**Pp6-FEH1** encodes an enzyme for degradation of highly polymerized levan and is transcriptionally induced by defoliation in timothy (*Phleum pratense* L.)

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Abstract

The ability of grasses to regrow after defoliation by cutting or grazing is a vital factor in their survival and an important trait when they are used as forage crops. In temperate grass species accumulating fructans, defoliation induces the activity of a fructan exohydrolase (FEH) that degrades fructans to serve as a carbon source for regrowth. Here, a cDNA from timothy was cloned, named *Pp6-FEH1*, that showed similarity to wheat fructan 6-exohydrolase (6-FEH). The recombinant enzyme expressed in *Pichia pastoris* completely degraded fructans that were composed mainly of β(2,6)-linked and linear fructans (levan) with a high degree of polymerization (DP) in the crown tissues of timothy. The substrate specificity of *Pp6-FEH1* differed from previously characterized enzymes with 6-FEH activity in fructan-accumulating plants: (i) *Pp6-FEH1* showed 6-FEH activity against levan (mean DP 20) that was 4-fold higher than against 6-kestotriose (DP 3), indicating that *Pp6-FEH1* has a preference for β(2,6)-linked fructans with high DP; (ii) *Pp6-FEH1* had significant activity against β(2,1)-linked fructans, but considerably less than against β(2,6)-linked fructans; (iii) *Pp6-FEH1* had weak invertase activity, and its 6-FEH activity was inhibited slightly by sucrose. In the stubble of seedlings and in young haplocorms from adult timothy plants, transcripts of *Pp6-FEH1* were significantly increased within 3 h of defoliation, followed by an increase in 6-FEH activity and in the degradation of fructans. These results suggest that *Pp6-FEH1* plays a role in the degradation of fructans and the mobilization of carbon sources for regrowth after defoliation in timothy.

Key words: Defoliation, fructan, fructan exohydrolase, levan, timothy (*Phleum pratense* L.).

Introduction

Many of the grass species used for forage, including the perennial grasses, have the ability to undergo rapid regrowth following defoliation by cutting or grazing. The meristems of forage grasses are situated near ground level, allowing the plants to regrow after defoliation. Studies on some temperate grass species have shown that defoliation-induced regrowth, following removal of photosynthetically active tissues, involves the degradation of fructans (Volenc, 1986; Gonzalez *et al.*, 1989). These fructose polymers are the main storage carbohydrates and are stored in basal tissues; regrowth rapidly stimulates fructan mobilization and alters carbon allocation. Morvan-Bertrand *et al.* (1999) reported that before the third day of regrowth, leaf growth in *Lolium perenne* mostly depends on fructan reserves, but, thereafter, it relies mainly on newly assimilated carbohydrates. The enzyme fructan exohydrolase (FEH) is responsible for the hydrolysis of fructans and releases the terminal fructose from a fructan molecule. When the degradation of fructans begins after defoliation, FEH activity increases in the stubble of temperate grasses (Mino and Maeda, 1976; Yamamoto and Mino, 1982; Prud’homme *et al.*, 1992).
Partially purified FEHs from the grasses Dactylis, Phleum, Festuca, and Lolium have been described (Simpson and Bonnett, 1993; Marx et al., 1997). Yamamoto and Mino (1989) reported that the increase in FEH (phleinase) activity in defoliated stem bases (pseudostems) of orchardgrass (Dactylis glomerata L.) derives from de novo synthesis of the enzyme. Over the past decade, identification and functional characterization of FEH cDNAs have been reported in several plant species. Based on analysis of the phylogenetic relationships of amino acid sequences, FEHs have been shown to be closely related to cell wall invertases, whereas fructosyltransferases (FTs), the enzymes responsible for synthesis of fructans, have similarity to vacuolar invertases (Van den Ende et al., 2009). Two types of FEH activity can be distinguished depending on the linkage form of fructan on which they act: fructan 6-exohydrolase activity hydrolyses (2,1)-linked fructans; and fructan 1-exohydrolase (1-FEH) activity hydrolyses (2,6)-linked fructans (Van den Ende et al., 2004). Temperate grasses mainly accumulate (2,6)-linked fructans (Bonnett et al., 1997). A 6-FEH cDNA has been identified in wheat, which predominantly accumulates graminan type (2,6)-linked compounds (Van Riet et al., 2006). In wheat, in addition to 6-FEH, other FEH homologues have been identified: 1-FEH (1-FEH w1 and w2), Van den Ende et al., 2003a; 1-FEH w3, Van Riet et al., 2008); 6&1-FEH (6&1-FEH w1 and w2), Kawakami et al., 2005), and 6-KEH (6-KEH w1 and w2, Van den Ende et al., 2005). 6-FEH cDNAs have also been found in Arabidopsis and sugar beet, non-fructan-producing species (Van den Ende et al., 2003b; De Coninck et al., 2005), but not in temperate forage grass species. In L. perenne, characterization of the expression of a 1-FEHa cDNA suggested that it might play a role in the trimming of fructans during synthesis rather than in fructan mobilization (Lothier et al., 2007).

