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RESEARCH PAPER

Identification and genetic characterization of a gibberellin 2-oxidase gene that controls tree stature and reproductive growth in plum

I. El-Sharkawy[1], W. El Kayal[2], D. Prasath[1], H. Fernández[3], M. Bouzayen[4], A. M. Svircev[5] and S. Jayasankar[1,*]

[1] University of Guelph, Department of Plant Agriculture. 4890 Victoria Av. N., PO Box 7000 Vineland Station, ON, L0R 2E0 Canada
[2] University of Alberta, Department of Biological Sciences, Edmonton, AB, T6G 2E9 Canada
[3] Laboratorio de Fisiología Vegetal Dpt. BOS, Universidad de Oviedo, c) Catedrático R Uría s/n, Oviedo, E-33071, Spain
[4] UMR 990 INRA/INPT-ENSAT 'Génomique et Biotechnologie des Fruits', Av. de l'Agrobiopole, BP 32607, F-31326 Castanet-Tolosan Cedex, France
[5] Agriculture and Agri-Food Canada, Southern Crop Protection and Food Research Centre. 4902 Victoria Av. N., PO Box 6000 Vineland Station, ON L0R 2E0 Canada

* To whom correspondence should be addressed. E-mail: jsubrama@uoguelph.ca

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Abstract

Several dwarf plum genotypes (*Prunus salicina* L.), due to deficiency of unknown gibberellin (GA) signalling, were identified. A cDNA encoding GA 2-oxidase (*PslGA2ox*), the major gibberellin catabolic enzyme in plants, was cloned and used to screen the GA-deficient hybrids. This resulted in the identification of a dwarf plum hybrid, designated as DGO24, that exhibits a markedly elevated *PslGA2ox* signal. Grafting 'Early Golden' (EG), a commercial plum cultivar, on DGO24 (EG/D) enhanced *PslGA2ox* accumulation in the scion part and generated trees of compact stature. Assessment of active GAs in such trees revealed that DGO24 and EG/D accumulated relatively much lower quantities of main bioactive GAs (GA_1 and GA_4) than control trees (EG/M). Moreover, the physiological function of *PslGA2ox* was studied by determining the molecular and developmental consequences due to ectopic expression in *Arabidopsis*. Among several lines, two groups of homozygous transgenics that exhibited contrasting phenotypes were identified. Group-1 displayed a dwarf growth pattern typical of mutants with a GA deficiency including smaller leaves, shorter stems, and delay in the development of reproductive events. In contrast, Group-2 exhibited a 'GA overdose' phenotype as all the plants showed elongated growth, a typical response to GA application, even under limited GA conditions, potentially due to co-suppression of closely related *Arabidopsis* homologous. The studies reveal the possibility of utilizing *PslGA2ox* as a marker for developing size-controlling rootstocks in *Prunus*.

Key words: Dwarf rootstocks, flower fertility, floral organogenesis, fruit development, GA deficiency, plum, rootstock–scion interaction.

Introduction

Modern fruit growing creates an increasing demand for tree size control that can offer numerous horticultural advantages. Trees with reduced stature allow high density cultivation, facilitate tree management, and minimize spray drift. In most temperate fruits, dwarf rootstocks can have profound effects on scions such as controlling tree size, flowering time, yield efficiency, and fruit quality (Janick *et al.*, 1996). However, the mechanism by which these effects are achieved is still not well understood. Attempts to explain how rootstocks cause dwarfing of trees, which focused on their effects on supply of mineral nutrients and water to the scion, have not provided any convincing

Abbreviations: EG/D, Early Golden/DGO24; EG/M, Early Golden/Myrobalan; L.1/G1, L.1/Group-1; L.4/G2, L.4/Group-2; LD, long day; PAC, paclobutrazol; SD, short day; WT, wild type.

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explanations (Jones, 1984; Ebel *et al.*, 2000). Other studies, which focused on the production and movement of endogenous hormones, demonstrated that dwarfism could be associated with deficiencies in gibberellin (GA) levels or signalling (Cristoferi and Filiti, 1981; Erez, 1984; Webster, 2004).

GA is an essential hormone that is involved in many aspects of plant growth and development (Fleet and Sun, 2005). To date, >100 GA forms have been identified in plants (MacMillan, 2002). However, only a small number of them are considered to be functional, while most other GAs are present in plant tissues as precursors for the active forms or due to deactivated metabolites. The GA biosynthetic pathway has long been studied, and the majority of genes encoding enzymes in each biosynthetic and catabolic step have been identified in the model species (Olszewski *et al.*, 2002).

In higher plants, the flux of active GAs is regulated by the balance between their rates of biosynthesis and deactivation. The GA20ox and GA3ox genes encode key enzymes of bioactive GAs synthesis, whereas GA2ox is the major GA inactivation enzyme (Yamaguchi, 2008). Modifying the regulation of genes controlling GA flux can subsequently alter the processes regulated by GA and, thus, plant architecture (Hedden and Phillips, 2000).

In plants, it is important to maintain optimal levels of phytohormones to ensure normal growth and development. Hence, it is essential that there is a mechanism in place to remove any excess active compounds or their biosynthetic precursors to ensure proper function of phytohormones. Such a strategy can modulate the signal produced and subsequently prevent the progressive accumulation of the hormones. A number of inactivation pathways have been identified for GA (Thomas and Hedden, 2006; Zhu *et al.*, 2006). However, based on the prevalence of 2β-hydroxylated GAs in many plant species (MacMillan, 2002), the most widespread mechanism for GA inactivation seems to be via 2-oxidation (Thomas *et al.*, 1999). Genes encoding GA 2-oxidases (GA2oxs) were first identified by screening cDNA expression libraries for 2β-hydroxylase activity (Martin *et al.*, 1999; Thomas *et al.*, 1999). Early characterized GA2oxs are active against C_{19}-GAs as substrates, including functional GAs and their immediate precursors. Later, a new type of GA2ox that catabolizes only the non-bioactive C_{20}-GAs was reported (Schomburg *et al.*, 2003). Apparently, this class of GA2ox is not involved in inactivation of functional GAs, but may be important in regulating GA biosynthesis through the removal of earlier intermediates in the pathway.

The physiological functions of GA2ox have been studied in a variety of plant species using different approaches; however, all these studies demonstrated that GA2ox is responsible for reducing the level of active GAs in plants. Overexpression of GA2ox enhances GA inactivation and thus induces dwarfism (Sakamoto *et al.*, 2001; Busov *et al.*, 2003; Appleford *et al.*, 2007; Dijkstra *et al.*, 2008). Loss-of-function mutation in a pea *PsGA2ox1* results in the hyper-elongated *slender* phenotype (Martin *et al.*, 1999). Similarly, in *Arabidopsis*, the *ga2ox* quintuple mutant results in plants behaving as the wild type (WT) supplemented with a high amount of GA (Rieu *et al.*, 2008*a*).

In this study, a dwarf plum hybrid (DGO24) that exhibits high levels of *PslGA2ox* and subsequently displays reduced bioactive GAs was identified. DGO24, when used as rootstock, reduces the scion ['Early Golden' (EG)] vigour and also causes several developmental defects; however, these could be temporarily restored by exogenous GA application. Moreover, the role of GA during different stages of fruit development was determined by studying the expression profile of *PslGA2ox* mRNA that reflects the alteration in GA accumulation. A model is proposed in which the role played by the plant hormone GA is as critical as that of auxin to ensure correct fruit development. Further, it was demonstrated that the overexpression of this gene results in a GA-deficient phenotype in *Arabidopsis* with growth traits similar to those found in EG/D (EG/DGO24) plum trees. In another case, the *PslGA2ox* transgene caused co-suppression of closely related *Arabidopsis* homologous, which triggered GA accumulation and resulted in a GA overdose phenotype. The results show that GA2ox can be used as a marker for the selection of dwarf rootstocks that might be suitable for the tender fruit industry.

Materials and methods

Plum tissues and post-harvest treatments

Flowers and fruits from different developmental stages were harvested from Japanese plum (*Prunus salicina* L.) cultivar EG as described previously (El-Sharkawy *et al.*, 2007). Leaves from 10-year old dwarf seedling DGO24 and 7-year old EG grafted onto DGO24 (EG/D) or onto vigorous rootstock Myrobalan (EG/M) were collected. All plant materials were frozen in liquid nitrogen and stored at –80 °C.

Isolation and in silico *analysis of plum* GA2ox *cDNA sequence*

Based on the sequence similarity of various *GA2ox* genes from different plant species, a pair of degenerate primers (primers 1 and 2, Supplementary Table S1 at *JXB* online) was designed from the conserved regions to amplify the *GA2ox* orthologues from *P. salicina*. The isolated fragment was cloned in the pGEM-T Easy vector (Promega, Madison, WI, USA), sequenced, and analysed using BLAST (Altschul *et al.*, 1997). Extension of the partial cDNA clone was carried out using the 3'- and 5'-RACE kit (Invitrogen, Burlington, ON, Canada). Full-length amplification of the cDNA sequence designated *PslGA2ox* was carried out using the Platinum Taq DNA Polymerase High Fidelity kit following the instructions provided by the manufacturer (Invitrogen). Alignment of the *PslGA2ox* predicted protein sequence and the Neighbor–Joining tree construction were performed as described previously (El-Sharkawy *et al.*, 2009).

Protoplast isolation and transient expression of PslGA2ox*–GFP fusion protein*

The coding sequence of *PslGA2ox* was cloned as a C-terminal fusion in-frame with green fluorescent protein (GFP) into the pGreenII vector using the *Bam*HI site, and expressed under the control of the *35S* promoter. Protoplasts used for transfection were obtained from suspension-cultured tobacco (*Nicotiana tabacum*) BY-2 cells. Protoplasts were transfected and analysed for GFP fluorescence by confocal microscopy as described previously

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(El-Sharkawy *et al.*, 2009). All transient expression assays were repeated at least three times.

