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Fructan exohydrolases in non-fructan plants

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de graad van Doctor in de Wetenschappen: Biologie

door
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LIST OF ABBREVIATIONS

3-D	three-dimensional
ACN	acetonitrile
ADP	adenosine diphosphate
AEC-PAD	anion exchange chromatography with pulsed amperometric detection
AGPase	adenine 5'-diphosphate glucose pyrophosphorylase
AOX	alcohol oxidase
At6-FEH	*Arabidopsis thaliana* fructan 6-exohydrolase
At(6&1)-FEH	*Arabidopsis thaliana* fructan (6&1)-exohydrolase
BB	brilliant blue
βF	beta-fructofuranosidase
bp	base pair
Bv6-FEH	*Beta vulgaris* fructan 6-exohydrolase
CaMV	Cauliflower mosaic virus
cDNA	complementary DNA
CID	collision induced dissociation
CINV	cytoplasmic invertase
CWINV	cell wall invertase
Col0	ecotype Columbia 0 of *Arabidopsis thaliana*
ConA	Concanavalin A
d	days
Da	Dalton
DNA	deoxyribonucleic acid
DP	degree of polymerization
dpi	days post inoculation
DTT	dithiothreitol
EB	extraction buffer
EC	Enzyme Commission
EF1α	elongation factor 1α
EPS	exopolysaccharides
ESI-Q-TOF	electrospray ionization quadrupole time-of-flight
FBE	fructan biosynthetic enzymes
FEH	fructan exohydrolase
FFT	fructan:fructan fructosyltransferase
F_n	fructosyl oligosaccharides with a degree of polymerization of n
FPLC	fast performance liquid chromatography
GF	sucrose
^{G}-FFT	glycosyl-fructosyl transferase
GFP	green fluorescent protein
GH	glycosyl hydrolase
h	hour
HMW	high molecular weigth
HPLC	high pressure liquid chromatography
INA	2,6 dichloroisonicotinic acid
INV	invertase
kDa	kilodaltons
KEH	kestose exohydrolase
K_i	inhibition constant
K_m	Michaelis constant
KO	knock-out
M	molar
mAU	milli absorbance units
MeJa	methyljasmonate

min	minutes
M_r	molecular weight
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MS/MS	tandem mass spectrometry
mU	milli units
n.d.	not determined
OD	optical density
OEX	overexpression
ORF	open reading frame
PCR	polymerase chain reaction
PEG	poly ethylene glycol
P_i	inorganic phosphate
pI	isoelectric point
PR	pathogenesis related
qRT-PCR	quantitative reverse transcriptase PCR
Ref	reference
RNA	ribonuleic acid
RT	reverse transcriptase
s	second
SA	salicylic acid
SCFA	short chain fatty acids
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SE	standard error
SFT	sucrose:fructan fructosyltransferase
SPP	sucrose 6'-phosphate phosphatase
SPS	sucrose 6'-phosphate synthase
SST	sucrose:sucrose fructosyltransferase
Suc	sucrose
SuS	sucrose synthase
TFA	trifluoroacetic acid
Tris	tris(hydroxymethyl)aminomethane
U	units
UTR	untranslated region
UV	ultraviolet
UDP	uridine diphosphate
VINV	vacuolar invertase
Ws	ecotype Wassilewskija of *Arabidopsis thaliana*
WT	wild-type

GENERAL INTRODUCTION

Plants have the capacity to capture energy from the sun and fix it in organic molecules by the process of photosynthesis. Virtually all life on earth depends, directly or indirectly, on the products of this process. A primary product of photosynthesis is sucrose. This disaccharide can be a substrate for biosynthesis of fructans, fructose-based polysaccharides. Fructans have been recognized for nearly 200 years and have been detected in ± 15% of vascular plants. Their prominence in the economically important families the Poaceae and the Asteraceae indicates that they cannot be considered as a pathway of minor evolutionary significance. Several fructan biosynthetic (SST, FFT, SFT…) and fructan degrading enzymes (1-FEH, 6-FEH) have been purified, characterized and their corresponding cDNAs have been cloned. Both group are evolutionary related to invertases, enzymes that hydrolyze sucrose.

This doctoral study covers a new research area in the fructan field, namely fructan exohydrolases (FEHs) in non-fructan plants. This work includes purification of 6-FEH proteins, cloning and heterologous expression of their corresponding cDNAs, and research on the physiological role of these enzymes in non-fructan plants by using transgenic plants.

In the first chapter an introduction to sucrose and fructan metabolism is given, with an emphasis on fructan breakdown. Chapter 2 and 3 consist of a detailed characterization of a *Beta vulgaris* (sugar beet) 6-FEH (Bv6-FEH). It starts with the detection of a 6-FEH activity in the taproots of this economically important crop. Next, the purification and characterization (substrate specificities, K_m, inhibition by sucrose, pH optimum) of this enzyme is described. Subsequently, the 6-FEH cDNA is cloned and heterologously expressed in the yeast *Pichia pastoris*. The properties of the native and recombinant enzymes are compared and their homology to cell wall invertases is discussed. Chapter 4 presents the occurrence of two specific FEHs in the model plant *Arabidopsis thaliana,* namely At6-FEH and At(6&1)-FEH. The cDNAs are cloned and heterologously expressed in *P. pastoris* and the native At6-FEH is partially purified. The substrate specificities of both recombinant and native enzymes are determined and some special characteristics of At(6&1)-FEH are emphasized. To end, chapter 5 presents work on the role of FEHs in non-fructan plants. Therefore, At6-FEH and At(6&1)-FEH knock-out and overexpression plants were investigated. Sugar extracts of both plants were analyzed for the presence of an endogenous substrate. Furthermore, the expression of At6-FEH and At(6&1)-FEH is examined after different treatments and the role of both enzymes during pathogenesis is studied.

CHAPTER I

LITERATURE REVIEW

I.1 Introduction

Vascular plants have the ability to store and translocate some of the carbon fixed during photosynthesis in order to sustain heterotrophic organs or to support survival of plants in periods or tissues where carbon fixation is insufficient or impossible. Therefore, plants accumulate a plethora of soluble and insoluble storage carbohydrates. The amounts and types of carbohydrates differ depending on developmental stage, species, organ and tissue. Starch and sucrose are by far the most abundant and studied storage carbohydrates. However, many advances have been made in our understanding of the metabolism of the less common carbohydrates such as fructans and raffinose oligosaccharides.

In this literature, a brief introduction will be given on sucrose and its metabolism. Next, the occurrence and metabolism of fructans will be treated in more detail, emphasizing the breakdown of these fructans.

I.2 Sucrose and sucrose metabolism in plants

I.2.1 Introduction

Carbon resource allocation is a fundamental process in higher plants. As much as 80% of the carbon acquired in photosynthetically active source tissues (mature leaves) can be transported by the phloem to photosynthetically less active or inactive sink tissues such as roots, fruits and tubers. The non-reducing disaccharide sucrose, a primary product of photosynthesis, is arguably the most important carbon metabolite in this phenomenon called "assimilate partitioning" (Chiou *et al.*, 1998; Roitsch, 1999). Sucrose can be used for the synthesis of the larger reserve carbohydrates such as starch and fructan or can be stored in the vacuole of storage organs e.g. sugar beet roots. In tomato fruits, however, sucrose in the vacuole is hydrolyzed and the formed hexoses are sequestered in this organelle (Nguyen-Quoc and Foyer, 2001). During the last decade, convincing evidence shows that sucrose not only provides the fuel for plant growth but that sucrose and its cleavage products also play a major role in signalling. Sucrose can modulate the expression of genes that are implicated in many metabolic and developmental processes (Lunn and MacRae, 2003).

Sucrose is the only dominant sugar in higher plants that is capable of fulfilling all the above mentioned roles: transport sugar, storage reserve and signalling compound. In certain plant families, oligosaccharides of the raffinose series or sugar alcohols are major products of photosynthesis and are important in transport and storage. However, even where such compounds are clearly of major importance, they do not replace sucrose entirely (Ap Rees, 1984). The universal occurrence of sucrose in plant cells is still a mystery and the question 'why sucrose?'(and e.g. not trehalose) is still unanswered (Salerno and Curatti, 2003).

I.2.2 Sucrose metabolism

I.2.2.1 Biosynthesis of sucrose (Fig. I.1)

Sucrose synthesis is restricted to the cytosol. The principal sucrose-biosynthesis route involves the action of two enzymes: sucrose-phosphate synthase (SPS; EC 2.4.1.14) catalyzes the formation of sucrose-6-phosphate that is hydrolyzed by sucrose phosphatase (SPP; EC 3.1.3.24) yielding free sucrose and inorganic phosphate (P_i). This irreversible pathway provides an efficient production of sucrose even at low substrate

concentrations. SPS activity is controlled by protein phosphorylation in response to environmental signals (Winter and Huber, 2000). Overexpression of the maize SPS gene in tomato leaves resulted in reduced levels of starch and increased levels of sucrose (Worrell *et al.*, 1991).

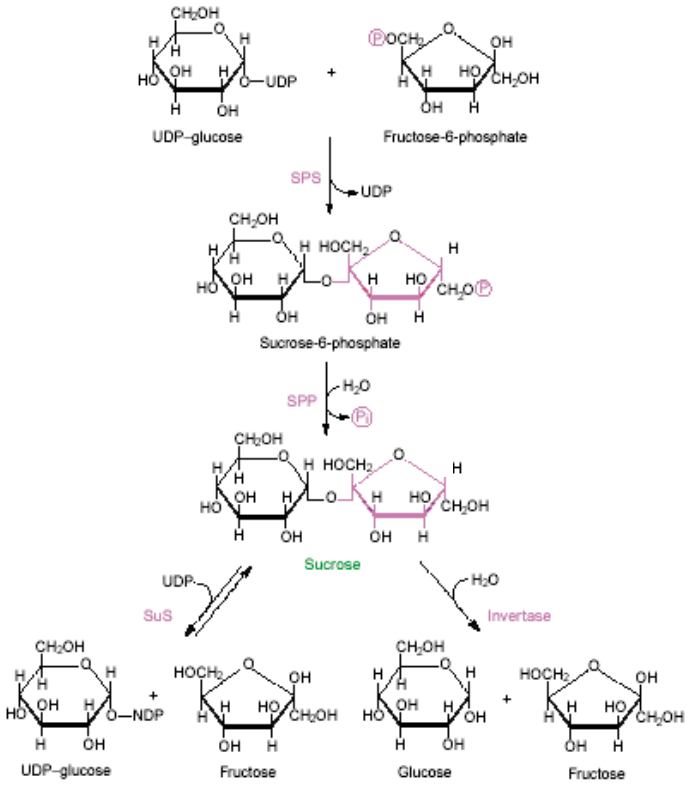


Figure I.1 Sucrose metabolism in plants (Salerno and Curatti, 2003). SPS, sucrose-phosphate synthase; SPP, sucrose-phosphate phosphatase; SuS, sucrose synthase; P_i, inorganic phosphate; UDP, uridine diphosphate.

I.2.2.2 Breakdown of sucrose (Fig. I.1)

Sucrose breakdown is vital to higher plants, not only to provide cells with fuel for respiration and with carbon and energy for the synthesis of new compounds but also for facilitating carbon allocation and for the initiation of hexose-based sugar signals (Sturm, 1999; Koch, 2004). It has been postulated that in general, hexoses favour cell division and expansion, whereas sucrose favours differentiation and maturation (Koch, 2004). So, sucrose-cleaving enzymes can alter plant development and growth.

Sucrose cleavage in plants can be catalyzed by invertases (INV; EC 3.2.1.26), producing glucose and fructose or sucrose synthases (SuS; EC 2.4.1.13), yielding UDP-glucose and

fructose. The latter reaction is reversible, so SuS could be involved in sucrose synthesis. However under most physiological conditions SuS plays a role in sucrose cleavage. SuS is localized in the cytosol whereas invertases can be found in the vacuole, cell wall and cytosol. This different localization and action pattern of the sucrose-cleaving enzymes sustains a distinct profile of sugar signals resulting in a different contribution of invertases and sucrose synthases during development. It has been postulated that invertase activity is linked to sink initiation and cell expansion and that SuS activity is associated with storage and maturation phases (Winter and Huber, 2000; Koch, 2004).

Because of the evolutionary homology between invertases and fructan enzymes (§I.3.3.3), the next section will deal only with invertases and not sucrose synthases.

Different types of invertases

As mentioned before, a variety of invertases have been reported even from a single plant species e.g. 17 in *Arabidopsis thaliana* and 19 in *Oryza sativa* (rice). Based on their subcellular localization, pH optima, iso-electric points (pI) and solubility, invertases can be divided into three categories (Tymowska-Lalanne and Kreis, 1998a; Kim *et al.*, 2000):

1) soluble cytoplasmic invertase (CINV) with a neutral to alkaline pH optimum
2) soluble vacuolar invertase (VINV) with an acidic pH optimum
3) insoluble cell wall bound and soluble apoplastic or extracellular invertase (CWINV) with an acidic pH optimum

Vacuolar invertases (VINVs) and cell wall invertases (CWINVs) have very similar properties. Their activity is inhibited by heavy metals and they are N-glycosylated proteins having an acidic pH optimum. Both enzyme types are classified in the group of β-fructofuranosidases (βF) who are defined as enzymes that hydrolyze terminal non-reducing beta-D-fructofuranoside residues in beta-D-fructofuranosides. In general, invertases are also able to hydrolyze other β-fructose-containing oligosaccharides such as raffinose, stachyose and 1-kestose, although to a much lesser extent. This category is characterized by the NDPN-G/A motif (βF-motif) (Sturm, 1999). The amino acid sequences of VINVs and CWINVs share a high degree of homology (40-60% identical) and contain the conserved amino acid motifs NDPN-G/A and WEC-P/V-D (Goetz and Roitsch, 1999).

The intron-exon structure (six to eigth exons) of both enzymes is very similar and has a special feature, namely the occurrence of a mini-exon DPN (three amino acids in the conserved motif). The amino acid sequence of VINV has a longer N-terminal propeptide and a C-terminal extension, lacking in (almost) all CWINVs sequences (Michiels *et al.*, 2004). Most CWINVs have a high pI. Only few of them show a low pI, supporting the idea

that the latter ones are soluble. Different isoforms of VINVs and CWINVs (based on sequence homology) can be found in the same plant e.g. *A. thaliana* contains two vacuolar invertases (AtVINV1-2) and six cell wall invertases (AtCWINV1-6) (Sherson *et al.*, 2003). *O. sativa* contains 9 CWINVs (OsCIN1-9) and two VINVs (OsVIN1-2) (Ji *et al.*, 2005).

Information on cytoplasmic invertases (CINV) or neutral invertases is very poor. Their activity is rapidly lost *in vitro* and their purification is very difficult. CINV are not glycosylated and not inhibited by heavy metals; in contrast they are strongly inhibited by Tris, glucose and fructose (Sturm, 1999). Sucrose appears to be the sole substrate. Although a lot of isoforms exists in plants e.g. 9 in *A. thaliana* (Vargas *et al.*, 2003; Ji *et al.*, 2005) and 8 in *O. sativa* (Ji *et al.*, 2005), the role of these enzymes in plants is not known.

These two families of acid invertases (CWINVs and VINVs) and neutral/alkaline invertases are unrelated and probably have a different evolutionary origin. The plant neutral invertases are closely related to cyanobacterial invertases (Vargas *et al.*, 2003), while the plant acid invertases have a clear homology with the invertases of respiratory eukaryotes such as yeast and aerobic bacteria as *Bacillus* spp. (Sturm and Chrispeels, 1990; Ji *et al.*, 2005).

Roles and regulation of acid invertases in plants

Many authors already confirmed that the expression pattern of different invertase isoforms from one plant can vary depending on organ and development-stage e.g. in *Daucus carota* (carrot) (Sturm *et al.*, 1995), *A. thaliana* (Tymowska-Lalanne and Kreis, 1998a; b; Sherson *et al.*, 2003), *O. sativa* (Ji *et al.*, 2005) and *Lycopersicon esculentum* (tomato) (Godt and Roitsch, 1997).

Both gene expression and enzyme activity of invertases are regulated by a variety of intracellular and extracellular factors such as sugars, hormones, pathogen infection, wounding, light and inhibitors (Tymowska-Lalanne and Kreis, 1998a). The different family members can show contrasting responses to these factors and the expression of one gene can differ markedly with tissue and organ (Koch, 2004). In the following paragraph some examples of invertase regulating factors will be given:

- sugars: Both inhibition and activation by sugars have been demonstrated, e.g. Roitsch *et al.* (1995) reported the induction of a CWINV from *Chenopodium rubrum* by glucose; vacuolar invertases however were not affected.

- hormones: In most cases the effect of hormones is related to an increased demand for carbohydrates for growth (Roitsch and González, 2004). It has been reported that both auxin and gibberellic acid play an important role in regulating invertase activity (Tymowska-Lalanne and Kreis, 1998a). The extracellular invertases from *Chenopodium rubrum* (Ehness and Roitsch, 1997) and *L. esculentum* (Godt and Roitsch, 1997) are induced by cytokinins. Balibrea Lara *et al*. (2004) demonstrated that extracellular invertase is an essential component of the cytokinin-mediated delay of senescence. When invertase activity is inhibited, cytokinin is not able to delay senescence, probably because invertase is necessary to provide assimilates.
- wounding and infection: The expression of the first cloned CWINV cDNA from carrot roots is highly upregulated after wounding and pathogen infection with *Erwinia carotovora* (Sturm and Chrispeels, 1990). Mechanical wounding induced the accumulation of CWINV mRNA from *Pisum sativum* in leaves, stems and roots (Zhang *et al*., 1996). AtcwINV1 from *A. thaliana* is upregulated after infection with *Albugo candida* (Chou *et al*., 2000) and the fungal pathogen *Erysiphe cichoracearum* (Fotopoulos *et al*., 2003). It has been postulated that CWINVs alter the source/sink status and that this stress-related induction of invertase is due to the need for extra carbohydrates, providing additional metabolic energy to activate the cascade of defense reactions (Roitsch *et al*., 2003). In addition to this, the formed hexoses are supposed to play a signalling role, hereby inducing other stress-related genes. In contrast to this theory, Quilliam *et al*. (2006) reported no difference in the sink status and gene expression of wounded wild-type and CWINV deficient *A. thaliana* plants.
- light: Transcripts of a vacuolar invertase from *Beta vulgaris* showed circadian oscillation with maximal accumulation during the light period (González *et al*., 2005).
- invertase inhibitors: In addition to transcriptional regulation, invertase activities also depend on post-translational mechanisms. The most important one seems to be complex formation of invertases with specific proteinaceous inhibitors (Rausch and Greiner, 2004).

As mentioned earlier and as suggested by the different regulation systems summarized, invertases play a major role in development and growth of the plant, especially during initiation and expansion of sink structures. During these developmental stages, hexose production is maximized, enhancing respiration and osmoregulation. Because of their localization, vacuolar and cell wall invertases fulfil different functional roles. In summary,

vacuolar invertases are important in the control of sugar composition in fruits and storage organs, for osmoregulation and in response to abiotic stress. Cell wall invertases play a major role in the regulation of sucrose partitioning, regulation of pollen and seed development and in the response to abiotic and biotic stress (Roitsch and González, 2004). Extracellular invertase is one of the key enzymes of the apoplasmic phloem unloading pathway, maintaining a sucrose gradient between sieve element and sink apoplasm (especially in seeds and pollen). The resulting hexose monomers are then imported into the sink cell by monosaccharide transporters. Several striking examples of this function were found in maize, carrot and tobacco. The maize mutant *miniature-1* is deficient in an endosperm-specific CWINV (Cheng *et al*., 1996). The lack of this invertase activity causes a gap between the pedicel and endosperm, interrupting the transport of photo-assimilates into the developing kernel. As a consequence, seeds are only a fifth of their normal weigth. After antisense inhibition of a carrot cell wall invertase (Tang *et al*., 1999), tap root formation was prevented and an increase of foliar growth was observed. Expression of a CWINV antisense construct under the control of its own promoter in transgenic tobacco plants resulted in inhibited pollen development and proved an efficient method to introduce male sterility (Goetz *et al*., 2001; Roitsch *et al*., 2003).

I.3 Fructans in plants

I.3.1 Fructan structure

Fructans are soluble, linear or branched oligo- or polymers of fructose molecules and most often one glucose molecule. If present, the glucopyranose unit is linked to the terminal fructopyranose unit by an α-1,2 bond as in sucrose, forming a non-reducing carbohydrate. The smallest non-reducing fructans (trisaccharides) are built up by adding a fructofuranosyl unit to one of the primary hydroxyl groups of sucrose, resulting in 1-kestose, 6-kestose or neokestose (Fig. I.2). Further elongation of these trisaccharides by different enzymes will produce inulin-type, levan-type, mixed-type (graminans) or neokestose-based fructans (Pollock and Chatterton, 1988). Sometimes fructans lack the glucose-unit, forming the reducing F_n-type fructans (termed inulo-*n*-ose or levan-*n*-ose). In general DPs of 30 to 50 are found in plant fructans, but occasionally DPs can exceed 200 (Vijn and Smeekens, 1999). Different micro-organisms are also able to produce fructans. Their occurrence and metabolism will be discussed in §I.4.

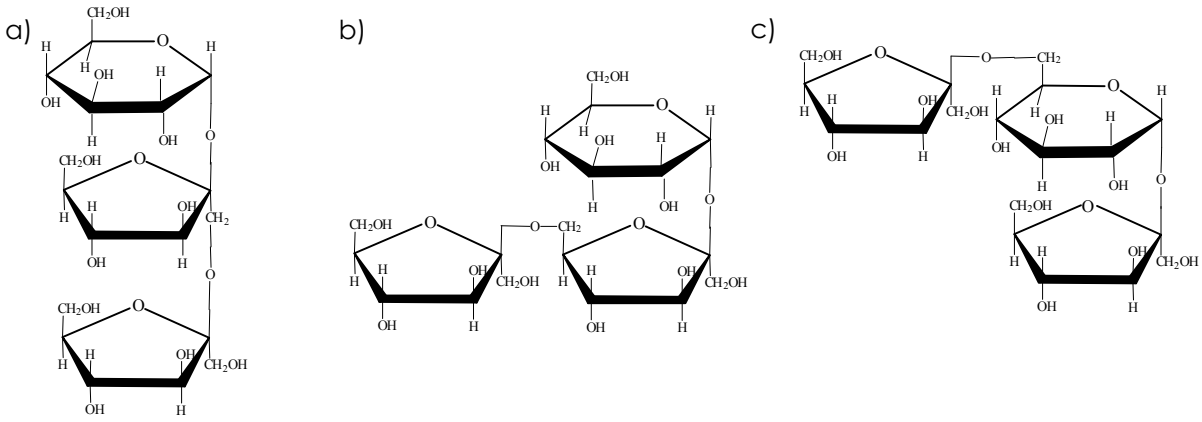


Figure I.2 Structure of the three basic fructan oligosaccharides. a) 1-kestose, b) 6-kestose and c) neokestose

I.3.1.1 Inulin-type fructans: G1,2-F1,(2-F-1)$_n$,2-F

Inulin is built up from 1-kestose (1-kestotriose; nomenclature, Lewis 1993) by adding β-2,1 linked fructose units (Pollock and Chatterton, 1988). This simple and linear fructan is found in dicotyledonous plant families like the Asteraceae, Campanulaceae and Boraginaceae.

The reducing inulo-*n*-oses (F_n-type fructans) have been found *in vivo* e.g. in chicory roots at the end of the growing season, during storage, forcing and sprouting. The smallest fructan in this series is the disaccharide inulobiose (Van den Ende *et al.*, 1996a).

I.3.1.2 Levan-type fructans: G1,2-F6,(2-F-6)$_n$,2-F

Levan is built up from 6-kestose (6-kestotriose) by adding β-2,6 linked fructose units. In literature this linear plant levan is often called phlein (Pollock and Chatterton, 1988) and is found in a few grasses (e.g. *Dactylis glomerata*, Chatterton *et al.,* 1993; *Poa secunda,* Chatterton and Harrison, 1997). The smallest reducing fructan with a β-2,6 linkage is levanbiose (cfr. inulobiose).

I.3.1.3 Mixed-type fructans or graminans

Graminans contain both β-2,1 and β-2,6 linked fructose units. Bifurcose ((1&6)-kestotetraose) (Fig. I.3) is the tetrasaccharide from which they can be built up. This fructan-type is found e.g. in *Triticum aestivum* and *Hordeum vulgare*, two species belonging to the Poaceae (Carpita *et al.*, 1989; Bonnett *et al.*, 1997).

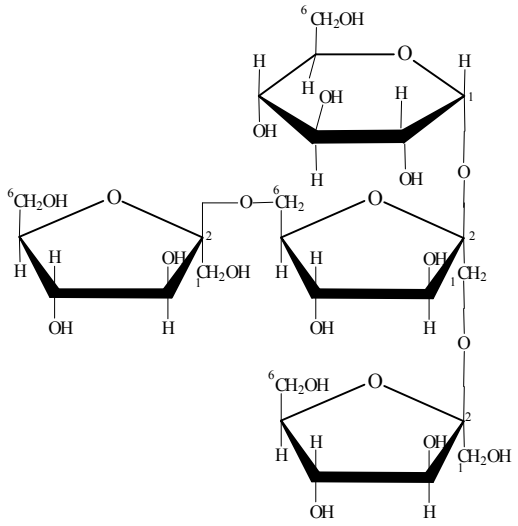


Figure I.3 Structure of bifurcose

I.3.1.4 Neokestose-based fructans = neoseries

Fructans in which fructose chains occur on both ends of the glucose molecule are neokestose (6G-kestotriose)-based fructans or neoseries. Inulin neoseries (*Asparagus officinalis* and *Allium cepa* in the Liliaceae) (Shiomi, 1989; 1993) and levan neoseries can be found, depending on the type of linkage. Both series are present in the Poaceae species *Avena sativa* and *Lolium perenne* (Livingston *et al.*, 1993; Pavis *et al.*, 2001).

I.3.2 Fructan Biosynthesis

The synthesis of fructans from sucrose occurs by the action of different biosynthetic enzymes. In the next paragraph the different enzymes are described according to the fructan-type they synthesize (Fig. I.4).

I.3.2.1 Inulin biosynthesis (Fig. 1.4)

Inulin metabolism has been extensively studied. In 1968, Edelman and Jefford proposed a model for the biosynthesis of inulin in *Helianthus tuberosus*. Two enzymes were involved namely sucrose:sucrose 1-fructosyltransferase (1-SST) and fructan:fructan 1-fructosyl-transferase (1-FFT). This model was called the 1-SST/1-FFT model and has been further corroborated in some species of the Asteraceae like *H. tuberosus* (Koops and Jonker, 1996, Lüscher *et al.*, 1996) and *Cichorium intybus* (Van den Ende and Van Laere, 1996a; b).

Starting from two sucrose molecules, 1-SST (E.C. 2.4.1.99) (Table I.1) produces 1-kestose releasing a glucose moiety. In this initial step sucrose serves both as donor and acceptor molecule. The reaction is essentially irreversible. *In vitro* 1-SST can produce small amounts of longer fructans (max. DP 5-6) and shows a low β-fructosidase activity. The 1-kestose produced can further be elongated by the action of 1-FFT (EC 2.4.1.100) (Table I.1), transferring a fructose moiety from 1-kestose (or longer fructans) to sucrose or other fructans. This reaction is reversible. Preferred donor and acceptor substrates for 1-FFTs differ between plant species and might be responsible for different inulin profiles (e.g. *Cynara* vs *Helianthus*; Hellwege *et al.*, 1998; e.g. *Cichorium* vs *Echinops*; Vergauwen *et al.*, 2003).

In *Cichorium intybus* 1-FFT is also responsible for the production of inulo-*n*-oses (F_n-type) by using free fructose as the acceptor substrate (Van den Ende *et al*., 1996a).

1-SST: GF + GF → GFF + G

1-FFT: $GF(F)_n + GF(F)_m \leftrightarrow GF(F)_{n-1} + GF(F)_{m+1}$ with $n \geq 1$ en $m \geq 0$

$GF(F)_n + F(F)_m \leftrightarrow GF(F)_{n-1} + F(F)_{m+1}$ with $n \geq 1$ en $m \geq 0$

Different 1-SST and 1-FFT enzymes have been (partially) purified: 1-SST and 1-FFT from *Helianthus tuberosus* (Koops and Jonker, 1996; Lüscher *et al*., 1993a) and *Cichorium intybus* (Van den Ende *et al.,* 1996a, b), a 1-SST from *Hordeum vulgare* (Lüscher *et al*., 2000a) and a 1-FFT from *Taraxacum officinale* (Lüscher *et al.*, 1993b) and *Echinops ritro* (Vergauwen *et al*., 2003). The masses of the enzymes were estimated around 70 kDa and after SDS-PAGE two bands were visible (except for 1-FFT from *Helianthus* and *Taraxacum*), suggesting a heterodimeric nature of these biosynthetic enzymes. The glycosylation and acidic pH optimum (pH 5 to 6) support a vacuolar localization (Frehner *et al.*, 1984).

Both *1-SST* and *1-FFT* cDNAs have been cloned from *Helianthus tuberosus* (Van der Meer *et al*., 1998), *Cichorium intybus* (de Halleux and Van Cutsem, 1997; Goblet, unpublished results), *Cynara scolymus* (Hellwege *et al*., 1997; 1998) and *Triticum aestivum* (Kawakami and Yoshida 2002; 2005). Other *1-SST* cDNAs have been cloned from *Taraxacum officinale* (Van den Ende *et al*., 2000a), *Allium cepa* (Vijn *et al*., 1998), *Festuca arundinacea* (Lüscher *et al*., 2000b), *Lolium perenne* (Chalmers *et al*., 2003) and *Hordeum vulgare* (Nagaraj *et al*., 2004). The *1-FFT* cDNAs, responsible for high DP inulin formation in *Viguera discolor* and *Echinops ritro* have been cloned (Van den Ende *et al*., 2005a; 2006). The *1-SST* genes from *Triticum aestivum* (Yoshida *et al*., 2004), *Festuca arundinacea* (Lüscher *et al*., 2000b) and *Lolium perenne* (Chalmers *et al*., 2003) have been cloned and contained 3 introns in the former and 2 introns in the latter two plants.

I.3.2.2 Levan biosynthesis (Fig. I.4)

Levan biosynthesis like in *Dactylis glomerata* and *Poa secunda* (Chatterton and Harrison, 1997) starts with the action of sucrose:fructan 6-fructosyltransferase (6-SFT; EC 2.4.1.10) (Table I.1), producing 6-kestose from two sucrose molecules. It is still uncertain whether synthesis occurs by this single enzyme or that specific sucrose:sucrose 6-fructosyltransferase (6-SST; Table I.1) and fructan:fructan 6-fructosyltransferase (6-FFT; Table I.1) enzymes are also involved. 6-SST activity has been reported from *Poa secunda* (Wei *et al*., 2002). However, the isolation and functional characterization of the genes encoding *6-SST* or *6-FFT* activities have not yet been reported (Chalmers *et al*., 2005).

I.3.2.3 Graminan or mixed-type fructan biosynthesis (Fig. I.4)

For the synthesis of bifurcose both 1-SST and 6-SFT enzymes are required. 1-SST produces 1-kestose, which is subsequently used as acceptor by 6-SFT to synthesize bifurcose. Nagaraj *et al.* (2004) suggested that the much higher regulatory responsiveness of 1-SST, as compared to 6-SFT, indicates that 1-SST plays the role of pacemaker for fructan synthesis in barley leaves. Further elongation to graminans is probably established by the action of 6-SFT and/or 1-FFT (in *Triticum aestivum*, Kawakami and Yoshida, 2005). As mentioned before 6-FFT could also play a role during elongation. *6-SFT* cDNAs have been cloned from *Hordeum vulgare* (Sprenger *et al.,* 1995), *Agropyron cristatum* (Wei and Chatterton, 2001), *Triticum aestivum* (Kawakami and Yoshida, 2002) and *Poa secunda* (Wei *et al*., 2002). A 1561 bp promoter region of the *6-SFT* gene from *Hordeum vulgare* has been cloned (Nagaraj *et al*., 2001). The promoter is switched on in responses to treatments with continuous light or sucrose.

I.3.2.4 Neoseries biosynthesis (Fig. I.4)

The synthesis of the neoseries requires fructan:fructan 6^G-fructosyltransferase (6^G-FFT) (Table I.1). This enzyme produces neokestose by using 1-kestose as donor molecule and sucrose as acceptor molecule. Neokestose formation depends again on the availability of 1-kestose that is synthesized by 1-SST. Further elongation of neokestose can occur on either side of the internal glucose by the activity of a 1-FFT (inulin neoseries) or 6-SFT (levan neoseries). Pavis *et al.* (2001) also postulated the existence of 6-FFT for the synthesis of levan neoseries but till now this could not be proven. In asparagus, three enzymes are involved in the neoseries synthesis namely 1-SST, 1-FFT and 6^G-FFT (Shiomi, 1989). However, the need for a separate 1-FFT activity in both onion and *Lolium* has been questioned. After heterologous expression of the *6^G-FFT* cDNA from onion in By2 tobacco suspension cultures (Ritsema *et al*., 2003) and from *Lolium perenne* in *Pichia pastoris* (Lasseur *et al*., 2006), the recombinant enzymes produced an array of fructans from the inulin series and inulin neoseries using 1-kestose as substrate. Moreover the recently purified 6^G-FFT from onion bulbs also showed an additional 1-FFT activity (Fujishima *et al*., 2005).

Table I.1 Summary of fructosyl donors and fructosyl acceptors of the different fructosyl transferases

Fructosyl transferase	Fructosyl donor	Fructosyl acceptor
1-SST	sucrose	sucrose
1-FFT	fructan	sucrose / fructan
6-SFT	sucrose	sucrose / fructan
6^G-FFT	1-kestose	sucrose / fructan
6-SST ?	sucrose	sucrose
6-FFT ?	fructan	sucrose / fructan
invertase	sucrose	water
FEH	fructan	water

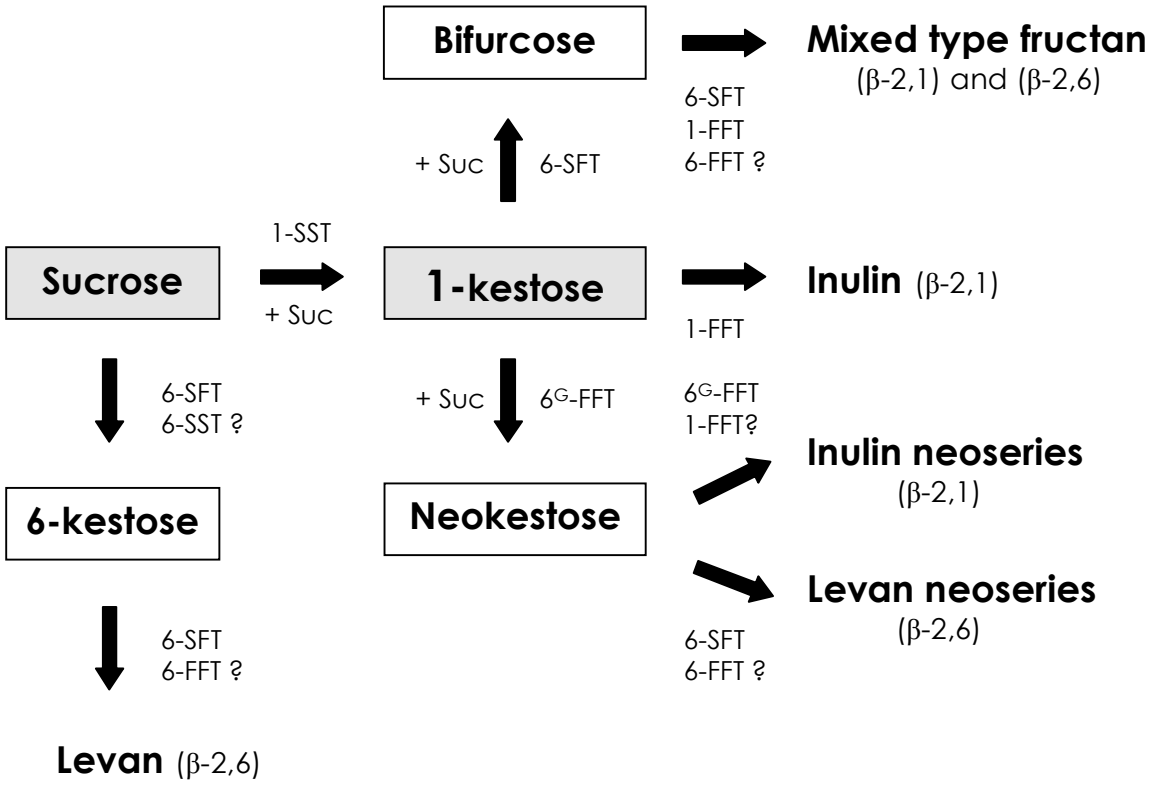


Figure I.4 Schematic model of the enzymology of fructan biosynthesis (adapted from Vijn and Smeekens, 1999 and Van den Ende *et al*., 2002)

Fructan biosynthetic enzymes (FBE) most probably evolved from vacuolar-type invertases (Fig. I.5, §I.3.3.3). This happened by relatively few mutational changes. FBE and vacuolar invertases from monocots occur in a clade separate from those of the dicots. This supports the idea that plant fructan genes evolved independently after the divergence of monocotyledons and dicotyledons (Wei and Chatterton, 2001). The phylogenetic tree reveals also that fructosyltransferases show clusters of enzymes catalyzing the same reaction, but derived from different plants (Ritsema and Smeekens, 2003).

I.3.3 Fructan breakdown[1]

In contrast to the occurrence of both exo- and endohydrolases in bacteria (§I.4.1.2), the breakdown of plant fructans is catalyzed by Fructan ExoHydrolases (FEHs). During the 40 years of research on fructan breakdown in plants, no fructan endohydrolases have been detected. The hydrolysis of fructans is a multi-chain mechanism, releasing one terminal fructose molecule at a time (FEH; $GF_n + H_2O \rightarrow GF_{(n-1)} + F$ with $n > 1$). This was concluded by several authors and Simpson and Bonnett (1993) summarized it as follows: sucrose appeared at a very late stage of degradation of fructan with a DP of 30 and is not appearing at all with substrates with a high DP (up to 250).

I.3.3.1 Biochemical properties and occurrence of FEHs in monocots and dicots

Table I.2 gives a - to our knowledge - complete overview of all FEHs found up till now in monocots (a) and dicots (b). This table is adapted from Simpson and Bonnett (1993) and has been updated where necessary. The FEH-activities were either measured in crude extracts, partially purified or electrophoretically pure fractions. All studied FEHs bind to a Concanavalin A column suggesting that they are glycoproteins (Wagner and Wiemken, 1986; Henson, 1989; Claessens *et al*. 1990; Marx *et al*., 1997a, b; De Roover *et al*., 1999; Van den Ende *et al*., 2003a). The presence of glycosyl chains was later confirmed by mass spectrometry on different FEHs. Their pH optimum indicates that FEHs prefer an acidic environment. The molecular mass varies from 57 to 72 kDa. Two smaller molecular masses have been found for an FEH from *Hordeum vulgare* (33 kDa) (Henson and Livingston, 1998) and one from *Avena sativa* (47 kDa) (Henson and Livingston, 1996). The authors

[1]The data in this paragraph are presented as part of a review in Fructo-oligosaccharides Research:

De Coninck B., Van den Ende W. and Le Roy K. (2007). Fructan exohydrolases in plants : properties, occurrence and 3-D structure. *In*: Advances in Fructo-oligosaccharides Research (Ed. N. Shiomi, N. Benkeblia and S. Onodera). *In press*

suggest that the small molecular mass of the *Hordeum vulgare* FEH is due to its homodimeric nature. However, all other FEHs from fructan accumulating plants found untill now appear to be monomers of 60-70 kDa which is in accordance with cDNA-derived molecular masses (see below) (Claessens *et al*., 1990; Marx *et al.,* 1997a, b; De Roover *et al.*, 1999; Van den Ende *et al*., 2001; Van den Ende *et al*., 2003a; Van Riet *et al.,* 2006). So, perhaps these smaller masses represent proteolytic artefacts.

