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Original article

β-xylosidase activity and expression of a β-xylosidase gene during strawberry fruit ripening

Gustavo A. Martínez [a,*], Alicia R. Chaves [b], Pedro M. Civello [a]

[a] *Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús (IIB-INTECH) (CONICET-UNSAM), Camino de Circunvalación Laguna Km 6, 7130 Chascomús, Argentina*
[b] *Centro de Investigaciones en Criotecnología de Alimentos (CIDCA) (UNLP-CONICET), 47 y 116, 1900 La Plata, Argentina*

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Abstract

Strawberry fruit shows a marked softening during ripening and the process is associated with an increment of pectin solubility and a reduction of the molecular mass of hemicelluloses. In this work, we report the activity of β-xylosidase and the expression of a β-xylosidase gene in strawberry fruit. We have cloned a cDNA fragment encoding a putative β-xylosidase (*FaXyl1*) from a cDNA library obtained from ripe strawberry fruit. The analysis of the deduced amino acid sequence revealed that *FaXyl1* is closely related to other β-xylosidases from higher plants. The expression of *FaXyl1* was strongly associated to the receptacle tissue although a low expression level was detected in achenes and ovaries. The accumulation of *FaXyl1* mRNA is ripening-related, starting in white fruit, reaching the maximum at 25–50% red fruit and decreasing thereafter. The total β-xylosidase enzyme activity was detected in all ripening stages with the maximum in 25–50% red fruit. The low activity level detected in immature stages, where no expression of *FaXyl1* was found, suggests the presence of other β-xylosidases-like genes. Both the expression of *FaXyl1* and the total β-xylosidase activity were down regulated by auxins, as occurs for most of the ripening-related processes in strawberry fruit. A putative role of *FaXyl1* and β-xylosidase is discussed.

Keywords: Fruit ripening; Strawberry; Xylosidase

1. Introduction

Firmness is one of the main factors that determine the quality of fresh fruit. The disassembly of cell wall structure that occurs during fruit ripening apparently facilitates the attack of pathogens because ripened fruit show increased susceptibility to decay. Strawberry is a non-climacteric fruit showing a high softening rate, which contributes to its fast post-harvest decay. The biochemical basis of cell wall degradation in strawberry fruit has not been clearly established yet. The percentage of water-soluble pectins increases during strawberry ripening [12], although pectin depolymerization is slight [24], in accordance with the low polygalacturonase (PG) activity detected [23]. Redondo-Nevado et al. [30] reported a PG gene specific to strawberry fruit, but they suggested that the gene might be involved in the production of oligosaccharins, molecules involved in fruit ripening activation, rather than in the degradation of pectins. Nogata et al. [24] proposed that the pectin solubilization in strawberry could be due to the cleavage of pectin polymer side chains. Supporting this hypothesis, a decrease of cell wall of galactose and arabinose, which are usually located in pectin side chains, correlates with strawberry ripening [29] and a β-galactosidase-like gene with an enhanced expression in ripe strawberries was described [36]. Together, these data suggest that the cleavage of homogalacturonans does not play a significant role in softening of strawberry fruit. However, strawberry transgenic lines with antisense suppression of a putative pectate lyase gene, encoding an enzyme that would catalyze a β-elimination reaction that would cleave homogalacturonans showed a significant reduction of softening [14].

Abbreviations: NAA, naphthalene acetic acid; PG, polygalacturonase; PVPP, polyvinyl polypyrrolidone; SDS, sodium dodecyl sulfate.

* Corresponding author.
E-mail address: gmartinez@intech.gov.ar (G.A. Martínez).

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In contrast to pectin metabolism, the hemicelluloses of strawberry fruit show substantial reduction of molecular mass during ripening [12]. This finding correlates with the increased cellulase activity [1] and expression of endo β-1,4-glucanases genes [10,16] along the course of ripening. These results could suggest that hemicellulose depolymerization could be the main cause of softening in strawberries. However, strawberry transgenic lines with down-regulated expression of cel1, which encodes for an endo β-1,4-glucanase, showed no appreciable reduction of ripening-related fruit softening [40].