Quantification of bioactive GAs

Approximately 3 g of fresh leaf and stem tissues from field-grown EG/M, DGO24, and EG/D plum trees was collected. Each sample was frozen in liquid nitrogen, lyophilised, and finally stored at –20 °C until analysed. Plant materials were homogenized in 15 ml of cold 80% methanol containing 50 ng of deuterated GA_1 and GA_4, and extracted overnight in darkness at 4 °C. After filtration, the residue was re-extracted with 5 ml of 80% methanol for 2 h and re-filtered. The extract was purified through a C18 Sep-pack cartridge and the eluate was absorbed onto 0.5 g of Celite 545 (ProLab), and purified by step-elution silicic acid (SiO_2) partition chromatography running into an ethyl acetate:hexane (95:5) buffer. The samples were then evaporated under reduced pressure at room temperature until dry. Phytohormones were analysed by high-performance liquid chromatography/mass spectrometry (HPLC/MS) carried out on a Varian 1200l triple quadrupole, working in positive electrospray ionization mode (ESI+) with a capillary voltage of 5500 V and acid voltage of 40 V. HPLC/MS analysis was carried out by MRM (multiple reaction monitoring) ion detection mode working with three transitions for each compound. Liquid chromatography (LC) was performed using a Polaris 3 µm, 150×2.1 mm I.D. analytical column, maintained at 40 °C. The mobile phases consisted of water/0.1% formic acid (A) and methanol/acetonitrile 25/75 (B). The flow-rate was 0.2 ml min^{-1}. In each case, 20 µl of sample was injected. The gradients used for GAs were: t=0 min (90% A, 10% B); t=1 min (75% A, 25% B); t=10 min (0% A; 100% B); and t=15 min (0% A; 100% B). The levels of phytohormones in the plant samples were determined from the area ratios of endogenous to corresponding deuterated phytohomones. A curve was prepared always with the same quantity of labelled isotope added to samples and with concentrations from 1 ppb to 250 ppb for the compounds analysed. The minimum quantification level was 1 ppb (1 ng ml^{-1}) for each compound. All experiments were carried out in three independent replicates.

Plasmid construction and plant transformation

For the generation of the *35S::PslGA2ox* construct, a high fidelity PCR system was used to amplify the full-length sequence using specific primers 3 and 4 (Supplementary Table S1 at *JXB* online), subcloned in pGEM-T Easy vector, and then introduced into the *Bam*HI site of the pGreen0029 binary vector (Hellens *et al.*, 2000). The resulting vector was introduced into *Agrobacterium tumefaciens* strain C58 by the freeze–thaw method (Holsters *et al.*, 1978) and then employed for *Arabidopsis* transformation using the floral dip method (Clough and Bent, 1998). The *PslGA2ox* gene under the control of the *35S* promoter was introduced into the WT *Arabidopsis* background Col-0. Six T_3 homozygous independent lines showing significant *PslGA2ox* accumulation were identified based on transgene levels along with phenotype characteristics. These lines were pooled into two groups based on their growth behaviour, and a representative from each group was selected for further experimental use (L.1/G1 and L.4/G2). All plants were divided into four groups (24 plants per group); three groups were grown under a long day (LD) photoperiod (16:8 h light/300 µmol m^{-2} s^{-1}; 23:18 °C, and 65% relative humidity): control, treated with 100 µM GA_3 or 10 µM paclobutrazol (PAC). The fourth group was transferred to short day (SD) conditions (8:16 h light and 20:18 °C). The plant materials were frozen in liquid N_2 immediately after collection and stored at –80 °C until use.

RNA isolation

Total RNA from plum was extracted using the methods described by Meisel *et al.* (2005). For *Arabidopsis*, total RNA was extracted using a Plant Total RNA Purification kit (Norgen, Thorold, ON, Canada). All RNA extracts were treated with DNase I (Promega) then cleaned up with anRNeasy mini kit (Qiagen, Mississauga, ON, Canada).

Real-time quantitative RT-PCR

DNase-treated RNA (5 µg) was reverse transcribed in a total volume of 50 µl using SuperScript III Reverse Transcriptase (Invitrogen). Gene-specific primers were designed using Primer Express (v3.0, Applied Biosystems, Carlsbad, CA, USA) (primers 5–30, Supplementary Table S1 at *JXB* online). Quantitative reverse transcription PCRs (qRT-PCRs) were performed using 20 ng of cDNA and 300 nM of each primer in a 20 µl reaction volume with SYBR GREEN PCR MasterMix (Qiagen, Mississauga, ON, Canada). Three biological and three technical replicates for each reaction were analysed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) with a first step of 95 °C for 15 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting curves were generated using the following program: 95°C for 15 s, 60 °C for 15 s, and 95 °C for 15 s. Transcript abundance was quantified using standard curves for both target and reference genes, which were generated from serial dilutions of PCR products from corresponding cDNAs. Transcript abundance was normalized to the reference genes [*PsAct* (EF585293) and *AtAct* (NM_121018)] that show high stability across the different treatments.

Results

Isolation and structural characterization of PslGA2ox *cDNA*

PCR amplification resulted in the isolation of a partial DNA fragment with the expected size. Sequence analysis of the PCR product indicated that this fragment encoded a part of the *GA2ox* gene family. Extension of the partial cDNA clone resulted in a full-length cDNA containing an open reading frame of 1482 bp encoding a protein of 342 amino acids and hence designated *PslGA2ox*. Alignment of the PslGA2ox amino acid sequence with that of other reported GA2oxs revealed that the predicted protein shares sequence identity ranging from 53% to 68% with closely related homologues and highlighted a number of conserved motifs and structural similarities that are common within the dioxygenase family of GA catabolic enzymes (Supplementary Fig. S1 at *JXB* online) (Valegård *et al.*, 1998). In order to classify the PslGA2ox sequence among the various GA2oxs, a phylogenetic tree was constructed (Fig. 1). The dendrogram analysis defines that the GA2ox gene family could be divided into three main classes based upon sequence conservation. PslGA2ox is a member of class I that (as well as class II), act as a major C_{19}-GA deactivator (Thomas *et al.*, 1999). However, tested members of class III can only catabolize C_{20}-GA2oxs (Schomburg *et al.*, 2003). Additionally, analysis of various GA2ox amino acid sequences, characterized so far, revealed the absence of any obvious targeting sequence that can signify the localization of this protein in the plant cell. The results show that the GFP distribution in both control and *PslGA2ox*–GFP is spread throughout the cytoplasm and nucleus (Supplementary Fig. S2 at *JXB* online).

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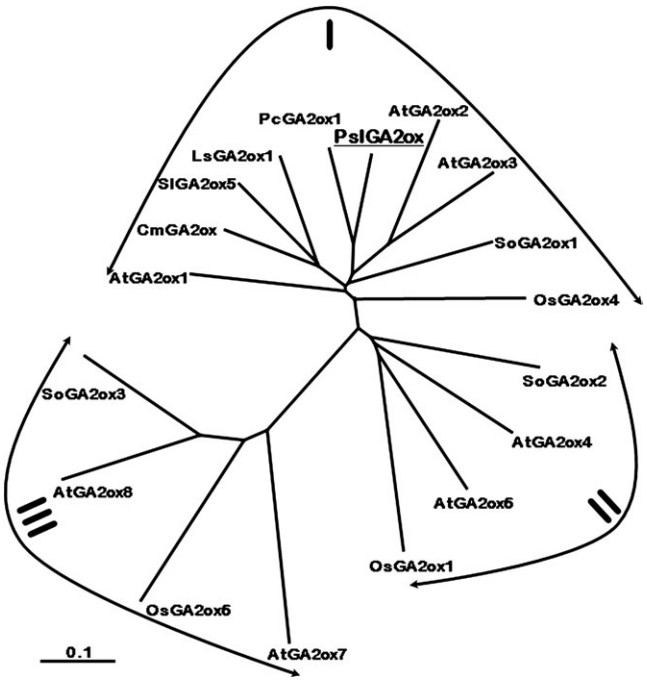

Fig. 1. Phylogenetic relationships between *Prunus salicina* PslGA2ox (HM021156), *Arabidopsis thaliana* AtGA2ox1 (CAB41007), AtGA2ox2 (CAB41008), AtGA2ox3 (CAB41009), AtGA2ox4 (AAG51528), AtGA2ox6 (AAG00891), AtGA2ox7 (AAG50945), AtGA2ox8 (CAB79120), *Oryza sativa* OsGA2ox1 (BAB40934), OsGA2ox4 (AAU03107), OsGA2ox6 (CAE03751), *Spinacia oleracea* SoGA2ox1 (AAN87571), SoGA2ox2 (AAN87572), SoGA2ox3 (AAX14674), *Solanum lycopersicum* SlGA2ox5 (ABO27636), *Lactuca sativa* LsGA2ox1 (BAB12442), *Phaseolus coccineus* PcGA2ox1 (CAB41036), and *Cucurbita maxima* CmGA2ox (CAC83090) based on the full-length amino acid sequence. Bootstrap confidence values from 1000 replicates are indicated. I, II, and III represent the three different GA2ox protein classes.

Identification of a GA2ox-dependent dwarf plum line

Twenty-five dwarf individual plum hybrids, produced through controlled hybridizations in a breeding program, were identified phenotypically based on their compact stature with short internodes and small dark leaves. In order to determine if the dwarf phenotype is due to deficiency in bioactive GAs, they were treated with GA_3. GA application showed phenotypic effects in only 10 hybrids as determined by their internode length and leaf size. To establish if the dwarfism in these hybrids is associated with *PslGA2ox*, they were screened against a vigorously growing plum, EG/M, for the accumulation of *PslGA2ox* (Supplementary Fig. S3 at *JXB* online). The expression results defined that only one hybrid, designated DGO24, displayed significantly high *PslGA2ox* mRNA levels; therefore, this was selected for further analysis. Then, the full-length *PslGA2ox* cDNA was isolated from DGO24 to determine whether there are divergences within the amino acid sequence that can elucidate the hyper accumulation of transcripts. However, sequence analysis revealed a nucleotide sequence identical to that isolated from EG/M trees. Compared with EG/M control trees (tree height 3.73±0.22 m), DGO24 displayed very short trees along with a slow growth rate (tree height 1.37±0.15 m). Grafting EG onto DGO24 (EG/D) resulted in shoots that exhibited shorter internodes and reduced stem elongation (tree height 2.06±0.25 m) than EG/M (Supplementary Fig. S4 at JXB online). To determine whether *PslGA2ox* accumulation is the cause of the EG scion's phenotype, its expression was studied in EG/M, DGO24, and EG/D shoots. A strong signal was detected in both DGO24 and EG/D trees; however, the expression was weak in EG/M (Fig. 2). It was thus decided to investigate bioactive GA accumulation within the three different plum trees with the aim to determine whether there are dissimilarities in GA content that can account for the diversity in growth behaviour. Quantification of active GAs revealed that DGO24 and EG/D shoots exhibited at least 6- and 8-fold lower concentrations of the bioactive forms GA_1 and GA_4, respectively, than EG/M (Table 1).