FEHs are classified according to the linkages they hydrolyze: 1-FEHs (β-2,1 linkages) and 6-FEHs (β-2,6 linkages). Recently a (6&1)-FEH has been cloned from *Triticum aestivum* (Table I.3) and characterized by heterologous expression; the corresponding native enzyme, however, has not yet been purified (Kawakami *et al*., 2005). Six monocot FEHs have been purified to homogeneity: an FEH that prefers fructans with β-2,1 linkages from *Hordeum vulgare* (Henson and Livingston, 1998), a 6-FEH from *Lolium perenne* (Marx *et al.*, 1997a), a 6-FEH from *Avena sativa* (Henson and Livingston, 1996), two 1-FEHs and a 6-FEH from *Triticum aestivum* (Van den Ende *et al*., 2003a; Van Riet *et al.,* 2006). FEHs preferring β-2,1 or β-2,6 linkages were detected in the same species (e.g. *Hordeum vulgare*, *Lolium rigidum*, *Triticum aestivum*). As shown in Table I.2a, several monocot FEHs are able to hydrolyze *in vitro* both β-2,1 and β-2,6 linked fructose molecules to some degree (usually very low) but the purity of the enzyme preparations and whether this also happens *in planta* can be questioned. Four dicot FEHs have been purified to homogeneity (Table I.2b): one 1-FEH from *Helianthus tuberosus* (Marx *et al.,* 1997b) and three 1-FEHs from *Cichorium intybus* (Claessens *et al*., 1990; De Roover *et al.*, 1999; Van den Ende *et al*., 2001).

The substrates used to assay FEH activity include both fructans extracted from the same plant species and various other commercially available fructans. Because of the complex structure of fructans in different grass species, it is very difficult to use well-defined substrates for monocot FEHs. As can be deduced from Table I.2a, native fructans are most often the best substrates, but it is difficult to determine exact K_m and V_{max} values because of the limited availability and solubility of the different substrates. Moreover, the recent finding of highly specific FEHs in wheat - e.g. 6-KEH (6-kestose exohydrolase) (Van den Ende *et al.,* 2005b)- emphasizes the importance of using well-defined and specific substrates. The presence of fructans with only β-2,1 linked fructose molecules in dicots makes it easier to define the best substrate although the low solubility of inulin and the limited availability of DP>4 substrates remain problematic.

Different FEHs (partially purified) show some activity against sucrose. It is however difficult to determine that this activity is due to the enzyme itself and not to contaminating invertase activities in the fractions.

Except for an FEH from *Taraxacum officinale* (Rutherford and Deacon, 1972), a 1-FEH from *Cichorium intybus* (Claessens *et al*., 1990), an FEH from *Hordeum vulgare* (Wagner and Wiemken, 1986) and a 6-FEH from *Triticum aestivum* (Van Riet *et al*., 2006) all other FEHs are inhibited to some extent by sucrose. Other disaccharides and free fructose had no inhibitory effect on the FEH activity (Edelman and Jefford, 1964; Marx *et al.*, 1997a; De Roover *et al.*, 1999). It was postulated that sucrose directly regulates the activity of some FEHs (De Roover *et al*., 1999) and this was recently confirmed by co-crystallization of sucrose and chicory 1-FEH IIa (Verhaest *et al*., 2007).

Although homologous in sequence, the substrate specificity, expression profile, regulation and kinetic properties of FEHs can greatly vary. For instance, 1-FEH I and 1-FEH IIa of *C. intybus* share 51% sequence identity. While 1-FEH I is poorly inhibited by sucrose, 1-FEH IIa is strongly inhibited. The most striking differences can be found in their expression: 1-FEH IIa is abundantly expressed in cold-stored, forced and defoliated roots and in Belgian endive leaves. It could not be found in leaves and roots of very young chicory plants. 1-FEH I is only expressed in cold-stored roots. So, it can be concluded that cold is a trigger for both 1-FEH I and 1-FEH IIa expression. The expression of 1-FEH IIa is also regulated by other triggers such as environmental stress (Van den Ende *et al*., 2001; Michiels, unpublished results).

FEHs are beta-fructofuranosidases, hydrolyzing terminal non-reducing beta-D-fructofuranoside residues in beta-D-fructofuranosides. In the early 1990s literature mentioned that beta-fructofuranosidases can be classified into 4 categories (Simpson and Bonnett, 1992 based on Avigad and Bauer, 1966).

1) Beta-fructofuranosidases which hydrolyze sucrose more rapidly than fructans. These enzymes are described as invertases and belong to EC 3.2.1.26
2) Non-specific beta-fructofuranosidases capable of hydrolyzing both sucrose and β-2,1-linked and β-2,6-linked fructans. These enzymes are classified under EC 3.2.1.80
3) Beta-fructofuranosidases preferentially hydrolyzing β-2,1-linked fructans without degrading sucrose
4) Beta-fructofuranosidases preferentially hydrolyzing β-2,6-linked fructans without degrading sucrose

For decades, no own EC number had been dedicated to categories 3 and 4. Plant FEHs were always classified under EC 3.2.1.80 although it is not clear if such enzymes even occur in plants. Because untill now no plant FEHs have been found that degrade both fructans and sucrose (at a similar rate) (in contrast to bacterial fructofuranosidases), new EC numbers were appointed to category 3 (EC 3.2.1.153) and 4 (EC 3.2.1.154), respectively. Very recently, however, a (6&1)-FEH (Table I.3) that does not degrade sucrose but hydrolyzes both β-2,1-linked and β-2,6-linked low DP fructans to a similar extent was found in *Triticum aestivum* (Kawakami *et al.*, 2005). Perhaps an additional distinctive EC number should be appointed to this kind of enzymes.

In conclusion:

Why such a large and complex variety of fructan structures and enzymes occur in plants is unknown. It might be a consequence of the different physiological needs of the plants, but it most probably reflects several evolutionary origins of fructan biosynthesis in different plant families by duplication events of an ancestral invertase gene.

Table I.2a Summary of biochemical properties of fructan exohydrolases purified (partially or to homogeneity) from monocotyledonous species

Source of enzyme	Plant parts	Purification + isoforms	- Mr (kDa) - Temp. opt. (°C) - pH optimum	Activity against sucrose	Activity against different substrates	Sucrose inhibition (substrate)	Km with corresponding substrate	reference
Phleum pratense	haplocorms	Crude extract	/ / 5.5					Mino and Maeda, 1976
Festuca arundinacea	Culm bases	Crude extract + $(NH_4)_2SO_4$ precipitation	/ / 4.5	Not possible to measure[a]	Native fructan (levan)[b] >>> 0.1% inulin [c]	n.d.[d]	n.d.[d]	Smith, 1976
Dactylis glomerata	Stem bases after defoliation	Partial purification [e]	57 40 5.5	Not detected	1 mM native fructan (phlein) > 1 mM triticin from *Agropyron repens* > 1 mM inulin[f]	n.d.[d]	91 mM for native phlein DP 60	Yamamoto and Mino, 1985
Hordeum vulgare	Primary leaves	Crude extract	/ 35 5.2	Not possible to measure[a]	1% native fructan (β-2,1 + β-2,6) > 1% phlein from *P. pratense* > 1% inulin from *Dhalia sp.* >>> levan from *Aerobacter levanicum*	No inhibition (1 % native fructan)	n.d.[d]	Wagner and Wiemken, 1986
	stems	Partial purification [e]	62.5 / 5.5-6	Very low	0.2% native fructan (β-2,1 + β-2,6) > 5% inulin from *C. intybus* > 5% inulin from *Dahlia sp*. >>> levan from *A. levanicum*	n.d.[d]	- 0.798 mM for barley fructan (DP 17) - 15.2 mM for inulin (DP 30[g])	Henson, 1989
	stems	Purification to homogeneity	33kDa / /	Low	β-2,1 linkages in 1.5 mM 6^G,1 -kestotetraose > 1.5 mM 1&6^G-kestotetraose > 1.5 mM 1,1-kestotetraose > 2mM kestotriose >> β-2,6 linkages 2 mM 6^G-kestotriose > 1.5 mM 6^G,6-kestotetraose	Inhibition (≠ concentrations inulin from *C. intybus*)	n.d.[d]	Henson and Livingston, 1998
Lolium rigidum	Shoots (10-d old seedlings)	Crude extract	/ 25 /	Not possible to measure[a]	4% HMW native fructan[h] >> 4% inulin from *H. tuberosus* > 1% levan from *A. levanicum*	n.d.[d]	n.d.[d]	Smouter and Simpson, 1991

Table I.2a continued. Summary of biochemical properties of fructan exohydrolases purified (partially or to homogeneity) from monocotyledonous species

Source of enzyme	Plant parts	Purification + isoforms	- Mr (kDa) - Temp. opt. (°C) - pH optimum	Activity against sucrose	Activity against different substrates	Sucrose inhibition (substrate)	Km with corresponding substrate	reference
Lolium rigidum	Mature plants (stems + leaves – inflorescences)	fraction A	/	High	20 mM Sucrose > 1% HMW native fructan[i] > 1% fructan from *Urginea maritima*[j] > 1% inulin from *C. intybus* >> 1% fructan from *A. levanicum*	Inhibition (≠ concentrations inulin from *C. intybus*)	n.d.[d]	Bonnett and Simpson, 1993
		fraction B, C	/	Very high	100 mM Sucrose >> all other fructans (5 %)	n.d.[d]		
		fraction D	68kDa	Not detected	5% fructan from *U. maritima* > 5% inulin from *C. intybus* > 5% native fructan	Inhibition (5% inulin from *C. intybus* and 5% native fructan)		
		fraction E (All partially purified)	68kDa	High	100 mM sucrose > 5% inulin from *C. intybus* > 5% fructan from *U. maritima* > 5% native fructan	Inhibition (5% inulin from *C. intybus* and 5% native fructan)		
	Mature plants (stems + leaves – inflorescences)	fraction A			≠ concentrations: 6,6,6-kestopentaose > native fructan >> inulin from *C. intybus*	Inhibition for all fractions (10 mM native fructan)	No K_m possible[k] (kestopentaose and inulin from *C. intybus*)	Bonnett and Simpson, 1995
		fraction B			All substrates 10mM: (6,6,6)-kestopentaose > native fructan > (1,1,1)-kestopentaose > inulin from *C. intybus*	Inhibition for fractions B-E[l] (10 mM inulin from *C. intybus*)	n.d.[d]	

Table I.2a continued. Summary of biochemical properties of fructan exohydrolases purified (partially or to homogeneity) from monocotyledonous species

Source of enzyme	Plant parts	Purification + isoforms	- Mr (kDa) - Temp. opt. (°C) - pH optimum	Activity against sucrose	Activity against different substrates	Sucrose inhibition (substrate)	Km with corresponding substrate	reference
Lolium rigidum		fraction C			All substrates 10mM: (6,6,6)-kestopentaose > (1,1,1)-kestopentaose native fructan > inulin from *C. intybus*		n.d.[d]	
		fraction D			≠ concentrations: (1,1,1)-kestopentaose > inulin from *C. intybus* > native fructan and (6,6,6)-kestopentaose		- 18 mM for (1,1,1)-kestopentaose	
		fraction E (All partially purified)			≠ concentrations: (1,1,1)-kestopentaose > inulin from *C. intybus* > native fructan > (6,6,6)-kestopentaose		- 5,6 mM for (1,1,1)-kestopentaose - 17 mM for native fructan (DP 30) - 29 mM for (6,6,6)-kestopentaose	
Lolium temulentum	Primary leaves	- Crude extract	/ / 4.8 - 5.5	Not possible to measure[a]	All substrates 1%: HMW native fructan[h] > phlein from *D. glomerata* > fructan from *A. sativa* > levan from *P. pratense*> inulin from *H. tuberosus* > inulin from *C. intybus* > inulin from *Dhalia sp*.		- 37% (w/v) for *C. intybus* inulin - 24% for *L. temulentum* fructan	Simpson *et al.*, 1991

Table I.2a continued. Summary of biochemical properties of fructan exohydrolases purified (partially or to homogeneity) from monocotyledonous species

Source of enzyme	Plant parts	Purification + isoforms	- Mr (kDa) - Temp. opt. (°C) - pH optimum	Activity against sucrose	Activity against different substrates	Sucrose inhibition (substrate)	Km with corresponding substrate	reference
Lolium temulentum		- Partial purification [a] : fraction B	/ / /	Low	Tested only with 5% inulin from *C. intybus*	Inhibition (≠ concentrations inulin from *C. intybus*)		
Lolium perenne	stubble	Purification to homogeneity	65 40 5.1 – 5.6	Low	All substrates 5 mM: 6-kestotriose > 6,6-kestotetraose > 1&6-kestotetraose > 6^G-kestotriose > fructan from *T. aestivum* (DP>7) > 1,1-kestotetraose > 1-kestotriose	Inhibition (1 and 5 mM 6,6 - kestotetraose)	No K_m possible[k] (6,6-kestotetraose)	Marx *et al.*, 1997a
Avena sativa	Lower 2 nods + internode of main culms and tillers	Purification to homogeneity	43 / 4.5 – 5	Not detectable	- All substrates 1.25 %: 6^G,6-kestotetraose >> inulin from *C. intybus* > levan from *A. levanicum* -more in detail with native fructans (0.1%): 6^G,6-kestotetraose > 6^G,1&6-kestopentaose >> 1&6^G-kestotetraose > 6^G-kestotriose	n.d.[d]	- 2.8 % (w/v) for neokestin DP 7-14 - 5.6 % (w/v) for 6^G,6-kestotetraose	Henson and Livingston, 1996
Triticum aestivum	Senescing wheat stems	Purification to homogeneity (2 fractions) 1-FEH w1 1-FEHw2	70 30-40 4.5-5.5	Very low	1-kestose > 1,1-nystose > inulin from *C. intybus* >> low M_r levan > neokestose > 6-kestose[b]	Inhibition K_i = 0.9 mM (inulin 12 mM)	- 7 mM for 1-kestose - No K_m possible[k] for inulin	Van den Ende *et al.*, 2003a
	Spike tissue	Purification to homogeneity 6-FEH	70	Very low	All substrates 1 mM: 6-kestose > levan (DP 72) > phlein > levanbiose > neokestose >>> 1-kestose, inulin and 1,1-nystose	No inhibition (1 mM levan)	n.d.[d]	Van Riet *et al.*, 2006

Table I.2a continued. Summary of biochemical properties of fructan exohydrolases purified (partially or to homogeneity) from monocotyledonous species

Source of enzyme	Plant parts	Purification + isoforms	- Mr (kDa) - Temp. opt. (°C) - pH optimum	Activity against sucrose	Activity against different substrates	Sucrose inhibition (substrate)	Km with corresponding substrate	reference
Triticum aestivum	Wheat crowns, stems and ears at anthesis stage	Partial purification 6-KEH	72	Very low	All substrates 5mM: 6-kestose >>> inulin, levan and sucrose	n.d.[d] for native enzyme recombinant enzyme: no strong inhibition (4 mM 6-kestose)	n.d.[d] for native enzyme recombinant enzyme: 29 mM for 6-kestose	Van den Ende *et al.*, 2005b

[a] due to working with a crude extract, invertase enzymes are still present; [b] concentration not mentioned; [c] pyrogen-free inulin (Dahlin : Alanin) supplied by Nutritional Biochemical crops, plant source not specified; [d] not determined; [e] FEH not yet purified till homogeneity; [f] supplied by Merck, plant source not specified; [g] this DP is an assumed value; [h] linkages not mentioned or determined in this article; [i] assumed to be β-2,6-linked (Sims *et al.*, 1992); [j] β-2,1 backbone with β-2,6-linked chains; [k] No K_m possible for the indicated substrate because of the almost linearly increasing velocity. Use of higher substrate concentrations was impossible because of solubility problems or low availability.

Table I.2b Summary of biochemical properties of fructan exohydrolases purified (partially or to homogeneity) from dicotyledonous species

Source of enzyme	Plant parts	Purification + isoforms	- Mr (kDa) - Temp. opt. (°C) - pH optimum	Activity against sucrose	Activity against different substrates	Sucrose inhibition (substrate)	Km with corresponding substrate	reference
Taraxacum officinale	roots	Partial purification [a]: Hydrolase A Hydrolase B	/ / 4	Not detected	All substrates 4% and (DP7-14) *H. tuberosus* oligosaccharides > *T. officinale* oligosaccharides > *C. intybus* oligosaccharides	No inhibition (4 mM inulin[b])	15,4 mM for inulin (MW 5600)[c]	Rutherford and Deacon, 1972
Helianthus tuberosus	tubers	Partial purification [a]: Hydrolase A Hydrolase B	/ / A: 5; B: 5.2	Very low	Inulin from *C. intybus* >> levan from *B. subtilis*[d]	B more inhibited than A (10 mM inulotetraose)	Hydrolase B: - 33 mM for 1-kestose - 33 mM for inulin (MW 5000)[c] - 22 mM for inulopentaose	Edelmann and Jefford, 1964
	tubers	Purification to homogeneity	75 ± 5 / 5.2	Not detected	All substrates 5 mM 1,1,1-kestopentaose > inulin oligomers from chicory (DP>7) > 1,1-kestotetraose > 1-kestotriose > inulobiose and (1&6)-kestotetraose > 6,6-kestotetraose, 6-kestotriose and 6_G-kestotriose	Inhibition (2,5 and 5 mM 1,1-kestotetraose)	- 8.2 mM for kestotetraose - 12 mM for kestopentose	Marx *et al.*, 1997b
Cichorium intybus	roots	Purification to homogeneity = 1-FEH I	72 / 5	Not detected	1% inulin from *C. intybus* >> levan from *A. levanicum*[d,e]	Low inhibition (inulin[d])	6% for inulin (DP 25[c]) → 15mM	Claessens *et al.*, 1990
	Forced roots	Purification to homogeneity = 1-FEH II	60 35 5.5	Not detected	All substrates 1 mM: inulotetraose > 1-kestose > inulin (DP>10) > 1,1-nystose >> levan oligosaccharides and levanbiose	Inhibition K_i = 5.9 mM (3 % inulin)	- 58 mM for 1-kestose - 64 mM for 1,1-nystose - K_m not possible (inulin)	De Roover *et al.*, 1999

Table I.2b continued. Summary of biochemical properties of fructan exohydrolases purified (partially or to homogeneity) from dicotyledonous species

Source of enzyme	Plant parts	Purification + isoforms	- Mr (kDa) - Temp. opt. (°C) - pH optimum	Activity against sucrose	Activity against different substrates	Sucrose inhibition (substrate)	Km with corresponding substrate	reference
Cichorium intybus	Belgian endive leaves + forced roots	Purification to homogeneity = 1-FEH IIa + b	Cfr 1-FEH II	Cfr 1-FEH II	Cfr 1-FEH II	Cfr 1-FEH II	Cfr 1-FEH II	Van den Ende *et al.*, 2001

[a] FEH not yet purified till homogeneity; [b] Supplied by Hophin and Williams Ltd., Chadwell Heath, Essex, U.K.; plant source not specified; [c] this DP or mass is an assumed value; [d] concentration not mentioned; [e] prepared from *Aerobacter levanicum*.

I.3.3.2 Molecular properties of FEHs

At the start of this thesis project only the 3 *FEH* cDNAs from *Cichorium intybus* had been cloned (Van den Ende *et al*., 2000b; Van den Ende *et al*., 2001) (Table I.3). Since then, 7 *FEH* cDNAs from *Triticum aestivum* (Van den Ende et al., 2003a; Van den Ende *et al*., 2005b; Kawakami *et al*., 2005; Van Riet *et a*l., 2006; Van Riet, unpublished results) have been published, including two specific 6-KEHs and a (6&1)-FEH. A *1-FEH* cDNA from *Campanula rapunculoides* has also been cloned (K. Le Roy, unpublished results). The functionality of these cDNAs has been proven by either heterologous expression and/or by purification of the corresponding native protein (supported by Q-TOF analysis on tryptic fragments of the protein). Chalmers *et al.* (2005) mentions the cloning of a *Lolium perenne 1-FEH* (DQ073968). In the *EMBL Database* a partial cDNA of a *Lolium perenne 1-FEH* (AY693396) and a full length cDNA of a *Hordeum vulgare 1-FEH* (AJ605333) are also identified as putative FEHs. However, the functionality of these cDNAs remains to be confirmed. The iso-electric points (pI) of the deduced amino acid sequences vary from 4.8 to 6.5 and their deduced molecular weight (non glycosylated) is around 60 kDa.
Recently, the whole gene for *1-FEH IIa* from *C. intybus* has been cloned (Michiels *et al.,* 2004). Like plant invertase genes, the gene has seven exons, including the DPN mini-exon.

Table I.3 Summary of cloned cDNAs from FEHs

Plant species	Enzyme + accession number	pI (pH)	cDNA-derived Mr (kDa)	Length of ORF	Potential glycosylation sides (N-X-S/T)	Confirmation heterologous expression	Reference
Cichorium intybus	1-FEH I AJ242538	6.5	60.5	568	10	*Solanum tuberosum*	Van den Ende *et al.*, 2000b
	1-FEH IIa AJ295033	5.24	61	581	3	*Pichia pastoris*	Van den Ende *et al.*, 2001
	1-FEH IIb AJ295034	5.24	61	581	3	No	Van den Ende *et al.*, 2001
Triticum aestivum	1-FEH w1 AJ516025	4.79	61.2	597	4	No	Van den Ende *et al.*, 2003a
	1-FEH w2 AJ5058387	4.78	61.2	596	4	No	Van den Ende *et al.*, 2003a
	1-FEH w3 AJ564996	4.88	61	596	4	*Pichia pastoris*	Van Riet, unpublished results
	6-FEH AM075205	6.5	65	590	5	*Pichia pastoris*	Van Riet *et al.*, 2006
	6-KEH w1 AB089271	4.91	61	589	5	*Pichia pastoris*	Van den Ende *et al.*, 2005b
	6-KEH w2 AB089270	4.88	61	587	5	*Pichia pastoris*	Van den Ende *et al.*, 2005b
	6&1 FEH AB089269	5.2	61	595	5	*Pichia pastoris*	Kawakami *et al.*, 2005
Campanula rapunculoides	1-FEH AJ509808	5.1	61	578	1	*Pichia pastoris*	Le Roy, unpublished results

I.3.3.3 Evolution and structure of FEHs

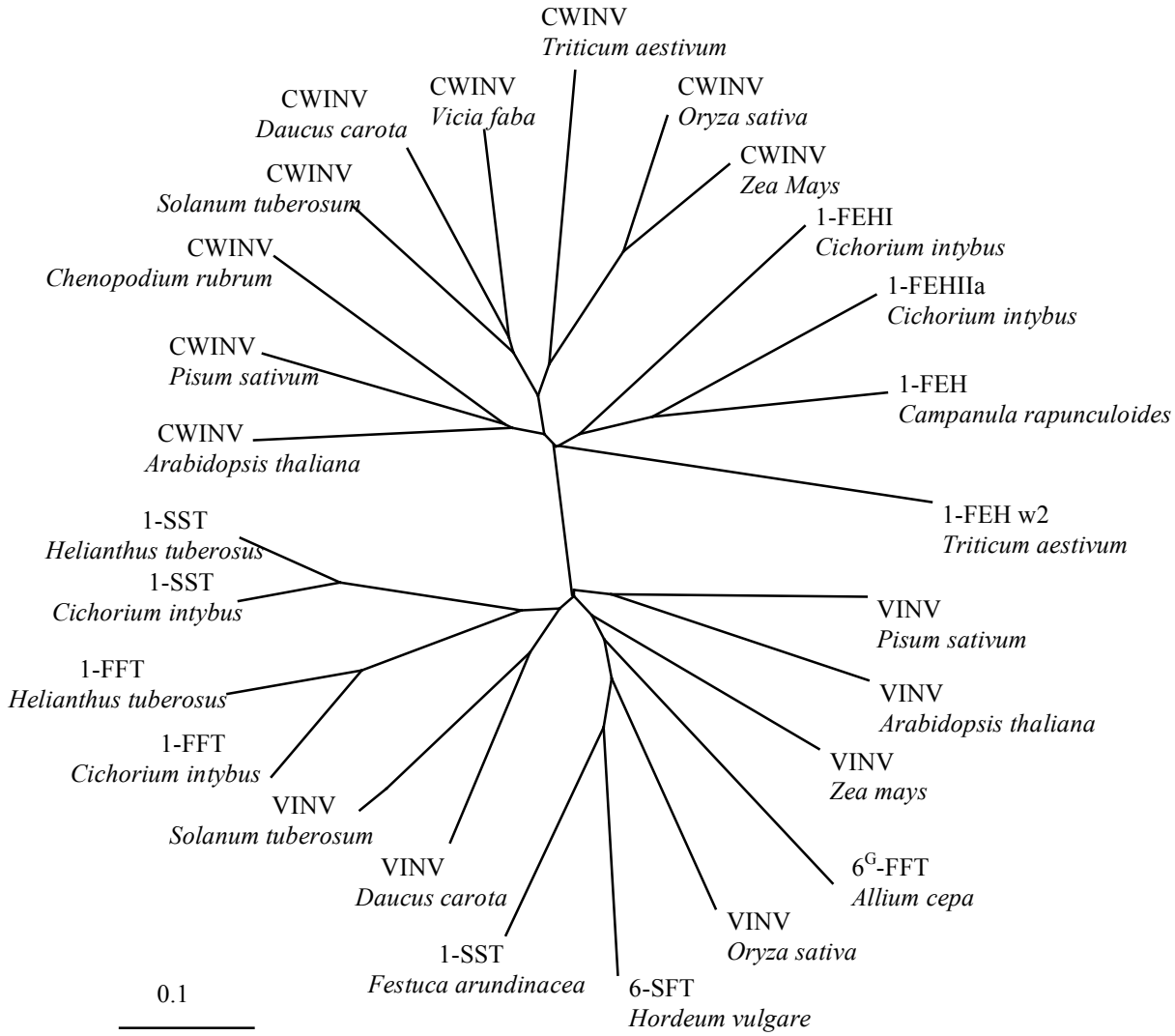


Figure I.5. Unrooted phylogenetic tree of cDNA deduced amino acid sequences of some vacuolar- and cell wall-type invertases, fructan biosynthesis and breakdown enzymes. Accession numbers are between brackets in the legend. VINV, vacuolar invertase; CWINV, cell wall invertase. VINV *Pisum Sativum* (AY112702), VINV *Oryza sativa* (AF276704), VINV *Arabidopsis thaliana* (Q39041), VINV *Daucus carota* (Y18707), VINV *Zea mays* (U16123), VINV *Solanum tuberosum* (L29099), 6-SFT *Hordeum vulgare* (X83233), 6G-FFT *Allium cepa* (Y07838), 1-FFT *Cichorium intybus* (U84398), 1-SST *Cichorium intybus* (U81520), 1-SST *Festuca arundinacea* (AJ297369), 1-SST *Helianthus tuberosus* (AJ009757), 1-FFT *Helianthus tuberosus* (AJ009756), 1-FEHIIa *Cichorium intybus* (AJ295033), 1-FEHI *Cichorium intybus* (AJ242538), CWINV *Arabidopsis thaliana* (X74514), CWINV *Daucus carota* (M58362), CWINV *Pisum sativum* (AF063246), CWINV *Chenopodium rubrum* (X81792), CWINV *Triticum aestivum* (AF030420), CWINV *Vicia faba* (Z35162), 1-FEHw2 *Triticum aestivum* (AJ508387), 1-FEH *Campanula rapunculoides* (AJ509808), CWINV *Oryza sativa* (AB073749), CWINV *Zea mays* (AF050129), CWINV *Solanum tuberosum* (Z22645). The scale bar indicates a distance value of 0.1.

A phylogenetic tree (Fig. I.5) containing some (but not all) deduced amino acid sequences from vacuolar invertases, cell wall invertases, fructan biosynthetic and breakdown enzymes reveals that fructan breakdown enzymes are more related to cell wall invertases (41-63% identical) than to FBE (31–43% identical) (deduced from ClustalW results). This finding was quite surprising because FEHs are supposed to be localized in the vacuole (Frehner *et al.*, 1984; Wiemken *et al.*, 1986) and, in contrast to FEHs, most cell wall invertases have a high pI. Plant FEHs apparently evolved from a cell wall invertase-like ancestor gene that later obtained a vacuolar targeting signal (Van Laere and Van den Ende, 2002). It has been suggested that FEH activity can be detected in the apoplast (Livingston and Henson, 1998; Wang and Nobel, 1998), but this needs further support.

All plant fructan enzymes and acid invertases belong to glycoside hydrolase (GH) family 32 in the carbohydrate active enzyme database (http://afmb.cnrs-mrs.fr/CAZY), a classification based on overall amino acid sequence similarities (Henrissat and Davies, 1997). The bacterial levansucrases and invertases are grouped in GH family 68. The first 3-D structure of a plant GH family 32 that has been resolved was that of 1-FEH IIa from *Cichorium intybus* (Verhaest *et al.*, 2005). The crystal structure consists of an N-terminal fivefold β-propeller domain and a C-terminal domain formed by two β-sheets (Fig. I.6a). The active site of the enzyme is located in the β-propeller domain and is composed of three amino acids (two D and one E) (Fig. I.6b) occurring in the highly conserved regions N**D**PNG (D22), R**D**P (D147) and W**E**CPD (E201) of GH family 32. They act as the catalytic nucleophile, the transition-state stabilizer and the general acid-base or proton donor respectively. A cleft at the interface between the N- and C-terminal domains is believed to play a very important role in the binding of inulin (Verhaest *et al.*, 2005). This structure provides a template for all plant members of GH family 32 and recently also the 3-D structure of a cell wall invertase of *Arabidopsis thaliana* (AtCWINV1) was elucidated (Verhaest *et al.*, 2006). These two structures will help us to determine which amino-acids are involved in the different substrate specificity of FEHs and CWINVs.

a) b)

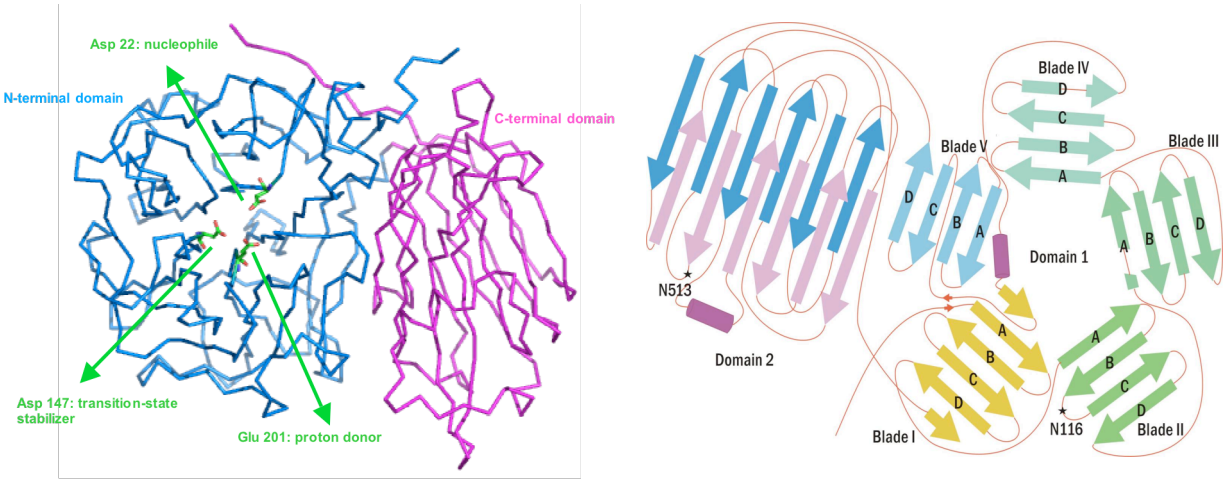


Figure I.6 a) Schematic overview of the active site of 1-FEH IIa. Figure provided by K. Le Roy **b) Schematic diagram of the topology of 1-FEH IIa**. β-strands are depicted by arrows, α-helices by cylinders. Asterixes represent glycosylation sites. Verhaest *et al.*, 2005.

I.3.4 Physiological role of fructans

I.3.4.1 Reserve carbohydrate

The main physiological role of fructans is to serve as a reserve carbohydrate. Fructans accumulate in different plant parts according to the plant species. Whenever needed, fructans are mobilized by FEHs to produce energy and carbon skeletons. Net fructan hydrolysis can occur when consumption in sinks exceeds supply of carbohydrates from photosynthesis. The fructose probably is converted to sucrose for phloem loading or phosphorylated to enter glycolysis.

In the next paragraph, some examples will be given of fructans as a reserve carbohydrate and their need for remobilization:

- In tubers of *H. tuberosus* and rhizophores of *Vernonia herbacea,* 1-FEH activity was high during sprouting in spring. On the other hand, FEH activity was barely detectable during other periods of growth (Marx *et al.*, 1997b; Asega and Carvalho, 2004). A similar result has been seen in *T. officinale*, where flowering was accompanied by an increase in FEH activity (Rutherford and Deacon, 1974).
- An extreme example of remobilization of fructans can be found in the alpine plant *Phippsia algida* during flower development. This small plant is found on late snow-sites. Flower development is very fast; within 3 weeks flowers develop and seeds are produced. When conditions are favourable (this can take several years)

fructan content of the plants with inflorescence primordia drops rapidly during flower development. So, exhaustion of this storage pool during the fast flower and fruit development constitutes an essential part of the plant adaptation to a very short growing season (Solhaug and Aares, 1994).

- Different grasses that are well adapted to grazing and cutting show induction of FEH after defoliation, indicating that carbohydrates for re-growth are supplied by fructan hydrolysis, e.g. in *P. pratense* (Mino *et al*., 1978), *D. glomerata* (Yamamoto and Mino, 1987) and *L. perenne* (Morvan *et al*., 1997).
- Excision of aerial organs of *V. herbacea* and defoliation of young plantlets of *C. intybus* also resulted in fructan breakdown by 1-FEH, establishing a temporary change of the rhizophore and chicory root from a sink organ to a source organ (Asega and Carvalho, 2004; Van den Ende and Van Laere, 2002). Surprisingly, this observation could not be duplicated in mature, field-grown chicory roots. This can probably be explained by the fact that no sprouting occurred after defoliation (because the season is unfavourable) and only minor decreases in the bulk fructan content were necessary for re-growth.
- During the forcing process of chicory roots in the dark, Belgian endive leaves are produced at the expense of root inulin which is degraded by 1-FEHs (Van den Ende and Van Laere, 1996c).
- Damage caused by typhoon winds results in a decrease of fructan levels and increase of 1-FEH activity in the underground reserve organs of *Polymnia sonchifolia* (Yacon) (Fukai *et al*., 1997).
- Mobilization of fructans in wheat stems occurs from approximately 4 weeks after anthesis; the assimilates formed are used as an energy source supporting the kernel development (Willenbrink *et al*., 1998).
- Circadian rhythms in fructan content also occur. Fructans synthesized during the end of the light period in barley primary leaves are immediately mobilized after transition to the dark period (Sicher *et al*., 1984).

I.3.4.2 Other possible functions

As noticed decades ago plant species capable of fructan biosynthesis did not lose their capacity for starch metabolism, not even within the same organ. E.g. similar amounts of fructan and starch occur within the bulbs of *Lachenalia minima* (Orthen, 2001). Starch, however, can not be detected in the tubers of *Helianthus tuberosus*, but is present in the leaves (Pollock, 1986). Why does an alternative for starch exist in plants? Is it possible that fructan accumulation gives an advantage to certain plants? Since the discovery of fructans, these questions have been addressed by different authors. They all agree that the role of fructans is wider than just storage, but untill now, no clear-cut answers could be given.

The presence of fructosyltransferases and fructans in the vacuole has been shown convincingly (Wagner and Wiemken, 1986; Frehner *et al*., 1984). Vacuolar fructan synthesis could lower the sucrose concentration in the cytosol and prevent sucrose-induced feedback inhibition of photosynthesis. In leaves, fructan accumulation can reach levels up to 70% of the dry weight without inhibiting photosynthesis. Hendry (1987) studied the carbohydrate content of 130 British species. Although fructan was only detected in 20 of those species, 9 of these appeared in the top 12 with the highest reserve carbohydrate content. Only three species accumulated starch to anything approaching the concentration of fructans.

Fructan metabolism may affect the tolerance of plants to cold, frost or drought. Starch biosynthesis diminishes strongly when temperature drops below 10°C. Fructan production, however, is less sensible to low temperatures (Pollock, 1986). So, fructan accumulation would be advantageous for plants that stay photosynthetically active during winter or early spring. 1-FEH IIa from *C. intybus* is induced in field-grown roots when temperature drops and in cold-stored roots. Expression patterns of the *1-FEH IIa* promoter-luciferase construct indicates that *1-FEH IIa* is highly expressed in cold-stored roots. This has already been demonstrated in Van den Ende *et al*. (2002) in which the expression of *1-FEH IIa* was investigated by northern blots. Promotor deletion and sequence analysis revealed the presence of cold responsive elements that may be involved in the temperature and tissue-specific control of its expression (Michiels *et al*., 2004). A role for FEH in cold protection is, however, not obvious since depolymerization of vacuolar fructans does not result in a spectacular freezing point depression (0.2 - 0.5°C; Hendry, 1987). Livingston and Henson (1998), however, described the occurrence of fructans with low DP in the

apoplast of *Avena sativa* after second phase hardening. Because of the small volume of the apoplast this might result in a more significant freezing point depression.

An increased tolerance to drought caused by fructans was reported by Pilon-Smits *et al.* (1995), who introduced a bacterial levansucrase into tobacco, a non-fructan plant. These fructan producing transgenic plants were found to be more resistant to PEG-induced drought stress. These results, however, should be interpreted carefully because introducing non-plant fructans might induce a general stress response, resulting in a higher resistance to drought. De Roover *et al*. (2000) subjected chicory plantlets to drought stress and Kerepesi *et al*. (1998) did the same with wheat seedlings. Both authors reported induction of fructan synthesis by such treatment.
Reports on an interaction between lipid monolayers and high molecular weight bacterial levans (Demel *et a*l., 1998) and Dahlia or chicory inulin (Hincha *et al*., 2000), suggest that fructans play an important role in stabilization of membranes during freezing or drying. It is well known that membranes are the primary targets of both freezing and desiccation injuries in cells.

Fructan hydrolysis drives petal expansion in both *Hemerocallis* (daylily) (Bieleski, 1993) and *C. rapunculoides* (Vergauwen *et al*., 2000). Up to the stage of flower opening fructan concentrations in petals were high. During a short period (2-3d), all fructan is degraded. The high sugar concentration (mainly fructose) resulting from this breakdown lowered the water potential, and by osmotically attracting water, resulted in rapid expansion of the petals and flower opening.

I.3.5 Heterologous expression

Different systems have been used for heterologous expression of both FBE and breakdown enzymes, depending on goals envisaged:

- In order to examine the functionality of cDNAs encoding possible fructan metabolizing enzymes, heterologous expression has often been performed in the methylotrophic yeast *Pichia pastoris*. Hochstrasser *et al.* (1998) was the first study using this system for a 6-SFT from *Hordeum vulgare*. The recombinant enzyme, however, showed a pronounced additional 1-SST activity when incubated with sucrose. Other enzymes expressed in *P. pastoris* were e.g. 1-SST from *Festuca arundinacea* (Lüscher *et a*l., 2000b), a 1-SST, 6-SFT and 1-FFT from *Triticum aestivum* (Kawakami and Yoshida, 2002; 2005), a 1-SST from *Lolium perenne* (Chalmers *et al*., 2003), 1-FFTs from *Viguera discolor* and *Echinops ritro* (Van den Ende *et al*., 2005a,

2006) and a 6^G-FFT from *Asparagus officinalis* (Ueno *et al.*, 2005). Not all recombinant enzymes were compared to their native enzymes, so it is not always possible to conclude if an additional side-activity for the recombinant enzymes is reminiscent of the native properties. However, additional side-activity should be considered when performing mutational analysis on the recombinant enzymes. Recently, this expression system has also been used for breakdown enzymes such as 1-FEH IIa from *Cichorium intybus,* a 6-KEH, 6-FEH and (6&1)-FEH from *Triticum aestivum* (Verhaest *et al*., 2005; Van den Ende *et al*., 2005b; Van Riet *et al*., 2006; Kawakami *et al*., 2005). Another approach to investigate the functionality of the cDNAs is heterologous expression in tobacco protoplasts or in other non-fructan accumulating plants. These methods are time-consuming but probably more accurate because the expression and posttranslational modifications (e.g. glycosylation) can be expected to be more plant-like: e.g. 1-FFT from *H. tuberosus* has been introduced in tobacco protoplasts (Hellwege *et al*., 1998) and 1-FEH I from *C. intybus* has been expressed in potato tubers (Van den Ende *et al.,* 2000b).

- In order to study possible advantages of fructan storage and to understand their physiological role in stress resistance, biosynthetic enzymes have been introduced in non-fructan accumulating plants. An increased tolerance to drought was reported by Pilon-Smits *et al*. (1995) who introduced a bacterial levansucrase into tobacco plants, a non-fructan plant. However, the use of plant fructan enzymes is preferable to investigate stress resistance. Not much research has been done on this topic yet. Recently, Hisano *et al*. (2004) reported that transgenic *Lolium perenne* expressing wheat fructosyl-transferase genes accumulated increased amounts of fructan and showed increased tolerance to freezing.
- Introducing FBE in non-fructan accumulating plants might be used for the production of fructans for industrial purpose. Transformed sugar beet has been used to produce low-DP inulin-type fructans (Sévenier *et al*., 1998). In the taproot of sugar beet transformed with 1-SST from *H. tuberosus*, the stored sucrose was almost completely converted into low molecular weight fructans. The specific advantage of fructan oligosaccharides over sucrose is that it cannot be degraded by human digestive enzymes, it stimulates growth of bifidogenic bacteria and it has a lower caloric value.

