Plant Biotechnology 28, 497–501 (2011)
DOI: 10.5511/plantbiotechnology.11.0823a

Note

The promoter from tomato sucrose synthase gene *TOMSSF* drives stamen-specific gene expression in *Chrysanthemum*

Koji Tanase[1,a,*], Ryutaro Aida[1], Akio Ohyama[2], Takashi Onozaki[1]

[1] National Institute of Floricultural Science, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-8519, Japan; [2] National Institute of Vegetable and Tea Science, National Agriculture and Food Research Organization, Tsu, Mie 514-2392, Japan
* E-mail: tanase@affrc.go.jp Tel: +81-29-838-6801 Fax: +81-29-838-6841

Received June 28, 2011; accepted August 23, 2011 (Edited by H. Shimada)

Abstract Anther-specific promoters have been extensively studied in terms of the genetic engineering of male-sterile plants. Here, we fused the 5′ upstream promoter regions of a sucrose synthase gene from tomato (*TOMSSF*) to the β-glucuronidase (*GUS*) gene and used the construct to transform *Chrysanthemum* plants. Histochemical analysis of two transformants showed high GUS activity in ray florets and tubular florets. Analysis of the staining pattern in these florets reveled that staining was stamen-specific. *GUS* gene expression was highest in the stamen and remained at a steady-state level throughout the experiment. These results suggest that the *TOMSSF* promoter has high activity in the stamen of tubular florets and would be useful as a high stamen-specific promoter in *Chrysanthemum*.

Key words: *Chrysanthemum*, promoter analysis, sucrose synthase.

Transgenic research of ornamental flowers seeks to produce transgenic plants with disease tolerance, flower longevity, and modified color, morphology, or fragrance, all of which are of value to the flowers. These characteristics can be attained by introducing certain genes into target plants by use of recombinant DNA techniques. However, problems arise if the introduced gene derived from the genetically modified plants spreads horizontally in the environment through pollen. Male sterility is a useful trait to prevent this problem. Male sterility has been studied for the production of hybrid (F_1) varieties (Williams 1995), and the recent development of recombinant DNA and transformation techniques has created the potential to engineer male sterility. Antisense expression of anther-specific genes or the use of anther-specific or preferential promoters fused to cytotoxic genes are useful methods for producing male sterility (Koltunow et al. 1990; Mariani et al. 1990; Muschietti et al. 1994; Xu et al. 1995). In some plant species, anther-specific gene expression has been detected, and other studies suggest that the temporal and spatial regulation of anther-specific gene expression is controlled primarily at the transcriptional level (Goldberg et al. 1993). For some anther-specific genes, promoter activity has been confirmed by using transgenic approaches, such as chimeric gene fusion between the promoter of the anther-specific gene and a reporter gene.

Chrysanthemum morifolium Ramat. is a popular cut, pot, and garden flower worldwide. Its genetic transformation would be a useful tool for breeding of future cultivars. There have been many reports of *Chrysanthemum* transformation (Deroles et al. 2002). Efforts have focused on finding efficient promoters for transgenic expression in *Chrysanthemum* by using the *GUS* gene as a reporter, because the *35S* promoter functions poorly in transgenic *Chrysanthemum* (Wordragen et al. 1992). Recently, the tobacco *EF1α* promoter was shown to be effective for transgene expression in *Chrysanthemum* plants (Aida et al. 2005). Moreover, some studies have reported the production of transgenic *Chrysanthemum* plants containing useful transgenes. Narumi et al. (2005), for example, reported that overexpression of mutated ethylene receptor genes in transgenic plants reduced sensitivity to ethylene and delayed leaf yellowing caused by exogenous ethylene. Similarly, the carotenoid cleavage dioxygenase gene, *CmCCD4a*, was cloned from white petals of *Chrysanthemum* (Ohmiya et al. 2006), and the introduction of an RNAi construct of *CmCCD4a* turned the white petals yellow in transgenic plants. Shinoyama

Abbreviations: EF1α, elongation factor 1α; GUS, β-glucuronidase; MS, Murashige and Skoog; 4-MU, 4-methylumbelliferone; RT, reverse transcription; SE, standard error; SuSy, sucrose synthase; TOMSSF, tomato fruit–type SuSy.

[a] Present address: National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-8517, Japan

This article can be found at http://www.jspcmb.jp/
Published online October 20, 2011

et al. (2003, 2008) produced insect-resistant transgenic lines that contain a modified *cry1Ab* gene from *Bacillus thuringiensis*. Before these transgenic plants can be introduced into cultivation fields, however, a method for induction of male sterility and prevention of transgene flow via pollen is needed.

