Running head: β(2,6) linked fructans in *Pachysandra*

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Biochemical processes and macromolecular structures
Unexpected presence of graminan- and levan-type fructans in the evergreen frost-hardy eudicot *Pachysandra terminalis* (Buxaceae). Purification, cloning and functional analysis of a 6-SST/6-SFT enzyme

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Abstract

About 15% of flowering plants accumulate fructans. Inulin-type fructans with $\beta(2,1)$ fructosyl linkages typically accumulate in the core eudicot families (e.g., Asteraceae), while levan-type fructans with $\beta(2,6)$ linkages and branched, graminan-type fructans with mixed linkages predominate in monocot families. Here, we describe the unexpected finding that graminan and levan-type fructans, as typically occurring in wheat and barley, also accumulate in *Pachysandra terminalis*, an evergreen, frost-hardy basal eudicot species.

Part of the complex graminan- and levan-type fructans as accumulating in vivo can be produced in vitro by a sucrose: fructan 6-fructosyltransferase (6-SFT) enzyme with inherent sucrose: sucrose 6-fructosyltransferase (6-SST) activity, and some minor 1-SST activity. This enzyme produces a series of cereal-like graminan- and levan-type fructans from sucrose as a single substrate. The 6-SST/6-SFT enzyme was fully purified by classic column chromatography. In-gel trypsin digestion led to RT-PCR based cDNA cloning. The functionality of the 6-SST/6-SFT cDNA was demonstrated after heterologous expression in *Pichia pastoris*. Both the recombinant and native enzymes showed rather similar substrate specificity characteristics, including peculiar temperature-dependent inherent 1-SST and 6-FEH side activities. The finding that cereal-type fructans accumulate in a basal eudicot species further confirms the polyphyletic origin of fructan biosynthesis in nature. Our data suggest that the fructan syndrome in *Pachysandra terminalis* can be considered as a recent evolutionary event. Putative connections between abiotic stress and fructans are discussed.
Introduction

About 45000 species of angiosperms, approximately 15% of the flowering plants, store fructans, fructose (Fru) based oligo- and polysaccharides derived from sucrose (Suc). Fructans are known to occur in the highly evolved orders of the Poales (Poaceae), Liliales (Liliaceae), Asparagales, Asterales (Asteraceae and Campanulaceae) and Dipsacales as well as within the Boraginaceae (Hendry, 1993). Fructans are believed to accumulate in the vacuole (Wiemken et al., 1986) although fructans and fructan degrading enzymes (fructan exohydrolases or FEHs) have also been reported in the apoplast (Livingston and Henson, 1998; Van den Ende et al., 2005). To explain this observation, it was hypothesized that fructans can be transferred from the vacuole to the outer side of the plasma membrane by vesicle-mediated exocytosis (Valluru et al., 2008 and references therein), especially under stress. Fructans might protect plants against freezing/drought stresses (Valluru and Van den Ende, 2008), by stabilizing membranes (Vereyken et al., 2001; Hincha et al., 2002, 2003). Recent studies on transgenic plants carrying fructan biosynthetic genes (Parvanova et al., 2004; Li et al., 2007; Kawakami et al., 2008) suggest that the enhanced tolerance of these plants is associated with the presence of fructans. Their reduced lipid peroxidation levels indicate that fructans, similar to Raffinose Family Oligosaccharides (RFOs; Nishizawa et al., 2008), might directly act as reactive oxygen species (ROS) scavengers too (Van den Ende and Valluru, 2009; Bolouri et al., 2010; Stoyanova et al., 2010).

While dicotyledonous species were believed to exclusively store inulin-type fructan consisting of linear $\beta(2,1)$ linked fructofuranosyl units, $\beta(2,6)$ levan-type and mixed-type fructans predominate in monocots (Vijn and Smeekens, 1999). Different types of fructan biosynthetic enzymes (also termed fructosyltransferases or FTs) have now been characterized that can readily explain the diversity of fructans in plants (Lasseur et al., 2006; Tamura et al., 2009). They can be classified in S-type FTs (using Suc as donor substrate) and F-type FTs (using fructans as donor substrate) (Schroeven et al., 2009). Two different enzymes (1-SST and 1-FFT) are required to synthesize the most common and best studied inulin-type fructans as occurring in Asteracean species (Edelman and Jefford, 1968; Van den Ende and Van Laere, 2007). Depending on the species, a more complex cocktail of FTs (1-SST, 1-FFT, 6-SFT, 6G-FFT) is needed within the monocots (Yoshida et al., 2007; Prud’homme et al., 2007). Structure-function relationships have been found explaining the evolutionary differences between vacuolar invertases and FTs on the one hand (Schroeven et al., 2008; Altenbach et al., 2009) and between different types of FTs.
on the other hand (Lasseur et al., 2009; Schroeven et al., 2009). Clearly, the capacity for
fructan biosynthesis arose many times in the course of evolution, in bacteria, fungi and
higher plants (Ritsema et al., 2006; Altenbach and Ritsema, 2007). In plants, FTs evolved
from vacuolar invertases (Wei et al., 2001; Schroeven et al., 2008) while FEHs probably
evolved from cell wall invertases (Le Roy et al., 2007). Like other Suc splitting enzymes,
S-type FTs can fulfil important roles in regulating source/sink balances in plants (Ji et al.,
2010).

