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RESEARCH PAPER

Functional analysis of homologous and heterologous promoters in strawberry fruits using transient expression*

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Abstract

The isolation and characterization of fruit-specific promoters are critical for the manipulation of the nutritional value and quality of fruits by genetic engineering. The analysis of regulatory sequences of many ripening-related genes has remained elusive for many species due to their low transformation efficiency and/or lengthy regeneration of a small number of transgenic plants. Strawberry is an important crop and represents one of the most widely studied non-climacteric model systems. However, until recently, its difficult regeneration has limited the functional study of promoters by stable transformation. A protocol based on biolistic transient transformation has been developed in order to study the function of promoters in a fast and efficient manner in strawberry fruits. The protocol has been applied to the study of the *GalUR* promoter, a gene involved in the biosynthesis of vitamin C in this fruit. The activity of the *GalUR* promoter is restricted to the fruit, being strictly dependent on light. The analysis of deletion series revealed the presence of a minimum activation region 397 bp upstream of the gene with a putative G-box motif, and a negative regulatory region between −397 and −518 bp, where an I-box was identified. The transient assay has been used to study the activity of the tomato *polygalacturonase* and the pepper *fibrillin* promoters in strawberry fruits. Whereas slight activity was observed with the fibrillin promoter, no significant activity was found with the polygalacturonase promoter. The *GalUR* promoter in transiently transformed ripe tomato fruits showed no activity, indicating the presence of regulatory sequences specific for its function in strawberry fruit.

Key words: D-galacturonate reductase, fibrillin, fruit promoters, polygalacturonase, strawberry, transient expression.

Introduction

The study of fruit development and ripening has received considerable attention mainly due to their uniqueness as plant developmental processes as well as for the importance that fruits have in the human diet. Extensive genetic and molecular analyses have provided substantial information about genes participating in several aspects of fruit ripening, such as the disassembly of the cell wall structure, changes in soluble sugars, pigment biosynthesis, or the production of vitamins, antioxidants, flavour, and aromatic volatiles (Giovannoni, 2001). However, there are many questions that remain unanswered regarding the genetic regulatory elements that control this process. Analyses of regulatory sequences related to fruit ripening have been the focus of a number of studies, but in most cases they have been restricted to tomato (Giovannoni, 2001). This species has also been used as a heterologous system to study promoters from other plants such as apple, pepper, or kiwifruit (Atkinson *et al*., 1998; Kuntz *et al*., 1998; Wang *et al*., 2000).

Although the majority of fruits display similar changes during ripening, two major types of fruits can be distinguished: climacteric, such as tomato, and non-climacteric fruits, such as pepper and strawberry. Broadly, the difference between these types is that only the former requires ethylene for complete and co-ordinated ripening. In non-climacteric fruits other developmental and non-ethylene-mediated regulation occurs which may be common with an additional ethylene-independent control in climacteric

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fruits (Giovannoni, 2001). In strawberry, a non-climacteric fruit, the application of ethylene apparently does not affect ripening although a low level is produced throughout the development of the fruit (Given *et al*., 1988). Thus, it is likely that gene expression in non-climacteric fruits is differently regulated and promoters from strawberry genes may contain different regulatory elements not active in climacteric fruits such as tomato. It has been found that auxins produced in the achenes govern the growth of the receptacle in strawberry and that a decline in the concentration of auxin in expanded green fruits induces fruit ripening (Given *et al*., 1988; Lis *et al*., 1978). Many ripening-specific cDNA clones have been isolated in strawberry (Agius *et al*., 2003; Aharoni *et al*., 2002; Manning, 1998; Medina-Escobar *et al*., 1997). However, analysis of their promoter regions has been scarce and only recently have the promoters of two endo β1-4 glucanases and *STAG1*, a putative *AGAMOUS* homologue from strawberry, been analysed (Rosin *et al*., 2003; Spolaore *et al*., 2003). Spolaore *et al*. used a method based on the injection of an *Agrobacterium* suspension into the fruits that allows quantitative assays to be carried out in strawberry fruit (Spolaore *et al*., 2001), whereas Rosin *et al*. (2003) analysed the expression level in different strawberry tissues from transgenic plants.

Traditional analysis of regulatory sequences requires well-established protocols of plant transformation. Therefore, in many plants in which permanent transformation is difficult, this is a limiting factor for a functional analysis of promoters. Alternatively, transient gene expression systems allow direct comparison of the activity of different promoters and can be used for a wide range of research applications in plant molecular biology and plant biotechnology (Baum *et al*., 1997; Manzara *et al*., 1994). Protoplasts have frequently been used for transient expression analyses of regulatory sequences in promoters activated by external stimuli (Abel and Theologis, 1994). However, the results obtained in intact tissues and organs can provide more valuable information since they reflect the *in vivo* situation in plants. Thus, particle bombardment of intact plant organs allows the analysis of the transcriptional regulation of organ-specific genes in their native environment (Langenkämper *et al*., 2001; Manzara *et al*., 1994; Xu *et al*., 1996). The rapidity of analysis is the major advantage of these techniques compared with stable transformation, particularly in species with limited and variety-specific regeneration capacity such as strawberry (El Mansouri *et al*., 1996). Only very recently, has efficient regeneration been shown in a number of strawberry cultivars (Passey *et al*., 2003).