Sucrose Synthase (SuSy; EC 2.4.1.13) is a key enzyme of sucrose metabolism in plants, providing carbohydrates for respiration as well as for cell wall and starch synthesis (Tanase and Yamaki 2000). The 5′ upstream promoter regions of the tomato SuSy gene *TOMSSF* have been isolated and characterized (Ohyama et al. 2010). The *TOMSSF 5′* upstream promoter regions, which include an intron of approximately 1.6 kbp in the 5′ untranslated region, were fused to the β-glucuronidase (*GUS*) gene and then used to transform tomato. Histochemical analysis of transgenic plants showed GUS activity in the anther, stem, ovary, fruit, and seed. In potato and rice, the 5′ upstream fragments of the *SuSy* gene had promoter activity in the anther and in pollen (Fu et al. 1995a; Fu et al. 1995b; Fu and Park, 1995). Therefore, the possibility exists for the promoter of a *SuSy* gene to function in the anther of the *Chrysanthemum* plant. Here, we report on the activity of the tomato *SuSy* promoter, *TOMSSF 5′*, for transgene expression in *Chrysanthemum*.

Chrysanthemum 'Sei-Marine' was used for transformation in this study. The plants were grown *in vitro* in Murashige-Skoog medium with half-strength minerals (1/2 MS) (Murashige and Skoog 1962), solidified with 0.2% (w/v) gellan gum, at 25°C under a 16-h light/8-h dark photoperiod with fluorescent light (70 μmol s^{-1} m^{-2}). The promoter region (approximately 3.4 kbp in length) of the *SuSy* gene isolated from tomato (*TOMSSF*: DDBJ/GenBank/EMBL accession number AJ011535 and L19762) was used for vector construction (Ohyama et al. 2010). The vector plasmid, pTSSGHm (Figure 1), was derived from pIG121Hm (Hiei et al. 1994) by replacing the CaMV 35S promoter with the tomato *SuSy* promoter. The 5′ upstream regions of *TOMSSF* (*TOMSSF 5′*) were inserted upstream of the *GUS* gene of pIG121Hm by using standard molecular techniques. The resultant plasmid was designated as pTSSGHm (*TOMSSF 5′*:: *GUS*). *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens*) strain EHA105 (Hood et al. 1993) was also used for this study. Transformation was performed as previously described (Aida et al. 2004). Transgenic *Chrysanthemum* plants were grown in a contained greenhouse and eleven transgenic plants with pTSSGHm were tested for GUS staining intensity. We selected two lines (978-15 and 978-17) as having high-GUS expression plants based on histochemical staining (described below). We obtained a transformation efficiency of 5.5% (11 paromomycin-resistant plants per 200 explants), which was the same as that in our previous study (Aida et al. 2004). However, the percentage of GUS-positive plants among the paromomycin-resistant plants (18.2%) was slightly lower than that in the previous study.

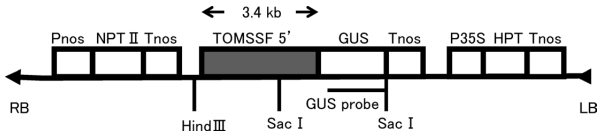

Figure 1. Structure of the T-DNA region of the binary vector, pTSSGHm. The chimeric genes were inserted between the right and left border sequences of T-DNA. The plasmid was designated as pTSSGHm (*TOMSSF 5′:: GUS*). RB and LB, right and left border sequences of T-DNA; Pnos and Tnos, promoter and terminator of the nopaline synthase gene; TOMSSF 5′, 5′ upstream region of *TOMSSF*; NPT II, coding region of the neomycin phosphotransferase II gene; GUS, coding region of the β-glucuronidase gene; HTP, hygromycin phosphotransferase. Restriction sites for *Hin*dIII and *Sac*I are shown.

Southern blotting was performed by using a DIG-High Prime and DIG Luminescent Detection Kit for nucleic acids (Roche Diagnostics, Mannheim, Germany). Total DNA was extracted from the leaf tissues of 978-15 and 978-17; 10 μg of DNA digested with *Hin*dIII was electrophoresed in a 0.6% agarose gel and transferred to a positively charged nylon membrane. The *GUS* gene (Figure 1), which was amplified by using PCR, served as a probe.

