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ORIGINAL PAPER

Engineering gibberellin metabolism in *Solanum nigrum* L. by ectopic expression of gibberellin oxidase genes

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Abstract Gibberellins (GAs) control many aspects of plant development, including seed germination, shoot growth, flower induction and growth and fruit expansion. Leaf explants of *Solanum nigrum* (Black Nightshade; Solanaceae) were used for *Agrobacterium*-mediated delivery of GA-biosynthetic genes to determine the influence of their encoded enzymes on the production of bioactive GAs and plant stature in this species. Constructs were prepared containing the neomycin phosphotransferase (*npt*II) gene for kanamycin resistance as a selectable marker, and the GA-biosynthetic genes, their expression under the control of the CaMV 35S promoter. The GA-biosynthetic genes comprised *AtGA20ox1*, isolated from *Arabidopsis thaliana*, the product from which catalyses the formation of C_{19}-GAs, and *MmGA3ox1* and *MmGA3ox2*, isolated from *Marah macrocarpus*, which encode functionally different GA 3-oxidases that convert C_{19}-GAs to biologically active forms. Increase in stature was observed in plants transformed with *AtGA20ox1*, *MmGA3ox2* and *MmGA3ox1* + *MmGA3ox2*, their presence and expression being confirmed by PCR and RT-PCR, respectively, accompanied by an increase in GA_1 content. Interestingly, *MmGA3ox1* alone did not induce a sustained increase in plant height, probably because of only a marginal increase in bioactive GA_1 content in the transformed plants. The results are discussed in the context of regulating plant stature, since this strategy would decrease the use of chemicals to promote plant growth.

Keywords *Agrobacterium* transformation · Gibberellin 3-oxidase · Gibberellin 20-oxidase · *Arabidopsis thaliana* · *Marah macrocarpus* · *Solanum nigrum*

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Introduction

Gibberellins (GAs) are endogenous plant hormones that influence many aspects of plant development, including seed germination, shoot growth resulting from internode extension through enhancement of cell division and elongation, flower induction and fruit expansion (Thomas and Hedden 2006). Since these developmental processes are relevant to agriculture and horticulture, manipulating GA status in plants through application of GAs or inhibitors of GA biosynthesis that act as growth retardants, has long been common practice in plant husbandry. For example in horticulture, GA_3 is used to alter architecture in ornamentals such as *Colocasia*, *Caladium*, *Dieffenbachia*, *Spathiphyllus* and *Syngonium* (Brooking and Cohen 2001), with several ready-to-use GA formulations such as 'Promalin', being commercially available. Furthermore, GAs can prevent post-harvest leaf yellowing in cut flowers, such as lilies and alstromeria. Inhibitors of GA biosynthesis, such as daminozide (Alar), chlormequat chloride (Cycocel, CCC) and paclobutrazol (Benzi), are used as growth retardants (Rademacher 2000). Dwarf or semi-dwarf mutants with altered GA biosynthesis or signalling have been selected in a number of important crops, prominent examples being the 'green revolution' dwarfing genes in rice and wheat. The latter compromise GA biosynthesis

and GA signal transduction, respectively, in these crops (Hedden 2003).

Gibberellin biosynthesis (Hedden and Phillips 2000; Yamaguchi 2008) and the early stages of GA signal transduction (Hirano et al. 2008) are known in detail. In the latter, binding of GA to the GIBBERELLIN INSENSITIVE DWARF 1 (GID1) receptor causes a conformational change that promotes the interaction of GID1 with the growth-repressing DELLA proteins. The GID1-GA-DELLA complex is recognized by the F-box component of a Skp1-cullin-F-box (SCF) ubiquitin ligase, resulting in polyubiquitination, thereby targeting the DELLA protein for degradation by the 26S proteasome (Harberd et al. 2009). Removal of the DELLA proteins allows growth to occur by releasing the inhibition of transcription factors (Daviere et al. 2008). The biosynthesis of GAs from their diterpenoid precursor geranylgeranyl diphosphate requires the activity of terpene cyclases, cytochrome P450 monooxygenases and 2-oxoglutarate-dependent dioxygenases. The dioxygenases, comprising the biosynthetic enzymes GA 20-oxidase (GA20ox), GA 3-oxidase (GA3ox) and the inactivating enzymes GA 2-oxidase (GA2ox), are important sites of regulation (Figs. 1, 2). In contrast to the enzymes involved in the early stages of the pathway, the dioxygenases are encoded by multiple genes with differential expression and regulation by endogenous and environmental cues, including temperature and light (Hedden and Phillips 2000). As a consequence of their relatively high impact on the content of bioactive GAs, the dioxygenase genes are prime targets for genetic modification of growth and development (Phillips 2004; Radi et al. 2006).

