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A Japanese plum α-L-arabinofuranosidase/β-D-xylosidase gene is developmentally regulated by alternative splicing

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ABSTRACT

A full-length cDNA clone named *PsARF/XYL* was obtained from *Prunus salicina* Lindl., and determined to encode a putative α-L-arabinofuranosidase/β-D-xylosidase belonging to glycoside hydrolase (GH, EC 3.2.1.-) family 3. Two related *PsARF/XYL* cDNAs were amplified, one from a fully-spliced transcript (*PsARF/XYLa*) and another one from an intron-retained transcript (*PsARF/XYLb*). The protein deduced from *PsARF/XYLb* is a truncated peptide at C-terminus that conserves the active-site amino acid sequence. High levels of *PsARF/XYLa* and *PsARF/XYLb* transcripts are detectable in several plant tissues. *PsARF/XYLb* transcripts accumulate progressively during the phase of exponential fruit growth but they become barely noticeable during on-tree ripening, or after a 6-h exposure of preclimacteric full-size plums to ethylene. In contrast, *PsARF/XYLa* is expressed throughout fruit development, and transcript accumulation parallels the climacteric rise in ethylene production during ripening. *PsARF/XYLa* expression is strongly induced in preclimacteric full-size plums after a 6-h treatment with physiologically active concentrations of ethylene. These findings suggest that *PsARF/XYL* gene is post-transcriptionally regulated by alternative splicing during development and that ethylene may be involved in this regulation. The isolation of a partial cDNA clone, *PsARF1*, is also reported. It encodes a putative cell-wall α-L-arabinofuranosidase, and its transcription is rapidly inhibited by ethylene in mature green plums.

1. Introduction

Japanese plums (*Prunus salicina* Lindl.) are soft-fleshed and highly perishable fruits mainly grown for fresh consumption. The onset of ripening and rapid softening renders them susceptible to pathological breakdown and physical damage. Achievement of high-quality fruit with long shelf life has been a major breeding goal for plums [1]. Genetically-programed metabolic changes occur during stone fruit ripening, which determine a major drop in firmness due to the disassembly of the cell wall [2]. A coordinated series of modifications of the polysaccharide components of the primary cell wall and middle lamella results in a weakening of the wall structure [2]. The molecular mechanisms that control the complex of those cell-wall modifications are still unclear. In 'Gigaglia' plums, arabinose proved to be the principal neutral monosaccharide constituent in cell walls during growth and the most dynamic neutral sugar in pectic fractions [3]. A conspicuous loss of arabinose from tightly bound pectins was found to be a relatively early feature in the sequence of cell-wall biochemical modifications, thus strongly suggesting a softening-related role during Japanese plum ripening [3].

α-L-Arabinofuranosidases (α-Araf; EC 3.2.1.55) and β-D-xylosidases (β-Xyl; EC 3.2.1.37) are enzymes involved in cell-wall neutral sugar metabolism. Many cDNAs encoding α-Araf and β-Xyl have been described in fruits from different woody species, such as apple [4,5], Japanese pear [6,7], peach [8–10], and kiwifruit [11]. Hayama et al. [9] described a peach *PpARF/XYL* cDNA, encoding a putative bifunctional α-Araf/β-Xyl, which was proposed to be a softening-related gene with an ethylene-dependent expression. In fact, peach *PpARF/XYL* transcripts accumulate at different developmental stages and plant tissues, even when ethylene biosynthesis is hardly detectable, but this does not exclude a softening-related role since cell-wall genes like *PpARF/XYL* might be post-transcriptionally regulated according to cellular needs [10]. In Japanese plums, genes involved in the arabinose release from ionically and tightly bound cell wall pectins have not been reported as yet [3], and their contributions to fruit softening have to be explored.

Abbreviations: AS, alternative splicing; α-Araf, α-L-arabinofuranosidase; DAFB, days after full bloom; GH, glycoside hydrolase; 1-MCP, 1-methylcyclopropene; β-Xyl, β-D-xylosidase.

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Most eukaryotic genes include nucleotide sequences named introns that must be correctly spliced from pre-mRNA to give rise to functional mRNAs. An efficient highly-regulated splicing is needed for correct gene expression in plants and other eukaryotes [12]. Alternative splicing (AS) is a mechanism that joins exons and introns selectively and produces different mRNAs from the same gene. Some AS events are constitutive, with constant ratios of mRNA variants in the same cells, whereas others are regulated in response to developmental or physiological signals [13]. The synthesized proteins may have different substrate specificities, spatial localization or temporal expression conferring strategic functional diversity for a unique coding sequence. An AS event has been defined as functional "if it is required during the life cycle of the organism and activated in a regulated manner" [14].

The number of known alternatively spliced plant genes is expanding rapidly, and it is likely that this post-transcriptional regulation plays a major role in the control of gene expression in plants and in other eukaryotes. AS proved to be a significant mode of genetic regulation in perennial fruit crops, such as apple [15] and *Vitis vinifera* berries [16]. In fact, Zenoni et al. [16] identified AS events for 385 grape genes and suggested that transcript complexity could be higher than appreciated in advance. These authors were the first to use high-throughput sequencing analysis (RNA-Seq technology) to gain insight into the wide range of transcriptional responses associated with fruit growth and ripening in a woody vine species. In *Prunus persica*, this massive sequencing methodology, combined with the recent sequencing of the complete genome [17], offers new opportunities to detect AS events [18]. Using RNA-Seq technology, Wang et al. [19] analyzed five types of AS events in the peach transcriptome, and identified 10,835 AS events in 5520 transcribed regions, with exon skipping being the most abundant AS event. Results of genome analysis revealed differences between plant species in the frequency of the AS types. Intron retention accounts for ~40% of the AS events found in *Arabidopsis thaliana* [20] but it is a rare phenomenon in peach since it accounts for ~2.8% of all the observed splicing events [19]. AS has been detected in 359 genes after *Plum pox virus* infection in peach leaves [21]. In fruits, some relevant AS events have also been reported such as the *PpETR1* gene that encodes a receptor involved in ethylene perception [22]. Different accumulations of three *ETR1* mature transcripts occur in fruits subjected to wounding experiments but not in other plant organs such as leaves, revealing that changes in ethylene sensitivity may take place specifically during fruit wounding by means of AS. To the best of our knowledge, no AS events operating in Japanese plums have been reported.

The first objective of this work was to clone and characterize a new Japanese plum *PsARF/XYL* gene and two related splicing cDNA variants encoding a putative cell-wall-related α-Araf/β-Xyl, and to analyze their expression in growing and ripening plums, as well as in other plant organs. A second objective was to isolate and characterize the expression pattern of a partial cDNA clone named *PsARF1*, encoding a putative α-Araf from Japanese plum. Changes in the expression profiles after exposure to ethylene and 1-methylcyclopropene (1-MCP) were also examined. As far as we know, this is the first report of an AS event in *P. salicina*.

2. Materials and methods

2.1. Plant material

Japanese plums 'Gigaglia' were randomly picked from the orchard of the Facultad de Agronomía, Universidad de Buenos Aires (34°35′S; 58°29′W). 'Gigaglia' is an early hybrid, Japanese-type plum, involving a cross to *P. salicina* Lindl. Trees were grown on Myrobalan plum rootstocks and trained to an open-vase system. Different organs and tissues were collected, including seedling roots, intact mature leaves and open flowers that were dissected in sepals, petals, stamens (filaments plus anthers) and non-pollinated ovaries. Samples were frozen in liquid nitrogen and stored at −80 °C for later RNA analysis.