A biolistic transient gene expression assay has been developed here that allows a rapid analysis of promoters in strawberry fruits. Using this assay, the expression of several promoters has been evaluated. First the tomato polygalacturonase promoter was analysed whose expression is fruit-specific and regulated by ethylene (DellaPenna *et al*., 1986; Grierson *et al*., 1986; Sitrit and Bennett, 1998). Second, the expression of the fibrillin promoter from pepper, a non-climacteric fruit, was analysed (Deruère *et al*., 1994*a*, *b*). Third, the expression of the *GalUR* promoter from strawberry was analysed. This gene encodes a D-galacturonic acid reductase, an enzyme involved in the biosynthesis of vitamin C in strawberry fruits (Agius *et al*., 2003). The expression of *GalUR* is fruit-specific and increases during the ripening process to reach very high level in mature red fruits. Therefore, the promoter region of *GalUR* has a clear biotechnological interest. Analyses of the *GalUR* promoter using the transient assay developed here in strawberry fruits indicated a level of expression similar to that of the constitutive CaMV 35S promoter. Deletion analyses of the promoter also identified a ~400 bp region required for expression in fruits.

Materials and methods

Plant material and treatments

Strawberry plants (*Fragaria*×*ananassa* cv. Chandler) were grown under field conditions in Huelva in the southwest of Spain. Ripe red fruits and leaves were harvested on the day of bombardment. Dark treatments were performed by covering individual fruits with aluminium foil (while attached to the plant) for 24 h and 48 h under the same field conditions. Five days under low-light (30–40 μmol m^{-1} s^{-1}) or high-light (150–200 μmol m^{-1} s^{-1}) exposures were performed with plants that had been grown in pots in a growth chamber under a 16/8 h light/dark cycle at 22 °C. Cherry tomato fruits (*Lycopersicon esculentum* cv. Josefina) were harvested on the day of bombardment from plants growing under standard greenhouse conditions. All the fruits were harvested, immediately frozen in liquid N_2, and stored at −80 °C until analysed.

Reporter gene constructs

A 5′-proximal 3.5 kb fragment of the *PG* promoter (Nicholass *et al*., 1995) was excised with *Eco*RI and *Bam*HI restriction enzymes from plasmid pPGGUSI and subcloned in the polylinker of the pBluescript SK vector (Stratagene) digested with the same enzymes. The resulting plasmid was digested with *Bam*HI, filled in with Klenow and digested with *Kpn*I. The promoter fragment was purified and inserted in the *Kpn*I and *Sma*I sites of the pGL3 vector (Promega), upstream of the luciferase (*LUC*) reporter gene (Ow *et al*., 1986).

A 2.3 kb *Xho*I/*Nco*I fragment containing the *fibrillin* promoter (Kuntz *et al*., 1998) was excised from plasmid pSIGN and inserted upstream of the *LUC* reporter gene in the pGL3 vector (Promega).

The *GalUR* promoter was isolated by screening a genomic library of *Fragaria*×*ananassa* using 750 bp of the 5′ cDNA as probe. Hybridizing plaques were purified and phage DNA prepared as described by Sambrook *et al*. (1989). A fragment of 1.2 kb corresponding to the sequence upstream of the ATG of *GalUR* was subcloned into the pBluescript SK vector (Stratagene). The *GalUR* promoter fragment was ligated into the polylinker of the pGL3 vector, upstream of the *LUC* reporter gene. Deletion fragments of the promoter were generated by PCR using the following forward primers: Primer 1, AGGTACCCATCACATCACCAGTTA; Primer 2, AGGTACCGATCGAGGATCGGAGTC; Primer 3, AGGTACCGCCGTTTAAAGACTATGA; Primer 4, AGGTACCAATCTCACGGTTATAAATGATGAT; Primer 5, AGGTACCTGGAAATTGATGACCAA; Primer 6, AGGTACCTGGCACTCTGAGGTTT, all with a *Kpn*I site added

(underlined), and the reverse primer PRO3′ (AAAGCTTTGAGCTAGTTTACAATTGAAAT, with a *Hin*dIII site added (underlined)). These primer combinations generated fragments of 1200 bp, 950 bp, 678 bp, 518 bp, 397 bp, and 168 bp, respectively. The PCR-generated promoter deletions were cloned into the pBluescript vector (Stratagene) previously digested with *Eco*RV and sequenced. Then the promoter fragments were excised with *Kpn*I and *Hin*dIII and ligated into the pGL3 vector.

A constitutive promoter construct was generated as a chimeric gene fusion between the cauliflower mosaic virus 35S promoter (Odell *et al*., 1985) and the *LUC* reporter gene in the pGL3 vector (Promega).

