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Research article

α-L-Arabinofuranosidase from strawberry fruit: Cloning of three cDNAs, characterization of their expression and analysis of enzymatic activity in cultivars with contrasting firmness

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ABSTRACT

Softening of fleshy fruits during ripening is associated to catabolism of cell wall components. In strawberry, pectin degradation, as well as loss of neutral sugars (mainly arabinose), increases during ripening, and probably contributes to fruit softening. In this work, we report the activity of α-L-arabinofuranosidase (α-L-arafase) and the expression of related genes in strawberry. Activity of α-L-arafase was measured during ripening of cultivars with contrasting firmness. An important increment in the specific activity of α-L-arafase was detected during ripening in both cultivars. However, in the softest one (Toyonoka) the specific activities were higher than in the firmest (Camarosa). A combination of semi quantitative reverse transcriptase-PCR (RT-PCR) with degenerate primers and a screening of a cDNA library allowed the isolation and cloning of three cDNAs encoding putative α-L-arafases (*FaAra1*, *FaAra2* and *FaAra3*). The deduced proteins revealed that *FaAras* belong to the glycoside hydrolase family 51 and not to glycoside hydrolase family 3. Expression studies, carried out by means of Northern-blot and semi quantitative RT-PCR, revealed that *FaAras* were predominantly expressed in fruit tissue and detected over the entire ripening process. Due to similarity of *FaAras* sequences, Northern-blot analysis probably grouped the expression of the three genes. The expression was high at small green stage, decreased at white stage and increased thereafter. The increment of the expression from white to 50% red stage was more evident in the softest cultivar (Toyonoka). Semi quantitative RT-PCR analysis allowed determining the expression of individual *FaAras*. The expression of the three genes was detected in all developmental and ripening stages. However, differences in expression levels could be detected between cultivars. In the softest cultivar, the expression of the three *FaAras* was higher at 50% and 75% red stages, and in the case of *FaAra3* a higher expression was found also at 100% red stage. Overall, specific activity of α-L-arafase was higher in the softest cultivar; such activity reflects the expression of at least three putative *FaAra* genes.

1. Introduction

Development and ripening of fresh fruit involve modifications of the cell wall components leading to textural changes that contribute to decay and loss of quality. Primary cell walls present in fruit consist mainly of an assembly of cellulose and hemicelluloses embedded in a pectin-rich matrix, and their modifications are mediated by several cell wall metabolizing enzymes and proteins as well as other regulators including non-enzymic chemical species [27]. A common feature during ripening is the solubilisation and depolymerisation of some pectic and hemicellulosic polysaccharides [6,7,20,37]. Nevertheless, some of these events might be minimal or null in certain species [4].

Loss of neutral sugar residues from the cell wall is observed during ripening of different fruits, though the extent and the specificity of sugar loss is highly dependant on the species considered [16,18,32,33]. This loss occurs almost exclusively from complex pectins including arabinans and galactans as side chains, which are firmly bound to cell wall and can be extracted with concentrated alkali along with hemicelluloses [4]. Furthermore, it

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has been shown that pectin side chains can bind in vitro to cellulose and that the maximum adsorption affinity found was through debranched arabinans [50]. In strawberry, neutral sugar loss during ripening has been mainly attributed to loss of arabinose and galactose residues [18,24,32] and the solubilisation of pectic polymers has been associated to hydrolysis of residues present in their side chains [24].

The enzyme α-L-arafase (EC 3.2.1.55) catalyzes the hydrolysis of terminal non-reducing α-L-arabinofuranosil residues from various pectic and hemicellulosic homo- (arabinans) and heteropolysaccharides (arabinogalactans, arabinoxylans, arabinoxyloglucans, glucuronoarabinoxylans, etc.) as well as from different glycoconjugates [3,36,41]. Changes of α-L-arafase activity have been studied in several fruit: Japanese pear [43,44], European and Chinese pear [30], apple [17,48], apricot [9], peach [5,23], tomato [22,40], avocado [42], kaki [47], and banana [49]. In some of these fruits (Japanese pear, apple, apricot and banana) it has been proposed the association between α-L-arafase activity and the modification of the cell wall architecture during fruit softening. In this study, we have characterized the α-L-arafase activity and expression levels during development and ripening of strawberry fruit. In addition, three putative α-L-arafase coding cDNAs were isolated and the expression levels of their corresponding genes were analyzed.

2. Materials and methods

2.1. Plant material

Strawberry (*Fragaria x ananassa* Duch.) fruit were obtained from local producers (La Plata, Buenos Aires Province, Argentina). Two cultivars were selected by their different fruit firmness: Camarosa and Toyonoka [35]. Fruit were harvested at different ripening stages according to the external coloration degree and size: Small Green (SG), Large Green (LG), White (W), 50% red (50% R), 75% red (75% R) and 100% red (100% R). Other tissues were collected, including: petal (P), leaf (L), sepal (S) and achenes from LG and 75% R fruit. Samples were washed, drained and, after removing the calyx and peduncle, frozen with liquid nitrogen and stored at −80 °C until used.

2.2. Firmness

Fruit firmness was determined using a Texture Analyzer (TA.XT2, Stable Micro Systems Texture Technologies, Scarsdale, NY) fitted with a 3 mm flat probe. The fruit was penetrated 7 mm at a constant speed of 0.5 mm $s^{-1}$and the maximum force developed during the assay was recorded. Each fruit was measured twice in opposite sides of its equatorial zone and 30 berries of each cultivar, at each ripening stage, were assayed.

2.3. Determination of α-L-Arafase specific activity

Frozen strawberries (approximately 10 g) were homogenized in an Omni-Mixer (OCI Instruments) with 30 mL of 0.05 mol L^{-1} sodium acetate/acetic acid (pH 6.0), 1 mol L^{-1} NaCl, 0.05% (v/v) Triton X-100, 10 mmol L^{-1} EDTA, 2 mmol L^{-1} PMSF, 1% (w/v) PVPP. The mixture was incubated for 4 h at 4 °C in an orbital shaker, and then centrifuged at 10,000× *g* for 30 min. The supernatant was used to determine α-L-arafase activity, using 4-nitrophenyl-α-L-arabinofuranoside as substrate according to Tateishi et al. [43] with slight modifications. The following reaction mixture was prepared: 250 μL of 3 mmol L^{-1} 4-nitrophenyl-α-L-arabinofuranoside in 150 mmol L^{-1} citrate buffer (pH 4.5) and 300 μL of enzymatic extract. The mixture was incubated at 37 °C, aliquots of 130 μL were taken at 0, 15, 30 and 45 min, and the reaction was stopped by freezing with liquid nitrogen. Aliquots were stored at −20 °C until used for quantification. For the colorimetric assay, 50 μL of the reaction mixture were mixed with 150 μL of 0.4 mol L^{-1} Na_2CO_3. The amount of 4-nitrophenol released was determined by measuring the optical density at 410 nm using *p*-nitrophenol to perform a standard curve.