Xylose is an abundant component of plant cell wall, being present in both hemicelluloses and pectins. Hemicelluloses principally include xyloglucans, polymers of β-1,4-linked D-glucosyl residues substituted with α-linked D-xylosyl residue side groups and xylans and arabinoxylans, composed of a backbone of β-1,4-linked D-xylosyl residues, without or with arabinosyl residue side groups [5]. Xyloglucan backbones are probably degraded by endoglucanases [4], while xylans backbones are degraded by the action of endo β-1,4-xylanases, which cleave the β-1,4 glycosidic bonds between adjacent D-xylosyl residues to produce xylooligosaccharides which would then be further degraded by β-xylosidases (EC 3.2.1.37), which release single xylosyl residues from oligosaccharides [7]. Among pectins, xylose is present in xylogalacturonan, a polymer with an α-1,4-linked D-galactosyluronic acid backbone (homogalacturonan) with β-D-xylosyl residues attached to C-3 of the backbone residues [5].

Genes encoding xylanases and β-xylosidases have been extensively studied in fungal pathogens [19,25,37], while there is scarce information in plants. The presence of β-xylosidase has been reported in pears [2]; cucumbers [21,22] and wheat flour [7]; and the participation of both enzymes has been proposed to be implicated in the ripening of avocado fruit [31].

In strawberry fruit, xylose represents around 29% of hemicelluloses and is a minor component of neutral sugars in pectins [15,24]. Since the amount of hemicellulosic glucose is similar, it should be expected that 75% of xylose were α-xylose (xyloglucan component) and only 25% were β-xylose (xylan or xylogalacturonan). Thus, the β-xylose should be 7% of hemicellulosic sugars approximately. Although the percentage of β-xylose is low, it could be relevant in the structure of cell wall, as was demonstrated in *Arabidopsis thaliana* where down-regulation of a β-xylosidase gene (*AtBXL1)* induced alteration of the cell wall composition and plant development [9].

In the present paper, we report the expression pattern of a β-xylosidase putative gene (*FaXyl1*) and the total β-xylosidase enzyme activity during strawberry fruit ripening. We also describe the influence of auxins on both, the expression of *FaXyl1* gene and the total β-xylosidase activity in strawberry fruit.

2. Results and discussion

2.1. Identification of FaXyl1

FaXyl1 was isolated during the screening of a cDNA library from ripe strawberry fruit, trying to find the strawberry PG gene with an heterologous probe from tomato (fragment of *pTOM6,* probe 1). The search was successful and a clone encoding a putative PG was isolated (unpublished data). The screen using probe 1 identified another clone having high identity with reported β-xylosidases; this second clone was named *FaXyl1*. This unexpected hybridization between probe 1 and *FaXyl1* could be due to the low stringency used in the wash steps. *FaXyl1* had a size of 1699 bp, which is shorter than most of the cDNAs encoding β-xylosidases cloned to date (open reading frames of 2.1–2.5 kbp) [13,25,28]. Therefore, we assumed that the cloned insert probably did not contain the full-length cDNA of *FaXyl1*. The translation of *FaXyl1* over the three reading frames rendered a compatible amino acid sequence only in the third frame, which showed a 3′ untranslated region of 224 bp. Phylogenetic analysis of the deduced amino acid sequence of *FaXyl1* showed higher identities with sequences of β-xylosidases obtained from higher plants (*Prunus persica:* 79%; *Hordeum vulgare:* 41.8%; *A. thaliana:* 39.7%; *Oryza sativa:* 38.1%) and clearly diverged from those sequences belonging to fungi and oomycetes (Fig. 1). Moreover, the highest similarity was observed with the sequences reported in peach fruit [32]. In the phylogenetic analysis were also included several β-glucosidases, since the family 3 of glycosil hydrolases is formed not only by β-xylosidases but also by β-glucosidases. Clearly, *FaXyl1* has higher proximity to other β-xylosidases, rather than to the divergent group of β-glucosidases (Fig. 1). Conserved cysteine and tryptophan residues, which are present in β-xylosidases from higher plants, are present in the deduced amino acid se-

