expression shown to be regulated by endogenous and environmental cues (Yamaguchi and Kamiya, 2000). GA 20-oxidases, GA 3-oxidases, and GA 2-oxidases are 2-oxoglutarate-dependent dioxygenases encoded by small gene families. For instance, in *Arabidopsis*, five *GA20ox*, four *GA3ox*, and seven *GA2ox* genes (Yamaguchi, 2008) and in tomato at least four *GA20ox*, two *GA3ox*, and five *GA2ox* genes (Serrani *et al*., 2008) have been reported.

Tomato (*Solanum lycopersicum* L.) is one of the most studied fleshy fruits due to its considerable commercial importance. Fruit-set normally is induced by fertilization and precedes fruit development, which in tomato takes place in two consecutive phases: active cell division lasting ~7–10 d post-anthesis, followed by cell expansion until fruit maturation (Gillaspy *et al*., 1993). The following evidence supports the contention that fruit-set in tomato depends on GA signalling: (i) GA application to non-pollinated ovaries induces parthenocarpic fruit growth (Fos *et al*., 2000; Serrani *et al*., 2007*a*, *b*); (ii) GA content increases in the ovary upon pollination (Bohner *et al*., 1988; Koshioka *et al*., 1994; Serrani *et al*., 2007*b*); (iii) the application of GA biosynthesis inhibitors prevents fruit-set of pollinated ovaries, and this effect is reversed by GA application (Sjut and Bangerth, 1982/83; Fos *et al*., 2000; Serrani *et al*., 2007*b*); and (iv) post-transcriptional antisense silencing of the *SlDELLA* gene, a nuclear repressor whose action is suppressed by GA (Schwechheimer, 2008), induces the production of parthenocarpic fruits (Martí *et al*., 2007). Pollination induces up-regulation of *SlGA20ox1*, *SlGA20ox2*, and *SlGA20ox3* genes, associated with the increase in GA_1 content (Serrani *et al*., 2007*b*). An increase in *SlGA20ox1* gene expression in the parthenocarpic *pat* mutant 24 h after pollination has also been reported (Olimpieri *et al*., 2007). Fruit-set in tomato is also controlled by auxin (Pandolfini *et al*., 2007; Serrani *et al*., 2007*a*; De Jong *et al*., 2009), the action of which is mediated at least in part by GAs through increasing *GA20ox* transcript content (Serrani *et al*., 2008, 2010). These observations suggest that *GA20ox* expression may play a key role in tomato fruit-set.

In this work tomato transgenic lines overexpressing a citrus gene encoding a GA20ox, previously shown to produce substantial enhancement of stem elongation when expressed in tobacco (Vidal *et al*., 2001), have been produced and characterized. In addition to the expected elongated shoot phenotype, it was found that the transgenic tomato lines had increased yield and displayed parthenocarpic growth capacity, supporting the idea that the regulation of *GA20ox* expression is important for fruit-set and development in tomato.

Materials and methods

Plant material and growth conditions

Tomato (*S. lycopersicum* L.) cvs Micro-Tom (MT), MT with an introgressed *D* gene (MT-D; see Serrani *et al*., 2010), Ailsa Craig, and UC-82 (seeds from Centro de Conservación y Mejora de la Agrodiversidad Valenciana, UPV, Valencia, Spain) were used in the experiments. For plant transformation, sterilized seeds of MT were germinated and grown in 600 ml glass jars (~9 cm diameter×9 cm height) (10–15 seeds per jar) covered with transparent lids, containing 50 ml of germination medium (GM) [Murashige and Skoog (MS) salts, 1% (w/v) sucrose, and 0.8% (w/v) agar], in a growth chamber at 26±2 ºC under 16 h light (photon fluence of 115 μmol m^{-2} s^{-1})/8 h dark conditions. For phenotype evaluation, seeds were pre-germinated in Petri dishes in the growth chamber, and selected seedlings were transferred to 600 ml pots (one seedling per pot) containing a mixture of peat:vermiculite (1:1), and cultured in the greenhouse under 24 ºC (day)/20 ºC (night) conditions, irrigated daily with Hoagland's solution. Natural light was supplemented with Osram lamps (Powerstar HQI-BT, 400 W) to achieve a 16 h light photoperiod.

Production of transgenic lines

A construct containing the citrus *CcGA20ox1* gene under the control of a double *Cauliflower mosaic virus* (CaMV) 35S promoter, and the marker gene *npt*II under the CaMV 35S promoter (as described in Vidal *et al*., 2001), was used for transformation using the *Agrobacterium tumefaciens* strain LBA4404. CcGA20ox1 has been shown to metabolize equally well *in vitro* both [^{14}C]GA_{12} and [^{14}C]GA_{53} substrates (Vidal *et al*., 2003).

MT transformation was carried out essentially as described in Ellul *et al*. (2003). Briefly, tomato first leaf sections (from 20-day-old seedlings grown under sterile conditions) were cultured for 2 d in the dark in Petri dishes containing solidified pre-culture (PC) medium [MS salts supplemented with vitamins, 3% (w/v) sucrose, 100 mg l^{-1} myo-inositol, 4 mg l^{-1} indole acetic acid (IAA), 4 mg l^{-1} kinetin, and 0.8% (w/v) agar]. Then they were immersed in bacterial suspensions (OD_{600nm}=0.4) containing 200 μM acetosyringone for 10 min. Blotted explants were cultured in the dark for 2 d in solidified PC medium with 200 μM acetosyringone, washed in washing medium [MS salts, 2% (w/v) sucrose, 100 mg l^{-1} myo-inositol, and 500 mg l^{-1} cefatoxime], and cultured for 2 d in the dark in solidified PC medium. Then the explants were transferred to PC medium containing 1 mg l^{-1} zeatin, 300 mg l^{-1} cefatoxime, and 100 m l^{-1} kanamycin. Explants developing resistant calli produced shoots, which were excised and placed on rooting medium [MS salts, 2% (w/v) sucrose, 100 mg l^{-1} myo-inositol, 1 mg l^{-1} thiamine, 0.1 mg l^{-1} IAA, and 0.8% (w/v) agar]. Rooted explants were cultured in pots containing vermiculite, watered with Hoagland's solution, and conditioned in a growth chamber before transferring to the greenhouse. Progeny from the transgenic plants were obtained by selfing under controlled conditions.

