Antisense down-regulation of the strawberry β-galactosidase gene FaβGal4 increases cell wall galactose levels and reduces fruit softening

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Abstract

Strawberry softening is characterized by an increase in the solubilization and depolymerization of pectins from cell walls. Galactose release from pectin side chains by β-galactosidase enzymes has been proposed as one reason for the increase in soluble pectins. A putative β-galactosidase gene, FaβGal4, has been identified using a custom-made oligonucleotide-based strawberry microarray platform. FaβGal4 was expressed mainly in the receptacle during fruit ripening, and was positively regulated by abscisic acid and negatively regulated by auxins. To ascertain the role of FaβGal4 in strawberry softening, transgenic plants containing an antisense sequence of this gene under the control of the CaMV35S promoter were generated. Phenotypic analyses were carried out in transgenic plants during three consecutive growing seasons, using non-transformed plants as control. Two out of nine independent transgenic lines yielded fruits that were 30% firmer than control at the ripe stage. FaβGal4 mRNA levels were reduced by 70% in ripe fruits from these selected transgenic lines, but they also showed significant silencing of FaβGal1, although the genes did not share significant similarity. These two transgenic lines also showed an increase in pectin covalently bound to the cell wall, extracted using Na2CO3. The amount of galactose in cell walls from transgenic fruits was 30% higher than in control; notably, the galactose increase was larger in the 1 M KOH fraction, which is enriched in hemicellulose. These results suggest that FaβGal4 participates in the solubilization of covalently bound pectins during ripening, reducing strawberry fruit firmness.

Key words: β-galactosidase, cell wall, Fragaria × ananassa, fruit ripening, fruit softening, pectins.

Introduction

Softening is one of the most characteristic aspects of the fruit ripening process. In soft fruits, such as strawberry, the soft melting texture is highly appreciated by consumers, but it poses a major problem for strawberry producers, determining
the short postharvest shelf life of this fruit and limiting its storage and postharvest transport. During ripening, fruit cell walls are extensively modified, and these changes are the most important factor leading to fruit softening. In general, cell wall modifications that accompany softening involve the solubilization of pectin polymers, the depolymerization of pectins and matrix glycans, and the loss of neutral sugars from pectin side chains (Brummell, 2006; Goulao and Oliveira, 2008; Mercado et al., 2011). All these processes often occur concurrently during fruit ripening, although the extension of these modifications greatly depends on the kind of fruit (Brummell, 2006; Mercado et al., 2011).

Strawberry softening is characterized by a moderate increase in pectin solubilization—i.e. an increase in the amount of pectins loosely bound to the cell wall—and depolymerization (Posé et al., 2011). Functional studies of genes encoding pectinase enzymes, such as polygalacturonase (Quesada et al., 2009) orpectate lyase (Jiménez-Bermúdez et al., 2002; Youssef et al., 2009; Youssef et al., 2013), support a key role of pectin disassembly in strawberry softening. Transgenic fruits with low expression levels of these genes were significantly firmer than control fruits and displayed a reduction in solubilization and depolymerization ofpolyuronides (Santiago-Domènech et al., 2008; Posé et al., 2013; Posé et al., 2015). The molecular mechanism underlying pectin disassembly is unclear (Paniagua et al., 2014). Many fruits, including strawberry, show a loss of neutral sugars, mainly arabinose andgalactose, during ripening (Gross and Sams, 1984; Reddwell et al., 1997). The removal of these carbohydrates fromrhamnogalacturonan I (RG-I) polyuronides by β-galactosidases has been suggested as one of the possible reasons for the increase of soluble pectins (Koh and Melton, 2002; Brummell, 2006).

β-galactosidases (EC 3.2.1.23) are glycosyl hydrolases characterized by their ability to hydrolyse terminal, non-reducing β-D-galactosyl residues from numerous β-D-galactoside substrates (Pérez-Almeida and Carpita, 2006; Tateishi, 2008). In most fruits, β-galactosidases are encoded by small gene families with different patterns of expression that could play distinct roles in fruit development (Smith and Gross, 2000; Mwaniki et al., 2005; Tateishi et al., 2007; Othman et al., 2011). Functional analyses of β-galactosidases genes using transgenic plants have only been carried out in the climacteric model fruit tomato (Solanum lycopersicum) with contrasting results. Antisense TBG4 tomato fruits displayed reduced TBG4 mRNA levels and free cell wall galactose only at the onset of ripening, but softening of ripe fruits decreased by 40% (Smith et al., 2002). By contrast, neither the silencing of TBG1 (de Silva and Verhoeyen, 1998) nor TBG3 (Carey et al., 2001) modified tomato fruit firmness. In TBG6 antisense tomato plants, fruit firmness was also similar to that of control plants, but these fruits showed structural alterations in the cuticle that increased fruit cracking (Montezuma et al., 2003).

In strawberry (Fragaria × ananassa), a non-climacteric fruit, β-galactosidase activity increases during fruit development and remains high in ripe fruit (Trainotti et al., 2001; Figueroa et al., 2010). At the molecular level, Trainotti et al. (2001) isolated three full-length cDNAs encoding β-galactosidase genes FaβGal1 to FaβGal3. Although all of them could be detected in fruit and vegetative tissues, only FaβGal1 showed an expression pattern that could be related to the fruit ripening process, with the other two genes expressed mainly in green, immature fruits. Transcriptomic studies performed in our research group have identified a large group of genes whose expression increases throughout strawberry fruit ripening. One of these genes, FaβGal4 (accession number KR189030), displays significant sequence homology with putative β-galactosidase from higher plants. The main goal of this study was the functional characterization of this gene. For this purpose, transgenic strawberry plants carrying an antisense sequence of FaβGal4 were generated and the effects of FaβGal4 down-regulation in fruit firmness and cell wall structure were analysed.

