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β-Galactosidases with a lectin-like domain are expressed in strawberry[1]

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Abstract

Strawberry fruits (*Fragaria*×*ananassa* Duch.) undergo a marked softening during their ripening, and the process is accompanied by a release of free sugars with galactose among them. In this work total β-galactosidase activity was measured in cell wall proteins from strawberry fruits at different developmental stages. Three full-length cDNAs (Faβgal1, Faβgal2 and Faβgal3, respectively) encoding different β-galactosidases (EC 3.2.1.23) were isolated from a library representing red fruit transcripts. All of them could be detected both in fruits and in vegetative tissues. However, only Faβgal1 showed an increasing expression during the ripening stages up to a maximum in the red fruits, while the other two (Faβgal2 and Faβgal3) were mostly found in green fruits and became barely detectable during ripening proper. The three β-galactosidase-encoding cDNAs were expressed in the yeast *Pichia pastoris*, and it was thus possible to demonstrate that each of them encode a β-galactosidase. The expression of the three β-galactosidase genes appears to be down-regulated by auxin, as already observed for other ripening-related genes of the non-climacteric strawberry. An unusual characteristic of two strawberry β-galactosidases (Faβgal1 and Faβgal2) is that at the C-terminus of the enzymes a domain is found which is structurally related to known animal peptides with a sugar-binding ability.

Key words: Fruit ripening, β-galactosidase, lectin-like domain, strawberry.

Introduction

Early studies aimed at an understanding of fruit softening demonstrated that the integrity of the fruit cell walls was greatly affected, and that the structural weakening could be related to increases in the activity of one or more cell wall-degrading enzymes. The application of molecular biology techniques to the study of fruit softening revealed that the complexity of this physiological process is greater than previously supposed. A striking example of such complexity was met in tomato plants where, on the basis of biochemical data, fruit softening was supposed to be caused essentially by polygalacturonase activity (Crookes and Grierson, 1983). Surprisingly, this did not prove to be the case in transgenic tomatoes with suppressed expression of the ripening polygalacturonase activity (Smith *et al*., 1988; Giovannoni *et al*., 1989), thus suggesting that cell wall modifying enzymes other than polygalacturonase could play a significant role in the loss of firmness that occurs in ripening tomatoes. Actually, the high sensitivity of the molecular techniques evidenced that many different genes encoding cell wall modifying enzymes could be expressed throughout the softening process (Rose and Bennett, 1999), some of them at levels so low as to make their products undetectable by means of biochemical analyses.

When studying the ripening of fleshy fruits, a frequent observation was a significant loss of neutral sugars, and common among them was galactose (Bartley, 1976; Yamachi *et al*., 1979; Gross and Sams, 1984; Redgwell *et al*., 1992; Cutillas-Iturralde *et al*., 1993; Rose *et al*., 1998; Chin *et al*., 1999).

Galactose residues can be found both in hemicelluloses and, more abundantly, in pectins (Brett and Waldron, 1996). This sugar is mostly located at the level of the side chains that depart from the main polymeric molecules, so

[1] The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers AJ278703 (Faβgal1), AJ278704 (Faβgal2) and AJ278705 (Faβgal3).

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its possible role in cell wall stability has long been difficult to perceive. However, the remarkable release of galactose during dismantling of fruit cell walls has led many researchers to study galactosidases due to their apparent involvement in the mobilization of galactose.

To this purpose, many studies have been carried out with fruits, although significant research has also been made with germinating seeds and growing tissues. The biochemical approach to the study of galactosidases has demonstrated that both α- and β-enzymes can be found in plants, and that they can act either in an exo- or in an endo- manner (Reid and Meyer, 1973; Burns, 1990; Kontos and Spyropoulos, 1996; Valero and Labrador, 1996).

Galactosidases have been characterized biochemically in many different fruits (Pressey, 1983; Biles *et al*., 1997; Kitagawa *et al*., 1995; Ross *et al*., 1993; Ali *et al*., 1995; Ranwala *et al*., 1992). By contrast, less is known from a molecular point of view. In particular, β-galactosidase (EC 3.2.1.23) encoding cDNAs have been isolated and their expression studied in apple (Ross *et al*., 1994) and tomato (Carey *et al*., 1995; Smith *et al*., 1998; Smith and Gross, 2000) fruits, besides asparagus spears (King and Davies, 1995) and carnation petals (Raghothama *et al*., 1991).

As regards the softening of strawberry, a limited exo-polygalacturonase activity has been reported in young green strawberry fruits which then decreased to about one-fifth of the initial value in ripe fruits (Nogata *et al*., 1993). However, Neal was able to detect pectin methyl esterase activity (Neal, 1965) and, more recently, a pectate lyase mRNA (Medina-Escobar *et al*., 1997) has been demonstrated to be expressed during ripening of strawberries, thus suggesting an involvement of pectin degradation in the softening process. On the basis of the high amounts of expression of two different endo-β-1, 4-glucanase genes (Manning, 1998; Harpster *et al*., 1998; Llop-Tous *et al*., 1999; Trainotti *et al*., 1999*b*), hemicellulose degradation is expected to occur during softening of strawberries. Taken together, the above data are well related to the massive degradation of the matrix parietal components observed by electron microscopy (Knee *et al*., 1977; Trainotti *et al*., 1999*a*).

Loss of either galactans (Neal, 1965) or galactose (Knee *et al*., 1977; Redgwell *et al*., 1997) has been reported during ripening of strawberries, therefore galactosidase activity might play a role in the softening of these fruits. Besides evidencing β-galactosidase activity in ripening fruits, the possible expression of β-galactosidase genes in the ripening of strawberries has been studied here. To this purpose, three cDNAs encoding different β-galactosidases have been isolated and characterized and their expression pattern has been analysed. The polypeptides deduced from the nucleotide sequences show different characteristics, and it is particularly interesting that a C-terminus domain appears to be found in two of them. The β-galactosidase activity of the enzymes encoded by the three cDNAs has been demonstrated in experiments with recombinant proteins expressed by the yeast *Pichia pastoris*.

