Structure-function analysis of plant fructosyltransferases

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
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Abbreviation List

1-FFT  fructan:fructan 1-fructosyltransferase
1-k    1-kestose
1-SST  sucrose:sucrose 1-fructosyltransferase
6G-FFT fructan:fructan 6G-fructosyltransferase
6-k    6-kestose
6-SFT  sucrose:fructan 6-fructosyltransferase
6-SST  sucrose:sucrose 6-fructosyltransferase
AOX1   alcohol oxidase 1
aclINV Allium cepa vacuolar invertase
bif    bifurcose
cDNA   complementary DNA
ConA   concanavalin A
DNA    deoxyribonucleic acid
DP     degree of polymerisation
faSST  Festuca arundinacea 1-SST;
      re-classified as Schedonorus arundinaceus
FEH    fructan exohydrolase
fru    fructose
FT     fructosyltransferase
HPAEC  High Performance Anion Exchange Chromatography
LSLB   Low Salt Luria Bertani medium
LsuB   large subunit of faSST
MES    2-morpholinoethanesulfonic acid
mRNA   messenger RNA
nys    nystose
PCR    polymerase chain reaction
PEG    polyethylene glycol
pPICZaC Pichia pastoris shuttle vector
SDS-PAGE sodium dodecyl sulphate-polyacrylamide gel electrophoresis
suc    sucrose
YPDS   yeast peptone dextrose sorbitol
Summary

Fructans are an important class of plant carbohydrates that consist of linear or branched chains of fructosyl moieties. Their synthesis requires fructosyltransferases (FTs) that catalyze the transfer of fructosyl units from a donor substrate (sucrose or fructan) to an acceptor substrate (sucrose or fructan). The fructosyltransferases involved in fructan metabolism are related to acid invertases, enzymes that cleave sucrose into glucose and fructose. An invertase can be considered a fructosyltransferase which transfers the fructose moiety to water. The aim of the present work was to elucidate what determines the different catalytic activities of this enzyme group, by use of molecular methods. In order to study such structure-function relationships we artificially introduced mutational changes and constructed chimeric FTs (enzymes with exchanged regions). The goal was to detect the determining regions or single amino acids. For this purpose we optimized the expression of FTs in the methylo trophic yeast *Pichia pastoris* and developed the methodology to create the chimeric constructs. Conventional cloning using conveniently located restriction sites and the method of overlapping PCR was used.

In a first part domain exchanges between two closely related FTs from cereals were analyzed by expressing the corresponding constructs in *Pichia* (Chapter 2). The two subunits of FTs (N-terminal large subunit and C-terminal small subunit) were exchanged between *Festuca arundinacea* (re-classified as *Schedonorus arundinaceus*) sucrose:sucrose 1-fructosyltransferases (1-SST) and *Hordeum vulgare* sucrose:sucrose 6-fructosyltransferase (6-SFT). The study revealed that it is the large subunit that carries the structural features responsible for enzyme specificity.

In a second part we focused on the conserved motifs (S/N)DPNG, RDP and EC, located on the large subunit, that are presumably essential in the active site of plant FTs. For this purpose two other SST-SFT-chimeras with exchanged N-termini encompassing these motifs, as well as *Festuca* 1-SST carrying single amino acid substitutions in the RDP- and EC-motif were analyzed (Chapter 3). This study revealed the importance of the
three hypothesized active site motifs for the transfructosylation reaction. All three of them were shown to be important for enzyme activity and/or for specificity.

In a third part, we addressed the question what structural components determine the relative transferase and hydrolase activities of FTs and vacuolar invertases via a targeted mutational analysis based on sequence comparisons between vacuolar invertases and 1-SSTs, the latter an example of a sucrose-using FT (Chapter 4). We chose *Allium cepa* invertase and *Festuca arundinacea* 1-SST for our analysis. Nine amino acids dispersed along the sequence could be identified correlating with either invertase or 1-SST activities. The selected amino acids of onion invertase were mutated to the corresponding amino acids in *Festuca* 1-SST and *vice versa*. For both enzymes, the mutations were analyzed independently. Functional expression in *Pichia* revealed shifts in the catalytic specificity and activity, demonstrating the importance of these amino acids outside the three highly conserved motifs (S/N)DPNG, RDP, and EC for the enzymatic reaction (Chapter 4).

This work helped to narrow down the region potentially responsible for enzyme specificity in plant FTs. We could pinpoint the importance of the regions with the highly conserved motifs, and of some additional characteristic single amino acids dispersed along the sequence, for enzyme activity and specificity.
Chapter 1

General introduction

1.1. Fructans: occurrence, structure and physiological function

The disaccharide sucrose consists of glucose and fructose and is the main transport sugar in all plants. It can furthermore serve as reserve carbohydrate. Sucrose – dissolved in large quantities in the vacuole – and starch – stored in insoluble form in the amyloplasts or temporary in the chloroplasts – are by far the most common reserve carbohydrates in higher plants. Apart from these, about 15 % of flowering plants use fructans as reserve carbohydrate (Hendry, 1993; Wiemken et al., 1995; Vijn and Smeekens., 1999; Ritsema and Smeekens, 2003a; Ritsema and Smeekens, 2003c). Fructans are “extensions of sucrose”: They consist of linear or branched fructose chains attached to sucrose. As highly water soluble molecules fructans are predominantly stored in the vacuole (Wiemken et al., 1995). Depending on the plant species, fructans mostly reach a DP of 10 to 200 and are very diverse in structure (Vijn and Smeekens., 1999).

In plants fructans occur in many prominent orders like the Asterales, the Liliales, and the Poales, among which are representatives of economic importance (e.g. wheat, barley) (Hendry, 1987; Hendry, 1993). Fructans are classified according to their differences in glycosidic linkage type (Fig. 1). Linear fructans called inulins are composed of $\alpha$(2-1) linked fructosyl units. They typically occur in the order of Asterales (e.g. chicory). Linear fructans containing primarily or exclusively $\alpha$(2-6) linkages occurring in many forage grasses (Poaceae), are called phleins (Waterhouse and Chatterton, 1993). Grasses often contain mixed fructan-types where $\alpha$(2-1) and $\alpha$(2-6) fructosyl linkages are combined within one molecule. These fructans which occur for example in wheat and barley, are called graminans (branched fructans). Graminans sometimes are of even more complex structures where the fructose chains, linked $\alpha$(2-1) and $\alpha$(2-6), are elongated on two sites of the starter sucrose, at the C1 of the fructose,
and/or at the C6 of the glucose residue (e.g. *Lolium perenne*; Pavis et al., 2001; Fig. 1). These fructans, called neo-series fructan, most often occur as inulin neo-series and are widespread in the order of Liliales and Asparageles (e.g. onion and garlic and asparagus; Shiomi, 1989).

In plants, fructan mainly serves as a reserve carbohydrate. Storing fructan instead of sucrose as soluble reserve carbohydrate has several advantages: as soluble polysaccharide fructans are osmotically less active than sucrose, and can therefore be stored in much higher concentrations. Since fructans are highly water soluble and accumulate in the vacuole, the largest cell compartment, storage of very large quantities is possible. In sink organs like roots, tubers, bulbs or stems, as well as in source organs like mature leaves, high fructan concentrations (up to 70% of dry weight) can be stored (Wiemken et al., 1995). Generally fructans are stored if photosynthetic carbon production exceeds demands, and are mobilized if carbon and energy is needed. An example is the rapid breakdown of fructan stored in the leaf base upon defoliation of grasses, providing energy and building stones for the re-growth of leaves (Wagner et al., 1986; Schnyder and Nelson, 1987; Morvan-Bertrand et al., 2001).

If carbon fixation in a leaf exceeds export and demands, accumulation of sucrose can lead to a feedback inhibition of photosynthesis. In this situation the ability to synthesize fructan is a physiological advantage, since vacuolar fructan synthesis lowers the concentration of sucrose in the cell and thus, prevents sugar-induced feedback inhibition of photosynthesis (Housley & Pollock, 1993). For most plants the main reserve carbohydrate is starch. It can accumulate in “source”-leaves as transitory starch in the chloroplasts, or in reserve organs in amyloplasts. Like the accumulation of fructan, storage of transitory starch can lower the sucrose concentration in the leaf, but storage of equivalent amounts of starch as observed with fructans, would inevitably obstruct the chloroplasts and consequently interfere with photosynthesis (Wiemken et al., 1995). Starch biosynthesis decreases dramatically when the temperature drops below 10 °C, whereas photosynthesis and fructan production are much less sensitive to low temperature (Pollock, 1986). Thus, plants having the possibility to accumulate not only starch, but also sucrose and/or fructan can optimally react to environmental conditions and therefore have physiological advantages.
In addition to their function as short and long term storage compounds, fructans presumably protect against drought and freezing stress. This assumption is supported by the fact, that fructan-accumulating plants are especially abundant in temperate and arid climate zones with seasonal frost or drought periods, and are almost absent in tropical regions (Hendry, 1993). Because the cell membranes are primary targets for both freezing and desiccation injuries (Vereyken et al., 2001), fructans are supposed to be involved in the stabilization of membranes. Indeed, it was shown using in vitro systems, that fructooligosaccharides enhance membrane stability during freezing and cellular dehydration through their affinity to phospholipids (Hincha et al., 2000; Vereyken et al., 2001; Vereyken et al., 2003; Hincha et al., 2002). In planta it was shown, that transgenic sugar beet (Beta vulgaris) and tobacco (Nicotiana tabaccum) transformed with a bacterial levansucrase, had enhanced drought and freezing resistance (Pilon-Smith et al., 1999; Konstantinova et al., 2002). Another functions of fructan metabolism is partitioning of assimilates induced by biotic or abiotic factors (Pollock and Cairns, 1991; Suzuki and Chatterton, 1993). A rapid sequence of accumulation and breakdown of fructans in the growth zone of barley leaves (Roth et al., 1997) and during anthesis in Campanula rapunculoides (Vergauwen et al., 2000) and daylily (Bieleski et al., 1993) flowers are examples that lead to the assumption that fructans play a role in cell expansion. Depolymerization of fructans probably contributes to the osmotic driving force involved in cell expansion.

Interestingly also a few algae (green algae or Chlorophyta) and some microorganisms are capable of synthesizing fructans (Hendry, 1993). Bacterial strains such as Bacillus, Actinomyces and Streptococcus produce fructan extracellularly (Cote & Algren, 1993; Hendry, 1993). Bacterial fructans are called levans and are generally composed of (2-6) linked fructosyl residues linked to a terminal sucrose and can reach a DP of up to 100’000. Remarkably, also (2-1) linked fructans and branched fructans are found. There are a few reports on the synthesis of extracellular fructans by fungi, e.g. by Aspergillus, Penicillium and Fusarium (Hendry and Wallace, 1993).
In microorganisms, fructans which are extracellularly produced might act as adhesive material around plant roots or leaves (Leigh and Coplin, 1992). Also for oral fructan synthesizing streptococci such as *Streptococcus mutans*, fructans are believed to serve as a glue and readily mobilized carbohydrate, enhancing the formation of dental plaque (Suzuki and Chatterton, 1993).

Interest in fructans increased during the last decade due to health-promoting effects of fructans for humans. Inulin, mainly isolated from chicory roots, is added to a variety of products like yoghourt and “muesli” as a food additive. Long chain fructans act as emulsifiers and give a better mouth feeling to products like fat-free yoghurt. Short chain fructans and oligofructose can serve as sweeteners. Fructans act as “soluble food fibers”, because the human digestive tract contains no enzymes to degrade \( \beta(2-1) \) and \( \beta(2-6) \) glycosidic linkages. Therefore, fructans pass from the small intestine into the large intestine without being absorbed. Only in the bowel fructans are utilized preferably by the beneficial bifidobacteria. This effect of advantageously altering the balance in the bacterial flora of the intestine is thought to increase gut health (Kleessen et al., 2001). Further beneficial effects of fructan to human health are reported such as an increased calcium resorption, or a lowering of the concentrations of insulin and cholesterol (Jackson et al., 1999; Delzenne and Kok, 2001).
Fig. 1 Representation of sucrose and the derived first representatives of different fructan types. The enzymes for synthesis are indicated using their abbreviations. Arrows indicate the possible sites of fructose chain elongation for the different groups. Abbreviations: 1-SST (sucrose:sucrose 1-fructosyltransferase); 6-SST (sucrose:sucrose 6-fructosyltransferase); 6-SFT (sucrose:fructan 6-fructosyltransferase); 1-FFT (fructan:fructan 6-fructosyltransferase); 6-FFT (fructan:fructan 6-fructosyltransferase); 6G-FFT (fructan:fructan 6G-fructosyltransferase).
1.2. **Enzymes involved in fructan metabolism**

Sucrose is the starting point of fructan metabolism (Fig. 1). Sucrose is synthesized in the cytoplasm from the sequential actions of sucrose-phosphate synthase and sucrose-phosphate phosphatase, and it can be reversibly cleaved by sucrose synthase, or irreversibly hydrolyzed by invertases (Winter and Huber; 2000). Invertases in plants exist in several isoforms with different biochemical properties and subcellular locations (Sturm, 1999). Acid invertases (vacuolar invertases and cell-wall invertases) that are also named β-fructosidases and neutral/alkaline invertases (cytosolic invertases) can be distinguished in plants (Sturm, 1999). Besides cleaving sucrose, also hydrolysis of low DP fructans as well as of raffinose and stachyose has been detected with acid invertases (Marx, 1995). In contrast, alkaline invertases located in the cytosol, are sucrose specific (Sturm, 1999). For the understanding of fructan metabolizing enzymes the acid invertases are of special importance.

Plant fructans are derived from sucrose (Fig. 1). Their synthesis requires fructosyltransferases (FTs) that catalyze the transfer of fructosyl units from a donor substrate (sucrose or fructan) to an acceptor substrate (sucrose or fructan). Synthesis is always initiated by 1-SST (sucrose:sucrose 1-fructosyltransferase), producing the shortest fructan with a (2-1) linkage called 1-kestose besides glucose, from two molecules of sucrose. In this case sucrose serves as both a fructosyl donor and acceptor (Fig. 1; Fig. 2). Chain elongation to higher DP fructan occurs via the action of 1-FFT (fructan:fructan 1-fructosyltransferases), 6-SFT (sucrose:fructan 6-fructosyl transferases) and/or 6G-FFT (fructan:fructan 6G-fructosyltransferases), depending on fructan types synthesized by different plant species. 1-FFT, 6-SFT and 6G-FFT are named according to the glycosidic bond they form.

Inulin type fructans are synthesized by the elongation of 1-kestose via successive attachment of fructosyl units by the action of 1-FFT (Edelman & Jefford, 1968; Fig.2). 1-FFT uses one fructan as a fructosyl donor and attaches it to another fructan or sucrose, thereby shortening one fructan and elongating another one. Sucrose can be used as fructosyl acceptor but not as donor substrate.
The two enzymes, 1-SST and 1-FFT, can only form β(2-1) linked fructans, the inulin, but cereals such as wheat and barley form other types of fructan, the graminans, that have primarily β(2-6) linkages between the fructosyl units (Simmen et al., 1993). The only enzyme so far known to form β(2-6) linkages in cereals, the 6-SFT, has been purified and cloned first from barley (Duchateau et al., 1995; Sprenger et al., 1995). The preferred substrates of 6-SFT are sucrose and 1-kestose leading to the formation of the tetrascarharide bifurcose which is the smallest branched fructan, and glucose (Fig 1; Fig. 2). In the presence of sucrose as the only substrate, the activity of 6-SFT is mainly hydrolytic, leading to the production of glucose and fructose, and only 20% of total activity is directed into the production of 6-kestose. Evidence for the existence of a 1-FFT in barley was obtained by Lüscher and coworkers while purifying barley 1-SST (Lüscher et al., 2000). Studies of oat fructan showed, that also fructans of the neo-series occur in grasses (Livingston et al., 1993). Nevertheless no 6G-FFT (Fructan:fructan 6G-fructosyltransferase), the enzyme known to form these fructans in liliaceae has been cloned to date in grasses. However, such an enzyme has been cloned from onion (Vijn et al., 1997). It can use both, sucrose and low DP inulin as fructosyl acceptors, whilst fructosyl donors can be 1-kestose and low DP inulin, but not sucrose. Although the enzyme shows a high sequence similarity to onion invertase, it cannot hydrolyze sucrose (Vijn et al., 1997). The two FTs known in onion are 1-SST and 6G-FFT. It was shown, that transgenic tobacco BY2 cells expressing onion 6G-FFT and incubated with 1-kestose produced the same fructan-pattern as it is found in onion bulbs (Ritsema et al., 2003). Thus, no 1-FFT seems to be needed in onion for the formation of higher DP fructans of the neo-series. In contrast, in the neo-series producing plant asparagus, 1-SST, 6G-FFT and 1-FFT have been shown to be involved in fructan synthesis (Shiomi, N., Fructan 2004).

It is not much known what determines the typical chain lengths patterns found in different plant species. In one case it has been shown that biochemical differences of two 1-FFTs, namely the FFT from chicory and the one form globe thistle, are determinants of the specific chain lengths synthesized in the two plant species (Vergauwen et al., 2003; Hellwege et al., 1998).
Fructosyltransferases play a key role in the synthesis and breakdown of fructans, which are significant carbohydrate reserves in many plant species. The degree of polymerization (DP) is indicated for fructans with \( m \geq 3 \) and \( n \geq 2 \).