Particle bombardment

Fruits were cut into 1–2 mm thin transverse slices that were incubated for 5 min in CPW12 medium (CPW salts [Power and Chapman, 1985] supplemented with 12% [w/v] mannitol and 20 mM MOPS pH 7.0), unless otherwise indicated. For strawberry, individual slices of fruits or ~6 small leaves were placed on Petri dishes, containing CPW12 medium and 0.7% agar, for bombardment. In the case of tomato, seeds and locular tissue were removed and the pericarp of each slice was cut into three sections that were incubated for 5 min in CPW12 medium and placed together in the centre of a Petri dish containing CPW12 medium and 0.7% agar. Gold particles (1.6 μm diameter) were prepared at a concentration of 60 mg ml^{-1}, as described previously (Sanford *et al*., 1993), and 50 μl aliquots of particle suspension were stored at 4 °C until use. For bombardment, one aliquot was mixed with 5 μl plasmid DNA (~1 μg μl^{-1}), 50 μl 2.5 M $CaCl_2$, and 20 μl 0.1 M spermidine. After 3 min of vortexing, particles were centrifuged for 10 s, washed with 250 μl ethanol and resuspended in 60 μl ethanol. One 6 μl aliquot of DNA-coated gold particles was used for each bombardment in a helium-driven Biolistic PDS-1000 System (BioRad) using 28 mm Hg vacuum. The distance between the rupture disc and the macrocarrier was 4 cm (level 2), and the distance between the macrocarrier and the sample was 4 cm (level 4). Rupture discs of 1800 psi (BioRad) were used, unless indicated otherwise. Strawberry and tomato fruits were bombarded twice, once on each side, whereas strawberry leaves were bombarded once on the abaxial side. Bombarded fruit slices and leaves were incubated in the CPW12 medium dishes at 25 °C with continuous light (~200 μmol m^{-1} s^{-1}) or in the dark, before being frozen in liquid nitrogen and stored at −80 °C.

Luciferase activity assay

Frozen tissue was ground to a powder in liquid nitrogen and homogenized in 1 ml cell culture lysis reagent 1× (CCLR 1×, Promega) supplemented with 1 M TRIS-phosphate pH 7.8 to a final concentration of 300 mM, unless otherwise indicated. The extract was centrifuged at 13 000 rpm for 20 min at 4 °C. Luciferase activity was determined using the Promega Luciferase Assay System and the light emission was measured for 30 s in a luminometer.

Protein concentration in the extracts was determined using the Bio-Rad Protein Assay kit.

Gel blot analysis

Total RNA was isolated from strawberry red and green fruit as described previously (Manning, 1991). Twenty μg of total RNA was separated in 6% formaldehyde–1.2% agarose gels and blotted onto Hybond-N membrane (Amersham Pharmacia Biotech). The membranes were hybridized with *GalUR* cDNA probes synthesized by random primer labelling using [α-^{32}P] dCTP and the High-Prime Kit (Roche Diagnostics) and hybridized at 60 °C for 16 h (hybridization buffer contained 0.3 M sodium phosphate buffer pH 7.2, 7% w/v SDS, 1 mM EDTA, and 1% w/v BSA). The membranes were washed with 2× SSC and 0.1% SDS at room temperature for 15 min and then washed twice with 0.2% SDS and 0.1% SDS at 60 °C for 20 min. After washing, membranes were exposed to X-ray films (X-Omat AR, Kodak) at −80 °C.

Western blot

Protein extracts used for SDS-PAGE analyses were prepared from strawberry tissues according to Agius *et al*. (2003). Polyclonal antibodies against the GST-GalUR recombinant protein were used for immunoblotting. Immunodetection was conducted with a chemiluminescence ECL kit (Amersham Pharmacia Biotech) in accordance with the manufacturer's instructions.

Results

Biolistic transient transformation of red strawberry fruit

To optimize the transient assay, the CaMV 35S promoter was fused to the *LUC* gene. This promoter has been shown to be functional in transgenic strawberry fruits (El Mansouri *et al*., 1996; Jiménez-Bermúdez *et al*., 2002). The main parameters that can be optimized in the biolistic transformation of plant tissue are the size of the gold particle, the distance between the sample and the macro-carrier, the amount of DNA used in each bombardment, as well as the number of shots per sample (Sanford *et al*., 1993). All of them were optimized for red strawberry fruits (data not shown). The addition of an osmoticum to the bombardment medium has been shown to increase the efficiency of transformation in a number of tissues, since it provides osmotic support for the tissue and minimizes cell damage (Baum *et al*., 1997; Sanford *et al*., 1993). In the case of red strawberry fruits, which contain large cells, the incubation of fruit slices in 12% mannitol prior to and during bombardment was found to be essential (data not shown). The particular fruit texture and cell composition of ripe strawberry meant that the bombardment pressure and the ionic strength of the lysis buffer were critical in the transformation process. As shown in Fig. 1A, 1800 psi bombardment pressure was needed to detect activity of the reporter gene over the background level. Since strawberry fruits are highly acidic, it was found that the buffer capacity of the commercial lysis reagent CCLR was not enough to maintain the pH optimum for assaying LUC activity. As shown in Fig. 1B, only when the concentration of TRIS-phosphate buffer in the lysis reagent was raised from 25 mM to 300 mM was the luciferase activity detected above background (Fig. 1B).