Southern analysis

A 10 μg aliquot of total DNA from young leaves (extracted according to Dellaporta *et al*., 1983) were digested with *Nco*I, separated by 1% agarose electrophoresis, and transferred to Hybond N^+ membrane (Amersham Biosciences) by capillarity with 20× SSC. Filters were hybridized at 50 ºC in a solution containing 0.5 M Na HPO_4 pH 7.2, 1 mM EDTA, 7% SDS, and a 691 bp DNA probe labelled with [^{32}P]dCTP using the Ready-To-Go Labelling Beads kit (Amersham Biosciences). After hybridization, the filters were washed with 1× SSC, 0.1% SDS at 55–60 °C then with 0.1× SSC, 0.1% SDS at 60 ºC for 10–15 min and exposed for autoradiography (Kodak X-OMAT LS, Amersham Biosciences). The DNA probe covering part of the promoter and part of the transgene was produced by PCR amplification using the *35S:CcGA20ox1* construct DNA, the specific primers: 5'-GATTCCATTGCCCAGCTATC-3' (sense) and 5'-TCCATGTAACGGTGAGCATC-3' (antisense), and the following thermocycling conditions: 95 ºC for 5 min, 35 cycles of 95 ºC for 45 s/58 ºC for 45 s/72 ºC for 45 s, and a final extension of 72 ºC for 5 min.

RT-PCR and qPCR

RT-PCR was carried out to estimate transgene expression (Supplementary Fig. 2B at *JXB* online) using total RNA, isolated using TRIZOL®

Reagent according to the manufacturer's instructions (Invitrogen™ Life Technologies, Barcelona, Spain), and the primers: 5'-TTATCAGG ACTACTGCGAGTCGATGAG-3' (sense) and 5'-TCAGCTGTTTTT TCTGTTGAAGCCAG-3' (antisense), amplifying a band of 581 bp spanning the second intron. Thermocycling conditions used were 95 ºC for 5 min followed by 30 cycles of 95 ºC for 45 s/60 ºC for 45 s/72 ºC for 45 s, and a final 5 min extension of 72 ºC.

Absolute amounts (molecules of single-stranded RNA ng^{-1} total RNA) of transgene and transcript levels of endogenous *SlGA20ox*, *SlGA3ox*, and *SlGA2ox* were also determined by qPCR using the external standard curve procedure and primers described in Serrani *et al*. (2008). For transgene transcript quantification, PCR fragments of 149 bp of the transgene were amplified with the specific primers: 5'-AAAAGCCTTGTGTTAATGCACC-3' (sense) and 5'-CGATTAGGCTTGAATCAACTCCA-3' (antisense). Absolute amounts of mRNA in the samples were quantified using three independent biological replicates.

Plant hormone applications

Seeds were germinated in 0.6 ml glass jars (15 seeds per jar) covered with transparent lids, containing GM medium and 5×10^{-5} M GA_3 (Duchefa, Haarlem, The Netherlands) and/or 10^{-7} M paclobutrazol (PAC; Duchefa). The seedlings were grown in a growth chamber under the conditions described before until complete hypocotyl growth (~10 d).

Application of GA_3 was also carried out by spraying the aerial part of plants grown in the greenhouse with 10^{-5} M aqueous solution containing 0.1% Tween-80 (Sigma-Aldrich, St Louis, MO, USA). Control plants were sprayed with the same solvent solution. Hormone application was carried out daily, starting when the first leaf was just appearing above the cotyledons.

Quantification of gibberellins

GAs were quantified following the protocol described in Fos *et al*. (2000). In summary, aliquots from apical shoots including the three youngest developed leaves from plants just before flowering (3–10 g fresh weight) and of 10-day-old fruits (~3 g fresh weight) 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 before higp-performance liquid chromatography (HPLC) and gas chromatography–mass spectroscopy (GC-MS) analysis. [17,17-^{2}H]GAs (purchased from Professor L. Mander, Australian National University, Canberra) were added to the extracts as internal standards for quantification, which was carried out by GC-SIM. The concentrations of GAs in the extracts were determined using calibration curves. The analyses were carried out using three biological replicates.

Determination of parthenocarpic capacity

To determine parthenocarpic capacity, two or three emasculated flowers per truss and the 2–3 trusses were left in at least 12 plants per line, in two independent experiments. Flower emasculation was carried out 2 d before anthesis (d –2) to prevent self-pollination, and all non-selected flowers were removed. The percentage of fruits developed and their weight were determined at maturity.

Determination of Brix index and concentration of soluble solids

Brix index values were determined in the squeezed juice of ripened pollinated tomatoes, five randomly selected fruits per plant, 12 plants per line, using a portable refractometer (model N1, Atago Co Ltd, Honcho, Itabashi-ku, Tokyo, Japan).

The concentration of citric acid, glucose, and fructose in the juice was quantified by HPLC, using a Transgenomic ICSep ION-300 ion exclusion column (300 × 7.8 mm) coupled to a refractive index detector (Waters 2414). The juice was centrifuged at 12 000 rpm for 5 min, and 20 µl of the supernatant was isocratically eluted at 60 ºC with 5 mM H_2SO_4 at 0.3 ml min^{-1}, over a total run of 35 min. The compounds were quantified using external standard curves (Waters Breeze software).

Results

Isolation of transgenic tomato lines overexpressing CcGA20ox1

Tomato leaf explants were infected with *Agrobacterium tumefaciens* LBA4404 harbouring the *35S*:*CcGA20ox1* construct, and kanamycin-resistant shoots were isolated. Twelve independent diploid lines were grown in pots and cultured in the greenhouse. Five of them, all presenting a significantly taller stature than control plants and non-serrated leaves, and expressing the transgene (data not presented) were selected to isolate homozygous lines. Two homozygous overexpressor lines (*GA20ox*-OE; L4 and L19), carrying only one transgene insertion as shown by Southern analysis (Supplementary Fig. S2A at *JXB* online), and displaying high expression of the transgene in vegetative tissues (Supplementary Fig. S2B), were chosen for further characterization.

Phenotypic characterization of GA20ox-*OE lines*

Seedlings of the two isolated tomato transgenic lines overexpressing *GA20ox* had longer hypocotyls and roots, and the plants were taller, with longer internodes and thinner stems compared with controls (Table 1A, Fig. 1A, 1B). Furthermore, the aerial part of fully developed transgenic plants was heavier, particularly in L19 (Table 1A). The leaves of transgenic plants lacked the characteristic serrated borders of wild-type (WT) leaves (Fig. 1C). Most of the leaves had fewer leaflets or were of reduced size when compared with WT leaves of similar age and position on the plant (Fig. 1C).