2.4. Size exclusion chromatography

Frozen Toyonoka fruit (approximately 30 g) in white and 100% R ripening stage were homogenized in an Omni-Mixer with 90 mL of buffer A (0.05 mol L^{-1} sodium acetate/acetic acid pH 6.0, 10 mmol L^{-1} cystein, 10 mmol L^{-1} EDTA, 2 mmol L^{-1} PMSF) with 1% (w/v) PVPP. The supernatant was discarded and the pellet was washed with 90 mL of buffer A and centrifuged for 15 min at 10,000× *g*. This step was repeated twice. The resulting pellet was extracted for 4 h under constant agitation with 50 mL of buffer A added with 10 mmol L^{-1} sodium azide, 1 mol L^{-1} NaCl and 0.05% (v/v) Triton X-100. After centrifugation, the supernatant was dialyzed against 0.05 mol L^{-1} sodium acetate/acetic acid (pH 6.0) and 10 mmol L^{-1} EDTA during 3 h, replacing the buffer with fresh one once. A first acetone precipitation was done at 20% (v/v) and the supernatant was precipitated at a final concentration of 60% (v/v). After centrifugation, the pellet was dissolved in 3 mL of buffer B (0.15 mol L^{-1} citric acid/sodium citrate buffer pH 4.5, 1 mol L^{-1} NaCl). An aliquot of 1.7 mL of protein extract was fractionated on a Sephacryl S-100 column (40 × 1.6 cm) pre-equilibrated in buffer B. The column was eluted with the same buffer at a flow rate of 0.4 mL min^{-1}. Fractions of 0.8 mL were collected and 200 μL of each fraction was utilized for assaying α-arabinofuranosidase activity at 37 °C.

2.5. Cloning of α-L-arafase cDNA

A pair of degenerate primers were designed for α-L-arabinofuranosidase with CODEHOP online program [34] (L2Ara and R2Ara 5′, Table 1) using plant protein sequences available in the GenBank. A cDNA library (Stratagene, La Jolla, CA, USA) constructed from 25–75% R strawberry fruit (cv Chandler) was used as template [11]. The temperature program was 1 cycle of 4 min at 95 °C; 30 cycles of 1 min at 95 °C, 1 min at 50 °C, 1 min at 72 °C; 1 cycle of 7 min at 72 °C. PCR product was analyzed on 1.2% (w/v) agarose gel, recovered using GFX kit (Amersham Biosciences, Little Chalfont Buckinghamshire, UK) and cloned into pGEM-T easy vector (Promega, Madison, WI). Once the sequence was determined using a sequencer Applied Biosystems ABI 377 (DNA Sequencing Service, Instituto de Investigaciones Biotecnológicas, Universidad Nacional

Table 1
Primers used for probe synthesis (library screening and Northern-blotting) and for semi quantitative RT-PCR.

Name	Sequence
L2Ara	5′-GACCAAAGGCTTTTGTTTCTGARTAYGCNGT-3′
R2Ara	5′-GAATAACCTTATTTGGTTCATTAAAAGAATTYTCRTCCAT-3′
LAra	5′-TCGGGCTTGAGAAAAACAGCG-3′
RAra	5′-GAGATAGAGGAAGAATCAGCG-3′
FaAra1L	5′-TCGGGCTTGAGAAAAACAGTG-3′
FaAra1R	5′-AGAGATAGAGGAAGAATTAGTG-3′
FaAra2L	5′-TCGGGCTTGAGAAAAACAGCG-3′
FaAra2R	5′-GAGATAGAGGAAGAATCAGCG-3′
FaAra3L	5′-GAGTACTTCCACTGTGTTCC-3′
FaAra3R	5′-GTGCAGAAAGTCCAACATCC-3′
Rib5	5′-ACCGTAGTAATTCTAGAGCT-3′
Rib3	5′-CCACTATCCTACCATCGAAA-3′

de General San Martín, Argentina), and its homology with other α-L-arabinofuranosidases was confirmed, this fragment was used to generate a probe for screening the cDNA library mentioned above. For screening, 4.2×10^5 pfu were plated and plaque lifts were performed with Hybond-N+ nylon membranes (Amersham, Pharmacia). The membranes were cross-linked with an UV-Stratalinker Model 1800 (Stratagene) and prehybridized for 4 h at 42 °C in a solution containing 50% (v/v) formamide, 6× SSPE, 5× Denhart's solution, 150 μg mL^{-1} denatured salmon sperm DNA, and 0.5% (w/v) SDS. Then, the ^{32}P-labeled probe was added, and the membranes were hybridized overnight at 42 °C. The membranes were washed once for 30 min at 42 °C and twice for 30 min at 50 °C in 25 mL of 1× SSC with 0.1% (w/v) SDS. The blot was exposed to X-ray film (X-OMAT AR, Kodak) with an intensifying screen at −80 °C, and the film was developed according to manufacturer's recommendation. Positive plaques were carried through two additional rounds of screening for purification. After this, phagemid DNA was excised and the clones were sequenced. Nucleotide and amino acid sequences were compared with the GenBank database using the Blast program [1]. Three clones of genes encoding putative α-L-arabinofuranosidases, named *FaAra1*, *FaAra2* and *FaAra3*, were obtained.

2.6. RNA isolation and Northern-blotting

Total RNA was isolated from fruit at different developing and ripening stages, petiole, leaf, sepal, LG and 75% R achenes by using the hot borate method [46]. Each RNA sample (10 μg) was analyzed by electrophoresis in a 1.1% (w/v) agarose and 1% (v/v) formaldehyde denaturing gel. After running, RNA was transferred to a Hybond-N+ nylon membrane (Amersham, Pharmacia), and cross-linked with a UV-Stratalinker Model 1800 (Stratagene). Membranes were prehybridized with 25 mL of hybridization solution at 42 °C for 4 h and then hybridized overnight at 42 °C with the denatured radiolabelled probe. As template for probe synthesis, a PCR product recovered from gel was used. In this case, the template was the 25%–75% R fruit cDNA library, mentioned above, and the primers used were LAra and RAra (Table 1). The probe hybridized with the three *FaAras* described in this work. The membranes were washed once at 42 °C and twice at 50 °C for 30 min each time in 25 mL of 1× SSC with 0.1% (w/v) SDS. The blot was exposed to X-ray film (X-OMAT AR, Kodak) with an intensifying screen at −80 °C, and the film was developed according to manufacturers' recommendation. rRNAs stained with ethidium bromide were used as loading control.