Analysis of ripening-regulated promoters in strawberry and tomato fruits

In a search for promoters that could be used in strawberry fruits in addition to the CaMV 35S, two heterologous fruit ripening-regulated promoters were analysed. One was the tomato *polygalacturonase* (*PG*) promoter whose expression is confined to the tomato fruit and which is transcriptionally activated during ripening (Nicholass *et al*., 1995). This *PG* activity during ripening appears to be regulated by

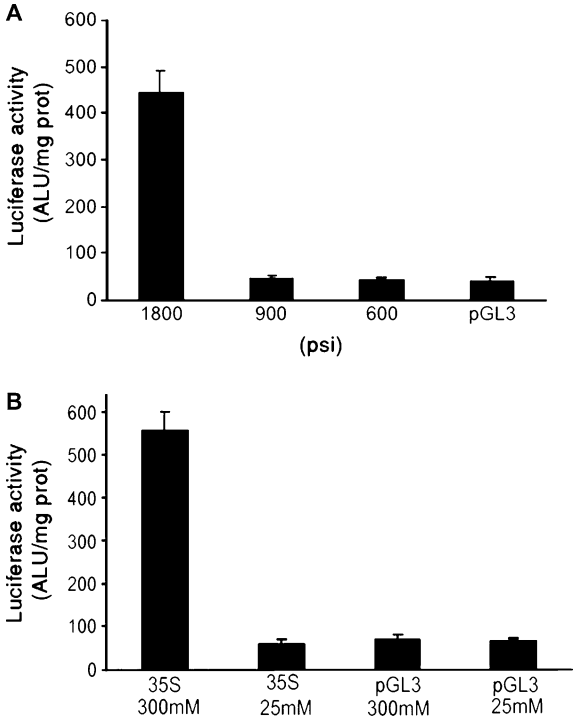

Fig. 1. Optimization of the biolistic transient transformation of ripe strawberry fruit. (A) Effect of the helium pressure on luciferase activity of the reporter gene. (B) Effect of the concentration of the lysis buffer on the luciferase activity. The mean values and standard errors correspond to three samples, each resulting from combined tissue of three bombardments from which equal size aliquots were pooled and processed. Luciferase activity is expressed as Arbitrary Light Unit (ALU) mg^{-1} protein.

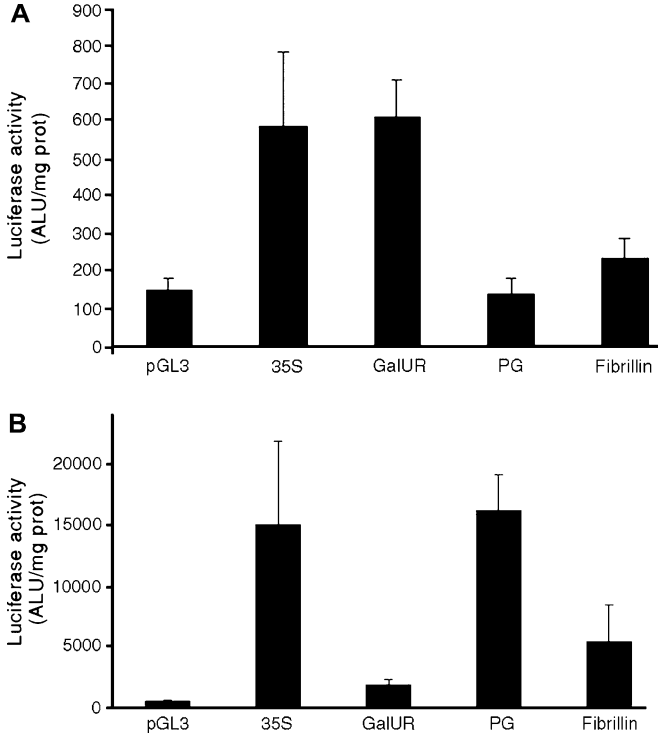

Fig. 2. Analysis of promoters in ripe strawberry and tomato fruits. (A) Luciferase activity in extracts of ripe strawberry fruits transformed with the *35S*, the tomato *polygalacturonase* (*PG*), the pepper *fibrillin*, and the strawberry *GalUR* promoters in the expression cassette of the plasmid pGL3. Transformation with the empty vector (pGL3) was used as the control. Mean values and standard deviations correspond to three different experiments. (B) Luciferase activity in the extracts of ripe tomato fruits transformed with the *35S*, the tomato *polygalacturonase* (*PG*), the pepper *fibrillin*, and the *GalUR* promoters in the expression cassette. Transformation with the empty vector (pGL3) was used as the control. Mean values and standard deviations correspond to three different experiments. Luciferase activities are expressed as Arbitrary Light Unit (ALU) mg^{-1} protein.

ethylene (Sitrit and Bennett, 1998). The second promoter was from the *Fibrillin* (*Fib*) gene, which encodes a protein that accumulates during ripening in the chromoplasts of red bell pepper fruits (Deruère *et al*., 1994*a*, *b*). More recently, it has been shown that *Fib* mRNA can be induced in leaves as a consequence of stresses such as wounding or drought (Chen *et al*., 1998; Langenkämper *et al*., 2001). Using the transient assay on strawberry fruit, no significant *PG* promoter activity was found, whereas the *Fib* promoter was active in strawberry but at a significantly lower level than the 35S promoter (Fig. 2A). The activity of these promoters was analysed next in tomato fruit. The protocol used for transient analysis in tomato was optimized to these experimental conditions and is described in the Materials and methods. As shown in Fig. 2B, transient assays using the CaMV 35S promoter revealed much larger activity in ripe tomato fruits relative to ripe strawberry fruit. Thus the luciferase activity increased around 25 times (from ~600 ALU mg^{-1} protein to ~15 000 ALU mg^{-1} protein). The *35S* and *PG* promoters showed similarly high levels of activity, indicating that the *PG* promoter used in this study had all the regulatory elements necessary for a high expression in tomato. It was also found that the *Fib* promoter had significant activity in ripened tomato fruits, as described previously (Kuntz *et al*., 1998). These results suggest that, although these promoters could be used in tomato fruits, they could not drive high expression of a gene of interest in strawberry fruits.

