Regulation of fructan metabolism in barley leaves

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SUMMARY

Fructans, the polymers of fructose (Fru), are major non-structural storage carbohydrates in the vegetative tissues of many higher plants including temperate forage grasses and cereals, as well as major crop plants such as wheat and barley. Fructans play an important role in assimilate partitioning, plant development, environmental stress tolerance etc. Fructans also have a vast application potential in nutrition and medicine. The main focus of this dissertation is the fructan biosynthetic pathway in barley leaves. Its major aspects are the identification of sucrose:sucrose 1-fructosyltransferase (1-SST) as a pacemaker enzyme, regulation of the promoter of sucrose:fructan 6-fructosyltransferase (6-SFT) - one of the main fructosyltransferases (FTs) and the role of vacuolar invertases during fructan metabolism.

Excised barley leaves exposed to continuous light accumulate large amounts of fructans containing β(2-6) linkages with β(2-1) branches, the so-called graminans. The pathway for graminan biosynthesis has not been well characterised, but it has been proposed that the successive action of two main enzymes, 1-SST and 6-SFT is involved (1-SST/6-SFT model). To demonstrate the validity of this model, excised leaves were subjected to a light-dark regime known to sequentially induce fructan accumulation and mobilization. The pattern of accumulation of soluble carbohydrates, the level of 1-SST and 6-SFT activities, and the expression of the corresponding genes, all indicate that the diversion of sucrose (Suc) into the pathway fructan synthesis is initiated by 1-SST induction. The stability of transcripts and enzyme activities of 1-SST and 6-SFT were compared, using appropriate inhibitors. The transcripts of 1-SST and enzymatic activity are subject to a rapid turnover and respond more quickly than 6-SFT. The much higher responsiveness of 1-SST to

regulatory processes clearly indicates that it plays the role of the pacemaker enzyme of fructan synthesis in barley leaves.

Plants regulate fructan synthesis in response to several internal and external stimuli primarily through the modulation of gene expression of FTs. Little is known about signal perception and transduction events that control the expression of FT genes. The regulatory sequences of FT genes are valuable tools to decipher the underlying signaling events. Using PCR-based genome walking procedures, the promoter of 6-SFT gene corresponding to 1.6 kb of the upstream region of the coding sequence, was cloned. The promoter activity of the cloned sequence was investigated in transient assays by fusing it to a reporter gene [*uidA* encoding β-glucuronidase, (GUS)] and by microprojectile bombardment of excised barley leaves. Strong expression of the GUS gene was observed in leaves induced for fructan biosynthesis by Suc and light, indicating that the cloned sequence contains the necessary *cis* acting elements conferring Suc and light induction of 6-SFT transcription.

Arabidopsis thaliana has been extensively used to study the sugar induced signal transduction pathways in plants. In order to investigate the signaling events involved in the activation of the 6-SFT promoter, stably transformed Arabidopsis plants harboring the 6-SFT promoter driving the expression of the GUS reporter gene, were obtained. Though Arabidopsis is a non-fructan producing plant, the sugar-regulated activation of the barley 6-SFT promoter is maintained in Arabidopsis. The inhibitors of protein phosphatases and protein kinases, and a chelator of calcium, known to block Suc induction of 6-SFT gene expression in wheat, were effective in Arabidopsis too, suggesting that this signal transmission process seems to be conserved between cereals and Arabidopsis. These transgenic plants are valuable to

study the activity of the barley 6-SFT promoter further and identify the transcription factors that interact with the key promoter elements.

Invertases play a central role in the metabolism of Suc, the main product of photosynthesis and substrate for the synthesis of the fructans. Soluble acid invertase (SAI) isoforms are present in the vacuoles and are believed to be the ancestors of fructosyltransferases FTs. No SAI sequences are available from barley yet. In the present work, a soluble acid invertase cDNA was cloned from barley (HvSAI) and functionally characterized by heterologous expression in *Pichia pastoris*. Furthermore, the expression of HvSAI gene was studied in excised leaves and roots. The recombinant HvSAI cleaves Suc efficiently, but despite very high amino acid sequence similarity to FTs, is devoid of FT or fructan hydrolase like side activities. Compared to the FTs, the activity of the recombinant HvSAI is relatively easily saturable (Km of 13.5 mM for Suc) and possesses a higher temperature optimum (10°C more that 1-SST). The mRNA levels of HvSAI are constitutive and not affected much by enhanced sugar levels in excised leaves and roots, by Suc supply or continuous illumination of cut leaves. The cloning of SAIs will help to investigate their role in the regulation of fructan metabolism and decipher the structure-function relationship between SAI and FTs.

ZUSAMMENFASSUNG

Fruktane, die Polymere der Fruktose sind bedeutende, unstrukturierte Speicherkohlenhydrate im vegetativen Gewebe zahlreicher höherer Pflanzen, einschliesslich Futtergräser und Getreidepflanzen der gemässigten Breiten und bedeutende Feldfrüchte, wie Weizen und Gerste. Fruktane spielen eine wichtige Rolle bei der Verteilung von Photosynthese- Assimilaten, der pflanzlichen Entwicklung, der Toleranz gegenüber umweltbedingtem Stress usw. Fruktane haben auch ein enormes Anwendungspotential in Ernährung und Medizin. Der Hauptblickpunkt dieser Dissertation ist der Biosyntheseweg von Fruktanen in Gersteblättern. Die wichtigsten Aspekte sind hierbei die Identifikation der Saccharose: Saccharose 1-Fruktosyltransferase (1-SST) als Schrittmacher-Enzym der Fruktan- Biosynthese, sowie die Regulation des Promoters der Saccharose: Fruktan 6-Fruktosyltransferase (6-SFT) – eine der bedeutendsten Fruktosyltransferasen (FTs) - und die Rolle der vakuolären Invertasen im Fruktan- Metabolismus.

Abgeschnittene Gerstenblätter, die Dauerlicht ausgesetzt werden, akkumulieren grosse Mengen an Fruktanen, welche β(2-6) Bindungen mit β(2-1) Verzweigungen ausbilden. Dabei handelt es sich um sogenannte Graminane. Der Weg der Graminan-Biosynthese ist noch unzureichend charakterisiert, aber es wurde vorgeschlagen, dass die aufeinander folgende Aktivität der beiden bedeutendsten Enzyme, der 1-SST und 6-SFT, dabei involviert ist (das 1-SST/6-SFT Modell). Um die Gültigkeit dieses Systems zu demonstrieren, wurden abgeschnittene Gerstenblätter unter speziellen Licht-Dunkel-Bedingungen gehalten, deren Abfolge gemäss Literatur die Anreicherung und Mobilisierung von Fruktanen hervorruft. Das Modell der Anreicherung löslicher Kohlenhydrate, die Aktivität von 1-SST und 6-SFT, sowie die

Expression der entsprechenden Gene deuten insgesamt darauf hin, dass die Fruktan-Biosynthese aus Saccharose durch Induktion von 1-SST herbeigeführt wird. Die Stabilität der RNA-Transkripte und die Aktivität der Enzyme 1-SST und 6-SFT wurden unter Zuhilfenahme geeigneter Inhibitoren getestet. Die Transkripte von 1-SST und die enzymatische Aktivität des entsprechenden Proteins unterliegen einer raschen Erneuerung und reagieren auf Änderungen schneller als 6-SFT. Die weitaus höhere Empfindlichkeit von 1-SST gegenüber regulatorischen Prozessen deutet stark darauf hin, dass 1-SST die Rolle des Schrittmacher-Enzyms in der Fruktan-Biosynthese in Gerstenblättern einnimmt.

Pflanzen regulieren die Synthese von Fruktanen - als Antwort auf zahlreiche interne und externe Stimuli - vor allem durch die Modulation der Genexpression der FTs. Es ist jedoch bis jetzt wenig bekannt über die Ereignisse der Perzeption und Transduktion der Signale, welche die Expression der FT-Gene regulieren. Die regulatorischen Sequenzen der FT-Signale sind sehr wertvolle Hilfsmittel zur Entschlüsselung der zugrunde liegenden Signalereignisse. Unter Zuhilfenahme der *PCR-based genome walking* Methode, konnte der Promoter des 6-SFT Gens kloniert werden, welcher 1.6 Kilobasen der upstream gelegenen Region der kodierenden Sequenz entspricht. Die Promoteraktivität der klonierten Sequenz wurde in transienten Assays untersucht, in welchen der Promoter mit einem Reporter-Gen fusioniert wurde [*uidA*, welches für Glucuronidase (GUS) kodiert]. Diese Konstrukte wurden durch Mikroprojektil-Bombardierung in abgeschnittene Gerstenblätter eingeführt. Eine starke Expression des GUS Gens wurden in Blättern beobachtet, welche durch Saccharose und Lichteinfluss zur Synthese von Fruktanen angeregt wurden. Dies deutet darauf hin, dass die klonierte Sequenz in der Tat die benötigten, in *cis*-Position befindlichen Elemente besitzt, welche die Induktion der RNA-

Transkription von 6 –SFT durch Saccharose und Licht überträgt.

Arabidopsis thaliana ist ein weithin etabliertes Modellsystem zur Analyse von Zucker-induzierten Signaltransduktionswegen in Pflanzen. Um die Signalereignisse zu untersuchen, die mit der Aktivierung des 6-SFT Promoters zusammenhängen, wurden stabil transformierte *Arabidopsis* Pflanzen hergestellt, welche den 6-SFT Promoter beinhalten, der die Expression eines GUS- Promoters antreibt. Obgleich *Arabidopsis* keine Fruktan-produzierende Pflanze ist, wird eine Zucker-regulierte Aktivierung des Gerste- 6-SFT Promoters in *Arabidopsis* beibehalten. Inhibitoren von Proteinphosphatasen und Proteinkinasen, sowie ein Kalzium-Chelator, deren inhibitorische Wirkung auf die Saccharose-Induktion der Genexpression von 6-SFT in Weizen bekannt ist, sind auch in *Arabidposis* funktionstüchtig, was uns vermuten lässt, dass die generellen Signaltransduktionsprozesse zwischen Getreide und *Arabidosis* konserviert zu sein scheinen. Die transgenen Pflanzen sind ein wertvolles Hilfsmittel um die Aktivität des Gerste- 6-SFT Promoters eingehender zu studieren und Transkriptionsfaktoren aufzuspüren, welche mit den Schlüsselelementen des Promoters interagieren.

Invertasen spielen eine bedeutende Rolle im Metabolismus von Saccharose, das Hauptprodukt der Photosynthese und sind gleichzeitig das Substrat zur Synthese von Fruktanen. Isoformen der löslichen sauren Invertasen [Soluble acid invertases (SAI)] wurden in den Vakuolen identifiziert. Sie werden als Vorgänger der Fruktosyltransferasen (FTs.) betrachtet. Bis jetzt sind noch keine SAI Sequenzen aus Gerste veröffentlicht worden. In dieser Arbeit wurde eine SAI cDNA aus Gerste kloniert (*HvSAI*) und durch heterologe Expression in *Pichia pastoris* funktionell charakterisiert. Des Weiteren wurde die Expression des *HvSAI* Gens in abgeschnittenen Gersteblättern und Gerstewurzeln untersucht. Das rekombinante

HvSAI spaltet Saccharose mit hoher Effizienz, doch trotz sehr grosser Ähnlichkeit zu FTs auf Ebene der Aminosäuren, besitzt das Protein keine FT- oder Fruktanhydrolase-ähnliche Nebenaktivität. Verglichen mit FTs, ist die Aktivität des rekombinanten HvSAI sehr leicht saturierbar, (Km beträgt 13.5 mM für Saccharose). Zudem zeigt sie ein höheres Temperaturoptimum (ca. 10°C mehr als 1-SST). Die mRNA Mengen des HvSAI-Gens sind konstitutiv und werden weder durch erhöhte Zuckermengen in abgeschnittenen Gersteblättern und Gerstewurzeln, noch durch Zugabe von Saccharose, oder durch Dauerbeleuchtung beeinflusst. Die Klonierung der SAIs wird von grossem Nutzen sein, wenn es darum geht, ihre Rolle bei der Regulation des Fruktan-Metabolismus zu untersuchen und Struktur-Funktions-Verhältnisse zwischen SAIs und FTs zu entschlüsseln.

(German translation by Thorsten Fritzius and Philipp Raab)

SCOPE OF THIS THESIS

While significant progress has been made in understanding the biochemistry and enzymology of inulin metabolism, graminans have been relatively less studied. The objective of this dissertation is to gain new insights into the synthesis of graminans in barley leaves, the regulation of the main fructosyltransferases (1-SST and 6-SFT), the activity of 6-SFT promoter and the physiological significance of SAIs during fructan metabolism.

This thesis contains 6 chapters, starting with a general introduction. An attempt has been made to introduce the reader to the basic information on fructans and the current progress made in the field has been reviewed. Chapters 2 to 5 describe the experimental work undertaken and is presented in the form of independent research articles. Each of these chapters also contains an introduction to provide a background to the work that follows. Since published articles (chapter 2 and 3) or those intended for submission (chapter 4 and 5) have been almost directly inserted into this thesis, some parts are redundant with respect to summary, general introduction and general discussion chapters.

Chapter 2 has been published in New Phytologist **161:** 735-748 (2004) with the title: “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” and the work involves contributions from the authors Nagaraj VJ, Altenbach D, Galati V, Luscher M, Meyer AD, Boller T and Wiemken A. This study extends the previous work of purification of barley 1-SST (Lüscher et al., 2000) by employing a reverse genetics approach to clone the 1-SST cDNA. This sequence formed the bases for an extensive comparison of the regulation

of 1-SST with the other key enzyme, 6-SFT whose cloning was reported earlier (Sprenger et al., 1995). Graminan biosynthesis in the excised leaf system, is demonstrated to occur *via* the previously proposed 1-SST/6-SFT model (Wiemken et al., 1995). Evidence is also presented suggesting 1-SST as the primary and tightly controlled pacemaker enzyme by which the diversion of sucrose into the fructan synthesis pathway is regulated in barley leaves.

The cloning and activity of the first gene sequence representing a FT promoter is described in chapter 3. This work has been published in the Journal of Plant Physiology **158:** 1601-1607 (2001) under the tile "Light and sugar regulation of the barley sucrose:fructan 6-fructosyltransferase promoter" with **N**agaraj VJ, Riedl R, Boller T, Wiemken A and Meyer AD as the contributing authors. Using genome-walking techniques, the upstream region of the coding sequence of the barley 6-SFT was identified. The activity of the 6-SFT promoter region in response to light and sucrose is demonstrated during transient assays in excised leaves.

Transgenic *Arabidopsis thaliana* plants carrying the 6-SFT promoter driving the expression of a GUS reporter gene were obtained to study the signalling events controlling the activity of the 6-SFT promoter (chapter 4). Transformation of Arabidopsis involved collaboration with Dr. Sjef Smeekens, University of Utrecht, The Netherlands. Studies involving the regulation of promoter activity in Arabidopsis leaves using inhibitors of protein phosphatases, protein kinases and calcium chelators where done with valuable input from Ms. Giselle Martinez Noel, Centro de Investigaciones Biológicas, Fundación para Investigaciones Biológicas Aplicadas, Mar del Plata, Argentina M.

Chapter 5 deals with the cloning and characterization of a barley SAI cDNA (HvSAI) and is a continuation of the work initiated by Virginie Galati during her

ATA diploma (1998). SAIs are closely related to FTs with respect to sequence, subcellular localization and biochemical properties. The amino acid changes leading to the evolution of FTs, the differences in the biochemical properties of recombinant HvSAI and barley 1-SST, and the role of SAIs during fructan metabolism are discussed in this chapter.

Chapter 6 is a general discussion of all the new findings reported in this dissertation. The open questions related to the work done and future scenarios for fructan research are also dealt with in this chapter. The complete list of references cited in the various parts of the thesis is available at the end.

CHAPTER 1

General introduction

Importance of fructans

Each year it is estimated that more than 100 billion metric tons of CO_2 and H_2O are converted into carbohydrates by plants and algae through the process of photosynthesis. Carbohydrates play an important role in fulfilling man's basic needs of food, clothing and shelter and are increasingly finding novel applications in engineering, nutrition and medicine. One such carbohydrate is fructan, a polymer of Fru. Fructans are considered as functional food ingredients because of their beneficial effects on health. Experimental studies have shown that fructans act as bifidogenic agents by encouraging the development of beneficial intestinal bacteria (Kruse *et al.*, 1999) and decreasing the levels of pathogenic bacteria in the intestine (Kleessen *et al.*, 2001). Fructans can reducing the risk of osteoporosis by increasing calcium absorption (Scholz-Ahrens and Schrezenmeir, 2002) and minimize the risk of atherosclerosis (Roberfroid and Delzenne, 1998). In rats fructans curb the incidence of colon cancer and even aid cancer therapy (Taper and Roberfroid, 2002). Fructans are naturally present in the food we consume (e.g. garlic, leak, onion etc) and are used as an additive by the food industry to modify the texture and taste due to its properties as gelling agents, fat substitutes, soluble dietary fibers and low calorie sweeteners (Kaur and Gupta, 2002).

The main industrial sources of fructans are tubers of Jerusalem artichoke (*Helianthus tuberosus*) and chicory roots (*Chicorium intybus*). Though monocots can accumulate large amounts of fructans, they have not yet been used for industrial extractions. Oligosaccharides (degree of polymerization, DP $\leq$ 10) and long chain

fructans (up to DP 60) with different types of linkages between the Fru moieties have unique properties and many prospective applications. Improving our understanding of the chemistry, biochemistry, physiology and molecular biology of fructans will help in the better exploitation of its vast application potential and may eventually allow large-scale production of tailor-made fructans.

Occurrence of fructans

In nature, the occurrence of fructans is widespread in bacteria and flowering plants, and to a lesser extent, in liverworts, algae and some other organisms (Hendry, 1993). Fructan producing bacteria include plant and animal pathogens, as well as those making up the gut and dental flora of terrestrial vertebrates. *Bacillus, Pseudomonas, Erwinia and Actinomyces* are examples of bacterial genera in which fructan producing strains can be found. There are few reports on the synthesis of fructans in fungi. *Aspergillus*, *Penicillum* and *Fusarium* are known to synthesize fructans (Hendry, 1993).

It is estimated that about 15% of all species of flowering plants contain fructans and the majority of them belong to orders that are considered to be highly evolved (Hendry, 1993). Among the plants that store fructans, many are of significant economic importance, such as cereals (e.g. wheat, oats and barley), vegetables (e.g. chicory, onion and lettuce), ornamentals (e.g. dahlia and tulip) and forage grasses (e.g. *Lolium* and *Festuca*) (Hendry, 1993). Fructans in the dicots Jerusalem artichoke (Edelman and Jefford, 1968) and Chicory (Van Laere and Van den Ende, 2002) have been extensively studied. Among grasses, fructans accumulate to high concentration in just one of the major sub-families, the *Pooideae* or northern grasses which include widely cultivated cereals (Hendry, 1993). While fructans are almost absent from C4

species (Pollock and Cairns, 1991), some CAM plants (e.g. agave) are known to contain fructans (Wang and Nobel, 1998). Wider chemotaxonomic surveys with respect to presence (or absence) of fructans in plants, employing modern analytical tools, taking into account the environmental conditions in which they thrive, could provide better clues to the physiological role of this carbohydrate.

Chemical nature of fructans

Fructans as polymers of Fru form a remarkably diverse group of natural polysaccharides that differ widely in chemical structure and degree of polymerisation. But only in the recent past, many improvements in the techniques for purifying, analyzing and characterizing fructan molecules have contributed significantly to our knowledge on the molecular structure, nature and distribution of branching and DP. Earlier studies were based on acid hydrolysis of fructan extracts and analysis of the products. Later, paper chromatographic techniques ensured crude separation of the components in the fructan extract based on size. Size exclusion chromatography has been employed to estimate the molecular size of the extracts. The use of thin layer chromatography ensured higher resolution and revealed considerable additional complexity and identification of different isomeric oligosaccharides. Methylation analysis, followed by separation and analysis of partially methylated alditol acetates by gas chromatography/mass spectrometry has permitted structures to be assigned to each of these isomers. In some cases, NMR has also been used to investigate the structural details. High performance liquid chromatography (HPLC) using anion exchange column with pulsed amperometric detection resolves individual oligosaccharides easily up to DP 30 or higher.

Based on the chemical structure, Fructans can be classified into five main types (Vijn and Smeekens, 1999). The nomenclature used here is according to Lewis (1993)

1. **Inulin** consists of a linear chain of β(2-1) linked Fru residues attached to the fructosyl moiety of Suc. The shortest inulin molecule is the trisaccharide 1-kestose (1-K) or isokestose. Inulin has been found in some bacteria like *Streptococcus* (Rosell and Birkhed, 1974) and *Lactobacillus* (van Hijum *et al.*, 2002). Fungal inulin synthesis has been reported from *Aspergillus* (Heyer and Wendenburg, 2001). In plants, inulin is mainly found in dicotyledonous plants and particularly in the species belonging to Asteraceae (E.g. *Chicorium, Helianthus, Taraxacum* and *Cynara*) (Van Laere and Van den Ende, 2002).
2. **Levan** consists of a linear chain of β(2-6) linked Fru residues attached to the fructosyl moiety of Suc. 6-kestose (6-K) is the shortest levan molecule. Levans are produced by bacteria and can have a high DP of up to 100,000. *Dactylis glomerata* (Bonnet *et al.*, 1997) and big bluegrass (Wei *et al.*, 2002) produce short levans. Plant levans are referred to as phleins.
3. **Mixed levan** consists of β(2-6) linked chains of Fru units with β(2-1) branches attached to the fructosyl moiety of Suc. Bifurcose [Bif (1&6 kestotetraose)] is the shortest molecule of this group. Gramineae, including the cereals barley and wheat, are typical examples of plants producing mixed levan (Bonnet *et al.*, 1997). Therefore this type of fructans are called graminans.
4. **Levan Neoseries** has levan-type chains attached to both the 1st and 6th C of the glucose (Glc) moiety of Suc. This type of fructans are found in *Avena stiva* (Livingston *et al.*, 1993) and *Lolium temulentum* (Sims *et al.*, 1992)

5. **Inulin Neoseries** has β(2-1) linked Fru chains attached to both the 1st and 6th C of the Glc moiety of Suc. Neokestose (N-K) is the shortest neoseries fructan. Many members of *Liliaceae* produce this type of fructans (Shiomi, 1992).

Apart from these major types, there exist other kinds of fructans like inulo-*n*-oses etc (Lewis, 1993; Ernst *et al.*, 1998; Timmermans *et al.*, 2001).

Fructan biosynthesis in plants

Starch, by virtue of being the most abundant storage carbohydrate in the plant kingdom, has received a lot of research attention. The biochemistry of fructans on the other hand has been less intensively investigated. Among plants the most well-known and studied fructan is inulin. *Helianthus tuberosus* (Jerusalem artichoke) (Edelman and Jefford, 1968) and *Chicorium intybus* (chicory) (Van Laere and Van den Ende, 2002) have been used as model plants to elucidate the biochemistry of its synthesis and breakdown. Much of the work on inulins has been based on the Edelman and Jefford's model for fructan metabolism in higher plants derived from studies with Jerusalem artichoke (Edelman and Jefford, 1968). The model proposes sucrose (Suc) to be both the "donor" and ultimate "acceptor" of the fructosyl moieties of fructans. Without any sugar-phosphate or nucleotide-sugar intermediates, Suc is converted to fructans in a process involving two different 'fructosyltransferase' enzymes (FTs). 1-SST transfers a Fru moiety from a Suc to the C-1 of a Fru in another Suc molecule, leading to the synthesis of the trisaccharide 1-K. The enzyme fructan:fructan 1-fructosyltransferase (1-FFT) transfers Fru moieties from 1-K (or larger fructans) to either Suc, 1-K or larger fructans. The presence of these two distinct enzymes (1-SST and 1-FFT) in Jerusalem artichoke has been demonstrated by purifying them to

homogeneity (Koops and Jonker, 1994, 1996). *In vivo* type inulins can be synthesized *in vitro* by the two distinct enzymes from physiologically relevant Suc concentrations (Lüscher et al., 1996). Edelman & Jefford's 1-SST/1-FFT model has been further validated by the cloning of 1-SST and 1-FFT cDNAs from Jerusalem artichoke (van der Meer *et al.*, 1998) and the transforming non-fructan producing plants such as potato (Hellwege *et al.*, 2000) and sugar beet (Sevenier *et al.*, 1998) into inulin synthesizers by introducing the FT cDNAs.

The 1-SST/1-FFT model is inadequate to explain the synthesis of levan-type or neoseries-type fructans (Cairns, 1993). Studies on the synthesis of inulin neoseries from onion (Ernst *et al.*, 1998) and asparagus (Shiomi, 1992) show that 1-SST initiates fructan synthesis by the production of 1-K. Subsequently the formation of a 6-Glc linked chain is commenced by fructan:fructan 6Glc-fructosyltransferase (6G-FFT), which uses 1-K as a fructosyl donor and transfers the Fru unit to the Glc residue of Suc leading to the formation of Neokestose (N-K). Further linking of Fru residues to N-K at either of its Fru residues with β(2-1) bonds results in the synthesis of inulin neoseries and involves the action of 1-FFT and 6G-FFT (Vijn *et al.*, 1998).

Fructan synthesis in monocots is complicated and has not been understood very well (Pollock and Cairns, 1991; Ritsema and Smeekens, 2003). The synthesis of phleins, containing exclusively β(2-6) linkages, could occur through the combined action of SST-type and FFT-type enzymes. Using Suc as substrate, sucrose:sucrose 6-fructosyltransferase (6-SST) could lead to the synthesis of 6-K and further chain elongation could be catalyzed by sucrose:fructan 6-fructosyltransferase (6-SFT) (Wei et al., 2002). Though crude protein extracts from *Poa secunda* show 6-SST and 6-SFT activities, phlein synthesis has been attributed to only one enzyme, the 6-SFT (Wei *et al.*, 2002). It is possible that the initial 6-K is produced by ETP [the elongation-

trimming pathway (Bancal *et al.*, 1992)] i.e. through a specific hydrolysis of Bif, and indeed, Bif can be detected in very low amounts in these plants (Wei *et al.*, 2002). Certain grasses have mixed fructans of the levan neoseries (Chatterton and Draper, 1990; Livingston III et al., 1993; Pavis et al., 2001b) but only a partial biochemical characterization of the biosynthetic pathway has been done (Pavis et al., 2001a). Four enzymes have been predicted to be necessary to account for the synthesis of mixed fructans in *Lolium perenne*, namely 1-SST, 1-FFT, 6G-FFT and 6-FFT or 6-SFT (Pavis et al., 2001a).

Barley and wheat have been used as model plants to study the graminan biosynthetic pathway (Simmen et al., 1993; Penson and Cairns, 1994; Wiemken et al., 1995). Leaves of barley can be induced to accumulate large amounts of fructans when exposed to low temperature, or by continuous illumination, or by sugar feeding of excised leaves and the sequence of synthesis of soluble carbohydrates has been used to obtain clues about the graminan biosynthetic pathway (Wagner et al., 1986). The various models proposed initially for the synthesis of fructans in cereals have been conflicting (Penson and Cairns, 1994). While it was reported that 1-K is the initial and predominant trisaccharide formed (Simmen *et al.*, 1993), in other studies, 6-K was the only trisaccharide found to accumulate in excised illuminated barley leaves (Smeekens *et al.*, 1991). In wheat leaves, the timing of *in vivo* 6-K appearance and the involvement of the ETP pathway for its synthesis through the catabolism of Bif (Bancal *et al.*, 1992) have been challenged (Penson and Cairns, 1994). Enzyme extracts from illuminated wheat and barley leaves were capable of 6-K synthesis directly from Suc (Penson and Cairns, 1994) suggesting that an enzyme with 6-fructosyltransfer (6-FT) activity is involved. The rapidly induced SST activity in the excised barley leaves can be separated into two distinct forms, the 1-SST and 6-SST

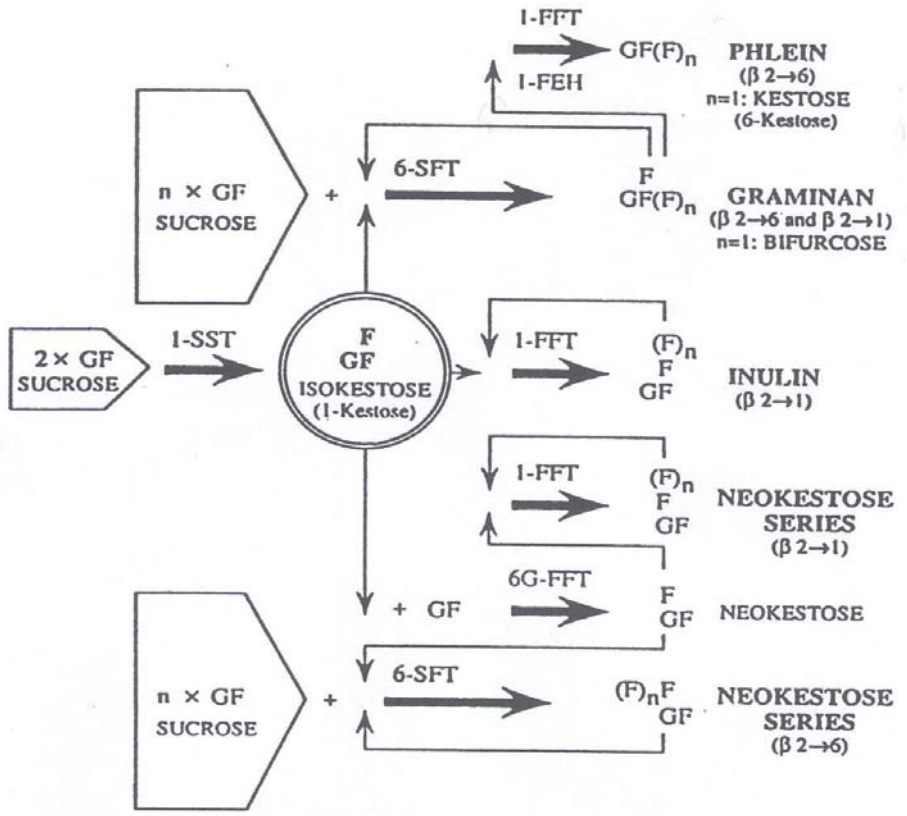

Figure 1.1. Model for the biosynthetic pathways of different fructans in plants. All the species of fructans reported to occur in barley and wheat can potentially be synthesized by a concerted action of four enzymes, 1-SST, 6-SFT, 1-FFT or/and 1-FEH (upper part of the figure). Note the central position of 1-K produced by 1-SST, the primary and crucial enzyme responsible for triggering fructan synthesis. After 1-K formation the flow of fructosyl residues from Suc to fructans is determined by 6-SFT, producing at first Bif. Fructan species of the phleins-type including 6-K are presumably also derived from Bif upon debranching by 1-FFT and 1-FEH. Inulin type fructans in barley and wheat may be produced according to the 1-SST/1-FFT model (Edelman and Jefford, 1968). Synthesis of the neokestose series (lower part of the figure) requires one additional enzyme activity, e.g. a 6G-FFT (Shiomi, 1992) producing neokestose from 1-K and Suc. (Figure and legend from Wiemken et al., 1995).