Dwarfism is reversed by GA_3 application

In addition to the compact stature, short internodes, and small dark leaves, EG/D trees exhibited malformed flowers and small fruit at harvest (Fig. 3A, B) along with significant delay in fruit development compared with EG/M (10±2.74 d). EG/D fruit were ~36% smaller than EG/M fruit in size and weight (Fig. 3B, C). In order to confirm that these developmental deformities are due to insufficient GA necessary to coordinate plant growth, EG/D trees were sprayed with GA_3. GA application restored the growth of compact EG scions to near normal, as determined by internode length, accelerated the fruit development process to levels comparable with those of EG/M fruits, and resulted in proper flower organogenesis (Supplementary Fig. S5 at *JXB* online; Fig. 3A). Moreover, GA treatment caused significant increases in fruit size and weight, and these were up to ~2-fold greater at harvest than their counterparts from untreated EG/D trees (Fig. 3B, C).

PslGA2ox expression during fruit ontogeny

PslGA2ox transcripts were consistently expressed at moderate levels during different stages of fruit development in EG/D. In order to elucidate the physiological role of *PslGA2ox* and subsequently the contribution of GAs in fruit development, its expression profile was analysed during different stages of EG/M fruits (Fig. 4). *PslGA2ox* transcripts were initially low in flower buds, but greatly increased soon after flowering [~4 days after bloom (DAB)] followed by sharp inhibition of its mRNA levels after fertilization, ~7 DAB. The accumulation of *PslGA2ox* mRNA at bloom represented the highest transcript abundance during the whole experiment. During early fruit development, 7–15 DAB, *PslGA2ox* transcripts increased gradually in young fruits. Stone fruits (*Prunus* spp.) exhibit a typical double sigmoid growth pattern during fruit development, with four distinct stages, S1–S4 (El-Sharkawy *et al.*, 2007). Within the first stage, intense cell division is predominant, while during

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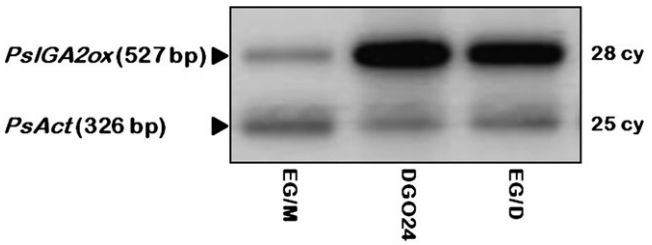

Fig. 2. *PslGA2ox* accumulation on EG/M, DGO24, and EG/D. All RT-PCR experiments were repeated at least three times with three different cDNA synthesized from three different RNA extractions for the same sample. The required number of cycles necessary for exponential, but non-saturated PCR amplification was determined using the cDNA from the highest expressing sample (DGO24).

Table 1. Concentrations of bioactive GAs in plum shoots

Plum tree	GA concentration (ng g^{-1} DW)	
	GA_1	GA_4
EG/M (control)	2.81±0.91	16.83±2.2
DGO24	0.5±0.18**	2.0±0.84**
EG/D	0.66±0.08**	4.53±0.95**

The GA concentration is given in ng g^{-1} dry weight. The values are the average of three replicates. Statistically significant differences from the applicable control are indicated by (**) for the probability level ($P < 0.01$).

S2 phase there is hardly any increase in fruit size but the endocarp hardens to form a solid stone. Throughout these developmental periods (22–52 DAB), *PslGA2ox* transcription showed a significant and gradual increase, reaching relatively maximal levels by the end of the S2 stage. The S3 stage (62–72 DAB) is accompanied by a considerable increase in fruit size, which is mostly attributable to cell expansion. In S3 phase, when the pulp readily separates from the seed, *PslGA2ox* levels increased in abundance within the pulp tissue, but consistently decreased in the seeds during maturity. Throughout the S4 stage, where most metabolic changes due to fruit ripening take place, *PslGA2ox* accumulation was generally low. As ripening progressed, the expression level of *PslGA2ox* increased in abundance within the pulp tissue, reaching relatively high levels at the post-climacteric phase (~83 DAB); however, in the seeds, its transcription increased to a peak at the pre-climacteric stage (~80 DAB) and slightly decreased thereafter.

Ectopic expression of PslGA2ox *in WT* Arabidopsis

GA2ox proteins play repressive roles in plant growth and development through catabolizing bioactive GAs, in particular GA_4, into inactive forms (Thomas *et al.*, 1999; Schomburg *et al.*, 2003). The present results suggest the involvement of *PslGA2ox* in the dwarf plum (DGO24); however, to confirm this hypothesis, the *PslGA2ox* gene was overexpressed in *Arabidopsis*, where GA_4 is the major active GA controlling different aspects of plant development (Xu *et al.*, 1999). Ectopic expression of *PslGA2ox* led to a wide range of disturbances in general growth and development behaviour. Six independent transgenic lines were confirmed after PCR analysis and then divided into two groups based on their phenotypic and molecular characteristics (Fig. 5). Group-1, including L.1, L.5, and L.7, exhibited a typical dwarf phenotype due to a significant decline in the length of all stem growth-related characters, resulting in ~60% reduction in overall plant height, which was associated with considerably high *PslGA2ox* levels. Group-2 that includes L.2, L.3, and L.4 showed a hypergrowth pattern. The plants were ~41% taller than the WT due to notably shorter, but numerous internodes, which was associated with low but significant *PslGA2ox* accumulation (Supplementary Table S2 at *JXB* online; Fig. 5A, B). Analysis of the transgene profile in mutant plants showed that the level of *PslGA2ox* correlated negatively with plant height. The tallest plants had the lowest transcription levels while the expression was weaker in longer plants. Thus a homozygous representative from each group (L.1/G1 and L.4/G2) was selected for further studies. To assess whether the overexpression of *PslGA2ox* can disturb the GA response pathway, the transcription levels of a number of genes that are induced (*At2g21220*, *AtGA2ox2*, *AtGA2ox3*, and *AtGA2ox8*) or repressed (*AtGA20ox1* and *AtGA3ox1*) by GA were determined (Phillips *et al.*, 1995; Thomas *et al.*, 1999; Schomburg *et al.*, 2003; Nemhauser *et al.*, 2006). Expression of the various GA-responsive genes was considerably different in the two lines (Fig. 5C). All GA-inducible transcripts were suppressed by 21–57% in L.1/G1, and the GA-repressed genes, *AtGA20ox1* and *AtGA3ox1*, increased by ~4.7- and ~5.5-fold, respectively. In contrast to the expected results, L.4/G2 accumulated more of the GA-up-regulated transcripts, while *AtGA2ox2* and *AtGA2ox3* were barely detected. Application of GA_3 that cannot be metabolized by GA2oxs (Sakamoto *et al.*, 2001) resulted in a differential response in the treated plants (Supplementary Table S2 at JXB online; Fig. S6). GA treatment rescued L.1/G1 height as a result of increasing internode number and length. However in L.4/G2, GA treatment caused a significant plant height reduction (~47%). To evaluate the effect of GA, the expression of the different GA-regulated genes was assessed (Fig. 5D). In L.1/G1, the accumulation profile of the different GA-responsive genes was similar to that in the WT, while in L.4/G2, only a modest increase in the various GA-up-regulated transcripts was observed; however, *AtGA2ox2* and *AtGA2ox3* remained undetectable. Generally, the GA biosynthesis inhibitor (PAC) and SD conditions reduced stem elongation and shoot growth of all treated plants (Supplementary Fig. S6 at *JXB* online). However, this effect was very pronounced in L.1/G1, and half of the plants were dead before completing their life cycle.

Developmental phenotypes of PslGA2ox *lines*

In order to validate the previous data, the *PslGA2ox* lines were phenotypically characterized for some well known

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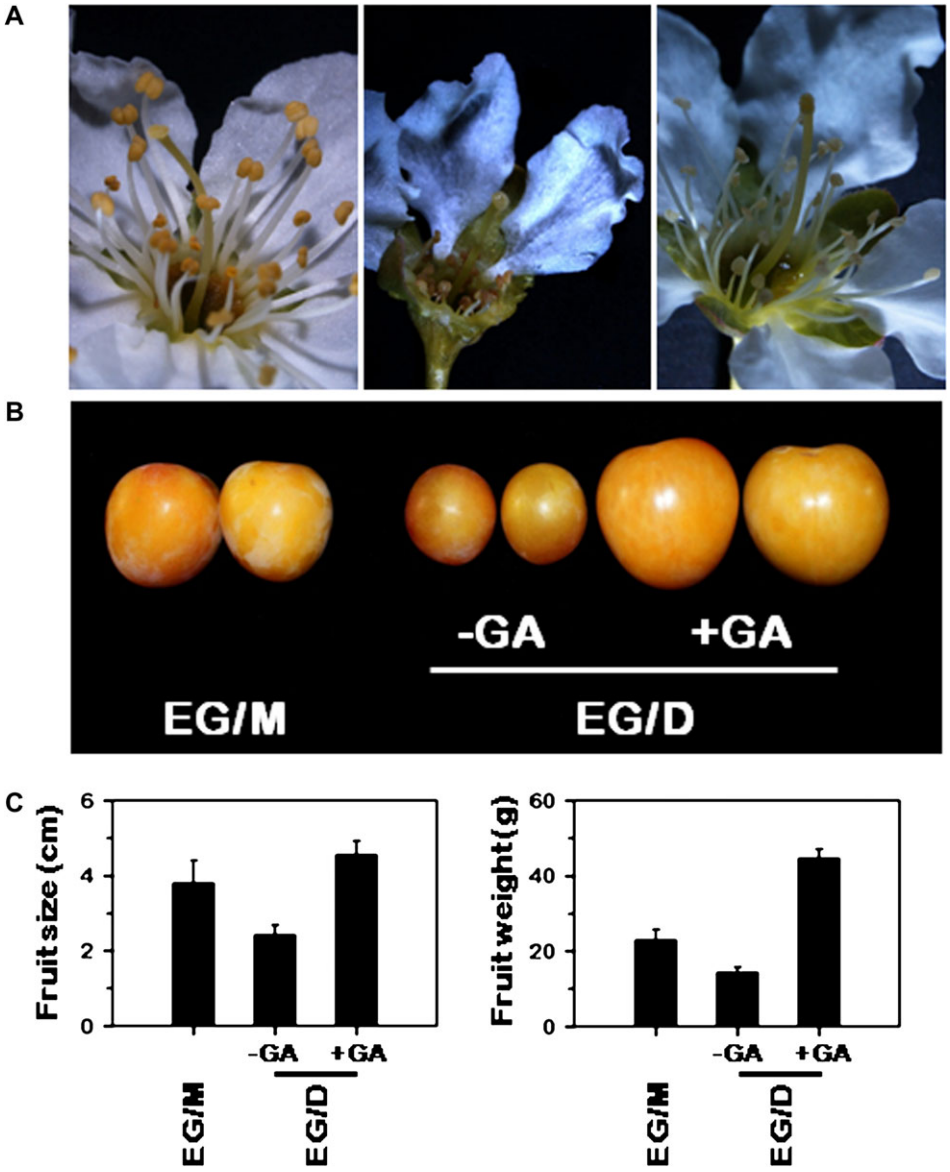

Fig. 3. (A and B) Close-up views of EG/M and EG/D flowers and fruits, respectively, before and after GA application. (C) The changes in EG/D fruit size and weight, before and after GA treatment, compared with their counterpart in EG/M fruit.

