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RESEARCH PAPER

Regulation of two germin-like protein genes during plum fruit development

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Abstract

Germin-like proteins (GLPs) have several proposed roles in plant development and defence. Two novel genes (*Ps-GLP1* and *2*) encoding germin-like protein were isolated from plum (*Prunus salicina*). Their regulation was studied throughout fruit development and during ripening of early and late cultivars. These two genes exhibited similar expression patterns throughout the various stages of fruit development excluding two important stages, pit hardening (S2) and fruit ripening (S4). During fruit development until the ripening phase, the accumulation of both *Ps-GLP*s is related to the evolution of auxin. However, during the S2 stage only *Ps-GLP1* is induced and this could putatively be in a H_2O_2-dependent manner. On the other hand, the diversity in the *Ps-GLP*s accumulation profile during the ripening process seems to be putatively due to the variability of endogenous auxin levels among the two plum cultivars, which consequently change the levels of autocatalytic ethylene available for the fruit to co-ordinate ripening. The effect of auxin on stimulating ethylene production and in regulating *Ps-GLP*s transcripts was also investigated. These data, supported by their localization in the extracellular matrix, suggest that auxin is somehow involved in the regulation of both transcripts throughout fruit development and ripening.

Key words: Auxin, ethylene, expression profile, flowering, germin-like protein, plum fruit development and ripening, subcellular localization.

Introduction

Germins and germin-like proteins (GLPs) constitute a large and highly diverse family of ubiquitous plant proteins. Germin was first identified in germinating wheat embryos (Thomson and Lane, 1980) and was shown to play an important role in the plant defence response (Lane *et al.*, 1986) as well as to possess oxalate oxidase activity (Lane *et al.*, 1993). Proteins with sequence similarity to germins have been identified in various plant species and these are termed 'germin-like proteins' (GLPs). GLPs have a global low sequence identity with germins (Bernier and Berna, 2001). Despite the considerable sequence heterogeneity between different GLPs, they all contain localized amino acid sequence 'signatures' (Lane *et al.*, 1991). Germins and the GLP gene family can be divided into two distinct groups. Members of the first group (the true germins) have relatively homogeneous sequences (Lane, 2000). However, the members of the second group (GLPs) are much more numerous and show high sequence divergence (Bernier and Berna, 2001). GLPs can be further divided into three subgroups based upon sequence conservation (Carter and Thornburg, 2000).

Plant GLPs have been found in various organs (leaves, roots, and floral tissues) and under different physiological conditions (seed germination, stress, and pathogen attack) (Membré *et al.*, 1997; Wojtaszek *et al.*, 1997). So far, extracellular manganese superoxide dismutase enzyme activity has been associated with many GLPs (Yamahara *et al.*, 1999; Carter and Thornburg, 2000). Several putative

Abbreviations: DAB, days after bloom; EG, 'Early Golden'; GLP, germin-like protein; SH, 'Shiro'; TIBA, 2,3,5-triiodobenzoic acid.

roles have been suggested for GLPs. They were assumed to be structural proteins as a consequence of their localization in the extracellular matrix. On the other hand, other functions have also been proposed, including epidermal-specific protein (Wei *et al.*, 1998), rhicadhesin receptor (Swart *et al.*, 1994), low-affinity auxin-binding protein (Ohmiya *et al.*, 1993), involved in circadian rhythms and floral induction (Heintzen *et al.*, 1994; Ono *et al.*, 1996; Vallelian-Bindschedler *et al.*, 1998), and disease resistance (Park *et al.*, 2004; Zimmermann *et al.*, 2006; Godfrey *et al.*, 2007).

In general, Japanese plums are climacteric fruits characterized by significant diversity for the date and rate of ripening (Abdi *et al.*, 1997). It has been proposed that auxin can stimulate climacteric ethylene biosynthesis by activating *ACS* and *ERF* transcription (El-Sharkawy *et al.*, 2008, 2009). Evaluation of the auxin content in peach fruits demonstrated that, concomitant with the climacteric ethylene production, a significant increase in IAA concentration could be detected in the mesocarp tissue (Miller *et al.*, 1987). Thus, it seems that auxin is also a part of the mechanism that acts upstream of ethylene and controls the ripening of climacteric fruits by stimulating the onset and production of autocatalytic ethylene.

The isolation and characterization of two putative proteins encoding a germin-like protein in plum is reported here. *Ps-GLP*s gene expression in flowers, during fruit development as well as their protein localization was studied. The ability of auxin and auxin inhibitor to promote and inhibit, respectively, ethylene biosynthesis in fruit was investigated.

Materials and methods

Plant material and post-harvest treatments

Fruits from two Japanese plum (*Prunus salicina* L.) cultivars 'Early Golden' (EG) and 'Shiro' (SH) were harvested and treated as described previously (El-Sharkawy *et al.*, 2007). Other tissues such as flowers and early developmental stages were collected from the 'SH' cultivar. All plant materials were frozen in liquid nitrogen and stored at –80 °C.

Fruit treatments

In order to evaluate IAA-induced ethylene production in plum fruit, mature 'SH' fruit were harvested as late as possible before fruit ethylene production had risen. The fruit were sterilized and treated with auxin-IAA (1, 10, and 100 μM) or with the auxin transport inhibitor, TIBA (50 μM). Fruit without any treatment were used as the control. After treatments, fruits were incubated at room temperature and ethylene production levels were measured daily. Mixed tissues of two fruit displaying a similar ethylene production at the same age were frozen for further analysis.

RNA isolation

Total RNA from fruit samples was extracted using the methods described by Boss *et al.* (1996). For vegetative tissues and flowers, total RNA was extracted using the Plant Total RNA purification kit (Norgen, Thorold, ON, Canada), as per the manufacturer's instructions. All RNA extracts were treated with DNase I (Promega, Madison, WI, USA) then cleaned up with RNeasy mini kit (Qiagen, Mississauga, ON, Canada).