Fruit developmental stages were defined by using the first derivative of the growth cumulative curve [3]. During growth, plums were harvested and assigned to different physiological stages (S1, S2 and S3), and their age (days after full bloom, DAFB), fresh weight, and ethylene production were recorded. Physiological stages were defined as follows: S1, fruit set, initial phase of exponential growth (12, 16, 20 and 27 DAFB); S2, lag phase (31, 38 and 42 DAFB); S3, cell expansion phase (49, 58, 65 and 72 DAFB; 72-DAFB plums were used as preclimacteric fruit). Additionally, five ripening stages (R1–R5) were defined on the basis of their age (DAFB), flesh firmness (N), epicarp color (h°: hue angle values) and ethylene production (nL h^{-1} g^{-1}), as follows: R1 (75 DAFB, 46 ± 4.63 N, $h^\circ = 106 \pm 3.14$, 0.91 ± 0.39 nL h^{-1} g^{-1}), R2 (76 DAFB; 36 ± 1.91 N; $h^\circ = 91 \pm 4.09$; 1.75 ± 0.57 nL h^{-1} g^{-1}), R3 (77 DAFB; 26 ± 1.73 N; $h^\circ = 65 \pm 7.91$; 4.34 ± 0.68 nL h^{-1} g^{-1}), R4 (78 DAFB; 15 ± 2.69 N; $h^\circ = 47 \pm 5.34$; 1.41 ± 0.27 nL h^{-1} g^{-1}), R5 (79 DAFB; 11 ± 1.17 N; $h^\circ = 29 \pm 2.1$; 1.12 ± 0.25 nL h^{-1} g^{-1}). At least 10 fruits were collected per sampling date. Three mm-thick mesocarp pieces, obtained from the equatorial region of each fruit at a site equidistant from the epicarp and the endocarp, were sampled and immediately frozen in liquid nitrogen and stored at −80 °C for later RNA analysis.

2.2. Ethylene, firmness and color assessment

Ethylene production was quantified on a Hewlett Packard 5890 Series II gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA, USA) fitted with a flame ionization detector and a stainless steel Porapak N column [23]. Fruit firmness was determined with an Instron Universal Testing Machine (Model 3342, Canton, MA, USA) as previously detailed [24]. Hue angle values (h°) were measured on the equatorial region of intact fruit with a CR-300 chromameter (Minolta, Osaka, Japan) as described elsewhere [3]. At least ten fruits were used per sampling date.

2.3. 1-MCP and ethylene application

Preclimacteric full-size plums were harvested and ethylene production was assessed. Only the fruits that yielded non-detectable ethylene production were used and randomly assigned to different treatments. Fruit free from decay and defects were randomized to provide three experimental units per treatment, with at least three fruits per unit. Each unit was placed in a sealed 67-L container at 20 °C. The experimental units were kept as untreated controls, or treated with 1 μL L^{-1} ethylene for 3, 6 or 24 h, or with 0.5 μL L^{-1} 1-MCP–one of the most effective antagonists of ethylene action in climacteric plums [25]—for 24 h. The required concentration of 1-MCP was obtained from SmartFresh™ powder (active ingredient 0.14%, AgroFresh, Inc., Rohm and Haas, USA) as previously described [26]. After treatments, the skin was removed and two mesocarp pieces located on opposite sides of the fruit were excised into liquid nitrogen and stored at −80 °C until use.

2.4. Isolation and characterization of cDNA clones

To clone cDNAs encoding α-Araf/β-Xyl from *P. salicina* 'Gigaglia', a set of degenerate and specific primers, were designed against conserved amino acid residues of fruit α-Araf/β-Xyl from the following GenBank accession numbers: AB195230 (peach *PpAz152*); AB195230 (Japanese pear *PpARF2*), and AB264280 (peach *PpARF/XYL*). These primers were used in standard PCR reactions containing cDNA from plum mesocarp as template.

Polyadenylated RNA was extracted from fruit mesocarp by using the mRNA Isolation Kit (Roche Molecular Biochemicals, Gmbh, Mannheim, Germany) and cDNA was made up with 50 ng of mRNA sample by means of RevertAid™ M-MuLV Reverse Transcriptase system (Fermentas International Inc., Burlington, Ontario, Canada). PCR conditions were as follows: 4 min at 94 °C (first cycle); 45 s at 94 °C, 45 s at 53–55 °C, and 1 min at 72 °C (28–35 cycles); and 7 min at 72 °C (last cycle). Overlapping sequences were obtained by using three different combinations of primers that covered the cDNA coding region except for the 5′ and the 3′ end. The partial sequences were aligned and a 1645 bp contig sequence was obtained. Isolation of *PsARF1* clone was carried out using primers designed on the basis of a previously described peach cDNA clone sequence (*PpARF1*, [10]). The primers used and the RT-PCR product sizes for the *PsARF/XYL* and *PsARF1* clones are listed in supplementary data (Table S1). PCR products were separated on 1.5% agarose gels, stained with SYBR Green (Invitrogen, Carlsbad, CA, USA) and visualized by the UVP Doc-It LS Image Acquisition Software. For sequencing, PCR products were cloned into the pGEM®-T Easy vector system (Promega Corp., Madison, WI, USA).

Poly(A)$^+$ RNA was obtained from mesocarp of Japanese plum as described above and used as template in the 5′ and 3′-RACE reactions. PCR amplifications were performed according to the 3′and 5′ RACE System for Rapid Amplification of cDNA Ends kits (Invitrogen, Carlsbad, CA, USA). To obtain the 3′ end of the *PsARF/XYL* sequence, the first cDNA strand was primed with an oligo-dT adapter primer that targets the Poly(A)$^+$ tail region. For cDNA amplification, separate PCR reactions were carried out by using different forward gene-specific primers (GSP) and reverse adapter primer. A UAP (Universal Amplification primer) was used to anneal the adapter sequence of the adapter primer. The primers used were GSP1 (F1) for *PsARF/XYLa* transcript and GSP1′ (Fin) for *PsARF/XYLb* transcript. Nested GSP was not required due to the specificity of the first PCR product amplified. To obtain the 5′ end of the *PsARF/XYL* sequence, first strand cDNA synthesis was carried out by use of a GSP1 (R22). SuperScript™ II RT at 48 °C was used to increase the specificity of first strand cDNA synthesis. Two nested PCR reactions were performed according to the manufacturer's instructions, using GSP2 (RIO) and GSP3 (NstGSP). The GSPs used are listed in supplementary data (Table S2). The PCR products were purified and sequenced as described below.

2.5. Gene cloning and sequence analysis

'Gigaglia' leaves (100 mg) were ground in liquid nitrogen with a mortar and pestle until a fine powder was obtained. Genomic DNA isolation was carried out using ChargeSwitch® genomic DNA isolation kit and MagnaRack® magnetic particle separator (Invitrogen, Carlsbad, CA, USA). DNA integrity was evaluated through agarose gel electrophoresis and DNA yield by use of Qubit® Fluorometer and Qubit® dsDNA BR Assay Kit (Invitrogen, Carlsbad, CA, USA). Ten nanogram of genomic DNA were used as template for standard PCR reactions. Primers that cover the complete coding region were used to amplify *PsARF/XYL* gene. The primers and the expected product sizes are listed in supplementary data (Table S3). PCR products were separated on 1.5% agarose gels, stained with SYBR Green (Invitrogen, Carlsbad, CA, USA) and visualized by the UVP Doc-It LS Image Acquisition Software. The PCR products were cloned into the pGEM®-T Easy vector system (Promega Corp., Madison, WI, USA) and sequenced. The partial sequences were aligned and a 4040 bp contig sequence was obtained.

Sequence analysis was carried out on an ABI PRISM® 377 DNA Sequencer (Applied Biosystems, USA). The complete cDNA sequences were analyzed and homology searches were performed using NCBI BLAST algorithm [27] on GenBank databases. Deduced amino acid sequence alignment and comparison were made by means of ClustalW [28]. Signal peptides and their cleavage sites were predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP [29]). The subcellular location was predicted with TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP [30]). The theoretical isoelectric point (pI) and mass values for mature peptides were calculated by means of the Compute pI/Mw tool [31]. The presence of exon and intron sequences, donor and acceptor splice sites, and branch points were analyzed using the *NetGene2* splice-site prediction web server [32]. Molecular phylogenetic analysis was performed using the maximum parsimony method (100 bootstrap replicates; Molecular Evolutionary Genetics Analysis [MEGA] software Version 5) [33].

