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Enzymatic and Expression Analysis of Timothy *PpFT1* Encoding a Fructosyltransferase for Synthesis of Highly Polymerized Levans

Ken-ichi Tamura[1*] • Akira Kawakami[2] • Yasuharu Sanada[1] • Kazuhiro Tase[1] • Toshinori Komatsu[1] • Midori Yoshida[1]

[1] National Agricultural Research Center for Hokkaido Region, Hitsujigaoka 1, Toyohira, Sapporo 062-8555, Japan
[2] National Agricultural Research Center, Kannondai 3-1-1, Tsukuba, Ibaraki 305-8666, Japan

Corresponding author: * tamuken@affrc.go.jp

ABSTRACT

Although fructosyltransferase (FT) genes have been isolated in a range of plant species, sucrose:fructan 6-fructosyltransferase (6-SFT) cDNAs have been functionally characterized in only few species, such as barley and wheat. In this study we characterized the cDNA of an FT homolog, *PpFT1*, from timothy (*Phleum pratense* L.) which accumulates high-DP fructans. A recombinant PpFT1 protein expressed in *Pichia pastoris* showed 6-SFT and sucrose:sucrose 1-fructosyltransferase (1-SST) activities with optimal pH of 5.2. The recombinant enzyme produced linear β(2,6)-linked levans from sucrose with higher DPs (>50) than those of fructans produced by wheat recombinant 6-SFT at low temperature (7°C). We also confirmed that excised timothy leaves had elevated levels of *PpFT1* transcripts during the accumulation of fructans under an illuminated condition. Our results suggest that timothy *PpFT1* is involved in the synthesis of highly polymerized levans with unique enzymatic properties different from those of previously cloned plant 6-SFTs.

Keywords: degree of polymerization (DP), fructan, sucrose:fructan 6-fructosyltransferase (6-SFT)
Abbreviations: DP, degree of polymerization; **FT**, fructosyltransferase; **HPAEC-PAD**, high-performance anion exchange chromatography and pulsed amperometric detection; **6-SFT**, sucrose:fructan 6-fructosyltransferase; **1-SST**, sucrose:sucrose 1-fructosyltransferase; **6-SST**, sucrose:sucrose 6-fructosyltransferase

INTRODUCTION

Timothy (*Phleum pratense* L.) (**Fig. 1**) is used as a forage plant in temperate grasslands with harsh winters because it has better winter hardiness than alternative crops such as perennial ryegrass. It predominantly accumulates simple β(2,6)-linked fructans called levans with a higher degree of polymerization (DP) than those of wheat, barley, oat, *Lolium* spp. and *Festuca* spp. (Spollen and Nelson 1988; Suzuki 1989; Cairns and Ashton 1993). Timothy has been reported to have a DP of up to 90 in leaf tissue (Cairns *et al.* 1999). An enzyme with fructan polymerization activity was partially purified and characterized from excised and illuminated leaves of timothy (Cairns *et al*. 1999). This enzyme preparation showed sucrose:fructan 6-fructosyltransferase (6-SFT) activity which transfers a fructose unit from sucrose to a fructan by a β(2,6) linkage, and produces linear β(2,6)-linked fructans from sucrose with a DP of up to 50. However, to date, isolation of 6-SFT cDNA has not been reported for timothy. Moreover, the genes for fructosyltransferase (FTs) involved in the synthesis of levans with a high DP have not been cloned from any plant specie.

Fig. 1 Timothy plant grown in the field.

Functional characterizations were reported on 6-SFT cDNAs isolated from barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) which accumulate mixed levans, called graminans, composed of branched-type fructans containing β(2,6)- and β(2,1)-linked fructose residues (Carpita *et al*. 1989; Bonnett *et al*. 1997). The Triticeae 6-SFT enzymes preferentially transfer a fructose unit to 1-kestotriose, rather than to 6-kestotriose, to produce a branched fructan, bifurcose (Sprenger *et al*. 1995; Kawakami and Yoshida 2002). The 6-SFT homologs have also been identified in *Poa secunda* (Wei *et al*. 2002), *Lolium perenne* (Hisano *et al*. 2008) and *Lolium temulentum* (Gallagher *et al*. 2004) although the enzymatic activities of the encoded enzymes have yet to be reported.