Flowers from transgenic plants were of similar size to those from the WT, although a certain number of the former had exserted stigmas protruding above the staminal cone due to a longer style and/or larger ovary (Fig. 1D, 1E). The percentage of this kind of flower varied from batch to batch of plants for unknown reasons (values obtained in a typical experiment were 4% in L4 and 10% in L19), but they were never found in WT plants. Interestingly, the number of leaves before the first inflorescence was higher in the transgenic lines (9–10 versus ~7 leaves in the WT) (Table 1A), associated with a delay of flowering time as determined by the time to anthesis of the first opened flower (~5 d later in the transgenics) (Table 1B). Fruit ripening time (determined by colour break of the first ripened fruit) was also delayed in the transgenic lines (~10 d) (Table 1B). Although the transgenic plants had slightly lower numbers of flowers (at least in the first inflorescence; see Table 1B), their total number of fruits was higher and these had a similar mean fresh weight to WT fruits, thus leading to higher overall yields (Table 1B). The number of seeds per fruit was reduced in the transgenics (from 36 in the WT to 24–30) (Table 1B), but the final germination percentage was not affected.

The Brix index is an indication of the amount of total soluble solids (expressed as a percentage of sucrose equivalent),

Table 1. *Phenotype of the wild type (WT) and* GA20ox *transgenic tomato lines (L4 and L19)*

Data are means of 10–12 seedlings or plants, except when otherwise stated, ±SE.

(A) Vegetative phenotypes							
Line	**Hypocotyl length (mm)**	**Root length[a] (mm)**	**Height to first inflorescence (cm)**	**Internode length[b] (cm)**	**Stem diameter[c] (mm)**	**Leaves to first inflorescence (*n*)**	**Aerial part[d] (g plant^{-1})**
WT	15.5±0.3	38.8±1.3	10.8±0.7	1.5±0.1	7.2±0.2	7.2±0.2	86±4
L4	25.2±0.5	45.8±1.7	25.0±0.3	2.7±0.2	5.0±0.1	9.7±0.2	95±6
L19	23.8±0.4	46.1±1.5	28.0±0.1	2.8±0.3	5.2±0.1	9.3±0.3	115±9

(B) Reproductive phenotypes								
Line	**Flowers per first inflorescence (*n*)**	**Fruits per plant(*n*)**	**Fruit weight (g/fruit)**	**Fruit production (g plant^{-1})**	**Seeds per fruit[e] (*n*)**	**Days to anthesis[f] (*n*)**	**Days to colour break[f] (*n*)**	**Brix index[g]**
WT	8.3±0.3	16±1	3.9±0.3	64±5	36±2	31.6±0.2	70.7±0.3	5.7±0.2
L4	6.9±0.3	29±2	4.0±0.2	116±10	30±3	36.9±0.3	80.8±0.4	6.6±0.3
L19	6.9±0.3	22±2	3.6±0.2	80±4	24±3	37.1±0.6	82.3±0.3	7.4±0.2

[a] Root length (*n*=20) was measured 48 h after transferring the plates with seeds from 4 ºC to 24 ºC.
[b] Mean internode length between cotyledons and the first truss.
[c] Stem diameter corresponds to that of the fifth internode.
[d] Weight of aerial part corresponds to plants after fruit harvest.
[e] The number of seeds per fruit corresponds to the first five mature seedy fruits (*n*=8).
[f] Days to anthesis and to colour break correspond to those of the first opened flower and first fruit breaker, respectively.
[g] Brix index was determined in the first five ripened fruits from 10 plants.

and it is an important economic factor in the tomato industry. Interestingly, Brix index values of the juice were higher in transgenic fruit (6.6–7.4 versus 5.7 in WT fruit) (Table 1B), due to a higher content of citric acid, but not of glucose or fructose (Supplementary Table S1 at *JXB* online).

The phenotype of GA20ox*-OE lines was mimicked by GA_3 application to WT plants*

GA_3 application enhanced hypocotyl elongation of WT seedlings to values similar to those of the transgenic lines, but it had no effect in transgenic seedlings (Fig. 2A, 2B). Hypocotyl length was reduced by application of the GA biosynthesis inhibitor PAC, in both control and transgenic seedlings, and this effect was reversed when GA_3 was also included in the culture medium (Fig. 2A, 2B).

WT plants sprayed with GA_3 had a similar height to L4 and L19 (Fig. 2C, 2D), as well as having a similar leaf shape, particularly the absence of serrated borders (Fig. 2E, 2F). In WT plants the number of leaves before the first inflorescence increased after GA_3 application, as occurred by *CcGA20ox1* overexpression, whereas it was decreased by PAC application, the effect of which was reversed by GA_3 (control=7.2±0.1, GA_3=8.0±0.0, PAC=5.3±0.2, and PAC+GA_3=6.2±0.3 leaves; *n*=10).

The transgenic lines have facultative parthenocarpic capacity

Interestingly, while no parthenocarpic fruits were found in WT plants, a low and variable number of seedless (parthenocarpic) fruits (<10%) developed in *GA20ox*-OE plants. It is known that fruit-set of pollinated tomato ovaries depends on GA (Serrani *et al*., 2007*b*). For this reason, it was also of interest to determine whether non-pollinated ovaries from *GA20ox*-OE lines grow in the absence of pollination. No difference in fruit-set and weight of fruits developed from pollinated ovaries was found between control and transgenic plants (Table 2). In contrast, while non-pollinated ovaries did not grow in WT plants, a certain number of non-pollinated ovaries (from 14% to 30%, depending on the line and experiment) developed into seedless mature fruits in emasculated flowers from the two transgenic lines (Table 2, Fig. 1F). The weight of parthenocarpic fruits was less than half that of pollinated fruits (Table 3). Interestingly, the locular cavities of those parthenocarpic fruits were filled with locular tissue (Fig. 1F), in contrast to parthenocarpic fruits induced by GA_3 application (Serrani *et al*., 2007*a*).

The transgenic lines have altered GA concentration

Many of the phenotypic characteristics, as well as the results obtained with GA_3 and PAC applications described above, prompted the determination of whether the *GA20ox* transgenic plants contained higher GA levels than WT plants. The concentrations of GAs in shoots and developing fruit of the dwarf cultivar MT (Martí *et al*., 2006) were first compared with those of two tall cultivars: Ailsa Craig (indeterminate) and UC-82 (determinate). Importantly, all GAs from both early-13-hydroxylation and non-13-hydroxylation pathways (Supplementary Fig. S2 at *JXB* online) were detected and quantified in the three cultivars, although some differences were found between them, with higher concentrations of the active GA_1, GA_3, and GA_4 in MT than in Ailsa Craig and UC-82 (Supplementary Table S2).

