RESEARCH PAPER

Engineering of gibberellin levels in citrus by sense and antisense overexpression of a GA 20-oxidase gene modifies plant architecture

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Abstract

Carrizo citrange (Citrus sinensis × Poncirus trifoliata) is a citrus hybrid widely used as a rootstock, whose genetic manipulation to improve different growth characteristics is of high agronomic interest. In this work, transgenic Carrizo citrange plants have been produced overexpressing sense and antisense CcGA20ox1 (a key enzyme of GA biosynthesis) under control of the 35S promoter to modify plant architecture. As expected, taller (sense) and shorter (antisense) phenotypes correlated with higher and lower levels, respectively, of active GA1 in growing shoots. In contrast, other phenotypic characteristics seemed to be specific to citrus, or different from those described for similar transgenics in other species. For instance, thorns, typical organs of citrus at juvenile stages, were much longer in sense and shorter in antisense plants, and xylem tissue was reduced in leaf and internode of sense plants. Antisense plants presented a bushy phenotype, suggesting a possible effect of GAs on auxin biosynthesis and/or transport. The main foliole of sense plants was longer, although total leaf area was reduced. Leaf thickness was smaller in sense and larger in antisense plants due to changes in the spongy parenchyma. Internode cell length was not altered in transgenic plants, indicating that, in citrus, GAs regulate cell division rather than cell elongation. Interestingly, the phenotypes described were not apparent when transgenic plants were grafted on non-transgenic rootstock. This suggests that roots contribute to the GA economy of aerial parts in citrus and opens the possibility of using the antisense plants as dwarfing rootstocks.

Key words: Carrizo citrange, dwarfing, gibberellin 20-oxidase, transgenic plants.

Introduction

Gibberellins (GAs) form a group of tetracyclic diterpenes, some of which are biologically active, that act as hormones in higher plants by controlling diverse growth and developmental processes such as shoot elongation, expansion and shape of leaves, flowering, seed germination, and fruit development through promoting cell division and elongation (Olszewski et al., 2002; Sponsel and Hedden, 2004). Manipulation of GA levels is widely used in agriculture, for instance to stimulate fruit growth in seedless grapes, to delay fruit senescence in oranges and lemons, to increase fruit set in mandarins, apples, and pears, to increase stem elongation in sugarcane, or to decrease growth in cotton, canola, and apple (Hedden and Phillips, 2000a).

The GA biosynthetic pathway has been elucidated and its key components identified (Hedden and Phillips,

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Abbreviations: GA, gibberellin; GA20ox, gibberellin 20-oxidase.

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GA levels in plants are mainly regulated by transcriptional control of metabolic genes. The last reactions of the GA biosynthesis pathway are catalysed by three soluble 2-oxoglutarate-dependent dioxygenases, GA 20-oxidase (GA20ox), GA3ox and GA2ox (Fig. 1A) that constitute small multigene families. Multifunctional GA20ox catalyses the stepwise conversion of the C20 gibberellins GA_{12}/GA_{53}, by three successive oxidations to GA_{9}/GA_{20}, which are converted by the action of GA3ox to the active gibberellins GA_{4} and GA_{1}, respectively. GA2ox catalyses the conversion of GA_{9}/GA_{20} and GA_{3}/GA_{1} to the inactive GA_{53}/GA_{52} and GA_{33}/GA_{36}, respectively. Biochemical and molecular data showed that GA20ox transcript levels are regulated by bioactive GAs through a negative feedback mechanism (Hedden and Kamiya, 1997) and by diverse environmental factors (Kamiya and García-Martínez, 1999; Vidal et al., 2003), indicating that they may control the content of active GAs.

Overexpression of AtGA20ox1, -2, and -3 in Arabidopsis led to elongated hypocotyls, increased shoot growth, and early flowering, and a 2–3-fold increase in active GA_{1} or GA_{4} content in the case of AtGA20ox1 (Huang et al., 1998; Coles et al., 1999). Conversely, antisense expression of GA20ox in Arabidopsis reduced plant height and GA_{4} content (Coles et al., 1999). These results demonstrated that GA levels and, consequently, plant growth and development, could be modulated by genetic engineering of GA20ox and opened a way for biotechnological manipulation of plant architecture by altering GA metabolism. Eriksson et al. (2000) have reported that overexpression of AtGA20ox1 in hybrid aspen led to higher levels of active GAs and enhanced growth. Transgenic hybrid aspen trees also showed increased biomass and more and longer xylem fibres, a desirable trait in paper production. More recently, a fragment of a wild apple (Malus pumila) GA20ox gene has been isolated and overexpressed in sense or antisense in the dessert apple variety Greensleeves. In this case, co-suppression of the corresponding endogenous gene led to dwarf fruit trees with reduced internode length and number, and reduced leaf area, as a result of lower GA_{1} content (Bulley et al., 2005).

Carrizo citrange (Citrus sinensis L. Osbeck×Poncirus trifoliata L. Raf.) is an important rootstock of citrus, widely used in several countries, including Spain where around 90% of the new plantations are grafted onto citranges. In this work, Carrizo citrange has been transformed to constitutively express either sense or antisense copies of CcGA20ox1 (a GA20ox from Carrizo citrange; Vidal et al., 2003) with the aim of modulating its plant growth habit. As a rootstock, this might eventually also affect the development of the scion and facilitate diverse cultural practices (e.g. pruning, pesticide applications, and harvesting). The ectopic overexpression of CcGA20ox1 in tobacco has been shown previously to produce an extremely tall phenotype associated with an increase of GA_{4} content (Vidal et al., 2001). It is shown here that it is possible to modify the architecture of citrus plants by increasing or reducing their GA_{1} content in actively growing shoots through the overexpression (sense plants) and down-regulation (antisense plants) of CcGA20ox1. Interestingly, sense and antisense transgenic plants did not show any

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**Fig. 1.** (A) Scheme of the gibberellin biosynthetic pathway showing the last reactions catalysed by GA dioxygenases. (B) Schematic representation of CcGA20ox1 sense and antisense chimeric gene constructs used in Agrobacterium-mediated transformation experiments.
phenotype when grafted onto non-transgenic Carrizo citrange rootstocks. This suggests that unknown factors from the rootstock may be transported to the aerial parts and negate the altered GA levels in the transgenic scion.

Materials and methods

Plant material and vector construction

Carrizo citrange (*Citrus sinensis* L. Osbeck×*Poncirus trifoliata* L. Raf.) seeds from a single tree of the IVIA Citrus Germplasm Bank were peeled, removing the two seed coats, sterilized in 0.5% (w/v) sodium hypochlorite solution containing 0.1% (v/v) Tween-20 for 10 min, and rinsed three times with sterile distilled water. Seeds were introduced into the disarmed *Citrus* transformation and regeneration *CcGA20ox1* cDNA clone from Carrizo citrange (Vidal et al., 2001; Genbank accession number AJ250187) was subcloned into the unique BamHI site of the binary vector pBinJIT in sense or antisense orientation between the CaMV 35S double-enhanced promoter and the CaMV polyadenylation sequence to produce the pBinJIT-*CcGA20ox1*-sense and pBinJIT-*CcGA20ox1*-antisense plasmids (Fig. 1B). The empty plasmid pBinJIT was used to get transgenic control plants. These vectors also contained in the T-DNA the selectable neomycin phosphotransferase II (*nptII*) gene driven by the nopaline synthase (*nos*) promoter and terminator sequences (Fig. 1B). The three plasmids were introduced into the disarmed *Agrobacterium tumefaciens* strain EHA105 by electroporation. Bacteria were cultured overnight in an orbital shaker (200 rpm) at 28 °C in LB medium containing 25 mg l⁻¹ kanamycin and 25 mg l⁻¹ naldixic acid, pelleted at 3500 rpm for 10 min, and resuspended and diluted to 4×10⁷ cells ml⁻¹ in liquid inoculation medium (IM) (consisting of MS salt solution, 0.2 mg l⁻¹ thiamine hydrochloride, 1 mg l⁻¹ pyridoxine hydrochloride, 1 mg l⁻¹ nicotinic acid, and 3% (w/v) sucrose, pH 5.7) before using for transformation.

