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M.-A. D'Aoust · B. Nguyen-Quoc · V.-Q. Le · S. Yelle

Upstream regulatory regions from the maize *Sh1* promoter confer tissue-specific expression of the β-glucuronidase gene in tomato

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Abstract A promoter fusion (Sh35) combining upstream regulatory regions from the maize *Sh1* promoter with a truncated 35S promoter, Δ9035 (–90 to +8) has been compared with the original *Sh1* promoter for its capacity to promote expression of the β-glucuronidase (GUS) gene in stably transformed tomato plants. For both promoters, very faint GUS expression was detected in the vegetative tissues, and no expression was detected in the fruit pericarp tissues. However, in the seed, *Sh1* promoted low GUS expression but Sh35 directed 25-fold higher GUS expression. For both constructs, the profile of GUS expression was similar to that of endogenous sucrose synthase activity, but maximal GUS activity was reached 15 days after the peak of sucrose synthase activity.

Key words Maize · Promoter · *Shrunken-1* · Sucrose synthase · Tomato

Abbreviations *DAA* Days after anthesis · *GUS* β-Glucuronidase · *Susy* Sucrose synthase

Introduction

In maize, the expression of sucrose synthase (Susy) is under the control of two distinct genes, *Sh1* and *Sus1* that encode two different isoforms, SS1 and SS2, respectively (Chourey 1981; McCarty et al. 1986; Werr et al. 1985). Based on enzyme localization, mutant phenotypes, and promoter studies, Susy isoforms have been proposed to play distinct physiological roles in plants. The SS1 isoform, the product of the *Sh1* gene, is usually associated with phloem and starch-synthesizing tissues (Chen and Chourey 1989; Heinlein and Starlinger 1989; Rowland et al. 1989) while the SS2 form, the product of the *Sus1* gene, is found in rapidly growing tissues (Nguyen-Quoc et al. 1990). In tomato, only one Susy gene has been described (Wang et al. 1993) but there are indications of the presence of at least two tomato Susy genes (Nguyen-Quoc et al. 1995) as found in other dicots like *Arabidopsis* (Chopra et al. 1992; Martin et al. 1993) and potato (Fu and Park 1995). Preliminary studies have been carried out to test the capacity of the *Sh1* and *Sus1* promoters from maize to drive tissue-specific expression of the β-glucuronidase (GUS) gene in transgenic tomato plants. *Sh1* drives expression in the vascular tissues of the leaves and in the roots of in vitro plants, whereas *Sus1*-driven GUS expression is restricted to the base of the leaf (X. F. Huang, B. Nguyen-Quoc and S. Yelle, unpublished data). Unfortunately, the level of expression obtained in each transformant was often near the histochemical GUS detection limit and did not allow precise studies on temporal and spatial variations in gene expression.

The Δ9035S promoter region is now routinely used for the study of putative enhancer regions from monocot promoters (Luan and Bogorad 1992; Maas et al. 1990; Thomas and Flavell 1990) and dicot promoters (Benfey et al. 1990; Fujiwara and Beachy 1994; Lam et al. 1990; Poulsen and Chua 1988). This region of the 35S promoter was first recognized as essential for the high expression level of the promoter. Later, the region of the 35S extending to –90 was recognized as essential for a high level of expression in tobacco leaves when combined to the leaf-specific *rbcS*–8B upstream regulatory regions (Poulsen and Chua 1988). Alone, the Δ9035S promoter section is known to drive expression in the radicle and in the radicle pole of the endosperm (Benfey and Chua 1989) but the level of expression conferred by this promoter section alone is low and even near the detection limit of the fluorometric measurement method (Fujiwara and Beachy 1994; Slocombe et al. 1994; Thomas and Flavell 1990).