In shoots of transgenic plants the contents of GA_{44}, GA_{19}, GA_{20}, and GA_{29}, from the early-13-hydroxylation pathway were reduced (particularly the first two), while those of GA_1 and GA_8 increased slightly (Table 3A). Changes were much higher in GAs

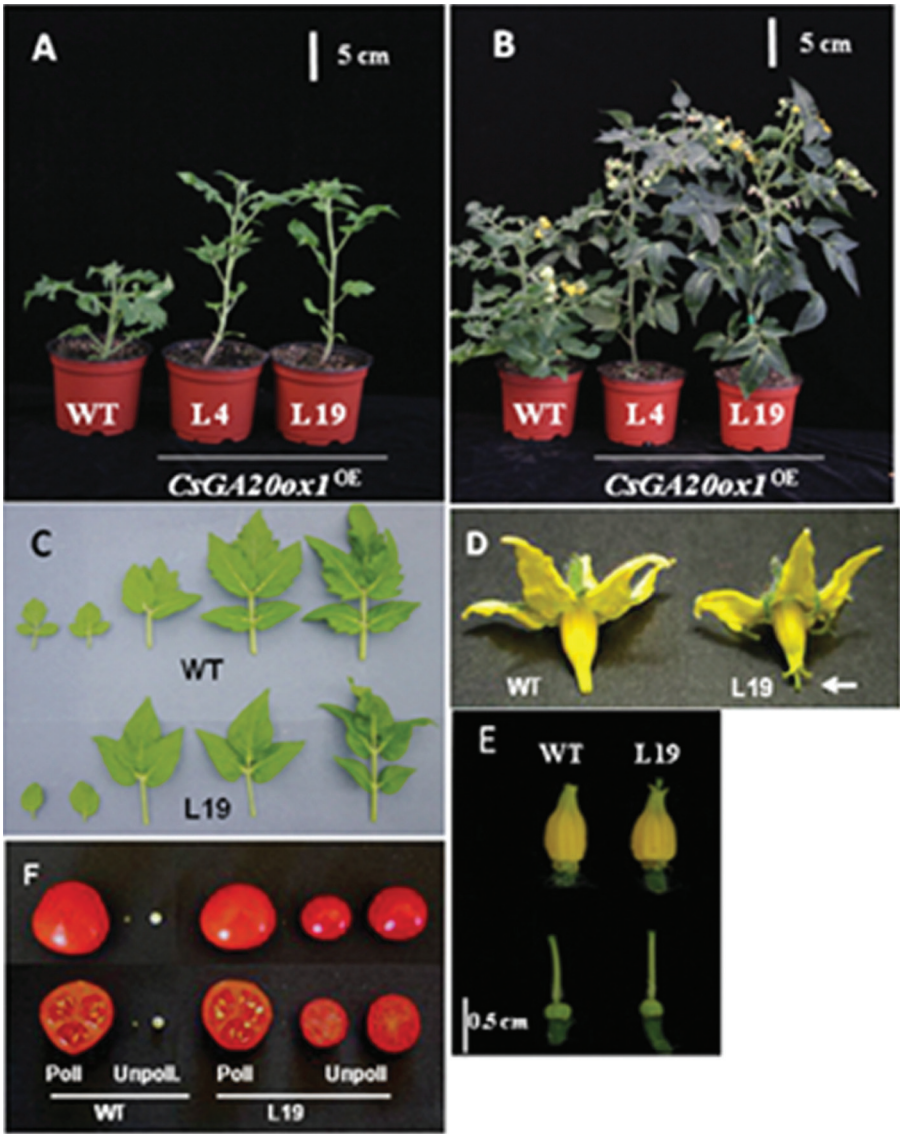

Fig. 1. Photographs of representative WT and *GA20ox*-OE lines (L4 and L19) of tomato. (A) Plants before flowering. (B) Plants with mature fruits. (C) Leaves at different positions on the plant (first to fifth, left to right). (D) Flowers at anthesis. The arrow indicates the protruding stigma. (E) Stamen cones and pistils. (F) Pollinated and non-pollinated fruits. WT, wild type; Poll., pollinated; Unpoll., non-pollinated. (This figure is available in colour at *JXB* online.)

from the non-13-hydroxylation pathway, where concentrations of the active GA_4 and its immediate precursor GA_9 in the transgenic lines were at least 10-fold higher than in the control, and that of GA_{34} (a GA_4 metabolite) about double (Table 3A). We also analysed GA content in 10-day-old fruits, which in the case of transgenic plants consisted of a mixture of pollinated and parthenocarpic fruits because they are not easy to distinguish due to little seed development at that age. As occurred in the shoot, concentrations of all the GAs from the early-13-hydroxylation pathway analysed (GA_{19}, GA_{20}, GA_{29}, and GA_1) were lower in the transgenic fruits, whereas those of GA_4 and GA_{34}, from the non-13-hydroxylation pathway were higher (Table 3B). GA_9 could not be quantified in fruits due to the presence of interfering compounds.

GA_1 is considered to be the main active GA in tomato (Fos *et al*., 2000; Serrani *et al*., 2007*b*). Thus, to investigate whether the increase in GA_4 content (produced normally from a minor GA biosynthetic pathway in tomato) had physiological relevance, GA_4 activity was compared with that of GA_1. As shown in Table 4, both GAs were similarly active in enhancing hypocotyl growth and inducing parthenocarpic fruit growth. Both effects (including the percentage of parthenocarpic fruits developed) were proportional to the amount of GA applied.

Expression of genes of GA metabolism in transgenic plants

The increase in GA_4 content in the fruit from transgenic plants compared with controls was much less than in the shoot (2–3 times compared with ~10 times in the latter). To determine whether this was the result of different levels of transgene expression, transgene transcript abundance was determined by