- **1-SST**: \[ \text{sucrose} + \text{sucrose} \rightarrow 1\text{-kestose} + \text{glucose} \]
- **1-FFT**: \[ \text{fructan}_{DP=m} + \text{fructan}_{DP=n} \rightarrow \text{fructan}_{DP=m-1} + \text{fructan}_{DP=n+1} \]
- **6-SFT**: \[ \text{sucrose} + \text{sucrose} \rightarrow 6\text{-kestose} + \text{glucose} \]
  \[ \text{sucrose} + 1\text{-kestose} \rightarrow \text{bifurcose} + \text{glucose} \]
- **6G-FFT**: \[ \text{fructan}_{DP=m} + 1\text{-kestose} \rightarrow \text{neokestose} + \text{fructan}_{DP=m-1} \]
- **FEH**: \[ \text{fructan}_{DP=m} \rightarrow \text{fructan}_{DP=m-1} + \text{fructose} \]
- **INV**: \[ \text{sucrose} \rightarrow \text{glucose} + \text{fructose} \]

**Fig. 2** Main activities of fructosyltransferases in plants. The degree of polymerization (DP) is indicated for fructans with \( m \geq 3 \) and \( n \geq 2 \).

Breakdown of fructan is thought to proceed *via* fructan exo-hydrolases (FEH; Fig. 2), since increased FEH activity correlates with fructan breakdown (Marx et al., 1997). FEHs degrade fructan polymers by splitting off terminal fructosyl residues. Up to now, no evidence for fructan endohydrolases has been found in plants. FEHs preferentially hydrolyzing \([2-1]\)-bonds (1-FEH) or \([2-6]\)-bonds (6-FEH) have been distinguished (Van Damme et al., 1983). Generally sucrose has a strong inhibitory effect on FEHs and seems not to be hydrolyzed (Bonnet and Simpson, 1993). Surprisingly, FEH genes and activities have been recently detected in non-fructan plants, where they might play a role in defense, acting on microbial (exogenous) fructans (Van den Ende et al., 2004).
In plants sugars are not only central metabolites but play a role as signal molecules functioning as regulators of gene expression (Koch, 1996). Thus, by altering sucrose availability, invertases, FEHs and FTs may be indirectly involved in the control of cell differentiation and plant development.

Interestingly, bacterial fructan is generally synthesized by one single enzyme called levansucrase (sucrose:fructan 6-fructosyltransferase). The enzyme cleaves sucrose, releases the glucose and attaches the remaining fructosyl molecule initially to the fructosyl residue of another molecule of sucrose and subsequently, to the growing fructan chain (Cote and Imam, 1989). A side activity of levansucrase is the hydrolysis of sucrose into glucose and fructose.

Glycoside hydrolases have been classified into 87 families based on the similarity of their overall amino acid sequences, assuming that this reflects both structural and mechanistic relationships (Henrissat, 1991). The database of “carbohydrate-active enzymes” (CAZY, [http://afmb.cnrs-mrs.fr/-cazy/CAZY/index.html](http://afmb.cnrs-mrs.fr/-cazy/CAZY/index.html)) groups bacterial invertases and levansucrases into glycoside hydrolase (GH) family 68, whilst fungal and plant invertases and FTs fall into GH family 32. Both these families are members of the glycoside hydrolase clan GH-J (Henrissat, 1991; Pons et al., 2000). The overall sequence homology between the two families is less than 15% although they catalyze very similar reactions.
1.3. The evolution of fructosyltransferases

In the plant kingdom invertases with different biochemical properties and subcellular locations are omnipresent (Sturm, 1999; see introduction 1.2.). In contrast, FTs are of rather limited distribution, restricted to a few although partially very large plant families (Hendry, 1993). Comparison of the amino acid sequences of plant FTs and acid invertases revealed a very high degree of identity (see alignment in appendix and Fig. 5 (chapter 1.5.); Sprenger et al., 1995; Vijn & Smeekens, 1999). The occurrence in unrelated and young plant families as well as the close homology of the enzymes indicate that the capacity for fructan synthesis is a relatively novel trait that most probably developed independently in several genera.

If vacuolar invertases are presented in a phylogenetic tree together with FTs, FEHs and cell-wall invertases, they cluster with FTs whilst FEHs cluster with cell-wall invertases (Fig. 3). The following evolutionary process from invertases to FTs was proposed (Wei and Chatterton, 2001): An ancestral invertase gene duplicated before the divergence of monocots and dicots. One duplicate evolved into cell-wall invertase isoforms and FEHs, and the other evolved into the vacuolar invertases and various FTs. Because of the high degree of similarity of the amino acid sequences, it was speculated that vacuolar invertases were recruited for generating FTs by means of small mutational changes (Hendry, 1993; Sprenger et al., 1995; Pons et al., 1998; Vijn and Smeekens, 1999; Van Laere and Van den Ende, 2002).
Fig. 3 (picture taken from Ritsema and Smeekens; 2003a): Phylogenetic tree of fructosyltransferases, fructan exohydrolases and invertases in monocots (m) and dicots (d). acGFT, Allium cepa 6G-FFT (Y07838); acINV, Allium cepa invertase (AJ006067); acSFT, Agropyron cristatum 6-SFT (AF211253); acSST, Allium cepa 1-SST (AJ006066); aoINV, Asparagus officinalis invertase (AF002656); asSST, Allium sativum 1-SST (AJ006066); atINV, Arabidopsis thaliana invertase (AY142666); ciCIN, Cichorium intybus cell wall invertase (Y11124); ciFEH1, Cichorium intybus 1-FEH I (AJ242538); ciFEH2, Cichorium intybus 1-FEH II (AJ295033); ciFFT, Cichorium intybus 1-FFT (U84398); ciINV, Cichorium intybus invertase (AJ419971); ciSST, Cichorium intybus 1-SST (U81520); csFFT, Cynara scolymus 1-FFT (AJ000481); csSST, Cynara scolymus 1-SST (Y09662); faSST, Festuca arundinaceae 1-SST (AJ297369); htFFT, Helianthus tuberosus 1-FFT (AJ009756); htSST, Helianthus tuberosus 1-SST (AJ009757); hvSFT, Hordeum vulgare 6-SFT (X83233); leCIN, Lycopersicon esculentum cell wall invertase (AF506006); leINV, Lycopersicon esculentum invertase (D11350); lpSFT 6-SFT Lolium perenne (AF494041); lpSST, Lolium perenne 1-SST (AF492836); osCIN, Oryza sativa cell wall invertase (AB073749); osINV, Oryza sativa invertase (AF019113); psSFT, Poa secunda 6-SFT (AF192394); taCIN, Triticum aestivum cell wall invertase (AF030420); taFEH, Triticum aestivum FEH (AJ508387); taSFT, Triticum aestivum 6-SFT (accession number AB029887); taSST, Triticum aestivum 1-SST (AB029888); toSST, Taraxacum officinale 1-SST (AJ250634); zmCIN, Zea mays cell wall invertase (U17695); zmINV, Zea mays invertase (U16123).
1.4. Molecular and biochemical properties of plant fructosyltransferases and acid invertases

Both, vacuolar invertases and FTs, are formed as vacuolar targeted preproenzymes with an N-terminal signal sequence and a propeptide that are cleaved off after protein folding and final targeting (Sturm and Chrispeels, 1990; Sprenger et al., 1995). Typically they are encoded by one single mRNA (Fig. 4). The common feature of plant acid invertases and FTs is to consist of an approximately 80 kD protein, which is cleaved during maturation yielding a N-terminal large subunit and a smaller C-terminal subunit (Fig. 4; Sturm, 1999; Sprenger et al., 1995; Lüscher et al., 2000a; Koops and Jonker, 1996; Van den Ende et al., 1996a and 1996b; Van der Meer et al., 1998)). Whether or not this cleavage has a physiological function is not clear, and also the functions of individual subunits are unresolved. In contrast to these acid invertases and plant FTs which are heterodimers, FEHs are not cleaved in two subunits. Also bacterial fructosyltransferases consist of one subunit only.

Generally plant FTs and acid invertases are glycoproteins. The number of potential glycosylation sites (Asn-X-Ser/Thr) varies between the different proteins. Evidence for N-glycosylation was experimentally proven via binding to ConA/Sepharose columns (Sprenger et al., 1995, Koops and Jonker, 1996). The hypothesized impact of N-glycosylation on protein folding and stability still remains to be proven (Ritsema and Smeekens, 2003a).

At the biochemical level, further similarities between plant FTs and vacuolar invertases are evident. These enzymes differ in their preferential fructosyl donor and acceptor substrates, but generally catalyse also fructosyl- transfers from and to alternative substrates, albeit at a lower efficiency, depending on the type and substrate concentrations provided, the temperature and the ionic strength (Simmen et al., 1993; Cairns 1995; Vijn & Smeekens, 1999). For example barley (*Hordeum vulgare*) 6-SFT and tall fescue (*Festuca arundinacea*; re-classified as *Schedonorus arundinaceus*) 1-SST.
exhibit invertase activity in addition to their main activity, depending on the environment in which they are active. When sufficient amounts of sucrose and 1-kestose are present, 6-SFT guides 80% of its total activity into fructan synthesis, forming bifurcose, and only 20% into the hydrolysis of sucrose. If only sucrose is available as substrate, 6-SFT acts almost purely as a hydrolase (Sprenger et al., 1995). Thus the enzyme is not only highly homologous to vacuolar invertases at the level of amino acid sequence but also retains considerable invertase activity. On the other hand invertases are well known to exhibit some FT activity under certain conditions, forming 1-kestose from sucrose (Obenland et al., 1993; Vijn et al., 1998). In biochemical terms, FTs harbor the intrinsic capacity to act as hydrolases and vice versa.

**Fig. 4** (A) Scheme of barley 6-SFT cDNA. (B) Amino acid sequence of a typical plant FT (6-SFT). Highly conserved motifs are indicated.
The potential close relatedness of enzymes with hydrolyzing and polymerizing activity of glycosidic bonds was experimentally demonstrated in the case of a *Bacillus subtilis* levansucrase (Chambert and Petit Glatron, 1991). The activity of the enzyme could be readily modulated by site directed mutagenesis: if the arginine 331 was mutated to histidine, the ratio between polymerase and hydrolase activities decreased significantly. Moreover, the substitution of arginine 331 with lysine, leucine or serine led to an enzyme that formed trisaccharides but no levans besides hydrolytic activity. In attempts of sequence alignments with acid invertases and plant FTs, the arginine 331 of levansucrase could not be assigned to a certain amino acid, but this is not surprising since the bacterial levansucrases belong to GH family 68 and the plant enzymes to GH family 32.
1.5. Structure-function relationships of fructosyltransferases

Comparison of the amino acid sequence of plant FTs and acid invertases reveals a high degree of identity (Sprenger et al., 1995; Vijn and Smeekens, 1999). An alignment of peptide sequences (Fig. 5) and structural analysis reveals several conserved motifs (Vijn and Smeekens, 1999) for members of protein family GH32 (containing besides invertases and FTs also inulinases and levanases).

The general enzymatic mechanism proposed for glycoside hydrolases of GH family 32 and 68 is a ping-pong mechanism via an enzyme-fructosyl intermediate (Chambert and Gonzy-Treboul, 1976; Song and Jacques, 1999, Vergauwen et al., 2003). It involves the protonation of the glycosidic oxygen followed by a nucleophile attack on the anomeric carbon of the sugar substrate by a carboxylate group. The reaction requires three acidic amino acids: (i) The catalytic nucleophile for the covalent binding of the fructose residue, (ii) an acid/base catalyst that functions as a proton donor and, (iii) an amino acid that is not directly involved in catalysis but acts as a transition state stabilizer (Ozimek et al., 2004).

Alignments and experimental studies with yeast extracellular invertase and bacterial levansucrase, such as affinity labelling, site-directed mutagenesis and random mutagenesis propose three conserved regions involved in the reaction containing the following motifs: The $\beta$-fructosidase motif (Fig. 5; region A), the EC-motif (Fig. 5; region G) and the RDP motif (Fig. 5; region F). The $\beta$-fructosidase motif (first described and defined by Reddy and Maley in 1990) consists of the amino acids NDPNG for all known acid invertases, whereas it is quite variable in FTs (NDPNG, SDPNG, ADPNA, GDPNA etc.). Since the FTs analyzed in this work either carry the motif NDPNG (Festuca arundinacea 1-SST and Allium cepa invertase) or SDPNG (Hordeum vulgare 6-SFT), we subsequently name the $\beta$-fructosidase motif (N/S)DPNG motif.
Fig. 5 (Picture taken from Vijn and Smeekens, 1999): Comparison of amino acid sequences of plant fructosyltransferases with those of acid invertases and cell wall invertases. Alignment of well-conserved regions of invertases and fructosyltransferases. Letters in bold type show almost perfectly conserved amino acids. Numbers above the comparisons represent the amino acid sequence of onion (Allium cepa) 1-SST (Ac1-SST). Region A contains the so-called Suc-binding box NDPNG with the well-conserved Asp. Region G contains the well-conserved Glu, which, together with the Asp, is involved in Suc hydrolysis in invertases. The following sequences were included: 1-SST from onion (accession no. AJ006066), artichoke (accession no. Y09662), Jerusalem artichoke (accession no. AJ009757), and chicory (accession no. U81520); 1-FFT from Jerusalem artichoke (accession no. AJ009756) and artichoke (accession no. AJ00481); 6G-FFT from onion (accession no. Y07838); 6-SFT from barley (accession no. X83233); acid/vacuolar invertases of onion (accession no. AJ006067), asparagus (accession no. AF002656), carrot (accession no. A67163 [DcINV] and accession no. X75351 [DcINV1-1]), tulip (accession no. X95651), tomato (accession no. D22350), bean (accession no. U92438), mung bean (accession no. D10265), potato (accession no. X70368); and cell wall invertases from Arabidopsis (accession no. X78424), tobacco (accession no. X81834), carrot (accession no. X78424), tomato (accession no. AB004558), wheat (accession no. AJ224681), and fava bean (accession no. Z35162).

The $\beta$-fructosidase motif was shown to be essential for activity in yeast invertase (GH32) where Asp23 was identified as the catalytic nucleophile (Reddy and Maley, 1990; Reddy and Maley, 1996). With respect to the catalytic nucleophile in GH68,
mutational studies have been reported for FTs of *Lactobacillus reuteri* 121 and *Bacillus subtilis* levansucrase (Ozimek et al., 2004; Meng & Fütterer, 2003). Changing the putative catalytic nucleophile resulted in a heavily reduced total sucrose hydrolyzing activity.

The Glu204 of the EC-motif in yeast invertase (GH32) is proposed as proton donor during sucrose hydrolysis (Reddy and Maley, 1990; Reddy and Maley, 1996). This conclusion is strengthened further by site-directed mutagenesis and structural analysis of *Bacillus subtilis* levansucrase (GH68), where the mutation of Glu342 to Ala completely abolished enzyme activity. The mechanism proposed by Reddy and Maley for the cleavage of sucrose, basically involves the above mentioned nucleophile, attacking the fructose of sucrose, and a proton donor which might be the glutamate of the EC-motif. In the first step glucose is released and fructose is bound to the nucleophile in an ester linkage. Then this ester linkage can be hydrolyzed using water and thereby releasing the fructose. This mechanism for sucrose hydrolysis is also proposed for the polymerizing activity of members of GH family 32 and GH family 68.

All studies available concerning the highly conserved RDP motif were done with enzymes from family GH68. Studies in bacterial levansucrase propose that the Asp in this motif forms a transient covalent fructosyl intermediate or acts as a transition state stabilizer (Batista et al., 1999; Song and Jacques, 1999; Yanase et al., 2002). Some amino acids in the vicinity of the RDP motif, namely Arg331 in *Bacillus subtilis* levansucrase and the His-296 in *Zymomonas mobilis* levansucrase, were shown to be important for maintaining the enzyme’s polymerizing activity. The hydrolyzing activity was not affected by changing these amino acids (Chambert and Petit-Glatron, 1991; Yanase et al., 2002). In addition, conserved aromatic amino acid residues should be considered as potential candidates influencing the hydrolytic and the transfructosylation activities of glycoside hydrolases and/or polymerases (T. Ritsema; personal communication).

The above described studies were performed using extracellular yeast invertase or bacterial FTs, enzymes showing relatively low overall homologies to plant acid invertases and FTs. But since the \(\beta\)-fructosidase motif, the EC-motif and the RDP motif
are also very conserved in plant FTs, a similar mechanism for sucrose hydrolysis and/or the fructosyl-transfer to sucrose and/or fructan can be expected. Similarly as proposed in the model of yeast invertase, the corresponding glutamate (EC-motif) of plant FTs could serve as a proton donor after a nucleophile attack on sucrose and/or fructan by the aspartate of the \( \beta \)-fructosidase motif. So far only the role of the \( \beta \)-fructosidase motif has been experimentally studied by mutational analysis in plant FTs (Ritsema et al., 2004). The EC- and RDP- motifs of plant FTs have not been investigated so far. It remains to be shown which amino acids are involved in the transfructosylation process and in determining enzyme specificity.
1.6. Three-dimensional structures

Common sequence motifs of GH family 32 and 68 are found also in GH family 43, a family comprising \( \beta \)-xylosidases and \( \beta \)-L-arabinofuriosidases. By means of modelling studies a common six-bladed \( \beta \)-propeller structure for the GH families 32 and 68 has been predicted (Pons et al., 2000), which is homologous to the structure of neuraminidase. The first elucidation of the structure of a GH43 enzyme, namely of *Cellvibrio japonicus* \( \beta \)-L-arabinase A43 (Arb43A), revealed a novel five-bladed \( \beta \)-propeller fold (Nurizzo et al., 2002).

For GH68 the crystal structure from *Bacillus subtilis* levansucrase and for GH32 the crystal structure of the invertase from the bacterium *Thermotoga maritima* were recently elucidated (Meng and Fütterer, 2003; Alberto et al., 2004). Within GH family 32 the structures of the exo-inulinase from *Aspergillus awamori* and of the fructan-exohydrolase (FEH) from chicory followed (Nagem et al., 2004; Verhaest et al., 2005). *Bacillus subtilis* levansucrase was crystallized in both, the ligand-free and -bound (sucrose) state. The exo-inulinase from *Aspergillus awamori* was crystallized in the fructose-bound state, and the invertase from *Thermotoga maritima* and the FEH from chicory in the ligand-free states only.