I.3.6 Health aspects of fructans

Although not directly related to the research reported here, the use of fructans in food applications is worth mentioning. Inulin and oligofructose have been recognized as a beneficial food ingredient because they have prebiotic qualities. The term oligofructose is used for a mixture of small inulins with an average DP of 4, varying from DP 2 to 20. They have a sweet taste whereas longer inulin chains (with an average DP of 25, varying from 11 to 60) form emulsions with a neutral taste and a similar texture as fat, but with much lower caloric value (Roberfroid, 2005). Therefore inulin is used as a low-calorie replacement for fat in e.g. yoghurts, chocolate and ice-cream.

Inulin and oligofructose escape digestion in the upper gastrointestinal tract and reach the large intestine virtually intact. There they are fermented by several bacteria encouraging the growth of beneficial bacteria such as *Lactobacilli* and *Bifidobacteria* species. As a consequence, the human intestinal pathogenic bacteria become less abundant (Gibson *et al.*, 2005). The trisaccharide neokestose even seems to be a better prebiotic than chicory inulin (Kilian *et al*., 2002). Predominance of *Lactobacilli* and *Bifidobacteria* species in the gut is important for the prevention of many diseases and for maintaining good health. The short chain fatty acids (SCFA) produced by bacteria e.g. improve the absorption of calcium in the colon and this may be useful in the prevention of osteoporosis (reviewed in Coxam, 2005).

I.4 Fructans in micro-organisms

Fructans are produced by many different gram-negative bacteria, gram-positive bacteria and fungi including yeasts. Literature on fructans and fructan-metabolizing enzymes in micro-organisms is vast and quickly accumulating. In this chapter only a short introduction on this topic is presented.

I.4.1 Bacterial fructans

I.4.1.1 Synthesis of fructans in bacteria

Numerous bacterial species synthesize levan, a β-2,6 linked polyfructan but with frequent β-2,1 linked branching points, from exogenous sucrose by the action of extracellular levansucrase (EC 2.4.1.10). In contrast to the need for at least two enzymes in plants to produce fructans (1-SST and 1-FFT, 6-SFT or 6^G-FFT), this enzyme catalyzes the transfer of the fructosyl unit of sucrose to a number of acceptors including sucrose, water (resulting in hydrolysis) and fructan polymers. The size of bacterial levans varies from 20 kDa to several MDa. Levansucrases were found e.g. in the gram-negative bacteria *Bacillus subtilis* (Dedonder, 1966), *Erwinia amylovora* (Geier and Geider, 1993), *Pseudomonas syringae pv. glycinea* (Li and Ullrich, 2001), *pv. phaseolicoa* (Hettwer et al., 1995), *Paenibacillus polymyxa* (Bezzate *et al*., 2000), *Gluconacetobacter diazotrophicus* (Arrieta *et al*., 1996), *Zymomonas mobilis* (Lyness and Doelle, 1993) and in the gram-positive bacteria *Streptococcus salivarius* (Simms *et al*., 1990) and *Lactobacillus reuteri* (van Hijum *et al*., 2004). Levansucrases show a substrate-independent expression. Bereswill *et al*. (1997) however demonstrated a temperature-dependent expression of levansucrase for some natural *E. amylovora* strains deficient in levan synthesis.

Few bacteria, all belonging to the gram-positive bacteria, synthesize inulin. The enzyme responsible for this synthesis is inulosucrase (EC 2.4.1.9). Inulosucrases have been found in e.g. *Lactobacillus reuteri* (van Hijum *et al.,* 2003; Ozimek *et al*., 2005) and *Leuconostoc citreum* (Olivares-Illana *et al*., 2002).

The degree of branching of fructans can differ enormously between bacterial species. *Lactobacillus reuteri* produces a levan with a low degree of branching while most levans, such as those of *Streptococcus salivarius,* contain up to 30% β-2,1 branches. *Streptococcus mutans* Ingbritt A strain produces a fructan which contains only β-2,1 linked

fructose units (Baird *et al.*, 1973) while *Streptococcus mutans* JC-2 produces inulin with 5% β-2,6 branches (Rosell and Birkhed, 1974).

I.4.1.2 Breakdown of fructans in bacteria

Levan and inulin can be broken down by bacterial levanases and inulinases, respectively. Most levanases function as fructose- or levanbiose-producing exohydrolases. Fructose-releasing levanases (EC 3.2.1.65) have been identified from e.g. *Bacillus subtilis* (Wanker *et al.*, 1995) and *Paenibacillus polymyxa* (Bezzate *et al.*, 1994). In addition to levan these enzymes are also capable of hydrolyzing inulin, raffinose and sucrose to a certain extent. Levanbiose releasing levanases (EC 3.2.1.64) e.g. in *Pseudomonas* sp No. 43 (Kang *et al.*, 1999) and *Streptomyces exfoliates* F3-2 (Saito *et al.*, 2003) hardly hydrolyze these alternative substrates. A levan-heptaose producing exolevanase and a levanoctaose-producing exolevanase, with absolute substrate specificity, were secreted by *Pseudomonas* K-52 and *Streptomyces* sp. 366L, respectively (Kang et al., 1998; Lim *et al.*, 1998). An endo-levanase from *Bacillus* sp. No. 71 splits levan more randomly to yield levanheptaose and oligomers with higher degrees of polymerization.
Because inulin is not the predominant fructan in bacteria (in contrast to fungi) not much research has been done on inulinases. An exo-inulinase (EC 3.2.1.80) and endo-inulinase (EC 3.2.1.7) are respectively secreted by *Bacillus* sp snu-7 and *Paenibacillus* sp CDB 300 (Kim *et al.*, 2004; Gern *et al.*, 2001). Bacteria of the genus *Arthrobacter* are reported as inulin-fructotransferase producers (inulinase II), which is an enzyme used to produce difructose-dianhydrid.

In contrast to plant fructosidases (invertases and FEHs) and microbial fructanases (inulinases and levanases) that are grouped in the glycoside hydrolase family 32 (GH32), levansucrases and several invertases from bacterial origin are very different and are clustered in the glycoside hydrolase family 68 (GH68) (http://afmb.cnrs-mrs.fr/CAZY/).

I.4.1.3 Function of fructans in bacteria

Fructans from bacteria might perform different physiological roles:

- Levans from e.g. *Pseudomonas syringae* and *Erwinia amylovora* are thought to play a role in the plant-pathogen interaction. Together with other exopolyssacharides (EPS), they can enhance bacterial survival by generating a hydrogenated matrix to minimize host recognition or by providing a detoxifying

barrier against plant defense compounds. Additionally, EPS formation may protect bacterial cells from desiccation and improve attachment to surfaces (Li and Ullrich, 2001). On the other hand the simple sugar structure of levan may not allow an effective protection of bacteria against plant defenses (Bereswill *et al*., 1997). The formation of levan from leaking plant sucrose by an extracellular levansucrase provides the bacteria with glucose, which can be immediately be used as a carbon source.

- Disruption of the *Paenibacillus polymyxa* levansucrase gene impairs its ability to aggregate soil in the wheat rhizosphere. So, levan is implicated in the aggregation of root-adhering soil on wheat, which is very important for growth and nutrition of the plant (Bezzate *et al*., 2000).
- A variety of oral bacteria e.g. *Streptococcus mutans* and *salivarius* utilize sucrose to produce fructans. The ability to synthesize these polymers within oral biofilms is believed to allow organisms to capture a greater proportion of dietary sucrose by converting it to a non-diffusing, extracellular storage polysaccharide which can be catabolized when exogenous sources are lacking (Burne *et al*., 1999).

I.4.2 Fructans in fungi and algae

Some fungi such as *Aspergillus*, *Penicillium* and *Fusarium* species have been reported to produce either low- or high-molecular mass inulin. Little is known about the physiological significance of fructans in fungi.

A *1-SST* with the highest sequence homology to levanases was cloned from *Aspergillus foetidus* (Rehm *et al.,* 1998). Heyer and Wendenburg (2001) cloned an oligosaccharides producing fructosyltransferase from *Aspergillus sydowi*, with very low homology to other fructosyltransferases.

Because of their possible industrial importance much more research has been done on inulinases. Fungal inulinases can be divided into endo- or exo-acting enzymes. 'Exo-inulinase' splits the terminal fructosyl linkage present in sucrose, raffinose and inulin, so this enzyme can be defined as a β-fructofuranosidase. In contrast endo-inulinases are specific for inulin and hydrolyze the internal linkages in inulin to liberate inulotriose, -tetraose, and -pentaose as the main products (Akimoto *et al*., 2000). Both enzymes have been reported in the same species e.g. *Aspergillus niger* and *Penicillium purpurogenum*. It has been suggested that exo- and endo-inulinases evolved independently (Moriyama *et al*., 2003).

Inulin has been widely investigated as a source for the production of fructose-syrups through enzymatic hydrolysis by either the sole action of exo-inulinase or the synergic action of exo-and endo-inulinases (Nakamura *et al.*, 1995). Fructose formation from inulin instead of starch offers advantages since it involves only a single enzymatic step yielding up to 95% fructose (Pandey *et al.*, 1999). Endo-inulinases can produce fructose-oligosaccharides (FOS), which have been proven to be beneficial for human health.

Fructans have also been detected in some green algae such as species from the Cladophorales and Dasycladales (Hendry and Wallace, 1993).

CHAPTER II

INTRODUCTORY CHAPTER

The work described in this chapter was published as:
Van den Ende W., De Coninck B., Clerens S., Vergauwen R. and Van Laere A. (2003b). Unexpected presence of fructan 6-exohydrolases (6-FEHs) in non-fructan plants: characterization, cloning, mass mapping and functional analysis of a novel 'cell-wall invertase-like' specific 6-FEH from sugar beet (*Beta vulgaris* L.). *Plant J.* 36: 697-710.

II.1 Introduction

Fructans have been found in 12 to 15% of the vascular plants (Hendry, 1987; 1993). Several plants that do not accumulate this polysaccharide have been used for heterologous expression of fructosyltransferases (reviewed in Cairns, 2003; §1.3.5). The complete absence of fructan exohydrolases in those plants has been considered as trivial. This doctoral research proves that this 'certainty' has to be rejected. A first indication of the possible occurrence of FEHs in non-fructan plants was found during a heterologous expression experiment in which potatoes were transformed with a cDNA coding for *1-FEH*. Low but measurable 1-FEH activities were detected in potatoes transformed with an empty vector. At first, this was ignored as a side activity of ubiquitous invertase enzymes. However, some further observations enticed our curiosity:

- High levels of FEH activity where found in etiolated leaves of *Cichorium intybus* (a fructan plant), while fructans were absent in this tissue (Van den Ende *et al.*, 2001).
- A phylogenetic tree of CWINVs and FEHs (see further § III.2.1.3, Fig. III.3) demonstrates that some CWINVs are more related to FEHs than to other CWINVs e.g. a putative invertase from *Arabidopsis thaliana* (AtcwINV6), from *Beta vulgaris* (CWINV2) and from *Cichorium intybus*. Moreover, these putative CWINVs show a low pI like all known FEHs.

These findings led us into starting a project with the aim of finding out if there are indeed genuine FEHs in non-fructan plants. In this introductory chapter some results prior to this work obtained by various people in the lab (Van den Ende *et al.,* 2003b) are given in order to situate this doctoral study. *Beta vulgaris* (sugar beet) was initially chosen to work with because the taproot of this plant is an ideal bulky material for protein purification purposes and it contains a 'putative CWINV' (CWINV2) closely related to FEHs.

II.2 Results

II.2.1 Activity profiles of 6-FEH, 1-FEH and invertase in *Beta vulgaris*

To get more insight in the activity profiles of the enzymes in sugar beet, 6-FEH, 1-FEH and invertase activities were measured in the taproots (Fig. II.1a), petioles (Fig. II.1b) and leaf parenchyma (Fig. II.1c) throughout the growing season on the field (June-October).

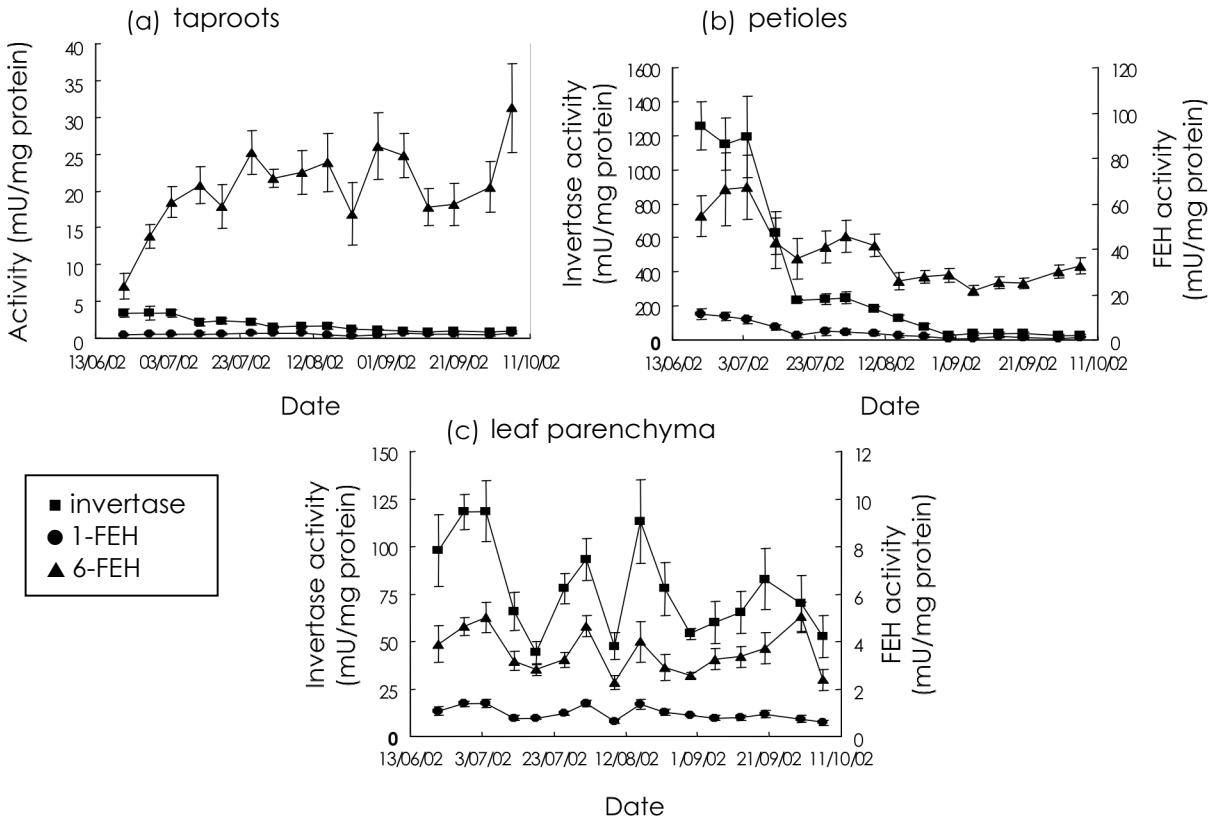


Figure II.1 Activity profiles of 6-FEH (▲), invertase (■) and 1-FEH (●) in sugar beet taproots (a), petioles (b) and leaf parenchyma (c) throughout the growing season. Note the different scales for invertase and FEH in (b) and (c).

Except for the first sampling date, the 6-FEH activity in the taproot was much higher than 1-FEH and invertase activities. Very high invertase activities were detected in the petioles of young plants, but these activities strongly decreased during the growing season (Fig. II.1b). The 6-FEH activity in the petioles was much lower than the invertase activity, but still much higher than the 1-FEH activity. In the leaf parenchyma, invertase and 6-FEH activities greatly fluctuate together (Fig. II.1c), with FEH activities about 20 times less than invertase activities. Most probably, the observed 6-FEH activities in leaf parenchyma and

the apparent higher 6-FEH activity in young petioles are due to a side activity of vacuolar invertases. However, in mature leaf petioles (Fig. II.1b), the 6-FEH/invertase ratio was much higher than in the leaf parenchyma and the 6-FEH activity remained rather constant throughout the growing season at about the same level as observed in taproots.

II.2.2 Purification of a 6-FEH enzyme from sugar beet taproots

II.2.2.1 Purification procedure

As deduced from above results, the best material to purify 6-FEH is the sugar beet taproot. The purification procedure (Materials and Methods § VII.2.4.1) was based on $(NH_4)_2SO_4$ precipitation, lectin affinity chromatography (Con A), anion- and cation exchange chromatography (Table II.1).

Table II.1 Purification of 6-FEH from 3.5 kg sugar beet taproots. Elution pHs on Mono Q and S are indicated. Activity tested with 2 mM levan.

Purification step	Protein (mg)	Total activity (U)	Recovery (%)	Specific activity (U mg^{-1} protein)	Purification (fold)
Crude extract	246.4	3.91	100	0.016	1
30-80% $(NH_4)_2SO_4$	241.5	4.25	109	0.044	2.8
Con A	14.0	1.20	30.7	0.23	14.2
Mono Q (7.0)	1.95	1.09	27.9	0.56	35.2
Mono S (4.5)	0.045	0.26	6.8	5.87	370
Mono Q (8.5)	0.014	0.13	3.4	9.21	581
Mono Q (8.7)	0.002	0.025	0.64	12.5	788

The total activity slightly increased after $(NH_4)_2SO_4$ precipitation, perhaps because of the removal of an inhibitor. Activity losses occurred after each further purification step. A maximal purification of almost 800-fold was obtained and the enzyme had a high specific activity of 12.5 U mg^{-1} protein. The pure enzyme (Q 8.7 fraction) was used for enzyme characterization (§II.2.2.2) and for electrospray ionization (ESI)-Q-TOF MS analysis (Materials and Methods §VII.2.5), whereas the Q 8.5 fraction was used to visualize protein components on SDS-PAGE (Fig. II.2). The three low molecular weight bands of 19, 20 and 36 kDa were found to originate from a 'putative CWINV', as proven by ESI-Q-TOF MS analysis and they were shown to make up the complete sugar beet 6-FEH (see further §III.2.1.2, Table III.3). The peptides will be used to construct degenerated primers for cloning of the *6-FEH* cDNA (Chapter III).

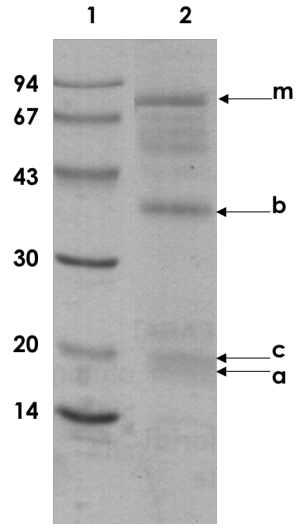


Figure II.2 SDS-PAGE of sugar beet 6-FEH. Lane 1: Molecular mass marker proteins, numbers on the left indicate their mass in kDa. Lane 2: SDS-PAGE of 3 µg of partially purified sugar beet 6-FEH (Mono Q pH 8.5 fraction). Arrows indicate (a) 19, (b) 20 and (c) 36 kDa polypeptides. The 75 kDa band fragment (m) is not related to 6-FEH and peptide analysis points to an α-mannosidase.

II.2.2.2 Native 6-FEH substrate specificity and properties

The native sugar beet 6-FEH did not hydrolyze sucrose (Table II.2) and no other products than fructose were found after using levan as substrate, indicating that the enzyme is indeed a fructan exohydrolase and not an endohydrolase or invertase. Next, the enzyme was incubated with different fructan substrates (Table II.2). It most efficiently hydrolyzed fructans with β-2,6 linkages: levan, phlein, neokestose, 6-kestose and levanbiose are the best substrates while β-2,1-type fructans are poor (1-kestose) or no substrates at all (1,1-nystose and inulin). Therefore, the enzyme can be designated as a real fructan 6-exohydrolase (= Bv6-FEH).

Table II.2. Substrate specificities of the native sugar beet 6-FEH. Results are shown as values relative to the activity with the substrate levan. The substrates were used at a concentration of 1 mM.

Substrate	DP	Native activity (%)
sucrose	2	<< 1
levanbiose	2	63
1-kestose	3	12
6-kestose	3	77
neokestose	3	93
1,1-nystose	4	<< 1
phlein	4-12	84
inulin	>10	<< 1
levan	± 72	100

The purified Bv6-FEH has a pH optimum around 5.0 (Fig. II.3a) and a temperature optimum around 30°C (Fig. II.3b). Sucrose is not a strong inhibitor of the enzyme (Fig. II.3c), a K_i of 134 mM with 10 mM levan was estimated. It was not possible to use levan as substrate for K_m determination because of the lower solubility of this substrate. With 6-kestose as substrate, a K_m value of 77 mM was obtained (Fig. II.3d).

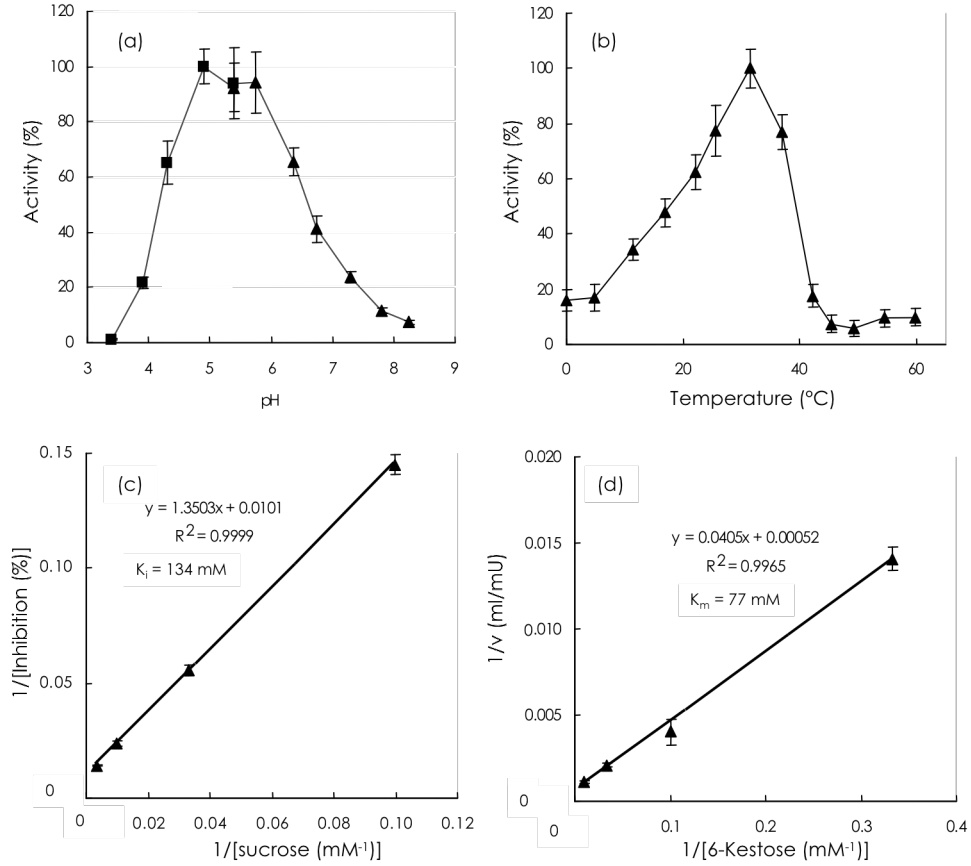


Figure II.3 Properties of the native 6-FEH. (a) Effect of pH on the activity of purified sugar beet 6-FEH. 6-FEH was incubated for 1h with 2 mM levan at different pH values using 100 mM Na-acetate buffer (■) and Na-phosphate buffer (▲). Activity is expressed as a percentage of the maximal activity. (b) Effect of incubation temperature on the activity of sugar beet 6-FEH. 6-FEH was incubated with 2 mM levan for 1 h at different temperatures between 0 and 60°C. (c) A plot of the percentage of inhibition of sugar beet 6-FEH as a function of the sucrose concentration. The fitted linear regression line and parameters are indicated. From this, a K_i was derived. (d) A plot of 6-FEH activity against the concentration of 6-kestose. The fitted linear regression line and its parameters are presented. The K_m value was estimated based on these parameters.

In conclusion:

A 6-FEH activity was detected in sugar beet taproots and the enzyme was purified and characterized. We remained cautious about the existence of specifc 6-FEHs in non-fructan plants as plant endophytes or bacterial pathogens also could produce 6-FEHs. However, the inability of the enzyme to hydrolyze sucrose (in contrast to bacterial FEHs) and the homology of the peptides, yielded by Q-TOF MS, with plant CWINVs and FEHs proved the existence of a specific plant 6-FEH in sugar beet. Results in this chapter will be discussed in Chapter III, together with the cloning and heterologous expression of the corresponding *6-FEH* cDNA.

CHAPTER III

CLONING AND HETEROLOGOUS EXPRESSION OF A 6-*FEH* CDNA FROM *BETA VULGARIS*

The work described in this chapter was published as:
Van den Ende W., De Coninck B., Clerens S., Vergauwen R. and Van Laere A. (2003b). Unexpected presence of fructan 6-exohydrolases (6-FEHs) in non-fructan plants: characterization, cloning, mass mapping and functional analysis of a novel 'cell-wall invertase-like' specific 6-FEH from sugar beet (*Beta vulgaris* L.). *Plant J.* 36: 697-710.

III.1 Introduction

The occurrence of a specific 6-FEH in non-fructan plants is a completely new fact in the fructan field. In this chapter, the cloning and heterologous expression of the corresponding sugar beet 6-*FEH* cDNA will be described. For heterologous expression the yeast *Pichia pastoris* was used. At the start of this doctoral study, this expression system had been used for fructan biosynthetic enzymes (Hochstrasser *et al.,* 1998; Kawakami and Yoshida 2002). At that moment, our laboratory successfully expressed the first fructan breakdown enzyme in *P. pastoris*, namely 1-FEH IIa from *Cichorium intybus* (Verhaest *et al.*, 2004).

III.2 Results

III.2.1 Cloning and heterologous expression of the *6-FEH* cDNA

III.2.1.1 Cloning strategy

An RT-PCR-based strategy (Materials and Methods §VII.4.1) was used for cloning the *6-FEH* cDNA. The primers used were based both on internal 6-FEH peptides generated by Q-TOF MS and on conserved regions. The 3' part was cloned with an oligo dT-based primer and the missing 5' part was cloned by PCR on a sugar beet root cDNA library by combining vector and 6-FEH specific primers. After sequencing of the 5' and 3' cDNA parts, final primers were used to amplify the whole cDNA (Fig. III.1) by RT-PCR. The tryptic peptides retrieved from the purified 6-FEH covered 31% of the cDNA derived amino acid sequence, with an identity of 100% (Table III.1 and Fig. III.1). The cDNA coding region is preceded by a short 53 bp 5' untranslated part and followed by a longer 141 bp untranslated 3' region. The sugar beet *6-FEH* cDNA contains a single ORF of 606 codons. Comparison of the cDNA-derived amino acid sequence with the presumptive N-terminal sequence of the mature sugar beet 6-FEH enzyme (Table III.1), demonstrates that the primary translation product has a 50 amino acid signal peptide, which is post-translationally removed. The estimated pI of the polypeptide encoded by the *Bv6-FEH* was calculated at 5.0 which is in agreement with its behavior on anion and cation exchange columns (§II.2.2.1). The derived polypeptide of the *Bv6-FEH* cDNA contains 9 potential glycosylation sites (N-X-S/T). Extensive glycosylation might explain the observed difference between the cDNA-derived M_r (62.9 kDa) and the M_r of 75 kDa (19+36+20) as estimated from SDS-PAGE.

MAPNNGSWLVLSISMMLLSHGMIIIAKDQAIHHHDDDHDDMLINDHQMINDDDP***YRTAYHF***QSPKNWM***NDPN***GPM
IYKGIYHLFYQYYPYD***PVWHTEI***VWGHSTSTDLINWTQQPIALSPSEPYDINGCWSGSITILPQNKPVILYTGIN
NKNYQVQNLALPKNLSDPYLKEWIKLPQNPLMAGTPTNNNNINASSFRDPSTAWQLSDGKWRVIVGTQQGKRGLA
VLFTSDDFVKWNNTGNPLHSTEGNGI***WE***C***PD***FFPVYVGKSLGADTS***IIGDDVK***HVLKLSLFDTQYEYYTI***GRYDI***
EKDIYVPDEGSIESDLGLRYDYGKFYAS***KSFFDDE***TNR***RILWGW***VNESSIQADDIKKG***WSGVQAI***PRTVVLDKSG
KQL***VQWPLAE***VDMLRENDVELPSQVIKGGSLVEISQITASQADVEISFKIPESNYVEELDSTCTNPQILCSQKGA
SIKGRFGPFGLLTLASMGLEEYTAVFFRIFKGPNKYVVLMCSDQTRSSLNPTTDKLSFGIFVDVDPINEDLSLRI
LIDHSIVESFSAKGKSCITARVYPTMAINDKAKLYV***FNNGT***EDVKITKLSAWSMKKAQINLSTDNTSNMSYNSNK
VEKEEF

Figure III.1 Deduced amino acid sequence of the *6-FEH* cDNA from sugar beet. Potential glycosylation sites are underlined. The peptide regions where the cloning primers were chosen are indicated in bold and italics (§VII.4.1). The N-terminal amino acid of the mature protein is indicated in grey. The three carboxylic acids that are believed to be crucial for enzyme activity are boxed.

III.2.1.2 Heterologous expression of Bv6-FEH cDNA in Pichia pastoris

Next, the *Bv6-FEH* cDNA was heterologously expressed in the yeast *Pichia pastoris* in order to confirm the functionality of the *6-FEH* cDNA and to compare it with the native enzyme. An expression plasmid containing the mature 6-FEH protein part (starting from DDDPYR) was constructed. After transformation of *P. pastoris* with the expression plasmid and growth of the yeast in an expression medium (Materials and Methods §VII.5.2), the recombinant enzyme was secreted outside the cells. After protein precipitation the recombinant 6-FEH enzyme was subjected to SDS-PAGE and Q-TOF MS in order to confirm the presence of the enzyme. In contrast to the native 6-FEH, the heterologously expressed enzyme is a monomer after SDS-PAGE (data not shown). Table III.I gives an overview of the fragment ions detected in Q-TOF after tryptic digest of the recombinant and native 6-FEH.

Table III.1 Fragment ions detected in Q-TOF after tryptic digest of sugar beet 6-FEH, with calculated matches to theoretical digest of virtual cDNA derived protein and confirmation of identity by tandem MS/MS sequencing. Glycosylation sites NX(S/T) are presented in italics and underlined. t, native purified enzyme, no electrophoresis; a, SDS-PAGE band of 19 kDa; b, SDS-PAGE band of 20 kDa; c, SDS-PAGE band of 36 kDa; h, heterologous expressed enzyme.

Observed mass	Charge state	Calculated mass (presumptive 6-FEH fragment ion)	MS/MS sequence (from N- to C-terminus)
780.4 (a)	2+	780.32 [T1+2H]2+ (partial)	1 DDDPYR 6
1078.6 (t,a,h)	3+	1078.53 [T2+3H]3+	7 TAYHFQSPK 15
1579.8 (t,a,h)	2+	1579.70 [T3+2H]2+	16 NWMNDPNGPMIYK 28
1595.8 (a)	2+	No match	16 NWMoxNDPNGPMIYK 28 and 16 NWMNDPNGPMoxIYK 28 (T3 derived)
1287.8 (t,a,h)	2+	1287.71 [T5+2H]2+	103 NYQVQNLALPK 113
2323.0 (a)	3+	No match	T6 N-linked glycopeptide: 114 *NLS*DPYLK 121 (Pen)1 (HexNAc)1 (Deo)1 (Hex)3 (HexNAc)2
1958.0 (a)	3+	No match	T6 N-linked glycopeptide: 114 *NLS*DPYLK 121 (Pen)1 (Deo)1 (Hex)2 (HexNAc)2
2976.4 (h)	3+	No match	T6 N-linked glycopeptide: 114 *NLS*DPYLK 121 (Hex)7 (Hex)3 (HexNAc)2
4336.3 (h)	3+	No match	T9 N-linked glycopeptide: 126 LPQNPLMAGTPTNNNNI*NAS*SFR 148 (Hex)6 (Hex)3 (HexNAc)2
3085.6 (c)	3+	No match	Partial T9-10 or T9-11 N-linked glycopeptide: 138 NNNNI*NAS*SFRDPSTAWQLSDGK 160 (HexNAc)2 (Pen)1 (Deo)1 (Hex)3 (HexNAc)2 or 141 NI*NAS*SFRDPSTAWQLSDGKWR 162 (HexNAc)2 (Pen)1 (Deo)1 (Hex)3 (HexNAc)2
1304.6 (c,h)	2+	1304.61 [T10+2H]2+	149 DPSTAWQLSDGK 160
1647.1 (c)	3+	1646.79 [T10-11+3H]3+	149 DPSTAWQLSDGKWR 162
1412.0 (c,h)	2+	1411.75 [T14+2H]2+	173 GLAVLFTSDDFVK 185
1391.0 (t,c,h)	2+	1390.71 [T16+2H]2+	215 SLGADTSLLGDDVK 228
1869.0 (t,h)	2+	1868.91 [T18+2H]2+	233 LSLFDTQYEYYTIGR 247

Table III.1 continued. Fragment ions detected in Q-TOF after tryptic digest of sugar beet 6-FEH

Observed mass	Charge state	Calculated mass (presumptive 6-FEH fragment ion)	MS/MS sequence (from N- to C-terminus)
667.4 (c,h)	2+	667.33 [T19+2H]2+	248 YDIEK 252
1878.1 (t,c,h)	2+	1877.91 [T20+2H]2+	253 DLYVPDEGSLESDLGLR 269
645.4 (c,h)	2+	645.29 [T21+2H]2+	270 YDYGK 274
615.4 (c,h)	2+	615.31 [T22+2H]2+	275 FYASK 279
1130.6 (t,c,h)	2+	1130.47 [T23+2H]2+	280 SFFDDETNR 288
1286.7 (c)	2+	1286.58 [T23-24+2H]2+	280 SFFDDETNRR 289
4117.9 (c)	3+	No match	T25-26 N-linked glycopeptide: 290 ILWGWV*NES*STQADDIKK 307 (Hex)7 (Hex)3 (GlcNAc)2
1070.4 (t,c,h)	2+	1070.57 [T27+2H]2+	308 GWSGVQAIPR 317
674.6 (c,h)	2+	674.41 [T28+2H]2+	318 TVVLDK 323
946.6 (c)	3+	946. 56 [T28-29+2H]3+	318 TVVLDKSGK 326
1698.2 (t)	2+	1697. 90 [T30+2H]2+	327 QLVQWPLAEVDMLR 340
1370.8 (t,c,h)	2+	1370.72 [T31+2H]2+	341 ENDVELPSQVIK 352
962.4 (b,h)	2+	962.48 [T40+2H]2+	447 SSLNPTTDK 455
1151.6 (b,h)	2+	1151.58 [T45+2H]2+	497 VYPTMAINDK 506
1167.6 (b)	2+	No match	497 VYPTMoxAINDK 506 (T45 derived)
2626.3 (b)	3+	No match	T47 N-linked glycopeptide: 509 LYVFN*NGT*EDVKITK 520 (Pen)1 (HexNAc)1 (Man)3 (GlcNAc)2
2829.4 (b)	3+	No match	T47 N-linked glycopeptide: 509 LYVFN*NGT*EDVKITK 520 (Pen)1 (HexNAc)2 (Man)3 (GlcNAc)2
3425.8 (h)	3+	No match	T47 N-linked glycopeptide: 509 LYVFN*NGT*EDVKITK 520 (Hex)7 (Man)3 (GlcNAc)2

The recombinant Bv6-FEH enzyme was incubated with various fructan substrates and sucrose (Table III.2). Like for native Bv6-FEH, levan is hydrolyzed to fructose; sucrose and inulin are not hydrolyzed (Fig. III.2), demonstrating that the enzyme retained its 6-FEH specificity. A comparison of both native and heterologous enzymes demonstrated very similar substrate specificities, although the heterologous 6-FEH seems to have a higher affinity towards neokestose (Table III.2). Moreover, neokestose seems to be a better substrate than levan. Both pH and temperature optimum are the same for the native and recombinant 6-FEH enzyme (data not shown). The specific activity of the recombinant enzyme however was calculated at 2-3 U/mg protein and this value differed from the specific activity of the native enzyme (12.5 U/mg protein).

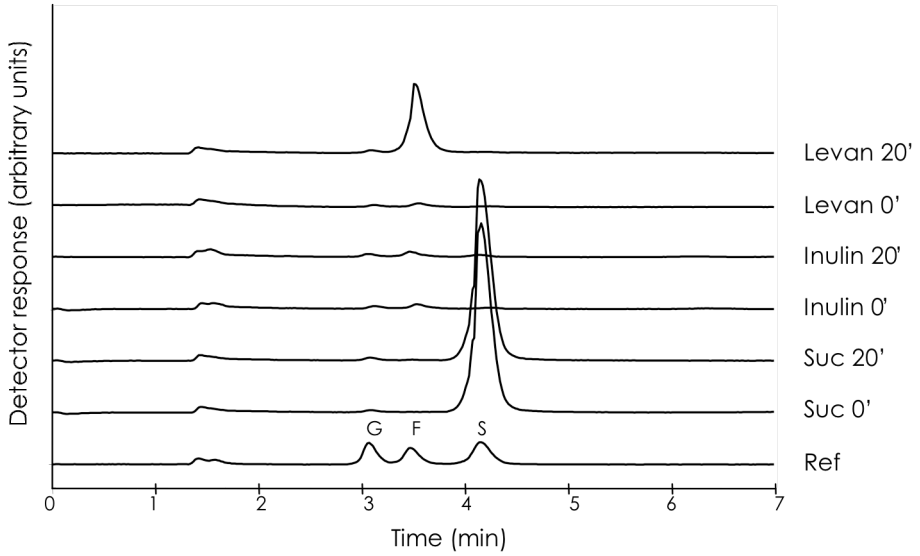


Figure III.2 Substrate specificity of the recombinant 6-FEH. AEC-PAD chromatograms of the reaction mixtures of the recombinant sugar beet 6-FEH from *Pichia pastoris* together with Suc (sucrose), inulin or levan (1 mM) after 0 and 20 min of incubation at 30°C. Inulin and levan are not visible on the chromatogram because their elution time is later than 7 min. Reference (Ref) includes glucose (G), fructose (F) and sucrose (S).

Table III.2 Substrate specificities of the recombinant Bv6-FEH. Results are shown as values relative to the activity with the substrate levan. The substrates were used at a concentration of 1 mM. Native specificities are repeated from §II.2.2.2. (Table II.2) for comparison.

Substrate	DP	Recombinant activity (%)	Native activity (%)
sucrose	2	<< 1	<< 1
levanbiose	2	62	63
1-kestose	3	13	12
6-kestose	3	68	77
neokestose	3	111	93
1,1-nystose	4	<< 1	<< 1
phlein	4-12	92	84
inulin	>10	<< 1	<< 1
levan	±72	100	100

III.2.1.3 Homology with other fructan metabolizing enzymes and invertases

As expected and deduced from sequence alignments (CLUSTALW; www.ebi.ac.uk/clustalw), *Bv6-FEH* is more homologous to cell wall invertases and other FEHs (42-59% of amino acid identity) than to vacuolar invertases and fructan biosynthetic enzymes (33-41% of amino acid identity). Sequence homology with microbial fructan hydrolases was much lower (below 28% of amino acid identity). Five different branches can be discerned in an unrooted, radial phylogenetic tree of cDNA-derived amino acid sequences of putative cell wall invertases and FEHs (Fig. III.3). This tree is constructed with cDNAs found at the end of this doctoral study. *Bv6-FEH* was the first cloned 6-*FEH* cDNA in both fructan and non-fructan plants. Branch I contains CWINVs from monocots such as *Oryza sativa* (rice) and *Zea mays* (maize). Branch II contains CWINVs from dicots such as *Solanum tuberosum*, *Daucus carota*, *Arabidopsis thaliana* and *Lycopersicon esculentum*. All the enzymes in those two groups have a high iso-electric point. Until now branch III contained only cell wall invertases from dicots. The 6-FEH enzyme from sugar beet has now been added as the first FEH member of this group. Except for Bv6-FEH and CWINV3 from *A. thaliana* all enzymes in this group have basic iso-electric points. Branch IV, containing only enzymes from monocots, can be divided in two subgroups: subgroup IVa consists of FEHs from *Triticum aestivum*, putative FEHs from *Lolium perenne* and *Hordeum vulgare* and one CWINV (CWINV5) from the non-fructan plant *Oryza sativa*; subgroup IVb contains CWINVs from *Zea mays* and *Oryza sativa* and one 6-FEH from *Triticum aestivum*. The enzymes in both subgroups have an acidic pI. Branch V contains 1-FEHs and some CWINVs from dicots all having an acidic pI.

