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# Isolation and Characterisation of a Strawberry Fruit-Specific Promoter

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## ABSTRACT

In order to achieve specific expression of transgenes in strawberry fruits, the availability of tissue- (receptacle) specific promoter sequences is desired. For this reason, 5′-upstream sequences of the strawberry expansin gene *FaExp2*, which is expressed in a fruit-specific manner, have been isolated. To characterise the promoter activity of the isolated sequences, fragments of 0.7 kb (0.7p*FaExp2*) and 1.6 kb (1.6p*FaExp2*) have been fused to the β-glucuronidase reporter gene (*gus*). In transgenic strawberry plants transformed with either 0.7p*FaExp2-gus* or 1.6p*FaExp2-gus,* a fruit-specific expression pattern was observed for both promoter constructs. However, quantitative RT-PCR revealed that *gus* expression levels driven by the 1.6p*FaExp2* promoter fragment were much more higher. In addition to the expression in fruits, both promoter fragments also seemed to direct gene expression in the achenes and to some extent in epidermal and subepidermal tissues of petioles and stems of flowers and fruits. It is concluded that both promoter sequences are suitable for directing transgene expression in strawberry fruits in a specific way.

**Keywords:** expansin gene, *Fragaria x ananassa*, gene expression, receptacle
**Abbreviations:** ***CaMV35S***, cauliflower mosaic virus 35S; ***dbp***, putative DNA binding protein from *Arabidopsis thaliana*; ***FaExp***, strawberry expansin gene; **GUS**, β-glucuronidase; ***gus***, β-glucuronidase reporter gene; **X-gluc**, 5-bromo-4-chloro-3-indolyl β-glucuronide

## INTRODUCTION

Genetic modification of crop plants is gaining importance. A key feature of genetic modification is that a cultivar of particular interest can undergo improvement of one or a few traits, while the cultivar's own characteristic properties are in principle not disturbed. Most currently cultivated genetically modified crops have been modified by introducing genes that provide the plant with resistances to herbicides, bacterial or fungal pathogens or insects. In these crops generally strong heterologous constitutive promoters like the cauliflower mosaic virus 35S (*CaMV35S*) promoter have been employed, but the advantages of regulating transgene expression more finely and specifically are increasingly being recognised (Potenza *et al.* 2004).

Breeding of improved strawberry cultivars is difficult and time-consuming. Amongst others, breeding of strawberries is hampered because of their octoploid, hybrid and highly heterozygous genome (Shaw and Famula 2005). In addition, the availability of genetic resources is limited for many important traits such as disease resistance. Genetic modification of strawberry looks promising for a relatively quick improvement of existing important strawberry cultivars (Nehra *et al.* 1990a, 1990b). Depending on the cultivar of interest, the production of transgenic strawberry plants is rather easy to realise. However, the number of suitable genes and specific regulatory sequences that will result in the desired improvements is still rather limited.

Initially, we described the possibility of two heterologous promoter sequences to direct gene expression in strawberry fruits (Schaart *et al.* 2002). However, for more specific and higher levels of gene expression, other promoters, for example promoters that are highly strawberry receptacle-specific, have to be identified and isolated from strawberry itself. Only for a few strawberry genes the isolation and characterisation of promoter sequences have been described. Spolaore *et al.* (2003) demonstrated differences in timing and levels of expression of two endo-β-1,4-glucanase genes and studied promoter activities of the corresponding regulatory sequences using *gus*-reporter gene fusions. It was concluded that both promoter sequences could be candidates for genetic modification approaches involving modification of fruit-specific characteristics. Unfortunately, no data showing spatial expression patterns of both promoters were shown. The strawberry *AGAMOUS* homolog *STAG1* showed a low level of fruit-specific expression as determined by Northern blot analysis (Rosin *et al.* 2003). A detailed expression study was performed using transgenic strawberry plants containing *STAG1*-promoter-*gus* fusions. Histochemical GUS staining revealed that *STAG1*-promoter activity was mainly localised in the achenes and in vascular strands leading to the achenes. In mature fruits a faint GUS staining was observed throughout the cortex and the pith of the strawberry fruit. This promoter may, therefore, be suitable for certain applications for which low expression levels are required. Agius *et al.* (2005) analysed the activity of two heterologous promoters and the homologous *GalUR* promoter, which regulates the expression of the D-galacturonate reductase gene in ripe strawberry fruits. Using biolistic transient transformation of ripe strawberry fruit tissue it was shown that the *GalUR* promoter drives luciferase reporter gene expression to a similar high level as the *CaMV35S* promoter did. Recently, the isolation of a fruit-specific promoter of the strawberry β-xylosidase gene was also reported (Bustamante *et al*. 2009), but in this study no expression analysis of the isolated promoter sequence was performed.