_Pachysandra terminalis_ Sieb & Zucc (Japanese spurge - Buxaceae) is a frost-hardy
evergreen plant originating from Japan and China, but became increasingly popular as a
ground-cover plant in Europe and North America (Zhu and Beck, 1991; Zhou et al., 2005).
_Pachysandra_ can survive freezing temperatures up to -33°C (plants.usda.gov). Together
with _Sarcococca, Styloceras, Buxus_ and _Notobuxus_, the genus _Pachysandra_ belongs to the
Buxaceae family within the basal eudicots (Hoot et al., 1999; von Balthazar et al., 2000;
Anderson et al., 2005; Jiao and Li, 2009). _Pachysandra_ and other members of the
Buxaceae contain alkaloids with antibacterial, antiviral and anticancer properties
(Kinghorn et al., 2004; Devkota et al., 2008). Moreover, _Pachysandra terminalis_ was
found to be an effective vole repellent (Curtis et al., 2002).

To the best of our knowledge, so far the presence of fructans has not been reported in
_Pachysandra terminalis_ or any other species within the basal eudicots. To date, the
presence of graminan- and levan-type fructans with predominant β(2,6) linkages was
thought to be restricted to monocot plant species only. In this paper, we demonstrate to our
knowledge for the first time that cereal-type levans and graminans are present in
_Pachysandra terminalis_, a representative of the primitive eudicots. We report on the
purification and characterization of the main FT involved, and on the cloning and
heterologous expression of the cDNA encoding this enzyme, showing peculiar substrate
specificity characteristics as a function of the incubation temperature and time.

**Results and discussion**

_Pachysandra terminalis_ accumulates both graminan and levan-type fructans
Linear β(2,1) inulin-type fructans with 1-kestotriose as backbone, as occurring _e.g._ in
chicory roots (Fig. 1), are the most studied fructans and become more and more popular as
prebiotics in food (Roberfroid and Delzenne, 1998; Roberfroid, 2007). Linear levan-type
fructans (backbone: 6-kestotriose) accumulate in a few grass species including _Dactylis_
glomerata (Fig. 1). Graminan-type fructans, with 1&6-kestotetraose (also termed bifurcose) as backbone, are predominant in cereals such as wheat (Triticum aestivum in Fig. 1; Yoshida et al., 2007). As a result of a major screening of > 100 plant species belonging to different families in the plant kingdom, by means of HPAEC-PAD, the rhizomes of Pachysandra terminalis were found to contain a complicated mixture of so far unidentified oligo- and polysaccharides in (Fig. 1). When a neutral, carbohydrate containing fraction of Pachysandra terminalis rhizomes was subjected to mild acid and enzymatic hydrolysis with chicory 1-FEH IIa (Van den Ende et al., 2001) and sugar beat 6-FEH (Van den Ende et al., 2003a), a massive production of Fru was observed (not shown). A closer inspection of the oligosaccharides from Pachysandra terminalis on the HPAEC-PAD chromatograms revealed that it contained 1) a series of peaks corresponding to the graminan-type fructans as occurring in wheat and 2) another series with elution times identical to the linear levan-type fructans as accumulating in Dactylis glomerata (Fig. 1). These findings already suggested that Pachysandra terminalis accumulates a mixture of both graminan- and levan-type fructans, as well as some 1-kestotriose. High DP levan-type fructans occur both in Pachysandra and in Dactylis (arrows in Fig. 1). Hard proof of the putative fructan identities was generated by manually collecting individual compounds (a-m in Fig. 1) during elution from a preparative HPAEC-PAD column. Enzymatic and mild acid hydrolysis treatments indeed confirmed the unique presence of the same graminan- and levan-type fructan structures (Table I) as earlier described in wheat (Bancal et al., 1993; Van den Ende et al., 2003b; Yoshida et al., 2007). However, the levan/graminan ratio is higher in Pachysandra compared to wheat (Fig. 1) but lower than the one observed in Dactylis glomerata (Maleux and Van den Ende, 2007) and in Phleum pratense (Tamura et al., 2009). At the end of a long cold period, rhizomes of Pachysandra terminalis accumulated up to 12.3 % of fructans on a fresh weight basis. For comparison, commercial chicory root cultivars can accumulate up to 18 % inulin-type fructans (Van Waes et al., 1998; Van den Ende and Van Laere, 2007).

Purification and properties of a 6-SST/6-SFT involved in graminan and levan synthesis
The dominant character of graminan- and levan-type fructans in rhizomes of Pachysandra terminalis suggested the presence of a prominent enzymatic activity responsible for the formation of β(2,6) Fru-Fru linkages. Preliminary in vitro incubations with Suc as a single substrate indeed showed a prominent formation of 6-kestotriose compared to 1-kestotriose and 6G-kestotriose (neokestose), and the formation of 1&6-kestotetraose from Suc and 1-
kestotriose (not shown). Therefore, the enzyme was provisionally termed a 6-SST/6-SFT. The β (2,6) forming enzyme was purified by monitoring its 6-SST activity (quantification of 6-kestotriose production from Suc by HPAEC-PAD). The results of a typical purification are presented in Table II. Although considerable activity losses occurred on Con A, Mono S pH 4.5 and Mono S pH 4.0, a maximal purification of about 88-fold and a specific activity of 2.1 U mg⁻¹ protein were found (Table II), similar to the purified 1-SST from chicory roots (Van den Ende et al., 1996a).