2.7. Semi quantitative RT-PCR

First strand of cDNA was obtained by using the following mixture: total RNA 1 μg, 0.03 mmol L^{-1} dNTPs, 1 μL Moloney murine leukemia virus RT (200 U μL^{-1}; Promega), 5 μL 5× reaction buffer (250 mmol L^{-1} Tris–HCl, 375 mmol L^{-1} KCl, 15 mmol L^{-1} $MgCl_2$, 50 mmol L^{-1} DTT, pH 8.3), 330 pmol of random primers (Biodynamics S.RL., Buenos Aires, Argentina) up to a total volume of 25 μL. The reaction mixture was incubated at 38 °C for 1.5 h. PCR amplification was done with 2.4 μL of reverse transcription reaction as template, 4 μL of 10× buffer Taq DNA polymerase (500 mmol L^{-1} KCl; 100 mmol L^{-1} Tris–HCl; 15 mmol L^{-1} $MgCl_2$, pH 9.0), 50 μmol L^{-1} dNTPs, 0.5 mmol L^{-1} $MgCl_2$, 1 U Taq DNA polymerase (Promega), and 0.1 μmol L^{-1} of primers. For each pair of primers, different concentrations of $MgCl_2$ and temperature gradients were performed using minipreps of each clone at a final concentration of 0.62 pmol L^{-1}, to find the PCR conditions in which no cross-amplification occurred. For *FaAra1* (primers FaAra1L and FaAra1R, Table 1) the temperature program was one cycle for 5 min at 95 °C; 23, 27, 31 or 35 cycles of 45 s at 95 °C, 1 min at 60.0 °C, 1 min at 72 °C; 1 cycle at 72 °C for 7 min. In the case of *FaAra2* (primers FaAra2L and FaAra2R, Table 1) the temperature program was one cycle for 5 min at 95 °C; 23, 27, 31 or 35 cycles of 45 s at 95 °C, 1 min at 62.5 °C, 1 min at 72 °C; 1 cycle at 72 °C for 7 min. For *FaAra3* (primers FaAra3L and FaAra3R, Table 1) the temperature program was one cycle for 5 min at 95 °C; 31, 34, 37 or 40 cycles 45 s at 95 °C, 1 min at 60.0 °C, 1 min at 72 °C; 1 cycle at 72 °C for 7 min. For template correction, 18S rRNA from strawberry was used (primers Rib5 and Rib3, Table 1) with 1.5 mmol L^{-1} $MgCl_2$, 50 μmol L^{-1} dNTPs and 0.3 μmol L^{-1} of each primer. Conditions of amplification were 5 min at 95 °C; 16, 20, 24 or 28 cycles of 45 s at 95 °C, 1 min at 62 °C, 1 min at 72 °C and 1 cycle of 7 min at 72 °C. PCR products were run in 0.7% (w/v) agarose gels. The gels were submerged in denaturalization solution (1.5 mol L^{-1} NaCl, 0.5 mol L^{-1} NaOH) during 30 min with gentle agitation and then in neutralization buffer (1.5 mol L^{-1} NaCl, 0.5 mol L^{-1} Tris–HCl pH 7.5) for 30 min. DNA was then transferred by capillary blotting onto a Hybond-N+ nylon membrane (Amersham Biosciences, Little Chalfont, UK) and cross-linked by UV irradiation at a dose of 7 μJ m^{-2} (UV-Stratalinker Model 1800, Stratagene). Membranes were prehybridized for 4 h at 42 °C in a solution containing 50% (v/v) formamide, 6× SSPE, 5× Denhart's solution, 150 μg mL^{-1} denatured salmon sperm DNA, and 0.5% (w/v) SDS. For *FaAra1* and *FaAra2* detection, a probe was prepared using a gel-recovered PCR product generated with L FaAra2, R FaAra2 (see above) and 25–75% R fruit cDNA library as template. In the case of *FaAra3* the template was generated using L FaAra3 and R FaAra3 as primers (see above) and the same cDNA library. Once the ^{32}P-labeled probe was added, hybridization was carried out overnight at 42 °C. Membranes were washed once for 30 min at 42 °C and twice for 30 min at 50 °C in 25 mL of 1× SSC with 0.1% (w/v) SDS. The blot was exposed to X-ray film (X-OMAT AR, Kodak) with an intensifying screen at −80 °C. The films were scanned and analyzed with Gel-Pro Analyzer program (Media Cybernetics, Maryland, USA) to determine in which cycles there was linear amplification. The cycles chosen were 24, 31, 31 and 37 for 18S RNA, *FaAra1*, *FaAra2* and *FaAra3*, respectively. New gels were made with loading corrections performed, after analysis of 18S RNA band intensity, to establish the expression pattern.

2.8. Sequence comparisons and phylogenetic analysis

An alignment of predicted *FaAra1*, *FaAra2* and *FaAra3* protein sequences with putative α-L-arabinofuranosidases from other fruits was made. The alignment was generated by ClustalW method [45] using GenomeNet service of Kyoto University homepage (http://align.genome.jp). For phylogenetic analysis, the same homepage was used to perform an alignment including predicted protein sequences from members of plant glycoside hydrolase families 3, 43 and 51. To generate phylogenetic tree, MEGA3 software was used [26]. The phylogeny reconstruction analysis was performed by Neighbor-Joining method, with amino Poisson correction model and bootstrap with 1025 replicates.

2.9. Statistic analysis

Fruit firmness and α-L-arafase specific activity was analyzed by ANOVA, and the means were compared by the LSD test at significance level of 0.05.

3. Results

3.1. Fruit firmness

For both strawberry cultivars, fruit softened rapidly as stages progressed to full maturity (Fig. 1). From the selected varieties,

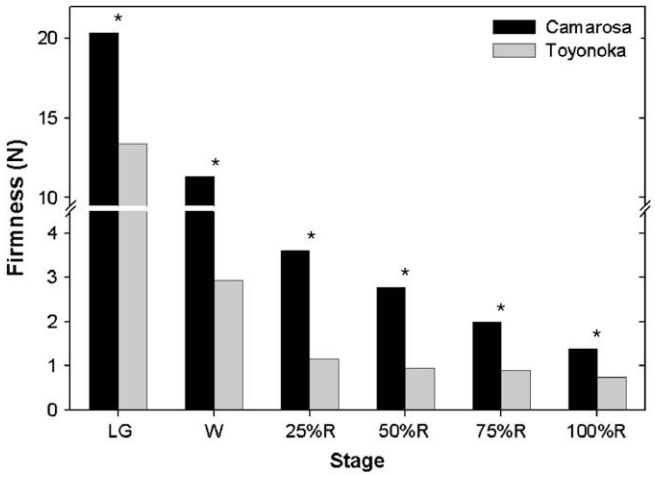

Fig. 1. Change of fruit firmness during ripening of two strawberry cultivars. Asterisks indicate significant differences when cultivars were compared at the same stage ($P = 0.05$).