Isolation and in silico *analysis of plum cDNA sequences*

Based on the sequence similarity of various GLPs, a pair of degenerate primers, GLP-F and GLP-R (primers from 1 and 2 in Table 1), was designed from the conserved regions to amplify the *GLP* orthologues from *P. salicina*. The isolated fragments were cloned in pGEM-T Easy vector (Promega), sequenced, and compared with database sequences using the BLAST program (Altschul *et al.*, 1997). Extension of the partial cDNA clones was carried out using the 3′- and 5′- RACE kit (Invitrogen, Burlington, ON, Canada). Full-length amplification of cDNA sequences, designated as *Ps-GLP1* and *Ps-GLP2*, was carried out using the Platinum *Taq* DNA Polymerase Alignment of *Ps-GLP* predicted protein sequences and the Neighbor–Joining tree construction was performed as described previously (El-Sharkawy *et al.*, 2009).

Real-time quantitative RT-PCR

All RT-PCRs were performed as described previously (El-Sharkawy *et al.*, 2007). Mx4000 v 4.20 software (Stratagene, La Jolla, CA, USA) was used to design gene-specific primers (primers from 3 to 8 in Table 1). To determine relative fold differences for each sample in each experiment, the *Ct* value for the two genes studied was normalized to the *Ct* value for β-actin and was calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$. The calibrator is the sample that exhibited the minimum level of transcripts in the whole experiment ('SH' fruits 105 DAB for *Ps-GLP1* and *2*).

Promoter isolation

Genomic DNA was extracted from immature leaves using the DNeasy Plant Maxi Kit (Qiagen). Promoters of Ps-GLP1 and Ps-GLP2 were isolated using the Universal Genome Walker Kit (Clontech, Palo Alto, CA, USA). Promoter sequence analysis was performed using PLACE Signal Scan Search program (Prestridge, 1991; Higo *et al.*, 1999).

Protoplast isolation and transient expression of Ps-GLP::GFP fusion proteins

The coding sequences of *Ps-GLP1* and *2* were cloned as a C-terminal fusion in frame with the GFP into the pGreen vector (Hellens *et al.*, 2000) and expressed under the control of the *35S* promoter. A high fidelity PCR system was used to amplify the ORFs using the following primers: Ps-GLP1(FG) and Ps-GLP1(RG) or Ps-GLP2(FG) and Ps-GLP2(RG) (primers from 9 to 12 in Table 1). The corresponding ORFs of Ps-GLP1 and 2 were cloned using the *Bam*HI restriction site of the pGreen vector. Protoplasts used for transfection were obtained from

Table 1. The oligonucleotide primers used

Name	Oligonucleotide sequence
1- GLP-F	5′-GCHGCAGTBACCCCTGCATTC-3′
2- GLP-R	5′-RCCACCAAGAACACCCTTMAGCTT-3′
3- Ps-GLP1(F)	5′-GCCCAATTTCCTGGTGTG-3′
4- Ps-GLP1(R)	5′-CGGGGTGAGTGTGAAAGG-3′
5- Ps-GLP2(F)	5′-CCCGGGTCTCCAAATTCT-3′
6- Ps-GLP2(R)	5′-CCCCCAAGAACACCCTTC-3′
7- Ps-actin(F)	5′-CTGGACCTTGCTGGTCGT-3′
8- Ps-actin(R)	5′-ATTTCCCGCTCAGCAGTG-3′
9- Ps-GLP1(FG)	5′-CGCGCGGATCCATGATTTTCCCTATCTTC-3′
10- Ps-GLP1(RG)	5′-CGCGCGGATCCCATTAGTGCCACCAAGAA-3′
11- Ps-GLP2(FG)	5′-CGCGCGGATCCATGCGCCAGGCAACGATG-3′
12- Ps-GLP2(RG)	5′-CGCGCGGATCCCATTAGTACCCCCAAGAA-3′

suspension-cultured tobacco (*Nicotiana tabacum*) BY-2 cells according to the method described by Leclercq *et al.* (2005). Protoplasts were transfected by a modified polyethylene glycol method and analysed for GFP fluorescence by confocal microscopy as described by El-Sharkawy *et al.* (2009). All transient expression assays were repeated at least three times.

Results

Gene structure and organization

In the present work, two putative full-length germin-like protein cDNAs (*Ps-GLP1* and *2*) were isolated. Their deduced nucleotide and amino acid sequences exhibited strong identity to the peach GLPs *Pp-ABP19* and *Pp-ABP20*, respectively (Ohmiya *et al.*, 1998). The *Ps-GLP1* sequence is 893 bp in length with a predicted open reading frame (ORF) encoding a protein of 209 amino acids. The 5′-, 3′-non-coding, and poly (A^+) sequences were 42, 206, and 17 bp, respectively. The full-length *Ps-GLP2* cDNA is 862 bp in length with a predicted ORF encoding a protein of 214 amino acids. The 5′-, 3′-non-coding, and poly (A^+) sequences were 63, 132, and 24 bp, respectively. Multiple alignments of *Ps-GLP* sequences with other reported true germin/GLP genes highlighted a number of conserved motifs and structural similarities that are common to the plant GLP subfamily (Bernier and Berna, 2001). The deduced amino acid sequences of Ps-GLP1 and 2 comprise a conserved extracellular targeting peptide signal located at the N-terminus that is characteristic of the GLP gene family (see Supplementary Fig. S1 at *JXB* online). A signal peptide search (Nielsen *et al.*, 1997) showed that a cleavage prediction of the hydrophobic signal targeting peptide would occur after Ala-18 and Ala-23 of the Ps-GLP1 and 2, respectively. In addition, the predicted protein of both sequences contains the three highly conserved germin/GLP oligopeptides characteristic of GLPs (Bernier and Berna, 2001). Box-A (QDFCVAD), includes a cysteine residue (Cys-24 and Cys-29 for Ps-GLP1 and 2, respectively), which is followed by a second cysteine (Cys-39 and Cys-44 for Ps-GLP1 and 2, respectively) believed to form an internal disulphide bridge of the extracellular domain (Woo *et al.*, 2000). Box B is (G-P-H-HPGASEXXXXX-G) and box C is (GXXHFQXNG), in which X corresponds to any hydrophobic amino acid. Boxes B and C contain the three histidines and the glutamate residues involved in heavy metal ion-binding site (Dunwell and Gane, 1998; Woo *et al.*, 1998). Consistent with GLP from other plant species (Carter *et al.*, 1998), a single potential N-glycosylation site was identified in both cDNAs. The RGD-like tripeptides (KGD) motif sequence, thought to be involved in protein–protein interactions, was also present in both sequences (Labouré *et al.*, 1999).