In the present study, the impact on growth of ectopic expression of the GA 20-oxidase gene *AtGA20ox1* from

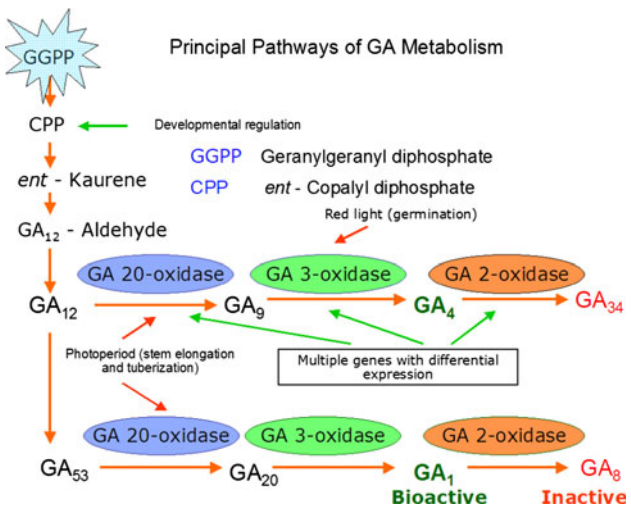

Fig. 1 GA biosynthetic pathway and targets for genetic manipulation

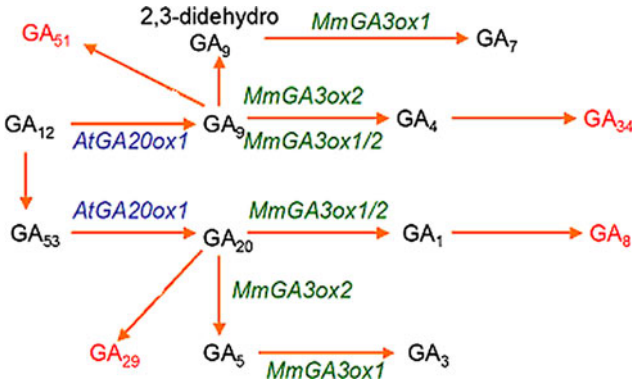

Fig. 2 Mode of action of GA genes employed in the present investigation

Arabidopsis thaliana was compared with expression of *GA3ox* genes from *Marah macrocarpus.* The protein encoded by *AtGA20ox1* catalyses the formation of GA_9 or GA_{20} from GA_{12} or GA_{53}, respectively, in a rate-limiting reaction in GA biosynthesis. Thus, Coles et al. (1999) found that over-expression of *AtGA20ox1* in *Arabidopsis* increased GA_4 content and stature. The genes from *M. macrocarpus, MmGA3ox1* and *MmGA3ox2*, encode functionally different GA 3-oxidases. *MmGA3ox1* converts GA_9 or GA_{20} to the bioactive GA_4 or GA_1, respectively, while *MmGA3ox2* functions mainly as a desaturase, forming 2,3-didehydro GA_9 or GA_5, respectively, as major products from these two substrates (Ward et al. 2010). In combination, these enzymes produce the unsaturated bioactive GAs, GA_7 or GA_3, from GA_9 or GA_{20}, respectively. These genes were expressed, alone or in combination, in the easily transformable model species *Solanum nigrum.*

Materials and methods

Plant material

Plants of *S. nigrum* (Black Nightshade) were grown from seed and maintained under glasshouse conditions in 6:6:1:1 by volume of a mixture of Levington M3 compost (Scotts UK, Ipswich, UK), John Innes No. 3 compost (J. Bentley, Barrow-on-Humber, UK), Perlite (Silvaperl, Gainsborough, UK) and Vermiculite (Silvaperl). Natural daylight in the glasshouse was supplemented with 16 h of fluorescent illumination (195 µmol m^{-2} s^{-1}; TLD/58 W 35 V 'Daylight' fluorescent tubes; Phillips, Croydon, UK) with day and night temperatures of 25 ± 1°C. Material for transformation was obtained from the uppermost fully expanded leaves excised from 4- to 10-week-old plants and surface sterilised by immersion in 10% (*v*/*v*) 'Domestos' bleach (Diversey Lever Ltd., Northampton, UK) for 10 min, followed by three washes with sterile, reverse osmosis water.