Camarosa was the firmest at all development and ripening stages analyzed. The main firmness reduction in the softest cultivar (Toyonoka) was observed between large green (LG) and white (W) stages, while in Camarosa the main firmness decrease was found between W and 25% red (25% R) stages.

3.2. α-L-arafase specific activity

Preliminary data regarding the extraction method suggested a Zn^{2+}-dependance of α-L-arafases from different species [41] (and refs. therein). Consequently, extraction buffers containing or not 1 mmol L^{-1} $ZnCl_2$ were assayed to evaluate the requirement of Zn^{2+}. Simultaneously, the effect of the presence or not of 0.05% (v/v) Triton X-100 was also investigated. Clearly the detergent was essential for optimum extraction but the presence of $ZnCl_2$ did not affect such process (data not shown). Different extraction times were assayed (4 and 10 h), but no differences were found (data not shown).

Except for small green stage (SG), α-L-arafase specific activity was detected in all stages of both cultivars (Fig. 2). When progressing from W to 50% red (50% R) stages, both in Camarosa and Toyonoka a remarkable raise in the specific activity took place, which led to values that doubled those observed in W stage. Nevertheless, in Camarosa the activity level was nearly half of that found in Toyonoka through this progression as well as in the rest of the ripening process. The specific activity in Camarosa increased from W to 75% red (75% R) stages and then remained constant. In the case of Toyonoka, the specific activity increased continuously from W to 100% red (100% R) stages.

In tomato, different isoforms with α-L-arafase activity could be detected by size exclusion chromatography [41]. Considering this fact, we analyzed concentrated extracts from strawberry by using Sephacryl S-100. Protein extracts of Toyonoka cultivar at the beginning of ripening (W) and at an advanced ripening stage (100% R) were employed. A single broad peak was found ranging from 50 to 70 kDa and no shift in the size distribution was observed when ripening (Fig. 3).

3.3. Identification of FaAra1, FaAra2 and FaAra3

Degenerate primers were designed based on sequences of putative fruit α-L-arabinofuranosidases available in the GenBank. Most of these sequences belong to the family 51 of glycoside hydrolases. A PCR was performed using degenerate primers and a cDNA library from 25–75% R strawberry fruit as template. A 528 bp PCR product was cloned, sequenced and a high sequence homology with other fruit α-L-arafases present in the GenBank was found. Three positive clones encoding for three putative strawberry α-L-arafases were identified by hybridization of this PCR product to the strawberry fruit cDNA library. To minimize sequencing mistakes, several copies of each clone were sequenced at least 3 times.

The *FaAra1* clone was 2,328-bp long and contained a 142-bp 5′ untranslated region, an open reading frame of 1989, and 197 bp prior to the poly(A+) sequence. A 663-amino acid long immature protein was predicted for this clone.

FaAra2 clone was 2380-bp long, and contained a 254 bp 5′ untranslated region, an open reading frame of 1995, and a 131 bp prior to the poly(A+) sequence. The immature protein expected for this clone contained 665 amino acids.

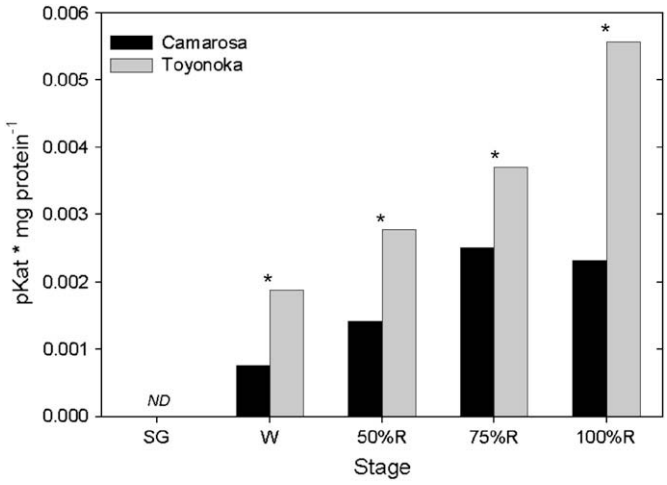

Fig. 2. Changes of α-L-arafase specific activity during development and ripening of strawberry. The assay was performed using 4-nitrophenyl-α-L-arabinofuranoside as substrate, and the activity expressed as pkat per mg of protein (pkat * mg protein^{-1}). Asterisks indicate significant differences when cultivars were compared at the same stage ($P = 0.05$). *ND*, not detected.

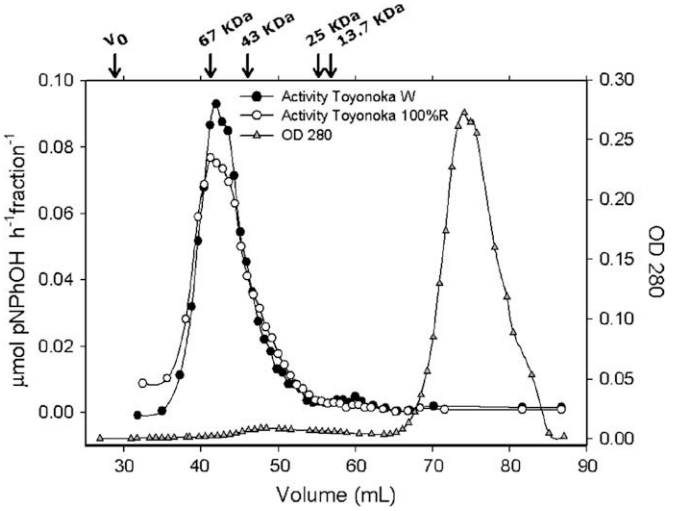

Fig. 3. Size exclusion chromatography of protein extracts with α-L-arafase acitvity in W and 100% R stages of Toyonoka cultivar. Elution standards include: bovine serum albumin (67 kDa), ovoalbumin from hen egg (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa), both from bovine pancreas. Fractions (800 µL) were collected and α-L-arafase was assayed as in Fig. 2. V_0, void volume.

A 2504-bp long clone of *FaAra3* was obtained. The sequence was analyzed and a 259 bp untranslated 5′ region was found. An open reading frame of 1446 bp was interrupted by a stop codon which was present in an insert of 45 bp that was not observed in the other two clones. After this insert, the sequence continued 549 bp until reaching a 211-bp long 3′ untranslated region. Translation of the larger open reading frame resulted in a 482-amino acid long immature protein. Noticeably, after the stop codon present in this sequence, translation in the same reading frame resulted in a sequence similar to those of the other two *FaAras*. It should be pointed out that the insertion found in *FaAra3* clone could be amplified, using one of the primers spanning this region and RT-PCR product as template.