Materials and methods

Plant material and hormone treatments

Strawberry plants (*Fragaria*×*ananassa* Duch. cv. Chandler) were grown at farms near Verona (Consorzio Verde Europa) and S. Giuseppe di Comacchio (C.I.V.). Fruits were selected at five developmental stages: small green (average diameter *c*. 1 cm), large green (average diameter *c*. 2 cm), white (fruit showing depleted levels of chlorophyll, average diameter *c*. 3 cm), pink, and red (commercially ripe) (Huber, 1984). Other tissues were young elongating stolon tips and their proximal parts, young plantlets, flowers at anthesis, mature leaves, young expanding leaves.

Fruits that had to be used in the hormone-treatment experiments were first dipped in an antifungal solution containing promycidon (0.06 g l^{-1}). The synthetic auxin 1-naphthalene acetic acid (NAA, 2 mmol l^{-1} and Silwet L-77 as surfactant, 200 µl l^{-1}) was sprayed on a pool of fruits every 12 h over a period of 48 h. Ethylene treatment was achieved by flushing fruits, placed in an air-tight chamber, with the gaseous hormone (100 µl l^{-1} in air) at a flow rate of 6.0 l h^{-1}. Control fruits were treated in the same way omitting the hormones.

All fruits used in this work (i.e. either treated or untreated) were quartered, frozen in liquid nitrogen and stored at −80 °C for subsequent use.

Protein extraction and β-galactosidase assay

Protein extraction was carried out by grinding 5 g of fruit tissues in a mortar in the presence of liquid nitrogen. The tissue powder was then homogenized with 20 ml of cold washing buffer (25 mmol l^{-1} NaOAc, pH 4.5) added with 1% polyvinylpolypyrrolidone (PVPP), 50 µl of a cocktail of proteinase inhibitors (Plant cell inhibitor cocktail, Sigma, USA) and ascorbic acid at a final concentration of 1 mmol l^{-1}. The slurry was centrifuged at 22 000 *g* for 15 min at 4 °C and the pellet washed twice with 15 ml of cold washing buffer (without PVPP); this procedure was used in order to minimize the content of soluble sugars and cytosolic proteins. After centrifugation the pellet was resuspended in 15 ml of cold extraction buffer (25 mmol l^{-1} NaOAc, 1 mol l^{-1} NaCl, pH 4.5) added with ascorbic acid at a final concentration of 1 mmol l^{-1} and stirred for 16 h at 4 °C. After extraction, the insoluble material was removed by centrifugation, while the enzyme extracts were used directly for the β-galactosidase assays (Pressey, 1983). In particular, β-galactosidase activity was assayed by measuring the rate of *p*-nitrophenyl-β-galactoside (PNPG, Sigma) hydrolysis. The reaction mixture (1.5 ml) consisted of 25 mmol l^{-1} NaOAc, pH 4.5, 0.8 mg l^{-1} PNPG (2.66 mmol l^{-1}) and 400/700 µl of the protein extracts. At time 0 and at different times over a period of 2 h, 350 µl of the reaction mixture were added to 1.35 ml of sodium carbonate (0.42 mol l^{-1}) and the liberated *p*-nitrophenol was measured with a spectrophotometer at 405 nm. The absorbance values obtained were used to construct a straight line, the slope of which could be converted to pmol of *p*-nitrophenol released in the reaction over a time unit (min).

These values (obtained in triplicate) were correlated to the protein contents.

RNA extraction and analysis

RNA was extracted using the Nucleon PhytoPure system (Amersham Pharmacia Biotech, UK) following the manufacturer's instructions. Since this system was actually developed to obtain DNA, the total RNA was separated from it by means of an overnight precipitation in 2 mol l^{-1} LiCl at 4 °C. When using fruit tissues, the amount of Nucleon PhytoPure resin had to be doubled and the supernatants obtained after the chloroform extractions had to be clarified with a 1 h centrifugation at 11 000 *g* in order to obtain high quality RNA ($OD_{260/280} > 1.8$).

Ribonuclease protection assays (RPAs) were performed with 40 μg of total RNA. Lower amounts of RNA (20 μg) were specifically used to avoid signal saturation in the hormone experiments relative to the probes Faβgal1 (only on the white fruits) and Faβgal3 (only on small green fruits). Gene-specific riboprobes were prepared from PCR products obtained after the amplification of the untranslated regions (UTRs) of the three β-galactosidase cDNA clones. Oligos LT224 (TGATTCTGAGTCTCAAGTA) with LT225 (TAATACGACTCACTATAGGGAGGATAACAAGTTGATGCTA) and LT227 (CCTTTACAACCATAGCCAA) with LT228 (TAATACGACTCACTATAGGGAGGACTCTATATTGATTTCTCA) were used to amplify 492 and 203 bp of the Faβgal1 and Faβgal3 3′ UTRs, respectively. In the case of Faβgal2, the 5′ UTR and a small portion of the transcribed region (352 bp in total) was amplified with oligos RS39 (GTATTAGTGACCCTGCTGACGCCT) and LT226 (TAATACGACTCACTATAGGGAGGCACCACTAGCTTAAACTCA). To one oligo of each pair (LT225, LT228 and LT226) a T7 phage promoter was added, so that the PCR products could be directly used for the riboprobe synthesis without any further manipulation. Therefore, the resulting probes would only be a few bases longer than the protected fragments. Probes were labelled with [α-^{32}P]CTP (800 Ci $mmol^{-1}$, Amersham) and T7 RNA polymerase (Ambion Inc., Austin, TX, USA), purified by gel electrophoresis and used in hybridization experiments using the RPA III kit (Ambion). Hybridizations were carried out at 45 °C overnight. The RNase digestion was performed with the RNaseA/T1 mix supplied with the kit at a 1:100 dilution for 30 min at 37 °C. The protected RNA fragments were separated on denaturing 5% acrylamide/8 mol l^{-1} urea gels. After electrophoresis, the gels were placed on Whatman 3 MM paper, dried in a gel dryer and exposed to X-ray films (Biomax MS, Kodak) at −80 °C.