We aimed at the identification of a strawberry promoter sequence, which is able to direct transgene expression to a high level in a receptacle-specific way. For this, we selected the strawberry expansin 2 gene (*FaExp2*), which showed a high expression level in strawberry fruit during ripening

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(Civello *et al.* 1999; Aharoni *et al.* 2002; Salentijn *et al.* 2003; Dotto *et al.* 2006; Figueroa *et al.* 2009), and cloned and characterised its 5′-upstream genomic DNA fragments.

## MATERIALS AND METHODS

### Isolation of nucleic acids

Genomic DNA was isolated from young folded leaves from greenhouse plants according to the method described by Doyle and Doyle (1987), including 1% (w/v) polyvinylpyrrolidone-10 (Sigma-Aldrich) in the DNA extraction buffer. Total RNA was isolated from young leaves, roots and small green, green-white, large white, turning and orange and red ripe fruits as described by Asif *et al.* (2000).

### Isolation of promoter fragments

Isolation of 5'-upstream sequences was performed as described by Rosin *et al.* (2003). In short, for the isolation of expansin promoter fragments, genomic DNA libraries of the strawberry cultivar 'Elsanta' were used which had been constructed using the Universal Genome Walker™ kit (BD Bioscience). For the primary and nested PCR the *FaExp2* gene specific primers GSP1 (5′-CCAGAA GCATCACCACCTCCATAGA-3′) and GSP2 (5′-GATACCAG-AAGAGTAATAGCCAAGC-3′) were used, together with the corresponding adapter primers (AP1 and AP2, respectively). PCR conditions used were as described into the Universal Genome Walker™ kit user manual. Three cloned PCR fragments of respectively 400, 700 and 1600 bp were obtained from a *Sca*I-, *Stu*I- and *Dra*I-digested genomic DNA library, and were completely sequenced.

### Sequence-specific PCR

Because in strawberry different expansin gene sequences have been identified, sequence- specific PCR was applied to check correlation of the obtained promoter fragments with the different expansin gene sequences. For this PCR, forward primers unique for each of the 700 and 1600 bp promoter fragments (5′-TTCTGC TTCTTACAATCCACCAC-3′ and 5′-TTCTGCTTCGAGTTC TCATTATCC-3′, respectively; **Fig. 2**) were combined with reverse primers, which have been described by Harrison *et al.* (2001) and which are specific for six different expansin genes (FaExp 2 –7).

### Construction of transformation vector

To study promoter activities of the 700 bp and 1600 bp *FaExp2* promoter fragments, both were cloned upstream of the β-glucuronidase (GUS) reporter gene (*gus*) that was equipped with a nopaline synthase terminator sequence. The promoter-*gus* fusions, which are indicated as 0.7p*FaExp2-gus* and 1.6p*FaExp2-gus*, were cloned into the binary vector pBinplus (van Engelen *et al.* 1995) (**Fig. 1**), and the ultimate construct was subsequently transferred to the supervirulent *Agrobacterium tumefaciens* strain Agl0 (Lazo *et al.* 1991).

### Strawberry transformation

For expression analysis of the cloned promoter fragments, transgenic strawberry plants of the cultivar 'Calypso' harbouring T-DNA with the described constructs were produced according to Schaart *et al.* (2002). As a control, transgenic plants with the *gus* gene under the control of the constitutive *CaMV35S* promoter were produced. From the obtained transgenic strawberry plants containing 0.7p*FaExp2-gus* or 1.6p*FaExp2-gus,* respectively three and four independent transgenic lines, showing intense GUS staining in red fruit tissue were selected for further analysis. One *CaMV35S-gus* plant showing intense blue staining in red fruit tissue was included as control.

### DNA gel-blot analysis

Ten microgram of genomic DNA of the different transgenic lines and a non-transgenic strawberry plant was digested with the restriction enzymes *Eco*RI or *Hin*dIII. After electrophoretic separation of the fragmented DNA on a 0.9% agarose gel, the DNA was transferred onto Hybond-N+ membranes (Amersham Biosciences). Overnight hybridisation at 65°C with an alkaline phosphatase-labelled *npt*II probe, stringent washing [2 × (1.0 SSC + 0.1% SDS) + 1 × (0.1 SSC + 0.1% SDS)] at 65°C and chemiluminescent detection was performed according to the 'gene images AlkPhos direct labelling and detection system' (Amersham Biosciences).

