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Cloning of the promoter region of β-xylosidase (*FaXyl1*) gene and effect of plant growth regulators on the expression of *FaXyl1* in strawberry fruit

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ABSTRACT

Expression and activity of cell wall modifying enzymes involved in fruit softening may be regulated by hormones and/or other signal molecules. In strawberry, *FaXyl1* encodes for a fruit-specific β-xylosidase probably associated to hemicellulose degradation. In this work, we have isolated and analysed the promoter region of *FaXyl1* gene. Analysis of the sequence revealed the presence of *cis*-acting elements associated with hormone, light and stress-related responses. Several treatments were done on fruit in order to prove the responsiveness of *FaXyl1* to plant growth regulators related to the regulatory elements identified in the promoter region (abscisic acid, auxins and gibberellins) and others associated with the ripening process (ethylene and nitric oxide). The effect of each treatment on *FaXyl1* expression, the corresponding protein levels and the β-xylosidase activity was evaluated. ABA (abscisic acid) stimulated *FaXyl1* expression and protein levels. In contrast, expression levels of *FaXyl1* gene decreased after treatments with NAA (naphthalene acetic acid), GA_3 (gibberellic acid) and ethephon, an ethylene-generating compound. SNP (sodium nitroprusside), a NO donor, did not affect *FaXyl1* mRNA and protein levels. The effect of 1-MCP (1-methylcyclopropene), an ethylene perception inhibitor, on *FaXyl1* expression was consistent with the effect observed with ethephon. β-xylosidase activity was down regulated by NAA, whereas GA_3, ABA, ethephon, 1-MCP and SNP had no effect on it.

1. Introduction

Fruit ripening is a complex process that involves numerous changes in colour, aroma, flavour and texture of the flesh. Texture changes occur through modifications of pectins, hemicelluloses, cellulose and proteins that compose the cell wall. These modifications could be mediated by expansins and hydrolases, the latter affecting mostly selected glycosidic bonds present in cell wall polysaccharides [1]. The participation of these enzymes during ripening is reflected in changes in the physical properties and molecular composition of the cell wall [2].

Strawberry is characterized by high softening rate, short post-harvest life and fast decay. Ripening of this fruit is associated with an increase of pectin solubility and a reduction of hemicellulose content [3]. Hemicelluloses mainly include xyloglucans and xylans. The latter consist of β-D-xylopyranosyl residues that form a core backbone, which may be substituted with α-L-arabinofuranosyl (arabinoxylans) and, to a lesser extent, with α-D-glucuronic acid (glucuronarabinoxylans) residues [4]. Xylan degradation occurs through the coordinated action of several enzymes, including the endo-β-1,4-xylanases (EC 3.2.1.8), which cleave the β-1,4-glycosidic bonds between D-xylose residues in the main chain to produce xylooligosaccharides, and β-xylosidases (EC 3.2.1.37), which cleave xylooligosaccharides to release xylose [5]. Although genes encoding for fungal β-xylosidases have received much attention, little is known about the possible role of these enzymes during fruit ripening. The activity of β-xylosidase enzyme has been reported in stone fruits, olive, tomato and Japanese pear [6–9], and it has been proposed that the enzyme is involved in the ripening process of avocado and strawberry fruits [10–12].

Strawberry is considered as non-climacteric fruit, since it does not exhibit a peak in respiration rate and ethylene production [13]. However, recent studies have put in doubt this statement. Ethylene production was detected during ripening, but its influence is not fully understood [14]. Strawberries can ripen without exogenous ethylene treatment, but exogenous ethylene

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Abbreviations: 1-MCP, 1-methylcyclopropene; ABA, abscisic acid; ACC, aminocyclopropane-1-carboxylic acid; CTAB, hexadecyltrimethyl-ammonium bromide; EDTA, ethylenediaminetetraacetic acid; GA_3, gibberellic acid; NAA, naphthalene acetic acid; NO, nitric oxide; SDS, sodium dodecyl sulfate; SNP, sodium nitroprusside; SSC, salt sodium citrate.

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induces the acceleration of fruit colour development and softening [14,15]. In addition, Trainotti et al. [13] cloned and characterized several genes involved in ethylene metabolism and suggested a possible involvement of this hormone in strawberry ripening. On the contrary, a clear role was established for auxin in relation to their effects on ripening. Auxins are produced by the achenes and are the key phytohormones controlling the growth and ripening of strawberry receptacles. Auxin stimulates receptacle expansion during fruit development, and later inhibits fruit ripening [16]. As strawberry fruit ripens, the diminution of auxin level activates the expression of ripening-related genes [17]. Earlier studies on the physiology of strawberry ripening have indicated that gibberellic acid (GA_3), abscisic acid (ABA) and nitric oxide (NO) could also modulate ripening [18–20].

In strawberry, the hormonal regulation of genes encoding cell wall modifying enzymes has not been elucidated in detail. *FaXyl1* encodes a fruit-specific β-xylosidase, an enzyme associated with cell wall disassembly, which could be implicated in strawberry fruit softening [12]. In this work, we isolated and analysed the promoter region of *FaXyl1* gene in order to find putative hormone response elements and study the effects of different hormone treatments on *FaXyl1* expression, protein levels and β-xylosidase activity.