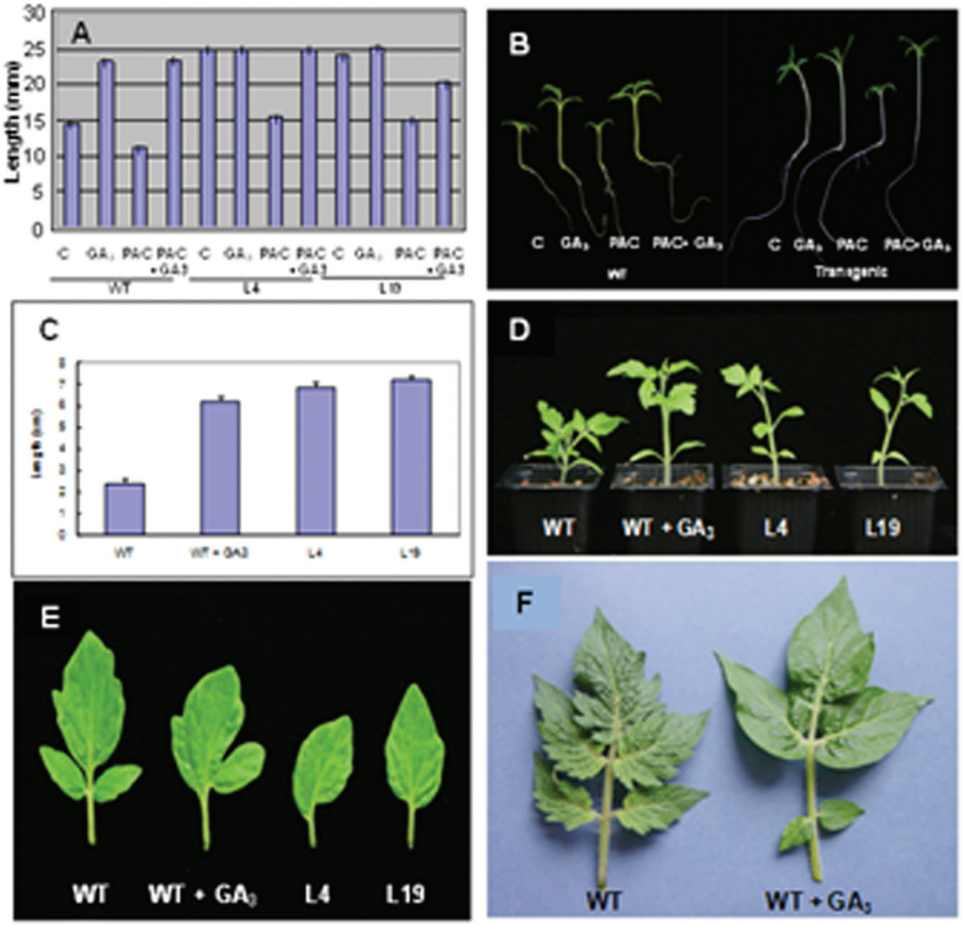

Fig. 2. Effect of GA_3 and PAC application on the phenotype of WT plants compared with that of *GA20ox*-OE lines (L4 and L19). (A) Mean hypocotyl length ±SE (n=15). (B) Photograph of representative seedlings. The seedlings were cultured in vessels containing solid medium in a growth chamber, and the hormones applied in the culture medium at 5×10^{-5} M GA_3 and 10^{-7} M PAC. (C) Length from cotyledons to fourth leaf. Data are means of ten 2-week-old plants ±SE. (D) Photograph of representative 2-week-old plants. (E) Photograph of representative first leaves. (F). Photograph of representative fifth leaf of adult WT plants untreated and treated with GA_3. Plants were sprayed daily with a 10^{-5} M GA_3 solution from the time of appearance of the first leaf above the cotyledons. C, control; PAC, paclobutrazol; WT, wild type. (This figure is available in colour at *JXB* online.)

Table 2. *Parthenocarpic capacity of wild- type (WT) and transgenic* GA20ox-*OE lines (L4 and L19) in two independent experiments*

In the case of pollinated ovaries, the values come from 60 flowers tagged in 12 plants. In the case of non-pollinated fruits, fruit-set was determined by emasculating flowers (2–3 trusses per plant, 2–3 ovaries per truss, in at least 12 plants). Values in parentheses indicate the number of ovaries set over total number of non-pollinated ovaries. Weight data are means of developed fruits larger than 1 g ±SE.

	Line	Pollinated ovaries		Non-pollinated ovaries	
		Fruit-set (%)	Fruit weight (g fruit^{-1})	Fruit-set (%)	Fruit weight (g fruit^{-1})
Experiment I	WT	100	5.5±0.2	0 (0/72)	–
	L4	100	5.2±0.2	13.9 (10/72)	2.2±0.3
	L19	100	5.4±0.2	16.7 (12/72)	2.1±0.1
Experiment II	WT	–	–	0 (0/40)	–
	L4	–	–	24.1 (28/116)	2.4±0.8
	L19	–	–	29.8 (25/84)	1.8±0.4

qPCR in leaves and seeded fruits (pericarp and seeds separated). The results obtained showed that *35S:GA20ox* was expressed in all the tissues analysed and that, contrary to expectation, it was expressed more highly in the reproductive (at least in the pericarp) than in vegetative tissues (Fig. 3A). Therefore, attempts were made to explain this paradox by determining the effect of ectopic *GA20ox* overexpression on transcript levels for endogenous GA metabolism enzymes *SlGA20ox*, *SlGA3ox*, and *SlGA2ox*

Table 3. *Gibberellin concentration (ng g FW^{-1}) in apical shoots (A) and 10-day-old fruits (B) of wild-type (WT) and* GA20ox-*OE lines (L4 and L19)*

Results are means of three biological replicates ±SE.

(A) Apical shoots									
Line	**GA_{44}**	**GA_{19}**	**GA_{20}**	**GA_{29}**	**GA_1**	**GA_8**	**GA_9**	**GA_4**	**GA_{34}**
WT	2.3±0.7	2.2±0.4	4.3±0.1	1.1±0.1	0.37±0.02	4.9±0.4	0.50±0.20	1.20±0.1	2.2±0.2
L4	<0.01	0.06±0.04	3.7±0.4	0.50±0.1	1.10±0.50	6.1±1.3	16.3±2.3	15.1±3.4	4.9±0.2
L19	<0.01	0.05±0.03	3.6±0.5	0.40±0.1	0.50±0.02	6.1±1.5	17.8±0.8	14.8±3.4	4.1±0.3
(B) Ten-day-old fruits									
Line		**GA_{19}**	**GA_{20}**	**GA_{29}**	**GA_1**	**GA_8**	**GA_9**	**GA_4**	**GA_{34}**
WT		1.3±0.2	5.6±0.8	3.7±1.2	1.0±0.1	6.8±0.8	NR	2.8±0.6	2.9±0.4
L4		0.60±0.30	2.9±0.8	1.5±0.2	0.40±0.20	4.2±0.6	NR	8.0±0.6	8.0±0.9
L19		0.06±0.06	1.3±0.2	1.3±0.1	0.20±0.05	2.2±0.2	NR	6.8±0.5	8.2±0.9

NR, no result due to weak signals or interfering ions.

Table 4. Effect of GA_1 and GA_4 application on hypocotyl length (mm) (A) and parthenocarpic growth (g fruit^{-1}) (B) of tomato cv Micro-Tom Hypocotyl lengths (A) are means of 28 seedlings ±SE. Fruit weights (B) are means of developed fruits higher than 1 g ±SE.