In temperate grasslands that have severe winters, timothy (Phleum pratense L.) is used as a forage plant due to its high nutritive quality and good winter hardiness. It predominantly accumulates simple (2,6)-linked fructans, levan with higher degrees of polymerization (DPs) (up to 90) than in other grass species (Suzuki, 1968; Cairns et al., 1999). A cDNA encoding an FT involved in the synthesis of (2,6)-linked fructans with high DPs has been identified (Tamura et al., 2009). In timothy, after wintering, the lower basal internodes swell and thicken to form a structure called a haplocorm. The haplocorm stores nutrients and is useful for vegetative regrowth after cutting or grazing, because fructans comprise up to 40% of its dry matter (Sheard, 1967). Defoliation-induced fructan degradation and FEH activity in the haplocorm of timothy has been confirmed (Mino and Maeda, 1976). However, little is known about the mechanism of FEH induction, and no FEH cDNAs have been identified to date in timothy.

This study seeks to elucidate the mechanism of FEH induction after defoliation in temperate grasses by (i) cloning and functional characterization of a timothy cDNA encoding an FEH, and (ii) investigating changes in transcript levels of this FEH gene in the stubble of timothy seedlings and in haplocorns of adult plants after defoliation.

**Materials and methods**

**Plant materials**

Seeds of the timothy cultivar ‘Hokushu’ were grown in soil in a controlled climate chamber (16 h light, 150 μmol m⁻² s⁻¹ photon flux density, 22 °C) for 6 weeks after germination. They were then used for the defoliation experiment: the seedlings were cut 5 cm above the ground 1.5 h after the beginning of the light period. Three biological replicates were sampled using 8–10 seedlings on each occasion; tissues were sampled from the cut seedlings at 2 cm above root tissues at each sampling time. Tissues from non-defoliated seedlings were used as the control.

To study haplocorns, ‘Hokushu’ plants were grown outdoors in 4.8-cm square pots in Sapporo, Japan from October 2009 to April 2010, and transferred to 15-cm depth pots in a greenhouse (natural light conditions) to allow additional growth. The plants were maintained at an average temperature of 17.4 °C during this period, and also after defoliation. After 6 weeks, mature plants before heading were defoliated at 5 cm above the ground at 10:00 a.m. Three biological replicates of young haplocorns originating from different genotypes were sampled at each sampling time. Haplocorns from non-defoliated plants were used as the control.