Similar to all other plant FTs purified so far (Van den Ende and Van Laere, 2007), the retention on Con A confirmed that this enzyme is also a glycoprotein. The molecular mass of the native protein was about 70 kDa as determined by gel filtration (not shown). The most pure fraction (Mono S pH 4.0; Table II), resulted in 53 and 22 kDa subunits after SDS-PAGE (Fig. 2). Such heterodimeric nature is a typical characteristic for FTs derived both from dicot and monocot species (Altenbach et al., 2004; Van den Ende and Van Laere, 2007), although a few monomeric FTs have also been reported (Koops and Jonker, 1994). Within GH32 plant enzymes, the large subunit contains the catalytic triad (Lammens et al., 2009), but the small subunit is also essential to preserve the biologic activity of FTs (Altenbach et al., 2004). The pH optimum of the 6-SST/6-SFT was between 5.0 and 5.5 (Fig. 3A), consistent with other Suc splitting plant FTs (Van den Ende and Van Laere, 2007) and a presumptive vacuolar localization. The temperature optimum was close to 20°C (Fig. 3B), which is considerably lower than the 30-35°C of the 1-kestotriose and 6-kestotriose forming enzymes in barley (Simmen et al., 1993) but comparable with the 20-25°C of the 1-SST from Helianthus tuberosus (Koops and Jonker, 1996). The high activity at 0°C (40% of the maximum) might represent a specific adaptation to increase fructan accumulation at lower temperatures, as occurring within the Pooideae (Sandve and Fjellheim, 2010) including cereals (Yoshida et al., 2007) and other grasses (Hisano et al., 2004; 2008).

Mass fingerprint of the purified Pt 6-SST/6-SFT

The two subunits were excised, trypsinized in-gel and the masses of ZipTip eluted tryptic peptides were determined by Q-TOF. A theoretical tryptic digest of the cDNA-derived 6-SST/6-SFT sequence (obtained later, see Fig. 4) yielded 50 peptides (designated T1-T50 from N- to C-terminus). Most masses matched - within the acceptable mass measurement error of +/- 0.2 Da - with one of the theoretical fragments (Table S1). Two fragments, representing the N and C terminal parts of the enzyme, did not match with the masses of
the theoretical fragments, demonstrating the existence of an alternative N-
(YPWSNAQLSWQR and YPWSNAQLSWQR) and C-terminal (IWEMNSAFIQPFH)
processing *in planta*. To the best of our knowledge, such (differential) N- or C-terminal
processing has not been reported before for plant FTs.

Cloning a cDNA encoding the Pt 6-SST/6-SFT and phylogenetic analysis

A full 6-SST/6-SFT cDNA from *P. terminalis* was obtained by a combination of RT-PCR,
PCR and 5’ and 3’ RACE RT-PCR. On the one hand, the Pt 6-SST/6-SFT cDNA-derived
amino-acid sequence (EMBL accession no. FN870376) is aligned (Fig. 4) with the most
identical sequence available in the literature (65 % identity with *Pyrus pyrifolius* vacuolar
invertase, Yamada *et al.*, 2007). On the other hand, it is compared to the functionally
related 6-SFT enzymes from *Hordeum vulgare* and *Phleum pratense* (Sprenger *et al.*,1995;
Tamura *et al.*, 2009). Five potential glycosylation sites [N-X-(S/T)] were detected (Fig. 4).
The estimated molecular weight of the derived polypeptide, without taking into account
putative glycosylations, was predicted at 60.7 kDa. Its predicted pI was 5.2 which is typical
for all vacuolar FTs and invertases (Van den Ende and Van Laere, 2007). The
NWMNDPNG (or β-fructosidase) motif is changed into SWMSDPDG (Fig. 4). An altered
β-fructosidase motif is typical for all FTs described so far (Altenbach *et al.*, 2009; Van den
Ende *et al.*, 2009). Another characteristic of FTs is the presence of an altered W(A/G)W
motif (Altenbach *et al.*, 2009). Pt 6-SST/6-SFT is an exception since it contains a full
WAW motif (Fig. 4). Intriguingly, in Pt 6-SST/6-SFT, a “Asp/Gln” couple (DEEQ) is
observed instead of a “Asp/Arg” couple as observed in *Pyrus pyrifolius* VI (DDDR) and in
the *Phleum pratense* and *Hordeum vulgare* 6-SFTs (DDER) (Fig. 4). Perhaps the Asp/Gln
couple can fulfil a similar role for stabilizing Suc (Lasseur *et al.*, 2009).

An unrooted phylogenetic tree was constructed containing the Pt6-SST/6-SFT cDNA-
derived amino-acid sequence (see arrow, Nr. 1 in Fig. 5) and many other vacuolar-types of
plant invertases and FTs. Three distinct groups can be clearly distinguished (Fig.5). Group
I and II contain dicotyledonous enzymes and group III contains monocotyledonous
enzymes. Hitherto, it was believed that all dicot FTs (see 1-SSTs and 1-FFTs in subgroup
IIb, Fig. 5) evolved from “Type II” vacuolar invertases (Van den Ende *et al.*, 2002) by
changes in their WMNDPNG and WGW motifs (Schroeven *et al.*, 2008; Altenbach *et al*.,
2009). Here, we demonstrate for the first time that a FT (the Pt 6-SST/6-SFT, Nr 1 in Fig.
5) shows higher identity to “Type I” vacuolar invertases (52-65% identity to 2-12 in Fig 5)
than to “Type II” of vacuolar invertases (51-57% identity to 13-18 in Fig. 4) suggesting
that the basal eudicot Pt 6-SST/6-SFT evolved from a Type I vacuolar invertase or, alternatively, from an ancestral vacuolar invertase before the separation of the two vacuolar invertase subgroups by duplication. Further sequencing of vacuolar invertases of lower plants and a number of basal eudicot species is necessary to discriminate between these two possibilities. However, care should be taken since gene losses might further complicate the interpretation of such phylogenetic analyses. For instance, the two only vacuolar invertases in the genome of *Arabidopsis thaliana* (Nrs 10 and 11 in Fig. 5) belong to Type I vacuolar invertases, suggesting that the group II Type gene was lost during evolution and the group I gene was duplicated in this species. Four subgroups (IIIa-d) can be differentiated within monocots. The subgroups IIIb and d contain vacuolar invertases. Subgroup IIIc harbours both vacuolar invertases and FTs from Asparagales. Subgroup IIIa contains mainly FTs but the presence of a genuine invertase in this subgroup (Nr. 34 shows an intact WMNDPNG and WGW motif, Schroeven et al., 2008; Lasseur et al., 2009) can not be excluded. This subgroup further splits up in enzymes that biosynthesize β(2,1) fructosyl linkages (1-SSTs) and those that make β(2,6) linkages (6-SFTs and a putative 6-FT). Taken together, the phylogenetic tree (Fig. 5) suggests that the capability to create fructans was gained at at least 4 times throughout plant evolution: within the basal eudicots and within the Asterales, Poaceae and Asparagales. In this scenario, it can be speculated that different FTs were created by small number of mutations (Schroeven et al., 2008, 2009). It was recently speculated that this evolution occurred in two steps: first the development of S-type FTs (1-SST, 6-SFT; still using Suc as donor substrate) from vacuolar invertases followed by the development of F-type FTs (1-FFT, 6G-FFT, using fructan as donor substrate) from S-type FTs (Schroeven et al., 2009).