Isolation of β-galactosidase cDNA clones

Different oligonucleotides (BGfor1: 5′ CARACNTAYGTNTTYTGGAA 3′; BGfor2: 5′ TGYGCNGARTGGAAYTW 3′; BGrev1: 5′ CARTARAANCCRTTRCANGT 3′; BGrev2: 5′ TCNGTCCACATYTTNGGYTT 3′), to be used as primers, were prepared by back-translating some conserved peptides (TYVFWN, CAEWN, TCNGFYC and PKMWTE, respectively) evidenced by aligning several plant β-galactosidases. 2 μg of total RNA were used as the starting material for the RT-PCR experiments. The first-strand synthesis was carried out with the 'SuperScript' kit (Life Technologies, USA) using oligo-dT as primer. An aliquot (2 μl) of the first-strand reaction was used for the subsequent PCR amplification. PCR reactions with 200 pmol of the possible four primer combinations and 2.5 mmol l^{-1} $MgCl_2$ were performed in 50 μl vol using a 'DNA Thermal Cycler' (Perkin Elmer, USA) apparatus. Denaturation, annealing and extension temperatures were 94 °C for 1 min, 48 °C for 1 min and 72 °C for 45 s, respectively. This cycle was repeated 35 times. The PCR products were separated by gel electrophoresis and the fragments of interest cloned in the pCR2.1 vector using the TA Cloning kit (Invitrogen, USA).

The cDNA library used for the isolation of the full-length cDNA clones had been prepared starting from 5 μg of poly A^+ enriched RNA extracted from red strawberry fruits (Trainotti *et al*., 1999*b*). The screening was carried out using as probes the three β-galactosidase fragments obtained from the RT-PCR experiments. After isolation, the positive phagemids were excised *in vivo* by means of a helper phage.

DNA sequencing and analysis

DNA sequencing was performed at the University of Padua sequencing facility (CRIBI) using a PCR-based dideoxinucleotide terminator protocol and an automated sequencer (Applied Biosystems 377). Sequences were determined on both strands using, when necessary, chemically synthesized oligonucleotides. Sequence manipulations, analyses and alignments were performed using the 'Lasergene' software package (DNASTAR, USA). Multiple sequence alignments were performed with the CLUSTALW computer program (Thompson *et al*., 1994). Aligned sequences were used to build an evolutionary tree according to the Neighbour–Joining method (Saitou and Nei, 1987), as implemented in the TREECON program ver. 1.3b (Van de Peer and De Wachter, 1994). Bootstrap resamplings (Felsenstein, 1985) were performed to test the robustness of trees. 1000 replicates were done. Maximum parsimony analyses were performed only on informative characters. The program settings used in the different analyses are detailed in the legend of the figure. Protein sequences used to construct the sequence alignments were deduced from nucleic acid ones and were the following (with EMBL/GeneBank accession numbers): strawberry Faβgal1 (AJ278703), Faβgal2 (AJ278704) and Faβgal3 (AJ278705); *Cicer arietinum* (CAA06309; CAA09457;); apple (L29451); papaya (AAC77377); *Lupinus* (CAA09467); carnation (X57171); *Asparagus* (P45582); tomato (Tomβgal1: X83854; Tomβgal4: AF020390 and Tomβgal3: CAA10173); *Arabidopsis* (CAB39679; AAD21482 and AAC04500); *Anthocidaris crassispina* (P22031); *Oncorhynchus mykiss* (BAA92255); *Caenorhabditis elegans* (CAA91091); *Parasilurus asotus* (BAA87860); and *Bos taurus* (AAD05309).

Heterologous expression of the strawberry β-galactosidases and activity assays

The *Pichia pastoris* expression system was chosen to test the enzymatic activity of the proteins encoded by the three isolated cDNA clones due to its efficacy to express cell wall modifying enzymes in an active form (Ferrarese *et al*., 1998). For the secretion of the recombinant enzymes, both the native signal peptides and the mouse α-factor secretion signal (supplied with the *Pichia* expression kit; Invitrogen) were used. In the case of the native signal peptides, the vector of choice was pPIC3, while pPIC9 was used to have fusion proteins with the mouse α-factor secretion signal. In both cases, the vectors were prepared by cutting them with Sna BI and Not I in order to have a blunt end at the 5′ terminus and a Not I compatible end at the 3′ terminus. PCR reactions were carried out with primers designed to have amplification products containing the entire open reading frames (ORFs) of the three β-galactosidases, with the addition

of a Not I restriction site at their 3′ ends to be used for a directional cloning in the Sna BI-Not I cut pPCI3 vector. Oligonucleotides were: for Faβgal1 LT188 (GGGTTTGAGGCTTGTAATGTGGAA) and LT190 (NNN**GCGGCCGC**ATTAGCTGCAAATGGCCTCCACA); for Faβgal2 RS42 (GGCTGTGGCAATGAGAGGAGTT) and RS56 (AGC**TGCGGCCGC**TCTATTTACAAGAAGCTTCT); for Faβgal3 RS45 (GTGGAACTTCAACATGTCGA) and RS 41 (NNN**GCGGCCGC**TCAACTTCTTGCTACCAAAGAA). Bold characters in the oligomers indicate the Not I restriction site. The obtained recombinant plasmids were named Faβgal1PIC3, Faβgal2PIC3 and Faβgal3PIC3, respectively.

pPIC9 was chosen as recipient vector to have fusion proteins with the mouse α-factor and PCR amplifications were carried out with 5′ primers designed to remove the native βgal signal peptide (calculated according to von Heijne, 1983). Therefore, LT189 (TCTGTTTCTTATGATTCCAAGGC) for Faβgal1, RS43 (GTGATCGATGGAAAGCGCAGAG) for Faβgal2 and RS46 (ATCATAGTCAATGGAAAGAGA) for Faβgal3 were the oligos respectively used, while the 3′ primers were the same as for pPIC3. The recombinant plasmids obtained were named Faβgal1PIC9, Faβgal2PIC9 and Faβgal3PIC9, respectively.