See the Materials and methods for experimental details.

(A) Hypocotyl length (mm)					
	GA concentration (M)				
	0	**10^{-8}**	**10^{-7}**	**10^{-6}**	
GA_1	17.7±0.2	17.0±0.4	20.3±0.4	26.1±0.5	
GA_4		16.3±0.3	17.6±0.4	25.0±0.3	
(B) Parthenocarpic growth (g fruit^{-1})					
	GA dose (ng)				
	0	**2**	**2**	**200**	**2000**
GA_1	0 (0)	1.0±– (1)	1.8±0.2 (5)	1.6±0.2 (7)	2.1±0.1 (9)
GA_4		1.0±0.1 (2)	1.5±0.1 (2)	2.2±0.1 (7)	2.6±0.1 (9)

in the shoot and fruit pericarp, to determine whether GA homeostasis was different in these organs.

Expression of most of the GA metabolism genes analysed differed in the transgenic lines compared with controls, although the changes were not always consistent with the expected GA feedback regulation. Contrary to expectations, transcript content for *SlGA20ox1* and *SlGA20ox3* was higher in the transgenic shoots and pericarp compared with the WT organs (Fig. 3B, 3C), while *SlGA20ox2* expression was also slightly higher in the shoot, but decreased in the pericarp (Fig. 3B); *SlGA20ox4* expression was reduced in some cases, although it was always at a very low level (Fig. 3C). Compared with the WT, transcript levels of *SlGA3ox1* were higher and those of *SlGA3ox2* unchanged in the shoot of transgenic lines, but in the pericarp *SlGA3ox1* expression was higher (in L19) while that of *SlGA3ox2* was lower (in both lines) (Fig. 3D, 3E). The expression of all *SlGA2ox* genes increased in the shoot of the two transgenic lines, as expected (Fig. 3F). In contrast, in the pericarp, only the expression of *SlGA2ox1* in L19, and that of *SlGA2ox4* in L4, was increased, while the expression of *SlGA2ox2*, *SlGA2ox3*, and *SlGA2ox5* decreased in most cases (Fig. 3F).

Therefore, it was of interest to compare those results with the response of vegetative tissues of WT plants to GA_3 and PAC application, the usual method for testing the GA feedback regulation of GA metabolism genes. After 46 d of continuous hormone application to the roots, the plants showed the expected elongated (in the case of +GA_3) and dwarf (in the case of +PAC) phenotypes (Supplementary Fig. S3A at JXB online). At that time, using shoots similar to those in the experiment for Fig. 4, it was found that the effects of the treatments on transcript levels for *SlGA20ox1*, *SlGA20ox2*, *SlGA20ox3*, *SlGA3ox1*, and *SlGA3ox2* were consistent with the expected negative feedback regulation (Supplementary Fig. S3B–D), but not with the observed effects of transgene expression. In the case of *SlGA20ox2*, *SlGA20ox3*, and *SlGA3ox3*, negative feedback regulation was only observed with PAC application. The levels of *SlGA20ox4* transcripts were very low, but increased upon GA_3 treatment, as in the transgenic plants, and decreased upon PAC treatment, contrary to expectations (Supplementary Fig. S3C). The five *SlGA2ox* genes displayed positive feed-forward regulation (Supplementary Fig. S3F, G), as expected, also observed in most cases in the transgenic shoots.

Discussion

GA20ox *overexpression alters vegetative and reproductive phenotypes in tomato*

The overexpression of *CcGA20ox1* in tomato produced the characteristic phenotype observed in plants from diverse species overexpressing *GA20ox* (Eriksson *et al*., 2000; Vidal *et al*., 2001; Phillips, 2004): longer hypocotyls and roots, and taller plants with longer and thinner internodes. The growth of leaves without serrated borders is a phenotype not previously described in tomato *GA20ox*-OE plants, although it is similar to that found in GA_3-treated WT plants (Fig. 2D; Martí *et al*., 2006). In addition, the reduced number or size of leaflets, both in the transgenics and in GA_3-treated plants, in leaves at ontogenic positions

