

## Characterization of Fruit-Type Sucrose Synthase Gene Promoters Isolated from Tomato and Japanese Pear

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### Abstract

We isolated the 5' upstream promoter regions of the fruit-type sucrose synthase (SS) gene from tomato and Japanese pear by inverse PCR. The 5' region of the tomato SS gene (*TOMSSF*) contained an intron approximately 1.6 kbp long in the 5' untranslated region, whereas the equivalent 5' region in the Japanese pear SS gene (*PypSUS1*) had no intron. Each region was fused to the  $\beta$ -glucuronidase (*GUS*) gene and then each construct (*TOMSSF* 5':*GUS* or *PypSUS1* 5':*GUS*) was used to transform tomato. Histochemical analysis of GUS activity of all transformants showed high GUS activity in the fruit. Analysis of the staining pattern in the fruits of all transformants showed staining specific to vascular tissues and testae. The highest level of GUS activity and GUS mRNA was found in fruits at earlier stages of development in all transformants. However, the increase in the level was not observed in the ripening fruits of *PypSUS1* 5':*GUS* lines, indicating that the expression patterns of *PypSUS1* 5':*GUS* tomato were different from those of *PypSUS1* in Japanese pear. It may ascribe to the differences in genetic background between tomato and Japanese pear that relate to the mechanisms contributing to the sucrose synthesis during ripening.

**Discipline:** Biotechnology

**Additional key words:** promoter, sucrose synthase (EC 2.4.1.13)

### Introduction

Sucrose synthase (SS) is an agriculturally important enzyme that catalyses the first mobilization reaction of transported sucrose in sink tissues, including crop seeds<sup>1,7,22</sup>, potato tubers<sup>4,14</sup>, and various fruits<sup>18,20</sup>. It is generally thought that SS participates in supplying fructose and uridine diphosphoglucose by cleaving sucrose to meet the metabolic demand of processes including starch synthesis, cell wall synthesis and energy generation<sup>17</sup>.

Multiple forms of SS exist in a range of different plants<sup>7,17,18</sup>. The activity of fruit-type SS in tomato, known as the TOMSSF enzyme, is high in fruit at a young developmental stage and then decreases as the fruit matures<sup>9,13,20,23</sup>.

Therefore, production of the TOMSSF enzyme is believed to accompany the transient starch accumulation observed in young green fruit<sup>13,16,20</sup>. By comparison, production of the fruit-type SS in Japanese pear, known as the PypSUS1 enzyme, increases in fruit during the young developmental stage, declines as the fruit enlarges, and then increases again during the ripening process<sup>19</sup>. This unique expression profile of PypSUS1 implies that the increase in the enzyme level in ripening fruit is associated with sucrose resynthesis<sup>18,19</sup>.

Despite intensive expression analyses of the SS genes in fruits, including tomato and Japanese pear<sup>5,19-21</sup>, the promoters of these genes have not been well characterized. Fruit is a large sink organ to be harvested. Promoters driving genes in fruit are important tools for molecular improvement of fruit characteristics and molecular farming for production

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of functional and/or valuable components in fruit. Here we report the isolation and characterization of the 5' upstream promoter region of the fruit-type SS gene in tomato and Japanese pear. The localization of SS activity was analyzed in tomato plants transformed with promoter-*GUS* fusion gene constructs.

## Materials and methods

### 1. Plant materials

Leaves of Japanese pear (*Pyrus serotina* Rehder var. *culta* Rehder cv. Hosui) were kindly provided by Mie Prefecture Agricultural Research Institute (Matsusaka, Mie). Leaves of tomato (*Solanum lycopersicum* cv. Syugyoku) were harvested at a greenhouse of the National Institute of Vegetable and Tea Science.