A histochemical GUS assay was performed according to the procedure reported by Aida et al. (2004) with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid as a substrate. Quantitative GUS activity in the flowers and leaves was also examined according to the methods reported by Aida et al. (2004). The GUS assay buffer contained 20% methyl alcohol to eliminate endogenous GUS activity. GUS activity is expressed as picomoles of 4-methylumbelliferone (4-MU) produced at 37°C per milligram of protein per minute (pmol 4-MU mg^{-1} protein min^{-1}).

We determined the levels of *GUS* mRNA in flower organs (stamen, pistil, petals of tubular florets, and petals of ray florets) by using quantitative RT-PCR analysis. Tubular florets were harvested at three different stages (<stage G1, stage G2 and G3, and stage G4). These stages were defined by Kuklina (2003) as follows: G1, growth of anther stalks and maturation of pollen grains in anthers; G2, dehiscence of anthers inside closed florets, corolla with separate petals, and stigma located below the split anther; G3, growing pistil pushes mature pollen out of the whorl of five glued anthers; G4, open mature stigma, and anthers located below the mature stigma. The "<stage G1" stage represents the G1 stage together with the events prior to G1. Total RNA was extracted, and 1-μg aliquots were used for the synthesis of the first-strand cDNA. To confirm the amount of RNA template, a fragment of the *Chrysanthemum* elongation factor-1 alpha gene (*Cm-EF1*α) (Yang et al. 2009)

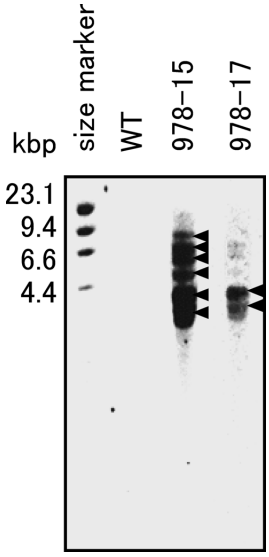

Figure 2. Southern blot analysis of two GUS-positive plants transformed with *TOMSSF 5′:: GUS*. The coding region of the *GUS* gene served as a probe (Aida et al. 2005). Each band is marked with a triangle.

served as an internal control.

As stated above, the 5′ upstream region of the sucrose synthase gene from tomato (*TOMSSF 5′*) was fused to the *GUS* gene and the chimeric gene was used to transform *Chrysanthemum* plants. Two lines (978-15 and 978-17) were selected as having high-GUS expression plants and the presence of the transgenes in them was confirmed by Southern blot analysis (Figure 2). The number of bands detected by *Hin*dIII digestion and the *GUS* probe reflected the number of copies of the *GUS* gene in the plants, since the T-DNA of pTSSGHm has a single *Hin*dIII site (Figure 1). Southern blot analysis of the *GUS* gene revealed six bands of different sizes in the 978-15 line and two bands of different sizes in the 978-17 line. This result suggests that multiple-copy integration of the *GUS* gene occurred in the genome of these transgenic plants.

We then examined the pTSSGHm-transformed *Chrysanthemum* plants for quantitative GUS activity in flowers (ray florets and tubular florets), leaves, and stems. Flowers were harvested when stigmas were observed in the tubular florets. A high level of GUS activity was found in the ray florets and tubular florets of 978-15 and 978-17, but GUS activity was low in the leaves and stems (data not shown). Wild-type (WT) plants showed low GUS activity in all of the organs examined.

The GUS staining pattern in the flowers, leaves, and stems of transgenic plants is shown in Figure 3. GUS-stained regions were observed in the tubular florets of 978-15 and 978-17 plants whose petals were open (Figure 3c–f). Strongly stained regions included the stamens in the tubular florets of both transformants