Light is required for the activity of the GalUR *promoter*

Previous studies have shown that *GalUR* is expressed specifically in fruits during ripening (Agius *et al*., 2003). Additional studies have shown that its expression is very high in red strawberry fruits (~0.01% abundance in the mRNA population) and specific to the receptacle (data not shown). As a result it was decided to isolate and analyse the promoter region that drives the expression of *GalUR*. A region of 1.2 kb upstream of the ATG initiation codon of the *GalUR* cDNA was cloned from a strawberry genomic library (see Materials and methods). The promoter activity of this fragment in ripe strawberry fruit was comparable to the 35S promoter (Fig. 2A). In tomato fruit there was no induced expression of the reporter gene under the 1.2 kb fragment of the *GalUR* promoter (Fig. 2B).

Computer analysis of the promoter sequence identified *cis* elements with significant homology to the light responsive elements G-Box, I-box, and GT-1 (Terzaghi and Cashmore, 1995), and to the wound-responsive element Wun (Matton *et al*., 1993) (Table 1). A putative TATA-box was located −174 bp upstream of the ATG initiation codon. The significance of these putative light-responsive elements in the promoter activity of *GalUR* was tested by the transient expression assay. It was found that the *GalUR* promoter was active in red strawberry fruits only when the bombarded tissue was maintained in continuous light prior to the LUC assay (Fig. 3A). Luciferase activity was ~5-fold higher in fruits incubated in these conditions than in fruits incubated during the same period in the dark, whose activity was not significantly different from the control. A general effect of light on gene expression was excluded since the activity of the 35S promoter was similar in dark and light conditions (Fig. 3A). The *GalUR* promoter also contains regulatory regions that are potentially induced by mechanical damage, like the Wun-motif elements (Table 1). However, the absence of luciferase activity in the dark (Fig. 3A), when there is some wounding caused by the bombardment, seems to exclude any functionality to this wun box in the absence of light. Northern analyses showed expression of *GalUR* in ripening fruits but not in leaves (Agius *et al*., 2003). The biolistic assay developed here confirmed this result, since no luciferase activity was observed after bombardment of strawberry leaf (Fig. 3B), by contrast with the activity under the CaMV 35S promoter that was used as positive control.

A requirement for the activity of the *GalUR* promoter has been shown to be light (Fig. 3A). To determine whether this reflects the *in vivo* expression of *GalUR*, it was next examined if light regulates mRNA and protein accumulation of *GalUR*. Red fruits were maintained in the dark *in planta* by covering them with aluminium foil for 24 h and 48 h. Red fruits that remained uncovered were used as the control. There was a continuous decrease of *GalUR* mRNA after 24 h and 48 h in fruits maintained in the dark, in accordance with the data obtained from the promoter analysis (Fig. 4A). While a slight reduction of GalUR protein was also observed in the covered fruits after 24 h and 48 h (Fig. 4B), no significant changes were observed in either *GalUR* mRNA or protein levels in uncovered fruits (Fig. 4C, D). It was further determined whether light intensity regulates GalUR expression *in vivo*. It was found that the level of GalUR protein is dependent on light intensity as observed in plants that were maintained for 5 d under low-light (LL, 30–40 μmol m^{-1} s^{-1}) or high-light (HL, 150–200 μmol m^{-1} s^{-1}) exposures (Fig. 4E).

Table 1. Cis-*acting regulatory elements in the strawberry* GalUR *promoter*

The *GalUR* promoter sequence was screened against a database of plant transcription *cis* elements (http://intra.psb.ugent.be:8080/PlantCARE/). Only *cis* element sites with a point score >94.0 are shown. The positions are given relative to the ATG initiation codon in the strawberry *GalUR* gene.

Cis-elements	Motif and position	Putative function
G-box-like consensus: CACGTG	−183 TACGTG −178	*Cis*-acting regulatory element involved in light responsiveness
I-box consensus: GATAAGAT	−455 GATAAGTA −446	Light-responsive element
GT-1 consensus: GGTTAA	−655 GGTTAA −649	Light-responsive element
Wun-motif consensus: AATTTCC	−759 AATTTCC −752 −769 AATTTCC −762 −24 CATTTCC −18	*Cis*-acting regulatory element involved in wound responsiveness

Analysis of cis*-regulatory elements required for the expression of* GalUR *in strawberry fruit*

As shown in Fig. 5, the 1.2 kb promoter region and five 5′-deletions of the *GalUR* promoter were analysed. The CaMV 35S–LUC construct and the empty vector were used as positive and negative controls, respectively. Reduced activity of the promoter was observed when the −1200 to −950 fragment was removed indicating the presence of positive *cis*-acting elements in this region (Fig. 5, column a).

