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RESEARCH PAPER

Isolation and promoter analysis of two genes encoding different endo-β-1,4-glucanases in the non-climacteric strawberry[1]

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Abstract

Two endo-β-1,4-glucanase (EGase; EC 3.2.1.4.) genes, highly expressed during ripening of the non-climacteric strawberries (*Fragaria×ananassa* Duch. cv. Chandler), were isolated. Serial promoter deletions of both genes (i.e. *FaEG1* and *FaEG3*) fused to GUS were transiently assayed in strawberry fruits by using a technique recently developed in this laboratory. Although differences were observed with the short fragments, GUS activity became comparable with the largest fragments of both promoters. The apparently similar strength of the two largest promoter fragments was in contrast with previous results of Northern analyses which demonstrated different transcripts amounts for the two genes. The inclusion of the 3′ flanking region of both genes in the transient assays showed that, in the case of *FaEG3*, the 3′ region had a down-regulating effect on the expression of GUS, and this might account for the lower amount of *FaEG3* mRNA usually observed in ripe fruits compared to that of *FaEG1*. Downstream instability elements might be involved in such down-regulation.

Key words: Endo-β-1,4-glucanase genes, *Fragaria×ananassa* Duch., strawberry, transient promoter analysis.

Introduction

Higher plants endo-β-1,4-glucanases (EGases; EC 3.2.1.4.), also known as 'cellulases', have long been studied in events where the separation of cells is required. Although such events are found in a wide range of physiological processes, particular attention has been given to the abscission of organs and the softening of fleshy fruits due to their economic relevance. As regards the involvement of endo-β-1,4-glucanases in the softening of fruits, a good deal of work at the molecular level has involved, among others, the fruits of tomato (reviewed in Rose and Bennett, 1999), avocado (Fisher and Bennett, 1991; Abeles *et al*., 1992), pepper (Ferrarese *et al*., 1995; Harpster *et al*., 1997), and strawberry (Harpster *et al*., 1998; Manning, 1998; Llop-Tous *et al*., 1999; Trainotti *et al*., 1999).

Tomato has been, and still is, a model plant for a number of reasons. Among them is the fact that its berry is a climacteric fruit. This characteristic makes tomato interesting for studies of ripening since the appearance of the climacteric peak is usually considered to mark the onset of ripening. Accordingly, in their review of the cell wall modifications that occur in growing and ripening tomato fruits, Rose and Bennett (1999) made a distinction between genes encoding cell wall modifying enzymes that are expressed during fruit development and are up-regulated by auxin, and genes encoding cell wall modifying enzymes that are expressed during ripening and are up-regulated by ethylene.

The absence of both respiratory and ethylene climacteric peaks to mark a possible boundary between growth and ripening makes the non-climacteric fruits more intriguing. However, the pattern of endo-β-1,4-glucanase gene expression in the non-climacteric strawberry fruits seems partly to contradict the idea that in these fruits the passage from growth to ripening is smooth and gradual.

In strawberry, two endo-β-1,4-glucanase genes are significantly expressed in fruits (Llop-Tous *et al*., 1999; Trainotti *et al*., 1999) and one of them (*FaEG3*) is also

[1] The nucleotide sequence data are reported in the EMBL, GenBank and DDBJ Nucleotide Sequence databases under the Accession Numbers AJ414708 (*FaEG3*) and AJ414709 (*FaEG1*).

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expressed in growing vegetative tissues (Trainotti *et al*., 1999). In fruits, *FaEG3* is switched on at a very young stage and shows an increasing expression up to a maximum in red ripe fruits. In other words, its gradual expression in fruits seems to parallel the gradual evolution of fruits from growth to ripening. The second endo-β-1,4-glucanase gene (*FaEG1*) is fruit-specific and starts to be expressed at the stage of white fruits, that is when the fruits stop their growth and enter into the ripening phase. The well-defined start of expression of *FaEG1* suggests that some yet unknown signal might mark the beginning of ripening, as the climacteric peak does in the climacteric fruits.

The different spatial and temporal expression pattern of the two strawberry endo-β-1,4-glucanase genes makes them valuable. In particular, the fruit ripening specificity and the high rate of expression of *FaEG1* make its promoter a good candidate for biotechnological uses that require a fruit and ripening specific expression of a given gene in strawberry. As regards *FaEG3*, the possibility that its early expression in fruits might lead to the production of oligosaccharides acting as signals for the subsequent start of ripening (Dunville and Fry, 2000) might offer a tool to modulate the timing of fruit ripening.