The DP of fructans differs among plant species (Vijn and Smeeken 1999). Asteraceae species accumulate inulins [β(2,1)-linked fructans] with variable DPs. Differences in the DP of these inulin-type fructans have been attributed to

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differences in substrate specificity of their fructan:fructan 1-fructosyltransferases (1-FFTs) (Vergauwen *et al*. 2003). To date, however, there is relatively little information on the relationship between the DP of fructans and the enzymatic properties of 6-SFT in the synthesis of β(2,6)-linked fructans.

In this study, we characterized the enzymatic properties of the recombinant protein derived from *PpFT1*, a timothy FT cDNA (Tamura *et al*. 2009). We also confirmed the relationship between the expression level of the timothy FT gene, FT activity, and fructan content in the excised leaves.

MATERIALS AND METHODS

Assay for FT activity of recombinant proteins

Preparation of the recombinant protein of PpFT1 and wheat 6-SFT (Wft1) expressed in methylotrophic yeast *Pichia pastoris* has been described by Tamura *et al.* (2009) and Kawakami and Yoshida (2002). To analyze the reaction product with sucrose as substrate, 20 μl of concentrated medium (BMMY) containing recombinant enzyme was incubated with 20 μl of 2 M sucrose for 48 h at pH 5.2 (20 mM citrate-phosphate buffer) and 7°C. Half of the PpFT1 reaction mixture was further incubated for 48 h following the addition of 10 μl of fresh recombinant enzyme solution and 10 μl of 2 M sucrose solution. The sugar products were analyzed by high-performance anion exchange chromatography and pulsed amperometric detection (HPAEC-PAD) (DX-500, Dionex) as described by Tamura *et al.* (2009). Levan series fructans (linear β(2,6)-linked fructans) were putatively identified by comparison of the HPAEC retention times with fructan oligomers extracted from crown tissues of cold-acclimated timothy in the field in December 2007. For the determination of the effects of pH, 4 μl of enzyme solution was incubated with 1 M sucrose at 25°C and pH 3.1, 4.1, 4.7, 5.2, 5.9, 6.3 and 7.2, respectively. The FT activities were extrapolated by subtracting the amount of fructose released from that of glucose produced after 2 hours incubation. Measurements of fructose and glucose were performed using HPLC as described by Yoshida *et al*. (1998).

Analysis of gene expression, enzyme activity and carbohydrates content in excised leaves

Seedlings of the timothy cv. 'Hokushu' were grown in a controlled climate chamber (16 h light, 150 μmol $m^{-2} s^{-1}$ PFD, 22°C) for 3 weeks after germination. The leaves of the timothy seedlings were excised, placed with the cut ends in water, and kept in continuous light (150 μmol $m^{-2} s^{-1}$ PFD, 22°C). *PpFT1* expression analysis using quantitative RT-PCR, assay of sucrose:sucrose 6-fructosyltransferase (6-SST) activity and measurement of carbohydrates were performed as described by Tamura *et al.* (2009).

RESULTS AND DISCUSSION

PpFT1, a 6-SFT homolog isolated from timothy which accumulates high-DP fructans

We isolated a cDNA clone *PpFT1* (AB436697), similar with plant FTs, from the cold acclimated crown tissues of timothy (Tamura *et al.* 2009). This clone was deduced to encode 623 amino acids containing conserved amino acid motifs suggested to be essential for β-fructosidase activity (Verhaest *et al*. 2005). A phylogenetic comparison of the amino acid sequences of PpFT1 and of FTs and vacuolar invertases from a range of species indicated that *PpFT1* was a member of a group of genes coding (putative) 6-SFTs (**Fig.**