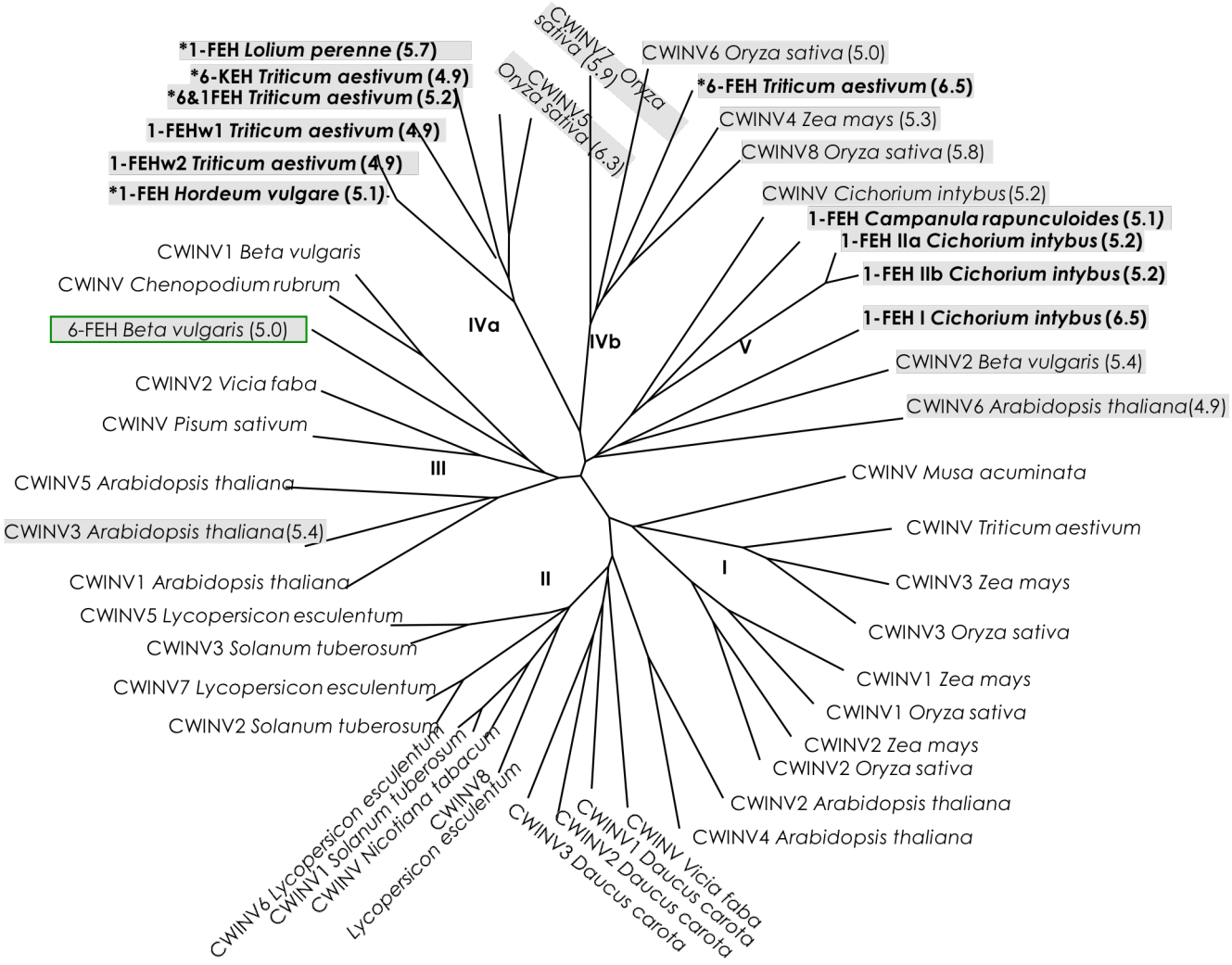

*1-FEH Lolium perenne (5.7)
*6-KEH Triticum aestivum (4.9)
*6&1FEH Triticum aestivum (5.2)
1-FEHw1 Triticum aestivum (4.9)
1-FEHw2 Triticum aestivum (4.9)
*1-FEH Hordeum vulgare (5.1)
CWINV7 Oryza sativa (5.9)
CWINV5 Oryza sativa (6.3)
CWINV6 Oryza sativa (5.0)
*6-FEH Triticum aestivum (6.5)
CWINV4 Zea mays (5.3)
CWINV8 Oryza sativa (5.8)
CWINV Cichorium intybus (5.2)
1-FEH Campanula rapunculoides (5.1)
1-FEH IIa Cichorium intybus (5.2)
1-FEH IIb Cichorium intybus (5.2)
1-FEH I Cichorium intybus (6.5)
CWINV2 Beta vulgaris (5.4)
CWINV6 Arabidopsis thaliana (4.9)
CWINV Musa acuminata
CWINV Triticum aestivum
CWINV3 Zea mays
CWINV3 Oryza sativa
CWINV1 Zea mays
CWINV1 Oryza sativa
CWINV2 Zea mays
CWINV2 Oryza sativa
CWINV2 Arabidopsis thaliana
CWINV4 Arabidopsis thaliana
CWINV Vicia faba
CWINV1 Daucus carota
CWINV2 Daucus carota
CWINV3 Daucus carota
CWINV8 Lycopersicon esculentum
CWINV Nicotiana tabacum
CWINV1 Solanum tuberosum
CWINV6 Lycopersicon esculentum
CWINV2 Solanum tuberosum
CWINV7 Lycopersicon esculentum
CWINV3 Solanum tuberosum
CWINV5 Lycopersicon esculentum
CWINV1 Arabidopsis thaliana
CWINV3 Arabidopsis thaliana (5.4)
CWINV5 Arabidopsis thaliana
CWINV Pisum sativum
CWINV Chenopodium rubrum
CWINV1 Beta vulgaris
6-FEH Beta vulgaris (5.0)
IVa
IVb
V
III
II
I

Figure III.3 Unrooted phylogenetic tree of cDNA deduced amino acid sequences of cell wall-type invertases and fructan breakdown enzymes. Accession numbers are between brackets in the legend. CWINV, cell wall invertase. The tree is divided in five groups. Group I, cell wall invertases from monocots: *Musa acuminata* (AY180200), CWINV *Triticum aestivum* (AF030420), CWINV3 *Zea mays* (AF043346), CWINV3 *Oryza sativa* (AY578160), CWINV1 *Z. mays* (U17695), CWINV1 *O. sativa* (AB073749), CWINV2 *Z. mays* (AF050631), CWINV2 *O. sativa* (AY578159). Group II, cell wall invertases from dicots: CWINV2 *Arabidopsis thaliana* (AK118343), CWINV4 *A. thaliana* (AB049617), CWINV1 *Vicia faba* (Z35162), CWINV1 *Daucus carota* (X69321), CWINV2 *D. carota* (X78424), *D. carota* (X78423), CWINV8 *Lycopersicon esculentum* (AF506004), CWINV *Nicotiana tabacum* (X81834) , CWINV1 *Solanum tuberosum* (Q43171), CWINV6 *L. esculentum* (AF506005), CWINV2 *S. tuberosum* (Q9M4K7), CWINV7 *L. esculentum* (AJ272306), CWINV3 *S. tuberosum* (Q9M4K8), CWINV5 *L. esculentum* (AY173050). Group III, cell wall invertases and one 6-FEH from dicots: CWINV1 *A. thaliana* (AY079422), CWINV3 *A. thaliana* (AB029310), CWINV5 *A. thaliana* (BAB01929), CWINV *Pisum sativum* (X85327), CWINV2, *V. faba* (Z35163), 6-FEH *Beta vulgaris* (AJ508534), CWINV *Chenopodium rubrum* (X81792), CWINV1 *B. vulgaris* (AJ422052). Group IV, cell wall invertases and FEHs from monocots divided in subgroup IVa and IVb: 1-FEH *Hordeum vulgare* (AJ605333), 1-FEHw2 *T. aestivum* (AJ508387), 1-FEHw1 *T. aestivum* (AJ513025), 6&1FEH *T. aestivum* (AB089269), 6-KEH *T. aestivum* (AB089271), 1-FEH *Lolium perenne* (DQ073968), CWINV5 *O. sativa* (AY578161), CWINV7 *O. sativa* (AY578162), CWINV6 *O. sativa* (AY578163), 6-FEH *T. aestivum* (AM075205), CWINV4 *Z. mays* (AF043347), CWINV8 *O. sativa* (AY578164). Group V, CWINV *Cichorium intybus* (Y11124), 1-FEH *Campanula rapunculoides* (AJ509808), 1-FEHIIa *C. intybus* (AJ295033), 1-FEHIIb *C. intybus* (AJ295034), 1-FEHI *C. intybus* (AJ242538), CWINV2 *B. vulgaris* (AJ422053), CWINV6 *A. thaliana* (AY060553). Enzymes with low pI (indicated next to species) are marked by grey boxes. FEHs are indicated in bold. FEHs that were cloned after *Bv6-FEH* are indicated by an asterix *.

III.3 Discussion

In chapter II and III we described the unexpected presence of a 6-FEH in the taproots of the non-fructan plant *Beta vulgaris*. For the first time a 6-FEH has been purified, cloned and heterologously expressed. During this doctoral study, other authors cloned *6-FEH* cDNAs from fructan plants (Fig. III.3).

III.3.1 Biochemical and molecular properties of Bv6-FEH

FEH activity was detected in the non-fructan plant *Beta vulgaris* but instead of an expected 1-FEH activity, a pronounced 6-FEH activity was measured. This was surprising because in dicot fructan plants only 1-FEHs had been characterized and a putative CWINV cDNA from sugar beet (CWINV2) was known to cluster with the 1-FEHs. Most 6-FEH activity was found in the taproots (Fig. II.1) and the enzyme seems to be constitutively active during the growing season. The occurrence of a 6-FEH cannot be ruled out in the petioles. In contrast, the existence of a specific 6-FEH in the leaf blades is most unlikely (Fig. II.1).

A purification protocol resulted in a small amount of pure enzyme as confirmed by ESI-Q-TOF MS analysis (Table III.1). The use of degenerated primers and primers based on internal peptides proved to be a good strategy to clone the corresponding *6-FEH* cDNA (Fig. III.1). Sequence alignments with other plant and bacterial invertases and FEHs confirmed the plant origin of the enzyme. Absolute certainty about the functionality of the clone was obtained by heterologous expression of the *6-FEH* cDNA in *Pichia pastoris*.

Both native and recombinant enzymes are inactive against sucrose, prefer fructans with β-2,6 linkages (Table III.2) and are poorly inhibited by sucrose (Fig. II.3). Because of its inability to hydrolyze sucrose, in contrast to microbial beta-fructosidase (EC 3.2.1.80), the 6-FEH enzyme is classified under the recently introduced category EC 3.2.1.154. The fact that sucrose does not inhibit the activity of 6-FEH supports the idea that two major classes of plant FEHs can be distinguished based on their inhibition by sucrose (§I.3.3.1). Interestingly, neokestose is also a very good substrate indicating that a fructose-fructose linkage is not necessary for enzymatic breakdown by Bv6-FEH. Both pH optimum and molecular mass are comparable to other FEHs (§I.3.3.1). The heterotrimeric nature of the Bv6-FEH is unique in comparison with the monomeric nature of other FEHs and cell wall invertases. It is, however, not clear whether these polypeptides represent proteolytic

artefacts or real enzyme subunits *in vivo*. The monomeric recombinant enzyme, however, kept the same substrate activities as its native heterotrimeric counterpart.
The properties of native and recombinant enzyme are very similar, indicating that the *P. pastoris* expression system is a very good tool to verify the functionality of fructan breakdown enzymes. The only difference between the two enzymes was their specific activity (12.5 vs 3 U/mg protein). However, the high specific activity of the native enzyme could be an overestimated value due to the difficulty of measuring very low protein concentrations (0.002 mg) and the lower specific activity of the heterologous enzyme could be an underestimated value since the enzyme was not completely purified from the Pichia supernatant.

A phylogenetic tree of CWINVs and FEHs (Fig. III.3) reveals a rather unexpected localization of the *Bv6-FEH* sequence. The enzyme did not cluster with other known FEHs but could be found in a group with only putative cell wall invertases. The function, however, of most cDNAs in the phylogenetic tree has not been confirmed by heterologous expression and since the absence of FEHs in non-fructan accumulating plants has been considered as trivial, it is logic that those translated cDNAs were listed as cell wall invertases based only on their homology. Due to the existence of a 6-FEH in sugar beet, it is premature to classify translated cDNAs as cell wall invertases when the cDNAs have been identified by no other means than homology.

Most cell wall invertases have basic pIs (from pH 7 to 9.7) and are positively charged to form ionic bonds with apoplastic components (Goetz and Roitsch, 1999). On the contrary, all FEHs have more acidic pIs (from pH 4.9 to 6.5). Based on vacuole isolation experiments it is accepted that fructans and fructan metabolic enzymes are located in the vacuoles of fructan plants (Wiemken *et al*., 1986). Recently, different 'cell wall invertase-like' enzymes with a low pI from non-fructan plants have been identified e.g. CWINV5, 6, 7 and 8 from *O. sativa* (Ji *et al*., 2005), CWINV3 and 6 from *A. thaliana*, CWINV4 from *Z. mays* (Kim *et al.,* 2000) and CWINV2 from *B. vulgaris* (www.ncbi.nlm.nih.gov). This is probably an indication that these enzymes are not ionically bound to the cell wall. It is, however, not unlikely that some of these so-called invertases are genuine FEHs and not invertases. The localization of Bv6-FEH is uncertain, but preliminary experiments (Rudy Vergauwen, personal communication) do not support an apoplastic localization.

After transformation of sugar beet taproots with *1-SST* cDNA from *Helianthus tuberosus* (Sévenier *et al*., 1998), 40% of the dry weight consisted of low molecular weight fructans (GF_2, GF_3 and GF_4). Because of the β-2,1 nature of these fructans, the newly discovered

6-FEH could not have an effect on accumulation of those fructans. During this doctoral study, however, Weyens *et al*. (2004) introduced *1-SST* and 6^{G}*-FFT* cDNAs from onion in sugar beet taproots. This resulted in high-level accumulation of onion-type fructans including 1-kestose, neokestose, nystose, 1&6^{G}-kestotetraose and 1,6^{G}-kestotetraose. Since neokestose is a good substrate for 6-FEH, the vacuolar localization of Bv6-FEH could be questioned. An alternative explanation for accumulation of neokestose is that the low constitutive expression and very low amount of 6-FEH (0.6 μg/kg) are unable to degrade such high amounts of formed neokestose.

III.3.2 Putative functions of Bv6-FEH

The occurence of a 6-FEH in *Beta vulgaris* is not obvious and raises a lot of questions about a more general occurrence of this enzyme in other non-fructan plants and its physiological role. Fructans do not accumulate in sugar beet, so a working hypothesis on the role of this enzyme in degrading endogenous substrates does not seem straightforward. It is not the first time that plant enzymes have been described that apparently lack an endogenous substrate. In the next paragraph, some examples are given of enzymes that, at first sight, have no endogenous plant substrate. Their function is mostly more complicated than first thought:

- Different classes of chitinases occur in both vacuoles and apoplasts of higher plants. Chitinases catalyze the cleavage of the β-1,4 linked N-acetylglucosamine residues in chitin. This substrate is, however, not present in plants. Since they are induced upon infection, chitinases are classified as pathogenesis-related (PR) proteins. Evidences have been found that their function during fungal pathogen attack is twofold (Kasprzewka *et al.*, 2003 and references therein). The apoplastic chitinases play an important role during early stages of infection by releasing elicitor molecules from fungal chitin resulting in activation of defence mechanisms, including synthesis of vacuolar chitinases. After penetration and destruction of the cells by the hyphae, vacuolar chitinases further degrade the fungal chitin, causing repression of fungal growth. It is also noteworthy that Nod-factors, bacterial signal molecules produced during legume nodulation, consist of chito-oligosaccharides. Based on some striking results, it has been suggested that chitinases also play a role in plant growth and developmental processes. Endochitinases can rescue somatic embryogenesis in a *Daucus carota* mutant unable to complete embryo development (de Jong *et al*., 1992). The search for an endogenous substrate started and van Hengel *et al.* (2001) demonstrated that arabinogalactan proteins contain glucosamine and N-acetyl-glucosamine residues (0.2%) and that they

were sensitive to endochitinase cleavage. Remarkably, in *A. thaliana* a mutation of a certain chitinase resulted in ethylene overproduction (in contrast to what occurs after infection) and an aberrant phenotype (shorter internodes and roots), not fully correlated with this ethylene overproduction, was seen. In this view it can be hypothesized that this chitinase is not only involved in the ethylene pathway but also in other developmental processes (Zhong *et al.*, 2002). Furthermore, induction of chitinases has also been detected in germinating seeds (Hodge *et al.*, 1996). These examples prove that the role of chitinases is not only restricted to pathogen attack, but they most likely also fulfil a function in plant growth and development.

- Trehalase, an enzyme that hydrolyzes trehalose, a glucose-containing disaccharide, is ubiquitous in higher plants. For long trehalose could not be detected in those plants except for the 'resurrection plants' *Selaginella lepidophylla* and *Myrothamnus flabelifolia*. Now it is proven that trace amounts of trehalose can be detected in most plants (Müller *et al.*, 2001). Moreover, the discovery of functional genes encoding trehalose-biosynthesis enzymes and the presence of 21 putative trehalose-biosynthesis genes in the *A. thaliana* genome suggest that trehalose metabolism in higher plants is more common and important than first expected (Leyman *et al.*, 2001; Eastmond *et al.*, 2003). Evidence has been found that trehalose, and even more its precursor trehalose-6-phosphate, play crucial regulatory roles in plant growth and development (Eastmond *et al.*, 2002; Schluepmann *et al.*, 2003).

Since different bacteria are surrounded by levan polymers, a straightforward working hypothesis (in analogy to chitinases) might be that plant 6-FEHs participate in hydrolysis of this levan preventing or reducing infection by phytopathogenic bacteria like e.g. *Pseudomonas* or *Erwinia*. However, the possibility that trace amounts of an endogenous substrate occur in plants must not be omitted.

The putative function of FEHs in non-fructan plants will be further discussed in Chapter V.

In conclusion:

In Chapter II and Chapter III the purification, characterization, cloning and heterologous expression of a 6-FEH in the non-fructan plant *Beta vulgaris* has been described. In Chapter IV it will be investigated if this novel enzyme also occurs in the model plant *Arabidopsis thaliana.*

CHAPTER IV

THE OCCURRENCE OF SPECIFIC FRUCTAN EXOHYDROLASES IN *ARABIDOPSIS THALIANA*

Part of the work described in this chapter was published as:
De Coninck B., Le Roy K., Francis I., Clerens S., Vergauwen R., Halliday A., Smith S.M., Van Laere A. and Van den Ende W. (2005). Arabidopsis AtcwINV3 and 6 are not invertases but are fructan exohydrolases (FEHs) with different substrate specificities. *Plant Cell Environ. 28: 432-443.*

IV.1 Introduction

The presence of a specific 6-FEH in the non-fructan plant sugar beet opens a completely new research area in the fructan field. Resolving their physiological function is not straightforward and in order to find out more about their function it was decided to look for FEHs in the model plant *Arabidopsis thaliana*. This plant has many advantages because of the known genome and the availability of a wide range of molecular techniques.

In this chapter, we set out to determine if some known putative cell wall invertase cDNA sequences could encode an FEH rather than a genuine invertase. As discussed in Chapter III most cell wall invertases were identified by no other means than sequence homology with other cell wall invertases. In the phylogenetic tree of cell wall invertases and fructan exohydrolases (Fig. III.3), the known dicotyledonous FEHs (Branch V) are positioned in a different subgroup than Bv6-FEH (Branch III). Therefore, we will focus on the presumed 'so-called invertases' from these two subgroups. Six putative cell wall-type invertase genes (*AtcwINV1-6*) with distinct patterns of expression have been identified in the *A. thaliana* genome (revised nomenclature, Sherson *et al.,* 2003). *AtcwINV1*, *3* and *5* are situated in branch III of the phylogenetic tree. Amongst the different putative invertases in *A. thaliana*, the expression level of *AtcwINV1* (At3g13790) is the highest (Fig. IV.1) and even increases after infection (Fotopoulos *et al.,* 2003). The enzyme has a high iso-electric point typical for cell wall bound invertases and is believed to be a sucrose hydrolyzing enzyme. *AtcwINV5*, also having a high pI, is expressed only in flowers (Fig. IV.1). *AtcwINV3* (At1g55120) has a low pI like all FEHs and its expression seems to be rather low in the different tissues of healthy plants (Fig. IV.1). *AtcwINV6* (At5g11920), also with a low pI, is situated in branch V together with the 1-FEHs and has a moderate expression (Fig. IV.1) (Sherson *et al*., 2003). AtcwINV2 and 4 are situated in the lower part of the phylogentic tree where, up till now, no FEHs have been found (Fig. III.3). None of the enzymes corresponding to AtcwINV1-6 have been purified and properly characterized.

It has to be noticed that the expression level and profile of *AtcwINV1-6* mentioned here were based on Figure IV.1 from Sherson *et al*. (2003). During this work, however, own results and newly published articles revealed some differences in the expression level of the enzymes compared to Sherson *et al*. (2003). This will be further discussed in Chapter V.

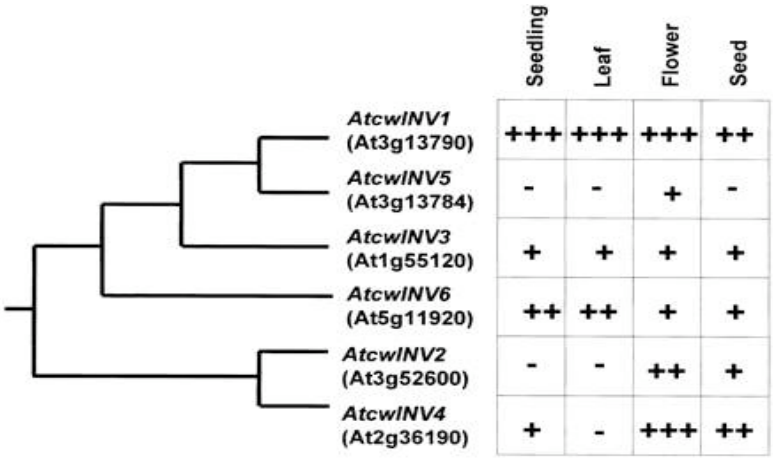


Figure IV.1 The Arabidopsis cell wall invertase gene family. The levels of expression of each gene were determined by RT-PCR using gene-specific primers and RNA from 3d-old seedlings, expanding rosette leaves, flowers and developing seeds removed from green siliques. (+++) high, (++) moderate, (+) low and (-) no expression detected. Figure from Sherson *et al.,* 2003.

With all above facts in mind, three of those six cell wall invertases from *A. thaliana* were chosen for a thorough characterization. *AtcwINV1, 3* and 6 cDNAs were heterologously expressed in *Pichia pastoris* and the functionality of the corresponding enzymes was tested.

In order to confirm the presence of FEH in *A. thaliana*, experiments were performed to test for endogenous FEH activity by comparing the hydrolytic activity towards the soluble reserve carbohydrates sucrose, inulin and levan, in the soluble and insoluble fractions of different parts of *A. thaliana*. Next, a purification has been set up using *A. thaliana* plants in order to separate FEH activity from invertase activity and to obtain enzyme for Q-TOF mass-spectrometric analysis (Q-TOF).

Finally, some special characteristics of AtcwINV3 and AtcwINV6 will be discussed.

IV.2 Results

IV.2.1 Heterologous expression of *AtcwINV1, 3* and 6 cDNAs

IV.2.1.1 Substrate specificities of the recombinant AtcwINV1, 3 and 6

At the start of this doctoral study, *AtcwINV1* and 6 cDNAs were available from cDNA stock centres. The *AtcwINV3* cDNA was at that time not available and was cloned from leaves (Materials and Methods, §VII.4.2). Like for the *6-FEH* cDNA from sugar beet, the *Pichia pastoris* expression system was used to express the *AtcwINV1, 3* and 6 cDNAs. Three expression plasmids containing the mature AtcwINV1, 3 or 6 protein parts were constructed. After transformation of *P.* pastoris with the expression plasmids and growth of the yeast in an expression medium (Materials and Methods § VII.5.2), the recombinant enzymes were secreted outside the cells. After protein precipitation, an SDS-PAGE was performed and the 72 kDa bands were cut out, trypsinized and subjected to ESI Q-TOF MS to confirm the presence and identity of the enzymes. A theoretical tryptic digest of the cDNA-derived AtcwINV1, 3 and 6 protein sequences yields 68, 56 and 54 peptides respectively (designated T1-T68 from N- to C-terminus). Most masses detected matched - within the acceptable mass measurement error of +/- 0.2 D - with one of the theoretical fragments. CID MS/MS analysis yielded a number of sequence tags which proved the identity of the tryptic peptides (data not shown). These data confirm the presence of the enzymes in the supernatant.

The recombinant enzymes AtcwINV1, 3 and 6 were incubated with different substrates and results are shown in Table IV.1. Essentially, AtcwINV1 greatly prefers sucrose as substrate but can degrade 1-kestose and neokestose to a limited extent. Longer fructans, especially of the inulin-type, are very poor substrates. In marked contrast, AtcwINV3 prefers fructans as substrate and the enzyme is essentially inactive against sucrose, demonstrating that it is not an invertase but a genuine FEH. Of all fructans tested, the recombinant enzyme hydrolyzes β-2,6 linkages (levan, DP4-12 phlein, neokestose, 6-kestose and levanbiose) most efficiently. The enzyme is less active upon fructans with β-2,1 linkages (1-kestose, 1,1-nystose and inulin).

Sucrose is also a very poor substrate for AtcwINV6. Instead, the enzyme hydrolyzes both β-2,6 and β-2,1 type fructans, although the 2,6 type fructans are degraded more efficiently. The best substrates for this enzyme are phlein oligomers and high DP bacterial

levan. In contrast to AtcwINV3, AtcwINV6 hydrolyzes 1-kestose and 6-kestose at a similar rate and has a higher activity with inulin.

Table IV.1 Comparison of substrate (1 mM) specificities of the heterologously expressed *Arabidopsis thaliana AtcwINV1, 3 and 6* cDNAs in *Pichia pastoris.* Results are shown as values relative to the activity with the substrate sucrose for AtcwINV1 and levan for AtcwINV3 and 6.

Substrates	DP	Activity of recombinant AtcwINV1 (%)	Activity of recombinant AtcwINV3 (%)	Activity of recombinant AtcwINV6 (%)
sucrose	2	100	<< 1	<< 1
levanbiose	2	6	75	73
1-kestose	3	38	26	41
6-kestose	3	6	83	43
neokestose	3	15	114	87
1,1-nystose	4	7	13	23
phlein	4-12	9	115	102
inulin	>10	1	8	34
levan	±72	10	100	100

Incubations of AtcwINV3 and 6 with different concentrations of sucrose show that even at high concentrations, sucrose hydrolysis is only minor compared to levan breakdown (Fig. IV.2a). AtcwINV3 and 6 show FEH activities that are respectively 70 and 150 times higher than their invertase activity. Figure IV.2b shows an incubation of AtcwINV1 with different sucrose concentrations, its K_m for sucrose (< 1 mM) is much lower than the sucrose K_m of AtcwINV3 and 6 (> 200 mM).

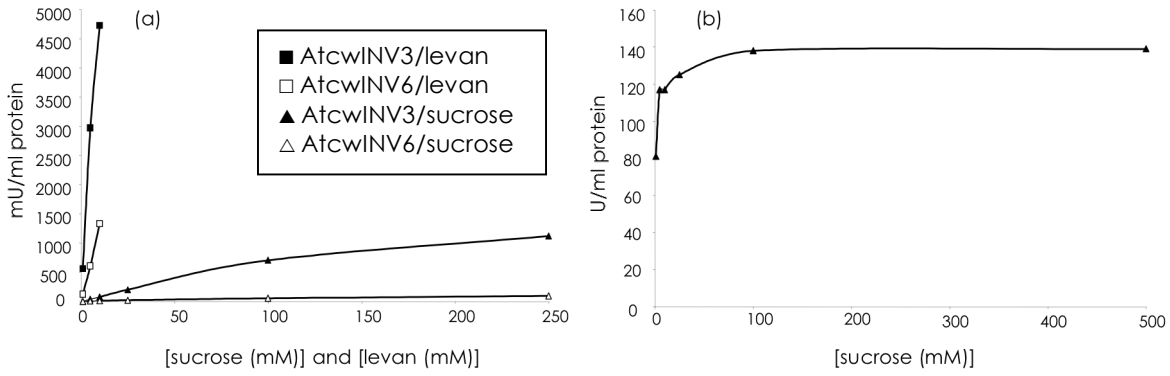


Figure IV.2 Activities of AtcwINV1, 3 and 6. (a) AtcwINV3 (closed symbols) and 6 (open symbols) incubated with sucrose (triangles) and levan (squares) and (b) AtcwINV1 incubated with sucrose.

When AtcwINV3 and AtcwINV6 are incubated with levan in combination with sucrose, almost no inhibitory effect of sucrose (up to 30 mM) is detected (Fig. IV.3). With 100 mM sucrose and 5 mM levan, the enzymes lost ± 30% of their activity. Compared to this 1-FEH IIa from *C. intybus* lost 60% of its activity after incubation with 10 mM sucrose (Verhaest *et al.,* 2007). So it can be concluded that AtcwINV3 and 6 are almost not inhibited by sucrose.

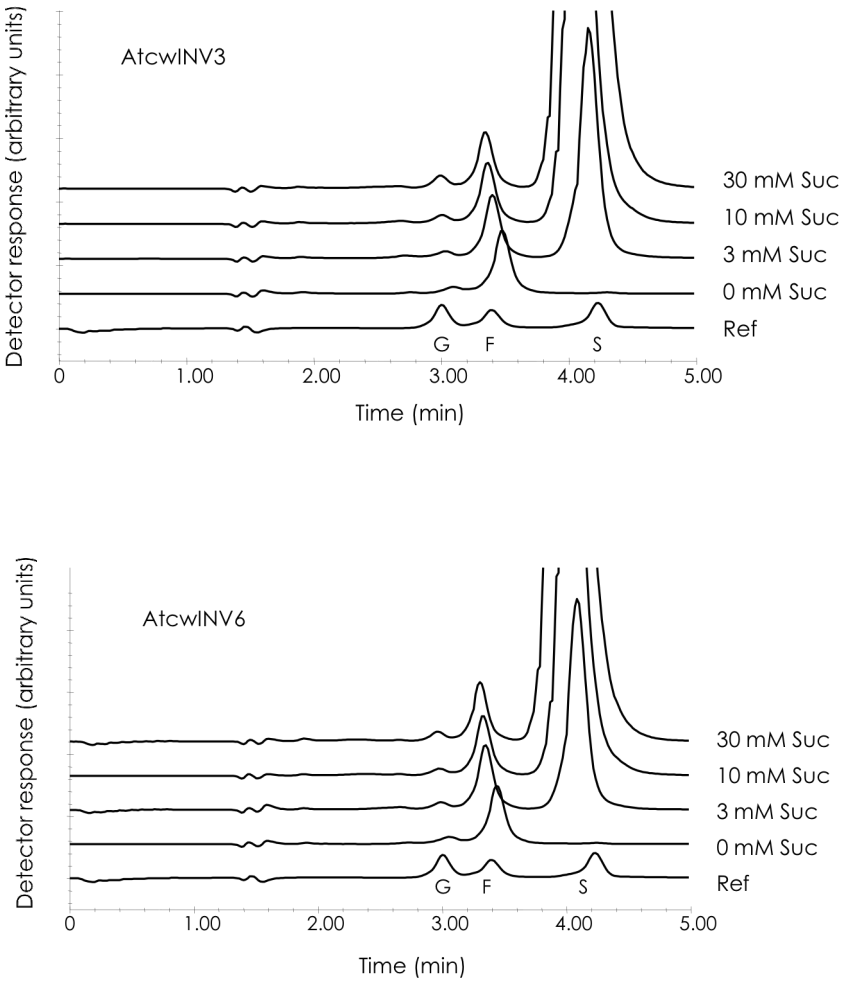
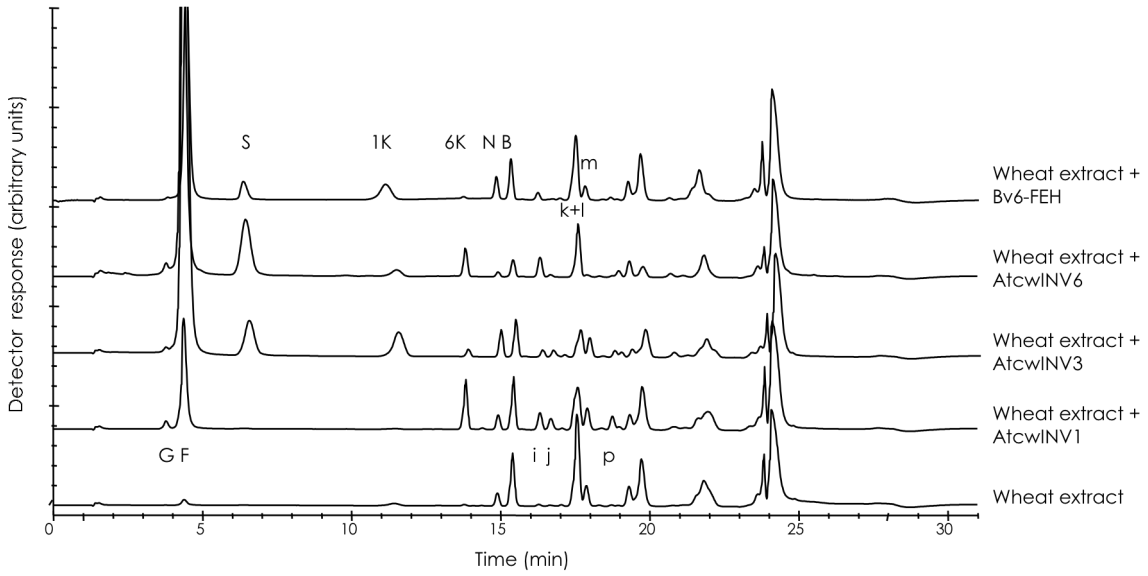


Figure IV.3 Activity of AtcwINV3 and AtcwINV6 in the presence of sucrose. AEC-PAD chromatograms of incubation (40 min, 30°C) of heterologous AtcwINV3 and 6 with 5 mM levan in combination with 0, 3, 10 and 30 mM Suc. Inulin and levan are not visible on the chromatogram because their elution time is later than 5 min. Reference includes glucose (G), fructose (F), sucrose (S).

As already deduced from Table IV.1, AtcwINV3 and 6 have different specificities towards fructans with β-2,1 and β-2,6 linkages. The differences in substrate specificity between AtcwINV1, 3 and 6 are demonstrated in Figure IV.4. We incubated these enzymes with a wheat fructan extract, containing no glucose, fructose and sucrose, but graminan type fructans with β-2,6 and β-2,1 linkages. This wheat extract is a poor substrate for AtcwINV1 since this enzyme is a genuine invertase. The accumulation of 6-kestose (6K), DP4- (j) and DP5-levan (p) and the degradation of 1&6,1 kestopentaose (l) demonstrate that the enzyme has some activity against β-2,1 but not β-2,6 fructans. Although both heterologous AtcwINV3 and 6 show no invertase activity (Table IV.1), their effect on wheat fructans is clearly different. Due to the complexity of this chromatogram it is very difficult to make clear statements about the specificity of the enzymes and therefore only some observations will be given. The accumulation of sucrose (S), 1-kestose (1K) and 1,1-nystose (N) but not 6-kestose (6K) shows that AtcwINV3 prefers β-2,6-linked fructans. From the hydrolysis patterns it can also be deduced that bifurcose (B) and 1&6,6 kestopentaose (m) are better substrates for AtcwINV6 than for AtcwINV3. Sucrose, 1-kestose and 6-kestose accumulation was detected for AtcwINV6, but in contrast to AtcwINV3 more 6-kestose than 1-kestose was found. Nystose accumulates for AtcwINV3 (probably from degradation of 6&1,1 kestopentaose (k)), whereas 6,1 kestotetraose (i) accumulates for AtcwINV6 (probably from degradation of 1&6,1 kestopentaose (l)), indicating that AtcwINV6 has less difficulties with β-2,1-linked fructose molecules than AtcwINV3.

Also in this graph, a wheat fructan extract incubated with 6-FEH from sugar beet is depicted. This chromatogram resembled more the chromatogram of AtcwINV3 than that of AtcwINV6. Indeed, when comparing Table IV.1 of the recombinant enzymes of *A. thaliana* and Table II.4 of the recombinant Bv6-FEH, AtcwINV3 and Bv6-FEH are most related.



(1K) 1-kestose: G-F-F

(6K) 6-kestose: G-F
F

(N) 1,1-nystose: G-F-F-F

(B) bifurcose: G-F-F
F

(i) 6,1 kestotetraose: G-F
F-F

(j) 6,6 kestotetraose: G-F
F
F

(k) 6&1,1 kestopentaose: G-F-F-F
F

(l) 1&6,1 kestopentaose: G-F-F
F-F

(m) 1&6,6 kestopentaose: G-F-F
F
F

Figure IV.4 Comparison of AtcwINV1, 3, 6 and sugar beet 6-FEH. AEC-PAD chromatograms of incubation mixtures (overnight incubation) of heterologously expressed AtcwINV1 (4 U for sucrose), 3, 6 and sugarbeet 6-FEH (0.05 U for levan) with wheat kernel fructans. G (glucose), F (fructose), S (sucrose), p (6,6,6 kestopentaose). Structures of branched wheat fructans were derived from the work of Bancal *et al.* (1993) and reconfirmed by Van den Ende *et al*. (2003a).

In conclusion, AtcwINV3 and 6 are not cell wall invertases in contrast to AtcwINV1. Moreover, AtcwINV3 and 6 are FEHs with different substrate specificities. Therefore, we suggest to use the terms At6-FEH and At(6&1)-FEH for AtcwINV3 and AtcwINV6, respectively.

IV.2.1.2 Specific activity of AtcwINV1, At6-FEH and At(6&1)-FEH

Specific activities were first calculated by using the concentration of precipitated protein from the *P. pastoris* supernatant, resulting in specific activities around 34 U/mg protein, 0.4 U/mg protein and 3 U/mg protein for respectively AtcwINV1, At(6&1)-FEH and At6-FEH. Because of the discrepancy between the different values, especially the low value for

At(6&1)-FEH, it was decided to purify the enzymes from the supernatant (Materials and Methods §VII.2.4.3). After precipitation, proteins were applied on a ConA column and the eluate was used for FPLC chromatography using the cation exchange column Mono S pH 7.5 for AtcwINV1 and the anion exchange column Mono Q pH 7.5 for At6-FEH and pH 7 for At(6&1)-FEH.

Comparing the different chromatograms in Figure IV.5 makes it clear that the purification was successful for AtcwINV1 but not for At6-FEH and At(6&1)-FEH. For AtcwINV1, activity was concentrated in one big peak (fractions 25 to 33). The newly calculated specific activity (40 U/mg protein) matched more or less the previous value. So, almost all protein in the supernatant corresponds to AtcwINV1. For At6-FEH, activity was found from fraction 11 to 27, with the major activity in fraction 13. For At(6&1)-FEH, activity was found from fraction 23 to 31 with the major activity in fraction 26. Calculating new specific activities for At6-FEH and At(6&1)-FEH is now very difficult because of the inability to measure exact protein concentrations.