All structures show a five-bladed \( \beta \)-propeller with a deep negatively charged central pocket (Fig. 6). One propeller consists of five \( \beta \)-sheets that adopt the classical “W” topology of four antiparallel \( \beta \)-strands that are named A to D. The N-terminal A strand lines the central cavity, and the C-terminal D-strand the periphery. The \( \beta \)-sheets are packed face-to-face and show a characteristic propeller blade-like twist. In contrast to the crystallized levansucrase, the structures from enzymes of the GH family 32 are composed of two modules, namely the five-bladed \( \beta \)-propeller and the C-terminal \( \beta \)-sandwich (Fig. 6). The \( \beta \)-sandwich consists of two sheets of six \( \beta \)-strands. The two modules are linked via a ten residue linker. The structures provide a template for all members of GH family 32 including acid invertases and also plant FTs (Alberto et al., 2004). The C-terminal \( \beta \)-sandwich module corresponds to the C-terminal small subunit
found in plant vacuolar invertases and FTs, where it usually consist of the C-terminal third of the protein.

![Central pocket: NDPNG; RDP; EC (I)](image)

The active site in the available structures is positioned at one end of the cavity at the center of the β-propeller, with a funnel like opening towards the surface of the molecule. This central pocket is heavily negatively charged. The pocket in the crystal structure of *B. subtilis* accommodates a single sucrose molecule in the substrate-bound structure of an inactive levansucrase mutant (Meng and Fütterer, 2003). The fructosyl unit of sucrose is located at the bottom of the pocket and the glucose moiety on top. The pocket is composed almost exclusively of amino acids that belong to highly conserved sequence motifs. Included are three highly invariant acidic residues, D86, D247 and

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**Fig 6 A.** *Thermotoga maritima* invertase. Picture taken from Alberto et al., J. Biol. Chem. 2004; 279:18903-18910.

The central pocket is the active site. Involved in enzymatic activity are D in the β-fructosidase motif (N/S)DPNG, D in the RDP-motif and E in the EC-motif.

**Fig 6 B.** *Cichorium intybus* FEH IIa. Picture taken from Verhaest et al., The Plant J. 2005; 41:400-411.
E342. D86 corresponds to the catalytic nucleophile of the -fructosidase motif and E342 to the general acid/base catalyst of the EC-motif that were described in more detail in chapter 1.5. The corresponding acidic amino acids can be determined via sequence comparison for members of GH32 and correspond to D17 and E190 in Thermotoga maritima invertase, D41 and E241 in Aspergillus awamori exo-inulinase, and D22 and E201 in chicory FEH where they also flank the active site. The third acidic amino acid, D247 of B. subtilis levansucrase is also flanking the active site in the other structures and corresponds to the aspartate of the highly conserved RDP motif. It is proposed to be a transition state stabilizer. In all structures the three highly conserved acidic amino acids flanking the central pocket are members of the conserved motifs (N/S)DPNG, RDP and EC within the GH32.

Thermotoga maritima invertase (Fig. 6A) and chicory FEH (Fig. 6B) were crystallized only in the ligand-free state, but since the catalytic modules of invertase, FEH and levansucrase are structurally related, the comparison allowed the modeling of the position of the substrate in the active site. The crystal structures of invertase and FEH revealed a glycerol molecule that was present in the active site. It mimics the O4' and O6' hydroxyl-groups of the fructose-moiety of sucrose or inulin respectively. This feature helped furthermore in defining the precise position of the modeled substrate molecule in the active site.

The crystallographic data show that the aspartate of the -fructosidase motif and the glutamate of the EC-motif are in the ideal position to be in close contact with their substrate sucrose or fructan respectively.

No crystal structure of a plant fructan synthesizing enzyme or a plant acid invertase is yet available. These plant enzymes are only up to 15% identical to members of GH68 such as B. subtilis levansucrase and should rather be compared to members of their own family, namely GH32. From the available structures, chicory FEH is the closest relative to plant FTs as well as to plant acid invertases with an amino acid sequence identity of 30 to 40%.

Mutational studies (Chapter 1.5.) combined with the available knowledge concerning crystal structures provide further insight into the mode of action of members of GH family 32 and 68. The question what determines enzyme specificity remains unknown.
1.7. Expression systems for fructosyltransferases

The fact that vacuolar invertases show FT activity under certain condition led to a considerable debate in the literature about the existence of specific fructan-synthesizing enzymes (Cairns, 1995). Clear evidence for the existence of specific FTs has appeared only with the purification of the corresponding enzymes (Simmen et al., 1993; Van den Ende et al., 1996, Sprenger et al., 1995). The activities of the purified FTs are clearly different from the activities of invertases. However, until today the allocation of a sequence to either vacuolar invertases or FT is rather speculative. A reliable classification requires functional analysis which can be achieved by the use of a gene expression system.

The first successfully used expression system for FTs was transient expression in tobacco (Nicotiana plumbaginifolia) protoplasts (Sprenger et al., 1995), tobacco being a non fructan producing plant. Expression of FTs in plant cells has advantages over bacterial or fungal expression systems. As vacuolar enzymes, FTs are N-glycosylated and potentially the glycosylation has an effect on the activity and specificity of the enzyme. Bacteria do not N-glycosylate proteins and in fungi, like yeast, the pattern of glycosylation differs from that in plants with respect to the kind and amount of sugar residues added (Elbein, 1991). Furthermore, enzyme processing during targeting differs in plant cells from that in bacterial- or yeast-cells. Barley 6-SFT was successfully expressed as first FT in tobacco protoplast (Sprenger et al., 1995). After a short lag phase, the protoplasts steadily accumulated an activity that formed 1-kestose from sucrose, and bifurcose from sucrose and 1-kestose. The experiment unequivocally proved that the cloned cDNA encoded a functional 6-SFT. However, the protoplasts did not accumulate higher DP fructans, and a possible hydrolytic activity of the expressed enzyme could not be determined since the hydrolytic background activity in this expression system was too high.
In order to further characterize barley 6-SFT, it was stably expressed in the non-fructan plant tobacco and in the inulin type fructan accumulating plant chicory (Sprenger et al., 1997). Comparing these two plants was of special interest, since the \textit{in vivo} effect of having sucrose as the sole substrate (tobacco) could be compared to having both, sucrose and 1-kestose as substrates (chicory). Transformants of tobacco synthesized the trisaccharide 6-kestose and a series of higher DP fructans of the phlein type. Transformants of chicory, a plant naturally only producing inulin, synthesized in addition branched fructans of the graminan type. The main branched fructan found was the tetrasaccharide bifurcose, which also represents the dominant fructan found in barley leaves. Similarly as barley 6-SFT, onion 6G-FFT was expressed in chicory and tobacco plants as well as in tobacco protoplasts (Vijn et al., 1997). A low activity of this enzyme was found in protoplasts and tobacco plants, and in chicory the results were ambiguous because of the interfering FT activities. Recently, another plant system, tobacco BY2-cells, was successfully used to express onion 6G-FFT (Ritsema et al., 2003). The limitation of this system was again the high background invertase activity.

The disadvantage of using plant expression systems for FTs is that all plants contain interfering invertases. This feature is especially inconvenient since the relative hydrolase and transferase activities of FTs are of special interest. The question what structural components are determining the balance between the polymerase or hydrolase activities of these enzymes is a key question. For FTs it has furthermore to be shown what determines enzyme specificity. A convenient tool for such structure-function studies is a suitable gene expression system.

In 1998, Hochstrasser and Co-workers introduced the \textit{Pichia pastoris} yeast expression system for FTs. The big advantage of the methylotrophic yeast \textit{Pichia pastoris} compared to plant expression systems is that it does not secrete sucrose metabolising enzymes such as invertase. Due to the special design of the \textit{Pichia} expression system plasmids, the recombinant enzymes are secreted to the medium where no invertases interfere with the activity of interest. The responsible element for targeting to the extracellular space is the secretion signal of the \textit{Saccharomyces cerevisiae} $\alpha$-factor.
*Pichia* expression vectors, like the one that was used in this work (pPICZ\(\alpha\)C), have the secretion signal coding region 5’ to the cloning sites, so that the sequence of the gene of interest can be cloned as translational fusion. A methanol inducible promoter (AOX1) is located on the plasmid 5’ of the secretion signal (Fig. 7). By feeding *Pichia* in induction culture with methanol as the carbon source, enzyme production is induced. The *Pichia* shuttle vector carries the gene for Zeocin resistance. *Pichia* transformed with the corresponding vector can be selected against Zeocin that functions as an inhibitor of DNA replication.

In comparison to the conventional yeast *Saccharomyces cerevisiae*, *Pichia* has the advantage that it does not hyperglycosylate secreted proteins. Both *Saccharomyces cerevisiae* and *Pichia pastoris* have a majority of N-linked glycosylation of the high-mannose type, but the length of the oligosaccharide chains added by *Pichia* (average 8-14 mannose residues per side chain) is much shorter than that in *Saccharomyces cerevisiae* (50-150 mannose residues). Thus, the glycosylation of secreted proteins in *Pichia* is closer to plants (Grinna & Tschopp, 1989; Cregg et al., 1993). Very little O-linked glycosylation has been observed in *Pichia*.

![Fig. 7 Scheme of the expression unit of FTs translationally fused to the myc-epitope and a 6xHistidine tag cloned into the *Pichia* shuttle vector, pPICZ\(\alpha\)C.](image)

The expression system furthermore offers the possibility to tag enzymes with a myc-epitope and a 6xHistidine tag translationally fused to the C-terminus of the enzymes (Fig. 7). The 6-SFT from barley and the 1-SST from *Festuca* were initially expressed as untagged versions in *Pichia* (Hochstrasser et al., 1998; Lüscher et al., 2000b) (Fig. 8A). Tagging of FTs carries a potential risk since the tags could disturb the correct folding of a protein due to the changes they cause in size and charge. A first trial for the expression of
a tagged FT was done with barley 6-SFT (Fig. 8; Altenbach, Diplomathesis 2000). The tags had no influence on enzyme activity. Tagged 6-SFT retained all characteristics of its untagged recombinant counterpart. In this work we expressed all constructs with a myc-epitope and a 6xHistidine tag (Fig. 8B).

Previous expertise showed that the expression of FTs in *Pichia* is especially successful, when only the sequence corresponding to the mature proteins were cloned between the secretion signal and the tagging epitopes (Hochstrasser et al., 1998). The expression level of recombinant 6-SFT carrying the entire N-terminus including the plant vacuolar targeting signal, was found to be heavily reduced in comparison to the expression of the sequence encoding the mature protein only. Possibly, the combination of the α-factor secretion signal with the sequence of the vacuolar targeting signal led to mistargeting of the proteins in the secretion process. Therefore all subsequent expression studies were performed with the sequences encoding mature proteins.

![Diagram](image.png)

**Fig. 8** Constructs introduced into plasmid pPICZαC for expression in *P. pastoris*. (A) Original constructs with the natural stop codon. (B) Constructs where the stop codon was changed to an XbaI site, yielding recombinant proteins with C-terminal tags.
This expression system was the ideal tool to study structural features of plant FTs determining the balance between their specific polymerase or hydrolase activities. An optimized expression procedure of recombinant tagged FTs was achieved in this work, and enabled the analysis of the effect of mutational changes (Chapter 2, Chapter 3, Chapter 4) as well as domain exchanges (Chapter 2, Chapter 3; Nüesch, Diplomathesis 2003) between different FTs.
1.8. Aim of the thesis

Many plant FTs have been sequenced during the last few years, and the data clearly indicated that a high homology exists between the different FTs and the vacuolar invertases. At the biochemical level further similarities between plant FTs and vacuolar invertases are evident. Depending on conditions, FTs harbor the intrinsic capacity to act as hydrolases and *vice versa*. Because of the high degree of similarity at the molecular and biochemical level it was speculated, that vacuolar invertases were recruited for generating FTs by means of small mutational changes. It is also speculated that the distinct fructosyl donor and acceptor specificities of different FTs are determined by slight modifications of the amino acid sequence.

If in nature a few amino acid changes were the basis for the evolution of FTs from invertases, it should be possible to artificially influence FT activity and specificity by introducing mutational changes and/or by exchanging regions between different FTs. In order to pinpoint structural requirements for enzyme activity and/or specificity, we functionally characterized chimeric FTs (Chapter 2; Chapter 3), and FTs carrying mutational changes (Chapter 3; Chapter 4), *via* expression in the yeast *Pichia pastoris*. 
Chapter 2

The large subunit determines catalytic specificity of barley sucrose:fructan 6-fructosyltransferase (6-SFT) and fescue sucrose:sucrose 1-fructosyltransferase (1-SST)

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2.1. Abstract  Plant fructosyltransferases are highly homologous in primary sequence and typically consist of two subunits but catalyse widely different reactions. Using functional expression in the yeast *Pichia pastoris*, we show that the substrate specificity of festuca sucrose:sucrose 1-fructosyltransferase (1-SST) and barley sucrose:fructan 6-fructosyltransferase (6-SFT) is entirely determined by the large subunit. Chimeric enzymes with the large subunit of festuca 1-SST (LSuB) and the small subunit of barley 6-SFT have the same catalytic specificity as the native festuca 1-SST, and *vice versa*. If the LSuB is expressed alone, it does not yield a functionally active enzyme, indicating that the small subunit is nevertheless essential.

*Key words*: Cereals; Enzyme specificity; Fructosyltransferase; Pichia pastoris; Sucrose:sucrose 1-[]-D-fructosyltransferase (1-SST); Sucrose:fructan 6-[]-D-fructosyltransferase (6-SFT)

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2.2. Introduction

Fructans are an important class of carbohydrates in plants [1-3]. The enzymes characteristic of plant fructan metabolism, fructosyltransferases and fructan hydrolases, have been found to be highly homologous to the plants' soluble acid [-fructosidases (invertases) belonging to glycoside hydrolase family 32 [2,4]. Indeed, the first plant fructosyltransferase to be cloned, the 6-SFT from barley (Hordeum vulgare), displayed both 6-SST/6-SFT and [-fructosidase activity [5]. In contrast, the first 1-SST of grasses to be cloned, the one of tall fescue (Festuca arundinacea), produced almost exclusively 1-kestose and glucose when supplied with sucrose and had very little [-fructosidase activity [6]. In view of their striking homologies, fructosyltransferases may have evolved from [-fructosidases by relatively few mutational changes [4,5,7]. However, it is unknown which changes are essential for changes of catalytic specificity.

Typically, plant acid [-fructosidases and fructosyltransferases are synthesized as a primary translation product of ~85 kDa but then are cleaved into a large N-terminal subunit of ~60 kDa and a small C-terminal subunit of ~25 kDa [5,8-11]. The large subunit contains putative catalytic motifs for sucrose binding and hydrolysis, namely the [-fructosidase motif, the RDP motif and the EC-domain [3,10]. The importance of these motifs was experimentally proven for the [-fructosidase motif and the EC-motif in the case of yeast invertase [12] and for the RDP motif in the case of a bacterial fructosyltransferase [13].

We have previously established a convenient heterologous expression system in Pichia pastoris to study fructosyltransferases of plants [6,14]. In the present work, we describe how this system can be optimised, and we use it to investigate the catalytic activity of chimeric enzymes generated by exchanging the large and small subunit of 6-SFT and 1-SST, respectively. Our results show that it is the large subunit of the enzyme which determines its catalytic properties.
2.3. Materials and Methods

2.3.1. Microbial strains and vectors used for cloning and heterologous expression

*Escherichia coli* strain DH5α was used for amplification of the recombinant plasmids pK18, pBluescript KS+ (Stratagene, Amsterdam, The Netherlands), pPICZαC. *Pichia pastoris* strain X-33 (wild type), and the pPICZαC shuttle vector were obtained from Invitrogen BV (Leek, The Netherlands).

2.3.2. Cloning and mutagenesis

Constructs P1 and F1 in pPICZαC, representing the native coding sequences of barley 6-SFT (EMBL X83233) and festuca 1-SST (EMBL AJ297369), respectively (Fig. 1A,B), were described earlier [6,14]. To obtain myc- and 6xhis-tagged versions of the two enzymes, their coding regions were excised with *Eco*RI and *Xba*I and subcloned into pK18. The stop codons were then altered to *Xba*I restriction sites by PCR, using primers P3f and P3r for 6-SFT and SST001 and SST002 for 1-SST (Table 1). The resulting PCR products were purified and digested with *Age*I and *Xba*I for 6-SFT and with *Nru*I and *Xba*I for 1-SST, respectively, ligated into the correspondingly digested parent plasmid, and excised from the plasmid by *Eco*RI and *Xba*I. These fragments were cloned in frame with the myc-epitope and his-tag into the *Pichia* shuttle vector, leading to pPICZαC-P3 and pPICZαC-F2 (Fig. 1C,D). Note that we used the tags here simply to verify that the recombinant proteins are expressed, but that we would like to use them, in the future, to purify high levels of recombinant proteins to produce antibodies against them; there are still no specific antibodies against invertases or plant fructosyltransferases available.
Fig. 1 Constructs introduced into plasmid pPICZ\textsuperscript{a}C for expression in \textit{P. pastoris}. A, B: Original constructs with the natural stop codon. C,D: Constructs where the stop codon was changed to an Xba\textsuperscript{i} site, yielding recombinant proteins with C-terminal tags. E: Swap1: large subunit of festuca 1-SST with the small subunit of barley 6-SFT fused to it. F: Swap2 large subunit of barley 6-SFT with the small subunit of festuca 1-SST fused to it. G: large subunit of festuca 1-SST expressed alone.
Table 1
Oligonucleotides used for cloning

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<td>SST006</td>
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</tbody>
</table>

Mutant P3[DTN], in which the first three amino acids (EAD) of the small subunit of barley 6-SFT are changed into the motif DTN typical of FFTs, was generated by site directed mutagenesis using standard procedures (QuikChangeTM Site-Directed Mutagenesis Kit, Stratagene), based on the pK18-clone containing the tagged version of barley 6-SFT. Primers ADDA008 and ADDA009 (Table 1) were designed to introduce the desired mutation and an additional restriction site (AclI).