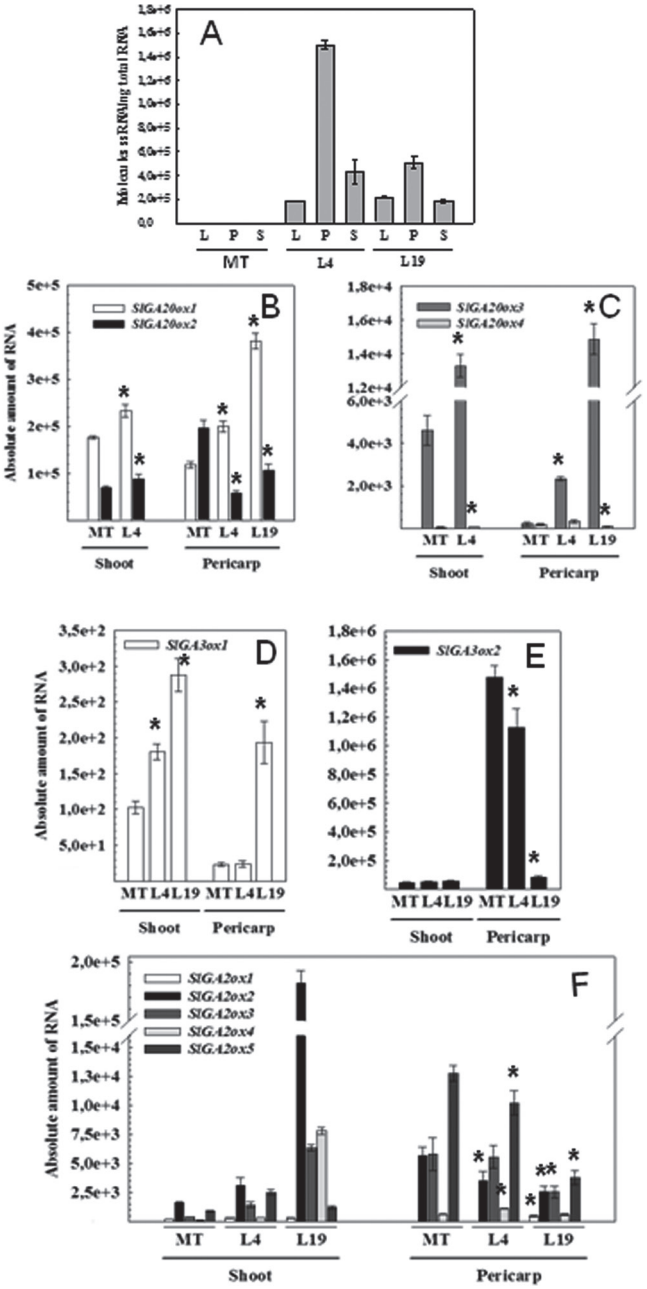

Fig. 3. Absolute amounts (molecules of single-stranded RNA ng total RNA^{-1}) of the transgene and GA metabolism gene transcripts in shoots and fruit of MT (wild type) and *GA20ox*-OE lines (L4 and L19) of tomato. (A) *CcGA20ox1* transgene in leaves, pericarp, and seeds of pollinated fruits. (B, C) *SlGA20ox1*, *SlGA20ox2*, *SlGA20ox3*, and *SlGA20ox4*. (D, E) *SlGA3ox1* and *SlGA3ox2*. (F) *SlGA2ox1*, *SlGA2ox2*, *SlGA2ox3*, *SlGA2ox4*, and *SlGA2ox5*. RNA was extracted from leaves of plants just before flowering, and from pericarp and seeds of 10-day-old pollinated fruits. Values are means of three biological replicates ±SE. Asterisks denote a significant difference ($P < 0.05$; Student's *t*-test) between MT and the corresponding transgenic line). All values of *SlGA2ox* transcripts in the shoot of both lines are significantly higher than those corresponding in MT, but asterisks are not given in this case for the purpose of clarity. L, leaves; P, pollinated fruits; S, seeds.

similar to in the untreated WT is consistent with the published role of GAs in determining leaf architecture (Singh *et al*., 2010, and references within). LANCEOLATE activity, responsible for precocious tomato leaf differentiation and development, has been shown to be partially mediated by changes in GA levels (Yanai *et al*., 2011).

Although the size of petals, sepals, and stamens was not altered, the stigma protruded in some transgenic flowers due to a longer style. This indicates that pistil growth is regulated by GAs, and agrees with the observation of Martí *et al*. (2007) that flowers of tomato overexpressing antisense *SlDELLA* had a longer style, completely hindering self-pollination. The tomato *procera* mutant, which contains a single base mutation in *SlDELLA* leading to a constitutive GA response phenotype (Bassel *et al*., 2008; Jasinski *et al*., 2008), also produces flowers with exserted stigmas (Carvalho *et al*., 2011). This suggests that DELLA proteins may play a role in the control of style elongation.

Interestingly, flowering in *GA20ox*-OE tomato lines was slightly delayed (Table 1B). This inhibitory effect of GA on tomato flowering was confirmed by GA_3 and PAC application experiments. Delay of flowering by GA was also found in other species such as grape (Boss and Thomas, 2002). However, these results are in contrast to those found with tobacco, where application of GA_3 or *GA20ox* overexpression did not affect flowering time (Vidal *et al*., 2001; Gallego-Giraldo *et al*., 2007), and with *Arabidopsis*, where GA advances flowering under long-day conditions (Blázquez and Weigel, 2000; Rieu *et al*., 2008*b*). This means that while it is generally accepted that GAs have an enhancing effect on stem elongation, their role in flowering is clearly more complex and depends on the species.

GA20ox *overexpression induces parthenocarpic fruit-set capacity and increased yield in tomato*

Ovaries of *GA20ox*-OE tomato plants displayed parthenocarpic capacity. This was shown first by the presence of seedless fruits in plants with self-pollinated flowers, probably associated with absence of pollination due to the long style phenotype described above. The parthenocarpic capacity of *GA20ox*-OE plants was further demonstrated by emasculating the flowers and thus preventing self-pollination. In this case, up to 30% of the ovaries produced parthenocarpic fruits (Table 2). The relatively low rate of parthenocarpy may be a consequence of the constitutive expression of the *GA20ox* transgene. It is known that there is negative interaction between vegetative and reproductive growth, and that application of GA biosynthesis inhibitors reduces shoot vigour and favours fruit growth in some crops, such as grape (Gianfagna, 1995). Therefore, the parthenocarpic capacity of tomato might be further increased by specific overexpression of *GA20ox* in the ovary, thus preventing the enhanced vegetative growth occurring in constitutively *GA20ox*-OE plants. This is supported by the observation that localized application of GA_1 and GA_4 to unpollinated WT ovaries induced up to 100% fruit-set (see Table 4).

The higher yield (total weight production of seeded plus seedless fruits) from the *GA20ox*-OE lines is notable, particularly because it was associated with increased fruit numbers without the reduction of mean fruit weight (Table 1) normally resulting

from higher fruit numbers. In tomato, as in other species producing many flowers, the strong competition for nutrients established between the developing fruits reduces fruit size and limits the growth of all self-pollinated ovaries (Bangerth, 1989; Serrani *et al*., 2007*a*). The higher yield in *GA20ox*-OE lines was the consequence of bearing more fruits, in spite of the transgenic plants producing slightly fewer flowers and the seeded fruits bearing fewer seeds. It was found that 10-day-old fruits from transgenic plants contained 2–3 times more GA_4 than those from WT plants (Table 3B). Therefore, this additional GA_4 synthesized in the ovary of transgenic plants may produce stronger sink capacity of pollinated ovaries, as well as the development of parthenocarpic fruits in the case of ovaries with exserted styles that prevent self-pollination. It has been found that overexpression of *GA20ox* in citrus enhances their leaf photosynthetic capacity (Huerta *et al*., 2008). Therefore, it is also possible that leaves of *GA20ox*-OE tomato plants provide more sugars to the reproductive tissues, which would still be competing, but for more resources.

In ripe tomato fruits, the most important compounds present in the so-called soluble solids fraction are sugars and organic acids (Tanksley and Hewitt, 1988). It is of interest that although fruit juice from transgenic lines had higher Brix index values (Table 1B), it did not contain higher concentrations of glucose and fructose, the two main sugars of tomato juice. This was surprising because GA application induces higher invertase activity in ovaries of different species during fruit-set (Estruch and Beltrán, 1991; Pérez and Gómez, 2000; Zhang *et al*., 2007). In contrast, in the case of *GA20ox*-OE plants, the fruit juice contained a significantly higher concentration of citric acid. The regulation of the citric acid cycle in plants is still not clear, and the present results suggest a possible role for GAs in that process during fruit ripening.