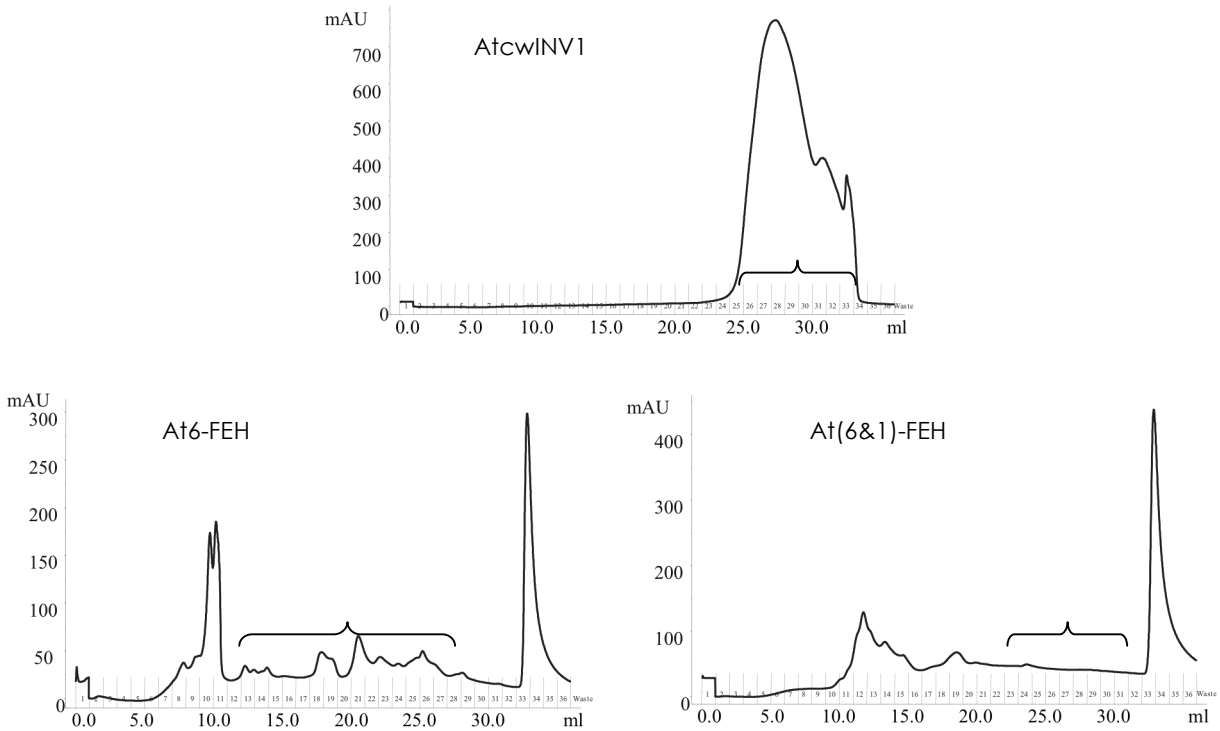


Figure IV.5 FPLC chromatograms of heterologous AtcwINV1, At6-FEH and At(6&1)-FEH. Y-axis indicates UV (in mAU). The different fractions (1ml/min) are displayed on the X-axis. The salt gradient used: from 0 M to 0.3 M NaCl in 30 min, followed by a complete elution using 100% NaCl. The black line indicates the fractions that exhibited activity.

IV.2.2 Native FEH in *Arabidopsis thaliana*

IV.2.2.1 Invertase and FEH activities in different plant parts of Arabidopsis

In order to test for FEH activity in *A. thaliana*, we compared the production of fructose from inulin, levan and sucrose in the soluble and insoluble fraction of extracts from different parts of *A. thaliana* plants (Fig. IV.6). Invertase activity was high in flowers but lower in roots and leaves. Most invertase activity is due to a soluble form. 6-FEH activity was measurable in the soluble fraction of all plant parts but especially in the flowers. Activity distribution of 1-FEH was comparable to 6-FEH, but approximately four times lower (not shown). No FEH activities were detected in the insoluble fractions.

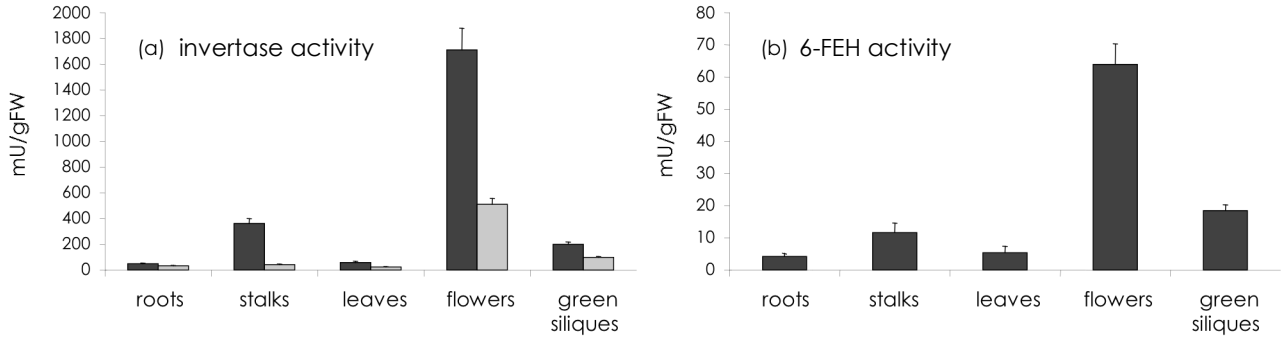


Figure IV.6 Invertase and 6-FEH activities in *A. thaliana*. Production of fructose from sucrose (a) and levan (b) were measured in soluble (black bars) and insoluble fractions (grey bars) of different plant parts of *A. thaliana*. Results are the mean (±SE) for n=3.

It is a matter of discussion if the 6-FEH activity measured in the different plant parts is due to a genuine FEH or rather a side-activity of soluble invertase. Invertase and 6-FEH activity fluctuate together among the different plant parts and invertase activity is approximately 25 times higher than 6-FEH activity. Therefore, it is not possible to state that this activity is due to a genuine 6-FEH.

IV.2.2.2 Purification and subcellular localization of At6-FEH

To exclude the possibility that FEH activities of At6-FEH and At(6&1)-FEH are due to the heterologous *P. pastoris* expression system, we attempted to purify a specific 6-FEH from whole *A. thaliana* plants. Because of their low pI, only At6-FEH and At(6&1)-FEH and the two vacuolar invertases Atβfruct3 and Atβfruct4 are expected in the soluble plant extract.

The purification was based on $(NH_4)_2SO_4$ precipitation, lectin affinity chromatography (ConA) and anion and cation exchange chromatography (Materials and Methods §VII.2.4.2). After Mono S (pH 4.5) and Mono Q (pH 7.5), a protein fraction with 6-FEH activity and free of any invertase activity was found. After SDS-PAGE (not shown), a band of 72 kDa was cut out, trypsinized and peptide masses were determined by Q-TOF and compared to the masses of theoretical peptides from At6-FEH and At(6&1)-FEH. Most masses detected matched – within the acceptable mass measurement error of ±0.2Da – with one of the theoretical fragments from At6-FEH. Collision-induced dissociation (CID) MS/MS analysis yielded a number of sequence tags that proved the identity of the tryptic peptides (Table IV.2). Other fragments can be explained by the occurrence of oxidized methionines or by the occurrence of a miss-cleavage.

Table IV.2 Fragment ions detected in Q-TOF after tryptic digest of the native At6-FEH. Calculated matches to theoretical digest of virtual cDNA derived protein, and confirmation of identity by tandem MS/MS sequencing are shown.

Observed mass	Charge state	Calculated mass (presumptive 6-FEH fragment ion)	MS/MS sequence (from N- to C-terminus)
545.8	2+	545.78 [T2+2H]2+	7 TGYHFQPLK 15
860.4	2+	No match	16 NWMoxNDPNGPMoxIYK 28 (T3 derived)
798.3	2+	No match	16 NWMNDPNGPMoxIYK 28 (T3 derived)
860.4	3+	860.42 [T7+3H]3+	122 EWSKPPQNPLMTTNAVNGINPDR 144
508.8	2+	508.76 [T9+2H]2+	147 DPTTAWLGR 155
331.7	2+	331.65 [T10+2H]2+	156 DGEWR 160
524.8	2+	524.76 [T11+2H]2+	161 VIVGSSTDDR 171
602.8	2+	No match	161 VIVGSSTDDRR 172 (T11-12)
496.7	2+	No match	163 VGSSTDDRR 171 (partial T11-T12)
389.3	2+	389.24 [T13+2H]2+	172 GLAILYK 178
715.7	3+	715.68 [T17+3H]3+	233 VSLIETLHDYYTIGSYDR 250
444.2	2+	444.21 [T22+2H]2+	280 TFYDDVK 286
508.3	2+	No match	280 TFYDDVKK 287
567.8	2+	567.78 [T28+2H]2+	308 GWSGLQSFPR 317
494.3	2+	494.25 [T32+2H]2+	341 GQQVNWQK 348
743..4	2+	743.37 [T38+2H]2+	381 ADVIEPSWTDPQK 393
554.3	3+	554.28 [T47+3H]3+	481 TLIDHSIVESYGGGGR 496
775.4	2+	775.4 [T50+2H]2+	507 LAIGENANLFVFNK 520
1019.0	2+	No match	503 VYPKLAIGENANLFVFNK 520 (T49-50)

The 'purified' enzyme was incubated with different fructan substrates and with the wheat fructan extract. A comparison of native and heterologous At6-FEH showed very similar substrate specificities (Table IV.3, Fig. IV.7). The native enzyme, however, has a higher activity with neokestose and phlein. A possible explanation is the extensive glycosylation of the heterologous enzyme (4 glycosylation sites) resulting in some hindrance for these substrates.

Table IV.3 Comparison of native and recombinant At6-FEH. Comparison of substrate specificities of heterologously expressed and native At6-FEH. The substrates were used at a concentration of 1 mM. Results are shown as values relative to the activity with levan.

Substrates	DP	Activity of recombinant At6-FEH (%)	Activity of native At6-FEH (%)
sucrose	2	<< 1	<< 1
levanbiose	2	75	76
1-kestose	3	26	23
6-kestose	3	83	77
neokestose	3	114	151
1,1-nystose	4	13	12
phlein	4-12	115	156
inulin	>10	8	4
levan	±72	100	100

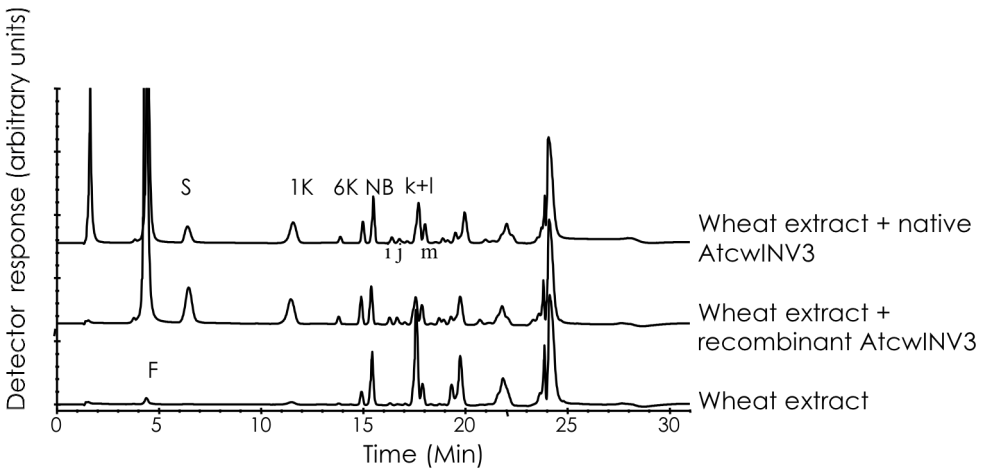


Figure IV.7 Comparison of native and recombinant At6-FEH. AEC-PAD chromatograms of incubation mixtures (overnight incubation) of heterologously expressed and native At6-FEH (1 unit) with endogenous wheat kernel fructans. Abbreviations as in Fig IV.4.

Since FEHs are most probably localized in the vacuole, it can be questioned if this also holds for FEHs in non-fructan plants. Preliminary work on *Beta vulgaris* did not suggest an apoplastic localization for Bv6-FEH. Carter *et al.* (2004) characterized the vegetative vacuole proteome from *Arabidopsis thaliana*. Beside both VINVs, At6-FEH was identified; At(6&1)-FEH was not recovered. So, this strongly suggests that At6-FEH, formerly categorized as a 'cell wall invertase', is not located in the cell wall and moreover does not act as an invertase.

IV.2.2.3 Special features of At(6&1)-FEH

Purification of At(6&1)-FEH and putative localization

During the purification of native At6-FEH, we also attempted to purify At(6&1)-FEH, but no other fractions with 6-FEH activity could be detected in the glycosylated fraction (Con A) where cell wall and vacuolar enzymes normally remain. Remarkably, the deduced amino acid sequence of the *At(6&1)-FEH* cDNA is approximately 25 amino acids shorter at the N-terminus compared to most other CWINVs and FEHs (Fig IV.8).

```
(6&1)-FEH A. thaliana ----------------------------------------MAD--VMEQNL 9
CWINV C. intybus      ----------------------------------------MSNSSNASESL 11
1-FEHIIa C.intybus    MKKS-----------LSSFIVLCFLVIILET-----GRVKATSRNLNDVI 34
CWINV C. rubrum       ---------------MASYKLPKQVILLLVS-----LLFFCYGVVELQAA 30
6-FEH A. thaliana     MAKLN----------RSNIGLSLLLSMFLAN----------FITDLEASS 30
CWINV5 A. thaliana    MANIV----------WCNIAMFLLVSLFLTDDAV------VVLDALDNVP 34
CWINV1 A. thaliana    MTKEV----------CSNIGLWLLLTLLIGNYVVNLEASHHVYKRLTQST 40
CWINV2 A. thaliana    MSAP-----------KFGYVLLLIVLINISN---NGVDAFHKVFKKLQSK 36
CWINV4 A. thaliana    MAISN----------VISVLLLLLVLINLSNQNIKGIDAFHQIYEELQSE 40
CWINV1 D. carota      MGVTIRNRNYDHGSLPFLQSLLAILLVTTTTLHINGVEAFHEIHYNLQSV 50
6-FEH B. vulgaris     MAPNNGS------WLVLSISMMLLSHGMIIIAKDQAIHHHDDDHDDMLIN 44
1-FEHw1 T.aestivum    MAQAWAFLLPVLVFGSYVTSLFFPSYISGPLCGGDGGGRSLFLCAQAPKD 50

(6&1)-FEH A. thaliana LQT--AVLN--RTSFHFQPQRNWLNDPNAPMYYKGFYHLFYQNNPLAPEF 55
CWINV C. intybus      FPA--TSEQPYRTAFHFQPPQNWMNDPNGPMCYNGVYHLFYQYNPFGPLW 59
1-FEHIIa C. intybus   MLANQQIEQPYRTGYHFQPPSNWMNDPNGPMLYQGVYHFFYQYNPYAATF 84
CWINV C. rubrum       QSP--PSNQPYRTAYHFQPRKNWINDPNGPMLFKGIYHLFYQYNPNGVKL 78
6-FEH A. thaliana     HQDL---NQPYRTGYHFQPLKNWMNDPNGPMIYKGIYHLFYQYNPYGAVW 77
CWINV5 A. thaliana    NNIK---NQPYRTGYHFQPPKNWMNDPNGPMIYKGIYHLFYQWNQNGAVM 81
CWINV1 A. thaliana    NTKSPSVNQPYRTGFHFQPPKNWMNDPNGPMIYKGIYHLFYQWNPKGAVW 90
CWINV2 A. thaliana    STSLESVSPLHRTAYHFQPPRHWINDPNAPMLYKGVYHLFYQYNPKGAVW 86
CWINV4 A. thaliana    --SVESVNHLHRPSFHFQPPKHWINDPNGPVYYKGLYHLFYQYNTKGAVW 88
CwINV1 D. carota      G--AENVKQVHRTGYHFQPKQNWINDPNGPMYYKGVYHLFYQYNPKGAVW 98
6-FEH B. vulgaris     DHQMINDDDPYRTAYHFQSPKNWMNDPNGPMIYKGIYHLFYQYYPYDPVW 94
1-FEHw1 T. aestivum   QDPSPAVSTMYKTAFHFQPAKNWMNDPSGPMYFNGFYHEFYQYNLNGPIF 100
```

Figure IV.8 Multiple alignment of *At(6&1)-FEH* and some highly related translated cDNAs. Alignment of the deduced amino acid sequences of the N-terminal of *6-FEH* cDNA from *B. vulgaris*, cell wall invertase 1, 2, 4, 5, *(6&1FEH)* and *6-FEH* cDNA from *A. thaliana*, *1-FEH IIa* cDNA from *C. intybus*, *1-FEHw1* cDNA from *T. aestivum* and cell wall invertase cDNAs from *C. intybus, Daucus carota* and *Chenopodium rubrum*.

This means that this protein lacks the signal peptide necessary for transport to the endoplasmic reticulum and subsequent glycosylation. Therefore, At(6&1)-FEH might have escaped in our routine protocol based on affinity chromatography. Indeed, SignalP (www.cbs.dtu.dk/services/SignalP), an analysis program that predicts the presence and location of signal peptide cleavage sites, did not detect any signal peptide. This could mean that At(6&1)-FEH does not reside in the cell wall or any other organelle, but in the cytoplasm. TargetP (www.cbs.dtu.dk/services/TargetP), a prediction program for subcellular localization, did not target At(6&1)-FEH to the chloroplast, the mitochondria nor the secretory pathway. In contrast, both At6-FEH and AtcwINV1 were targeted to the secretory pathway.

These findings could suggest a cytoplasmic localization for At(6&1)-FEH. However, contrary to this hypothesis, heterologous At(6&1)-FEH has an acidic pH optimum of 5.4 (Fig. VI.9), like other FEHs and CWINVs.

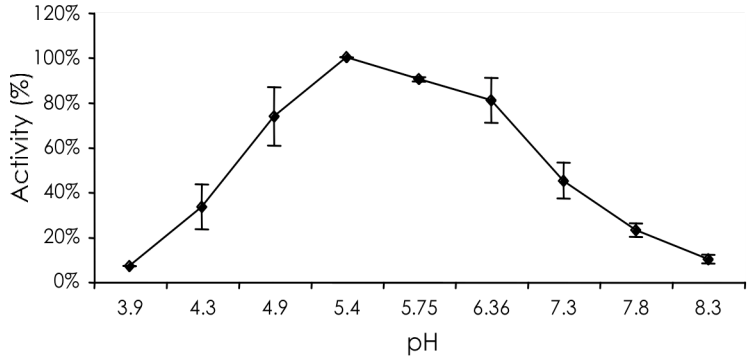


Figure IV.9 pH optimum of At(6&1)-FEH. Heterologous At(6&1)-FEH was incubated with 5 mM levan for 1h at 30°C at different pH values. Activity is expressed as the percentage of the maximal activity.

If At(6&1)-FEH is indeed cytoplasmic and not glycosylated *in vivo*, glycosylations due to the *P. pastoris* expression system can result in aberrant activity properties. Therefore, prior to activity measurements, recombinant At(6&1)-FEH and At6-FEH were incubated with $EndoH_f$ (Fig. IV.10) (Materials and Methods § VII.2.2), a glycosidase cleaving the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins. The 6-FEH activity of At(6&1)-FEH increased slightly (1/3) whereas At6-FEH activity was marginally affected.

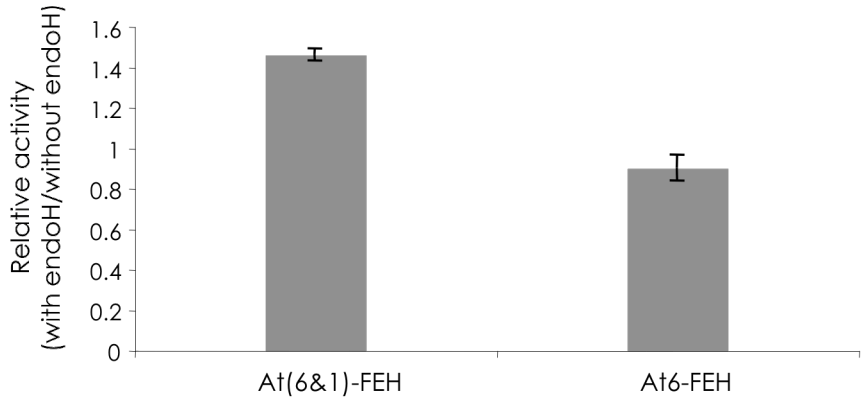


Figure IV.10 6-FEH activity of heterologous At(6&1)-FEH and At6-FEH after incubation with EndoH$_f$. Heterologous At(6&1)-FEH and At6-FEH were incubated with 5 mM levan for 1h at 30°C after 1h incubation at 37°C of both enzymes with U EndoH$_f$.

Inhibition by Tris

So, to continue with the purification of native At(6&1)-FEH, the flow through of the ConA column (non-glycosylated fraction) was loaded on a Mono Q column equilibrated with Tris-HCl pH 7.5. To our surprise no activity was found in any of the fractions of the eluate. In order to find out what happened with the At(6&1)-FEH enzyme during purification, activity of the different recombinant enzymes was checked in combination with 5 and 20 mM Tris (pH was kept at 5) (Materials and Methods §VII.2.2). All enzymes except At(6&1)-FEH more or less kept their activity (Fig. IV.11). With 20 mM Tris At(6&1)-FEH lost 90% of its activity, indicating that this enzyme is inhibited by Tris.

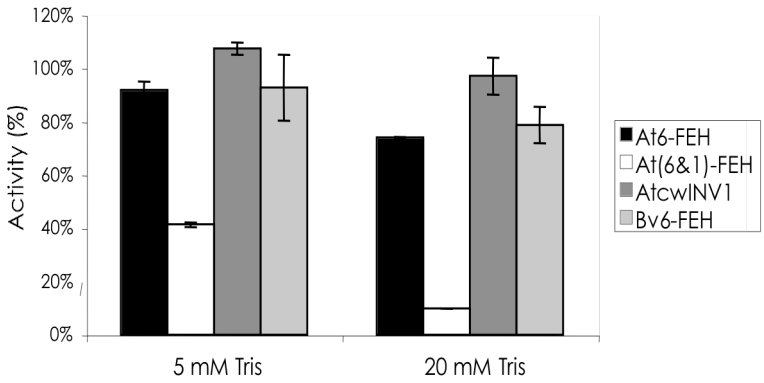


Figure IV.11 Relative activity of heterologous At6-FEH, At(6&1)-FEH, AtcwINV1, Bv6-FEH in combination with Tris. Enzymes were incubated with 4 mM levan and 5 or 20 mM Tris. Y-axis represents activity values relative to activity measurements with 0 mM Tris.

Tris acts as an inhibitor of At(6&1)-FEH with a K_i of 4.38 mM (calculated with 4 mM levan) (Fig. IV.12).

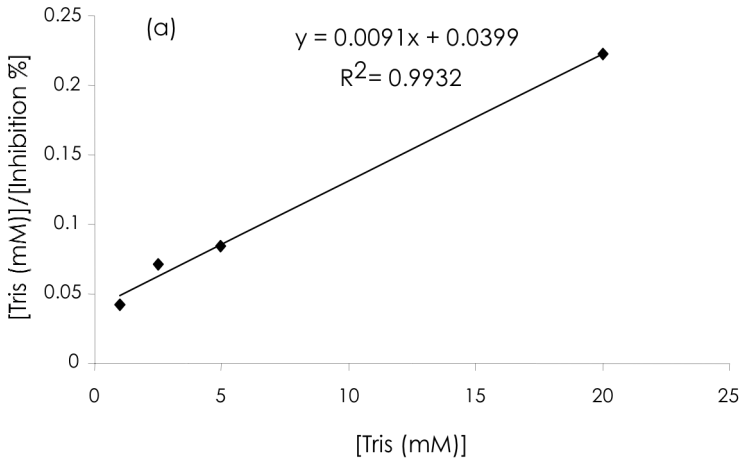


Figure IV.12 Inhibition of heterologous At(6&1)-FEH by Tris. A plot of the ratio Tris conctration/% inhibition of At(6&1)-FEH as a function of the Tris concentration. The fitted linear regression line and parameters are indicated. From this, a K_i was derived.

IV.3 Discussion

Cell wall invertases and fructan exohydrolases are very similar in amino acid sequence. A phylogenetic tree of both enzymes clusters the known monocot and dicot 1-FEHs from fructan plants in two different subgroups, respectively branch IV and V (Fig. III.3). Bv6-FEH, the first characterized and cloned 6-FEH from a non-fructan plant (Chapter II, III) is located in a different subgroup (branch III) containing cell wall invertases only. This new enzyme raised questions about the functionality of the cDNAs categorized as CWINVs by means of sequence homology.

In order to proof that FEHs also exist in non-fructan plants other than sugar beet, it seemed worthwhile to investigate the functionality of some known CWINV cDNAs. The focus in this chapter was on CWINVs from *A. thaliana*. At the start of this doctoral research, not much was known about the amino acids that determine substrate specificity of invertases and FEHs. So, based on amino acid sequence it was impossible to predict the functionality of the enzymes. Therefore, three CWINVs out of six (AtcwINV1, 3 and 6) were chosen based on their position in the phylogenetic tree, their pI (low or high) and their expression level (§IV.1).

IV.3.1 AtcwINV1

Heterologous expression of AtcwINV1 revealed the genuine invertase nature of this enzyme (Table IV.1). Sucrose is the best substrate and with a K_m below 1 mM this enzyme will still function optimally at low sucrose concentrations. Although the active site of AtcwINV1 is well designed to bind and hydrolyze sucrose, this is intrinsically linked to an efficient 1-kestose binding and degradation.

The high yield and homogeneity (Fig. IV.5) of this enzyme after heterologous expression made this protein suitable for crystallization; this was achieved by Verhaest *et al*. (2006) (Chapter I, §I.3.3.3). So, two 3-D structures of plant GH family 32 enzymes were elucidated and comparing them will help to determine which amino acids determine substrate specificity (Le Roy *et al*., submitted).

The expression level of AtcwINV1 is the highest among the six CWINVs from *A. thaliana*. Tymowska-Lalanna and Kreis (1998b) reported expression in roots, stems, leaves and flowers. Micro-array results (www.genevestigator.ethz.ch) more or less confirm these results

(see further §V.2.3). Major expression was found in the different parts of the roots, moderate expression was detected in leaves and seedlings and minor expression was found in flowers. AtcwINV1 is also induced after infection with *Erisyphe cichoracearum* (Fotopoulos *et al.*, 2003) and *Albugo candida* (Chou *et al.*, 2000) and after wounding (Quilliam *et al.*, 2006). The roles of cell wall invertases during development and infection or wounding are discussed in Chapter I (§I.2.2.2). In conclusion, AtcwINV1 is expressed in a developmental stage and organ-specific manner; moreover its expression level is also regulated by environmental factors.

IV.3.2 AtcwINV3 and AtcwINV6

In contrast to AtcwINV1, AtcwINV3 (At6-FEH) and AtcwINV6 (At(6&1)-FEH) are not genuine invertases. Although both degrading fructans, the enzymes are not identical in specificity towards β-2,1 and β-2,6 linkages (Table IV.1, Fig. IV.4).

The substrate specificity of At6-FEH resembles that of Bv6-FEH, which means that the enzyme prefers fructans with β-2,6 linkages. At6-FEH and Bv6-FEH are located in the same branch of the phylogenetic tree (Fig. IV.13); they share 53% identity compared to 47% identity between At(6&1)-FEH and Bv6-FEH. Therefore, it can be suggested that At6-FEH from *A. thaliana* is the orthologue of Bv6-FEH. The only difference between the two enzymes is the lack of activity of Bv6-FEH against inulin and 1,1-nystose (Table III.2). In fructan plants, only one 6-FEH (wheat; Van Riet *et al.*, 2006) has been cloned and characterized. Contrary to At6-FEH and At(6&1)-FEH, the wheat enzyme degraded levan, phlein and 6-kestose at a similar rate but 1,1-nystose and inulin were not subject to degradation. So, the wheat 6-FEH acts more specifically than the 6-FEHs found in non-fructan plants.

At(6&1)-FEH was even less specific towards the different substrates than At6-FEH and Bv6-FEH, although the enzyme still prefers fructans with β-2,6 linkages. Remarkably, 1-kestose and 6-kestose are degraded at a similar rate. At(6&1)-FEH is the first plant enzyme characterized that hydrolyzes both β-2,1 and β-2,6-linked fructans. It can be concluded that this enzyme has unique properties among all plant GH family 32 members. During this doctoral study, Kawakami *et al*. (2005) cloned and characterized an FEH from wheat with similar properties as At(6&1)-FEH. This enzyme is also able to degrade both β-2,1 and β-2,6 linkages and prefers β-2,6 linkages but, contrary to At(6&1)-FEH, wheat (6&1)-FEH is hardly able to degrade fructans with higher DP (levan and inulin).

Interestingly, for both recombinant Arabidopsis enzymes (At(6&1)-FEH: 87%; At6-FEH: 114%) and especially for the native At6-FEH (151%), neokestose is a very good substrate. The efficient breakdown of neokestose was also noticed for Bv6-FEH. So, FEHs in non-fructan plants are able to remove very easily the β-2,6 glucose-bound fructose unit but not the α-1,2 glucose-bound fructose unit. This result demonstrates that the presence of a fructose-fructose linkage is not a prerequisite for enzymatic activity. In contrast, neokestose is not efficiently broken down by 6-FEH (39 %) and 6-KEH from wheat (<< 1%) (Van Riet *et al.*, 2006; Van den Ende *et al.*, 2005b). Because of its extreme specificity towards 6-kestose, 6-KEH is unique among all known fructosyltransferases.

Several FEHs are inhibited by sucrose (§I.3.3.1, Table I.2). It was postulated that sucrose directly regulates the activity of some FEHs (De Roover *et al.*, 1999) and this was recently confirmed by co-crystallization of sucrose and chicory 1-FEH IIa (Verhaest *et al.*, 2007). Like 1-FEH from *Taraxacum officinale* (Rutherford and Deacon, 1972), 1-FEH I from *Cichorium intybus* (Claessens *et al.*, 1990), a FEH from *Hordeum vulgare* (Wagner and Wiemken, 1986), 6-FEH from *Triticum aestivum* (Van Riet *et al.*, 2006), and Bv6-FEH, At6-FEH and At(6&1)-FEH are almost not inhibited by sucrose. So, up to now, none of the FEHs in non-fructan plants are inhibited by sucrose. From the fact that some FEHs are inhibited by sucrose and some are not, it can be hypothesized that both groups fulfil different physiological roles. Perhaps FEHs in fructan plants that are not inhibited by sucrose fulfil the same role as FEHs in non-fructan plants.

IV.3.3 Purification of At6-FEH and At(6&1)-FEH

IV.3.3.1 Purification of recombinant enzymes

Purification of heterologous AtcwINV1, At6-FEH and At(6&1)-FEH from the *P. pastoris* supernatant indicates that there is not always an optimal yield of secreted proteins. Moderate yield of protein can have several causes: low stability, misfolding, retention (no extracellular secretion) (Ritsema *et al.*, 2006) etc. The wide distribution of activity (for At6-FEH and At(6&1)-FEH) amongst the different fractions makes it difficult to calculate specific activities. The *P. pastoris* expression system uses post-translational modifications similar to plant systems; the glycosylation chains, however, are longer and could mask ionized groups, causing heterogeneity in the elution pattern of FPLC.

It is, however, very important to keep in mind that the low specific activity of At6-FEH and At(6&1)-FEH for levan could be the result of using the wrong substrate for those enzymes. This will be discussed in Chapter V.

IV.3.3.2 Purification of native enzymes

During an enzyme purification procedure, a 6-FEH activity was separated from invertase activities and it was proven that the origin of this activity was due to the At6-FEH enzyme. This indicates that *A. thaliana* plants indeed contain 6-FEHs although activities in crude extracts were low and fluctuate together with invertase activities (Fig. IV.6). Since the native enzyme has the same substrate specificities as the recombinant enzyme, it can be concluded that the *P. pastoris* expression system is working well to distinguish fructan enzymes and invertases.

As explained in §IV.2.2.3, purification of At(6&1)-FEH could not be accomplished. The recombinant protein showed some special features: lack of an N-terminal signal sequence and inhibition by Tris. Two other putative cell wall invertase lack the N-terminal signal peptide (SignalP; www.cbs.dtu.dk/services/SignalP), namely a CWINV form *C. intybus* (Fig. IV.8) and CWINV7 from *Oryza sativa*. Like for At(6&1)-FEH TargetP (www.cbs.dtu.dk/services/TargetP) did not target either protein to the secretory pathway. Interestingly, both enzymes have a low pI and the CWINV from *C. intybus* clusters together with the 1-FEHs (branch V). CWINV7 from *O. sativa* is located in branch IVb of the phylogenetic tree (see further, Fig. IV.13), together with 6-FEH from wheat.

Normally fructan enzymes, VINVs and CWINVs are not inhibited by Tris. On the other hand, Tris inhibits neutral invertases (Van den Ende and Van Laere, 1995; Chen and Black, 1992). Van den Ende and Van Laere (1995) reported the purification of a neutral invertase from *Cichorium intybus* for which Tris acted as a competitive inhibitor ($K_i \approx 1.2$ mM). The specific mechanism of Tris inhibition has not been elucidated. There is no sequence homology between fructan enzymes, CWINVs, VINVs on the one hand and neutral invertases on the other hand. Both enzyme groups belong to different GH families; respectively 32 and 100.

IV.3.4 Phylogenetic tree

FEHs are restricted to the upper part of the phylogenetic tree (Fig. IV.13) and are located in different subgroups. It has been hypothesized that FEHs evolved from CWINVs (Van den Ende *et al.*, 2002). Both enzymes have the same acceptor substrate (water), but differ in donor substrate (sucrose or fructan). AtcwINV1 has a relatively high side-activity for 1-kestose and site-directed mutagenesis of only one amino acid turned AtcwINV1 into a 1-KEH. Kinetics indicate that the enzyme largely lost its ability to degrade sucrose but not 1-kestose (Le Roy *et al.*, submitted). So, FEHs could have evolved from cell wall invertases with very little mutations.

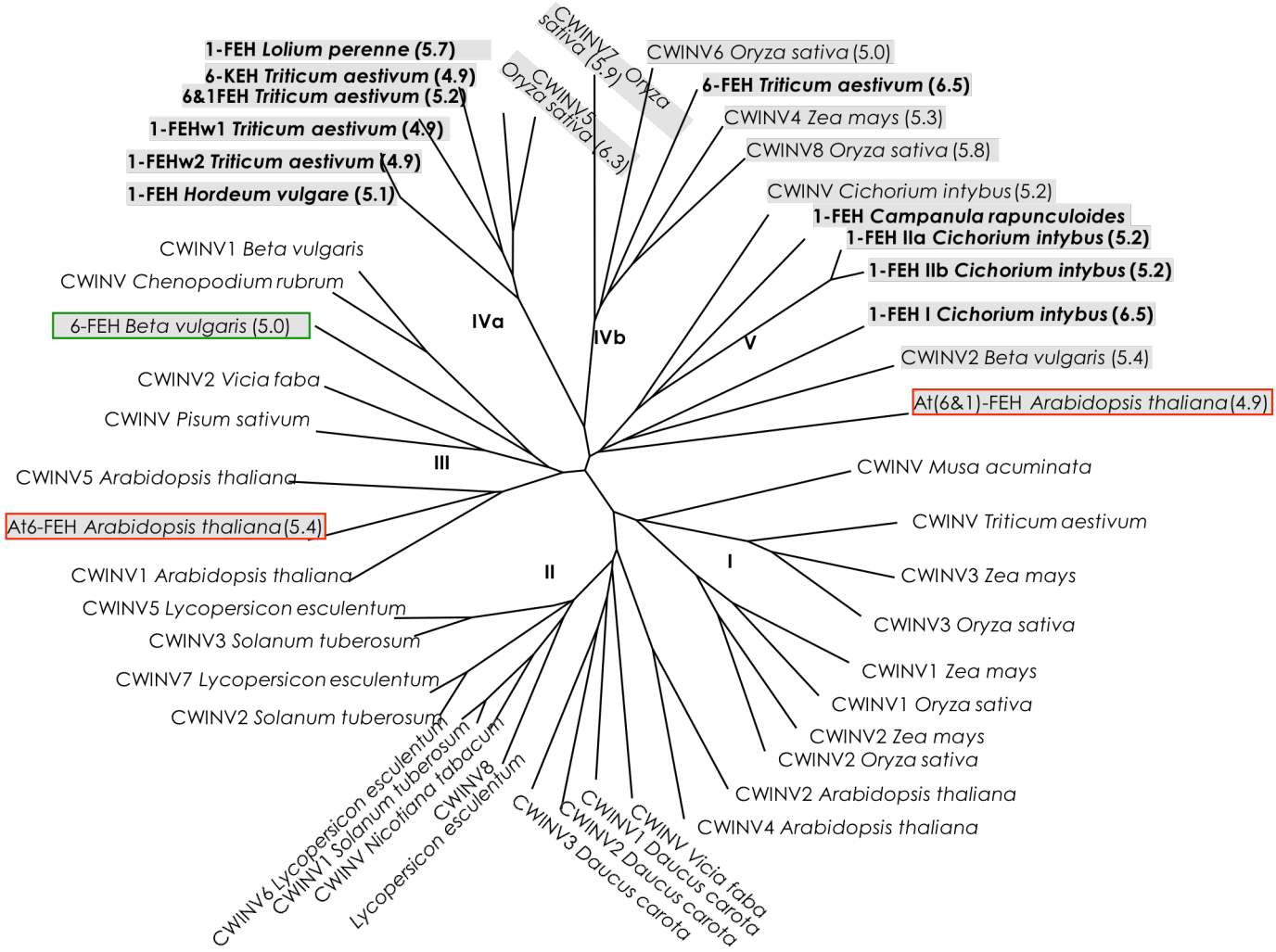


Figure IV.13 Unrooted phylogenetic tree of cDNA deduced amino acid sequences of cell wall-type invertases and fructan breakdown enzymes. Legend as in Fig. III.3.

Since AtcwINV1 and At6-FEH cluster together in the phylogenetic tree and since they have a similar genomic organization (Fig. IV.14), it can be speculated that At6-FEH evolved from AtcwINV1, losing its ability to degrade sucrose. At(6&1)-FEH, however, is located in a different branch. Its genomic organization does not differ drastically from AtcwINV1 and A6-FEH. It can be hypothesized that At(6&1)-FEH and AtcwINV1 evolved independently by duplication from an ancestral beta-fructosidase with both invertase and fructan-hydrolase activity.

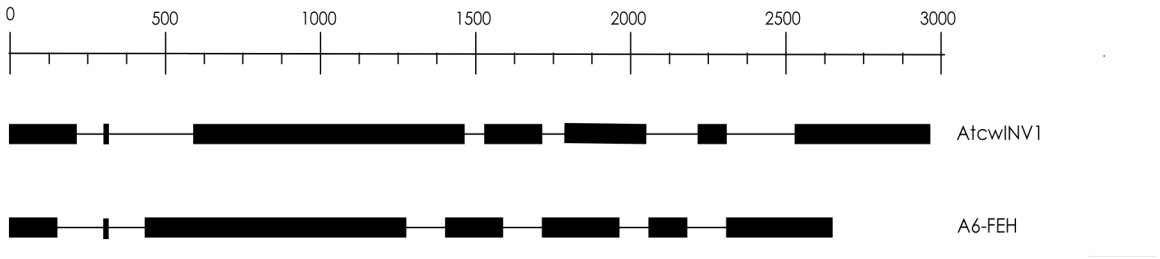


Figure IV.14 Gene structure of AtcwINV1 and At6-FEH. Intron (line)/Exon (black bars) structure in nucleotide coordinates relative to the start of the gene.

Up to now, all FEHs including those from non-fructan plants where found to have a low pI. The phylogenetic tree also displays some other CWINVs with a low pI. It has been suggested that these soluble enzymes belong to a second type of CWINVs, namely 'mobile' apoplastic invertases (Roitsch and Gonzàlez, 2004). However, the three characterized CWINVs with a low pI proved to be FEHs. This might point out that other putative CWINVs with low pI are FEHs instead of genuine invertases. Moreover the suggested existence of soluble 'invertases' in the cell wall can be questioned, since At6-FEH is most probably localized in the vacuole (Carter *et al.*, 2004). In this context we noticed the longer C-terminal extension of At6-FEH and Bv6-FEH (Fig. IV.15). It is known that vacuolar targeting signals can be located in the C-terminal extension (Matsuoka and Neuhaus, 1999). Some other FEHs might even be cytoplasmic given the lack of a normal signal peptide (e.g. At(6&1)-FEH). To elucidate once and for all the exact localization of FEHs in both fructan and non-fructan plants, GFP-fusion protein studies are strongly recommended.

```
1-FEHIIa C. intybus    FNNGTQNVKISEMSAWSMKNAKFVVDQSVKSAA------------ 581
(6&1)-FEH A. thaliana FNDGENGVIMTSLEAWSMRNAQINSNPTY---------------- 550
6-FEH A. thaliana      FNKGTQSVDILTLSAWSLKSAQINGDLMSPFIEREESRSPNHQF- 594
CWINV5 A. thaliana     FNKGSQNVNVLSLSAWSMKSSL---------------------- 572
CWINV1 A. thaliana     FNYGYQSVDVLNLNAWSMNSAQIS--------------------- 584
6-FEH B. vulgaris      FNNGTEDVKITKLSAWSMKKAQINLSTDNTSNMSYNSNKVEKEEF 606
CWINV2 A. thaliana     FNNGSQPVTVESLNAWNMQKPLKMNQGAK---------------- 590
CWINV4 A. thaliana     FNNGTQTVTIESLNAWNMDRPLQMNDGAL---------------- 591
CwINV1 D. carota       FNNGSETITVENLDAWSMKKPLRMN-------------------- 592
```

Figure IV.15 Multiple alignment of some highly related translated cDNAs. Alignment of the deduced amino acid sequences of the C-terminal of *6-FEH* cDNA from *B. vulgaris*, cell wall invertase 1, 2, 4, 5, *(6&1)-FEH* and *6-FEH* cDNA from *A. thaliana*, *1-FEH IIa* cDNA from *C. intybus* and a cell wall invertase cDNA from *Daucus carota*.