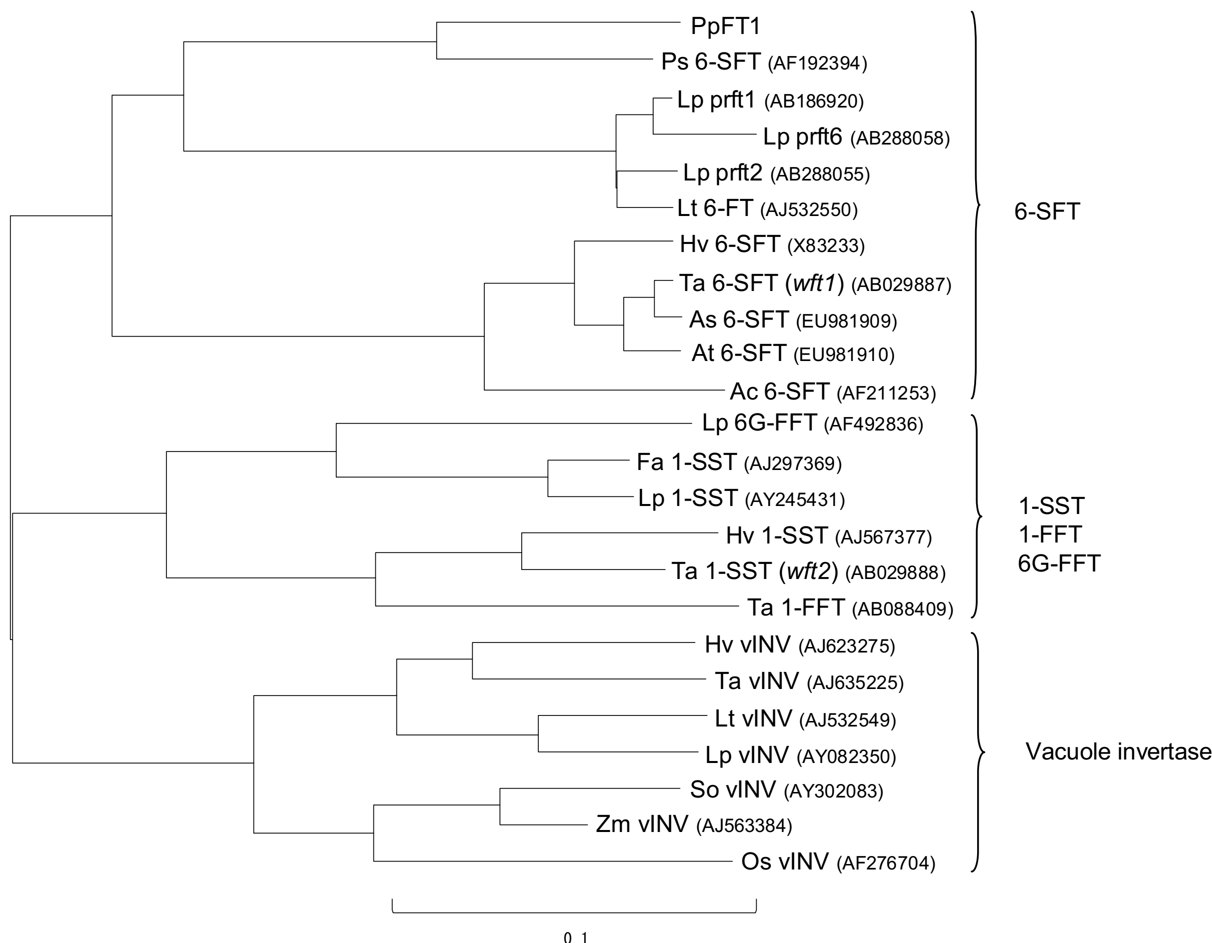

Fig. 2 Phylogenetic tree of FTs and vacuolar invertases (vINV) of Poaceae species based on predicted amino acid sequences. Phylogenetic tree was constructed by the neighbor joining method, using the CLUSTALW program. Scale bar indicates branch length. Abbreviations for species names indicate: *Ac*, *Agropyron cristatum*; *As*, *Aegilops searsii*; *At*, *Aegilops tauschii*; *Fa*, *Festuca arundinacea*; *Hv*, *Hordeum vulgare*; *Lp*, *Lolium perenne*; *Lt*, *Lolium temulentum*; *Os*, *Oryza sativa*; *Ps*, *Poa secunda*; *So*, *Saccharum officinarum*; *Ta*, *Triticum aestivum*; *Zm*, *Zea mays*.

