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Influence of plant growth regulators on *Expansin2* expression in strawberry fruit. Cloning and functional analysis of *FaEXP2* promoter region

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ABSTRACT

FaEXP2 encodes a fruit-specific expansin that is known to be associated with cell wall loosening and fruit softening in strawberry (*Fragaria* × *ananassa* Duch.). In spite of its relevant role in fruit ripening, little is known about the hormonal regulation of this gene. In the present study, we isolated a 650 bp fragment of the *FaEXP2* gene promoter and the *in silico* analysis revealed the presence of *cis*-acting elements associated with hormones, light and stress-related responses. With the aim of characterizing the hormonal regulation of *FaEXP2* expression, strawberry fruit were treated with plant growth regulators and changes in transcript levels were analyzed by qPCR. *FaEXP2* expression levels were significantly higher than control in fruit treated with abscisic acid (ABA), and when the endogenous source of auxins (achenes) was removed. In contrast, transcript accumulation was not affected by exogenous applications of gibberellic acid (GA_3), naphtalenacetic acid (NAA), ethylene or 1-methylcyclopropene (1-MCP). Also, functional analysis of *FaEXP2* gene promoter was performed by transient and permanent strawberry transformation. Histochemical GUS staining analysis showed that the 650 bp-promoter fragment was able to drive the reporter gene expression specifically to the receptacle and along ripening. Our results provide new insights into the hormonal regulation of *FaEXP2* expression.

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1. Introduction

Fruit ripening has been extensively studied in tomato (*Solanum lycopersicum*), a climacteric fruit, whose regulation differs in many aspects from non-climacteric fruits as strawberry (*Fragaria* × *ananassa* Duch.) (Giovannoni, 2001). While climacteric fruits undergo a transient increase in respiration and ethylene production that triggers the onset of ripening, in non-climacteric fruits these peaks are smooth or not detected (Giovannoni, 2001). Instead, it has been proposed a significant role of auxins in relation to their effects on ripening in non-climateric fruits, such as strawberry. The auxins produced by the achenes at the early stages of development delay strawberry fruit ripening, and the effect can be reversed experimentally by fruit deachenation to eliminate the endogenous auxin source (Given et al., 1988; Manning, 1994). Moreover, the expression of many ripening specific genes can be down-regulated by treatments with exogenous auxins (Aharoni et al., 2002; Villarreal et al., 2009; Bustamante et al., 2009). To a lesser extent, it has been also shown that gibberellic acid treatment has an inhibitory effect on strawberry fruit ripening, evidenced by a delay in chlorophyll degradation and anthocyanin synthesis (Martínez et al., 1994; Villarreal et al. 2009; Csukasi et al., 2011).

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Although no single growth regulator appears to play a positive role for strawberry fruit similar to that played by ethylene in climacteric fruits, it has been reported that ethylene and abscisic acid (ABA) promote processes associated with strawberry fruit ripening, such as color development and cell wall disassembly (Jiang and Joyce, 2003; Villarreal et al., 2010; Merchante et al., 2013). Interestingly, it has been shown that down-regulation of a 9-*cis*-epoxycarotenoid dioxygenase gene, which encodes a key enzyme in ABA biosynthesis pathway, results in a significant decrease in ABA levels and uncolored strawberry fruit (Jia et al., 2011).

Fruit ripening is a complex process that involves cell wall changes leading to flesh softening (Brummell and Harpster, 2001; Vicente et al., 2007). Cell wall disassembly is determined by the coordinated action of several enzymes and non-hydrolytic proteins acting on the different polymers that support its structure (Cosgrove, 2000; Minic, 2008). Expansins are non-hydrolytic proteins involved in plant cell wall loosening that affect different processes in developing organs (McQueen-Mason and Cosgrove, 1995), including the softening of fleshy fruits (Brummell et al., 1999; Hayama et al., 2003). In strawberry, seven expansin genes have been identified (*FaEXP1–FaEXP7*), each of them with a particular expression pattern during the course of fruit ripening (Civello et al., 1999; Harrison et al., 2001). The expression of *FaEXP2* is fruit specific and closely related to fruit ripening (Civello et al., 1999; Dotto et al., 2006). It has been reported that *FaEXP2* expression begins at the white stage and increases to a maximum in fully ripe fruit (Dotto et al., 2006). Moreover, a correlation between *FaEXP2* expression levels and softening rate among strawberry cultivars was found, and a cultivar having softer strawberry fruit showed higher expression levels of *FaEXP2* than firmer ones (Dotto et al., 2006).

In spite of the new insights on the effect of plant hormones on cell wall genes expression, information about the hormonal regulation of expansin genes is still scarce. Only one previous study in strawberry showed that *FaEXP2* transcript accumulation was not affected by auxin or ethylene treatments (Civello et al., 1999).

Regulation of gene expression is mainly controlled by gene promoters and their contributing *cis*-acting elements. Thus, the analysis of the promoter region of a particular gene provides valuable information about the regulation of its expression at the transcriptional level. The information about promoter regions of ripening related genes in non-climacteric fruits, as strawberry, is quite limited. Most fruit-specific promoters currently available have been isolated from tomato (Cordes et al., 1989; Hirai et al., 2011), but these promoters may not be appropriate to be used to drive gene expression in strawberry transgenic plants. For instance, Mathews et al. (1995) used the *E4* tomato fruit-specific promoter in strawberry and did not detect expression of the reporter gene in the receptacle. Also, the tomato polygalacturonase (*PG*) promoter was not able to drive significant transient expression of a reporter gene in strawberry fruit (Agius et al., 2005). Therefore, the isolation of a strawberry promoter able to direct the expression of a gene of interest specifically to the fruit is relevant, not only because it would provide a tool for a biotechnological strategy to improve fruit quality, but also would facilitate the functional analysis of a particular gene during strawberry fruit ripening.

In the present work, a short fragment of *FaEXP2* promoter (650 bp) was isolated and analyzed *in silico* to identify potential response elements. Taking this analysis into account and with the aim of improving the characterization of a relevant gene in strawberry fruit softening, the effect of different plant growth regulators on *FaEXP2* transcript levels was analyzed. Finally, after a preliminary functional analysis of the promoter region using a biolistic method, the 650 bp fragment of the *FaEXP2* promoter was used to generate permanent transgenic strawberry plants. The promoter ability to direct the expression of the β-glucuronidase reporter gene (GUS) in a particular strawberry plant tissue was evaluated and compared to a constitutive promoter.

2. Materials and methods

2.1. Plant material

Strawberry leaves (*Fragaria* × *ananassa cv.* Toyonoka) were used for isolation of the promoter fragment of the strawberry *Expansin2* gene (*FaEXP2*; AF159563). For assays employing plant growth regulators, fruits from the cultivar Camarosa at the white stage were used. For strawberry transformation, plants from the cultivar Camarosa were grown under standard greenhouse conditions with a 16/8 h light/dark cycle at 25 °C and a photon flux density of 40 μmol m^{-2} s^{-1}. After the selection process performed *in vitro*, plants were acclimated and then grown in greenhouse under natural light conditions in southern Spain (Málaga). All plants were vegetatively propagated by stolons every season. Fruits from control lines and transgenic plants were harvested at three different developmental stages: small green (SG), white (W), and red (R).