(Simmen *et al.*, 1993). Detailed biochemical characterisation of the enzyme 6-SST revealed that it used Suc exclusively as a fructosyl donor but prefers fructans as fructosyl acceptors and this led to its re-naming as 6-SFT (Duchateau et al., 1995). In the new pathway for graminan synthesis that emerged [1-SST/6-SFT model (Wiemken *et al.*, 1995)], 1-SST was proposed to be the primary and crucial enzyme responsible for triggering fructan synthesis through the synthesis of 1-K. 6-SFT determines the subsequent flow of fructosyl residues from Suc to fructans. In this model, phleins like 6-K are presumed to be derived by a debranching process involving 1-FFT or fructan exohydrolase (1-FEH), the neokestose series through the action of 6G-FFT and the inulins by 1-FFT (Wiemken *et al.*, 1995).

Properties of plant fructosyltransferases

The hypothesis that FTs are not unique enzymes but just side activities of invertases (Cairns, 1993, 2003) has been comprehensively dispelled. The activities of several plant FTs have been separated from invertases and purified to homogeneity. Some of the genes encoding such plant FT genes have been cloned. Heterologous expression of these genes confirmed that they indeed encode specific FTs. It has now become clear, based on the deduced amino acid sequence, that SAIs are evolutionarily closely related to FTs (Vijn and Smeekens, 1999) and are grouped in the same gene family (glycosidase hydrolase family 32). Invertases mainly catalyse the hydrolytic cleavage of Suc into Glc and Fru (Sturm, 1999) but can also have FT-like side activities (Van den Ende and Van Laere, 1993). Invertase-like side activities of FTs and *vice versa* may be a result of their high sequence homology (Sprenger *et al.*, 1995). The cloning and characterisation of SAI invertases are important to understand

the amino acid changes that occurred during the evolution of FTs which resulted in the modulating of their catalytic properties.

Subsequent to the cloning of the first plant FT, the 6-SFT from barley (Sprenger *et al.*, 1995), cDNA sequences of several FTs are now available in public databases (http://afmb.cnrs-mrs.fr/CAZY/GH_32.html). The deduced amino acid sequences of plant FTs show that they are probably formed as longer precursors typically about 650 amino acids in length. Plant FTs have been localized in the vacuole (Wagner et al., 1983; Frehner et al., 1984; Wagner and Wiemken, 1986a) and the N terminal region (the initial 60 to 100 residues) appears to contain information required for vacuolar targeting but is poorly conserved among FTs. However, by sequence comparisons, the signal peptide region of both SAIs and FTs is characterised by the presence of a conserved sequence R[G/A/P]XXXGVS[E/D/M]K[S/T/A/R] (Van den Ende et al., 2002). Heterologous expression studies with the recombinant 6-SFT show that the inclusion of the vacuolar sorting domain considerably reduces the activity of the enzyme but does not alter the reactions catalysed (Hochstrasser *et al.*, 1998). It is not clear if the signal peptide is retained on the FTs in their final form in the vacuole and it is predicted that cleavage occurs during posttranslational processing (Sprenger *et al.*, 1995). Though fructan hydrolases (FEH) have also been localized in the vacuole (Frehner et al., 1984; Wagner and Wiemken, 1986a), they have a shorter signal peptide than FTs (Van den Ende et al., 2001; Van den Ende et al., 2003) and the conserved signal peptide sequence present in FTs and SAIs is absent. It would be interesting to know if the sub cellular targeting route or mechanism or even destination may be different in case of the FEHs as compared to the FTs. Based on the sequence information and localization studies, it is unlikely that the fructan metabolism enzymes are anchored to the

tonoplast. Vacuoles are highly dynamic compartments (Boller and Wiemken, 1986) and the presence of more than one type of vacuole has been reported in some plant cells (Swanson *et al.*, 1998). It is not known if the different FTs and FEHs are targeted separately to specialized vacuoles for regulation of fructan metabolism by spatial separation of certain events.

The length of the mature peptide sequence of FTs (and SAIs) is about 500 to 550 amino acids. Usually, from this single translation product, a heterodimer is generated by proteolytic cleavage. The N terminal part of the mature peptide constituting the large subunit is at least 350 residues long. The small subunit (about 150 amino acids) starts with the conserved residues EADV, which are probably recognized by specific proteases responsible for cleavage. The large and small subunits show a size of approximately 50 and 25 kDa in SDS-PAGE (Sprenger et al., 1995; Lüscher et al., 2000). Based on studies with the recombinant tall fescue 1-SST, cleavage seems to be non-essential for FTs to be functional (Lüscher et al., 2000). Plant fructosyl transferases like SAIs are glycoproteins containing several potential N-linked glycosylation sites (Asn-X-Ser/Thr) and bind to ConA/Sepharose columns. Glycosylation may be important for subcellular targeting, enzyme stability, conformation and specific activities (Ritsema and Smeekens, 2003).

Based on studies on yeast invertase, the mechanism proposed for the hydrolysis of Suc involves an aspartic acid as a nucleophile in the Suc binding domain and a glutamic acid located in the EC domain acting as an acid/base catalyst (Reddy and Maley, 1996). The Suc binding region of plant FTs has found to contain the residues H-x(2)-(PTV)-x(4)-(LIVMA)-(NSCAYG)-(DE)-P-(NDSC)-(GA) (Pons *et al.*, 2000). Along with the EC domain, the arginine residue of the RDP motif, important for polymerase activity in bacterial leavan sucrase (Chambert and

PetitGlatron, 1991) is present in plant FTs too. The crystal structure of the *Bacillus subtilis* levan sucrase shows a five fold β-propeller topology with a deep negatively charged pocket. The central pocket contains the nucleophile, acid/base catalyst and stabilizers of the transition state critical for catalysis. Adjacent to this pocket, but in an exposed site lays the arginine involved in polymerase activity (Meng and Futterer, 2003). These residues are conserved in the respective domains of the plant invertases and FTs and hence are thought to play a similar role (Ritsema and Smeekens, 2003). Interestingly all these important residues are present on the large subunit. Domain swapping studies between barley 6-SFT and tall fescue 1-SST have shown that the large subunit determines the specific activities of FTs (Nüesch, 2003).

The enzyme 1-SST (EC 2.4.1.99) is responsible for the synthesis of the trisaccharide 1-K using two molecules of Suc as substrates. It is the initiator of fructan synthesis. The reaction catalysed by 1-SST is essentially irreversible; the enzyme cannot use 1-K as a donor or Glc as an acceptor. 1-SST has been purified and characterised from several plants producing inulins, graminans and neoseries sugars (Shiomi and Izawa, 1980; Koops and Jonker, 1996; Vandenende et al., 1996; Lüscher et al., 2000). In grasses the purification of 1-SST is tricky since the enzyme is highly unstable and its activity declines rapidly, especially after the affinity chromatography step (John et al., 1997; Lüscher et al., 2000). Nevertheless, the cloning and heterologous expression of several 1-SST cDNAs have been reported (Hellwege et al., 1997; Vijn et al., 1998; Lüscher et al., 2000). Though the amino acid sequences of 1-SST and SAIs are highly similar, the two enzymes can be distinguished by their unique properties. 1-SST uses Suc not only as a fructosyl donor but also as the preferred acceptor substrate. Hence 1-SST does not obey the Michaelis-Menten kinetics and the enzyme activity is not saturated even at very high Suc concentrations.

The pH optimum of the barley 1-SST (5.7) is also acidic but markedly different from SAIs (4.8). The thermal stability and temperature optimum of barely 1-SST are also distinct from SAIs (Wagner *et al.*, 1983). Nevertheless SAIs and 1-SSTs share several similar properties like Suc and fructan hydrolase like activities and the synthesis of l low DP inulin (Van den Ende and Van Laere, 1993; Cairns, 1995; Koops and Jonker, 1996; Vandenende et al., 1996; Lüscher et al., 2000). In barley two 1-SST isoforms showing different 1-k hydrolase activities can be detected. The physiological significance of the existence of these 1-SST isoforms remains unclear (Lüscher et al., 2000). The purified enzyme preparation of tall fescue 1-SST shows an additional 6G-FFT activity that was absent in the recombinant enzyme (Lüscher et al., 2000).

The synthesis of inulins requires the action of 1-FFT (EC 2.4.1.100) in addition to that of 1-SST (Edelman and Jefford, 1968; Hellwege et al., 2000). The isolation and characterization of 1-FFTs from several plants has been achieved (Lüscher *et al.*, 1993; StJohn *et al.*, 1997) and a number of 1-FFT cDNAs have been cloned from dicots (Hellwege *et al.*, 1998; van der Meer *et al.*, 1998). However, so far no 1-FFT genes have been cloned from grasses. Through the β(2-1) linkage of Fru residues, the elongation of inulin chains as well as redistribution of fructosyl units among fructan units is carried out by 1-FFT. Since the products of 1-FFT are also its substrates, enzyme activities are difficult to characterise. The ratio of 1-SST to 1-FFT influences the DP of inulin (Vandenende et al., 1996; VandenEnde and VanLaere, 1996) and the species-specific changes in the pattern of fructans within Asteraceae has been attributed to the differences in the properties of their respective 1-FFTs (Hellwege *et al.*, 1998). Suc cannot be used as a fructosyl donor by 1-FFTs but can act as an inhibitor of its activity (Edelman and Jefford, 1968; Penson and Cairns, 1994) . The 1-FFT of plants containing comparatively high DP inulin (e.g. globe

thistle) has a low affinity for Suc, Fru and 1-K and a high affinity for inulin as fructosyl acceptors. In plants containing low DP inulin (e.g. chicory), Suc, Fru and 1-K also act as good fructosyl acceptors leading to enhanced redistribution of Fru residues from large to small fructans during active fructan synthesis (Vergauwen *et al.*, 2003). The ping-pong mechanism of fructosyl transfer activity by 1-FFTs is analogous to bacterial levansucrases (Vergauwen *et al.*, 2003).

The necessity of 1-FFT for the synthesis of neoseries-type fructans has been questioned, since the combined action of 1-SST and 6G-FFT is sufficient to synthesis all the fructans present in onion (Ritsema et al., 2003). The activity of 6G-FFT was first described in onion (Henry and Darbyshire, 1980) and the purification of this enzyme was reported from asparagus (Shiomi, 1981). The onion cDNA for 6G-FFT has also been cloned (Vijn *et al.*, 1997). Using 1-K as substrate, the recombinant 6G-FFT displays multiple activities and can synthesize an array of fructans of the inulin series and inulin neo-series with prolonged incubations resulting in high DP fructans (Ritsema et al., 2003).

The synthesis of levan and mixed levan through the formation of β(2-6) linkages is mainly due to the activity of 6-SFT (EC 2.4.1.10). This enzyme was first purified to homogeneity from barley (Duchateau *et al.*, 1995), and the corresponding cDNA was cloned (Sprenger *et al.*, 1995). 6-SFT is a multifunctional enzyme and can assume different roles depending on the substrates available. It acts mainly as an invertase (80% of its activity) in the presence of Suc alone, but can also synthesise 6-K (20% activity). If 1-K is present along with Suc, then the invertase activity is suppressed and the enzyme mainly acts as a 6-SFT leading to Bif synthesis. With Bif as the sole substrate 6-SFT acts like a 6-FFT and FEH. Glc can be fructosylated by the transfer of a Fru residue from Suc or Bif i.e. SGT or FGT activity. Even a minor

1-SST activity was also detected. However since Suc is the preferred donor and 1-K is the preferred acceptor, it has been named as 6-SFT (Duchateau *et al.*, 1995). This enzyme can, in theory, bring about synthesis of the whole spectrum of fructans found in barley by transfructosylation and has therefore been proposed to be the key fructan biosynthetic enzyme in this plant (Sprenger *et al.*, 1995). For grasses containing only β(2-6) linked fructans, such as *Poa secunda*, it has been suggested that a 6-SFT, exhibiting a prominent 6-SST activity, could be the sole enzyme responsible for fructan synthesis (Wei *et al.*, 2002).

The breakdown of fructans in the vacuole is due to the activity of FEH. (Wagner and Wiemken, 1986a). Specific enzymes that can degrade β(2-1) and β(2-6) linkages, the 1-FEH and the 6-FEH respectively, have been purified from *Lolium perenne* (Marx *et al.*, 1997, 1997). The cloning of cDNAs encoding 1-FEH has been reported from Jerusalem artichoke, chicory and wheat (Van den Ende et al., 2001; Van den Ende et al., 2003). Unlike FT genes that originated from vacuolar-type invertases, FEHs seem to have evolved from a cell wall invertase ancestor gene that later obtained a low iso-electric point and a vacuolar targeting signal (Van Laere and Van den Ende, 2002). Unlike invertases, sulfhydryl groups are not required for fructan hydrolytic activity (Henson, 1989). The 1-FEH is a glycoprotein, and the pH optimum of this enzyme is generally between 5.5-6.0, which is in the range expected for vacuolar enzymes. The purified barley stem 1-FEH exhibits typical Michaelis-Menten kinetics (Henson, 1989). Generally, plant FEHs seem to be devoid of invertase activity (Bonnett and Simpson, 1993; Marx et al., 1997, 1997; De Roover et al., 1999). It has been suggested that 1-FEH plays a role in fructan synthesis as a specific β(2-1) trimmer (Bancal *et al.*, 1992), and substantial amounts of a 1-FEH isoform were detected in fructan biosynthesizing wheat stems (Van den Ende et al.,

2003). Recently the cloning of a 6-FEH cDNA has been reported, interestingly from a non fructan producing plant (sugar beet) and it has been hypothesized to have a role in plant-pathogen interactions (Van den Ende et al., 2003). Endo-inulinases (E.C. 3.2.1.7) have so far been characterised only from fungi (Ohta et al., 1998).

Heterologous expression of Fructosyltransferases

Many expression systems have been used to identify the genes encoding FTs and to characterize the properties of FTs. Protoplasts prepared from tobacco (*Nicotiana plumbaginifolia*) were used as an expression system to asses the functionality of cloned barley 6-SFT (Sprenger *et al.*, 1995) and onion 6G-FFT (Vijn *et al.*, 1998) cDNAs. The fast growing and high yielding suspension culture derived from Bright Yellow 2 tobacco variety (BY2) cells has been used to characterize the activities of 6G-FFT (Ritsema et al., 2003). However, very high inherent invertase activity in the protoplasts makes it difficult to detect any additional invertase activity from the expression of transgenes besides resulting in a background FT activity of the protoplast native invertases. The methylotropic yeast (*Pichia pastoris*) does not secrete Suc metabolizing enzymes such as invertases (Sreekrishna *et al.*, 1987) and has proved to be a convenient system to express invertase cDNAs from rice (Fu *et al.*, 2003) and sweet potato (Huang *et al.*, 2003), barley 6-SFT cDNA (Hochstrasser *et al.*, 1998), tall fescue 1-SST cDNAs (Lüscher et al., 2000) and conduct structure-function studies of FTs (Nüesch, 2003). The N-glycosylation in *Pichia* is similar to plants and the possibility of tagging recombinant proteins allows detection as well as purification (Cregg *et al.*, 2000). However, compared to the activities of the native 6-SFT, substantial differences in the minor activities were observed in the *Pichia* derived enzyme (Hochstrasser *et al.*, 1998).

Role of fructans in plants

The diversity and frequency of the occurrence of fructan metabolism among highly evolved families of vascular plants indicates that fructan biochemistry may have developed recently, perhaps in response to one or several new selective pressures, and is not a minor pathway of declining evolutionary significance. While starch is the most common reserve carbohydrate in higher plants, some species that synthesize starch also maintain the ability to produce fructans. Due to its subcellular location, solubility properties and the insensitivity of fructan synthesis enzymes to low temperature, fructans are probably not an alternative to starch but rather an exceptional carbohydrate with unique advantages to certain plants (Chatterton and Asay, 1989). Yet, the physiological role of fructans in plants is not fully understood (Vijn and Smeekens, 1999). The pattern of distribution of fructan producing plants in different climatic zones, the developmental stages and environmental conditions that influence fructan metabolism in the plants are all indicators of the various functions of fructans in higher plants.

Fructans appear to play a role in plant development. Inulin accumulation in the tubers of *Helianthus tuberosus* (Jerusalem artichoke) and the tap roots of *Chicorium intybus* (chicory) clearly has a function as a long time reserve, which supports growth after overwintering (Van Laere and Van den Ende, 2002). Ubiquitous presence of inulin in the tissues of *Campanula rapunculoides* suggests that it has a housekeeping role in carbohydrate metabolism (Vergauwen *et al.*, 2000). The rapid hydrolysis of fructans during petal expansion plays an important role during flower opening in *Campanula* (Vergauwen *et al.*, 2000) and ephemeral daylily (Bieleski, 1993). In grasses, fructans are more important for intermediate and short-term storage. Fructans accumulate after anthesis in the stems of wheat and barley as intermediate

carbohydrate store, which thereafter, is mobilized for grain filling (Schnyder, 1993). During the initial stages of grain filling, fructans are synthesized also in wheat grains (Housely and Daughtry, 1987) but starch dominates the seed content of mature grains. In cool season grasses like *Lolium perenne*, the fructans are stored in the leaf sheaths and elongating leaf bases (Morvan-Bertrand et al., 1999) and serve as a source of carbon for fresh growth following defoliation (Morvan-Bertrand et al., 2001). In barley, the transitory accumulation of fructans in the growth zones plays a key role in the development of leaves (Roth *et al.*, 1997). Fructan accumulation in the leaf blades of grasses occurs when carbon supply exceeds demand, and can be induced by low temperature treatments and by illumination/sugar feeding of excised leaves (Wagner and Wiemken, 1989). Vacuolar fructan metabolism in the immediate vicinity of the site of photosynthesis lowers Suc concentration and prevents sugar-induced feedback inhibition of photosynthesis (Wagner and Wiemken, 1989).

The distribution of fructan-rich families is not just confined to colder zones of the world but spread over temperate through to sub-tropical regions. These plants may have to cope with periods of cold or drought stresses in order to survive in their habitat (Hendry, 1993). Drought stress studies using contrasting *Bromus* species (Puebla *et al.*, 1997) and tall fescue (Spollen and Nelson, 1994) have linked fructan accumulation to stress responses. However, it appears to contribute only indirectly to osmotic adjustment (Spollen and Nelson, 1994). Increased fructan accumulation occurred in wheat seedlings subjected to PEG mediated drought stress and NaCl mediated salt stress (Kerepesi and Galiba, 2000), and drought induced fructan synthesis in the roots and leaves of chicory (DeRoover *et al.*, 2000). The DP of the accumulated fructan may depend on the extent of water stress as seen in leafy liverwort *Porella platyphylla* (Marschall *et al.*, 1998). Fructans can play a significant

role in osmoregulation since the osmotically inert polymers can be rapidly converted into osmotically active monomers. Genetically engineering fructan synthesis in non-producing plants such as tobacco (Pilon-Smits et al., 1995) and sugar beet (Pilon-Smits et al., 1999) resulted in their enhanced performance under drought stress. Suggestions have also been made for a role of fructans in cold stress tolerance since its production is much less sensitive to low temperature (Pollock, 1986). Nitrogen and phosphorous deficiencies lead to fructan accumulation in barley (Wang and Tillberg, 1996; Wang and Tillberg, 1997). Though fructan accumulation during various stresses could be a side effect of reduced growth or sink activity under continued carbon assimilation, it could impart freezing and drought tolerance through enhanced membrane stability as experimentally shown using liposomes (Hincha *et al.*, 2002) and may help in osmotic adjustments (Pilon-Smits et al., 1999).

Fructans may play a role in vascular transport since the presence of oligofructans and FT activity were reported from the phloem sap and vascular tissues of *Agave deserti* leaves (Wang and Nobel, 1998). By fructan localization studies, it was shown that a preferential storage of fructans occurs in the phloem parenchyma cells and in the vicinity of secondary sieve tube elements (Van den Ende *et al.*, 2000). In *Taraxacum officinale* roots, it has been proposed that fructan synthesis in the phloem parenchyma might be the driving force to maintain a steep Suc gradient facilitating Suc transport to the vascular tissues (Van den Ende *et al.*, 2000). Fructans has also been reported in xylem parenchyma cells in roots of *Gomphrena macrocephala* (Vieira and Figueiredoribeiro, 1993) and *Vernonia discolor* (Tertuliano and Figueiredo-Ribeiro, 1993).

Regulation of fructan metabolism

Changes in fructan accumulation have been reported in response to several internal and external stimuli. These stimuli include sugars (Suc, Glc, Fru, trehalose etc), developmental changes, hormones (cytokinins, gibberellins, ABA), nutrient status (nitrogen, phosphorous etc), heavy metals (Ni, Cd, Hg etc), biotic (pathogens, symbionts) and abiotic factors (light, temperature etc). Our knowledge of how these factors are perceived specifically in the context of the signal transduction pathway leading to the synthesis of fructans is rudimentary. The growth zone of cereal leaves, the stems of cereals after anthesis and the mature leaf blades of grasses are the most commonly used tissues to study the regulation of fructan metabolism in grasses. The grass leaf blade is a particularly convenient model system and can be induced to accumulate large amounts of fructans by subjecting plants to cold stress and, very efficiently, by continuous illumination or feeding of sugars to excised leaves (Wagner et al., 1986). *De novo* gene expression is crucial for this process since the inhibitors of transcription and translation block fructan accumulation (Wagner et al., 1986; Cairns and Pollock, 1988).

The induction of FTs in response to various factors and treatments has been linked to elevated Suc concentration within the cell. Exogenously supplied Suc to excised barley leaves very efficiently induces FT activity (Wagner et al., 1986) and gene expression (Müller *et al.*, 2000). Suc was proposed to be a major means of control for fructan metabolism (Edelman and Jefford, 1968). Suc can regulate the expression of several plant genes (Koch, 1996) through a unique sensing and signal transduction mechanism (Smeekens, 2000). In barley, the regulation of fructan synthesis occurs independently of the hexokinase mediated control mechanism (Smeekens, 2000) and is probably based on the sensing of Suc and disaccharides

structurally related to Suc, such as trehalose that can replace Suc as a regulatory compound (Müller *et al.*, 2000). Suc sensing for the purpose of activation of fructan accumulation apparently is dependent on cell type. The parenchymatous bundle sheath cells, as compared to mesophyll cells, have a higher sensitivity for Suc and thus a lower Suc threshold can induce FT gene expression (Pollock *et al.*, 2003). The mechanisms that govern the cellular heterogeneity in Suc sensing leading to the induction of FTs have not been studied. However there is some evidence that, upon sensing of Suc, protein kinases and protein phosphatases take part in the chain of events that intervenes in the induction of FT gene expression (Noel *et al.*, 2001).

Quantitative and temporal differences seem to exist in the regulation of the various FTs during fructan metabolism. In chicory there are major differences in the expression of 1-SST and 1-FFT throughout the growing season and later during fructan degradation (Van Laere and Van den Ende, 2002). In barley, after 24 h of Suc feeding to excised leaves, 1-SST was stimulated by a factor of three while 6-SFT activity increased by a factor of more than 20 (Müller *et al.*, 2000). During induced fructan accumulation in onion leaves, the increase of 1-SST mRNA levels corresponded with Suc accumulation whereas 6G-FFT mRNA increase was delayed leading to suggestions that induction of 6G-FFT gene expression requires an additional signal (Vijn *et al.*, 1998). There is a possibility that the Suc threshold required for activation of FT gene expression is not only dependent on cell type but also is gene-specific.

It is not clear if the various stimuli leading to changes in fructan accumulation always use Suc as a signal initiator. The correlations between concentrations of Suc and fructan in leaf tissues from a large number of cool-season species are rather low (Chatterton and Asay, 1989). During drought stress studies in perennial rye grass,

fructan accumulation was not accompanied by an increase of Suc (Amiard *et al.*, 2003). The 1-SST activities induced by nitrogen starvation was not correlated with the stable Suc levels in barley leaves and differences were observed in the regulation of fructan metabolism in sink and source leaves (Wang and Tillberg, 1996). Using a heterologous probe from chicory, by Northern analysis, it was shown that nitrogen deficiency resulted in a moderate induction of the 1-SST as compared to a strong induction of 6-SFT (Wang *et al.*, 2000). In excised barley leaves, nitrate appears to be a negative signal for fructan synthesis. The effect of nitrate is independent from the positive sugar signalling and can even override it (Morcuende *et al.*, 2004). High Suc levels do not lead to fructan synthesis in all tissues and under all conditions in fructan producing plants. For instance, during cold treatment, high Suc concentrations failed to upregulate 6-SFT gene expression in barley seedlings (Wei *et al.*, 2001). Both SST and 6G-FFT did not increase under conditions of Suc accumulation in *Lolium perenne* leaf blades (Pavis *et al.*, 2001a). Elevated Suc level fails to induce the gene expression of 6-SFT and synthesis of fructans in epidermal cells (Koroleva *et al.*, 2001) and probably in other heterotrophic cells as well (Pollock *et al.*, 2003). Depending on the type of cell, there is a considerable heterogeneity on the role of Suc as a signal molecule in fructan metabolism (Pollock *et al.*, 2003). 'High' or 'low' sugar is not a good indicator of the real availability of sugars, since soluble carbohydrates have to be sensed in sub-cellular compartments and it has been suggested that, ideally, rather than sensing the amount of sugars present, plants should sense the inter/intra cellular 'flux' of sugars (Loreti *et al.*, 2001).

The degradation rates of different mRNAs in higher plants can be regulated by a variety of endogenous and exogenous stimuli (Abler and Green, 1996). Protein turnover is a specific and highly regulated process. Degradation of proteins occurs in

response to specific environmental and cellular signals (Estelle, 2001). Theoretically, apart from transcription, the regulation of FTs can be exercised at the mRNA and protein stability levels too. Besides, depending on the abundance of substrates, FTs can exhibit several different properties, leading to regulation at the enzyme activity level. The activity of invertases can be controlled through specific proteinaceous inhibitors (Scognamiglio *et al.*, 2003) and it is not known if such a mechanism of regulation has been inherited by the FTs too.

Fructan biotechnology

Fructans, because of their unique properties have the potential to be commercially used for a wide range of applications. The wealth of knowledge available on the chemistry of fructans, the properties of FTs from various organisms and the genes involved in fructan metabolism can be applied to produce tailor made fructans of defined structure and amounts. Molecular approaches are useful to study the physiological importance of fructans by repression, over production or by gain of function in transgenic plants. It is also useful to alter the quality and yield of fructan producers, or to convert plants into fructan producing factories.

The first fructan producing stable transgenic plants carried a bacterial levansucrase gene (Ebskamp *et al.*, 1994; Vandermeer *et al.*, 1994; Pilon-Smits *et al.*, 1995; Caimi *et al.*, 1996; Pilon-Smits *et al.*, 1999). These plants synthesised bacterial levans that are large polymers, not normally found in plants (Ebskamp *et al.*, 1994). Modifications in carbon flux and in some cases altered phenotypes were observed (Vandermeer *et al.*, 1994). However, transgenic fructan producers performed better in terms of growth rate and fresh weight during drought stress (Pilon-Smits *et al.*, 1995; Pilon-Smits *et al.*, 1999). The pattern of chicory fructans could be altered in transgenic

plants. In addition to the normally found inulin, chicory plants transformed with the barley 6-SFT gene synthesized mixed levans (Sprenger *et al.*, 1997), while the introduction of the onion 6G-FFT gene resulted in neoseries type fructans (Vijn *et al.*, 1997). The expression of a fungal FT in transgenic potato yielded fructans upto DP 40 (Heyer and Wendenburg, 2001). Inulin of DP upto 200 could be successfully produced in transgenic potato harbouring the 1-SST and 1-FFT genes of globe artichoke. While the amount of fructan produced was low, no adverse effects were reported on phenotype or tuber yield (Hellwege *et al.*, 2000). However the difficulty in obtaining defined chain length and desired branched fructans in transgenic plants has thus far limited their application (Vijn and Smeekens, 1999). The availability of a wider choice of plant FT cDNAs, use of efficient promoters to control the expression of the transgene, appropriate selection of host plants, efficient subcellular targeting of the expressed FTs and means to suppress fructan hydrolase activities in the transgenic plants will help to make the concept of industrial fructan production a reality.