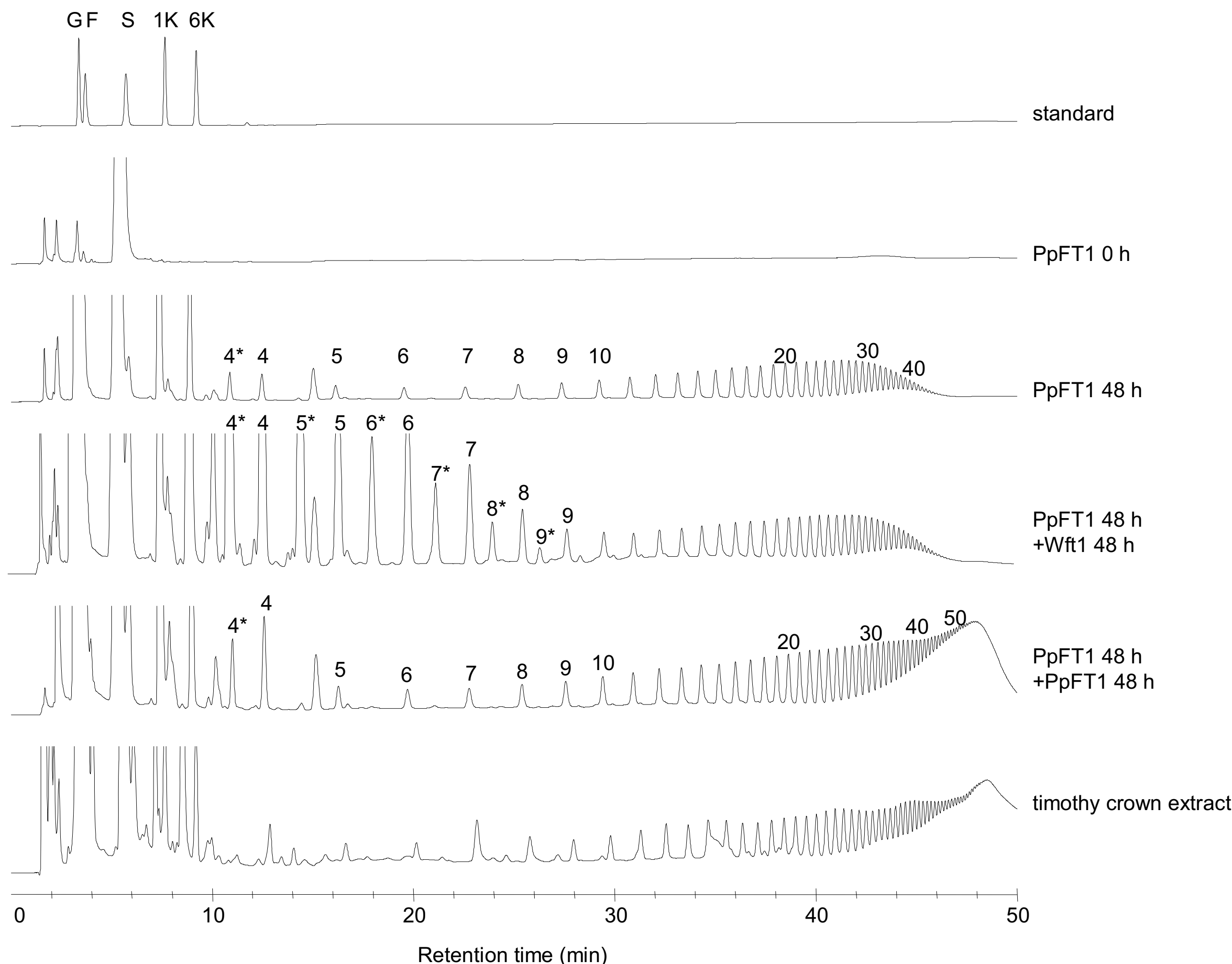

Fig. 3 Anion exchange HPLC analysis of fructans generated by the recombinant enzyme in the presence of sucrose. The enzymatic reaction with PpFT1 was performed with 1 M sucrose at 7°C for 48 h, and fresh enzyme of PpFT1 or Wft1 and 0.5 M sucrose were provided to the reaction solution at 48 h for the further 48 h incubation. Compounds were identified using pulsed amperometric detection and external standards. Soluble carbohydrates extracted from timothy crown tissues in December 2007 were used as standards for the oligo-fructans. Abbreviations for each sugar peak are: G, glucose; F, fructose; S, sucrose; 1K, 1-kestotriose; 6K, 6-kestotriose. The numbers indicate the putative DPs of β(2,6)-linked linear fructan oligomers. The numbers with asterisks indicate the putative DPs of β(2,6)-linked branched fructan oligomers produced by polymerization of fructose units linked to 1-kestotriose, e.g. '4*' indicates bifurcose (1&6-kestotetraose).

2). The predicted amino acid sequence of PpFT1 showed highest identity (86%) to a putative 6-SFT from *Poa secunda* (Wei *et al.* 2002). This phylogenetic tree indicates that Poaceae 6-SFTs fell into three groups: the Triticeae group, the *Lolium* spp. group, and the timothy and *Poa secunda* group (**Fig. 2**). These three plant groups accumulate fructans with different structural characteristics: the Triticeae group produces a branched type of fructan called graminan (Carpita *et al.* 1989; Bonnett *et al.* 1997), the *Lolium* spp. group mainly produces a levan neo-series (Chalmers *et al.* 2005), and the timothy and *Poa secunda* group produces linear β(2,6)-linked fructans with a high DP (Chatterton and Harrison 1997; Cairns *et al.