Citrus transformation and regeneration

Carrizo citrange epicotyl segments (about 1 cm long) from 5-week-old seedlings grown in vitro were incubated for 15 min with the bacterial suspension, blotted dry on sterile filter paper, and placed horizontally on co-cultivation media (CM). This medium consisted of IM plus 2 mg l⁻¹ indole-3-acetic acid, 1 mg l⁻¹ 2-isopentenyladenine, 2 mg l⁻¹ 2,4-dichlorophenoxyacetic acid, and 8 g l⁻¹ agar. After 3 d of cocultivation, the explants were transferred to shoot regeneration medium (SRM) containing the components of IM plus 3 mg l⁻¹ benzylaminoopurine and 10 g l⁻¹ agar, supplemented with 100 mg l⁻¹ kanamycin for selection of transgenic events, and 250 mg l⁻¹ vancomycin plus 500 mg l⁻¹ cefotaxime to prevent bacterial growth. Cultures were maintained in darkness for 2–4 weeks at 26 °C and then transferred to the following conditions: 16 h photoperiod (45 μM m⁻² s⁻¹ light intensity), 60% relative humidity and 26 °C. Explants were subcultured to fresh medium every 4 weeks (Cervera et al., 1998).

Two methods were used to recover transgenic plants: (i) apical portions of the shoots emerging from the explants were shoot-tip grafted *in vitro* onto Troyer citrange (*C. sinensis* (L.) Osbeck×*Poncirus trifoliata* (L.) Raf.) to promote rapid growth, and grafted again later onto vigorous greenhouse-grown rootstock to allow acclimatization and development of plants under greenhouse conditions (Peña et al., 1995); (ii) regenerated shoots from transformed epicotyl explants were excised and cultured for 10 d on IM medium supplemented with 0.2 mg l⁻¹ BAP and then on rooting medium (RM) (containing 10 mg l⁻¹ indole-3-butyric acid plus 250 mg l⁻¹ of cefotaxime).

Application of plant growth regulators to *in vitro*-grown plantlets

Sense and control shoots regenerating in a kanamycin-containing culture medium were excised from the explants and transferred to IM plus 0.2 mg l⁻¹ BAP for 10 d and, subsequently, to RM supplied or not with 0.1 mg l⁻¹ paclobutrazol (ICI, Bracknell, UK).

Stem pieces (15 cm long) from transgenic and control plants growing in the greenhouse were stripped of their leaves and thorns, then disinfected for 10 min in a 2% sodium hypochlorite solution containing 0.1% (v/v) of Tween-20, and rinsed three times with sterile water. Nodal segments of 1–2 cm length were cultured horizontally on IM plus 1 mg l⁻¹ BAP, so that the shoots could grow vertically. Once buds were sprouted, the cultures were transferred to IM plus 5% (w/v) sucrose for one month. Shoots originated from nodal explants were transferred to rooting medium (RM) supplied or not with paclobutrazol (0.1 mg l⁻¹) in sense and control shoots. A minimum of 50 shoots was analysed per treatment.

DNA and RNA extraction

Carrizo citrange genomic DNA was extracted from fresh fully expanded leaves of growing flushes according to Dellaporta et al. (1983) and its purity was evaluated on the basis of the UV absorption ratio at 260/280 nm.

For northern blot analyses, total RNA from 0.5 g of young leaves was extracted with water-saturated phenol and then fractionated with 6 M LiCl (Malmberg et al., 1985). For quantitative real-time RT-PCR, total RNA was extracted from young internodes using the RNeasy plant mini kit (Qiagen), and genomic DNA was removed during RNA purification with the RNase-free DNase kit (Qiagen). RNA was quantified using a NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies).

PCR, Southern, northern, and western blot analyses

Standard polymerase chain reaction (PCR) techniques were used to detect the presence of the *CcGA20ox1* transgene in the regenerated putative transgenic plantlets. The two specific primers used were: 5'-TTGATGGGCTTCTGGATTTATGACAT-3' (sense) and 5'-TAGATCCCTCCATTATGCAAGTT-3' (antisense), that generated an amplification product of 1217 bp. Reactions were performed in a thermal cycler under the following conditions: 94 °C for 2 min, 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 40 s at 72 °C, plus a final extension of 4 min at 72 °C.

Southern blot analysis was performed to confirm the stable integration of the *CcGA20ox1* transgene in kanamycin-resistant regenerated plants. BamHI-digested DNA samples (15 μg) were electrophoresed on 1% (w/v) agarose gels, transferred onto Hybond-N⁺ membranes (Amersham Biosciences, Cerdanyola, Barcelona, Spain), and fixed by UV irradiation. Filters were hybridized at 60 °C in a solution containing 5× SSC, 0.02% (w/v) SDS, 0.1% (w/v) N-lauroylsarcosine, and 1% (w/v) blocking reagent and were washed twice for 5 min at room temperature in 2× SSC, and then additionally twice more with 0.1× SSC, and 0.1% (w/v) SDS for 15 min at 68 °C. Filters were probed with a digoxigenin (DIG; Boehringer-Mannheim, Mannheim, Germany) labelled fragment of the coding region of *CcGA20ox1* prepared by PCR as described above.

Expression of antisense *CcGA20ox1* in antisense lines was analysed by northern blot. Aliquots (20 μg) of RNA were
Electrophoresed in 1% (w/v) agarose– formaldehyde denaturing gels, transferred by capillarity to nylon membranes, and fixed by UV irradiation in a cross-linking oven. Prehybridization and hybridization were carried out at 42 °C using the solution described for Southern blot analysis, but also including 50% deionized formamide. Filters were washed as described earlier before probing with a PCR-amplified DIG-labelled fragment of CcGA20ox1. Chemiluminescent detection, using CSPD substrate (Tropix, Bedford, MA, USA), of target RNA was conducted by using the non-radioactive DIG system from Boehringer-Mannheim following the manufacturer’s protocols.

Expression analysis of CcGA20ox1 in sense lines was carried out by determining the accumulation of CcGA20ox1 by western blot. Young leaf tissue was used to obtain crude protein extracts. Extraction buffer was 100 mM TRIS–HCl pH 6.8, 1 mM phenylmethylsulphonyl fluoride, and 0.3% (v/v) β-mercaptoethanol. Protein concentration of these crude extracts was quantified according to Bradford (1976), using the Protein Assay Dye Reagent (Bio-Rad, Hercules, CA, USA), and bovine serum albumin as standard. Aliquots (20 μg) of protein extracts were fractionated by electrophoresis on SDS-PAGE (12% polyacrylamide) and electrophobotted on to Immobilon-PVDF membranes (Millipore, Bedford, MA, USA) using a semi-dry transfer system (Bio-Rad), according to the suppliers’ instructions. Immunodetection was performed using a 1:12 000 (v/v) dilution of a CcGA20ox1 antiserum as primary antibody (Vidal et al., 2001) and alkaline-phosphatase-conjugated anti-rabbit IgGs (Boehringer-Mannheim, Mannheim, Germany) as secondary antibody at 1:5000 dilution.