In parallel with the good deal of work done with climacteric fruits, ripening related genes have mostly been studied in this type of fruits. In the present work, data are presented about the characterization of two genes encoding endo-β-1,4-glucanases in the non-climacteric strawberry. In particular, chimeric gene fusions of 5′- and 3′- flanking regions of both genes with a β-glucuronidase (GUS) gene were prepared. The different constructs were used in transient expression assays in strawberry fruits, also treated with the auxin analogue NAA.

Materials and methods

Plant material and auxin treatment

Strawberry fruits (*Fragaria*×*ananassa* Duch. cv. Chandler) were obtained from farms either near Verona or at Pergine (Trento). The different developmental stages of fruits were determined according to Huber (1984).

Fruits that had to be used in the experiments with auxin were first dipped in an antifungal solution containing promycidon (0.06 g l^{-1}). The synthetic auxin 1-naphthalene acetic acid (NAA, 2 mM), and Silwet L-77 (200 µl l^{-1}) as surfactant, were sprayed on a pool of fruits every 12 h over a period of 48 h. Control fruits were treated in the same way but NAA was omitted.

When not used immediately, fruits were quartered, frozen in liquid nitrogen and stored at –80 °C.

Nucleic acids extraction

When extracting DNA by using the Nucleon PhytoPure system (Amersham Pharmacia Biotech, England), RNA was obtained as a by-product of the DNA extraction by means of an overnight precipitation in 2 M LiCl at 4 °C.

Isolation of genomic clones, DNA sequencing and determination of the transcription start site

The genomic clones were isolated from a strawberry (*Fragaria*×*ananassa* Duch. cv. Chandler) genomic library constructed by cloning Mbo I partially digested DNA into the Xho I (partially filled-in) site of the Lambda FIX II vector (Stratagene, USA). The library was a gift from Dr Juan Munoz Blanco (University of Cordoba, Spain). The FaEG1 and FaEG3 cDNAs (Trainotti *et al*., 1999) were used as probes to screen the library following standard procedures (Sambrook *et al*., 1989). DNA from the purified lambda clones was extracted using a Qiagen kit (Qiagen, Germany), digested and, after electrophoresis and blotting, probed once more with either FaEG1 or FaEG3. The hybridizing bands were subcloned in the pGEM (Promega, USA) plasmid vector.

DNA sequencing was performed at the CRIBI sequencing facility of the University of Padua using a PCR-based dideoxynucleotide terminator protocol and an automated sequencer (Applied Biosystems 377). Sequences were determined on both strands using, as templates, plasmids containing inserts of different lengths prepared by progressive unidirectional deletions using the 'Erase-a-Base' system (Promega, USA) and, when necessary, chemically synthesized oligonucleotides. Sequence manipulations, analyses and alignments were performed using the 'Lasergene' software package (DNASTAR, USA).

The transcription start site of both genes was determined using the 'AMV Reverse Transcriptase Primer Extension System' (Promega). Each reaction was carried out with 200 fmol µl^{-1} of ^{32}P-end-labelled primer and 20 µg of total RNA from red fruits. A control without RNA was also included.

Preparation of glucuronidase gene constructs

Plasmids used for transformation experiments contained the GUS reporter gene interrupted by a plant intron described by Vancanneyt *et al*. (1990). This gene can be driven by the CaMV 35S promoter (35S) in plasmid p35SGUS-INT or it can be used without promoter, as in plasmid pPR97 (Szabados *et al*., 1995), to carry out negative controls. Plasmid pPR97 was also used as the cloning vector for both the *FaEG1* and *FaEG3* promoter deletions. These promoter deletions were prepared by PCR using a high fidelity polymerase (Pfu, Stratagene).