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M. A. D'Aoust · B. Nguyen-Quoc (✉) · S. Yelle
Centre de recherche en horticulture, Pavillon de l'Envirotron,
Université Laval, Sainte-Foy, Québec, G1K 7P4, Canada
e-mail: nguyenb@plg.ulaval.ca
Fax: +1-418-6567871

V.-Q. Le
Centre de recherche en biologie forestière,
Pavillon Charles-Eugène Marchand, Université Laval,
Sainte-Foy, Québec, G1K 7P4, Canada

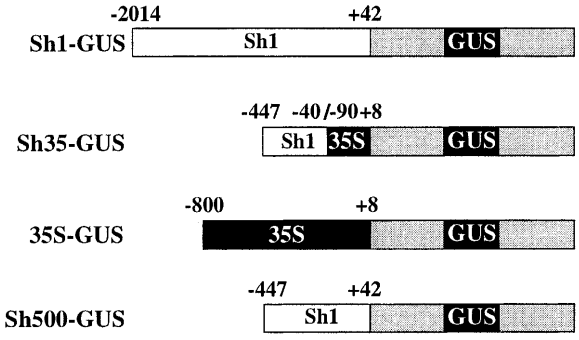

Fig. 1 Schematic representation of the transcriptional gene constructs. The *Sh1-GUS* gene contains the full length *Sh1* promoter fused to the GUS reporter gene. The *Sh35-GUS* gene contains the GUS coding sequence under the control of a regulatory region of the *Sh1* promoter fused to the Δ9035S promoter. The *35S-GUS* gene consists of the unmodified constitutive 35S promoter fused to the GUS gene. The *Sh500-GUS* gene is a 500 bp-truncated version of the *Sh1* promoter fused to the GUS gene. The *numbers* represent the position from the transcription initiation site. Details of the constructions are presented in the Materials and methods section

In this study, a promoter fusion was created combining a putative enhancer region from the *Sh1* promoter (Werr et al. 1985) and the Δ9035S promoter fragment. Our objective was to take advantage of the capacity of the Δ9035S section to serve as an efficient core promoter region that could enhance the level of expression without changing the tissue specificity conferred by the *Sh1* upstream regulatory regions. Compared to the original *Sh1* promoter, the resulting promoter fusion mediated a 25-fold increase in GUS expression in the endosperm tissue of the seed.

Materials and methods

Constructs

The constructs used in this study are illustrated in Fig. 1.

Sh1-GUS

The *Sh1* promoter, a maize genomic fragment from –2014 to +42 subcloned into pUC9, was kindly provided by Dr. W. Werr (Institute of Genetics, Cologne, Germany). The clone was linearized with *Eco*RI, blunt-ended by Klenow fragment treatment and the insert was cut off with *Bam*HI. This 5′-blunt-ended and 3′ *Bam*HI cohesive fragment was ligated to the pUC18 previously digested with *Xba*I, blunt-ended and cut off with *Bam*HI. After ligation, the recombinant plasmid was digested with *Hin*dIII and *Bam*HI and this fragment, containing the *Sh1* promoter, was inserted into pBI101 in the sense orientation.

Sh35-GUS

The vector pBI121 was digested with *Pst*I (at –800 of 35S) and *Bam*HI (at +8 of 35S) and the resulting fragment (35S promoter) was inserted into pUC19 between the same sites, thus creating pU1935. pU1935 was digested with *Eco*RV (at –90 of 35S) and *Bam*HI (at +8 of 35S) and inserted into pUC19 between the *Sma*I and *Bam*HI sites, resulting in the plasmid pU1990. For coupling the region of *Sh1* to the minimal promoter of 35S, pU1990 was digested with *Kpn*I, blunt ended, and digested with *Sac*I. The *Sh1* fragment between the *Sac*I (–447) and *Pvu*II (–40) sites was inserted inside the blunt end and the *Sac*I site of pU1990. The new plasmid, containing the final chimeric promoter is referred to as pUSh35. To create new sites around the promoter, pUSh35 was digested with *Sac*I, blunt-ended, digested with *Bam*HI and inserted between the *Hin*cII and *Bam*HI sites into pUC18, creating pUSh3518. To insert the promoter Sh35 in the binary vector pBI101, pUSh3518 was digested with *Hin*dIII and *Bam*HI, and inserted inside the same sites into pBI101.