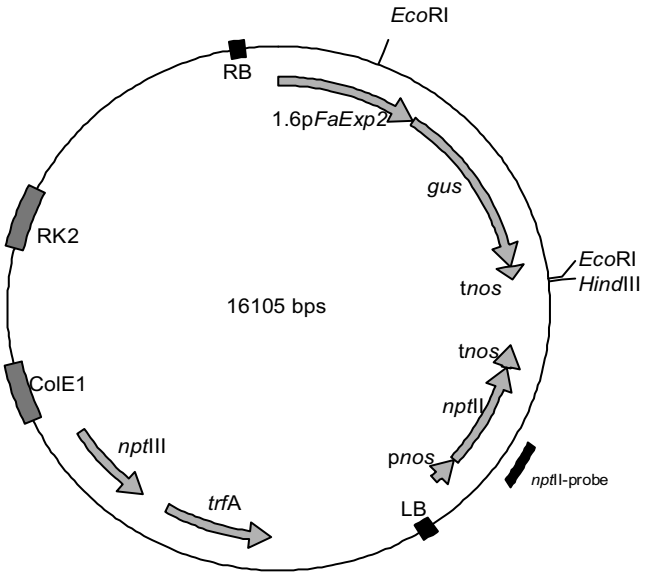


**Fig. 1 Schematic representation of the binary vector used for transformation of strawberry for expression analysis of the *FaExp2* promoter fragments.** For analysis of the shorter promoter fragment, the 1.6p*FaExp2* in the binary vector promoter sequence is replaced by 0.7p*FaExp2*. The *CaMV35S* promoter was used for control plants. The restriction enzyme sites *Eco*RI and *Hin*dIII and the indicated *npt*II probe are used in DNA-gel blot analysis.

### Histochemical GUS-assay

Histochemical GUS staining of leaf discs, root tips, cross-sections of petioles and longitudinal sections of petioles, flowers and fruits at various developmental stages was performed as described by Jefferson (1987) using a modified staining solution containing 1 mM 5-bromo-4-chloro-3-indolyl β-glucuronide (X-gluc; Duchefa) in 50 mM sodium phosphate buffer (pH 7.5), 10 mM EDTA, 0.1% (v/v) Triton X-100, 0.5 mM potassium ferricyanide and 5% (w/v) polyvinylpyrrolidone-40 (all from Sigma-Aldrich).

### Quantitative RT-PCR

For quantification of *gus* expression levels quantitative RT-PCR was performed. In order to limit the number of RNA isolations, for each tissue type RNA was isolated from pooled tissue of the different independent transformants produced with the same construct. cDNA was synthesised using the SuperScript first-strand cDNA synthesis system for RT-PCR (Invitrogen) according to the instruction manual. Quantitative RT-PCR was performed using the ABI Prism7700 Sequence Detection System (Perkin Elmer, Applied Biosystems) as described by Schaart *et al.* (2002), but instead of the fluorogenic TaqMan probes, SYBR Green was used for detection of PCR products. For amplification of *gus*, the forward and reverse *gus*-primers (5′-cggaagcaacgcgtaaactc-3′ and 5′-tgagcgtcgcagaacattacat-3′) were used (product size: 80 bp). As endogenous control, a strawberry gene encoding a DNA binding protein with high homology to a gene coding for a putative DNA binding protein from *Arabidopsis thaliana*, was selected as a reference gene. This gene, indicated as *dbp* (forward primer 5′-TTG GCAGCGGGACTTTACC-3′, reverse primer 5′-CGGTTGTGT GACGCTGTC-AT-3′, product size: 72 bp), has shown a similar level of expression in multiple strawberry tissues (Schaart *et al.* 2002). All PCR reactions were performed in triplicate. For each reaction the threshold cycle, $C_T$, which is defined as the PCR cycle