2.2. Isolation of FaEXP2 promoter fragment

For the isolation of the proximal promoter region of *FaEXP2*, the Universal Genome Walker kit (Clontech) was used. Briefly, 100 ng of genomic DNA was digested in four reactions with the restriction enzymes supplied with the kit (EcoRV, PvuII, DraI and SspI). Next, the provided adaptor was ligated to the digested DNA and four libraries were generated. Finally, PCR amplification was performed on the libraries using specific primers for *FaEXP2* and for the adaptor. A 650 bp promoter fragment was obtained and cloned in pGEM-T vector (Promega). The sequence obtained was verified at the Macrogen USA sequencing service.

2.3. Bioinformatics analysis

Identification of putative *cis*-acting regulatory elements was carried out by scanning a 650 bp of 5′ regulatory region of *FaEXP2*, using Plant CARE (Rombauts et al., 1999), PLACE (Higo et al., 1999) and Genomatix Matinspector (Cartharius et al., 2005) professional software.

2.4. Plant growth regulators treatments

The hormonal treatments were performed on harvested fruits from Camarosa cultivar at the white stage.

2.4.1. Auxins and gibberellins treatments

The peduncle of each fruit was cut at 30 mm from the receptacle base and submerged into sterile microcentrifuge tubes containing 1 mmol L^{-1} naphtalenacetic acid (NAA) or 1 mmol L^{-1} gibberellic acid (GA_3) solutions, prepared in distilled water. The controls were a set of fruit whose peduncles were submerged in distilled water. In order to further investigate the role of auxins on *FaEXP2* gene expression, we performed an assay removing achenes (the endogenous source of auxins) from a set of fruits. Achenes were removed from one-half of each fruit using sharp tweezers, maintaining the other half as control.

2.4.2. Abscisic acid (ABA) treatment

Peduncles of treated fruit were immersed in a solution containing 1 mmol L^{-1} ABA in 2% (v/v) ethanol, while the controls

were prepared by immersing the peduncles in a solution of 2% (v/v) ethanol.

2.4.3. Ethylene and 1-methylcyclopropene (1-MCP) treatments

The ethylene-releasing agent ethephon (2-chloroethyl-phosphonic acid) was used to perform the treatment. The whole fruit were submerged during 5 min in a solution of 2 mmol L^{-1} ethephon in 0.02% (v/v) Tween, 1% (v/v) ethanol, prepared immediately before use. Control fruit were submerged during the same time in 0.02% (v/v) Tween, 1% (v/v) ethanol. The effect of the inhibitor of ethylene perception, 1-MCP, was also assayed. Fruit were treated with 1 μl L^{-1} 1-MCP in a hermetic container during 10 h at 22 °C, while controls were kept in the same conditions without 1-MCP. During and after treatments, the fruit peduncles were submerged in microcentrifuge tubes containing distilled water in order to avoid dehydration. After treatments, fruits were stored at 22 °C during 48 h (ethylene and 1-MCP treatments) or 72 h (GA_3, NAA, achene/deachened, ABA). After storage, the calyx and peduncle were removed and treated and control fruit were dissected, frozen in liquid nitrogen and stored at −80 °C until use.

2.5. RNA isolation and reverse transcription

Total RNA was isolated from frozen fruit using the 2-butoxyethanol method (Manning, 1991), treated with DNAase I (Promega) and then purified with chloroform:octanol (24:1). First strand of cDNA was obtained by using the following mixture: 1×10^{-6} g of total RNA, 0.03 mmol L^{-1} dNTPs, 1×10^{-3} mL of Moloney murine leukemia virus RT (200 U μL^{-1}; Promega), 5×10^{-3} mL of 5× reaction buffer (250 mmol L^{-1} Tris–HCl, 375 mmol L^{-1} KCl, 15 mmol L^{-1} $MgCl_2$, 50 mmol L^{-1} DTT, pH 8.3), 330 pmoles of random primers (Biodynamics S.R.L., Buenos Aires, Argentina) and distilled water up to a total volume of 25×10^{-3} mL. The reaction mixture was incubated during 1 h at 38 °C and 5 min at 95 °C. Three independent RNA extractions were performed for each condition.

2.6. Real-Time PCR assays

First strand cDNA from strawberry, obtained as described above, was used for Real-Time PCR assays. The primers used were: *FaEXP2Fw* (5′-AGCTTTCTTTTGGGTTCTCTCTC-3′), *FaEXP2Rv* (5′-CCTTAACAACCAAAGCAGATGGT-3′), *FaGAPDH1Fw* (5′-TCCATCACTGCCACCCAGAAGACTG-3′) and *FaGAPDH1Rv* (5′-AGCAGGCAGAACCTTTCCGACAG-3′). The sizes of the amplified fragments were 152 bp for *FaEXP2* and 96 bp for *FaGAPDH1* gene. The amplification reactions were performed using FastStart Universal SYBR Green Master Rox 2× (Roche) according to the manufacturer's instructions, in a Step One Plus Real-Time PCR System (Applied Biosystems). PCR conditions were: 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 60 °C for 60 s; at the end of each amplification, a melting curve from 60 °C to 95 °C at 0.3 °C increments was performed. A dilution series was built to estimate the amplification efficiency of each primer pair, using a cDNA from fruit samples as template. Each reaction was performed in triplicate, and a negative water control was included in each run. Fluorescence was measured at the end of each extension step. In each experiment, the relative expression level corresponds to the mean of four biological replicates, normalized against the expression level of *FaGAPDH1* gene, which shows constant expression level throughout all fruit developmental stages. Expression levels were calculated according to the method described by Pfaffl (2001) and expressed in arbitrary units ± SEM.

2.7. Cloning of FaEXP2 promoter fragments and strawberry transient transformation

The 650 bp fragment of the *FaEXP2* promoter region (KF958250), as well as deletions consisting of 300 and 150 bp, were obtained by re-amplification using forward primers containing a Xho I recognition site and a reverse primer containing a Hind III recognition site (5′-GAAGCTTGCTAGAAGGAGAAG-3′). These sites were used to clone the different promoter regions into the multiple cloning site of the pGL3 Basic vector (Promega), driving the expression of the luciferase gene. Constructs obtained were named pFaEXP2-650:luc (forward primer: 5′-GCTCGAGACAGCAAACAAG-3′), pFaEXP2-300:luc (forward primer: 5′-GCTCGAGATGCATGTGCAG-3′) and pFaEXP2-150:luc (forward primer: 5′-GCTCGAGGCTCTCTCTCAC-3′). As control of transient transformation assays, the same vector containing four tandem copies of CaMV-35S promoter (4xCaMV35S:luc) or the empty vector (CONTROL) were used.

The transient transformation assays were performed on white or fully ripe fruit disks, using bombardment conditions described before (Agius et al., 2005).

2.8. FaEXP2 promoter:GUS fusion

The 650 bp fragment of the *FaEXP2* promoter region was cloned upstream the β-glucuronidase gene (GUS), contained into the binary vector pKGWFS7 (Karimi et al., 2002). To this aim, forward primer 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAagcaaacaagtaatattattcttg-3′ and reverse primer 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTAgaaggagaagaaggtggag-3′ were used for amplification of the promoter region. Recombination sites attB1 and attB2 (indicated in capitals) were engineered into the upstream region of the forward primer and into the downstream region of the reverse primer. The product from the PCR assay was purified and inserted in pDONR vector (Invitrogen) using the enzyme BP Clonase (Invitrogen). The sequence obtained was verified at the Macrogen USA sequencing service. The *FaEXP2* promoter region was then cloned into the binary vector pKGWFS7 (Karimi et al., 2002) by recombination between the attL sites of the mentioned construct and the attR site of the destination vector, using the LR clonase (Invitrogen).