2. Materials and methods

2.1. Plant material and hormone treatments

Strawberry fruit (*Fragaria* × *ananassa*, cv. Camarosa) were obtained from local producers (La Plata, Buenos Aires Province, Argentina). Fruit were harvested at the white stage with intact peduncles, sorted on the basis of size and absence of physical damage, and randomly divided into lots of at least 10 fruit for each treatment. The effects of hormone treatments were evaluated by utilizing the *in vitro* ripening assay described by Given et al. [16], with slight modifications. Fruit peduncles were trimmed to a uniform length of 3 cm and immersed in ca. 1.5 mL of hormone solution held in a microcentrifuge tube. For naphthalene acetic acid (NAA) and gibberellic acid (GA_3) treatments, peduncles were immersed in 1 mmol L^{-1} NAA and 1 mmol L^{-1} GA_3 for 3 d at 20 °C, respectively. Peduncles of control fruit were immersed in distilled water. For abscisic acid (ABA) treatments, peduncles of treated fruit were immersed in a solution containing 1 mmol L^{-1} ABA in 2% (v/v) ethanol for 3 d at 20 °C, while the controls were prepared by immersing the peduncles in a solution of 2% (v/v) ethanol. Treatments with ethephon, an ethylene-generating compound (2 mmol L^{-1} ethephon with 0.02% (v/v) Tween 20 and 1% (v/v) ethanol, 5 min), and SNP, a NO donor (5 μmol L^{-1} SNP, 2 h), were made as fruit dips. Fruit were then air-dried and stored at 20 °C for 2 d with the peduncle of each fruit immersed in distilled water to avoid dehydration. Control fruit for ethephon and SNP treatments were dipped in 0.02% (v/v) Tween 20 with 1% (v/v) ethanol and water, respectively and stored as above. In the case of 1-methylcyclopropene (1-MCP), a competitive inhibitor of ethylene action, fruit were treated with 1 μL L^{-1} 1-MCP in 80 L sealed jars for 10 h at 20 °C; after treatment, the jars were vented to air and the fruit stored for 2 d at the same temperature, with the peduncle immersed in distilled water to avoid dehydration. Control fruit were kept at similar conditions but in absence of the inhibitor. After each treatment, the calyx and peduncle were removed and treated and control fruit were frozen in liquid nitrogen and stored at −80 °C until use. The samples from each treatment were analysed in relation to their anthocyanin content, *FaXyl1* expression, the levels of the corresponding protein and β-xylosidase activity.

2.2. RNA isolation and Northern blotting

Total RNA was isolated from frozen fruit using the method described by Chang et al. [21]. Each RNA sample (10 μg) was analysed 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), 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 ^{32}P-labelled probe. 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.

2.3. Probe preparation

The β-xylosidase probe was prepared from the cDNA clone of *FaXyl1* (GenBank accession no. AY486104). The restriction of this clone with EcoRI produced a fragment of approximately 800 bp that was purified from the gel with GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences) and used as a template in a random priming labelling reaction using [^{32}P]dATP.

2.4. Western blot

Frozen strawberries (3 g) were homogenized in an Omnimixer with 3 volumes of 50 mmol L^{-1} Tris–HCl (pH 7.0), 2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 1 mmol L^{-1} EDTA, 5% (w/v) sucrose, and 1% (w/v) polyvinylpolypyrrolidone. The suspension was stirred for 40 min and centrifuged at 9000 × *g* for 30 min at 4 °C. The supernatant fraction was added with 0.1 volumes of 100% (w/v) trichloroacetic acid and incubated for 30 min at 4 °C. The suspension was centrifuged at 9000 × *g* for 5 min, the supernatant was discarded and the protein pellet was dissolved in 0.1 mol L^{-1} sodium hydroxide and 1% (w/v) SDS. Extracts containing 10 μg of proteins were separated by SDS-PAGE using 12% (w/v) polyacrylamide gels [22] and electroblotted onto nitrocellulose membranes. Immunodetection was carried out with the ECL Western blotting analysis system (Amersham-Pharmacia) by using a 1:1000 dilution of the *Fragaria* × *ananassa* FaXyl1 antibody [12].

2.5. Enzymatic activity assay

Frozen strawberries (5 g) were homogenized in an Omnimixer with 15 mL of the following extraction buffer: 0.05 mol L^{-1} sodium acetate/acetic acid (pH 6.0), 1 mol L^{-1} 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 activity, using *p*-nitrophenyl β-D-xylopyranoside as substrate. The following reaction mixture was prepared: 5 mmol L^{-1} *p*-nitrophenyl β-D-xylopyranoside, 1 mol L^{-1} NaCl, 0.05 mol L^{-1} sodium acetate/acetic acid (pH 6.0), 750 μL of enzymatic extract in a total volume of 1500 μL. The mixture was incubated at 55 °C, aliquots of 150 μL were taken at different times and the reaction was stopped by adding 500 μL of 1% (w/v) Trizma base solution. In the control reactions, 750 μL of buffer 0.05 mol L^{-1} sodium acetate/acetic acid (pH 6.0) plus 1 mol L^{-1} 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. β-xylosidase activity was expressed as nmol of *p*-nitrophenol released per second and per kilogram of fruit.

2.6. Anthocyanin content determination

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 1% (v/v) hydrochloric acid–methanol 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 calculated by using E_{molar} = 36,000 L mol^{-1} cm^{-1} [23]. Results were expressed as a percent of the anthocyanin content obtained in control fruit. Four replicates were done per each treatment analysed.