CHAPTER 2

Sucrose:sucrose 1-fructosyltransferase, the pacemaker enzyme for fructan synthesis in barley leaves

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Abstract

Fructans are important storage carbohydrates in many temperate grasses, including wheat and barley. We previously purified, characterized and cloned sucrose:fructan 6-fructosyltransferase (6-SFT) from barley (*Hordeum vulgare L.*), and we proposed that *de novo* synthesis of graminans typical for wheat and barley is brought about by the concerted action of two enzymes: (1) by sucrose:sucrose 1-fructosyltransferase (1-SST), as in other fructan producing plants and, (2) by 6-SFT, an enzyme so far detected only in grasses. Here we report the cloning of barley 1-SST, and we demonstrate that the two key enzymes for fructan synthesis in barley leaves differ remarkably with respect to regulation at the biochemical and molecular level. Using a reverse genetics approach, we cloned the cDNA of barley 1-SST and verified the activity of the encoded protein by expression in *Pichia pastoris*. As expected, barley 1-SST shared close homology with invertases and fructosyl transferases, and in particular with barley 6-SFT. The gene expression pattern of 1-SST and 6-SFT, along with the corresponding enzyme activities and fructan levels were investigated in excised barley leaves subjected to a light-dark regime well known to sequentially induce fructan accumulation and mobilization. Inhibitors of gene expression and proteolysis were applied to compare the turnover of transcripts

and enzyme activities of 1-SST and 6-SFT. We found the 1-SST transcripts and enzymatic activity to respond quickly being subject to a rapid turnover. In contrast, the 6-SFT transcripts and enzymatic activity were found to be much more stable. The much higher sensitivity and responsiveness of 1-SST, as compared to 6-SFT, to regulatory processes clearly indicate that 1-SST plays the role as the pacemaker enzyme of fructan synthesis in barley leaves.

Introduction

Fructans are major non-structural storage carbohydrates in the vegetative tissues of many higher plants including temperate grasses and cereals, as well as major crop plants such as wheat and barley (Hendry, 1993). In these plants, fructan metabolism is crucial in the photosynthetic partitioning process and ultimately determines yield (Schnyder, 1993; Wiemken *et al.*, 1995; Pollock *et al.*, 2003). Apart from their role as a storage carbohydrate, fructans may have other benefits to plants such as protection from damage by various stresses, including drought and cold (Ritsema and Smeekens, 2003b).

The simplest fructans are the inulins, consisting of β(2-1) linked chains of fructoses (Fru) attached to sucrose (Suc) (Bancal *et al.*, 1991). Inulins are present, for example, in many Asteraceae, and studies with *Helianthus tuberosus* led to the classic two-step model of plant fructan synthesis (Edelman and Jefford, 1968; Van Laere and Van den Ende, 2002). In the first step, responsible for *de novo* fructan synthesis, catalysed by 1-SST, two molecules of Suc act as donor and acceptor of a fructosyl unit, respectively, leading to the synthesis of the trisaccharide 1-K. In repeated subsequent steps, catalyzed by fructan:fructan 1-fructosyltransferase 1-FFT, Fru units are shuffled between 1-K and higher polymeric β(2-1) linked fuctan molecules, leading to fructans with β(2-1) linkages only. Fructans of barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.), called graminans, are structurally more complex and consist of β(2-6) linked Fru units with β(2-1) branches (Bancal *et al.*, 1991). Based on studies with barley, the 1-SST/6-SFT model for graminan biosynthesis in grasses was proposed (Wiemken *et al.*, 1995): As in the classic model, 1-SST catalyses the essential first step producing 1-K. In the second step, equally

important for *de novo* fructan synthesis in these plants, 6-SFT catalyzes the formation of bifurcose, using Suc as the fructosyl donor and 1-K as the favored fructosyl acceptor. In further steps, higher polymeric-graminans are synthesized by the same 6-SFT through β(2-6) linked chain elongation (Duchateau *et al.*, 1995). In this model, both 1-SST and 6-SFT act in combination to introduce new fructosyl units from Suc into fructans to bring about fructan synthesis, in contrast to the classic model where fructosyl units in the fructan are ultimately introduced only by the action of 1-SST. In further steps, the structural composition of the fructan synthesized can be modified by FFT's, through shuffling of Fru residues between fructan molecules, and by different fructan exohydrolases (FEH's), through specific trimming of fructosyl chains.

The two types of fructan differ also with respect to their physiological role and temporal metabolic regulation. In roots and tubers destined for over-wintering, inulin is usually a long-term reserve and gradually accumulates throughout the vegetative period; in chicory roots for example, the activity of 1-SST is prominent in the early vegetative stage and then declines while 1-FFT activity remains more or less constant throughout the growing season (Van Laere and Van den Ende, 2002). In contrast, phases of graminan synthesis and breakdown in temperate grasses alternate rapidly and frequently in response to developmental and environmental changes. For instance, transitory accumulation of fructan in the growth zone plays a key role in the development of elongating barley leaves (Roth *et al.*, 1997). Plants that are photosynthetically active but inhibited in growth, upon exposure to low temperatures build up carbohydrate reserves temporarily in the form of fructan. Fructan accumulated in leaf bases and stems of grasses, during autumn and early winter, serves as a reserve for quick re-growth during favorable conditions in spring, or to sustain re-foliation soon after defoliation (Morvan-Bertrand *et al.*, 2001). Frost-

tolerant cultivars of wheat achieve the arrest of fructan synthesis at the second stage of cold hardening by the downregulation of 1-SST and 6-SFT and degrade fructan, probably as a cryoprotective mechanism (Kawakami and Yoshida, 2002). Fully-grown cereal plants, limited in sink strength for photoassimilates at an early stage after anthesis, deposit large amounts of fructans in stems and later remobilize them for grain filling (Blacklow *et al.*, 1984; Schnyder, 1993; Gebbing, 2003). In general, fructan accumulates in grass leaf tissues when carbon supply exceeds demand, for example upon cold treatment of plantlets, upon illumination or feeding of sugars to excised leaves (Wagner et al., 1983; Wagner and Wiemken, 1989), upon restriction of growth by phosphorous or nitrogen starvation (Wang and Tillberg, 1997; Wang et al., 2000), or upon reduction of sugar export from leaves by cooling of roots (Koroleva *et al.*, 1998; Koroleva *et al.*, 2001).

Previously, the cloning of barley 6-SFT (Sprenger *et al.*, 1995), the purification and characterization 1-SST from barley (Lüscher *et al.*, 2000a) and the cloning of a 1-SST from tall fescue (Lüscher *et al.*, 2000b) has been reported. Here the cloning of a full-length 1-SST cDNA from barley and its heterologous expression in *Pichia pastoris* is described. Using the excised leaf system, the gene expression pattern of 1-SST and 6-SFT was compared, along with the corresponding enzyme activities and fructan levels, upon induction of fructan accumulation and subsequent mobilization by subjecting the leaves to a light-dark cycle. A clear evidence is provided in support of the 1-SST/6-SFT model for graminan synthesis in barley leaves and of the role of 1-SST as the pacemaker enzyme of this pathway.

Materials And Methods

Plant Material and Growth Conditions

Barley (*Hordeum vulgare* L. cv. Lyric) seeds were soaked overnight in running tap water, planted in a commercial soil mixture (UFA Haus und Garten, Bern, Switzerland) and grown for 7 days in a growth chamber. The conditions were: 14 h light (500 μE m^{-2} s^{-1}) at 22 °C, 10 h dark at 16 °C and a constant relative humidity of 70%.

Purification of 1-SST from Barley and Partial Sequencing of the Tryptic Digestion Products

Using the growth zone of barley, 1-SST was purified as described earlier (Lüscher *et al.*, 2000a), separated by SDS PAGE, electrotransferred onto a polyvinylidene difluoride membrane (Immobilon PVDF, Millipore, Bedford, MA) and visualized with Coomassie blue. The largest band was cut out, eluted and digested with trypsin. The resulting peptide fragments were separated and microsequenced as described (Lüscher *et al.*, 2000b).

Cloning of the Full Length 1-SST cDNA

The peptide sequences obtained from purified barley 1-SST were mostly identical to that of the barley 6-SFT, except for one (FTNLIQ). On the basis of this unique peptide, the primer 20414 as a "reverse primer" was designed and used it in combination with a general forward primer, 20124 (Table 2.1), representing a highly conserved consensus sequence in invertases and fructosyltransferases (FTs). The template for this PCR reaction was a cDNA preparation obtained with the Reverse

Transcription System (Promega, Madison, Wisconsin, U.S.A.) from total RNA isolated from the growth zones of barley shoots (RNeasy Plant Mini kit, Qiagen, Hilden Germany). Among the bands obtained, a 930 bp fragment was used as a template for nested PCR using primer 20124 and another degenerate primer, 20115 (Table 2.1) for FTs and invertases. This yielded a single product of 760 bp, which was sequenced. Using this sequence information, the primers 3'GSP1 and 3'GSP2 (Table 2.1) were designed to obtain the 3' end of the cDNA in conjuction with an oligo dT anchor primer by 3'-RACE. Attempts to obtain additional sequence information by 5'-RACE failed. However, about 250 bp of additional upstream sequence were obtained by PCR using a barley cDNA library directly as template (Sprenger *et al.*, 1995) with T3 and 20852 primers. This sequence contained the N-terminus of the mature protein and was used to construct the forward primer 21456. Using a reverse primer based on the lambda arm of the barley cDNA library (16160) along with the 21456 primer, PCR on the cDNA library resulted in a 1.9 kbp clone. This clone was further amplified by PCR using Pfor and Prev primers containing convenient restriction sites for cloning into a vector for expression in the Pichia system (see below). Next, a barley “genomic adapter library” (Nagaraj *et al.*, 2001) was used for genome walking by PCR (primers 5’GSP1 and 5’GSP2 together with AP1 and AP2, Table 2.1) to obtain sequence information up to the (putative) start codon. Finally, a previously described barley cDNA library (Sprenger *et al.*, 1995) was subjected to PCR with the primers "For" and "Rev" (Table 2.1) to amplify a full-length cDNA clone. Three full length clones derived from this PCR product were obtained in pGEMT vector (Promega) and sequenced; their nucleotide sequence (2157 bp) matched 100%. For steps 4 and 5, PCR was done using the Expand Long template PCR system (Boehringer Mannhein, Mannhein, Germany) supplemented with $MgCl_2$.

Table 2.1 PCR Primers. Restriction sites are indicated in italics (*Cla*I on Pfor and *Xba*I on Prev). Degenerate primers (20414, 20124 and 20115) have N (=A,C,G,T), Y(=C,T), R(=A,G), W=(A, T) and H(=A,C,T).

Primer	Sequence
Primers used for cDNA cloning	
20414:	TCY TCN TYN GGC CAY TGD AT
20124:	TGG TAY CAY HTN TTY TAY CAR TA
20115:	GCR WTY TTN CCC CAR TCR TA
3'GSP1:	CAC TCG GGC ATC GTC TTC TC
3'GSP2:	ATG TAC AAC TCG ACG GCG GA
20852:	AGC CGG TGA GCA CGC CCT T
21456:	GCA CGA GAC GGA GTT CCC GA
5'GSP1:	AGC AGG CCC CCG ATG TCT TCT CCG ACA CGC
5'GSP2:	GAC ACG CCG TGC TCC TTG CCC CTG CTC CTC
For:	CCT TCC TCC TCC GCC GAC GCC AAT
Rev:	CAT CCA TGC CAT TTT GAT TCC CAA CAA AAT
16160	CGA CGT TGT AAA ACG GCC AGT G
T3	CGC CAA GCT CGA AAT TAA CCC TCA C
AP1	GTA ATA CGA CTC ACT ATA GGG C
AP2	ACT ATA GGG CAC GCG TGG T
Primers used for cloning into the *Pichia* expression vector	
Pfor:	CAC TAG TGA TT*A TCG ATC* CAC GAG ACG GAG
Prev:	C TTG TAA GCG ATC A*TC TAG A*CC GAC GAC TAC
Primers used for RT PCR	
B1-SSTRT1:	AAC CGC GCC CTC ACC GAA
B1-SSTRT2:	GCA TAG ATG GCC TCG GTC GG
B6-SFTRT1:	TCC AAT GAG GAC GAT GGC ATG T
B6-SFTRT2:	AAT GCA TGC AAG CGA GGT
BubiRT1:	GCC AAG AAG CGC AAG AAG AAG A
BUBIRT2:	GGT CGA AGT GGT TGG CCA TGA

Expression of Barley 1-SST cDNA in *Pichia pastoris*

A cDNA encoding the mature 1-SST from barley was expressed in *Pichia pastoris* for activity characterization. Using primers Pfor and Prev, and the partial cDNA fragment (see above) as template, a cDNA fragment was amplified by PCR so as to introduce a ClaI site at the 5'-end and an XbaI site at the 3'-end. The resulting product was digested by the appropriate restriction enzymes and ligated in frame behind the α-factor signal sequence of the expression vector pPICZαC (Easy Select Pichia expression kit from Invitrogen BV, Leek, NL), to allow the entry into the secretory pathway. The insertion of the DNA in the shuttle vector led to the plasmid pPICZαC-V4. During secretion the α-factor is cleaved off the new protein (Clare *et al.*, 1991). The insert was sequenced three times with exactly identical results.

Expression in *P. pastoris* was performed as described (Hochstrasser *et al.*, 1998),with minor modifications. The *P. pastoris* strain X-33 was transformed with 4 μg of PmeI-linearized pPICZαC-V4 and plated on selective YPDS/Zeocin plates. Single colonies of transformants were inoculated on fresh YPDS/Zeocin plates. To screen for activity, some of the newly grown colonies were inoculated in liquid culture. For all further experiments, the best growing colony was chosen and used for induction with 1% (v/v) methanol. To maintain the methanol concentration over the induction period, the growth medium was supplemented again by 1% (v/v methanol at 15, 24, 36 and 42 h of induction). After 48 h of induction, the supernatant of the culture medium was concentrated from 50 ml to 1 ml by dialysis against solid PEG 35'000. The concentrate was desalted into 50 mM MES (NaOH) buffer (pH 5.75). The concentrated, desalted media of the transformant was tested for activity.

Induction of Fructan Biosynthesis

Primary leaves of one-week old barley plants were harvested just before the beginning of the light period by cutting at the ligule. The next 2 cm were excluded, and 4 cm of the center of the leaf blade were used for all the experiments so as to always have a uniform region of the leaf. The excised leaf system described earlier (Wagner and Wiemken, 1986) was modified for induction of fructan biosynthesis. The leaf bits were floated on a tray with water or inhibitor solutions with the adaxial surface illuminated (500 μE m^{-2} s^{-1}) under constant humidity 70% and temperature (22 °C) in a growth chamber. The light was switched off during the dark phase. The metabolic inhibitor solutions: 100 μM α-amanitin, 80 μM MG-132 (carbobenzoxy-leucinyl-leucinyl-leucinal), 300 μM leupeptin (acetyl-leucinyl-leucinyl-argininal) or water (control) were fed to the leaf by vacuum infiltration. Leaf bits were immersed in the corresponding solutions in a Falcon tube and the air pressure gradually lowered to 10 kPa and then held for 30 sec. Afterwards the leaf bits were floated again on the respective solutions, as described above. Incubation in the light was continued for the α-amanitin treatment while the leaves were transferred to darkness for the MG-132 and leupeptin treatments. The leaves were harvested at chosen time points, frozen in liquid nitrogen and kept at –80 °C until further analysis. For each time point, each treatment and for each analysis 4 separate samples were collected as replicates.

Extraction and Analysis of Fructans

Soluble carbohydrates were extracted according to procedures reported earlier (Roth *et al.*, 1997). One ml of 80% ethanol was added to 100 mg of leaf tissue and heated for 5 min at 80 °C. The leaf tissue was ground using an Eppendorf grinder and centrifuged at 15 K ***g*** for 5 min. The pellet was re-extracted twice with water, heated

and centrifuged as above. The three supernatants were combined, freeze-dried, redissolved in 200 µl water, filtered and analysed by high-performance liquid chromatography (HPLC) using an anion exchange column and pulsed amperometric detection as described (Lüscher *et al.*, 2000a). Using Suc, Glc, Fru, 1-K, 6-K and bifurcose as standards (Duchateau *et al.*, 1995), the corresponding components of the sugar extract were identified and quantified. Total fructan content was measured using the cysteine-carbazole reaction (Nakamura, 1968), correcting for Fru and Suc present in the extracts, but not for the Glc of the fructans that is not quantified by the fructose specific reagent; total fructan contents are therefore slightly underestimated

Protein Extraction and Enzyme Assays

Leaf samples (100 mg fresh weight) were homogenized with 50mM citrate (Na^+) buffer pH 5.8 (200 µL). The extract was centrifuged at 16 K ***g*** at 4 °C for 10 min and the supernatant desalted by passage through a column of Bio-Gel P-6 (Biorad). The 1-SST assay mixture (final volume 40 µL) consisted of 36 µL of the enzyme extract and 4 µL of 1 M Suc (final concentration: 100 mM) and was incubated at 27°C for 3 h. The reaction was stopped at 0 and 3 h by heating for 1 min at 95°C and analyzed by HPLC as described above. The production of 1-K was used as a measure of 1-SST activity.

For 6-SFT assays, 34 µL of the extract was mixed with 4 µL of 1 M Suc (final concentration: 100 mM) and 2 µL of 1 M 1-K (final concentration: 50mM) and incubated at 27 °C for 3 hours. The reaction was stopped at 0 hour and 3 hours by heating for 1 minute at 95 °C and analyzed by HPLC as described above. Bifurcose production was used as a measure of 6-SFT activity. Protein concentrations of the extracts were determined using the Bradford method (Bio-Rad protein assay reagent).

RNA Isolation and RT-PCR Analysis

RNA was isolated from the leaf tissue using the RNeasy Plant Mini kit (Qiagen). The RNA extract was treated with deoxyribonuclease I (MBI Fermentas) to remove contaminating DNA, subjected to phenol/chloroform extraction and quantified spectrophotometrically. One μg of RNA was used for synthesis of cDNA using the Reverse Transcription System (Promega). The cDNA was used as template directly for PCR reactions with 24 cycles using standard conditions.

Since the barley 1-SST and 6-SFT cDNAs have an identity of 75%, primers were designed so as to specifically detect 1-SST or 6-SFT (accession no. X83233) sequences. As an internal standard for plant cDNA, primers for the barley ubiquitin were designed so as to achieve a combined amplification of the mub1 and mub 2 genes. (Accession nos. M60175 and M60176). The lengths of the respective amplicon are 265 bp (1-SST), 171 bp (6-SFT) and 186 bp (Ubiquitin).

Results

Cloning of Barley 1-SST cDNA

A functional barley 1-SST cDNA was cloned by a combination of RT-PCR, RACE and genome walking by PCR in five steps . To begin with, a cDNA fragment was amplified by PCR using a reverse primer derived from short unique peptide sequence of the purified 1-SST protein along with degenerate invertase/FT primers. This amplicon was used for nested PCR and the sequence of the resulting product was used for designing RACE primers. While the 3' end of the cDNA was obtained by 3' RACE, 5' sequence information upto the start of the N terminal of the mature protein was obtained by PCR on a previously constructed barley cDNA library (Sprenger *et al.*, 1995). A partial cDNA clone encompassing the coding information of the mature protein was obtained by PCR on the cDNA library and when expressed in *Pichia pastoris* showed 1-SST activity (see below). Further upstream sequence upto the start codon and a small 5'UTR was obtained by Genome walking by PCR. Finally, the entire cDNA of 2157bp was directly amplified from the barley cDNA library (see Materials and Methods for details).

The barley 1-SST cDNA (EMBL accession number AJ567377), contains an ORF of 632 amino acids, starting at position 19 and ending at position 1914, followed by 243bp 3'UTR. The ORF starts with a typical signal sequence, as expected for a protein entering the secretory pathway. Like other well characterised FTs and invertases, barley 1-SST is most probably formed as a preproprotein and N-terminally processed in the secretory pathway. Based on sequence comparisons (Fig. 2.1), the mature polypeptide is expected to start at amino acid residue 77, and its molecular weight, without consideration of its probable glycosylation (see below), is predicted

to be 61.8 Da. Its calculated pI is 5.02, which is close to the pI of 4.9 determined experimentally, by analytical isoelectric focusing, for the plant protein (Lüscher *et al.*, 2000a). The plant protein, which has been localized to the vacuole (Wagner *et al.*, 1983), is glycosylated and 6 predicted

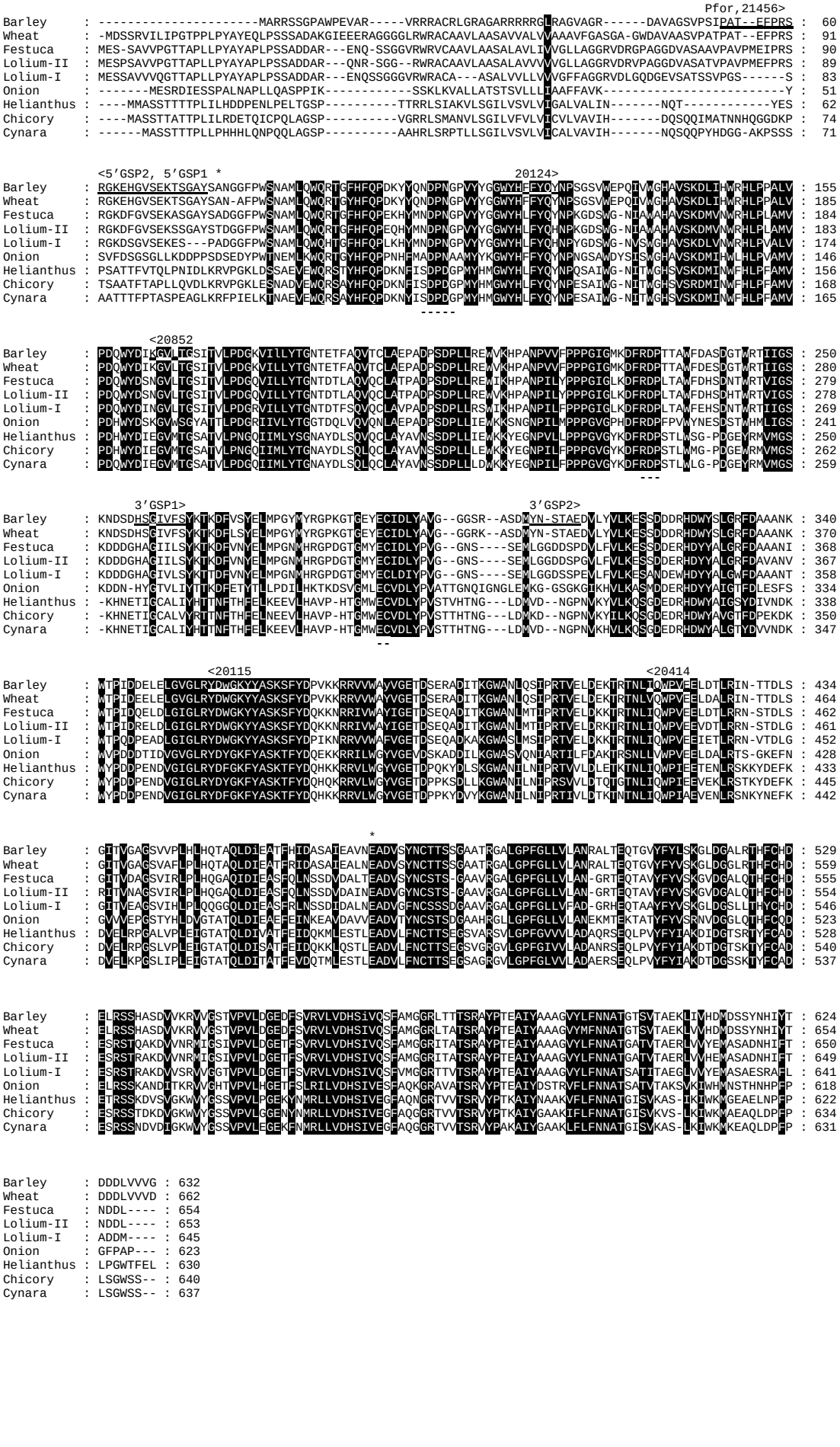
Pfor,21456>
<5′GSP2, 5′GSP1 *
20124>
<20852
3′GSP1>
3′GSP2>
<20115
<20414
*

Figure 2.1. Comparison of the amino acid sequence of 1-SST from *Hordeum vulgare* (Barley, AJ567377) with 1-SSTs from other plants: *Triticum aestivum* (Wheat, AB029888), *Festuca arundinacea* (Festuca, AJ297369), *Lolium perenne* (Lolium I, AF492836), *Lolium perenne* (Lolium II, AY245431), *Allium cepa* (Onion, AJ006066), *Helianthus tuberosus* (Helianthus, AJ 009757), *Cichorium intybus* (Chicory, U81520) and *Cynara scolymus* (Cynara, Y09662). The identical amino acids are highlighted in black. Asterisks indicate the putative N-termini of the large subunit and small subunit, respectively. The dotted line represents the known conserved domains among invertases and FTs: namely NDPNG (Suc binding box), the RDP motif and the EC domain. The amino acids shown in lower case are taken from the clone expressed in Pichia, which corresponds to the consensus sequence (see results for details). The underlined amino acid residues with names above indicate the location of the primers used for cloning the cDNA.

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glycosylation sites in the ORF were found. The barley 1-SST is probably proteolytically cleaved (likely at position 478), since two subunits of 50 and 22kDa were found upon SDS polyacrylamide gel electrophoresis, which is similar to the situation reported for other FT's and invertases (Sturm, 1999).

The 1-SST gene sequence was aligned with closely related sequences from grasses and two other monocots (onion and tulip), corresponding to putative FT's and invertases, and an unrooted phylogenetic tree was constructed (Fig. 2.2). This analysis stressed the difference between cell wall invertases, as compared to vacuolar invertases. Clearly, the barley 1-SST cloned here clusters well with other grass 1-

SST's and is more closely related to 6-SFT's, vacuolar invertases and other FT's from grasses than to the corresponding enzymes of the other two monocots or to the monocot cell wall invertases and FEH's (Fig. 2.2).