All the constructs, linearized with either Sal I (Faβgal1PIC3 and Faβgal1PIC9) or Dra I (the others) endonucleases, were used for the transformation of *Pichia* lines either GS115 (*his4*) to get mut+ clones or KM71 (*arg4 his4 aox1*) to get mut s clones. As negative controls, the pPIC3 and pPIC9 vectors linearized with the same enzymes were used for the transformation of the yeast. In order to discover the best β-galactosidase secreting clones, several hundreds of colonies were replica-plated onto minimal methanol (MM) plates and allowed to grow upside-down for 5 d at 30 °C; 200 µl of methanol were supplied every 12 h inside the lid of the plate to induce recombinant protein production. On the fifth day, the plates were overlaid with a Whatman 3 MM paper disc that was then wetted with 1 ml of citrate buffer (20 mmol l^{-1}, pH 4.6) containing 50 µl of 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-Gal, 50 mg ml^{-1}) and incubated at 28 °C until the appearance of the blue staining. The positive control consisted of a *Pichia* strain expressing the *Escherichia coli* β-galactosidase while the negative control was a strain expressing bovine serum albumin (BSA). From this screening 15 different lines (five for each strawberry β-galactosidase) were selected for subsequent analyses.

In particular, the selected clones were used for assays of β-galactosidase activity with nitrophenyl-β-D-galactopyranoside, in either *orto* (ONPG) or *para* (PNPG) configuration, as the substrate. *Pichia* cells grown on solid BMMY (Buffered Complex Methanol) medium were harvested with a toothpick, resuspended in 1 ml of citrate buffer (20 mmol l^{-1}, pH 4.6) and quantified by measuring the OD_{600} of this suspension. Thereafter, the cell suspension was added with 200 µl of either ONPG or PNPG (4 mg ml^{-1}) and allowed to incubate at 28 °C for either 2 h (Faβgal2) or 3.5 h (Faβgal1, Faβgal3 and the negative BSA control). At the end of the incubation period, 500 µl of Na_2CO_3 (1 mol l^{-1}) were added to each tube and, following clarification by centrifugation, the OD_{420} of the resulting supernatant was measured. β-galactosidase units were calculated as described previously (Miller, 1972).

Results and discussion

Total cell wall-associated β-galactosidase activity was measured in strawberry fruits at different stages of development and ripening (Fig. 1). This activity could be detected only from the large green stage and increased many fold thereafter. During ripening proper (i.e. white, pink and red fruits), β-galactosidase activity remained significant in accordance with the total galactose loss from cell wall material observed during strawberry ripening (Knee *et al*., 1977; Redgwell *et al*., 1997). β-galactosidase activity could not be detected in cytosolic protein extracts (not shown).

The use of degenerate primers in RT-PCR experiments with RNA from ripe strawberry fruits yielded three different cDNA fragments which, on the basis of comparisons with other sequences from data banks (not shown), code for different β-galactosidases. The three partial cDNAs were used as homologous probes to screen a cDNA library previously constructed with poly $(A)^+$ RNA from red strawberries. The results of the screenings were three different full-length cDNA clones which were named Faβgal1, Faβgal2 and Faβgal3, respectively. The three clones were then sequenced and further characterized.

The deduced amino acid sequences of the three strawberry β-galactosidases show a number of interesting peculiarities (Fig. 2). They all have an N-terminal peptide with the hydrophobicity characteristics of the signal peptides usually found in secreted proteins (von Heijne, 1983). The polypeptides encoded by Faβgal1 and Faβgal2 have two putative glycosylation sites each, while the third polypeptide has one such site at the level of the signal peptide, which suggests that the native protein might not be glycosylated.

β-galactosidases (EC 3.2.1.23) belong to family 35 of the glycosyl hydrolases and a consensus pattern for the putative active site has been determined (Henrissat *et al*., 1995). Interestingly, all three strawberry β-galactosidases are perfectly identical at this site (Fig. 2).

A notable characteristic of the strawberry β-galactosidases is that they have different lengths. In particular, Faβgal1 and Faβgal2 are much longer than Faβgal3 due

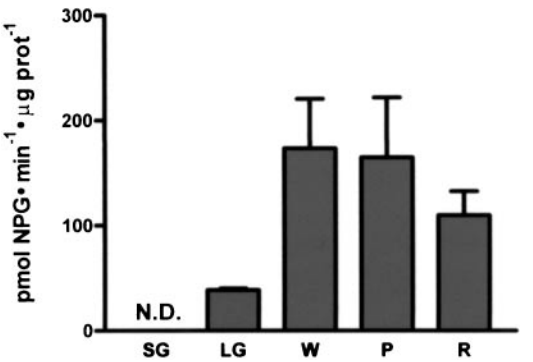

Fig. 1. β-galactosidase activity in strawberry fruits at various stages of development (SG, small green; LG, large green; W, white; P, pink; R, red). N.D.: not detected. Bars indicate standard deviations among three independent experiments.