The midribs were removed from each leaf and the laminae were cut into 1 cm^2 explants under axenic conditions.

Constructs for *Agrobacterium* transformation of leaf explants

MmGA3ox1 and *MmGA3ox2* The coding regions were amplified from *M. macrocarpus* by polymerase chain reaction (PCR) using gene-specific primers, i.e. *MmGA3ox1* forward 5′-CCCGATATCATGGCAGATCAGGAGATTACT-3′ and reverse 5′-GGGCTCGAGCTAAATTAAAGATGATATTTTTACGG-3′ (1,083 bp product), *MmGA3ox2* forward 5′-CCCGATATCATGGCCACCAAAATAACCGAC-3′ and reverse 5′-GGGCTCGAGTTAGCCTACTTTGACCTGACT-3′ (1,131 bp product). After sub-cloning into pCR2.1 (Invitrogen, Groningen, The Netherlands), genes were inserted separately into the *Sac*I site of the binary vector pLARS120 adjacent to the CaMV 35S promoter. The T-DNA of pLARS120 also contained the neomycin phosphotransferase (*npt*II) gene with the *nos* promoter, located next to the left border of the T-DNA (Fig. 3).

AtGA20ox1 The cDNA clone was amplified by PCR using the forward primer 5′-CGGTTTCTTCCTCGTGGTCA-3′ and the reverse primer 5′-GTGACTTCCTCTCGCTCTTG-3′ (677 bp product). The fragment was inserted into the *Xba*I site of the binary vector pCIB200, which contained the *npt*II gene as a selectable marker (Coles et al. 1999; Fig. 3).

Agrobacterium tumefaciens-mediated plant transformation

Agrobacterium tumefaciens strain LBA4404 was transformed as described (Sambrook et al. 1989), the binary vectors carrying *MmGA3ox1*, *MmGA3ox2* or *AtGA20ox1*, and cultured in 100-ml aliquots of Luria Broth (LB; Sambrook et al. 1989) with 40–50 mg l^{-1} kanamycin sulphate and 100 mg l^{-1} streptomycin (50 mg l^{-1}) in 500 ml

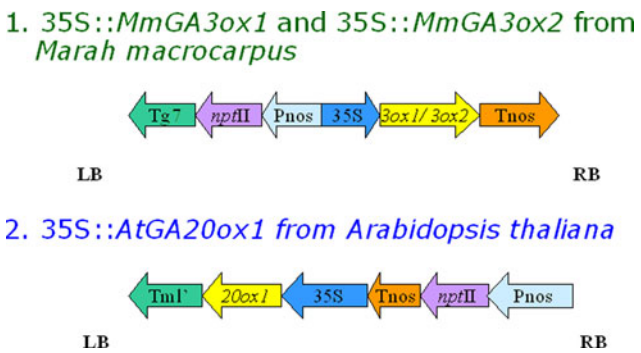

Fig. 3 Gene cassettes carrying *AtGA20ox1*, *MmGA3ox1* and *MmGA3ox2* genes

Erlenmeyer flasks. Cultures were maintained on a rotary shaker (150 rpm) at 27 ± 1°C for 16 h in the dark; those with an OD_{600nm} 0.7–1.2 were used to inoculate leaf explants.