2.3.3. Construction of recombinant tagged enzymes with exchanged large and small subunits

To obtain Swap1 (Fig. 1E), a construct with the large subunit of 1-SST at the N-terminus followed by the small subunit of 6-SFT, we made use of a PstI restriction site present close to the beginning of the small subunit in both festuca 1-SST and barley 6-SFT. In 1-SST there is another PstI site; therefore the 1-SST containing plasmid pPICZαC-F1 was digested using KpnI, XbaI, and the resulting SST-fragment
was ligated into pBluescript leading to pBluescript1. This plasmid was digested with \textit{PstI} and \textit{XbaI} to cut off the small subunit of the festuca 1-SST. Digesting of pK18-P3 with \textit{PstI} and \textit{XbaI} yielded the DNA-sequence corresponding to the small subunit of barley 6-SFT. This was ligated into pBluescript1 containing the \textit{KpnI-PstI}-fragment of festuca 1-SST, leading to the plasmid pBlueSwap1. To include the entire mature sequence of the large subunit of festuca 1-SST in the swap-construct, the plasmids pBlueSwap1 and pPICZ\textsc{a}C-F1 were digested \textit{KpnI} and \textit{XbaI}, and the fragments ligated. This resulted in plasmid pPICZ\textsc{a}C-Swap1.

For Swap2 (Fig. 1F), the large subunit of barley 6-SFT was coupled to the small subunit of festuca 1-SST by overlapping PCR [15], using the primer pairs SFT001/P4001 and SST006/P4002, respectively, to amplify the coding regions of the C-terminus of the large subunit of barley 6-SFT (from a conveniently located \textit{XmaI} site to the subunit cleavage site) and the small subunit of festuca 1-SST. The PCR-amplified fragments were mixed in equimolar amounts, denatured and re-annealed for a second PCR using SFT001 and SST006 as primers. The full-length product created in this way was digested with \textit{XmaI} and \textit{XbaI} and ligated into the \textit{XmaI/XbaI} digested pPICZ\textsc{a}C-P3, resulting in pPICZ\textsc{a}C-Swap2.

2.3.4. \textit{Cloning procedure to obtain the LsuB expressed alone}

A \textit{XbaI} site was introduced in the plasmid pK18-F2 5bp upstream of the beginning of the small subunit by site directed mutagenesis (QuikChangeTM Site-Directed Mutagenesis Kit, Stratagene), using primers SST004 and SST005 (table 1). This allowed construction of a tagged version of the large subunit (Fig. 1G). All inserts in pPICZ\textsc{a}C were sequenced after cloning and found to correspond exactly to the desired constructs.

2.3.5. \textit{Expression of fructosyltransferases in Pichia pastoris}

The sequences were all cloned in frame behind the \textsc{a}-factor signal sequence of the expression vector pPICZ\textsc{a}C, to allow entry into the secretory pathway. Competent
Pichia pastoris cells were transformed according to the EasyComp transformation protocol (EasySelect™ Pichia Expression Kit, Invitrogen BV).

Expression in Pichia pastoris was performed as described [14], with minor modifications. The P. pastoris strain X-33 was transformed with 4 μg of PmeI-linearized constructs and plated on selective YPDS/Zeocin plates. To screen for activity, some of the newly grown colonies were inoculated in liquid culture. The best-growing colony was selected and used for further experiments. This strain was then grown in liquid culture, and the transgene was induced with 1% methanol, added at 15, 24, 36 and 42h of induction. Generally, the cultures were used for experiments after 48h of induction. The culture medium was harvested by centrifugation, concentrated 50-fold by dialysis against solid PEG 35000, and then desalted using desalting columns equilibrated with 50 mM MES (NaOH) buffer (pH 5.75).

2.3.6. Characterization of recombinant fructosyltransferases

Enzyme assays were performed for 1-5h at 27°C, with 100 mM sucrose (suc) or with 50 mM of all other substrates and the products formed were analysed by anion exchange chromatography as described [16]. Catalytic specificity was tested with the following substrates: sucrose, 1-kestose, nystose and a combination of sucrose with 1-kestose. A maximum of 7% of the substrates was used up after 5 h of incubation. In order to keep the substrate concentration constant during the experiments, the amount of protein used per enzyme assays was ca 0.5 μg for festuca 1-SST and 5 to 10 μg for all other constructs.
2.4. Results and Discussion

2.4.1. Expression of recombinant plant fructosyltransferases in Pichia pastoris

Previously, we introduced *Pichia pastoris* as a heterologous expression system for plant fructosyltransferases such as barley 6-SFT [14] and festuca 1-SST [6]. The fact that *Pichia* does not produce any invertases or fructosyltransferases makes it especially suitable for this purpose since there are no background activities that might interfere with our activities of interest. In this early work, we used pPICZαC without making use of the potential of this plasmid to express a myc-epitope and a 6xhistidine tag at the C-terminus of the recombinant protein. We now constructed barley 6-SFT and festuca 1-SST containing these tags (Fig. 1C, D) and compared their expression levels and enzymatic specificities with the parent untagged versions (Fig. 1A, B). In both cases, the tagged versions were expressed to a similar degree as the corresponding untagged parent enzymes, and the tagged versions had the same catalytic specificity as their native parent enzymes (data not shown). Western blots with antibodies against the tags revealed a major band of approximately 85 kDa (data not shown), indicating that the recombinant enzymes are well expressed but not cleaved into two subunits, as demonstrated previously for recombinant untagged 1-SST in *Pichia pastoris*[6].

Since the yield of recombinant proteins in our original protocol [6,14] was relatively low, we attempted to improve it. Previously, we had used 0.5% (v/v) methanol, applied every 24h, to induce the transgenes (which are under control of the AOX1 promoter, see Fig. 1). Both with 1-SST and 6-SFT, we achieved an approximately 50 times higher yield of enzyme activity when we added methanol at 1% (v/v) final concentration at 0, 15, 24, 36, and 42h of induction (data not shown).

2.4.2. Study of the possible role of a conserved motif at the start of the small subunit

The putative catalytic domains ([β]-fructosidase motif, RDP motif, EC-motif) of fructosyltransferases are located on the large subunit. In order to search for domains possibly important for enzyme specificity on the small subunit, we compared
invertases and fructosyltransferases, and found the first three amino acids at the (putative) N-terminus of the small subunit to be conserved. In fact, for barley 6-SFT, the N-terminus of the small subunit was experimentally determined [5] and shown to start with the motif EAD. Remarkably, most of the related plant [-]fructosidases, 1-SSTs, 6-SFTs, and also 6G-FFTs have the triplet EAD, while none of the currently known fructan:fructan fructosyltransferases (FFTs) has this motif. The FFTs of *Cynara scolymus* (AJ000481) and *Cichorium intybus* (U84398) show the motif DTN at the corresponding position in sequence alignments. To investigate the importance of these differences on enzymatic specificity, we replaced the motif EAD in our construct P3 by DTN, using site-directed mutagenesis.

**Fig. 2** Catalytic activities of recombinant tagged barley 6-SFT (P3) with the native EAD motif (**A, C**) and comparison to the mutated version (P3[DTN]) (**B, D**). Time course of product formation over a period of 0 to 5 h with 100 mM sucrose (**A, B**) or 100 mM sucrose and 50 mM 1-kestose (**C, D**) as substrates.
The enzymatic activities of the tagged recombinant barley 6-SFT (P3, with the native EAD sequence) and its derivative, the mutated 6-SFT (P3[DTN]) were compared, using either sucrose alone or a mixture of sucrose and 1-kestose as substrate. When sucrose was offered as the sole substrate, fructose, 1-kestose and 6-kestose-levels linearly increased with incubation time in exactly the same way in both enzyme preparations (Fig. 2A,B). Similarly, with sucrose and 1-kestose as substrates, the products fructose, 6-kestose and bifurcose were formed by both enzymes in an almost identical manner (Fig. 2C,D). Thus the mutational change of the motif EAD to DTN does not affect enzymatic activity of recombinant 6-SFT.

2.4.3. Chimeric enzymes with exchanged large and small subunits

To learn more about the function of the two subunits of plant fructosyltransferases, we studied the enzymatic properties of chimeric enzymes which contained the large subunit of festuca 1-SST coupled to the small subunit of barley 6-SFT (Swap1) and vice versa (Swap2). Furthermore we also expressed the large subunit of festuca 1-SST alone. The activity of the hybrid enzymes was markedly reduced compared to the “wild-type”-enzymes. The small subunit might play a role in the level of activity and this in a cooperative way with its “own” large subunit. However the recombinant proteins with exchanged (swapped) subunits were functional fructosyltransferases and had catalytic activities that were qualitatively almost identical to the parent enzyme contributing the large subunit.

Thus, when incubated with 100 mM sucrose, Swap1 produced mainly 1-kestose, a small amount of fructose and traces of nystose, just like the parent enzyme contributing the large subunit, 1-SST (Fig. 3A). There was no measurable production of 6-kestose with Swap1, as might have been expected if the small subunit derived from 6-SFT contributed to catalytic specificity. Similarly, when Swap2 was incubated with 100 mM sucrose, it exhibited the same enzymatic activity as the parent enzyme contributing the large subunit, 6-SFT. Swap2 primarily acted as a β-fructosidase, yielding fructose, and produced 1-kestose and 6-kestose in the same proportions as the parent enzyme, 6-SFT (Fig. 3B).
Fig. 3 Relative enzyme activities of 1-SST (F2) / Swap1 (A, C) and of 6-SFT (P3) / Swap2 (B, D), using 100 mM sucrose (A, B) or 100 mM sucrose and 50 mM 1-kestose (C, D) as substrates. In incubations with 100 mM sucrose, the total activity of the protein was approximately 125 nkat/mg protein for F2, 0.75 nkat/mg protein for P3, 0.7 nkat/mg protein for Swap1 and 0.05 nkat/mg protein for Swap2. Black bars: recombinant tagged 1-SST and 6-SFT, respectively. Adjacent white bars: Chimeric tagged proteins with the same large subunit, but an exchanged small subunit. Data represent means and standard deviation (n= 6, corresponding to six different enzyme preparations from six separate induction experiments). The sum of products formed is set to 100%.
The same picture emerged when enzymatic activities were compared in the presence of sucrose and 1-kestose as substrates. Swap1 yielded nystose and fructose in the same proportions as the parent enzyme delivering the large subunit, 1-SST, but no bifurcose as would have been expected if the enzyme contributing the small subunit, 6-SFT, had a role in enzymatic specificity (Fig. 3C). *Vice versa*, Swap2 yielded bifurcose in the same proportion as the parent enzyme delivering the large subunit, 6-SFT, but no nystose as would have been expected if the enzyme contributing the small subunit, 1-SST, had a role in enzymatic specificity (Fig. 3D).

1-SST acts as a fructan exohydrolase when incubated with 1-kestose or nystose alone [6]. This is also true for Swap1, comprising the large subunit of 1-SST: Both the parent enzyme 1-SST and Swap1 yielded considerable amounts of fructose when incubated with 1-kestose or nystose (Fig. 4A,B). Interestingly, both the parent enzyme and Swap1 additionally act as a FFT when incubated with 1-kestose, as indicated by the fact that about 15% of the products represent nystose (Fig. 4A).

**Fig. 4** Relative enzyme activities of 1-SST (F2, black columns) and Swap1 (white columns), using 50 mM 1-kestose (A) or 50 mM nystose (B) as substrates. Data represent means and standard deviation (n= 6, corresponding to six different enzyme preparations from six separate induction experiments). The sum of products formed is set to 100%.
In contrast, both 6-SFT and recombinant Swap2, comprising the large subunit of 6-SFT, displayed no measurable product formation in incubations with either 50 mM 1-kestose alone or nystose alone (data not shown).

To obtain further insight into the importance of the small subunit, we attempted to express a tagged version of the large subunit of festuca 1-SST alone (LSuB). However, we could not observe any enzyme activity in the corresponding preparations from the \textit{P. pastoris} culture fluid (data not shown). In addition, immune blots with antibodies directed against the tags did not reveal any antigenic proteins in the preparations from the culture fluid; however, an immune-responsive protein of the correct size (approximately 50 kD) was present in preparations obtained by boiling induced cells in the presence of 0.5% SDS and 500 mM NaCl, followed by de-salting (data not shown). These data indicate that the large subunit alone is translated correctly in \textit{Pichia}, but not correctly folded and thus not secreted. The retained protein showed no activity in enzymatic assays using lysed cells (data not shown).

In conclusion, our data show that the small subunit of fructosyltransferases is essential for functional expression of the protein, but has no influence on the precise catalytic specificity of the enzyme. It is the large subunit alone which determines catalytic specificity and the nature of the products formed. We are currently performing more detailed domain-exchange experiments within the large subunit, in conjunction with site-directed mutagenesis of some of its highly conserved domains, in order to narrow down the regions important for catalytic specificity.

\textit{Acknowledgments:} We thank V. Mandak for excellent help with HPLC-analysis and P. Wille, M. Elfstrand and T. Ritsema for critical reading of the manuscript. This project was funded, in part, by the Swiss National Science Foundation.
2.4.4. References


Chapter 3

Mutational analysis of the active center of plant fructosyltransferases: *Festuca* 1-SST and barley 6-SFT

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Submitted to FEBS Letters

3.1. Abstract
The active center of the glycoside hydrolase family 32 contains the three characteristic motifs (N/S)DPNG, RDP, and EC. We replaced the N-terminal region including the (N/S)DPNG motif of barley 6-SFT (sucrose:fructan 6-fructosyltransferase) by the corresponding region of *Festuca* 1-SST (sucrose:sucrose 1-fructosyltransferase). The chimeric enzyme, expressed in *Pichia*, retained the specificity of 6-SFT. Attempts to replace a larger piece at the N-terminus including also the RDP motif failed. A point mutation introduced in the RDP motif of 1-SST abolished enzymatic activity. Interestingly, point mutations of the EC-motif resulted in an enzyme which had lost the capability to form 1-kestose and glucose from sucrose but still accepted 1-kestose, producing fructose and sucrose as well as nystose.
3.2. Introduction

Fructans are polymers of fructose used as storage carbohydrates by many plants. They are synthesized from sucrose by specific fructosyltransferases (FTs). The linkages between the fructosyl-residues are either exclusively of the β(2-1) or the β(2-6) type, or both linkage types occur in branched fructans [1]. The degree of polymerization and distribution of linkage types, depending on FT specificities, are characteristic of different plant species. Fructan synthesis in plants is initiated generally by a sucrose:sucrose 1-fructosyltransferase (1-SST) using sucrose both as fructosyl donor and acceptor to produce 1-kestose, the shortest β(2-1) linked fructan, and glucose. In the case of grasses such as wheat and barley, the next step of fructan synthesis is mediated by a sucrose:fructan 6-fructosyltransferase (6-SFT), which again uses sucrose as fructosyl donor but fructans, preferably 1-kestose, as acceptor, forming β(2-6) linkages. Thus, by fructosyl transfer to 1-kestose the smallest branched fructan, the tetrasaccharide bifurcose, is formed. It remains to be shown whether or not additional FTs are involved in fructan synthesis of grasses.

Many plant FTs have been sequenced during the last few years, and the data clearly indicate a high homology to the vacuolar, acid invertases (β-fructosidases). These enzymes are all members of the glycoside hydrolase family 32 (GH32) and share three highly conserved regions characterized by the motifs (N/S)DPNG (also called β-fructosidase motif), RDP, and EC [2]. A function in sucrose hydrolysis has been experimentally shown for the (N/S)DPNG- and EC-motifs in the case of yeast invertase, and for the RDP motif in the case of bacterial FTs belonging to the family GH68 [3-5]. An invertase of the bacterium Thermotoga maritima was the first GH32 member of which the crystal structure has been determined [6], followed by structural analyses of an Aspergillus awamori exo-inulinase and of a chicory fructan-exohydrolase [7,8]. These structures show all a five bladed β-propellers with a deep negatively charged central pocket and a β-sandwich. The three highly conserved motifs mentioned above flank this central pocket. The aspartate of the (N/S)DPNG motif is proposed to act as a nucleophile, the glutamate of the EC-motif as a proton donor, and the aspartate of the RDP motif as transition state stabilizer in the transfructosylation reaction [2].
From the GH32 members structurally elucidated, chicory FEH is the closest relative of plant FTs and vacuolar invertases, with an amino acid sequence identity of 30 to 40%. For plant FTs, no crystal structures are available yet, but the three conserved domains of chicory FEH flanking the central pocket, defined by the motifs (N/S)DPNG, RDP and EC, are highly conserved also in these FTs. Moreover, the (N/S)DPNG-motif has been shown by mutational studies to be essential for the activity of an onion FT [9]. For the RDP- and EC-motif of plant FT’s, such studies have not been made so far. Another common feature of plant FTs and vacuolar invertases is that they usually are composed of a large and a small subunit due to a posttranslational processing [10-12], as indicated in Fig. 1A. In a previous study using barley 6-SFT [10] and Festuca 1-SST [13], we found that the large subunit, which harbours all three conserved domains mentioned above, determines the catalytic specificity [14].