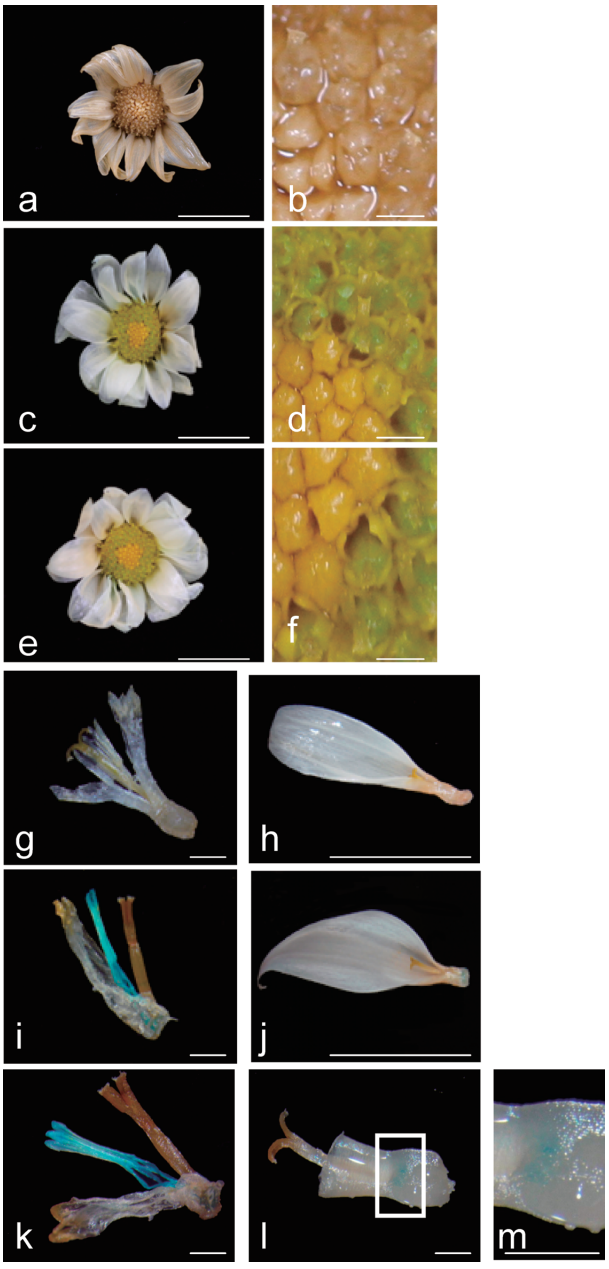

Figure 3. Histochemical analysis of GUS activity in the flowers of *Chrysanthemum* plants containing pTSSGHm (lines 978-15 and 978-17). Transformants were grown in a containment greenhouse. Flowers were harvested at full open stage and were sampled for GUS staining. Destaining with ethanol was performed until the green background of the sepals disappeared. The different background colors for the flowers of the WT and transgenic plants are because the observations were made at different times. However, the flowers of these transgenic plants did not show any obvious phenotypic changes. White bars represent 10 mm in a, c, e, h, and j. White bars represent 1 mm in b, d, f, g, i, k, l, and m. Wild-type flower: a, b, g, h; 987-15: c, d, i, j; 987-17: e, f, k, l, m. Whole flower: a, b, c, d, e, f; tubular floret: g, i, k; ray floret: h, j, l, m. "m" is a magnified view of the area surrounded by the white line in "l".

(Figure 3i–l), whereas no GUS staining was observed in the pistil and petals of the WT plants (Figure 3g, h). The lower part of the ray floret of 978-17, with the upper part

of the petal removed, is shown in Figure 3l. GUS staining was detected in the basal parts of the ray florets (Figure 3m), but not in any other part (Figure 3l). No GUS staining was observed in any of the florets of the WT plants. There were no GUS-stained regions in any of the leaves or stems of either transgenic or WT plants (data not shown). These results suggest that the *TOMSSF* promoter activity is highly specific to the stamen of *Chrysanthemum* plants and may be useful for expressing foreign genes in the stamen.