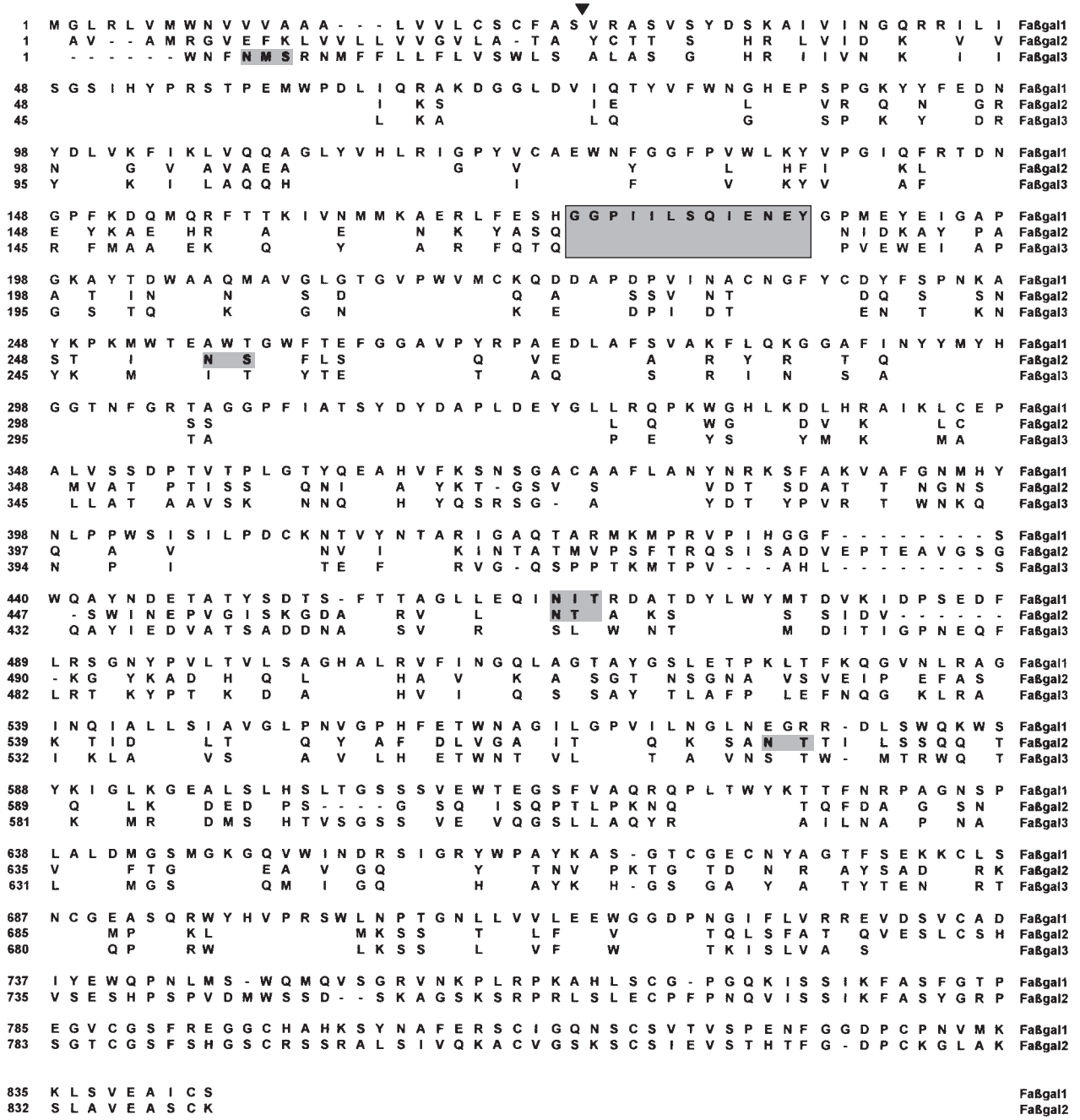

Fig. 2. Alignment and analysis of the three strawberry β-galactosidases. Dashes in the sequences have been introduced by the program to optimize the alignment. Identical amino acid residues have been omitted in the sequences of both Faβgal2 and Faβgal3. The arrowhead marks the putative cleavage site of the signal peptide as determined according to von Heijne (von Heijne, 1983). Potential Asn glycosylation sites (N-X-S/T) in the three sequences are indicated by shaded amino acids. The large shaded rectangle includes the putative active site for β-galactosidases according to Henrissat *et al.* (Henrissat *et al.*, 1995). At the C-terminus the first two sequences have an extra domain of about 120 amino acids.

to the presence of an extra peptide of about 120 amino acids at their C-terminus. Although this extra peptide is far away from the putative active site, and therefore might not affect the catalytic properties of the enzyme, it comprises a peculiar domain, i.e. a SUEL-type (sea urchin egg lectin, PROSITE accession no. PS50228) lectin domain that, if proven to be functional, could affect the biochemical characteristics of the enzymes containing it. A comparison of the extra peptide of the two long cDNAs (i.e. Faβgal1 and Faβgal2) to other known sequences, obtained from very divergent animal species (i.e. sea urchin, rainbow trout, nematode, catfish, and ox), shows the highly conserved position of seven cysteine residues in all the sequences (Fig. 3). Although the similarity among

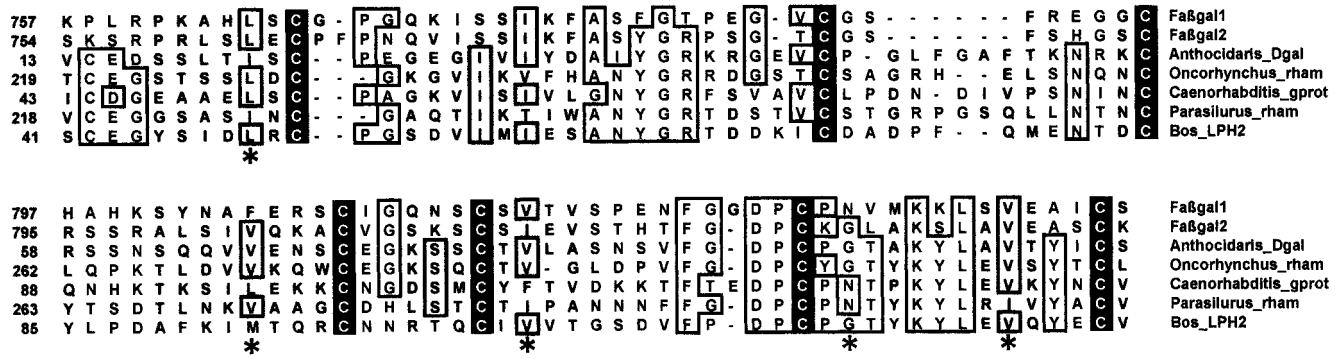

Fig. 3. Alignment of five sequences of animal lectins (sequence accession numbers are indicated in Materials and methods) with the C-terminal region of the two 'long' strawberry β-galactosidases. Black boxes show the conserved cysteines while clear boxes indicate four or more identical amino acids; asterisks indicate conservative amino acid substitutions.

the plant and animal sequences ranges from 16–33%, it is not too speculative to suggest that this common structure might also reflect a similarity of function. It has recently been demonstrated that the protein from catfish eggs acts as a lectin able to bind both D-galactose and, preferentially, L-rhamnose (Hosono *et al*., 1999). Moreover, in the case of sea urchin it was shown that the egg lectin exists as a disulphide-linked homodimer of two subunits. The dimeric form is required for the haem-agglutination activity, though the monomeric form retains its ability to bind carbohydrates (Ozeki *et al*., 1991). One would also have expected to see an alignment of the β-galactosidase C-terminal domains with known plant lectins, but this was not possible. In fact, plant lectins are quite different, for instance, no cysteine residues are present in the lectin-like protein PsNlec1 from pea (Kardailsky *et al*., 1996). However, this is not surprising considering that lectins form a class of structurally divergent proteins or protein domains whose common characteristics is their functional capability to bind carbohydrates (Rini, 1995).