GA-related traits. Furthermore, the plants were grown under various GA-unlimited or -limited conditions.

Root elongation

Recent studies suggested that GA inhibited root growth by suppressing lateral root formation (Eriksson *et al.*, 2000; Gou *et al.*, 2010). Relative to the WT, all Group-1 plants exhibited compact shoot growth associated with accelerated root formation. Root lengths of Group-1 plants were enhanced by ~30–61%. In contrast, all Group-2 plants displayed extended shoot length with roots significantly shorter than the WT, ~20–33% (Supplementary Table S3 at *JXB* online; Fig. 6A). GA application caused a rapid stem elongation with a concomitant suppression in root development in all treated plants; however, these responses were much less in the case of Group-1 (Supplementary Table S3 at JXB online; Fig. 6B).

Flowering characteristics

GAs are involved in the developmental events leading to reproductive competence, as well as in floral determination and commitment (Cheng *et al.*, 2004; Rieu *et al.*, 2008*b*). The flowering time was considerably delayed in Group-1 transgenics (+5.63±0.7 d) and accelerated in Group-2 (–6.93±0.7 d) relative to the WT (Supplementary Table S4 at *JXB* online). GA application noticeably restored flowering time in Group-1; however, it did not significantly influence Group-2. PAC and SD conditions substantially retarded the transition to flowering in Group-1 by ~20 d and ~65 d, respectively. However, Group-2 treated with PAC or exposed to SD started flowering ~10 d earlier than the corresponding WT.

WT *Arabidopsis* flowers exhibit a typical coordinated flower structure that ensures proper self-pollination. Group-1 plants displayed generally smaller flowers and their filaments

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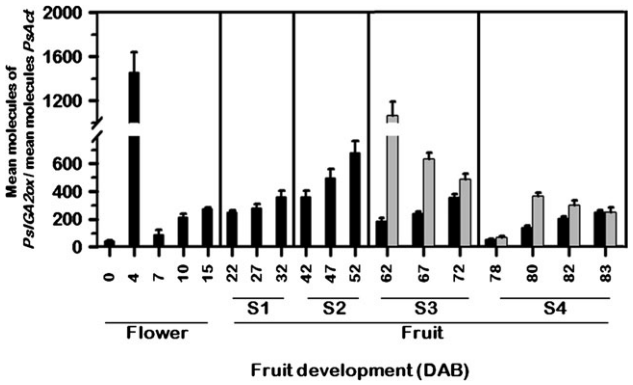

Fig. 4. Steady-state transcript levels of *PslGA2ox* mRNA assessed by qRT-PCR in EG/M flowers and throughout fruit ontogeny. During S1 and S2 of fruit development, the expression was determined in the whole fruit. However, during S3 and S4, the expression was determined in pulp (black filled bars) and in seeds (grey filled bars). The experiments were carried out in three biological replicates, and error bars represent the SD. The *y*-axis refers to the mean molecules of *PslGA2ox* per reaction/mean molecules of *PsAct*. The *x*-axis represents the developmental stages indicated by the number of days after bloom (DAB).

were usually shorter than their pistils. A similar flower structure was observed in Group-2, but this was largely due to acceleration of pistil growth (Supplementary Table S4 at *JXB* online; Fig. 7). Such variation between the stamens and pistil can cause a major reduction in fertility, especially in self-pollinated species. GA application visibly changed the flower structure. In Group-1, the flower size, pistil and stamen lengths were mostly restored; however, the stamen remained shorter than the pistil. In contrast, GA reduced overall Group-2 flower size without a visible change in structure. In general, PAC and SD treatments reduced the size of the various floral organs. In the case of Group-1, SD conditions significantly affected pistil elongation, resulting in filaments much longer than the pistils. Although Group-2 pistil length was also reduced due to SD, the flowers were generally larger than SD-grown WT.

Role of PslGA2ox *in floral organ patterning*

Arabidopsis flowers consist of a precise pattern of organs arranged in four concentric whorls. In addition to the regular flowers, all Group-1 members also exhibited fused flowers (Fig. 8A). These abnormal flowers hold eight short stamens with often fasciated filaments (Fig. 8B). In some instances, the stamens were completely malformed beyond recognition (Fig. 8C). Additionally, they also had two separate, functional pistils, as they progressed towards maturity (Fig. 8B, D). Such fused flowers were alternatively arranged in a pre-set pattern along with regular flowers within the whole plant (Fig. 8E). In spite of these defects, both the pollen and pistil seemed to be at least partially viable as pollen germination and occasional seed set could be observed. These results indicate that Group-1 plants exhibited major disorder in the floral organ identity procedure, while GA treatment along with LD conditions was largely enough to recover all defects in plant development including floral organogenesis (Supplementary Fig. S7A at *JXB* online). To investigate the cause of floral organogenesis deformities, the expression profile of a set of genes involved in floral patterning, such as *LFY* and the floral homeotic genes from classes B (*AP3* and *PI*) and C (*AG*), were studied (Parcy *et al.*, 1998; Lohmann and Weigel, 2002). Relative to the WT, accumulation of all studied transcripts dramatically decreased in L.1/G1 (Supplementary Fig. S7B at JXB online). Thus, it was decided to examine further the potential involvement of GA in the promotion of floral-related gene expression. Interestingly, GA treatment restored the regular flower patterning as well as the levels of all studied genes (Supplementary Fig. S7A, B at JXB online). Growing Group-1 mutants under SD increased the disturbance in the floral organs (Fig. 9). The whole plant exhibited flowers with reduced number of stamens that were not consistent either in thickness or in length. Some flowers displayed fasciations of stamens at filaments and/or anthers (Fig. 9A). In other flowers, the stamens fused to other floral organs such as petals or carpel, and the anthers were malformed (Fig. 9B, C). In yet other cases, the filament was totally absent, resulting in anthers fused directly to the base (Fig. 9D). The stamens also show other deformities such as rudimentary anthers on unusual thick filamentous structure (Fig. 9E), such that the filament and the anther could not properly differentiate. Similarly, an abnormal short and thick stigma was also observed. In such cases, the ovary is often split, exposing the ovules (Fig. 9E). Consequently, such disruptions in the flower organogenesis caused complete sterility. The results showed that all these major disorders were associated with low or almost undetectable floral patterning transcripts. Interestingly, treatment of Group-1 plants under SD conditions with GA recovered the different aspects of the plant growth pattern to near normal (Supplementary Fig. S7C, D at *JXB* online).

Fruit growth and development

The time from pollination to silique occurrence was not significantly altered in Group-2 mutants compared with the WT in different growth conditions or treatments. In contrast, silique formation was delayed by ~4.1 d in Group-1, which could be partially restored by GA. However, both PAC and SD caused a dramatic delay in silique emergence of Group-1 plants (Supplementary Table S4 at *JXB* online). Moreover, WT and Group-2 siliques matured at about the same time, while Group-1 siliques shattered at least ~7.2 d later. GA treatment delayed silique maturity; however, both mutant groups displayed a substantially longer time than GA-treated WT to reach maturity. PAC and SD treatments remarkably delayed Group-1 silique maturation (~13 d and ~27 d, respectively). In contrast, Group-2 siliques significantly shattered earlier. Furthermore, both silique length and seed number were drastically reduced in both mutant groups (Supplementary Table S5 at *JXB* online; Fig. S8). Siliques of Group-1 and -2 were