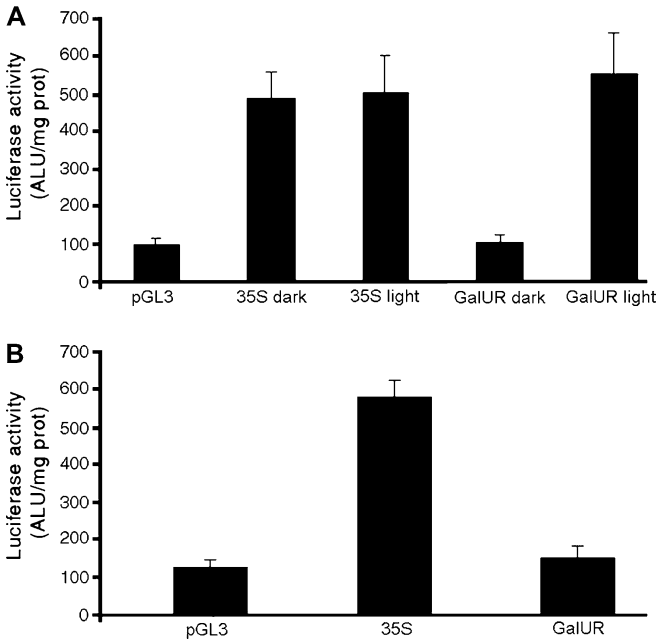

Fig. 3. Analysis of the *GalUR* promoter in fruit and leves of strawberry. (A) Luciferase activity measured in fruit incubated in the dark (dark) and in continuous light (light) transformed with the *35S–LUC* and *GalUR–LUC* constructs. Transformation with the empty vector (pGL3) was used as a negative control. (B) Luciferase activity in leaves (incubated in 16/8 h light/dark photoperiod) transformed with *GalUR–LUC* construct. Values corresponding to leaf transformed with the empty vector (pGL3) and the *35S–LUC* construct (incubated in 16/8 h light/dark photoperiod) are also shown. Luciferase activity is expressed as Arbitrary Light Unit (ALU) mg^{-1} protein.

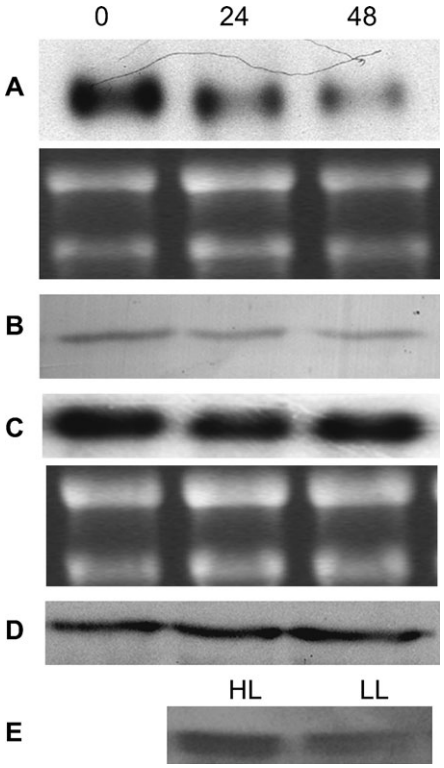

Fig. 4. Expression analysis of the *GalUR* gene. (A) Northern blot of 20 μg of total RNA of strawberry red fruits maintained in darkness (covered) on the plant for 24 h and 48 h. Ribosomal RNA is shown as a control of loading. (B) Immunoblot analysis of protein extracts from strawberry fruit maintained in darkness up to 48 h in the same conditions as for northern analysis. Samples with 20 μg of protein in the SDS extracts were separated on a 12% SDS polyacrylamide gel, electroblotted, and made to react with the antiserum against the GalUR protein. (C) Northern blot of 20 μg of total RNA of strawberry red fruits maintained in the light (uncovered) on the plant during 24 h and 48 h. Ribosomal RNA loading is shown. (D) Immunoblot analysis of protein extracts from strawberry fruit maintained in the light (uncovered) for 24 h and 48 h. Samples with 20 μg of protein in the SDS extracts were separated on a 12% SDS polyacrylamide gel, electroblotted, and probed with the antiserum against GalUR protein. (E) Immunoblot analysis of protein extracts from strawberry fruits maintained on the plant and grown under high-light (HL) and low-light conditions (LL) for up to 5 d. Samples from SDS extracts containing 20 μg of protein were separated on a 12% SDS polyacrylamide gel, electroblotted, and probed with antiserum against the GalUR protein. Experiments were performed twice with similar results.