**Materials and methods**

**Plant material**

Strawberry plants (Fragaria × ananassa Duch., cv. Camarosa) were grown under field conditions in Huelva (south-west Spain). Fruits were harvested at different developmental stages: small-sized green fruits (G1, 2–3 g), medium-sized green fruits (G2, 3–5 g), full-sized green fruits (G3, 4–7 g), white fruits (W, 5–8 g), and full-ripe red fruits (R, 6–10 g). Vegetative tissues, such as runners, flowers, and expanding leaves, were also harvested. For the genetic transformation, in vitro micropropagated plants, cv. Chandler, were used. All tissues and fruit samples were immediately frozen in liquid nitrogen and stored at −80°C.

Cloning and sequence analysis of full-length cDNA of FaβGal4

The full-length cDNA corresponding to the FaβGal4 gene was isolated from a Fragaria × ananassa R stage fruit cDNA library (Medina-Escobar et al., 1997). The deduced amino acid sequence and the phylogenetic tree construction were performed using the Lasergene software package (DNASTAR).

**Auxin and nordihydroguaiaretic acid treatments**

Achene of two sets of 50 middle-sized green fruits (G2) each, still attached to the plant, were carefully removed using the tip of a scalpel blade. One set of deacheden G2 fruits was treated with the synthetic auxin 1-naphthalenacetic acid (NAA in lanolin paste (1 mL) with 1 mM NAA in 1% (w/v) DMSO. The other set of G2 deacheden fruits (control group) were treated with the same paste, but without NAA. Both treatments were applied over the whole fruit surface. All fruits were harvested 5 days after treatment, immediately frozen in liquid nitrogen, and stored at −80°C. During the course of the assays, the fruits reached the G3–W developmental stages.

Nordihydroguaiaretic acid (NDGA) is an ideal inhibitor of the 9-cis-epoxycarotenoid dioxygenase enzyme and was used to block abscisic acid (ABA) biosynthesis (Creelman et al., 1992). The lowest NDGA concentration that completely blocks ABA accumulation is 100 μM, as determined by preliminary tests in tomato fruit (Zhang et al., 2009). Strawberry fruits (Fragaria × ananassa, cv. Elsanta) were used at the
mature G–W stages for the purposes of this study. All fruits (ten fruits per treatment) were carefully injected with 1–2 mL of NDGA (100μM) sterile solution or sterile water (control fruits) using a hypodermic syringe. Three replications were conducted for each treatment. The samples were harvested after 8 days of treatment, when the fruits reached the R developmental stage, then frozen in liquid nitrogen and stored at −80°C. Fruits treated with NDGA were white, whereas control fruits were red. These samples were used to determine relative expression of FaβGal4.

RNA isolation
Total RNA was isolated from independent pools of strawberry fruits at different growth and ripening stages and from vegetative tissues, in accordance with Asif et al. (2000). Achenes were always removed from fruit and only receptacle RNA was extracted and purified. RNA obtained was treated with RNase-free DNase I (Invitrogen) and purified through the RNaseasy Mini kit (Qiagen). RNA concentration and purity were evaluated using a NanodropTM spectrophotometer ND-1000 (Thermo Scientific) and by 1% agarose gel electrophoresis.

Expression analysis by quantitative real-time PCR
Gene expression analysis of FaβGal4 was performed by quantitative real-time (qRT)-PCR through an iCycler (BioRad) device, as previously described (Benitez-Burraco et al., 2003). FaβGal4 gene primer sequences for quantitative amplification were 5′-CAG CCA CCC ACT CCT CTA TAA CCA GTT-3′ and 5′-GGC AAG CAG TAA AAT ACC AAG CAA AGC-3′. Each reaction was performed at least in triplicate and the corresponding cycle threshold (Ct) values were normalized using the Ct value corresponding to an intersperser 26S-18S strawberry RNA gene (housekeeping gene) (Benitez-Burraco et al., 2003; Molina-Hidalgo et al., 2013). All of these values were subsequently used to determine the relative increase or decrease in FaβGal4 expression in the samples in comparison to that of the control gene in accordance with Pedersen (2001). Intersperser 26S-18S (primers: 5′-ACC GTT GAT TCG CAC AAT TGG TCA TCG-3′ and 5′-TAC TGC GGG TCG CTC GCA AAT ACC CCT CCA CCA-3′) was selected as the control gene owing to its constitutive expression throughout all of the different experimental conditions tested. The efficiency of each particular qRT-PCR and the melting curves of the products were also analysed to ensure the existence of a single amplification peak corresponding to a unique molecular species. The expression levels of the different β-galactosidase genes in control and transgenic antisense FaβGal4 fruits were measured by qRT-PCR as described above, using the following primers: FaβGal1 5′-AAA GCC AAG CAC GAC ATA CC-3′ and 5′-CCA TAA CAT CAG CCC AAT ACC CC-3′; FaβGal2 5′-TTC ATG GCT CTC TGC TT-3′ and 5′-ACA TCC AAG CCT CCA TCT T-3′; FaβGal3 5′-TTC ATG GCT CTC TGC TT-3′ and 5′-ACA TCC AAG CCT CCA TCT T-3′; FaβGal4 5′-GAT GCT TCT CGG TAT CC-3′ and 5′-TGT AAT CGC TTC TTC TGT TCC T-3′.

Binary vector for antisense FaβGal4 silencing and generation of transgenic strawberry plants
A 300-bp non-conserved region of the FaβGal4 gene in antisense orientation was cloned into the pK7WG2 binary vector using Gateway technology (Invitrogen, Darmstadt, Germany) for antisense silencing of FaβGal4. The forward primer 5′-AGA GGA GAT GCT CGG TCT CGG TAT C-3′ and reverse primer 5′-TGG CAT AGC GCT TAA ATA GTT CAT TCA GTT-3′ were used. The resulting fragment was cloned into pCR/GWTOPO (Invitrogen) and then transferred to the Gateway pK7WG2 vector by way of a specific recombination of both plasmids using LR clonase (Invitrogen). The resulting plasmid (pK7WG2-FaβGal) was tested through sequencing and restriction analyses prior to strawberry plant transformation. The plasmid was introduced into Agrobacterium tumefaciens strain AGL1 by electroporation.

