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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

Claudia A. Bustamante [a], Pedro M. Civello [a,b], Gustavo A. Martínez [a,b,*]

[a] IIB-INTECH (Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús) (CONICET-UNSAM), Camino de Circunvalación Laguna Km 6, (B7130IWA) Chascomús, Argentina
[b] Facultad de Ciencias Exactas, Universidad Nacional de La Plata (UNLP), 47 y 115, (1900) La Plata, Argentina

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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

* Corresponding autor at: Facultad de Ciencias Exactas, Universidad Nacional de La Plata (UNLP), 47 y 115, (1900) La Plata, Argentina. Tel.: +54 2241 424049; fax: +54 2241 424048.
E-mail address: gmartinez@intech.gov.ar (G.A. Martínez).

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