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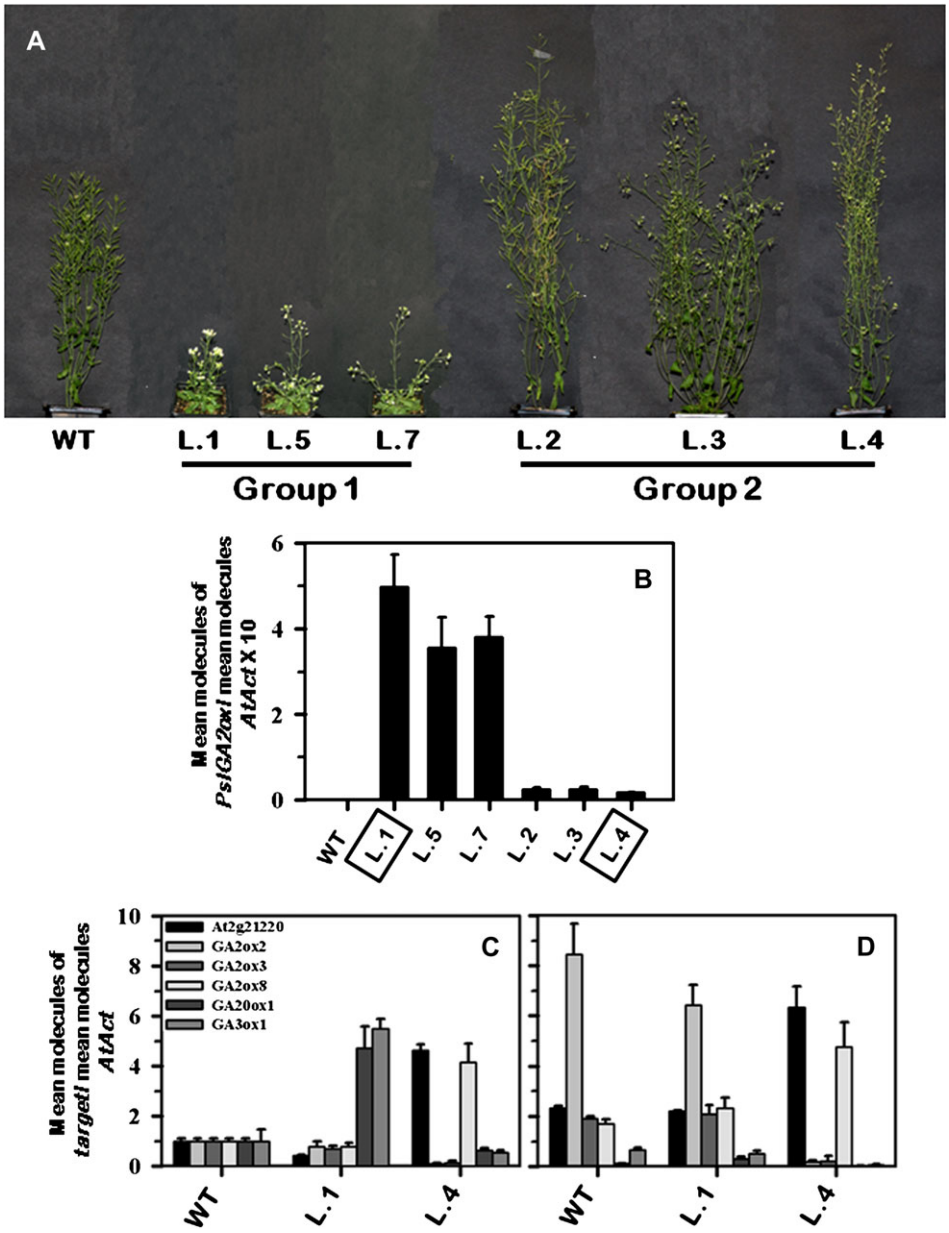

Fig. 5. (A) Aerial portions of WT and the two phenotype groups resulting from ectopic expression of *PslGA2ox* in *Arabidopsis* under LD conditions. (B) *PslGA2ox* accumulation in the WT and the different transgenic mutants. (C and D) The expression of the different GA-responsive genes in the WT and a representative from each group of mutants (L.1 and L.4) in the absence (C) and presence (D) of GA. Transcripts accumulation was determined using qRT-PCR on three biological replicates. Standard curves were used to calculate the numbers of target gene molecules per sample, which were then normalized relative to *AtAct* expression.

reduced in length by ~68% and 30%, respectively. Even more strikingly, both mutants exhibited a reduction in seed content by ~96% and 61%, respectively. Most Group-1 seeds were not completely developed as determined by their flat shape and low germination. Both mutants strongly responded to GA application; however, their responses were contradictory. In Group-2, GA caused inhibition in silique length and seed number. In contrast, Group-1 siliques elongated greatly along with increased seed content; however, both traits remained less than in the WT. PAC and SD significantly suppressed both traits, although Group-2 exhibited more tolerance for these GA-limiting conditions.

In addition, all Group-1 siliques were short, thin, usually seedless, and ~42% of them exhibited a twisted shape (Supplementary Fig. S9 at JXB online); and this phenotype continued throughout the entire plant ontogeny. GA treatment resulted in full recovery of silique shape. Apparently, Group-1 bent siliques seem to be a result of unequal distribution of GA levels between the two silique sides resulting in differential elongation rate.

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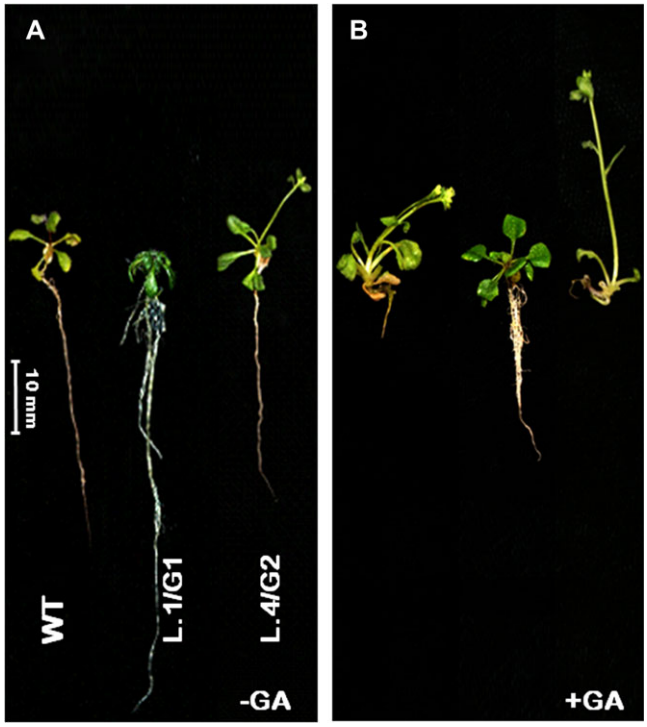

Fig. 6. Representative 15-day-old seedlings primary roots of WT, L.1, and L.4 genotypes. Plants were grown in MS medium (Murashige and Skoog, 1962) without or with (100 μM) GA_3. Bar=10 mm.

Group-2 mutants show sensitivity to exogenous GA

Endogenous GA levels are the result of an antagonistic reaction between GA biosynthesis and inactivation mechanisms (Hedden and Phillips, 2000). Therefore, any disturbance in this machinery can modify the accumulation of active GAs (Rieu *et al.*, 2008*a*, *b*). Previous data illustrated that Group-2 exhibited much higher sensitivity to GA than the WT. Application of GA caused a major reduction in silique length of both the WT and L.4/G2. In order to confirm this inhibitory consequence of GA, WT and L.4/G2 plants were treated with gradually increasing concentrations of GA (Supplementary Fig. S10A at JXB online). Significant reduction in L.4/G2 silique elongation occurred with a GA concentration of 5 μM; however, the WT responded only to 50 μM GA concentrations (Supplementary Fig. S10B).

Discussion

Ten dwarf plum hybrids due to deficiency of unknown GA signalling were identified. Then, a cDNA sequence encoding GA 2-oxidase (*PslGA2ox*), the major GA catabolic enzyme in plants, was used to screen the 10 hybrids. This resulted in the identification of a plum hybrid (DGO24) that showed extremely high *PslGA2ox* accumulation, concomitant with low accumulation of bioactive GA_1 and GA_4. The irregular growth pattern of DGO24, including the compact stature and deformed flowers, is transmissible to the scion in grafted trees, to a certain extent. Thus, it is possible to interpret this to mean that DGO24 rootstock may be involved in the inactivation of bioactive GAs within the scion part, which is further supported by the low amount of active GAs in EG/D compared with control trees.

PslGA2ox is a member of class I GA2oxs, which catalyse the conversion of active C_{19}-GAs into inactive forms by 2β-hydroxylation. As a consequence of lack of any apparent targeting sequence in GA2ox proteins, they were assumed to be cytosolic enzymes (Sun, 2008). The present results showed that PslGA2ox protein is localized in both the cytoplasm and the nucleus. Interestingly, the GA receptors (GID1s) also exhibit a similar localization behaviour (Ueguchi-Tanaka *et al.*, 2005), suggesting that GA2oxs act as GID1s by binding the bioactive GAs with high affinity, but to convert them into inactive forms.

In tree fruit crops, dwarfism induced due to GA deficiency is an advantage; however, synchronized levels of endogenous GA are still very important to ensure correct fruit development and production (Serrano *et al.*, 2007). EG/D trees exhibited a significant delay in fruit development. The re-establishment of fruit development is dependent on the availability of sufficient GA in the appropriate developmental stages, when the requirements for GAs are essential. Dunberg and Odén (1983) showed that the active GA_4 is the most effective GA form leading to flowering promotion and reproductive growth. Thus, the scarcity of overall active GA content, particularly GA_4, within EG/D trees can explain the distortion in flower structure and the delay in flowering events as well as the shift of the overall fruit ripening date. Consequently, determining the role of GA during fruit ontogeny has convenient implications in understanding and controlling the fruit development process.

Studies on the effect of GA on plant growth and development have been hindered by their low abundance and variation in forms, time, and localization. However, examining the expression of genes encoding enzymes involved in GA biosynthesis and catabolism provides an alternative approach for such studies. Interestingly, the evolution of *PslGA2ox* accumulation was generally aligned with the quantification of bioactive GAs during plum fruit development (Yamaguchi and Takahashi, 1976; Bukvoac and Yuda, 1979; this study). Analysis of the *PslGA2ox* expression profile indicated that GAs play important roles in fruit development, mainly throughout immature stages before ripening. In flowers, the abundance of *PslGA2ox* transcripts suggested a dominant task of GA in promoting flowering and elucidated the role of PslGA2ox enzyme in regulating GA accumulation during this stage (Dunberg and Odén, 1983; Pharis and King, 1985).

