Analysis of sugar transport-related gene products expressed in developing seeds of *Vicia faba* and *Hordeum vulgare*

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Summary

Studies on expression and localization of a sucrose transporter, a hexose transporter and a sucrose binding protein-like protein in faba bean

Sugar transport and its regulation are of great importance during seed development. In this work, sugar transporters of faba bean were studied. To enable detailed studies on the expression and the localisition of sugar transporters during seed development of faba bean, antibodies were raised against a sucrose transporter (VfSUT1), a hexose transporter (VfSTP1), as well as a sucrose binding protein-like protein (VfSBPL).

The highest expression of the VfSTP1 protein was found in mid-stage cotyledons, closely correlated to mitotic activity. The VfSUT1 protein was detected mainly in later stages of developing cotyledons, suggesting a correlation to storage activity. The VfSBPL cDNA was isolated from a cDNA library of faba bean cotyledons. The sequence shows high homology to the sucrose binding protein of soybean (Grimes et al.*, 1992). Increasing amounts of the VfSBPL protein were detected during development. In dry seeds, the protein is present in appreciable amounts, but it degrads during germination similar to 50kD-vicilin. The VfSBPL protein has been localized on the sub-cellular level by immunological methods. It was found mainly in protein bodies during developmentally late stages. Taken together, the VfSBPL protein behaved more like a storage protein than a membrane associated sucrose-binding protein as expected from the sequence analysis.*

Studies on sugar transporters in barley

In order to understand the role of sugar transporters during barley seed development, two full length cDNAs encoding sucrose transporters (HvSUT1 and HvSUT2) were cloned from a barley caryopsis library. The HvSUT1 and HvSUT2 cDNAs possess open reading frames of 1694 and 1521 bp encoding 523 and 507 amino acids, respectively. Both deduced proteins have the typical 12 membrane-spanning domain structure. The HvSUT1 cDNA sequence is only 42% identical to HvSUT2, whereas it is more than 80% homologous to a rice sucrose transporter, which is the only sucrose transporter known so far from a monocot species. For functional expression and analysis in yeast, these two cDNAs were cloned into the yeast expression vector pNEV. Sugar uptake experiments with yeast cells indicated that both HvSUT1 and HvSUT2 cDNA encode sucrose transporters. In barley, transcripts of both genes were found in sink and source organs. HvSUT1 mRNA levels were very high in the caryopsis between 7 to 11 days after flowering (DAF), but much lower in sink and source leaves and roots. Compared to HvSUT1, the amount of HvSUT2 mRNA was higher in younger and older caryopsis. Furthermore, in situ *hybridization shows that HvSUT1 is mainly expressed in the nucellar projection and in the endospermal transfer cells, closely corresponding to the sucrose level. Therefore, HvSUT1 is most probably involved in the accumulation of sucrose.*

Because of the specific expression pattern of the HvSUT1 gene in developing barley caryopses, the genomic structure including the promoter of this gene were studied. The results indicated that the HvSUT1 is a single copy gene. 4862 bp were sequenced including about 1.4kb of HvSUT1 gene promoter region, the coding region (9 exons) and 8 introns. The 1.4kb fragment of the HvSUT1 promoter region was cloned into the promoter-less pRT103GUS vector. The promoter activity of the 1.4kb fragment was confirmed using a transient expression system. Direct plant transformation is needed for further promoter studies.

The HvSUT2 protein has sucrose transport function, but it has low homology to most of the sucrose transporters analyzed up to now. Therefore, the localization of the HvSUT2 protein was studyed to get additional information on it's specific. To localize the HvSUT2 protein, antibodies were raised. The HvSUT2 protein was overexpressed, purified and used to prepare polyclonal antibodies. The specificity of the antibody was confirmed by dot blot and Western blot experiments. By using this antibody, the HvSUT2 protein was localized in the endospermal transfer cell layer and it was found mainly in the plasma membrane layer of barley protoplasts.