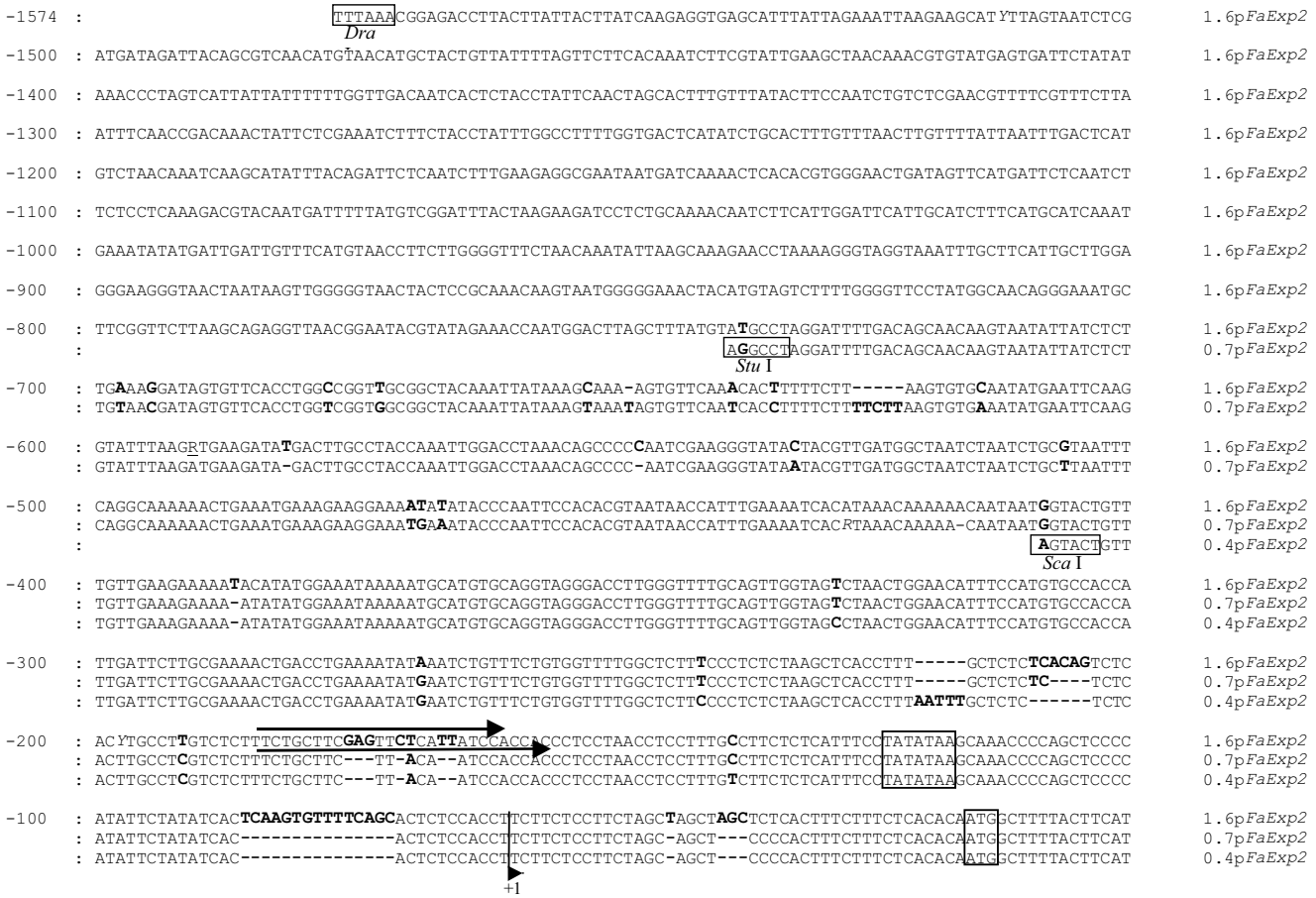


**Fig. 2 Sequence alignment of the 0.4, 0.7 and 1.6 kb *FaExp2* promoter fragments.** Nucleotides in bold indicate polymorphic sites, hyphen indicates deletion. The restriction enzyme sites, *Dra*I, *Stu*I and *Sca*I, which have been used in the construction of strawberry genome-walking libraries, are boxed as well as a TATATAA-box and the first ATG of the *FaExp2*-gene. The putative start of transcription is indicated by an arrowhead. Forward primers used for the sequence-specific PCR are indicated by arrows above the 1.6p*FaExp2* and 0.7p*FaExp2* sequences.

at which a statistically significant increase of $\Delta R_n$ is first detected, was determined. The relative quantification was done using the comparative $C_T$-method (User bulletin #2, ABI PRISM 7700 Sequence Detection System, December 1997, Perkin-Elmer, Applied Biosystems) in which the differences in the $C_T$ for the *gus*-amplicon and the $C_T$ for the endogenous control *dbp*, called $\Delta C_T$, were calculated to normalise for the differences in the total amount of cDNA present in each reaction and the efficiency of the RT step. For comparison of two samples the $\Delta C_T$ values were subtracted from each other, giving a $\Delta\Delta C_T$ value and finally the relative amount of *gus* mRNA copies was calculated by $2^{-\Delta\Delta CT}$.

## RESULTS

### Isolation of *FaExp2* promoter fragments

In order to isolate a strawberry receptacle specific promoter, the strawberry expansin 2 gene (*FaExp2*) was selected. The expression of this gene was described to be relatively strong and highly strawberry fruit-specific (Civello *et al.* 1999; Aharoni *et al.* 2002; Harisson *et al.* 2002; Salentijn *et al.* 2003; Dotto *et al.* 2006; Figueroa *et al.* 2009). Furthermore, its expression was demonstrated not to be affected by either auxin (Civello *et al.* 1999; Aharoni *et al.* 2002) or ethylene treatment (Aharoni *et al.* 2002) which made this gene a good candidate for isolation of a strawberry receptacle-specific promoter sequence. Following a genome walking approach using gene specific primers for *FaExp2*, three different 5′-upstream genomic DNA sequences of 400, 700 and 1600 bp have been amplified from *Sca*I, *Stu*I, and *Dra*I-digested 'Elsanta' genomic libraries, respectively.

DNA sequence alignment of the three different fragments showed a high degree of similarity. The sequences differed in several SNPs and small indels (inserts/deletions) and a larger (15 bp) indel (**Fig. 2**). SNPs in the *Sca*I and *Stu*I-restriction sites, that were responsible for promoter fragment length polymorphisms, could be traced back in the larger sequences (**Fig. 2**).