Five promoter fragments were prepared for each gene. In the case of *FaEG1*, the fragments were amplified from either lambda or plasmid DNA using oligo SS11 (5′-TTTTTTTTCTCTCTCG-TTTTTGCTGG-3′, annealing from base 3213 to base 3188 of the antisense strand) at the 3′ end and either sequence-specific or universal oligos (annealing to vector sequences) at the 5′. The resulting fragments contained 46 bp of the 5′ untranslated region (UTR) of the *FaEG1* mRNA and different portions of the 5′ untranscribed region. These fragments were cloned into the vector pPR97, a few bases upstream of the starting ATG of the GUS gene. The resulting constructs were named to reflect both the length and the origin of the inserted promoter fragment. So pEG1 refers to the promoter fragments of the *FaEG1* gene. pEG1-30 contains 2980 bp, pEG1-20 2113 bp, pEG1-10 953 bp, pEG1-4.6 462 bp, and pEG1-2 216 bp of the 5′ untranscribed region of *FaEG1*. Constructs pEG1-30-3′ and 35S-3′EG1 were obtained by replacing the NOS polyadenylation sequence present in constructs pEG1-30 and 35SGUS-INT with the 1281 bp of *FaEG1* ranging from 6825 to 8105, and corresponding to the 3′ UTR plus 1028 bp of 3′ untranscribed region. Also in this case the fragments were prepared by means of PCR with appropriate restriction sites, useful for the subsequent cloning steps, added at the 5′ end of the oligos used for the amplification.

To produce the five promoter fragments of gene *FaEG3*, the 3′ oligo was SS21 (5′- ACTAAAACACTGGTCTATACTA-3′, annealing from base 3379 to base 3358 of the antisense strand). The resulting fragments contained 134 bp of the 5′ UTR of the FaEG3 mRNA and different portions of the 5′ untranscribed region. Also these fragments were cloned into the vector pPR97 and the resulting constructs were named pEG3-n (i.e. promoter of gene *FaEG3*). In particular, pEG3-30, pEG3-20, pEG3-14, pEG3-8, and pEG3-4 contained 3025, 2025, 1420, 814, and 490 bp of the *FaEG3* untranscribed region, respectively. Constructs pEG3-30-3′ and 35S-3′EG3 were obtained by replacing the NOS polyadenylation sequence present in constructs pEG3-30 and 35SGUS-INT with the 1458 bp of *FaEG3* ranging from 6733 to 8190, and corresponding to the 3′ UTR plus 957 bp of 3′ untranscribed region. Also in this case the fragments were prepared by means of PCR with appropriate restriction sites, useful for the subsequent cloning steps, added at the 5′ end of the oligos used for the amplification.

Analysis of transient gene expression

A new protocol, developed in the laboratory (Spolaore *et al*., 2001), was used to test the strength of the different promoter deletions of the two strawberry endo-β-1,4-glucanase genes. In brief, *Agrobacterium* cells were transformed with plasmids containing the constructs to be transiently assayed. Afterwards, a suspension of transformed *Agrobacterium* cells was evenly injected throughout the entire fruits by using a sterile 1 ml hypodermic syringe. After 2 d incubation at 22 °C with a 16 h light photoperiod, the injected fruits were frozen and ground in a mortar. The extracted proteins were then used to assay both GUS and luciferase activities. In the case of the auxin treatments, fruits were injected after 24 h from the first hormone treatment; thereafter, the *Agrobacterium* infiltrated fruits were treated with auxin every 12 h throughout the incubation period.

Proteins for reporter gene assay were extracted and assayed as previously described (Spolaore *et al*., 2001). In order to compare values obtained in different infiltration experiments, the GUS activities measured were normalized to the corresponding luciferase activities, so the plotted values are expressed as nmol 4-MU released min^{-1} pg^{-1} of luciferase.

Results and discussion

The two endo-β-1,4-glucanase encoding cDNAs (FaEG1 and FaEG3) previously characterized in the laboratory (Trainotti *et al*., 1999) were used as probes to screen a strawberry genomic library. Two DNA fragments, each containing one of the two EGase genes, were the result of this screening. In the case of *FaEG1*, 8627 bp were sequenced, while 8215 were the bases sequenced for *FaEG3*. The sequences of the two genes are not shown here and are available in public databases with the following accession numbers: AJ414708 (*FaEG3*) and AJ414709 (*FaEG1*).

The transcription start site of *FaEG1* was determined by the 5′ primer extension technique and it was found to correspond to the **T** in position –46 from the initial ATG. One TATA box was found at position –26 from the transcription start site (–72 from the initial ATG). The transcription start site of *FaEG3* corresponds to the **C** in position –134 from the initial ATG. No standard TATA box could be evidenced, though a putative one (TATATA) was found at –27 bp (relative to the transcription start site, –161 bp from the initial ATG).