Leaf discs of strawberry plants, cv. Chandler, micropropagated in vitro were used as explants for Agrobacterium-mediated transformation experiments, as described in Barceló et al. (1998). Explants were inoculated with a diluted culture of A. tumefaciens and selected in 25 mg L−1 kanamycin. After 7–8 months of selection, kanamycin-resistant shoots were acclimated and transferred to the greenhouse. Transgenic mother plants were propagated by runners and the daughter plants were used for phenotypic analysis. The presence of the transgenes in these putative transgenic lines was confirmed by PCR amplification of a 220-bp fragment belonging to the nptII gene.

Phenotypic analysis of transgenic plants
Transgenic plants were evaluated during three consecutive growing seasons, using non-transformed plants, cv. Chandler, as control. Plants were grown in a greenhouse under natural temperature and light conditions and fruit was collected from March to July. During the first year, nine independent antisense FaβGal4 lines were analysed. Eight plants per line and a minimum of 10 ripe fruits per line were evaluated. During the second and third year, two selected lines showing higher fruit firmness than control were evaluated. Thirty plants per line and 50–100 ripe fruits per line were analysed in both years. Fruits were harvested at the stage of full ripeness, when the fruit surface was completely red, and the weight, size, colour, soluble solids, and firmness were recorded. Colour was measured using a colorimeter (Minolta Chroma Metre CR-400, Osaka, Japan). The instrument was calibrated with a standard white and a standard black reflective plate before use. The L* a* b* colour space parameters (lightness, redness, yellowness) were recorded. Soluble solids were measured using a refractometer (Atago N1), and firmness using a hand penetrometer (Effeji) with a cylindrical needle of 9.62 mm² area.

DNA extraction and enzyme assays
Genomic DNA was extracted from young strawberry leaves using Qiagen DNeasy Plant Kit. Previously, plant material had been washed three times with washing buffer solution consisting of 100 mM sodium acetate buffer (pH 5), 20 mM EDTA, 0.2% sorbitol, 2% polyvinylpyrrolidone (PVP, molecular weight 40 000), and 1% β-mercaptoethanol (Mercado et al., 1999). β-galactosidase activity was measured in ripe fruits according to Figueroa et al. (2010). Protein extraction was carried out by grinding 10 g of strawberry fruits under liquid nitrogen into a fine powder. The powder was homogenized using an Ultra-Turrax (Janke & Kunkel) with 20 mL of cold extraction buffer [0.05 M Na-acetate buffer, pH 6, 1% (w/v) polyvinylpyrrolidone (PVP), containing 1 M NaCl]. Homogenates were stirred at 4°C for 3 h and then centrifuged at 11 000 g for 30 min. The supernatant was used to determine β-galactosidase activity by using p-nitrophenyl-β-D-galactopyranoside (Sigma) as the substrate. To measure exogalactanase activity, fruit tissue was extracted as described by Trainotti et al. (2001). Briefly, the powder was homogenized with cold extraction buffer, centrifuged at 11 000 g for 15 min, and the pellet washed with 10 mL of extraction buffer without PVP. After centrifugation, the pellet was resuspended in 0.05 M Na-acetate buffer, pH 6, containing 1 M NaCl and stirred for 5 h at 4°C. After extraction, the insoluble material was removed by centrifugation and the supernatant was dialyzed against 0.05 M Na-acetate buffer, pH 4.5. A lupin galactan pretreated with α-L-arabinofuranosidase
(Megazyme) was used as the substrate for exo-galactanase activity as described by Carey et al. (1995). The galactosidase released in the assay was estimated by measuring the release of reducing sugars using 2-cyanoacetamide (Gross, 1982). Protein extractions were performed in triplicate.

Cell wall analysis

The cell walls were extracted from frozen ripe fruits following the method of Redgwell et al. (1992) with some modifications as previously described by Santiago-Doménech et al. (2008). Briefly, 20 g of fruit were homogenized in a 40 ml mix of phenol, acetic acid, and water (PAW, 2:1:1, w:v:v). The homogenate was centrifuged at 4000 g for 15 min and the supernatant filtered through Miracloth (PAW fraction). The pellet was treated overnight with 90% DMSO to solubilize the starch. The extract was then centrifuged at 4000 g and the pellet washed twice with distilled water. The water fraction was discarded and the pellet, the cell wall material (CWM), was lyophilized and weighed. After extraction, the CWM was sequentially fractionated following the procedure of Santiago-Doménech et al. (2008). Briefly, 300 mg of the CWM was sequentially extracted overnight with deionized water, 0.05 M Na2CO3 containing 0.02 M NaBH4, M KOH containing 0.02 M NaBH4, and 4 M KOH containing 0.02 M NaBH4. All fractions were extensively dialyzed against pure water. Then, the extracts were concentrated with a rotary evaporator and freeze-dried. Three independent fractionations per CWM sample were performed.

The uronic acid (UA) content was measured using the carbazole method (Filisetti-Cozzi and Carpita, 1991) using galacturonic acid as the standard. For neutral sugar analysis, the CWM and the different cell wall fractions were hydrolysed with 72% (w/w) sulphuric acid. After neutralization with ammonia, carbohydrates were derivatized to alditol acetates and analysed by gas chromatography with flame ionization detection (Carey et al., 1983).

Hormonal regulation of FaβGal4

It has been proposed that the ABA to auxin content ratio in strawberry fruit receptacles constitutes a signal that triggers the fruit ripening process (Perkins-Veazie, 1995). Thus, we investigated whether FaβGal4 expression was under the control of these two hormones. Because it is known that auxins present in strawberry receptacles are synthesized by the achenes, a comparative gene expression analysis was carried out between control and de-achened green fruits at the G2 stage that were or were not externally treated with NAA. A clear increase in expression was under the control of FaβGal4 transcript was detected in de-achened fruits (Fig. 3A). This increase did not occur, however, when de-achened fruits were treated with a lanolin paste containing NAA. These results clearly indicate that the expression of this gene was negatively regulated by auxins.