2.9. Strawberry transformation

Transformation and regeneration of strawberry plants was performed according to the protocol described by Barceló et al. (1998). Strawberry leaf disks (4 × 4 mm) were transformed with *Agrobacterium tumefaciens* (strain LBA4404) containing the construct described above (Section 2.8) or the empty vector (pKGWFS7, Karimi et al., 2002). At least 200 leaf disks per transformation were placed with the adaxial surface in contact with regeneration medium and stored in the dark for one week in order to induce the organogenic capacity of the leaf disks prior infection with *A. tumefaciens*. After this induction period, explants were placed into 50 mL tubes, inoculated with an overnight grown *A. tumefaciens* culture diluted to DO_{600} 0.2–0.3 in MS basal medium, and gently shaken for 15 min. The infected explants were cultivated on shoot regeneration medium in the dark for one week, and then transferred to selection medium with 500 mg L^{-1} carbenicillin and 25 mg L^{-1} kanamycin (the selection antibiotic), for growth under standard culture conditions. Explants were transferred to a fresh medium every 4 weeks. Shoots regenerated after 16–20 weeks were multiplied in $N_{30}K$ medium supplemented with kinetin (4.7 mg L^{-1}, for root development) and kanamycin (25 mg L^{-1}) in glass tubes with plastic caps. One shoot per callus

Table 1
Main regulatory motifs found within the promoter sequence of *FaEXP2*.

Motif	Function	Strand	Distance from ATG	Sequence (5′–3′)
TATA-box	Core promoter element around −30 of transcription start	+	−88	TATATAA
CAAT	Common *cis*-acting element in promoter and enhancer regions	+	−588; −409; −365; −134	CAAT
		−	−514; −251	
CCAF	Circadian control factor	−	−526	CAAGTCTATC
GATA-box	Light responsive element	−	−495	GATAGGG
LREM	Light responsive element	+	−466	ATCT
GBOX	Light responsive element	+	−393	GATA
MBS factor	MYB binding site involved in drought-inducibility	+	−275	TAACTG
MBS factor	MYB binding site involved in drought-inducibility	+	−287	CAGTTG
MBS factor	MYB binding site involved in drought-inducibility	−	−388	GTTTAC
ABRE	Abscisic acid responsive element	−	−399	TACGTG
GARE	Giberellin responsive element	−	−413	AAACAGA
WRKY71OS	W Box family, binding site for WRKY factors	+	−233	TGAC

was selected for further analysis of independent transgenic lines. Control lines were obtained by *in vitro* regeneration of plants transformed with the empty vector.

2.10. Histochemical GUS-assay

Histochemical GUS staining of leaf, stolons, flowers and different developmental stages of fruit was performed as described by Jefferson et al. (1987) using a modified staining solution containing 1.9 mmol L^{-1} 5-bromo-4-chloro-3-indolyl β-glucuronide (X-gluc) in 100 mmol L^{-1} sodium phosphate buffer (pH 7.0), 20% (v/v) methanol, 0.3% (v/v) Triton X-100, 0.5 mmol L^{-1} potassium ferricyanide and 1% (w/v) polyvinylpyrrolidone-40. Tissues were excised with a razor blade and then vacuum infiltrated with staining solution three times during five minutes each. Then the samples were incubated at 37 °C for 3 or 7 h in the case of fruit, and 22 h for the rest of the tissues analyzed. After this, tissues were kept in absolute ethanol and then hydrated with 20% (v/v) glycerol before being photographed. When it was necessary, 0.2 mmol L^{-1} L-cysteine was added to avoid tissue oxidation.

2.11. Statistical analysis

Each hormone treatment was performed on 15 fruit, while another 15 fruit were maintained untreated as controls. The whole experiment was repeated three times. For Real-Time PCR assay, four independent RNA extractions were made for each condition analyzed. Data were analyzed comparing means by Student's *t*-test at a significance level of $P \leq 0.05$. For transient transformation assays, an average of 15 fruit disks were bombarded twice, once on each side. The reported values are the average of two independent transformation events. Data were analyzed comparing means by ANOVA (Tukey's *post-hoc* comparison) at a significance level of $P \leq 0.05$. Statistical analysis was done using the GraPh-PadPrism version 5.03 software program (GraPh-Pad, San Diego, CA, USA).

3. Results and discussion

3.1. Cloning and sequence features of the FaEXP2 promoter region

Regulation of gene expression involves a complex regulatory system where each gene contains a unique combination of *cis*-acting regulatory elements in the promoter region that determines its temporal and spatial expression (Ibraheem et al., 2010). These regulatory elements control many biological processes and stress responses. Here, a fragment of 650 bp upstream of the translation start codon of *FaEXP2* was isolated from strawberry genomic DNA and *in silico* analysis was performed to search for putative *cis*-acting regulatory elements. Three different softwares were used and as a result, 19 responsive elements were found by PLANTCARE (Rombauts et al., 1999), whereas 54 and 43 responsive elements were found by PLACE (Higo et al., 1999) and MatInspector (Genomatix database, http://www.genomatix.de) softwares, respectively. In Table 1, the *cis*-acting elements found for at least two of the three mentioned programs are shown. The analysis revealed

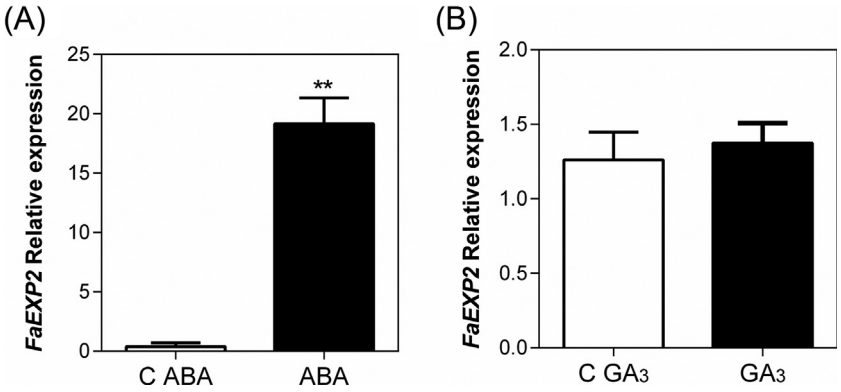

Fig. 1. Effect of abscisic acid and gibberellins on *FaEXP2* expression. (A) Abscisic acid (ABA) treatment. Strawberry peduncles of fruit from Camarosa cultivar at white stage were immersed in a solution of 1 mmol L^{-1} ABA in 2% (v/v) ethanol. Peduncles of control fruit (C ABA) were immersed in a solution of 2% (v/v) ethanol. Fruit samples were collected after 72 h at 20 °C. (B) Gibberellic acid (GA_3) treatment. Strawberry peduncles of fruit from Camarosa cultivar at white stage were immersed into a 1 mmol L^{-1} GA_3 solution while peduncles of control fruit (C GA_3) were immersed in distilled water. Fruit samples were collected after 72 h at 20 °C. Relative expression values represent the expression level of *FaEXP2* normalized against *FaGAPDH1* abundance. Bars represent the mean ± SEM of three replicates. Asterisks indicate significant differences between each treatment respect to the control ($P \leq 0.01$).