The three *FaAras* had high sequence similarity in the coding region, nevertheless, clear differences were found at the 5′ UTR, especially at the region most distant from the beginning of the open reading frame. Differences among the three clones were also found at the 3′ UTR (data not shown).

FaAra sequences had similarity with other plant α-L-arafases present in the GenBank: *Prunus persica* (ABF22680), *Malus domestica* (AAP97437), *Pyrus communis* (BAF42035) and *Pyrus pyrifolia* (BAC99303), with identity percentage ranging from 78 to 84%.

From the deduced amino acid sequence, four N-glycosylation sites were predicted for *FaAra1* and *FaAra2*, and three sites for *FaAra3* (Fig. 4). These four sites were also found in the aligned α-L-arafases from other species. PSORT [31] as well as TargetP programs [14] predicted that the three putative proteins would be exported to the apoplast, indicating enzymes that would act on the cell wall. A signal peptide was predicted in the three proteins, and its cleavage would result in 70.5, 70.4 and 51 kDa proteins, for *FaAra1*, *FaAra2* and *FaAra3*, respectively.

Protein sequences were analyzed in order to find conserved domains or amino acids typical of this group of hydrolases. In the three *FaAras* a carbohydrate binding module (CMB) CMB_4_9 (pfam 02018) with an *E* value of 10^{-5} could be found, between the amino acids 60 and 224 for *FaAra1* and *FaAra3*, and between 62 and 226 for *FaAra2*. In *FaAra1* and *FaAra2* also an α-L-arafase C-terminus domain (Alpha-L-AF_C, approximately 200 residues long) was found, with an *E* value of 10^{-45}, between positions 450 and 638, and 452 and 640, respectively. Contrastingly, *FaAra3* contained a minimal portion of this domain. Nevertheless, both glutamic acid residues proposed as the catalytic ones [15] were found in *FaAra3* predicted mature protein as well as in the other two *FaAras* (Fig. 4).

A phylogenetic tree was built using alignments of accessions from plant glycoside hydrolase (GH) families 3, 43 and 51 (http://www.cazy.org, [12]) (Fig. 5). The deduced proteins revealed that the three *FaAras* were grouped into GH family 51 (http://afmb.cnrs-mrs.fr/CAZY) and not in GH family 3. In this case, members of GH 43 were included as an outgroup, since α-L-arafase sequence information can be found in this group [44] but it is clearly not closely related to GH 51.

3.4. Expression of FaAras in different stages and tissues

The expression of *FaAras* was analyzed by the mRNA abundance in vegetative tissues and in developing and ripening fruit. Due to sequence similarity, the probe used probably hybridized with mRNAs of the three genes studied, as well as with mRNAs of other putative *FaAras* that may not have been described yet. Hybridization was observed in 75% R receptacle, however only a diffuse signal was observed in other tissues (petal, sepal and achenes) analyzed (Fig. 6). Sepal was the tissue in which a slight signal was detected. This pattern suggests that the expression of *FaAras* prevails in fruit tissue.

When expression was studied over development and ripening (Fig. 7), hybridization was detected in all stages for both cultivars analyzed. A strong signal was detected in Camarosa at SG which decreased at W. A marked increase of intensity was found when turning from W to 50% R, but from then on, this increase continued with a smaller slope. In Toyonoka, the reduction of the intensity between SG and W was less drastic than in Camarosa, but the increase observed when turning from W to 50% R was higher. From 50% R to 100% R, the intensity in Toyonoka remained high and relatively constant.

3.5. Expression analysis by RT-PCR

In order to establish if the primers selected for the RT-PCR were specific, combinations of primers and templates (plasmids containing each clone) were assayed using the PCR conditions and number of cycles chosen for each pair of primers. No cross-amplification was detected even when the detection was performed with radiolabeled probes (data not shown). Expression of the three genes could be detected in all developmental and ripening stages analyzed for both cultivars (Fig. 8). The general expression profiles found were different, supporting the concept that three different putative *FaAras* cDNAs had been cloned.

Expression of *FaAra1* in the softest cultivar, Toyonoka, was found to be high at SG but the intensity diminished when turning to W stage. Between W and 75% R the intensity clearly raised, but decreased abruptly from 75% R to 100% R. The expression in Camarosa increased when turning from SG to W, and had a relatively constant level from W to 75% R but diminished at 100% R.

The relative band intensity for *FaAra2* in Camarosa decreased gradually along development and ripening. On the other hand, in Toyonoka a clear maximum was observed at 50% R, reaching higher values than in Camarosa.

In Camarosa, the higher intensity of *FaAra3* was found at W and thereafter the levels remained lower and constant until reaching 100% R. Toyonoka presented a different pattern with high levels at SG and a minimum at W. In this cultivar the expression increased until reaching full ripeness. From 50% R to 100% R, Toyonoka had higher intensities than those of Camarosa.

4. Discussion

To our knowledge this is the first report about α-L-arafase activity and expression in strawberry development and ripening. In other species, it was found that α-L-arafases are dependent on the presence of ions. In tomato, particularly, the isoform α-Af I was found to be Zn-dependent and probably associated with modification of the cell wall during tomato fruit growth [41]. In other systems, such as carrot cell cultures, the activity of α-L-arafase was stimulated by Zn^{2+} as well as by Ca^{2+} ions [25]. The same activation by these cations was observed when activity from soybean cotyledons was studied [19]. Though the specific role of Zn^{2+} on certain α-L-arafase isoforms is not clear, the main role of structural Zn^{2+} is to stabilize protein tertiary structures [41]. In our case, we found that α-L-arafase specific activity was not dependent on the presence of Zn^{+2} neither in the extraction buffer nor in the reaction medium.

We analyzed α-L-arafase specific activity along fruit development and ripening, and found a clear rise through these processes. This pattern was also monitored for an isoform of α-L-arafase in tomato [41], peach [5,23], apricot [9], and avocado [42], among others. In our case, we made a comparative study using two strawberry cultivars with contrasting fruit firmness. The activity values in the softest strawberry cultivar nearly doubled those of the firmest one (Fig. 2). *In vivo* substrates of α-L-arafase could be α-L-arabinofuranosyl residues of pectin side chains. Arabinose loss is first evidenced early during the ripening process. In other words, between W and pink stages the arabinose content diminishes in