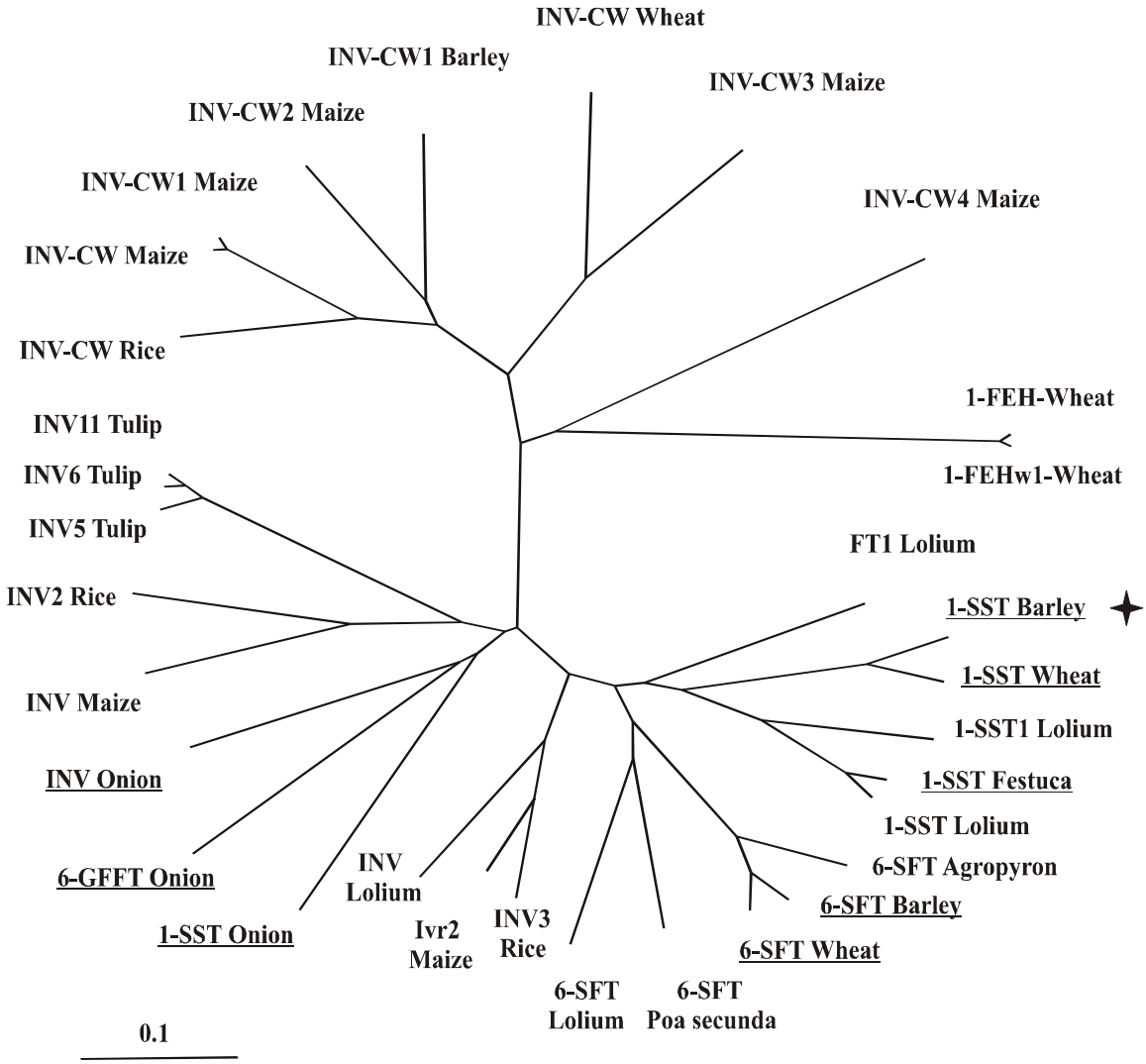


Figure 2.2. Unrooted phylogenetic tree of protein sequences of some invertase and fructan metabolism genes from grasses and two other monocots (onion and tulip), including the newly cloned barley 1-SST. The sequence of fructan exohydrolases are more similar to cell wall invertases, while the fructosyl transferases (1-SST, 6-GFFT, FT, 6-SFT) group together with vacuolar invertases. Fructosyltransferases that have been functionally characterised by heterologous expression have been underlined. The subgroups, sequence names, their origin and accession numbers are: **Cell Wall Invertases and Fructan Exohydrolases**; INV-CW1 Maize (*Zea mays*, AF050129), INV-CW2 Maize (*Zea mays*, AF050128), INV-CW3 Maize (*Zea mays*, AF043346), INV-CW4 Maize (*Zea mays*, AF043347), INV-CW Maize (*Zea mays*, U17695), INV-CW Rice (*Oryza sativa*, AB073749), INV-CW Wheat (*Triticum aestivum*, AF030420), 1-FEH wheat (*Triticum aestivum*, AJ508387), 1-FEHw1 wheat (*Triticum aestivum*, AJ516025). INV-CW1 Barley (*Hordeum vulgare*, AJ534447). **Vacuolar Invertases**; INV Lolium (*Lolium perenne*, AY082350), INV Onion (*Allium cepa*, AJ006067), AF069309), INV3 Rice (*Oryza sativa*, AF276704), INV2 Rice (*Oryza sativa*, AF276703), INV Maize (*Zea mays*, U16123), Ivr2 Maize (*Zea mays*, U31451), INV5 Tulip (*Tulipa gesneriana,* X97642), INV6 Tulip (*Tulipa gesneriana,* X97643), INV11 Tulip (*Tulipa gesneriana,* X95651). **Fructosyltransferases**; 6-GFFT Onion (*Allium cepa*, Y07838), 1-SST Onion (*Allium cepa*, AJ006066), FT1 Lolium (*Lolium perenne*, AF481763), 6-SFT Wheat (*Triticum aestivum*, AB029887), 6-SFT Barley (*Hordeum vulgare*, X83233), 6-SFT Agropyron (*Agropyron cristatum*, AF211253), 6-SFT Poa secunda (*Poa secunda*, AF192394), 6-SFT Lolium (*Lolium perenne*, AF494041), 1-SST Wheat (*Triticum aestivum*, AB029888), 1-SST Barley (*Hordeum vulgare*, AJ567377), 1-SST Lolium (*Lolium perenne*, AF492836), 1-SST1 Lolium (*Lolium perenne*, AY245431), 1-SST Festuca (*Festuca arundinacea*, AJ297369).

Expression of Barley 1-SST in *Pichia pastoris*

Expression studies were done on the basis of the partial cDNA coding for the mature 1-SST protein. This cDNA fully represents the 1-SST cDNA described above, except for 179 bp of the 5' end; since the 3'-UTR regions are exactly the same, the two cDNAs represent products of the same gene. There were, however, seven nucleotide differences (consistently observed in three independent sequencing runs and therefore most likely corresponding to PCR artefacts) between the two cDNAs at the following positions of the ORF, (nucleotide in full-length clone/nucleotide in partial clone): 242:A/G, 557:C/T, 988: G/A, 1090:C/T, 1157:G/A:, 1385:C/T, 1715: G/T. In all cases, one of the two resulting codons matched the consensus of the four other 1-SST sequences available from grasses (see Fig. 1.1), and was thus considered to reflect the true sequence. These differences occurred at the following positions: (amino acid in derived full-length sequence/amino acid in derived partial sequence, consensus in boldface) 75:**Y**/C, 180:P/**L**, 324:**D**/N, 358:R/**W**, 380:C/**Y**, 456:T/**I**, 566:S/**I**). Note that there were only two changes from the consensus in the partial sequence used for the expression study (positions 75 and 324), which were both located apart from the conserved and well known domains of plant FTs that are presumably of particular functional importance (Ritsema and Smeekens, 2003c). The coding region of this cDNA was cloned into *Pichia pastoris*, using a plasmid containing the necessary expression signals as well as the coding information for the N-terminal signal sequence of the α-factor.

Upon incubation of the heterologously expressed enzyme with 100 mM Suc, a marked 1-SST activity was observed, as seen by the massive production of 1-K and Glc. There was only a marginal production of Fru, indicating that the enzyme preparation had almost no invertase activity (Table 2.2). When the same enzyme

preparation was incubated with 50 mM 1-K alone, it acted mainly as a FEH, leading to the formation of Fru and Suc. Furthermore, an appreciable amount of nystose was formed indicating some 1-FFT activity. When incubated with a combination of 100 mM Suc and 50 mM 1-K, the production of Fru and nystose seen in the presence of 1-K alone was repressed, indicating that under these conditions, the enzyme acted mainly as 1-SST producing 1-K, as indicated by the large amount of Glc formed, and had very little 1-FEH and 1-FFT activities. The small amount of nystose formed under these conditions could be due to minor 1-SFT and/or 1-FFT activity (Table 2.2). None of the enzyme assays yielded any 6-kestose (6-K) or bifurcose, demonstrating that the enzyme is devoid of 6-SFT activity. A corresponding concentrated and desalted induction medium of *Pichia* strain X33 transformed with the parent plasmid pPiCZαC (vector control) showed no detectable product formation with any of the substrates utilized (activity <0.01 nkat/mg protein, based on the detection limit for 1-K in HPLC).

Table 2.2. Activities of the enzyme produced by heterologous expression of the 1-SST clone in *Pichia pastoris*, incubated with 100 mM Suc, 50 mM 1-K and a combination of 100 mM Suc plus 50 mM 1-K. All assays were performed in triplicates (standard deviation of the mean is indicated)

Substrate	Product formation (nkat/mg protein)				
	Glc	Fru	Suc	1-K	Nys
Sucrose	2.13 ± 0.14	0.11 ± 0.02	n.a.[a]	1.8 ± 0.11	n.d.[b]
1-Kestose	0.12 ± 0.05	1.49 ± 0.09	1.34 ± 0.07	n.a.[a]	0.33 ± 0.03
Sucrose + 1-kestose	1.86 ± 0.05	0.21 ± 0.07	n.a.[a]	n.a.[a]	0.11 ± 0.01

[a] n.a., not assayed (added as substrate)

[b] n.d., not detectable in the HPLC analysis

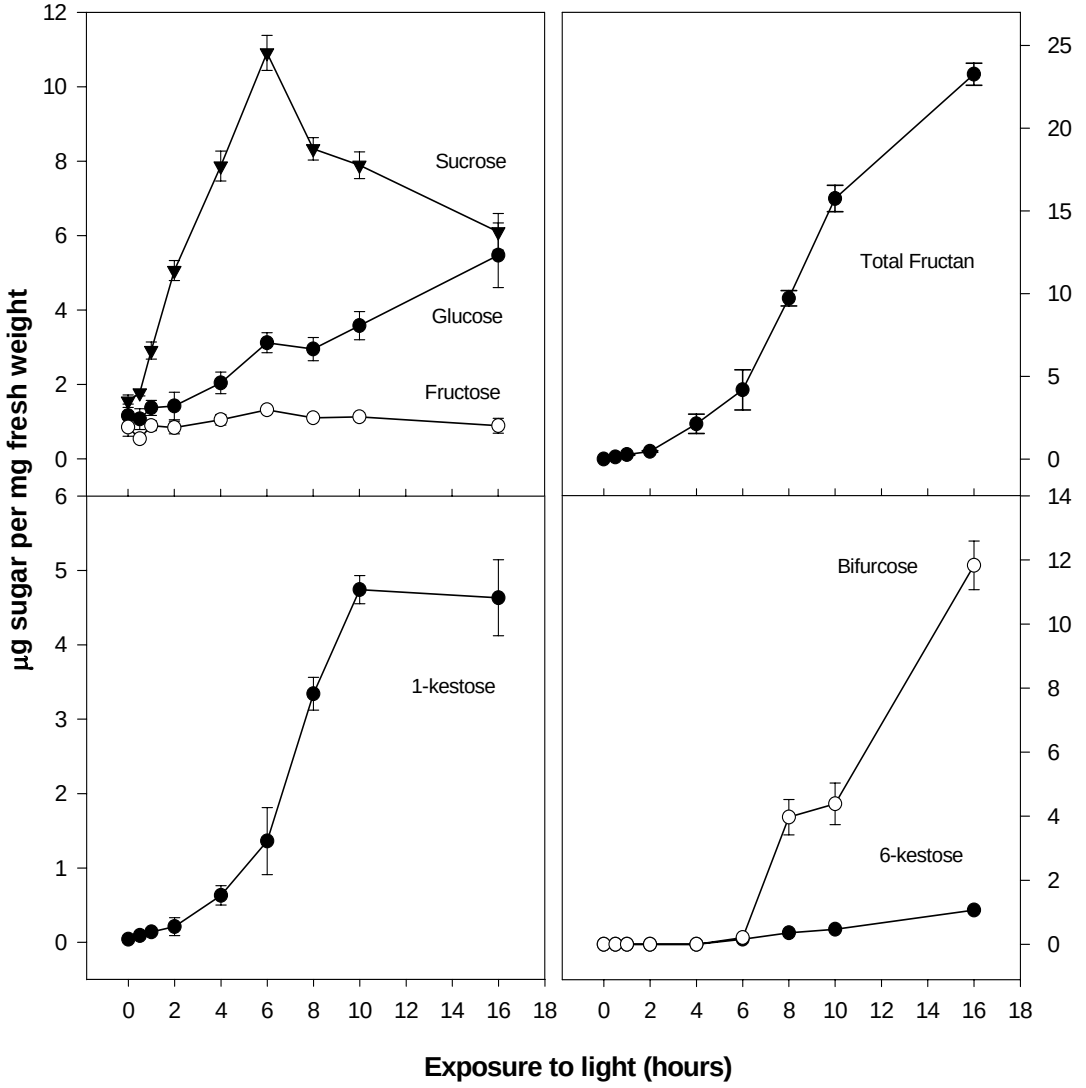


Figure 2.3. Accumulation of soluble carbohydrates in excised leaves of barley upon exposure to continuous illumination. Error bars represent the standard error of the mean (n = 4).

Induction of Fructan Biosynthesis

Excised barley leaves were exposed to continuous light to study the accumulation of soluble carbohydrates, the level of 1-SST and 6-SFT activities and the pattern of expression of the corresponding genes.

At the beginning of the light treatment, the leaf pieces contained relatively small amounts of Glc, Fru and Suc while fructans were completely absent. Upon illumination, Suc synthesis was rapidly induced after 1 h and increased about 6-fold over the next 6 h. Subsequently, Suc levels dropped slightly for the rest of the illumination period (Fig. 2.3). Levels of Fru did not change during the experiment, while Glc steadily increased during illumination. The total amount of fructan increased almost exponentially between 2 and 10 h of illumination and reached higher levels than Suc after ca. 8 h (Fig. 2.3). The earliest formed fructan was 1-K. Its rate of accumulation steadily increased as well, yielding an almost exponential accumulation pattern between 0 and 8 h of illumination (Fig. 2.3). 6-K was formed only at a later stage and remained a minor fructan throughout the experiment. Bifurcose was first detected after 6 h and then accumulated rapidly, becoming the most prominent fructan after 16 h of illumination (Fig. 2.3). Higher DP fructans were detected by 24 h (data not shown).

Looking at the time course of induction of enzyme activities (Fig. 2.4), 1-SST activity was found at low levels already at the start of the light phase, possibly representing a marginal side activity of invertase. After a lag phase of ca. 2 h, it steadily increased for the next 8 h and subsequently continued to increase at a slower rate. There was no detectable 6-SFT activity at the beginning of the experiment. This enzyme started to increase slowly between 4 and 10 h of illumination, and it reached

high levels only after 16 h (Fig. 2.4). Note that invertase was constitutively present in the leaves and did not change in the course of the experiment.

The rate of fructan accumulation in the leaves (1.45 μg mg^{-1} h^{-1}) during the 16 h illumination period was comparable with that calculated from the combined fructosyl transfer activities of 1-SST and 6-SFT (2.02 μg mg^{-1} h^{-1}).

The sequence of the changes of transcript levels of 1-SST and 6-SFT, as detected by RT-PCR, preceded the changes observed of the enzyme activities. The level of 1-SST mRNA was very low at the beginning of the experiment, increased markedly already within 1 h and reached a new steady state level after 2 h (Fig. 2.5). In contrast, the 6-SFT expression remained at a constant low level during the first 2 h and started to increase only thereafter (Fig. 2.5). The level of transcripts encoding ubiquitin remained constant throughout the course of the experiment (Fig. 2.5).

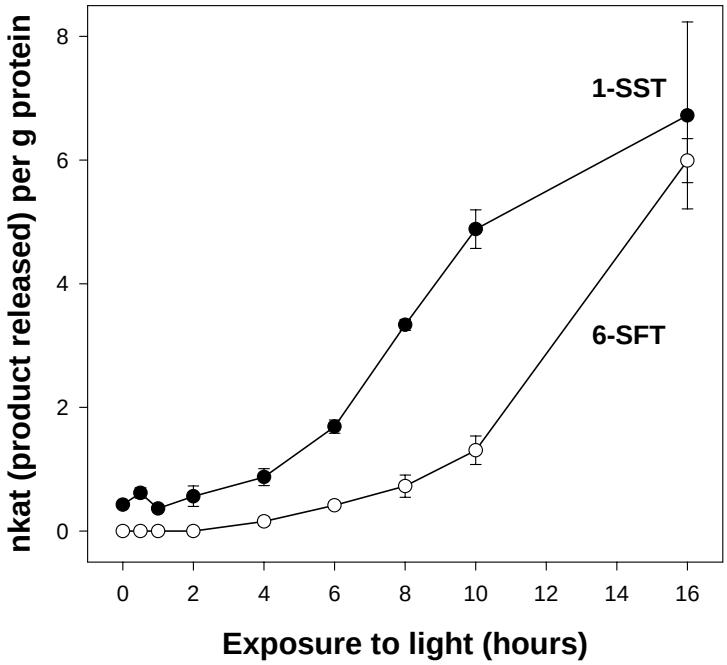


Figure 2.4. Activities of 1-SST and 6-SFT in excised leaves of barley upon exposure to continuous illumination. Error bars represent the standard error of the mean (n = 4).

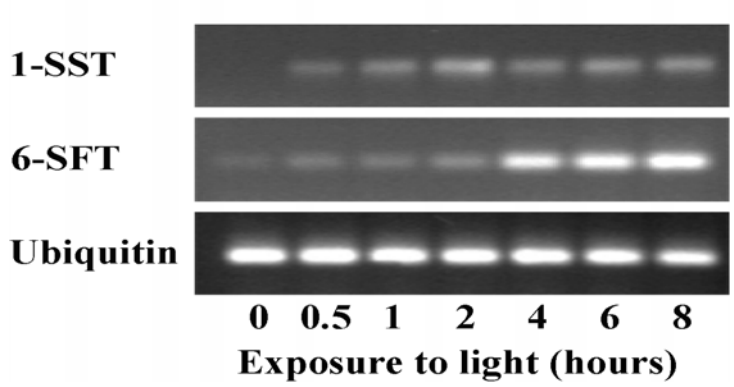


Figure 2.5. Expression of 1-SST and 6-SFT transcripts in excised primary leaves of barley during continuous illumination, as compared to ubiquitin transcript expression. Ethidium bromide-stained gels after RT-PCR amplification (24 cycles).

Fructan Metabolism in the Dark Phase

After continuously illuminating the excised leaves for 24 h, the lights were switched off and the depletion of fructans, enzyme activities and steady state levels of transcripts in the dark were followed. After the onset of the dark phase, Suc remained at a constant high level for about 1 h but then dropped rapidly by about 50% between 1 and 4 h of the dark period. The Glc pool dropped faster but to a lesser extent than Suc, while Fru levels were little affected (Fig. 2.6). Total fructan was even slightly increasing at the beginning of the dark phase and then was degraded only gradually (Fig 2.6). The most rapid decrease was that of 1-K: this pool started to drop immediately and reached levels of only 30% within 4 h (Fig 2.6). Bifurcose, like total fructan, displayed an increase during the first 2 h and then decreased slowly but steadily; 6-K remained low and approximately constant during the dark period (Fig. 2.6).

The activity of 1-SST dropped very rapidly as soon as the dark phase started, with a half-life of less than 1 h, and appeared to reach a new steady state after approximately 4 h (Fig. 2.7). This corresponded well to the immediate drop of 1-K mentioned above. In contrast, 6-SFT activity stayed approximately constant for the first hour and then decreased slowly but steadily with a half-life of about 5 h (Fig. 2.7).

When mRNA levels were considered, transcripts corresponding to 1-SST disappeared very quickly after the onset of the dark phase (Fig. 2.8). In sharp contrast, transcripts corresponding to 6-SFT remained almost constant during the first 8 h of the dark phase and decreased only thereafter (Fig. 2.8).

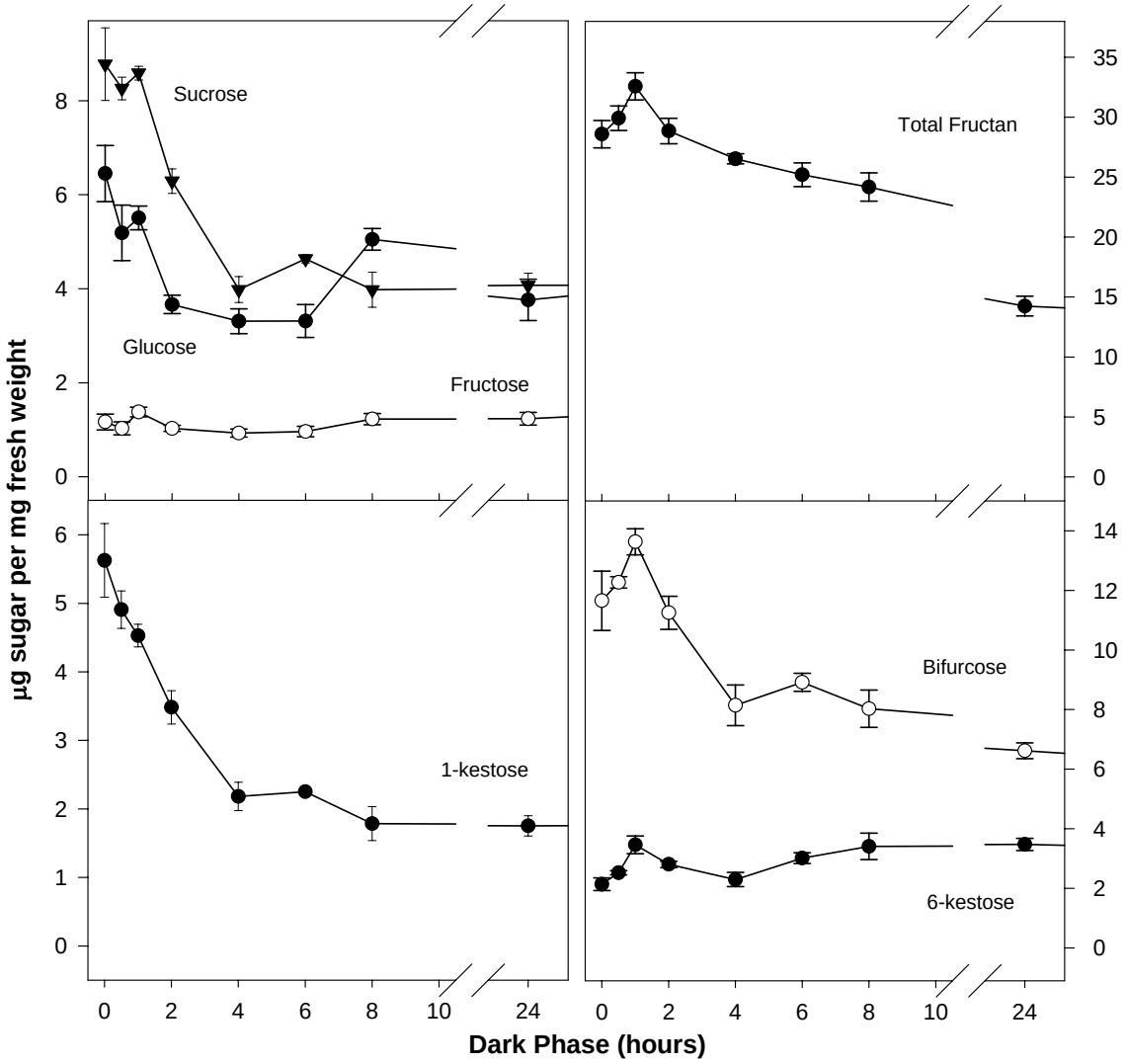


Figure 2.6. Amounts of soluble carbohydrates in excised primary leaves of barley after continuous illumination for 24 h and subsequent transfer to darkness at time zero. Error bars represent the standard error of the mean (n = 4).

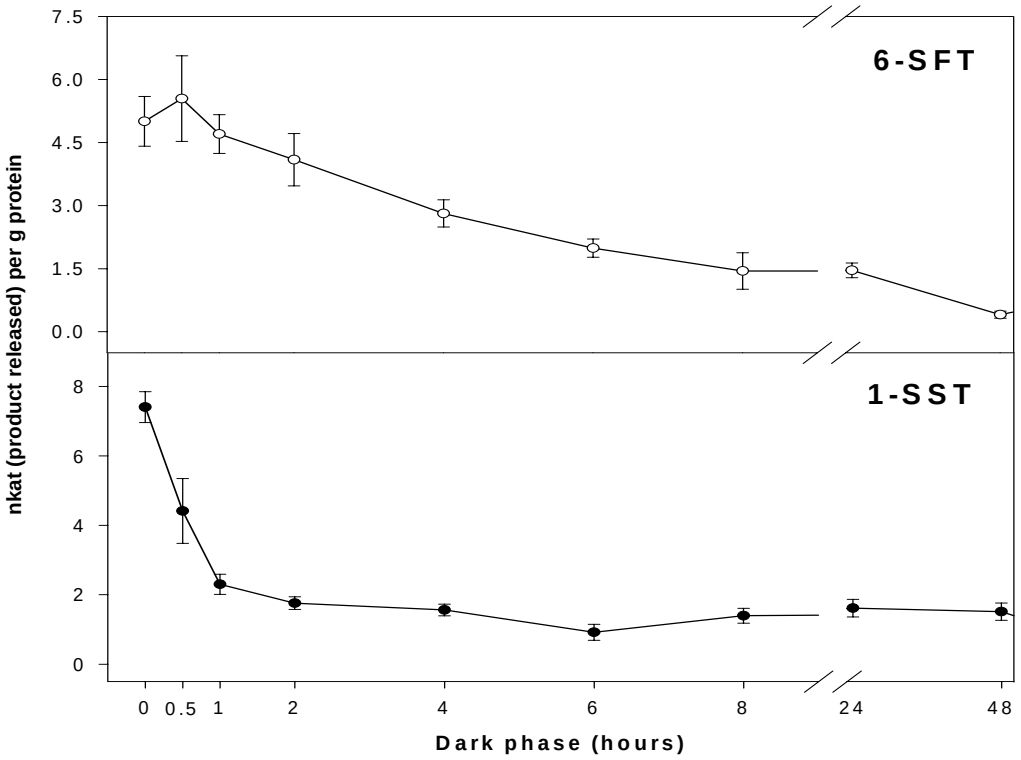


Figure 2.7. Activities of 1-SST and 6-SFT in excised primary leaves of barley after continuous illumination for 24 h and subsequent transfer to darkness at time zero. Error bars represent the standard error of the mean (n = 4).

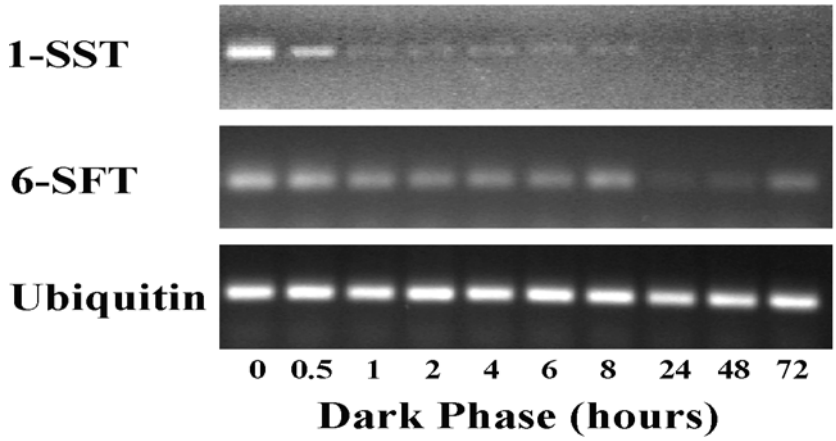


Figure 2.8. Transcript levels of 1-SST and 6-SFT in excised primary leaves after continuous illumination for 24 h and transfer to darkness at time zero, as compared to ubiquitin transcript expression. Ethidium bromide-stained gels after RT-PCR amplification (24 cycles).

Effect of α-Amanitin, an Inhibitor of Transcription

After 16 h of light, α-amanitin (100 μM), an inhibitor of RNA polymerase II, was fed to the leaves by vacuum infiltration, and illumination of the leaves was continued with the leaves floating on a solution of the inhibitor. The levels of mRNAs encoding 1-SST, 6-SFT and ubiquitin were then followed over the next 6 h by RT-PCR analysis to compare the stability of the transcripts (Fig. 2.9). Transcripts corresponding to 1-SST decreased very rapidly and were barely detectable after 2 h. In contrast, transcripts corresponding to 6-SFT remained constant for about 2 h and then appeared to slightly decrease. Ubiquitin mRNA disappeared more slowly than 1-SST mRNA but more rapidly than 6-SFT mRNA (Fig. 2.9).

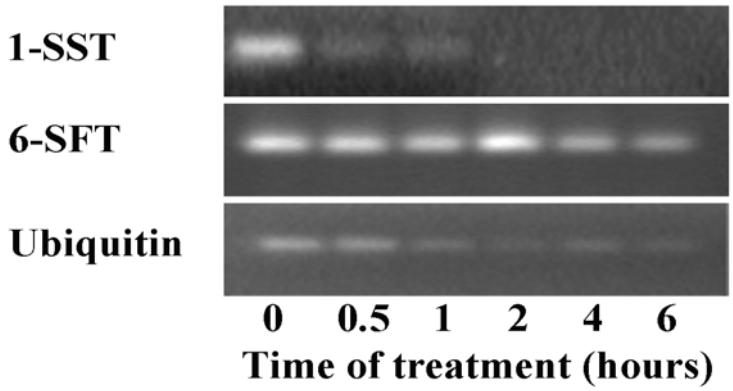


Figure 2.9. Transcript levels of 1-SST and 6-SFT in excised barley primary leaves, kept under continuous illumination, during a treatment with α-amanitin, as compared to ubiquitin transcript expression. The inhibitor α-amanitin (100 μM) was applied at time zero, i.e. after 16 h of continuous illumination. Ethidium bromide-stained gels after RT-PCR amplification (24 cycles).

Effect of Leupeptin and MG132, two Inhibitors of Proteolysis

Previous work showed that an unspecified SST activity of barley was undergoing rapid turnover and that this could be stopped by the protease inhibitor leupeptin (Obenland *et al.*, 1991). In accordance leupeptin was found to block the decay of 1-SST induced by a dark treatment at least during the first 2 h (Fig. 2.10). MG-132, a similar peptidyl aldehyde inhibiting various plant cysteine proteases (Woffenden *et al.*, 1998; Cotelle *et al.*, 2000), was even more efficient: in its presence, 1-SST activity slightly increased initially and then remained constant during the 6 h duration of the treatment (Fig. 2.10). The comparatively slow decrease of 6-SFT activity upon transfer of the leaves to the dark was further reduced by leupeptin; in the presence of MG-132, 6-SFT activity increased slightly initially, as did 1-SST, and then it remained virtually the same for the remainder of the experiment (Fig. 2.10).