In conclusion:

The data described in this chapter indicate that the occurrence of FEHs is not restricted to sugar beet. After heterologous expression and characterization of the corresponding cDNAs, two enzymes from *A. thaliana* (AtcwINV3 and AtcwINV6), formerly categorized as putative cell wall invertases, have been identified as FEHs. AtcwINV3 and AtcwINV6 are now named At6-FEH and At(6&1)-FEH, respectively. Both enzymes differ strongly in substrate specificity and putative localization. In the next chapter, the physiological function of both enzymes is investigated.

CHAPTER V

PHYSIOLOGICAL ROLE OF FEHS IN NON-FRUCTAN PLANTS?

V.1 Introduction

Now that apparently FEHs occur in different species, the quest for their function can start. Their role in non-fructan plants is not obvious but two possible functions can be postulated a priori:

- FEH degrades minute amounts of endogenous fructans or any other yet unknown compounds containing one or more fructose molecules.
- FEH degrades exogenous fructans produced by pathogenic or symbiotic bacteria and prevents infection, in analogy with chitinases and β-glucanases hydrolyzing cell-surface components of fungal plant pathogens. In addition to this, FEHs might have a general detoxification role and prevent fructan accumulation, even after infection occurred.

Arabidopsis thaliana has emerged as the model organism of choice in plant biology research. As results from the previous chapter demonstrated that at least two FEHs occur in *A. thaliana* and since there were no indications for a clear physiological function of Bv6-FEH in sugar beet, *A. thaliana* was chosen to further investigate the role of these FEHs. A powerful approach to reach this goal is the use of transgenic plants. Therefore, this chapter starts with the construction of *At6-FEH* and *At(6&1)-FEH* knock-out (KO) and overexpression (OEX) plants. Next, sugar extracts of both knock-out and wild-type plants are investigated for the presence of an endogenous substrate. Subsequently, the expression of *At(6&1)-FEH* and *At6-FEH* is examined after different treatments of wild-type (WT) plants. Furthermore, micro-array results from public databases are analyzed and summarized. To investigate the possible role of FEHs during pathogenesis, OEX and KO plants have been challenged with *Pseudomonas syringae* pv. *tomato* DC3000. This pathogen is known to produce levans and the interaction between *A. thaliana* and *P. syringae* is well described (Katagiri *et al*., 2002).

V.2 Results and discussion

V.2.1 *At6-FEH* and *At(6&1)*-FEH overexpression and knock-out plants

V.2.1.1 Knock-out lines at6-feh and at(6&1)-feh

Expression and activity

The homozygous KO lines *at6-feh* (Ws background) and *at(6&1)-feh* (Col-0 background) were obtained from the Laboratory of Molecular Biology in Edinburgh (Dr. Steven Smith) (Materials and Methods, §VII.1.1). With semi-quantitative RT-PCR, the absence of At6-FEH and At(6&1)-FEH mRNA was reconfirmed (Fig. V.1).

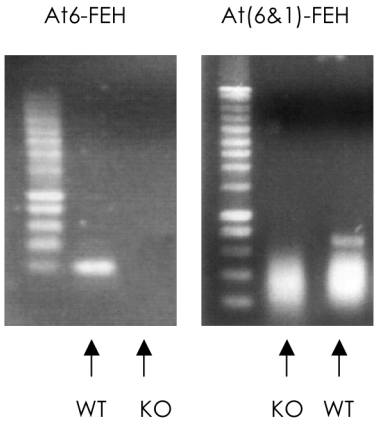


Figure V.1 Confirmation of knock-out lines. Expression of *At6-FEH* and *At(6&1)-FEH* in wild-type lines and KO lines *at6-feh* and *at(6&1)-feh*, respectively.

Next, 6-FEH and invertase activities were measured in both lines and compared to the activities in wild-type lines (Fig. V.2).

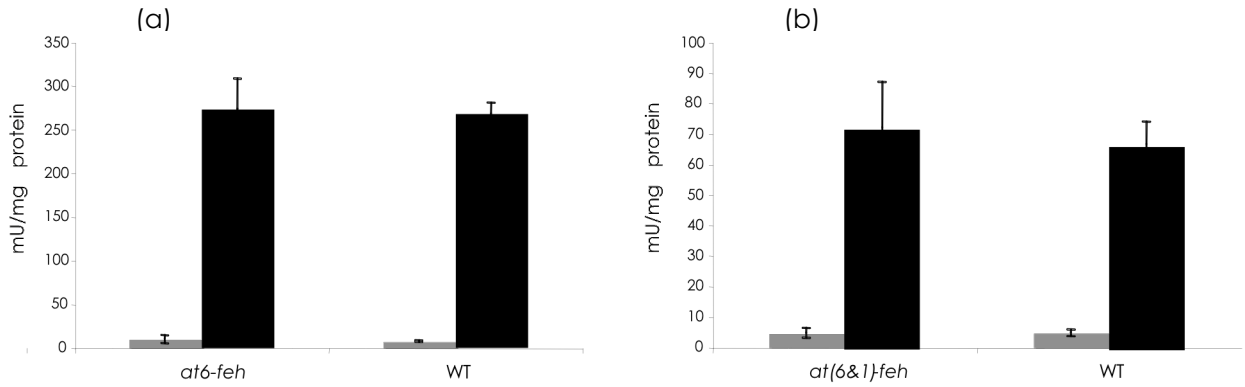


Figure V.2 6-FEH and invertase activities in whole Arabidopsis plants. Production of fructose from levan (grey bars) and sucrose (black bars) was measured in wild-type lines (a and b) and *at(6&1)-feh* (a) and *at6-feh* (b) 8-week old plants. Results are the mean (±SE) for n=3.

As expected, no difference was found in the invertase activity between the wild-type line and both KO lines. Remarkably, no change in 6-FEH activity could be detected either.

For both seedlings and mature plants no different phenotype was observed for the KO plants under normal conditions. Also some preliminary experiments, investigating growth of seedlings on Murashige Skoog medium, supplemented with different sugars, did not result in a different phenotype.

Discussion

As discussed in § IV.2.2.1, the FEH activity in the different plant parts of *A. thaliana* is very low and it is possible that this activity corresponds to a side activity of soluble invertases, explaining the similar 6-FEH activities measured in WT and KO lines. An alternative explanation is redundancy. Based on the fact that at least two FEH genes are present in *A. thaliana*, one enzyme could replace the other enzyme when the first one is absent. However, as discussed in §IV.3, At6-FEH and At(6&1)-FEH seem to have different characteristics and localization. Therefore redundancy in this situation is unlikely.

V.2.1.2 Overexpression lines 35S::At6-FEH and 35S::At(6&1)-FEH

Expression and activity

Overexpression lines were constructed in collaboration with Dr. Delauré from CMPG (Centre of Microbial and Plant Genetics, KULeuven, Prof. B. Cammue). Plant transformation vectors were constructed containing the coding region of *At6-FEH* or *At(6&1)-FEH*, the cauliflower mosaic virus 35S promotor (p*35S*), and a selection marker gene, resulting in resistance to the herbicide phosphinothricin (Basta, Hoechst, Agrevo) (Materials and Methods, §VII.6.1). After mobilization of the vectors into *Agrobacterium tumefaciens,* Arabidopsis plants were transformed by the floral-dip method (Clough and Bent, 1998). Subsequently, transgenic plants were selected on a sand/perlite mixture containing the Basta herbicide (Materials and Methods, §VII.6.2).

Gene-expression studies via semi-quantitative RT-PCR (Materials and Methods, §VII.3.2) were performed in order to select for the highest expressors. Unfortunately, not all high expressors exhibited higher 6-FEH activity than wild-type plants (data not shown). Therefore, in a next strategy several T1 (first transgenic line) plants were selected and the 6-FEH activity was measured. In Figure V.3 6-FEH activities in different *At6-FEH* and *At(6&1)-FEH* OEX plants are displayed. Only ± 25% of the transformed plants had higher activities than wild-type plants.

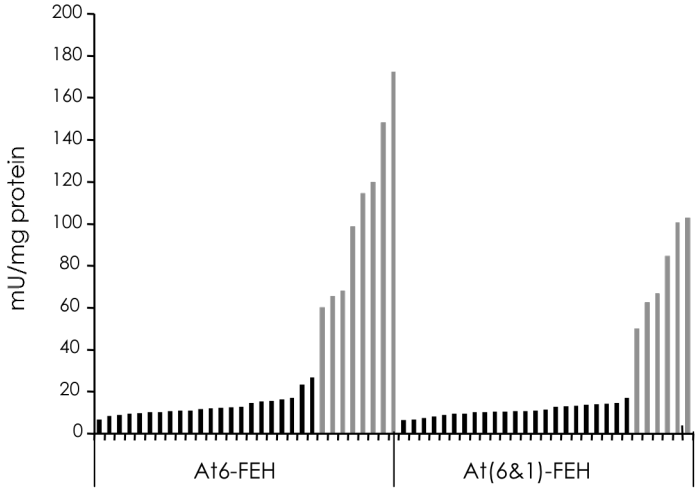


Figure V.3 6-FEH activity in *A. thaliana* leaves. Production of fructose from levan (5 mM) was measured in different *At6-FEH* and *At(6&1)-FEH* OEX plants. Grey bars present significantly higher 6-FEH activity than in wild-type lines.

Different OEX plants with high activity were chosen for self-pollination and T2 seedlings were grown and sprayed with Basta. The same was done for T3 seedlings. Based on the expected 3:1 segregation of the phosphinothricin selectable marker, homozygous plants could be selected. Finally two homozygous plants of each line were chosen for qRT-PCR (Fig. V.4; Materials and Methods, §VII.3.3) and activity measurements (Fig. V.5; Materials and Methods, §VII.2.2).

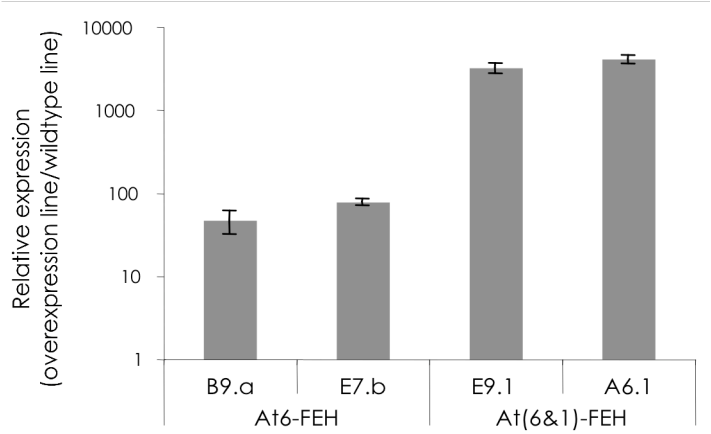


Figure V.4 Expression of *At6-FEH* and *At(6&1)-FEH* in leaves. Expression of *At6-FEH* and *At(6&1)-FEH* in the lines *35S::At6-FEH* (B9a and E7b) and *35S::At(6&1)-FEH* (E9.1 and A6.1) compared to the wild-type line. Results are the mean (±SE) for n=3.

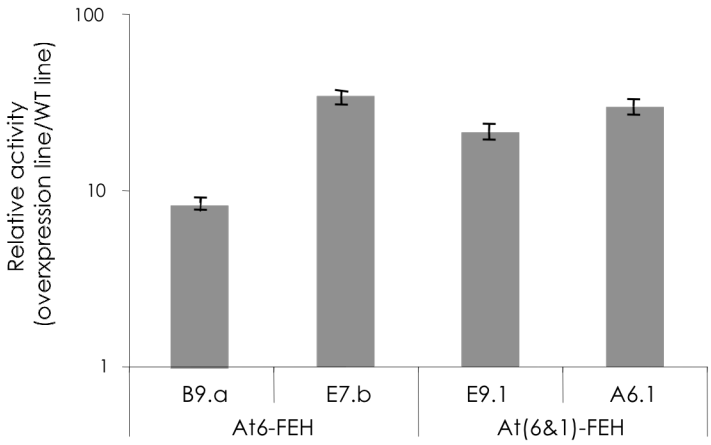


Figure V.5 6-FEH activities in *A. thaliana*. 6-FEH activity of crude extracts from *35S::At6-FEH* (E7b and B9a) and *35S::At(6&1)-FEH* (E9.1 and A6.1) plants compared to wild-type plants. Results are the mean (±SE) for n=3.

From the previous graphs (Fig. V.4; V.5), it can be concluded that for both OEX lines higher mRNA transcripts correlated with higher 6-FEH activities. Remarkably for *35S::At(6&1)-FEH* approximately 3500 times more mRNA transcripts (possibly due to the very low endogenous expression level) are required than the wild-type to give a 30-fold rise in 6-FEH activity. This is not the case for At6-FEH.

V.2.1.3 Growth of At(6&1)-FEH OEX plants

After self-pollination of highly expressing T1 *35S:: At(6&1)-FEH* plants, the resulting T2 seedlings germinated very poorly on sand/perlite substrate impregnated with basta (Fig. V.6a). This was not the case for the low expressors (Fig. V.6b).

(a) (b)

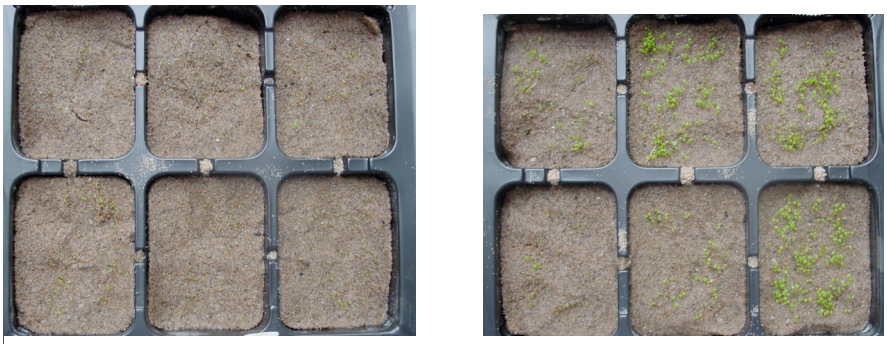


Figure V.6 Growth of *35S::At(6&1)-FEH* T2 seedlings. (a) six high expressors, verified in T1 generation, (b) six low expressors, verified in T1 generation. The medium is sand/perlite impregnated with a Basta solution.

To exclude the effect of Basta, seeds were grown on soil and after 2 weeks seedlings were sprayed with basta (Fig. V.7). For the high expressors approximately 25% of the seeds germinated. Based on this number, it was thought that those plants were the homozygous WT plants and that transgenic *35S::At(6&1)-FEH* did not germinate. However, after Basta spray ± 2% survived. Those two or three plants (out of 100) of each line all exhibited high 6-FEH activity.

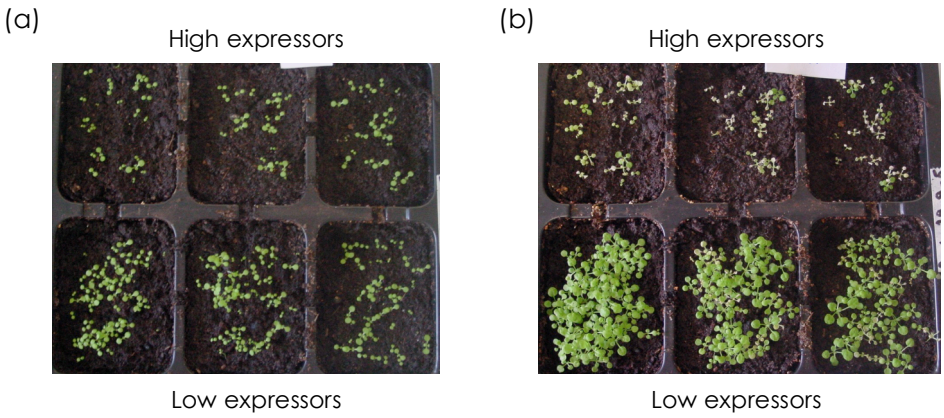


Figure V.7 Growth of *35S::At(6&1)-FEH* T2 seedlings. (a) three high expressors and three low expressors before Basta spray (b) three high expressors and three low expressors after Basta spray.

It was expected that seeds of the high expressor T2 generation would germinate normally. Figure V.8 however showed that only 22% of the homozygous *35S::At(6&1)-FEH* T3 generation germinated.

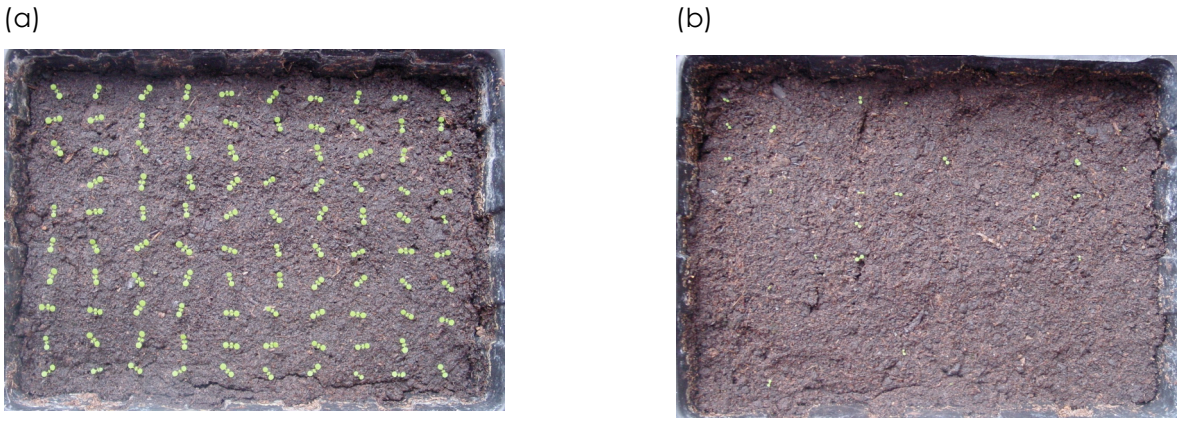


Figure V.8 Growth of *35S::At(6&1)-FEH* T3 seedlings. (a) Col0, (b) *35S::At(6&1)-FEH* line E9.1 cfr. (Fig. V.4 and V.5).

No explanation could be found for the bad germination of the seeds from high expressors of *35S::At(6&1)-FEH.* Since *At(6&1)-FEH* is constitutively expressed in those plants, a good strategy would be to use a construct with an inducible promoter. This could elucidate if *At(6&1)-FEH* is indeed playing a role during germination.

V.2.2 Endogenous substrate

Arabidopsis thaliana and *Beta vulgaris* belong to the family of the Brassicaceae and the Chenopodiaceae, respectively. Species from both families do not accumulate fructans. It can be speculated that fructan accumulation is not always detectable because of a high turnover. In contrast to this hypothesis no genes encoding homologues to SST and/or FFT (fructan biosynthetic enzymes) can be detected in the *A. thaliana* genome. It is, however, known that soluble invertases can produce some fructan trisaccharides from high sucrose concentrations (Cairns and Ashton, 1991 and references therein). In an extract of excised and illuminated leaves of *A. thaliana,* the fructan trisaccharides 1-kestose, 6-kestose and neokestose are synthesized after incubation with 200 mM sucrose (Fig. V.9). This is probably due to a side-activity of one or both soluble vacuolar invertases, Atβfruct3 and Atβfruct4, present in *A. thaliana* (Haouazine-Takvorian *et al.,* 1997). So, the possibility exists that FEH substrates could be synthesized under certain conditions.

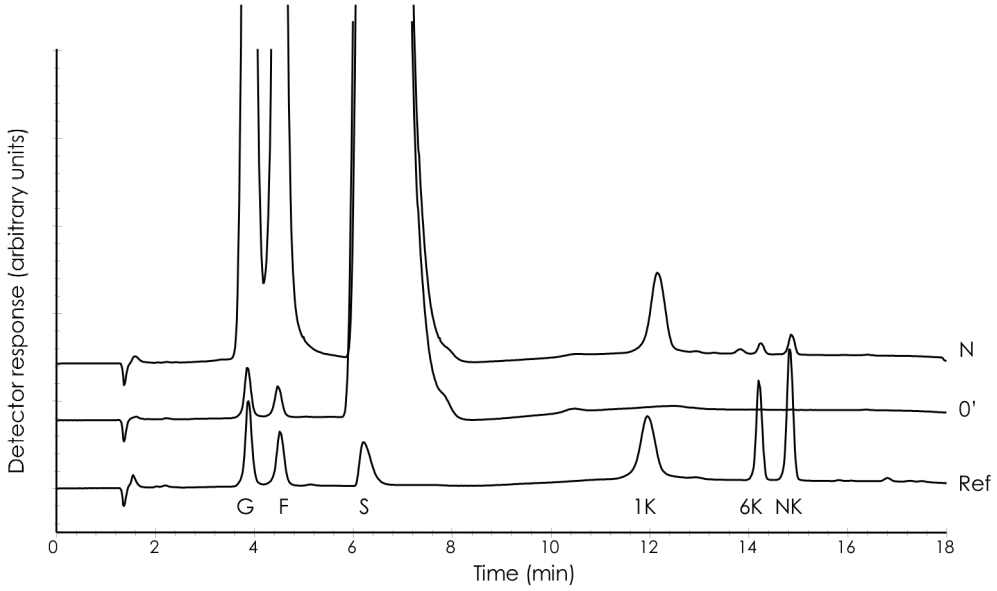


Figure V.9 *In vitro* synthesis of fructan trisaccharides. AEC-PAD chromatograms of incubation mixture of a soluble enzyme extract of excised and illuminated *A. thaliana* leaves with 200 mM sucrose. Ref (reference), N (overnight incubation), G (glucose), F (fructose), S (sucrose), 1K (1-kestose), 6K (6-kestose), NK (neokestose).

Because of the rather low specific activity of the FEHs towards kestoses, it can be hypothesized that other unknown fructosyl containing compounds are target of these enzymes. In figure V.10 a chromatogram of the low DP neutral sugar fraction (after passing through a dowex column) in WT and KO *A. thaliana* plants is depicted. No difference is seen in sugar composition of both lines. Absence of a detectable substrate was further verified by combining the sugar extract with recombinant At6-FEH and At(6&1)-FEH. No peaks are disappearing.

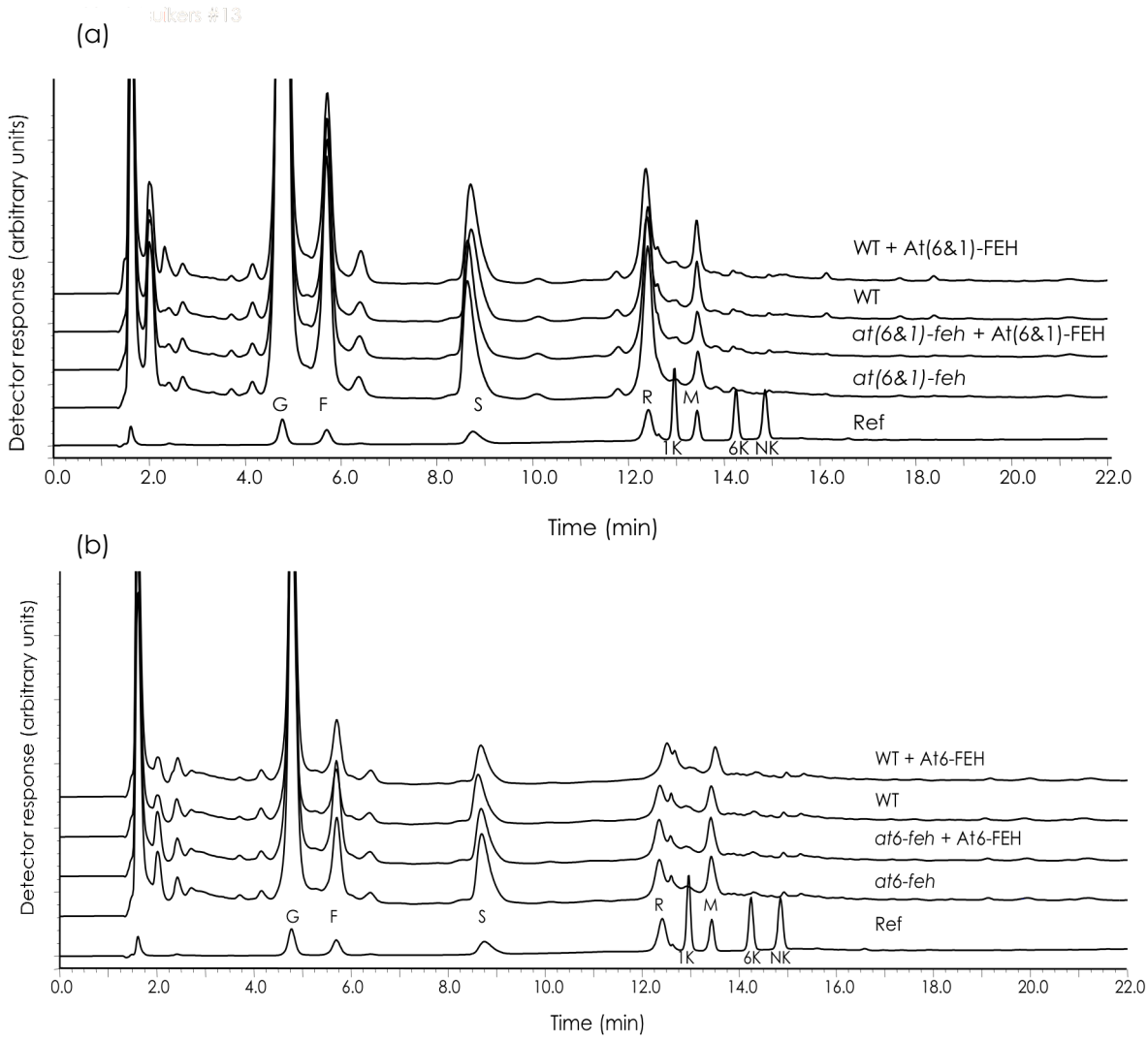


Figure V.10 Sugar content of *A. thaliana* plants. AEC-PAD chromatograms of sugar extracts of KO and WT plants of *At(6&1)-FEH* (a) and *At6-FEH* (b). Sugar extracts were also incubated with recombinant At(6&1)-FEH (a) and At6-FEH (b). Ref (reference), G (glucose), F (fructose), S (sucrose), R (raffinose), 1K (1-kestose), M (maltose), 6K (6-kestose), NK (neokestose).

Other experiments where sugar extracts from WT plants were analyzed before using a dowex column or after passing through only dowex Ac^- or dowex H^+ did not reveal new substrates (data not shown). Also after removing glucose and fructose from the sugar samples, no fructose peak is appearing when samples where incubated with recombinant At6-FEH or At(6&1)-FEH. From all these results it can be deduced that no detectable endogenous substrates could be found in healthy *A. thaliana* plants. Sugar extracts were also made for OEX plants (data not shown), but as expected no additional sugar compounds have been found.

V.2.3 Expression profiles of *At6-FEH* and *At(6&1)-FEH*

Sherson *et al*. (2003) determined the relative expression levels of *At6-FEH* and *At(6&1)-FEH* in leaves, seedlings, flowers and seeds with semi-quantitative RT-PCR. These data suggested that (1) both enzymes are expressed in all tissues, but at a lower level than *AtcwINV1* and (2) that expression of *At(6&1)-FEH* is higher than *At6-FEH* in seedlings and leaves. In the following graph (Fig. V.11) expression profiles of *AtcwINV1*, *At6-FEH* and *At(6&1)-FEH* amongst the different tissues are reproduced from micro-array results obtained by the Gene Atlas tool from Genevestigator (www. Genevestigator .ethz.ch; Zimmerman *et al*., 2004).

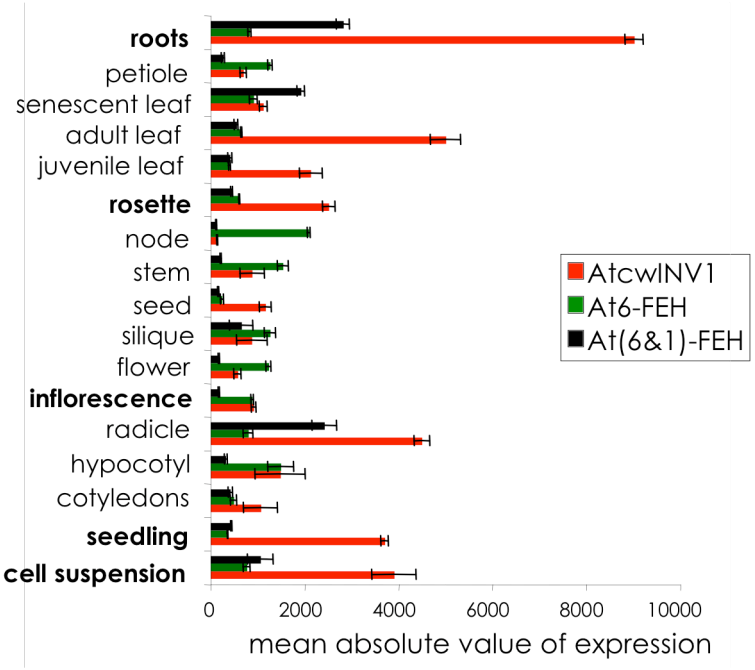


Figure V.11 Expression profiles of *At6FEH*, *At(6&1)-FEH* and *AtcwINV1* in the different tissues. Organs are given in bold under their different parts. Results obtained from Genevestigator.

Expression of *AtcwINV1* is low in inflorescences. In roots, adult and juvenile leaves, seedlings and cell suspensions the expression of *AtcwINV1* is much higher than the other two enzymes. The highest expression level of *At(6&1)-FEH* is found in roots, senescent leaves, radicles and cell-suspension cultures. Only in these tissues expression of *At(6&1)-FEH* is much higher than *At6-FEH*. Expression of *At6-FEH* is more equally distributed among the different tissues and its expression is higher than that of *At(6&1)-FEH* in stems, nodes, hypocotyls, inflorescences, flowers, siliques and petioles. From these data it is very difficult to formulate hypotheses about the role of FEHs in non-fructan plants. *At(6&1)-FEH* seems to be expressed in rapidly dividing tissues such as radicles, roots and cell suspension cultures. But oppositely to this, it is also expressed in senescent leaves.

In the following graph (Fig. V.12), expression profiles of *AtcwINV1*, *At6-FEH* and *At(6&1)-FEH* after different treatments are reproduced from micro-array results obtained by the Response Viewer tool from Genevestigator (www. Genevestigator .ethz.ch).

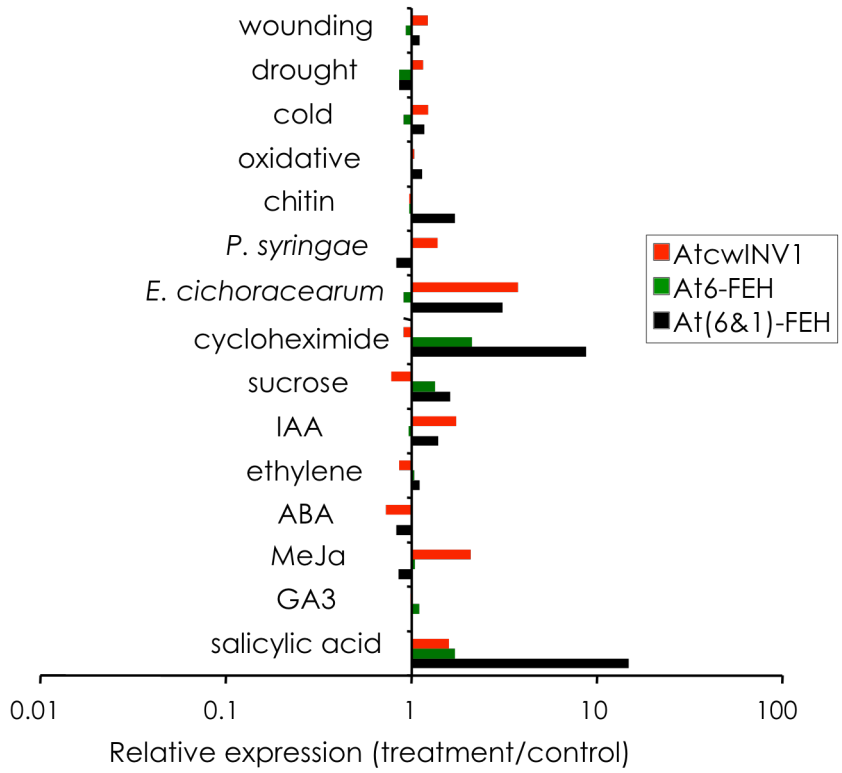


Figure V.12 Expression profiles of *At6FEH*, *At(6&1)-FEH* and *AtcwINV1* during different stress treatments.

Expression of *At6-FEH* did not change significantly under any of the condition tested. For *AtcwINV1* only *E. cichoracearum* induced its expression. Fotopoulos *et al.* (2003) confirmed the latter result.

Although micro-array results can give a good idea about the expression of a gene, these data should be considered with care. For example, no induction of *AtcwINV1* was seen after wounding. Quilliam *et al.* (2006), however, reported a fast induction of *AtcwINV1* after mechanical wounding. The same authors also reported a significant expression of *AtcwINV3 (At6-FEH)* in untreated leaves, but could not detect high levels of *AtcwINV6 (At(6&1)-FEH)*. This correlated well with our findings during several qRT-PCRs and semi-quantitative RT-PCRs.

Expression of *At(6&1)-FEH* increased after treatment with salicylic acid and cycloheximide. Inoculation with *E. cichoracearum* resulted in a moderate increase of *At(6&1)-FEH* mRNA. Salicyclic acid (SA) is an important signal molecule in plant defense reactions. Cycloheximide (CHX) is a protein synthesis inhibitor. Induction by CHX has for example been reported for auxin and SA early response genes (Abel *et al.,* 1995). Induced expression by both SA and CHX has been described for a transcription factor called Dof protein (DNA binding with one finger) (Kang and Singh, 2000).

Expression of *At(6&1)-FEH* and *At6-FEH* was verified with semi-quantitative RT-PCR after treatment with INA (an inducer of resistance, similar effect as SA) (Fig. V.13a). The *A. thaliana* housekeeping gene elongation factor 1α (*EF*) was used as internal control.

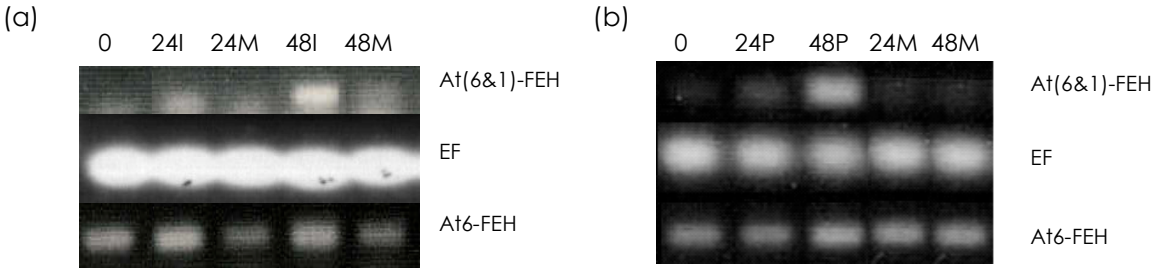


Figure V.13 Expression of *At(6&1)-FEH* and *At6-FEH* after treatment of *A. thaliana* leaves with INA (a) and paraquat (b). 0: 0h; 24I, 48I: 24h and 48h after treatment with INA; 24M, 48M: 24h and 48h after mock treatment (water); 24P, 48P: 24h and 48h after treatment with paraquat.

Induction of *At(6&1)-FEH* was also seen after treatment with paraquat, a chemical that induces the production of reactive oxygen species. Both chemicals did not significantly influence the expression of *At6-FEH*.

To verify some of the micro-array results, a qRT-PCR was performed after treatment of 4 week old *A. thaliana* plants with different hormones, *P. syringae* and paraquat. Results of *At(6&1)-FEH* are shown in figure V.14.

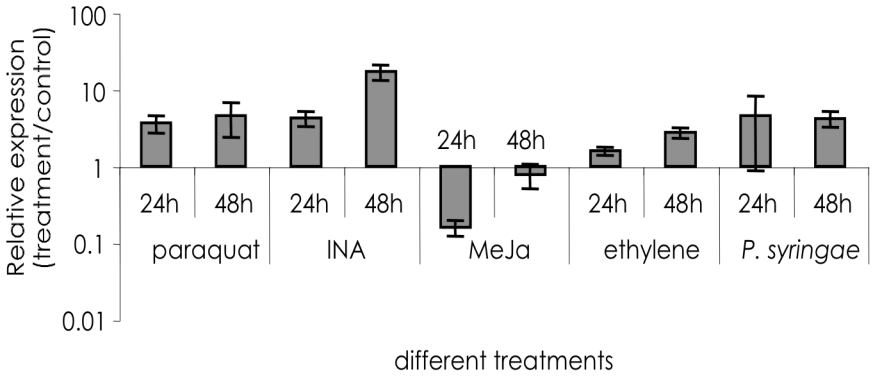


Figure V.14 Expression of *At(6&1)-FEH* in *A. thaliana* leaves. Relative expression of *At(6&1)-FEH* in wild-type *A. thaliana* plants at 24h and 48h after chemical treatment as compared to control (mock-treated) plants. Results are the mean (±SE) for n=3.

The data in Figures V.13 and V.14 confirm the induction of *At(6&1)-FEH* after treatment with SA or INA as seen in the micro-array results (Fig. V.12). The micro-array was performed on seedlings and induction was noticed after 3h. The experiments done during this doctoral study showed an induction of *At(6&1)-FEH* in 4 week old plants 48h after treatment with INA. Low inductions were seen after treatment with paraquat and *P. syringae* (48h). Expression of *At(6&1)-FEH* seems to be repressed with MeJa. However, since expression of *At(6&1)-FEH* is so low in untreated leaves, this repression could be of little importance.

Since the expression of *At(6&1)-FEH* increased after challenging with INA we wanted to test for elevated 6-FEH activity in those plants. No elevated 6-FEH activity was detected in the treated plants (Fig. V.15). However, this is perhaps not so unexpected knowing that 3500 times more mRNA is necessary to give a 30-fold rise in activity (Fig. V.4, V.5).

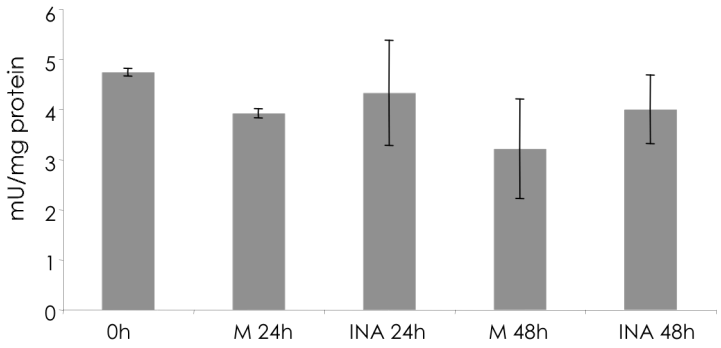


Figure V.15 6-FEH activities in *A. thaliana* leaves. 6-FEH activity of crude extracts from mock-treated (M, water) and INA-treated plant leaves at 24h and 48h.

In conclusion, the expression patterns of *At6-FEH* and *At(6&1)-FEH* differed significantly. *At6-FEH* is not induced by any of the treatments (data not shown). *At(6&1)-FEH* is significantly induced by SA (and INA). SA signalling is very important during plant defence and its pathway works via a complex network (reviewed in Shah, 2003). Occurrence of a hypersensitive response or attack by certain pathogens triggers activation of SA-dependent signalling. SA levels rise, leading to SAR (systemic acquired resistance) associated with activation of defence effector genes (e.g. PR1). SA signalling is mediated by two mechanisms: an *NPR1* (non-expressor of PR1) dependent and *NPR1* independent pathway (Glazebrook, 2005). SA signalling plays an important role in limiting biotrophic *P. syringae* growth. Besides defences depending on SA, other defence pathways depending on jasmonic acid and ethylene have been described. Especially necrotrophic bacteria (e.g. *Erwinia carotovora)* induce this pathway. Both pathways, however, seem to act antagonistically and synergistically (Thatcher *et al*., 2005).