The promoter sequence from Ps-GLP1 (1194 bp, EU310511) and Ps-GLP2 (1031 bp, EU310510) contain several predictive *cis*-acting elements presumed to be involved in transcriptional regulation. Both Ps-GLP 5′-UTR regions hold ARF binding element (SAUR 15a, TGTCTC) located at positions –170 and –761 for Ps-GLP1, and –408 for Ps-GLP2 (Ulmasov *et al.*, 1999). The ARF motif is found in the promoter of genes up-regulated by IAA (Goda *et al.*, 2004). Also, a single binding element (LECPLEACS2, TAAAATAT) in Ps-GLP1 (at position –900) and two in Ps-GLP2 (at positions –627 and –898), proven to regulate *ACC* synthase gene expression in tomato (Matarasso *et al.*, 2005), were identified. In addition, an identical sequence to tobacco NtBBF1 (ACTTA) was detected uniquely in the Ps-GLP1 promoter region at position –91. The NtBBF1 motif box is believed to be required for auxin induction (Baumann *et al.*, 1999).

A phylogenetic tree, comprising 28 germin/GLP sequences from 16 species, was generated (Fig. 1). The dendrogram analysis defines that germins/GLPs could be divided into two main groups, true germins and GLPs. However, the members of the GLPs could be further divided into three main subfamilies (I, II, and III). Establishing a correlation between phylogenesis and functional properties of the encoded proteins based on published data allow a number of functional characteristics to be outlined. The true germin subfamily comprises most of the proteins that have oxalate oxidase enzyme activity (Lane *et al.*, 1993; Le Deunff *et al.*, 2004). GLP gene members corresponding to subfamilies I and II comprise GLP proteins exhibiting superoxide dismutase activity (Carter *et al.*, 1999; Yamahara *et al.*, 1999; Zimmermann *et al.*, 2006; Godfrey *et al.*, 2007). The *Pisum sativum* GLP, Ps-GER1, belongs to subfamily II and has been reported to function as a Rhicadhesin receptor (Swart *et al.*, 1994). Ps-GLP1 and 2 are members of subfamily III that may have a common role as regulatory proteins involved directly or indirectly in auxin metabolism (Khuri *et al.*, 2001). This group includes low-affinity auxin-binding proteins from peach (Ohmiya *et al.*, 1993, 1998), cotton GLP1 important for cell wall expansion (Kim *et al.*, 2004), as well as GLPs known to have a role linked to circadian rhythms and floral induction in *A. thaliana* (Staiger *et al.*, 1999), *Hordeum vulgare* (Vallelian-Bindschedler *et al.*, 1998), *Sinapis alba* (Heintzen *et al.*, 1994), and *Pharbitis nil* (Ono *et al.*, 1996). Despite strong sequence diversity between the different gene members of the four groups, many of them confer resistance against pathogen infection (Carter and Thornburg, 2000; Park *et al.*, 2004; Zimmermann *et al.*, 2006; Godfrey *et al.*, 2007).

Ps-GLP gene expression in flower and during fruit development

In flower and during early fruit development (0–15 DAB), both transcripts showed a similar pattern of accumulation. *Ps-GLP* mRNAs were highly expressed at bloom and decreased slightly afterward (~4 DAB). The expression of the two *Ps-GLP*s was considerably re-stimulated after fertilization (~7 DAB) and gradually declined thereafter, 10–15 DAB (Fig. 2).

The stone fruit development period could be divided into three different stages (El-Sharkawy *et al.*, 2007). The first stage (Stage 1, 22–37 DAB) illustrated by intense cell division and differentiation, and rapid growth caused by massive