In addition, when the receptacle ABA content was depleted by adding NDGA, a significant reduction in the amount of FaβGal4 and other cell wall enzyme transcripts, e.g. FaRGlyase1, was observed when compared against control fruits (Fig. 3B). This indicates that FaβGal4 expression could be activated by ABA.

Phenotypic analysis of antisense FaβGal4 plants

Nine independent kanamycin-resistant shoots carrying the antisense sequence of the FaβGal4 gene were recovered, yielding an average transformation rate of 2%. The transgenic nature of the plants was confirmed by PCR amplification of a 220-bp fragment belonging to the nptII gene (results...
FaβGal4 silencing reduces strawberry softening

Transgenic FaβGal4 plants showed a vegetative growth pattern similar to that of the control plants. However, during the first year of analysis, fruit yield was reduced by 60–80% in most of the lines compared with the control line. Furthermore, the proportion of malformed and small fruits in these lines was higher than in control. To estimate fruit quality, control and transgenic fruits were harvested at the stage of full ripening, and the weight, length, width, colour, soluble solids, and firmness were recorded. Results obtained

Fig. 1. Phylogenetic analysis of selected fruit β-galactosidase proteins. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events. GenBank accession numbers and sources for the respective sequences are: TomβGal4 (AF020390; Solanum lycopersicum); CjβGal4 (AJ277377; Carica papaya); FaβGal1 (AJ278703; Fragaria × ananassa); FaβGal2 (AJ278704; Fragaria × ananassa); FaβGal3 (AJ278705; Fragaria × ananassa); FaβGal4 (KR189030; Fragaria × ananassa); JpβGal3 (AB046543; Pyrus pyrifolia); MdβGal (L29451; Malus domestica); PaβGal (AB061017; Persea americana); TomβGal1 (X83854; Solanum lycopersicum); and TomβGal3 (CAA10172; Solanum lycopersicum). Sequences were aligned using MegAlign (Windows 32; MegAlign 5.0; DNASTAR).

Fig. 2. Expression of the strawberry FaβGal4 gene during fruit receptacle development (A), achenes from fruits at different developmental stages (B), and in different vegetative tissues (C). Fl: flower; G1: small-sized green fruit; G2: medium-sized green fruits; G3: full-sized green fruit; L: leaf; R: red fruit; Rn: runner; W: white fruit. Quantification was based on Ct values as described in Materials and Methods. The increase in the mRNA value was relative to the G1-Ct value, which was assigned an arbitrary value equal to unity. Mean values ± SD of five independent experiments are shown. In all figures, statistical significance with respect to the reference sample (G1 achenes in B and G1 fruit receptacle in A and C) was determined by the Student’s t-test. **P-value < 0.01 and ***P-value < 0.001.
on this observation, these two lines were selected for further studies.

Fruit quality parameters in selected transgenic lines were evaluated during two additional growing seasons, using each year's daughter plants derived from vegetative propagation by runners. Results obtained in the two growing seasons were similar to those observed during the first year of analysis (Table 2). Transgenic fruits were smaller than control and showed higher soluble solid contents, especially those from line β-Gal37. Colour was also slightly modified in both transgenic lines. With respect to fruit firmness, both transgenic lines displayed significantly higher firmness values than control in the two years of analysis (Table 2): the average increase of firmness was 28%. Fruit yield was significantly reduced in both transgenic lines, with mean values of 179.2 ± 31.2, 63.7 ± 10.9, and 102.2 ± 29.2 g of fruit per plant in control, non-transformed plants, and β-Gal28 and β-Gal37 transgenic lines, respectively. This decrease was due to a reduction in fruit weight but also a decrease in the number of fruits produced.

FaβGal4 gene expression and enzyme activity in transgenic fruits

The expression level of FaβGal4, as well as the other three β-galactosidase genes described in strawberry fruit (FaβGal1–3), was analysed in red fruits from control and selected β-Gal28 and β-Gal37 transgenic lines by qRT-PCR (Fig. 4). A significant down-regulation of FaβGal4 was observed in ripe fruits from the two transgenic lines, with a similar level of silencing in the transgenic genotypes, close to 70%. Despite the FaβGal4 sequence sharing a low similarity with the rest of the β-galactosidase genes analysed, both transgenic lines also showed a 70% decrease in the amount of FaβGal1 mRNA levels (Fig. 4). Furthermore, β-Gal28 transgenic fruits showed an additional silencing of FaβGal3. By contrast, mRNA levels of FaβGal2 were not modified in transgenic lines.

β-galactosidase enzymes are characterized by their ability to hydrolyse terminal, non-reducing β-galactosyl residues from numerous substrates. In this research, the term β-galactosidase activity refers to an enzyme that hydrolyses a β-galactosyl residue linked to a variety of aglycones, e.g. nitrophenyl-[β-D-galactopyranoside (NPG), whereas exo-galactanase refers to an enzyme that is specific for the non-reducing end of galactan (Smith et al., 2002). Both activities were measured in triplicate in protein extracts from ripe fruits of the two anti-FaβGal4 selected lines. Total β-galactosidase activity was similar in control and transgenic fruits, 0.6 ± 0.1 μmol NPG·g FW⁻¹·h⁻¹ in control versus 0.7 ± 0.1 and 0.9 ± 0.2 μmol NPG·g FW⁻¹·h⁻¹ in fruits from β-Gal28 and β-Gal37, respectively. Exo-galactanase activity, measured against a lupin galactan, was low, and no differences between control and transgenic lines were observed (1.1 ± 0.5 μg·g FW⁻¹·h⁻¹ in control versus 0.8 ± 0.1 and 2.3 ± 1.3 μg·g FW⁻¹·h⁻¹ in fruits from β-Gal28 and β-Gal37, respectively).
Table 1. Characteristics of ripened fruits in control and transgenic β-galactosidase plants. Fruits were harvested at the stage of full ripeness and data represent mean ± SD of a minimum of ten fruits per line, evaluated during the first year of analysis.