### 2. Isolation of 5' upstream regions of the SS genes

Genomic DNA was isolated from leaves of tomato and Japanese pear by the standard method<sup>8</sup>. The 5' upstream promoter regions of fruit-type SS genes were isolated from tomato and Japanese pear by inverse PCR (IPCR)<sup>3</sup>. For isolation of the 5' upstream region of the tomato SS gene, *TOMSSF* (DDBJ/GenBank/EMBL accession numbers AJ011535 and L19762), genomic DNA of tomato was digested by *MunI* and then self-ligated. The self-ligated DNA was digested by *AflII* and then used as a template for IPCR. The 5' region of *TOMSSF* (*TOMSSF* 5') was PCR amplified by using the primers LeSUS485-505 (5'-CACTCAGGTAATTCGGGTTTG-3') and LeSUS60-41 (5'-TAGTTGATTCAGCAGATGGG-3'). For isolation of the 5' upstream region of the Japanese pear SS gene, *PypSUS1* (accession number AB045710), genomic DNA of Japanese pear was digested by *Clai* and then self-ligated. The self-ligated DNA was digested by *AflII* and then used as a template for IPCR. The 5' region of *PypSUS1* (*PypSUS1* 5') was PCR amplified by the primers PypSUS148-166 (5'-GACACTCTCTCCGACCACC-3') and PypSUS54-34 (5'-CTTCGTCTGTGTTCTGCAAGT-3'). A thermal cycle consisting of 20 s at 95°C, 30 s at 62°C, and 5 min at 72°C was repeated 25 times. PCR products were cloned into the pGEM-T vector (Promega KK, Tokyo, Japan) and sequenced by an automated sequencer (model 373A; Applied Biosystems, Foster City, CA, USA). The cloned 5' upstream regions from *TOMSSF* (approximately 3.4 kbp in length) and from *PypSUS1* (approximately 1.8 kbp in length) were designated as pTSPM1 and pPSP511-5, respectively.

### 3. Estimation of transcription start sites of the promoters by 5'-RACE analysis

Transcription start sites of the *TOMSSF* 5' and *PypSUS1* 5' were estimated by 5'-rapid amplification of cDNA ends

(5'-RACE) using the SMART RACE cDNA amplification kit (Clontech Laboratories, Inc., Palo Alto, CA, USA). RNA samples of young Japanese pear fruit (cv. Hosui) were kindly provided by Dr. S. Yamaki of Nagoya University. Tomato total RNA was isolated from Micro-Tom fruit at 35 to 45 days after anthesis (DAA) according to the method of Ohyama et al.<sup>11</sup>. Primers used for 5'-RACE analysis of *TOMSSF* and *PypSUS1* mRNAs were TSPRACE+200a (5'-AATTGCATCGAACTCAGCCAAAAG-3') and Py-PSSRACE+400a (5'-CGGTCAATTCCTCCACACTTAG-3'), respectively. The PCR-amplified 5' region of the cDNA was cloned into the pT7 Blue vector (Novagen, Inc., Madison, WI, USA) and then sequenced.

### 4. Construction of binary vectors

The 5' upstream regions from *TOMSSF* in pTSPM1 and from *PypSUS1* in pPSP511-5 were inserted into the upstream region of the *GUS* gene of pBI101<sup>6</sup> using standard molecular techniques. The resultant plasmids were designated as pTSSG-7 (*TOMSSF* 5'::*GUS*, Fig. 1) and pPSSG-8 (*PypSUS1* 5'::*GUS*, Fig. 1), respectively.