Quantitative real-time PCR

For quantification of endogenous CcGA20ox1 transcript levels, expression analysis of CcGA20ox1 sense lines was carried out by determining the accumulation of CcGA20ox1 by western blot. Young leaf tissue was used to obtain crude protein extracts. Extraction buffer was 100 mM TRIS–HCl pH 6.8, 1 mM phenylmethylsulphonyl fluoride, and 0.3% (v/v) β-mercaptoethanol. Protein concentration of these crude extracts was quantified according to Bradford (1976), using the Protein Assay Dye Reagent (Bio-Rad, Hercules, CA, USA), and bovine serum albumin as standard. Aliquots (20 μg) of protein extracts were fractionated by electrophoresis on SDS-PAGE (12% polyacrylamide) and electrophobotted on to Immobilon-PVDF membranes (Millipore, Bedford, MA, USA) using a semi-dry transfer system (Bio-Rad), according to the suppliers’ instructions. Immunodetection was performed using a 1:12 000 (v/v) dilution of a CcGA20ox1 antiserum as primary antibody (Vidal et al., 2001) and alkaline-phosphatase-conjugated anti-rabbit IgGs (Boehringer-Mannheim, Mannheim, Germany) as secondary antibody at 1:5000 dilution.

Phenotypic assessment of transgenic plants

Three to 13 rooted cutting replicates from five sense, six antisense, and two control transgenic lines were transferred to pots under greenhouse conditions. About 6–7 months after the buds from the cuttings started to sprout, the number of internodes, branches, and leaves was counted, and the lengths of the stems and internodes from all the plants were measured. Leaf area from three sense, three antisense, and one control line (in total 48–208 leaves per line, with a minimum of 15 leaves randomly chosen from each plant) was determined by using a scanner and the program Matrox Inspector version 2.2. Length of thorns was measured in one selected control, sense, and antisense plant (minimum of five branches per plant, 42 thorns per branch). Diameter of the main stem and branches was measured in two sense, two antisense, and two control representative lines (one measurement at the basal, medium, and apical main stem, and a minimum of 12 measurements in branches per plant).

One plant from four sense (S6, S8, S22, and S23), four antisense (A4, A8, A10, and A33), and two control transgenic lines were grafted on non-transgenic Carrizo citrange rootstocks and a similar phenotypic assessment was made. Lengths of main stems and of internodes (about 150–200 measurements per plant) and the number of branches and leaves from all the plants were measured. Ten leaves randomly chosen from each plant were used to determine leaf area (20–40 leaves per line). Thorn length was measured in five sense, six antisense, and two control representative lines (22 thorns per line). Diameter of main stem and branches was measured in four sense, four antisense and one control line (one measurement at the basal, medium, and apical main stem, and a minimum of six measurements in branches per plant).

The data set was analysed by using the SAS statistical software package (SAS Institute, Cary, NC).

Ploidy analysis was performed by flow cytometry in leaves of nine selected antisense and two control transgenic lines using a ploidy analyser (PARTEC® PA) and a high resolution DNA kit (PARTEC®, Münster, Germany).

Quantification of GAs

GAs were quantified in actively growing shoots (about 2 cm apical portions, carrying leaves with the main folioli width 2 cm long) collected from individual trees, frozen in liquid N2, and kept at −20 °C lyophilized until analysis. Rooted transgenic control and CcGA20ox1 sense and antisense Carrizo citrange plants were analysed when they were about 2-years-old, while transgenic scions grafted onto non-transgenic rootstocks were analysed 2 months after grafting. Plant material (3–6 g dry weight) was extracted and purified essentially as described in Talon and Zeevaart (1992). Deuterated GAs ([17-2H2]GA1, [17-2H2]GA4, [17-2H2]GA20 purchased from Professor L Mander, Australian National University, Canberra), and ABA (1 μg; Sigma) were added to combined filtrates as internal standards. ABA, detected by absorbance at 254 nm was used to check the reproducibility of GA retention times. In brief, GAs in the aqueous phase of 80% methanol extracts were purified by partitioning against 0.1 M potassium phosphate buffer pH 8.2, n-hexane, and diethyl ether. The aqueous phase was then passed through a charcoal–Celite column, subjected consecutively to polyvinylpolypyrrolidone and QAE-Sephadex A-25 (Sigma, St Louis, MO) anion exchange chromatography, and finally passed through a C18 cartridge (Sep-Pak, Waters). GAs were fractionated by HPLC using a 25 cm×0.46 cm i.d. Hypersil C18 column and a 40 min linear gradient of 20–100% methanol gradient in 1% aqueous acetic acid, at 1 ml min−1. HPLC fractions (1 ml) were collected and grouped according to retention times relative to that of ABA used as an internal standard.

Dried samples from HPLC were methylated with ethereal diazomethane and trimethylsilylated at room temperature for at least 1 h with 10 μl of bistrimethylsilyl trifluoroacetamide. GAs were quantified by GC-MS using a gas chromatograph (Model 9800, Fisons, Danvers, MA) equipped with a fused silica capillary column (30 m x 0.25 mm x 0.25 μm film thickness; DB-5MS, J&W Scientific, Folsom, CA) coupled to a quadrupole mass detector (800; Fisons). Positive ion electron impact masses at 70 eV were acquired in selected ion monitoring (SIM) mode, and GA quantification was based on the use of internal standards and the method of sequential extractions (Ben-Cheikh et al., 1997).
standards added to the extractions were successively adjusted in consecutive extractions until similar to those of endogenous GAs in the samples. In general, three extractions per sample were performed.

Light microscopy and morphometrical analysis

Samples of leaves and internodes from representative sense (S23) and antisense (A8) transgenic lines were taken at the level of the third and fourth internode (top to bottom). Samples were fixed and embedded in Micro-Bed resin (Electron Microscopy Sciences, Fort Washington, Pa., USA) according to Tadeo et al. (1995). Sections (about 1 μm thick) were cut with a Leica RM2255 microtome (Leica Microsystems, Wetzlar, Germany) using glass knives and fixed to microscope slides. Cross-sections of leaf blades and longitudinal- and cross-sections of internodes were stained with 0.05% Toluidine Blue O (CI 52040; Merck, Darmstadt, Germany) and examined and photographed with a Leica DM LA microscope (Leica Microsystems, Wetzlar, Germany). The morphometrical analysis was performed on highly contrasted micrographs with the Leica IM software (Leica Microsystems, Wetzlar, Germany).

Results

Phenotype of Carrizo citrange plants overexpressing sense and antisense CcGA20ox1

To investigate the possibility of overexpressing and down-regulating GA 20-oxidase in citrus, epicotyl segments from Carrizo citrange seedlings were transformed with Agrobacterium tumefaciens carrying the binary vector pBinJIT empty (control), or with the construct 2×35S::CcGA20ox1 in sense or antisense orientation (Fig. 1B). Transformed epicotyls were transferred to an organogenesis-promoting culture medium, and after 4–12 weeks kanamycin-resistant shoots started to regenerate. The buds were either left to elongate attached to the explants or micrografted in vitro onto non-transgenic stocks (see Materials and methods). Sense shoots elongating directly from the explants were longer than controls (Fig. 2A). This phenotype was even more apparent when the shoots were excised from the explants and rooted in vitro, exhibiting longer and thinner stems compared with controls (Fig. 2B). Shoots generated directly from antisense explants were similar to controls (results not presented), but showed a clearly reduced stem growth when excised and rooted in vitro (Fig. 2B). Rooting of sense shoots was generally difficult and application of paclobutrazol did not improve rooting efficiency (results not shown). For instance, whereas 46% of control and 42% of antisense cuttings produced roots, only 26% of the sense cuttings rooted. Moreover, probably related to their poor rooting capacity, buds from sense cuttings started to sprout about one month later than those of antisense and control cuttings. By contrast, while all antisense shoots rooted earlier, lines with the most extreme (dwarf) phenotype barely elongated.