_Heterologous expression of the Pt 6-SST/6-SFT cDNA in Pichia pastoris. Comparison of the native and heterologously produced enzymes_

Heterologous expression in *Pichia pastoris* is considered as a reliable method to study the functionality within the GH32 family (Van den Ende et al., 2009). We are confident that the native 6-SST/6-SFT enzyme was > 99% pure since the same enzyme preparation was used for enzyme crystallization and 3D structure determination (Lammens et al., in preparation). Short-term incubations with the native and recombinant Pt 6-SST/6-SFT and Suc showed the production of Glc and 6-kestotriose (Fig. 6). When using 1-kestotriose as a single substrate (not shown), or when combining Suc and 1-kestotriose, no 1,1-nystose was
produced, showing that the (native and recombinant) enzymes lack 1-FFT activity. By combining Suc and 1-kestotriose as substrates, 1&6-kestotetraose (bifurcose) is the main fructan product formed by both the native and heterologous enzyme (Fig. 6). Only low amounts of 6-kestotriose were produced, indicating that 1-kestotriose is a more preferential acceptor substrate than Suc (Fig. 6). This was further corroborated by kinetic studies (both at 0°C and 30°C) on the recombinant enzymes at varying Suc concentrations (6-SST activity) and at a constant Suc concentration (150 mM) combined with varying 1-kestotriose concentrations (6-SFT activity). With Suc as single substrate, no saturation was observed, not even at 1.2 M Suc (not shown). The Suc to 1-kestotriose transfer reaction was clearly saturable (Fig. 7; Fig. S1). The apparent $K_m$ ‘s for 1-kestotriose as an acceptor substrate were estimated at 35 mM (0°C) and 76 mM (30°C), respectively. These apparent $K_m$ ‘s are surprisingly low compared to those derived for other plant FTs (Schroeven et al., 2008). Inhibition of 1&6 kestotetraose formation was observed at 0°C but not at 30°C (Fig. 7). The native enzyme shows only minor 1-SST side activities at 0°C but higher 1-SST side activities at 30°C (Fig. S2), probably contributing to the difference in fructan patterns (graminan/levan ratio) generated from Suc as a single substrate (Fig. S2). However, this temperature-dependent difference in graminan/levan ratio was found to be less prominent for the recombinant enzyme (Fig. 8).

Hydrolytic activities of 6-SST/6-SFT: a multifunctional “premature” FT?

Evolutionary considerations

Besides limited sucrolytic activities (Fig. 6), prolonged incubation times (substrate depletion) surprisingly demonstrated that the Pt 6-SST/6-SFT also showed intrinsic 6-FEH activities (Fig. 8). Typically, the hydrolase/transferase ratio increases with increasing temperature, as observed for the chicory 1-SST and 1-FFT (Van den Ende et al., 1996a,b). Therefore, it seems reasonable to assume that the intrinsic 6-FEH activity of Pt 6-SST/6-SFT might increase at higher temperatures, explaining why higher DP levans do not accumulate at 30°C (Fig. 8B) compared to 0°C (Fig. 8A). This finding is in line with previous observations that some vacuolar invertases show intrinsic FEH activities (Van den Ende et al., 2003a; De Coninck et al., 2005; Ji et al., 2007), suggesting that perhaps many vacuolar invertases have such characteristics. This new point of view sheds a different light on some former data and conclusions. Indeed, leaves of many grasses, typically showing huge soluble invertase activities, also show high in vitro FEH activities, even during periods of active fructan synthesis (Lothier et al., 2007 and refs therein).
Therefore, it should be re-investigated whether these prominent FEH activities represent genuine FEH activities rather than FEH side activities of vacuolar invertases. Perhaps these vacuolar invertase side activities are strong enough to play a role in planta. Whether genuine 6-FEH type enzymes occur in the vacuoles of fructan plants is still not clear since the characterised 6-FEH from wheat is believed to be apoplastic (Van Riet et al., 2006).