2.6. Semi quantitative RT-PCR analysis

Expression analysis of *PsARF/XYL* and *PsARF1* was carried out through semi-quantitative RT-PCR reactions. Total mRNA was obtained from 200 mg of plant tissue and cDNA synthesis was carried out as described above after DNAse I treatment of mRNA samples. For DNase I treatment, 1 unit of DNase I, RNase-free (Fermentas International Inc., Burlington, Ontario, Canada) per 50 ng of total mRNA or per 1 μg of total RNA was used following manufacturer's instructions prior to cDNA synthesis. To check DNA contamination, a negative control (hereafter called "No RT reaction") was performed using RNA without Reverse Transcriptase as PCR template. PCR conditions were as follows: 4 min at 94 °C (first cycle); 45 s at 94 °C, 45 s at 54 °C, and 1 min at 72 °C (30–35 cycles); and 7 min at 72 °C (last cycle). Expression analysis was performed with the primers listed in supplementary data (Table S4). Actin expression was selected as an internal control for normalization of the amount of starting template because of its consistent transcript level in all tissues and conditions tested in comparison with other housekeeping candidate genes. PCR conditions were as described above except for the annealing temperature (55 °C) and the number of cycles (25). PCR conditions for each primer pair were optimized empirically to determine the linear range of amplification. Each gel is representative of several replications. Total RNA was extracted following a previously described protocol [34].

3. Results

3.1. Isolation of splicing cDNA variants for PsARF/XYL gene and PsARF1 cDNA clone

RT-PCR reactions using plum cDNA as template yielded two predominant products that differ in 116 bp. These products were detected in floral organs (petals, sepals, stamens and carpels), roots and leaves and were referred to as ***a*** and ***b*** (Fig. 1A). Product ***b*** included a putative intron that was predicted by sequence and bioinformatics analysis. The detection of product ***b*** persisted in RT-PCR reactions after DNAse I treatment of RNA samples, composed of total or polyadenilated RNA (Fig. 1B). No product in no-reverse transcriptase control reactions was amplified. The full-length cDNA sequences were obtained (GenBank accession number JX455818). Based on homology searches, the full-length cDNA sequences corresponding to products ***a*** and ***b*** were named *PsARF/XYLa* and *PsARF/XYLb*, respectively. To compare the 3′ UTR of *PsARF/XYLa* and *PsARF/XYLb* transcripts, a forward primer (Fin) that anneals intron VI sequence (Fig. 1C, line 1) or (F1) that anneals an exon sequence upstream of intron VI (Fig. 1C, lines 2) were designed as Gene Specific Primer 1 (GSP1) and used in RACE 3′ reactions. Further cloning and sequence analysis of RACE 3′ products determined identical 3′ UTR in *PsARF/XYLa* and *PsARF/XYLb* transcripts. RACE 3′ reactions performed using cDNA from an ethylene-treated pre-climacteric fruit as template showed that *PsARF/XYLb* transcripts were less abundant than *PsARF/XYLa* transcripts. Bioinformatics

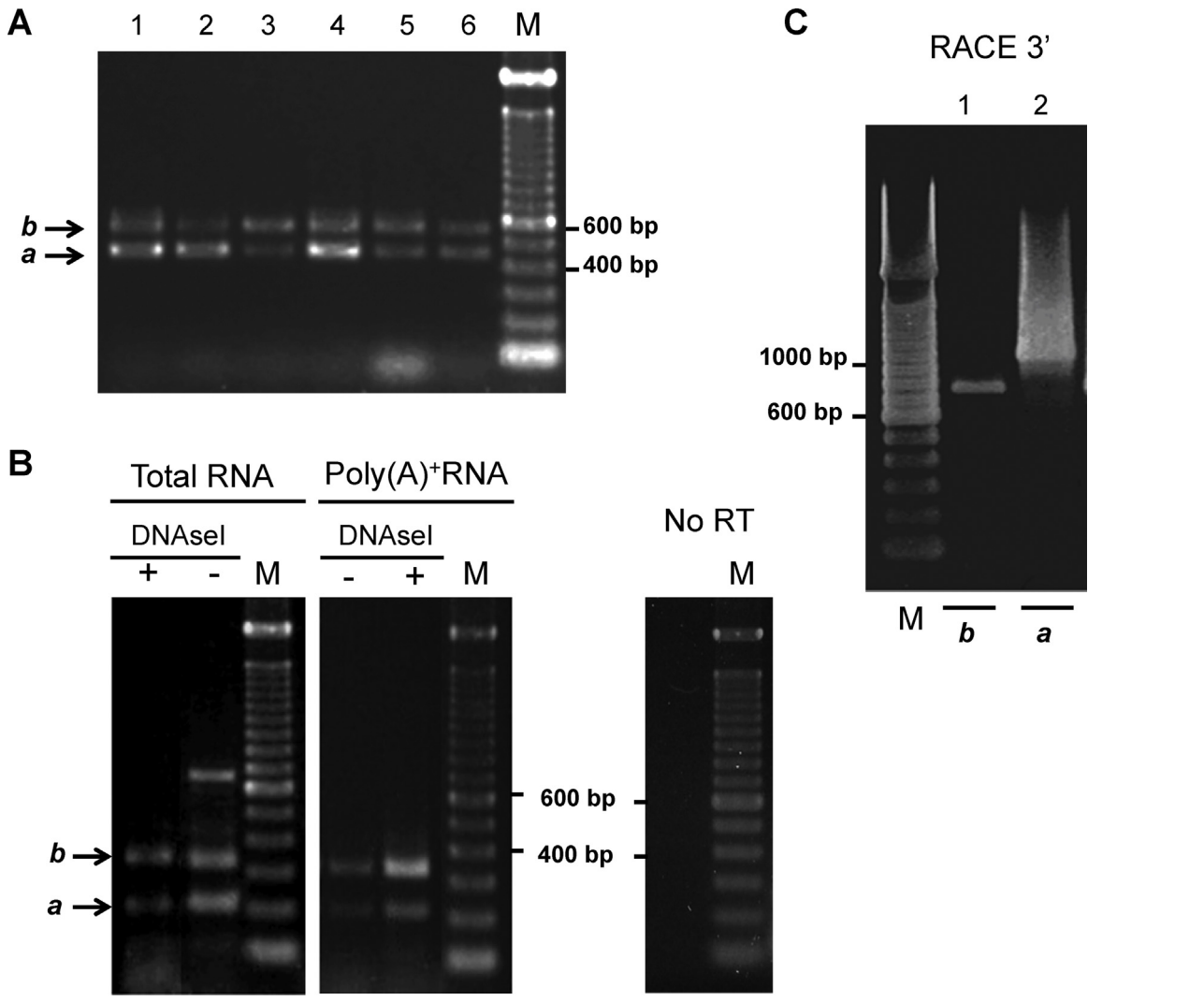

Fig. 1. Isolation of two alternative transcripts from *PsARF/XYL* gene. (A) RT-PCR products amplified by using primers F2-R3 flanking intron VI and cDNA template from plum tissues in the following order: petals, sepals, stamens, carpels, leaves, and roots, respectively (1–6); M, 100 bp DNA ladder used as molecular weight standard. Arrows indicate *PsARF/XYLa* and *PsARF/XYLb* RT-PCR products as follows: ***a***, fully-spliced transcript; ***b***, intron retained transcript. (B) RT-PCR amplification of two alternative transcripts from plum RNA. Total RNA and polyadenilated RNA extraction were subjected to DNAse I treatment. "No RT reaction" is a negative control for DNA contamination, as described in Materials and Methods. (C) RACE 3′ products obtained using forward primers that specifically amplify *PsARF/XYLb* (1) or *PsARF/XYLa* (2) cDNAs.

analysis revealed that the predicted mature protein of *PsARF/XYLa* includes 747 amino acids with a molecular mass of 81 kD and a theoretical pI of 8.84. As a consequence of intron retention, predicted mature protein of *PsARF/XYLb* is truncated at the 3′ end and includes 591 amino acids with a molecular mass of 65 kD and a theoretical pI of 8.33. The SignalP and SMART software predicted the presence of a signal peptide with a cleavage site at position 28 in both *PsARF/XYLa* and *PsARF/XYLb* mature proteins. TargetP 1.1 indicated that these proteins could be exported to the apoplast. Another cDNA clone related to cell-wall GH was partially isolated (GenBank accession number DQ822467) and BLASTX analysis of this sequence showed its homology with members of GH family 51 in general and with other plant α-Araf in particular (see below). On the basis of these findings the clone was named *PsARF1*.