**Cloning of FEH cDNA**

A cDNA library was prepared previously from timothy crown tissues (see Tamura et al., 2009). Partial fragments of putative FT and invertase cDNAs were obtained by PCR amplification from the cDNA library using the primers 6SFT-5F (5'-GAGATGCTG-CAGTTGGCAGCG-3') and FT-1R (5'-CCNARYGMTAG-TAGTCGGT-3'). The primer sequences were designed using DNA sequences conserved among Poacae FT genes. Three amplified fragments of different lengths were cloned into pgEM-T Easy Vector (Promega) and the nucleotide sequences of the fragments were determined using the CEQ 8000 Genetic Analysis System (Beckman Coulter). A 725-bp fragment (AB436698, probe A) that had high similarity to a partial sequence of Poaceae FT genes (also used in Tamura et al., 2009), and a 1167-bp fragment (AB583556, probe B) that showed similarity to vacuolar invertase genes and included a putative 460-bp insertion without homology to any other genes, were used as probes. These fragments were labelled using a PCR DIG Probe Synthesis Kit (Roche) and used for cDNA library screening by plaque hybridization with a DIG Luminescent Detection Kit for Nucleic Acid (Roche). Approximately 4.0×10⁴ recombinant plaques were screened using probe A and probe B. Plaques positive for probe B but either negative or slightly positive for probe A were isolated. After plaque purification, in vivo excision of the pBluescript SK- phagemid vector was performed in the Escherichia coli XLOLR strain. The nucleotide sequences of both strands of the inserts were determined. Multiple sequence alignments and phylogenetic trees were constructed by the neighbor-joining method, using the CLUSTALW program of DNASIS®Pro ver. 2.0 software (Hitachi Software Engineering).

**Expression of recombinant protein in methylotrophic yeast**

The isolated cDNA was expressed in the methylotrophic yeast Pichia pastoris (EasySelect Pichia Expression kit, Invitrogen) after cloning into the secretory expression vector pPICZαA. The DNA sequence corresponding to the putative mature protein...
region predicted by SignalP 3.0 (Bendtsen et al., 2004) was amplified using the primers tyft81-1F (5' -CGAATTCTCTCGAGTCCTGACC-3') and tyft81-1R (5' -CCCCCTCTAGATACCTCAGCGCGCGCG-3'). The amplification product was digested with EcoRI and XbaI, and ligated into pPICZα-A, transformed by electroporation using 10 μg of the PmeI-linearized construct, and transformants were selected on YPD/S/Zeocin plates. A 3-ml preculture medium (BMGY, pH 6.0) was inoculated with freshly prepared single colonies and cultured for 2 d at 30 °C with vigorous shaking (200 rpm). The cells were collected by centrifugation, resuspended in 20 ml of inoculum medium (BMMY, pH 6.0), and incubated at 30 °C with shaking at 200 rpm. Methanol (400 μl) was added to the culture medium every day. After 5 d of induction, the culture was centrifuged and the resulting supernatant medium was recovered and tested for enzyme activity. A 16-ml aliquot of the medium was concentrated to 260 μl, diluted with 12 ml of 20 mM citrate–phosphate buffer (pH 5.2), and concentrated by ultrafiltration on an Amicon Ultra-15 with a cut-off of 10 kDa (Millipore). The dilution/concentration process was repeated twice. After Biospin column 30 (Bio-Rad) treatment, protein concentration was measured using the Protein Assay reagent (Bio-Rad). SDS–PAGE was performed on a 10% polyacrylamide gel that was stained with Coomassie Brilliant Blue-R-250.

Enzyme assay of the recombinant enzyme

Unless otherwise specified, recombinant enzyme preparations were incubated with substrates in 20 mM citrate–phosphate buffer (pH 5.2), 0.02% sodium azide, at 30 °C. For the determination of substrate specificity and effects of pH and temperature, enzyme amount and incubation time were adjusted to achieve linear production of fructose during the incubation period. The reaction was terminated by heating at 95 °C for 5 min. Timothy carbohydrate extracts were prepared from cold-acclimated timothy crown tissues sampled in the field in Sapporo during December 2007. One gram fresh weight (FW) of the tissues was boiled in 5 ml of distilled water. Levan for use as a substrate was prepared by 80% EtOH precipitation of products generated by the PpFT1 (timothy 6-SFT) recombinant enzyme with 1 M sucrose (Tamura et al., 2009). Inulin, 1-kestotriose, 1,1-kestotriose, and 1,1,1-kestotetraose (Wako Pure Chemical Industries, Ltd) were used as substrates. The mean DP of levans and of inulin was estimated as the ratio of the amounts of fructose to glucose after hydrolysis with 0.06 M HCl. The enzymic products generated from timothy substrates. The mean DP of levan and of inulin was estimated as