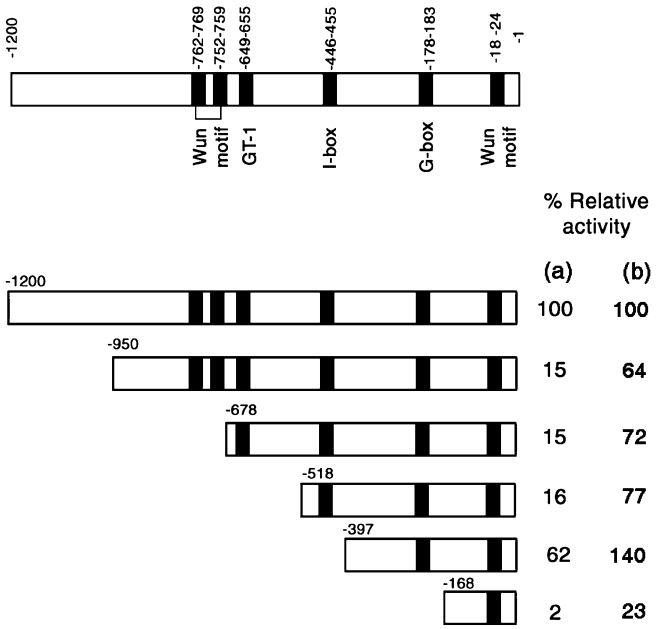

Fig. 5. Deletion analysis of the *GalUR* promoter. A schematic representation of the1200 bp *GalUR* promoter region is shown at the top. The numbering represents positions in bp with respect to the ATG initiation codon. The G-box, I-box, GT-1, and Wun motif are indicated by shaded boxes. The 5′ deletion constructs containing different *GalUR* upstream regions were analysed by transient expression. The numbers in the diagram refer to the 5′ end of the *GalUR* fragments upstream of the ATG codon. The mean values of activities corresponding to six independent transformations by biolistic (a) and infiltration with *Agrobacterium* (b) are shown. Activities are expressed as a percentage of the value obtained with the complete 1.2 kb fragment.

However, computer analysis of the *GalUR* promoter did not identify putative regulatory *cis*-elements in this region with significant homology to others in the databank (Table 1). The decreased activity in the −518 bp, −678 bp, and −950 bp constructs compared with the −397 construct indicates the presence of a negative regulatory element in the region from −397 to −518 bp of the *GalUR* promoter (Fig. 5). In this region between −518 and −397, the promoter contains an I-box-like sequence that could be a negative *cis*-acting element. The construct containing 397 bp upstream of ATG showed 62% of the activity of the 1.2 kb *GalUR* promoter, indicating that the −397 bp fragment upstream of the ATG is sufficient to confer high expression in red fruits. Therefore, this region could be defined as the minimum sequence that showed promoter activity for the *GalUR* gene, and included a G-box like element.

The transient expression using the biolistic technique developed here was compared with the method previously reported in strawberry fruits that used Agro-infiltration (Spolaore *et al.*, 2001). The expression cassettes were identical for both methods with the difference that the constructs for the infiltration method were inserted in a binary vector. As shown in Fig. 5 (column b) similar results were obtained using this transient assay method when compared with the biolistic method developed here (column a). Again, a reduced activity of the promoter was observed when the −1200 to −950 fragment was removed confirming the presence of positive *cis*-acting elements. Also, putative negative regulatory elements in the region from −397 and −518 bp of the *GalUR* promoter were detected.

Discussion

Transient transformation of intact plant organs by particle bombardment has proved to be a useful approach to study the promoter activity of organ-specific genes, as the

transformed cell can be monitored in its native organ environment (Goff *et al*., 1990; Potrykus, 1991). The use of biolistics has the advantage of allowing a fast analysis, but it has to be adapted to the specific characteristics of the plant tissue. Ripe fleshy fruits usually have large vacuolated cells whose walls undergo marked changes in their structure as a result of the presence of many cell wall hydrolytic enzymes. These physiological and anatomical peculiarities must be considered when fruit is transformed by particle bombardment. Results obtained in tomato fruits (Baum *et al*., 1997) showed that osmotic treatment of the fruit slice before, during, and after particle bombardment, together with optimization of bombardment conditions resulted in a 100-fold increase in the transient expression of luciferase driven by the ribulose 1,5-*bis*phosphate carboxylase/oxygenase small subunit promoter (Manzara *et al*., 1994; Montgomery *et al*., 1993). It was observed that osmotic treatments of red strawberry fruit slices before, during, and after bombardment were critical for the detection of luciferase activity. These results might be explained by a loss in the turgor of the cell at the cut surface and the subsequent improved viability of the cells hit by the gold particles, as was observed in tomato fruit (Baum *et al*., 1997). Thus, using the osmotic treatment together with optimized bombardment conditions and two shots per slice with the CaMV 35S promoter, a reporter gene activity that was about 7–10-fold higher than the background was consistently obtained. Co-bombardment with a 35S–GUS construct to be used as an internal control among different experiments could not be used due to the internal fluorescence of the red fruit tissue that made it difficult to assay the GUS activity. Therefore, the intrinsic variability of the protocol was minimized by increasing the number of replicates. Another important parameter to be optimized was the assay of the luciferase activity in the transformed strawberry tissue. The optimum pH for luciferase is 7.8; however, it was found that the pH of the extract prepared from strawberry red fruit ranged from 5.0 to 5.4. This suggested the need to increase the buffering capacity of the TRIS-phosphate extraction buffer by raising its concentration to 300 mM. The same requirements were reported in the transient expression of tomato red fruits transformed with *35S–LUC*, *RBSC3b–LUC* and *HMG2–LUC* (Manzara *et al*., 1994).

The strawberry *GalUR* gene that encodes a D-galacturonate reductase involved in the biosynthesis of L-ascorbic acid in strawberry fruit has been isolated and characterized (Agius *et al*., 2003). The results suggested that the activity of this protein regulates the levels of vitamin C. Northern studies revealed that the expression of *GalUR* was restricted to the ripening fruit. It has been shown here that 1.2 kb of the promoter region contains *cis* elements that determine a high and specific activity of the promoter in strawberry fruit. Other plant promoter regions, with a size in the range of several hundreds bp to 1 kb, have also been found to reproduce faithful expression patterns of reporter genes *in vivo* (Yamagata *et al*., 2002). An important characteristic of the *GalUR* promoter is that its activity seems to be strictly dependent on light. This light-dependence was confirmed by following the levels of the gene expression products during the dark/light treatments. Slight discrepancies between the mRNA and protein levels may reflect differences in the turnover rate of mRNA versus the protein. Nevertheless, the result is consistent with the old finding that the content of vitamin C in strawberry fruit is dependent on light intensity (Hansen and Waldo, 1944).