* 1999). In fact, wheat and barley 6-SFTs preferentially transfer fructosyl units to 1-kestotriose, produced by 1-SST activity, to synthesize bifurcose, the starting tetrasaccharide required for the formation of graminans (Sprenger *et al.* 1995; Kawakami and Yoshida 2002). On the other hand, as described below, PpFT1 produces β(2,6)-linked linear fructans with high DPs. Further studies are needed to clarify the relationship between 6-SFT genes, or their proteins, and the structures of the fructans they produce.

Characterization of PpFT1 recombinant protein

Enzymatic properties of the *PpFT1* cDNA expressed in *P. pastoris* were investigated at low temperature (7°C). An HPAEC-PAD analysis showed that the reaction mixtures with PpFT1 recombinant enzyme and 1 M sucrose contained 6-kestotriose and other, more polymerized products (**Fig. 3**). The peaks of PpFT1 products eluted after 6-kestotriose have identical retention times of those in extracts from timothy crown tissues, which contained mainly linear β(2,6)-linked fructans (Cairns *et al.* 1999) (**Fig. 3**). The production of 6-kestotriose and putative β(2,6)-linked fructans (levans) from sucrose indicated that the PpFT1 recombinant enzyme exhibited 6-SST and 6-SFT activity. The predominant production of linear β(2,6)-linked fructans indicates that PpFT1 preferentially transfers fructose units to 6-kestotriose rather than to 1-kestotriose. This substrate specificity of PpFT1 is clearly different from that observed in wheat and barley 6-SFTs, i.e., they preferentially use 1-kestotriose as an acceptor to make bifurcose (Sprenger *et al.* 1995; Kawakami and Yoshida 2002).