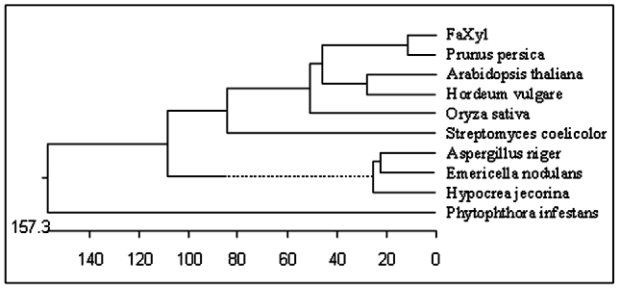

Fig. 1. Phylogenetic analysis of deduced amino acid sequences of β-xylosidases and β-glucosidases. The tree was constructed using the Megalign program, Clustal method, with a PAM250 residue weight table. The GenBank accession numbers of the sequences are: *A. thaliana* (1) (xylosidase): AAF17692; *A. thaliana* (2) (glucosidase): NM179517; *A. thaliana* (3) (glucosidase): AK117809; *Aspergillus niger:* CAB06417; *Emericella nodulans* (xlnD): Y13568; *Fragaria* x *ananassa* (FaXyl1): AY486104; *H. vulgare:* AYO29260; *Hypocrea jecorina* (bxll): CAA93248; *O. sativa:* BAB55751; *Phytophthora infestans* (BGX1): AF352032; *Polygonum tinctorium:* ABO03039; *P. persica* (PpAz152): ACO22521; *Ralstonia solanacearum:* NCO03295; *Secale cereale:* (AF293849); *Streptomyces coelicolor:* CAB55650; *Zea mays:* U33816.

quence of *FaXyl1,* as well as an amino acid residue (Asp-13) that is present in the active site of the β-glycosidase family [13]. Moreover, a conserved amino acid motif corresponding to a family of glycosyl hydrolases, is present at amino acids residues 118–306 (Fig. 2). Together, these sequence features strongly suggest that *FaXyl1* cDNA encodes a β-xylosidase protein. We are aware of only a few reports on purification or characterization β-xylosidases from fruit [31] and only six cDNAs encoding β-xylosidases have been cloned from higher plants, two of which are fruit specific of Japanese pear [13] and peach [32].

2.2. Expression of FaXyl1 in strawberry tissues and during ripening

Northern blot analysis of total RNA isolated from different strawberry tissues and ripening stages was used to evaluate the mRNA abundance of *FaXyl1*. Probe 2, prepared from a fragment of *FaXyl1,* showed a strong hybridization signal with mRNA present in 25% red fruit, while a faint signal was observed in ripe fruit, achenes and ovaries (Fig. 3). Instead, no expression of *FaXyl1* was detected in vegetative (root, leaf, stem) and reproductive (sepal, petal and stamen) tissues. This pattern suggests that the expression of *FaXyl1* is strongly related to fruit tissues, as it has been reported in Japanese pear [13].

The expression of *FaXyl1* was also analyzed in different ripening stages (Fig. 4). In this case, *FaXyl1* mRNA was absent in small and large green fruit and increased in white fruit, reaching the maximum at 25–50% red fruit and diminishing thereafter. A very low expression of *FaXyl1* was detected in ripe fruit. The expression pattern in ripening strawberry fruit is clearly different from those patterns described in Japanese pear and peach. In pear, the highest accumulation of β-xylosidase mRNA was found in overripe fruit [13]. Similarly, the gene encoding a peach fruit β-xylosidase is highly expressed in the last stages of ripening and also in senescing leaves [32]. Most of strawberry genes encoding cell wall degrading proteins show a maximum expression in ripe and overripe fruit. That is the case of endo β-1,4-glucanases [10,16,35,40], pectate lyase [20], β-galactosidase [36] and expansins [6,11]. However, the expression of *spG,* a strawberry PG gene [30], shows a maximum at white stage and then decreases in ripe strawberries. Similarly, *Faβgal3,* a β-galactosidase gene [36], has the highest expression in small and large green fruit with only a barely detectable amount of transcript in ripe fruit. The expression of different cell wall modifying proteins at different times is a very common pattern in fruit [4], which suggests that prior action of some of the enzymes is necessary before other enzymes can act on a give wall-localized polymer substrate or that different isoforms of a given activity play distinct roles at different developmental times. We do not know if there are additional β-xylosidase-encoding genes in strawberry.