Transformation of leaf explants

Explants were immersed for 5 min in suspensions of *A. tumefaciens*, the latter being diluted immediately before use 1:10 (*v*:*v*) with liquid Murashige and Skoog (1962) (MS)-based culture medium (containing 30 g l^{-1} sucrose, but lacking growth regulators; designated MS0) at pH 5.8. Control explants were treated similarly, except that the bacteria were omitted from the inoculation medium. Following inoculation, explants were blotted dry on sterile filter paper and transferred to the surface of 25 ml aliquots of MS-based medium containing 1.0 mg l^{-1} zeatin and semi-solidified with 8 g l^{-1} agar (designated MSZ; 8 explants/9 cm Petri dish). Inoculated explants were maintained at day/night temperatures of 22 ± 1 and 20 ± 1°C, respectively, with a 16 h photoperiod and a light intensity of 19.5 µmol m^{-2} s^{-1} 'Daylight' fluorescent tubes (Thorn EMI Ltd, Hayes, UK). After 2 days, leaf explants were transferred to semi-solid MSZ medium supplemented with cefotaxime (500 mg l^{-1}) and kanamycin sulphate (50 mg l^{-1}). Uninoculated explants were transferred to medium either lacking antibiotics to regenerate non-transformed plants for comparison with transgenic plants, or to medium with antibiotics, as used for explants inoculated with *A. tumefaciens*. Cultured leaf explants, which formed callus after 8–10 weeks, were transferred to 175 ml screw-capped jars (Beatson Clarke and Co., Rotherham, UK), each containing 50 ml of semi-solid MSZ medium, supplemented with the same antibiotics as used previously. Regenerated shoots were excised from leaf-derived calli and rooted on semi-solid (0.8% *w/v* agar) MS0 medium with kanamycin at 50 mg l^{-1}. Rooted plants were potted in the same mixture as used for seedlings. Potted plants were covered with 17 cm × 15 cm plastic bags and transferred to the glasshouse under natural daylight. The tops of the bags were opened progressively over a 14-day period.

Phenotypic analyses

The phenotypic characteristics of each plant (height, internode length, leaf length and width) were recorded 42 days after acclimation of the plants to glasshouse conditions. Transgenic ($n = 9$) and control ($n = 6$) plants were allowed to flower and self-pollinate. One hundred ripe berries were collected from each plant for fresh weight determinations. Berries from individual plants were combined, macerated and incubated in 30 ml aliquots of 1 M HCl to remove enveloping tissues. Seeds were stirred in

running tap water and collected on a nylon sieve (1 mm^2 mesh), dried overnight on filter paper (No. 1; Whatman International Ltd., Maidstone, UK) at 23 ± 2°C and weighed. Seeds were sown in compost and plants maintained for subsequent generations.

PCR analysis

Primers were manufactured and sequenced by MWG Biotech, Ebersberg, Germany. The sequences used were forward 5′-GCTCTTCGCTCTTCCAAC-3′ and reverse 5′-ACCT GTCTGCTAAACCCTTC-3′ (*MmGA3ox1*), forward 5′-CC CGATATCATGGCCACCAAAATAACCGAC-3′, reverse 5′-GGGCTCGAGTTAGCCTACTTTGACCTGACT-3′ (*MmGA3ox2*), forward 5′-GAGAATTCAAAATGGCCGT AAAGTTTCG-3′, reverse 5′-CGCTCTAGAACTAGTGG ATC-3′ (*AtGA20ox1*), forward 5′-AGACAATCGGCTGCT CTGAT-3′, and reverse 5′-ATACTTTCTCGGCAGGAG CA-3′ (*npt*II). Template genomic DNA was extracted from plants using a GenElute Plant Miniprep kit (Sigma-Aldrich, Missouri, USA). PCR was performed using RED Taq Ready Mix (Sigma-Aldrich) according to the manufacturer's instructions. Amplification was performed in a DNA Thermal Cycler 480 (Perkin Elmer Applied Biosystems Division, Warrington, UK), with initial denaturing (1 cycle, 94°C, 3 min), denaturating (35 cycles, 94°C, 1 min), primer annealing [35 cycles; 57°C (*npt*II), 53°C (*AtGA20ox1*) or 61°C (*MmGA3ox1* and *MmGA3ox2*), 1 min], primer extension (35 cycles, 72°C, 90 s), final extension (1 cycle, 72°C, 10 min) and holding at 4°C (5 min to ∞).

For reverse transcriptase-PCR (RT-PCR) analysis, RNA was extracted from 100 mg leaf samples of putatively transformed and non-transformed plants using an RNeasy® Plant Mini Kit (Qiagen, Crawley, UK). RT-PCR employed a One-Step RT-PCR Kit (Qiagen) according to the manufacturer's instructions. The amplification programme involved reverse transcription (1 cycle, 50°C, 30 min), polymerase activation (1 cycle, 94°C, 15 min), denaturation (35 cycles, 94°C, 1 min) and primer annealing (35 cycles, 55°C, 1 min). Subsequent conditions were as for PCR analysis.