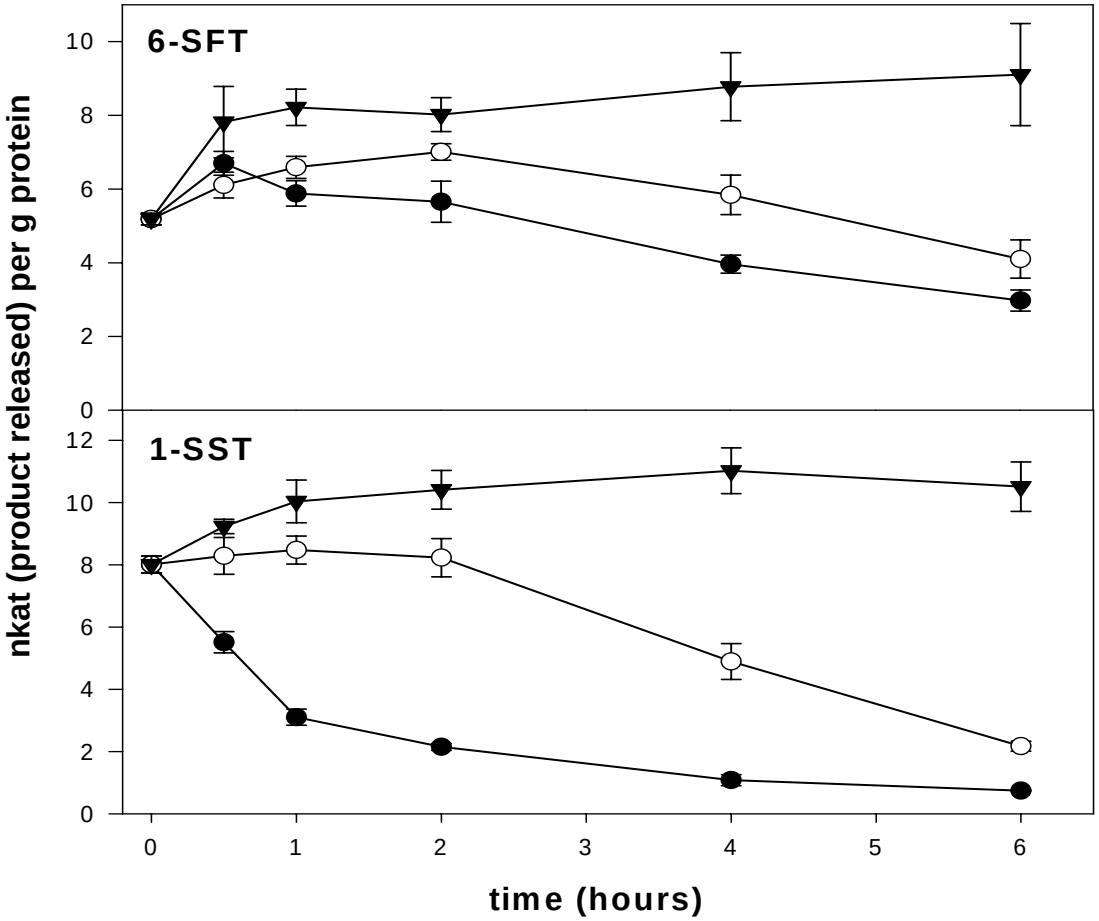


Figure 2.10. Enzyme activities in excised primary leaves of barley after continuous illumination for 24 h and subsequent transfer to darkness at time zero, in the absence (black circles) or presence of proteinase inhibitors: 300μM leupeptin (white circles) or 80 μM MG-132 (inverted black triangles). Error bars represent the standard error of the mean (n = 4).

Discussion

In the present work, a reverse genetics approach has been used to clone a cDNA from barley encoding 1-SST, and 1-SST activity of the encoded protein has been verified by heterologous expression in *Pichia pastoris*, as in the previous work with *Festuca* 1-SST (Lüscher *et al.*, 2000b). Interestingly, the Pichia-expressed enzyme behaved biochemically just like the purified barley 1-SST (Lüscher *et al.*, 2000a) and the heterologously expressed *Festuca* 1-SST (Lüscher *et al.*, 2000b) with regard to the following key properties: (i) very little invertase activity, as judged from the production of Fru from Suc, (ii) marked 1-K hydrolase activity, as judged from the production of Fru from 1-K, and (iii) a strong inhibition of 1-K hydrolase activity by Suc. High protein sequence similarity of the barley 1-SST to other grass 1-SST's [wheat: 91%, (Kawakami and Yoshida, 2002); Festuca: 79% (Lüscher *et al.*, 2000b)] along with interesting differences in comparison to 6-SFTs [e.g. barley: 69% similarity, (Sprenger *et al.*, 1995)] and to various other FT's and vacuolar invertases from grasses (Poaceae) may help to elucidate the crucial domains responsible for the different activities and substrate specificities of these closely related enzymes.

Several grass FT and invertase protein sequences have been published recently in public databases, but for most of them the actual function of the proteins encoded is still putative and remains to be established. Nevertheless, a comparison of the available sequences from grasses in an unrooted phylogenetic tree reveals several distinct groups: the 1-SST's, the 6-SFT's, the vacuolar invertases and putative FT's and, as a quite separate group, the cell wall invertases and FEH's. Remarkably, sequences of functionally related enzymes from other monocots, (onion and tulip) are grouping apart from the sequences of the corresponding enzymes of grasses,

indicating that fructan synthesis evolved at least twice independently in these monocots, in both cases on the basis of FTs recruited from vacuolar invertases.

In the time course study with excised and illuminated barley leaves, Suc levels were found to increase rapidly, reaching a peak after 6 h. During this time, the trisaccharide 1-K was essentially the only fructan formed. 1-K accumulated at an accelerating rate up to 8 h, in accordance with the rapid induction of 1-SST gene expression and enzyme activity. This finding indicates that the diversion of Suc into the pathway of fructan synthesis is initiated by 1-SST induction. Synthesis of the branched tetrasaccharide bifurcose started only after a marked delay. This corresponds to a similar delay in the induction of 6-SFT gene expression and the corresponding enzyme activity. After 10 h, the level of 1-K did not increase further while bifurcose as well as higher fructan polymers continued to accumulate, indicating that only in the presence of 6-SFT - in addition to that of 1-SST - a large drain is formed drawing photosynthates in direction to fructan. This sequence of events agrees well with previous biochemical studies showing that the preferred fructosyl acceptor substrate of 6-SFT is 1-K, and that higher polymeric fructans can serve as acceptors as well whilst Suc is an unsuitable acceptor (only minor 6-SST activity), particularly in the presence of 1-K (Simmen *et al.*, 1993; Duchateau *et al.*, 1995; Wiemken *et al.*, 1995). In fact, the level of 6-K remained low throughout the experiment.

The promotor of 6-SFT has previously been shown to be activated by Suc (Nagaraj *et al.*, 2001), and the induction of 6-SFT expression in the illuminated excised leaves occurs most likely in response to Suc accumulation. The results obtained now corroborate and extend these findings. It is striking that the 1-SST transcript level increased already within the first 30 min after illuminating the excised

leaves, much earlier than the level of 6-SFT transcripts, and already at a time at which the pool of Suc was still low. This clearly indicates that 1-SST is regulated in a different way than 6-SFT at the transcript level. Nevertheless, Suc may still be the regulatory molecule: Suc is well known to be compartmentalized both at the subcellular and cellular levels and it must be assumed that only a part of the total Suc measured in the leaves, most probably the part constituting the small and therefore rapidly responding cytosolic pool, has a regulatory function whereas a larger part of Suc generally resides in the vacuoles exerting a storage function (Boller and Wiemken, 1986). Cytoplasmic Suc may be the inducing factor for both genes, but the 1-SST gene may have a lower threshold for activation by Suc than the 6-SFT gene. The threshold for activation by Suc may also depend on cell types: When single cells from barley leaves were sampled, a lower Suc level appeared to be required for inducing 6-SFT expression and fructan accumulation in the parenchymatous bundle sheath cells than in the mesophyll cells of barley leaves (Koroleva *et al.*, 2001; Pollock *et al.*, 2003), and similar observations were made in a comparison of different regions of the uppermost internode of wheat (Gebbing, 2003). There is evidence that the signal transduction pathway responsible for regulation of fructan synthesis is independent from the hexokinase associated sugar sensing system (Müller *et al.*, 2000) and involves protein kinase/phosphatase activities (Noel *et al.*, 2001).

A pacemaker function of 1-SST was most evident in the experiment where the excised and illuminated leaves were transferred from the light to darkness. Under these conditions the 1-SST, in contrast to the 6-SFT, was instantaneously down regulated at the level of mRNA and at the level of enzyme activity. Remarkably, this fast regulatory response occurred again much before the large Suc pool had changed appreciably, precluding a regulatory role of this pool as a total. In accordance with the

much faster drop of the 1-SST activity as compared to the drop of the 6-SFT activity, 1-K dropped immediately whereas bifurcose and total fructan contents initially increased further and started to decrease only after a considerable delay. Upon depletion of 1-K, 6-SFT could act as an invertase, cleaving remaining vacuolar Suc (Duchateau *et al.*, 1995) and, thus, curtailing any further use of Suc for fructan synthesis. In this context it is worth mentioning that the removal of the vacuolar Suc pool might be a prerequisite for allowing a mobilization of the fructan reserves; this is because Suc has been found to act as an efficient inhibitor for some FEH's (Van den Ende *et al.*, 2003a). Total FEH activity has been shown to be more or less constitutive in barley leaves and, like the fructans, it is located in the vacuoles of barley leaves (Wagner *et al.*, 1986).

The rapid down-regulation of 1-SST in the dark was found to proceed at the level of mRNA as well as at the level of protein. The short half-life of the 1-SST transcripts observed after treating with α-amanitin suggests a tight regulation of 1-SST at the level of mRNA turnover. This could be due to specific sequences in the UTR known to influence transcript stability (Green, 1993). It is worth noting that the 5' and 3' UTR of the 1-SST and 6-SFT transcripts share very low sequence similarity (36%), which is in contrast to the high similarity (71%) of their translated regions. Further investigations are needed to find out if these differences are responsible for the distinct mRNA decay rates observed.

The rapid loss of 1-SST activity in the leaves upon transfer from the light to darkness is most probably due to a fast protein turnover, as suggested already in an earlier study on unspecified SST activities (Obenland *et al.*, 1991). The two protease inhibitors applied, leupeptin and MG-132, completely blocked the loss of 1-SST and 6-SFT activity for at least 2 h after transition to darkness. Both these inhibitors are

peptide aldehydes with a strong potential to inhibit cysteine proteases. Although MG-132 has been used as an inhibitor of the proteasome in animal cells, it is a potent inhibitor of various cysteine proteases in plants (Cotelle *et al.*, 2000) including the well-characterized autolytic tracheary element proteases believed to be vacuolar (Woffenden *et al.*, 1998). Since 1-SST is in all likelihood a vacuolar enzyme (Wiemken *et al.*, 1995) it is degraded presumably by the classic vacuolar cysteine proteases (Boller and Wiemken, 1986).

In conclusion, based on the finding of a rapid regulation of the 1-SST at multiple levels and, in contrast, a much slower regulation of the 6-SFT, both under conditions of induction and repression of fructan synthesis, 1-SST is likely to play a crucial role as pacemaker enzyme controlling the flow of carbon into fructan in barley leaves.

CHAPTER 3

Light and Sugar Regulation of the Barley Sucrose:fructan 6-fructosyltransferase Promoter

Vinay Janthakahalli Nagaraj, Ralph Riedl, Thomas Boller, Andres Wiemken and Alain Denis Meyer

Abstract

Fructans are a class of major storage compounds in many plants including several economically important cereals. Earlier we characterized and cloned the sucrose:fructan 6-fructosyltransferase (6-SFT) of barley (*Hordeum vulgare* L.), a key enzyme for the synthesis of branched fructans (graminans) typical of many cereals and temperate forage grasses. Here, we describe the cloning of the barley 6-SFT promoter region, by PCR-based genome walking procedures. Using a promoter-reporter gene construct (*uidA* encoding β-glucuronidase) and microprojectile bombardment of excised barley leaves, we show that the cloned sequence contains the necessary *cis* acting elements conferring sucrose and light induction of 6-SFT transcription.

Introduction

Fructans occur in ca. 15% of the flowering plant species, including many members of the *Poaceae* (Hendry, 1987). In several temperate forage grasses and cereals, fructans are the major storage carbohydrates in vegetative organs (Pollock and Cairns, 1991). Barley (*Hordeum vulgare* L.), for example, can accumulate branched fructans with β(2 -1) and β(2-6) linkages called graminans. These fructans are synthesized from sucrose through the consecutive action of two enzymes: 1-SST,

producing the trisaccharide 1-K, and 6-SFT, which adds fructosyl units to 1-kestose to produce first the branched tetrasaccharide bifurcose and then longer-chain graminans (Wiemken et al., 1995; Vijn and Smeekens, 1999).

Excised primary leaf blades of barley represent an excellent model system to study the regulation of fructan synthesis (Wagner et al., 1986c). They do not contain fructans initially, but they start to accumulate large amounts of fructan upon illumination or supply of sucrose, and they concomitantly display strongly enhanced activities of 1-SST and 6-SFT (Simmen et al., 1993).

Using this model system, our lab previously purified and characterized 6-SFT and cloned the corresponding cDNA (Sprenger et al., 1995). Previous data showed that induction of 6-SFT activity was paralleled by an accumulation of 6-SFT transcripts, indicating transcriptional regulation of 6-SFT (Sprenger et al., 1995; Müller et al., 2000).

Here, the cloning of the promoter region of 6-SFT, comprising 1561 bp upstream of the ORF encoding 6-SFT, and an analysis of its function by transient expression of a promoter-reporter gene construct in excised barley leaves is described. The cloned 6-SFT promoter was found to be inactive in non-induced barley leaves that do not accumulate fructans, but that it is active in leaves that accumulate fructans in response to treatments with continuous light or sucrose. Thus, the promoter region contains the necessary *cis* elements to drive light- or sucrose-dependent expression.

Materials And Methods

Plant material and growth conditions

Barley (*Hordeum vulgare* L. cv. Lyric) seeds were soaked for 24 hours in running tap water, planted in a commercial soil mixture (UFA Haus und Garten, Bern, Switzerland) and grown for 7 days in a growth chamber. The conditions were: 16 h light (200 µmole photons $m^{-2}s^{-1}$) at 26°C, 8 h dark at 20°C and a constant relative humidity of 76%.

Induction of Fructan Biosynthesis

Primary leaves of one-week old barley plants were harvested in the middle of the light period and cut such as to leave about half of the leaf sheath and the complete leaf blade. Using the excised leaf system (Wagner et al., 1986c), fructan synthesis was induced by continuous illumination of the leaves (300 µmole photons $m^{-2}s^{-1}$) or by feeding them with sugars as indicated. After the treatments, the leaves were harvested, frozen in liquid nitrogen and kept at –80°C until further analysis.

Extraction and Analysis of Fructans

Soluble carbohydrates were extracted according to procedures reported earlier (Roth et al., 1997) and analyzed by high-performance liquid chromatography (HPLC) using an anion exchange column and pulsed amperometric detection as described (Lüscher et al., 2000a).

RNA Isolation and Analysis

Total barley leaf RNA was isolated (Chomczynski and Sacchi, 1987) and 30 μg RNA from each sample was separated on a formaldehyde-containing agarose gel (Sambrook et al., 1989) and blotted onto Hybond N+ Nylon membrane (Amersham Pharmacia, Little Chalfont, UK). Filters were hybridized with [α-^{32}P]CTP-labelled 6-SFT antisense-RNA probes by transcribing the cDNA [B5, (Sprenger et al., 1995), accession # X83233] at 60°C as described by Meyer and coworkers (Meyer et al., 1995). Blots were finally washed twice for 20 min at 65°C with 0.1X SSC (1X SSC is 0.15 M NaCl, 15 mM Na-citrate) and 0.5% SDS and signals were visualized by storage phosphorescence imaging in a Molecular Imager (Bio-Rad).

Cloning of the 6-SFT Promoter

A combination of PCR based genome walking procedures was used to clone 1.56 kb laying upstream of the 6-SFT coding sequence. Genomic DNA was extracted from barley leaves using the Nucleon PhytoPure Genomic DNA Extraction Kit (Amersham Pharmacia). About 1.2 kb of the sequence 5` to the start codon was cloned using the Universal Genome Walker Kit (Clonetech). Barley genomic DNA was digested with five enzymes *Dra* I, *Eco* RV, *Pvu* II, *Sca* I and *Stu* I, separately, and ligated to adapters in order to construct “libraries”. The libraries served as template for PCR using primers GSPI-1: 5’-TGCACGGCCTGCTCCATCCTCAATCC-3’ and GSPI-2: 5’-TTGTAGGCGTACGGTAGCGGTGGCTTG-3’ designed according to the 5’end of the 6-SFT coding sequence (Sprenger et al. 1995) along with primers from the adapter sequence. Products were cloned into pGEM-T Easy vectors (Promega) and

sequenced. Primers GSPII-1: 5'-TTGGCGTGCCATGTGTGTGAAAATGTGTG-3' and GSPII-2: 5'-GTGTTAGGCTCGGCTGTGATTTACCAT-3' made from the newly obtained sequence, were used to clone the flanking 700 bp up to the *Stu* I site, from the respective *Stu* I library. The remaining 400 bp were cloned using "genomic mini plasmid libraries" which were constructed with genomic DNA digested with different restriction enzymes and the fragments cloned into the Bluescript vector. The genomic mini plasmid libraries served as templates for nested PCR, in which two reverse primers from the previously deduced sequence, GSPIII-1: 5'-CGATGAGGGTCTCTCGCCGAGTTTTGCTTC-3' and GSPIII-2: 5'-CGCCGAGTTTTGCTTCCCTCATGTACCAG-3' was used with two vector specific forward primers V-1: 5'-CACACAGGAAACAGCTATGACCATG-3' and V-2: 5'-CGCCAAGCTCGAAATTAACCCTCAC-3'. The different sequences were assembled and new primers Pfor: 5'-TATGAACGCTCTGTTAGCAAGTC-3' and Prev: 5'-CGATTTTGTAGGCGTACGGTA-3'were designed and the complete 1.6 kb was directly amplified with Barley Genomic DNA as template and cloned into the pGEMT-T Easy Vector (Promega). All PCR reactions were done using the Expand Long template PCR System (Boehringer Mannheim) and $MgCl_2$ was supplemented.

Plasmid Constructs for Transient Assays

The 1.6 kb *Eco* RI / *Nco* I fragment from the pGEM-T easy vector was subcloned into the pUbiGUS plasmid [J.Fütterer, unpublished, (Christensen et al., 1992)], so as to replace the Polyubiquitin1 promoter controlling the GUS reporter gene. Plasmid pUbiGUS was directly used as a control construct for the transient assay.

Transient Expression Studies by Microprojectile Bombardment

One week old primary leaves of barley were cut into 3 cm sections and floated on 10% sucrose (w/v) for 3 hours in the dark prior to bombardment. Gold particles (1.5-3 µm, Sigma-Aldrich, St. Louis, MS, USA) were coated with the plasmid constructs and delivered into the sectioned leaves using a particle inflow gun as described (Vain et al., 1993). For bombardment, the leaf segments were placed into small petri dishes (5 cm diameter) with 10 ml of 0.5 strength MS basal medium (Murashige and Skoog, 1962; Sigma-Aldrich) containing 8 g agarose per l and the following supplements: none (for light and dark treatment), 0.5 M sucrose (for sugar treatment) or 0.5 M mannitol (as control). After bombardment, 2 ml of water (light induction) or 2 ml of 0.5 M sucrose or 0.5 M mannitol (sugar induction) were added to the petri dishes and the leaf segments floating on these solutions were incubated for 24 hours at 25^{o} C in the dark (sugar induction and control of light induction) or in the light (90 µmole photons $m^{-2}s^{-1}$).

To assess promoter activity, after the 24 hours incubation, the leaf segments from all treatments were stained for GUS activity (Jefferson et al., 1987). To compare the relative activities of the 6-SFT and polyubiquitin1 promoters under the various conditions, the stained cells (foci) were counted in 10 randomly chosen fields of 4 mm^2 under the binocular with leaf segments from three leaves out of two independent microprojectile bombardment experiments.

Results

Cloning of the Barley 6-SFT Promoter

Genome Walking by PCR was used to clone 1.56 kb of genomic DNA of barley (*Hordeum vulgare* L. cv. Lyric) laying upstream of the 6-SFT coding sequence (Fig. 3.1). Co-linearity of the cloned sequence with the 6-SFT coding region is indicated by 100 bp of perfect sequence overlap with the 5' end of the 6-SFT cDNA sequence (Sprenger et al., 1995; accession # X83233). A 1.56 kb fragment from genomic DNA of a different barley cultivar (Express) was also amplified, cloned and sequenced and difference was found (data not shown). The 6-SFT promoter sequence has been submitted to EMBL (accession # AJ306962).

The PLACE database (Higo et al. 1999) was used to detect potential *cis*-acting regulatory elements. A putative TATA box (at position 1417) and a CAAT box (1021) were found as well as several boxes, including those involved in light, cold temperature, abscisic acid (ABA) and drought regulation (Fig. 3.1).

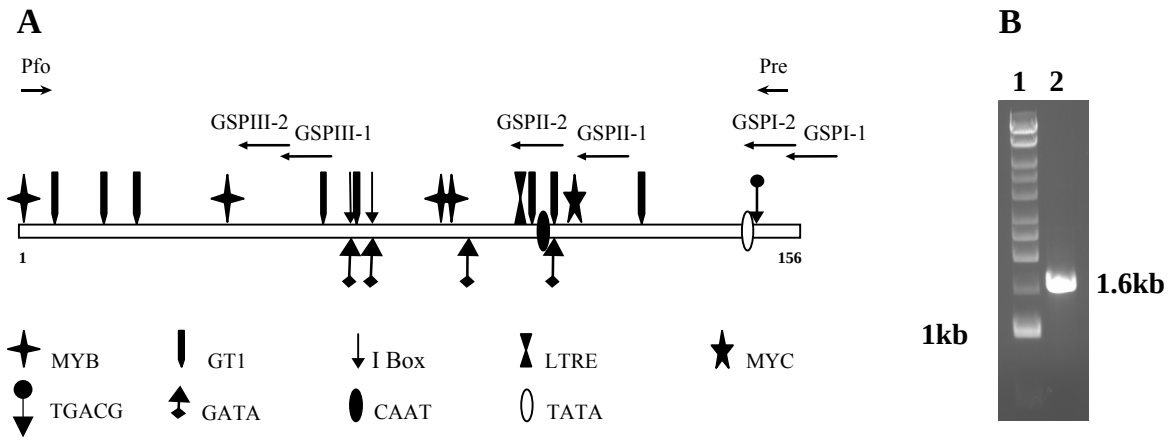


Figure 3.1. **A**. Architecture of 1.6 kb sequence upstream of the 6-SFT coding region. The symbols indicating the positions of the promoter elements found with the PLACE database (Higo et al. 1999) are explained below the scheme. Above the scheme, the positions of the primers used for cloning are indicated. **B** Agarose gel showing PCR amplification of the promoter fragment. Lane 1: 1 kb Molecular weight marker. Lane 2: PCR product.

Transcriptional Regulation of the 6-SFT

In order to test the transcriptional regulation of the 6-SFT, the well-established excised-leaf system was employed (Wagner et al., 1986c). Fructan synthesis was induced by light and with the sucrose treatment (Fig. 3.2, traces L and S). The presence of 1-K, 6-K, Bif and higher DP fructans indicates that the enzymatic activities of at least 1-SST and 6-SFT were induced by both treatments. In contrast, no

fructans were detected in extracts of the leaves after treatment in the dark without sugar or with mannitol (Fig. 3.2, traces D and M).

Total RNA was size fractionated, blotted and hybridized with an antisense probe representing the 6-SFT coding region (Fig. 3.3). In both RNA samples from light and sucrose treatments, the probe hybridized to a band of approx. 2 kb, in good agreement with the expected size of the 6-SFT transcript (1863 bases ORF plus 5' and 3' untranslated regions and polyadenylation). No signal was obtained by northern hybridization in the samples of the control treatments, indicative of a tight transcriptional control of the 6-SFT.

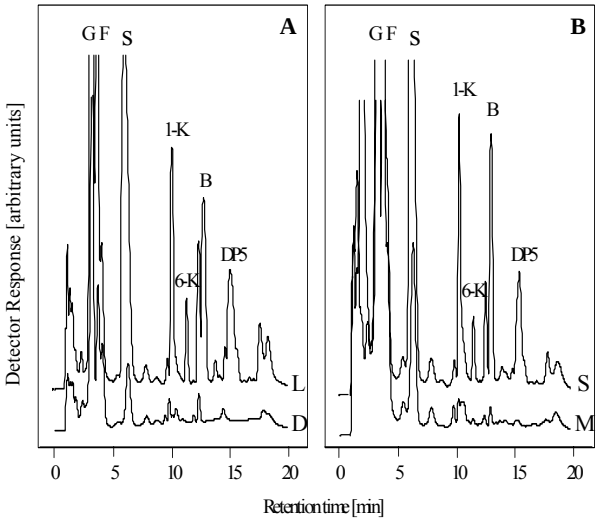


Figure 3.2. HPLC profiles of sugar extracts from excised barley leaves incubated for 24 h under the following conditions: **A**. Light of 350 $\mu Em^{-2}s^{-1}$ (trace L) and dark (trace D). **B**. Sucrose (trace S) and mannitol (trace M) at 500 mM each. Peaks are indicated as follows: Glucose (G), fructose (F), sucrose (S), 1-kestose (1-K), 6-kestose (6-K), bifurcose (B) and (DP5) fructan with degree of polymerization 5.

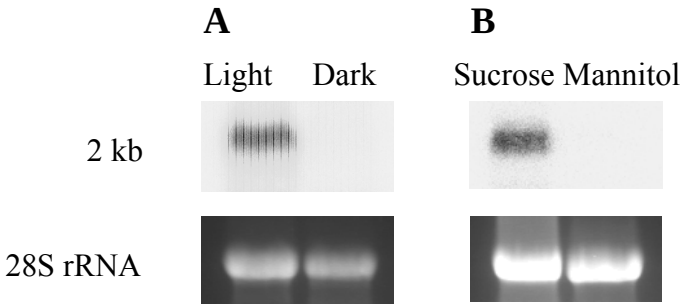


Figure 3.3. RNA blot analysis of 30 µg total RNA prepared from excised barley leaves incubated for 24 h under the following conditions: **A**. Light of 350 µmole photons $m^{-2}s^{-1}$ (L) and dark (D). **B**. Sucrose (S) and mannitol (M) at 500 mM each in the dark. Upper panel: blots hybridized with the 6-SFT antisense RNA probe. The approximate size of the hybridizing transcript in kb is indicated on the left. Lower panel: Ethidium bromide staining of the agarose gel.

Promoter Activity in Transient Expression Studies

In order to assess whether the cloned 1.5 kb fragment contained the necessary regulatory elements responsible for the light- and sucrose-mediated accumulation of 6-SFT mRNA, the fragment was fused to a *uidA* reporter gene encoding β-glucuronidase (GUS) and excised barley leaf segments were bombarded with this construct ($P_{6\text{-}SFT}$GUS). Thereafter, upon the light and sugar treatments, the leaves were stained for GUS activity and examined under a microscope. GUS activity was detected only in sucrose (Fig. 3.4C) and light (Fig. 3.4E) treated leaves but not in leaves incubated without sucrose and in the dark (Fig. 3.4A). Leaves bombarded with a control reporter gene construct containing GUS controlled by the maize polyubiquitin promoter (P_{Ubi}GUS) had blue spots (foci) in leaves from all treatments

(Fig. 3.4 B,D, F and J). The great majority of stained cells were mesophyll cells (Fig. 3.4H). However, some epidermal cells (Fig. 3.4G) along with guard cells (Fig. 3.4I) were also stained upon induction by light and sucrose, suggesting that they were also competent for 6-SFT promoter activation under the experimental conditions. In the segments of basal region of the detached leaves, there were noticeably more blue spots. With the P_{Ubi}GUS construct, stained cells (example in Fig. 3.4J) were scattered evenly over the whole shooting area.

Enumeration of blue foci (Fig. 3.5) indicated that leaves shot with the $P_{6\text{-}SFT}$GUS construct resulted in a mean of 2.0 ± 0.6 (±SEM: standard error of the mean; N=10) after induction with light but zero blue foci were found in leaves incubated in the dark. After induction with sucrose, leaves bombarded with the $P_{6\text{-}SFT}$GUS construct showed a mean of 3.2 ± 0.9 blue foci per field as compared to zero blue foci in the leaves incubated on mannitol. In leaves bombarded with the P_{Ubi}GUS construct, 1.9 ± 0.9 and 2.5 ± 0.9 foci were counted after incubation in light and in the dark, respectively and 1.7 ± 0.9 and 2.6 ± 1.1 blue foci after the treatments with sucrose or mannitol.

Statistical analysis using the Mann-Withney U test revealed that P_{Ubi}GUS expression did not differ significantly in response to the various treatments of the leaves. Therefore, the total absence of $P_{6\text{-}SFT}$GUS expression in leaves incubated in the dark or with mannitol is due to the regulated light or sucrose dependent activity of the 6-SFT promoter.

The promoter contains elements that have been suggested to provide ABA regulation in other systems. However, no evidence was found for ABA induction of the 6-SFT promoter: In bombardment experiments with ABA-treated leaves, the $P_{6\text{-}SFT}$GUS construct did not yield any blue spots representing promoter activity, while

the P_{Ubi}GUS construct had its normal activity (data not shown). Furthermore, ABA failed to induce accumulation of 6-SFT mRNA or fructan (data not shown).