Throughout fruit development, it is almost certain that the series of modifications that make the fruit proceed through the consequent developmental stages involve many different metabolic pathways. So far, only the hormone auxin has been demonstrated to be involved in the developmental program of *Prunus* fruit (Miller *et al.*, 1987; El-Sharkawy *et al.*, 2008, 2009, 2010). However, previous studies suggested cross-talk between GA and other hormones in the regulation of different plant development

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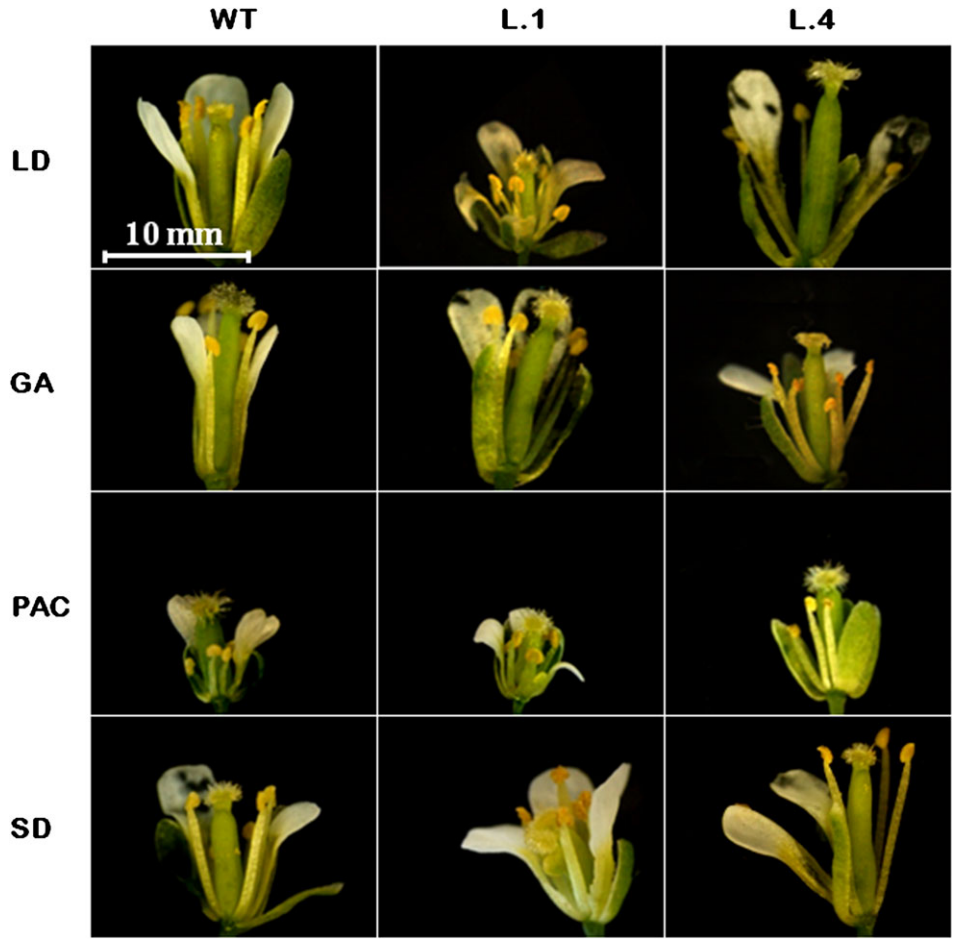

Fig. 7. Close-up views of WT, L.1, and L.4 flowers from plants exposed to several growth conditions: LDs, GA, PAC, and SDs. Sepals and petals were removed to reveal the anthers and pistil. Bar=10 mm.

events, in particular with auxin in processes such as fruit set and development (Fleet and Sun, 2005; de Jong *et al.*, 2009; Csukasi *et al.*, 2011). Additionally, in *Prunus* spp., the evolution of the plant hormones auxin and GA was found to exhibit a similar pattern during fruit development (Yamaguchi and Takahashi, 1976, Miller *et al.*, 1987). Furthermore, earlier studies in *Prunus* showed the stimulatory effect of exogenous auxin and GA on enhancing fruit development (Jackson, 1968; El-Sharkawy *et al.*, 2010; this study). Taken together, apparently during plum fruit growth, in particular during S1, S3, and S4 phases, the actions of auxin and GA are not independent of each other but are coordinated to regulate the progression of fruit development, as has been proposed previously in other plant systems (Serrani *et al.*, 2007; Csukasi *et al.*, 2011). Recent studies reported that the growth of seeded tomato fruits is coordinated by a delicate balance between auxin and GA (de Jong *et al.*, 2009), where auxin is needed to mediate the rate of cell division, and GA is required to organize cell expansion. The mutual effect of the two phytohormones was further validated in the development of parthenocarpic fruit. Either auxin or GA treatment can promote parthenocarpic tomato fruit growth, whereas neither of them alone was able to maintain the growth rate to the end of ripening. Only the joint application of both hormones resulted in parthenocarpic fruits similar to those obtained by pollination (Serrani *et al.*, 2007). This is accurate for the different developmental stages, excluding the S2 phase. As mentioned previously, during the S2 stage there is hardly any increase in fruit size (no evidence of cell division and the expansion process), which coincided with a significant reduction in auxin content (Miller *et al.*, 1987). Therefore, the accumulation of GAs during the S2 stage seems to be due to the lignification of the endocarp to form a solid stone, which is the only developmental process occurring during this stage. Biemelt *et al.* (2004) demonstrated that GA mediates lignin formation and deposition by polymerization of pre-formed monomers.

Further, in terms of gene expression, it was noted that during fruit maturation and ripening (S3 and S4) when the seed separated from the pulp, the signal of *PslGA2ox* detected in the developing seeds was almost 6-fold higher than its counterpart in the pulp. Also, *PslGA2ox* displayed a contrasting accumulation profile between fruit pulp and seed. The up-regulation of the transcript in the pulp usually coincided with its down-regulation in the seed. Accordingly, it seems that seed is mainly responsible for GA biosynthesis within the plum fruit; however, both seed and pulp could be

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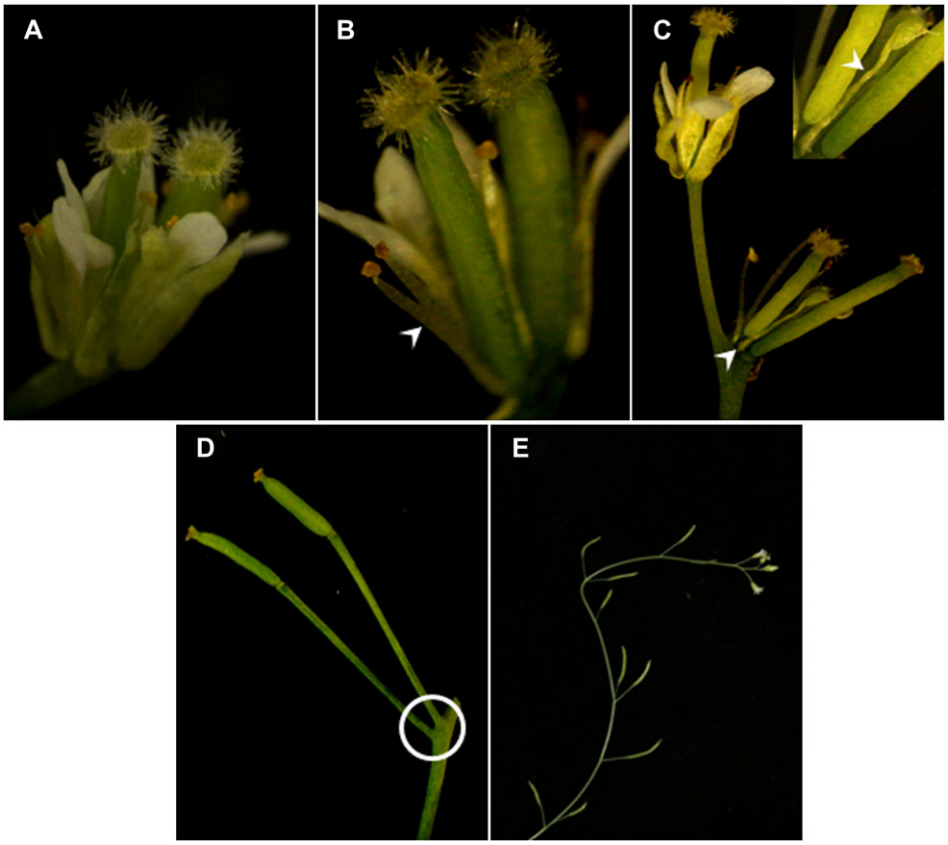

Fig. 8. Close-up views of defective L.1 flowers under LD (A). The arrowheads indicate the fused filaments (B) and stamen-like organ (C). The circle in (D) indicates the connection region between the twin siliques. (E) The frequency of the twin silique pattern in an inflorescence.

the site of action of this hormone depending on GA requirement. Previous studies suggest that as seed germination proceeds, the GA-dependent transcriptional events are not restricted to the sites of GA biosynthesis (Ogawa *et al.*, 2003). However, there are other cases where bioactive GA is produced at their site of action (Kaneko *et al.*, 2003; Csukasi *et al.*, 2011). Further, the role of seeds in stone fruit development was determined by Jackson (1968) and Miller *et al.* (1987) who provided evidence that seeds stimulate fruit growth and ripening by providing auxins and GAs.

Ectopic expression of *PslGA2ox* in *Arabidopsis* generated two groups of plants that exhibited two contradictory phenotypes. Group-1 displayed a typical GA-deficient phenotype and consequently exhibited substantial disorder in all GA-regulated transcripts. Interestingly, both Group-1 mutants and EG/D trees displayed many common GA-deficient growth traits, including compact vegetative growth and general disturbance in reproductive development events, which is indicative of the role of *PslGA2ox* in producing this phenotype. The previous growth properties along with the accelerated root formation are a common behaviour in GA-deficient mutants (Koornneef and van der Veen, 1980; Griffiths *et al.*, 2006; Rieu *et al.*, 2008*b*; Gou *et al.*, 2010). Although the disturbances in the flowering characteristics were the most pronounced outcome in Group-1 mutants, it still can produce flowers. Previous reports indicated that the development of floral organs is usually interrupted in GA-deficient plants (Goto and Pharis, 1999; Cheng *et al.*, 2004) or in plants incapable of responding to GA (Griffiths *et al.*, 2006), which triggers flower infertility. However, most of these mutants remained leaky to some degree, and can produce small amounts of active GAs, sufficient to induce flowering even under severe GA growth conditions such as SDs (Wilson *et al.*, 1992). In *Arabidopsis*, development of floral organs is under the control of homeotic genes that must be accessible to maintain the typical organogenesis process (Weigel and Meyerowitz, 1994). In Group-1 flowers under LDs or SDs, transcripts of the different floral organ identity genes were significantly lower, which can explain the abnormal flower formation. However, the plant phenotype along with the expression profile of homeotic genes before and after GA treatment suggests its essentiality to maintain their accumulation and subsequently have a correct flower patterning (Lohmann and Weigel, 2002; Eriksson *et al.*, 2006; this study). *Arabidopsis* is a facultative LD plant and its flowering is controlled by the interplay between three different pathways: LDs, an autonomous pathway, and the GA pathway. However, only the GA pathway plays a central role in the control of flower initiation under SDs (Mouradov *et al.*, 2002; Boss *et al.*, 2004; Putterill *et al.*, 2004). Apparently growing Group-1