V.2.4 The role of At6-FEH and At(6&1)-FEH during pathogen attack

V.2.4.1 Introduction

As mentioned before it can be hypothesized that FEHs in non-fructan plants play a role in plant-pathogen interaction. Many bacteria produce extracellular polysaccharides (EPS) in culture and in host plants during pathogenesis or symbiosis. EPS can be homo-polysaccharides such as levan, cellulose, glucan and alginate or heteropolysaccharides that have repeating subunit structures with carbohydrate and non-carbohydrate substances such as amylovoran, stewartan and xanthan (reviewed in Leigh and Coplin, 1992; Denny, 1995). During pathogenesis, EPS might benefit the pathogen by promoting colonization, reducing contact with toxic molecules and minimizing contact with the plant. Levans are produced by some bacterial plant pathogens such as *P. syringae*, and *E. amylovora*. Like plant chitinases (§III.3.2) FEHs, could degrade the exogenous fructans resulting in a reduced virulence of the pathogens. In this paragraph, the *in vitro* antimicrobial activity of the recombinant enzymes At6-FEH and At(6&1)-FEH is tested. Next, the occurrence of levans in *Erwinia carotovora* is examined and finally OEX and KO plants are challenged with *P. syringae*.

V.2.4.2 In vitro antimicrobial activity of At6-FEH and At(6&1)-FEH

To test for *in vitro* antimicrobial activity of At6-FEH and At(6&1)-FEH, the enzymes were incubated with different bacteria. For this experiment the recombinant enzymes, precipitated and desalted from a *P. pastoris* culture, were used. A dilution series of the enzymes was incubated with different pathogenic bacteria such as *Erwinia amylovora*, *Erwinia carotovora* and *Pseudomonas syringae*. A protein extract from *P. pastoris* transformed with an empty vector was used as a negative control. Before the actual experiment, growth of the different bacteria was tested at pH 5 (optimal pH for FEH activity) and with 5% sucrose in the medium (to induce synthesis of fructans). It was verified that these conditions did not have negative effects on the bacterial growth (data not shown).

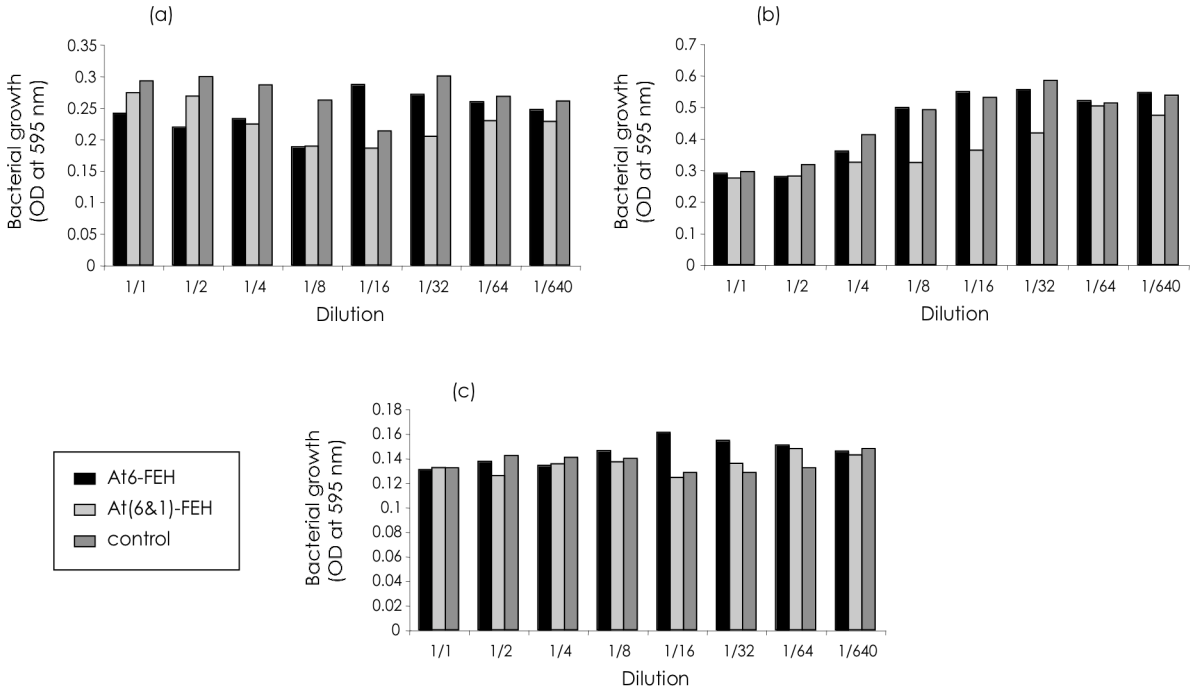


Figure V.16 Growth of bacteria. Determination of growth (18h) of *Erwinia carotovora* (a), *Erwinia amylovora* (b) and *Pseudomonas syringae* (c) after different dilutions of the recombinant enzymes At6-FEH, At(6&1)-FEH and a control.

The data in figure V.16 suggest that FEHs do not have an *in vitro* antimicrobial activity against the three chosen bacteria. For *E. amylovora* (b) there is an inhibitory effect of the protein extract, but this is also reflected in the negative control, indicating that FEH is not responsible for this inhibition. These results were more or less expected and do not exclude

a role for FEHs during pathogen attack. Levans are most probably not essential for bacterial growth *in vitro* although *P. syringae* and *E. amylovora* are both known to produce levans *in vitro* when 5% sucrose is added to the medium (Fett and Dunn, 1989; Gross *et al.,* 1992).

V.2.4.3 Erwinia carotovora and levans

Sturm and Chrispeels (1990) detected an induction of an extracellular beta-fructosidase in *Daucus carota* after infection with *Erwinia carotovora*. The authors suggested that this enzyme might hydrolyze the bacterial levan slime coat, making the pathogen susceptible to further defence reactions. In the beginning of this doctoral study we assumed that *Erwinia carotovora* produced levans. However, during this work the genome of this bacterium was published (Bell *et al.,* 2004) and no levansucrase gene could be found, suggesting that *E. carotovora* is not able to produce levans. Nevertheless, in the next experiment, the presence of fructans was examined. Both *E. carotovora* and *E. amylovora* (control) were grown on plates containing 5% sucrose. In a first test (Du and Geider, 2002), liquid medium was inoculated with the bacteria and after overnight growth the supernatant was incubated with 500 mM sucrose. Samples were taken after 4h and sugars were analyzed by AEC-PAD chromatography (Fig. V.17). The enzymes in the supernatant of *E. amylovora* are able to produce oligosaccharides from sucrose and the high glucose/fructose ratio suggests the production of fructans (GF + GF → GFF + G). Invertase and fructosyltransferase activities were absent in *E. carotovora*.

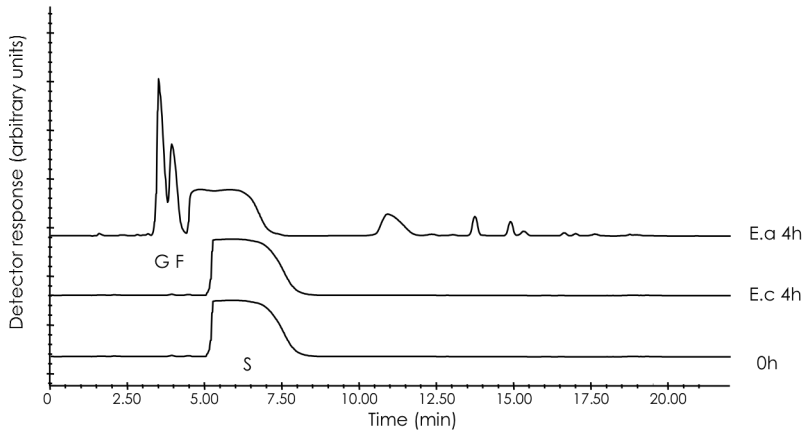


Figure V.17 Transferase activities in *Erwinia* species. Sugar pattern of the supernatant of *E. amylovora* (E.a) and *E. carotovora* (E.c) after 4h incubation with 500 mM sucrose (S). Glucose (G), Fructose (F).

In a second test (Fig. V.18), liquid medium containing 5% sucrose was inoculated with the bacteria. Samples were taken after 0h, 17h and 42h and centrifuged. The supernatant as such and hydrolyzed supernatant were analyzed (Fig. V.18). In case of *E. amylovora* (Fig. V.18a) sucrose disappears from the medium. After 17h more glucose is formed by the action of levansucrase. After sucrose depletion glucose is subsequently used by the bacteria (42h). The fructose found after hydrolysis of the sample taken after 42h confirms the production of fructans. In case of *E. carotovora* (Fig. V.18b), no fructan production could be observed.

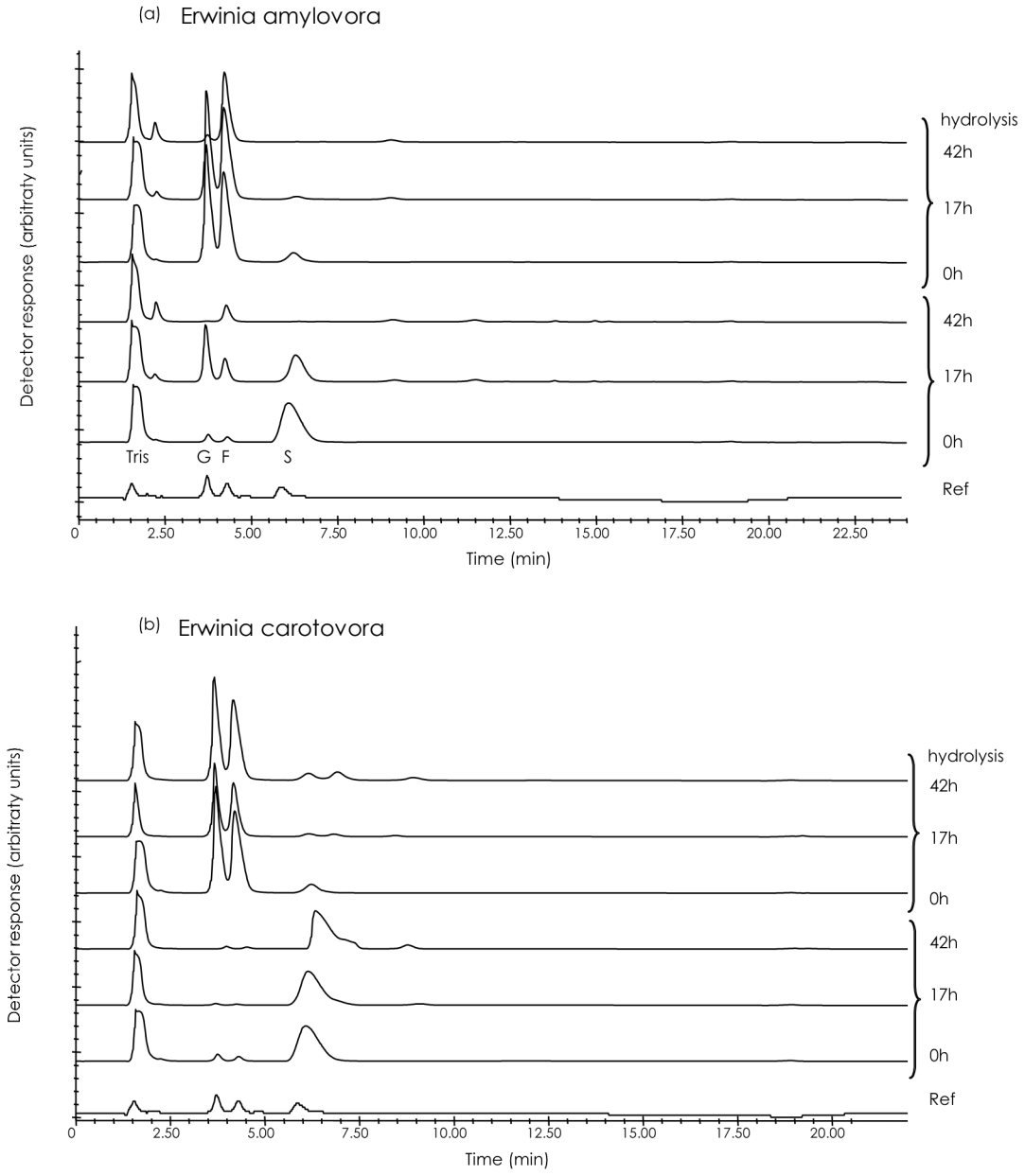


Figure V.18 Occurrence of fructans in *Erwinia* species. Sugar pattern of the supernatant of *E. amylovora* (a) and *E. carotovora* (b) cultures before and after acid hydrolysis. Samples were taken at 0h, 17h and 42h. Glucose (G), Fructose (F), Sucrose(S), Tris (buffer used).

In conclusion, *Erwinia carotovora* most probably does not synthesize fructans.

V.2.4.4 Study of At6-FEH and At(6&1)-FEH OEX and KO plants after inoculation with P. syringae pv. tomato DC3000

Since *E. carotovora* is not able to produce levans, this pathogen is not suited to test the hypothetical role of FEHs in degradation of extracellular fructans. Not so many bacterial pathogens are known to produce levans but it can be suggested to work with *Erwinia amylovora*. *E. amylovora* causes the devastating disease fire blight on e.g. apple and pear. *E. amylovora* produces both levan and amylovoran as EPS. Amylovoran is known as an important factor in pathogenesis of these bacteria because amylovoran deficient mutants lack pathogenic ability (Belleman and Geider, 1992; Oh and Beer, 2005). Although the effect of levan during pathogenesis is less pronounced, mutants for levansucrase showed retarded development of necrotic symptoms on inoculated pear seedlings. This delay in symptoms was not seen after inoculation of immature pear slices (Geier and Geider, 1993; Bereswill *et al.,* 1997). The levansucrase genes and their regulator gene were activated during infection of immature pear tissue (Zhao *et al*., 2005). So, *Erwinia amylovora* and its levan deficient mutant could be very interesting bacteria to test the hypothesis. However, several attempts to infect *A. thaliana* with *E. amylovora* were unsuccessful (Beer S.V., personal communication). The many advantages of working with *A. thaliana* and the proved presence of two FEHs in this plant, made us decide not to change strategy by working on e.g. pear or apple.

Experiment

In order to investigate the role of FEH during pathogenesis, FEH OEX and KO plants were infected with *P. syringae* pv. *tomato* DC3000. The interaction between *P. syringae* and *A. thaliana* is well-studied and levansucrase is present in *P. syringae*. If the proposed hypothesis is correct, then KO plants should be more susceptible and OEX plants should be more resistant to *P. syringae*. In a first test *at(6&1)-feh* and *at6-feh* KO plants were challenged with this pathogen (Fig. V.19). For each test 36 plants distributed over 6 trays were inoculated. Each tray also contained 6 control plants. As control, an azygous line (wild-type) from the same generation as the homozygous KO lines was used. After inoculation, the percentage of infected leaves was scored at specific time points.

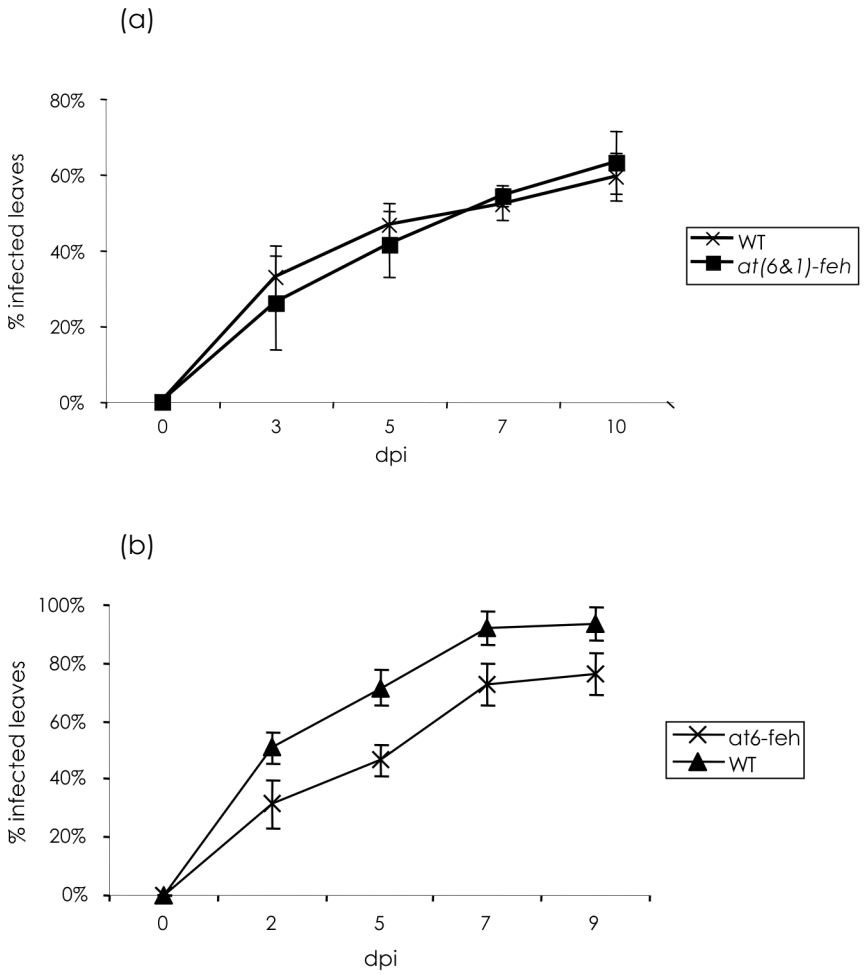


Figure V.19 Percentage infected leaves after inoculation of KO lines with *P. syringae*. (a) *at(6&1)-feh* (b) *at6-feh*. dpi (days post inoculation).

Several inoculation tests were carried out and they gave similar results as in figure V.19. For At(6&1)-FEH it is obvious that no difference is seen between the KO and WT line. For At6-FEH there is a small difference, but this was not always significant in the different tests. The WT plants seem to be a little more susceptible to infection with *P. syringae*. This is contradictory to our hypothesis. So, it can be concluded that knock-out plants are not more susceptible to *P. syringae* pv. *tomato* than wild-type plants.

Next, FEH OEX plants were challenged with *P. syringae* (Fig. V.20). The same strategy as for KO plants was used. The experiment was done on the T1 generation of the OEX plants. As control, a T1-transgenic *A. thaliana* Col0 with a pFAJ337 bar-expressioncassette (FAJ) was used.

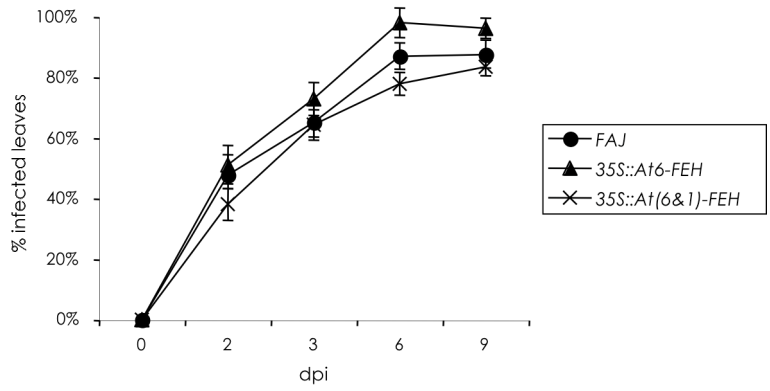


Figure V.20 Percentage infected leaves after inoculation of OEX lines with *P. syringae*. dpi (days post inoculation).

Several inoculation tests were carried out and all of them gave similar results. As deduced from the graph in figure V.20 no difference was seen between the different lines.

The infection tests were also performed on the T3 generation of *At6-FEH* overexpression plants. Also in this test, no difference in symptom development was seen (data not shown). Since T3 *At(6&1)-FEH* plants germinated so poorly, the experiment was not repeated for this generation.

Overall, it can be concluded that overexpression plants are not more resistant to *P. syringae* pv. *tomato* than wild-type plants.

Discussion

Results in this chapter do not sustain our hypothesis that FEHs play a role during plant-pathogen interaction. However, some considerations, discussed in the next paragraph, should be made before making conclusions.

P. syringae produces alginate and levan as EPS. Three genes encoding levansucrase, required for the production of levan, were identified in *P. syringae* pv. *tomato* DC3000, the pathogen used in this study (Buell *et al*., 2003). Fett and Dunn (1989) reported levan production for *P. syringae* pv. *tomato in vitro;* however, no levan biosynthesis could be found *in vivo* after inoculation of young bean leaves. In analogy to this, Boch *et al.* (2002) did not report induction of levan biosynthetic enzymes in *P. syringae* pv. *tomato* after inoculation of *A. thaliana* leaves. In contrast, alginate biosynthetic enzymes were indeed induced. The fact that no differences were seen after inoculation with *P. syringae* of both

overexpression and knock-out plants compared to wild-type plants could be consistent with the few data found in literature.

Since *At(6&1)-FEH* expression is highest in roots, perhaps the enzyme plays a role during infection of roots instead of leaves. *P. syringae* is largely an epiphytic foliar bacterium but *P. syringae*'s root pathogenicity has been described by Bais *et al.* (2004). Biofilm formation is important in root pathogenesis and production of EPS generally plays an essential role in these biofilms (Ramey *et al.*, 2004).

In conclusion: do FEHs play a role in pathogen attack?
Unfortunately, this question could not be answered during this doctoral study. When returning to sugar beet, this plant is especially susceptible to the fungi *Fusarium oxysporum*, *Cercospora beticola*, *Rhizoctonia solani* and the bacterium *Erwinia carotovora*. However, none of its important pathogens produces levans.

CHAPTER VI

CONCLUSIONS AND PERSPECTIVES

VI.1 Conclusions

VI.1.1 Occurrence of FEHs in non-fructan plants

In this doctoral study, the occurrence of FEHs in non-fructan plants has been established. A *Beta vulgaris* 6-FEH (Bv6-FEH) has been purified, characterized, cloned and heterologously expressed. Subsequently two putative CWINVs from *Arabidopsis thaliana* were identified as a genuine 6-FEH (At6-FEH) and a (6&1)-FEH (At(6&1)-FEH) after heterologous expression and overexpression in *P. pastoris* and *A. thaliana*, respectively. Bv6-FEH and At6-FEH have the same characteristics and are located in the same branch of the phylogenetic tree, suggesting that they are orthologues. Preliminary data (not shown in this thesis) demonstrate the presence of a 6-FEH in the roots of *Daucus carota* and a 1-KEH in roots of *Solanum tuberosum*. From these data, it can be concluded that FEHs occur in different non-fructan plants.

Based on their sucrose inhibition FEHs can be divided in two groups. Bv6-FEH, At6-FEH and At(6&1)-FEH are not inhibited by sucrose. In contrast, despite several exceptions, most FEHs in fructan plants are inhibited by sucrose. It was suggested that these FEHs, occurring in the vacuole, are effectively inhibited by normal physiological sucrose concentrations. However, these enzymes could be activated immediately when sucrose concentrations fall (by damage, wounding, cutting, grazing or other stresses). Since FEHs from non-fructan plants are not inhibited, activation of these enzymes by a drop of sucrose concentrations is not needed. As a consequence, those enzymes can function in sucrose-rich environments. Perhaps FEHs in fructan plants that are not inhibited by sucrose (e.g. 1-FEH I from *C. intybus*) fulfil the same physiological role as FEHs in non-fructan plants.

VI.1.2 FEHs from *Arabidopsis thaliana*

The model plant *Arabidopsis thaliana* has many advantages including a small genome, short life cycle, small stature, prolific seed production and ease of transformation. In addition, a wealth of genomic resources exists, such as a completely sequenced genome, a near saturation insertion mutant collection, a genome array that contains the entire transcriptome…. Therefore, this plant rather than sugar beet was chosen to elucidate the physiological role of FEHs in non-fructan plants.
In table VI.1 the characteristics of At6-FEH and At(6&1)-FEH are summarized. Compared to At6-FEH, At(6&1)-FEH shows some special features: (1) the enzyme can degrade both

levan and inulin and its activity is inhibited by Tris, (2) its cDNA is located in a different branch of the phylogenetic tree and lacks an N-terminal signal sequence, (3) the enzyme has a very low specific activity, (4) *At(6&1)-FEH* expression is induced by SA/INA and (4) a poor germination is noticed in OEX plants. The poor germination of *35S::At(6&1)-FEH* seeds suggests that this enzyme has a regulatory role while its induction by SA suggests a role during infection.

Table VI.1 Characteristics of At6-FEH and At(6&1)-FEH.

	At6-FEH	**At(6&1)-FEH**
Phylogenetic tree	Branch III, together with Bv6-FEH and AtcwINV1	Branch V, together with 1-FEHs from dicotyls
Activity	Prefers fructans with β-2,6 linkages	Prefers fructans with β-2,6 linkages but is also able to hydrolyze fructans with β-2,1 linkages
Specific activity	Rather low	Very low
Tris	No inhibition	Strong inhibition
Sucrose	No inhibition	No inhibition
Subcellular localization	Probably vacuole, C-terminal extension	Probably cytoplasm, no N-terminal signal sequence
KO plants	No aberrant phenotype, no difference after infection with *P. syringae*	No aberrant phenotype, no difference after infection with *P. syringae*
OEX plants	No aberrant phenotype, no difference after infection with *P. syringae*	No aberrant phenotype in T1 generation, very poor germination of the T2 seedlings, no difference after infection with *P. syringae*
Expression pattern	Highest in nodes, stems and hypocotyls	Highest in roots, senescent leaves and radicles
SA/INA	No induction	Induction

No endogenous substrate was found in healthy wild-type and knock-out plants. In my opinion, the search for an endogenous substrate should not be stopped. In this doctoral study, we looked at whole plant sugars, but following experiments should concentrate on the different organs, especially roots, and different stress conditions. Sugars of other species like *D. carota* and *S. tuberosum* could also be examined.

The results obtained in this thesis do not support a role for FEHs in the *A. thaliana* - *P. syringae* interaction. However, this does not exclude a role for FEHs in plant-microbe interaction. As discussed in Chapter V the well-studied *A. thaliana* leaves - *P. syringae*

interaction is perhaps not the best system to check involvement of plant FEHs on exogenous bacterial fructans during pathogen attack. Levan production is most favourable under high sucrose concentrations. In this regard, the sugar beet taproot, containing high sucrose concentrations, is an ideal material to test. Several levan producing bacteria occur within the roots of sugar beet or in its rhizosphere (Tallgren *et al.* 1999; Lilly *et al*., 1996). FEHs could play a role in stabilizing the interaction between micro-organisms and the plant, preventing them to become pathogenic or reducing the possible toxicity of levans. A detrimental effect of levan was detected after heterologous expression of a *Bacillus amiloliquefaciens* levansucrase in the cytosol of potato tubers and tobacco leaves (Caimi *et al*., 1997). During preliminary experiments in which wild-type and *35S::At6-FEH* plants were infiltrated with 10 mM levan, leaves of wild-type plants seemed to be more affected by the infiltration than leaves from *35S::At6-FEH* (data not shown).

The differences between At6-FEH and At(6&1)-FEH could indicate that the enzymes have a different physiological function. At6-FEH and Bv6-FEH have most probably the same physiological function. Both enzymes also seem to be constitutively expressed.

The putative cytoplasmic localization, low specific activity towards levan and inhibition by Tris of At(6&1)-FEH are a-typical compared to other FEHs. In this view, the presumptive catalytic role of this enzyme *in vivo* could be questioned. During the past years it became clear that an increasing number of proteins have additional roles that are not enzymatic but rather structural or regulatory. These proteins most often possess the ability to assemble into multiprotein complexes and mediate sophisticated biological functions such as integrating signals (Jeffery, 1999). A well-studied example is hexokinase (HXK). This enzyme mediates the first step in glycolysis, namely phosphorylation of glucose. In addition to its metabolic role, this enzyme plays an important role in sugar signalling. Glucose signalling in plants requires the presence of hexokinase. Moreover, this signalling is not dependent on its catalytic activity (Moore *et al.,* 2003). Very recently, some isoforms of SuS (from maize) and neutral invertase (from rice), cytoplasmic enzymes that are more related to our At(6&1)-FEH, have been reported to be localized in the mitochondria (Subbaiah et al., 2006; Murayama and Handa, 2006). Subbaiah *et al.* (2006) proposed a novel non-catalytic role for SuS in mitochondria: (1) the enzyme has a high K_m for sucrose, (2) limited levels of sucrose are expected in mitochondria, (3) yeast two-hybrid analysis and co-immunoprecipitation studies indicate an interaction of SuS and Voltage-dependent Anion Channel (VDAC). Since the *in vitro* translated neutral invertase

exhibited a catalytic neutral invertase activity, Murayama and Handa (2006) remained more cautious about a non-catalytic function of this enzyme.

VI.2 Perspectives

This doctoral work comprises the identification and characterization of FEHs in *B. vulgaris* and *A. thaliana*. In the last chapter, several experiments were performed to find clues about the physiological function of these FEHs. Unfortunately, these data were too preliminary to draw conclusions.

A major drawback in the research on FEHs in both fructan and non-fructan plants is the lack of certainty about the localization of those enzymes. Although Wiemken *et al.* (1986) detected FEH activity in the vacuoles some other authors (Livingston and Henson, 1998) reported FEH activity in the apoplast. The localization of FEHs in non-fructan plants is even a bigger mystery: At6-FEH and Bv6-FEH are probably localized in the vacuole while At(6&1)-FEH could be localized in the cytoplasm. GFP-fusion protein studies probably could once and for all elucidate the localization of FEHs and these experiments are necessary to help resolve the function of FEHs in non-fructan plants.

The purification to homogeneity and cloning of the FEHs from *D. carota* and *S. tuberosum* will further strengthen the overall occurrence of FEHs in non-fructan plants. The enzyme in *S. tuberosum* is most probably a 1-KEH, indicating the presence of FEHs with different substrate specificities in non-fructan plants.

As mentioned before, no endogenous substrate was found but a thorough investigation of sugar extracts from different growth stadia, different plant parts and plants under stress conditions is needed. Infection experiments could be repeated with e.g. root pathogens and the expression levels of some genes involved in pathogen attack could be examined in OEX and KO plants. The experiments in which leaves were infiltrated with levan should be extended. A yeast two-hybrid assay would be very interesting, especially for At(6&1)-FEH, to find clues about a possible non-catalytic function.

CHAPTER VII

MATERIALS AND METHODS

In this chapter, the terminology At6-FEH and At(6&1)-FEH will be used for respectively AtcwINV3 and AtcwINV6, although some experimental procedures were performed prior to the discovery of the functionality of those genes.

VII.1 Biological material

VII.1.1 Plant Material

Beta vulgaris L. (cv. Opus) was sown in a local field with sandy loam soil on April 26, 2002. For RT-PCR, a number of sugar beet roots were uprooted on September 30, 2002. For purification, fresh September-October roots were taken as well as roots that were stored at +1°C for various time-intervals starting from November 7, 2002. For enzyme activity measurements, representative samples of field-grown sugar beet plants (hypocotyls and leaves) were collected weekly between June 21 and October 5, and samples were stored at -80°C until further analysis. Samples for RNA and for enzyme activity measurements were taken from both root and leaf material.

Seeds of *Arabidopsis thaliana* wild-type ecotype Columbia (Col0), originating from the European Arabidopsis stock Centre (NASC, http://nasc.nott.ac.uk/home.html), were provided by the Centre of Microbial and Plant Genetics (Leuven). The homozygous mutant lines for At(6&1)-FEH and At6-FEH were obtained from the laboratory of Dr. Steven Smith (Laboratory of Molecular Biology, Edinburgh). The KO plant *at(6&1)-feh* (N5136281) was derived from the wild-type parental Columbia ecotype and was obtained from SALK (http://signal.salk.edu/tabout.html). The KO plant *at6-feh* was derived from the wild-type parental Wassilewskija (Ws) ecotype and was obtained from Wisconsin.
Arabidopsis seeds were sown on 'Zaai-en Stekgrond' (DCM, Sint-Kathelijne-Waver, Belgium) and grown in a growth chamber (Centre of Microbial and Plant Genetics) at 21°C day-time temperature and 18°C night-time temperature, 75% humidity and with a 12h day-light cycle (120 μmol m^{-2} s^{-1}). Plants for transformation were grown in the greenhouse under similar conditions.

For the enzyme activity measurement in the mature *A. thaliana* wild-type plant (8 week old), different parts (roots, stalks, leaves, flowers and green siliques) were collected. Enzyme activity measurements in knock-out and overexpression plants were done on leaves from 4 week old plants. Sugar extracts were obtained from the entire mature plants (8 week old).

VII.1.2 Micro-organisms

The following bacterial strains were used: *Pseudomonas syringae* pv. *tomato* (DC3000), *Erwinia carotovora* (LMG6663) and *Erwinia amylovora*. *P. syringae* pv. *tomato* was grown on solid King's B medium supplemented with 25 μg/ml rifampicin. *E. carotovora* and *E. amylovora* were grown on solid LB-medium. All bacteria were grown overnight at 30°C. Prior to the subsequent experiments, bacteria were grown overnight in liquid medium at 30°C in the corresponding medium. 5% sucrose was added to both solid and liquid medium for the *in vitro* antimicrobial activity experiment and the experiment in which the presence of levan in *Erwinia carotovora* was tested (Gross *et al*., 1992).

VII.2 Biochemical analyses

VII.2.1 Substrates

Commercial inulin was from chicory roots (Sigma-Aldrich, St Louis, USA). 1,1-Nystose, levanbiose and wheat extract were prepared earlier in the laboratory (Van den Ende *et al.,* 2003a). Low molecular weight bacterial levan, 6-kestose and 1-kestose were provided by Dr. Iizuka (Iizuka *et al.,* 1993). Neokestose and phlein were provided by Dr. Chatterton (Forage and Range Res. Lab, Utah State University). The mean DP of levan and phlein was estimated from the fructose/glucose ratio after mild hydrolysis in 60 mM HCl at 70°C for 75 min. Subsequently, molar concentrations were estimated based on this mean DP and glucose liberation.

VII.2.2 Enzyme activity determinations

Sugar beet and *A. thaliana* tissues (stored at -80°C) were ground with mortar and pestle until a very fine powder was obtained. Subsequently, an equal amount of extraction buffer (EB; 50 mM sodium acetate buffer pH 5.0 containing 10 mM $NaHSO_3$, 1 mM mercapto-ethanol and 0.1% (w/v) Polyclar AT; Serva, Heidelberg, Germany) was added and homogenized with mortar and pestle. The samples were centrifuged to pellet the cell debris (16,000 g; 5 min). $(NH_4)_2SO_4$ was added to the supernatant with a saturation of 80% in order to precipitate proteins. The pellet was washed four times with 50 mM sodium acetate buffer pH 5.0 saturated with 80% $(NH_4)_2SO_4$. Subsequently, the pellet was re-dissolved in 50 mM sodium-acetate buffer pH 5.0.

To measure 1-FEH, 6-FEH and invertase activities of sugar beet, aliquots were incubated with 3% (w/v) inulin, 10 mM levan or 100 mM sucrose in 50 mM sodium acetate buffer pH 5.0 for different time intervals at 30°C. To measure 1-FEH, 6-FEH and invertase activities of *A. thaliana*, aliquots were incubated with 5 mM sucrose, 3% (w/v) inulin or 5 mM levan in 50 mM sodium acetate buffer pH 5.0 for different time intervals at 30°C. 5 mM levan was used to calculate specific activities of the recombinant enzymes and to measure activities in knock-out and overexpression plants. Other substrate concentrations used are indicated in text.

To test the inhibition of Tris, different concentrations of Tris were added to the incubation reactions (with 4 mM levan). A final concentration of 200 mM sodium acetate buffer pH 5.0 was added to the samples in order to keep pH at 5.0.

Deglycosylation of recombinant proteins was achieved by adding 2000 U EndoH$_f$ (New England Biolabs, Beverly, MI) to 20 μg of protein. This reaction was incubated for 1h at 37°C. Next, the deglycosylated enzyme was incubated with 5 mM levan.

To test for FEH or invertase activity fructose formation was determined by Anion Exchange Chromatography with Pulsed Amperometric Detection (AEC-PAD) (Dionex, Sunnyvale, USA). Samples were injected on a Carbo-Pac PA-100 column equilibrated with 90 mM NaOH. The flow rate was 1 ml/min. The elution conditions were either 96% 90 mM NaOH and 4% 500 mM Na-acetate or 100% 90 mM NaOH. Elution time depended on the samples analyzed.

Enzymes were kept on ice and 0.02% sodium-azide (w/v) was added to all buffers to prevent microbial growth. Proteins were measured by the method of Sedmak and Grossberg (1977). Enzymatic activity is expressed in units (U), defined as the amount of enzyme which formed 1 μmol of fructose per min at 30°C.

VII.2.3 Carbohydrate analysis

Total *A. thaliana* plants and sugar beet samples were homogenized with mortar and pestle and an equal amount of water was added. After boiling for 15 min and cooling, samples were centrifuged at 16,000 g for 5 min. 200 μl of the supernatant was passed through a 500 μl bed volume of Dowex-50 H^+ and a 500 μl bed volume of Dowex-1-acetate. The column was rinsed 6 times with 200 μl distilled water. 0.02% azide was added and pH was adjusted

to 7 with Tris 1 M. Samples were analyzed by Anion Exchange Chromatography with Pulsed Amperometric Detection (AEC-PAD) (§VII.2.2).

VII.2.4 Purification of enzymes

VII.2.4.1 Native Beta vulgaris 6-FEH

Both fresh (harvested from the field in October) and stored (1-4 months at 4°C) sugar beet hypocotyls were used as a starting material for purification. A total of 3.5 kg hypocotyls were washed, cut in small pieces and subsequently homogenized with a Waring blender in 1.75 l EB. The homogenate was squeezed through cheese cloth. An $(NH_4)_2SO_4$ precipitation step with a saturation of 30% was done in order to pellet cell-debris, followed by an 80% $(NH_4)_2SO_4$ precipitation step. The precipitate was collected and re-dissolved in 150 ml 50 mM Na-acetate buffer pH 5.0 containing 1 mM of $CaCl_2$, $MgCl_2$ and $MnCl_2$. Undissolved material was pelleted for 10 min (40,000 *g*; 4°C). The supernatant was applied on a ConA Sepharose column (25×100 mm). After equilibration (200 ml) of the column with the previous buffer elution was performed with 60 ml 50 mM sodium acetate buffer pH 5.0 containing 1 M methylmannoside. Fractions with FEH activity (already free from invertase activity at this point) were pooled (10 ml). Next, a combination of anion- and cation exchange chromatography was performed. So, the pooled ConA fractions were adjusted to pH 7.0 with concentrated Tris and applied on a Mono Q column (Pharmacia Biotech HR 5/5, Uppsala, Sweden) equilibrated with 20 mM Tris-HCl buffer pH 7.0 (20 ml). Active fractions were pooled (3 ml), diluted 5 times with 50 mM sodium acetate buffer pH 4.5 and the pH was adjusted to pH 4.5 with concentrated acetic acid. Subsequently, the enzyme mixture was loaded onto a pre-equilibrated (20 ml) Mono S column (Pharmacia Biotech HR 5/5, Uppsala, Sweden). The active fractions (3 ml) were diluted 10 times with 50 mM HEPES buffer pH 8.5. These were applied on a pre-equilibrated (20 ml) Mono Q column. Only the most active fraction was re-chromatographed on a next Mono Q column equilibrated with 50 mM HEPES buffer pH 8.7 (20 ml), resulting in a very small amount of pure protein (as proven after Q-TOF analysis).

VII.2.4.2 Native Arabidopsis At6-FEH

A total of 750 g of whole *Arabidopsis thaliana* Col0 plants were homogenized with a Waring blender in 500 ml EB. Further handlings were as described in § VII.2.4.1. After ConA chromatography, the eluate (50 ml) was adjusted to pH 4.5 with 1 M acetic acid and loaded onto a Mono S column equilibrated with 50 mM sodium acetate buffer pH 4.5 (20

ml). Fractions with FEH activity and without invertase activity, were pooled and the pH was adjusted to pH 7.5 with 1 M Tris. Subsequently, the enzyme mixture was loaded onto a pre-equilibrated (20 ml) Mono Q column.

For both sugar beet and *A. thaliana*, proteins were eluted from Mono Q and S columns by using a linear gradient from 0 to 0.3 M NaCl in 30 min (flow rate 1ml/min) and fractions of 1 ml were collected. Throughout purification, aliquots were taken for activity determination (§VII.2.2) with 2 mM levan as substrate. To visualize proteins SDS-PAGE was performed on 12.5% (w/v) polyacrylamide gels and stained with Coomassie Brilliant Blue (BB)-R250.