We propose here that intrinsic FEH activities in vacuolar invertases can have important physiological and evolutionary implications. In Poaceae it is believed that the fructan syndrome was initiated shortly after a global super-cooling period at the Eocene–Oligocene boundary (Sandve and Fjellheim, 2010). When vacuolar invertase develops transferase capacity (by creating extra space and interaction capacity for binding the sugar acceptor substrate, Altenbach et al., 2009), the fructans that are produced need to be broken down at a later time-point, consistent with the idea that fructans mainly act as reserve compounds. Therefore, co-evolution of a cell-wall invertase into specific FEHs (Le Roy et al., 2007) is needed as well as the recruitment of a vacuolar targeting signal to translocate the FEHs from the cell wall to the vacuole (Van den Ende et al., 2001). However, maturation of vacuolar invertases to genuine FTs as well as the subsequent development of specific FEHs probably require extended time-periods throughout evolution. This means that there is probably an evolutionary window in which fructans are accumulating, while specific vacuolar FEHs are not yet available. We speculate that the Pt 6-SST/6-SFT - probably representing a “premature” or “evolutionary young” FT still containing many side activities (invertase, FEH, 1-SST) - might be involved in vacuolar fructan polymerization reactions as long as the Suc concentration is high enough. However, when the Suc concentration decreases, e.g. after defoliation or arrest of photosynthesis, the enzyme might play a role in fructan degradation as well. In chicory, in which the fructan syndrome developed a very long time ago (Fig. 5; Schroeven et al., 2009), a similar kind of Suc-based regulation is observed by means of very specific, Suc inhibited 1-FEHs (Van den Ende et al., 2001). Such depolymerization reactions might provide hexoses for re-growth, contribute to osmotic adjustments or adjust the DP of the fructans to create a sugar mix that is optimal for membrane and protein stabilization under stress (Livingston et al., 2009 and refs therein).

It is well-known that graminan and levan-type (2,6 linkage forming) FT genes are induced by cold in grasses (del Viso et al., 2009; Tamura et al., 2009; del Viso et al., 2010). We demonstrate here that the β(2,6) linkage forming Pt 6-SST/6-SFT enzyme shows an excellent activity even at lower temperatures (Fig. 3B) and a good levan polymerization
rate at 0°C (Fig. 8A), and both might represent important physiological adaptations to lower temperatures. Particularly, the presence of high DP fructans with β(2,6) linkages, as occurring in *Pachysandra* (Fig. 1), might play an important role in these processes, as suggested before (Volaire *et al*., 1998). A recent extensive screening of 42 annual bluegrass ecotypes, accumulating levan-type fructans, highlighted a strong correlation between the concentrations of high DP fructans and adaptation to subfreezing temperatures (Dionne *et al*., 2009). Noteworthy, the results also revealed that other known cryoprotectants such as sucrose and proline were not closely correlated with variations in cold tolerance in this species. Combined with the results presented here, the data bring a new contribution to the current debate on the adaptive value of fructans for cold adaptation in grasses and fructan accumulating basal eudicots.

**Conclusion**

So far, graminan and levan-type fructans with predominant β(2,6) linkages were believed to be absolutely restricted to monocot plant species. The discovery of cereal-type fructans in *Pachysandra terminalis*, a basal eudicot cold-tolerant species, changed this point of view and confirms the polyphyletic origin of fructan biosynthesis. Enzyme characterization of the native and recombinant versions of an important 6-SST/6-SFT type of FT involved in the synthesis of fructans in *Pachysandra* revealed some remarkable properties, consistent with the point of view that it represents a “premature” FT involved in the regulation of fructan levels in this species. The enzyme shows peculiar, temperature-dependent properties, urging deeper research into the side activities of vacuolar invertases and premature FTs of other fructan plants.

**Materials and Methods**

**Plant material and carbohydrate analyses**

*Pachysandra terminalis* (Buxaceae) was grown on a private field in the Leuven area (Belgium) and harvested in the winter period (February). Soluble sugars were extracted from its rhizomes and analyzed by analytical high-performance anion exchange chromatography and pulsed amperometric detection (HPAEC-PAD DX-300, Dionex, Sunnyvale, CA, USA) as described (Vergauwen *et al*., 2000). Individual fructans were ...
collected manually by using preparative HPAEC-PAD. For some compounds (e.g. peaks f and g, Fig. 1), repeated injections were necessary to collect enough material for further analysis. All the isolated fructans were subjected to enzymatic incubations with sugar beet 6-FEH and chicory 1-FEH IIa (10 U) in reaction mixtures buffered at pH 5.0 with 100 mM NaAc. After several time periods, the reaction was stopped by boiling for 5 min. All assays were analyzed by analytical HPAEC-PAD. In parallel, the isolated fructans were subjected to mild acid hydrolysis as described in Vanhaecke et al. (2006). The degree of polymerization (DP) was determined by the Fru to glucose (Glc) ratio (Table I).