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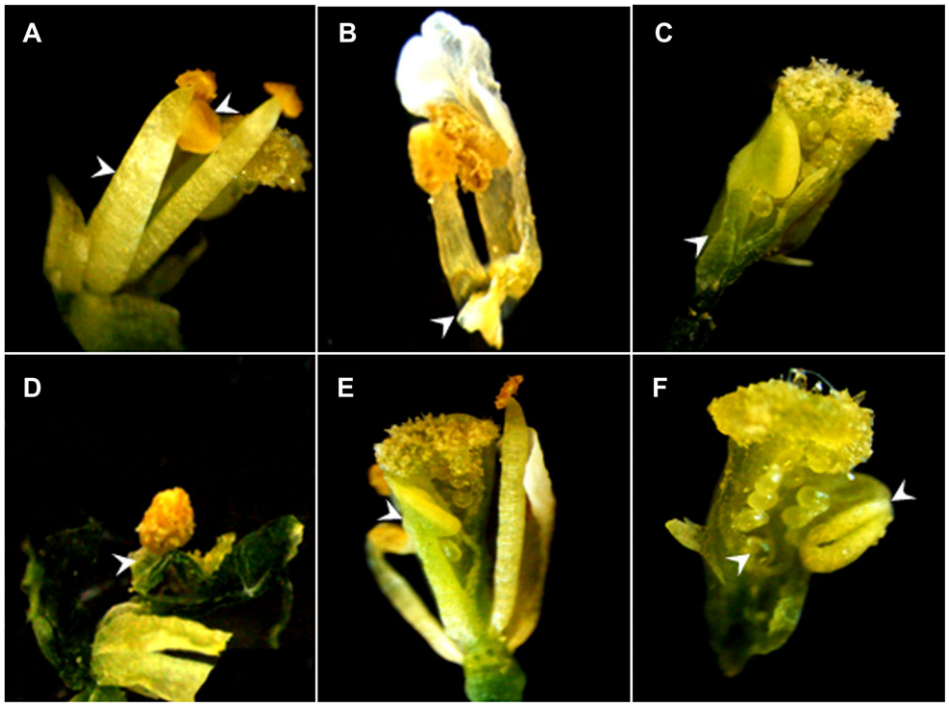

Fig. 9. Developmental defects of L.1 floral organ structure due to SDs. The arrowheads indicate the fused filaments and anthers (A), stamens fused to petals (B), the stamen-like organ fused to the carpel (C), the anther fused directly to the base (D), abnormal stamen structure (E), and stamen formed inside the ovules accompanied by opened ovule phenotype.

members under LDs alone can relatively minimize, but not prevent, the disturbance in the floral organogenesis procedure (Weigel *et al.*, 1992). However, the disorders under SDs were more severe, and this is largely due to the critical *LFY* levels that are the target of photoperiodic regulation (Blázquez *et al.*, 1997) and GA (Eriksson *et al.*, 2006). Consequently, GA treatment resulted in proper re-establishment of most Group-1 growth deformities under both LDs and SDs, including vegetative growth and floral organogenesis through restoring the accumulation profile of the various GA-regulated transcripts especially those of floral organ identity mRNAs.

In contrast, in Group-2 plants, *PslGA2ox* insertion somehow caused co-suppression of the closely related *Arabidopsis* homologous *AtGA2ox2* and *AtGA2ox3* that probably lead to an overall increase in active GAs (Rieu *et al.*, 2008*a*). Consequently, the different GA-related transcripts accumulated in a manner resembling unlimited GA conditions. Further, at the phenotypical level, Group-2 mutants demonstrated a characteristic 'GA overdose' growth pattern (Sun, 2000). Fleet and Sun (2005) reported that plants exhibiting a GA overdose phenotype showed excessive growth and increased sterility, suggesting the importance of optimal GA levels to ensure proper growth and development. The overall growth pattern of Group-2 plants resembled that of *ga2ox* quintuple mutant (Rieu *et al.*, 2008*a*) or those of GA-treated WT plants. The present results showed that any additional GA caused contradictory responses in Group-2, probably due to reaching lethal levels of the hormone. Synchronized elongation of the pistil and filament is essential to ensure efficient pollination within the flower. High levels of GA can induce male sterility and cause excessive elongation of the pistil (Sawhney and Shukla, 1994; Colombo and Favret, 1996). Plants carrying mutations in the biosynthetic (GA20oxs) or catabolic genes (C_{19}-GA2oxs) exhibited extended pistils, resulting in partial infertility (Rieu *et al.*, 2008*a*, *b*). A loss of fertility has also been described for WT plants treated with GA (Jacobsen and Olszewski, 1993) and for double mutants in the GA signalling repressors, RGA and GAI (Dill and Sun, 2001). Here, it is shown that Group-2 plants behaved similarly, which appears to be due to increased length of the pistil relative to the stamen that consequently reduces the self-pollination efficiency. Apparently, many GA-dependent mechanisms might be quite saturated within this group. Any additional GA results in serious disruptions of growth and development. The inhibitory effect of GA was clearly demonstrated by treating L.4/G2 with gradually increasing GA concentrations, which caused a significant reduction in silique length using 10-fold lower GA concentrations compared with the WT. The growth pattern of Group-2 plants under GA-deprived conditions further confirms the GA overdose phenotype. Rieu *et al.* (2008*a*) observed that PAC-treated *ga2ox* quintuple mutant exhibited a general growth spurt in comparison with treated WT. Further, PAC-treated and SD-grown L.4 plants exhibited earlier flowering onset, earlier silique maturation, and larger siliques than the WT exposed to the same conditions.

Rootstocks play a key role in improving and eventually stabilizing productivity in perennial crops, as they can adapt to diverse environmental conditions, which makes rootstock breeding as important as creating new varieties.

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Manipulation of plant stature, through classical plant breeding or use of plant growth regulators, has long been a major goal in tree fruit horticulture. Though the use of 'anti-GA' growth regulators showed success in controlling plant stature, efficient size reduction in perennial species, such as plums, requires repeated application of synthetic chemicals, which can be costly both commercially and environmentally. Therefore, selection and use of dwarf rootstocks that exhibits GA deficiency provides an environmentally attractive approach.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Amino acid sequence alignment of PslGA2ox with closely related GA2ox sequences using the ClustalX program.

Figure S2. The full-length *PsGA2ox* gene was fused to the GFP tag. *Nicotiana tabacum* protoplasts were transfected with the following constructs: (A) the control 35S::GFP and (B) *PslGA2ox*::GFP.

Figure S4. View of EG/M, DGO24, and EG/D trees under field conditions.

Figure S5. Branches from EG/M (A) and EG/D before (B) and after GA application (C).

Figure S6. Aerial portions of WT, L.1, and L.4 plants exposed to several growth conditions, sprayed with 100 μM GA_3, treated with 10 μM PAC, and short days (SD).

Figure S7. Close-up view of L.1 plants and defective flowers which resulted under LD (A) or SD (C) conditions with and without GA treatment. (B and D) Transcript accumulation of the *Arabidopsis* floral meristem identity gene (*LFY*) and floral homeotic genes (*AP3*, *PI*, and *AG*) assessed by qRT-PCR in inflorescence apices of WT and L.1 plants grown under LDs (B) or SDs (D) with and without GA treatment.

Figure S8. Close-up views of WT, L.1, and L.4 siliques from plants exposed to several growth conditions, LDs, GA, PAC, and SDs.

Figure S9. Close-up views of twisted L.1 siliques and the suppression of such a phenotype by GA application during different stages of silique development.

Figure S10. Representative image (A) and growth pattern (B) of the inhibitory effect of GA on WT and L.4 silique elongation.

Table S1. Oligonucleotide primers.

Table S2. Stem growth phenotype characterization of WT and transgenic *Arabidopsis* plants expressing *PslGA2ox* as shown in Fig. 5.

Table S3. The effect of GA treatment on root elongation of WT and transgenic *Arabidopsis* plants expressing *PslGA2ox* as shown in Fig. 6.

Table S4. Flower growth phenotype characterization of WT and transgenic *Arabidopsis* plants expressing *PslGA2ox*.

Table S5. Silique growth phenotype characteristics of WT and transgenic *Arabidopsis* plants expressing *PslGA2ox* as shown in Supplementary Fig. S7.

References

Appleford NEJ, Wilkinson MD, Ma Q, *et al*. 2007. Decreased shoot stature and grain α-amylase activity following ectopic expression of a gibberellin 2-oxidase gene in transgenic wheat. *Journal of Experimental Botany* **58,** 3213–3226.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25,** 3389–3402.

Biemelt S, Tschiersch H, Sonnewald U. 2004. Impact of altered gibberellin metabolism on biomass accumulation, lignin biosynthesis, and photosynthesis in transgenic tobacco plants. *Plant Physiology* **135,** 254–265.

Blázquez MA, Soowal LN, Lee I, Weigel D. 1997. *LEAFY* expression and flower initiation in *Arabidopsis*. *Development* **124,** 3835–3844.

Boss PK, Bastow RM, Mylne JS, Dean C. 2004. Multiple pathways in the decision to flower: enabling, promoting, and resetting. *The Plant Cell* **16,** S18–S31.

Bukvoac MJ, Yuda E. 1979. Endogenous plant growth substances in developing fruit of *Prunus cerasus* L. *Plant Physiology* **63,** 129–132.

Busov VB, Meilan R, Pearce DW, Ma CP, Rood SB, Strauss SH. 2003. Activation tagging of a dominant gibberellin catabolism gene (GA 2-oxidase) from poplar that regulates tree stature. *Plant Physiology* **132,** 1283–1291.

Cheng H, Qin L, Lee S, Fu X, Richards DE, Cao D, Luo D, Harberd NP, Peng J. 2004. Gibberellin regulates *Arabidopsis* floral development via suppression of DELLA protein function. *Development* **131,** 1055–1064.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16,** 735–743.

Colombo N, Favret EA. 1996. The effect of gibberellic acid on male fertility in bread wheat. *Euphytica* **91,** 297–303.

Cristoferi G, Filiti N. 1981. Comparison of hormonal levels in normal and dwarf peaches. *Acta Horticulturae* **120,** 244.

Csukasi F, Osorio S, Gutierrez JR, *et al*. 2011. Gibberellin biosynthesis and signalling during development of the strawberry receptacle. *New Phytologist* **191,** 376–390.

de Jong M, Mariani C, Vriezen WH. 2009. The role of auxin and gibberellin in tomato fruit set. *Journal of Experimental Botany* **60,** 1523–1532.