For strawberry several homologous expansin genes have been identified and especially *FaExp2* and *FaExp7* show a high degree of similarity (Harrison *et al.* 2001). In order to check the origin of the obtained promoter sequences, sequence-specific PCR was performed using specific forward primers for the two longest promoter fragments in combination with reverse primers that are specific for six different strawberry expansin genes (see Harrison *et al.* 2001). Only for the combination of both forward promoter primers together with the reverse primer specific for *FaExp2*, amplification of a fragment of expected size was obtained (**Fig. 3**), indicating that both different 5′-upstream sequences belong to the *FaExp2* gene and represent most likely allelic sequences.

### Construction of the reporter vector and introduction into strawberry plants

To study the promoter activity of two longest fragments obtained, the 700 and 1600 bp fragments were fused to the *gus* reporter gene and transferred to the strawberry cultivar 'Calypso' using *A. tumefaciens* mediated transformation. For comparison, a construct in which the heterologous *CaMV35S*-promoter was combined with the *gus* reporter gene was introduced in the same strawberry cultivar. For all constructs several transgenic lines expressing *gus* in fruit tissue have been produced, and three 0.7p*FaExp2-gus* (A-C) and four 1.6p*FaExp2-gus* (A-D) lines, showing a relatively high intensity of histochemical GUS staining in ripe fruits, were selected for further analysis. DNA gel-blot

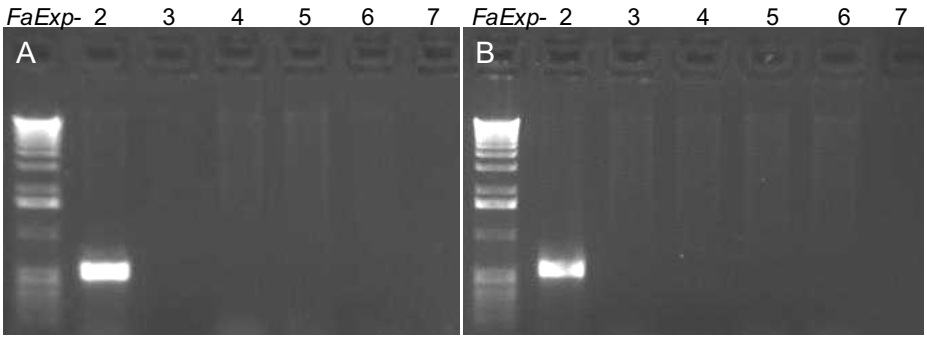


**Fig. 3 Sequence-specific PCR using forward primers specific for 0.7p*FaExp2* (A) or 1.6p*FaExp2* (B) and reverse primers specific for the strawberry expansin genes *FaExp2-7*.**