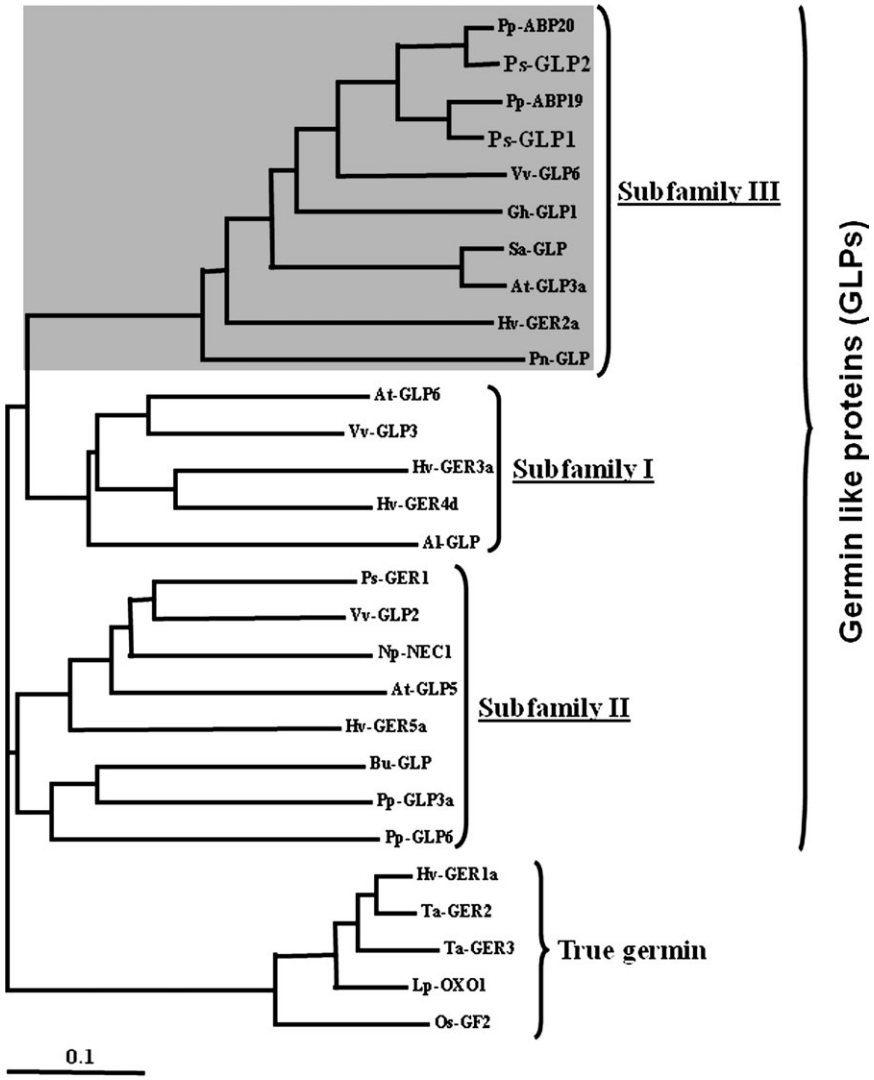

Fig. 1. Phylogenetic relationships between *P. salicina* [Ps-GLP1 (EU310513), Ps-GLP2 (EU310512)]; *P. persica* [Pp-ABP19 (U79114), Pp-ABP20 (U81162)]; *V. vinifera* [Vv-GLP2 (ABH09468), Vv-GLP3 (AAQ63185), Vv-GLP6 (ABL60875)]; *G. hirsutum* [Gh-GLP1 (AAO92740)]; *S. alba* [Sa-GLP (P45854)]; *A. thaliana* [At-GLP3a (P94072), At-GLP5 (AAB51569), At-GLP6 (P92997)]; *H. vulgare* [Hv-GER1a (ABG46232), Hv-GER2a (ABG46233), Hv-GER3a (ABG46234), Hv-GER4d (ABG46236), Hv-GER5a (ABG46237)]; *P. nil* [Pn-GLP (P45853)]; *A. lentiformis* [Al-GLP (BAA78563)]; *P. sativum* [Ps-GER1 (CAB65369)]; *N. plumbaginifolia* [Np-NEC1 (Q9SPV5)]; *B. unguiculata* [Bu-GLP (BAC53790)]; *P. patens* [Pp-GLP3a (BAD86499), Pp-GLP6 (BAD86502)]; *T. aestivum* [Ta-GER2 (P15290), Ta-GER3 (P26759)]; *L. perenne* [Lp-OXO1 (CAC19429)]; and *O. sativa* [Os-GF2 (ABF98325)] based on full-length amino acid sequence. True germin, germin like protein (GLP) I, II, and III correspond to the different germin/GLP gene subfamilies.

quantities of auxin (Miller *et al.*, 1987). During this period of fruit development, both transcripts gradually increased to a peak (~32 DAB) and declined thereafter (Fig. 3; Stage 1). In the second stage (Stage 2, 42–52 DAB); the endocarp hardens to form a solid stone, both genes clearly showed significant inhibition in their transcription levels (Fig. 3; Stage 2). However, the inhibition of *Ps-GLP2* transcripts was more brutal than those of *Ps-GLP1*. A slight increase in *Ps-GLP1* mRNA was detected throughout stage 2 (~47 DAB) and declined thereafter, whereas that of *Ps-GLP2* was weakly expressed. During Stage 3 (57–77 DAB), when the pulp (mesocarp) was separated from the seed (endocarp+embryo) both *Ps-GLP*s accumulated similarly in the pulp or seed (Fig. 3; Stage 3). However, their pattern of expression in the pulp was totally different from that in the seed. In the pulp, both transcripts peaked at ~67 DAB, whereas, in the seed, both mRNAs were strongly detected at the beginning (~57–62 DAB) and declined thereafter to reach their basal levels at the end of Stage 3, ~77 DAB. Interestingly, the start of inhibition of both transcripts in the seed coincided with their accumulation peak in the pulp, ~67 DAB.

The expression of Ps-GLPs during fruit ripening

To evaluate the possible role of the two Ps-GLP proteins in fruit ripening, QRT-PCR analysis was carried out to determine their accumulation profile throughout ripening of early 'EG' and late 'SH' plum cultivars.

In both cultivars the fruits produced ethylene throughout ripening. 'EG' fruit displayed an early, rapid ripening, and a short and rapid (maximal 5 d) ethylene production profile. 'SH' fruit exhibited a suppressed climacteric pattern. The fruit ripened slower and later than 'EG'. The ethylene production in 'SH' fruit reached a maximum at 11 d after the onset of ethylene emission (Fig. 4).