| Genotype | Weight (g) | Length (mm) | Width (mm) | Colour | Soluble solids (ºBx) | Firmness (N) |
|----------|------------|-------------|------------|--------|---------------------|-------------|
|          |            |             |            | L*     | a*                  | b*          |
| Control  | 11.2 ± 2.2 | 35.3 ± 4.8  | 26.2 ± 3.1 | 36.5 ± 1.9 | 38.0 ± 3.4          | 20.4 ± 3.0  | 8.3 ± 1.7 | 3.2 ± 0.6 |
| β-Gal15  | 9.5 ± 1.8* | 33.0 ± 3.0  | 23.8 ± 2.2*| 38.7 ± 3.3* | 37.3 ± 4.4          | 21.9 ± 5.2  | 8.8 ± 1.7 | 3.4 ± 0.5 |
| β-Gal18  | 7.8 ± 1.6* | 28.4 ± 3.1* | 24.4 ± 2.2*| 38.8 ± 3.6* | 39.5 ± 5.1          | 21.3 ± 3.3* | 9.0 ± 1.7 | 3.0 ± 0.6 |
| β-Gal19  | 7.8 ± 1.8* | 28.0 ± 4.4* | 24.0 ± 2.6*| 39.3 ± 3.5* | 37.8 ± 3.6          | 22.3 ± 3.5* | 9.0 ± 1.8 | 3.1 ± 0.7 |
| β-Gal21  | 10.2 ± 3.1 | 32.2 ± 5.4* | 25.0 ± 2.9 | 38.6 ± 2.5* | 37.9 ± 3.3          | 23.1 ± 3.9* | 8.9 ± 2.2 | 3.2 ± 0.7 |
| β-Gal24  | 7.3 ± 2.1* | 30.3 ± 5.6* | 22.9 ± 3.4*| 39.9 ± 6.3* | 39.8 ± 5.5          | 26.0 ± 5.5* | 9.6 ± 2.9 | 3.5 ± 0.6 |
| β-Gal25  | 5.2 ± 2.0* | 24.4 ± 4.3* | 22.6 ± 3.1*| 44.9 ± 5.8* | 40.7 ± 4.7          | 28.1 ± 4.2* | 13.8 ± 2.6* | 3.5 ± 0.9 |
| β-Gal27  | 11.9 ± 2.5 | 36.2 ± 4.7  | 26.9 ± 2.9 | 33.8 ± 2.7* | 36.9 ± 3.2          | 18.1 ± 3.5* | 7.5 ± 2.0* | 3.4 ± 0.6 |
| β-Gal28  | 9.7 ± 3.0* | 28.8 ± 4.1* | 27.0 ± 3.8 | 40.0 ± 4.1* | 43.2 ± 3.8          | 26.1 ± 4.3* | 10.0 ± 2.7* | 4.7 ± 0.8* |
| β-Gal37  | 5.9 ± 1.4* | 22.7 ± 3.8* | 23.4 ± 1.9*| 40.5 ± 3.2* | 40.9 ± 2.8          | 25.8 ± 3.2* | 10.8 ± 2.5* | 4.2 ± 0.9* |

*Mann–Whitney U test shows significant difference from control at P = 0.05

Table 2. Characteristics of ripened fruits in control and selected transgenic β-galactosidase plants. Fruits were harvested at the stage of full ripeness and data represent mean ± SD of a minimum of 50 fruits per line, evaluated during the second and third year of analysis. Mean separation within each year was performed by Tamhane T2 test at P = 0.05.

| Genotype | Weight (g) | Length (mm) | Width (mm) | Colour | Soluble solids (ºBx) | Firmness (N) |
|----------|------------|-------------|------------|--------|---------------------|-------------|
|          |            |             |            | L*     | a*                  | b*          |
| Second year |      |            |            |        |                     |             |
| Control  | 12.2 ± 3.8a| 36.2 ± 4.8a | 27.5 ± 3.8a| 36.1 ± 3.5b| 38.4 ± 3.7b        | 21.0 ± 4.2a | 6.9 ± 1.5c | 2.6 ± 1.0b |
| β-Gal28 | 8.2 ± 2.6b | 29.6 ± 3.7b | 25.8 ± 2.8b| 36.8 ± 4.2b| 40.2 ± 4.7a        | 22.5 ± 5.4b | 7.9 ± 1.3b | 3.4 ± 0.6a |
| β-Gal37 | 6.3 ± 1.5c | 24.0 ± 2.5c | 23.8 ± 2.2c| 38.8 ± 4.1a| 38.7 ± 4.4b        | 23.5 ± 4.8b | 8.9 ± 1.7a | 3.5 ± 0.5a |
| Third year |      |            |            |        |                     |             |
| Control  | 14.3 ± 5.3a| 39.5 ± 6.2a | 29.3 ± 4.6a| 35.5 ± 2.2b| 36.6 ± 3.7a        | 19.5 ± 3.5b | 6.4 ± 1.4c | 3.6 ± 0.8b |
| β-Gal28 | 9.9 ± 3.4b | 34.7 ± 5.8b | 25.7 ± 4.1b| 38.9 ± 4.2a| 42.9 ± 4.1b        | 25.4 ± 5.1a | 8.1 ± 1.5b | 4.6 ± 1.0a |
| β-Gal37 | 7.3 ± 2.3c | 26.0 ± 3.9c | 24.9 ± 3.2b| 40.1 ± 4.5a| 38.6 ± 4.9c        | 24.3 ± 3.8a | 9.2 ± 1.9a | 4.4 ± 0.8a |

Fig. 4. Relative FaβGal4 expression, estimated by qRT-PCR, in ripe fruits from control and transgenic β-galactosidase lines. Bars represent mean ± SD of three independent RNA quantifications. Statistical significance with respect to the control line was determined by Dunnett’s multiple comparison test. ***P-value < 0.001 and **P-value < 0.01.