3.2. Structural organization of PsARF/XYL gene

The complete genomic sequence of *PsARF/XYL* was isolated (GenBank accession number JX455817.1) and the exon–intron organization determined by cDNA-genomic alignments. Fig. 2 shows a schematic representation of the exon–intron organization of *PsARF/XYL* and the alternative spliced transcripts found in this work. cDNA-genomic alignments showed that the *PsARF/XYL* gene is composed of seven exons and six introns that cover a 4040-Kb region (Fig. 2, Table 1). *PsARF/XYLb* includes a 116 bp insertion that corresponds to intron VI of the genomic sequence (Fig. 2). Exon sizes were more variable and larger (99–601 bp) than intron sizes (93–116 bp), except for intron I that covers 1201 bp (Table 1), a significant portion of the gene. As in other dicots, introns of *PsARF/XYL* are AT rich (~61–78%, Table 1). Both the alternatively spliced intron (intron VI) and the constitutively spliced introns (introns I–V) showed the 5′ and 3′ splice sites in agreement with consensus sequences in dicots (data not shown) and had similar levels of AU content (Table 1).

3.3. Bioinformatics analysis of two cDNA clones, PsARF/XYLa and PsARF/XYLb

The proteins deduced from *PsARF/XYL* were aligned and compared (Fig. 3). TargetP (version 1.1) predicted that the proteins could be exported to the apoplast, thus suggesting their potential involvement in cell-wall arabinosyl metabolism. The program

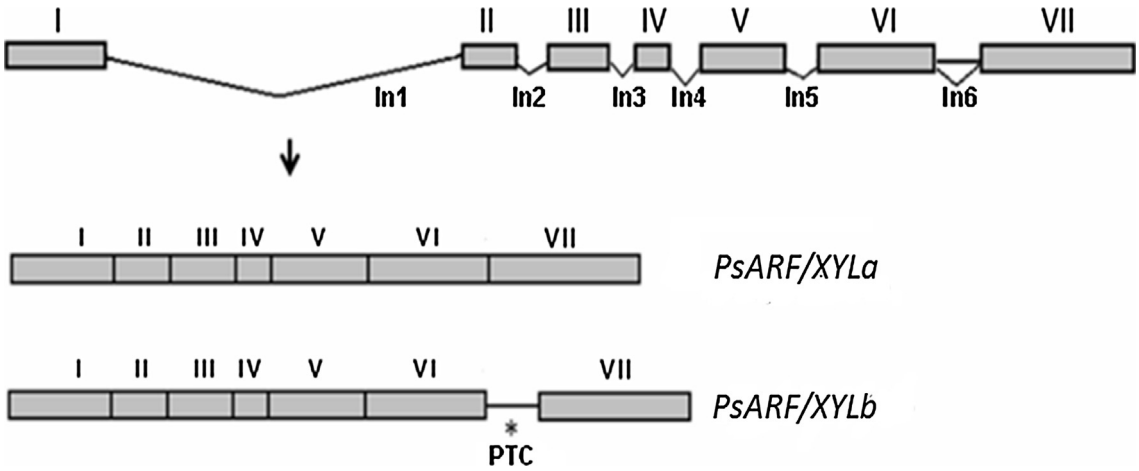

Fig. 2. Schematic representation of the exon–intron distribution in *PsARF/XYL* gene (Gen bank accession number JX455817.1), and two alternatively spliced mRNA variants. The exon and intron sequences are represented by boxes and lines, respectively. The retained intron in the *PsARF/XYLb* transcript includes a premature termination codon (PTC) indicated by an asterisk.

also predicted that the signal peptide could be cleaved at position 28–29 between the A and R residues, respectively. These proteins showed N-terminal and C-terminal catalytic domains conserved in GH family 3 (Pfam 00933 and Pfam 01915), and a fibronectin type III domain with unknown functions (Pfam 14310). BLASTp analysis showed that the protein deduced from *PsARF/XYLa* displays 59% amino acid identity with a barley bifunctional glycoside hydrolase family 3 (ARA-I) with proven α-Araf and β-Xyl activity (query cover: 95%, *E* value: 0.0). The action of retaining glycosidases on glycosides is in most cases mediated by two key active site amino acid residues, the catalytic nucleophile and the catalytic acid/base. Based on the results of these alignments, Asp-267 was predicted to be the putative catalytic nucleophile for the mature protein of *PsARF/XYLa*, while Glu-469 or Glu-471 were identified as the two candidate amino acid residues for the catalytic acid/base (Fig. 3). The retained intron in the *PsARF/XYLb* transcript introduced an in-frame premature termination codon (PTC). The protein deduced from *PsARF/XYLb* lacks a 156-amino acid sequence at C-terminus and includes a 41-amino acid sequence which is absent in the protein deduced from *PsARF/XYLa* (Fig. 3). The putative amino acids of the active site were found in the proteins deduced from both *PsARF/XYLa* and *PsARF/XYLb*.

3.4. Phylogenetic analysis

Phylogenetic analysis showed that the amino acid sequence of the protein deduced from *PsARF/XYLa* is closely related to other members of the GH family 3, while the partial sequence of the protein deduced from *PsARF1* showed high homology with members of GH family 51 in general and with other plant α-Araf in particular (Fig. 4).

3.5. Expression of PsARF/XYL and PsARF1 transcripts during plum growth and ripening

Growth and ripening behavior of 'Gigaglia' plums was evaluated during a period of 12 weeks, and DAFB, fresh weight changes and ethylene production were recorded throughout fruit ontogeny (Fig. 5). In addition, changes in epicarp color and flesh firmness were assessed at five ripening stages named R1, R2, R3, R4 and R5 (Fig. 6). 'Gigaglia' plums showed a typical double sigmoid growth pattern during fruit development and ripening (Fig. 5). Different stages of growth were determined: S1 (cellular proliferation, 10–30 DAFB), S2 (endocarp lignification, 31–48 DAFB), S3 (exponential phase of growth, 49–74 DAFB). 'Gigaglia' plums displayed a sharp ethylene peak on day 77 DAFB (4.34 nL h^{-1} g^{-1}) and decreased in 78-DAFB fruits (1.41 nL h^{-1} g^{-1}). On the basis of firmness loss (Fig. 6), color changes (Fig. 6) and ethylene production (Fig. 5), ripening stages were defined as follows: R1 (75 DAFB, firmness = 46 N, h° = 106), R2 (76 DAFB, firmness = 36 N, h° = 91), R3 (77 DAFB, firmness = 26 N, h° = 65), R4 (78 DAFB, firmness = 15 N, h° = 47) and R5 (79 DAFB, firmness = 11 N, h° = 29). Expression analysis of *PsARF/XYL* and *PsARF1* during growth (S1, S2, S3) and ripening (R1, R2, R3, R4, R5) stages was performed (Fig. 7). *PsARF/XYLa* and *PsARF/XYLb* transcripts were amplified at three different stages during growth and primers were used to span the retained intron VI (F2-R3, Fig. 7). *PsARF/XYLa* and *PsARF/XYLb* were expressed at relatively similar levels at S1 and S2, but in 49-DAFB plums *PsARF/XYLb* expression was hardly noticeable. *PsARF/XYLb* expression increased throughout S3 (58, 65, 72 DAFB) and was higher than *PsARF/XYLa* expression. A similar *PsARF/XYLb* expression was found in all stages during growth when using F2-Rin primers, which anneal specifically to the retained intron region and avoid the effects of template competition (Fig. 7). *PsARF/XYLa* expression increased during S3 (58, 65, and 72 DAFB;

Table 1
Position, length, and A + U content of the exons and introns in the *PsARF/XYL* gene (Genbank accession number JX455817.1).