### 5. *Rhizobium*-mediated transformation of tomato

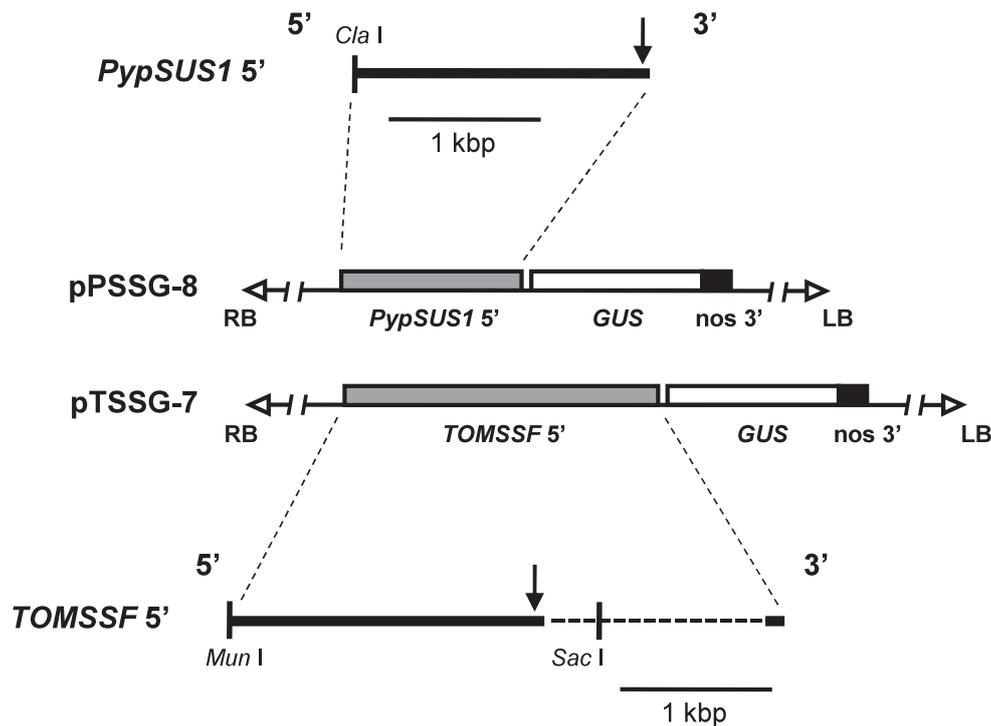
Transformation of tomato (*Solanum lycopersicum* cv. Micro-Tom) was performed as described previously<sup>12</sup>. *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens*) LBA4404 harboring pTSSG-7 or pPSSG-8 was used as a donor. Regenerated transgenic tomatoes were grown in a greenhouse during the autumn and winter seasons and flowers of transformants were tagged at anthesis.

### 6. Histochemical and enzymatic analyses of *GUS* activity

Histochemical and enzymatic analyses of transformed plants were performed as described by Ohta et al.<sup>10</sup>.

### 7. Northern blot analysis of *GUS* expression

Total RNA was extracted from transformed Micro-Tom fruit by the method of Ohyama et al.<sup>11</sup>. Samples of total RNA (5 µg/lane) was denatured by treatment with formaldehyde and fractionated by agarose gel electrophoresis<sup>15</sup>. The RNA was transferred to a nylon membrane (Hybond N+, GE Healthcare Bio-Sciences KK, Tokyo, Japan) and then probed with the *GUS* gene (1.8 kbp) labeled with digoxigenin (DIG, Roche Diagnostics GmbH, Mannheim, Germany). Labeling of the *GUS* gene was performed by PCR amplification with the DIG DNA labeling mixture, the pBI221 plasmid template<sup>6</sup>, the *GUS*-F primer (5'-ATGTTACGTCCTGTAGAAA-3'), and the *GUS*-R primer (5'-TCATTGTTTGCCTCCCTGCT-3') according to the manufacturer's protocol. Detection of hybridized DNA was performed by using the DIG Luminescent Detection Kit



**Fig. 1. Structure of the 5' upstream region of *TOMSSF* encoding tomato SS and *PypSUS1* encoding Japanese pear SS, and construction of the binary vectors, *pTSSG-7* and *pPSSG-8***

Arrows indicate the transcription start sites. The 5' regions were amplified by IPCR and then each region was inserted into the upstream of the *GUS* gene of pBI101<sup>6</sup>. Resultant plasmids were designated as *pTSSG-7* (*TOMSSF* 5':*GUS*) and *pPSSG-8* (*PypSUS1* 5':*GUS*). The construct *pTSSG-7* contained approximately 3.4 kbp of the *TOMSSF* 5', whereas *pPSSG-8* contained approximately 1.8 kbp of the *PypSUS1* 5'. LB and RB indicate the left and right borders of T-DNA, respectively. The dashed line indicates an intron approximately 1.6 kbp in length at the 3' end of the *TOMSSF* 5'. The restriction sites for *Cla*I, *Mun*I and *Sac*I are shown.

(Roche Diagnostics GmbH).