35S-GUS

The vector pBI121 (purchased from Clonetech Laboratories) was used without modification for the 35S-GUS.

Sh500-GUS

The *Sh1* promoter was digested with *Sac*I, blunt-ended, digested with *Bam*HI and inserted into pUC18 between the *Hin*cII and the *Bam*HI sites. The temporary plasmid was called pUSh500. The promoter Sh500 was inserted into pBI101 between the *Bam*HI and *Hin*dIII sites. Orientation and fidelity of the clonings were verified by digestion with restriction enzymes.

Transformation

Tomato plants (*Lycopersicon esculentum* Mill. var. Summerset) were transformed using the *Agrobacterium tumefaciens*-mediated transformation method as essentially described by McCormick (1991) with slight modifications. Briefly, well-expanded cotyledons of 10-day-old seedlings were cut and placed upside down on the induction medium. After 3 days of precultivation, green cotyledons which swelled in size were cut transversally and transferred to the diluted culture of *Agrobacterium* for 15 min. Following 3 days of cocultivation, the cotyledons were placed upside down in the selection medium. After 3 weeks of incubation in the selection medium, calli were excised from the cotyledons and transferred to organogenesis medium. The shoots grown from different calli were screened on the basis of enhanced ability to form roots on the medium containing kanamycin, and by PCR analysis.

Protein extractions and enzyme activity analysis

Tomato fruits were harvested and seeds were collected. Four developmental stages were considered for the analysis of GUS and Susy activities. The first seed stage was characterized by the early formation of the endosperm and the appearance of the embryo [corresponding to a fruit age of 10–15 days after anthesis (DAA)]. At the second stage of development, the embryo was well developed and the endosperm milky (25–35 DAA). At the third stage, the endosperm was harder, as cell walls had formed and the seed coat was partly sclerified (40–50 DAA). Finally, at the fourth stage, the seed was mature and seed coat wall sclerification was completed. Twenty seeds from each plant were opened and the embryo and endosperm tissues separated under a magnifying binocular. The tissues were immediately immersed into the extraction buffer. Embryos were rinsed twice to ensure that no endosperm was left in the extract. The pooled tissues were ground, centrifuged, and the supernatant was used for the enzyme assay. For the Susy activity assay, salts and nucleotides were removed from the extraction by Sephadex G-25 gel filtration. A HEPES buffer (200 mM HEPES pH 7.0, 0.5 mM Na_2EDTA pH 8.0, 0.5 mM PMSF, 0.5 mM DTT and 1 mM β-mercaptoethanol) was used for the extractions for Susy activity assays. A phosphate buffer (50 mM $NaHPO_4$ pH 7.0, 10 mM β-mercaptoethanol, 10 mM Na_2EDTA pH 8.0, 0.1% sodium lauryl sarcosine and 0.1% Triton X-100) was used in the extractions for GUS activity assays. Susy activity analyses were performed as described by Huber and Akazawa (1986). Histochemical and fluorometric analyses were performed as described by Jefferson et al. (1987).