2.7. Genomic DNA extraction

Total DNA was isolated from young strawberry leaves (cv. Toyonoka). Frozen fruit was ground with mortar and pestle in the presence of liquid nitrogen. Approximately 1 g of the resultant powder was poured into 10 mL of pre-warmed (65 °C) extraction buffer containing 3% (w/v) CTAB, 2% (w/v) polyvinylpyrrolidone, 1 mol L^{-1} Tris–HCl (pH 8.0), 20 mmol L^{-1} EDTA, 1.4 mol L^{-1} NaCl and 2% (v/v) 2-mercaptoethanol, mixed and incubated for 30 min at 65 °C with occasional mixing. The slurry was added with 1 volume of chloroform/isoamyl alcohol (24/1, v/v), mixed by inversion, and centrifuged for 10 min at 7500 × *g*. The upper phase was recovered and 1 volume of isopropanol was added and incubated for 2 h at −20 °C. The suspension was centrifuged for 10 min at 7500 × *g* and the pellet was dried and dissolved in 100 μL of deionized H_2O at 65 °C; 1 μL of 10 mg mL^{-1} RNAse was added and incubated at 37 °C for 15 min. The slurry was added with 100 μL of chloroform/isoamyl alcohol (24/1, v/v) and 100 μL of phenol and centrifuged for 10 min at 7500 × *g*. The supernatant was precipitated with 2.5 volumes of ethanol at −20 °C for 2 h and centrifuged at 7500 × *g* for 20 min. The pellet was air dried and dissolved in 35 μL of deionized H_2O at 65 °C. The integrity of genomic DNA was assessed by resolving DNA samples on a 0.7% (w/v) agarose gel by electrophoresis, followed by visualization with ethidium bromide staining.

2.8. Promoter cloning

Promoter region of the *FaXyl1* gene was cloned from strawberry genomic DNA by using the BD GenomeWalker™ Universal Kit (Clontech). Four libraries were made by digesting the high molecular weight DNA with *DraI*, *EcoRV*, *PvuII*, and *StuI*, followed by DNA purification, and ligation of genomic DNA to BD GenomeWalker™ adaptors according to the manufacturer's instructions. The Genome Walker libraries were used to amplify DNA upstream of the known *FaXyl1* sequence by using GSP1 primer (5'-CTTCTCCTGCAGTGTCAGCCGTCCGAT-3') and AP1 primer included in the kit and Platinum Taq DNA Polymerase High Fidelity (Invitrogen) according to the manufacturer recommendations. A nested PCR was performed using GSP2 primer (5'-GACGCGCATGTACCAGATTAAACAAGA-3') and AP2 primer (kit primer) as suggested in the manual. The PCR products were separated on a 1.5% (w/v) agarose gel and the dominant bands were excised and purified using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences). The purified products were cloned into TOPO TA Cloning Vector (Invitrogen) and sequenced using M13 forward and reverse primers.

2.9. DNA sequencing and bioinformatics analysis

DNA was sequenced by primer walking using T7, M13 reverse and internal primers. An Applied Biosystems ABI 377 sequencer was used (DNA Sequencing Service, Instituto de Investigaciones Biotecnológicas, UNSAM, Argentina). Sequence analyses were carried out using the Edit-Seq and SeqMan Programs included in the DNASTAR 4.05 software package. Identification of putative *cis*-acting elements was performed using PLANTCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PLACE (http://www.dna.affrc.go.jp/PLACE/) software.

2.10. Statistical analysis

Data of anthocyanin content and β-xylosidase specific activity were analysed by Student's *t*-test at a significance level of 0.05.

3. Results and discussion

3.1. Cloning and sequence features of the FaXyl1 promoter region

A fragment of 1825 bp (Genbank accession no. EU736110) of the region upstream of the translation start codon of *FaXyl1* was isolated from strawberry genomic DNA, and a promoter motif search of the *pFaXyl1* sequence was performed to reveal putative *cis* elements as indicated [24,25]. The sequence analysis detected the presence of a putative TATA-box at −61 bp from the ATG start codon and a number of CAAT-boxes. Potential regulatory elements associated with hormone, light and stress-related responses were also found in the *FaXyl1* promoter region (Table 1). Putative hormone responsive elements identified in the *FaXyl1* promoter region include an ABRE motif (involved in the abscisic acid responsiveness), an AuxRR-core (involved in auxin responsiveness), a GARE motif (gibberellin-responsive element) and a TCA-element (involved in salicylic acid responsiveness). The *cis*-acting elements involved in light responses include an ACE motif, four G-box sites, an MRE motif and an Sp1 motif. In addition, the *FaXyl1* promoter sequence contains a number of *cis*-elements related to stress responses, included an HSE motif (involved in heat stress responses), an LTR motif (involved in low-temperature responses), an MBS site (MYB binding site involved in drought-induction) and TC-rich repeats (involved in defence and stress responses). Finally, we detected a *cis*-acting element that confers high transcription levels (5UTR Py-rich stretch) (Fig. 1). The presence of these putative *cis*-elements indicates that the *FaXyl1* gene could be regulated by physiological (hormones) and environmental (light and stress) factors. With the aim to prove the responsiveness of *FaXyl1* to plant growth regulators, we investigated the expression levels of *FaXyl1* under several exogenous treatments, including the hormones related to elements identified in the *FaXyl1* promoter region (auxins, gibberellic acid, and abscisic acid) and others involved in the ripening process (ethylene and NO, provided by ethephon and SNP treatment, respectively).

3.2. Auxin and gibberellic acid treatments

As strawberry fruit mature, auxin levels decline in the receptacle, probably due to the decrease of auxin transport from the achenes. This triggers the ripening process, and it causes important changes in fruit properties such as pigmentation and texture loss [16]. The reduced auxin level is also accompanied by *de novo* synthesis of specific mRNAs [17]. Sequence analysis of the *FaXyl1* promoter region led to the identification of a putative AuxRR-core (auxin-responsive element) at position −443 relative to the ATG initiation codon, which consists of a 7 bp motif

Table 1
Main regulatory motifs found within the promoter sequence of *FaXyl1* gene.