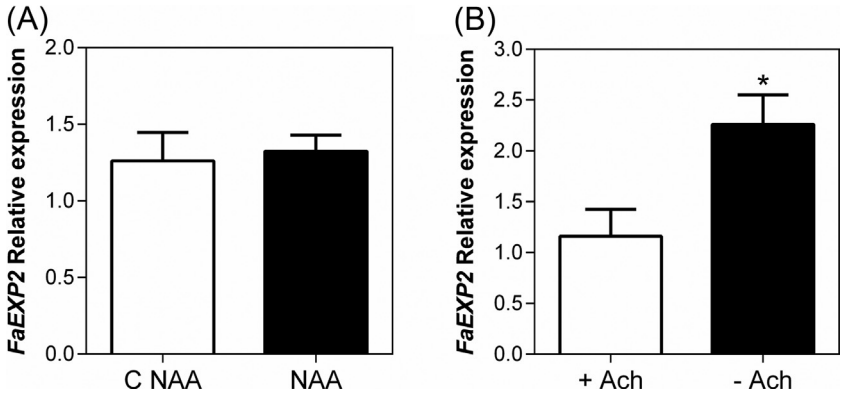

Fig. 2. Effect of auxins on *FaEXP2* gene expression. (A) Strawberry peduncles of fruit from Camarosa cultivar at white stage were immersed into a 1 mmol L^{-1} of naphtalene acetic acid (NAA) solution while peduncles of control fruit (C NAA) were immersed in distilled water. Fruit samples were collected after 72 h at 20 °C. (B) Auxin effect was also analyzed by removing the achenes from one-half of each fruit (−Ach), maintaining the other half as control (+Ach). Relative expression values represent the expression level of *FaEXP2* normalized against *FaGAPDH1* abundance. Bars represent the mean ± SEM of three replicates. Asterisk indicates significant differences between treatment respects to the control ($P \leq 0.05$).

several common regulatory sequences, such as a putative TATA-box at −88 bp from the ATG start codon and six CAAT-boxes. In addition, several light responsive elements (GATA-box, LREM and G-box motives) and an element involved in the circadian control of gene expression (CCAF) were predicted. Additionally, the 650 bp fragment of *FaEXP2* promoter contains a number of putative *cis*-elements related to stress responses, mainly associated to dehydration and salt stress. These MBS motives are binding sites for transcription factors involved in the response to drought induction (MYB binding sites) (Urao et al., 1993), which might include response to ABA (Abe et al., 2003). Regarding this hormone, PLANTCARE, PLACE and MatInspector recognized an ABRE motif, which is involved in the ABA responsiveness. In addition, a GARE motif, which is known to be related to gibberellin response, was also predicted (Table 1).

W-box elements are binding sites for WRKY transcription factors, which constitute a large family of regulatory proteins, many of them related to the response to wounding and to processes in which gibberellins and ABA are involved (Eulgem et al., 2000; Zhang et al., 2004; Xie et al., 2005). The three programs predicted a W-box element at −233 bp from the ATG of *FaEXP2* (Table 1).

The presence of these putative *cis*-elements in the *FaEXP2* promoter region here cloned suggests that the expression of this gene might be regulated by different physiological and environmental factors.

3.2. Plant growth regulators treatments

In silico analysis of the promoter region of *FaEXP2* revealed responsive elements associated with plant growth regulators. In order to contribute to the current knowledge about hormonal regulation of genes related to strawberry softening process, we investigated the expression levels of *FaEXP2* by Real-Time PCR assay under several exogenous treatments. We evaluated the effect of hormones related to elements identified in the 650 bp of *FaEXP2* promoter region (abscisic and gibberellic acids), as well as others that have been reported to be involved in the ripening process of strawberry fruit (ethylene and auxin).

3.2.1. Abscisic acid and gibberellins treatments

It has been reported that ABA acts as a signal molecule that promotes strawberry fruit ripening (Jia et al., 2011). This hormone up-regulates the expression of cell wall degrading genes in strawberry fruit such as xylosidase (*FaXyl1*; Bustamante et al., 2009) and xyloglucan endotransglycosylase (*FaXTH1*; Nardi et al., 2014). We found that strawberry fruits treated with 1 mmol L^{-1} ABA showed a significantly increased expression of *FaEXP2* relative to the control (Fig. 1A). It is worth to notice that potential regulatory elements for ABA were detected in the isolated promoter region of *FaEXP2* (Table 1).

The influence of gibberellins on the *FaEXP2* expression was evaluated by the application of 1 mmol L^{-1} GA_3. No significant

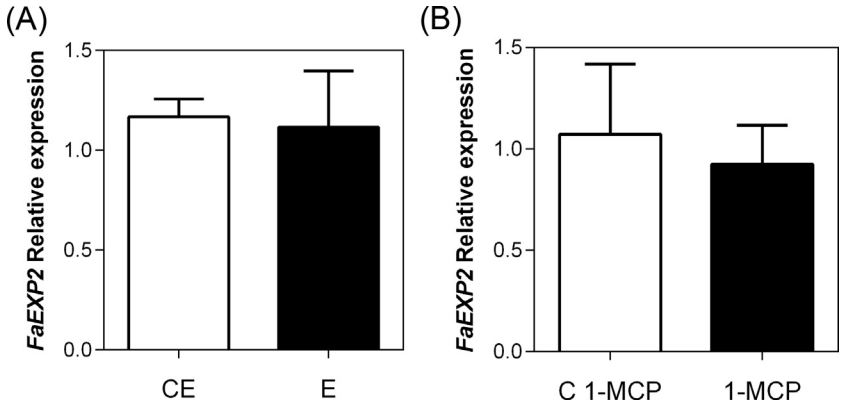

Fig. 3. (A) Effect of ethephon (E) and (B) 1-methylcyclopropene (1-MCP) treatments on *FaEXP2* expression. Strawberry fruit from camarosa cultivar at white stage were submerged in a solution containing 2 mmol L^{-1} ethephon or treated with 1 μL L^{-1} 1-MCP. Fruit samples were collected after 48 h at 20 °C. Relative expression values represent the expression level of *FaEXP2* gene normalized against *FaGAPDH1* abundance and compared to controls (CE or C 1-MCP). Bars represent the mean ± SEM of three replicates.

changes in *FaEXP2* transcripts accumulation were observed after GA_3 treatment (Fig. 1B). Previously, it was reported that GA_3 did not modify the expression of *FaPG1*, another cell wall related gene (Villarreal et al., 2009).

3.2.2. Effect of auxins on FaEXP2 expression

It is known that auxins produced in achenes in green strawberry fruits act as negative regulators of the initiation of receptacle ripening (Given et al., 1988). Accordingly, it has been reported that the expression of several strawberry cell wall degrading genes, associated with ripening, is down-regulated by this hormone (Aharoni et al., 2002; Bustamante et al., 2009; Villarreal et al., 2009).

In the present work, no significant differences in the accumulation of *FaEXP2* transcripts were observed when white fruit were treated with exogenous auxin (NAA) in relation to the control (Fig. 2A). However, when achenes (the endogenous auxin source) were removed from halves of a set of fruit, we detected a significant increase of *FaEXP2* expression in comparison with control (Fig. 2B). Previously, strawberry fruit treated with NAA also showed no differences in xyloglucan endotransglycosylase (*FaXTH1* and *FaXTH2*) transcripts accumulation regarding the control, but it was detected a significant increase in the expression of both genes after achenes removal (Nardi et al., 2014).