Purification of the 6-SST/6-SFT and Q-TOF MS

*Pachysandra terminalis* Sieb. & Zucc. leaves and stems were removed. Rhizomes were washed with cold tap water and 0.7 kg was homogenized in 0.9 L 50 mM NaAc buffer pH 5.0 containing 1 mM phenylmethylsulfonylfluoride, 1 mM mercaptoethanol, 10 mM NaHSO₃ and 0.1% (w/v) Polyclar AT. The homogenate was squeezed through cheesecloth. Ammonium sulfate was added to a saturation of 30% and stirred on ice for 30 min. After centrifugation for 20 min at 40,000 g the pellet was discarded. Ammonium sulfate was further added to the supernatant up to a final concentration of 80% and stirred on ice for 30 min. This time, the supernatant was discarded after centrifugation for 20 min at 40,000 g. The precipitate was collected and redissolved in 50 mM NaAc buffer (pH 5.0) supplemented with 1 mM CaCl₂, 1 mM MnCl₂ and 1 mM MgCl₂. Undissolved material was spun down for 10 min at 40,000 g. The supernatant was applied to a Concavanalin A - Sepharose column equilibrated with 50 mM NaAc buffer (pH 5.0) supplemented with 1 mM CaCl₂, 1 mM MnCl₂ and 1 mM MgCl₂. The column was washed with the same buffer. Bound proteins were eluted with 0.5 M methyl α-D-mannopyranoside in 50 mM NaAc buffer. The eluate was collected and proteins were loaded onto a Mono S (HR5/5; Pharmacia Uppsala, Sweden) cation-exchange column equilibrated with 50 mM NaAc buffer pH 4.5. Bound proteins were eluted using a linear gradient from 0.0 M to 0.3 M NaCl in 30 min. Fractions of 1 mL were collected. The most active fractions were re-applied onto the Mono S column at pH 4.0 and eluted as described above. SDS-PAGE of the most active fraction occurred in 12.5% (w/v) polyacrylamide gel, staining was performed with Coomassie Brilliant Blue-R250. The purification procedure was performed at 0-4 °C, and 0.02% Na-azide (w/v) was added to all buffers to prevent microbial growth. The SDS-PAGE protein bands (Fig. 2) were subjected to mass spectrometric (MS) identification. The Coomassie Brilliant Blue stained protein bands were excised,
trypsinized, extracted, desalted and analyzed on Q-TOF as earlier described (Van den Ende et al., 2001). Sequence information was derived from the MS/MS spectra with the aid of the MaxEnt 3 (deconvoluting and deisotoping of data) and PepSeq software of the Micromass BioLynx software package.

**Enzyme assays**

During purification of the 6-SST/6-SFT enzyme, aliquots (10-50 µL) were incubated in 50 mM NaAc buffer at pH 5.0 supplemented with 300 mM Suc (final concentration) and 0.02% (w/v) Na-azide. After 30 min incubation, the reaction was stopped by heating at 95°C for 5 min. Samples were diluted threefold with 0.02% (w/v) Na-azide. From these, 25 µL was automatically injected onto the HPAEC-PAD column. Enzymatic activity is expressed in units (U), defined as the amount of enzyme producing 1 µmol of 6-kestotriose per min with 300 mM Suc as a single substrate. The same assay was used to determine temperature and pH optima of the enzyme. The 6-kestotriose standard was a generous gift of Dr. M. Iizuka (Kobe Shoin Women's University, Japan) and was used as a standard. 1&6-kestotetraose (bifurcose) is not commercially available. Small amounts were obtained from wheat crown tissue by preparative HPAEC-PAD. Phlein consists of levan-type fructans ranging from DP4 to DP12, a kind gift of Dr. J. Chatterton (Utah State University, Logan, USA).

**Enzyme kinetics**

For the kinetical analyses, great care was taken to select time-points in the linear region, ensuring that < 10% of the original substrate was consumed. On the one hand, the recombinant 6-SST/6-FT was incubated at 0°C and 30°C in 50 mM sodium acetate buffer (pH 5.0) containing 0.02% (w/v) sodium azide and different Suc concentrations ranging between 50 and 1200 mM. Similarly, the enzyme was incubated together with 150 mM sucrose (donor substrate) and a range of 1-kestotriose concentrations (12.5-800 mM) as acceptor substrate. The incubation reactions were stopped by heating at 90°C for 5 min. The reaction products were analysed by HPAEC-PAD as described (Vergauwen et al., 2000). By comparing the peak areas of 1&6-kestotetraose (6-SFT activity) and 6-kestotriose (6-SST activity) with known amounts of standard compounds, the products were quantified. In the case of 6-SFT activity, an apparent $K_m$ could be estimated based on the linear Hanes plots (Fig. S1) as described before (Schroeven et al., 2008).
RNA preparation, cloning, sequencing and phylogeny

Fresh rhizomes were washed, peeled, cut in small pieces and mixed. Total RNA was isolated by using the Rneasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The peptide sequence YPWSNAQ (part of the N-terminus, see supplemental Table S1) was used to create the sense primer PTN (5’ TAYCCNTGGWSNAAYGCNCA 3’), while part of the C-terminus (WEMNSAF, Table S1) was used to design the antisense primer PTC (5’ AANGCNWRTTACATYTCCTCCA 3’). One-step RT-PCR with these primers (Access RT-PCR System, Promega, Madison, WI, USA) was performed. The RT reaction was performed at 48°C. PCR conditions: 94°C, 3 min; followed by 37 cycles: 94°C, 30 s; 50.5°C, 30 sec and 72°C, 2 min. Final extension was at 72°C, 10 min. A smear was detected after agarose gel electrophoresis. Therefore, a second nested PCR was performed on this reaction mixture by using conserved GH32 specific primers HFQP (5’ GSWTWYCAAYCYCARCC 3’) and CTERMINV (5’ CCNGTNGCRTTGTTRAA 3’). PCR conditions: 94°C, 3 min; followed by 30 cycles: 94°C, 30 s; 48°C, 30 s and 72°C, 2 min. Final extension was at 72°C, 10 min. A clear 1500 bp band was detected. The PCR mixture was ligated in the TOPO-XL vector and transformed to E. coli (TOPO-XL cloning kit, Invitrogen, Groningen, The Netherlands). Plasmids were extracted using the Qiaprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA) and the insert was sequenced. Two specific forward primers PAF1 (5’ GTGTGGGCTTGGACTAAGGA 3’) and PAF2 (5’ AGCTTTGTCAAGGTTGAAG 3’) were selected. In a first RT-PCR, PAF1 was combined with an oligo dT-based primer. The RT reaction was performed at 48°C. PCR conditions: 94°C, 3 min; followed by 37 cycles: 94°C, 30 s; 60°C, 30 s and 72°C, 2 min. Final extension was at 72°C, 10 min. Since multiple bands were still visible, a semi-nested PCR was set up with PAF2 and an oligo dT primer with identical PCR conditions except that an annealing temperature of 57°C was used and 30 cycles were accomplished. The obtained 500 bp PCR product was amplified, cloned in the TOPO-TA vector (TOPO-TA cloning kit, Invitrogen, Groningen, The Netherlands) and sequenced. To clone the 5’ part of the cDNA, the SMART PCR cDNA synthesis kit (Clontech) was used. Total RNA was isolated as described above and single stranded cDNA was produced by using the Takara Bio PrimeScript Reverse Transcriptase. Further manipulations were as described in the manufacturer’s manual. The advantage 2 polymerase kit (Clontech) was used for DNA amplification. In the process, the 5’ SMART primer 5’ CAACGCAGAGTACGCGGG 3’ was combined with the specific 6-SST/6-SFT antisense primer PtR1 (5’ GGATAGGGCGAGGCTCAACATCTC 3’). The obtained PCR product (~ 750 bp) was
cloned in the TOPO-TA vector and sequenced. The phylogenetic tree was created with TREEVIEW as described before (Van den Ende et al., 2005).