Motif	Function	Strand	Distance from ATG	Sequence
5UTR Py-rich stretch	*Cis*-acting element conferring high transcription levels	+	−283	TTTCTTCTCT
		+	−550	
ABRE	*Cis*-acting element involved in ABA responsiveness	+	−168	TACGTG
ACE	*Cis*-acting element involved in light responsiveness	+	−167	ACGTGGA
AuxRR-core	*Cis*-acting regulatory element involved in auxin responsiveness	+	−443	GGTCCAT
G-box	*Cis*-acting regulatory element involved in light responsiveness	+	−168	TACGTG
		−	−168	CACGTA
		−	−833	CACGAC
		−	−315	TAACACGTAG
GARE-motif	Gibberellin-responsive element	−	−266	AAACAGA
HSE	*Cis*-acting element involved in heat stress responsiveness	−	−1561	AGAAAATTCG
		−	−1394	AAAAAATTTC
LTR	*Cis*-acting element involved in low-temperature responsiveness	+	−1545	CCGAAA
		+	−1683	
MBS	MYB binding site involved in drought-inducibility	+	−318	CAACTG
MRE	MYB binding site involved in light responsiveness	−	−662	AACCTAA
Sp1	Light responsive element	+	−329	CC(G/A)CCC
		+	−1295	
TATA-box	Core promoter element around −30 of transcription start	+	−61	TATATAA
TC-reach repeats	*Cis*-acting element involved in defence and stress responsiveness	+	−1558	ATTTTCTTCA
TCA-element	*Cis*-acting element involved in Salicylic acid responsiveness	+	−430	CAATCTTTTT
CAAT-box	Common *cis*-acting element in promoter and enhancer regions	Strand + (number) 23		Strand − (number) 10

with the sequence GGTCCAT (Table 1). To evaluate the responsiveness of *FaXyl1* gene to auxins, we applied NAA, a synthetic auxin, on white fruit. Effect of NAA on ripening was evaluated through the accumulation of anthocyanins after 3 days of storage. Treatment with NAA clearly delayed the anthocyanin accumulation (Fig. 2A) and led to reduced β-xylosidase activity (Fig. 2B) in relation to the corresponding control. Western and Northern blot experiments were performed to evaluate the effect of auxins on FaXyl1 protein levels (Fig. 2C) and *FaXyl1* gene expression (Fig. 2D). After 3 days at 20 °C, it was detected a lower amount of mRNA in treated fruit in relation to controls. Moreover, FaXyl1 protein levels were almost undetectable in NAA treated fruit. In "Camarosa" fruit, *FaXyl1* transcript and protein levels are very low, showing a slight increase at the end of ripening, when auxin levels are probably very low [12]. Overall, these results indicate that auxins repress *FaXyl1* expression and produce a decrease in protein levels and β-xylosidase activity, as have been shown for other strawberry genes related to cell wall disassembly, such as pectate lyase, endo β-1,4-glucanase, polygalacturonase and β-galactosidase [26–29].

The influence of endogenous auxins is usually analysed by eliminating the achenes, the main auxin source, from one half of the fruit, maintaining the other half as control. Under these conditions, de-achenated halves receive less auxins and ripen faster than the other intact halves [16]. It has been reported that the expression of ripening-related genes such as *FaCel1* (endo β-1,4-glucanase) and pectate lyase is enhanced in de-achenated halves [30,26]. In the present study, 20 white fruit were de-achenated in one half and incubated at 20 °C during 2 d. Intriguingly, the elimination of the achenes did not promote ripening, measured as anthocyanin accumulation (data not shown). The treatment effect was also checked by analyzing the expression of *FaCel1* that is negatively regulated by auxins in strawberry fruit [30], in both achened and de-achened halves. Again, we found that de-achenation did not induce the expression of *FaCel1* (data not shown). We assumed that the lack of response of de-achenation could be associated to the cultivar utilized. It could occur that "Camarosa" cultivar was not suitable for experiments that involve de-achenation. Recent works have described that strawberry cultivars can differ in a particular metabolism. Our group has shown that cell wall metabolism can greatly vary among cultivars [12,31–33]. Moreover, differences among cultivars were also detected in content of sugars and level of antioxidants [34,35].

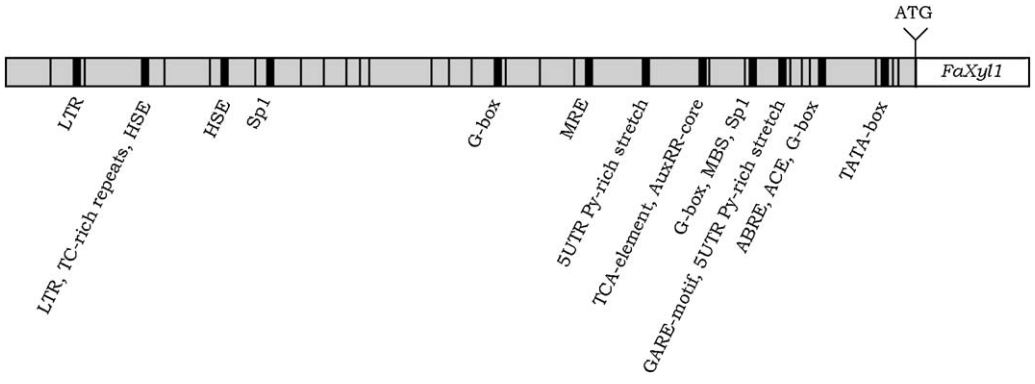

Fig. 1. Schematic representation of the 1861 bp *FaXyl1* promoter region. *cis*-elements are indicated by shaded boxes. Vertical lines indicate CAAT-boxes.