Cell wall analysis

The yield of CWM and soluble PAW fraction obtained per fresh weight of fruit was similar in control and transgenic lines, with average values of 0.93 g CWM·100 g FW⁻¹ and 0.09 g PAW·100 g FW⁻¹. CWM was sequentially fractionated with water, CDTA, Na₂CO₃, and KOH (1 M and 4 M) to extract fractions enriched in water-soluble pectins, ionically bound pectins, covalently bound pectins, and hemicellulosic polymers, as described by Santiago-Doménech et al. (2008). The yields of the different cell wall fractions obtained were similar to those described by Santiago-Doménech et al. (2008) and Posé et al. (2013) in fruits of the same cv., with no significant differences observed between control and transgenic fruits in any of the fractions obtained (data not shown).

UA content was measured in all fractions, and the results obtained are shown in Fig. 5. In both transgenic lines, the Na₂CO₃ fraction was enriched in UA when compared with control. A slight increase in UA was also observed in the transgenic water fractions. The amount of UA was also higher in the CDTA fraction from line β-Gal37, but not β-Gal28. The analysis of neutral sugars by gas chromatography revealed significant differences between the transgenic β-Gal37 line and control CWM. Transgenic CWM showed a 31% increase in Gal and Ara content (Table 3). The amounts of Rha and Fuc were also higher than control, although the differences were not statistically significant. By contrast, the amount of Xyl, Man, and Glc decreased...
slightly (Table 3). This general trend was observed in most cell wall fractions with some exceptions (Table 4). The control PAW fraction, which contained apoplastic free polymers solubilized by in vivo processes (Redgwell et al., 1992), displayed the highest proportion of Rha, but this carbohydrate was not detected in transgenic PAW. Additionally, this fraction, as well as that of 4 M KOH, showed a slight increase in Glc when compared with control. Polymers soluble in CDTA from transgenic fruits showed a slight decrease in Fuc, Ara, Xyl, and Glc content. Similarly, Na₂CO₃-soluble pectins displayed large decreases in Fuc, Xyl, and Man. Regarding Gal content, all cell wall fractions from transgenic fruits contained a higher proportion of this carbohydrate (Table 4), with the greatest increase in the water and, especially, the 1 M KOH fractions (see Supplementary Fig. 2 at JXB online). Interestingly, the Gal increase in this last fraction was concomitant with large decreases in Xyl and Man and a high increase in Rha (Table 4). The modification of the carbohydrate composition in the 1 M KOH fraction as a result of FaβGal4 silencing was confirmed by Fourier transform infrared spectroscopy (Fig. 6). Control fruits showed a spectral profile in the mid-infrared region at 1200–800 cm⁻¹, corresponding to the fingerprint region of carbohydrates, with peaks at 1014, 1026, 1045, 1077, and 1095 cm⁻¹. According to Kačuráková et al. (2000), homogalacturonan pectin profiles have maximum absorption bands at 1100 and 1017 cm⁻¹. RG-I shows the strongest peaks at about 1070 and 1043 cm⁻¹, and the main xyloglucan absorption band is at 1041 cm⁻¹. The 1 M KOH profile from control fruits could therefore be assigned to a mixture of RG-I and xyloglucans. The 1 M KOH profile from transgenic fruits was different, showing a main peak at 1038 cm⁻¹ and a shoulder at 1070 cm⁻¹. According to Kačuráková et al. (2000), these bands could be assigned to Ara and Gal, respectively, although the absorption infrared bands of other neutral

Table 3. Neutral sugar contents in CWM from ripe control and transgenic FaβGal4 ripe fruits. CWM was extracted from ripe fruits of control and antisense FaβGal4 selected line β-Gal37 and the neutral sugar content was estimated by gas chromatography. Data represent mean values of three independent measurements. Different letters within columns indicate significant differences by Student’s t-test at P = 0.05

| Amount (mg g CWM⁻¹) | Rha | Fuc | Ara | Xyl | Man | Gal | Glc |
|---------------------|-----|-----|-----|-----|-----|-----|-----|
| Control             | 6.9a| 5.7a| 32.5b| 48.4a| 30.0a| 78.5b| 307.5a|
| β-Gal37             | 10.6a| 8.2a| 44.1a| 45.5a| 26.4a| 103.4a| 281.1a|

Table 4. Neutral sugar composition in cell wall fractions from control and transgenic FaβGal4 ripe fruits. CWM from ripe control and transgenic selected line β-Gal37 fruits was sequentially fractionated with water, CDTA, Na₂CO₃, and 1 M and 4 M KOH. The neutral sugar composition of the different cell wall fractions, the residue after cell wall fractionation, as well as the material soluble in PAW was estimated by gas chromatography and expressed in mol%. Data represent mean values of three independent measurements

|          | mol% | Rha | Fuc | Ara | Xyl | Man | Gal | Glc |
|----------|------|-----|-----|-----|-----|-----|-----|-----|
| PAW      |      |     |     |     |     |     |     |     |
| Control  | 9.4  | 1.8 | 16.3| 18.5| 7.1 | 33.2| 13.8|
| β-Gal    | 0.0  | 2.3 | 21.6| 20.0| 5.6 | 34.9| 15.6|
| H₂O      | 2.2  | 1.4 | 27.5| 21.3| 14.2| 20.1| 13.3|
| CO₃      | 6.0  | 1.8 | 25.2| 13.8| 9.1 | 37.0| 7.2 |
| CDTA     | 4.8  | 2.0 | 32.2| 9.9 | 9.5 | 35.5| 6.2 |
| Na₂CO₃   | 8.1  | 1.7 | 27.6| 4.0 | 8.7 | 46.3| 3.6 |
| 1 M KOH  | 6.3  | 0.9 | 27.5| 1.9 | 2.0 | 59.4| 2.1 |
| 4 M KOH  | 1.6  | 2.1 | 15.2| 47.6| 7.7 | 12.9| 13.0|
| CWM      | 0.7  | 0.2 | 1.8 | 3.3 | 3.6 | 3.0 | 85.5|
| residue  | 0.6  | 0.2 | 2.4 | 3.3 | 3.5 | 2.2 | 87.9|

Fig. 5. UA content, expressed as milligrams of UA per 100 mg of CWM, in PAW and the different fractions isolated from cell walls from control and transgenic β-galactosidase ripe fruits. Bars represent mean ± SD of five independent measurements. Mean separation within each cell wall fraction by Dunnett’s multiple comparison test at P = 0.05.