Our present work continues the comparative study of barley 6-SFT and Festuca 1-SST and provides the first experimental evidence that the motifs of all the three conserved domains are of functional importance in the case of the two plant FT’s studied. We narrowed down the region of the proteins responsible for enzyme specificity by exchanging domains including the (N/S)DPNG-motif between Festuca 1-SST and barley 6-SFT, and by introducing point mutations in the EC- and RDP-motif of Festuca 1-SST.
3.3. Materials and Methods

Fig. 1. Constructs for expression in *P. pastoris*. A: *Festuca* 1-SST. Conserved motifs are indicated. B: Barley 6-SFT. C: Chimera1: N-terminus of mature *Festuca* 1-SST comprising the (N/S)DPNG motif, with the C-terminus of barley 6-SFT comprising the RDP and EC motifs fused to it. D: Chimera2: N-terminus of mature *Festuca* 1-SST comprising the (N/S)DPNG and RDP motif, with the C-terminus of barley 6-SFT comprising the EC motif fused to it. E, F: *Festuca* 1-SST with selected sites for amino acid substitutions. Numbers indicate positions in the corresponding amino acid sequence, counted from the N-terminus of the open reading frames.
3.3.1. Construction of recombinant chimeric enzymes, mutagenesis and expression

The *Pichia* shuttle plasmids pPICZ\[a\]C-F2 and pPICZ\[a\]C-P3 were described before [14]. They carry a Zeocin resistance gene and contain the following coding sequences, cloned in frame behind the [\]-factor signal sequence: pPICZ\[a\]C-F2, mature *Festuca* 1-SST (from *Festuca arundinacea*, re-classified as *Schedonorus arundinaceus*; EMBL AJ297369 [13]; Fig. 1A); pPICZ\[a\]C-P3, mature barley 6-SFT (from *Hordeum vulgare*; EMBL X83233 [10]; Fig. 1B). They were used for the construction of Chimera1 and Chimera2 (Fig. 1C and D).

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For Chimera1 (Fig. 1C), the N-terminal region of mature *Festuca* 1-SST was coupled to the C-terminus of barley 6-SFT by overlapping PCR [15], using primer pair SST-002/RDP-002 to amplify from the EcoRI cloning site to bp 639 in 1-SST and SFT-002/RDP-001 to amplify 6-SFT from bp 523 to bp 1162. The PCR-amplified fragments were used as templates in a second PCR with primers SST-002 and SFT-002. The full-length product created in this way was cloned into pPICZaC-P3 using EcoRI and XmaII resulting in pPICZaC-Chimera1.

Chimera2 (Fig. 1D) was created correspondingly using primers SST-002/EC-002 and SFT-002/EC-001 and primers SST-002 and SFT-002 in the second PCR, followed by EcoRI/XmaII cloning resulting in pPICZaC-Chimera2.

Mutant SST(D260A), in which the aspartate in the RDP motif of *Festuca* 1-SST was changed into alanine (Fig. 1E), as well as the mutant SST(E318A) and SST(E318D), in which the glutamate of the EC motif was changed into alanine or aspartate (Fig. 1F), respectively, were generated using the QuikChangeTM Site-Directed Mutagenesis Kit (Strategene), based on the pK18-clone containing the tagged version of *Festuca* 1-SST as described [14]. Primers SST023.2 and SST024.2 (Table 1) were designed to introduce the desired mutation for SST(D260A), SST017 and SST018 for SST(E318A) and SST015 and SST016 for SST(E318D). SST(D260A), SST(E318A) and SST(E318D) were cloned EcoRI/XbaI into the *Pichia* shuttle vector pPICZaC. All inserts in pPICZaC were sequenced after cloning and found to carry the desired mutation or the desired chimera. All constructs carry a C-terminal 6_histidine tag and are cloned behind the α-factor secretion signal.

Transformation to *Pichia*, expression and further processing were described before [14].

3.3.2. Characterization of recombinant fructosyltransferases

Enzyme assays were performed at 27°C with 100 mM sucrose (suc) or with 50 mM of all other substrates, and the products formed were analysed by anion exchange chromatography as described [16]. Catalytic specificity was tested with the following substrates: sucrose, 1-kestose, nystose and a combination of sucrose and 1-kestose. A maximum of 7% of the substrates was used up after the incubations. In order to keep
the substrate concentration constant during the experiments, the amount of protein used per enzyme assay was ca. 0.5 mg for *Festuca* 1-SST and 5 to 10 mg for all other constructs.

For western blots, methanol-chloroform precipitated enzyme preparations (5 to 10 mg each) were separated on a 10% gel by SDS-PAGE, electro blotted to a nitrocellulose membrane (Schleicher & Schuell GmbH) and probed with monoclonal anti-His and alkaline-phosphatase secondary antibodies.
3.4. Results

3.4.1. Heterologous expression in the yeast Pichia pastoris

To study structure-function relationships in plant FTs, the Pichia pastoris yeast expression system is a powerful tool [14]. The chimeric constructs were constructed with overlapping PCR using Festuca arundinacea 1-SST (Fig. 1A) and Hordeum vulgare 6-SFT (Fig. 1B). In Chimera1 (Fig. 1C), the N-terminal part encompassing the (N/S)DPNG motif is from 1-SST, whereas the C-terminal part encompassing the RDP motif and the EC-motif is from 6-SFT. In Chimera2 the N-terminal part encompassing the (N/S)DPNG- and RDP-motif is from 1-SST, whereas the C-terminal part encompassing the EC-motif is from 6-SFT (Fig. 1D). Point mutations were investigated using Festuca 1-SST (Fig. 1E, F).

Fig. 2. Western Blot analysis of recombinant methanol-chloroform precipitated enzyme preparations (5-10 μg each), probed with anti-His and alkaline-phosphatase secondary antibodies. The size of the bands is indicated in kD. P0: Pichia transformed with empty vector; 1-SST: recombinant 1-SST; 6-SFT: recombinant 6-SFT; Chimera1, Chimera2; SST(E318A), SST(E318D), SST(D260A): see Fig. 1.
To verify proper expression of the constructs, western blots were performed using antibodies against the C-terminal His tag. Extracts of *Pichia* transformed with the empty vector pPICZ\(\square\)C did not show any bands on the blots (Fig. 2, lane P0). In contrast, clear bands were revealed on blots with extracts of *Pichia* transformed with the various constructs. Normally, *Festuca* 1-SST and barley 6-SFT isolated form plants consist of two subunits and show two bands on SDS-PAGE \[10,13\]. However, upon expression in *Pichia*, most constructs yielded primarily a band of approximately 85 kD. This indicates that the recombinant proteins are well expressed but not cleaved into two subunits, as was already previously demonstrated for recombinant untagged SST \[13\]. A faint band at 35 kD was apparent for some of the constructs, indicating release of a fragment possibly corresponding to the small subunit in *Pichia* (Fig. 2); since the antibodies are directed against the C-terminal tag, fragments corresponding to the large subunit cannot be seen on this western blot. For Chimera2, the 85 kD is even fainter than the 35 kD band, indicating that this product is weakly expressed or unstable in *Pichia*.

3.4.2. The (N/S)DPNG motif does not determine enzymatic specificity

Chimera1 (Fig. 1C), with the NDPNG motif of 1-SST, was tested with different substrates and its activity was compared to the activities of recombinant 1-SST and 6-SFT, with the motif in the form of SDPNG (Fig. 3). In incubations with 100 mM sucrose it was mainly hydrolytically active, as can be seen by the predominant production of fructose (Fig. 3A). The other products formed by Chimera1 in incubations with sucrose were 1-kestose, 6-kestose and marginal amounts of bifurcose. The products formed and their relative amounts were the same as for recombinant 6-SFT. In corresponding incubations, recombinant 1-SST produces mainly 1-kestose (80%). Also in incubations with a combination of sucrose and 1-kestose, the products formed by Chimera1 were fructose, 6-kestose and bifurcose, and their relative amounts were the same as for recombinant 6-SFT (Fig. 3B). In contrast, the corresponding incubation with 1-SST lead to the production of fructose and nystose. These analyses of Chimera1 showed that it retained all the characteristics of recombinant 6-SFT. Its 1-SST derived N-terminal part, including the conserved \(-\)fructosidase motif NDPNG, does not influence enzymatic properties.
Preparations of Chimera2 (Fig. 1D) did not show any enzymatic activity. In addition, as shown above (Fig. 2), this chimera yielded only low amounts of protein, indicating that this highly conserved region between the EC-motif and the RDP motif is important for protein stability.

Fig. 3. Relative enzyme activities of recombinant 1-SST, 6-SFT and Chimera1 using 100 mM sucrose (A) or 100 mM sucrose and 50 mM 1-kestose (B) as substrates. In incubations with 100 mM sucrose, the total activity of the protein was approximately 125 nkat/mg protein for 1-SST, 0.75 nkat/mg protein for 6-SFT and 0.7 nkat/mg protein for Chimera1. Data represent means and standard deviation (n= 5, corresponding to five different enzyme preparations from five separate induction experiments). The sum of products formed is set to 100%. F: fructose, 1K: 1-kestose, 6K: 6-kestose, B: bifurcose, N: nystose.
3.4.3. *Amino acid substitutions in the RDP- and the EC-motif reveal their necessity for fructosyl-transfer*

In order to investigate the functional importance of the aspartate D260 in the RDP motif of 1-SST, it was changed to alanine in the mutant SST(D260A) (Fig. 1E). The resulting protein displayed a strong band of 85 kD on western blots and was equally well expressed and as stable as non-mutated recombinant 1-SST (Fig. 2). However, it had no detectable enzymatic activity, indicating the importance of the acidic residue in the transfructosylation reaction.

Typically 1-SST synthesizes glucose, little fructose and 1-kestose from sucrose (Fig. 4A). Like the mutant SST(D260A), the mutants SST(E318A) or SST(E318D), in which the glutamate of the EC motif was changed into alanine and aspartate, respectively, lost the ability to use sucrose as a fructosyl-donor and as an acceptor. After incubations of two days with 100 mM sucrose, neither breakdown of sucrose nor polymerization to fructan was observed (Fig. 4A). Western blotting showed that the enzymes were equally well expressed in *Pichia pastoris* as non-mutated 1-SST (Fig. 2). Wild-type 1-SST incubated with 1-kestose as the only offered substrate produced fructose, sucrose and nystose (Fig. 4B). Mutant SST(D260A) was inactive when incubated with 1-kestose. Interestingly, the mutants SST(E318A) and SST(E318D) kept the ability to use 1-kestose (Fig. 4B), but the activity was heavily reduced compared to non-mutated 1-SST. Time dependent product formation was followed for two days for SST(E318A) and SST(E318D) incubated with 50 mM 1-kestose (data are shown only for SST(E318A)). They produced increasing amounts of fructose, sucrose and very little nystose in a time dependent manner (Fig 4B). The two mutants were inactive in incubations with nystose (data not shown). In summary, the mutations of the glutamate of the EC-motif abolished the use of sucrose, but retained the ability to use 1-kestose. Thus the EC-motif has an influence on substrate usage in 1-SST.
Fig. 4. HPAEC-PAD profile of products formed in enzyme assays of 1-SST, SST(D260A) and SST(E318A). Recombinant enzymes were incubated with 100 mM sucrose (A) or 50 mM 1-kestose (B) as substrate. Products formed by 1-SST were analyzed after 1h of incubation. Products formed in incubations of SST(D260A) and SST(E318A) were analyzed from 0h to 2 days. G: glucose, F: fructose, S: sucrose, K: 1-kestose, Nys: nystose.
3.5. Discussion

Our biochemical data on the enzymatic activities of mutated plant fructosyltransferases are of interest in view of the recent elucidation of the structure of some related enzymes. Crystal structures became available recently for members of the glycoside hydrolase families GH32 and GH68, i.e. enzymes that catalyze similar reactions although they share only 15% overall sequence identity. They all form five bladed \( \beta \)-propellers with a deep negatively charged central pocket representing the active site [6-8,17]. Members of GH32 additionally have a \( \beta \)-sandwich attached to the propeller that possibly corresponds to the small subunit found in plant acid invertases and FTs. In the structure of \textit{Bacillus subtilis} levansucrase (GH68), the central pocket contains three acidic amino acid residues essential for the catalytic mechanism: the nucleophile (D86), the general acid/base catalyst (E342) and the transition state stabilizer (D247) [17]. These catalytic residues are also present in the active site of all enzymes of family GH32 for which structures are available, and they correspond to the conserved motifs (N/S)DPNG, EC and RDP within GH32.

In plant enzymes belonging to this family, i.e. the FTs and acid invertases, only the (N/S)DPNG (\( \beta \)-fructosidase) motif was biochemically characterized via domain-exchanges and point mutations. It was shown to be important for product specification but not for the specification of the type of fructosyl donor used; thus its influence on enzyme specificity seems to act via acceptor specification [9]. The mutation of the nucleophile itself (D85) resulted in an enzymatically inactive protein (Ritsema et al., submitted).

In our present work, the results with Chimera1 gave further insight into the function of the \( \beta \)-fructosidase motif, which has the usual sequence NDPNG in 1-SST but the less frequent sequence SDPNG in 6-SFT. The chimeric enzyme retained all the characteristics of recombinant 6-SFT although its N-terminal part, including the canonical NDPNG motif, was derived from 1-SST. 1-SST and 6-SFT can both use sucrose as an acceptor and donor substrate, but they differ in their preferential fructosyl acceptor substrate. Chimera1 prefers the same fructosyl acceptor substrates like barley 6-SFT. Since 1-SST and 6-SFT have the same preferred fructosyl-donor
(sucrose), exchanging the N-terminus containing the \([-\text{fructosidase motif might give no change in donor specificity. The C-terminal part of the chimera is influencing acceptor binding which is different for 1-SST and 6-SFT (sucrose or 1-kestose respectively).}

The point mutation SST(D260A) of the RDP motif yielded an inactive product. The aspartate residue of this motif has been proposed to act as transition state stabilizer [4,5,17,18]. It forms strong hydrogen bonds with the substrate sucrose, but is too far away from either the C2’ hydroxyl or the glycosidic oxygen of sucrose to be directly involved in the catalytic mechanism, i.e. in hydrolyzing the bond between glucose and fructose of sucrose [6,17]. The mutation (D260A) might increase the distance to the substrate and therefore be a hindrance for the formation of hydrogen bonds, but a conformational change of SST(D260A) cannot be excluded.

The acidic amino acids in the other two motifs have been found to be directly involved in catalysis. This was first described for yeast invertase, a member of the GH32 family: The aspartate in the (N/S)DPNG \([-\text{fructosidase motif (Asp23) was shown to be the catalytic nucleophile while the glutamate of the EC-motif (Glu204) was identified as the general acid/base catalyst [3]. Our results with the point mutation of the corresponding glutamate in 1-SST (SST(E318A); SST(E318D)) are particularly intriguing, since they indicate a resulting change in the enzyme's catalytic activity. Both mutated forms lost the ability to use sucrose as a substrate, showing neither cleavage into glucose and fructose nor polymerization to 1-kestose. This result is in accordance with the corresponding mutation of E204A in yeast invertase, where also the sucrose cleaving capacity was heavily reduced. Yeast invertase has only one substrate (sucrose) whereas Festuca 1-SST can also use 1-kestose. Wild type 1-SST produces fructose, sucrose and nystose when incubated with 1-kestose. The mutants SST(E318A) and SST(E318D)) formed these products from 1-kestose as well, although with markedly reduced efficiency. The 3D-structure of the active site of Bacillus subtilis levansucrase in the presence of sucrose shows that the glutamate of the EC-domain points towards the bond between the glucose and the fructose moiety of sucrose [17]. The fructosyl unit of sucrose is located at the bottom of the pocket and the glucose moiety on top. Since 1-kestose has two fructose moieties attached to
glucose, its terminal fructosyl residue may contact an alternative acid/base catalyst in
the active site. Due to the low overall homologies of *Festuca* 1-SST to the crystal
structures available, it cannot be predicted which glutamate might replace E318 in
catalysis.

The general glycosyl hydrolase reaction mechanism in members of the GH32
and GH68 families is based on three acidic amino acid residues in the active center.
Neither the available crystal structures nor the data obtained with mutational analysis
can unequivocally answer the question what structural components determine enzyme
specificity. Functional differences such as N-glycosylation or amino acid changes
outside active site residues may play a role in the selection of different substrates[8]
and will be investigated in the future.

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3.6. References


Chapter 4

Are fructosyltransferases modified invertases?

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4.1. Abstract

Enzymes polymerizing sucrose to fructans (fructosyltransferases, FTs) are highly homologous to enzymes hydrolyzing sucrose (acid invertases) and belong to glycoside hydrolase family 32. Recently, the first crystal structures were determined within this family. They consist of a five bladed β-propeller with a deep negatively charged central pocket representing the active site, and a β-sandwich. There are three highly conserved motifs flanking the central pocket that are functionally essential: the (N/S)DPNG-motif, the RDP-motif and the EC-motif. However, it remains unclear what structural components determine polymerization or breakdown of sucrose. Therefore we decided to perform a mutational analysis based on sequence comparisons between acid invertases and sucrose:sucrose 1-fructosyltransferases (1-SSTs), the latter an example of sucrose-using FTs, and chose Allium cepa invertase and Festuca arundinacea (re-classified as Schedonorus arundinaceus) 1-SST for our analysis. Nine amino acids dispersed along the sequence could be identified correlating with either invertase or 1-SST activity, respectively. The selected amino acids of onion invertase were mutated to the corresponding amino acids in Festuca 1-SST and vice versa. For both enzymes, the mutations were analyzed independently. Functional expression in the yeast Pichia pastoris revealed shifts in the catalytic specificity and activity, demonstrating the importance of amino acids outside the three highly conserved motifs ((N/S)DPNG, RDP, EC) for the enzymatic reaction.
4.2. Introduction

Fructans, are a class of highly water-soluble polysaccharides consisting of linear or branched fructose chains attached to sucrose. In plants, they occur in many prominent orders like the Asterales, the Liliales, and the Poales, among which are representatives of economic importance (e.g. wheat, barley) (Hendry, 1993). As highly water soluble molecules fructans are predominantly stored in the vacuole (Wiemken et al., 1995). Besides their function as a reserve carbohydrate, fructans are suggested to be involved in cell expansion (Bieleski, 1993) and enhanced tolerance to cold and drought via stabilization of cellular membranes (Hincha et al., 2000; Konstantinova et al., 2002; Vereyken et al., 2003).