The sense and antisense phenotypes described above did not appear in shoots grown from micrografted transgenic apices, regardless of the kind of construct used for transformation. However, when nodal segments from grafted transgenic lines were cultured in vitro and rooted, sprouted shoots from axillary buds were slender in all sense lines, and short in all antisense lines (Fig. 2C). As had happened with regenerated shoots from primary explants, sense and antisense axillary shoots from nodal segments were also difficult to root or to elongate, respectively.

Integration of the transgene into the Carrizo citrange genome was tested first by PCR analysis using DNA from

![Fig. 2. Carrizo citrange phenotype of shoots and plantlets overexpressing sense and antisense CcGA20ox1 early after in vitro regeneration, and Southern blot analysis of transgenic plants. (A) Shoots regenerating from a control explant compared with those regenerating from an explant transformed with CcGA20ox1 in sense orientation. (B) Representative control, sense, and antisense transgenic plantlets rooted in vitro, 150 d after starting the culture. (C) Representative control, sense, and antisense shoots regenerating from nodal segments cultured in vitro, 15 d after starting the culture. (D) Southern blot analysis of DNA digested with BamHI from control (lane C) and eight lines transformed with the CcGA20ox1 gene in sense (lanes S5–S23) or antisense (lanes A1–A33) orientations. M, DNA marker; P, plasmid pBinJIT-CcGA20ox1 used as positive control.](image-url)
leaves of grafted plants grown in test tubes (results not shown), and confirmed later by Southern blot analysis in 10-month-old plants grown in the greenhouse corresponding to eight independent lines carrying sense (S5, S6, S7, S8, S17, S20, S22, and S23) or antisense (A1, A4, A7, A8, A9, A10, A13, and A33) constructs. All sense and antisense plants showed the expected 1.2 kbp fragment corresponding to the hybridization of the BamHI-digested DNA with the CcGA20ox1 probe (Fig. 2D). No hybridizing band was detected in control lines transformed with the empty vector (Fig. 2D; lane C). CcGA20ox1 transgene copy number was determined in antisense plants by quantitative PCR, and there was variation between one copy in lines A10 and A33, and five copies in line A8 (results not shown).

CcGA20ox1 transgenic lines show alterations in plant growth and morphology

Selected Carrizo citrange lines overexpressing either sense or antisense CcGA20ox1 transgene were propagated by cuttings and grown in the greenhouse. Seven months after bud sprouting, sense and antisense plants displayed opposite growth behaviour. Overexpression of CcGA20ox1 produced longer internodes, consequently leading to taller plants compared with the control in four (S5, S8, S22 and S23) out of five lines investigated (Table 1A; Fig. 3A, B). By contrast, plants from antisense lines were slightly shorter than controls, although only line A8 was significantly less tall and had significantly shorter internodes (Table 1A; Fig. 3A, B). It is known that about 10% of Carrizo citrange seedlings germinated in vitro are autotetraploid (Cervera et al., 2000), and that this affects plant development causing dwarfism, among other alterations (Lee, 1988). It was checked that all antisense plants were diploid (data not presented), so ploidy changes were not causing the semi-dwarf phenotype of antisense plants, particularly in the case of the low expressor line A8.

Leaves from sense lines were longer and narrower, and total leaf area was significantly smaller than those from controls (Table 1A; Fig. 3C). These differences were more conspicuous when only the main foliole was compared (Fig. 3C). Down-regulation of CcGA20ox1, however, did not affect leaf shape or size (Table 1A). The growth of thorns was also dramatically affected, their mean length being almost double in sense (60.1 ± 1.0 mm; n=196), and about 20% shorter in antisense plants (27.2 ± 0.8 mm; n=237) compared with control plants (34.7 ± 1.6 mm; n=265) (Fig. 3D).

Interestingly, four (A4, A8, A10, and A33) out of six antisense lines investigated developed a higher number of branches than controls (Table 1A), which conferred them a characteristic bushy aspect (Fig. 3A, B). By contrast, overexpression of CcGA20ox1 did not alter the number of branches (Table 1A; Fig. 3A, B).

The diameter of the main stem and branches was measured in two representative sense (S8 and S23), antisense (A4 and A8), and control lines (minimum of 15 values per plant, 1 plant per line). Sense stems were much thinner (about half diameter) than those of control plants (Table 1B). After two years of growth in the greenhouse, most branches of sense plants were bent and the plants displayed a willow shape.

When sense and antisense transgenic plants were grafted onto non-transgenic Carrizo citrange rootstocks their phenotypes were completely lost, thus becoming indistinguishable from empty vector control plants, grafted in the same way for none of the parameters investigated, including main stem and internode length, number of branches and leaves, and leaf area (Table 2A), diameter of the main stem and branches (Table 2B), and length of thorns.

Table 1. (A) Phenotypic characteristics of transgenic CcGA20ox1 sense, antisense and control lines of Carrizo citrange

<table>
<thead>
<tr>
<th>Line (number of plants per line)</th>
<th>Branch length, higher than 5 cm (cm)</th>
<th>Internode length (mm)</th>
<th>Number of branches</th>
<th>Number of leaves</th>
<th>Leaf area (cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (12)</td>
<td>23.8 ± 2.8 (25)</td>
<td>26.1 ± 1.1 (25)</td>
<td>4.1 ± 0.7 (3–7)</td>
<td>141.1 ± 11.3</td>
<td>11.8 ± 0.7 (96)</td>
</tr>
<tr>
<td>S5 (3)</td>
<td>51.7 ± 12.7 a (7)</td>
<td>34.1 ± 2.7 (7)</td>
<td>2.3 ± 0.3 (2–3)</td>
<td>149.6 ± 49.8</td>
<td>6.7 ± 0.3 a (48)</td>
</tr>
<tr>
<td>S6 (4)</td>
<td>28.8 ± 3.2 a (14)</td>
<td>38.1 ± 1.9 a (14)</td>
<td>3.5 ± 0.3 (3–4)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S8 (9)</td>
<td>32.4 ± 3.3 a (31)</td>
<td>35.2 ± 9.4 a (31)</td>
<td>4.5 ± 1.0 (3–9)</td>
<td>163.8 ± 15.4</td>
<td>8.3 ± 0.4 a (112)</td>
</tr>
<tr>
<td>S22 (9)</td>
<td>33.5 ± 4.1 a (34)</td>
<td>32.8 ± 1.1 (34)</td>
<td>3.7 ± 0.5 (2–7)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S23 (9)</td>
<td>37.1 ± 3.7 a (36)</td>
<td>37.1 ± 1.1 a (36)</td>
<td>4.0 ± 1.1 (2–12)</td>
<td>164.3 ± 19.0</td>
<td>8.1 ± 0.4 a (128)</td>
</tr>
<tr>
<td>A4 (13)</td>
<td>18.0 ± 1.3 (92)</td>
<td>24.3 ± 0.7 (92)</td>
<td>7.0 ± 0.9 a (3–14)</td>
<td>122.3 ± 8.9</td>
<td>13.6 ± 0.9 (192)</td>
</tr>
<tr>
<td>A7 (12)</td>
<td>21.9 ± 1.9 (59)</td>
<td>27.2 ± 0.8 (59)</td>
<td>4.9 ± 0.8 (2–11)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>A8 (13)</td>
<td>11.7 ± 0.8 a (93)</td>
<td>19.9 ± 0.6 a (93)</td>
<td>7.2 ± 0.9 a (3–13)</td>
<td>147.0 ± 11.9</td>
<td>13.6 ± 0.6 (208)</td>
</tr>
<tr>
<td>A9 (7)</td>
<td>18.4 ± 1.9 (30)</td>
<td>30.6 ± 1.3 (30)</td>
<td>4.2 ± 0.3 (3–5)</td>
<td>132.7 ± 13.8</td>
<td>14.0 ± 0.8 (112)</td>
</tr>
<tr>
<td>A10 (9)</td>
<td>17.7 ± 1.3 (73)</td>
<td>31.2 ± 1.0 (73)</td>
<td>8. ± 1.2 a (3–15)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>A33 (12)</td>
<td>18.8 ± 1.9 (80)</td>
<td>23.0 ± 0.9 (80)</td>
<td>6.6 ± 0.9 a (3–15)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* In each column, values marked with *a* are significantly different compared to control plants (P < 0.05) according to Dunnett’s one way t test for internode and total stem length, and Poisson distribution for number of branches. In the case of leaf area the data were transformed (square root) to be converted into a normal distribution before applying ANOVA.
Table 1. (B) Diameter of main stem and branches (mm)