Quantitative real-time RT-PCR

RNA extraction, cDNA preparation, and real-time RT-PCR were performed as described in Tamura et al. (2009). The primer pairs used in the real-time PCR for Pp6-FEH1 were tyft81-1F (5' -GTTGTTAGCTAGTCGTG-3') and tyft81-1R (5' -ACTACCCGACAGAACACTAG-3'), and for the ubiquitin gene (the internal reference gene) were ubi-RT-2F (5' -CAAAGCCGAAGAGAGAGAC-3') and ubi-RT-2R (5' -GTCGTCGACCTTGTAGAAG-3'). The expression level of Pp6-FEH1 was normalized against ubiquitin gene expression and adjusted against the expression levels of 0-h samples, which were deemed to be 1.0.

Statistical analysis

Student t-tests were performed on gene expression levels, enzyme activities, and fructan content using JMP 9 software (SAS).

Results

Cloning of a candidate FEH cDNA from timothy

A cDNA library derived from crown tissues was probed with a partial cDNA that had high similarity to vacuolar invertases of Poaceae plants. In order to identify cDNAs from fructan-metabolism-related enzymes, in addition to the previously identified PpFT1 coding FT of timothy (Tamura et al., 2009), clones that were clearly positive for the probe used for PpFT1 cloning were eliminated. This screening strategy identified several cDNAs with similarity to vacuolar invertase and FT and three cell wall invertase-like cDNAs, two of which only had partial sequences. One of the clones was a full-length cDNA, designated Pp6-FEH1 (see below), that had 2139 bases and included an open reading frame of 1806 bases (AB583555). This clone was deduced to encode 601 amino acids containing three conserved amino acid motifs (WMS/NDPNG, RDP, and EC) that are suggested to be essential for β-fructosidase activity (Verhaest et al., 2005) (Fig. 1). The amino acid corresponding to aspartic acid 239 in Arabidopsis cwINV1, which is important for binding and hydrolysis of sucrose (Le Roy et al., 2007a), was substituted by methionine (278 from the first Met). An isoleucine (142) was located at the position corresponding to the serine 101 in Cichorium intybus 1-FEH IIa that is important for inhibitory binding of sucrose (Verhaest et al., 2004).
et al., 2007; Le Roy et al., 2008). The putative mature protein was predicted to have a pI of 5.0, molecular mass of 63.4 kDa, and have five N-glycosylation sites (N-X-S/T). The predicted amino acid sequence showed highest identity (69%) to wheat 6-FEH (Van Riet et al., 2006). A phylogenetic comparison of the amino acid sequences of vacuolar invertases and FEHs from a range of species indicated that Pp6-FEH1 was a member of a subgroup that includes wheat 6-FEH and several cell wall invertases in the Poaceae (Fig. 2).

Characterization of the Pp6-FEH1 recombinant protein

A partial cDNA containing the predicted mature protein region of Pp6-FEH1 (Fig. 1) was ligated in-frame behind the α-signal sequence and heterologously expressed in P. pastoris. The recombinant Pp6-FEH1 showed the expected mass of 78 kDa on an SDS–PAGE gel (Fig. 3). HPAEC-PAD analysis of the timothy carbohydrate extract showed peaks that putatively corresponded to levan with sequential DPs (up to >50), in addition to low molecular weight sugars such as sucrose (Fig. 4; Supplementary Fig. S1, available at JXB online). The reaction mixture with the recombinant Pp6-FEH1 showed a decrease in the areas of the peaks corresponding to levan and an increase in that for fructose (Fig. 4; Supplementary Fig. S1 at JXB online). This indicated that the recombinant Pp6-FEH1 had 6-FEH activity. The decrease in peak area corresponding to sucrose after 4 h, suggesting that the recombinant Pp6-FEH1 also had invertase activity, was also confirmed (Fig. 4; Supplementary Fig. S1 at JXB online). The recombinant Pp6-FEH1 showed the temperature and pH dependence expected of FEH: optimal temperature was 30 °C and optimal pH was 5.5 (Fig. 5). The activity of Pp6-FEH1 remained relatively high even at 4 °C, with up to 38% of its activity at 30 °C.