2.3. Change of total β-xylosidase enzyme activity during ripening

The solubilization of β-xylosidase required the use of NaCl in the extraction buffer, suggesting that the enzyme could be cell wall-associated. The activity of β-xylosidase was detected in all the ripening stages analyzed (Fig. 5). From small green to white stage, approximately 0.1 nkat per gram of fruit were detected. The activity increased c.a. threefold in 25–50% red fruits and then decreased to initial values in ripe fruit. β-Xylosidase specific activity showed a similar trend, except that the increase of activity from small green to 25–50% red fruit was c.a. 4.4-fold. The total enzyme activity profile correlates with the expression of *FaXyl1* described above. At 25–50% red, both mRNA *FaXyl1* and total β-xylosidase enzyme activity showed the highest accumulation. The low enzyme activity levels found in immature stages, where no accumulation of *FaXyl1* mRNA was detected, could be due to the expression of other genes encoding β-xylosidases. Increasing β-xylosidase activity has been also detected during ripening of Japanese pear [34] and avocado fruit [31]. For avocado, the highest β-xylosidase activity coincides with the maximum ethylene production, with activity decreasing in more mature stages.

Strawberry fruit ripening is associated with a significant reduction in the average molecular size of hemicelluloses [12]. Several endo β-1,4-glucanase genes whose expression is fruit specific have been reported in strawberry. All have enhanced expression at the ripe fruit stage [10,16,35,40], which supports their probable role in degrading xyloglucans. In addition to xyloglucans, dicots have small amounts of other hemicellulosic polymers, including β-1,4-xylans. Although the amount of xylans in the strawberry fruit cell wall has not been directly determined, a high xylose:glucose ratio in the hemicellulosic fraction has been reported [12,15,24], suggesting the presence of xyl-containing polymers. Xylans are degraded by the combined action of β-1,4-xylanhydrolases, which act as endo-hydrolases, and β-xylosidases, which attack the non-reducing ends generated by xylanhydrolases and release xylose [7]. Based on the different patterns observed in the expression of β-xylosidase and endo β-1,4-glucanase in strawberry, it is possible to hypothesize that the depolymerization of xylans could start earlier than the degradation of xyloglucans along the fruit ripening.

In strawberry cell walls, the amount of xylose is higher in the hemicellulose fraction than in the pectin fraction [15], but a pectin polymer target for the β-xylosidase might be a branched pectin. Schols et al. [33] have demonstrated the presence of xylogalacturonans in apple fruit (another member of Rosaceae) and reported that single β-xylosyl residues could be released from the xylogalacturonan backbone upon treatment with a fungal β-xylosidase.

2.4. Effect of auxin on FaXyl1 expression and total β-xylosidase activity

The growth and ripening of strawberry, a non-climacteric fruit, is regulated by the auxins produced in the achenes.

