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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′