## Results

The 3' terminal sequences of the IPCR fragments (3.4 kbp from tomato and 1.8 kbp from Japanese pear) matched the 5' untranslated region (5'-UTR) sequences of the fruit-type SS cDNAs for tomato and Japanese pear, respectively (Fig. 2). These data demonstrated that the amplified fragments contained the 5' upstream regions of the fruit-type SS genes of tomato and Japanese pear (Fig. 2). The transcription start sites of the isolated 5' regions of SS genes of tomato and Japanese pear were deduced by 5'-RACE analysis (Fig. 1, see also sequence data on the database: accession numbers AB455486 and AB455487). The analysis by 5'-RACE indicated that the amplified 5' region of tomato SS gene contained an intron approximately 1.6 kbp in length within the 5'-UTR (+100 to +1687), which is consistent with the report by García-Rodríguez et al.<sup>5</sup>. By comparison, the equivalent 5' region of the Japanese pear SS gene (*PypSUS1*) had no intron. The 5' regions of the tomato

and Japanese pear SS genes upstream of the transcription start sites contained known regulatory motifs<sup>24</sup> for phloem expression (Table 1). These two regions (approximately 3.4 kbp and 1.8 kbp, respectively) were fused to the *GUS* gene<sup>6</sup> and then each chimeric gene was used to transform tomato. Transgenic tomatoes were grown in a contained greenhouse and two plants transformed with *pTSSG-7* (*TOMSSF* 5':*GUS*, Fig. 1) and four plants transformed with *pPSSG-8* (*PypSUS1* 5':*GUS*, Fig. 1) were selected on the basis of *GUS* staining strength. The presence of the transgenes in the selected transgenic tomato plants was confirmed by PCR analysis (data not shown). These plants were used for further study.

Histochemical analysis of *GUS* activity in tomato plants transformed with *pTSSG-7* or *pPSSG-8* showed a high level of *GUS* activity in fruit and comparably weaker activity in flower tissues (Table 2). Analysis of the staining pattern in the fruit of all transformants showed staining specific to vascular tissues and the surface of developing seeds (Fig. 3). The strongly stained region in mature seeds was testa (Table 2, Fig. 3). A high level of *GUS* activity was

## A

**pPSP511-5 insert 3'**                    cttgtcgctcctcatccaacacttgcagaaacacagacgaag  
 \*\*\*\*\*  
**PypSUS1 mRNA 5'**                    cttgtcgctcctcatccaacacttgcagaaacacagacgaagaagaa (gac)<sub>5</sub> (gaa)<sub>2</sub> agatagttgaatc**ATGg**---

## B

**pTSPM1 insert 3'**                    agttgaactttgtctgaggatttcccatctgctgaatcaacta  
 \*\*\*\*\*  
**TOMSSF mRNA 5'**                    agttgaactttgtctgaggatttcccatctgctgaatcaactata**ATGg**---

**Fig. 2. Alignment of 3' sequences of IPCR fragments and 5'-UTR sequences of SS mRNAs from tomato and Japanese pear**

A: The pPSP511-5 insert is an IPCR product from Japanese pear. Asterisks indicate matched bases between the 3' sequence of IPCR product and the 5' sequence of Japanese pear SS (PypSUS1) mRNA. The underlined sequence shows the position of one primer (PypSUS54-34) used for IPCR. The translation initiation codon within the 5' sequence of PypSUS1 mRNA is shown in upper case font. B: The pTSPM1 insert is an IPCR product from tomato. Asterisks indicate matched bases between the 3' sequence of the IPCR product and the 5' sequence of tomato SS (TOMSSF) mRNA. The underlined sequence shows the position of one primer (LeSUS60-41) used for IPCR. The translation initiation codon within the 5' sequence of TOMSSF mRNA is shown in uppercase font.

**Table 1. Sequences in SS promoters similar to phloem-specific promoter regulatory elements**

Element type	Consensus sequence*	<i>TOMSSF</i> 5' location of element**	<i>PypSUS1</i> 5' location of element**
CCCC sequence	<u>CCCC</u>	NI	-183, -452, -457, -462, -676
ASL box	GCA(N) <sub>8-15</sub> <u>GCA</u>	NI	-201, -1073
GATA motif	A(N) <sub>3</sub> <u>GATA</u>	-157, -450, -1154	-787

\*: Sources of those sequences are rice tungro bacilliform virus promoter<sup>24</sup>.

\*\* : Location of underlined base of consensus sequence from transcription start site.