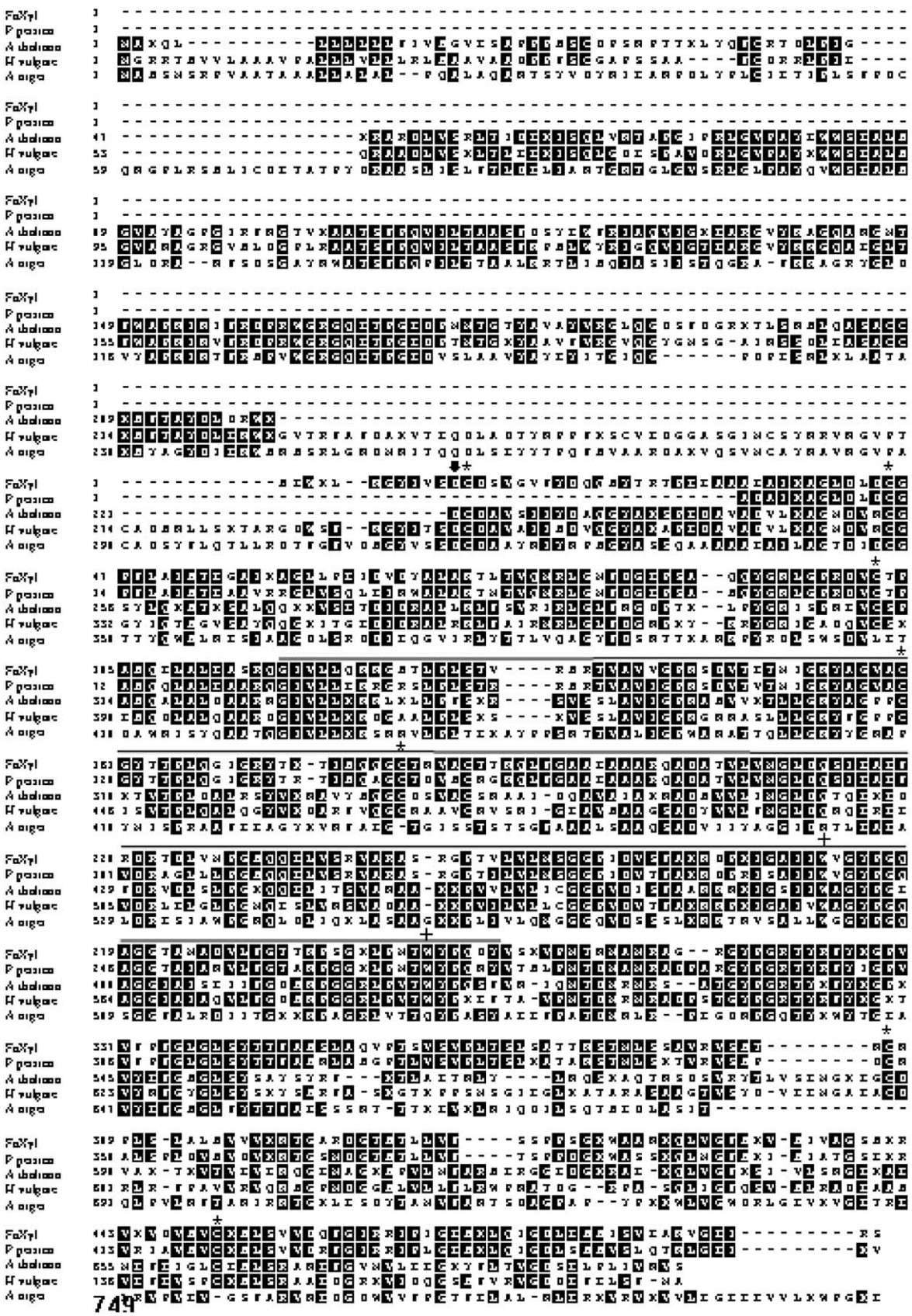

Fig. 2. Alignment and comparison of predicted amino acid sequence of *FaXyl1* and other β-xylosidases. The residues that are identical in at least two sequences are highlighted. The conserved active site "D" in the β-D-glucosidase family is marked by an arrow above the sequence. Conserved Cys and Trp residues are marked by an asterisk and a cross, respectively, above the sequence. A conserved amino acid motif of a family of glycosyl hydrolases is underlined.

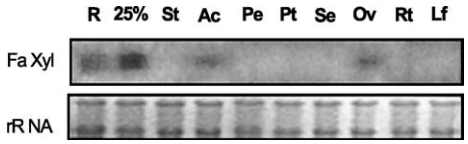

Fig. 3. Northern hybridization analysis of *FaXyl1* in different tissues. Total RNA (10 μg) from ripe fruit (R), 25% red fruit (25%), stamen (St), achenes (Ac), petals (Pe), petioles (Pt), sepals (Se), ovaries (Ov), roots (Rt) and leaves (Lf) was electrophoresed and then hybridized with radiolabeled probe 2. The blots 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 with an intensifying screen at –80 °C. The agarose gel was previously stained with ethidium bromide to check that there was approximately the same amount of RNA per lane (bottom panel).