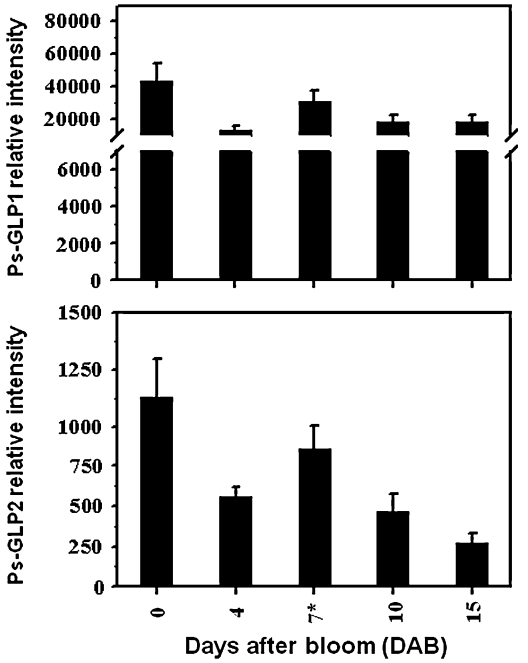

Fig. 2. Steady-state transcript levels of *Ps-GLP1* and *2* assessed by QRT-PCR during flower and early fruit development of the 'SH' cultivar. The fertilized flowers stage is marked with an asterisk. The experiments were carried out in triplicate. The *x*-axis represents the developmental stages indicated by the number of days after bloom (DAB). Relative intensity in the *y*-axis refers to the fold difference in gene expression relative to 'SH' fruits 105 DAB for *Ps-GLP1* and *2*.

Both plum cultivars showed a dramatic decrease in *Ps-GLP*s transcript levels throughout ripening. In 'EG' pulp, relatively high *Ps-GLP1* mRNA levels were detected in non-climacteric fruit (~78 DAB) and strongly declined thereafter (Fig. 4); however, *Ps-GLP2* was weakly detected. As ripening progressed and higher levels of autocatalytic ethylene produced, both transcripts increased relatively in the climacteric phase (~82 DAB). In 'EG' seed, *Ps-GLP1* mRNA was temporally quite constitutive during ripening, however, those of *Ps-GLP2* increased to a peak ~82 DAB as in the pulp but at much higher levels (Fig. 4). The peak of *Ps-GLP2* mRNA, in the whole 'EG' fruit, coincided with the climacteric peak of ethylene production.

Throughout ripening of the late cultivar 'SH' (90–105 DAB), *Ps-GLP1* mRNA remained at a minimal level or was almost undetectable in the whole 'SH' fruit. The accumulation pattern of *Ps-GLP2* in the whole 'SH' fruit (pulp and seed), coincided well with the evolution of ethylene. However, *Ps-GLP2* transcripts in the 'SH' pulp were at least three times lower than in 'SH' seeds (Fig. 4).

Auxin treatment stimulates ethylene biosynthesis and Ps-GLPs *transcription in late plum fruit*

The previous results suggest that the suppressed climacteric phenotype in 'SH' fruits could partially be due to inadequate quantities of auxin to co-ordinate ripening. To test this hypothesis, 'SH' fruits were treated with different concentrations of IAA and 50 μM of the auxin inhibitor, TIBA; untreated 'SH' fruit were used as the control. TIBA-treated fruits were unable to ripen autonomously and their ethylene production remained low even after 35 d post-treatment (data not shown). Untreated, control fruits displayed a climacteric peak after 10 d, with a corresponding

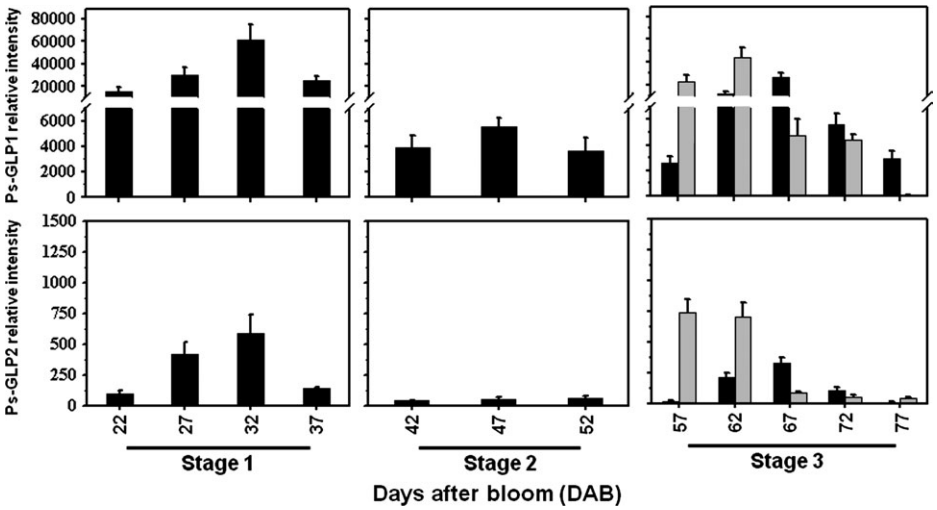

Fig. 3. Steady-state transcript levels of *Ps-GLP1* and *2* during Stage 1, Stage 2, and Stage 3 of 'SH' fruit development using the whole fruit (Stage 1 and Stage 2). However, during Stage 3 of fruit development the expression was determined in pulp (black bars) and in seeds (grey bars). The experiments were carried out in triplicate. The *x*-axis represents the developmental stage indicated by number of days after bloom (DAB) and by the name of stage. Other details are as described in Fig. 2.

