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

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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 (PsGA2ox), 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 PsGA2ox signal. Grafting ‘Early Golden’ (EG), a commercial plum cultivar, on DGO24 (EG/D) enhanced PsGA2ox 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 (GA1 and GA4) than control trees (EG/M). Moreover, the physiological function of PsGA2ox 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 PsGA2ox 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...
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 C19-GAs as substrates, including functional GAs and their immediate precursors. Later, a new type of GA2ox that catabolizes only the non-bioactive C20-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., 2008a).

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 homologues, 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 BamHI 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.
Arabidopsis background Col-0. Six T3 homozygous independent
35S dip method (Clough and Bent, 1998). The
and then employed for

m grown under a long day (LD) photoperiod (16:8 h light/300

behaviour, and a representative from each group was selected for

These lines were pooled into two groups based on their growth

strain C58 by the freeze–thaw method (Holsters

purified by step-elution silicic acid (SiO2) partition chromatography

and the eluate was absorbed onto 0.5 g of Celite 545 (ProLab), and

filtered. The extract was purified through a C18 Sep-pack cartridge

residue was re-extracted with 5 ml of 80% methanol for 2 h and re-

and extracted overnight in darkness at 4

PslGA2ox

was performed using a Polaris 3

ionization mode (ESI+) with a capillary voltage of 5500 V and acid

Varian 1200l triple quadrupole, working in positive electrospray

Bam

subcloned in pGEM-T Easy vector, and then introduced into the

For the generation of the

Plasmid construction and plant transformation

replicates.

specific primers 3 and 4 (Supplementary Table S1) at

PCR system was used to amplify the full-length sequence using

Total RNA from plum was extracted using the methods described

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 an RNaseq mini kit (Qiagen, Mississauga, ON, Canada).

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 GA1 and GA4,

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 (SiO2) 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 1200i triple quadrupole, working in positive electrospray

ionization mode (ESI+) with a capillary voltage of 5500 V and acid

cation 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-

cerate 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 correspond-

ing deuterated phytohormones. 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

BamHI 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 T3 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 GA1 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 N2

immediately after collection and stored at −80 °C until use.

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

dioxygenase family of GA catabolic enzymes (Supple-

dimentary 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 C19-GA deactivator
(Thomas et al., 1999). However, tested members of class III
can only catalyze C20-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).

DNAase-treated RNA (5 µg) was reverse transcribed in a total

volume of 30 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 PCR (qRT-PCR) 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 normalised to the reference genes [PslAct (EF585293) and

AtAct (NM_121018)] that show high stability across the different
treatments.
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 \textit{et al.}, 2007). Within the first stage, intense cell division is predominant, while during
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 (At2g12220, 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
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., 2008b). 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...
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 stamens 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 whors. 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 L1/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
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.

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.
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., 2008a, 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 GA1 and GA4. 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 C19-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 GA4 is the most effective GA form leading to flowering promotion and reproductive growth. Thus, the scarcity of overall active GA content, particularly GA4, 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
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
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 \( \text{PslGA2ox} \) in \( \text{Arabidopsis} \) 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 \( \text{PslGA2ox} \) 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., 2008b; 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 \( \text{Arabidopsis} \), 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). \( \text{Arabidopsis} \) 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

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.
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., 2008a). 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., 2008a) 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 (C19-GA2oxs) exhibited extended pistils, resulting in partial infertility (Rieu et al., 2008a, 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. (2008a) 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.

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.
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 ClustalW 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) 35S::PsGA2ox::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 GA3, 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**