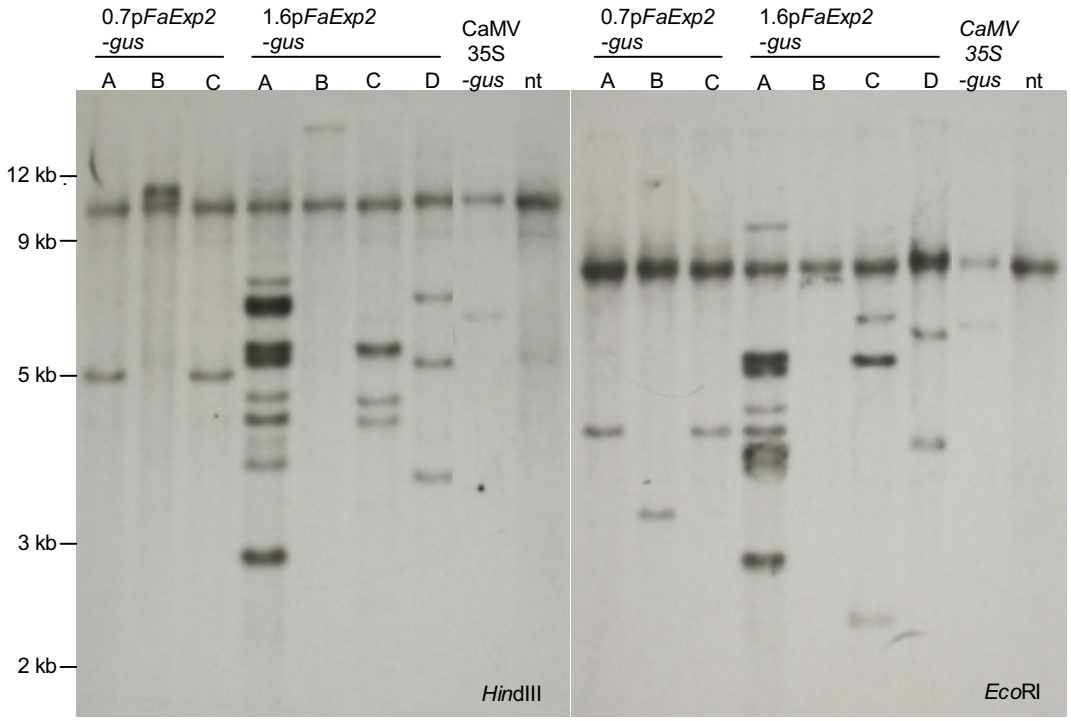


**Fig. 4 DNA-gel blot analysis of genomic DNA of independent transgenic strawberry lines harbouring the 0.7p*FaExp2-gus*, the 1.6p*FaExp2-gus* or the *CaMV 35S-gus* constructs.** DNA of a non-transgenic plant (nt) is included as control. Total DNA was digested with *Hin*dIII (left blot) or *Eco*RI (right blot) and the blotted DNA was hybridised with an *npt*II probe. The number of hybridisation bands indicates the integrated T-DNA copy number. An aspecific hybridisation band is present in all lanes, including the non-transgenic control lane.

analysis using genomic DNA of all transgenic lines was performed in order to determine the number of integrated T-DNA copies (**Fig. 4**). Both *Eco*RI and *Hin*dIII cut the T-DNA at one side of the *nptII* gene (**Fig. 1**), while the other relevant restriction site is located in the host DNA that flanks the *nptII* gene at the other side after T-DNA integration. Depending on the position of the restriction site in the host DNA, hybridisation bands of different sizes may be obtained after hybridisation with an *npt*II probe, representing different T-DNA integration events. For both blots an aspecific hybridisation band is visible at a similar position in all lanes (**Fig. 4**; approximately 10 kb for the *Hin*dIII-blot and 8 kb for the *Eco*RI-blot). Since this band is also present in the non-transgenic DNA lane, it should be left out in consideration when determining the T-DNA copy number. The DNA gel- blot analysis confirms the integration of the T-DNA, with a variable copy number, from 1 to at least 9 insertions, depending on the line.

## Histochemical GUS staining

For the selected 0.7p*FaExp2-gus* plants and 1.6p*FaExp2-gus* transgenic plants, a fruit-specific GUS staining pattern was observed (**Fig. 5**, 0.7p*Exp2* B, 1.6p*Exp2* A). In flowers and young fruits of these plants, no visible GUS staining was found in receptacle tissue, but in big white and red fruits a clear blue staining was observed. It was remarkable however, that the development of GUS staining in big white and red fruits of 1.6p*FaExp2-gus* transgenic plants was extremely fast as compared to similar fruits from 0.7p*FaExp2-gus* or *35SCaMV-gus* transgenic plants, indicating a high level of GUS activity in these fruits (results not shown). In addition to the GUS-staining found in receptacle tissue, blue staining was also found in achenes and in epidermal and sub-epidermal layers of petioles and stems of flowers and fruits. No GUS staining was observed in roots, and for leaf-discs only plants transformed with the 1.6p*FaExp2-gus* construct showed some GUS staining near the vascular tissue.

## *Gus* expression analysis

For the different transgenic lines made with p*FaExp2-gus* and *CaMV35S-gus* constructs the level of *gus* expression was determined by quantitative RT-PCR. In order to limit the number of RNA isolations, for each tissue type RNA