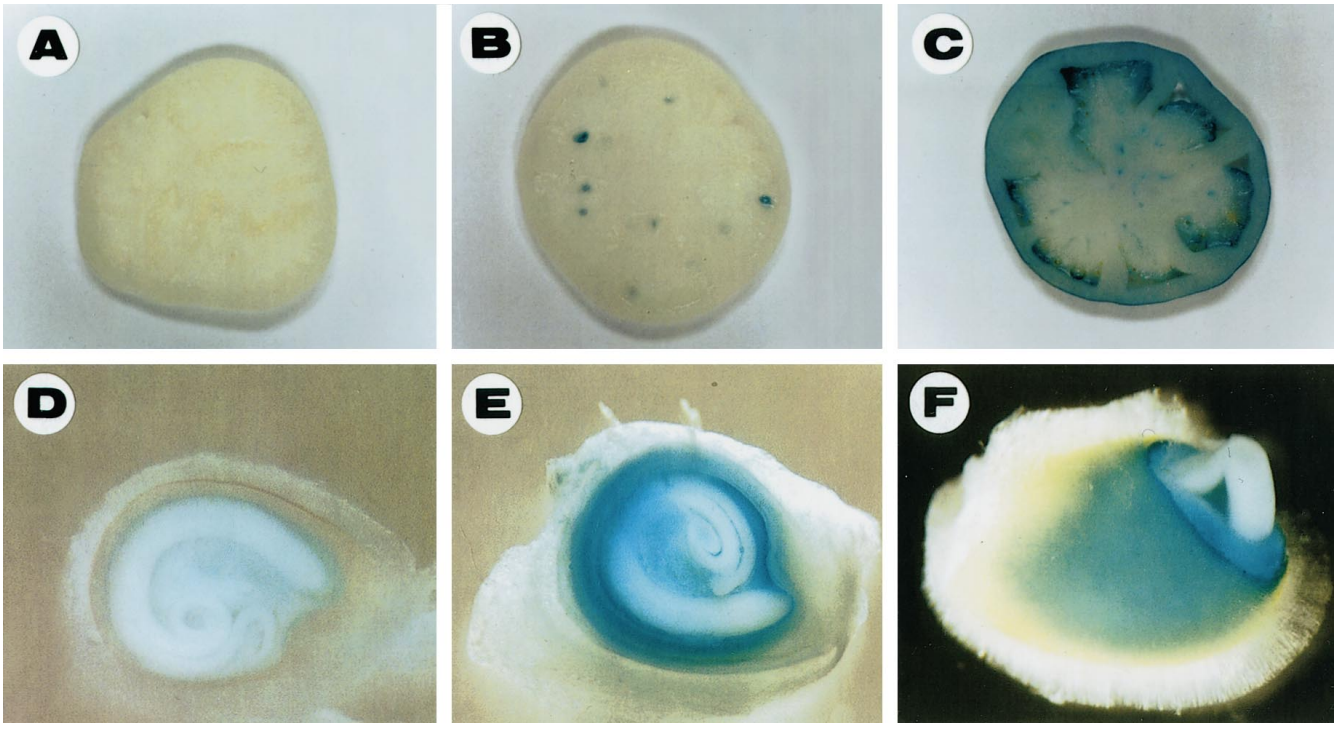

Figure 2A–F Histochemical detection of GUS activity in the transgenic tomato fruits at 25 DAA. **A** Transverse section of a *Sh1*-GUS fruit. **B** Transverse section of a Sh35-GUS fruit. **C** Transversal section of a 35S-GUS fruit. **D** Longitudinal section of a *Sh1*-GUS seed. **E** Longitudinal section of a Sh35-GUS seed. **F** Sh35-GUS seed

Results

Histochemical evaluation of GUS activity distribution

Transgenic tomato plants were transformed with the gene constructs shown in Fig. 1. The *Sh1* promoter comprised the complete promoter sequence cloned by Werr et al. (1985). In the Sh35 promoter, the enhancer region of the *Sh1* promoter extended from –447 to –40 and contained at least one of each regulatory element characterized by Werr et al. (1988). A short version of the *Sh1* promoter, named Sh500, was also analyzed. This short promoter comprised the original *Sh1* promoter truncated at –447, and hence contained the same regulatory elements as Sh35 but with the original core promoter sequences. Finally, the commercial version of the 35S promoter was also used for the transformations as a control for constitutive expression.

Transformants were obtained with each construct and in vitro plants were analyzed for the presence of GUS activity by histochemical coloration of vegetative tissues. The staining revealed that 19 of the 20 Sh35-GUS clones (95%) showed detectable GUS expression whereas only 30% (15 out of 50) of the Sh1-GUS plants allowed the detection of GUS activity in their vegetative tissues. When plants showing detectable GUS expression were compared, no visual difference in the tissue specificity or in the intensity of coloration could be detected between the plants transformed with Sh35 and those bearing the original *Sh1* promoter (data not shown). In all plants, GUS staining was found at the tip of the leaves and in the root tissues. No GUS activity could be detected in the vegetative tissues of the plants transformed with the Sh500-GUS construct.