VII.2.4.3 Purification of heterologous AtcwINV1, At6-FEH and At(6&1)-FEH

To purify heterologous AtcwINV1, At6-FEH and At(6&1)-FEH from the *P. pastoris* supernatant, we started with 16-fold normal *P. pastoris* culture (see further §VII.5.2). Further handling is as described in §VII.5.2. Next, the precipitate of each culture was re-dissolved in 20 ml 50 mM Na-acetate buffer pH 5.0 containing 1 mM of $CaCl_2$, $MgCl_2$ and $MnCl_2$. Undissolved material was pelleted for 10 min (40,000 *g*; 4°C). The supernatant was applied on a ConA column. After equilibration (200 ml) of the column with the previous buffer, elution was performed with 20 ml 50 mM sodium acetate buffer pH 5.0 containing 1 M methylmannoside. Subsequently, the pH of the AtcwINV1 eluate was adjusted to 7.5 with concentrated Tris and applied on a Mono S column equilibrated with 20 mM Tris-HCl buffer pH 7.5 (20 ml). The pH of the At6-FEH and At(6&1)-FEH eluates was adjusted with concentrated Tris to pH 7.5 and 7 respectively and applied on a Mono Q pH 7.5 for At6-FEH and pH 7 for At(6&1)-FEH. Elution was as described in §VII.2.4.2.

VII.2.5 Q-TOF analyses on tryptic fragments

The SDS-PAGE protein bands of native Bv6-FEH (36, 20 and 19 kDa) and heterologous AtcwINV1, At6-FEH and At(6&1)-FEH were subjected to mass spectrometric (MS) identification. The Coomassie BB stained protein bands were excised from the gel, washed in water and 50% acetonitrile (ACN). Next, the gel pieces were incubated for 16 h at 37°C in 25 mM NH_4HCO_3 (pH 8) containing 10% ACN and 0.1 μg trypsin (sequencing grade modified porcine trypsin, Promega, Madison, WI, USA).
DTT was added to a final concentration of 10 mM to pure native Bv6-FEH obtained from the final Mono Q 8.7 and the enzyme mixture was subsequently heated at 65°C for 1 h. After cooling, 10% (v/v) ACN and 0.1 μg of trypsin were added and the mixture was incubated for 16 h at 37°C. For both methods, resulting tryptic fragments were extracted twice with 50

µl 7.5% ACN containing 0.1% (v/v) trifluoroacetic acid (TFA). Further handlings were performed by Dr. Stefan Clerens (Laboratory of Neuro-endocrinology and Immunological Biotechnology). The samples were desalted and concentrated by using the standard C-18 ZipTip protocol (Millipore, Billerica, USA). Elution was done with 3 µl of 60% (v/v) ACN with 1% (v/v) formic acid in water. Samples were deposited into disposable capillaries (Protana engineering) that were fitted in the nano-electrospray source of the Quadrupole-time of flight- (Q-TOF) electron spray ionization instrument. An MS survey spectrum was acquired after which individual peptides were selected in the instrument's quadrupole and subjected to collision-induced dissociation (CID). Sequence information was derived from the MS/MS spectra with the aid of the MaxEnt 3 (deconvoluting and de-isotoping of data) and PepSeq software from the Micromass BioLynx software package (Micromass).

VII.3 General molecular analyses

VII.3.1 RNA extraction

RNA was isolated from field-grown sugar beet storage hypocotyls and *A. thaliana* leaves by using the Rneasy Plant Minikit (Qiagen, Valencia, USA) or Invisorb Spin Plant RNA Minikit (Invitek, Berlin, Germany) according to the manufacturer's instructions.

VII.3.2 RT-PCR and PCR reactions

The reverse transcription (RT) polymerase chain reaction (PCR) was performed by a two-step method (except for §VII.4) using 1 µg of RNA. The reverse transcription system from New England Biolabs (Beverly, MI) and an oligo-dT primer (5'-T_{25}V-3') were used, RNase inhibitor was added to the reaction. The cDNA synthesis was performed at 42°C for 55 min, followed by 95°C for 5 min. PCR reaction was performed with a Pfu Proofreading polymerase (Promega, Madison, USA) for cloning and normal Taq polymerase (Amersham Bioscience, Buckinghamshire, UK) for diagnostic analysis, both according to the instructions of the manufacturer. Primers used for knock-out analysis and semi-quantitative PCR are depicted in the table below (Table VII.1). Other primers used are mentioned in each section. For semi-quantitative RT-PCR equal amounts of cDNA of each sample were taken (± 100 ng/µl). PCR was performed with specific primers and with EF1α primers (Table VII.2).
The PCR program was composed of an initial denaturation for 3 min at 94°C, amplification cycles (number of cycles dependent on the cDNA cloned) of 30s at 94°C, 30s at x°C (x= dependent on the primers used) and 1 min at 72°C, and followed by a final elongation

step of 10 min at 72°C. 10 μl of each PCR reaction with 2 μl loading buffer was run on a 1.1% agarose gel and visualized with EtBr.

Table VII.1 Primers used for knock-out analysis, semi-quantitative PCRs or control of positive colonies

Gene of interest	Primer name	Primer sequence 5' → 3'	Annealing temperature
Bv6-FEH	BLEVF4	GGGAGGTATGACATTGAGAAGG	58°C
	BLEVR3	ACCCACCCCCATAGTATTCTT	
At6-FEH	CWI3F	CGAGTAATCGTCGGAAGCTC	60°C
	CWI3R	CTAACCTCGGAGCTGATTCG	
At(6&1)-FEH	ArafruF	CAAGTGGAAAGAAGCTGATG	56°C
	ArafruR	AACCGAGCTATCACGATCAAA	

VII.3.3 Real time quantitative PCR

RNA was isolated as described in §VII.3.1 and prior to cDNA synthesis (§VII.3.2) samples were subjected to DNase I (New England Biolabs, Beverly, MI) treatment to remove residual DNA. The start concentration of RNA was 5 to 10 μg. The reaction was performed at 37°C for 20 min and DNase I was heat inactivated for 10 min at 75°C. After cDNA synthesis (§VII.3.2), quantitative PCR was performed using the LightCycler real-time quantitative PCR apparatus (Roche Diagnostics, Basel, Switzerland). The reaction mixture was as described in the manufacturer's protocol. The *A. thaliana* housekeeping gene elongation factor 1α (*EF1α*; At5g60390) was used as internal control. Primers used for amplification of the genes are indicated in table VII.2. Forty-five cycles of amplification (95°C denaturation for 15s, annealing temperature for 5s and 72°C polymerization for 11s) were carried out.

Treshold values (Ct) and PCR efficiencies (Eff) were determined with LightCycler relative quantification software and LinRegPCR (University of Amsterdam, The Netherlands), respectively. To determine the induction or repression of the expression of the gene of interest (GI) following formula was used:

$[Eff_{(EF)}{}^{Ct_{(EF)}}(treatment)/Eff_{(GI)}{}^{Ct_{(GI)}}(treatment)] / [Eff_{(EF)}{}^{Ct_{(EF)}}(control)/Eff_{(GI)}{}^{Ct_{(GI)}}(control)]$

Table VII.2 Primers used for real-time quantitative PCR

Gene of interest	Primer name	Primer sequence 5' → 3'	Annealing temperature
At6-FEH	RTINV3F	AAACGAATCAGCTCCGAGGTTA	62°C
	RTINV3R	TTCAATCGGCCACTGTAGC	
At(6&1)-FEH	RTINV6F	CCGGACCAAAAGTGTTAGCC	62°C
	RTINV6R	TCGCATCATCAACTTGGTCAG	
EF1α	EFF	TGAGCACGCTCTTCTTGCTTTCA	62°C
	EFR	GGTGGTGGCATCCATCTTGTTACA	

VII.3.4 Subcloning, transformation of *E.coli* and sequencing

Fragments amplified by PCR were ligated into a TOPO-TA or TOPO-XL vector (Invitrogen, Paisly, UK) and transformed by heat shock to competent *E. coli* cells (TOP10 *E. coli* cells, Invitrogen) according to the supplier's instructions. Cells were grown on LB (Luria Bertani)-medium (1% tryptone, 1% NaCl, 0.5% yeast extract, 1.5% bacto agar) supplemented with 50 μg/ml kanamycin. Colonies were checked by PCR. Plasmids from positive colonies were extracted using the Qiaprep Spin Miniprep Kit (Qiagen, Valencia, USA). Sequencing was done by Baseclear (Leiden, The Netherlands).

VII.4 Cloning of different cDNAs

VII.4.1 Sugar beet 6-*FEH* cDNA

Four primers were constructed based on the conserved amino acid sequences found in sugar beet and other cell-wall type invertases:

- YRTAYHF: 5'-TACAGGACNGCNTAYCAYTT-3' (Fig.VI.1, VII.2)
- NDPNG: 5'-TGGATGAAYGAYCCNAAYGG-3' (Fig. VII.2)
- WECPD: 5'-GAATGTGGGARTGYCCNGA-3' (Fig. VII.1)
- VFNNGT: 5'-GGTNCCRTTRTTRAANAC-3' (Fig. VII.1)

Further, four 6-FEH based degenerated primers were designed based on the ESI-Q-TOF MS/MS internal sequences:

- IIGDDVK: 5'-ATHATHGGNGAYGAYGTNAA-3' (Fig. VII.1)
- KSFFDDE: 5'-AARTCTTTYTTYGAYGAYGA-3' (Fig. VII.3)
- WSGVQAI: 5'-GGAATNGCYTGNACNCCNGACCA-3' (Fig. VII.1)
- VQWPLAE: 5'-CYTCNGCNANNGGCCAYTGNAC-3' (Fig. VII.1, VII.2)

One-step RT-PCR was performed with primers YRTAYHF and VFNNGT (Access RT-PCR System, Promega, Madison, WI, USA) but no clear bands appeared at this stage. RT reaction was at 47°C. PCR conditions: 94°C, 3 min; followed by 35 cycles: 94°C, 40 s; 47°C, 40 sec and 72°C, 2 min. Final extension was at 72°C, 10 min. Subsequently, nested PCR (30 cycles, annealing temperature 54°C) was performed with MWECPD and VQWPLAE. Different bands still appeared and therefore a next PCR (30 cycles, annealing temperature 50°C) with IIGDDVK and WSGVQAI was performed resulting in a single band of about 300 bp.

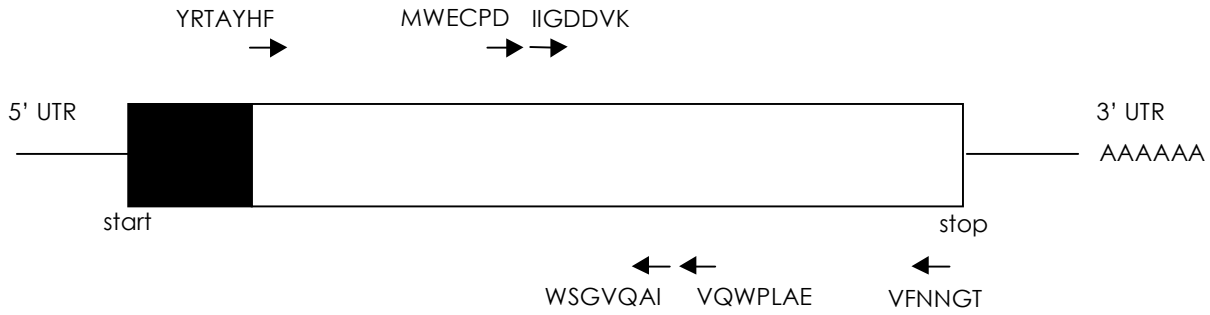


Figure VII.1 Primers used for cloning of *6-FEH* cDNA from *Beta vulgaris*. UTR, untranslated region

The 300 bp band was ligated in the TOPO-TA vector. Partial sequencing resulted in the development of two specific primers:

- GRYDIEK: 5'-GGGAGGTATGACATTGAGAAGG-3' (Fig. VII.3)
- RILWGW: 5'-ACCCACCCCCATAGTATTCTT-3' (Fig. VII.2, VII.3)

To obtain a longer fragment, RT-PCR was performed with primers YRTAYHF and VQWPLAE. Subsequently, nested PCR (30 cycles, annealing temperature 57°C) was performed with NDPNG and RILWGW.

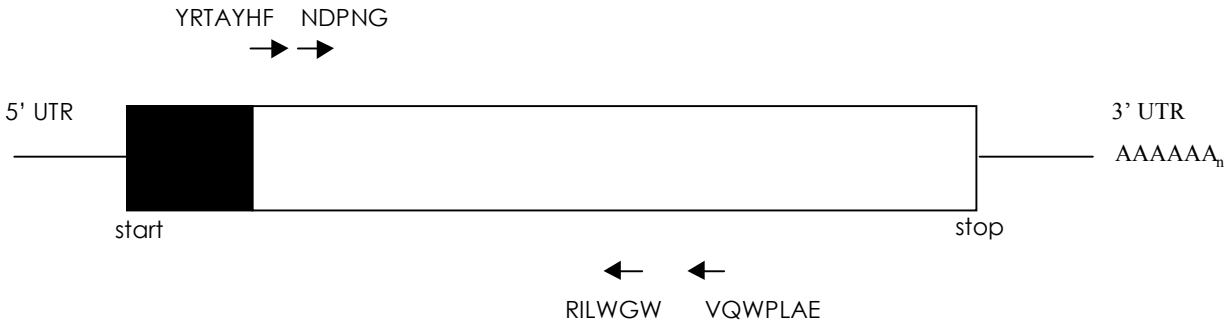


Figure VII.2 Primers used for cloning of *6-FEH* cDNA from *Beta vulgaris*.

From the sequence of this single 850 bp fragment, a highly specific antisense primer was selected:

- PVWHTE: 5'-TTTCAGTGTGCCATACAGGAT-3' (Fig. VII.3)

The 3' part of the cDNA was cloned by RT-PCR using the GRYDIEK and an oligo dT-based primer (35 cycles, annealing temperature 59°C) followed by semi-nested PCR (30 cycles, annealing temperature 52°C) with KSFFDDE and oligo dT. The missing 5' part of the cDNA was cloned by PCR on a sugar beet root cDNA library (received from Prof. M. McGrath, Michigan State University, USA) by combining the vector-specific primer M13F (5' GTAAAACGACGGCCAG 3') with RILWGW (30 cycles, annealing temperature 50°C), followed by a nested PCR with another vector-specific primer T7 (5' GTAATACGAC TCACTATAGGGC 3') and PVWHTE (30 cycles, annealing temperature 56°C).

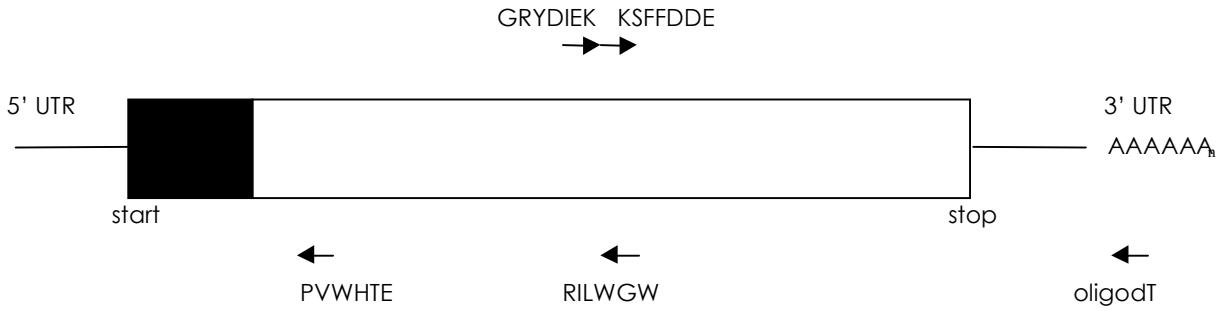


Figure VII.3 Primers used for cloning of *6-FEH* cDNA from *Beta vulgaris*.

After sequencing of the resulting 400 bp fragment, final primers BFINF and BFINR were designed from the outer 5' and 3' cDNA parts and used to amplify the whole cDNA by RT-PCR:

- BFINF: 5'-AACAATGGGAGTTGGTTAGTGCTG-3'
- BFINR:5'-TACGCAGCAACTAATTTGGGACAC-3'

First, total RNA was reverse-transcribed with AMV Reverse Transcriptase XL (TaKaRa) and BFINR at 50°C during 1 h. A number of independent PCRs was performed with BFINF and BFINR as primers. PCR conditions: 35 cycles of 94°C, 30 s; 64°C, 40 s and 72°C, 5 min. After A-tailing, fragments were ligated in the TOPO-XL vector. From a number of clones the full-length sugar beet *6-FEH* fragment was fully sequenced on both DNA strands. The sequence was deposited in the EMBL sequence library (accession number AJ508534).

VII.4.2 *AtcwINV1*, *At(6&1)-FEH* cDNAs and cloning of *At6-FEH* cDNA

The cDNA of the gene At3g13790 (*AtcwINV1*) was ordered from the Arabidopsis Biological Resource Centre (DNA stock centre) of the Ohio State University. This cDNA was delivered in the pUNI51-vector. The cDNA of the gene At5g11920 (*At(6&1)-FEH*) was ordered from the BioResource Centre of the Riken Tsukuba Institute in Japan. This cDNA was delivered in a modified bluescript vector FLC-1-B. The cDNA of the gene At1g55120 *(At6-FEH)* was cloned from mature *A. thaliana* leaves. After the reverse transcriptase reaction, primers were constructed depending on the expression system used (§VII.5, §VII.6).

VII.5 Heterologous expression in *Pichia pastoris*

For heterologous expression the EasySelect™ Pichia Expression Kit from Invitrogen (Paisly, UK) was used.

VII.5.1 Construction of the expression plasmids

To construct the expression plasmids p*Bv6FEH*, p*AtcwINV1*, p*At6-FEH* and p*At(6&1)-FEH*, containing the mature protein part, the N-termini had to be determined. The N-terminus of 6-FEH from sugar beet was determined by Q-TOF mass spectrometry. The N-terminal sequences of AtcwINV1, At6-FEH and At(6&1)-FEH are unknown. An estimation of the N-terminal position was made by comparison with homologous enzymes for which the N-termini were known (1-FEH IIa, 1-FEH IIb and 1-FEH from *Cichorium intybus* and *Daucus carota* CWINV1).

Table VII.3 Primers used for cloning into Pichia expression plasmid

Gene of interest	Primer name	Primer sequence 5' → 3'	Restriction enzyme	*Pichia* vector	Expression plasmid
Bv6-FEH	6FEHF	GCCG**GAATTC**GATGATGATCCATATAGAACAGC	*EcoRI*	pPICZαA	*pBv6FEH*
	6FEHR	GAAT**GCGGCCGC**GGTTGTCAGAACTCTTCTTTCTC	*Not*I		
AtcwINV1	PICAT1F	GGCCG**GAATTC**ACTAACACCAAATCTCCTTCCGTA	*EcoRI*	pPICZαA	*pAtcwINV1*
	PICAT1R	AAT**GCGGCCGC**GGTCAACTGATTTGGGCAGAGTTC	*Not*I		
At6-FEH	PCARA5F	GGGCCG**GAATTC**GCTTCTTCTCATCAAGATCTCAA	*EcoRI*	pPICZαA	*pAt6-FEH*
	PCARA5R	GGTCC**CCGCGG**TCAAAACTGATGATTAGGTGAGC	*Sac*II		
At(6&1)-FEH	PICATFF3	AAA**CTGCAG**TAATGGAACAGAATCTTCTTCAAACC	*Pst*I	pPICZαB	*pAt(6&1)-FEH*
	PICATFR3	GAAT**GCGGCCGC**GGTTGTTTTAGGGCAGTCCAAAG	*Not*I		

The coding region of *6-FEH* (TOPO-vector), *AtcwINV1* (pUNI51-vector), *At6-FEH* (directly from cDNA) and *At(6&1)-FEH* (bluescript vector FLC-1-B) were amplified with PCR (3 independent reactions) by using the corresponding primers indicated in Table VII.2:
- *Bv6-FEH*: annealing temperature 52°C for 5 cycles, followed by 30 cycles at 57°C.
- *At6-FEH*: annealing temperature 52 °C, 32 cycles.
- *AtcwINV1*: annealing temperature 53°C for 5 cycles, followed by 28 cycles at 58°C
- *At(6&1)-FEH*: annealing temperature 54°C for 5 cycles, followed by 28 cycles at 60°C
Both the amplified fragments and the pPICZα A or pPICZα B vectors (Invitrogen, Paisly, UK) were digested with the corresponding restriction enzymes (Table VII.1.). Subsequently, the DNA fragments were purified by using the Ultraclean DNA Purification Kit (MOBIO, Solana Beach, CA). The vectors were cut out of a 1,1% (w/v) agarose gel and purified according to the manufacturer's instructions. After de-phosphorylation of the vector by a Shrimp Alkaline Phosphatase (Promega, Madison, USA), PCR fragments were cloned into the vector resulting in the expression plasmid p*Bv6FEH*, p*AtcwINV1*, p*At6-FEH* and p*At(6&1)-FEH* having the corresponding cDNAs in frame behind the α-factor signal sequence of the vector. These plasmids were transferred to *E. coli* competent cells using heat-shock. Cells were plated on 2 × YT medium (1.6% bacto tryptone, 1% yeast extract, 0.5% NaCl and 1.5% bacto agar), supplemented with zeocine (30 µg/ml) as a selection marker. Isolation of plasmids from positive colonies was done following a Midiprep protocol (Quantum Prep™ Plasmid Midiprep kit, Bio-Rad).

VII.5.2 Expression and purification

After preparing *Pichia pastoris* strain X33 for electroporation, according to the supplier's instruction, competent cells were transformed with 20 µg of *Sac*I linearized p*Bv6FEH* p*AtcwINV1*, p*At6-FEH* or p*At(6&1)-FEH* and with empty vector (as a control). Cells were plated on YPDS medium (2% bacto peptone, 1% yeast extract, 2% glucose, 1 M sorbitol and 1.5% bacto agar) supplemented with zeocine (100 µg/ml) as a selection marker. Positive colonies (checked with PCR) were grown in order to express the protein. The growing procedure includes three steps:
- inoculation of the start-culture YPD (2% bacto peptone, 1% yeast extract, 2% glucose, 100 µg/ml zeocine) with a single colony (overnight, 30°C, 220rpm)
- transfer of 500 µl of this culture to 80 ml BMGY (2% bacto peptone, 1% yeast extract, 100 mM potassium phosphate pH 6, 1.34% YNB, 4x10^{-5}% biotin, 1% glycerol). Overnight growth in order to generate biomass (30 °C, 220rpm)
- harvesting of the cells (1000 g, 5 min, room temperature). Re-dissolving cells in 20 ml induction medium BMMY (2% bacto peptone, 1% yeast extract, 100 mM potassium

phosphate pH 6, 1.34% YNB, 4x10^{-5}% biotin, 0.5% methanol) (5 days, 30 °C, 220rpm). Each day, 2% methanol was added to the cells.

After 5 days, the yeast supernatant was collected (1,500 g, 10 min, 4°C). $(NH_4)_2SO_4$ (to 80% saturation) and citrate phosphate buffer pH 5.0 to a final concentration of 10 mM were added. Samples were put on ice for 45 min and next enzymes were pelleted (40,000 g, 20 min, 4°C). The pellet was washed three times with 20 mM Citrate phosphate buffer pH 5.0 saturated with 80% $(NH_4)_2SO_4$. Finally, the pellet was re-dissolved in 50 mM sodium acetate buffer pH 5.0. Diluted aliquots (depending on protein concentration and activity) of this supernatant were used during enzyme activity measurements (§V.2.2) and substrate concentrations are as indicated in text.

VII.6 Overexpression in *Arabidopsis thaliana*

VII.6.1 Construction of the expression plasmid

The overexpression constructs were engineered using the Gateway® technology (Invitrogen, Paisley, UK). This is a universal technology to clone DNA sequences for functional analysis and expression in multiple systems. It is based on site-specific recombination properties. In this doctoral study the LR-reaction system is used to facilitate recombination of an *att*L substrate (entry clone) with an *att*R substrate (destination vector) creating an *att*B-containing expression clone (Fig. VII.4). The reaction is catalyzed by LR Clonase™ enzyme mix. The vectors were constructed according to the manufacturer's instructions.

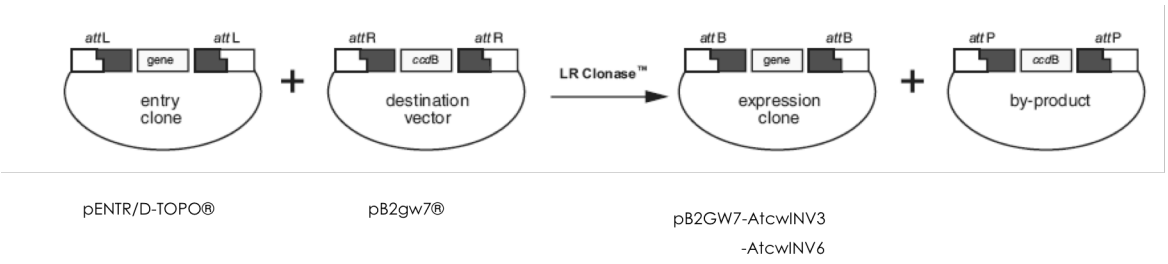


Figure VII.4 Outline of an LR Reaction.

First, the coding regions of the genes encoding At6-FEH (directly from cDNA) and At(6&1)-FEH (bluescript vector FLC-1-B) were amplified by PCR. The forward primer has four additional bases (CACC) (Table VII.4).

Table VII.4 Primers used for cloning into plant expression plasmid

cDNA	Primer name	Primer sequence 5' → 3'	Annealing temperature
At6-FEH	INV3FP	**CACC**ATGGCGAAATTAAATCGTTCC	57°C
	INV3RP	TCAAAACTGATGATTAGGTGAGC	
At(6&1)-FEH	INV6FP	**CACC**ATGGCTGATGTAATGGAACAGA	54°C
	INV6RP	CTAATAAGTTGGGTTTGAATTG	

In a next step, the amplified fragments were introduced in the recombination sites of the pENTR™/D-TOPO® vector (Invitrogen, Paisley, UK) according to the manufacturer's protocol. The overhang (GTGG) in the cloning vector invades the 5' end of the PCR product, anneals to the added bases and stabilizes the PCR product in the correct orientation. This cloning strategy is efficient to directionally clone blunt end PCR products into a vector. After transformation of *E. coli* with the plasmids and selection on LB-medium supplemented with kanamycin (50 μg/ml), colonies were checked with PCR. Plasmids from positive colonies were isolated. In a final step the desired DNA fragment was transferred to the destination vector pB2GW7 (Fig. VII.5, Karimi *et al.,* 2002) by means of an LR-recombination reaction (according to the manufacturer's instructions), resulting in the expression vectors pB2GW7-At6-FEH and pB2GW7-At(6&1)-FEH. pB2GW7 is a binary vector made for *Agrobacterium*-based plant transformation and it contains the Cauliflower Mosaic Virus 35S promotor (p35S), terminator (t35S) and the *bar* selection marker, which gives resistance against the herbicide phosphinothricin (Basta, Hoechst, Agrevo).

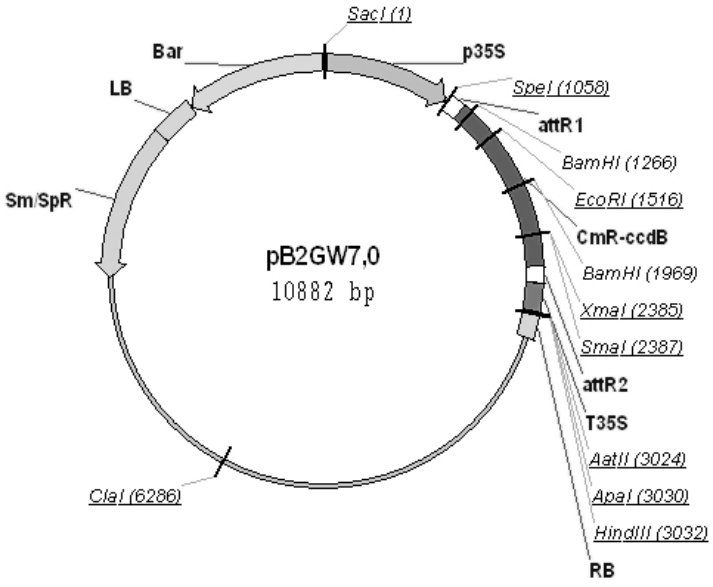


Figure VII.5 pB2GW7, vector used for Agrobacterium-based plant transformation. Important regions are explained in text.

After transformation of *E. coli* DH5α with the plasmids and selection on LB-medium supplemented with streptomycin (20 μg/ml) and spectinomycin (50 μg/ml), colonies were checked by PCR. Plasmids from positive colonies were isolated.

Subsequently, *Agrobacterium tumefaciens* pMP90 was transformed with the destination vectors by electroporation. Cells were grown on YEB (1% bacto peptone, 0.5% NaCl, 1% yeast extract, 1.5% bacto agar) medium containing 300 μg/ml streptomycin and 100 μg/ml spectinomycin. Presence of the plasmids was checked by PCR.

VII.6.2 Plant transformation and selection of transformants

Arabidopsis Col-0 plants were sown 6 to 7 weeks prior to transformation. Two days before transformation YEP-medium (5 ml; 1% bacto peptone, 0.5% NaCl, 1% yeast extract) was inoculated with transformed *A. tumefaciens* and grown overnight at 30°C. The next day this culture was added to 500 ml YEP-medium. After overnight growth at 30°C, cells were pelleted (3000g, 10 min). Bacteria were resuspended in 1 l infiltration medium (50 g/l sucrose). Plants were transformed using the flower dip method according to Clough and Bent (1998). The flower buds were dipped in an *Agrobacterium* suspension supplemented with 250 μl/l Silwett (Silwett-L77, van Meeuwen Chemicals BV, Netherlands). Plants were covered in order to keep high humidity for one night. After seed setting (4-6 weeks), seeds were collected and sown on sand/perlite medium irrigated with water containing Basta®-herbicide (25 μM) (Agrevo NV, Belgium). After 10 days transgenic seedlings were transferred to 'Zaai- en Stekgrond' and grown under the same conditions as described in §VII.1.1.

VII.7 Varia

VII.7.1 Inoculation of plants with bacteria

Prior to treatment of plants with *Pseudomonas syringae* pv. *tomato* DC3000, a liquid medium of King's B medium (1% Difco proteose peptone #3, 0.15% K_2HPO_4, 5 mM $MgSO_4$) supplemented with 50 μg/ml rifampicin and 20 μg/ml tetracycline was inoculated with the bacteria and grown overnight at 30°C. At the same day 4-5 week old plants were placed at 100% humidity. The next day cells were pelleted and resuspended in 10 mM $MgSO_4$ supplemented with 250 μl/l Silwett. Leaf inoculation was done through dipping the plants in the bacterial suspension. Control plants were inoculated with the same suspension but

without bacteria (= mock-treated plants). Plants were kept at 100% humidity throughout the experiment. The disease scoring was based on counting the infected leaves.

VII.7.2 Treatment of plants with chemicals

Plants were treated with INA (2,6-dichloroisonicotinic acid, a synthetic inducer of resistance; 1mg/ml; Novartis, Basel, Switzerland), methyl jasmonate (45 μM in ethanol; Serva), paraquat (25 μM; Sigma) and ethylene (50 ppm). 5 μl of each chemical (except ethylene) was dropped on different leaves of each plant. Control plants were inoculated with 5 μl water. For treatment with ethylene, plants were placed in an air-tight box (20 l) where 1 ml ethylene was injected through a septum in the chamber. After 24h and 48h, three leaves from three plants were taken and stored at -80°C until further processing.

Summary

Fructans, fructose-based reserve polymers (with maximum one glucose moiety) occurring in 12 to 15% of the vascular plants, are broken down by Fructan ExoHydrolases (FEHs). In dicotyledonous plants, inulin, a fructan with β-2,1-linked fructose molecules, is hydrolyzed by 1-FEH. Specific 6-FEHs hydrolyze the β-2,6-linked fructose molecules from the different monocotyledonous fructans. After complete degradation of fructans, sucrose is one of the end products and, in contrast to FEHs from microbial origin, plant FEHs do not hydrolyze sucrose. This disaccharide is hydrolyzed by invertases. FEHs and cell wall invertases (CWINVs) are both fructosyltransferases and they are very similar at the molecular level.

Prior to this study, relatively high 6-FEH activities were detected in the non-fructan plant *Beta vulgaris* (sugar beet). A specific 6-FEH was purified from the taproots and in this doctoral study the corresponding *Bv6-FEH* cDNA was cloned and heterologously expressed in the yeast *Pichia pastoris.* Both native and recombinant enzymes showed the highest activity with levan (fructan with β-2,6-linked fructose) as substrate; inulin and sucrose could not be hydrolyzed by the enzymes. This 6-FEH was the first cloned 6-FEH and the first FEH enzyme described in non-fructan plants. Since the amino acid sequences of CWINVs and FEHs are very homologous, we set out to determine if some known putative CWINV cDNA sequences from the model species *Arabidopsis thaliana* could encode an FEH rather than a genuine invertase. Three out of six CWINV cDNAs from *A. thaliana* (*AtcwINV1, AtcwINV3* and *AtcwINV6*) were heterologously expressed in *Pichia pastoris*. The following results were obtained: AtcwINV1 is a genuine invertase but AtcwINV3 and AtcwINV6 did not hydrolyze sucrose. Instead AtcwINV3 seems to be a true 6-FEH and AtcwINV6 a (6&1)-FEH preferentially degrading levan, but with a side activity against inulin. The terminology At6-FEH and At(6&1)-FEH was introduced for AtcwINV3 and AtcwINV6, respectively. Purification of At6-FEH from whole *A. thaliana* plants confirmed the specificities of the endogenous enzyme. *At6-FEH* and *Bv6-FEH* cluster in the same branch of a phylogenetic tree of CWINVs and FEHs. *At(6&1)-FEH* is located in another branch containing the known dicotyledonous 1-FEHs. Compared to other CWINVs and FEHs, At(6&1)-FEH showed some special features: it lacks an N-terminal signal sequence and the enzyme is inhibited by Tris.

Now that FEHs appeared to occur in different non-fructan accumulating species, research on its physiological function could be started. Two hypotheses can be put forward: (1) FEHs degrade minute amounts of endogenous fructans or another unknown

fructosylated substrate, (2) FEHs play a role in plant-microbe interactions by interfering with the fructose containing exopolysaccharides surrounding the bacteria. To resolve its function, it was decided to work with *At6-FEH* and *At(6&1)-FEH* knock-out and overexpression plants of *A. thaliana*. The transgenic plants did not show an aberrant phenotype. However, a very poor germination was noticed in the T2 generation of high *At(6&1)-FEH* expressors. Sugar extracts of both wild-type and knock-out plants did not reveal the presence of an endogenous substrate. As deduced from known micro-array analyses *At6-FEH* and *At(6&1)-FEH* RNAs show different expression levels amongst the various tissues. Expression analysis of *At(6&1)-FEH* suggests that it is induced by salicylic acid, but not by other hormones nor by *Pseudomonas syringae* infection. Disease assays with *P. syringae* could not confirm a higher susceptibility or higher resistance of knock-out and overexpression plants, respectively.

In conclusion, this doctoral study confirms the occurrence of a specific 6-FEH in sugar beet and describes the characterization of two FEHs in *A. thaliana*, formerly identified as putative CWINVs. The physiological function of these enzymes in non-fructan plants could not be resolved and their role in defence seems unlikely. Future work should perhaps be concentrated rather on a regulatory function. Since At6-FEH and At(6&1)-FEH have different characteristics, they could fulfil other physiological functions.

Samenvatting

Fructanen, reservesuikers opgebouwd uit fructose (met maximum 1 glucose), die voorkomen in 12 tot 15% van de vaatplanten, worden afgebroken door Fructan Exohydrolasen (FEH's). Dit zijn enzymen die terminale fructosyleenheden hydrolyseren. In dicotylen wordt inuline, een fructan met β-2,1-gebonden fructosemoleculen afgebroken door 1-FEH's. Specifieke 6-FEH's hydrolyseren β-2,6-gebonden fructosemoleculen bij verschillende fructanen van monocotylen. Na volledige hydrolyse van fructanen door FEH's blijft sucrose als eindproduct over. In tegenstelling tot microbiële FEH's, kunnen plant FEH's geen sucrose afbreken. Deze taak wordt vervuld door invertasen. FEH's en celwand invertasen zijn beide fructosyltransferasen en op moleculair niveau zijn ze sterk gelijkend.

Voorafgaand aan dit werk werden relatief hoge 6-FEH activiteiten gedetecteerd in de niet-fructan plant *Beta vulgaris* (suikerbiet). Vervolgens werd een specifiek 6-FEH gezuiverd uit de penwortel van deze plant. Tijdens deze doctorale studie werd het overeenkomstige *Bv6-FEH* cDNA gekloneerd en heteroloog tot expressie gebracht in de gist *Pichia pastoris.* Zowel het natieve als recombinant enzym vertoonden de hoogste specificiteit t.o.v. levan; inuline en sucrose werden niet gehydrolyseerd. Dit suikerbiet 6-FEH is het eerst gekloneerde 6-FEH en het eerste gevonden FEH in niet-fructan planten. Aangezien celwand invertasen en FEH's sterk gelijkend zijn op moleculair niveau, besloten we om dieper in te gaan op reeds gekende, als mogelijke celwand invertasen gecatalogeerde cDNA's van de modelplant *Arabidopsis thaliana*. Het zou immers niet verwonderlijk zijn dat sommige van hen coderen voor een FEH i.p.v. een invertase. Daarop werden drie van de zes celwand invertase cDNA's van *Arabidopsis thaliana* heteroloog tot expressie gebracht. Dit leverde volgende resultaten op: AtcwINV1 blijkt een echt invertase te zijn. AtcwINV3 en AtcwINV6 daarentegen breken geen sucrose af. AtcwINV3 vertoont een specifieke 6-FEH activiteit, AtcwINV6 hydrolyseert levan maar vertoont ook een relatief hoge zij-activiteit t.o.v. inuline. Hierop werd voor AtcwINV3 en AtcwINV6 een nieuwe terminologie geïntroduceerd, namelijk At6-FEH en At(6&1)-FEH, respectievelijk. Zuivering van At6-FEH uit *A. thaliana* planten bevestigde de functionaliteit van het endogeen enzym. *At6-FEH* en *Bv6-FEH* clusteren samen in een fylogenetische boom van celwand invertasen en FEH's. *At(6&1)-FEH* komt in een andere tak terecht, samen met de gekende dicotyle 1-FEH's. At(6&1)-FEH vertoont in vergelijking met celwand invertasen en andere FEH's enkele speciale eigenschappen: in het cDNA ontbreekt een N-terminale signaalsequentie en het enzym wordt geïnhibeerd door Tris.

Nu blijkt dat FEH's kunnen gevonden worden in verschillende species die geen fructanen bevatten, dringt het onderzoek naar de fysiologische rol van deze enzymen zich op. Twee hypothesen kunnen gevolgd worden: (1) FEH's breken kleine hoeveelheden endogene fructanen of een ander ongekend fructosylsubstraat af, (2) FEH's spelen een rol in de plant-microbe interactie door hun effect op de fructose-bevattende exopolysacharidenlaag die sommige bacteriën omgeeft. Om de functie van deze FEH's te ontrafelen, werden *A. thaliana At6-FEH* en *At(6&1)-FEH* knock-out en overexpressie-planten geconstrueerd. De transgene planten vertoonden geen duidelijk abnormaal fenotype. Opmerkelijk was echter wel de heel slechte kieming van T2 generatie planten van hoge *At(6&1)-FEH* expressoren. Er werd ook geen nieuw endogeen substraat gevonden bij vergelijking van suikerextracten van knock-out en wild-type planten. Uit micro-array analyses kon afgeleid worden dat *At6-FEH* en *At(6&1)-FEH* een verschillend expressiepatroon vertonen in de verschillende weefsels. Expressieanalyse van *At(6&1)-FEH* suggereert een inductie na toediening van salicylzuur. Andere hormonen en *P. syringae* infectie hadden geen effect. Ziekte-assays met *P. syringae* konden geen verhoogde gevoeligheid of resistentie aantonen bij knock-out en overexpressieplanten.

Ter conclusie, dit doctoraatswerk bevestigt de aanwezigheid van een specifiek 6-FEH in suikerbiet en beschrijft de karakterisatie van twee FEH's in *A. thaliana*, die vroeger geïdentificeerd werden als mogelijke invertasen. De fysiologische functie van FEH's in niet-fructan planten kon niet opgelost worden. Hun rol tijdens plant defensie is echter twijfelachtig. Toekomstig werk zou zich meer moeten focussen op een regulatorische rol. Aangezien At6-FEH en At(6&1)-FEH verschillende eigenschappen hebben, is het goed mogelijk dat beide enzymen een andere fysiologische functie vervullen.

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