In order to evaluate the regulatory capacity of the two promoters, serial promoter fragments were fused to the GUS reporter gene. Pink strawberry fruits were used for these experiments since ripening continued after the transformation with *Agrobacterium*, and the fruits became red by the time of tissue sampling for the reporter assays. To this purpose, it had previously been shown that the transition from pink to red fruits corresponds to the maximum rate of expression for both genes (Llop-Tous *et al*., 1999; Trainotti *et al*., 1999). Two different reporter genes were used, one of them (GUS-INT, Vancanneyt *et al*., 1990) for the promoter analysis and the other one (LUC-INT, Hanson *et al*, 1999) to check the transformation procedure. The different constructs and the results of the transient transformation assays are shown in Fig. 1. All the GUS values were normalized to the luciferase activity measured in the same protein extract.

In the case of the *FaEG1* promoter, whose fragments were named pEG1-n (Fig. 1, upper panel), GUS activity just above the background started to be detected with the smallest fragment (pEG1-2). Thereafter, in parallel with the increase in fragment size, the promoter strength increased steadily until about 1.0 kb (pEG1-10). The next promoter fragment (pEG1-20) caused a dramatic increase in GUS activity which appeared even higher than that obtained with the 35S promoter. Finally, a further increase in GUS expression was obtained with the largest promoter fragment (pEG1-30). Though 462 bp were sufficient to drive a significant expression of GUS, the above analysis demonstrated that a particularly high efficiency was obtained when the promoter fragment changed from about 1.0 kb to about 2.0 kb. Accordingly, the latter region might contain elements for a positive regulation of the *FaEG1* expression as observed for other genes (Montgomery *et al*., 1993; Nicholass *et al*., 1995; Atkinson *et al*., 1998).

The lower panel of Fig. 1 shows both the fragments of the *FaEG3* promoter (named pEG3-n) and the results of the transient expression assays. GUS activity above the background was detected with the fragment of about 0.5 kb (pEG3-4). Also in the case of the *FaEG3* promoter a steady GUS increase parallelled the increase in fragment size. However, in this case a jump in GUS activity was observed when the promoter size changed from about 2.0 kb to about 3.0 kb, and this activity was higher than that obtained with the 35S promoter. This finding suggests that elements involved in a strong positive regulation should be located in the distal 1.0 kb of the analysed *FaEG3* promoter, a situation different from that of the *FaEG1* promoter where the maximum regulatory capacity was found by adding the two most distal fragments.

With both promoters the strength of the largest 5′ fragments (about 3.0 kb) was greater than that of the