In order to characterize the enzymic properties of recombinant Pp6-FEH1 in detail, substrate specificities were investigated using a 1 mM concentration of different types of fructan (Table 1). Of the fructans tested, Pp6-FEH1 most efficiently hydrolysed levan (mean DP 20) with a relative activity four times greater than for 6-kestotriose (DP 3), which has the same linkage form as levan. Pp6-FEH1 also showed hydrolase activity for β(2,1)-linked fructans (DP 3 to mean 28), although the activity was lower than those for β(2,6)-linked fructans. Hydrolase activity for sucrose was quite low, but the occurrence of non-negligible activity towards 10 and 100 mM sucrose showed the intrinsic invertase activity of Pp6-FEH1. When a mixture of levan and sucrose is used as a substrate, an estimate of FEH activity is obtained by subtracting the amount of fructose released by invertase activity (approximated from the glucose production) from the total amount of fructose by hydrolase activity (see Table 1). Using this formula, the inhibitory effect of sucrose was evaluated as 4% at 10 mM sucrose and 23% at 100 mM sucrose.

Fig. 1. Alignment of the amino acid sequences of Pp6-FEH1 and FEHs from Poaceae species. The three boxed regions indicate conserved motifs that are crucial for β-fructosidase catalysis. The three carboxylic acids implicated in catalysis are in bold. The amino acids homologous to Ser101 of chicory 1-FEH IIa are indicated by §. † shows the amino acids homologous to Asp239 of AtcwINV1. The putative glycosylation sites of Pp6-FEH1 are underlined. The arrow indicates the predicted N-terminus of the mature Pp6-FEH1 protein.

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Analysis of Pp6-FEH1 transcripts in stubble and haplocorms after defoliation

In seedling stubble, the level of expression of Pp6-FEH1 rapidly increased (~7-fold) within 3 h of defoliation; expression then decreased but was nevertheless maintained at a significantly higher level than in non-defoliated tissues up to 72 h after defoliation (Fig. 6A). In the control (non-defoliated seedlings), the Pp6-FEH1 transcript level increased at 3 h but was significantly lower than in the defoliated stubble, and was then maintained at the same level as at 0 h (Fig. 6A). The increase in 6-FEH activity began within 3 h of defoliation, and at 24 h the activity was ~4-fold greater than at 0 h. In the control, 6-FEH activity remained at the same level as at 0 h throughout the tested period (Fig. 6B). Fructan content was significantly lower than that in control after 24 h (Fig. 6C). The mean lengths of newly elongating leaves after defoliation were 9.1 mm at 8 h and 22.0 mm at 24 h.

Next, changes in Pp6-FEH1 expression in young haplocorms after defoliation of timothy plants were investigated. An increase in Pp6-FEH1 expression was evident within 3 h of defoliation at ~7-fold of the control level; the amount of transcripts had rapidly increased by 7 h after defoliation (Fig. 7A; Supplementary Fig. S2A at JXB online). At 4 d after defoliation, Pp6-FEH1 transcripts were ~140-fold more abundant than those at 0 h (Fig. 7A; Supplementary Fig. S2A at JXB online). Thereafter, the transcript level decreased but, even at 14 d, was >15-fold higher than at 0 h. However, the change in Pp6-FEH1 transcript levels in control plants was much smaller than in defoliated plants (Fig. 7A; Supplementary Fig. S2A at JXB online). An increase in 6-FEH activity at 7 h after defoliation was present compared with the control; the significantly higher level of activity was maintained for 7 d (Fig. 7B; Supplementary Fig. S2B at JXB online). The amounts of fructans in defoliated haplocorms were significantly lower than those in the control after 4 d and were about one-ninth the abundance of those in control plants at 14 d (14 mg g⁻¹ FW compared with 130 mg g⁻¹ FW; Fig. 7C; Supplementary Fig. S2C at JXB online). The mean length of newly elongating leaves after defoliation was 9.7 mm at 7 h and 95.2 mm at 4 d.