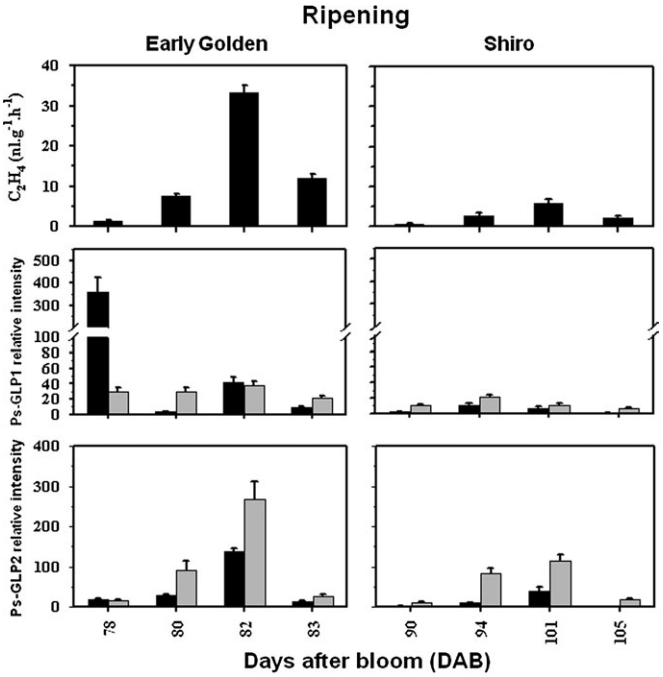

Fig. 4. Ethylene evolution and steady-state transcript levels of *Ps-GLP1* and *2* during early 'EG' (left panel) and late 'SH' (right panel) plum fruit ripening. The expression was quantified in pulp (black bars) and in seeds (grey bars). The experiments were carried out in triplicate. The *x*-axis represents the developmental stage indicated by number of days after bloom (DAB). Other details are as described in Fig. 2.

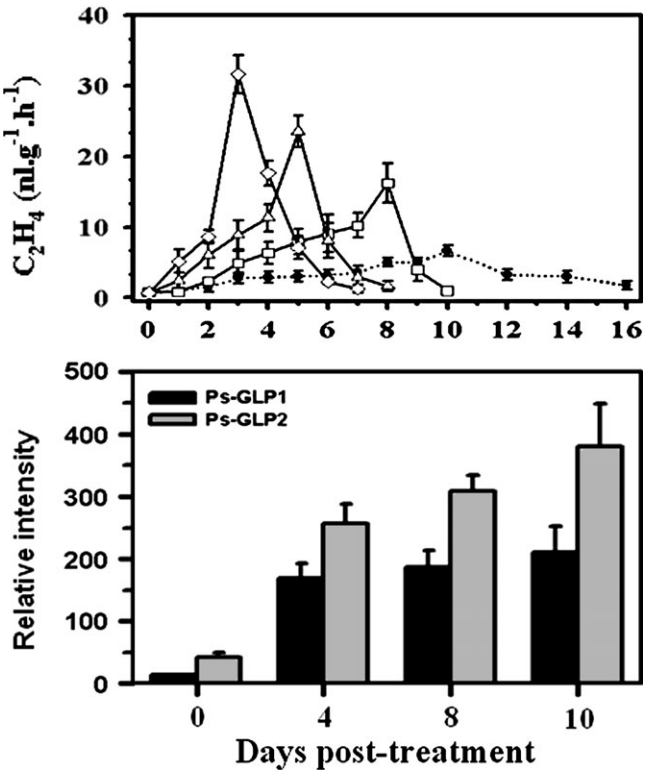

Fig. 5. (A) Ethylene production of 'SH' plum fruit treated with auxin (filled circles) 0 μM, (open squares) 1 μM, (open triangles) 10 μM, and (open diamonds) 100 μM. Fruit treated with 0 μM auxin served as the control. (B) The steady-state mRNA levels for *Ps-GLP1* and *2* in 'SH' fruit during ripening at room temperature after treatment with 10 μM IAA. The *x*-axis in each figure represents days after auxin treatment. Other details are as described in Fig. 2.

ethylene production at the peak of 6.6 nmol g^{-1} h^{-1} (Fig. 5A). Fruits that were treated with 1, 10, and 100 μM of IAA, exhibited a climacteric peak after 8, 5, and 3 d post-treatment, respectively; with a corresponding ethylene production at the peak of 16.2, 23.6, and 31.6 nmol g^{-1} h^{-1}, respectively (Fig. 5A).

'SH' fruits treated with 10 μM of IAA and 50 μM TIBA were selected to study the accumulation pattern of *Ps-GLP*s transcripts. TIBA treatment completely inhibited *Ps-GLP*s transcription in the fruit (data not shown). However, auxin treatment triggered a dramatic increase in the accumulation level and/or pattern of the two *Ps-GLP* transcripts, suggesting a possible role for auxin (Fig. 5B). In IAA-treated fruits, *Ps-GLP*s transcription were steadily augmented along with the progression of fruit ripening and continued to increase past the climacteric peak, reaching their maximal levels in post-climacteric fruit, 10 d after treatment.

Subcellular localization of Ps-GLP1 and 2 proteins

The presence of a putative signal peptide at the N-terminus of each Ps-GLP protein suggested their extracellular targeting localiztion. Ps-GLP1 and 2 coding regions were fused to the GFP tag and were transiently expressed in tobacco protoplasts. Fluorescence microscopy analysis demonstrated that control cells transformed with GFP alone displayed fluorescence ubiquitously as expected (Fig. 6A). By contrast, Ps-GLP1::*GFP* and Ps-GLP2::*GFP* fusion proteins were totally excluded from the nucleus and preferentially localized in the plasma membrane and the nuclear envelope (Fig. 6B, C).