Although no auxin-responsive element was found in the isolated promoter region of the here cloned *FaEXP2*, a search on the wild species *Fragaria vesca* genome (www.strawberrygenome.org), identified the complete promoter region of *F. vesca* expansin2 homolog (gene 21343). Analysis of the promoter sequence of this gene, 3408 bp, included a 650 bp-fragment with 92% of sequence identity to the fragment isolated here from *F. ananassa*. Analysis of the complete promoter sequence from *F. vesca* gene was performed with the softwares previously mentioned (Section 2.3). We found three auxin-response DNA elements (AuxRE) positioned at −807, −2001 and −3115 bp from the ATG. It should not be discarded that these elements could also be present in the promoter region of *FaEXP2*, upstream the sequence here cloned.

3.2.3. Ethylene and 1-MCP treatment

Even though strawberry is considered a non-climacteric fruit, endogenous ethylene production is detected during ripening and ethylene receptors have been cloned and characterized (Tian et al., 1997; Trainotti et al., 2005). In addition, although exogenous application of ethylene to strawberry fruit has not shown a remarkable effect on ripening, an effect on the expression of several ripening-related genes has been observed (Trainotti et al., 2001; Castillejo et al., 2004; Bustamante et al., 2009; Nardi et al., 2014). In order to study the influence of ethylene in *FaEXP2* expression, fruit from the Camarosa cultivar were treated with ethephon, a chemical reagent that releases ethylene once it is metabolized by plant tissues. As a second approach, ethylene influence on *FaEXP2* expression was evaluated by the application of 1-MCP, an inhibitor of ethylene perception. No differences in the expression level of *FaEXP2* were detected either with ethephon or 1-MCP treatments in comparison with the corresponding controls (Fig. 3A and B, respectively), suggesting that the expression of *FaEXP2* might be independent of ethylene. Similar results regarding ethylene treatment were reported by Civello et al. (1999) in Chandler cultivar by Northern blot assays.

3.3. FaEXP2 promoter functional analysis by transient transformation

The expression of *FaEXP2* is fruit specific and closely related to fruit ripening. Both characteristics were retained in the 650 bp promoter region isolated, which indicates a high biotechnological potential for this regulatory region. Functionality of the complete fragment (pFaEXP2-650) was analyzed, and also of two deletions of 300 and 150 bp from the ATG (pFaEXP2-300 and pFaEXP2-150). Transient transformation was performed by bombardment of fruit disks with constructs containing the fragments of interest driving the expression of the luciferase reporter gene (luc). Two controls were used, a construct including four copies in tandem of the constitutive promoter Cauliflower mosaic virus (CaMV) 35S fused to luc (4xCaMV35S:luc) and the empty pGL3 vector (CONTROL). Both deletions (pFaEXP2-300:luc and pFaEXP2-150:luc) were not able to drive luc gene expression in 100% red fruit disks (R) and their activity was comparable to that from the empty pGL3 vector (negative control) suggesting that these fragments lack necessary elements to be functional (Fig. 4A). However, the complete

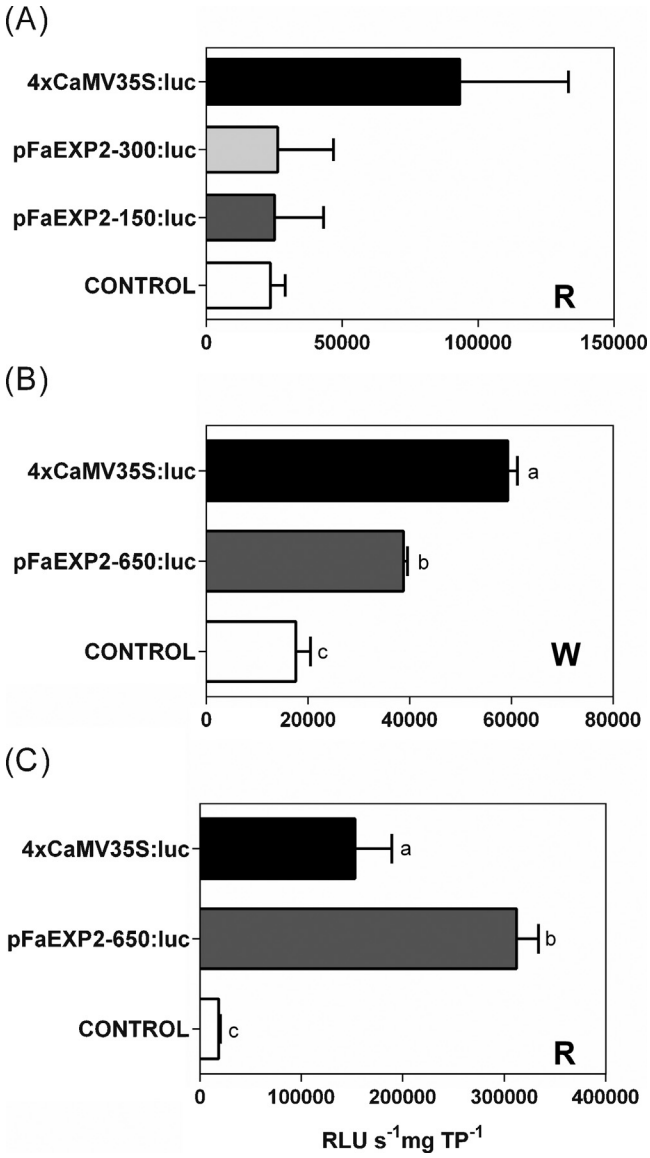

Fig. 4. Transient transformation of strawberry fruit disks with the 650 bp fragment of the *FaEXP2* promoter and two deletions (300 and 150 bp) driving the expression of the luciferase (luc) gene (pFaEXP2-650:luc, pFaEXP2-300:luc and pFaEXP2-150:luc) (A) Transient transformation of 100% red fruit disks (R) with pFaEXP2-300:luc and pFaEXP2-150:luc; (B) Transient transformation of white fruit disks (W) with pFaEXP2-650:luc. (C) Transient transformation of R fruit disks with pFaEXP2-650:luc. 4xCaMV35S:luc indicates the construct including four copies in tandem of CaMV-35S fused to luc. CONTROL: empty vector (pGL3). Bars indicate average value of luc activity (RLU s^{-1}) per mg of total protein (TP). The reported values are the average of two independent transformation events. Different letters represent significant differences in luc activity between constructs ($P \leq 0.05$).

fragment pFaEXP2-650:luc, resulted in high luciferase activity in both, white (W) and 100% red (R) fruit disks (Fig. 4B and C). It is worth to mention that luc activity when bombardment was achieved with pFaEXP2-650:luc in red fruit disks, was comparable to that observed with the 4xCaMV35S:luc construct, used as a positive control (Fig. 4C). Regarding this point, it should be emphasized that even though the lack of an internal control to perform the correction for transformation efficiencies does not allow us to make a quantitative comparison between pFaEXP2-650:luc and CaMV35S, our results indicates that the 650 bp promoter region contains the necessary elements to drive gene expression at high levels in fully ripe fruit. Taken these data together, the complete promoter fragment (pFaEXP2-650) was selected for further studies. It should be indicated that the *FaEXP2* fragment was cloned from the cv. Toyonoka, characterized by a high expression of the *FaEXP2* gene (Dotto et al., 2006) that contributes to a fast fruit softening.