Fructan synthesis in plants starts with the conversion of sucrose to the shortest $\beta(2,1)$ linked fructan 1-kestose. The enzyme that performs this reaction, sucrose:sucrose 1-fructosyltransferase (1-SST), is found in all fructan-producing plants. It uses two molecules of sucrose, one as fructosyl donor and the other one as fructosyl acceptor for the synthesis of the trisaccharide 1-kestose. In a second step, chain elongation to higher DP fructans occurs via the action of fructan:fructan 1-fructosyltransferase (1-FFT), sucrose:fructan 6-fructosyltransferase (6-SFT) and/or fructan:fructan 6G-fructosyltransferase (6G-FFT), depending on the species-specific type of fructan synthesized by the plant (Vijn and Smeekens; 1999). These fructosyltransferases (FTs) display different donor and acceptor specificities using either sucrose and/or fructan as fructosyl donor or acceptor, respectively, and are named according to the substrates they use and according to the linkage type they form ($\beta(2,1)$ or $\beta(2,6)$). Fructan cleavage is catalyzed by fructan exohydrolases (FEHs).

Functionally related to FTs are invertases, transferring the fructosyl residue from sucrose not to a saccharide-chain but to water, producing glucose and fructose. Vacuolar invertases resemble SSTs at the biochemical level: SSTs generally show a low but distinct hydrolase activity and vice versa invertases produce traces of 1-kestose, particularly at high sucrose concentrations ($\geq 100$ mM; Sprenger et al., 1995).
Invertases are ubiquitous in plants whilst fructosyltransferases are restricted to several unrelated families (Hendry, 1987). Peptide sequences of plant FTs generally have a very high similarity to amino acid sequences of different invertases (Sprenger et al., 1995; Van den Ende et al., 1996; Vijn et al., 1997). If vacuolar invertases are presented in phylogenetic trees with FTs, fructan exohydrolases (FEHs) and cell-wall invertases, the vacuolar invertases always cluster with FTs whilst the FEHs cluster with cell-wall invertases (Fig. 1; Wei and Chatterton, 2001). Close relationships at the biochemical and molecular level between fructan biosynthesizing enzymes and invertases support the idea, that the fructan synthesizing enzymes evolved from vacuolar invertases by relatively few mutational changes (Sprenger et al., 1995; Vijn and Smeekens, 1999). As the initiator of fructan synthesis, 1-SSTs are thought to be the closest relatives to vacuolar invertases. Both enzymes use sucrose as substrate: Invertases for hydrolyzation and SSTs for polymerization.

Plant fructan metabolizing enzymes and acid invertases are grouped in glycoside hydrolase family 32 (GH32), based on overall sequence homologies (Henrissat, 1991). Three highly conserved motifs are a characteristic for the GH32 family. They consist of the following conserved sequences: (N/S)DPNG (also called [-fructosidas motif), RDP (RDP motif) and EC (EC-motif). The acidic amino acids (marked in bold) of these motifs have been proposed to function as active site residues (Reddy and Maley, 1996; Pons et al., 2004). These three motifs can explain the cleavage of glycosidic bonds in general, but not what structural components determine enzyme specificity. It still has to be shown what features determine fructosyltransfer to a saccharide (FT activity) or to water (invertase activity).

To address the questions of structural components determining different enzyme specificities, we performed a targeted mutational approach, based on sequence comparisons between acid invertases and 1-SSTs. Sequence comparisons of acid invertases and 1-SSTs revealed nine amino acids dispersed along the sequence to correlate with either invertase or 1-SST activity respectively (Fig. 2). For our analysis we chose *Allium cepa* invertase (acINV) and *Festuca arundinacea* 1-SST (faSST; re-classified as *Schedonorus arundinaceus*), two enzymes that have been very efficiently
expressed in *Pichia pastoris* (Lüscher et al., 2000; acINV by T. Ritsema). The selected nine amino acids of acINV were mutated to the corresponding amino acid in faSST and *vice versa* (Fig. 3). For both enzymes, the mutations were analyzed independently. The functional expression of the mutated enzymes in the yeast system *Pichia pastoris* revealed shifts in the catalytic specificity and activity of invertase and 1-SST respectively.
4.3. Materials and Methods

4.3.1. Cloning and mutagenesis of Festuca 1-SST (faSST)

FaSST mutants carrying single mutations at specific sites of the sequence (A, B, C, D, E, F, G, H and X) were generated using the QuikChangeTM Site-Directed Mutagenesis Kit (Strategene), based on the pK18-clone containing the tagged version of Festuca 1-SST (Altenbach et al., 2004).

Primers SSTAf and SSTAr (Table 1) were designed to introduce the desired mutation at site A in faSST, SSTBf and SSTBr for the mutation at site B etc.

Wild type sequences and mutated sequences were cloned EcoRI/XbaI into the Pichia shuttle vector pPICZαC. Competent E. coli cells were transformed with pPICZαC-faSST-A, pPICZαC-faSST-B etc. respectively, and selected for Zeocin resistance. All inserts in pPICZαC were sequenced after cloning and found to carry the desired mutation.

4.3.2. Cloning and mutagenesis in onion invertase

The onion invertase present in pBlue (Vijn et al, 1998) was digested with SacI and XhoI and cloned into the mutagenesis vector pALTER-1 (Promega) digested with SacI and SalI (SalI and XhoI create the same overhang). Mutagenesis was performed using Altered Sites II (Promega) as described in the manual and mutations were checked by sequencing.

For cloning into the Pichia vector pPICZαC (Invitrogen) wild type invertase and mutants were PCRed with primers containing ClaI and XbaI restriction sites. After digestion of the product and the pPICZαC vector with ClaI and XbaI, they were ligated and the presence of inserts was confirmed by PCR.
### Table 1

Oligonucleotides used for cloning, mutations are indicated in bold

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4.3.3. Expression in Pichia pastoris

The wild-type enzymes and all mutants were present in frame behind the yeast α-factor signal sequence of the expression vector pPICZαC, to allow entry into the secretory pathway of Pichia pastoris. Competent Pichia pastoris cells were either transformed according to the EasyComp transformation protocol (EasySelect™ Pichia Expression Kit, Invitrogen BV) or by electroporation as described in the manual and selected for Zeocin® resistance.

Induction of protein expression in Pichia pastoris was performed as described (Altenbach et al., 2004) with minor modifications: The growth medium was supplemented by 1% (v/v) methanol at 16, 24 and 40h of induction. After 45h of induction the cultures were centrifuged (10 min, 3000 rpm) and the supernatants containing the recombinant enzymes were used for all subsequent steps.

4.3.4. Characterization of recombinant enzymes

To characterize the enzymes, the supernatants were diluted 1:10 to have a final concentration of 50 mM MES-buffer pH 5.6 and the desired sucrose concentration per assay. Enzyme assays contained sucrose concentrations as indicated (1 mM to 1 M). They were incubated for 20 minutes at 27°C and thereafter stopped by heating for 2 min at 95°C. The products formed were analysed by high performance anion exchange chromatography (HPAEC) as described (Lüscher et al., 2000).
4.4. Results

4.4.1. Molecular relationships of vacuolar invertases and sucrose:1-fructosyltransferases (1-SSTs)

Sequence similarity between 1-SSTs and vacuolar invertases reaches 80%. In a phylogenetic tree acid invertases and 1-SSTs exhibit separate but closely related clusters (Fig. 1). There are some exceptions: for example the vacuolar invertase from barley seems to be more closely related to SSTs and SFTs than to vacuolar invertases from Zea mais and Arabidopsis thaliana that are catalyzing the same reaction. SSTs from grass species (Festuca arundinacea, Lolium perenne, Hordeum vulgare and Triticum aestivum) build a cluster. They are more closely related to vacuolar invertases from monocotyledonous species (Hordeum vulgare, Allium cepa or Zea mais) than to the SSTs from dicotyledonous species included in the phylogenetic tree (Helianthus tuberosus and Chicorium intybus; Fig. 1).

Interestingly, enzymes from one species or family that confer different types of reactions are sometimes more closely related than enzymes that catalyze the same reaction but belong to different species or families. For example the different enzymes from Allium cepa (1-SST, 6G-FFT and the vacuolar invertase) are clustering with the SST form Allium sativum in the phylogenetic tree (Fig. 1). The tree furthermore illustrates that vacuolar invertases are more homologous to FTs than to cell-wall invertases and FEHs.
Fig 1. Phylogenetic tree of vacuolar invertases (INV), 1-SSTs (SST), 6-SFTs, 6G-FFT, cell-wall invertases (CWINV) and fructan exo-hydrolases (FEH). acINV, Allium cepa INV (AJ006067); acSST, Allium cepa SST (AJ006066); acGFT, Allium cepa 6G-FFT (ACY07838); asSST, Allium sativum SST (AY098442); atINV, Arabidopsis thaliana INV (S57951); atCWINV, Arabidopsis thaliana CWINV (NM_112232); atCWINV3 (re-classified as FEH; DeConinck et al., 2005), Arabidopsis thaliana CWINV3 (NM_104385); atCWINV6 (re-classified as FEH; DeConinck et al., 2005), Arabidopsis thaliana CWINV6 (NM_121230); aoINV, Asparagus officinalis INV (AF002656); bvFEH, Beta vulgaris FEH (AJ508534); ciINV, Cichorium intybus INV (AJ419971); ciCWINV, Cichorium intybus CWINV (Y11124); ciFEH, Cichorium intybus FEH (AY323935); ciFFT, Cichorium intybus FFT (U84398); cisST, Cichorium intybus SST (U81520); faSST, Festuca arundinacea (AJ297369); htSST, Helianthus tuberosus SST (AJ009757); hvINV, Hordeum vulgare INV (IAJ623275); hvSFT, Hordeum vulgare 6-SFT (X83233); hvSST, Hordeum vulgare SST (AJ567377); IpSST, Lolium perenne SST (AF492836); osINV, Oryza sativa INV (AF276703); psSFT, Poa secunda 6-SFT (AF192394); taCWINV, Triticum aestivum CWINV (AF030420); taSFT, Triticum aestivum 6-SFT (AB029887); taSST, Triticum aestivum SST (AB029888); vviINV, Vitis vinifera INV (AAB47172); zmlINV, Zea mays INV (U16123).
4.4.2. Selection of amino acids correlating with invertase or 1-SST activity respectively

We chose a directed mutational approach based on sequence comparisons between vacuolar invertases and SSTs for the elucidation of amino acids that possibly influence enzyme specificity. To this intend, sequences of vacuolar invertases and SSTs of monocotyledonous and dicotyledonous species were aligned and also vacuolar invertases from non-fructan producing species such as Arabidopsis thaliana (Fig. 2). We screened for amino acids in the sequence that specifically differed between invertases and SSTs. In particular, differences in amino acid polarity, hydrophobicity or hydrophilie and in the type of aromatic amino acid were considered. Nine amino acids dispersed along the sequence could be identified which correlated either with invertase or with 1-SST activity (Fig. 2 Sites A to H and X). These nine sites are located outside the highly conserved motifs (Fig. 3). Only site A flanks the highly conserved motif (N/S)DPNG, supposed to be the catalytic nucleophile in the transfructosylation reaction, and site E and F flank the EC-motif which is probably the proton donor needed for catalysis (Fig. 2, Fig. 3).

Remarkably, the majority of the amino acids found to be either invertase or SST specific turned out to be aromatic.

<table>
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<th>A</th>
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Fig 2. Alignment of the nine selected sites of 1-SSTs and vacuolar invertases. Abbreviations as in figure 1.
4.4.3. Functional characterization of wild-type and mutagenized enzymes expressed in *Pichia pastoris*

Studying structure function relationships of FTs and invertases demands an appropriate expression system. The expression of these enzymes in the yeast *Pichia pastoris* has been shown to be ideal, since this yeast is devoid of sucrose metabolizing enzymes such as invertases (Lüscher et al., 2000). For the mutational analysis we chose *Allium cepa* invertase (acINV) and *Festuca arundinacea* 1-SST (faSST), two enzymes that were known to be very efficiently expressed in *Pichia* (Lüscher et al., 2000; acINV by T. Ritsema). The amino acids at the nine selected sites of acINV were mutated to the...
corresponding amino acids in faSST and *vice versa* (Fig. 3, Table 2). For both enzymes these different mutations were analyzed separately.

Initially recombinant wild-type faSST and acINV were functionally characterized in order to subsequently detect possible activity shifts upon introducing the mutations. Invertases are saturable with sucrose and have Km-values from 2-20 mM. In contrast, fructosyltransferases do not show Michaelis-Menten kinetics and are essentially non-saturable, the Km has to be estimated (Ritsema et al., 2003; Van den Ende et al., 1996). It seems that SSTs need a higher sucrose concentration than invertases for proper activity. With increasing concentrations of sucrose, the recombinant faSST produced increasing amounts of glucose and 1-kestose besides small amount of fructose (Fig. 4A). The hydrolase activity was relatively low at all offered sucrose concentrations as compared to the polymerase activity. Saturation appeared only to be reached at sucrose concentrations $\geq 800$ mM. In contrast, recombinant acINV reached its saturation already at about 20 mM sucrose (Fig. 4B). At sucrose concentrations of $\geq 100$ mM, a distinct but very small amount of 1-kestose was found. In summary, faSST produces mainly 1-kestose and glucose, whereas acINV acts almost purely as a hydrolase. The differences in the saturation characteristics for sucrose of the two enzymes, and their relatively low side activities, namely hydrolase activity in the case of faSST, and polymerase activity in the case of acINV, are the ideal basis for analyzing possible shifts in enzymatic specificity upon mutational changes.

**Fig 4.** Characterization of recombinant faSST (A) and acINV (B). Enzyme preparations were incubated with increasing concentrations of sucrose (27°C, 20'). Product formation (nmol glucose, fructose, 1-kestose) was quantified on HPAEC-PAD.
4.4.4. Characterization of SST-mutants

Like the wild-type enzymes, genes encoding mutated enzymes were expressed in the yeast *Pichia pastoris*. Enzyme activity was assayed with sucrose. The faSST mutants at sites D and G were not active. At site D, tyrosine$_{248}$ was changed to valine (Table 2), representing a change from an aromatic amino acid with an OH-group to a hydrophobic amino acid. The site is located next to a highly conserved proline-triplet, that probably forms a turn in the tertiary structure (Fig. 2). In the inactive faSST mutant at site G, tyrosine$_{408}$ was changed to tryptophan, which is also an aromatic amino acid but a much bigger one, that might disturb the protein structure.

![Fig 5](image-url)

**Fig 5.** Relative hydrolytic activity of wild-type and mutated recombinant SSTs. The active faSST mutants at sites A, B, C, E, F, H and X (Table 2 gives an overview) were incubated with different concentrations of sucrose (sucrose concentrations are indicated in mmol on the x-axis). Relative activities of polymerization and hydrolysis of sucrose were determined, taking 1-kestose production as a measure for polymerization and fructose production as a measure for hydrolysis. The sum of polymerizing and hydrolyzing activities was set to be 100%, and % hydrolyase activity is shown. Standard deviations are indicated and are calculated from two independent induction experiments.
All the other single amino acid mutations resulted in active recombinant proteins. These active faSST mutants were tested by incubation with different concentrations of sucrose (Fig. 5). At site A, the amino acid tyrosine\textsubscript{133} of faSST was changed to tryptophan, as occurring in all known vacuolar invertases. The aligned SSTs carry a tyrosine or a phenylalanine at this site. Site A is in the vicinity of the highly conserved motif (N/S)DPNG. The mutant revealed no change in relative polymerase (80-90\%) or hydrolas (10-20\%) activities as compared to the recombinant wild-type faSST upon incubation with different concentrations of sucrose (Fig. 5). At site B, methionine\textsubscript{173} of faSST was replaced by leucine which is hydrophobic as well. The mutation at site E is like the one at site A, a change of a tyrosine\textsubscript{317} to tryptophan, and for the mutation at site F, leucine\textsubscript{322} which is aliphatic and apolar, was changed to the also apolar but aromatic phenylalanine. The mutations at sites A, B, E and F had no effect on faSST specificity as compared to the wild-type form.

The mutants at sites C and X in faSST had almost doubled relative hydrolytic activity in comparison to wild type faSST. I.e. fructose production doubled with respect to all sucrose concentrations offered (Fig. 5). At site C, the cysteine\textsubscript{203} of faSST was changed to asparagine, as occurring in all invertases of the alignment except for barley invertase (Fig. 2). This alteration can cause a loss of a disulfide bond. The mutant at site X also showed a doubling of the relative hydrolytic activity in comparison to wild-type faSST. The phenylalanine\textsubscript{362} in faSST was mutated to the omnipresent tyrosine of invertases in the corresponding site. The mutation represents an alteration from hydrophobic aromatic to polar aromatic.

In comparison to wild type faSST, the mutant at site H, where asparagine\textsubscript{425} was changed to serine, had almost exchanged ratios of hydrolysis\textit{ versus} polymerization. The enzymes relative hydrolytic activity was approximately 80\%, whereas its relative polymerizing activity was reduced to approximately 20\% (Fig. 5). Although the faSST carrying the mutation at site H was mainly hydrolytically active, it did not become a typical invertase. Its capacity to synthesize 1-kestose was still too high, and it was not saturated at low sucrose concentrations such as wild type acINV (Fig. 6, compare to Fig. 4).
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Fig 6. Characterization of the faSST mutant at site H. Enzyme preparations were incubated with increasing concentrations of sucrose (27°C, 20'). Product formation (nmol glucose, fructose, 1-kestose) was quantified on HPAEC-PAD.

4.4.5. Characterization of invertase-mutants

Genes encoding mutated onion invertase (acINV) were expressed in the yeast *Pichia pastoris* and enzymatic activity was assayed with sucrose similar to the wild-type enzyme. The acINV mutants at site D and X were not active. At site D, valine$_{276}$ was changed to tyrosine. Like in faSST carrying mutation D, the site for mutagenesis is located next to a highly conserved proline-triplet, probably representing a turn in the 3D-structure. At site X, tyrosine$_{394}$ was replaced in acINV with phenylalanine. This looks a relatively small change in polarity only, keeping the aromatic ring. Nevertheless, acINV carrying the mutation at siteX was inactive, and the faSST mutant at site X showed doubled relative hydrolytic activity.