The phenotypes of GA20ox-*OE plants are due to increased active* GA_4 *content*

The low GA levels of members of the non-13-hydroxylation pathway in young tomato fruit (Bohner *et al*., 1988; Koshioka *et al*., 1994) indicated that the main pathway in tomato is the early-13-hydroxylation pathway (producing GA_1). It was confirmed that both pathways are present in the shoot and fruit of MT, Ailsa Craig (indeterminate), and UC-82 (determinate) cultivars, and that the early-13-hydroxylation pathway predominates in the shoot, where the content of GA_1 was ~10-fold higher than that of GA_4. In contrast, the contents of GA_1 and GA_4 in the fruits were quite similar (Table 3). The higher concentration of GA_1 and GA_4 in MT, compared with Ailsa Craig and UC-82, may be due to their different genetic backgrounds, or possible differences in GA perception and/or signalling between the cultivars.

Although the early-13-hydroxylation pathway predominates in tomato, at least in vegetative tissues, overexpressing *CcGA20ox1* produced a much greater increase in the concentrations of GA_4 and its metabolite GA_{34} than in those of their 13-hydroxylated equivalents. In fact, the levels of the 13-hydroxyGAs were lower in transgenic fruit than in the WT. The expression product of the transgene *CcGA20ox1* acts *in vitro* on both GA_{12} and GA_{53} substrates (Vidal *et al*., 2003). Therefore, the above results are probably due to competition of the overexpressed GA 20-oxidase with the endogenous GA 13-hydroxylase for the GA_{12} substrate, leading to a greater relative flux through the non-13-hydroxylation pathway in the transgenic plants compared with the WT. Similar results were found in aspen (Eriksson *et al*., 2000) and tobacco (Vidal *et al*., 2001), also with a main endogenous early-13-hydroxylation pathway.

Importantly, the application of GA_4 to WT seedlings and non-pollinated ovaries showed that this GA was at least as active as GA_1 (the purported main active GA in tomato; Serrani *et al*., 2007*b*) in enhancing hypocotyl elongation and inducing parthenocarpic fruit growth (Table 4). Moreover, the fact that a very slight or no increase in GA_1 content occurred in the transgenic plants also supports the idea that GA_4, whose content increased in those plants, is active in tomato.

While overexpression of *CcGA20ox* produced a dramatic alteration of vegetative phenotype in tomato, the parthenocarpic capacity of the transgenic plants was limited (only 14–30% of non-pollinated ovaries developed into fruit) in comparison with GA-treated plants. This was probably due to the relatively lower increase of active GAs in young fruit (about three times more GA_4 but less GA_1), compared with vegetative tissues (~10-fold more GA_4 and twice as much GA_1), which would be insufficient to induce full parthenocarpic capacity. This hypothesis is supported by the observation that the percentage of parthenocarpic fruits developed from non-pollinated ovaries in WT plants was proportional to the amount of GA_1 and GA_4 applied per ovary, with 100% developed at the saturating (2 μg) dose (Table 4).

The different effect of the transgene on active GA content in shoots and fruit was not due to lower expression of the transgene in vegetative tissues than in ovaries, because expression in the pericarp (and also in the seeds of L4) was even higher than in leaves (Fig. 3A). It was found that most of the GA metabolism genes in the shoot showed the expected feedback regulation in plants treated continuously with GA_3 and PAC solutions (Supplementay Fig. S3 at *JXB* online). Therefore, to investigate the possibility that the different GA content was the result of GA homeostasis being less efficient in the shoot than in the fruit, transcript levels of all members of *SlGA20ox*, *SlGA3ox*, and *SlGA2ox* gene families were determined in the shoot and fruit pericarp. The results, however, were not consistent with the feedback regulation found in hormone-treated plants for many of the genes investigated. This implies that feedback regulation of GA metabolism genes may vary with the tissue and gene, and may also depend on other factors such as the method of modifying the GA content (exogenous application versus genetic modification of GA content). Differences in the tissue distributions of applied GA and 35S expression could also account for the lack of consistency of these two kinds of treatments. Absence of feedback regulation for some GA metabolism genes has also been reported before. For instance, *AtGA3ox2* (*GA4H*) in germinating seeds (Yamaguchi *et al*,. 1998), and *AtGA20ox4*, *AtGA20ox5* (Rieu *et al*., 2008*b*), and *AtGA2ox3* (Rieu *et al*., 2008*a*) in plants of *Arabidopsis*. In any case, the balance of those apparently contradictory effects on GA metabolism genes seems to favour higher accumulation of active GA in the shoot compared with the fruit. The decrease in *SlGA3ox2* expression in the pericarp, and the up-regulation of *SlGA3ox1* in the shoot of transgenic plants may result in GA 3-oxidase activity becoming limiting in the fruit

of the transgenic lines, thus reducing the induction of parthenocarpic capacity by *GA20ox* overexpression. Since the expression levels of *SlGA3ox1* were extremely low, other, unidentified *SlGA3ox* gene(s) could also contribute to GA homeostasis. Finally, it is possible that regulation of GA metabolism may also occur at the post-translation level and would therefore not be apparent from analysis only of transcript abundance.

The present results support the importance of GA 20-oxidase activity in controlling tomato fruit-set and development. In a report using RNA interference technology to reduce transcript levels of different *SlGA20ox* genes, no effect on flowering or fruit-set was observed (Xiao *et al*., 2006), probably because the reduction of transcript content obtained in this case was too low. In contrast, co-suppression of *SlGA20ox1* produced total absence of fruit-set associated with reduction of vegetative growth and pollen viability (Olimpieri *et al*., 2010), in agreement with the proposed role for this gene in fruit development. The hypothesis should be confirmed by expressing *GA20ox* using fruit-specific or inducible promoters to eliminate possible pleotropic effects fully.

Supplementary data

Supplementary data are available at JXB online.

Figure 1. Scheme of GA metabolic pathways.

Figure 2. Southern analysis (A) and transgene transcript levels (B) of WT and CcGA20ox transgenic lines L4 and L19 of tomato. Transcript levels were determined by amplifying a specific 581 bp region of the transgene by RT-PCR.

Figure S3. Feedback regulation of GA metabolism genes.

Table S1. Content of citric acid, glucose, and fructose (mmol ml–1) of tomato juice from the wild type and GA20ox-OE lines (L4 and L19).

Table S2. GA content (ng g FW–1) in tomato shoots (A) and 10-day-old fruits (B) of cultivars Micro-Tom (MT), Ailsa Craig and UC-82.

Acknowledgements

We thank Dr L.E.P. Peres for providing MT-D seeds, and Mrs T. Sabater for help with GA analysis. This work was supported by grants from the Ministerio de Ciencia y Tecnología of Spain (BIO2006-13437 and BIO2009-07968). EC was the recipient of a Ramón y Cajal grant. Rothamsted Research is sponsored by the Biotechnology and Biological Sciences Research Council of the UK.

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