Transformed plants were transferred to the greenhouse for the production of fruits. None of the plants transformed with either Sh1-GUS (Fig. 2A), Sh35-GUS (Fig. 2B) or Sh500-GUS (not shown) showed detectable GUS activity in the fruit pericarp. In contrast, transformation with the 35S-GUS construct led to high GUS activity in the fruit pericarp (Fig. 2C).

The *Sh1* promoter, which is known to yield high expression in maize endosperm, promoted very low but detectable GUS expression in the tomato seeds, with slightly higher expression in the endosperm than in the embryo (Fig. 2D). The Sh35 promoter also directed GUS expression in the seeds (Fig. 2E, F). Furthermore, as in Sh1-GUS plants, more GUS activity was observed in the endosperm tissue than in the embryo, but the intensity of the coloration was much greater than that obtained with the *Sh1* promoter. GUS activity was very high in both tissues of 35S-GUS seeds and was undetectable in the Sh500-GUS transformed seeds (not shown).

Quantitative determination of GUS activity in the seed tissues

Twenty seeds per plant were collected at the second stage of developement (25–35 DAA). The embryo and endosperm tissues from these seeds were separated and the pro-

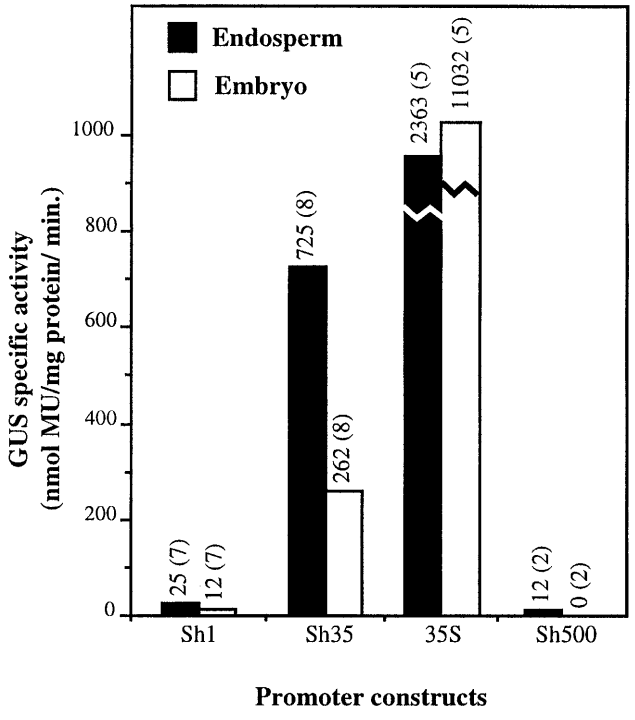

Fig. 3 GUS activity at the second stage of seed development (25–35 DAA). Values over the columns are medians for each contruct and tissue. The number of plants analyzed is indicated in parentheses

tein extracts from the pooled embryos and endosperms were used for fluorometric GUS activity analysis. For both Sh1-GUS- and Sh35-GUS-transformed plants, GUS activity in the endosperm was twice that in the embryo (Fig. 3). Moreover, Sh35-GUS plants showed over 25 and 20 times more GUS activity in the endosperm and the embryo, respectively, than the Sh1-GUS plants. The level of expression obtained with Sh35 was even 50 times higher than that obtained with Sh500, which contains a similar truncation of the *Sh1* promoter. The 35S-GUS-transformed seeds showed the highest GUS activity with 4 times more activity in the embryo than in the endosperm.