3.4. Expression of the β-glucuronidase (GUS) reporter gene in transgenic strawberry plants

So far, only a few promoters have been isolated from strawberry (Spolaore et al., 2003; Rosin et al., 2003; Agius et al., 2005; Schaart et al., 2011) and, in general, their functionality has been evaluated by transient expression assays (Spolaore et al., 2003; Agius et al., 2005). However, the information provided by transient expression analysis is restricted to the selected tissue. For this reason, it was important to obtain transgenic plants containing the regulatory sequence fused to a reporter gene.

A fusion was made between pFaEXP2-650 and the GUS reporter gene, using the binary vector pKGWFS7,0 (Karimi et al., 2002). The construct was further introduced into strawberry plants (pFaEXP2-650 plants) and a histochemical analysis for GUS staining was performed in different plant tissues. In all cases, a comparative analysis was performed with strawberry plants transformed with the GUS gene driven by the constitutive promoter CaMV-35S (CaMV-35S plants) and also with plants transformed with the empty vector (CONTROL).

3.4.1. Histochemical GUS staining of strawberry fruit at different ripening stages

Initially, GUS-staining was analyzed during fruit ripening as shown in Fig. 5. Three ripening stages were selected: small green (SG), white (W) and 100% red (R) and two independent pFaEXP2-650 transgenic lines (55 and 61) were analyzed, jointly with CaMV-35S and control plants. All the fruit were incubated during 7 h at 37 °C with GUS reagent. As expected, no GUS staining was observed at any ripening stage on control fruit (empty vector). In contrast, an intense and homogenous GUS staining was detected in all ripening stages for CaMV-35S plants. In the case of the two pFaEXP2-650 lines, the histochemical staining was observed at SG, W and R ripening stages, being more intense at the red stage. The same trend was observed in an additional transgenic line analyzed as shown in Supplemental Fig. S1. These observations are in agreement with the progressive increase of *FaEXP2* gene expression observed during strawberry fruit ripening (Dotto et al., 2006), thus indicating that the cloned promoter fragment retains the ability to be sensitive to changes associated with fruit ripening.

It is worth to mention that blue tissue staining was extremely faster in the case of pFaEXP2-650 fruits compared to CaMV-35S. Thus, in a parallel experiment, fruits were incubated during 3 h at 37 °C with the GUS reagent. After this time period, fruits from pFaEXP2-650 plants were fully stained, while fruits from the CaMV-35S plants showed a lower degree of staining (Fig. 6). It was necessary to wait for 7 h at 37 °C to have full blue coloration in the CaMV-35S fruits. This result might indicate a higher strength of the *FaEXP2* promoter isolated here, in terms of time of response, in comparison to the constitutive promoter CaMV-35S.

In order to determine the receptacle-specificity of the pFaEXP2-650 construct, a histochemical localization of GUS activity was performed in the achene and the surrounding receptacle of the transgenic fruits, at the three stages previously studied (Fig. 7). In the case of pFaEXP2-650 plants, GUS staining was detected only in the receptacle at all stages of ripening and no blue coloration due to GUS activity was observed in the achenes. In contrast, in plants transformed with the CaMV-35S constitutive promoter, GUS staining was observed in both achenes and receptacle in all stages analyzed. No GUS activity was detected in either receptacle or achenes in control fruits transformed with the empty vector (data not shown). These results show that the cloned fragment of *FaEXP2* promoter maintains the tissue specificity of this gene, whose expression in strawberry fruit, analyzed by Northern blot, was receptacle-specific (Civello et al., 1999).

Among plant promoters used in genetic engineering, constitutive promoters, which are active in most of the tissues and developmental stages, are commonly used. The CaMV-35S promoter from the cauliflower mosaic virus is the most widely utilized for basic research (Hernandez-Garcia and Finer, 2014) and biotechnological applications (Ye et al., 2000). However, the ectopic expression of the gene of interest might have a deleterious effect in the normal development of the plant (Hsieh et al., 2002a, b; Kasuga et al., 2004). Our results indicate that the regulatory region of *FaEXP2* isolated here is capable of directing the expression of a gene specifically to the receptacle and along ripening of the strawberry fruit.

3.4.2. Histochemical GUS staining in different tissues

In order to assess the tissue specificity of the promoter region of *FaEXP2*, the histochemical GUS-analysis was also performed on different tissues, including leaf, stolons and flowers (Fig. 8). As expected, no GUS activity was detected in any tissue analyzed for control plants transformed with the empty vector. In the case of CaMV-35S plants, blue staining was observed in the three tissues analyzed. Regarding pFaEXP2-650 plants, a slight staining due to the reporter gene activity was detected in leaves from line 55, whereas in line 61 no blue staining was observed. In stolons, no GUS activity was detected in the two lines analyzed of pFaEXP2-650 plants. When flower tissue was analyzed, the reporter gene activity was observed specifically in stigmas in both transgenic pFaEXP2-650 lines, although in line 61 a slight coloration was also observed in anthers as well. In the case of flowers from CaMV-35S plants, staining was also detected in petals and in the flower stem (Fig. 8). It must be emphasized that GUS activity detected in vegetative tissues of pFaEXP2-650 plants was evident only after 22 h of incubation at 37 °C with GUS reagent.

The main function of spatio-temporal promoters is to provide an accurate control of transgene expression that must be restricted to a given tissue or developmental stage (Hernandez-García and Finer, 2014). In the case of pFaEXP2-650 promoter, its activity was practically specific of receptacle and increased along ripening (Figs. 4–6), although a weak GUS activity was found in vegetative tissues after long incubation times (Fig. 8).

Schaart et al. (2011) have characterized the functionality of two fragments (700 and 1600 bp) of *FaEXP2* promoter region isolated from the cultivar “Elsanta”. Authors observed that the larger promoter fragment gave rise to an overall higher level of the reporter gene expression in comparison with the shorter one. However, histochemical GUS staining was not limited to fruit receptacle; instead both promoters directed GUS expression in the achenes and in epidermal and subepidermal tissues of petioles and stems of flowers and fruit as well. This differs from our results, probably because the region responsible for directing gene