All other single mutants of acINV were active. As before, the enzyme preparations of the mutants were analyzed with different concentrations of sucrose (Fig. 7) and the relative activities of hydrolysis versus polymerization were determined. Furthermore the Km for sucrose was evaluated (Fig. 8). AcINV mutated at sites B, C, E, F or H showed the same biochemical properties as the wild-type invertase, with respect to kestose production and Km-values. At site B, leucine$_{201}$ was replaced with methionine, both molecules are hydrophobic. At site C, asparagine$_{251}$ was changed to cysteine. The change at site E is like at site A, a change from tryptophan$_{343}$ to tyrosine, but such as the mutation at site E in faSST, showing no change in enzymatic specificity. In acINV
carrying the mutation at site F, the apolar character of phenylalanine\textsubscript{348} was unchanged, but the aromatic character was lost due to the mutation to leucine. For the acINV mutant at site H, that also showed unchanged enzymatic characteristics, serine\textsubscript{457} was mutated to the chemically more reactive asparagine.

**Fig 7.** Relative polymerizing activity of wild-type and mutated recombinant invertase. The active acINV mutants at sites A, B, C, E, F, G and H (Table 2 gives an overview) were incubated with different concentrations of sucrose (sucrose concentrations are indicated in mmol on the x-axis). Relative activities of polymerization and hydrolysis of sucrose were determined, taking 1-kestose production as a measure for polymerization and fructose production as a measure for hydrolysis. The sum of polymerizing and hydrolyzing activities was set to be 100%, and % polymerase activity is shown. Standard deviations are indicated and are calculated from two independent induction experiments with two assays per sucrose concentration.

**Fig 8.** Km-values for sucrose of invertase mutants. Onion invertase and the mutants were assayed with increasing concentrations of sucrose. The Michaelis-menten constant (Km) was calculated using Lineweaver-Burk-plots.
The invertase mutated at sites A or G, showed an increased kestose production as compared to recombinant wild-type acINV (Fig. 7). Also the Km-value for sucrose was significantly increased (Fig. 8). Site A is close to the highly conserved motif (N/S)DPNG. The conserved tryprophan in invertases at this site was mutated to tryrosine. Amazingly, the corresponding mutation in faSST had no, or only little effect. Like the mutations at site A and E, the mutation at site G represents a replacement of tryptophan at with tyrosine. This mutation in invertase led to an activity shift towards more polymerizing activity whilst in the case of faSST it led to inactivity.

In summary, SST mutants at sites C, H and X showed an increased relative hydrolytic activity and invertase mutants at sites A and G showed increased Km-values and increased fructosyltransferase activity, demonstrating the importance of amino acids outside highly conserved motifs for the enzymatic reaction.
4.5. Discussion

For GH family 32, where plant FTs and vacuolar invertases belong to, the acidic amino acids (indicated in bold) of three highly conserved motifs (N/S)DPNG, RDP and EC are proposed as active site residues (Pons et al., 2000; Ozimek et al., 2004; Verhaest et al., 2005). Recently the 3D-structures of three enzymes of this family were resolved: the invertase from the bacterium *Thermotoga maritima* (Alberto et al., 2004), the exo-inulinase from *Aspergillus awamori* (Nagem et al., 2004) and the fructan-exohydrolase (FEH) from chicory (Verhaest et al., 2005). Each structure shows a five bladed β-propellers with a deep negatively charged central pocket, representing the active site, and a β-sandwich. The three highly conserved motifs flank the central pocket, and can be brought in connection with the general reaction mechanism for the cleavage of glycosidic bonds. Nevertheless, how enzyme specificity is regulated is unknown. Despite the close molecular and biochemical relationships of vacuolar invertases and SSTs, and the availability of crystal structures, the question what structural features determine the hydrolyzing or polymerizing character of a distinct enzymes, remains unanswered.

In order to detect the amino acids in FTs and vacuolar invertases which are determining enzyme specificity, we performed a targeted mutational analysis. It was based on sequence comparisons between vacuolar invertases and SSTs. Upon a search in sequence alignments for amino acids correlating with either invertase or SST activities, nine amino acids dispersed along the sequence which fulfilled these selection criteria were identified. Three of these nine sites turned out to contain tryptophan in all aligned invertases, whilst at the corresponding site SST’s contain either tyrosine or phenylalanine at site A, tyrosine, leucine or tryptophan at site E and tyrosine, cysteine or phenylalanine at site G. Site A and site E flank the highly conserved motifs (N/S)DPNG and EC, respectively. Thus, the selection pressure to preserve tryptophan seems to be much higher in vacuolar invertases than in FTs, where more amino acid variation is found. Nevertheless also SSTs generally seem to keep the aromatic nature of the amino acids at
the above described sites. Maybe the replacement of the highly conserved tryptophans of invertases was a step in the evolution from vacuolar invertases towards FTs.

Interestingly, mutants at sites A and G in acINV, that showed increased kestose production and Km-values, were mutations of the highly conserved tryptophan to tyrosine. In faSST the mutation at site A, which changed the tyrosine to the larger aromatic molecule tryptophan, had no effect on enzyme specificity. The amino acid corresponding to site A was furthermore analyzed in onion 6G-FFT (Ritsema et al., 2005). Like in faSST, there is a tyrosine present at the corresponding site of 6G-FFT which was mutated to tryptophan. Corresponding to the result obtained for the faSST mutant at site A, also mutated onion 6G-FFT showed no changes in enzyme specificity. Whereas the mutant at site G of acINV showed an increased kestose production and an increased Km-value, the faSST mutated at site G was inactive. The two mutations leading to increased Km-values in invertase, at site A and at site G, also showed increased production of 1-kestose. Possibly the capacity to synthesize 1-kestose is linked to an increase in Km. If SSTs evolved from vacuolar invertases one evolutionary step could be the increase in Km for sucrose. A possible function of this biochemical feature could be to prevent fructan synthesis if sucrose concentrations are low and sucrose is needed for other physiological processes.

The mutations affecting SST activity towards increased relative hydrolytic activity were mutations at sites C, H and X. Here the mutation at site X involved aromatic amino acids. Phenylalanine was changed to tyrosine, the amino acid that is present in all invertases analyzed by the alignment. In general, the conservation of distinct amino acids in invertases seems to be much higher than in SSTs. AcINV mutated at site X was inactive. In summary, the majority of the sites we found to affect hydrolyzing activities versus polymerizing activities, turned out to be of aromatic character. In the literature mainly aromatic amino acids are discussed to have affinity to sugar molecules e.g. by constituting a channel for transport of long chain sugars across membranes in the maltoporines; the maltoporin appears to be passive while the sugar glides screwlike along an aromatic lane (Dutzler et al., 2002).
Whereas in the invertase only amino acid substitutions altering aromatic residues changed enzyme specificity, in SST also mutations at other sites, namely C and H, led to activity shifts. At site C the amino acid cysteine occurs predominantly in SSTs. An exception are the SSTs from *Allium cepa* and *Allium sativum*, carrying an asparagine at this site like the invertases compared, except the *Hordeum vulgare* invertase. A mutation at site C in faSST led to a doubling of the hydrolytic activity relative to transferase activity in comparison to wild-type faSST. In contrast, the acINV carrying the mutation at site C had unchanged enzymatic properties. Cystein has the potential to form disulfide bonds that can severely influence the tertiary structure of proteins. It is unclear if the tertiary structure was affected and led to a change in enzyme specificity in the faSST mutated at site C, or if the mutation of the cysteine to the chemically more reactive asparagine was the cause for the doubling in relative hydrolase activity. None of the 3D-structures within the GH32 family indicated disulfide bonds.

The alignment shows that SSTs predominantly carry asparagines at site H with two exceptions, *Allium cepa* SST and *Lolium perenne* SST, where a serine is present. The alignment revealed always a serine in the corresponding site of vacuolar invertases. Both, serine and asparagine are polar amino acids, but whereas serine can only interact with other amino acids or water molecules via its hydroxyl-group, asparagine is capable of undergoing reactions with either their carbonyl-group or the amino-group. The mutant at site H of faSST showed increased relative hydrolyzing activity of sucrose. Whereas wild-type faSST guided 80% of its total activity into the polymerization of sucrose to 1-kestose, the mutant was mainly hydrolytically active. The relative hydrolyzing activity of the mutant was 80%. Nevertheless, the faSST mutant at site H did not become a typical invertase, since it synthesized more 1-kestose than wild type invertase did, and it was essentially not saturable in incubations with sucrose. No alteration of enzyme specificity was detected for the acINV mutant at site H.

Mutations at site D led to inactivity of both, acINV and faSST. Most probably the activity was lost due to a disturbance of the tertiary structure. The site is located next to a highly conserved proline-triplet. The grouping of prolines in the amino acid sequence often leads to turns or loops. This is also seen in the 3D-structures (Alberto et al., 2004). Already
slight disturbance of the vicinity of this highly conserved area might be a reason for inactivity of our constructs.

For plant FTs and vacuolar invertases no crystal structure is yet available. The data obtained in this work were theoretically analyzed using the 3D-structure of the invertase from the bacterium *Thermotoga maritima*. This bacterial invertase was chosen for the interpretation of the data, since like onion invertase and *Festuca* 1-SST it is a sucrose metabolizing enzyme. The exo-inulinase from *Aspergillus awamori* and the FEH form chicory use inulin.

Based on an alignment of *Thermotoga maritima* invertase, onion invertase and *Festuca* 1-SST, the probable positions of the sites A, B, C, D, E, F, G, H and X could be localized in the sequence of *Thermotoga maritima* invertase. The sites are all located on the $\beta$-propeller domain in the apparent crystal structure, which carries the active site (data not shown). Usually vacuolar invertases and FTs consist of two subunits, a large and a small one. The $\beta$-propeller domain probably represents the large subunit that has been shown to determine enzyme specificity in barley 6-SFT and *Festuca* 1-SST (Altenbach et al., 2004). The fact, that the positions of the amino acids correlating either with invertase or with SST activity can be assigned to amino acids on the $\beta$-propeller domain, strengthens the argument that these amino acids could influence enzyme specificity.

It is probable that enzyme specificity of invertases and FTs is also depending on the N-glycosylation. This possibility is currently discussed and experimentally investigated (Verhaest et al., 2005; Le Roi et al., Fructan 2004). The 3D-structure of chicory FEH shows a $\beta$-propeller with a $\beta$-sandwich attached to it. Verhaest and co-workers could show that the cavity between the two domains forms a cleft, emerging from the active site, which is believed to be the inulin-binding site. This idea is supported by the presence of four glycerol molecules in this cleft. The cleft is also present in the structure of the invertase form *Thermotoga maritima*, but the groove is occluded near the active site. The role of the cleft present in chicory FEH was investigated via the introduction of an N-glycosylation site within the cleft (Le Roi et al., Fructan 2004). This glycosylation site is normally absent in FEHs, but present in invertases. The recombinant
glycosylated protein showed a strongly reduced activity with inulin. Thus, differences in glycosylation might influence substrate binding and therefore have an effect on enzyme specificity. None of our mutations (A to X) affected a glycosylation site.

Fig. 9 *Thermotoga maritima* invertase (Alberto et al., 2004). (A) View from the top into the active site. Active site residues (the aspartates of the motifs (N/S)DPNG and RDP, and the glutamate of the EC-motif) are indicated in green. Amino acids (at sites A, C, G, H and X) that influenced hydrolysis versus polymerization in onion invertase and *Festuca* 1-SST are indicated in red. (B) Side-view illustrating amino acid substitutions that led to a change in enzymatic specificity. They are located on the rim of the central pocket.

The assigned localization of the nine sites in the 3D-structure of *Thermotoga maritima* invertase revealed, that those amino acids that influenced hydrolysis versus polymerization, are located at the rim of the central pocket. They correspond to sites A and G in acINV and to sites C and H in faSST. Being located opposite the entrance of the central pocket, site X is an exception in this respect. Possibly the sites A, C, G, and H that are located at the rim of the active site, influence substrate recognition and/or guidance of the sugar substrate to the active site. Molecules located on this rim could hinder or facilitate the entry of substrates and thereby have an influence on substrate specificity.
Pons and coworkers used a sequence-structure compatibility search for the theoretical elucidation of sites that influence enzyme activity and specificity in different glycoside hydrolase families (Pons et al., 2004). They speculated that positions far away from the active site are involved in fructosyltransfer reactions as well as in the hydrolysis of long-chain glycans while positions close to the active site are important for the orientation of substrates and the hydrolysis of short glycans. These theoretical data are in accordance with our mutagenesis data with invertase and SST since both enzymes cleave a short glycan (sucrose) and transfer the fructosyl units either to water or another sucrose, respectively. Thus, the mutations of amino acids that are located close to the active site (A, C, G, and H) influence relative hydrolase versus transferase activities of a short glycan, namely sucrose.

The three conserved motifs (N/S)DPNG, RDP and EC could be brought in connection with the general mechanism for cleavage of glycosidic bonds within GH32. The present work reveals amino acids located outside these highly conserved motifs that influence enzyme specificity. The balance between polymerization and breakdown reactions by invertases and SSTs could separately be modulated via directed amino acid substitutions. We therefore speculate that differences in the amino acid sequence in combination with differences in glycosylation influence substrate recognition and/or binding and therefore have an effect on enzyme specificity.

It is unlikely that one single mutation is sufficient to change an invertase to an SST and vice versa. In order to get further insight into what components are important in polymerization and/or hydrolyzation using sucrose as a substrate, SST and invertase mutants carrying multiple mutations have to be investigated.
Chapter 5

5. General Discussion

5.1. Initial questions and experimental approach

The main goal of my thesis was to find structural components (motifs) important for the catalytic function and specificity of plant FTs and vacuolar invertases. Comparisons of the deduced primary amino acid sequences of plant FTs and vacuolar invertases, as well as related enzymes from fungi and bacteria, were the basis for the selection of potentially interesting sites or motifs. We used domain swaps and site directed mutagenesis, followed by expression of the resulting constructs in the yeast *Pichia pastoris*, to elucidate the relative importance of these sites or motifs for enzymatic function.
5. 2. “Acid invertase-like” enzymes

Utilization of sucrose as a source of carbon and energy depends on its cleavage into hexoses. Sucrose can be hydrolyzed to its components glucose and fructose by the enzyme invertase. In plants, invertases are ubiquitous. They occur as acid invertases (vacuolar invertases and cell-wall invertases) and alkaline (or neutral) invertases. If the protein sequence of any acid plant invertase is “blasted” against sequences of proteins from the kingdom of bacteria, the highest similarities are detected with bacterial invertases, levanases, inulinases, sucrose 6-phosphate hydrolases and levansucrases (Fig. 1). Similarly, “blasting” of an invertase sequence against proteins from the fungal kingdom, retrieves the sequences of invertases, inulinases, and of fructosyltransferases (Heyer and Wendenburg; 2001). If the protein sequence of a plant invertase is “blasted” against sequences of plant proteins, the first sequences retrieved correspond to invertases, FEHs and FTs. Interestingly, there are no “hits” when any invertase is “blasted” against all available sequences of animal proteins. Animals hydrolyze sucrose with the enzyme sucrase, a structurally totally unrelated enzyme. There are five “hits” when an invertase is blasted against sequences of proteins from the kingdom of Protista (e.g. *Leishmania*), and only one “hit” against sequences of Archaea proteins (*Haloarcula marismortui*).

![Fig. 1 Kingdoms harboring “acid invertase-like” enzymes.](image-url)
Plants contain an additional type of invertase, namely neutral/alkaline invertase. This enzyme shares no homology in its primary amino acid sequence with the acid invertase. Interestingly only neutral invertases and no acid invertases are reported for blue algae (cyanobacteria) such as for example *Anabaena variabilis* (Schilling and Ehrnsperger; 1985; Porchia et al., 1999). There are no homologues of neutral invertases in any other kingdom.

In summary, the class of “acid invertase-like” enzymes is predominantly found in the kingdoms of bacteria, fungi and plants. Their abundance in these only distantly related organisms implies an extremely large protein family. Thus the phylogenetic tree of plant acid invertases, FTs and FEHs, which is shown in chapter 4 (Fig. 1), represents only a very small branch of a huge tree of “acid invertase-like” enzymes. An example of an even smaller branch is provided by an unrooted tree of plant cell-wall invertases and FEHs (Fig. 2; Van den Ende et al., 2004). Although plant FEHs show no invertase activity, their amino acid sequences are highly homologous especially to cell-wall type invertases (Van den Ende et al., 2002). For example, the sugar beet 6-FEH does not cluster with the 1-FEHs from chicory plants in the tree, but appears in a different subgroup that also contains invertases from *Arabidopsis thaliana* (AtcwINV1, AtcwINV3 and AtcwINV5). AtcwINV6 groups together with FEHs from fructan plants in the phylogenetic tree. It was recently demonstrated that AtcwINV3 and AtcwINV6 are not invertases but are real FEHs with different substrate specificities. By contrast, AtcwINV1 was found to be a typical invertase (De Coninck et al., 2005). The tree illustrates the close relatedness of plant cell-wall invertases and FEHs.