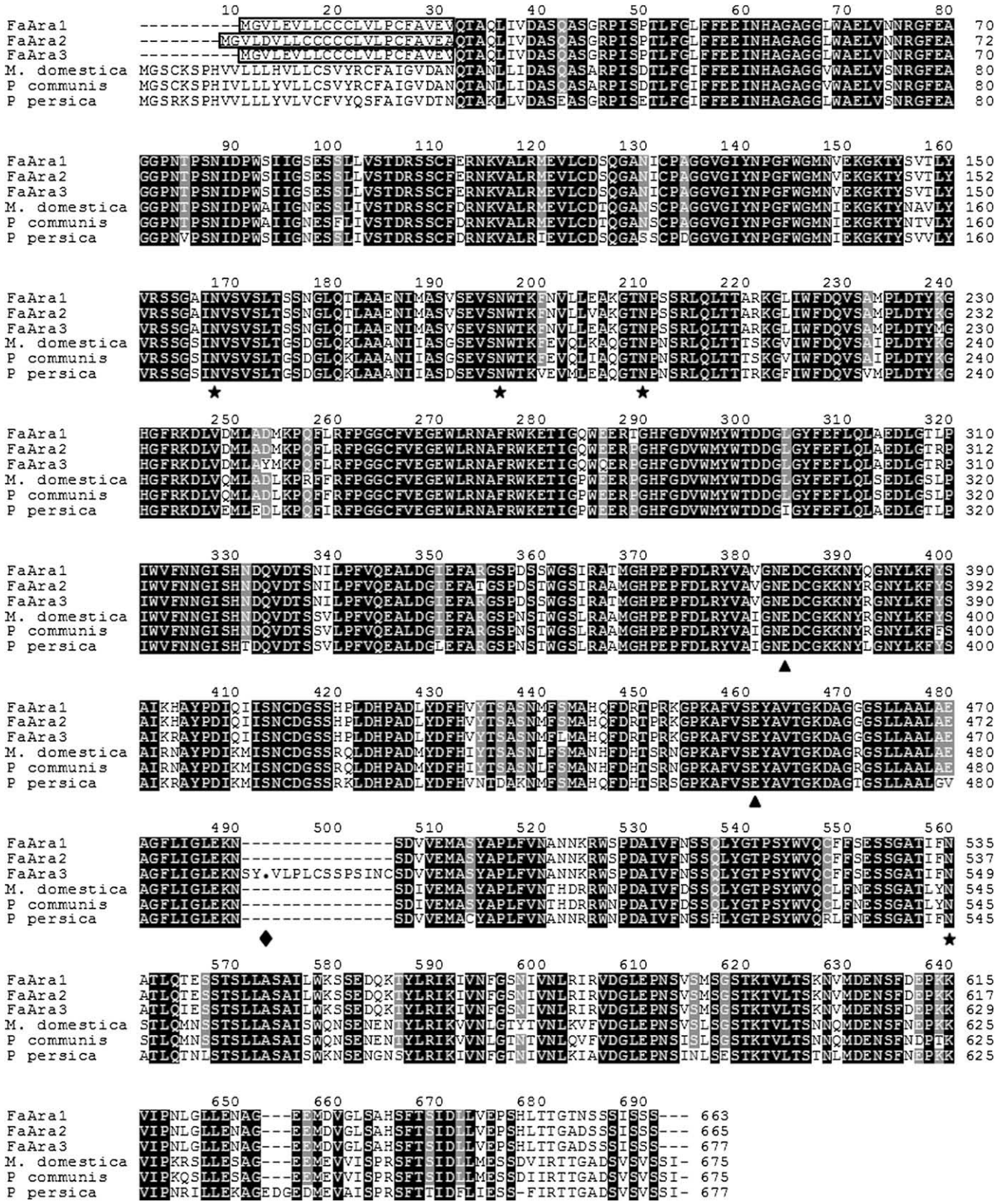

Fig. 4. Alignment of predicted FaAra1, FaAra2 and FaAra3 protein sequences with putative α-L-arabinofuranosidases from *Malus domestica* (AAP97437), *Pyrus communis* (BAF42035) and *Prunus persica* (ABF22680). The alignment was generated using ClustalW method [45]. Amino acids are numbered according to primary protein structure, with identical and similar amino acids represented with black and grey shaded boxes, respectively. Predicted FaAras signal peptide sequences are boxed. Symbols (stars and filled diamond) indicate putative N-glycosylation sites and the position of the stop codon present in *FaAra3*, respectively. The proposed catalytic acids Glu-374/Glu-376 and nucleophile residues Glu-451/Glu-453 are indicated by filled arrow heads.

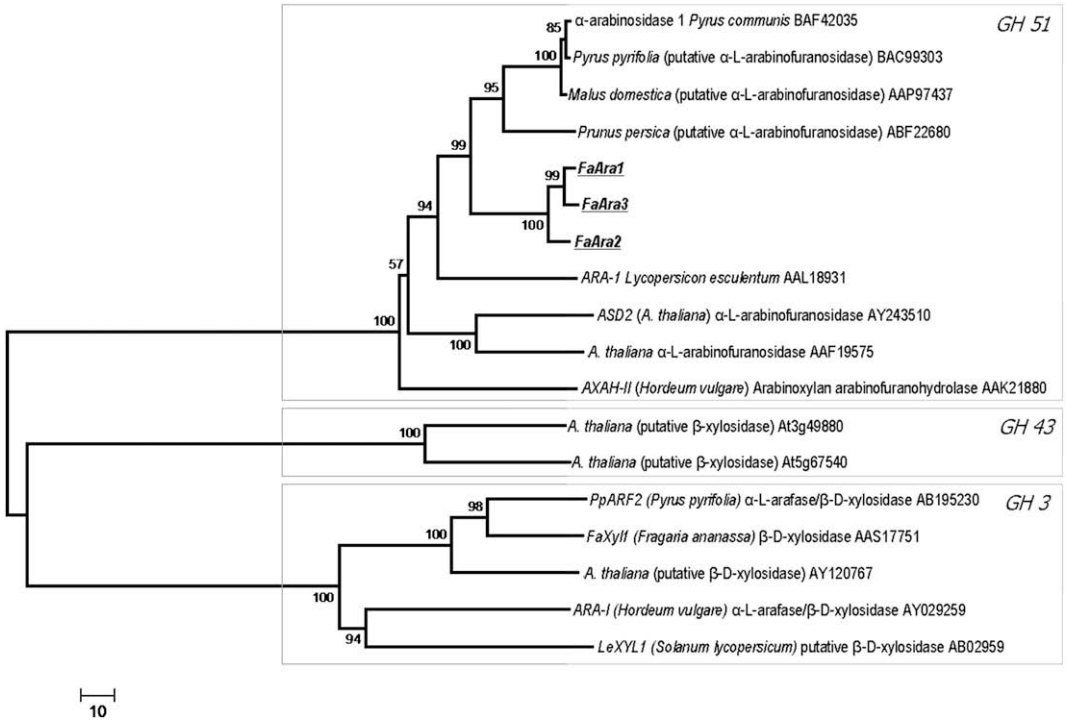

Fig. 5. Phylogenetic analysis including members of plant glycoside hydrolases families 3, 43 and 51. GenBank accession numbers are detailed beside each sequence name. The tree was constructed using ClustalW method [45] to generate the alignment subjected to a phylogeny reconstruction analysis with Neighbor-Joining method, amino Poisson correction model and bootstrap with 1025 replicates.