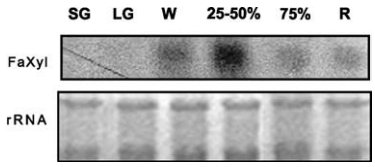

Fig. 4. Northern hybridization analysis of *FaXyl1* expression during strawberry fruit ripening. Total RNA (10 μg) from small green (SG), large green (LG), white (W), 25–50% red (25–50%), 75% red (75%), and ripe (R) fruit was electrophoresed and then hybridized with radiolabeled probe 2 and developed as described in the legend to Fig. 3.

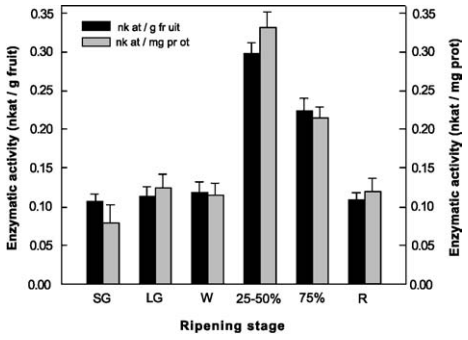

Fig. 5. Change of β-xylosidase activity during strawberry fruit ripening. Protein extracts were prepared from small green (SG), large green (LG), white (W), 25–50% red (25–50%), 75% red (75%), and ripe (R) fruit. Bars indicate standard deviations.

Auxins stimulate receptacle expansion during fruit development, and later inhibit fruit ripening [8,26]. As the strawberry fruit matures, a reduced tissue auxin concentration activates or de-represses the expression of ripening-related genes [17,18]. To evaluate the influence of auxins on *FaXyl1* expression and β-xylosidase activity we have eliminated the achenes or applied naphthalene acetic acid (NAA) to halves of white fruit. The treatment with NAA was chosen instead of 2,4 D or indole acetic acid due to its reported high effectiveness [10]. The progress of fruit ripening was followed by measuring the accumulation of anthocyanins (Fig. 6A). Untreated halves accumulated anthocyanin during incubation at 20 °C, and the final content was similar to that found in 75% red fruit. The NAA-treated halves showed lower anthocyanin accumulation than the corresponding controls. The absence of an endogenous auxin source after the elimination of the achenes in half of the fruit presumably removed the endogenous auxin source triggered ripening and increased anthocyanin synthesis in the deachened half. Northern-blot experiments were performed to evaluate the effect of auxins on *FaXyl1* expression (Fig. 6C). A clear increase in *FaXyl1* expression was detected in untreated halves after 3 days at 20 °C. In contrast, NAA-treated halves showed no increase in *FaXyl1* expression, with fruit maintaining an expression level similar to that detected in white fruit. The intact halves also showed an increase in the *FaXyl1* expression at 20 °C, but it was clearly lower than that detected in deachenated halves. Finally, the effect of auxins on total β-xylosidase activity is also analyzed (Fig. 6B). The activity of β-xylosidase increased in untreated halves during incubation at 20 °C, reaching a value comparable to that observed in 75% red fruit. A lower increase of β-xylosidase activity was found in the NAA-treated halves, reaching approximately 50% of the control activity. The elimination of the achenes increased the β-xylosidase activity in comparison with the value found in the achened fruit halves. Overall, these results indicated that auxin represses *FaXyl1* expression, fruit color development, and the increase of β-xylosidase activity. Similar results have been shown for other strawberry fruit genes related to cell wall disassembly, such as pectate lyase [20], endo β-1,4-glucanase [35], PG [30] and β-galactosidase [36]. However, the expression of *FaXyl1* increases only in