Diameters of the main stem (S) and branches (B) in sense (S8 and S23), antisense (A4 and A8) and control lines, represented according to the Principal Component Statistical Analysis. Each large circle represents the diameter measures from each group, according to the two principal components (PC1 and PC2). PC1 and PC2 explain 80.4% and 14.2% of data variance. The vectors ending in a small circle represent the variability of the different variables used in the assay (BS, basal main stem; MS, middle main stem; AS, apical main stem; BB, basal branch; MB, middle branch; and AB, apical branch).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>BS</th>
<th>MS</th>
<th>AS</th>
<th>BB</th>
<th>MB</th>
<th>AB</th>
</tr>
</thead>
<tbody>
<tr>
<td>S8</td>
<td>13.08</td>
<td>8.64</td>
<td>7.83</td>
<td>4.01</td>
<td>3.28</td>
<td>2.12</td>
</tr>
<tr>
<td>S23</td>
<td>14.56</td>
<td>8.53</td>
<td>7.21</td>
<td>4.22</td>
<td>3.54</td>
<td>2.59</td>
</tr>
<tr>
<td>A8</td>
<td>14.52</td>
<td>13.1</td>
<td>12.03</td>
<td>7.56</td>
<td>5.75</td>
<td>3.57</td>
</tr>
<tr>
<td>A4</td>
<td>12.92</td>
<td>11.65</td>
<td>10.94</td>
<td>6.22</td>
<td>5.19</td>
<td>4.53</td>
</tr>
<tr>
<td>Control 1</td>
<td>18.99</td>
<td>15.61</td>
<td>13.73</td>
<td>6.18</td>
<td>5.30</td>
<td>4.18</td>
</tr>
<tr>
<td>Control 2</td>
<td>16.40</td>
<td>15.94</td>
<td>14.96</td>
<td>6.50</td>
<td>5.65</td>
<td>4.59</td>
</tr>
</tbody>
</table>

(not shown). Interestingly, when sense and antisense scions were severely pruned after one year of growth in the greenhouse, the new sprouts were slightly more elongated and shorter, respectively, than control scions. However, both sense and antisense shoots progressively became phenotypically indistinguishable from control shoots one month after pruning (results not shown).

**Elongated and bushy phenotypes are correlated with the expression level of the CcGA20ox1 sense and antisense transgenes, respectively**

Transgene expression in sense lines was evaluated by western blot analysis. Western immunoblotting of protein extracts showed that most of the sense lines contained different levels of an approximately 42 kDa protein that immunoreacted with the CcGA20ox1 antiserum (Fig. 4A). A faint band was also detected in the control line. A second band of slightly lower molecular weight, present in all samples was probably a proteolytic product of the major 42 kDa band (Fig. 4A) (also found in tobacco over-expressing CcGA20ox1; Vidal et al., 2001). Accumulation of the CcGA20ox1 protein correlated apparently with internode length. For instance, only lines S6, S8, and S23, having the highest protein content (Fig. 4A) had significantly longer internodes than the control (Table 1A).

The effect of antisense CcGA20ox1 expression on CcGA20ox1 protein levels was difficult to determine because CcGA20ox1 content in controls was already very low (Fig. 4A). Indeed, CcGA20ox1 transcripts could not be detected by northern blot analysis in control lines under the hybridization conditions used (Fig. 4B). Levels of antisense transcripts accumulated in antisense lines as expected, mostly in A1, A4, A13, and A33 and much less in A7, A9, and A10. An exception was line A8, where transgenic transcripts could not be detected by northern blot (Fig. 4B), or by more sensitive RT-PCR analysis (results not shown).

Expression level was not correlated with mean length of branches nor with internode length. Better correlation was found between expression and branching. For instance, high expressor lines A4 and A33 were highly branched, while low expressor lines A7 and A9 had a similar number of branches as the control. Interestingly, lines A10 and A8 had relatively low and no transcript levels, respectively, but had the bushiest phenotype. Besides, A8 was the only antisense line being significantly smaller and having significantly shorter internodes (Fig. 3B; Table 1A). Quantitative real-time RT-PCR assay was performed to characterize CcGA20ox1 endogenous transcripts in A8 and A4 transgenic lines. Whereas the content of endogenous transcripts decreased only slightly in A4, it was reduced almost to one-half in A8, compared with the control (Fig. 4C). This probably explains the more conspicuous semi-dwarf phenotype of A8 than A4, including significantly shorter stem and internode length.

**Effect of altered expression of GA 20-oxidase genes on GA endogenous content**

Gibberellin analyses in 2-year-old shoots of rooted cuttings from selected transgenic lines were focused on GA20 and GA1 (from the pivotal pathway of GA biosynthesis in citrus, the 13-hydroxylation pathway), and on GA4 (from the non-13-hydroxylation pathway) (Table 3).

Young developing shoots from sense lines S8 and S23 had higher GA1 (the main active GA in citrus) levels (1.8–2.8-fold, respectively) while antisense lines A4 and A8 had lower levels (by 42% and 62%, respectively) than did the controls. The content of GA20 (the final metabolic product of GA 20-oxidase activity) showed a similar pattern (1.7–2.3-fold higher in sense genotypes, and 33–91% lower in antisense genotypes). The content of GA4 (another active GA) was, as GA1, also higher 1.6–2.1-fold in sense, and lower (50–56%) in antisense plants.

As described earlier, transgenic plants grafted onto non-transgenic Carrizo citrange rootstock did not show any phenotype (Table 2). Therefore, the GA content in young
developing shoots of the scion was determined in two grafted sense (S8 and S23) and two grafted antisense (A4 and A8) lines about two months after grafting, at a time when they showed no apparent phenotype. In contrast to rooted lines, little effect of \( \text{CcGA20ox1} \) over-expression (lines S8 and S23) on GA content was found in any case (Table 4). GA levels were higher in controls from grafted plants (Table 4) than in controls from rooted plants (Table 3) probably because the developing shoots were much younger in the former than in the latter case (2-month versus 2-year-old plants, respectively).