Although a functional analysis is required to establish the actual sugar-binding activity of the β-galactosidases with the lectin-like domain, on the basis of the highly conserved structural identity between the considered animal lectins and the C-terminal domains of the two strawberry β-galactosidases, it is suggested such a possibility exists for the two strawberry lectin-like domains. On the other hand, the presence in plants of genes encoding complex proteins with a domain having a specific activity and another domain involved in the recognition of sugars would not be new. A few years ago a putative receptor kinase with an extracellular lectin-like domain was characterized in *Arabidopsis thaliana* (Hervé *et al*., 1996).

A comparison of the three polypeptide sequences performed with the Lipman and Pearson method shows a surprisingly greater identity (70.2%) between Faβgal1 and Faβgal3 than between either Faβgal1 and Faβgal2 (53.7%) or Faβgal2 and Faβgal3 (56.1%) (Lipman and Pearson, 1985). In other words, in spite of its C-terminal extra domain, Faβgal1 resembles the shorter Faβgal3 more than the comparably long Faβgal2. Apparently, in these sequences, the region before the extra domain is of the most relevance in determining the percentage identity.

The complexity of the β-galactosidase structure is further demonstrated by the phylogenetic tree shown in Fig. 4 where a number of plant sequences have been compared by leaving out the C-terminal extra peptide and the putative signal peptide. From this analysis it appears that at least three groups can be considered. The first group, indicated by a bracket, is wholly formed by short sequences, while β-galactosidases with the extra domain gather in at least two groups. This analysis suggests that the group composed of the 'short' polypeptides is a natural one, while the 'long' polypeptides form a more heterogeneous group. Interestingly, the higher identity existing between Faβgal1 (long polypeptide) and Faβgal3 (short polypeptide), compared to Faβgal2 (long polypeptide), is not sufficient to locate those two sequences within the same group. Probably, in the region common to all the plant β-galactosidases there might be a yet unidentified characteristic sufficient to mark the difference between the long and the short polypeptides.

The expression pattern of the three β-galactosidase cDNAs was analysed in fruit at various stages of development and in vegetative tissues. A Northern analysis did not yield any results (not shown) so the more sensitive Ribonuclease Protection Assay (RPA) was used. As can be seen in Fig. 5, the expression pattern of the three β-galactosidase mRNAs is dissimilar and none of them has an expression limited to fruits.

In particular, the Faβgal1 related transcripts are highly expressed in expanding vegetative tissues, while an increasing progression is observed during development and ripening of fruits, so that the highest transcript amount is visible in softening strawberries (Fig. 5A).

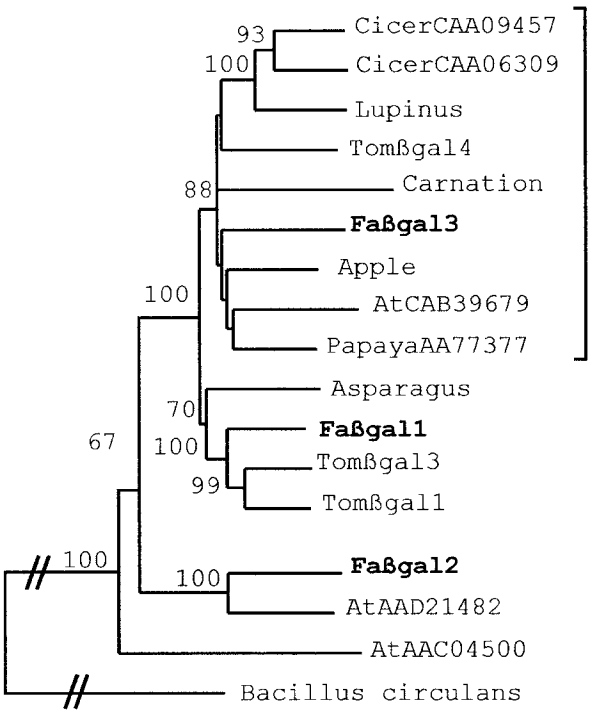

Fig. 4. Neighbour–Joining tree built with TREECON using an alignment of deduced amino acid sequences of a number of plant β-galactosidases and omitting both putative signal peptides and C-terminal extra peptide when present (references and accession numbers are indicated in Materials and methods). The following settings were used. (1) Distance estimation: (a) distance calculation: Poisson correction; (b) alignment position: all; (c) insertions and deletions: not taken into account. (2) Infer tree topology: Neighbour–Joining. Numbers appearing on the branches of the tree refer to the bootstrap values expressed as percentage after 1000 replicates; only values higher than 50% are reported. The group of the 'short' β-galactosidases (i.e. enzymes without the C-terminal extra peptide) is marked by the bracket.

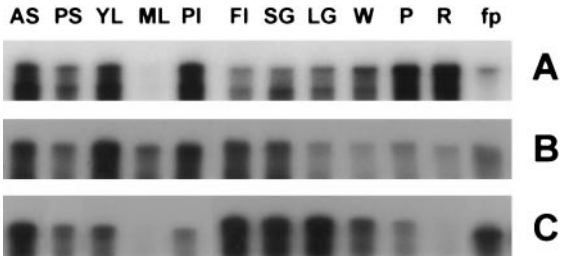

Fig. 5. Expression of the transcripts related to the three different β-galactosidase cDNAs analysed by Ribonuclease Protection Assay (RPA) in different tissues (AS: growing apex of stolons; PS: differentiated proximal region of stolons; YL: young expanding leaves; ML: mature leaves; Pl: young plantlets; Fl: flowers; SG: small green fruits; LG: large green fruits; W: white fruits; P: pink fruits; R: red fruits). fp indicates the free probe whose length is indicated in Materials and methods. 40 μg of total RNA were used in these analyses. Panel A: Faβgal1; Panel B: Faβgal2; Panel C: Faβgal3.