some of the cell wall fractions analyzed [18,24,32]. Particularly, it was described a decrease in the amount of these residues in covalently bound pectins during strawberry ripening. Under the light of these results it is not illogical to expect expression of related genes and activity of putative arabinose-releasing enzymes. The higher specific activity detected in Toyonoka cultivar is consistent with the fact that this cultivar had a more pronounced decrease in the amount of covalently bound pectins than Camarosa along ripening [35]. Different α-L-arafase activity profiles were also described in tomato when comparing different cultivars. When VF36 cultivar was analyzed, the activity increased as ripening proceeded [40]. On the other hand, in Ailsa Craig cultivar a clear diminution in the activity could be observed during development and ripening [22]. Mwaniki et al. [30] compared two pear types, European and Chinese, being the first one melting and the second one crispy. When analyzing crisp pear, no correlation could be established between α-L-arafase activity and softening. Nevertheless, in melting pear fruit, there was a correlation between α-L-arafase activity, climacteric ethylene production and the concomitant decrease in fruit flesh firmness.

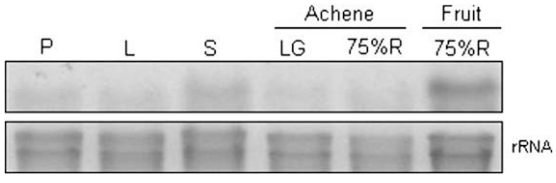

Fig. 6. Northern-blot hybridization analysis of *FaAras* in different tissues. Total RNA (10 μg) from petal (P), leaf (L), sepal (S), achenes from LG and 75% R fruit and 75% R fruit (as control) was electrophoresed and then hybridized with a radiolabeled probe that detected the three *FaAras* described in this work (see Materials and methods). rRNAs stained with ethidium bromide were used as loading control.

No α-L-arafase specific activity could be detected at very early developing stages (SG) in none of the cultivars studied, even when different extraction protocols were assayed. However, when we analyzed α-L-arafase expression by Northern-blot during ripening (Fig. 7), a strong hybridization was detected at SG stage for both cultivars. We hypothesize that there could be a post-transcriptional regulation, inhibitors' effect or technical difficulties that would be responsible for this discrepancy. Post-transcriptional regulation has also been suggested for different enzymes in other systems. Carrington et al. [10] studied LeMAN 4, an endo-(1,4)-β-mannanase, during tomato ripening. The corresponding mRNA levels were detectable in mature green stage, increased in breaker stage and remained at high levels thereafter. However, activity was almost undetectable in early stages and did not rise significantly until pink and red stages. The authors propose that possibly there would be a post-transcriptional regulation of this enzyme. More recently, Iglesias-Fernández et al. [21] studied a polygalacturonase gene from *Prunus domestica* (*Pd-PG1*) and found lack of correlation between gene expression and total PG activity that could be explained by the presence of more than one PG gene and/or a post-translational regulation.

Another possible post-translational regulation could be due to the presence of α-L-arafase inhibitors that remain active in the extracts used for activity assay. A glycoprotein inhibitor for pectin methylesterase was described in kiwi fruit [2]. Nonetheless, to date no natural α-L-arafase inhibitor has been reported. Some of these factors may explain the disagreement between gene expression and α-L-arafase specific activity; nevertheless, further experiments should be performed to confirm their incidence.

When α-L-arafase expression was analyzed in different tissues, no or weak signal was detected in non-fruit tissues, suggesting a fruit-predominant expression and that α-L-arafases might be involved in fruit cell wall modification during strawberry development and ripening.

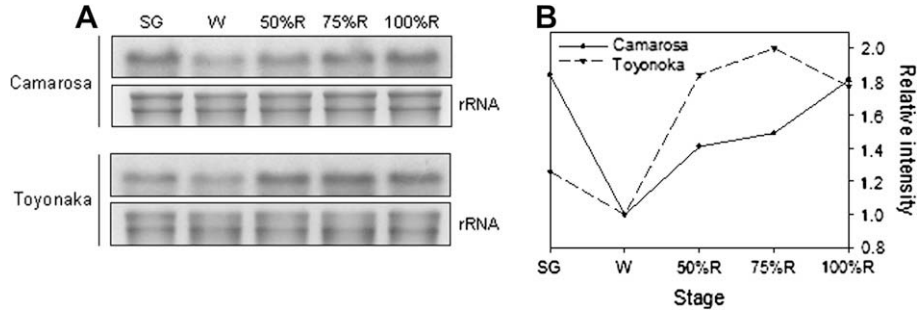

Fig. 7. Changes in the levels of transcripts hybridizing with *FaAras* probe and profile of relative intensity during fruit development and ripening. A, Northern-blot analysis for the two cultivars at different stages; Camarosa at the uppermost part and Toyonoka at the lowest. rRNAs stained with ethidium bromide were used as loading control. B, Signal intensities were determined with an imaging analyzer, and were made relative to the intensity found in the W stage in each cultivar.

We identified three putative strawberry α-L-arafases genes that presented sequence homology with α-L-arafases described in other species. Two of the putative α-L-arafases (*FaAra1* and *FaAra2*) when translated resulted in proteins that would be functional, but the other one (*FaAra3*) had a stop codon that would lead to a smaller protein containing a CBM, and part of the typical C-terminus of this family of hydrolases. Nevertheless, both putative catalytic acid and nucleophile residues were found in this smaller protein, so it would be possible that *FaAra3* were also a functional protein. Few sequence differences between coding regions of the three *FaAras* were found. However, untranslated regions of the three clones had important differences, and the analysis by semi quantitative RT-PCR revealed differences in the expression profiles of the three *FaAras* during ripening. Altogether, these results indicate that three different cDNAs were cloned, and that they are expressed during strawberry ripening. A similar case was reported by Shi et al. [39] working with β-1,3-glucanase genes from strawberry.