**Effect of CcGA20ox1 transgenic expression on the leaf blade and internode anatomy**

The effect of sense and antisense \( \text{CcGA20ox1} \) overexpression on leaf and internode anatomy was investigated in

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**Table 2. (A) Phenotypic characteristics of transgenic control and CcGA20ox1 sense and antisense Carrizo citrange plants grafted onto non-transgenic Carrizo citrange rootstocks**

The number of samples analysed is indicated in brackets.

<table>
<thead>
<tr>
<th>Lines</th>
<th>Branch length, higher than 5 cm (cm)</th>
<th>Internode length(^a) (mm)</th>
<th>Number of branches(^a)</th>
<th>Number of leaves</th>
<th>Leaf area(^a) (cm(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control: 1 and 2</td>
<td>16.0±1.0 (2)</td>
<td>23.5±2.7 (410)</td>
<td>7.5±1.5 (6–9)</td>
<td>235.0±75.0 (2)</td>
<td>12.4±1.7 (20)</td>
</tr>
<tr>
<td>Sense: S6, S8, S22, and S23</td>
<td>17.4±1.4 (4)</td>
<td>26.1±2.0 (792)</td>
<td>7.5±0.5 (6–8)</td>
<td>220.8±56.1 (4)</td>
<td>11.1±1.2 (40)</td>
</tr>
<tr>
<td>Antisense: A4, A8, A10, and A33</td>
<td>17.2±1.0 (4)</td>
<td>22.7±2.1 (685)</td>
<td>7.8±0.9 (6–10)</td>
<td>249.8±53.6 (4)</td>
<td>11.5±1.2 (40)</td>
</tr>
</tbody>
</table>

\(^a\) In each column, no significant differences compared to control were found applying ANOVA.
representative sense (S23) and antisense (A8) lines. Leaf blades and internodes of Carrizo citrange plants showed a typical anatomical organization (Schneider, 1968; Tadeo et al., 2003). Leaf blades consisted of a single layer of palisade parenchyma cells and about ten spongy parenchyma cell layers that constituted the leaf mesophyll delimited by the adaxial and abaxial epidermis (Fig. 5C). Leaves of S23 plants were thinner (about 10%) whereas those of A8 where thicker (about 20%) compared with control plants (Table 5). This change in leaf thickness was a consequence of mesophyll cellular changes. While the palisade parenchyma cell size increased in both S23 and A8 leaves, the spongy parenchyma cell size decreased in sense (about 19%), and increased (about 40%) in antisense leaves (Table 5). Moreover, cross-sections of A8 leaves showed an extra, although incomplete, palisade parenchyma layer, compared with control and S23 leaves (Fig. 5A, C, E). The spongy parenchyma cells were also more closely packed in S23 leaves than in control and A8 leaves due to a reduction of the intercellular air spaces (Table 5).

Leaf midvein in control plants consisted of a perfectly differentiated vascular system, with an externally localized phloem and an internally localized xylem enclosing the pith, and an external parenchyma tissue (Fig. 5D). Midvein vascular surface area was lower in S23 (more than 50% reduction), but was not affected in A8 leaves compared with control plants (Table 5). Vascular differentiation was also not affected (Fig. 5B, D, F). The size of the external parenchyma tissue was also reduced in sense leaves (Fig. 5B).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>BS</th>
<th>MS</th>
<th>AS</th>
<th>BB</th>
<th>MB</th>
<th>AB</th>
</tr>
</thead>
<tbody>
<tr>
<td>S6</td>
<td>28.4</td>
<td>21.6</td>
<td>20.8</td>
<td>12.15</td>
<td>11.96</td>
<td>11.53</td>
</tr>
<tr>
<td>S8</td>
<td>27.4</td>
<td>26.3</td>
<td>20.0</td>
<td>9.95</td>
<td>8.78</td>
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<td>29.9</td>
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<td>24.7</td>
<td>11.73</td>
<td>10.14</td>
<td>8.60</td>
</tr>
<tr>
<td>S23</td>
<td>34.3</td>
<td>30.4</td>
<td>25.6</td>
<td>14.14</td>
<td>13.91</td>
<td>13.73</td>
</tr>
<tr>
<td>A4</td>
<td>25.8</td>
<td>21.9</td>
<td>19.5</td>
<td>10.03</td>
<td>8.34</td>
<td>7.97</td>
</tr>
<tr>
<td>A8</td>
<td>39.9</td>
<td>34.8</td>
<td>34.4</td>
<td>12.48</td>
<td>9.52</td>
<td>7.08</td>
</tr>
<tr>
<td>A10</td>
<td>29.1</td>
<td>26.2</td>
<td>27.1</td>
<td>10.97</td>
<td>10.97</td>
<td>10.92</td>
</tr>
<tr>
<td>A33</td>
<td>29.7</td>
<td>24.8</td>
<td>24.2</td>
<td>11.33</td>
<td>10.55</td>
<td>9.64</td>
</tr>
<tr>
<td>Control</td>
<td>27.9</td>
<td>24.4</td>
<td>23.8</td>
<td>12.20</td>
<td>11.31</td>
<td>10.44</td>
</tr>
</tbody>
</table>

Table 3. Endogenous gibberellin content (ng g⁻¹ FW) in developing shoots (<2 cm apical tip) of transgenic control and CcGA20ox1 sense and antisense Carrizo citrange plants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>GA₂₀</th>
<th>GA₁</th>
<th>GA₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.8 ± 0.2</td>
<td>8.0 ± 0.8</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>S8</td>
<td>13.4 ± 0.8 b</td>
<td>14.6 ± 1.1 a</td>
<td>3.9 ± 0.1 a</td>
</tr>
<tr>
<td>S23</td>
<td>9.8 ± 1.4 b</td>
<td>22.3 ± 4.2 b</td>
<td>5.0 ± 1.0 b</td>
</tr>
<tr>
<td>A8</td>
<td>0.5 ± 0.3 b</td>
<td>3.0 ± 0.1 b</td>
<td>1.2 ± 0.1 b</td>
</tr>
<tr>
<td>A4</td>
<td>3.9 ± 0.2 b</td>
<td>4.6 ± 0.1 b</td>
<td>1.1 ± 0.7 b</td>
</tr>
</tbody>
</table>

* In each column, a (P < 0.10) and b (P < 0.05) indicate significant differences compared with the control. Data were analysed using a one-way ANOVA for each GA₂₀, GA₁ and GA₄.
Transverse sections of Carrizo citrange internodes presented a single-layered epidermis, a cortical layer divided into two zones (outer and inner cortex), and a vascular cylinder (external phloem, cambium, and internal xylem) enclosing the pith (Fig. 6E). As described before, internodes at the apical part of S23 plants were thinner than those of control (up to 55% reduction in cross-sectional area; Table 1) whereas those of A8 plants were not affected significantly (Fig. 6A, B; Table 6). Interestingly, the different tissues were not reduced equally in S23 internodes. Thus, although the percentage of pith surface area increased in S23 internodes (about 35%), the percentage of xylem surface area decreased (about 32%) (Table 6), accounting for the reduction in the internode diameter. Cell width of inner cortical cells increased in both S23 and A8 plants, whereas their cell length did not vary in A8 but increased in S23 internodes, although not significantly (Table 6).