Fig. 6. Attenuated total reflectance Fourier transform infrared spectroscopy absorbance spectra of the 1 M KOH fraction from control and transgenic β-Gal37 cell walls in the 1200–800 cm⁻¹ region.

Fig. 2 at JXB online.
sugars overlap in this region. This result suggests a change in the solubilization of RG-I present in the KOH fraction.

**Discussion**

A common feature of the cell wall disassembly process taking place during ripening of fleshy fruits is the loss of galactose from pectin side chains, supposedly due to the action of β-galactosidase enzymes (Smith and Gross, 2000; Brumell, 2006). In strawberry, Trainotti et al. (2001) identified three β-galactosidase genes. All of them were expressed in young expanding leaves, stolons, flowers, and green fruits, but only FaβGal4 showed increased expression during fruit ripening. Our comparative transcriptomic analysis between strawberry receptacles from immature (green) and ripened (red) strawberry fruits showed a sequence encoding a novel putative β-galactosidase gene, FaβGal4, whose expression was strongly up-regulated, suggesting that this gene could play a pivotal role in strawberry fruit cell wall degradation and fruit softening.

Bioinformatic analyses showed a low identity between FaβGal4 full-length cDNA, or its orthologue in F. vesca, and the three β-galactosidase genes previously described in strawberry. At the protein level, the predicted FaβGal4 shares the highest identity with FaβGal2. The deduced protein of FaβGal4 contains the consensus sequence pattern of the putative active site characteristics of the glycosyl hydrolase family 35 (Henriessat et al., 1995), and, interestingly, it is identical to the putative active site for the other three β-galactosidase genes described in strawberry (Trainotti et al., 2001). These three genes showed β-galactosidase activity when expressed in vitro (Trainotti et al., 2001) and, therefore, it is likely that the FaβGal4 product also has β-galactosidase activity. However, enzymatic activity assays should be performed in order to definitively address this question.

FaβGal4 is mainly expressed in ripe fruit receptacles and is regulated by auxin and ABA.

The expression pattern of FaβGal4 during receptacle fruit growth and maturation was clearly ripening-related, with maximum gene expression in red ripe fruits. This expression pattern was similar to those previously reported for other strawberry genes encoding pectin-degrading enzymes, e.g. pectate lyases, polygalacturonases, or rhamnogalacturonate lyase (Medina-Escobar et al., 1997; Redondo-Nevado et al., 2001; Benitez-Burraco et al., 2003; Quesada et al., 2009; Molina-Hidalgo et al., 2013).

During the development of strawberry fruit, the level of auxins produced by achenes and released into the receptacle declines, which induces the ripening process (Perkins-Veazie, 1995). Several studies have shown that the removing the achenes from the fruit surface induces the expression of many strawberry fruit ripening-related genes that encode cell wall enzymes (Medina-Escobar et al., 1997; Redondo-Nevado et al., 2001; Benitez-Burraco et al., 2003). This was also the case for FaβGal4. More recently, Chai et al. (2011) and Jia et al. (2011) provided molecular evidence indicating that ABA is a signal molecule that, at least, can promote the strawberry ripening-related production of anthocyanins. In fact, ABA levels gradually increase concomitant with the ripening process in strawberry fruits (Chai et al., 2011; Jia et al., 2011). When strawberry fruits were treated at the green stage with NDGA, an inhibitor that blocks the synthesis of ABA, FaβGal4 was significantly down-regulated, as has been observed for other cell wall enzymes (Molina-Hidalgo et al., 2013). Altogether, these results indicate a putative co-regulation of FaβGal4 with genes encoding other pectin-degrading enzymes, and support a physiological role related with the enzymatic degradation of the cell wall in ripe fruits leading to fruit softening.

**Antisense down-regulation of FaβGal4 reduces strawberry fruit softening**

Transgenic strawberry plants carrying a 300-bp antisense sequence of FaβGal4 under the control of the constitutive promoter CaMV35S were generated to get insight into the role of this gene on fruit softening and cell wall disassembly. In general, fruit yield and fruit weight were reduced in most transgenic lines, as has been observed in other transgenic lines with pectinases down-regulated, e.g. pectate lyase transgenic plants (Jiménez-Bermúdez et al., 2002; Youssef et al., 2009). Average fruit size and weight generally decrease when micropropagated strawberry plants are used directly for fruit production, but this side effect of the in vitro tissue culture phase disappears when the progeny of micropropagated plants is evaluated (Cameron et al., 1989; López-Aranda et al., 1994). In FaβGal4 transgenic plants, the reduction of fruit weight was stably maintained after three rounds of runner propagation in the greenhouse, suggesting that this effect was due to FaβGal4 silencing rather than the in vitro micropropagation of plants. In strawberry, fruit size depends mainly on the number of achenes per fruit, the flower position, and the receptacle sensitivity to auxin (Perkins-Veazie, 1995). In the case of the two transgenic lines selected, a marked reduction of the average number of achenes per fruit was observed, with mean values of 240.1 in control plants, 105.6 in β-Gal28, and 69.5 in β-Gal37. However, the mean number of achenes per gram of fruit was only statistically different to the control in the case of β-Gal28 fruits. These results suggest that the decrease in fruit size was mainly due to deficient fertilization in transgenic plants as a result of FaβGal4 silencing. Early and late male gametophyte development genes with high homology to β-galactosidases have been identified in pollen from tobacco, Arabidopsis, and rice (Tursun et al., 2003; Hrubá et al., 2005). The products of these genes may participate in cell wall loosening during young pollen expansion after microspore mitosis and/or may be associated with pollen germination and pollen tube penetration through the style (Hrubá et al., 2005).