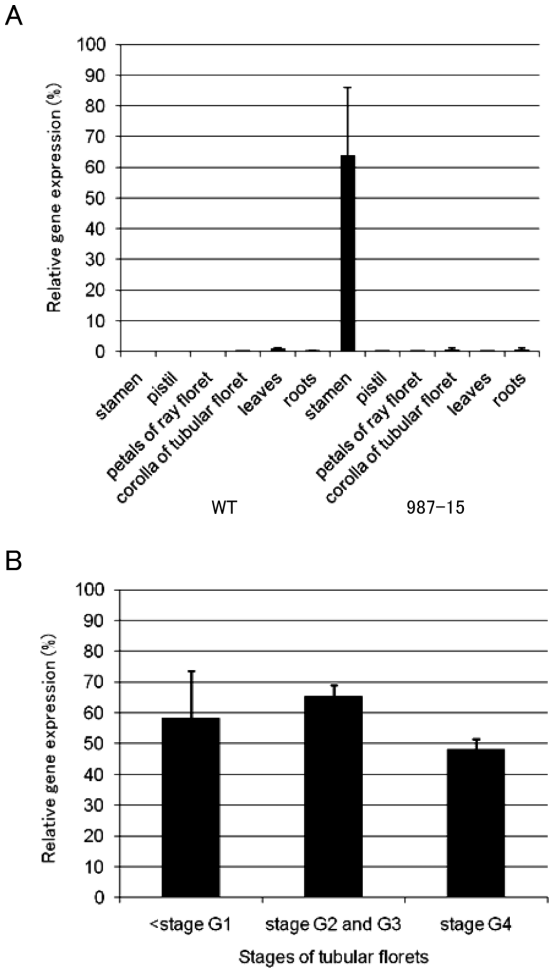

Figure 4. Quantitative RT-PCR analysis of *GUS* gene expression in transgenic *Chrysanthemum* plants (line 978-15). (A) Relative gene expression in the organs of transgenic plants (978-15). (B) Relative gene expression in <stage G1 tubular florets, stage G2 and G3 tubular florets, and stage G4 tubular florets of transgenic plants (987-15). Real-time RT-PCR was performed by using a LightCycler System (Roche Diagnostics, Mannheim, Germany). The following primers were used: *GUS* gene forward (5′-CCCTTACGCTGAAGAGATGC-3′) and *GUS* gene reverse (5′-TTTTTGTCACGCGCTATCAG-3′), *Cm-EF1α* forward (5′-AGACCACCAAGTACTACTGCAC-3′) and *Cm-EF1α* reverse (5′-CCACCAATCTTGTACACATCC-3′). PCR was performed by using the following program: 30 cycles at 94ºC, 10 s; 60ºC, 15 s; 72ºC, 20 s for the *GUS* gene; 30 cycles at 94ºC, 10 s; 60ºC, 10 s; 72ºC, 15 s for *Cm-EF1α*. Amplification of PCR products was monitored via intercalation of SYBR-Green. Relative amounts were calculated and normalized to the *Cm-EF1α* mRNA (=100%). Data represent the results of three independent experiments (±SE).

We also examined *GUS* gene expression in the flower organs (stamen, pistil, petals of tubular florets, and petals of ray florets), leaves, and roots of 978-15 plants by using quantitative RT-PCR. Generally, chrysanthemum plants are maintained by use of vegetative propagation, such as rooting. Unfortunately, the 978-17 line wilted and died during vegetative propagation. As a result, it was impossible to perform quantitative RT-PCR analysis with this line due to the lack of flowers. For 978-15 plants, levels of *GUS* gene expression were high in the stamen, but very low in the pistil of tubular florets, petals of tubular florets and ray florets, leaves, and roots (Figure 4A). In WT plants, the levels of *GUS* gene expression were very low in all flower organs, leaves, and roots. To clarify the changes in promoter activity of the *TOMSSF* during flower development, three different stages of tubular florets (<stage G1, stage G2 and G3, and stage G4) were assessed by use of quantitative RT-PCR analysis (Figure 4B). The levels of GUS mRNA at the early (<G1) stage in the florets were similar to those at the later stages. There have been several morphological studies of reproductive organs during floral initiation and development in *Chrysanthemum* (Fukai et al. 1997; Kuklina 2003). The basal parts of ray florets are stamens that stop developing. In ray florets, five stamen primordia appear inside the petal primordia and their development stops soon thereafter (Fukai et al. 1997). The pistil primordium appears inside the five stamen primordia and develops quickly. Finally, the inside of the ray florets contains the pistil and small traces of the stamen. Thus, the 5′ region of *TOMSSF* appears to show preferential promoter activity in the underdeveloped stamen of ray florets (Figures 3, 4) as well as in the stamen of tubular florets. These results suggest that the promoter activity of *TOMSSF 5′* remains high in the stamen of tubular florets during maturation of pollen grains.

Since the promoter of *TOMSSF* had high activity in the stamen of tubular florets, it may be useful for producing male sterility in *Chrysanthemum*. Male sterility has been produced by use of anther-specific promoters fused to cytotoxic genes, such as diphtheria toxin genes and RNase genes (Koltunow et al. 1990; Mariani et al. 1990; Muschietti et al. 1994; Xu et al. 1995). Moreover, transgenic tobacco plants expressing a mutant melon ethylene receptor (*Cm-ETR1/H69A*) that conferred ethylene insensitivity showed male sterility (Takada et al. 2005), and transgenic *Chrysanthemum* plants carrying the *Cm-ETR1/H69A* gene produced fewer pollen grains than untransformed control plants (Shinoyama et al. 2011). Therefore, expression of cytotoxic genes or the mutant ethylene receptor gene under the control of *TOMSSF 5′* could be a useful approach for producing male sterility in *Chrysanthemum*.

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