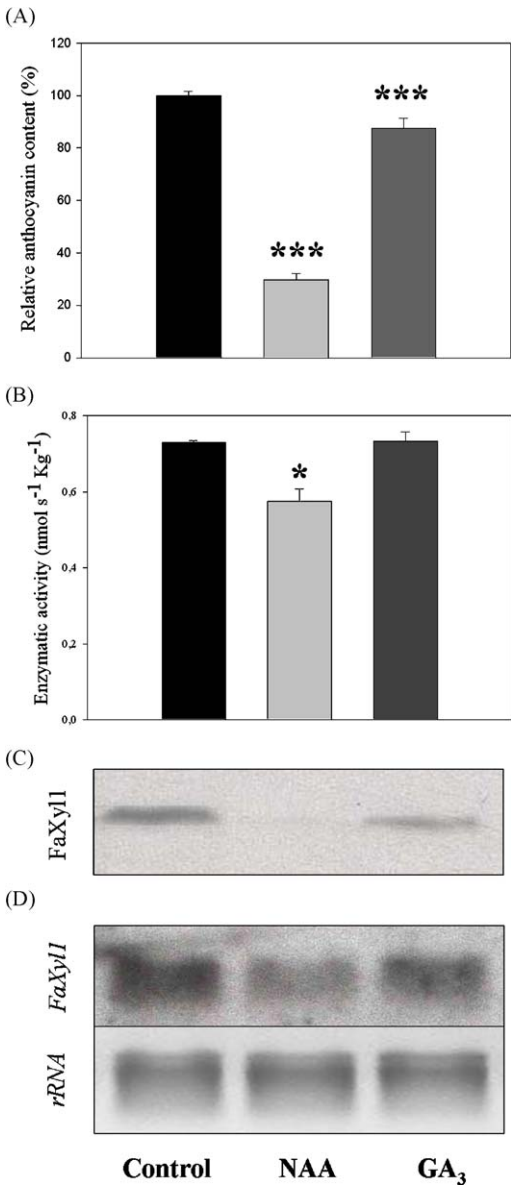

Fig. 2. Effect of auxin and gibberellic acid on anthocyanin content (panel A), β-xylosidase activity (panel B), FaXyl1 protein levels (panel C) and *FaXyl1* expression (panel D). For NAA and GA_3 treatments, fruit peduncles were immersed in 1 mmol L^{-1} NAA and 1 mmol L^{-1} GA_3 for 3 d at 20 °C, respectively. Peduncles of control fruit were immersed in distilled water. Bars indicate standard deviations. Statistical differences in the level of anthocyanin and the β-xylosidase activity between treatment and control according to Student's *t*-test are shown as $^{***}P < 0.001$ (for NAA and GA_3 respect control) and $^{*}P < 0.05$ (for NAA respect control), respectively.

Previous works have evaluated the effects of gibberellins on post-harvest ripening in strawberry through different biochemical parameters. Exogenous treatment of fruit at different ripening stages with GA_3 inhibited ripening, evidenced by a decrease in the respiratory activity and a delay in anthocyanin synthesis and chlorophyll degradation [18]. The promoter region of *FaXyl1* gene has been shown to contain a putative GARE motif (gibberellin-responsive element) (Table 1). This element was located at position −266 relative to the ATG initiation codon and consists of a 7 bp motif with the sequence AAACAGA. In order to test the putative biological significance of this *cis*-element, we evaluated the effect of exogenous application of GA_3 on *FaXyl1* by immersing the peduncle of white fruit in 1 mmol L^{-1} GA_3 for 3 d at 20 °C. After that period, GA_3-treated fruit showed a slight delay in anthocyanin accumulation (Fig. 2A), no significant change in the β-xylosidase activity (Fig. 2B) and a decrease in protein levels (Fig. 2C) and *FaXyl1* mRNA (Fig. 2D). Although to a lesser extent than NAA, these results indicate that GA_3 represses not only strawberry fruit ripening, but also *FaXyl1* expression. The effect of hormone GA_3 on the expression of *FaXyl1* supports a putative biological significance of the GARE motif present in the gene promoter region.

3.3. Abscisic acid treatment

It has been reported that the application of ABA can stimulate fruit ripening [19]. In climacteric fruits, like tomato and apple, the acceleration of ripening was attributed mainly to an enhanced ethylene biosynthesis that occurred after ABA application [36,37]. In strawberry (cv. "Everest", 90% full red colour) ABA treatment