GA quantitative analysis

Freeze-dried samples of leaf tissues (each 500 mg) were ground in a ball mill and resuspended in 100 ml of 80% (*v*/*v*) aqueous methanol. Subsequent stages, that included the addition of internal standards (a mixture of ^{2}H- and ^{3}H- labelled GAs), extraction, purification and analysis using combined gas chromatography-mass spectrometry, were as described in Dijkstra et al. (2008).

Statistical analysis

All phenotypic data were subjected to Student's *t* test. The *F* test was used with ANOVA when comparison was made of multiple distributed populations. Standard MINITAB version 15, statistical software (Minitab Inc., PA, USA) was employed in all *t* and *F* tests.

Results

Uninoculated leaf explants cultured on semi-solid MSZ medium with kanamycin, became necrotic and failed to regenerate shoots. Those on medium without kanamycin developed shoots that were used as controls. Negative segregants were also included as controls wherever feasible. However, no significant difference was found in growth and development parameters between control and negative segregant plants ($p = 0.05$). Dot blot analysis for the presence of transgenes was also carried out wherever feasible, for *MmGA3ox1, MmGA3ox2, MmGA3ox1* + *MmGA3ox2* and *AtGA20ox1* transformed plants. The transformation efficiency for *MmGA3ox1* was 10.2%, *MmGA3ox2* 3.9%, *MmGA3ox1* + *MmGA3ox2* 3% and *AtGA20ox1* 4%.

Plants transformed with *MmGA3ox1*

Four hundred and twelve leaf explants inoculated with *A. tumefaciens* strain LBA4404 carrying *35S::MmGA3ox1*, regenerated 82 putatively transformed shoots, of which 42 rooted plants were analysed for the presence of the *MmGA3ox1* and *npt*II genes. Twenty-five plants showed the presence of both genes. Student's *t* test on regenerated plants (T_0 generation) showed that plants expressing *MmGA3ox1* exhibited a marginal increase in height (Table 1a), but this was not sustained and was not significant in the T_2 generation. Two homozygous lines were identified. At least six plants per line were analysed in the subsequent generation. Transformed plants were more branched with a larger canopy compared to non-transformed plants (Fig. 4), this characteristic being retained in the T_2 generation. Stem girth, internode length, and leaf length and width, did not differ significantly in transgenic compared to control plants. Flower and fruit development were normal in the transformants. Plants with a single locus integration pattern of the *MmGA3ox1* gene, as determined by Mendelian inheritance, were selected for analysis. Transgenic plants were PCR- and RT-PCR-positive (Fig. 5). The GA content of selected transgenic plants showed only a marginal increase in GA_1 content (Table 1a).

Table 1 Concentrations of GAs in shoots of non-transformed (control), *MmGA3ox1*, *MmGA3ox2*, *MmGA3ox1* + *MmGA3ox2* and *AtGA20ox1* T_0 transformed plants (ng g^{-1} fresh weight)

Plant number	Plant height (cm)	GA_1	GA_3	GA_4	GA_8	GA_{19}	GA_{20}
a. *MmGA3ox1*							
Control	62.1	**2.28**			6.00		13.70
16	84.1	**3.75**			3.37		4.65
54	76.2	**nd**			2.26		2.83
b. *MmGA3ox2*							
Three-week-old leaf (composite) sample							
Control (1)	61.7	**24.7**	4.2	**3.4**	0.9	11.4	27.2
Control (2)	61.0	**26.1**	6.8	**2.6**	0.9	11.0	10.4
2	65.5	**59.0**	4.5	**13.0**	0.7	21.7	45.0
91	80.5	**51.0**	3.2	**5.1**	0.1	20.2	45.0
Six-week-old leaf (composite) sample							
Control	61.7	**1.62**	nd	**nd**	nd	4.67	0.53
2	65.5	**2.85**	nd	**nd**	nd	4.02	nd
91	80.5	**nd**	nd	**nd**	nd	2.36	nd
95	85.4	**4.00**	nd	**nd**	nd	2.78	5.70
c. *MmGA3ox1* + *MmGA3ox2*							
Control	67.0	**2.28**			6.00		13.70
29	78.1	**6.40**			nd		13.09
37	79.8	**nd**			1.13		4.02
84	78.4	**8.32**			3.54		20.00
125	87.2	**nd**			4.29		7.10