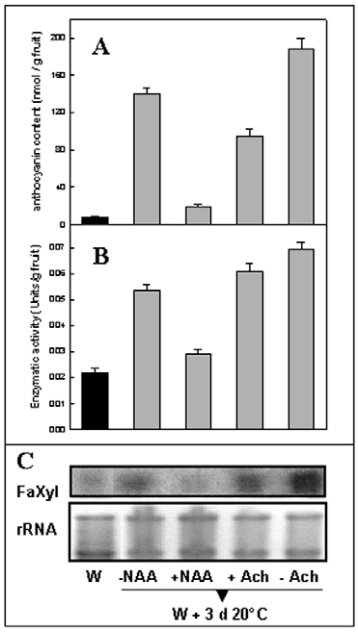

Fig. 6. Effect of auxin treatment and elimination of achenes on anthocyanin content (A), β-xylosidase activity (B) and *FaXyl1* expression (C). Halves of white fruit (W) were treated with NAA and incubated for 3 days at 20 °C (+NAA), maintaining the non-treated halves as control (–NAA). Similarly, halves of white fruit (W) were deachened and incubated in the same conditions (–Ach), maintaining the achened halves as controls (+Ach). After 3 days of incubation, the half fruit samples corresponding to each treatment were analyzed in relation to their anthocyanin content and β-xylosidase activity. Also, RNA (10 μg) from the half fruit samples was extracted and analyzed by northern hybridization with *FaXyl1*. An ethidium bromide stained RNA gel was used as a control (bottom panel). Bars indicate standard deviations.

white and 25–50% red fruit and decreases in more advanced ripening stages where auxin level is even lower. This particular expression pattern suggests that other regulation signal would contribute with the auxins in the regulation of *FaXyl1* and β-galactosidase enzyme activity.

3. Conclusion

The total β-xylosidase activity and the expression of *FaXyl1* (a fruit specific putative β-xylosidase gene) show a ripening-related pattern and a down-regulation by auxins. Although these results suggest a possible role of *FaXyl1* in fruit ripening, additional works are required to establish the in vivo substrates (xylans or xylogalacturonans) of the encoded protein and to determine whether the enzyme has a role in fruit softening.

4. Methods

4.1. Plant material

Strawberry fruits (*Fragaria* x *ananassa,* Duch., cv Selva), grown in greenhouses, were obtained from local producers. Fruit were harvested and classified according to external coloration and size into different ripening stages: small green (SG), large green (LG), white (W), 25% red (25% R), 50% red (50% R), 75% red (75% R) and ripe (R). The peduncle and calyx were removed, and the fruit were immediately used or frozen in liquid nitrogen and stored at –80 °C.

4.2. cDNA library screening

A cDNA library constructed from ripe strawberry fruit (cv Chandler) was screened with the original goal of searching for PG, using a heterologous probe from tomato. The library contained cDNAs cloned into the Uni-ZAP XR vector (Stratagene, La Jolla, CA, USA). For screening, 4.2×10^5 pfu were plated and plaque lifts were performed with Hybond-N+ nylon membranes (Amersham-Pharmacia). Then, the membranes were fixed by incubation for 2 h at 80 °C and cross-linked with an UV-Stratalinker Model 1800 (Stratagene). The 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. Then, the solution was replaced with a fresh aliquot labeled probe 1 (below) was added, and the membranes were hybridized overnight at 42 °C. The hybridized 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.

4.3. Nucleotide sequencing and analysis

DNA was completely sequenced using T3, T7 and internal primers. A sequencer Perkin-Elmer Applied Biosystems 377 with a 3700 and 3100 capillary column was used (HHMI Biopolymer and W.M. Keck Biotechnology Resource Laboratory, Yale University). The sequencing reactions utilized fluorescently labeled dideoxynucleotides (Big Dye Terminators) and Taq FS DNA polymerase in a thermal cycling protocol. Sequence analyses were carried out using the EditSeq and Megalign programs included in the DNASTAR 4.05 software package. Nucleotide and amino acid sequences were compared with the GenBank database using the Blast program [3]. A glycosyl hydrolase domain was identified in the GeneBank database using the Conserved Domain Database with Reverse Position Specific BLAST [3]. A phylogenetic analysis of deduced amino acid sequences was performed with the Megalign program, Clustal method of the DNASTAR 4.05 software package, with a PAM250 residue weight table.