Comparison of GUS expression with endogenous Susy activity during seed development

To relate the pattern of expression obtained with the *Sh1*-based promoters to that of endogenous tomato Susy, GUS activity was measured at four different stages of seed development and compared with endogenous Susy activity. Figure 4A, B shows the similar developmental profile of GUS activity in the seeds of Sh1- and Sh35-GUS-transformed plants. With both promoters, GUS activity reached a maximum at the third stage of development (40–50 DAA) and decreased during seed maturation. In both cases, the activity in the endosperm was approximately twice that in the embryo at every stage of development. For the first three stages of development, GUS activity was 21–38 times higher in the Sh35-transformed plants than in the *Sh1* plants. For

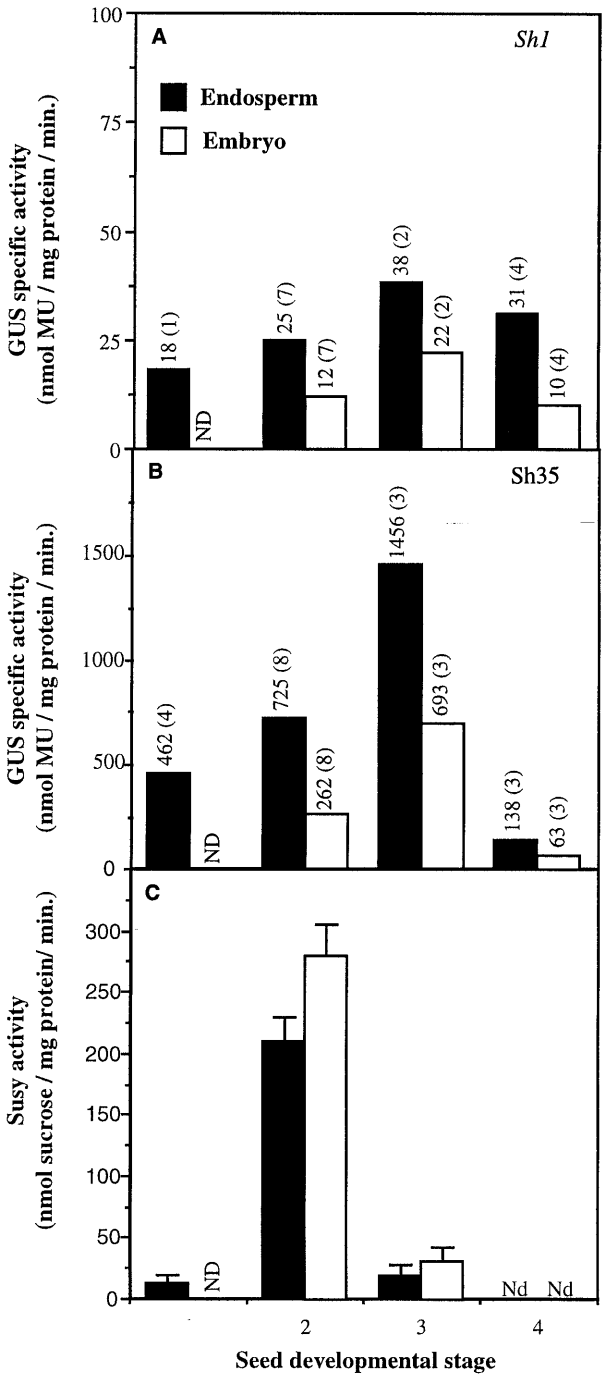

Fig. 4 Comparison of GUS activity in the Sh1-GUS- and Sh35-GUS-transformed plants and of endogenous Susy activity during seed development. Profile of GUS activity in the endosperm and embryo of Sh1-GUS (**A**) and Sh35-GUS (**B**) plants during seed development, and evolution of Susy activity in the endosperm and the embryo during tomato seed development (**C**) (*ND* not determined, *Nd* not detected). **A, B** Values over the columns are medians for each construct and tissue. The number of plants analyzed is indicated in parentheses. Each median value was obtained from 20 seeds per plant. **C** Each mean and SE value was obtained from the seeds of four to eight fruits

the fourth stage, the increase in expression level was smaller (4- to 6-fold) and this lower enhancement might be attributed to the difficulty of measuring lower GUS activity in the seeds. Due to the small size of the embryo at the first developmental stage, GUS activity was not determined.