It is interesting to note that such a pattern parallels the one previously described for the strawberry endo-β-(1,4)-glucanase-3 (Trainotti *et al*., 1999*b*).

In the case of the Faβgal2-related mRNA high amounts of expression are visible in expanding vegetative tissues (Fig. 5B). However, contrary to Faβgal1, high levels of transcripts appear also in flowers and in small green fruits, and a subsequent decrease up to barely detectable amounts occurs during fruit development and ripening. The expression pattern of the Faβgal3 related mRNA (Fig. 5C) is different again. In particular, the transcript amount appears to be very high in flowers and in young fruits (both small and large green ones), then it gradually decreases during fruit development and ripening and becomes almost undetectable in red fruits.

A correlation between the β-galactosidase activity measured in strawberry fruits and the expression of the three characterized cDNAs is not easy. In particular, based on the present data, it is impossible to associate the β-galactosidase activity measured in fruits to the expression of any of the three cDNAs characterized. On the other hand, an even more complicated situation resulted in tomato where the activity of isoform II was found to increase during ripening (Carrington and Pressey, 1996), while the expression of two genes (TBG4 and TBG5), one of them (TBG4) encoding isoform II, markedly decreased during ripening proper (Smith and Gross, 2000).

The three β-galactosidase-encoding cDNAs were used to have the related recombinant proteins expressed *in vitro* by the yeast *Pichia pastoris*. In all cases a significant activity was found (Fig. 6) and this is also confirmed by a comparison with proteins secreted from *Pichia* cells producing BSA (bovine serum albumin) instead of β-galactosidase. The peculiarity of the assay (activity is measured using intact yeast cells) is justified by the fact that the activity of the proteins secreted in the medium appeared to be very unstable, particularly for clones expressing β-gal1 and β-gal3. β-galactosidase activity of the recombinant enzymes was measured as soon as they had been secreted, that is when they could still be bound to the yeast cell wall. To this purpose it has to be mentioned that no increase in activity was observed when the yeast cells were lysated before the assay (not shown). Since no protein purification was made, it cannot be established whether the higher activity values obtained for Faβgal2 were actually due to a greater activity of this enzyme compared to the other two, or to a higher amount of enzyme produced by the yeast. However, what is important is that with all three recombinant proteins a β-galactosidase activity could be measured. In previous work performed with proteins purified from pepper fruits, it had been found that β-galactosidase activity assayed with *p*-nitrophenyl-β-D-galactopyranoside was higher compared to *o*-nitrophenyl-β-D-galactopyranoside (Biles *et al*., 1997). Interestingly, this was also true with the recombinant strawberry proteins. Probably the *para* configuration of the substrate might make it more accessible to the active site of the enzymes.

Hormones have been implicated in the maturation of fleshy fruits, and ethylene and auxin appear to be of

particular importance depending on the type of fruits (Abeles *et al*., 1992). Strawberry is a non-climacteric fruit and its ripening has been reported to be ethylene-insensitive (Abeles and Takeda, 1990), while auxin may delay the ripening process and the expression of ripening-related genes (Medina-Escobar *et al*., 1997; Harpster *et al*., 1998; Trainotti *et al*., 1999*b*). As for the climacteric fruits, though their ripening is commonly considered to be highly dependent on ethylene (Abeles *et al*., 1992), a more complex situation has recently been reported in climacteric melon fruits (Hadfield *et al*., 2000). In particular, the authors were able to show that both ethylene-dependent and ethylene-independent genes were expressed during the ripening of this climacteric fruit.

Due to this complexity, both hormones were used to treat strawberry fruits at either the small green (i.e. still growing) or the white (i.e. start of ripening) stage of development. However, while in the case of the small green fruits all three cDNAs were probed, for the start of ripening the assay was limited to Faβgal1 since it was the only one to show a significant and increasingly high expression along the ripening process.

The results of the experiments with small green fruits are shown in Fig. 7 where it can be seen that neither of the hormones had any apparent effect in the case of both Faβgal1 and Faβgal2 (Fig. 7A, B). The same result was obtained with fruits kept in air, which is in agreement with the finding that no relevant increase in gene expression was found at the subsequent stage of large green fruits (Fig. 5). A different result was obtained in the case of Faβgal3 (Fig. 7C). The continuation of fruit growth in air led to increased levels of this transcript, which is in accordance with a higher amount of the same transcript observed at the subsequent large green stage (Fig. 5C). Continuance of growth in the presence of ethylene did not seem to particularly affect the expression of Faβgal3, while the auxin analog NAA (1-naphthalene acetic acid) clearly decreased the rate of expression of this gene (Fig. 7C) as it occurred with Faβgal1 in NAA-treated white fruits (Fig. 8).

In white fruits, continuation of ripening in air led to an increased amount of the Faβgal1 transcripts; by contrast, both ethylene and, especially, NAA appeared to have a down-regulating effect on this gene expression (Fig. 8). The apparent down-regulating effect of ethylene on Faβgal1 gene expression is not completely unexpected as it mimics similar results obtained with the EGase FaEG3 (L Trainotti and G Casadoro, unpublished

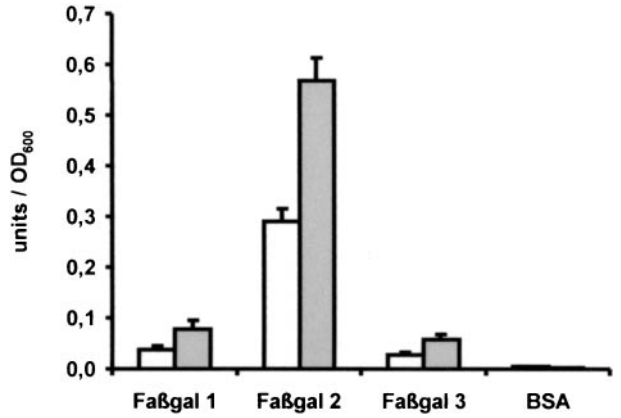

Fig. 6. Assays of β-galactosidase activity carried out on *Pichia pastoris* cells transformed with each of the strawberry β-galactosidase cDNAs. BSA refers to *Pichia* cells expressing bovine serum albumin instead of β-galactosidase. White rectangles indicate the *orto* (ONPG) while the shaded ones indicate the *para* (PNPG) configurations, respectively, of the nitrophenyl-β-D-galacto-pyranoside substrate. With all three enzymes the activity appeared to be higher when the *para* substrate was used. Bars indicate standard deviations among five independent experiments each with different *Pichia* strains.