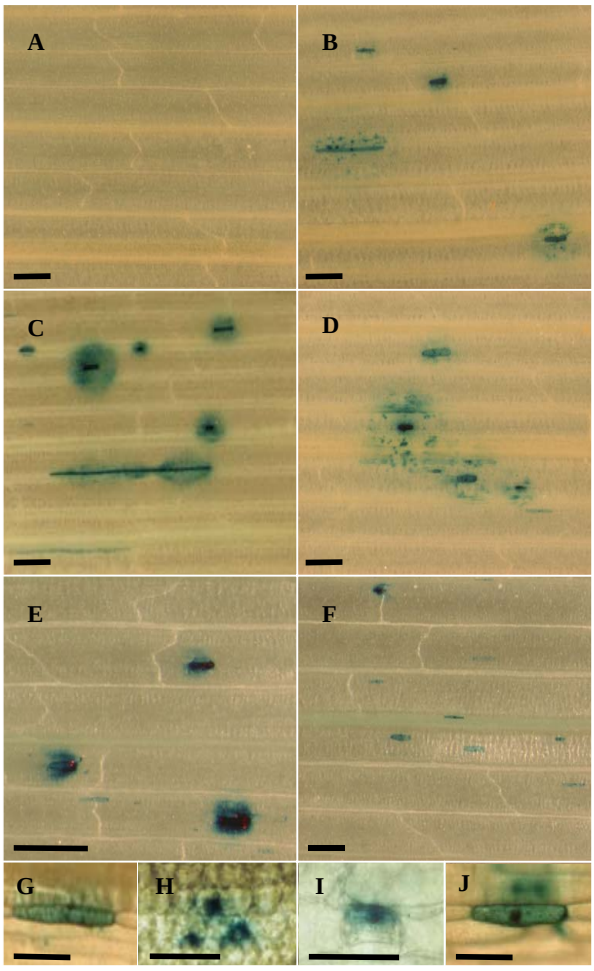


Figure 3.4. Histochemical assay of light and sugar induced expression of the GUS reporter gene after microprojectile bombardment of barley leaves with the $P_{6\text{-}SFT}$GUS construct (A,C,E,G,H and I.) or the P_{Ubi}GUS construct (B,D,F and J.). A and B: Leaves incubated in the dark and in the absence of sucrose. C and D: Leaves incubated with sucrose. E and F: Leaves exposed to light. G, H and I: An epidermal cell, a mesophyll cell and guard cells after bombardment with $P_{6\text{-}SFT}$GUS and

incubation in sucrose. J: Epidermal cell bombarded with P_{Ubi}GUS and incubated in sucrose. Size bars: in A to F: 500 µm and in G to J: 100 µm.

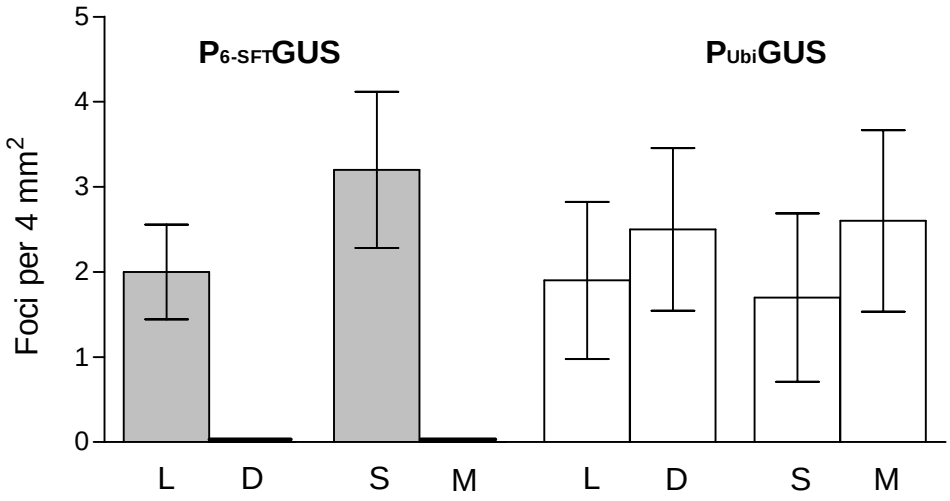


Figure 3.5. Light and sucrose dependent expression of the GUS reporter gene after microprojectile bombardment of excised barley leaves with the $P_{6\text{-}SFT}$GUS (shaded bars) or the P_{Ubi}GUS (white bars) constructs. After shooting, the leaves were incubated in the light (L) or dark (D) or with sucrose (S) or without sucrose but mannitol (M) as control. Blue foci on the leaves were counted under a binocular in 10 randomly chosen fields of 4 mm^2 from three leaf segments of two independent shooting experiments. Error bars indicate standard error of the mean (SEM).

Discussion

This study presents the cloning of 1561 bp of the barley 6-SFT promoter region by combined PCR based genome walking techniques. The identity of the promoter is apparent since there is a perfect match of all 100 nucleotides in the 3' region of the cloned sequence with the 5' end of the sequence of the 6-SFT cDNA clone (Sprenger et al., 1995). As the available evidence suggests the presence of only one copy of the 6-SFT gene in barley (Sprenger et al., unpublished), this promoter is clearly central in the control of 6-SFT expression. Microprojectile bombardment experiments demonstrate that the cloned sequence functions in expected ways as a regulated promoter in excised barley leaves: GUS activity in leaves can be observed after sucrose and light treatments, i.e. under the same conditions that caused accumulation of 6-SFT mRNA as well as of fructan, but not in control leaves in the absence of sucrose and light. Therefore the cloned 1.56 kb sequence contains the *cis* elements necessary for expression and regulation of the barley 6-SFT.

The cloned sequence contains the typical eukaryotic promoter elements such as CAAT and TATA boxes. The promoter region between 630 and 1040 contains several so-called I-Boxes (including I-Box cores), functionally vital in many light regulated-promoters (Terzaghi and Cashmore, 1995). In addition, several other putative *cis*-acting sequences like the GT-1 binding site (Zhou, 1999), GATA box and TGACG motif, shown to affect light mediated gene expression, are found. Apart from a putative LTRE, the promoter contains recognition sites for MYC and MYB proteins shown to participate in drought and or abscisic acid (ABA) regulated gene expression and in cell differentiation processes (Iwasaki et al., 1995; Terzaghi and Cashmore, 1995). The presence of these putative regulatory elements is in good agreement with

the observed regulation of fructan levels in plants and these sequences may be involved in mediating changes in fructan levels in response to biotic and abiotic factors and in response to developmental changes.

Light and sucrose are known to induce fructan synthesis (Wagner et al., 1986c; Obenland et al., 1991; Roth et al., 1997). The presence of light responsive elements in the 6-SFT promoter might suggest that the signal transduction pathways leading to light and sugar induction are separate. However, I-box elements have been shown to be recognized by several factors that depend on stimuli and regulations other than or in addition to light (Borello et al., 1993). Moreover, barley leaves placed in light but in conditions where photosynthesis was reduced because of low CO_2, did not accumulate fructan, nor did they induce 1-SST activity unless sucrose was added (Wagner et al., 1986c). These results indicated that a product of photosynthesis, possibly sucrose itself, mediates the induction of fructan synthesizing enzyme activities and initiation of fructan synthesis by light. However, the question whether light can independently regulate transcription of fructan synthesizing enzymes and at which level of gene expression sucrose or other sugars are required needs to be fully resolved. Several studies have shown that sugar signals, in turn, are closely related to ABA signal transduction pathways and that this relation involves hexose kinase mediated sugar-sensing (Smeekens, 2000). Although recent studies suggest that 6-SFT regulation is hexose kinase independent (Müller et al., 2000), it is notable that many of the putative *cis*-acting elements found in the 6-SFT promoter have been described to mediate ABA responses. However, ABA did not stimulate $P_{6\text{-SFT}}$ regulated GUS expression. Moreover, ABA did not induce accumulation of 6-SFT mRNA or fructan under the conditions tested. Hence, the role of these potential transcription factor binding sites remains unclear and awaits further investigation.

Developmental stage and cell differentiation specific factors control the cell's competence to respond to the induction factors light and sucrose with the synthesis of fructans. For example, fructan levels, fructan synthesizing enzyme activities (1-SST, 6-SFT), and 1-SST mRNA abundance change along developing leaves (Wagner and Wiemken, 1989; Roth et al., 1997; Lüscher et al., 2000a; Luscher et al., 2000b). Moreover, with increasing leaf age the light inducibility of fructan accumulation in excised leaves decreases (Wagner and Wiemken, 1989).

Surprisingly, although epidermal cells do not accumulate fructan (Koroleva et al., 2000), the 6-SFT promoter reporter gene experiments resulted in GUS expression under the inductive conditions with light and sucrose also in epidermal cells including guard cells. This indicates that other components of the cascade of events leading to fructan biosynthesis may be limiting in these cells. Alternatively, cell-specific repressors of 6-SFT transcription act *via cis*-elements outside the cloned promoter sequence used in the reporter gene construct. Finally, more stained cells were found in the lower zone of the leaf blade and in the sheath than towards the tip of the blade, reflecting gradients of inducibility in barley leaves observed earlier for fructan levels and 1-SST activity (Wagner and Wiemken, 1989).

Overall the results are in agreement with earlier physiological and enzymatic analyses of fructan regulation and provide further strong evidence for the transcriptional regulation of the 6-SFT. Hence, the 6-SFT promoter can be used as a molecular tool to isolate trans-acting factors involved in the signal transduction pathways causing fructan accumulation, and it may ultimately help in elucidating mechanisms of sugar-sensing.

CHAPTER 4

Regulation of the barley 6-SFT promoter in transgenic Arabidopsis

Vinay Janthakahalli Nagaraj, Giselle Martinez Noel, Thomas Boller and Andres Wiemken

Abstract

Fructan synthesis in excised barley leaves can be induced by continuous illumination or by sucrose (Suc) treatment in the dark. A key element of this process is the induction of sucrose:fructan 6-fructosyltransferase (6-SFT). We have isolated the gene encoding 6-SFT from barley and we have recently shown that the promoter contains the necessary *cis*-acting elements conferring Suc-mediated induction using transient expression of a β-glucuronidase (GUS) reporter construct. To study the regulation of this promoter in a model plant, *Arabidopsis thaliana* was stably transformed with a 6-SFT promoter-GUS reporter gene construct. Our results indicate that the barley 6-SFT promoter in Arabidopsis is regulated in a similar way as in barley and wheat-excised leaves: it is strongly induced by Suc in the dark and inhibitors of protein phosphatases and protein kinases affect induction. Our transgenic plants are a valuable tool to study the regulation of 6-SFT in a genetically tractable model.

Introduction

Suc is a major product of photosynthesis in the mesophyll and parenchymatous bundle sheath cells of barley (Koroleva *et al.*, 1998). Normally, upon synthesis, Suc does not accumulate but is translocated to the heterotrophic parts of the plant. If export from the phloem is blocked e.g by excision of leaves, Suc starts to accumulate and this leads to the synthesis of fructans, a process depending on the induction of 1-SST and 6-SFT (Wagner et al., 1986; Sprenger et al., 1995). It is apparent that these enzymes are induced by de-novo gene expression since inhibitors of transcription prevent Suc-induced accumulation of fructans (Cairns and Pollock, 1988).

In general, Suc is well known to act as a regulator of gene expression in plants (Smeekens, 2000). With respect to barely and fructan accumulation, the sensing of Suc and the subsequent information transmission events that lead to the activation of 6-SFT promoter is poorly understood. A hexokinase independent signal transduction pathway (Müller *et al.*, 2000) involving protein kinase and phosphatases (Noel *et al.*, 2001) is likely to be involved. Single cell sampling and analysis has revealed that the threshold Suc concentration for induction of 6-SFT gene expression and fructan synthesis in barley leaves is dependent on cell type and differs significantly between mesophyll and parenchymatous bundle sheath cells (Pollock *et al.*, 2003). The promoter region of barley 6-SFT, the key enzyme for fructan synthesis in barley (Duchateau *et al.*, 1995; Sprenger *et al.*, 1995), has been cloned and transient assays have shown that sucrose and light can induce the activity of promoter in barley leaves (chapter 3).

Arabidopsis thaliana has been used extensively as a model to investigate sugar sensing in plants, and several mutants with altered sugar sensing and signaling are

available (Smeekens, 2000). Arabidopsis has also proved to be useful to investigate the regulation of activities of sugar responsive promoters from other plants (Martin *et al.*, 1997). Here we shown that the barley 6-SFT promoter is regulated according to expectations in transgenic Arabidopsis plants in response to Suc. Furthermore we employ inhibitors to explore the sugar sensing and signal transduction known to interfere with the expression of 6-SFT in wheat, a close relative of barley (Noel *et al.*, 2001).

Materials And Methods

Arabidopsis growth conditions

Arabidopsis thaliana (ecotype Col-O) seeds were mixed with fine sand in a salt shaker and sprinkled evenly over the surface of a tray filled with soil mixture (garden soil: sand: vermiculite, 3:1:1). To maintain humidity, the tray was covered with aluminum foil and kept in the dark at 4°C for 3 days and then moved to a growth chamber. For the first 5 weeks, the plants were maintained under short day conditions (10h light and 14h dark) to favor vegetative growth. The plants were then transferred to long day conditions (16h light and 8h dark) to promote reproduction. The light intensity was 300μEm^{-2}s^{-1} and the temperature was 22°C through out the life cycle. When the siliques were mature and dry, they were harvested and crushed gently to release the seeds. The seeds were collected in Eppendorf tubes and stored under dry conditions at 4°C.

Plasmid construct for Arabidopsis transformation

The pCAMBIA3301 (CAMBIA, Canberra, Australia) *Agrobacterium tumefaciens* binary vector for plant transformation was a kind gift of Dr.Sjef Smeekens, Department of Molecular Plant Physiology, University of Utrecht, The Netherlands. The plasmid pP6SFT3301 (Fig. 4.1) was constructed by replacing the 35S promoter region of the binary vector pCAMBIA3301 with the barley 6-SFT promoter (AJ306962). The pCAMBIA3301 was first digested by *EcoR*I and *Nco*I to delete the 35S promoter region controlling the *uidA* (GUS) reporter gene. A 1.6kb *EcoR*I and *Nco*I fragment of the 6-SFT promoter was then ligated to the digested pCAMBIA3301 vector so that 6-SFT promoter controls the expression of the GUS

reporter gene. The pP6SFT3301 construct was sequenced to check for errors during cloning. The T-DNA region also has the *bar* gene which confers resistance to glufosinate (Basta) and enables selection of plants (Fig. 4.1). The plasmid has a kanamycin resistance gene for selection of bacterial transformants.

Arabidopsis transformation and selection of trangenic plants

Agrobacterium transformation of Arabidopsis was done in the lab of Dr.Sjef Smeekens. The pP6SFT3301 plasmid was introduced into *Agrobacterium tumefaciens* and Arabidopsis plants were transformed by dipping the developing floral tissues into a solution containing the *Agrobacterium* culture (Clough and Bent, 1998).

The seeds obtained were germinated as described above. Transgenic plants were selected on the basis of resistance to the herbicide Basta (Omya AG, Safenwil, Switzerland). Five day old seedlings were sprayed with 300µM Basta using a mist sprayer. Spraying was repeated every 2 to 3 days until healthy green plants were clearly distinguishable from moribund seedlings.

The healthy plants were then transplanted to new pots and further selection was done by PCR. DNA from Arabidopsis leaves extracted using the Alkai treatment method (Klimyuk *et al.*, 1993). To check for the presence of the 6-SFT promoter sequences (AJ306962), internal primers 6-SFTPro-1 (GGG AAG CAA AAC TCG GC) and 6-SFTPro-2 (GTG TTA GGC TCG GCT GTG ATT TAC CAT) amplifying a 603 bp fragment were used. To check for the presence of the GUS gene sequence, the GUSfor (TTT GCA AGT GGT GAA TCC GCA CCT) and GUSrev (AGT TTA GGC GTT GCT TCC GCC AGT) primers were used (expected amplicon size 632 bp). The annealing temperature was 55°C and amplification was done for 30 cycles.

Plants that were PCR positive for both 6-SFT and *uidA* sequences were used for promoter activity studies.

Barley 6-SFT promoter activity in Arabidopsis

To check for the activity of the barley 6-SFT promoter in transgenic Arabidopsis, rosette leaves of 4 week old Arabidopsis plants (having about 12 to 14 rosette leaves) were cut at the base. The leaves were floated on petri-plates containing 100mM Suc or 100mM Mannitol (Man) solution for 24h in the dark at 22°C and then stained for GUS activity (Jefferson et al., 1987). Treatment with the following inhibitor solutions: Okadaic acid (OA, 1μM), 1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra potassium salt (BAPTA, 5mM) and Staurosporine (ST, 2μM), were done as described (Noel *et al.*, 2001) but with some modifications. The leaves were put in falcon tubes with inhibitor solutions and subjected to vacuum (98 kPa) for 30s and then floated on the various inhibitor solutions for 2h. Afterwards the leaves were transferred to a 100mM Suc solution containing the respective inhibitor and incubated for another 24h in the dark. Treatments were also done without any inhibitors or Suc, but with only water (control) or 100mM Mannitol (osmotic control).

The leaves from all treatments were subsequently stained for GUS activity for 48h at 37°C (Jefferson *et al.*, 1987). For better visualization of blue spots chlorophyll was removed by destaining the leaves with 70% Ethanol at 60°C for 1h.

Results

Screening of transgenic Arabidopsis

The barley 6-SFT promoter is known to be activated by light and sucrose in barley leaves after transient transformation by particle bombardment as demonstrated with a promoter-GUS reporter construct (chapter 3). The activity of the promoter in response to Suc and the underlying signal transduction events were investigated further by stable transformation of the model plant *Arabidopsis thaliana*. The floral-dip method, which circumvents tissue culture or regeneration, was used to obtain transgenic Arabidopsis (Clough and Bent, 1998). The barley promoter region was fused to the GUS reporter gene (pP6SFT3301, Fig. 4.1) and introduced into Arabidopsis (T-0 plants) along with a gene encoding Basta resistance to enable selection of transgenic plants.

When progeny (T-1 generation) of the transformed Arabidopsis plants were sprayed with Basta, about 4 to 5% of the seedlings survived. Among the surviving plants, 50 healthy looking seedlings were transplanted into separate pots, labeled 1 to 50. Next, these plants were screened by PCR to check for the presence of the 6-SFT promoter and *uidA* sequences, and 22 were found to be PCR positive (data not shown). The leaves of only 9 PCR positive plants showed GUS activity in response to Suc feeding. Seeds were collected from four plants (1, 3, 7 and 10) showing relatively high GUS expression and sown in separate trays. Selection was done again at the T-2 generation of each of these four lines by spraying Basta and PCR. Positive plants (32 from each line) were transplanted into separate trays in rows (A to D) and columns (1 to 8).

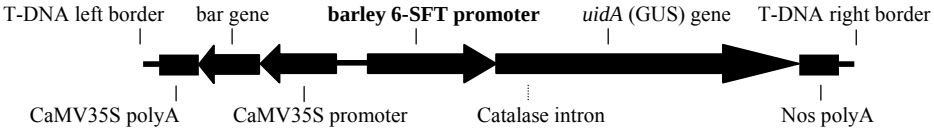


Figure 4.1. T-DNA region of the pP6SFT3301 plasmid containing the 6-SFT promoter-GUS-reporter gene construct used for Arabidopsis transformation. In comparison to the parent pCAMBIA3301 plasmid, the cauliflower mosaic virus (CaMV) 35S promoter driving GUS expression was replaced by the barley 6-SFT promoter. The T-DNA region also contains the bar gene expression cassette which imparts Basta herbicide resistance to transgenic plants.

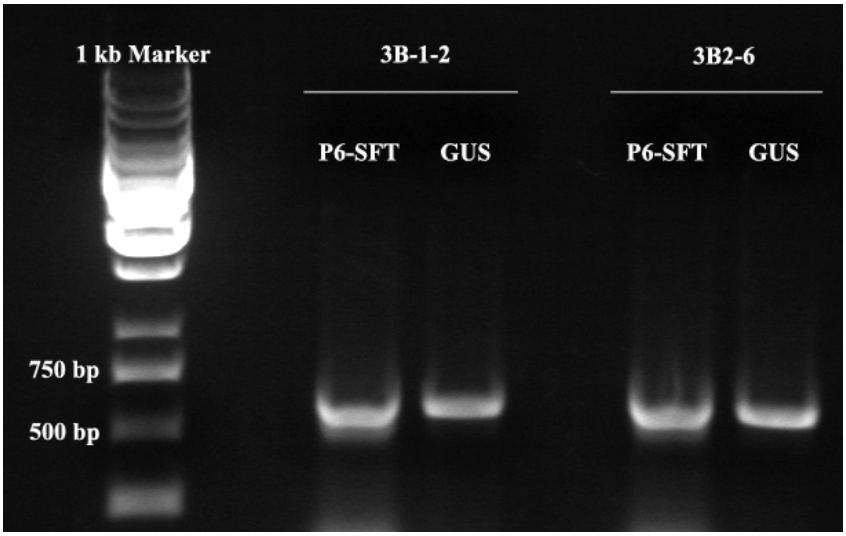


Figure 4.2. Ethidium Bromide stained agarose gel with the products of the PCR amplification of genomic DNA of transgenic Arabidopsis lines 3B1-2 and 3B2-6, using primers to amplify the barley 6-SFT promoter region and GUS gene.

Screeing of plants was done by GUS assays and all of them were found to have activtiy. From each line 5 plants were selected and seeds were collected. Line 3 showed consistently high GUS expression and was used for further experiments. All the progeny of 3B1 and 3B2 (i.e T-3 generation) were found to survive during Basta selection indicating that these lines were homozygous. Fig. 4.3 shows results from plants 3B1-2 and 3B2-6.

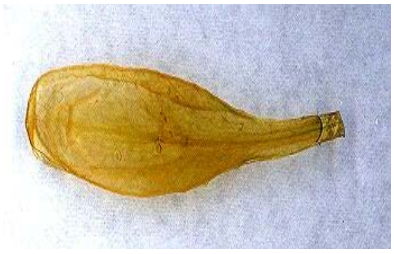
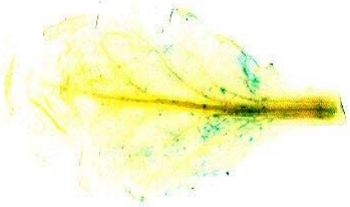
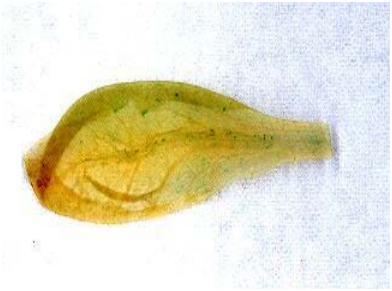
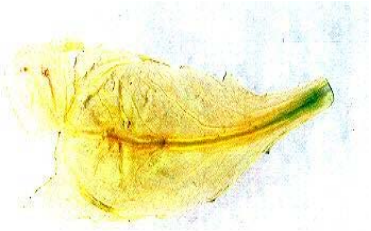
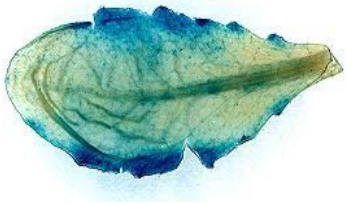
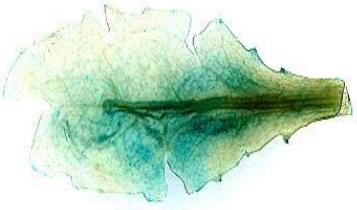
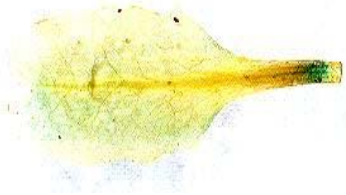
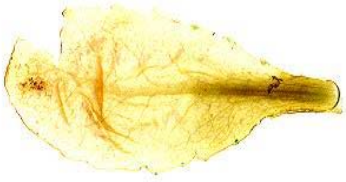
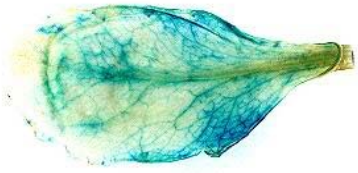
6-SFT promoter regulation in transgenic Arabidopsis

The promoter region of barley 6-SFT was able to drive the expression of the GUS reporter gene successfully in the leaves of transgenic Arabidopsis plants. The regulation of GUS expression in leaves was similar to that observed for the 6-SFT gene in barley leaves (Müller *et al.*, 2000). Rosette leaves of the two selected plants (3B1-2 and 3B2-6) floated on Suc (100mM) for 24h showed strong GUS activity (Fig. 4.3). There was almost no GUS activity in leaves floated on water or 100mM Man (Fig. 4.3). When the concentration of Suc was raised to 500mM, the leaves were clearly under osmotic stress and the activity of the promoter was reduced (data not shown). At 500mM Man, some GUS activity could be detected indicating that the promoter may be induced during hyper-osmotic stresses (data not shown).

In wheat, inhibitors of protein kinases and phosphatases interfered with Suc induction of 6-SFT gene expression and fructan synthesis (Noel *et al.*, 2001). Similar responses of the barley 6-SFT promoter in Arabidopsis suggest similarities in regulation. In rosette leaves of transgenic Arabidopsis, Okadaic acid (OA), an inhibitor of protein phosphatases effectively blocked the induction of the 6-SFT promoter by Suc (Fig. 4.3). Chelation of cytosolic calcium using BAPTA, a specific calcium chelator, had the same effect (Fig. 4.3). ST, which reduced the induction of

fructosyltranferases by about 50% in wheat with a significant suppression of 6-SFT gene expression (Noel et al., 2001) strongly reduced Suc-induced activation of the 6-SFT promoter in Arabidopsis leaves of line 3B2-6 and only marginally in the line 3B1-2 (Fig. 4.3).

Figure 4.3. Histochemical assay of the expression of the GUS reporter gene controlled by the barley 6-SFT promoter in transgenic Arabidopis leaves of plants 3B1-2 and 3B2-6 in response. The leaves were vacuum infiltrated with the inhibitor solutions or controls and floated on the respective solutions for 24h in the dark at 22°C, stained for GUS activity and then distained to remove chlorophyll. The experiment was repeated at least twice and pictures represent one of the trials done.

	3B1-2	3B2-6
Water		
100m Mannitol		
100mM Sucrose		
100mM Sucrose + 1µM Okadaic Acid		
100mM Sucrose + 5mM BAPTA		
100mM Sucrose + 2µM Staurosporine		

Discussion

Sugar sensing has received much attention recently (Loreti *et al.*, 2001). Genes involved in fructan synthesis appear to be regulated by Suc levels in barley leaves: an increase in Suc pools leads to the upregulation of 6-SFT gene expression and fructan accumulation (Müller *et al.*, 2000; Pollock *et al.*, 2003), and the promoter of 6-SFT is activated in response to Suc (chapter 3), as shown in transient assays using a promoter-reporter gene construct, (*uidA* encoding β-glucuronidase) and microprojectile bombardment. The 6-SFT promoter sequence appears to contain elements to conferring ABA and light induced transcription. But ABA failed to stimulate the promoter activity in transient assays in barley leaves (chapter 3). Continuous illumination of excised barley leaves in the presence of DCMU (a photosynthesis inhibitor) also failed to induce the expression of 6-SFT genes (Riedl, 2001). Prevention of Suc accumulation in excised illuminated actively photosynthesizing leaves by EDTA treatment also resulted in no 6-SFT mRNA accumulation (Riedl, 2001). But if Suc was supplied together with the inhibitors or ABA, 6-SFT gene expression was induced, suggesting that Suc is the main regulator of 6-SFT gene expression and the effects of light is mediated by Suc (Riedl, 2001). Sugars can override light as the major regulator of the light induced GLN2/ASN2 genes in light grown Arabidopsis (Thum *et al.*, 2003)

Sugar control of gene expression in plants may involve hexokinases as sugar sensors (Rolland *et al.*, 2002) or hexokinase independent sensing of disaccharides (Loreti *et al.*, 2001). The regulation of barley 6-SFT gene appears to be mediated by disaccharides rather than hexokinases (Müller *et al.*, 2000). It is not clear if Suc is sensed by receptors located in the apoplast or bound to membranes or present inside the cell. Thereafter the signal has to be transmitted to the nucleus to trigger gene

expression. Though studies in wheat using inhibitors point to the involvement of protein phosphatases and kinases (Noel *et al.*, 2001), our knowledge of the sugar sensing and signal transduction pathway for the regulation of the 6-SFT gene is limited.

In order to investigate the components of the above-mentioned signaling pathway transgenic Arabidopsis containing the 6-SFT promoter driving the expression of a GUS reporter gene were obtained. Under normal conditions, almost no GUS activity could be detected in leaves while Suc feeding resulted in strong promoter activity. Though Arabidopsis is a non-fructan producing plant, these results indicate that the sugar-regulated activation of barely 6-SFT promoter is maintained in transgenic Arabidopsis. The trans-acting Arabidopsis factors that interact with the *cis*-acting elements of the barley promoter sequence to regulate 6-SFT gene expression are probably conserved between the two plants.

Unlike the Suc sensing system that regulates the expression of the barley embryo α-amylase gene (Loreti *et al.*, 2000), a fructose moiety appears to be non-essential for disaccharide sensing controlling 6-SFT transcription. This is because trehalose, a non-reducing sugar consisting of two Glc residues, can induce 6-SFT gene expression (Müller *et al.*, 2000) and lead to fructan synthesis in excised leaves (Morcuende *et al.*, 2004). Maltose, another disaccharide of Glc but a reducing sugar, can also induce 1-SST activity and fructan accumulation in excised barley to levels comparable to Suc (Wagner and Wiemken, 1986). Hence the disaccharide-sensing pathway regulating 6-SFT transcription appears to be different from that operating for the α-amylase gene. The 6-SFT gene is also under the control of signalling pathways that act independently of Suc. Recent findings suggest that nitrate is a negative regulator of 6-SFT. The effect of nitrate is independent from the positive sugar

signalling and overrides it (Morcuende *et al.*, 2004). Analysis of Arabidopsis sugar sensing mutants has revealed intersections with hormone and nitrogen signaling and the various signaling networks are subject to a 'matrix effect' (Coruzzi and Zhou, 2001). It appears that a similar phenomenon also controls fructan synthesis.