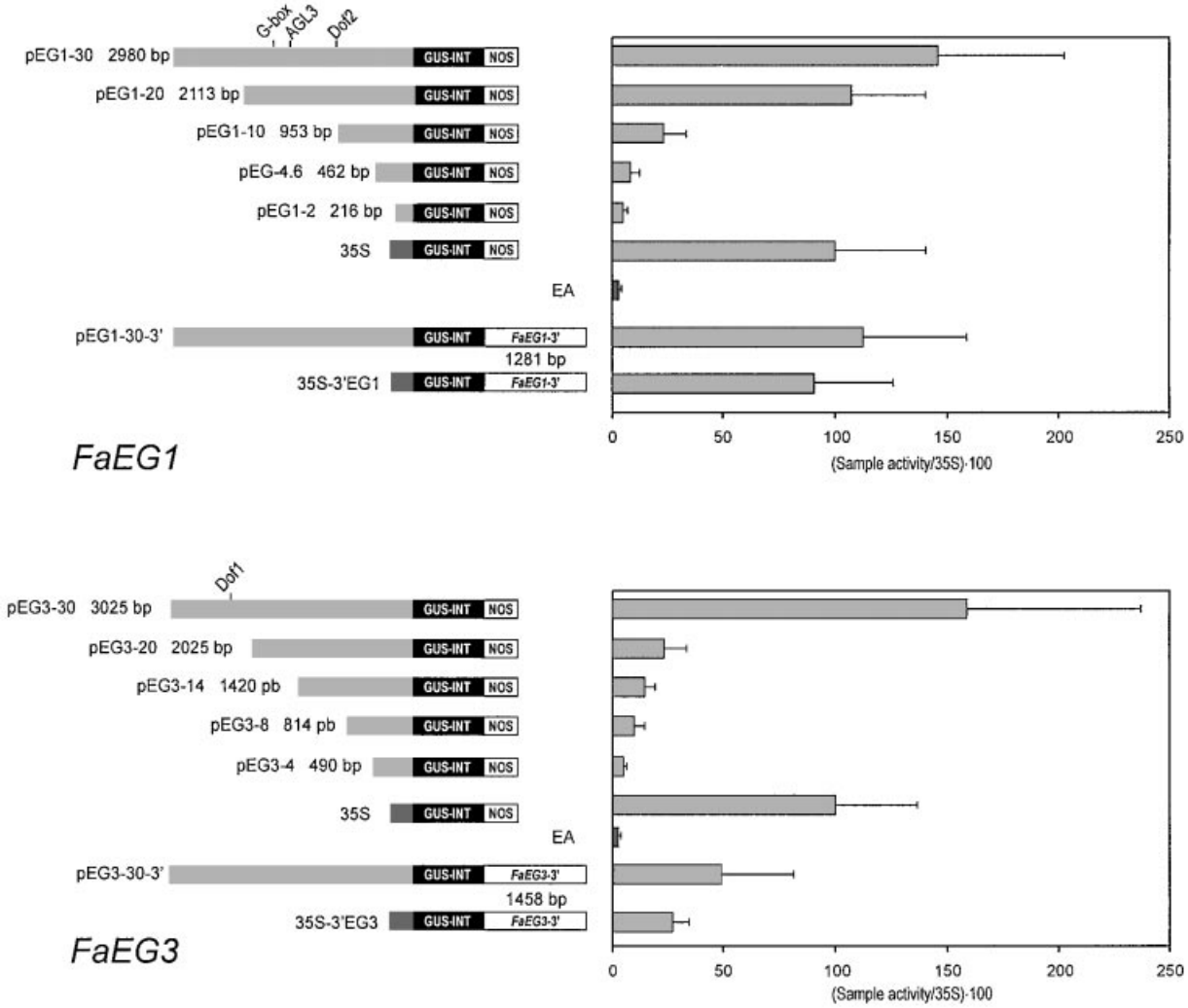

Fig. 1. GUS activity measured in protein extracts obtained from strawberries transformed at the pink stage with constructs harbouring different promoter deletions of *FaEG1* (top) and *FaEG3* (bottom). The different promoter fragments used in each construct are depicted on the left; the indicated bp length is meant from the starting ATG. Also putative *cis* elements are marked (G-box; AGL3: box recognized by Agamous-like proteins; Dof1 and Dof2: box recognized by Dof transcription factors). In the various experiments the GUS activity (expressed as nmol MU min^{-1} μg^{-1} protein) was normalized to the luciferase activity (expressed as pmol of luciferase in 1 μg of protein) measured in the same protein extract. The activities measured with the strawberry promoter fragments were expressed as a percentage of the activity obtained with the CaMV 35S promoter, set arbitrarily to 100%. EA: endogenous activity (i.e. GUS activity measured in fruits transformed only with a luciferase gene). All values are the average of four independent experiments. Bars represent standard errors.

constitutive 35S promoter. This result makes both EGase promoters useful for biotechnological applications aimed at the improvement of the strawberry fruit quality. However, the promoter of *FaEG1* might be especially interesting since the expression of this gene is both fruit- and ripening-specific, while *FaEG3* is also expressed in young fruits and in growing vegetative tissues.

A comparable regulatory capacity of the two largest promoter fragments seems to contrast with the amount of their related transcripts as determined by Northern analysis. In fact, previous research had shown that, in spite of an earlier start, expression of *FaEG3* led to an amount of mRNA in red fruits which was apparently lower than that of the transcript related to *FaEG1* (Llop-Tous *et al*., 1999; Trainotti *et al*., 1999).