Exon	Pos. 5′–3′	Length (bp)	(A + U) %	Intron	Pos. 5′–3′	Length (bp)	(A + U) %
I	1–415	415	47.58	1	416–1616	1201	68.94
II	1617–1897	281	49.11	2	1898–2008	111	61.26
III	2009–2178	170	50	3	2179–2275	97	65.31
IV	2276–2374	99	55.56	4	2375–2467	93	65.59
V	2466–2817	350	46.29	5	2818–2911	94	78.72
VI	2912–3324	413	47.46	6	3325–3340	116	70.69
VII	3441–4041	601	47.09				

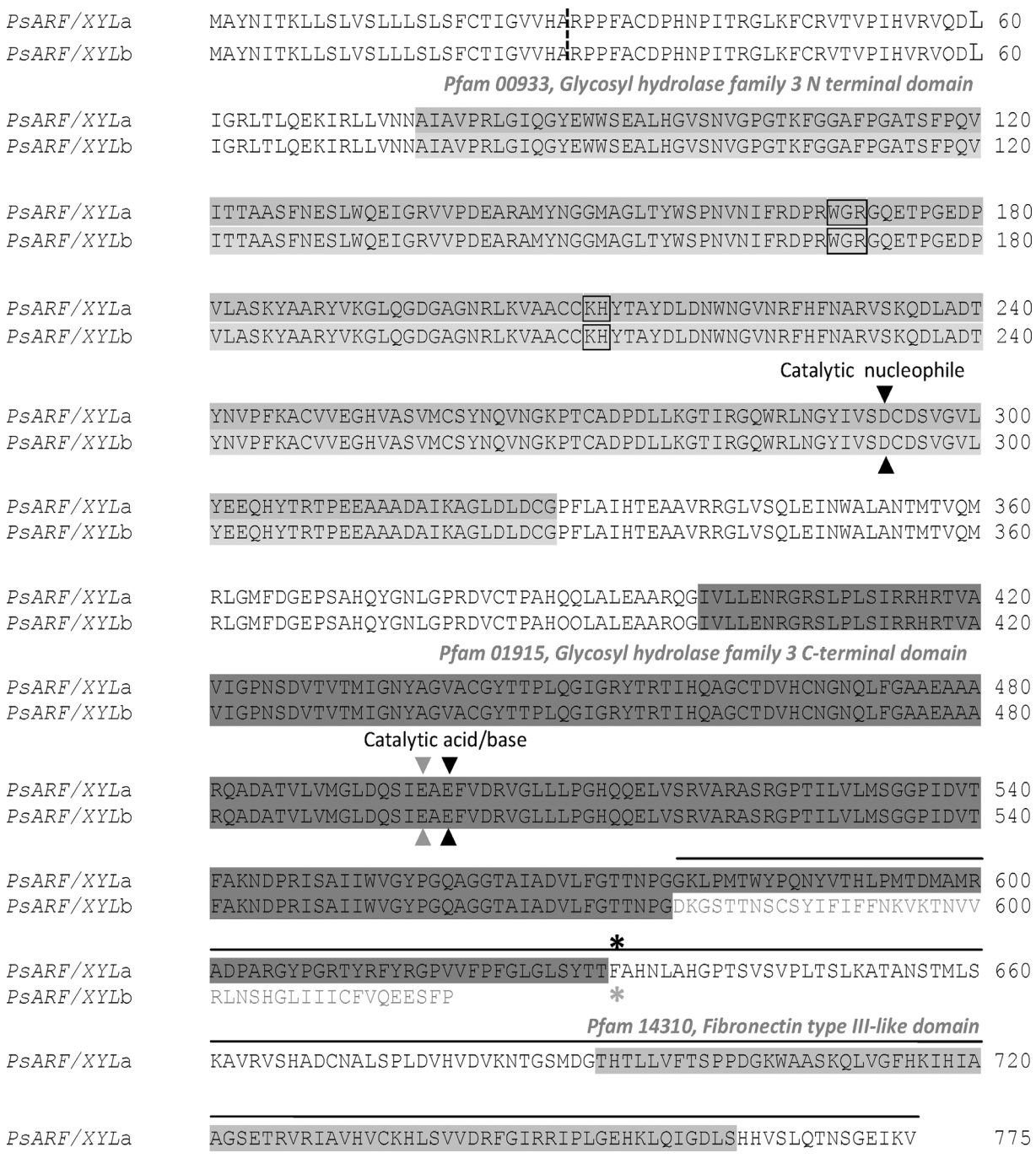

Fig. 3. (A) Alignment of the amino acid sequences of the proteins deduced from *PsARF/XYLa* and *PsARF/XYLb*. The dotted vertical line marks the putative cleavage site after the predicted signal peptide. Black arrowheads indicate putative catalytic nucleophile (Asp-267) and putative catalytic acid/base (Glu-471) for both predicted proteins. Grey arrowheads show an alternative catalytic acid/base residue (Glu-469). The solid overline above *PsARF/XYLa* indicates a 156 amino acid sequence that is absent in the hypothetical protein of *PsARF/XYLb* as a consequence of premature termination codon (PTC). Amino acids in grey indicate C-terminal differences between *PsARF/XYLb* and *PsARF/XYLa* due to the retained intron in *PsARF/XYLb* transcript. Conserved N-terminal and C-terminal domains are shaded in grey. Domain classification and associated pfam numbers are shown above in grey letters. Boxed amino acids indicate putative sites for carbohydrate binding. The black asterisk marks the C-terminus of barley ARA-I [37] (relative position). The grey asterisk marks the C-terminus of alfalfa MsXYL1 [38], and radish RsAraf1 [39] (relative position).

F2-R3; Fig. 7), but to a minor extend in *PsARF/XYLb* expression. A major *PsARF/XYLa* expression was observed in ripening R3 plums (Fig. 7), in concomitance with ethylene production peak (Fig. 5). In contrast, *PsARF/XYLb* expression relatively decreased during ripening when the F2-Rin specific primers were used, and was not detected using the F2-R3 primers. On the other hand, *PsARF1* expression – assessed with L2-R2 primers – was relatively high and similar during S1 and S2, and increased slightly during S3 (mainly at 58 and 65 DAFB). *PsARF1* expression strongly decreased in R1, R3 and R5 fruits compared to that in S3, R2 and R4 fruits (Fig. 7).

3.6. *PsARF1 and PsARF/XYL expression in ethylene- and 1-MCP-treated fruits*

To assess the role of ethylene on transcriptional changes during ripening, preclimacteric full-size plums were harvested and