Two out of the nine transgenic lines obtained produced fruit significantly firmer than control at the ripe stage. The firmer fruit phenotype was stably maintained during three growing seasons. Transgenic fruits of selected lines were on
Antisense FaβGal4 down-regulation increases cell wall galactose levels and reduces pectin solubilization

According to Redgwell et al. (1997), Gal levels decrease 18% in ripe strawberry fruit when compared with green immature fruit. The silencing of FaβGal4 increased Gal by 30% in transgenic ripe fruit, but also induced a significant increase in the Ara content. After cell wall fractionation, it was observed that all fractions contained higher amounts of Gal than control, with the largest increases present in the H₂O, CDTA, and especially the 1 M KOH fractions. KOH-soluble fractions comprise mainly hemicellullosic polymers, but also contain pectins (Koh and Melton, 2002; Santiago-Doménech et al., 2008; Posé et al., 2013). Interestingly, Redgwell et al. (1997) found that the highest Gal loss, 82.3% of total cell wall Gal, occurred in the KOH fraction and the CWM residue, while only 17.7% was lost from the CDTA/Na₂CO₃ polyuronide-enriched fractions. This trend was observed in most of the fruits analysed, including species where an increase of β-galactosidase activity during fruit ripening had previously been reported, e.g. tomato, kiwifruit, and avocado (Redgwell et al., 1997). The high Gal content in the 1 M KOH fraction from antisense FaβGal4 fruits suggests that Gal loss takes place mostly in arabinogalactan pectins loosely bound to xylolignans by covalent cross-links and/or physically entangled within the matrix glycan. It is noteworthy that this is the first report of the modification of cell wall Gal levels in fruit by suppression of a β-galactosidase gene. Total cell wall Gal content was not modified in antisense TBG4 tomato fruit, although decreased free galactose levels were observed in transgenic fruit at mature green stage, returning to wild-type levels during ripening (Smith et al., 2002).

The relationships among loss of neutral sugars, pectin solubilization, and fruit firmness are far from clear. Some fruits such as apple and nashi pear showed a marked loss of Gal but pectin solubilization was slight (Redgwell et al., 1997). Conversely, plum did not show any Ara/Gal loss but showed extensive pectin depolymerization (Redgwell et al., 1997). In FaβGal4-silenced fruits, the increase of cell wall levels of Gal was paralleled by a reduction in polyuronide solubilization, reflected in the higher amount of Na₂CO₃-soluble pectins. Additionally, a preliminary analysis of these pectins by atomic force microscopy showed larger polymers in transgenic samples than in control samples, suggesting a reduced depolymerization during ripening (unpublished results). Previous studies in transgenic strawberry plants with pectate lyase or polygalacturonase genes silenced demonstrated that the increased firmness in these transgenic fruits was a result of reduced pectin solubilization and depolymerization (Santiago-Doménech et al., 2008; Posé et al., 2013; Posé et al., 2015). In both cases, cell walls from transgenic fruit displayed higher amounts of ionically and covalently bound pectins, those soluble in CDTA and Na₂CO₃, respectively, than wild type. Whether the reduction in fruit softening in transgenic FaβGal4 fruits is directly due to the increase of cell wall Gal content or by an indirect effect on pectin solubilization needs to be elucidated. The reduced loss of Gal...
from hairy regions of pectins could limit the access of other cell wall-modifying proteins, such as polygalacturonase or pectate lyase, to their substrate, therefore limiting pectin solubilization and, likely, depolymerization. As an alternative hypothesis, galactosyl residues could have a direct effect on the mechanical properties of the cell wall. In pea (Pisum sativum) cotyledons, the increased pectin galactan content at late developmental stages correlated with an increase in firmness (McCartney et al., 2000). The Arabidopsis mutant MUR3 lacks a xyloglucan-specific galactosyl transferase and its cell walls exhibit reduced xyloglucan galactosidation (Peña et al., 2004). This modification markedly reduces cell wall strength during hypocotyl growth, although this effect could be related to a modulation of xyloglucan endo-transglycosidase/hydrolase activity (Peña et al., 2004). The ectopic expression of a fungal endo-β-galactanase in potato tuber reduces RG-I linear β-1,4-galactan and also modifies the physical properties of the tissue, making it more brittle when subjected to compression (Ulvskov et al., 2005). A putative role of FaβGal4 on the release of galactosyl-containing oligosaccharides that trigger the ripening process also cannot be discarded. Gross (1985) found that mature green tomato fruit infiltrated with free Gal produced ethylene and ripened earlier than control fruits. More recently, Lahaye et al. (2013) found indirect evidence for the role of Gal on fruit ripening; they observed positive correlations between some firmness quantitative trait loci and cell wall Gal content, galactosylated pectins, and galactosylated xyloglucans in tomato. This hypothesis could explain the pleiotropic effect of FaβGal4 silencing on fruit colour and on the expression of the other β-galactosidase genes.

Conclusions

The results obtained support a key role for FaβGal4 in the softening of strawberry fruit. Transgenic fruits with FaβGal4 silenced showed a 30% increase in fruit firmness at the ripe stage. At the cell wall level, these transgenic fruits contained more Gal in all cell wall fractions than wild type, but also displayed lower polyuronide solubilization. Although the removal of Gal from cell walls is a general feature of the ripening process in many fruits, its relationship with pectin solubilization and fruit firmness is controversial. Our results shed light on these processes and clearly indicate a close connection between Gal levels and polyuronide solubilization. The reduction of Gal loss from galactosyl-containing side chains in the wall might result in decreased porosity, obstructing the access of other cell wall enzymes to their substrates, leading to reduced softening. Alternatively, the increased level of galactosyl residues could have a direct effect on the mechanical properties of the cell wall. This work also indicates that FaβGal4 is a good candidate for the biotechnological or conventional improvement of strawberry texture.

Supplementary material

Supplementary data are available at JXB online.
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