A possible explanation for the discrepancy between Northern and promoter analyses could be obtained by including sequences at the 3′ of the genes in the analysis of both promoters. The 3′ flanking region of each EGase gene was also added to a 35S-GUS-INT construct in order to enucleate their own regulatory activity. While the 3′ sequence of *FaEG1* had a minor effect on the expression of the reporter gene, the 3′ sequence of *FaEG3* caused a marked decrease in the expression of GUS, and such a decrease was also shown with the 35S promoter (Fig. 1, upper and lower panels). Accordingly, the down-

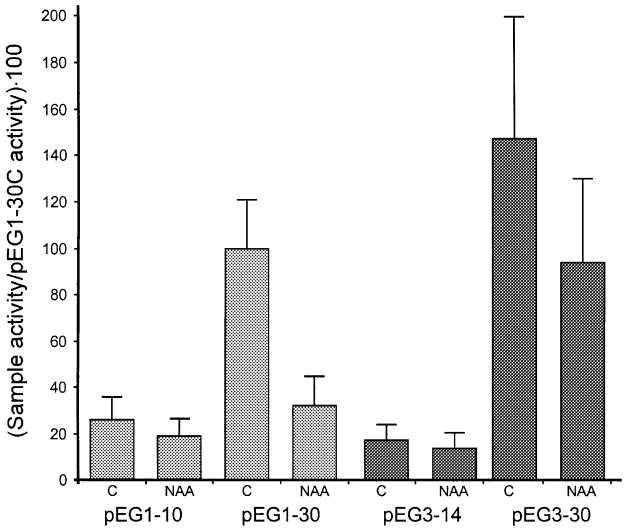

Fig. 2. GUS activity measured in protein extracts obtained from strawberries transformed at the white stage with constructs pEG1-10 and pEG1-30, respectively, for *FaEG1* and pEG3-14 and pEG3-30, respectively, for *FaEG3*. 36 h before transformation and throughout the subsequent incubation period, fruits have been treated with the auxin analogue naphthalene acetic acid (NAA). The GUS activity (expressed as nmolMU min^{-1} μg^{-1} protein) was normalized to luciferase activity (expressed as pmol of luciferase in 1 μg of protein) measured in the same protein extracts. All GUS activities were expressed as a percentage of the activity obtained with the CaMV 35S promoter, set arbitrarily to 100%. All values are the average of four independent experiments. Bars represent standard errors.

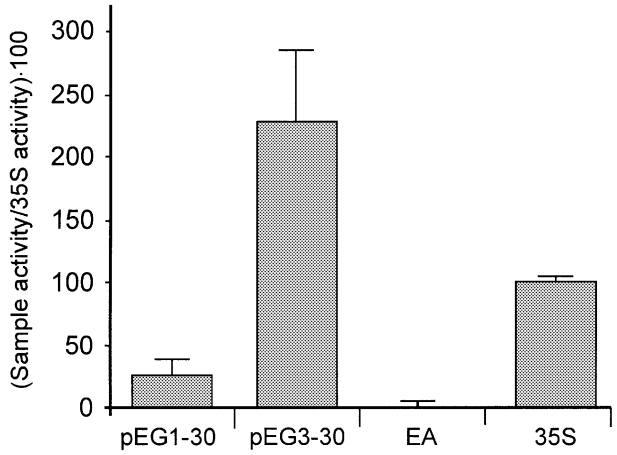

Fig. 3. GUS activity measured in proteins extracted from strawberries transformed at the white stage with different constructs (i.e. pEG1-30 and pEG3-30, respectively). The GUS activity (expressed as nmolMU min^{-1} μg^{-1} protein) was normalized to luciferase activity (expressed as pmol of luciferase in 1 μg of protein) measured in the same protein extract. Activities were expressed as a percentage of the activity obtained with the CaMV 35S promoter, set arbitrarily to 100%. EA: endogenous activity (i.e. GUS activity measured in fruits transformed only with a luciferase gene). All values are the average of four independent experiments. Bars represent standard errors.

regulating effect of the *FaEG3* 3′ flanking region might explain the observed lower amount of the *FaEG3* mRNA compared to that of *FaEG1*.

It is known that genes can contain sequences able to affect the stability of mRNAs (Abler and Green, 1996). In particular, it has been shown that the 3′ UTRs of *SAUR* transcripts contain a sequence [i.e. the DST (downstream) element] able to function as an instability element. This element consists of three separated subdomains, with the second having the sequence ATAGAT highly conserved in all the described *SAUR* DST elements (Abler and Green, 1996). A sequence analysis of the 3′ UTRs of the two strawberry EGase genes provided some clues as to the down-regulating effect shown by the *FaEG3* 3′ flanking region. The complete second subdomain ATAGAT and a partial third subdomain GTA were found in the *FaEG3* 3′ UTR, but not in the same region of the *FaEG1* gene. The presence of possible instability elements suggests that the *FaEG3* mRNA might be less stable than the *FaEG1* encoded transcript, and this could explain the apparent down-regulating effect of the 3′ flanking region of *FaEG3*. Of course, the authors are aware that other as yet unknown sequences might contribute to the different regulation of the two strawberry EGase genes.