Heterologous expression in Pichia pastoris
Because the N-terminal protein part was found (Table S1), the exact mature protein encoding region could be selected. An RT-PCR was performed by using the primers PicPtF (5’ AGCTGCAGTTCGATCCATGGAGCAAGCT 3’) and PicPtR (5’ CATCTAGATCAACGATGGAGGCTGAATG 3’). The Pfu Proofreading polymerase (Promega, Madison, WI, USA) was used (annealing temperature 57°C for 35 cycles). Three independently amplified PCR fragments were first subcloned in the TOPO-XL vector and fully sequenced (TOPO-XL cloning kit, Invitrogen, Groningen, The Netherlands). After vector amplification in E. coli, the DNA fragment was cut out with PstI and XbaI and ligated in the pPICZα B vector (Invitrogen, Groningen, The Netherlands). The resulting expression plasmid pPt6-SFT contains the mature protein part in frame behind the α-factor signal sequence. Further handlings and transformation were as described in Van den Ende et al. (2006).

Acknowledgments
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Literature cited


characterization, cloning, mass mapping and functional analysis of a novel 'cell-wall invertase-like' specific 6-FEH from sugar beet (*Beta vulgaris* L.). Plant J 36, 697–710


Figure legends

Figure 1.
HPAEC-PAD chromatograms of the neutral carbohydrate fractions derived from *Pachysandra terminalis*, *Triticum aestivum*, *Dactylis glomerata* and *Cichorium intybus*. A reference with sugar standards is also included. G, Glc; F, Fru; S, Suc; R, Raffinose; St, Stachyose; 1K, 1-kestotriose; M, maltose; 6K, 6-kestotriose; nK, neokestose or 6\(^G\)-kestotriose; N, 1,1-nystose; B, 1\&6-kestotetraose or bifurcose; F2, inulobiose; F3, inulotriose; IX: inulin with a degree of polymerization of X; LX, levan with a degree of polymerization of X; F2, inulobiose; F3, levantriose. Peaks a-m from *Pachysandra terminalis* were manually collected and further characterized (see Table I).

Figure 2.
SDS-PAGE (12%) of 3 µg native 6-SST/6-SFT enzyme from *P. terminalis* stained with Coomassie Brilliant Blue R250. The size of the molecular weight markers (Mr) in kD is indicated to the left.

Figure 3.
Effect of pH (A) and incubation temperature (B) on the 6-SST activity of the purified 6-SST/6-SFT enzyme from *P. terminalis*. Activities are expressed as a percentage of the maximal activity. Vertical bars indicate SE for n=3.