We added fresh enzyme of PpFT1 and sucrose to the reaction solution at 48 h and obtained further increase in fructans with an estimated DP of more than 50 (**Fig. 3**). On the other hand, the addition of wheat 6-SFT, Wft1 recombinant enzyme and sucrose to the levan oligomers in the PpFT1 reaction mixture at 48 h did not increase the DP of fructans, but increased the linear and branched β(2,6)-linked fructans with DPs lower than 10 (**Fig. 3**). These results indicate that PpFT1 has a higher affinity than Wft1 for linear β(2,6)-linked fructans with high DPs as acceptors. HPAEC-PAD chromatograms indicate that PpFT1 recombinant enzyme synthesized β(2,6)-linked fructans with DP similar to native

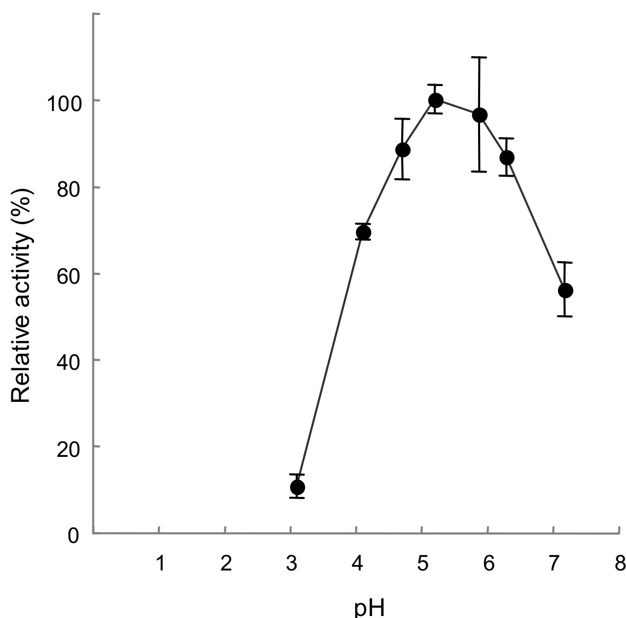

Fig. 4 Effect of pH on the fructosyltransferase activity of PpFT1. Activity is expressed as a percentage of the maximum activity calculated by subtracting the amount of fructose released from that of glucose after incubation with 1 M sucrose.

fructans extracted from cold acclimated timothy, although fructans with high DPs could not be clearly separated under this analytic condition (**Fig. 3**). This suggests that PpFT1 itself can synthesize fructans with a DP up to that of native timothy fructans. Nevertheless, we cannot exclude the possible involvement of other genes coding for FTs with fructan biosynthesis activity in timothy, because other cDNAs with high similarity to *PpFT1* were cloned from the same cDNA library (data not shown).

High levels of 1-kestotriose and fructose were also produced from sucrose by the PpFT1 recombinant enzyme (**Fig. 3**). This indicates that the PpFT1 recombinant enzyme has 1-SST and invertase activities in addition to 6-SST and 6-SFT activities. The optimal pH of the fructosyltransferase activity of PpFT1 was around 5.2 (**Fig. 4**). These enzymatic properties were shared by the fructan polymerase partially purified from timothy leaves by Cairns *et al.* (1999). They reported that fructan polymerase produced mainly linear β(2,6)-linked fructans with high DP (up to 30 by one enzymatic reaction). The similarities in enzymatic properties suggest that *PpFT1* encodes a protein similar to the partially purified enzyme reported by Cairns *et al.* (1999).

Analysis of *PpFT1* gene expression, enzyme activity, and carbohydrate content in excised timothy leaves under the illuminated condition

In general, fructans accumulate in grass leaf tissues when the carbon supply exceeds demand (Wagner and Wiemken 1989). Nagaraj *et al.* (2004) reported the accumulation of 6-SFT transcripts and fructans in excised leaves of barley exposed to continuous illumination. To investigate the relationship between the level of expression of the *PpFT1* gene and the accumulation of fructans in timothy, we assayed the *PpFT1* transcript levels, enzymatic activity, and sugar content in excised timothy leaves maintained under continuous illuminated conditions (**Fig. 5**). The level of expression of *PpFT1* and the fructan content of seedlings were very low prior to the excision of leaves. After the excision, the fructose, glucose and sucrose contents and *PpFT1* transcripts began to increase. Then, after 8 hours of excision treatment, the 6-SST activity and fructan content increased. At 24 h, the PpFT1 transcripts increased more than 80 times higher than in the non-excised leaves. These results suggest that *PpFT1* is involved in fructan biosynthesis in timothy. In barley, 6-SFT is transcriptionally induced by disaccharides such as sucrose (Müller *et al.* 2000). The increase of the *PpFT1* transcripts, fructosyltransferases activity and fructan content in timothy shoots was also observed under a high-sucrose condition (Tamura *et al.* 2009). Therefore, sucrose accumulation might be involved in the induction of *PpFT1* expression in excised leaves.