**Discussion**

GA 20-oxidase activity is a major determinant for active GA production in plants and therefore a putative target for genetic manipulation of GA biosynthesis. Here, transgenic plants were obtained of the citrus rootstock Carrizo citrange overexpressing sense and antisense *CcGA20ox1* and it has been shown that the phenotype of sense transgenic plants (longer internodes and thorns, and reduced leaf area) was associated with an increase in active GA content in growing shoots. By contrast, the semi-dwarf, branched, and short thorns phenotype of antisense plants was correlated with a reduction in GA content. The phenotype displayed by sense transgenic plants was also correlated with transgene *CcGA20ox1* expression. In the case of antisense plants, although the levels of endogenous *CcGA20ox1* protein was not determined, it was found that the bushy phenotype, but not plant height, correlated generally with antisense transcript level. Also, at least in the case of A4 and A8, the endogenous *CcGA20ox1* transcript levels were reduced (although significantly only in A8). These results agree with the phenotype of citrus plants treated with inhibitors of GA biosynthesis such as paclobutrazol (Mehouachi et al., 1996), and strongly support that the overexpression of *CcGA20ox1* in sense or antisense orientation had a relevant effect on endogenous GA metabolism in citrus plants.

A8 was the only antisense line, which, in addition to the bushy phenotype, exhibited a significant and strong dwarfing effect and shorter internodes. The high similarity between the phenotype of different antisense lines and that of A8 also suggests that, in this case, it was also induced by overexpression of the antisense transgene. Moreover, GA contents were much lower in this line than in controls. Interestingly, transcripts from the *CcGA20ox1* transgene

### Table 4. Endogenous gibberellin content (ng g⁻¹ FW) in actively growing shoots (≤2 cm apical portion) of transgenic control and *CcGA20ox1* sense and antisense Carrizo citrange plants grafted onto non-transgenic Carrizo citrange rootstocks

Data are means ±SE of three replicates; S, sense; A, antisense.

<table>
<thead>
<tr>
<th></th>
<th>GA₂₀⁺⁺⁺</th>
<th>GA₁⁺⁺⁺</th>
<th>GA₄⁺⁺⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.7±0.4</td>
<td>21.1±2.4</td>
<td>4.9±0.3</td>
</tr>
<tr>
<td>S8</td>
<td>6.5±0.6</td>
<td>20.5±0.5</td>
<td>5.1±0.1</td>
</tr>
<tr>
<td>S23</td>
<td>10.3±1.0</td>
<td>25.7±2.6</td>
<td>5.6±0.5</td>
</tr>
<tr>
<td>A8</td>
<td>6.4±0.5</td>
<td>29.4±4.0</td>
<td>6.1±0.6</td>
</tr>
<tr>
<td>A4</td>
<td>9.3±0.7</td>
<td>23.0±2.2</td>
<td>5.2±0.3</td>
</tr>
</tbody>
</table>

ns=non significant. In each column, values are not significantly different to the control. Data were analysed using a one-way ANOVA for each GA₂₀⁺⁺⁺, GA₁⁺⁺⁺ and GA₄⁺⁺⁺.

**Fig. 5.** Light micrographs of cross-sections from leaves attached to the third and fourth internode (top to bottom) in control (C, D) and *CcGA20ox1* transgenic sense (A, B) and antisense (E, F) Carrizo citrange plants. Anatomy of leaf blades (A, C, E) and leaf midvein (B, D, F) are shown. Abe, abaxial epidermis; ade, adaxial epidermis; p, pith; ph, phloem; pp, palisade parenchyma; sp, spongy parenchyma; x, xylem.
were not detected in line A8. In addition, it had integrated five copies of the CcGA20ox1 transgene, while one to three copies were detected in the rest of the transgenic antisense lines. Correlation between low or undetectable transgene mRNA levels and insertion of multiple T-DNAs in transgenic plants has been attributed to the tendency of multiple loci of the same transgene to produce mRNAs of both polarities that form dsRNAs which trigger post-transcriptional gene silencing in line A8 was further supported by the significant reduction in endogenous CcGA20ox1 mRNA levels (almost half compared to the control). By contrast, the high expressor line A4 only showed a moderate decrease in endogenous CcGA20ox1 transcript accumulation, probably reflecting the differences in dwarfing induction shown by these two lines.

The bioactive GA1 and GA4 are produced through two parallel pathways, the early-13-hydroxylation and the non-13-hydroxylation pathway, respectively (Fig. 1A), and are synthesized in different proportions, depending on the species. Transgenic overexpression of GA20ox increases the content of GA1 (Huang et al., 1998), GA4 (Coles et al., 1999), or both GAs (Eriksson et al., 2000) in different plants, usually depending on their predominance in their untransformed counterparts. However, in transgenic tobacco, where GA1 is the main bioactive GA, overexpression of CcGA20ox1 led to a 3–4 times increase in GA4 content and no increase or even a decrease in GA1 content, due to the enhancement of the non-13-hydroxylation pathway at the expense of the early-13-hydroxylation pathway (Vidal et al., 2001). In contrast, overexpression of CcGA20ox1 in Carrizo citrange increased the content of both GA1 (1.8–2.8 times), the main physiologically active gibberellin in citrus (Talón et al., 1992), and GA4 (1.6–2.1 times). Although the absolute increase of each active GA in citrus was lower than the increase in GA4 in tobacco using the same transgene, it was similar when considering GA1 plus GA4. As expected, the content in GA3β, the final product of GA20ox activity, also increased in sense and decreased in antisense plants. This is in contrast to the GA20 decrease found in CcGA20ox1 overexpressor tobacco (Vidal et al., 2001). The different effect of CcGA20ox1 overexpression in tobacco and Carrizo citrange may be due to a higher GA 13-hydroxylase activity in the latter, preventing the overexpression of CcGA20ox1 to divert GA12 to GA9.

Transgenic sense citrus plants overexpressing CcGA20ox1 were taller and rooted poorly, as also described in Populus, another tree species overexpressing AtGA20ox1 (Eriksson et al., 2000). However, striking differences were found between transgenic citrus and Populus GA20ox overexpressors regarding stem diameter, leaf area, and biomass, all of them smaller in citrus and larger in Populus. Stem diameter was smaller in sense transgenic citrus mainly as a consequence of a striking reduction in the xylem tissue. This is in contrast to the increase in diameter and number and length of xylem fibres found in Populus (Eriksson et al., 2000) and tobacco (Biemelt et al., 2004) overexpressing AtGA20ox1. These differences might be due to the different GA20ox used (an Arabidopsis one in the case of Populus and tobacco, and an endogenous one in the case of citrus). However, since xylem development was only apparent in older parts of the stem at least in tobacco (Biemelt et al., 2004), the possibility can not be discarded that CcGA20ox1 overexpressors may develop more xylem in more mature stem citrus tissues (difficult to analyse histologically). These results are also difficult to reconcile with observations in Populus suggesting that de novo GA biosynthesis occurs in the expanding xylem (Israëlsson et al., 2005). These results exemplify how constitutive overexpression of a GA20-oxidase transgene can have different effects on growth and development, and on GA homeostasis, depending on the plant and possibly on the specific transgene used.