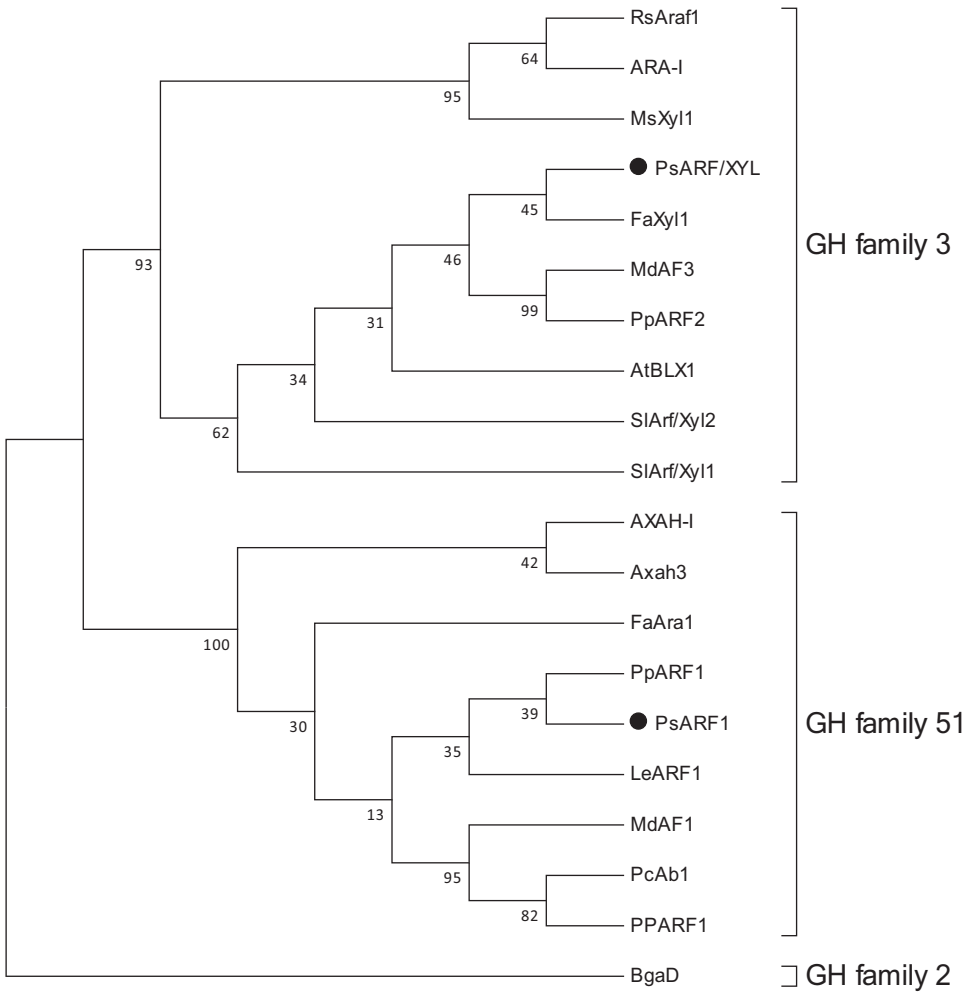

Fig. 4. Molecular phylogenetic analysis of the proteins deduced from *PsARF/XYL*, the partial clone *PsARF1*, and other plant GHs. Accession numbers of proteins are as follows: RsAraf1 from radish, BAE44362; ARA-I from barley, AY029259; MsXyl1 from alfalfa, ABQ45227; FaXyl1 from strawberry, AAS17751; MdAF3 from apple, ADI792081; PpARF2 from Japanese pear, BAD98523; AtBXL1 from Arabidopsis, NP_199747; SlArf/Xyl1 and SlArf/Xyl2 from tomato, BAL44717 and BAL44716 respectively; AXAH-I from rice, BAC10349; Axah3 from barley, AFF58881; FaAra1 from strawberry, ABV08815.1; PpARF1 from peach, ABF22680.3; LeARF1 from tomato, BAC99302; MdAF1 from apple, AAP97437; PcAb1 from *Pyrus communis*, BAF42035; PPARF1 from *Pyrus pirifolia*, BAC99303; and BgaD, encoding a β-galactosidase from *Aspergillus nidulans* (out-group), ADM36015. Numbers on the dendrogram branches show bootstrap probability values.

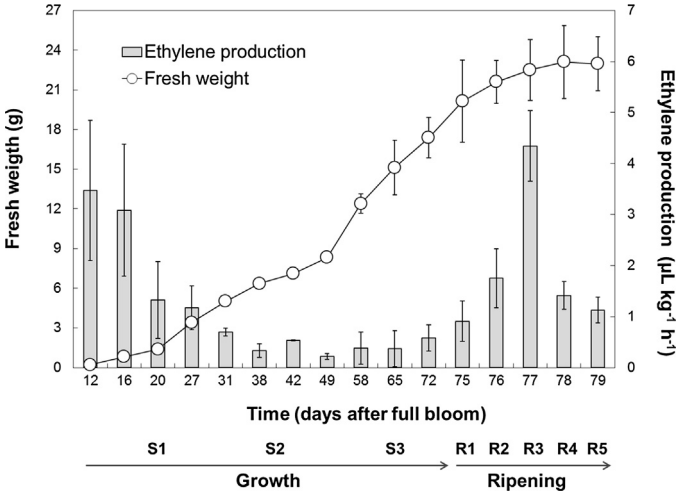

Fig. 5. Fresh weight and ethylene production of 'Gigaglia' plums during development and ripening. Values represent the means ± SD of 20 (fresh weight) and 5 (ethylene production) replicates. Where bars are not shown, the SD does not exceed the size of the symbol. Growth stages were designated as described in Materials and Methods.

treated with ethylene or 1-MCP. Ethylene exposure for 24 h sharply induced total accumulation of *PsARF/XYL* transcripts (Fig. 8, primers F1-R1). Total *PsARF/XYL* expression was lower and similar in control and 1-MCP treated plums after 24 h. However, when F2-R3 primers were used to differentiate *PsARF/XYLa* and *PsARF/XYLb* transcripts, only *PsARF/XYLa* expression was strongly induced in ethylene-treated plums after 24 h (Figs. 8 and 9). In contrast, *PsARF1* expression was weakly detected in 24-h ethylene-treated plums, whereas the gene was highly expressed in control and 1-MCP treated plums (Figs. 8 and 9). 1-MCP treatments showed a moderate increase of *PsARF/XYLa* expression (Fig. 8, primers F2-R3), a similar decrease of *PsARF/XYLb* expression (Fig. 8, primers F2-R3) and no changes in total *PsARF/XYL* expression (Fig. 8, primers F1-R1). Ethylene exposure for 3 h had almost no effects on the *PsARF/XYLa* and *PsARF/XYLb* expression, which was similar to that of control plums. Induction of *PsARF/XYLa* expression was evident after 6 h of ethylene application. *PsARF1* expression decreased after 3 and 6 h of ethylene application (Fig. 9).

4. Discussion

A full-length Japanese plum cDNA clone sequence encoding a putative cell wall α-Araf/β-Xyl enzyme was referred to as

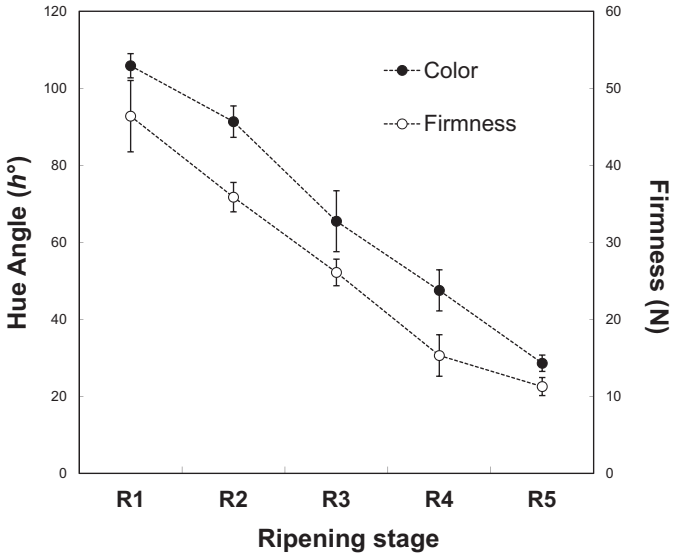

Fig. 6. Color (hue angle, ***h***°) and firmness (N) changes during ripening of 'Gigaglia' plums. Ripening stages were designated as follows: R1 (onset), R2 (early-ripe), R3 (mid-ripe, peak ethylene production), R4 (fully ripe), R5 (overripe). At least 10 independent samples per developmental stage were evaluated. Values represent the means ± SD of 10 independent replicates.

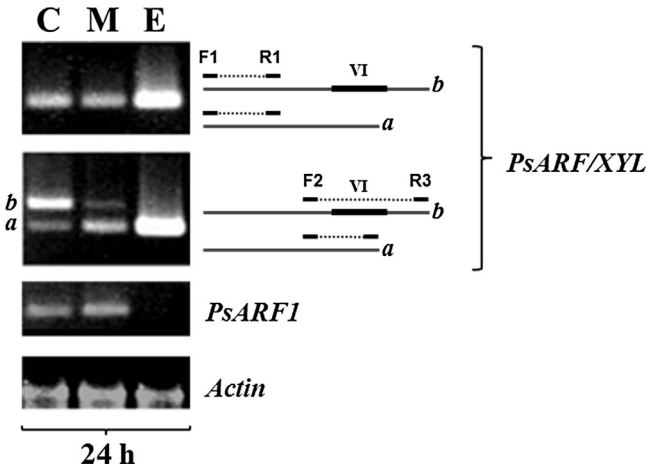

Fig. 8. *PsARF/XYL* and *PsARF1* expression profiles from mesocarp of preclimacteric 'Gigaglia' plums after ethylene or 1-MCP treatment. Harvested 72-DAFB fruits were exposed to 1 μL L^{-1} ethylene (E), 0.5 ppm 1-MCP (M), or a normal atmosphere (C) for 24 h at 20 °C. Total expression of *PsARF/XYL* was evaluated using F1 and R1 primers that span a sequence away from the retained intron VI (upper panel). Relative expression of *PsARF/XYLa* and *PsARF/XYLb* transcripts was evaluated with F2 and R3 primers that span retained-intron VI (middle panel). Total *PsARF1* expression was also assessed at the same experimental conditions (lower panel). Actin gene expression was assessed as an internal control of cDNA synthesis. Schematic representation of cDNAs amplified with F1-R1 and F2-R3 primers is indicated to the right of the expression panels.