Sequence analysis revealed the presence of G-box-, I-box-, and GT-1-like elements in the *GalUR* promoter; these have previously been identified in promoters of light-regulated genes (Terzaghi and Cashmore, 1995). The G-box motif is currently one of the best characterized *cis* regulatory elements in plants and has been identified in the promoters of a diverse set of unrelated genes, including those controlled by visible and UV light (Hartmann *et al*., 1998), ABA (Chandrasekharan *et al*., 2003), wounding (Rosahl *et al.*, 1986), and anaerobiosis (de Lisle and Ferl, 1990). A family of plant basic leucine zipper (bZIP) proteins has been identified that interacts with G-box elements to confer high promoter activity (Foster *et al*., 1994; Martínez-Hernández *et al*., 2002). The transient expression assay with −397 bp of the *GalUR* promoter fused to the *LUC* reporter gene suggests that the G-box is important for the fruit expression of *GalUR*, but also that some upstream sequences are necessary for full activity of the *GalUR* promoter. Most *in vivo* expression studies indicate that G-box elements cannot act on their own but require the presence of additional *cis* acting elements for their function (Foster *et al*., 1994; Martínez-Hernández *et al*., 2002). This analysis also indicates that a negative regulatory region is located between nucleotides −397 and −518 relative to ATG. This region contains an I-box-like element. This element has been shown to be involved in light-regulated and/or circadian clock-regulated expression of photosynthetic genes (Borello *et al*., 1993; Terzaghi and Cashmore, 1995). Another negative regulatory region in the *GalUR* promoter could be the GT-1-like box, since GT-1 sequences and sequences related to GT-1 can also act as negative regulators in different genes (Faktor *et al*., 1996; Zhou, 1999). However, an increase in activity was not observed when the fragment containing the GT-1-like sequence was deleted. Related GATA motifs are found in many other promoters, some of which are regulated by light (Anderson and Kay, 1995; Lam and Chua, 1989). In tomato, the I-box has been shown to be an activating *cis*-element of the *RBCS* in leaves (Baum *et al*., 1997, 1999). Recently, it has been demonstrated that I-box-like elements down-regulate gene expression in melon fruit, suggesting a new function of the I-box-like element as a negative regulator (Yamagata *et al*., 2002). This could be the case for the I-box element of the strawberry *GalUR* promoter.

Sugar and osmotic conditions can modulate gene expression (Jang *et al*., 1997; Mikolajczyk *et al.,* 2000). To determine whether the high osmotic solution used in the biolistic assays might affect the final result, the agroinfiltration-mediated transient gene expression method was also used (Spolaore *et al*., 2001, 2003). Similar data were obtained with the two methods, and this seems to exclude any effect of the experimental conditions on the final result. In addition, this supports the validity of the method developed in this work for the rapid analysis of fruit-specific promoters in strawberry. However, a drawback of this transient assay is that it cannot distinguish among specific tissues such as epidermis, vascular bundles, or achenes. The central part of the slice is hit more efficiently than the outer regions such as the epidermis of the receptacle and the achenes. Furthermore, induction by wounding cannot be completely eliminated due to the tissue damage by the bombardment. Therefore, this transient expression system cannot fully replace the permanent transformation system, which is more tissue-specific and sensitive.

Promoter regions of genes comprise highly divergent sequences visualized in modular terms whose activities are controlled by the combinatorial association of multiple proteins. Knowledge about this complex interaction that controls gene expression at the transcriptional level is still very limited. This makes it very difficult to predict the activity of a promoter in a different physiological context, either a different organ or a different species. In the case of strawberry this is more complex since the edible fruit is not a true fruit but an enlarged receptacle and the real fruits are the achenes. All this means that functional proofs are required to evaluate the validity of a promoter sequence as a biotechnological tool in each species. In the work described here, the proximal 3.5 kb of the tomato *PG* promoter, which has been described as pericarp-specific (Nicholass *et al*., 1995), showed no activity in strawberry fruits, whereas it was highly active when analysed in tomato fruits. Based on its tissue specificity in tomato, *PG* expression would be expected in the pericarp tissue (achenes) of strawberry. The petunia *Fbp-7* promoter has been shown to be active in the receptacle of petunia flowers and gives specific expression in the floral and receptacle tissue of transgenic strawberries (Schaart *et al*., 2002). This promoter may, therefore, be used to express genes of interest in strawberry fruit.

It was also found that the *Fib* promoter of pepper displays a significant activity in tomato fruits, as previously reported (Kuntz *et al*., 1998), but only a moderate activity in strawberry fruits. This low activity in strawberry would make this promoter of limited used for biotechnological purposes. Nevertheless, it has been established that the *GalUR* promoter is both highly specific and active in the receptacle of strawberry fruits and may well be used in the manipulation of fruit quality factors by genetic engineering.

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