Plant	GA_1	GA_{19}	GA_{20}
d. *AtGA20ox1*			
Control	**1.38**	1.9	0.2
20ox composite sample	**7.47**	3.2	++

Data for bioactive GAs are in bold

++ Very high; above calibration curve

Plants transformed with *MmGA3ox2*

Nine hundred leaf explants inoculated with *A. tumefaciens* strain LBA4404 carrying *35S::MmGA3ox2*, regenerated 343 plants; 71 putative transgenic plants were analysed for the presence of *MmGA3ox2* and the *npt*II genes. Thirty-five plants were characterized phenotypically (Fig. 4) and were PCR- and RT-PCR-positive for the *MmGA3ox2* and *npt*II genes. The mean height of transgenic plants in the T_0 generation was 70.1 cm (range 62.0–85.4 cm) compared to 61.0–61.7 cm in control plants, i.e. an increase of 14% (Table 1). Stem girth and internode length were increased by 7.2 and 4.0%, respectively, in the transgenic lines compared with the controls. Student's *t*-test confirmed that plants expressing *MmGA3ox2* showed a marginal increase in height that was retained in the T_1–T_3 generations. Three lines were identified with single locus integration and at least six plants per line were analysed in the T_2 and T_3 generations. Flower development in transgenic plants was comparable to controls; transgenic plants flowered and set fruit at the same time as the non-transformed plants. GA_1 and GA_4 in transgenic plants exhibited a significant increase ($p = 0.05$), which correlated with increased plant height (Table 1b).

Plants transformed with *MmGA3ox1* + *MmGA3ox2*

Nine hundred leaf explants inoculated with a mixture of *A. tumefaciens* LBA4404 strains carrying *35S::MmGA3ox1* and *35S::MmGA3ox2*, resulted in 26 plants that expressed

Fig. 4 Comparison of the phenotypes of *AtGA20ox1*, *MmGA3ox1*, *MmGA3ox2* and *MmGA3ox1* + *MmGA3ox2* transformed plants with the phenotype of a non-transformed plant at 42 days after potting. Pot size 13 cm diameter

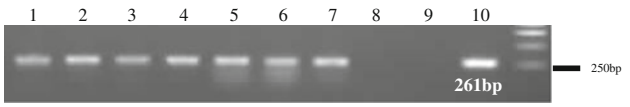

Fig. 5 Typical RT-PCR analysis of selected, kanamycin resistant plants of *S. nigrum* transformed with *MmGA3ox1* confirming expression of the transgene (*Lanes 1–7*). Non-transformed (control) plants did not express the transgene (*Lanes 8–9*). *Lane 10* shows positive control, PCR amplified *MmGA3ox1* construct

MmGA3ox1, *MmGA3ox2* and *npt*II. Thirty eight plants carried only *npt*II, 35 carried *Mm3ox1* and 29 had *Mm3ox2*. Three plants did not express any of the three transgenes. Transgenic T_0 generation plants expressing *MmGA3ox1* + *MmGA3ox2* increased by 10.4% in height compared to non-transformed plants (Table 1c); transgenic plants had a mean height of 68.6 cm compared to 61.0 cm for the controls. The mean internode length for transgenic plants was 8.1 mm (range 6.5–11.4 mm). Transgenic plants also exhibited a 37% increase of canopy diameter over controls, this being 36.9 cm in transgenic plants (31.6–54.9 cm) compared to 27.0 cm (24.8–29.2 cm) for controls (Figs. 4, 6). The GA content of selected transgenic plants increased significantly ($p = 0.05$), which correlated with the increase in plant height (Table 1c).