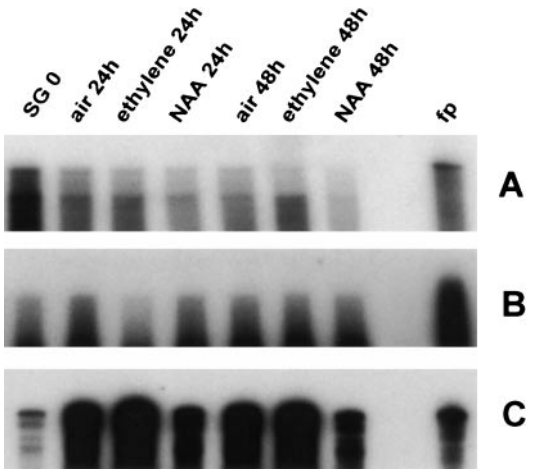

Fig. 7. Effect of either auxin (NAA) or ethylene treatments on the expression of the different Faβgal cDNAs in small green (SG) fruits. fp indicates the free probe whose length is indicated in Materials and methods. In order to avoid a possible signal saturation, the amount of RNA was halved (i.e. 20 μg) in the case of the Faβgal3 probe. While expression of transcripts related to both Faβgal1 and Faβgal2 appeared to be more or less insensitive to both hormones, in the case of Faβgal3 the auxin treatment had a down-regulating effect on its expression.

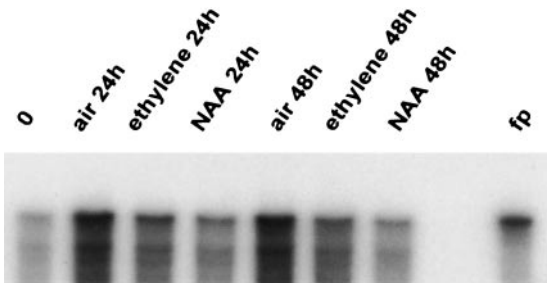

Fig. 8. Effect of NAA and ethylene treatments on the expression of the Faβgal1 related transcripts in white fruits. Lane fp indicates the free probe whose length is indicated in Materials and methods. The amount of RNA was reduced to 20 μg in order to avoid possible signal saturation. While continuance of growth in air brought about an increase in transcript amount, treatment with hormones, especially auxin, apparently had a down-regulating effect on expression of the Faβgal1 mRNA.

results). So, in the non-climacteric strawberry fruits gene expression can be either down-regulated or insensitive (Civello *et al*., 1999) to ethylene. On the other hand, the negative effect of auxin on gene expression in ripening strawberries confirms previous data (Medina-Escobar *et al*., 1997; Harpster *et al*., 1998; Trainotti *et al*., 1999*b*).

Conclusions

Three full length cDNAs have been isolated that, on the basis of sequence comparisons and expression of the encoded proteins in the yeast *Pichia pastoris*, code for different β-galactosidases. The measured β-galactosidase activity appears in agreement with the net galactose loss previously observed during fruit ripening (Knee *et al*., 1977; Redgwell *et al*. 1997). However, both these data and those obtained in tomato (Carrington and Pressey, 1996; Smith and Gross, 2000), make it difficult to find a clear correlation between gene expression and enzyme activity for this class of cell wall enzymes.

The pattern of expression of the three different β-galactosidase cDNAs showed that none of them had an expression limited to either fruits or vegetative tissues. Moreover, expression of two cDNAs (Faβgal2 and Faβgal3) throughout ripening proper decreased sharply to barely detectable amounts in ripe fruits, while the opposite occurred with Faβgal1 whose expression increased dramatically from white to red fruits. Therefore, of the three strawberry β-galactosidase cDNAs characterized in this work, only Faβgal1 can reasonably be related to the softening process.

It had previously been reported that in the non-climacteric strawberry the expression of some genes could be down-regulated by auxin (Reddy and Poovaiah, 1990; Manning, 1994; Medina-Escobar *et al*., 1997; Harpster *et al*., 1998; Trainotti *et al*., 1999*b*), therefore this hormone could negatively regulate some aspects of ripening in strawberry. These data were also confirmed in the case of the ripening-induced Faβgal1 since its expression appeared to be down-regulated by auxin in white fruits where ripening proper starts.

The presence of multiple forms of β-galactosidase in the same plant has been determined by means of molecular techniques (Smith and Gross, 2000), but it has been especially studied biochemically (Pressey, 1983; Ranwala *et al*., 1992; Biles *et al*., 1997). The multiplicity of β-galactosidase forms in the same species might reflects the structural heterogeneity of the β-galactose containing polymers in different tissues (Brett and Waldron, 1996), so that a complex set of degrading enzymes would be required for the parietal metabolism in a given species.

The fact that the transcripts related to the three cDNAs had to be detected by RPA implies that the rate of their expression was not particularly high. Moreover, only one of them had a pattern of expression that could be regarded as relevant to ripening proper. It might therefore appear difficult to find an explanation for the observed enzyme activity and the release of free galactose, previously reported to occur during the ripening of strawberry fruits (Knee *et al*., 1977; Redgwell *et al*., 1997), unless it is supposed that there are one or more β-galactosidase genes not yet cloned or that the ripening β-galactosidase is a particularly active and efficient enzyme.

The possibility that the strawberry β-galactosidase might work in a particularly efficient manner is, in fact, suggested by the characteristics of the C-terminal extra peptide. So, it might use the lectin-like domain to anchor itself to specific sugars, thus increasing its efficiency in releasing galactose residues from the parietal polymers containing them. This hypothesis might not appear so extravagant if it is also considered that an endo-β-1, 4-glucanase with a putative cellulose-binding domain is expressed in ripening strawberries (Trainotti *et al*., 1999*b*). The possibility for an enzyme to be anchored while carrying out its function in a process as massive as the dismantling of the cell wall would make its activity particularly efficient without requiring high amounts of protein to be expressed.

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