Discussion

In this study, the molecular characterization of two putative genes encoded for germin-like protein from plum is reported. GLP genes are members of a large multigene family exhibiting diverse patterns of expression and function (Bernier and Berna, 2001). Although sequence identity can be as low as 40% among the different germins/GLPs, there are highly conserved signature elements. One of the most important conserved elements is the presence of a signal peptide in the N-terminal region, which supported the hypothesis of an apoplastic and/or plasma membrane localization. Our results showed that both proteins are, as expected, exclusively localized in the plasma membrane outside the nucleus.

In the dendrogram, a number of well-defined branches have both *Arabidopsis* and other plant species genes but lack *Prunus* sequences, suggesting that there are likely to be as yet unidentified *GLP* genes within the *Prunus* genome.

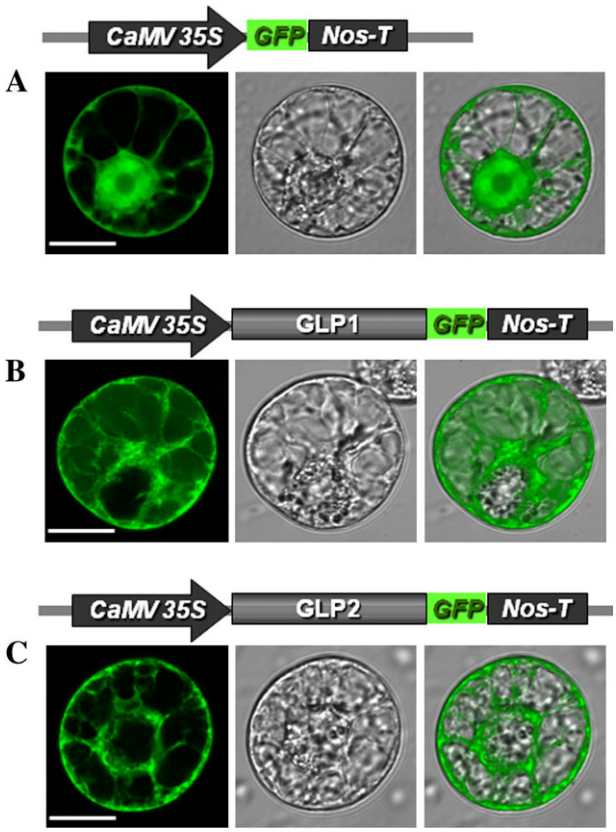

Fig. 6. Subcellular localization of Ps-GLP proteins fused to the GFP tag. Constructs consisting of either the control 35S::GFP, or 35S::*GLP1*-GFP or 35S::*GLP2*-GFP were used transiently to transform *N. tabacum* protoplasts. The subcellular localizations of the GFP protein under the control of 35S (A), the *GLP1*-GFP (B) or *GLP2*-GFP (C) fusion protein were analysed using confocal laser scanning microscopy. Light micrographs (centre panel) and fluorescence (left panel) images are merged (right panel) to illustrate the different locations of the two proteins. The length of the bar corresponds to 10 μm.

Twelve and seven *GLP*s that belong to the three different GLP subfamilies were characterized in *Arabidopsis* and grapes, respectively (Carter *et al.*, 1998; Godfrey *et al.*, 2007). Ps-GLP1 and 2 proteins belong to GLP subfamily III, which may consist of regulatory proteins with a possible involvement in auxin metabolism.

Assuming that the sequences isolated in this study encode functional GLP proteins, their expression profile was studied in different plant tissues and under various conditions in order to determine their role in fruit development. Bernier and Berna (2001) reported that at least one *GLP* mRNA has to be present in a specific organ and/or during a certain developmental stage throughout the entire plant life cycle. This simple analysis shows that *GLP*s are expressed in all plant parts and at all developmental stages.

Ps-GLP1 and *2* transcripts showed very close expression patterns in flowers and during early fruit development (0–37 DAB). Their strong accumulation during flowering reflects their important role in floral induction. *Arabidopsis GLP3* mRNA belongs to the same GLP group (subfamily III) and was found to be most abundant in leaves and flowers (Membré *et al.*, 1997). From the available data, it seems that both proteins are also required for regulating the abundant cell division during early embryo development, which occurs during this stage. Mathieu *et al.* (2006) demonstrated that hybrid larch GLP (LmGER1) played a central role in somatic embryo formation via the regulation of cell wall remodelling necessary for correct development.

Although both transcripts rapidly declined during S2, *Ps-GLP1* mRNA was still present in considerable levels, while, that of *Ps-GLP2* was totally absent. In order to determine the role of *Ps-GLP1*, it was essential to understand clearly the physiological aspects that characterize the S2 stage. As mentioned previously, during this 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) suggesting a minor role for auxin. The absence of auxin during this stage probably explains the significant reduction in *Ps-GLP*s levels. However, the only fruit development process during this stage is the lignification of the endocarp in a H_2O_2-dependent manner to form a solid stone (seed). The role of H_2O_2 in the lignification of the cell wall was clearly determined by Ros Barceló (2005). These results suggest that the transcription of *Ps-GLP1* could be altered due to the high levels of endogenous H_2O_2. The expression of both *Ps-GLP*s during this stage, along with their response to exogenous H_2O_2 treatment and the evolution of endogenous H_2O_2 content, suggest that only Ps-GLP1 protein can be involved in the lignification of the endocarp (see Supplementary Fig. S2 at *JXB* online).