Plants transformed with *AtGA20ox1*

Seven hundred explants inoculated with *A. tumefaciens* LBA4404 carrying *AtGA20ox1* produced 92 putative transgenic shoots on MSZ medium with kanamycin selection, 28 of which had integrated and expressed the *AtGA20ox1* and *npt*II genes as confirmed by PCR and RT-PCR. Nine plants expressing *AtGA20ox1* were significantly taller than the controls ($n = 3$) at ($p \geq 0.01$) using a one-way unpaired Student's *t* test, their heights ranging from 63.9 to 94.1 cm with a mean of 79.0 cm; non-transformed plants had a mean height of 61.0 cm. Mean height of transgenic plants was increased by 31% above the controls (Fig. 4), with a 48% increase in the tallest transgenic plant. The internode length of the transgenic plants was increased by 59%, with a mean of 9.8 cm for transgenic plants compared to 5.7 cm for controls. Mean stem girth of the transgenics was 14.9 mm compared to 10.7 mm in controls, with 13% more leaves and a 23% increase in the number of branches. *AtGA20ox1*-transformed plants had a canopy diameter of 36.7 cm compared to controls of 27.0 cm (Fig. 4). Flower development and pollen viability were unaffected. Figure 6 summarises plant height and canopy diameter from the transgenic and control plants. The heights of transgenic plants were consistent with their increased GA content (Table 1d). There was no difference in flowering time and root development between *Mm3ox1*, *Mm3ox2*, *Mn3ox1* + *Mm2ox1*, *AtGA20ox1* and non-transformed plants. The number of fruits and fruit weight were comparable and not statistically significant ($p = 0.05$; Fig. 6). The canopy diameter of transgenic plants was greater than that of non-transformed plants ($p = 0.05$).

Discussion

Alteration of plant stature can be achieved by modifying GA biosynthesis. Plants of *S. nigrum* transformed with *AtGA20ox1* exhibited the greatest increase in internode length and corresponding plant stature, typical of GA overproduction (Phillips 2004). Coles et al. (1999) and Huang et al. (1998) found that over-expression of *AtGA20ox1* resulted in a significant increase in plant height (25% more than in controls) in *A. thaliana*, the transgenic

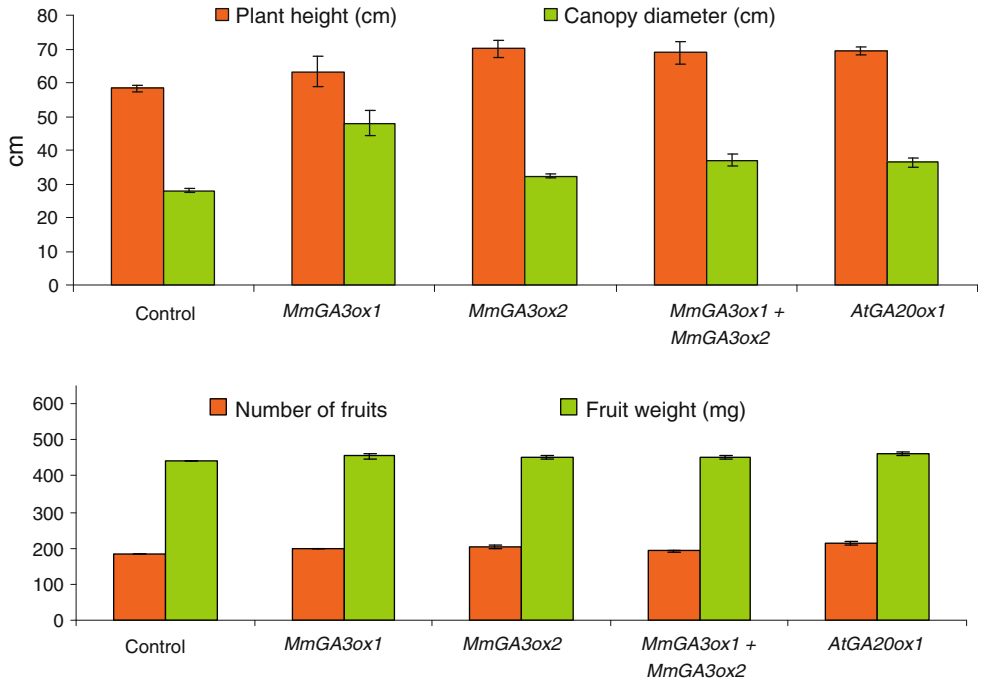

Fig. 6 Comparison of plant height, canopy diameter, number of fruits and fruit weight for *MmGA3ox1*, *MmGA3ox2, MmGA3ox1* + *MmGA3ox2* and *AtGA20ox1*, transformed plants compared to the same parameters for non-transformed plants at 42 days after potting