NI: not identified.

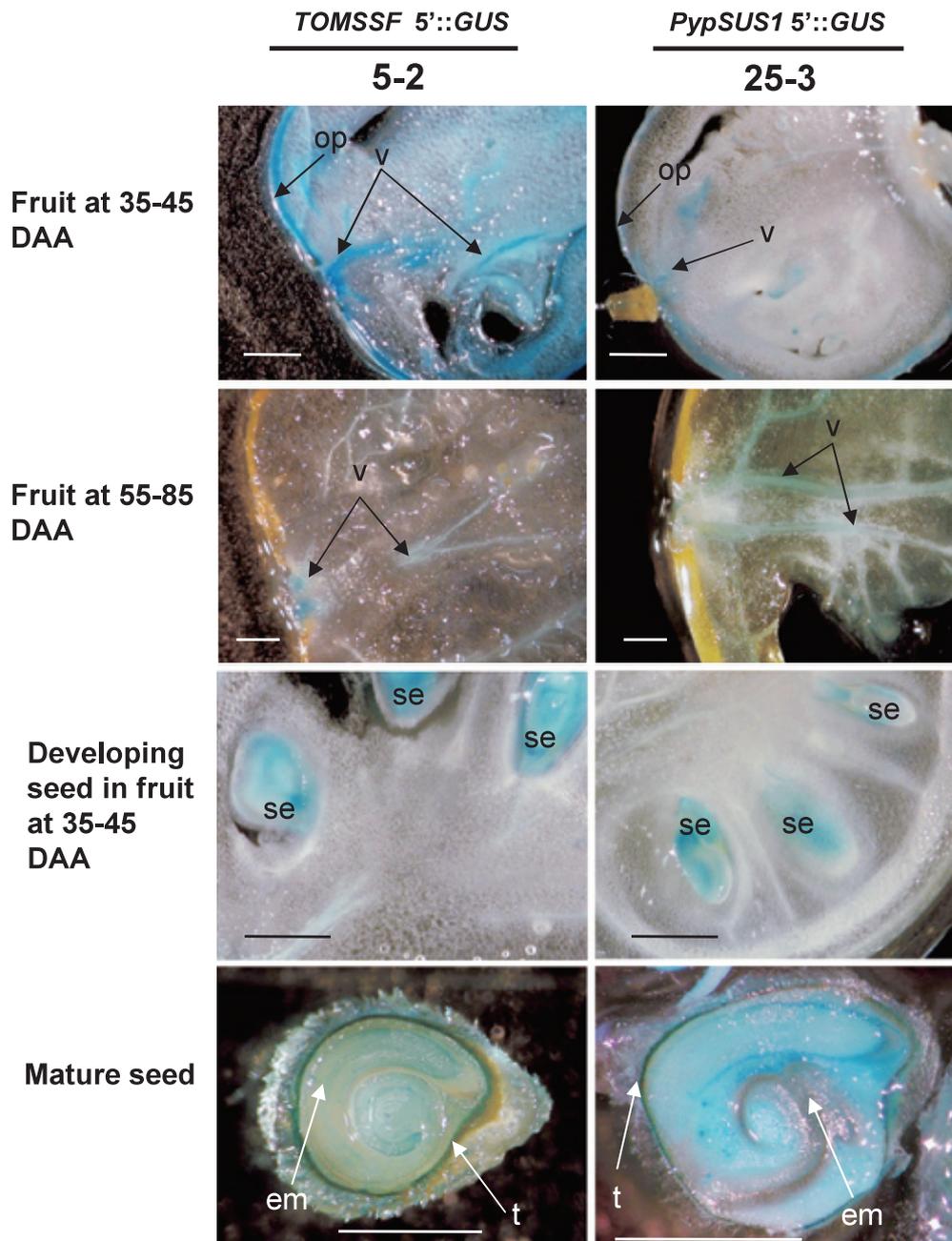
also observed in the outer pericarp of young (20-45 DAA) fruit of tomato plants transformed with pTSSG-7 but not with pPSSG-8 (Fig. 3).