Discussion

Pp6-FEH1 encodes a 6-FEH for highly polymerized levan

Phylogenetic analysis of amino acid sequences indicated that Pp6-FEH1 showed highest similarity to wheat 6-FEH (Van Riet et al., 2006) and was classified with other Poaceae cell wall invertases (Fig. 2). Pp6-FEH1 does not have the Asp239 homologue of AtcwINV1, which has been proposed as a reliable determinant for discriminating true invertases from defective invertases/FEHs (Le Roy et al., 2007a). The recombinant Pp6-FEH1 showed a higher substrate specificity for β(2,6)-linked fructans than for β(2,1)-linked ones; in particular, its activity for levan was 10-fold more than for inulin. This attribute indicates that Pp6-FEH1 can be classified as a 6-FEH. The preference of Pp6-FEH1 for

Fig. 2. Phylogenetic tree of FEHs and cell wall invertases (CW INV) of plants based on predicted amino acid sequences. Scale bar indicates branch length. GenBank accession numbers of the genes used in this analysis are given after the gene and species abbreviation. Abbreviations for the species are: At, A. thaliana; Bv, B. vulgaris; Ci, C. intybus; Cr, Campanula rapunculoides; Hv, Hordeum vulgare; Lp, L. perenne; Os, Oryza sativa; Pp, P. pratense; Ta, Triticum aestivum; Zm, Zea mays.
(2,6)-linked fructans as substrate is consistent with the fact that timothy mainly accumulates (2,6)-linked fructans. Indeed, the recombinant Pp6-FEH1 completely degraded the native fructans in timothy (Fig. 4).

In wheat, four genes, 6-KEH w1 and w2 (Van den Ende et al., 2005), 6&1-FEH (Kawakami et al., 2005), and 6-FEH (Van Riet et al., 2006) have been reported to encode FEHs that show considerable activity for degradation of (2,6)-linked fructosyl residues. However, Pp6-FEH1 showed a different substrate specificity from these wheat FEHs. Pp6-FEH1 had a clearly higher activity for (2,6)-linked fructans with high DPs than for those with a low DP (levan with mean DP 20 compared with 6-kestotriose with DP 3, Table 1). By contrast, wheat 6-FEH shows almost similar substrate specificities for (2,6)-linked fructans with different DPs such as 6-kestotriose, phlein (DP 4–12), and levan (DP ±72) (Van Riet et al., 2006). The remarkable difference in substrate specificity for (2,6)-linked fructans with different DPs is also shown by wheat 6-KEHs and 6&1-FEH (Kawakami et al., 2005; Van den Ende et al., 2005), which have a preference for certain small oligomers as substrates. The high affinity of Pp6-FEH1 for (2,6)-linked fructans with high DPs might be related to the fact that timothy accumulates mainly levan with high DPs (up to 90), in contrast to other temperate grasses (Suzuki, 1968; Cairns et al., 1999). In 1-FEH, it has been reported that the presence or absence of a glycosyl chain in the cleft between the N- and C-terminal domains is important for the binding of high-DP inulins; introduction of a glycosyl chain into C. intybus 1-FEH IIA prevents inulin binding and degradation (Le Roy et al., 2007b). However, 6-FEHs seem to bind to levan in a different way and at a different location (Le Roy et al., 2007b). Indeed, the predicted mass of Pichia recombinant Pp6-FEH1 from SDS–PAGE suggested the addition of glycosyl chains (Fig. 3). Pp6-FEH1 has a glycosylation site in the cleft region (Fig. 1, 341–343 from first Met) like other 6-FEHs and cell wall invertases (Le Roy et al., 2007b).