While apparently insensitive to ethylene (Abeles *et al*., 1992), in the non-climacteric strawberry the ripening process can be delayed by auxin (Given *et al*., 1988), a hormone that has been shown to have a down-regulating effect on the expression of a number of ripening-related genes (Reddy and Poovaiah, 1990; Manning, 1994; Medina-Escobar *et al*., 1997; Harpster *et al*., 1998; Trainotti *et al*., 1999). To ascertain whether the two isolated promoters contained some information involved in the hormonal control of both *FaEG1* and *FaEG3* gene expression, two different fragments for each promoter (pEG1-10, pEG1-30 and pEG3-14, pEG3-30, respectively) were used to drive expression of GUS in auxin-treated strawberry fruits. The effect of auxin treatments was analysed in white fruits, a stage where the expression of both EGase genes is far from having reached a maximum, thus allowing possible changes in reporter gene activity to be easily detected following the hormonal treatment. In all the analysed situations the auxin analogue NAA induced a decrease in the amount of reporter activity (Fig. 2). Interestingly, the auxin down-regulating effect is greater in percentage terms with the promoter of *FaEG1*, which is the gene more related to the ripening phase.

The pattern of spatial expression of the two EGase genes was shown to overlap in fruits by using the tissue printing technique (Trainotti *et al*., 2000), while differences were found with regard to the pattern of temporal expression (Trainotti *et al*., 1999). White fruits were, therefore, used in experiments aimed at understanding whether regulatory elements involved in this stage-specific expression might be present in the largest promoter fragments (i.e. pEG1-30 and pEG3-30, respectively) of the two genes. Contrary to

the comparable strength shown by these two promoters in the transition from pink to red fruits (see Fig. 1, upper and lower panels), in white fruits pEG3-30 showed a much greater strength than pEG1-30 (Fig. 3). The much higher amount of GUS activity observed with the *FaEG3* promoter is in accordance with the higher amount of the *FaEG3* mRNA observed in white fruits. This result suggests that sequences controlling the temporal expression of the two EGase genes should be present in both pEG1-30 and pEG3-30 promoter fragments. Another possibility is that *trans* acting factors regulating the expression of *FaEG1* might be available only at the ripening stage.

Transient expression analyses showed a different behaviour of the two EGase promoters, and suggested that important regulatory elements might be located in the 1.0 kb regions spanning from 1.0–2.0 kb of the *FaEG1* promoter and from 2.0–3.0 kb of the *FaEG3* promoter, respectively. An informatic analysis of the two promoters was therefore performed (Hehl and Wingender, 2001).

In the case of *FaEG3*, a number of putative domains involved in the binding of different transcription factors were found to be scattered throughout the promoter. However, in the most active region (i.e. from 2.0–3.0 kb) one DOF1 (DNA-binding with One Finger 1) domain was found (Fig. 1, lower panel), and it is known that Dof proteins can be involved in a number of regulatory circuits in plants. For instance, it has been shown that the pumpkin Dof protein AOBP binds to the promoter of an abscorbate oxidase gene (Kisu *et al*., 1998; Shimofurutani *et al*., 1998), while the maize Dof1 and Dof2 proteins control the expression of genes involved in carbon metabolism (Yanagisawa and Sheen, 1998; Yanagisawa, 2000). Accordingly, by binding to a yet unknown Dof protein, the DOF1 binding domain of the most distal 1.0 kb of the *FaEG3* promoter might be responsible for the observed high activity.

The analysis of the *FaEG1* promoter revealed that the most active promoter region (i.e. from 1.0–2.0 kb) contains important domains (Fig. 1, upper panel). Among them it is worth mentioning a domain for the binding of the AGL3 protein (Huang *et al*., 1995), and a G-box binding site (Sablowski *et al*., 1994). It is also interesting to note the presence of a DOF2 binding domain at the 5′ end of the pEG1-10 promoter fragment. Such a terminal localization might have made its possible binding to strawberry Dof proteins ineffective, while a binding and consequent effects on the transcription might have been effective in the case of the bigger pEG1-20 promoter fragment.

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