Figure 4C shows the profile of endogenous Susy activity in endosperm and embryo tissues of tomato seeds. The activity reached a peak at the second stage of development and rapidly decreased by the third stage. Susy activity was slightly higher in the embryo at every stage of development. The comparison of Susy activity with Sh35-driven GUS activity in the seeds revealed that the profile of both activities during seed development is similar but that Sh35-directed GUS expression was delayed. In addition, the tissue specificity of the expression was different: GUS activity was higher in the endosperm, while endogenous Susy activity was similar in the endosperm and embryo. Further research is needed to determine the number of genes responsible for Susy expression in the fruit pericarp and seed tissues, and their respective role in the normal development of these tissues.

Discussion

The Sh35 chimeric promoter directs a higher level of GUS expression in tomato without changing the tissue specificity and temporal expression pattern of the original *Sh1* promoter

To study the expression pattern of the maize *Sh1* promoter in tomato plants, a promoter fusion was obtained by combining regulatory regions from the *Sh1* promoter with the Δ9035S promoter. The high GUS activity found in the endosperm tissue of the tomato plants transformed with the Sh35 promoter is in accordance with previous experiments on the localization of the Susy peptide in maize (Chourey and Taliercio 1994). Similarly, it was shown that the *Sh1* promoter directed GUS expression in maize (Huang et al. 1998) and tobacco (Yang and Russel 1990) endosperm. The presence of GUS activity in the embryo of Sh1-GUS- and Sh35-GUS-transformed tomato plants contrasts with the endosperm specificity of the SS1 peptide in maize. Differences in the post-transcriptional regulation of the expression of GUS and Susy peptides may have caused this difference in tissue specificity. Post-transcriptional regulation of the *Sh1* gene in the embryo tissue was demonstrated by Chourey and Taliercio (1994) who showed the presence of SS1 mRNA in this tissue even in the absence of the corresponding peptide.

The *Sh1* promoter directs tissue-specific GUS expression which is not strictly linked to starch synthesis and sink status of the tissue

The expression of *Sh1* in maize tissues is restricted to high-starch-synthesizing tissues (Chen and Chourey 1989; Heinlein and Starlinger 1989; Rowland et al. 1989). In tomato, the *Sh1* promoter did not promote GUS expression in the fruit pericarp even if, at 25 DAA, the pericarp is an important sink tissue for carbohydrate, with high starch synthesis and endogenous Susy activities. A strict association between starch synthesis and the expression of *Sh1* should have led to the expression of the GUS gene in the fruit pericarp. Because the fruit pericarp is a net importer of sucrose, it is clearly a sink tissue with respect to its carbon balance. However, the expression of *Sh1* was not induced by the sink status of this tissue. These results suggest that the expression of *Sh1* is not sink but rather organ specific.

Control of Susy expression in tomato fruit tissues

The delayed GUS expression in Sh35-GUS plants in comparison to endogenous Susy activity can be attributed to the developmental difference between the maize and tomato seeds. While the endosperm of the maize seed increases in size during its development, that of tomato stops growing early and remains relatively small. Additionally, while the expression of *Sh1* was more important in the endosperm, endogenous Susy activity was similar in the tomato endosperm and embryo. The high expression of Susy in both the tomato endosperm and embryo can be attributed to differential expression of a single Susy gene or, as in maize, to the expression of two genes. Interestingly, the tomato seed Susy mRNA does not hybridize with the potato Susy cDNA, while, under the same conditions, the pericarp Susy mRNA does hybridize (Wang et al. 1994). Furthermore, none of the Susy mRNA found in the tomato fruit pericarp shares a high level of homology with *Sh1* or *Sus1* genes (Nguyen-Quoc et al. 1995, Wang et al. 1994). The presence of endosperm- and embryo-expressed Susy genes, and the relationship between these genes and fruit- or vegetative-tissue-expressed Susy remains to be elucidated.

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