PsARF/XYL and characterized. cDNA-genomic alignments showed that *PsARF/XYL* has seven exons and six introns. Sequence analysis suggested that retention of the last intron occurs alternatively in *PsARF/XYL* pre-mRNA producing both *PsARF/XYLb* (intron-retained transcript) and *PsARF/XYLa* (fully-spliced transcript). Although the copy number of *PsARF/XYL* in *P. salicina* genome has not yet been estimated, the coding sequence and the untranslated regions are identical in the *PsARF/XYLa* and *PsARF/XYLb* transcripts, strongly suggesting that the two transcripts originate from the same gene. *PsARF/XYLb* transcript harbored an in frame premature termination codon due to intron retention. The putative catalytic nucleophile, which is conserved in family 3 glycoside hydrolases [35], was predicted to be Asp-267 in the mature protein deduced from *PsARF/XYLa*. The two candidate amino acid residues for the catalytic acid/base of the mature protein of *PsARF/XYLa* were identified to be Glu-469, as proposed by Hrmova and Fincher [36], and Glu-471, in accordance with Lee et al. [37]. The proteins deduced from *PsARF/XYLa* and *PsARF/XYLb* have in common the putative amino acids of the active site previously described in GH family 3 [35,37]. Although *PsARF/XYLb* encodes a putative C-terminally truncated protein, it still contains the catalytic domain sequence that is a highly conserved feature common to all members of GH family 3. This suggests that *PsARF/XYLa* and *PsARF/XYLb* transcripts, if translated, could encode functional enzymes. Two GH family 3 enzymes purified from barley and alfalfa, which are closely related to the protein deduced from *PsARF/XYL*, are shorter at the C-terminus than predicted by their cDNA sequences [37,38]. Differences between the apparent molecular mass of the purified enzymes and that predicted from the corresponding cDNA sequence have been attributed to COOH-terminal processing that involves the removal of 130 amino acid residues from the primary translation

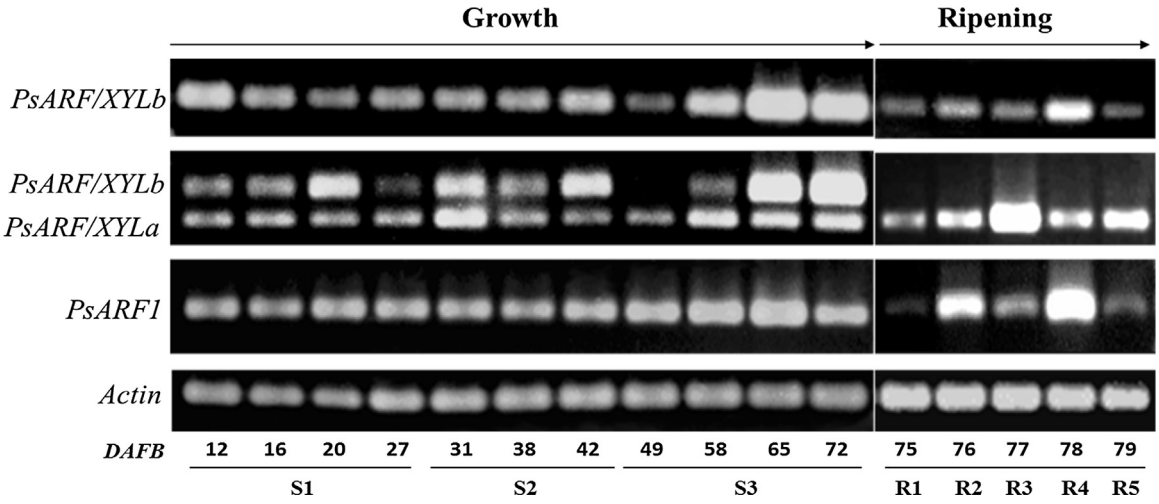

Fig. 7. *PsARF/XYLa*, *PsARF/XYLb* and *PsARF1* expression profiles during plum growth and ripening. RT-PCR reactions were performed using cDNA from developing plum mesocarp as template. Days after full bloom (DAFB) for each sample date are indicated. Specific *PsARF/XYLb* expression was analyzed using F1-Rin primers that anneal intron VI. *PsARF/XYLa* and *PsARF/XYLb* expression were analyzed using F2-R3 primers which span the region of included intron VI. Total *PsARF1* expression was also analyzed by L2-R2 primers. Actin gene was utilized as internal control of cDNA synthesis.

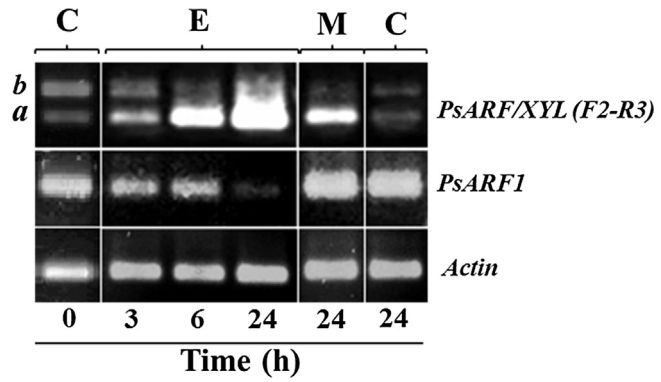

Fig. 9. Relative expression of *PsARF/XYL* transcripts (***a*** and ***b***) was evaluated in preclimacteric plums exposed to 1 $\mu L L^{-1}$ ethylene (E) for 3, 6 and 24 h. Expression profiles of 1-MCP-treated (M) and control plums (C) after 24 h were also assessed. *PsARF1* total expression was analyzed under the same experimental conditions. Actin gene expression was used as an internal control of cDNA synthesis.

product. Another example of such COOH-terminal processing has been described for an α-Araf/β-Xyl from immature radish seeds [39]. Taking into account the results of our study, the presence of alternatively spliced transcripts harboring in-frame PTCs could be another explanation for those observed differences. In fact, COOH-terminal processing described in barley, alfalfa and radish are in accordance with the truncation observed in the protein deduced from *PsARF/XYLb* as a consequence of the intron retention. However, the extent to which AS events play a role in shaping cell-wall architecture or regulating glycosidase expression has yet to be determined.

Phylogenetic analyses place the proteins deduced from *PsARF1* and *PsARF/XYL* in different clusters, within the plant GH family 51 and GH family 3, respectively. Amino acid sequence comparisons revealed that the predicted translation product from *PsARF1* shares 86% identity with that from *LeARF1*, another member of GH family 51. Tomato gene *LeARF1* is highly expressed during the early growth stages of the fruit and shows a drastic drop in its expression during ripening [40]. *LeARF1* may encode tomato β-Araf II, an enzyme that acts on tightly bound Na_2CO_3-soluble pectins during early growth [41]. Interestingly, both *LeARF1* [40] and *PsARF1* (this work) are negatively regulated by ethylene. On the other hand, BLASTX analysis revealed that the protein deduced from *PsARF/XYL* shares 85% identity with both PpARF2 [7] and MdAF3 [5], two enzymes potentially involved in different actions during fruit softening. PpARF2 is only detectable during Japanese pear ripening and found to be capable of releasing arabinose from native cell-wall polysaccharides prepared from *Pyrus pyrifolia* fruit [7]. MdAF3 was suggested to be the main contributor to α-Araf activity associated with apple mealiness, a cold-induced postharvest-related physiological disorder characterized by undesirable fruit softening and lack of juiciness [5]. Apparent parallels are not sufficient to suggest any mechanistic model as yet since fruit systems are diverse in terms of cell-wall architecture, constituent molecular changes and regulations; also, homologous proteins with phylogenetically close sequences do not necessarily evolve the same functions (e.g., identical substrate specificities or activities). On the other hand, members from families 3 and 51 may act coordinately under defective conditions in the arabinose metabolism. Tateishi et al. [42] have shown that the expression of gene *LeARF1*, which putatively codes for a GH family 51 member, increases in antisense fruit with suppressed expression of *SlArf/Xyl2*, a gene that encodes a GH family 3 enzyme. Also, the evidence that *PsARF1* and *PsARF/XYL* mRNA accumulate in fruit tissues during development is not enough to prove that the enzyme, if synthesized and effectively transported into the wall, comes into contact with its substrate and becomes involved in fruit cell-wall rearrangement or depolimerization. Nevertheless, *PsARF1* and *PsARF/XYL* are the only Japanese plum genes identified so far that could play a role in the cell-wall arabinose decrease that takes place in different domains during both fruit growth and ripening [3]. Changes in the arabinose/galactose ratio are particularly noticeable within the pectic fractions as the aging process occurs: a substantial loss of arabinose is more evident in the Na_2CO_3-soluble fraction – generally assumed to be enriched for covalently bound pectins – at S3. Conversely, ripening stages show an extensive decline in the arabinose content from the CDTA-soluble fraction, considered to be abundant for ionically bound pectins [3].