The highest level of GUS activity was found in fruit at the early and middle stages of development in all lines transformed with pTSSG-7 or pPSSG-8 (Fig. 4). The fruit of plants transformed with pTSSG-7 had a higher level of GUS activity compared with fruit of plants transformed with pPSSG-8 (Fig. 4). Northern blot analysis of the level of GUS mRNA in transformed fruits showed a similar trend to that of GUS activity in transformed fruits (Fig. 5). However, these trends differed slightly for GUS activity and the GUS mRNA level in fruit of the 25-3 line transformed with pPSSG-8 (Figs. 4 & 5). This difference may be due to differences in the maturity of the fruits used for Northern and enzymatic analyses.

## Discussion

We isolated the 5' upstream promoter region of the

fruit-type SS gene of tomato and Japanese pear by IPCR (Figs. 1 & 2). Expression characteristics of these promoters were analyzed in tomato carrying the promoter-*GUS* fusion gene (pTSSG-7 or pPSSG-8, Fig. 1). Histochemical analysis showed high GUS activity in the fruit of all transformants generated with pTSSG-7 and pPSSG-8 with activity specific to vascular tissues and testae (Table 2, Fig. 3). The presence of known regulatory motifs responsible for the phloem-specific expression in the 5' upstream regions supports our results of vascular expression of the SS promoters (Table 1). Furthermore, the results of vascular expression of the *TOMSSF* 5' (pTSSG-7 construct) are consistent with the results of *in situ* mRNA analysis<sup>21</sup>. These data indicate that both 5' regions have vascular type promoter activity in tomato. On the other hand, testae and vascular (phloem) tissues correspond to the transport pathways of assimilates. Although the exact localizations of TOMSSF and PypSUS1 enzyme in the vascular tissues are necessary to be clarified, it implies that TOMSSF and PypSUS1 enzymes localized in testae and vascular tissues (Table 2, Fig. 3) participate in the



**Fig. 3. Histochemical analysis of GUS activity in tomato plants (cv. Micro-Tom) carrying the *TOMSSF 5'::GUS* (line 5-2 transformed with pTSSG-7) or the *PypSUS1 5'::GUS* (line 25-3 transformed with pPSSG-8)**

Transgenic tomatoes were grown in a greenhouse and then fruit at the mature green stage (35-45 DAA) or red stage (55-85 DAA) were sampled for GUS staining (bar = 2 mm). Em, embryo; op, outer pericarp; se, seeds; t, testa; v, vascular tissue.

sucrose unloading for development of fruit and seeds. The report of D'Aoust et al.<sup>2</sup> that antisense inhibition of *TOMSSF* led to decrease in sucrose import capacity in the very young fruit may support this hypothesis.

Overall expression patterns of *TOMSSF 5'* were similar to those of *PypSUS1 5'* (Table 2, Fig. 3). However, a difference in the GUS expression between *TOMSSF 5'*

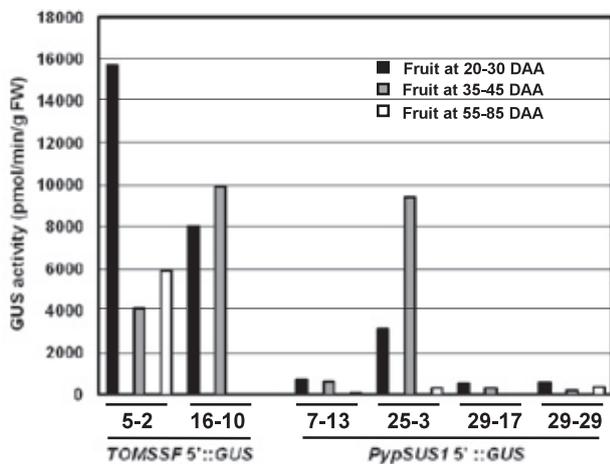
and *PypSUS1 5'* was observed in the outer pericarp (Fig. 3). It implies that the expression of *TOMSSF* leads to faster sucrose utilization in the growing pericarps of young tomato fruit as suggested by Obiadalla-Ali et al.<sup>9</sup>

The level of GUS activity in young (20–45 DAA) fruits transformed with pTSSG-7 was higher than that in fruits transformed with pPSSG-8 (Fig. 4). The relatively