The calculated molecular mass of FaAra1 and FaAra2 based on cDNA sequences were approximately 70 kDa. For *FaAra3*, the predicted mature protein has a molecular mass of 51 kDa. Sozzi et al. [41], working with tomato fruit, could distinguish different isoforms using size exclusion chromatography. In our case, a broad size distribution was found ranging from 50 to 70 kDa with a maximum at 60 kDa, which did not allow resolving possible

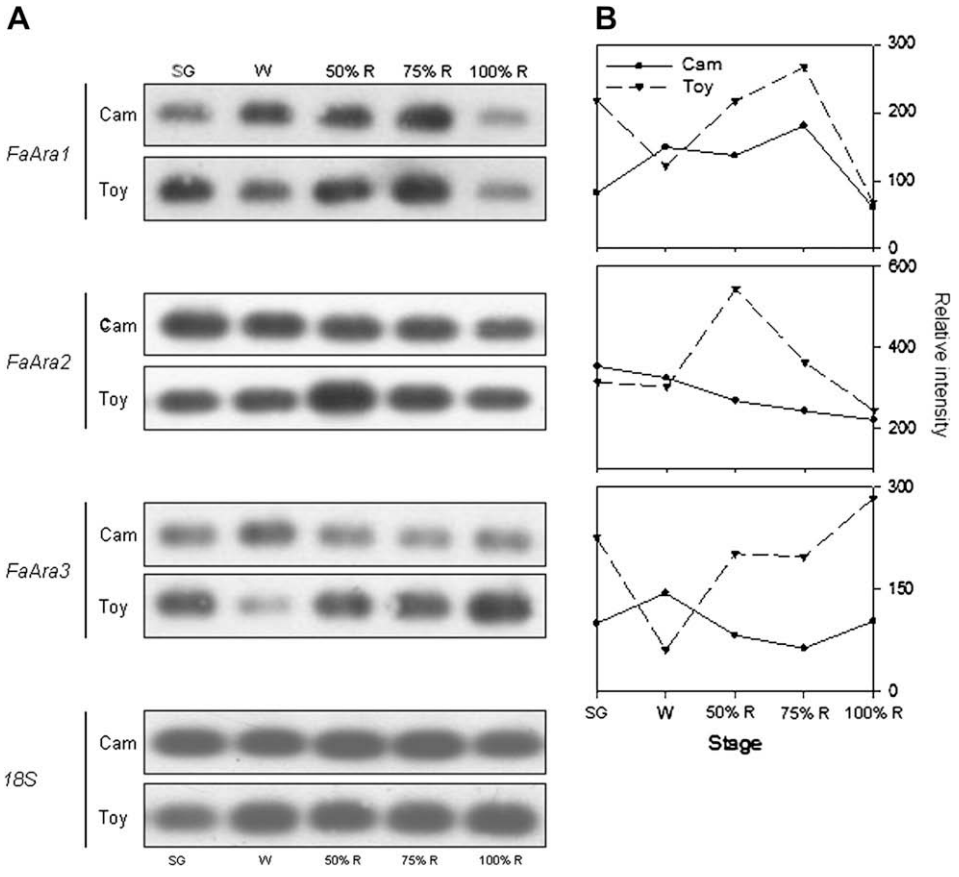

Fig. 8. RT-PCR analysis of *FaAras* expression during development and ripening of strawberry. A, Expression of *FaAra1*, *FaAra2* and *FaAra3* in different stages of Camarosa (Cam) and Toyonoka (Toy) strawberry cultivars. The bottom panel shows the expression of 18S gene, used as loading control. The cycles chosen were 24, 31, 31 and 37 for 18S RNA, *FaAra1*, *FaAra2* and *FaAra3*, respectively. B, Expression profiles for the three *FaAras*, showed as intensity of each band relative to the intensity of the corresponding loading control.

isoenzymes. Discrepancies between the molecular mass calculated for the mature protein and the molecular mass obtained in SDS-PAGE or size exclusion chromatography experiments was reported for other α-L-arafases and β-xylosidases [8,44]. Presumably there would be a post-translational cleavage at the C-terminal region that could explain the smaller sizes found in active proteins [28].

α-L-arafases were classified into five GH families (family 3, 43, 51, 54 and 62). In this work, degenerate primers were designed based on sequences of putative α-L-arafases belonging to the family 51 of glycoside hydrolases. As a consequence, the three cloned *FaAras* grouped with other α-L-arafases belonging to GH 51. Clearly, *FaAras* do not belong to the GH family 3 that includes β-xylosidases and bifunctional arabinofuranosidase/xylosidases. However, a member of GH 51, ARAf from *Arabidopsis thaliana*, has been proven to be active on different arabinose and xylose containing substrates, indicating that this enzyme has α-L-arafase/β-D-xylosidase activity [29]. Consequently, we cannot discard that the putative *FaAras* described in this work are also bifunctional. In a recent work, transgenic Arabidopsis plants with overexpression or suppression of a gene encoding for an α-L-arafase (ARAF1) that belongs to family 51 GH were analyzed [13]. The authors found that overall monosaccharide cell wall composition was only slightly altered in transgenic lines; nevertheless, in suppressed mutants an increase in arabinan associated signal (LM6 antibody signal) could be found. The authors suggested that arabinan-containing pectins are potential substrates for ARAF1 *in vivo*. Expression of genes of GH family 51 shows different patterns during ripening of different fruits. *LeARF1* from tomato was found to be expressed most highly during fruit development up to the time of formation of the mature green fruit. Thereafter, its expression declined markedly and was very low during the whole of fruit ripening [22]. Interestingly, *LeARF1* expression was negatively regulated by ethylene. In Chinese and European pear, the accumulation of *PcARF1* transcripts was detected at low levels during pre-ripening stage, but increased in both fruits during ripening [30]. Sekine et al. [38] analyzed several cell wall hydrolases from pear fruit (*P. communis*) with the purpose of linking their expression to the development of melting texture and softening. The authors found that *PcARF1* was constitutively expressed during ripening and proposed that this gene along with others might be involved in pear fruit softening, but not in the development of melting texture.

The analysis by RT-PCR performed on each putative gene indicated that transcripts of the three *FaAras* were present in both cultivars throughout development and ripening (Fig. 8), a fact that was consistent with the results obtained by Northern-blot. When analyzing the expression of each *FaAra* gene, differences were detected in the profiles between Camarosa and Toyonoka, mainly in *FaAra2* and *FaAra3*. While Camarosa had no significant expression rise for these transcripts during ripening, in Toyonoka a peak at 50% R for *FaAra2* and a clear rise for *FaAra3* was observed. Taking into account the expression of the three *FaAras* altogether, higher expression levels of *FaAras* can be detected in Toyonoka at the end of ripening (50%, 75% and 100% R), which correlates with the higher enzymatic activity found in this cultivar.

Finally, α-L-arafase specific activity was detected and was found to rise in strawberry during fruit ripening in both cultivars analyzed. This result is consistent with previous works where arabinose loss from cell walls during strawberry ripening was described [18,24,32]. The activity detected would reflect the expression of at least three putative *FaAra* genes (*FaAra1*, *FaAra2* and *FaAra3*) whose expression could be observed through all the development and ripening stages studied. Nevertheless in Toyonoka, the softest cultivar, the activity levels were higher than those found in Camarosa. This fact is in agreement with the findings that the levels of soluble pectins at the beginning of ripening were higher in Toyonoka cultivar [35]. These and previous results [18,24,32] would indicate that metabolism of arabinose could be of relevance in softening during strawberry fruit ripening.

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