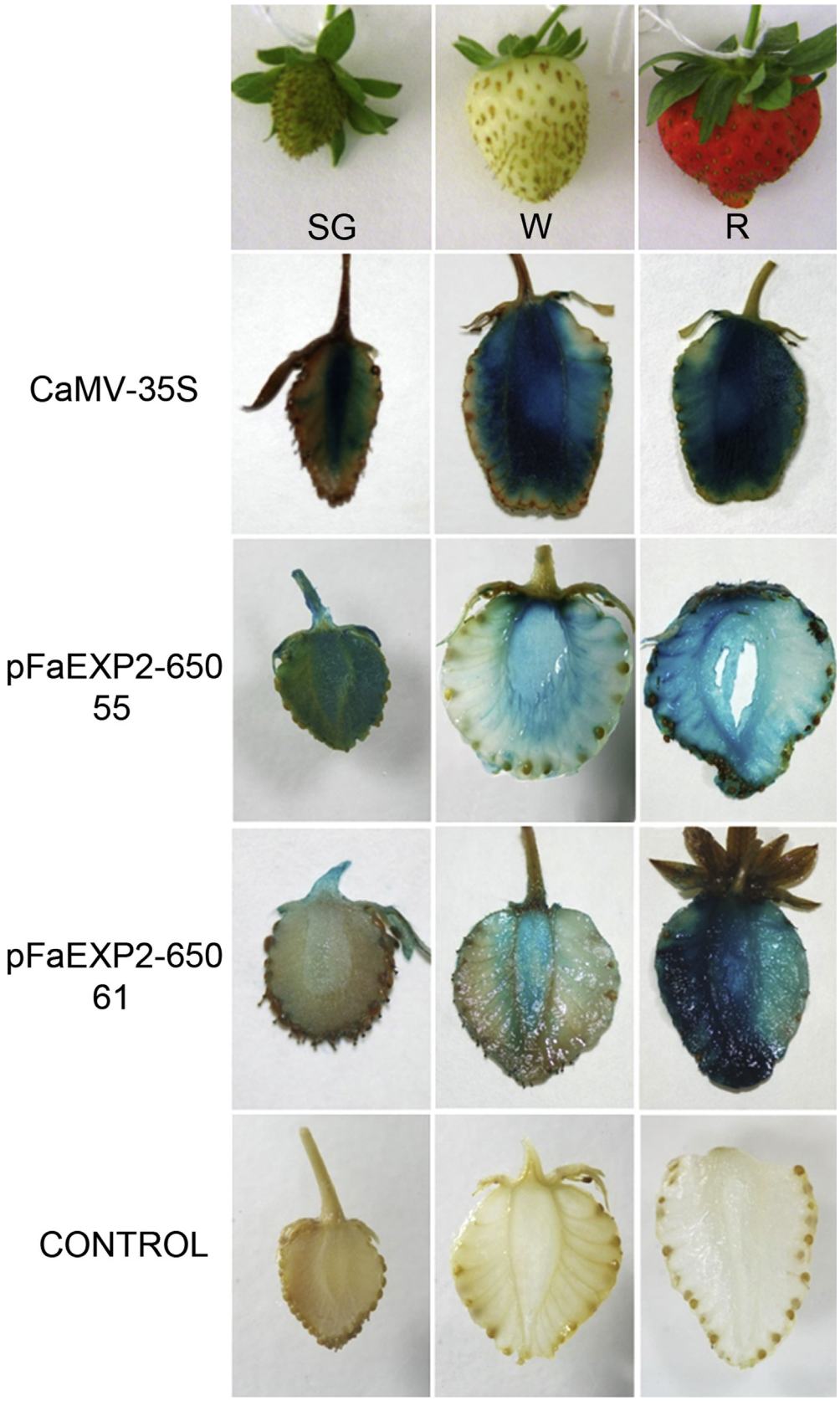

Fig. 5. Histochemical GUS staining of pFaEXP2-650 fruit at different ripening stages. Fruit were incubated for 7 h at 37 °C after GUS reagent treatment. CaMV-35S and plants transformed with the empty vector (CONTROL) were included as reference. Small green (SG), white (W) and 100% red (R) correspond to the stages of ripening analyzed. 55 and 61, are the two independent transgenic lines analyzed.

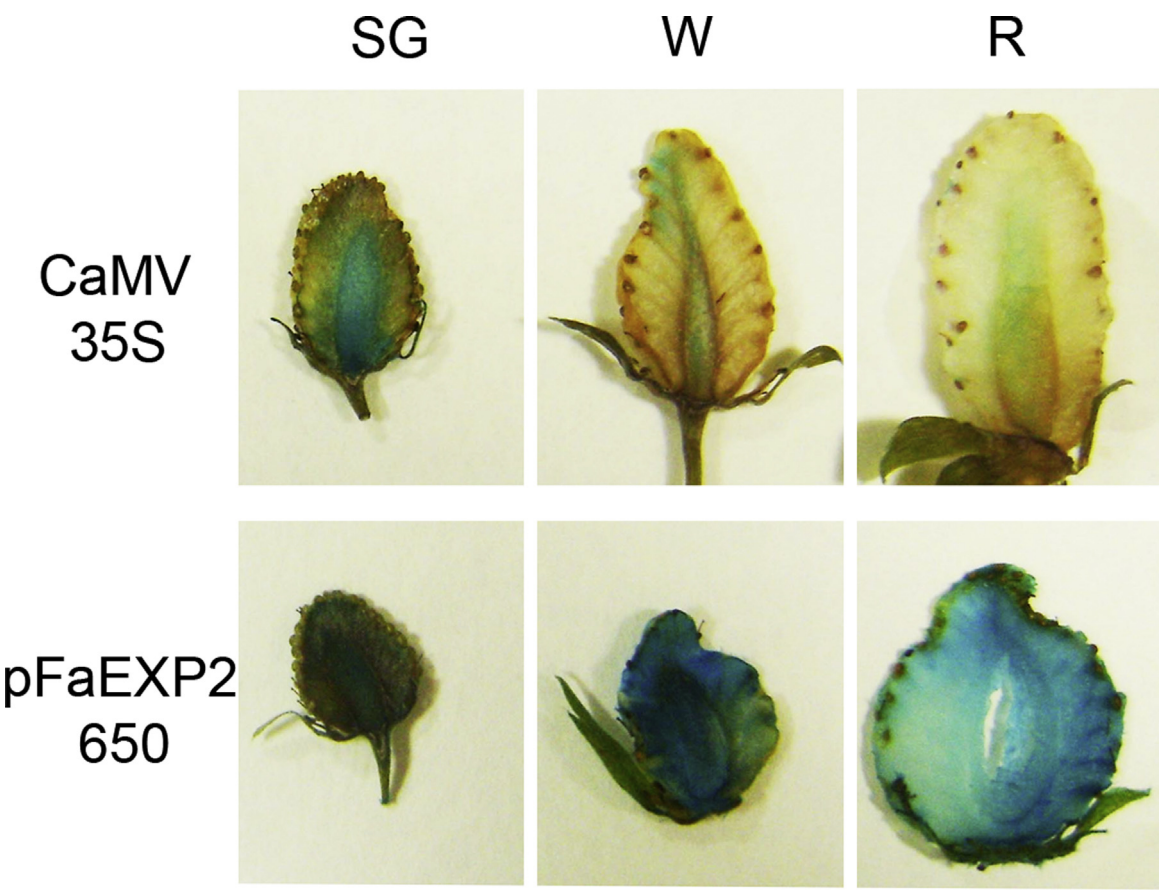

Fig. 6. Histochemical GUS staining of pFaEXP2-650 fruit incubated for 3 h at 37 °C. Fruit from CaMV-35S plants were included as reference. Small green (SG), white (W) and 100% red (R) correspond to the ripening stages analyzed.