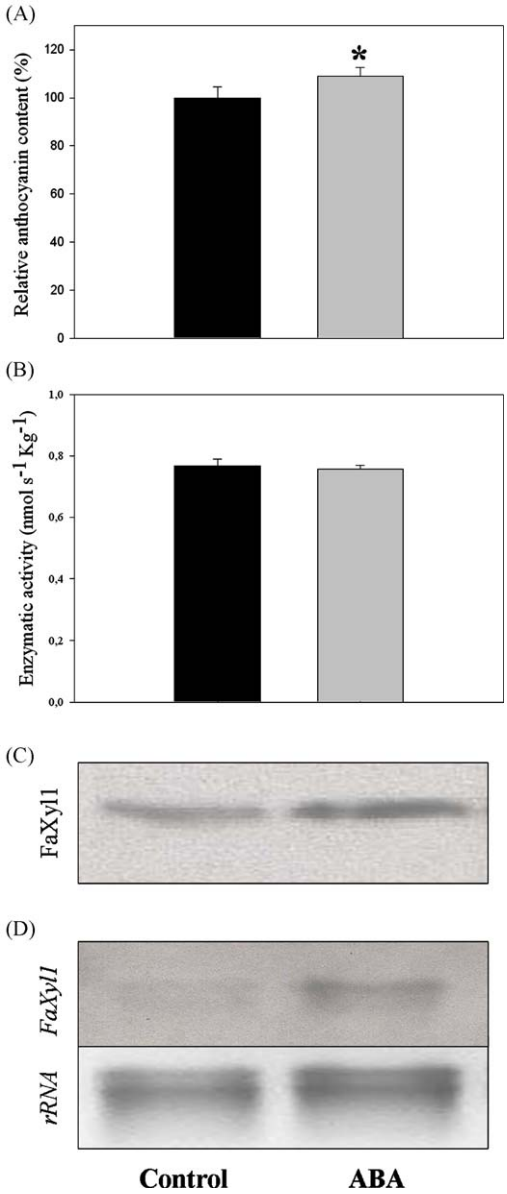

Fig. 3. Effect of ABA on anthocyanin content (panel A), β-xylosidase activity (panel B), FaXyl1 protein levels (panel C) and *FaXyl1* expression (panel D). Fruit peduncles were immersed in a solution consisted of 1 mmol L^{-1} ABA in 2% (v/v) ethanol for 3 d at 20 °C, using fruit treated with a solution of 2% (v/v) ethanol as control. Bars indicate standard deviations. Statistical differences in the level of anthocyanin between treatment and control according to Student's *t*-test are shown as $^{*}P < 0.05$.

accelerated fruit colour and softening through an up-regulation of ethylene production and an enhancement of phenylalanine ammonia-lyase (PAL) activity, a key regulatory enzyme of anthocyanin biosynthesis [19]. In our case, treatment with 1 mmol L^{-1} ABA for 3 d at 20 °C stimulated anthocyanin accumulation (Fig. 3A), *FaXyl1* expression (Fig. 3D) and protein levels (Fig. 3C), whereas it had no effect on the enzymatic activity (Fig. 3B). Most cell wall-modifying proteins are present as multi-gene families. Therefore, total enzymatic activity represents the combined activities of several isoforms. Moreover, in the case of β-xylosidase, other proteins can also contribute to enzymatic activity. For example, in *Arabidopsis thaliana* and barley, it has been reported the presence of α-L-arabinofuranosidases (EC 3.2.1.55) which exhibited bifunctional β-xylosidase/α-L-arabinofuranosidase activity [38,39]. In strawberry fruit, three genes encoding putative α-L-arabinofuranosidases have been isolated [40], and although their substrate specificity has no been determined, the presence of a bifunctional enzyme cannot be discarded. As a consequence, discrepancy between enzymatic activity and *FaXyl1* expression could be due to the presence of other proteins exhibiting β-xylosidase activity. When the promoter region of *FaXyl1* was analysed *in silico*, an ABRE site, *cis*-acting element involved in the abscisic acid response, was identified (Table 1). This element was located at position −168 relative to the ATG initiation codon and consists of a 6 bp motif with the sequence TACGTG. Although we have not performed a functional analysis of *FaXyl1* promoter, the presence of an ABRE site along with Northern and Western blot results suggests a possible responsiveness of the *FaXyl1* promoter region to ABA.