CONCLUSION

We isolated a novel FT cDNA from timothy, *PpFT1*, encoding fructosyltransferase that produce predominantly linear and β(2,6)-linked fructans with high DPs from sucrose. The substrate specificities of PpFT1 recombinant enzyme (affinity for DPs and linkage forms of fructans as acceptors) were different from the wheat and barley recombinant 6-SFTs previously reported. We also confirmed the coordinated induction of *PpFT1* transcripts, FT activity and fructan content in excised timothy leaves. *PpFT1* cDNA

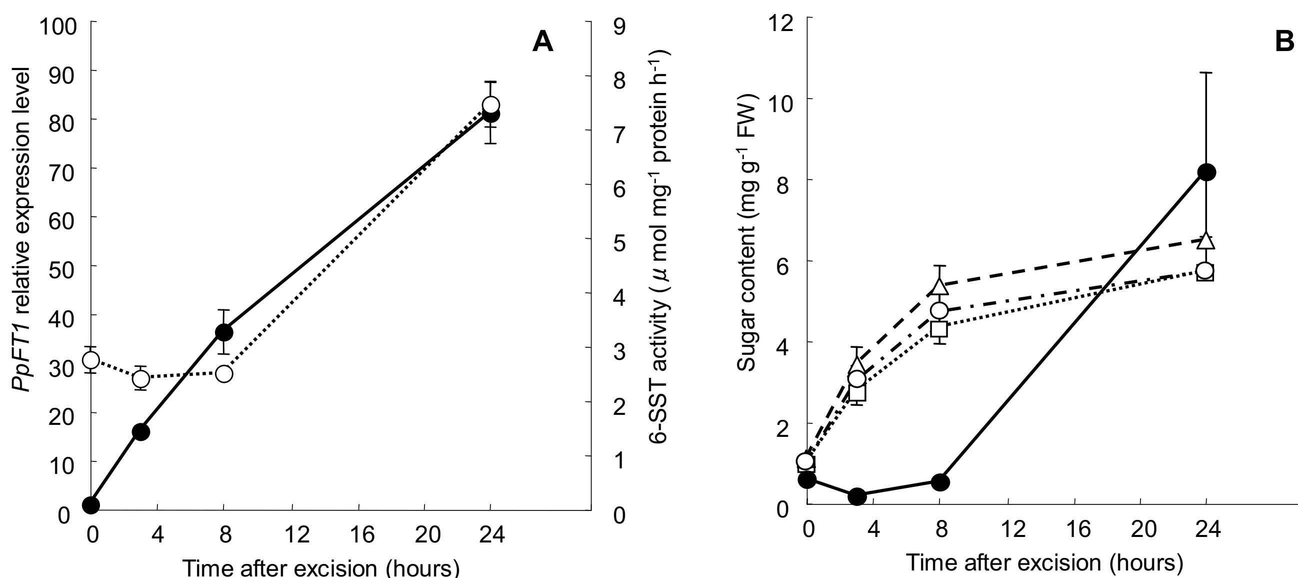

Fig. 5 *PpFT1* mRNA expression levels, 6-SST activities and carbohydrate contents of excised leaves of timothy under the illuminated condition. Expression levels of *PpFT1* relative to those of the α-tubulin gene were measured by quantitative real-time RT-PCR (**A**, *black circles*). Generation of 6-kestotriose by crude enzyme extracts incubated with sucrose was measured as 6-SST activities (**A**, *white circles*). The amounts of fructose (*white circles*), glucose (*squares*), sucrose (*triangles*) and fructans (*black circles*) were measured by HPLC (**B**). Results are given as the mean ± SD; n = 3.

would be a useful tool for transgenic approaches to elucidate the relationship of DPs or the structures of fructans and the physiological roles that fructans play in plants.

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