Diverse changes of leaf anatomy found in sense and antisense CcGA20ox1 transgenic citrus have not been described previously in other GA-transgenic species. For instance, the leaf midvein vascular system was less prominent in GA-overproducer Carrizo citrange plants (Fig. 5B; Table 5), although differentiation of the vascular system was not affected, suggesting that GAs

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**Table 5. Anatomical characteristics of leaves from transgenic control and CcGA20ox1 sense and antisense Carrizo citrange plants**

<table>
<thead>
<tr>
<th>Transgenic lines</th>
<th>Leaf thickness&lt;sup&gt;a&lt;/sup&gt; (μm)</th>
<th>Palisade parenchyma cell area&lt;sup&gt;b,d&lt;/sup&gt; (μm&lt;sup&gt;2&lt;/sup&gt;)</th>
<th>Spongy parenchyma cell area&lt;sup&gt;c&lt;/sup&gt; (μm&lt;sup&gt;2&lt;/sup&gt;)</th>
<th>Mesophyll intercellular air space&lt;sup&gt;a,b,d&lt;/sup&gt; (%)</th>
<th>Leaf midvein vascular area&lt;sup&gt;a&lt;/sup&gt; (mm&lt;sup&gt;2&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>249.5±7.9 b</td>
<td>267.5±5.0 b</td>
<td>698.8±24.9 b</td>
<td>30.0±1.5 a</td>
<td>0.197±0.024 a</td>
</tr>
<tr>
<td>Sense S23</td>
<td>223.2±4.8 c</td>
<td>341.0±5.8 a</td>
<td>568.9±15.4 c</td>
<td>22.5±1.4 b</td>
<td>0.086±0.008 b</td>
</tr>
<tr>
<td>Antisense A8</td>
<td>279.9±7.5 a</td>
<td>334.0±7.8 a</td>
<td>980.9±35.0 a</td>
<td>28.1±2.0 a</td>
<td>0.178±0.028 a</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentage of mesophyll cross-sectional area.<br>
<sup>b</sup> Number of determinations=5.<br>
<sup>c</sup> Number of determinations=60.<br>
<sup>d</sup> In each column, values with different letters were significantly different (P < 0.05). Data (mean ± SE) were analysed using a factorial analysis of variance and Duncan’s multiple range test.
only modulated vascular growth. The leaf blade of GA-overproducer Carrizo citrange plants was thinner (Fig. 5A; Table 5) while that of antisense plants was thicker (Fig. 5E, Table 5) due to changes in palisade parenchyma cell number and size, and cellular interspace volume. Opposite effects to those found in antisense citrus have been described in leaves from plants with constitutive expression of certain transcriptional factors that resulted in GA deficiency (Bereterbide et al., 2001; Rosin et al., 2003). Changes of leaf thickness similar to those found in Carrizo citrange were observed in tobacco overexpressing AtGA20ox1 (tall) and AtGA2ox1 (dwarf) (Biemelt et al., 2004), although no precise anatomical descriptions were carried out in these plants.

Cortical cell length in internodes from sense, antisense and control Carrizo citrange plants were similar (Fig. 6D, F, H; Table 6). Since internode length was altered in transgenic plants, this means that cell division was affected, but not cell elongation. Therefore, in citrus, the shorter and longer internodes produced by antisense and sense expression of CcGA20ox1, respectively, seems to be due to repression and induction of cell division. Similar results were obtained in transgenic AtGA20ox1 hybrid aspen plants (Eriksson et al., 2000). By contrast, in annual plants such as Arabidopsis or tobacco the elongated shoot growth induced by application of GAs or overexpression of GA biosynthetic genes (GA20ox), and the stunted phenotype produced by GA catabolic genes (GA2ox) were a result of an effect on cell elongation (Saibo et al., 2003; Biemelt et al., 2004). Therefore, changes in the GA endogenous amounts could regulate internode growth through modulation of both cell elongation and cell division. In annual plants, such as Arabidopsis and tobacco, with a short life cycle, internode length appears to be dependent on cell elongation, whereas in deciduous trees, such as hybrid aspen and citrus, cell division might be the process controlling internode length.

The lower GA1 levels in antisense citrus plants were consistent with their shorter phenotype during the first stages of development. However, after several months of growth in the greenhouse, the semi-dwarf characteristics of most antisense transgenic lines became attenuated and the main stem was not significantly shorter than controls. This may be the result of feed-back regulation leading to enhancement of expression of other CcGA20ox present in Carrizo citrange. Interestingly, the most striking change in tree architecture observed in antisense lines, maintained even in mature trees was their bushy shape, resulting apparently from the loss of apical dominance. This suggests that low GA levels may reduce auxin content and/or transport in citrus.

Reduction of plant height by modifying the expression of genes of GA biosynthesis and metabolism is potentially of high agronomic interest. Recently, a fragment of a GA20ox gene from wild apple has been used to produce dwarf apple cv. Greensleves through transgenic sense or antisense co-suppression. Importantly, transgenic scions retained their dwarf habit when grafted onto non-transgenic rootstocks (Bulley et al., 2005). By contrast, both sense
and antisense Carrizo citrange transgenic plants lost their elongated and semi-dwarf aspect, respectively, when grafted onto non-transgenic rootstocks of the same citrus genotype (Table 2). Interestingly, in this case, the concentrations of GA1, GA4 and GA20 were not significantly different between control and transgenic shoots (Table 4). The increase of GAs in the antisense scion may be the result of the transport of active GA or their precursors from the roots to aerial parts. Although there is no direct evidence for this kind of transport, transcripts of genes encoding diverse genes of GA biosynthesis have been detected in roots of different species, including Carrizo citrange (Vidal et al., 2003). Also, GA20 applied in the culture medium are absorbed and transported to the shoot in pea (Fichet, 2001). By contrast, the decrease of GA in the sense scion is not easy to explain. Probably other still unknown factors transported from the roots, in addition to GAs, may also regulate the GA homeostasis in the aerial part of the plant. Certainly, it remains to be investigated whether antisense transgenic Carrizo citrange used as a rootstock is able to reduce the size of non-transgenic scions.

In conclusion, it has been shown that it is possible to modify the architecture of citrus plants by genetic manipulation of endogenous GA20ox expression in transgenic plants. Potential reduction of scion plant stature by down-regulating GA20ox of a well-known and widely used rootstock would provide a considerable benefit to citrus culture by allowing higher planting density, easier management, and mechanical fruit harvesting, thus reducing labour costs.

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Table 6. Anatomical characteristics of internodes from transgenic control and CcGA20ox1 sense and antisense Carrizo citrange plants

<table>
<thead>
<tr>
<th>Transgenic lines</th>
<th>Internodal cross-sectional area (mm²) a,c</th>
<th>Xylem b,d,e</th>
<th>Pith b,d,e</th>
<th>Inner cortical cell length (μm) b,d,e</th>
<th>Inner cortical cell width (μm) b,d,e</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.402±0.830a</td>
<td>27.6±2.4a</td>
<td>22.7±1.6b</td>
<td>53.2±1.4a</td>
<td>22.3±0.5b</td>
</tr>
<tr>
<td>Sense S23</td>
<td>3.290±0.258b</td>
<td>18.9±1.0b</td>
<td>30.8±0.6b</td>
<td>57.2±1.6b</td>
<td>24.6±0.5b</td>
</tr>
<tr>
<td>Antisense A8</td>
<td>7.531±0.627c</td>
<td>27.8±2.1b</td>
<td>21.8±1.1b</td>
<td>53.3±1.5b</td>
<td>25.3±0.5b</td>
</tr>
</tbody>
</table>

a Number of determinations=5.
b Number of determinations=60.
c Percentage of internodal cross-sectional area.
d Obtained in longitudinal sections of the internodes.
e In each column, values marked with different letters were significantly different (P<0.05). Data (mean ±SE) were analysed using a factorial analysis of variance and Duncan’s multiple range test.

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Saibo NJM, Vriezen WH, Beemster GTS, Van Der Straeten D. 2003. Growth and stomata development of Arabidopsis are controlled by gibberellins and modulated by ethylene and auxins. The Plant Journal 33, 989–1000.