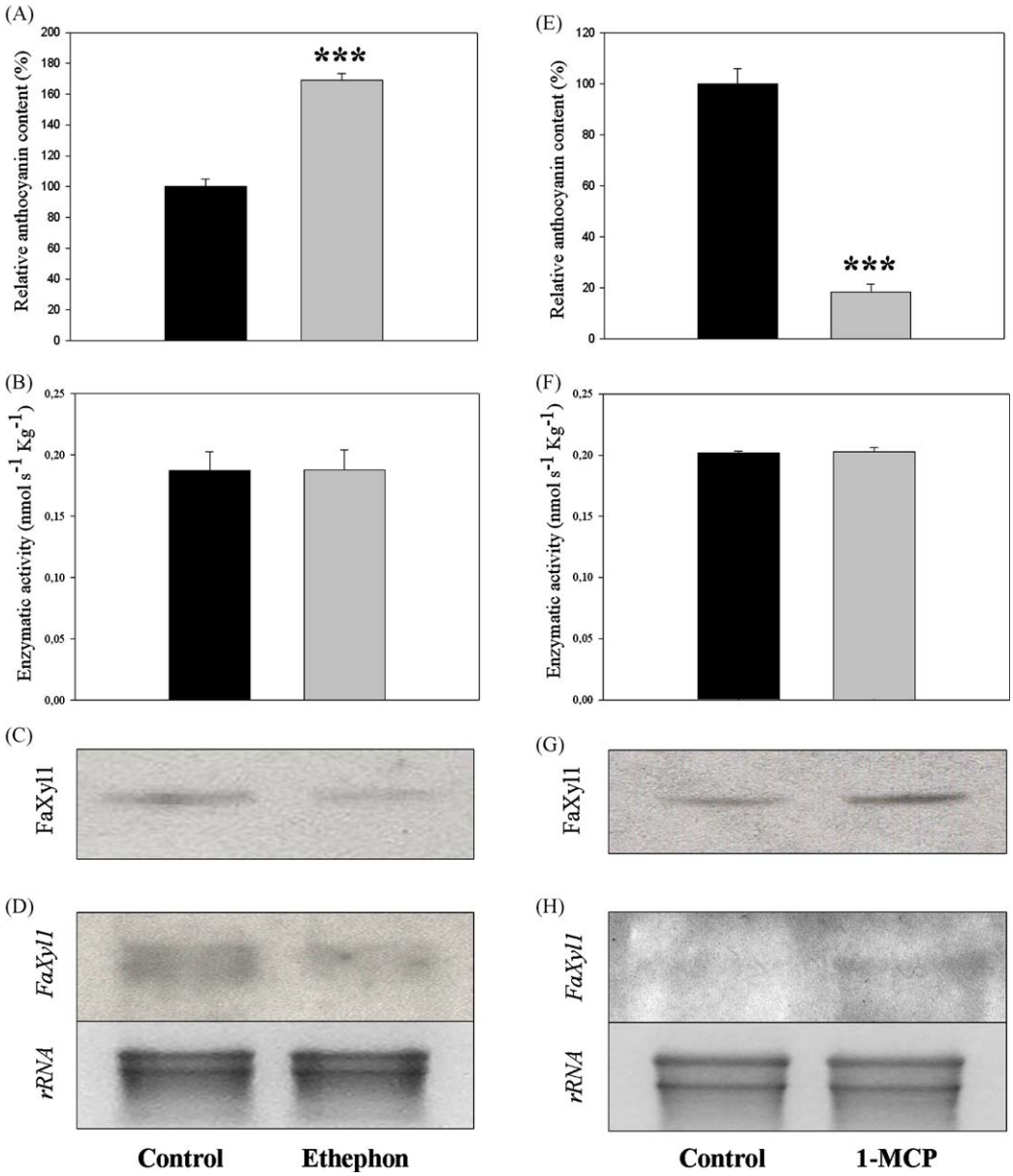

Fig. 4. Effect of ethephon on anthocyanin content (panel A), β-xylosidase activity (panel B), FaXyl1 protein levels (panel C) and *FaXyl1* expression (panel D). Effect of 1-MCP on anthocyanin content (panel E), β-xylosidase activity (panel F), FaXyl1 protein levels (panel G) and *FaXyl1* expression (panel H). For ethephon treatment, fruit were dipped in 2 mmol L^{-1} ethephon with 0.02% (v/v) Tween 20 and 1% (v/v) ethanol for 5 min. Control fruit were dipped in 0.02% (v/v) Tween 20 and 1% (v/v) ethanol. In the case of 1-MCP, fruit were treated with 1 μL L^{-1} 1-MCP in 80 L sealed jars for 10 h at 20 °C. Control fruit were kept in the same conditions but without the inhibitor. After treatments, fruit were stored at 20 °C for 2 days with the peduncle of each fruit immersed in distilled water to avoid dehydration. Bars indicate standard deviations. Statistical differences in the level of anthocyanin between treatment and control according to Student's *t*-test are shown as $^{***}P < 0.001$.

3.4. Ethylene and 1-MCP treatments

In non-climacteric fruits, ethylene is generally considered to have little or no effect on ripening [17]. In the case of strawberry, a fruit classified as non-climacteric, it was showed that treatment with ethylene inhibitors such as silver ions, norbornadiene and aminoethoxyvinylglycine in "Brighton" fruit did not affect ripening [16]. However, strawberries of "G-3" and "G-4" cultivars exposed to ethylene developed a more intense red colour and softened quicker than those stored in ethylene-free air [41]. Although the possible involvement of this hormone in the ripening of the non-climacteric fruits has been studied in different laboratories, results are still contradictory and no clear relationship between ethylene and strawberry ripening has been established yet. Jiang et al. [42] demonstrated that 1-methylcyclopropene (1-MCP), a competitive inhibitor of ethylene action, delays changes in strawberry fruit firmness and colour. In the present work, fruit treated with ethephon accumulated higher anthocyanin content than the control fruit (Fig. 4A), in accordance with previous works [14,15], but did not affect significantly β-xylosidase activity (Fig. 4B). However, we also observed that treated fruit showed a decrease in *FaXyl1* protein (Fig. 4C) and mRNA levels (Fig. 4D), even though no ethylene-responsive elements were found in the promoter region analysed. In order to validate our results, we performed a treatment with 1-MCP and analysed its effect on anthocyanin content, β-xylosidase activity and *FaXyl1* protein and mRNA levels. Treatment with 1-MCP clearly delayed anthocyanin accumulation (Fig. 4E) and stimulated the accumulation of *FaXyl1* protein (Fig. 4G) and transcript levels (Fig. 4H). As in the case of ethephon treatment, there was not effect on the enzymatic activity (Fig. 4F). These results suggest that the expression of *FaXyl1* would be negatively regulated by ethylene. Expression of most cell wall degradation genes is usually up-regulated by ethylene, so the repression of *FaXyl1* by this hormone could seem surprising. However, in strawberry, a similar behaviour has been reported for *FaPE1*, a pectin esterase related to pectin metabolism. Castillejo et al. [43] reported the repression of *FaPE1* by ethylene and proposed that this fact could be involved in textural changes occurring during fruit senescence.