It has been demonstrated that during the S3 stage the auxin content as well as various auxin-related genes starts to increase rapidly in the whole fruit (Miller *et al.*, 1987; Trainotti *et al.*, 2007). In our studies, a steady rise in the expression of both *Ps-GLP*s was observed during this stage suggesting that the up-regulation of the two transcripts is likely due to the increase in endogenous auxin content. Although, both transcripts have the same trend of accumulation in the pulp or in the seed, their expression seems to be tissue-specific since their accumulation profile in the pulp was totally different from those in the seed. To determine the role of *Ps-GLP*s during this stage, it is necessary to distinguish between the two tissues (pulp and seed). Accelerated cell division followed by cell expansion is a signature event of S3 that results in a significant increase in fruit size. Auxin regulates plant cell division, elongation, and differentiation through signal transduction (Taiz, 1984; Abel and Theologis, 1996; Christian *et al.*, 2006). Plum GLP1 and 2 could be components of this signal network that mediates cell division and expansion. Kim and Triplett (2004) showed that the highest accumulation of cotton GLP1 transcripts is positively correlated with the stages of maximal cotton fibre expansion. The finding of auxin-responsive *cis*-elements in some plant GLP promoters associated with the increase of their expression in response to auxin treatment give further credence to the idea that

GLPs may be involved in plant cell expansion (Berna and Bernier, 1997; this paper). Further, the findings that *Ps-GLP*s accumulation in the seed preceded that of pulp by at least 10 d indicate that seeds stimulate fruit growth and ripening by supplying auxin.

It is almost certain that the series of modifications that transform a mature green fruit into a ripe fruit occur during S3 (El-Sharkawy *et al.*, 2008, 2009) and involve many different metabolic pathways. Therefore, the factors that control the transition of a fruit from the end of growth to the onset of ripening are of primary importance. In climacteric fruits most aspects of the ripening process are triggered and maintained by ethylene (Lelièvre *et al.*, 1997). Previous studies showed that the endogenous auxin content significantly increased in the fruit during ripening, concomitant with the production of climacteric ethylene (Miller *et al.*, 1987). In addition, treatment of fruit with synthetic auxin enhanced both fruit development and ripening (Augustí *et al.*, 1999; Ohmiya, 2000). Furthermore, the evaluation of four different ethylene biosynthesis elements (*ACC* synthesis) and seven ethylene-responsive transcription factors (*ERF*s) in plum fruits (i.e. the same cultivars studied in the present work) revealed that auxin can affect ethylene production by increasing the transcription levels of different *Ps-ACS* and *Ps-ERF* mRNAs, which play an important role in determining the level of autocatalytic ethylene production and the capacity of the fruit to ripen (El-Sharkawy *et al.*, 2008, 2009). Finally, Arteca and Arteca (2008) observed that different parts of *Arabidopsis* plants produced various levels of ethylene in response to IAA treatment. The level of IAA-induced ethylene depends on the concentration of auxin, age of the tissue (the youngest leaves showing the greatest stimulation), and the organ position (IAA-induced ethylene occurred in the root and inflorescence tips more than the regions below this).

To investigate further a possible autonomous role played by auxin, the expression of *Ps-GLP1* and *2* was studied during 'EG' and 'SH' fruit ripening. Generally, the accumulation of the two *Ps-GLP* transcripts in the seed was higher or at least equal to their levels in the pulp during ripening of both plum cultivars. The *Ps-GLP1* expression pattern follow the ethylene evolution profile in 'EG' mesocarp. On the other hand, its mRNA was hardly detected in 'SH' fruit. However, *Ps-GLP2* accumulated in a similar pattern as that of ethylene production in the fruits of both cultivars. Although *Ps-GLP2* transcript abundance in the seed of both cultivars seems to be ethylene-dependent, it is not likely to be the case as the seed at this stage is much lignified and dry resulting in the inhibition of ethylene biosynthesis (Rodrìguez-Gacio and Matilla, 2001).

The differences in the accumulation levels and/or pattern of both transcripts throughout ripening of early and late fruits might be partially due to the variation in auxin contents and/or ethylene produced among the two plum cultivars. Such variations affect the capacity of the fruit to produce and respond to ethylene, which results in the differentiation in ripening behaviour thereafter (Trainotti *et al.*, 2007; El-Sharkawy *et al.*, 2008, 2009). The scarcity of auxin in the late cultivar can consequently affect the levels of autocatalytic ethylene available for the fruit to maintain ripening. Treatment of late 'SH' fruit with auxin significantly accelerated auxin-induced ethylene production and sensitivity. The fruits restored the typical climacteric pattern to a comparable level with those of early 'EG' fruits. Moreover, it seems that the concentration of auxin correlated positively with the precocity of the climacteric phase and the rate of ethylene production at the peak.

Taken together, auxin seems to be accumulated rapidly and in higher levels in the early cultivar, which leads to the up-regulation of different transcripts associated with auxin, including auxin and ethylene elements (Trainotti *et al.*, 2007; El-Sharkawy *et al.*, 2008, 2009). Such high levels of ripening-related proteins (auxin- and ethylene-related proteins) resulted in the early transition of the mature green fruit into the ripening stage. Once the fruit initiates autocatalytic ethylene, the ripening process will be enhanced in both auxin- and ethylene-dependent manners. Therefore, the possibility of regulating many ethylene-related genes by auxin is the best alternative that explains the significant accumulation of such transcripts during ripening in an ethylene-independent manner (El-Sharkawy *et al.*, 2008, 2009; Ziliotto *et al.*, 2008). However, the expression profiles of *Ps-GLP1* and *2* in response to auxin and auxin inhibitor application suggests that the two transcripts are putatively accumulated in the fruit during ripening in an auxin-dependent manner.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Amino acid sequence alignment of the *P. salicina* genes, Ps-GLP1 and Ps-GLP2 with closely related sequences from other species.

Supplementary Fig. S2. Hydrogen peroxide (H_2O_2) levels in developing plum fruits and the effect of externally applied H_2O_2 on the expression of Ps-GLP1 and Ps-GLP2 during the early stages of fruit growth.

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