Figure 4.
Alignment of the *Pachysandra terminalis* 6-SST/6-SFT (FN870376), *Pyrus pyrifolius* vacuolar invertase (the most identical GH32 member, BAF35859), *Hordeum vulgare* 6-SFT (X83233) and *Phleum pratense* 6-SFT (BAH30252). Potential glycosylation sites are underlined. The three carboxylic acids that are crucial for enzyme catalysis (Lammens *et al.*, 2009) are in bold and underlined. Boxes indicate motifs involved in donor and acceptor substrate specificity (Van den Ende *et al.*, 2009). The arrow indicates the N-terminus of the mature 6-SST/6-SFT protein. Consensus line: asterisks (*) indicate identical residues, colons (:) indicate conserved substitutions; and periods (.) indicate semi-conserved substitutions.
Unrooted phylogenetic tree of vacuolar invertases and FTs. Three main groups can be discerned. First group (I): “Type I” vacuolar invertases and a single FT from (basal and core) eudicots. 1, *Pachysandra terminalis* 6-SST/6-SFT (FN870376); 2, *Vitis vinifera* INV (Q9S943); 3, *Beta vulgaris* INV (AJ277455); 4, *Daucus carota* INV (Q42722); 5, *Cichorium intybus* INV (AJ419971); 6, *Pyrus pyrifolia* INV (BAF35859); 7, *Citrus unshiu* INV (AB074885); 8, *Phaseolus vulgaris* INV (O24509); 9, *Vicia faba* INV (Q43857); 10, *Arabidopsis thaliana* INV (AY039610); 11, *Arabidopsis thaliana* INV (AY046009); 12, *Brassica oleracea* INV (AF274298). Second group, subgroup IIA: Type II vacuolar invertases from core eudicots. 13, *Vitis vinifera* INV (QS944); 14, *Daucus carota* INV (X75352); 15, *Lycopersicon esculentum* INV (P29000); 16, *Capsicum annuum* INV (P93761); 17, *Ipomoea batatas* INV (AY037937); 18, *Ipomoea batatas* INV (AF017082). Second group, subgroup IIB: core eudicot fructan biosynthetic enzymes 1-SST and 1-FFT. 19, *Cynara scolymus* 1-SST (Y09662); 20, *Helianthus tuberosus* 1-SST (AJ009757); 21, *Taraxacum officinale* 1-SST (AJ250634); 22, *Cichorium intybus* 1-SST (U81520); 23, *Lactuca sativa* 1-SST (ABX90019); 24, *Echinops ritro* 1-FFT (AJ811624.1); 25, *Cichorium intybus* 1-FFT (U84398); 26, *Cynara scolymus* 1-FFT (AF000068); 27, *Helianthus tuberosus* 1-FFT (AJ009756); 28, *Viguiera discolor* 1-FFT (AJ811625). Third group: subgroup IIIa, FTs and a putative INV from Poaceae. 29, *Hordeum vulgare* 6-SFT (X83233); 30, *Triticum aestivum* 6-SFT (AB029887); 31, *Lolium perenne* putative 6-FT (AF494041); 32, *Poa ampla* 6-SFT (AF192394); 33, *Phleum pratense* 6-SFT (BAH30252); 34, *Lolium perenne* putative INV (AF481763); 35, *Lolium perenne* 6G-FFT (AF492836); 36, *Festuca arundinacea* 1-SST (AJ297369); 37, *Lolium perenne* 1-SST (AY082350); 38, *Triticum aestivum* 1-SST (AB029888); 39, *Triticum aestivum* 1-FFT (AB088409). Subgroup IIIb: monocot vacuolar invertases. 40, *Oryza sativa* INV; 41, *Lolium perenne* INV (AY082350); 42, *Triticum aestivum* INV (AJ65225); Subgroup IIIc: FTs from Asparagales; 43, *Allium cepa* 6G-FFT (AY07838); 44, *Asparagus officinalis* 6G-FFT (AB084283); 45, *Asparagus officinalis* INV (AF002656); 46, *Allium cepa* INV (AJ06067); 47, *Allium cepa* 1-SST (AJ06066); 48, *Agave tequiliana* 1-SST (DQ535031). Subgroup IIIId, monocot vacuolar INV. 49, *Tulipa gesneriana* putative INV (X97642); 50, *Zea mays* INV (P49175); 51, *Oryza sativa* INV (AF276703). The *Pachysandra terminalis* 6-SST/6-SFT is indicated with an arrow. The scale bar indicates a distance value of 0.1.
**Figure 6.**

In vitro fructan synthesis by the purified native and heterologous 6-SST/6-SFT (1 µg/ 80 µl) when incubated with 300 mM Suc and with 300 mM Suc + 300 mM 1-kestotriose. Incubation times: 0h and 13h. Incubation temperature 0°C. A reference with sugar standards is included. G, Glc; F, Fru; S, Suc; 1K, 1-kestotriose; 6K, 6-kestotriose; N, 1,1-nystose. B: 1&6 kestotetaose or bifurcose.

**Figure 7.**

Substrate-velocity plots for the production of 1&6-kestotetraose (abbreviated Bif) from 150 mM Suc and a range of 1-kestotriose concentrations. Reaction temperatures: 0°C and 30°C. The corresponding linear Hanes plots are shown in supplemental Fig. S1. Vertical bars indicate SE for n=3.

**Figure 8.**

Long-term in vitro fructan synthesis by the native 6-SST/6-SFT at 0°C (A, 2.5 µg/ 80 µl) and at 30°C (B, 1 µg/ 80 µl) with 300 mM Suc. A reference with sugar standards, phlein and the carbohydrate profile of *Pachysandra terminalis* are also included for comparison. G, Glc; F, Fru; S, Suc; 1K, 1-kestotriose; 6K, 6-kestotriose; N, 1,1-nystose. B: 1&6-kestotetaose or bifurcose. LX, levan with a degree of polymerization of X.

**Supplementary figure legends**

**Figure S1.**

Linear Hanes plots derived from the data presented in Fig. 7. A: 0°C; B: 30°C.

**Figure S2.**

Long-term in vitro fructan synthesis by the purified native 6-SST/6-SFT at 0°C (A, 2.5 µg/ 80 µl) and at 30°C (B, 1 µg/ 80 µl) with 300 mM Suc. The carbohydrate profiles of *Pachysandra terminalis* and *Dactylis glomerata* are also included for comparison. 1K, 1-kestotriose; 6K, 6-kestotriose; B: 1&6-kestotetaose or bifurcose. LX, levan with a degree of polymerization of X.
Figure 3.
Effect of pH (A) and incubation temperature (B) on the 6-SST activity of the purified 6-SST/6-SFT enzyme from *P. terminalis*. Activities are expressed as a percentage of the maximal activity. Vertical bars indicate SE for *n*=3.
A

Detector response (arbitrary units)

Time (min)

native, Suc, 0h
native, Suc, 13h
native, Suc + 1K, 0h
native, Suc + 1K, 13h
heterologous, Suc, 0h
heterologous, Suc, 13h
heterologous, Suc + 1K, 0h
heterologous, Suc + 1K, 13h
reference

G F S 1K 6K N B
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<tr>
<th>Peak</th>
<th>1-FEH generated products</th>
<th>6-FEH generated products</th>
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Table I. Characterisation of fructans in *Pachysandra terminalis*
**Table II** A typical purification of the 6-SST/6-SFT from *Pachysandra terminalis* rhizomes

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<th>Activity (U)</th>
<th>Recovery (%)</th>
<th>Specific activity (U (mg protein)^{-1})</th>
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