Dijkstra C, Adams E, Bhattacharya A, *et al*. 2008. Over-expression of a gibberellin 2-oxidase gene from *Phaseolus coccineus* L. enhances gibberellin inactivation and induces dwarfism in *Solanum* species. *Plant Cell Reports* **27,** 463–470.

Dill A, Sun TP. 2001. Synergistic derepression of gibberellin signaling by removing RGA and GAI function in Arabidopsis thaliana. *Genetics* **159,** 777–785.

Dunberg A, Odén PC. 1983. Gibberellins and conifers. In: Crozier A, ed., *The biochemistry and physiology of gibberellins*. New York: Praeger, 221–295.

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Ebel RC, Cayor AW, Pitts JA, Wilkins BS. 2000. Mineral nutrition during establishment of Golden Delicious 'Smoothee' apples on dwarfing rootstocks and interstems. *Journal of Plant Nutrition* **23,** 1179–1192.

El-Sharkawy I, Kim WS, El-Kereamy A, Jayasankar S, Svircev AM, Brown DCW. 2007. Isolation and characterization of four ethylene signal transduction elements in plums (*Prunus salicina* L.). *Journal of Experimental Botany* **58,** 3631–3643.

El-Sharkawy I, Kim WS, Jayasankar S, Svircev AM, Brown DCW. 2008. Differential regulation of four members of ACC synthase gene family in plum. *Journal of Experimental Botany* **59,** 2009–2027.

El-Sharkawy I, Mila I, Bouzayen M, Jayasankar S. 2010. Regulation of two germin-like protein genes during plum fruit development. *Journal of Experimental Botany* **61,** 1761–1770.

El-Sharkawy I, Sherif S, Mila I, Bouzayen M, Jayasankar S. 2009. Molecular characterization of seven genes encoding ethylene-responsive transcriptional factors during plum fruit development and ripening. *Journal of Experimental Botany* **60,** 907–922.

Erez A. 1984. Dwarfing peaches by pruning and by paclobutrazol. *Acta Horticulturae* **146,** 235–242.

Eriksson ME, Israelsson M, Olsson O, Moritz T. 2000. Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. *Nature Biotechnology* **18,** 784–788.

Fleet CM, Sun TP. 2005. A DELLAcate balance: the role of gibberellin in plant morphogenesis. *Current Opinion in Plant Biology* **8,** 77–85.

Goto N, Pharis RP. 1999. Role of gibberellins in the development of floral organs of the gibberellin-deficient mutant, *ga1-1, of Arabidopsis thaliana*. *Canadian Journal of Botany* **77,** 944–954.

Gou J, Strauss SH, Tsai CJ, Kai F, Chen Y, Jiang X, Busov VB. 2010. Gibberellins regulate lateral root formation in Populus through interactions with auxin and other hormones. *The Plant Cell* **22,** 623–639.

Griffiths J, Murase K, Rieu I, *et al*. 2006. Genetic characterization and functional analysis of the GID1 gibberellin receptors in *Arabidopsis*. *The Plant Cell* **18,** 3399–3414.

Hedden P, Phillips A. 2000. Gibberellin metabolism: new insights revealed by the genes. *Trends in Plant Science* **5,** 523–530.

Hellens RP, Edwards AE, Leyland NR, Bean S, Mullineaux P. 2000. pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. *Plant Molecular Biology* **42,** 819–832.

Holsters M, de Waele D, Depicker A, Messens E, van Montagu M, Schell J. 1978. Transfection and transformation of *Agrobacterium tumefaciens*. *Molecular and General Genetics* **163,** 181–187.

Jackson DI. 1968. Gibberellin in the growth of peach and apricot fruits. *Australian Journal of Biological Science* **21,** 209–215.

Jacobsen S, Olszewski N. 1993. Mutations at the *SPINDLY* locus of *Arabidopsis* alter gibberellin signal transduction. *The Plant Cell* **5,** 887–896.

Janick J, Cummins JV, Brown SK, Hemmat M. 1996. Apples. In: Janick J, Moore JN, eds. *Fruit breeding*. New York: John Wiley & Sons, 1–78.

Jones OP. 1984. Mode-of-action of rootstock/scion interactions in apple and cherry trees. *Acta Horticulturae* **146,** 175–182.

Kaneko M, Itoh H, Inukai Y, Sakamoto T, Ueguchi-Tanaka M, Ashikari M, Matsuoka M. 2003. Where do gibberellin biosynthesis and gibberellin signaling occur in rice plants? *The Plant Journal* **35,** 104–115.

Koornneef M, van der Veen JH. 1980. Induction and analysis of gibberellin-sensitive mutants in *Arabidopsis thaliana* (L.) Heynh. *Theoretical and Applied Genetics* **58,** 257–263.

Lohmann JU, Weigel D. 2002. Building beauty: the genetic control of floral patterning. *Developmental Cell* **2,** 135–142.

MacMillan J. 2002. Occurrence of gibberellins in vascular plants, fungi and bacteria. *Journal of Plant Growth Regulation* **20,** 387–442.

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

Meisel L, Fonseca B, González S, Baezayates R, Cambiazo V, Campos R, Gonzalez M, Orellana A, Retamales J, Silva H. 2005. A rapid and efficient method for purifying high quality total RNA from peaches (*Prunus persica*) for functional genomics analyses. *Biological Research* **38,** 83–88.

Miller AN, Walsh CS, Cohen JD. 1987. Measurement of indole-3-acetic acid in peach fruits (*Prunus persica* L. Batsch cv Redhaven) during development. *Plant Physiology* **84,** 491–494.

Mouradov A, Cremer F, Coupland G. 2002. Control of flowering time: interacting pathways as a basis for diversity. *The Plant Cell* **14,** S111–S130.

Murashige R, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15,** 473–497.

Nemhauser JL, Hong FX, Chory J. 2006. Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses. *Cell* **126,** 467–475.

Ogawa M, Hanada A, Yamauchi Y, Kuwahara A, Kamiya Y, Yamaguchi S. 2003. Gibberellin biosynthesis and response during *Arabidopsis* seed germination. *The Plant Cell* **15,** 1591–1604.

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

Parcy F, Nilsson O, Busch MA, Lee I, Weigel D. 1998. A genetic framework for floral patterning. *Nature* **395,** 561–566.

Pharis RP, King RW. 1985. Gibberellins and reproductive development in seed plants. *Annual Review of Plant Physiology* **36,** 517–568.

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

Putterill J, Laurie R, Macknight R. 2004. It's time to flower: the genetic control of flowering time. *Bioessays* **26,** 363–373.

Rieu I, Eriksson S, Powers SJ, *et al*. 2008*a*. Genetic analysis reveals that C_{19}-GA 2-oxidation is a major gibberellin inactivation pathway in *Arabidopsis*. *The Plant Cell* **20,** 2420–2436.

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Rieu I, Ruiz-Rivero O, Fernandez-Garcia N, *et al*. 2008*b*. The gibberellin biosynthetic genes *AtGA20ox1* and *AtGA20ox2* act, partially redundantly, to promote growth and development throughout the *Arabidopsis* life cycle. *The Plant Journal* **53,** 488–504.

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

Sawhney VK, Shukla A. 1994. Male sterility in flowering plants—are plant growth substances involved? *American Journal of Botany* **81,** 1640–1647.

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

Serrani JC, Sanjuán R, Ruiz-Rivero O, Fos M, García Martínez JL. 2007. Gibberellin regulation of fruit set and growth in tomato. *Plant Physiology* **145,** 246–257.

Serrano M, Martínez-Romero D, Zuzunaga M, Riquelme F, Valero D. 2007. Calcium, polyamine and gibberellin treatments to improve postharvest fruit quality. In: Dris R, Jain SM, eds. *Production practices and quality assessment of food crops. Vol. 4. Postharvest treatment and technology*. Dordrecht: Kluwer Academic Publishers, 55–68.

Sun TP. 2000. Gibberellin signal transduction. *Current Opinion in Plant Biology* **3,** 374–382.

Sun TP. 2008. Gibberellin metabolism, perception and signaling pathways. The Arabidopsis Book, vol. doi: 10.1199/tab.0103, American Society of Plant Biologists, 1–28.

Thomas SG, Hedden P. 2006. Gibberellin metabolism and signal transduction. In: Hedden P, Thomas SG, eds. *Plant hormone signaling*. Oxford: Blackwell, 147–184.

Thomas SG, Philips AL, Hedden P. 1999. Molecular cloning and functional expression of gibberellin 2-oxidases, multifunctional enzymes involved in gibberellin deactivation. *Proceedings of the National Academy of Sciences, USA* **96,** 4698–4703.

Ueguchi-Tanaka M, Ashikari M, Nakajima M, *et al*. 2005. GIBBERELLIN INSENSITIVE DWARF1 encodes a soluble receptor for gibberellin. *Nature* **437,** 693–698.

Valegård K, van Scheltinga AC, Lloyd MD, *et al*. 1998. Structure of a cephalosporin synthase. *Nature* **394,** 805–809.

Webster AD. 2004. Vigour mechanisms in dwarfing rootstocks for temperate fruit trees. *Acta Horticulturae* **658,** 29–41.

Weigel D, Meyerowitz EM. 1994. The ABCs of floral homeotic genes. *Cell* **78,** 203–209.

Weigel D, Alvarez J, Smyth DR, Yanofsky MF, Meyerowitz EM. 1992. *LEAFY* controls floral meristem identity in *Arabidopsis*. *Cell* **69,** 843–859.

Wilson NR, Heckman WJ, Somerville RC. 1992. Gibberellin is required for flowering in *Arabidopsis thaliana* under short days. *Plant Physiology* **100,** 403–408.

Xu Y-L, Li L, Gage DA, Zeevaart JAD. 1999. Feedback regulation of GA5 expression and metabolic engineering of gibberellin levels in *Arabidopsis*. *The Plant Cell* **11,** 927–936.

Yamaguchi I, Takahashi N. 1976. Change of gibberellin and abscisic acid content during fruit development of *Prunus persica*. *Plant and Cell Physiology* **17,** 611–614.

Yamaguchi S. 2008. Gibberellin metabolism and its regulation. *Annual Review of Plant Biology* **59,** 225–251.

Zhu Y, Nomura T, Xu Y, *et al*. 2006. *ELONGATED UPPERMOST INTERNODE* encodes a cytochrome P450 monooxygenase that epoxidizes gibberellins in a novel deactivation reaction in rice. *The Plant Cell* **18,** 442–456.

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