Purified native enzyme and the Pichia recombinant wheat 6-FEH showed extremely low activity against (2,1)-linked fructans at least lower than 1/100 compared with bacterial levan (DP ±72) and phlein (DP 4–12) (Van Riet et al., 2006). However, the Pichia recombinant Pp6-FEH1 had significant activity against (2,1)-linked fructans of 6–18% of that against levan. This indicates that Pp6-FEH1 has weak 1-FEH activity in addition to its 6-FEH activity. Wheat 6&1-FEH has both 6-FEH and 1-FEH activity with a substrate preference for bifurcose (1&6-kestotetraose), although it shows little or no activity for high-DP fructans (Kawakami et al., 2005), and is therefore different from Pp6-FEH1.

In contrast to wheat 6-FEH, which is not inhibited by sucrose (Van Riet et al., 2006), a small decrease in 6-FEH activity was found when Pp6-FEH1 was incubated with
C. intybus amino acid corresponding to Ser101 in a relationship between sucrose inhibition and the single genesis. FEHs with a small amino acid, such as glycine or dimensional structural analysis and of site-directed muta-

IIa. This proposal was based on the results of three-
P. pastoris expressed in young haplocorms (Figs 6, 7; Supplementary S2 at online). Verhaest et al. (2007) recently demonstrated the induction of 1-FEH II transcription within 1 d of defoliation. The present study provides the first indication of defoliation-induced transcription of FEH in grasses. The chicory root shows induction of 1-FEH II transcription within 1 d of defoliation (Van den Ende et al., 2001), while induction of 1-FEH transcripts occurs in the rhizophore of Vernonia herbacea 20 d after defoliation (Asega et al., 2008). This induction response is similar to that found here for Pp6-FEH1 in timothy stubble.

Table 1. Substrate specificity of recombinant Pp6-FEH1 expressed in P. pastoris

Activity is shown as the value relative to activity with 1 mM levan as substrate. The value of 100% corresponds to 11.6 μmol mg⁻¹ protein min⁻¹.

| Substrate | Linkage form | DP | Relative activity (%) |
|-----------|--------------|----|----------------------|
| 1 mM 6-kestotriose | (2,6) | 3 | 25 |
| 1 mM levan | (2,6) | 20<sup>a</sup> | 100 |
| 1 mM 1-kestotriose | (2,1) | 3 | 7 |
| 1 mM 1,1-kestotetraose | (2,1) | 4 | 18 |
| 1 mM 1,1,1-kestopentaose | (2,1) | 5 | 18 |
| 1 mM inulin | (2,1) | 28<sup>ab</sup> | 6 |
| 1 mM sucrose | 2 | <<1 |
| 10 mM sucrose | 2 | 2 |
| 100 mM sucrose | 2 | 15 |
| Mixture of 1 mM levan+10 mM sucrose | 97 |
| Mixture of 1 mM levan+100 mM sucrose | 87 |

<sup>a</sup> Mean DP.

It was demonstrated that transcription of Pp6-FEH1 was induced within 3 h of defoliation, and was followed by an increase in 6-FEH activity in both seedling stubble and young haplocorms (Figs 6, 7; Supplementary S2 at online). With regard to the enzymic function of Pp6-FEH1, these findings suggest that induction of Pp6-FEH1 expression plays a role in the initiation of fructan degradation and mobilization after defoliation in timothy. They also support the report of Yamamoto and Mino (1989) of de novo synthesis of FEH enzyme after defoliation. The present study provides the first indication of defoliation-induced transcription of FEH in grasses. The chicory root shows induction of 1-FEH II transcription within 1 d of defoliation (Van den Ende et al., 2001), while induction of 1-FEH transcripts occurs in the rhizophore of Vernonia herbacea 20 d after defoliation (Asega et al., 2008). This induction response is similar to that found here for Pp6-FEH1 in timothy stubble.