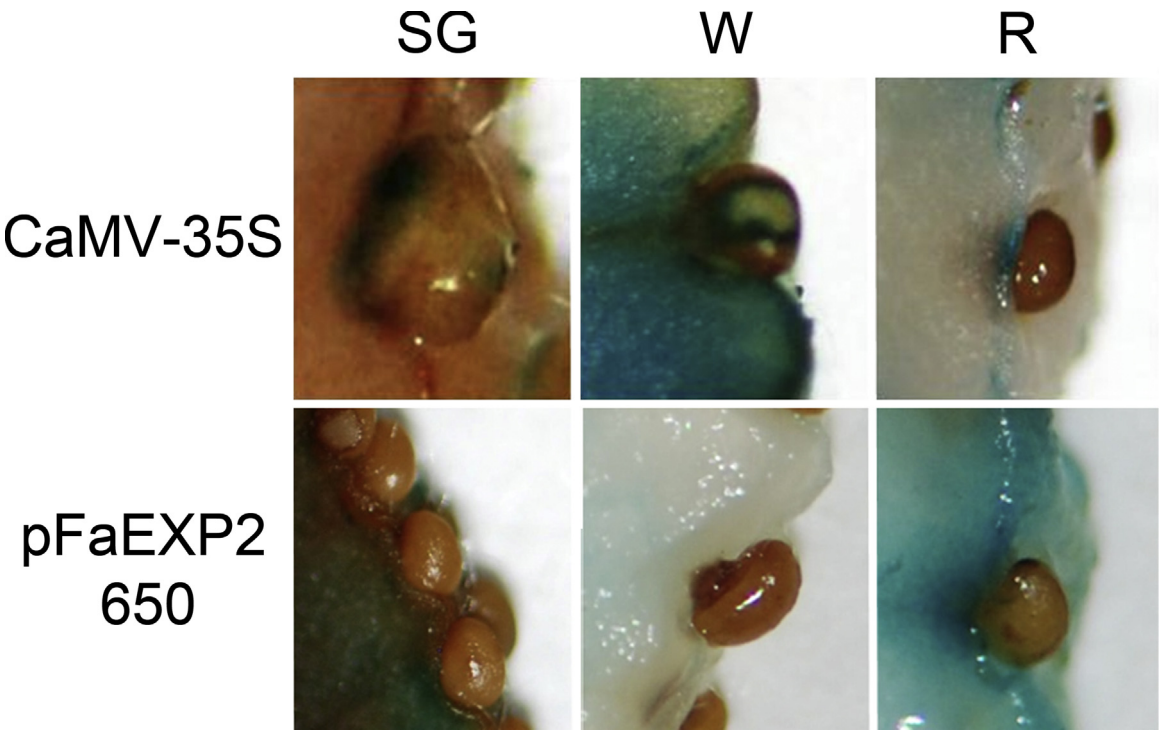

Fig. 7. Histochemical GUS staining of receptacle and achenes of pFaEXP2-650 plants. Fruit from CaMV-35S plants were included as reference. Small green (SG), white (W) and 100% red (R) correspond to the ripening stages analyzed.

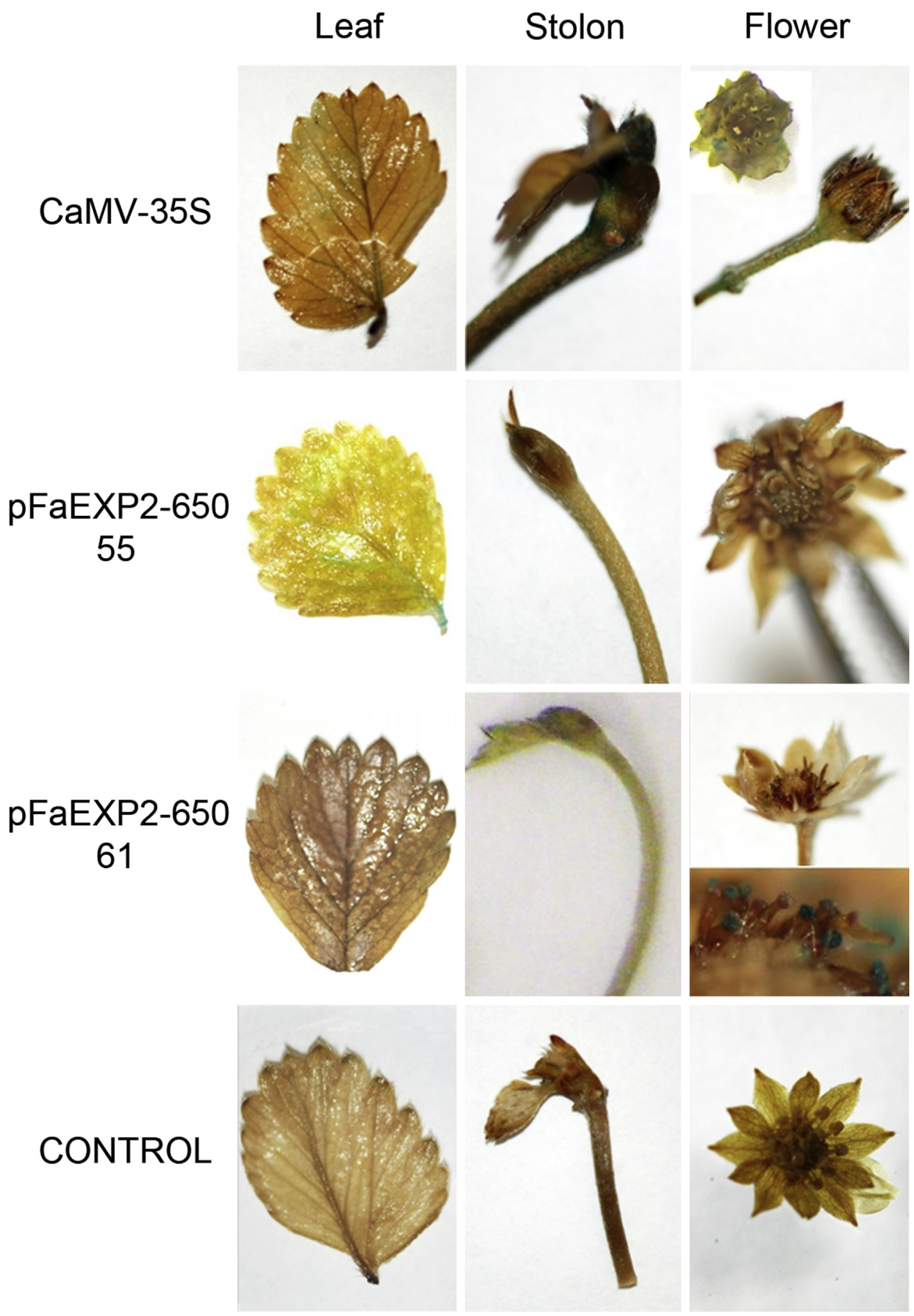

Fig. 8. Histochemical GUS staining of leaves, stolons and flower tissues of pFaEXP2-650 plants, incubated for 22 h at 37 °C. Tissues from CaMV-35S and plants transformed with the empty vector (CONTROL) were included as reference. 55 and 61, are the two independent transgenic lines analyzed.

expression to achenes is absent in pFaEXP2-650 promoter. On the other hand, differences described here might be due to polymorphisms detected between our sequence and Schaart et al. (2011), since both sequences were obtained from different cultivars.

4. Conclusions

The present work reports the characterization of a promoter fragment from *FaEXP2* gene. Bioinformatic analysis and gene expression measurements provides valuable information on the hormones involved in the regulation of expansin *FaEXP2* expression, a relevant gene involved in strawberry fruit softening. The promoter pFaEXP2-650 retains the capacity of driving an increasing gene expression during ripening of the strawberry receptacle, and its activity is very low in other tissues. This 650 bp-promoter fragment could be a valuable tool to drive the study of expression of genes of interest to improve quality, nutraceutical properties and shelf life of strawberry fruit, since so far, few strong and specific promoters have been developed to be used in non-climacteric fruits.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.postharvbio.2015.11.008.

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