It has been proposed that the accelerated changes in colour development and softening of strawberry produced by ABA treatment can be attributed to ethylene. However, in relation to *FaXyl1* expression, the results obtained with ethephon and ABA treatments do not support this observation since ABA stimulated *FaXyl1* expression. Therefore, it is possible that the stimulation of *FaXyl1* expression by ABA could be through an ethylene-independent way.

3.5. NO treatment

Nitric oxide (NO) has been described as an important second messenger in animal cells and accumulating evidence suggests that it is also important in plant cells as well [44]. In addition, NO is involved in vegetative stress and senescence of horticultural products. Post-harvest exogenous applications of NO, either by direct fumigation in an O_2-free atmosphere or by means of NO releasing chemicals, such as N-tert-butyl-α-phenylnitrone and 3-morpholino sydnonimine, do markedly extend shelf life of strawberry and other horticultural products [45]. The emission of NO was negatively related with ethylene production in the process of maturation and senescence of fruits. For example, in non-climacteric strawberry and climacteric avocado fruit it has been demonstrated that the maturation process is clearly accompanied by a marked decrease of NO concomitant with an increase of C_2H_4 [46]. Moreover, ripe strawberry fruit treated with 5 μmol L^{-1} sodium nitroprusside (SNP), a NO donor, significantly

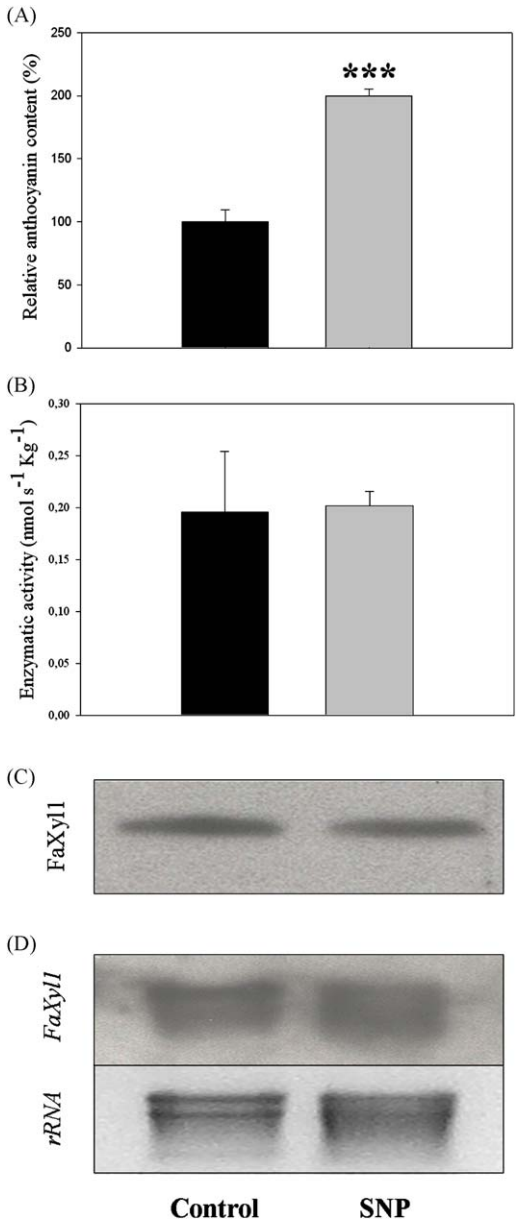

Fig. 5. Effect of NO on anthocyanin content (panel A), β-xylosidase activity (panel B), FaXyl1 protein levels (panel C) and *FaXyl1* expression (panel D). For SNP treatment, fruit were dipped in 5 μmol L^{-1} SNP for 2 h, air-dried and stored at 20 °C for 2 d with the peduncle of each fruit immersed in distilled water to avoid dehydration. Control fruit were dipped in distilled water. Bars indicate standard deviations. Statistical differences in the level of anthocyanin between treatment and control according to Student's *t*-test are shown as $^{***}P < 0.001$.

inhibited ethylene production, respiration rate, ACC synthase activity and ACC content, but did not affect ACC oxidase activity [20]. To explore the possible effect of exogenous application of NO on *FaXyl1*, white fruit were dipped in SNP aqueous solution for 2 h, air-dried and stored at 20 °C for 2 d with the peduncle of each fruit immersed in distilled water to avoid dehydration. Intriguingly, NO-treated fruit showed higher anthocyanin amount than the corresponding control (Fig. 5A). Since NO applications were done at white stage in our case and at ripe stage in previous work [20], discrepancies between results could be due to variations in responsiveness of fruit at different ripening stages. In addition, as it was mentioned previously, different cultivars can vary in their responses to a given hormonal application. SNP did not affect the β-xylosidase activity (Fig. 5B), protein levels (Fig. 5C) and *FaXyl1*

expression (Fig. 5D). Hence, NO might not regulate *FaXyl1* in strawberry fruit. To our knowledge, this is the first report on the effects of NO on the expression and protein levels of a gene related to cell wall disassembly.

4. Conclusions

Earlier studies on strawberry fruit ripening have shown that plant growth regulators such as auxins, GA_3, ABA, ethylene and SNP can modulate fruit softening. Our results suggest that *FaXyl1* expression and protein levels could be induced by ABA, and repressed by NAA, GA_3 and ethylene. The possible influence of ethylene on the expression of *FaXyl1*, and probably on other ripening-related genes, should not be underestimated in strawberry fruit ripening. Nitric oxide seems not to affect *FaXyl1* mRNA and protein levels. Although these results suggest a possible regulation of *FaXyl1* by ripening-related hormones, additional works are required to establish the *in vivo* action of this enzyme and its possible role in fruit softening.

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