In addition, a series of EST sequences, highly homologous to sugar transporters, were found by screening an EST database of barley caryopses. Two EST clones were identified coding for hexose transporters by their homology to known plant hexose transporters. The full length cDNAs were isolated from a barley caryopses cDNA library, and designated as HvSTP1 and HvSTP2. The HvSTP1 and HvSTP2 cDNAs possess open reading frames of 2232 and 2175 bp encoding 743 and 724 amino acids, respectively. HvSTP1 is a single copy gene. HvSTP2 seems to be a member of a small gene family. Gene expression of HvSTP1 was rather low at very early stages, but detectable during the whole developmental process with a maximum expression at 6 DAF. The HvSTP2 gene was mainly expressed in young grains between 1 to 4 DAF, with a second smaller peak at 8 to 10 DAF. By in situ *hybridization, the two transcripts were localized in both maternal and filial grain tissues. A clear parallel in the mRNA expression of the two hexose transporters was seen for the nucellar projections and the transfer cells. HvSTP1 may have a specific function for the early endosperm. A specific function for HvSTP2 in the early pericarp can be deduced.*

Zusammenfassung

***Untersuchungen zur Expression und Lokalisierung eines Saccharosetransporters, eines Hexosetransporters und eines Saccharosebindeprotein-ähnlichen Proteins der Ackerbohne* (Vicia faba L.)**

Der Zuckertransport und seine Regulation sind für die Entwicklung von Samen von großer Bedeutung. Dennoch sind noch viele Aspekte dieser Prozesse im Detail nicht geklärt. Die vorliegende Arbeit widmet sich Fragen der Expression und subzellulären Lokalisation von Proteinen des Zuckertransports bei der Entwicklung von Samen der Ackerbohne.

Diese Untersuchungen wurden vor allem mit immunologischen Methoden durchgeführt, für die zunächst Antikörper gegen einen Saccharosetransporter (VfSUT1), einen Hexosetransporter (VfSTP1) und ein Saccharosebindeprotein-ähnliches Protein (VfSBPL) hergestellt wurden. Mit diesen Sonden wurde gezeigt, daß der Expressionsverlauf des VfSTP1-Proteins in mittleren Stadien der Kotyledonenentwicklung ein Maximum zeigte und eng mit der mitotischen Aktivität dieses Organs korrelierte. Das VfSUT1-Protein wurde vor allem in späteren Entwicklungsstadien gefunden und stand somit in engerer Beziehung zur Realisierung der kotyledonaren Speicherfunktion.

Aus einer cDNA-Bank, die aus reifenden Kotyledonen der Ackerbohne angelegt worden war, wurde eine cDNA für VfSBPL isoliert. Die ermittelte Sequenz zeigt hohe Homologie zu einem Saccharosebindeprotein der Sojabohne (Grimes *et al*., 1992). Während der Kotyledonenentwicklung wurden zunehmende Mengen des VfSBPL-Proteins nachgewiesen. Auch in trockenen Samen wurden erhebliche Mengen dieses Proteins entdeckt, die allerdings im Verlaufe der Keimung ähnlich wie ein 50kD-Vicilin abgebaut wurden. Die immunologische Untersuchung der subzellulären Lokalisation des VfSBPL-Proteins zeigte, daß dieses Protein in den Proteinkörpern später Entwicklungsstadien von Kotyledonen konzentriert ist. Auf Grund dieser Ergebnisse hat das VfSBPL-Protein eher den Charakter eines Speicherproteins und nicht so sehr den eines membranassoziierten Saccharosebindeproteins, der durch die Sequenzhomologie nahegelegt wurde.

***Untersuchungen von Zuckertransportern der Gerste* (Hordeum vulgare)**

Aus einer cDNA-Bank heranreifender Gerstenkaryopsen wurden zwei Saccharosetransporter (HvSUT1 und HvSUT2) kloniert. Die cDNA-Klone mit 1694 und 1521 Bp kodieren für offene Leseraster von 523 und 507 Aminosäuren. Die abgeleiteten Proteinsequenzen zeigen die für Saccharosetransporter typische Domänenstruktur mit 12 transmembranen Helices. Die cDNA von HvSUT1 zeigt nur 42 % Übereinstimmung mit der HvSUT2-Sequenz, aber mehr als 80 % Identität mit einem Saccharosetransporter aus Reis als bisher einzigem bekannten Vertreter aus monokotylen Pflanzen.