In wheat leaves, treatment with OA, an inhibitor of protein phosphatases, prevents Suc induction of 6-SFT gene expression (Noel *et al.*, 2001). Similar results are shown here for the transgenic Arabidopsis. In sugar beet leaves, OA inhibits the activity of a plasma membrane Suc transporter by maintaining it in a phosphporylated form thus reducing Suc uptake (Roblin *et al.*, 1998). If similar mechanisms operate in Arabidopsis, then enough Suc may not have entered the cell to trigger the signaling events leading to 6-SFT promoter activation. One of the participants of the ensuing signaling cascade appears to be Ca^{2+}, since BAPTA a specific chelator of Ca^{2+} was able block GUS gene expression. Signal transduction seems to involve protein kinases as well since the inhibitor ST restrains the effects of Suc. Regulation of the promoter in Arabidopsis clearly has similarities to the regulation of 6-SFT in wheat and barley.

Transgenic Arabidopsis with promoters from sugar-regulated genes are valuable tools for the isolation of altered sucrose response mutants. The 'reduced sucrose response' mutants (Martin *et al.*, 1997) were identified by mutagenesis and screening of the progeny of the transgenic lines. The regulatory sequences of the sugar inducible ADP-glucose pyrophosphorylase subunit ApL3 were fused to a negative selection marker and 'impaired sucrose induction' mutants (Rook *et al.*, 2001) were isolated. The 6-SFT promoter controlling the expression of a reporter gene in Arabidopsis may also allow a novel approach to investigate the sugar sensing and signaling pathway. But first, further experiments are needed to confirm if there

exist any differences in the regulation of the 6-SFT promoter in barley and Arabidopsis, in response to various internal and external stimuli.

CHAPTER 5

Cloning and functional characterization of a cDNA encoding barley soluble acid invertase (HvSAI)

Vinay Janthakahalli Nagaraj, Virginie Galati, Marcel Lüscher, Thomas Boller and Andres Wiemken

Abstract

Invertases play a central role in the metabolism of sucrose, the main product of photosynthesis and substrate for the synthesis of the carbohydrate polymer fructans. The soluble acid invertase (SAI) isoforms are present in the vacuoles and are believed to be the ancestors of fructosyltransferases (FTs). Earlier we have purified SAIs, cloned and characterised the FTs, sucrose:sucrose 1-fructosyltransferase (1-SST) and sucrose:fructan 6-fructosyltransferase (6-SFT) from barley. Here we describe the cloning of a soluble acid invertase cDNA from barley (HvSAI), its functional characterization upon heterologous expression in *Pichia pastoris* and gene expression pattern in excised leaves and roots. The recombinant HvSAI cleaves Suc efficiently but despite very high amino acid sequence similarity to FTs, is devoid of FT or fructan hydrolase like side activities. Compared to the FTs, the activity of the recombinant HvSAI is relatively easily saturable (Km of 13.5 mM for Suc) and possesses a higher temperature optimum (10°C more that 1-SST). The mRNA levels of HvSAI are constitutive and not affected much by enhanced sugar levels in excised leaves and roots by sucrose supply or continuous illumination of cut leaves. The cloning of SAIs will help to investigate their role in the regulation of fructan metabolism and decipher the structure-function relationship between SAI and FTs.

Introduction

In most plants, Suc is the primary molecule for carbon and energy flow from source to sink tissues (Salerno and Curatti, 2003). Furthermore, Suc can influence the expression of a large number of genes and thereby regulate many metabolic and developmental processes (Koch, 1996). Hence the control of Suc metabolism is vital for the normal functioning for plant cells. Invertases catalyze the irreversible cleavage of Suc into Glc and Fru. Plants posses vacuolar, cell wall and cytosolic forms of invertases and each of these exist in several isoforms and with different biochemical properties and subcellular locations. Soluble acid invertases (SAI) have been localized in the vacuoles, have a pH optimum of about 5 and have an acidic pI. Cell wall invertases (CWI) are also most efficient in the acidic pH range but with a basic pI value and are bound to cell walls. Alkaline invertases are cytoplasmic and cleave Suc at neutral or alkaline pH but have an acidic pI (Sturm, 1999).

When carbon supply exceeds demand or when export is restricted, Suc begins to accumulate in cells. Upon reaching a threshold (Pollock et al., 2003), in fructan accumulating plants, Suc is utilised as a substrate for the synthesis of inulins by the action of 1-SST/1-FFT (Van Laere and Van den Ende, 2002) or graminans by 1-SST/6-SFT (Duchateau et al., 1995) or other kinds of fructans (Vijn and Smeekens, 1999). Fructans as well as the enzymes responsible for its metabolism, the FTs and FEHs have been localized to the vacuole (Wagner et al., 1983; Wagner and Wiemken, 1986) and may co-exist with SAI. Fructan metabolism enzymes and invertases share many similarities. Based on amino acid sequence comparisons, FTs appear to be evolutionarily related to SAIs while FEHs may have been derived from CWIs (Van Laere and Van den Ende, 2002). Biochemical studies have shown that FTs like the barley 1-SST (Luscher et al., 2000) and 6-SFT (Duchateau et al., 1995) exhibit Suc-

cleaving (invertase) activities *invitro*. Invertases can have 1-SST activity at high Suc concentrations (Van den Ende and Van Laere, 1993) and also exhibit FEH-like activity (Johnson et al., 2003).

Upon illumination of excised barley leaves, fructan biosynthesis is initiated by a rapid increase of 1-SST gene expression and enzyme activity leading to the synthesis of 1-K. Subsequent induction of 6-SFT is associated with the accumulation of Bif and higher degree of polymerization fructans. The dark phase is marked by a rapid loss of 1-SST activity and instant drop in 1-K levels, as compared to a gradual decrease of 6-SFT activity and Bif levels (chapter 2). The activity of unspecified vacuolar SAIs in excised barley leaves remains almost constant during fructan synthesis as well as breakdown (Wagner et al., 1986). In ryegrass, the expression of a vacuolar SAI (LpFT2) having considerable FEH-like activity (Johnson et al., 2003), has even been reported to be highest in plant organs with active fructan metabolism, with their gene expression profile matching that of ryegrass fructosyltransferases (Lidgett et al., 2002). During fructan biosynthesis in the vacuole, it is not known how Suc is preferentially utilized by the inducible FTs that coexist with the apparently constitutive or induced SAI isoforms. Within the leaf tissues, there exists considerable heterogeneity with respect to spatial distribution of carbohydrate metabolism and sucrose cleaving activities (Pollock et al., 2003). The activity of SAI in mesophyll as well as bundle sheath cells of barley is very low (Koroleva et al., 1997). Acid invertase proteins in barley leaves appear to be located predominantly in the vascular regions (KingstonSmith and Pollock, 1996) and high activity of a SAI isoform has been localized in the abaxial epidermis especially in the basal half of barley leaves (Obenland et al., 1993). Nevertheless, mesophyll protoplasts of fructan-enriched

barley leaves still contain high SAI in addition to the expected FT activity (Wagner et al., 1983).

We are interested in the properties of fructan metabolism enzymes and SAIs and their role in the regulation of fructan and Suc metabolism in grasses. We have purified, partially sequenced and characterised SAI isoforms from barley (Obenland et al., 1993) and separated their activities from FTs (Simmen et al., 1993), 1-SST (Luscher et al., 2000)and 6-SFT (Duchateau et al., 1995). The barley genes for 1-SST (AJ567377), 6-SFT (Sprenger et al., 1995) and 1-FEH (AJ605333) have been cloned. The regulation of gene expression and enzyme activities if 1-SST and 6-SFT has been studied during fructan synthesis and breakdown using the excised leaf system. Here we report the cloning of a SAI gene from barley (HvSAI), confirm its specific invertase activity by heterologous expression in *Pichia pastoris* and describe the biochemical properties of the recombinant enzyme. We show that, in contrast to FTs, the expression of the SAI gene is more or less constitutive in plant tissues. Biochemical properties of the recombinant enzyme have been investigated. While sequence comparison shows that SAIs and FTs are highly similar, there are specific differences that may provide clues to elucidate the crucial domains responsible for the different activities and substrate specificities of these closely related enzymes.

Methods

Plant material and growth conditions

Barley (*Hordeum vulgare* L.) seeds were planted in a commercial soil mixture (UFA Haus und Garten) and grown for 7 days in a growth chamber. The conditions were: 16 h light (500 μE m^{-2} s^{-1}) at 20 °C, 16 h dark at 18°C and a constant relative humidity of 70%.

Cloning of a barley cDNA encoding a SAI

The primary leaves were cut at the ligule after 7 days of growth and were frozen at –70°C. Total RNA was isolated from primary leaves of 7 day old barley seedlings using the "Hot phenol" extraction method (Reference) and cDNA was prepared using the Reverse Transcription System (Promega). The cDNA served as a template for PCR with degenerate forward (9177) and reverse (20115) primers for FTs and SAIs. A product of 840 bp was cloned and sequenced (Fig. 5.1: step 1) and found to be a SAI/FT-like sequence but different from 1-SST and 6-SFT. Using this sequence information, the forward primers 3'GSP1 and 3'GSP2 were designed to obtain the 3' end of the cDNA, by 3'RACE, in conjuction with an oligo dTAnchor and Anchor reverse primers (Fig. 5.1: step 2). Based on the sequence of the resulting 900 bp, the reverse primer INVRev was designed and used along with a new forward primer, INVfor1, to amplify a 1.7kb fragment (Fig. 5.1: step 3). Attempts to obtain additional sequence information by 5'-RACE failed. An additional 370 bp upstream sequence was obtained by PCR using a barley cDNA library (Sprenger et al., 1995) directly as template with 5'GSP1 reverse primer and T3 forward primer located on the lambda arm (Fig. 5.1: step 4). With the newly designed InvFor2 primer and the reverse primer from step3, a 2kb clone was amplified by PCR (Fig. 5.1: step 5).

Further 5' sequence up to the start codon was obtained using InvGSP2 and T3 vector primer (Fig. 5.1: step 6) and full-length cDNA clone of 2038 bp (EMBL Acc. No AJ623275) was obtained using InvFor3 and InvRev primers (Fig. 5.1: step 7). The sequences of all the primers are in Table 5.1.

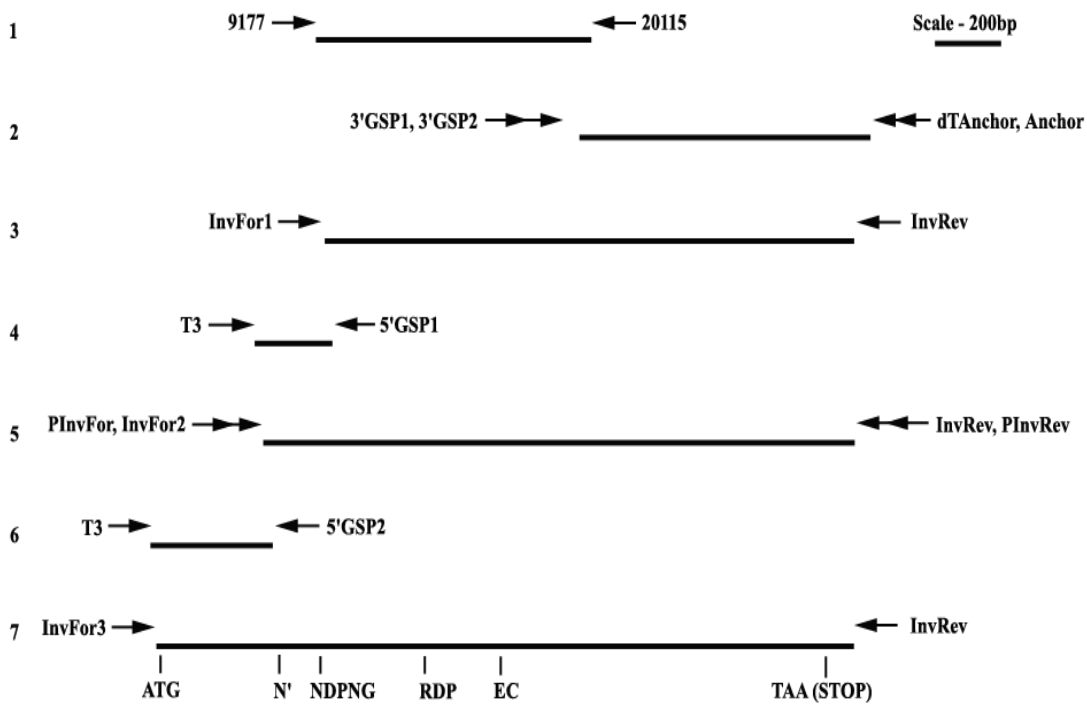


Figure 5.1. Cloning of the HvSAI cDNA. Step 1. A 840bp sequence was amplified from cDNA prepared from one week old barley primary leaves by PCR with degenerate invertase/FT forward (9117) and reverse (20115) primers. Step 2. Cloning of the 3'end of the cDNA by RACE using 3'GSP1 and 3'GSP2 forward primers and dTAnchor and Anchor reverse primers. Step 3. Amplification of a 1.7kb sequence directly using sequence information from steps 1 and 3. Step 4. Cloning of 370bp 5' region by PCR on barley cDNA library (see Material and Methods). Step 5. Amplification of the HvSAI sequence encompassing the mature peptide and introduction of restriction sites for cloning into a vector for heterologous expression in *Pichia pastoris*. Step 6. Cloning of 5' sequence upto the putative start codon of the cDNA by PCR on barley cDNA library (see Material and Methods). Step 7. Amplification of the full-length clone of 2039 bp (EMBL Acc. No. AJ623275) using InvFor2 and InvRev primers and barley cDNA library as template.

Table 5.1. PCR primers. Restriction sites are indicated in italics (*Cla*I on Pfor and *Xba*I on Prev). Degenerate primers (9177, 20115 and Anchor) have N (=A,C,G,T), Y(=C,T), R(=A,G), W=(A, T) V (= A, C, G) and S(=C,G).primer sequence

Primer	Sequence
Primers for cDNA cloning	
9177	TAY AAG ACS GCS TTY CAY TTY CAR CC
20115	ATR CTR ACC CCN TTY TWR CG
3'GSP1	GTG TTG CAC GTG ATG AAG GAG
3'GSP2	TCG ACG CCG ATC TCG GCA T
InvFor1	TTC CAC TTC CAG CCC GAG AAG AAC TGG
5'GSP1	CAC CCC TTG TAG TAA ACC GGA CCG TTG
InvFor2	GCC AGC CGA CGA CAG TAG ACG TGG
5'GSP2	GTC GGC ACC ACG TCT ACT GTC GTC
InvFor3	GTC GAG ATC GAG GGC GTC CTT CTT
InvRev	AAT GTA AGG CTT GGT TGG TTA GGA AGA ATC CAT TT
DTAnchor	TCT AGA GCG GCC GCG AAT TCT_{25}V
Anchor	TCT AGA GCG GCC GCG AAT TCT
T3	CGC CAA GCT CGA AAT TAA CCC TCA C
Primers for Pichia Expression	
PinvFor	ACT CGT ACC GC*A TCG ATC* CCG ACG ACG AGC ACC AGC
PinvRev	CGC TGT AAG CGA T*TC TAG A*TC GGA AGA ATC CAT TTC
Primers for RT-PCR	
InvRT-For	CCT CGA CGG TGG CTT GCA TAC A
InvRT-Rev	GTG ACT GAA TTT CCC GTG GCA TGG

Expression of HvSAI cDNA in *Pichia pastoris*

A construct was designed to express the mature form of SAI from barley encoded by the new cDNA clone, in *Pichia pastoris*. Using primers PInvFor and PinvRev (Fig. 5.1: step 5) a cDNA fragment was amplified by PCR so as to introduce a ClaI site at the 5'-end and a XbaI site at the 3'-end. The resulting product was digested by the appropriate restriction enzymes and ligated in frame behind the α-factor signal sequence of the expression vector pPICZαC (Easy Select Pichia expression kit from Invitrogen), to allow the entry into the secretory pathway. The insertion of the DNA in the shuttle vector led to the plasmid pPICZαCHvSAI. Expression in *P. pastoris* was performed as described (Hochstrasser et al., 1998), with minor modifications. The *P. pastoris* strain X-33 was transformed with 4 μg of PmeI-linearized pPICZαCHvSAI and plated on selective YPDS/Zeocin plates. Single colonies of transformants were inoculated on fresh YPDS/Zeocin plates. To screen for activity, some of the newly grown colonies were inoculated in liquid culture. The best growing colony was used for induction with 1% (v/v) methanol. To maintain the methanol concentration over the induction period, the growth medium was supplemented by 1% (v/v) methanol at 15, 24, 36 and 42 hours of induction. After 48h of induction, the supernatant of the culture medium was concentrated from 50 mL to 1 mL by dialysis against solid PEG 35000. The concentrate was desalted into 50 mM MES (NaOH) buffer (pH 5.75). The concentrated, desalted media of the transformant was tested for activity or stored in glycerol at -20°C. Recombinant 1-SST (chapter 2) was used for activity comparisons.

Treatment of plant tissues

Primary leaves of one-week-old barley were harvested just before the beginning of the light period by cutting 2 cm above the ligule. The next 4 cm of the leaf blade, corresponding to the center part, were used for the experiment. For Suc treatment the leaf bits were floated on 300 mM Suc solution in a tray with the adaxial surface facing upwards. Samples were collected at 0, 8h and 24h. After 24h the leaves were rinsed well in water and transferred to water and incubated for another 4h. For light induction, the leaves floating on water were illuminated (500 μE m^{-2} s^{-1}) and samples were collected at 0, 8 and 24h. After 24h of light, the leaves were placed in the dark for another 4 h. Light and Suc treatments were done at 70% humidity and 20°C.

Roots of one-week-old plants grown on moist filer paper were separated into two parts. The first 3cm from the base of the stem (older root) were cut and separated from the lower part (young roots). The root parts were incubated in falcon tubes with a vent and containing 300mM Suc solution. Samples were collected at 0h, 8h and 24h. After 24h the roots were rinsed well with water and transferred to falcon tubes with water and incubated for another 4h. The Suc treatment for roots was done at 20 °C in an incubator-shaker at 150 rpm.

Extraction and analysis of soluble sugars

Soluble carbohydrates were extracted according to procedures reported earlier (chapter 2). Briefly, one ml of 80% ethanol was added to 100 mg of leaf tissue and heated for 5 min at 80 °C. The leaf tissue was ground using an Eppendorf grinder and centrifuged at 15 K ***g*** for 5 min. The pellet was re-extracted twice with water, heated and centrifuged as above. The three supernatants were combined, freeze-dried,

redissolved in 200 µl water, filtered and analysed by high-performance liquid chromatography (HPLC) using an anion exchange column and pulsed amperometric detection as described (Luscher et al., 2000). Using Suc, Fru, 1-K and Bif as standards (Duchateau et al., 1995), the corresponding components of the sugar extract were identified and quantified.

Analysis of gene expression

RNA was isolated from the leaf tissue using the RNeasy Plant Mini kit (Qiagen). The RNA extract was treated with deoxyribonuclease I (MBI Fermentas, Vilnius, Lithuania) to remove contaminating DNA, subjected to phenol/chloroform extraction and quantified spectrophotometrically. One µg of RNA was used for synthesis of cDNA using the Reverse Transcription System (Promega). The cDNA was used as template directly for Real time PCR reactions (see below). Primers (Table 5.1) InvRT-for and InvRT-rev were designed to specifically amplify the HvSAI gene and used along with the primers to detect barley 1-SST (AJ567377), 6-SFT (X83233) and Ubiquitin (M60175 and M60176) genes (chapter 2).

Real time PCR was performed with a Gene Amp 5700 Sequence Detection System (Applied Biosystems, CA, U.S.A). The thermal profile was, 1 cycle 2 min at 50° C, 1 cycle 10 min at 95° C, 40 cycles 15 s at 95° C, 58° C 15 s and 1 min at 60° C. A 25 µL reaction volume consisted of 12.5 µL SYBR Green PCR master mix (Applied Biosystems), 8.5 µL water, 1.5 µL of 5 µM gene specific forward primers, 1.5 µL of 5 µM gene specific backward primers and 1 µL of cDNA preparation diluted 1:3. Copy numbers were calculated from amplification plots of known standards for all the genes. Ubiquitin transcript levels in the different samples were used to normalize the amounts of HvSAI, 1-SST and 6-SFT.

Results

Cloning of a soluble acid invertase cDNA from barley

A vacuolar soluble acid invertase cDNA (HvSAI) from barley was cloned employing a PCR based strategy. Using cDNA prepared from the primary leaves of one week old seedlings as template for PCR using degenerate SAI/FT forward and reverse primers, an 840bp fragment was cloned whose sequence was unique from 1-SST and 6-SFT but similar to vacuolar invertases. The 3' end of the cDNA was cloned by 3'RACE and the 5' end was obtained by two separate rounds of PCR using a cDNA library (Sprenger et al., 1995) as template (Fig. 5.1).

The barley full length HvSAI cDNA (EMBL Accession No AJ623275) is 2038bp long and contains an ORF of 657 amino acids, starting at position 47 and ending at 2017, followed by a 21bp 3'UTR. Sequence comparisons show that the N terminal region of the HvSAI ORF, like other SAIs, has a longer signal peptide when compared to CWI and Fructan hydrolases. The 5' region of the deduced amino acid sequence of HvSAI contains the conserved residues R[G/A/P]XXXGVS[E/D/M]K[S/T/A/R] typical of SAIs and FTs targeted to the vacuole (Van den Ende et al., 2002). The mature polypeptide is expected to start at amino acid residue 107 and its molecular weight, without consideration of its probable glycosylation, is predicted to be 61.1 kDa. Its pI is calculated to be acidic (5.15), which is typical for SAIs that have been localized in the plant vacuole. All previous studies on SAIs in barley (Karuppiah and Kaufman, 1989; Obenland et al., 1993) have identified the soluble isoforms to be glycoproteins. This appears to be true for the present invertase as well: four glycosylation sites were predicted in the ORF. The FTs from barley are proteolytically cleaved into two subunits of approximately 50 and 22kDa (Sprenger et al., 1995; Lüscher et al., 2000). Though the cleavage site

is fully conserved in the deduced HvSAI sequence (Fig. 5.2), the native barley SAI proteins purified were not found to be split into subunits (Karuppiah and Kaufman, 1989; Obenland et al., 1993).

An alignment of closely related 1-SSTs, 6-SFTs and SAIs from grasses shows (Fig. 5.2) that certain amino acids were completely conserved during the evolution of FTs from SAI. Amino acids unique to either Inv or 1-SST or 6-SFT could be determinants of their specific activities and are potential targets for introducing mutations to understand the structure function of these enzymes. Interestingly, residues conserved only among the FTs or between Inv & SST or Inv & SFT were also found.

Heterologous expression of HvSAI cDNA in *Pichia pastoris*

A fragment of HvSAI cDNA corresponding the sequence encoding the mature form of SAI with an additional an additional 26 amino acids upstream of the predicted N terminal was cloned into *Pichia pastoris*, using a plasmid containing the necessary expression signals as well as the coding information for the N-terminal signal sequence of the α-factor. Constructs which had only the complete mature peptide sequence (predicted to start from position 107, Fig 5.2) or shorter did not show any activity in Pichia (data not shown).

Upon incubation of the heterologously expressed enzyme with 100 mM Suc, a marked invertase activity was observed, as seen by the massive production of Glc and Fru. The synthesis of 1-K was very low, indicating that the enzyme had almost no 1-SST activity (Table 5.2). Fru was released from raffinose and stachyose while melibiose was not cleaved. When the enzyme preparation was incubated with 100 mM 1-K alone, almost no FEH activity was observed. When incubated with a

combination of 100 mM Suc and 50 mM 1-K, the invertase activity seen in the presence of Suc alone was reduced indicating that 1-K could act as an inhibitor under these conditions. None of the enzyme assays yielded any nystose, Bif or 6-kestose demonstrating that the enzyme is devoid of 1-SFT/1-FFT/6-SFT activities. As expected, invertase activity was strongly inhibited by 10mM pyridoxal phosphate and completely inhibited by 5μM $HgCl_2$. The Km of the recombinant enzyme for Suc was estimated to be 14 mM (Fig. 5.3). The temperature optimum of purified SAI from barley was found to be much higher than that of 1-SST (Simmen et al., 1993). Similar results were found with the heterologously expressed HvSAI. Its temperature optimum was at 40°C while that of the recombinant 1-SST (chapter 2) was 30°C (Fig. 5.4).

Table 5.2: Activities of the enzyme produced by heterologous expression of the HvSAI clone in *Pichia pastoris*. The Fru released from different substrates was used as a measure of invertase activity. (* and ° , 1-kestose and Bif formation was measured). Note: incubations were done overnight (14h) at 27°C.

Enzyme Activity	Substrates	% Activity
Cleavage of Suc	100mM Suc	100
Cleavage of Raffinose	100mM Raf	34.4
Cleavage of Stachyose	100mM Sta	13.5
Cleavage of Melibiose	100mM Mel	0
Cleavage of 1-K	100mM 1-K	0
Synthesis of 1-K*	100mM Suc	1.4
Synthesis of Bif °	100mM Suc + 50mM 1-K	0
Cleavage of Suc in the presence of potential inhibitors;	100mM Suc and;	
1-K	50mM 1-K	64
Pyridoxal phosphate	10mM Pyridoxal phosphate	11
H_gCl_2	5µM H_gCl_2	0

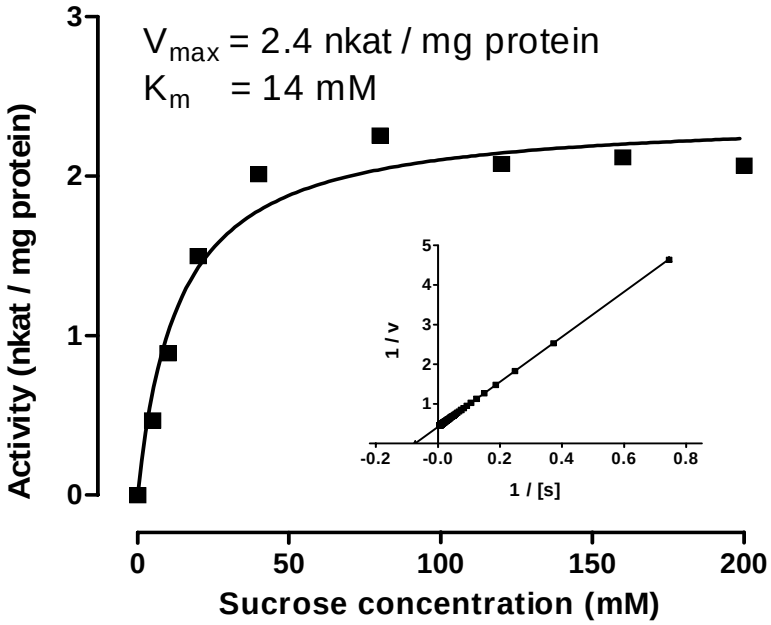


Figure 5.3. A substrate-velocity curve for the recombinant HvSAI and Lineweaver-Burk plot (embedded linear curve). The recombinant protein was incubated with 0-200mM Suc for 2 hours at 27° C and Fru released was used as a measure for invertase activity of the enzyme (nkat per mg protein).

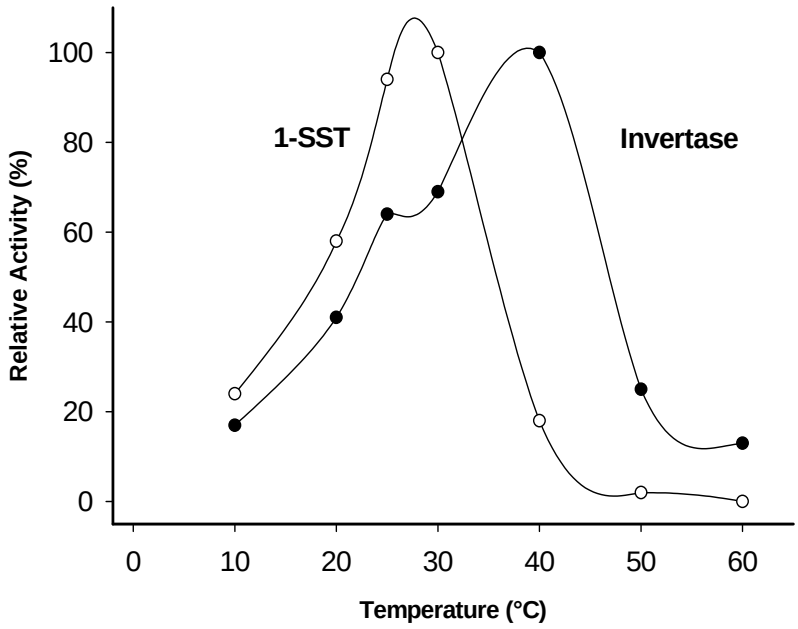


Figure 5.4. Comparison of the temperature dependence of HvSAI with 1-SST activities. The recombinant enzymes were incubated with 100mM Suc for 2h at the various temperatures indicated and Fru formation was used as a measure the invertase activity. The highest activity was considered as 100% and the activities at all other temperatures are expressed relatively.