Two additional “acid invertase-like” enzymes are included in this unrooted tree which are named microbial FEHs (Van den Ende et al., 2004): from bacterial origin, the levanase from *Bacillus subtilis* (6-FEH), and from fungal origin, the exo-inulinase from *Aspergillus awamori* (1-FEH). These enzymes from bacteria or fungi catalyze almost the same reaction as the plant enzymes but are located far away in the phylogenetic tree. The illustration of the inulinase from *Aspergillus awamori* and the levanase from *Bacillus subtilis* in the tree nicely shows that functionally closely related enzymes are widely dispersed on a huge tree of “acid invertase-like” enzymes (Fig. 2).
Fig 2. (taken from Van den Ende et al., 2004): Phylogenetic tree of plant cell wall invertases and FEHs. For comparison, two microbial FEHs are included as outliers (green branches). FEHs are indicated in green. Enzymes that have a low iso-electric point are underlined. The six Arabidopsis sequences (AtCWINV1–AtCWINV6) are boxed. For a detailed list of the Accession numbers, see http://www.kuleuven.ac.be/bio/dev/nederlands/cwtree.htm. The cell wall-type glycosyl hydrolases are tentatively divided into three major groups A–C. Group A is further separated into two subgroups I (monocots) and II (dicots). Group C is separated into three subgroups I (dicots), II (monocots) and III (monocots). The phylogenetic relationships were analyzed using ClustalW (http://www.ebi.ac.uk/clustalw/) and TREEVIEW [43]. The scale bar indicates a distance value of 0.1. Abbreviations: FEHs, fructan exohydrolases; INV, cell wall invertase; INV?, putative cell wall invertase (functionality not determined).
For each of the three distantly related kingdoms that harbor “acid invertase-like”
proteins (bacteria, fungi and plants) crystal structures became available recently: (i) the
invertase from the bacterium *Thermotoga maritima* (Alberto et al., 2004), (ii) the exo-
inulinase from the fungus *Aspergillus awamori* (Nagem et al., 2004) and (iii), the fructan-
exohydrolase (FEH) from chicory (Verhaest et al., 2005). All structures show a five-
bladed $\beta$-propeller with a deep negatively charged central pocket and a $\beta$-sandwich
attached to it (for details see chapter 1.6.). Thus there is a large family of “acid invertase-
like hydrolases” dispersed along different kingdoms of organisms, with almost identical
three-dimensional structures. Through a superposition of the structures, it was observed
that the active site is located in the $\beta$-propeller domain. It seems to be a characteristic that
the length of the proteins is conserved. The $\beta$-sandwich appears to be necessary for a
functional enzyme, although it is distant from the active site. The N-terminal $\beta$-propeller
corresponds to the N-terminal large subunit, and the C-terminal $\beta$-sandwich module to
the C-terminal small subunit found in plant vacuolar invertases and FTs, where it usually
consists of the C-terminal third of the protein.

Our results provide further information with respect to the function of the two subunits:
The analysis of chimeras between *Festuca* 1-SST and barley 6-SFT (Chapter 2) showed
that the product forming activity was entirely determined by the large N-terminal subunit.
This agrees well with the fact that the active site is located on the $\beta$-propeller in all crystal
structures elucidated so far. If only the large subunit of *Festuca* 1-SST was expressed in
*Pichia*, no FT activity could be detected. The amount of the recombinant protein found in
the *Pichia* culture filtrate was very low, possibly because of incorrect targeting in the
yeast cells or because of degradation by *Pichia* proteases. The small subunit might thus
have an effect on protein folding, proper post-translational processing, cellular targeting
or on the control of enzyme activity, but it is not involved in determining enzyme
specificity.
5.3. Enzyme specificity of “acid invertase-like” enzymes

Enzyme activities are manifold among “acid invertase-like” enzymes. They can basically be subdivided into fructosylhydrolases and fructosyltransferases. According to our view, the catalytic specificity of these enzymes basically involves an active site for the fructosyl donor and an active site for the fructosyl acceptor.

The simplest reaction is catalyzed by invertases. They initially bind the fructosyl moiety of sucrose (donor) and, in a second step, release it and transfer it to water (acceptor). 1-SSTs also initially cleave the bond between the glucose and fructose moiety of sucrose and bind the fructosyl residue, but then transfer it to another sucrose (acceptor). In contrast to invertases, 1-SSTs must therefore harbor a binding-site for the fructosyl acceptor sucrose. This can be considered as a first step in the evolution from vacuolar invertases towards FTs. A third enzyme which is also capable of using sucrose as fructosyl donor is 6-SFT. Like invertases, 6-SFTs cleave the bond between glucose and fructose of sucrose, bind the fructosyl moiety, but then transfer it to either sucrose or fructan (forming kestose or bifurcose). Like 1-SSTs, 6-SFTs must therefore have a binding-site for a fructosyl acceptor, but with a different specificity than 1-SSTs. Thus a further step in the evolution of specific SFTs was likely the emergence of a preference for a fructan as fructosyl acceptor. FFTs lost the capacity to use sucrose as fructosyl donor, but instead use a fructan of DP≥3 as donor. The fructosyl acceptor can be either sucrose or a fructan. The basic difference of FFTs compared to the previously described enzymes is that the donor-binding site can only accept fructan.

What can be learned from the crystal structures published up to now? Based on the extensive overlap of the active site of the available 3D-structures of “acid invertase-like” enzymes, the fructosyl donor binding site can be relatively well derived, because the exo-inulinase from Aspergillus awamori was crystallized in the fructose-bound state. In contrast, the invertase from Thermotoga maritima and the FEH from chicory were crystallized in the ligand-free states only. The donor binding site includes the three motifs that were also emerging from biochemical studies, namely the (N/S)DPNG, the RDP and the EC-motif. The reaction scheme in the case of 1-FEH has been interpreted as follows...
(Verhaest et al., 2005): a fructan of the inulin type initially binds to the active site, where the glycosidic oxygen of the inulin is protonated by the glutamate of the EC-motif. This step is followed by a nucleophile attack of the aspartate in the (N/S)DPNG motif, forming a covalent fructosyl-intermediate. The third step of the reaction is the hydrolysis of the fructosyl-intermediate releasing fructose and the enzyme in its ligand-free state. The same scheme can be applied to invertases and plant FTs, where instead of inulin, sucrose, 1-kestose or another fructan acts as fructosyl donor and binds to the aspartate of the (N/S)DPNG motif. Thus the fructosyl donor binding step is basically solved. However, it is currently unknown what structures account for donor preferences. Furthermore, it is unknown where the fructosyl acceptor binds and what determines acceptor preferences.

Our results show that small changes in amino acid sequence can be the basis for differences in donor and acceptor preferences.
1. The proteins with mutations of the glutamate of the EC-motif in Festuca 1-SST, were unable to use sucrose, but retained the ability to use 1-kestose (Chapter 3). Our results are particularly intriguing, since they indicate a change in the enzyme's catalytic activity caused by one single amino acid substitution.
2. Both, invertases and 1-SSTs use sucrose as fructosyl donor, but differ in their fructosyl acceptor which is water or another sucrose, respectively. In our mutational analysis of nine single amino acid residues in onion invertase and Festuca 1-SST, the mutated enzymes revealed changed catalytic specificity, gradually demonstrating the importance of single amino acids on donor and acceptor preferences (Chapter 4).
5.4. Outlook

We could show that the large N-terminal subunit entirely determines enzyme specificity in barley 6-SFT and *Festuca* 1-SST. The C-terminal small subunit was shown to have no impact on catalytic specificity, but might play a role in protein folding and stability or the control of enzyme activity. It would be interesting to further study the function of the small subunit by exchanging the subunits of enzymes which are less related than 1-SST and 6-SFT, e.g. by using enzymes with completely different substrate preferences like invertases and FFTs or SSTs and FEHs etc.

Furthermore, the functions of the post-translational cleavage process, if any, remains elusive. FTs which are cleaved in the two subunits *in planta*, are basically uncleaved but nevertheless active if expressed in *Pichia*. Specific plant factors, such as proteolytic enzymes in the plant vacuole might direct the cleavage process of plant FTs; such factors might be absent in *Pichia pastoris*. The expression of plant FTs in *Pichia* provides a “natural” tool for differences in post-translational modifications. Cellular targeting and glycosylation patterns differ in plants and the yeast system. These post-translational differences might have an impact on enzyme specificity and hence explain the differences in the activities between plant purified and recombinant FTs, as seen for barley 6-SFT (Hochstrasser et al., 1998). Studying translational modifications of FTs in *Pichia* might provide important knowledge of structure-function relationships. A promising approach for structure-function elucidations would be a mutational analysis of the potential glycosylation sites present in plant FTs and vacuolar invertases.

Our studies of conserved motifs revealed the importance of the (N/S)DPNG-, the RDP- and the EC-motif in the transfructosylation reaction of plant FTs, and helped to narrow down the region responsible for enzyme specificity. We could show *via* the analysis of chimeric enzyme, that the region responsible for enzyme specificity is located between the (N/S)DPNG motif and the small subunit in barley 6-SFT and *Festuca* 1-SST. More detailed domain exchanges within the large subunit, in conjunction with other FTs such as FFTs and FEHs and in conjunction with site-directed mutagenesis studies of
some of the highly conserved regions in these enzymes should further narrow down the regions important for enzyme specificity.

We could pinpoint the importance of the acidic amino acids of the highly conserved motifs (N/S)DPNG, EC and RDP using a mutational approach. Furthermore we could alter the relative hydrolase and transferase activities of onion invertase and Festuca 1-SST via directed amino acid substitutions.

It is interesting that the mutation in Festuca 1-SST of the EC-motif resulted in a mutant which had lost the capacity to use sucrose, but retained the ability to use 1-kestose. We conclude that there must be an alternative glutamate replacing the glutamate of the EC-motif for the activity with 1-kestose. It is impossible to predict which glutamate could act as alternative acid/base catalyst, due to the low overall homologies of the plant FTs to the crystal structures available. In order to discuss such key questions, one of the main goals in the near future should be the structural elucidation of a plant FT. A possible hindrance in the crystallization of a plant FT might be protein N-glycosylation. Chicory FEH, with only two glycosylation sites, is the only member of GH family 32 to be successfully crystallized so far. Crystallizing Festuca 1-SST, an enzyme carrying six potential glycosylation sites, might be a tedious but path-breaking task.
References


Porchia, A.C., Currati, L. and Salerno, G.L. (1999): Sucrose metabolism in cyanobacteria: sucrose synthase from Anabena sp. strain PCC 7119 is remarkably different from the plant enzymes with respect to substrate affinity and amino-terminal sequence. Planta 210, 34-40


| 90 | ------------------------ | SRGKDFGVSEKASGA | ------- | YSADGG | FPWSNA | faSST |
| 83 | ------------------------ | SRGKDSGVSEKENES | ------- | PADDG | FPWSNA | lpSST |
| 60 | ------------------------ | SRGKEGVSEKTSGA | ------- | YSANGG | FPWSNA | hSTT |
| 91 | ------------------------ | SRGKEHGVSEKTSGA | ------- | YSAN-A | FPWSNA | taSST |
| 67 | ------------------------ | SDSE | ------- | -------- | DYPWTNE | acSST |
| 83 | ------------------------ | GTGSDK | ------- | AD-A | FPWSNA | psSPT |
| 62 | ------------------------ | VDEEAA | ------- | AG-G | FPWSNE | hvSFT |
| 61 | ------------------------ | VDEEAA | ------- | GG | FPWSNE | taSST |
| 56 | ------------------------ | VDN | ------- | -------- | EFPWTND | acINV |
| 73 | ------------------------ | EKSFIRYSQ | ------- | -------- | TD | htFTT |
| 74 | ------------------------ | EKSSAQHYQ | ------- | -------- | SD | ciFTT |
| 83 | ------------------------ | SRCPEGVSEKTSAGAHGMLGADAG-NAPFWSNA | hvINV |
| 89 | ------------------------ | MLQWQRTGFHFQPEKNWM | NDPNG | PVYYKGWYHFLFYQYNPKGDWSW--NIAWAHAVSKDMV | faSST |
| 107 | MLQWQRTGFHFQPEKNWM | NDPNG | PVYYGGWYFLFYQHNPYGDSW--NVSWGHAVSKDLV | lpSST |
| 117 | MLQWQRTGFHFQPEKNWM | NDPNG | PVYYGGWYHFQPEKNWM--QIVWGHAVSKDLV | hvSST |
| 79 | ------------------------ | TDGVEEVGSSSKGAGF-GLLTSVSHG--QYPWTKN | aoINV |
| 89 | ------------------------ | TPFAVRLG--G | ------- | -------- | GAVR | DYAWTNS | osINV |
| 92 | ------------------------ | RGVEKGVSEKSFHP | ------- | LLGADN | SFPWSNN | ciINV |
| 3 | ------------------------ | HEKVE | ------- | -------- | AFPWNNNT | atINV |
| 19 | ------------------------ | PY | ------- | -------- | ciCWINV |
| 50 | ------------------------ | PY | ------- | -------- | atCWINV |
| 17 | ------------------------ | PY | ------- | -------- | atCWINV6 |
| 54 | ------------------------ | PY | ------- | -------- | bvFEH |
| 44 | ------------------------ | PY | ------- | -------- | ciFEH* |
| 1 | ------------------------ | | ------- | -------- | tmINV* |
| 22 | ------------------------ | | ------- | -------- | aaINU* |

(N/S)DPNG-motif

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| 117 | MLQWQRTGFHFQPLKHYN | NDPNG | PVYYGGWYHFQHNPYGDSW--QIVWGHAVSKDLV | hvSST |
| 78 | MLKWQRTGFHFQPLKHYN | NDPNG | PVYYGGWYHFQHNPYGDSW--QIVWGHAVSKDLV | taSST |
| 82 | MLKWQRTGFHFQPLKHYN | NDPNG | PVYYGGWYHFQHNPYGDSW--QIVWGHAVSKDLV | taSST |
| 77 | MLKWQRTGFHFQPLKHYN | NDPNG | PVYYGGWYHFQHNPYGDSW--QIVWGHAVSKDLV | hvSST |
| 75 | MLKWQRTGFHFQPLKHYN | NDPNG | PVYYGGWYHFQHNPYGDSW--QIVWGHAVSKDLV | taSST |
| 66 | MLKWQRTGFHFQPLKHYN | NDPNG | PVYYGGWYHFQHNPYGDSW--QIVWGHAVSKDLV | hvSST |
| 56 | MLKWQRTGFHFQPLKHYN | NDPNG | PVYYGGWYHFQHNPYGDSW--QIVWGHAVSKDLV | hvSST |
| 43 | ML KWQRTGFHFQPLKHYN | NDPNG | PVYYGGWYHFQHNPYGDSW--QIVWGHAVSKDLV | hvSST |
| 21 | ML KWQRTGFHFQPLKHYN | NDPNG | PVYYGGWYHFQHNPYGDSW--QIVWGHAVSKDLV | hvSST |
| 52 | ML KWQRTGFHFQPLKHYN | NDPNG | PVYYGGWYHFQHNPYGDSW--QIVWGHAVSKDLV | hvSST |
| 17 | ML KWQRTGFHFQPLKHYN | NDPNG | PVYYGGWYHFQHNPYGDSW--QIVWGHAVSKDLV | hvSST |
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| 17 | ML KWQRTGFHFQPLKHYN | NDPNG | PVYYGGWYHFQHNPYGDSW--QIVWGHAVSKDLV | hvSST |
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| 52 | ML KWQRTGFHFQPLKHYN | NDPNG | PVYYGGWYHFQHNPYGDSW--QIVWGHAVSKDLV | hvSST |
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372 ---TVEDE--ATNRIRAFL------DSCSVFEPFN-DSIAFSFRIHEN------VJNI tmINV*
468 GPLETP S T--GVVKL SIEFV------DRSSVEFGQGGETT LTQA IFPS SSD-A---VHAR aaINV*
**Fig.** Alignment of vacuolar invertases (INV), 1-SSTs (SST), 6-SFTs (SFT), 6G-FFT (GFT), cell-wall invertases (CWINV), fructan exo-hydrolases (FEH) and exo-inulinase (INU). acINV, Allium cepa INV (AJ006067); acSST, Allium cepa SST (AJ006066); acGFT, Allium cepa 6G-FFT (ACY07838); atINV, Arabidopsis thaliana INV (S57951); atCWINV, Arabidopsis thaliana CWINV (NM_112232); atCWINV6 (re-classified as FEH; DeConinck et al., 2005), Arabidopsis thaliana CWINV6 (NM_121230); aaINU, Aspergillus awamori INU (AJ315793); bvFEH, Beta vulgaris FEH (AJ508534); ciINV, Cichorium intybus INV (AJ419971); ciCWINV, Cichorium intybus CWINV (Y11124); ciFEH, Cichorium intybus FEH (AY323935); ciSST, Cichorium intybus SST (U81520); faSST, Festuca arundinacea (AJ297369); htFFT Helianthus tuberosus FFT (AJ009756); htSST, Helianthus tuberosus SST (AJ009757); hvINV Hordeum vulgare INV (IAJ623275); hvSFT, Hordeum vulgare 6-SFT (X83233); hvSST, Hordeum vulgare SST (AJ567377); lpSST, Lolium perenne SST (AF492836); osINV, Oryza sativa INV (AF276703); psSFT, Poa secunda 6-SFT (AF192394); tmINV, Thermotoga maritima INV (AJ001073); taINV, Triticum aestivum INV (AJ635225); taCWINV, Triticum aestivum CWINV (AF030420); taSFT, Triticum aestivum 6-SFT (AB029887); taSST, Triticum aestivum SST (AB029888). For three enzymes of GH family 32 crystal structures are available. They are marked with an asterisk (*).
**Publications**

03 Distinct regulation of sucrose-sucrose-1-fructosyltransferase (1-SST) and sucrose-fructan-6-fructosyltransferase (6-SFT), the key enzymes of fructan synthesis in barley leaves: 1-SST as the pacemaker. New Phytologist 04 (161: 735-748)
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04 The large subunit determines catalytic specificity of barley sucrose:fructan 6-fructosyltransferase (6-SFT) and fescue sucrose:1-fructosyltransferase (1-SST)
FEBS Letters 04 (567: 214-218)
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05 Mutational analysis of the active center of fructosyltransferases: Festuca 1-SST and barley 6-SFT. Submitted
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**Conferences**

00 Fructan 2000, Arolla (CH)
Poster presentation of the Diploma Thesis

04 Fructan and Human Health, Lugano (CH)
Oral presentation: Modifying plant fructosyltransferase activities using domain-shuffling

04 Fructan 2004, Havana (Cuba)
Poster presentation: the collaborative work with the University Utrecht: Are fructosyltransferases adapted Invertases?