Pp6-FEH1 plays a role in fructan mobilization after defoliation
The maximum transcript level of \textit{Pp6-FEH1} after defoliation, compared with 0 h, was much higher in haplocorms than in seedling stubble: ~140-fold increase in haplocorms at 4 d compared with 7-fold in stubble at 3 h after defoliation. However, the level of induction of FEH activity showed less difference: 15-fold at 7 d in haplocorms compared with 4-fold at 24 h in seedling stubble. Post-transcriptional regulation of \textit{Pp6-FEH1} or other FEH enzymes might be involved in the different relationship between \textit{Pp6-FEH1} transcript level and 6-FEH activity in the two tissues. The induction of \textit{Pp6-FEH1} transcripts in haplocorms was maintained for 4 d after defoliation, whereas in the stubble of seedlings the transcript level dropped rapidly by 8 h. The rate of decrease of fructan content in the haplocorms and seedling stubble appeared to correlate with the fluctuations in levels of \textit{Pp6-FEH1} transcripts (Figs 6, 7).

What factors might affect the induction of \textit{Pp6-FEH1} expression? Exogenous application of sucrose, fructose, or glucose to excised pseudostems of orchardgrass appears to suppress any increase in FEH activity (Yamamoto and Mino, 1987). In the present study, these sugars rapidly decreased in the stubble and haplocorms of timothy after defoliation (data not shown). The decrease in photosynthetic assimilates after defoliation might induce an increase in \textit{Pp6-FEH1} transcripts in order to mobilize carbon sources. Morvan et al. (1997) reported that FEH activity in \textit{L. perenne} was inhibited by uniconazole, an inhibitor of the biosynthesis of gibberellin, and also
showed an increase in activity following treatment with gibberellic acid. However, an increase in gibberellin content in stubble after defoliation has not been confirmed (Morvan-Bertrand et al., 2001). In orchardgrass, exogenous gibberellic acid, cytokinin, or 8-bromo-cAMP all increased FEH activity in pseudostems (Yamamoto and Mino, 1998). A future study of the transcriptional analysis of Pp6-FEH1, including identification of inducing factors and cis-acting elements in the promoter, should help to elucidate the mechanism of transcriptional regulation of FEH genes after defoliation.

The regulation of latent FEH enzyme activity by means of sucrose inhibition could not be completely excluded in timothy, but at least for the Pp6-FEH1 enzyme the effect of such inhibition would be relatively small compared with that of induction of transcripts. In L. perenne, a grass species frequently used for grazing due to its high regrowth performance, activity of both 6-FEH and 1-FEH is strongly inhibited by sucrose (Marx et al., 1997; Lothier et al., 2007). The major advantage of sucrose inhibition of FEH activity is a rapid shift from net fructan biosynthesis to net fructan degradation when sucrose is lacking (Verhaest et al., 2007). It will be of interest to determine whether the difference in the regulation mechanism of FEH activity in forage grass species affects the speed of regrowth after defoliation.

Subcellular localization of Pp6-FEH1

As fructans are mainly accumulated in the vacuole, it is generally believed that fructan-metabolizing enzymes must be present there (Wanger and Wiemken, 1986). Two factors suggest localization of Pp6-FEH1 in the vacuole: a low pI (5.0), which is characteristic of vacuolar enzymes; and a weak, but intrinsic, response to inhibition by sucrose, which is present at a high concentration in the vacuole. However, the possible localization of Pp6-FEH1 to the apoplast cannot be excluded as FEH activity in the apoplast has been reported by several studies (Livingston and Hensen, 1998; Van den Ende et al., 2005).

Supplementary data

Supplementary data can be found at JXB online.

Fig. S1. HPAEC-PAD chromatograms corresponding to those in Fig. 4 but redrawn using an extended y axis scale.

Fig. S2. Bar graph representation of the line graphs in Fig. 7.

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