Sugar levels and the expression of the HvSAI gene in barley seedlings

The expression of SAI and FT genes respond to sugar levels in different tissues of plants (Koch, 1996; Pollock et al., 2003). The expression of the cloned HvSAI gene was compared to 1-SST and 6-SFT, both known to be upregulated by Suc (Muller et al., 2000), in a time course experiment in excised leaves and roots under elevated sugar levels. HPLC was used to quantify the amount of Suc, Fru, 1-K and Bif present in the plant tissues while gene expression was analysed by the Real-Time PCR technique.

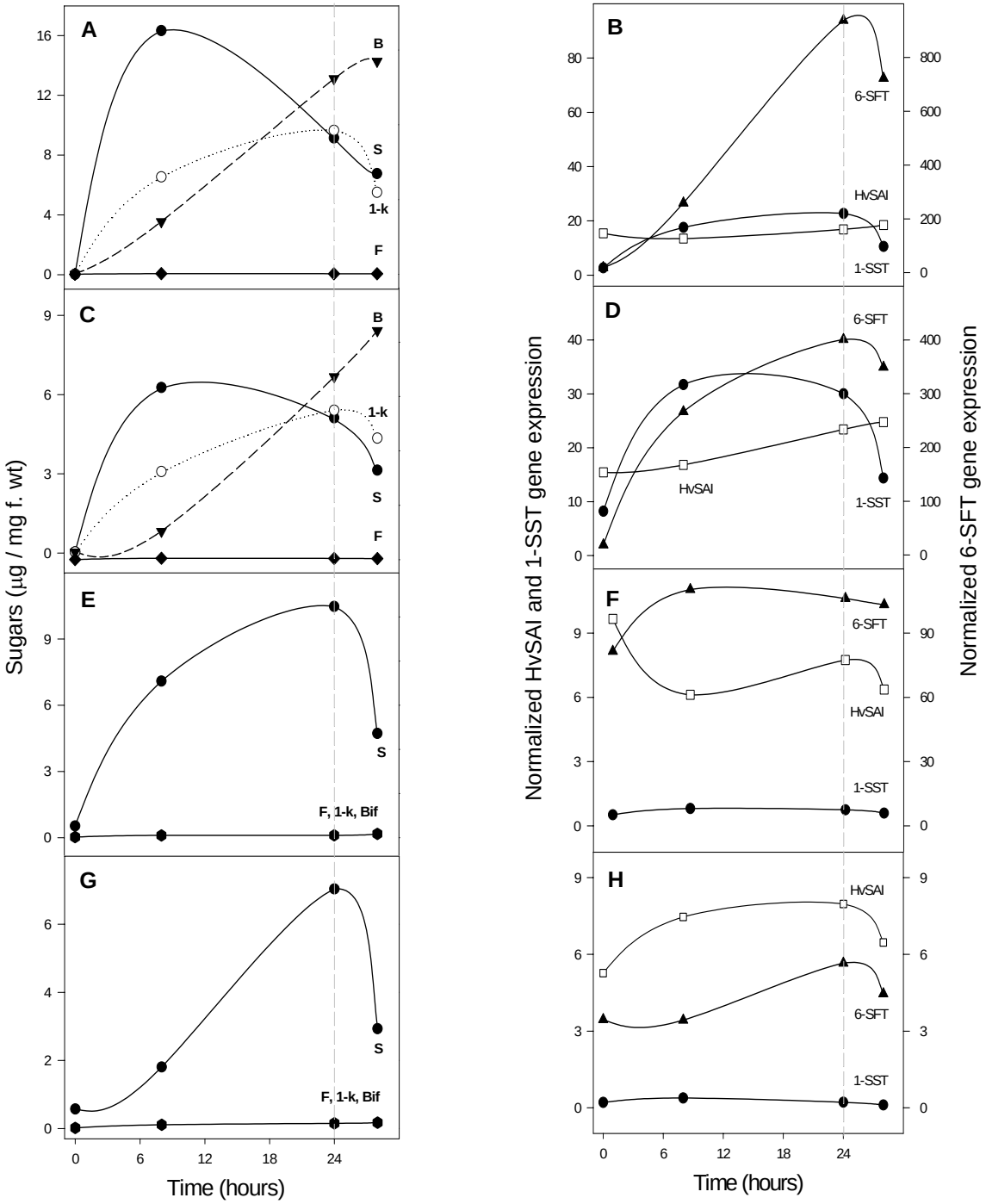
Fructan metabolism in excised leaves continuously exposed to light for 24 h and then transferred to darkness for 4 h (Fig. 5.5A) matched well with previous studies (chapter 2). Very little Suc, Fru, 1-K and Bif were present at the start of the experiment. Suc accumulated rapidly upon illumination, and so did 1-K; Bif increased with a certain delay but was the most abundant sugar after 24 h (Fig. 5.5A). In parallel, expression of 1-SST and 6-SFT transcripts were strongly induced; 1-SST transcripts were fully induced already after 4 h and dropped to background levels within 4 h of darkness, while 6-SFT transcripts increased more slowly and remained at a high level also after 4 h of darkness (Fig. 5.5B). Expression of HvSAI, in contrast, remained constant throughout the course of the experiment (Fig. 5.5B).

When excised leaves were kept in the dark but fed with sucrose, fructan metabolism displayed the same pattern as in the continuously illuminated leaves (Fig. 5.5C). Expression of 1-SST and 6-SFT transcripts increased rapidly, as reported previously (chapter 2). However, expression of HvSAI transcripts remained constant (Fig. 5.5D).

We also examined the response of excised roots to exogenous Suc feeding. Under our incubation conditions, Suc accumulated in the roots, both in its upper parts

(Fig. 5.5E) and in its lower parts (Fig. 5G). However, there was no accumulation of 1-K or Bif in the roots (Fig. 5.5E,G). Expression of 6-SFT was high in the roots but was not affected by sucrose finding, and expression of 1-SST in the roots remained at a very low level throughout the experiment (Fig. 5.5F,H). HvSAI was expressed constitutively in the roots, and it was barely affected by sucrose feeding (Fig. 5.5F,H).

Figure 5.5. A, C, E & G, soluble sugars (sucrose, S, fructose, F, 1-kestose, 1-K, bifurcose, Bif) and **B, D, F and H**, gene expression of HvSAI, 1-SST and 6-SFT (normalized to ubiquitin levels). **A & B**, excised barley leaves illuminated continuously for 24h and subsequently placed in the dark for 4 h. **C & D**, excised leaves floated on 300mM Suc solution in the dark for 24h and thereafter for 4h on water. **E & F**, upper part of the roots incubated in 300mM Suc for 24h and subsequently for 4h in water. **G & H**, lower part of the roots incubated in 300mM Suc for 24h and subsequently for 4h in water. The vertical dotted line in gray indicates the 24h time point.


A
B
S
1-k
F
C
B
1-k
S
F
E
S
F, 1-k, Bif
G
S
F, 1-k, Bif
Sugars (µg / mg f. wt)
Time (hours)
B
6-SFT
HvSAI
1-SST
D
6-SFT
HvSAI
1-SST
F
6-SFT
HvSAI
1-SST
H
HvSAI
6-SFT
1-SST
Normalized HvSAI and 1-SST gene expression
Normalized 6-SFT gene expression
Time (hours)

Discussion

Vacuolar SAIs have a prominent influence on diverse plant functions through the control of sugar composition and metabolic fluxes. Previously, we have purified three SAIs from barley leaves and obtained partial N-terminal peptide sequence for two isoforms, Hv-I and Hv-IIB (Obenland et al., 1993). A cDNA representing the Hv-I peptide sequence (AJ605333) was cloned, but found to have 94% identity to 1-FEH from wheat (Van den Ende et al., 2003) and high similarity to apoplastic invertases. In order to clone the vacuolar isoform of barley invertase, a PCR based strategy using degenerate primers was used (Fig.5.1), and we cloned a 2038bp cDNA (HvSAI, Acc. No. AJ623275) highly similar to putative SAI genes from grasses.

Since the sequence of SAIs and FTs are highly identical it is difficult to assign functions to newly cloned genes purely based on sequence similarity. The mature peptide sequence of HvSAI shows high identity to 1-SST (66%) and 6-SFT (64%) from barley, but upon heterologous expression in *Pichia pastoris* the recombinant protein shows properties that are clearly characteristic of vacuolar SAIs. The high Suc hydrolyzing activity and the ability to release Fru from raffinose indicates that the enzyme is a β fructofuranosidase (EC 3.2.1.26). But FEH-like activities found in β fructofuranosidases from bifidobacteria (Warchol et al., 2002) and fructan producing plants like ryegrass (Johnson et al., 2003) was absent in the recombinant HvSAI. It has been proposed that invertases, in general, can also catalyse the synthesis of fructans from Suc (Cairns, 1993). However almost no FT-like side activities were observed, namely synthesis of 1-K, Bif or other fructans even after prolonged incubations with Suc, suggesting that HvSAI is a pure invertase. Inhibition by Hg implies that a sulphydryl group may be required for HvSAI activity. Pyridoxal and its

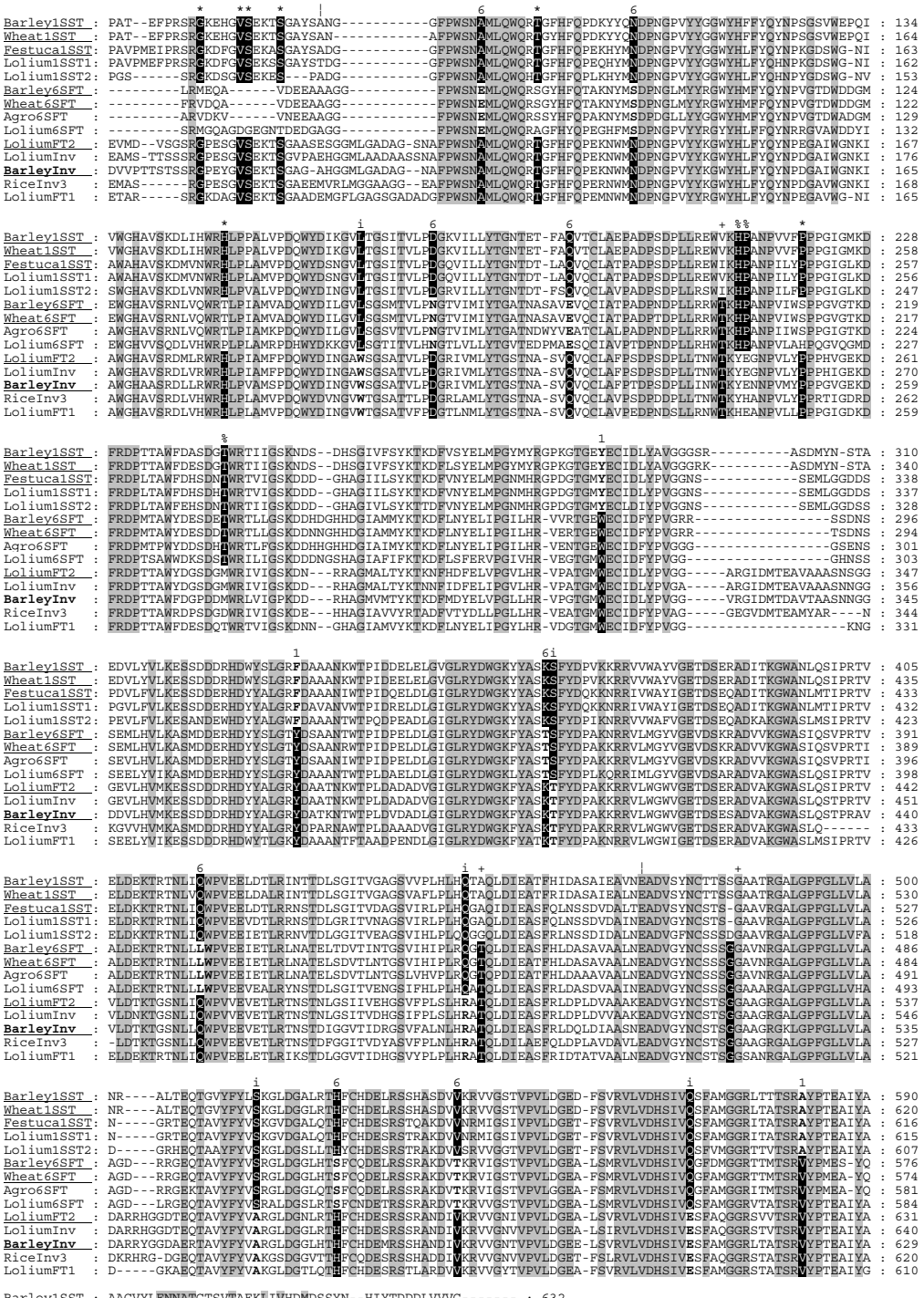


Figure 5.2. Alignment of HvSAI peptide sequence with other closely related SAI and the FTs (1-SST and 6-SFT) from grasses. Columns shaded in gray represents identical amino acid residues among the SAI and FTs. Column indicated with ¦ is the region where the large and small subunit of the peptides start. Columns shaded in black contain residues conserved for SAI/1-SST, SAI/6-SFT and 1-SST/6-SFT are specified by *, + and % respectively. 1, 6 and i represent conserved columns with a "signature amino acid" (shown in bold) for 1-SST, 6-SFT and SAI respectively. The underlined sequences have been functionally characterised by heterologous expression. The accession numbers of the sequences are: 6-SFTs; Barley (*Hordeum vulgare*, X83233), Wheat (*Triticum aestivum*, AB029887), 6-SFT Agropyron (*Agropyron cristatum*, AF211253), Lolium (*Lolium perenne*, AF494041), 1-SSTs; Barley (*Hordeum vulgare*, AJ567377), Wheat (*Triticum aestivum*, AB029888), Lolium1 (*Lolium perenne*, AF492836), Lolium2 (*Lolium perenne*, AY245431), Festuca (*Festuca arundinacea*, AJ297369), SAIs, LoliumFT2 (*Lolium perenne*, AY082350), LoliumInv (*Lolium temulentum*, AJ532549) Rice (*Oryza sativa*, AF276704), Barley (*Hordeum vulgare*, AJ623275).

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analogues can inhibit invertases (Pressey, 1968) but has no effect not the activity of FTs (Simmen *et al.*, 1993). We found that pyridoxal PO_4 effectively blocked the activity of the recombinant HvSAI. Purified SAI from barley was found to have a

higher thermal stability and temperature optimum than FTs (Wagner et al., 1983; Simmen et al., 1993). The temperature optimum of recombinant HvSAI was found to be 40°C, about 10°C higher than that of 1-SST. The Km of FTs (like the barley 1-SST) for Suc, is very high since the enzyme activity is nonsaturable even at substrate concentrations as high as 500 mM (Luscher et al., 2000). A substrate-velocity curve for the recombinant HvSAI (Fig. 5.3) shows the activity of the enzyme is saturable and the Km for Suc was estimated to be 14 mM using the Lineweaver-Burk analysis (Fig. 5.3), which is similar to the Km (12mM) of SAI purified from elongating stem tissue of barley (Karuppiah and Kaufman, 1989).

Suc levels in plant tissues is known to regulate the gene expression of invertases (Koch, 1996). Vacuolar SAIs of maize fall into two categories; those upregulated by sugar depletion and those whose expression is enhanced by supplying sugars (Xu et al., 1996). To test how HvSAI expression responds to sugars, we compared its expression to 1-SST and 6-SFT, both known to be regulated positively by Suc (Muller et al., 2000), by providing excised leaves and roots with Suc or illumination of excised leaves. The transcript levels of HvSAI was not affected much, both in roots and leaves, in contrast to a considerable upregulation of 1-SST and 6-SFT in the leaves, suggesting that there may exist a class of SAIs apparently not modulated by sugar levels. The expression of putative SAI grass gene was also found not to change in excised leaves fed with Suc (Pollock et al., 1999).

Changes in the barley leaf SAI activity have been reported in response to certain conditions. The barley SAI activity is leaf-age-dependant (Wagner and Wiemken, 1989) with variations along the leaf blade (Roth et al., 1997), increases when leaves are infected with powdery mildew (Scholes et al., 1994) and decreases during nitrogen deficiency induced fructan accumulation (Wang et al., 2000). But the

activity of SAI in barley leaves was found not to change when induced for fructan accumulation by illumination (Wagner et al., 1986) or during phosphorous deficiency (Wang and Tillberg, 1997). There exist several isoforms of SAIs in barley leaves (Obenland et al., 1993) the activities of which remain unresolved in assays with crude protein extracts. The abaxial epidermis of barley leaves contains a large part of the total leaf invertase and this activity is represented exclusively by the Hv-IIB isoform (Obenland et al., 1993). Since the amino acid sequence of the newly cloned HvSAI is different from that of Hv-IIB, it is likely to be located in leaf-cells where fructans metabolism occurs i.e within the vacuoles of mesophyll or parenchymatous bundle sheath cells. The possibility of HvSAI compartmentalization away from the site of active fructan metabolism needs investigation since the cells of some barley tissues can contain more than one kind vacuoles (Paris et al., 1996; Swanson et al., 1998). Unless spatially separated or regulated by other mechanisms, co-localization could lead to a competition for Suc between HvSAI and FTs during fructan biosynthesis

The regulation of SAIs could occur at the activity level too. Vacuolar proteinaceous inhibitors, similar to the one isolated from apricot (Scognamiglio et al., 2003), may modulate SAI activities in barley too. Fru has been shown to be a weak inhibitor of barley SAI (Kingston-Smith et al., 1999) but we found its levels during fructan synthesis in the leaf very low to be effective. The partial inhibition of Suc hydrolysis *in vitro* by the recombinant SAI activity in the presence of the first fructan molecule synthesized, 1-K, could favor Suc to be preferentially channeled in the direction of fructan synthesis in the vacuole. Other SAI isoforms that can synthesize and/or hydrolyze fructans, not considered in the present study, may be regulated differently since they can play a more direct role in fructan metabolism as compared to invertases devoid of side activities.

CHAPTER 6

General discussion

Fructan biosynthesis in grasses

Fructans of barley and wheat, called graminans, are structurally complex and consist of β(2-6) linked chains of Fru units with β(2-1) branches (Bancal et al., 1991). The various pathways proposed for graminan synthesis have been conflicting (Penson and Cairns, 1994). While earlier studies found that 6-K was the only trisaccharide formed in excised illuminated barley leaves induced for fructan accumulation (Smeekens et al., 1991), others found 1-K to be the primary fructan synthesized (Simmen et al., 1993). It was suggested that two inducible enzymes, 1-SST and 6-SST, act in concert to initiate fructan accumulation in barley leaves (Simmen et al., 1993; Wiemken et al., 1995). Based on a detailed biochemical analysis of its activities, the purified 6-SST was renamed as 6-SFT. It was shown that most of the flux of carbon from Suc to fructan can be mediated by 6-SFT and hence it was proposed to occupy a central position in graminan synthesis (Duchateau et al., 1995). The role of 6-SFT was further characterised by the cloning of the corresponding cDNA from barley (Sprenger et al., 1995). In a new model proposed for graminan synthesis, 1-SST catalyses the essential first step producing 1-K by β(2-1) linkage. In the second step, equally important for *de novo* fructan synthesis in these plants, 6-SFT catalyzes the formation of Bif (1&6 kestotetraose), using Suc as the fructosyl donor and 1-K as the favored fructosyl acceptor. In further steps, higher polymeric-graminans are synthesized by the same 6-SFT through β(2-6) linked chain elongation (Wiemken et al., 1995). The structural composition of the fructan synthesized can be modified by FFT's, through shuffling of Fru residues between fructan molecules, and

by different fructan exohydrolases (FEH's), through specific trimming of fructosyl chains. However the introduction of new fructosyl units from Suc into fructans to bring about fructan synthesis is only through the action of 1-SST and 6-SFT (Wiemken et al., 1995).

The very presence of a unique 1-SST and its need for fructan synthesis in grasses has been questioned since invertases from various sources can catalyse the formation of 1-K, 6-K and N-K under appropriate conditions (Pollock and Cairns, 1991). However, the activity of 1-SST has been convincingly separated from invertases (Simmen et al., 1993) and the cloning of the cDNAs encoding 1-SST from grasses has been achieved recently (Luscher et al., 2000; Kawakami and Yoshida, 2002; Chalmers et al., 2003). In the present work, the role and regulation of 1-SST during graminan biosynthesis was investigated by cloning a functional 1-SST cDNA. In time course studies with excised and illuminated barley leaves, Suc levels were found to increase rapidly. The only trisaccharide that accumulated initially was 1-K and was associated with a rapid induction of 1-SST gene expression and enzyme activity. There was a marked delay in the induction of 6-SFT gene expression and the corresponding enzyme activity. Subsequently, the level of 1-K did not increase further indicating that it was being diverted for the synthesis of Bif as well as higher fructan polymers. Hence the introduction of Suc into the pathway of fructan synthesis is initiated by 1-SST induction. But only in the presence of 6-SFT - in addition to that of 1-SST - a large drain is formed drawing photosynthates in direction to fructan. This sequence of events agrees well with previous biochemical studies showing that the preferred fructosyl acceptor substrate of 6-SFT is 1-K, and that higher polymeric fructans can serve as acceptors as well whilst Suc is an unsuitable acceptor (only minor 6-SST activity), particularly in the presence of 1-K (Simmen et al., 1993;

Duchateau et al., 1995; Wiemken et al., 1995). The synthesis of 6-K occurred much later than 1-K and its levels remained low throughout the experiment.

When the continuously illuminated leaves were transferred from light to darkness, a swift down regulation of 1-SST was observed at the level of mRNA and enzymatic activity. The rapid down regulation of 1-SST during fructan mobilization is associated with unstable transcripts as well as high protein turnover. Earlier studies have also suggested a rapid constant proteolytic degradation of 1-SST protein in the vacuole (Obenland et al., 1991). By the rapid regulation of 1-SST at multiple levels and, in contrast, a much slower regulation of 6-SFT both under conditions of induction and repression of fructan synthesis, 1-SST appears to play a crucial role as a pacemaker enzyme controlling the flow of carbon into fructan in barley leaves.

Apart from the main enzymes 1-SST and 6-SFT, the other components that determine the final graminan composition are 1-FFT, 6G-FFT, 1-FEH and 6-FEH (Wiemken et al., 1995). These enzymes need to be isolated and their cDNAs cloned to understand graminan metabolism and its regulation better. It is not fully understood how the length of the graminan chain and the type and extent of its branching are determined. Work in this direction has been initiated by the cloning of a cDNA with very high homology to an isoform of 1-FEH abundant in fructan biosynthesizing wheat stems (Van den Ende et al., 2003). Experiments are underway to assess its precise role.

Regulation of fructan metabolism

In previous studies of the regulation of fructan biosynthesis in barley leaves, the gene expression and activity of FTs increased rapidly upon the feeding of Suc or treatments that caused accumulation of photosynthates (Müller et al., 2000). Since

surplus sugars in the leaf appear to be involved in the induction of FTs, sugar sensing may be important for the regulation of fructan biosynthesis in grasses. The upregulation of 6-SFT gene expression appears to be based on the sensing of Suc and its disaccharide analogs like trehalose (Müller et al., 2000) . It is not clear if the expression of 6-SFT is associated only with 'high' sugar levels, since several other internal and external stimuli may exert a direct influence, bypassing the sugar signal. Recently it has been shown that metabolites like nitrate (Morcuende et al., 2004) can modulate 6-SFT gene expression independently from sugar signaling.

Since the regulation of 6-SFT occurs primarily at the level of transcription, the promoter region of this gene is central to the control of gene expression. About 1.6 kb of the 5'-upstream region of the barley 6-SFT gene was cloned. The sequence contains several interesting conserved regulatory elements shown to be functionally vital in plant genes expressed in response to abiotic and biotic factors such as light, low temperature, drought, ABA and developmental changes. During transient expression studies in excised barley leaves, strong promoter activity was observed during Suc and light treatments, conditions known to induce FT activity and fructan synthesis (Wagner et al., 1986). Activities of the promoter in leaf epidermal cells including guard cells, which are not known to synthesize fructans (Koroleva et al., 2000) are intriguing and need further investigation.

The various FTs fulfill specific roles during fructan metabolism (Wiemken et al., 1995) and there exists a clear differences in the regulation of 6-SFT and 1-SST genes. The Suc threshold required for FT induction is probably gene-specific. However, it remains to be seen if major differences exist in signalling pathways that control the expression of the different FT genes. Barley 1-SST is regulated rapidly and the cloning of its promoter will facilitate comparative studies on the regulation of

gene expression. The tall fescue 1-SST gene promoter has been cloned and there are no significant sequence similarities with the barley 6-SFT promoter.

An important approach to study sugar sensing and signaling is the introduction of sugar responsive promoter-reporter constructs into plants (Smeekens, 2000). While stable transformation of cereals such as barely is yet to become a routine technique, promoter analysis by transient assays poses constraints for a comprehensive analysis of activity. Valuable information has been obtained on the Suc specific induction of genes in heterologous transgenic systems, such as the patatin class I (B33) promoter in Arabidopsis (Martin et al., 1997; Hellmann et al., 2000) and the rolC promoter in tobacco (Yokoyama et al., 1994). The various signal transduction events involved in sugar mediated gene expression and the interaction of sugar sensing and signaling with other pathways has been investigated exhaustively in the model plant Arabidopsis (Smeekens, 2000). To benefit from information already available on sugar sensing and the relative ease at which Arabidopsis can be transformed, the barley 6-SFT promoter was fused to the GUS reporter gene and transgenic Arabidopsis plants were obtained. Even though Arabidopsis is not a fructan producer, high GUS expression in Arabidopsis leaves was induced in response to Suc feeding, indicating that the regulation of the 6-SFT promoter is highly similar to that in barley excised leaves. Using appropriate inhibitors, the activities of protein kinase, protein phosphatase and calcium were shown to be involved in the chain of events that intervenes in the Suc induction of 6-SFT (Noel et al., 2001). Leaves of transgenic Arabidopsis showed similar responses and it appears that the relevant signaling events are conserved between cereals and Arabidopsis. Deletion studies of the 6-SFT promoter will help to identify the functional cis-acting elements controlling gene expression. Better insights into the regulation of promoter activity can be gained by

the identification of the trans-acting factors instrumental in regulating 6-SFT gene expression by interacting with the cis-elements. These transgenic plants can be further used as tools to select and characterize sugar-unresponsive or sugar hyperresponsive mutants. The tissue-specific promoter responses to developmental stages, drought, nutrient deficiency, low temperature stress, pathogen attack and hormones will contribute to our understanding of the physiological role of fructans in general.

An important component of future studies on the regulation of fructan metabolism should be the control of FTs at the posttranscriptional and posttranslational level. Suc can suppress the translation of ATB2 mRNA (Rook et al., 1998) and may interfere with the Suc symporter transport activity (Smeekens, 2000). Glucose can destabilize α-amylase transcripts (Loreti et al., 2000). These sugars are found to be present in considerable amounts in the cell during fructan synthesis and their levels change rapidly during different stages of fructan metabolism. The potential of sugars to exert their regulatory function on fructan metabolism at multiple levels needs further investigation. Wheat glucanotransferases responsible for starch (amylopectin) branching are controlled *via* interaction with other proteins (Tetlow et al., 2004) and it remains to be seen if FTs are amenable to similar regulatory controls. What also remains to be seen is the precise impact of altered glycosylation on the activity of FTs and if this contributes to their regulation.

Invertases and fructan metabolism enzymes

The close relationship between invertases and FTs at the biochemical and molecular levels is strong evidence for the notion that FTs evolved from acid invertases by relatively few mutational changes (Vijn and Smeekens, 1999). Phylogenetic analyses suggest that the mutational changes to invertases occurred

independently in several phyla. Hence the amino acid sequences of indigenous invertases provide the best clues to decipher the precise mutational changes that causes the conversion of a predominantly Suc hydrolyzing enzyme into a fructan synthesizing enzyme. The protein domains that are important for the specificity of the respective fructosyltransferases have not yet been identified. In this context, a functional barley vacuolar invertase was cloned and found to possess high identity to 1-SST (66%) and 6-SFT (64%) from barley. Interestingly, residues conserved only between Inv & SST or Inv & SFT or among the FTs are found especially within the domains characterized to be important for functionality in the yeast invertase (Reddy and Maley, 1996) and bacterial levan sucrase (Meng and Futterer, 2003). These are obvious targets for future studies introducing mutations to understand the structure-function relationship of these enzymes. There was not much variation of HvSAI mRNA during fructan synthesis and breakdown, and similar results were obtained when invertase activity was monitored in the vacuole (Wagner et al., 1986). Future studies on fructan biosynthesis should also address how invertase activity is controlled so that Suc is utilized for anabolic reactions by the FTs. The physiological significance of constitutive SAIs during fructan metabolism needs to be clarified.

A valuable approach to understand FTs and functional importance of fructans in plants would be to identify and characterize mutants with altered fructan metabolism. Novel technologies like the silencing of specific genes through siRNA (Arenz and Schepers, 2003), homologous recombination mediated knockout (Puchta, 2002) and change-of-function studies in transgenic plants offer exiting prospects to investigate the role fructans in plants. Gene chips for barley containing about 25,500 unique sequences are now commercially available (Affymetrix, Santa Clara, USA) and may prove to be handy, along with proteomics approaches, to identify the

components of the various signaling pathways regulating fructan metabolism in barley.

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