As depicted in the expression profiles of *PsARF/XYL*, *PsARF/XYLa* and *PsARF/XYLb* transcripts accumulate similarly during the initial phase of growth S1 and the lag phase of development S2. *PsARF/XYLb* transcripts are almost undetectable at the beginning of the exponential phase of growth (day 49) and show a remarkable accumulation towards the end of S3 (days 65–72). During plum ripening, *PsARF/XYLb* expression is negatively regulated whereas *PsARF/XYLa* expression is positively induced. Besides, *PsARF/XYLa* transcript accumulation parallels endogenous ethylene levels during plum ripening. Taken together, these results indicate that *PsARF/XYLa/PsARF/XYLb* ratio is regulated in a developmental-dependent manner. Ethylene is regarded as the main regulator of Japanese plum ripening and the accumulation of *PsARF/XYLa* transcripts is coincident with the autocatalytic ethylene production during ripening. Conversely, other factors may also play a role in *PsARF/XYLa* expression, since transcripts accumulate at different developmental times and organs even when ethylene biosynthesis is barely detectable. Apart from ethylene, there has been little research on other natural plant growth regulators and transcription factors that could play a role during Japanese plum ripening. Nevertheless, some theoretical considerations may be extrapolated from other *Prunus* drupes since a high degree of sequence conservation has been found within the *Prunus* genus. Auxin signaling has been reported in peach mesocarp during ripening, and some auxin receptors and auxin response factors showed to have enhanced expression during the ethylene-induced ripening process [43]. Besides the independent roles played by each plant growth regulator, an active interplay between auxin and ethylene was detected in peach, with genes in the auxin domain regulated by ethylene and genes in the ethylene domain regulated by auxin [43]. Different plant growth regulators may also be involved in a seed-pericarp cross-talk [44]. Auxins, cytokinins and gibberellins may be candidate regulators operating either directly or indirectly as signals during early developmental stages – when the organ cross-talk is more active – whereas ABA, known to play antagonistic roles with respect to auxin, may be potentially involved in hormone signals during the later stages of peach mesocarp development [44]. Nevertheless, a species-specific regulation cannot be discarded. In apricot, a considerable number of genes encoding IAA protein in action regulators and heat shock proteins were found to be highly up-regulated at early and late ripening stages but showed a different pattern from that in peach during the last stages of on-tree fruit development [45]. Together these observations suggest that *PsARF/XYLa* expression and ethylene influence may be modified through altered auxin or other hormone synthesis and/or signaling.

PsARF1 is highly expressed during fruit growth and decreases at R1, as previously described for tomato *LeARF1* [40]. Nevertheless, while *LeARF1* developmental expression declines even further as wild-type tomato fruit ripening progresses [40], *PsARF1* transcripts accumulate at specific stages during on-tree plum ripening, namely R2 and R4. Also, a drastic decrease in transcript abundance is apparent in R3, when the climacteric peak of ethylene production reaches its maximal value, and in R5 when fruit is fully ripe. On the other hand, it is clear that ethylene perception can negatively affect *PsARF1* gene expression under controlled conditions. Altogether,

these results do not question the responsiveness of *PsARF1* to ethylene but suggest that transcription of this gene may also be regulated by factors other than ethylene (*e.g.* auxins, gibberellins, or ABA). Further research is required to identify those factors involved in *PsARF1* transcription during on-tree ripening.

Two criteria have been defined to establish the role of ethylene in the regulation of specific gene expression: significant changes in mRNA levels should be induced at physiologically active concentrations of ethylene, and the response to ethylene should be fast so as to know whether changes in gene expression are a primary response to ethylene or the result of a cascade of events initiated by ethylene. In tomato, Lincoln et al. [46] showed that ethylene-regulated genes display a response at a level of increased mRNA accumulation within 8 h. Polygalacturonase mRNA was also detected within 6 h following ethylene treatment [47]. In our study, *PsARF1* mRNA decreased ~47% within a 3-h exposure to a physiologically active level of ethylene (1 $\mu L\,L^{-1}$), and almost 90% within a 24-h exposure. While a very faint signal for *PsARF1* gene expression was detected in Japanese plums after ethylene treatment, high-level expression of *PsARF1* was observed both in control fruit and in fruit treated with a single dose of 0.5 $\mu L\,L^{-1}$ 1-MCP for 24 h, similar to that described for tomato *LeARF1* [40]. This type of response suggests that *PsARF1* gene expression is highly sensitive to ethylene.

Also, treatment of preclimacteric *P. salicina* fruits with ethylene results in mRNA accumulation of the fully spliced form *PsARF/XYLa* and a decrease of the intron-retained transcript *PsARF/XYLb*. *PsARF/XYLa/PsARF/XYLb* ratio increased as much as 20-fold after a 6 h exposure to ethylene, and 24-fold after 24 h. Time and magnitude of this response strongly suggest that intron processing can be highly induced by ethylene during ripening. In 1-MCP treated fruit, *PsARF/XYLa/PsARF/XYLb* ratio also increased but to a lesser extent. Altogether, results suggest that the ratio between *PsARF/XYLa* and *PsARF/XYLb* transcripts is subjected to AS regulation by intron retention, and that this ratio is positively regulated by ethylene during ripening. Only a few studies have described AS events in ripening fruit from woody perennial species [15,16,22], or alternative spliced genes regulated by ethylene [48,49], or involved in ethylene-dependent developmental processes [50]. A critical question is whether the alternative transcript has a biological function in Japanese plum fruit. During ripening, it is less abundant than the fully spliced form *PsARF/XYLa*, as indicated by the weak bands on the gel electrophoresis of the RT-PCR, and could be targeted for non-sense-mediated mRNA decay, since more than one-third of AS events may be coupled with this type of RNA surveillance system [51]. However, it is possible that the generation of this transcript has a gene regulatory effect. Its synthesis could reduce the concentration of the functional transcript, thereby negatively regulating the gene expression. If the alternatively spliced transcript is translated, the modified protein may have novel characteristics, modifying its cellular roles or allowing it to take part in other molecular pathways.

Recent reviews have described the substantial insights been made into the mechanistic basis of ethylene biosynthesis, perception and signaling, as well as the identity of key regulators of ripening that act upstream of, or in concert with a regulatory pathway mediated by this plant growth regulator [52–54]. AS is one of the mechanisms to be considered as part of the entire transcriptome [55]. Although 22.6% of peach genes have been observed to undergo AS [19], the incidence of splicing events in the fruit ripening process has remained elusive, and the links that connect ethylene, AS and downstream processes are far from being fully understood. Here, evidence is shown that *PsARF/XYL* gene may be post-transcriptionally regulated by AS through intron retention during Japanese plum development and that ethylene may be involved in this regulation during ripening. Due to the taxonomic proximity of *Prunus* species, the phenomenon of intron retention in Japanese plum is probably as rare as reported for peach [19]. To obtain information from the reference peach genome released by the International Peach Genome Initiative (http://www.rosaceae.org/species/prunus_persica/genome_v1.0; [17]), BLAST searches were performed on the Genome Database for Rosaceae using the sequence of cDNA clone *PsARX/XYLa* as query. The presence of a possible ortholog in peach was determined (Id: ppa001718m.g, 98% of protein identity) which has a similar genomic organization and UTRs. However, no alternative transcripts corresponding to this locus were found in the genome annotations. In any case, a high-quality draft genome sequence of peach is available that allows to determine a large number of AS events [17], and peach genome sequencing can be combined with RNA-Seq, a powerful tool for transcriptomics, to provide an integrated view of gene expression and regulation in *Prunus* [18,19]. Thus, other similar events may become evident when the on-going large-scale transcriptome sequencing projects are completed that allow the identification of alternative intron splicing, an overlooked aspect in fruit ripening.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2014.12.001.

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