4.4. Preparation of probes

Probe 1 was prepared from a cDNA clone of PG from tomato fruit (*pTOM6*). This clone was cut with *Hin*dIII and the fragment of approximately 740 bp was purified and used as a template in a random primer labeling reaction using [^{32}P]dATP. This probe was used for the screening of the cDNA library. Probe 2 was prepared from the isolated cDNA clone of β-xylosidase (*FaXyl1*). The restriction of this clone with *Eco*RI produced a fragment of approximately 800 bp that was purified and used as a template in a random primer labeling reaction using [^{32}P]dATP. Probe 2 was used in northern blotting.

4.5. RNA isolation and northern blotting

Total RNA was isolated from frozen fruit and other tissues by using the hot borate method [38]. Each RNA sample (10 μg) was analyzed by electrophoresis in a 1.1% (w/v) agarose and 1% (v/v) formaldehyde denaturing gel. To ensure that equal amounts of RNA per lane were loaded, gels were stained with ethidium bromide and individual lanes evaluated for comparable fluorescence levels upon exposure to a short UV light source. After running, RNA was transferred to a Hybond-N+ nylon membrane (Amersham-Pharmacia Biotech UK, Buckinghamshire, UK), fixed by incubation for 2 h at 80 °C 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 radiolabeled probe 2. 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.

4.6. Auxin treatment

The auxin treatment was performed with fruit halves, maintaining the other halves as controls. Sixteen fruit at the white stage were used in each experiment. In a set of experiments, the achenes were removed from one half of the surface of each fruit using a sharp forceps, maintaining the other half as control. In other experiments, a lanolin paste containing 1 mM NAA and 1% (v/v) dimethyl sulfoxide was applied over one half of the surface of each fruit, while a similar paste without NAA was applied over the other half as a control. The peduncle of each fruit was immersed in a microcentrifuge tube containing distilled water to avoid dehydration, and then the fruits were incubated for 3 days at 20 °C. After the treatment, the calyx and peduncle were removed, and the treated and control halves were cut apart, frozen in liquid nitrogen and stored at –80 °C until use. The samples from each treatment were analyzed in relation to their anthocyanin content, total β-xylosidase activity and *FaXyl1* expression.

4.7. Enzymatic activity assay

Fresh or frozen strawberries (10 g) were homogenized in an Omnimixer with 30 ml of the following extraction buffer: 0.05 M sodium acetate/acetic acid (pH 6.0), 1 M NaCl, 1% (w/v) PVPP. The mixture was left under stirring for 2 h and then centrifuged at 9000 × *g* for 30 min. The supernatant was used to determine β-xylosidase (EC 3.2.1.37) activity, using *p*-nitrophenyl β-D-xylopyranoside as substrate according to Cleemput et al. [7], with slight modifications. The following reaction mixture was prepared: 5 mM *p*-nitrophenyl β-D-xylopyranoside, 1 M NaCl, 0.05 M sodium acetate/acetic acid (pH 6.0), enzymatic extract 750 μl in a total volume of 1500 μl. The mixture was incubated at 55 °C and the reaction was stopped mixing 150 μl of the mixture with 500 μl of 1% (w/v) Trizma base solution. In the control reactions, 750 μl of buffer 0.05 M sodium acetate/acetic acid (pH 6.0) plus 1 M NaCl was added instead of enzymatic extract. The amount of *p*-nitrophenol released was determined measuring the optical density at 410 nm and comparing with a calibration curve prepared from *p*-nitrophenol.

4.8. Measurement of anthocyanin content

Frozen fruit were ground with mortar and pestle in the presence of liquid nitrogen. Approximately 0.3 g of the resultant powder was poured into 3 ml of HCl–methanol (1%, v/v) and held at 0 °C for 10 min. The slurry was centrifuged at 1500 × *g* at 4 °C for 10 min, the supernatant was saved and its absorbance at 515 nm was measured. The amount of anthocyanins was expressed as nmole of pelargonidine-3-glucoside per gram of fruit, by using $E_{molar} = 36{,}000\ M^{-1}\ cm^{-1}$ [39].

4.9. Protein dosage

The extract protein concentration was measured by the modified Lowry method described by Potty [27], using bovine albumin as standard.

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