plants resembling wild-type plants treated with GA. Subsequently, similar results were reported in tobacco (Beimelt et al. 2004; Vidal et al. 2001), potato (Carrera et al. 2000), poplar (Eriksson 2000; Eriksson and Moritz 2002) and Carrizo citrange (Fagoaga et al. 2007). Transgenic poplar plants had a greater stem girth than their control counterparts, but stem girth was reduced in Carrizo citrange, which also exhibited a reduction in the length of thorns and poor rooting. Thus, ectopic expression of GA 20-oxidase may cause some subtle phenotypic differences, depending on the target species and also on the level of gene expression (Fagoaga et al. 2007).

An increase in canopy diameter of transgenic *AtGA20ox1* plants of *S. nigrum* was the result of the more vigorous growth of branches. Ectopic expression of GA 20-oxidase resulted in an increase in internode length without an increase in the number of internodes in aspen hybrids (Isrealsson 2004), which could be attributed to the role of GA in stimulating cell elongation. García-Martínez et al. (1997) suggested that since GA 20-oxidase catalyses the rate-limiting step in GA biosynthesis, *GA3ox* expression may not increase GA production sufficiently to increase plant stature, as found for *MmGA3ox1* in the present study. However, Radi et al. (2006) found that ectopic expression of a *GA3ox* gene (*CmGA3ox1*) in *Arabidopsis* did increase plant height, although in contrast, Phillips (2004) reported that *GA3ox* expression did not change plant phenotype in the same target plant. These differences may have been due to the very high levels of expression obtained by Radi et al. (2006), who used an enhanced 35S promoter to drive transgene expression. In the present work, ectopic expression of *MmGA3ox1*, which encodes a 3β-hydroxylase (Ward et al. 2010) resulted in only a small increase in GA_1 and plant height, whereas expression of *MmGA3ox2*, the product of which possesses desaturase as well as 3β-hydroxylase activity, resulted in much greater increases in GA content and plant stature. In this case, there were also greater concentrations of the precursors GA_{19} and GA_{20}, indicating enhanced GA biosynthesis, although the reason for this is unclear. A greater content of bioactive GAs might be expected to reduce GA 20-oxidase activity through feedback regulation, which would result in lower concentrations of intermediates. However, Radi et al. (2006) found increased concentrations of GA_{24} and $GA_{9,}$ the non-13-hydroxylated analogues of GA_{19} and GA_{20}, after over-expression of a pumpkin *GA3ox* gene in Arabidopsis. Combined expression of *MmGA3ox1* and *MmGA3ox2*, which might be anticipated to increase the production of 1,2-desaturated GAs that are resistant to inactivation by GA 2-oxidases (Ward et al. 2010), also produced an increase in plant height and GA_1 content. Unfortunately, for technical reasons it was not possible to determine the GA_3 concentrations in these plants. These unexpected results may have been due to interactions

between the activities of the endogenous and introduced enzymes.

Future research should be directed towards identifying tissue-specific or specific inducible promoters by screening microarray databases and, subsequently, in developing constructs to drive gene expression in vegetative tissues such as internodes and petioles, thus ensuring that flowering and seed set are not affected. The possibility of using constructs carrying GA oxidase genes, but lacking selectable maker gene(s), should be explored to facilitate the acceptance of this genetic manipulation technology to regulate plant height. It may also be possible to use sequences similar to T-DNA sequences, but which have originated from target plant genomes to develop cisgenic plants (Chandler and Tanaka 2007). Additionally, vectors that incorporate selectable markers of plant origin and recombinase recognition sequences (*Cre-lox* system), could also be beneficial (Wang et al. 2003; Zuo et al. 2001). The use of co-transformation (Komari et al. 1996) or the use of chimeric plants (De Vetten et al. 2003) may be considered.

In conclusion, the enhancement of plant stature and modification of shoot architecture in *S. nigrum* was achieved by over-expressing selected GA-biosynthesis genes. Over-expression of GA 20-oxidase (*AtGA20ox1*) induced the greatest increase in plant height, with over-expression of the 3-oxidases *MmGA3ox1* and *MmGA3ox2* producing a more limited response. This transformation approach may provide a strategy to reduce the requirement of agrochemicals to regulate plant stature which could be applicable to the ornamental horticulture industry.

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