**Table 2. Relative strength of GUS histochemical staining**

Tissue	<i>TOMSSF 5'::GUS</i>		<i>PypSUS1 5'::GUS</i>	
	Line 5-2	Line 16-10	Line 25-3	Line 29-29
Leaf	-	-	-	-
Stem	+	±	±	NE
Ovary	+	++	+	-
Style	-	NE	+	-
Stigma	-	NE	+	-
Anther	+	NE	+	+
Petal	-	NE	-	-
Sepal	-	NE	+	-
Fruit (20-30DAA)	++	-	±	-
Fruit (35-45DAA)	+++	++	+	++
Fruit (55-85DAA)	+	++	++	++
Mature seed (testa)	+++	+++	+++	+++
Root	-	-	-	-

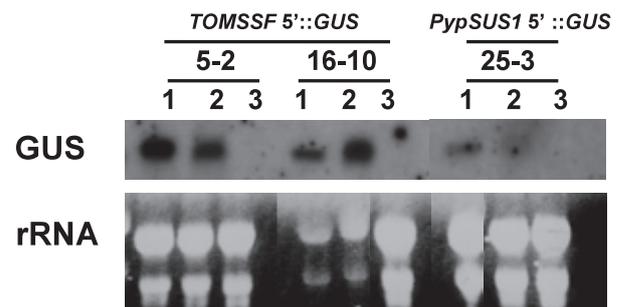
NE: not examined; +++: strong activity detected; -: no activity detected; DAA: days after anthesis.



**Fig. 4. GUS activity in fruit of tomato lines transformed with pTSSG-7 (*TOMSSF 5'::GUS*) or pPSSG-8 (*PypSUS1 5'::GUS*)**

Fruit at various developmental stages (20–30 DAA, 35–45 DAA, and 55–85 DAA) were sampled and then the GUS activity in each fruit was measured.

high level expression of GUS in pTSSG-7 (*TOMSSF 5'::GUS*) fruits may be due to the presence of the intron<sup>10</sup> in the 5'-UTR of *TOMSSF* (Fig. 1). The highest level of GUS activity and GUS mRNA was found in fruit at the early and middle stages of development in all transformed lines (Figs. 4 & 5). A few reports describe promoters that can drive the expression of foreign genes in young fruit of tomato. The promoters isolated in the present study can be considered as candidate tools for the genetic engineering of tomato plants



**Fig. 5. Northern blot analysis of tomato fruits transformed with pTSSG-7 (*TOMSSF 5'::GUS*) or pPSSG-8 (*PypSUS1 5'::GUS*)**

Total RNAs were extracted from fruit at various developmental stages (1: 20–30 DAA, 2: 35–45 DAA, 3: 55–85 DAA), electrophoresed under denaturing conditions, blotted onto a membrane, and then the GUS mRNA was detected as described in Materials and methods. The rRNA bands on the same gel were visualized by ethidium bromide staining as a loading control.

to achieve vascular expression of foreign genes at earlier stages of fruit development.

The level of GUS expression driven by the *TOMSSF 5'* was high at the young to middle stages of fruit development (Figs. 4 & 5). Our results for the expression patterns of GUS are consistent with the endogenous expression of *TOMSSF* mRNA in tomato reported by Wang et al.<sup>21</sup>. The level of *PypSUS1* protein in Japanese pear fruit is reported to be high at the young stage of development then to decline

with fruit enlargement and increases again with maturation of the fruit<sup>19</sup>. Although both *TOMSSF 5'* and *PypSUS1 5'* conferred dominant promoter activities on tomato, we did not observe a rise in the level of GUS activity or GUS mRNA in the ripening tomato fruit of plants carrying *PypSUS1 5'::GUS* (Figs. 4 & 5). The production of PypSUS1 in ripening Japanese pear fruit is thought to be associated with sucrose synthesis<sup>19</sup>. By comparison, a rise in SS activity and associated sucrose synthesis are not observed in ripe tomato fruit<sup>13,16,23</sup>. Therefore, it is possible that the absence of a rise in GUS expression in ripening tomato fruit transformed with pPSSG-8 (*PypSUS1 5'::GUS*) is due to differences in genetic background between Japanese pear and tomato that relate to the mechanisms contributing to sucrose synthesis in ripening fruit.

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