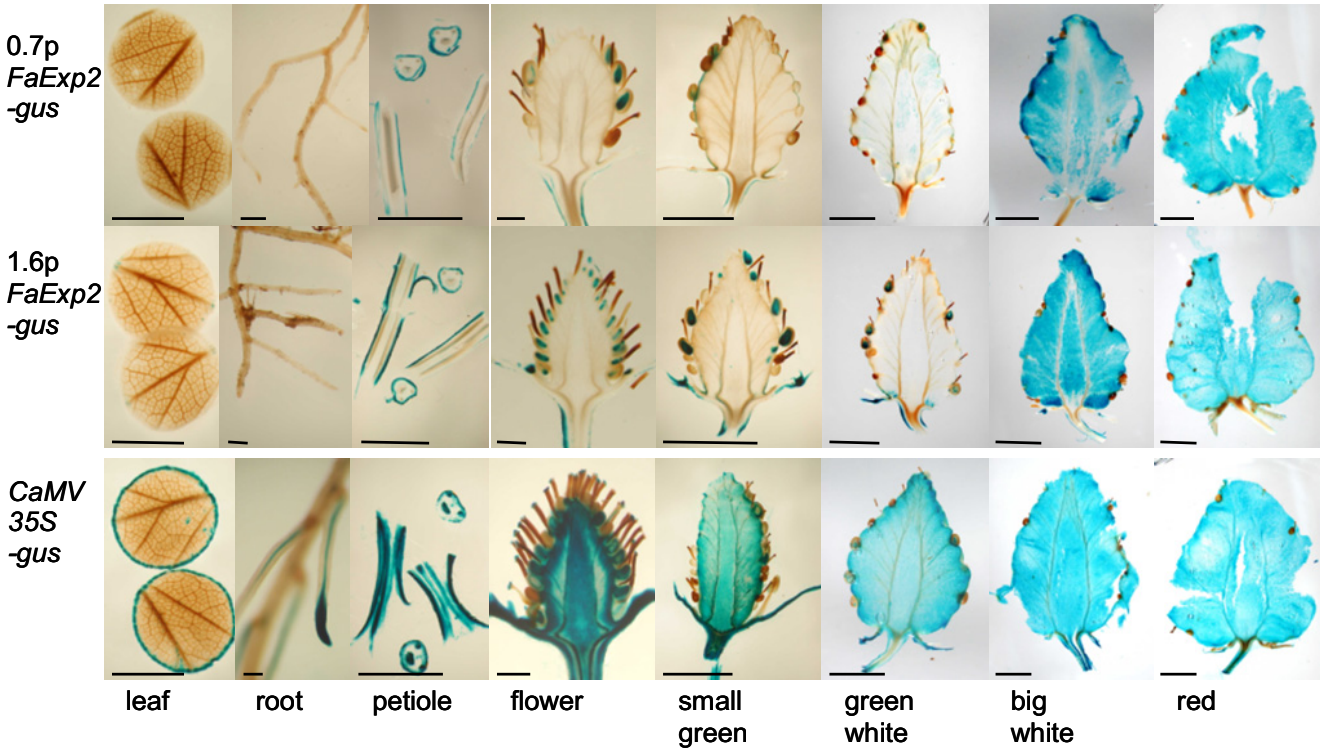


**Fig. 5 Histochemical GUS staining of leaf, root, petiole and flower tissue and of fruits at different developmental stages.** Typical GUS staining patterns for the 0.7p*FaExp2* and the 1.6p*FaExp2* promoters are represented by tissues of plant 0.7p*FaExp2-gus* B and plant 1.6p*FaExp2-gus* A, respectively. Tissue of a *CaMV35S-gus* transgenic strawberry plant is included as reference. Size bars: 1.0 mm for roots and flowers; 5.0 mm for all other tissues.

was isolated from pooled tissue samples from independent transgenic lines harbouring the same construct. Therefore, this will give average expression levels for the different constructs, rather than specific expression results for each transgenic line. **Fig. 6** shows that for 0.7p*FaExp2-gus* plants (**Fig. 6**, lower panel) as well as for 1.6p*FaExp2-gus* plants (**Fig. 6**, upper panel) expression levels are upregulated in ripening fruits, starting with big white fruits. For 1.6p*FaExp2-gus* transformants there was also considerable expression in the green-white fruit stage. For *CaMV35S-gus* plants *gus* expression was not correlated with any fruit stage and highest expression levels were observed for leaf and root tissue.

## DISCUSSION

For tissue-specific expression of genes in strawberry, the availability of specific regulatory sequences is desirable. Because *FaExp2* was known to be tightly regulated during ripening (Civil *et al.* 1999; Aharoni *et al.* 2002; Salentijn *et al.* 2003; Dotto *et al.* 2006; Figueroa *et al.* 2009), we selected this gene for the isolation of a strawberry receptacle and ripening-specific promoter sequence. Using a genome walking approach we cloned three highly homologous *FaExp2* 5′-upstream PCR fragments of different lengths. PCR-analysis using a specific forward primer for the 0.7 and 1.6 kb *FaExp2* promoter fragments in combination with sequence specific reverse primers for six of the seven known strawberry expansin genes (*FaExp2-7*), revealed that both promoter sequences belong to the *FaExp2* gene. However, whether the promoter fragments belong to different alleles or different gene copies of *FaExp2*, could not be determined from this experiment.

In general, promoter sequences do not have strictly defined borders. For some genes regulatory elements as far as 20 kb upstream from the transcription start have been identified (Potenza *et al.* 2004). However, most promoter sequences used for regulating transgene expression range between 0.5-2.0 kb in length (Potenza *et al.* 2004). In order to investigate the promoter activity of the 0.7 and 1.6 kb *FaExp2* promoter sequences, both fragments were fused to the *gus* reporter gene and the resulting gene fusion was transferred to the strawberry genome by means of *Agrobacterium*-mediated transformation.

Histochemical GUS staining showed that both 5′-flanking fragments of *FaExp2* were sufficient to confer fruit-specific and ripening-regulated expression of *gus*. For white and red fruits of transgenic plants containing the 1.6 kb promoter fragment fused to the *gus* reporter gene, a much faster development of blue staining was observed than for the 0.7 kb fragment, suggesting a higher GUS activity in these fruits. However, no differences in the tissue-specific GUS staining pattern were observed between fruits of the 0.7p*FaExp2-gus* and 1.6p*FaExp2-gus* transgenic strawberry plants. Although the 1.6p*FaExp2-gus* plants showed quite some variation in T-DNA copy number as determined by DNA-gel blot analysis, these differences do not seem to have much influence on the *gus* expression pattern. GUS staining of petiole tissue and achenes indicated that the expression of *FaExp2* is not completely restricted to ripening strawberry fruits. Aharoni and O'Connell (2002) determined, using DNA microarrays, that the expression level of *FaExp2* in receptacle of red fruits was 13-fold higher than in achenes. For a number of other expansin genes from strawberry, pear and tomato an overlapping expression has also been reported (Brummel *et al.* 1999; Harrison *et al.* 2001; Hiwasa *et al.* 2003). Possibly, it is a common feature of certain expansin genes to be expressed in different tissues, suggesting variable functions for these genes.

Quantitative *gus* expression analysis showed a large difference in *gus* transcript level in ripening fruits of 0.7p*FaExp2-gus* and 1.6p*FaExp2-gus* plants. This suggests that in the 1.6p*FaExp2* promoter sequence one or more additional positive regulatory *cis*-acting elements were present as compared to the 700 bp fragment, which gave rise to an overall higher level of gene expression. The temporal and spatial control of gene transcription is generally mediated by the interaction of negative and positive regulatory elements. For example, for a fruit-specific promoter of the tomato polygalacturonase gene, deletion analysis indicated

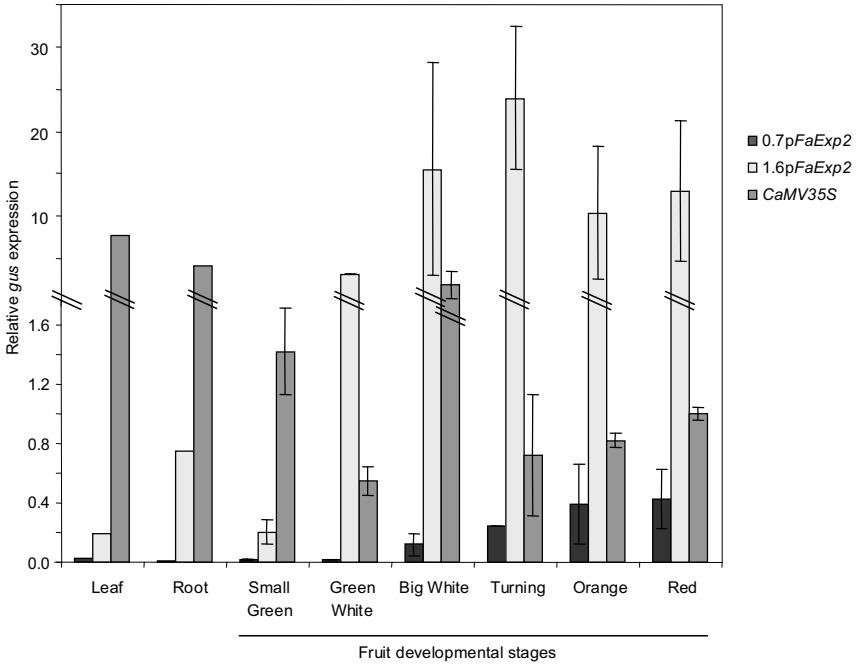


**Fig. 6 Gus gene expression analysis by quantitative RT-PCR.** Due to large differences in gus transcript levels, the data are plotted against two different scales. Two oblique lines indicate change in the scale on the Y axis. Expression levels of all samples are related to the *gus* expression level in red fruits of the *CaMV35S*-gus control plant. Error bars represent ± SE values. All fruit sample values are replicates in two-fold. Leaf and root samples have been analysed once. RT-PCRs for 0.7p*FaExp2-gus* and 1.6p*FaExp2-gus* have been performed on RNA samples obtained from pooled tissues of different transgenic lines transformed with the same construct.

the presence of different positive and negative regulatory regions which modulated tissue-specific gene expression (Montgomery *et al.* 1993). For the *FaExp2* promoter it is most likely that *cis*-acting elements, which promote the level of gene expression, are located in the proximal 900 bp region. However, the observed differences in expression could also be due to polymorphisms that discriminate the two promoter sequences in the first 700 bp (**Fig. 2**). In order to clear up the location of relevant regulatory elements, more detailed experimental data are required.

## CONCLUSION

In order to achieve strawberry fruit-specific expression of transgenes, 5′-upstream sequences of *FaExp2*, a strawberry gene which is expressed in a fruit-specific manner, have been isolated and two different fragment lengths have been characterized. Depending on the desired level of expression, both lengths of the *FaExp2* promoter may be good candidates to direct *trans*- (or intra-)gene expression in ripening strawberry receptacle tissue. Currently, we investigate the suitability of the 1.6p*FaExp2* promoter to direct the expression of an antifungal gene with the aim to enhance the resistance level to fruit rot caused by *Botrytis cinerea*.

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