Für eine funktionale Charakterisierung wurden beide Klone in Hefe exprimiert. Versuche zur Saccharoseaufnahme in transgene Hefezellen bestätigten, daß beide Proteine die Funktion von Saccharosetransportern erfüllen.

Bei der Gerste wurden Transkripte beider Transporter sowohl in „sink"- als auch in „source"-Organen nachgewiesen. Der Gehalt an HvSUT1-mRNA war besonders hoch in Karyopsen in der Zeit zwischen dem 7. und 11. Tag nach der Bestäubung, während er in „sink"- und „source"-Blättern sowie in Wurzeln erheblich niedriger war. Verglichen dazu war der mRNA-Gehalt von HvSUT2 in jungen und älteren Karyopsen höher. Durch *in situ*-Hybridisierung wurde gezeigt, daß die HvSUT1-Transkription in der nucellaren Projektion und den endospermalen Transferzellen am höchsten ist. Aus der Korrelation zwischen Saccharose gehalt der Karyopsen und HvSUT1-mRNA-Expression, kann abgeleitet werden, daß HvSUT1 wahrscheinlich an der Akkumulation von Saccharose beteiligt ist.

Weiterhin wurden die genomische Struktur sowie die Promotorregion des HvSUT1-Gens untersucht. Die aus einer BAC-Bank der Gerste erhaltenen Ergebnisse zeigen, daß HvSUT1 ein singuläres Gen darstellt. Seine Länge umfaßt 4862 Bp unter Einschluß einer Promotorregion von 1.4 kBp sowie der durch 8 Introns getrennten 9 Exons. Zur Untersuchung der Promotorfunktion wurde ein transientes Expressionssystem benutzt. Dazu wurde das 1.4 kBp-Promotorfragment als Kontrollelement in den promoterlosen pRT103GUS-Vector kloniert und mit diesem Konstrukt eine biolistische Transformation von Protoplasten durchgeführt, die aus einer Zellkultur von *Nicotiana plumbaginifolia* hergestellt worden waren. Auf diese Weise konnte eine transiente Glucuronidase-Aktivität nachgewiesen werden.

Auch das HvSUT2-Protein transportiert nach heterologer Expression in Hefe einen Saccharosetransport, hat aber andererseits nur geringe Homologie zu den meisten bisher sequenzierten Saccharosetransportern. Um weitere Anhaltspunkte für die spezifische

Funktion dieses Transporters zu erhalten, sollte seine Lokalisierung mit immunologische Methoden erfolgen. Zur Herstellung eines Antigens wurde das Protein in Hefe überexprimiert und nach entsprechender Reinigung zur Produktion von Antikörpern eingesetzt, deren Spezifität durch verschiedene Kontrollen sichergestellt wurde. In anschließenden Versuchen wurde gezeigt, daß das HvSUT2-Protein vor allem in der Plasmamembran konzentriert ist.

Schließlich wurden über ein Screening-Verfahren in einer Datenbank von Gerstenkaryopsen noch zahlreiche ESTs mit hoher Homologie zu Zuckertransportern aufgefunden. Zwei dieser Sequenzen stellen auf Grund ihrer Homologie wahrscheinlich Hexosetransporter dar. Die entsprechenden vollständigen Klone wurden aus einer cDNA-Bank isoliert und als HvSTP1 und HvSTP2 bezeichnet. Sie enthalten offene Leseraster von 2232 und 2175 Bp und kodieren für Proteine von 743 und 724 Aminosäuren laenge. HvSUT1 ist ein singuläres Gene. HvSPT2 ist als Mitglied einer kleinen Genfamilie zu betrachten. Das HvSPT2-Gen wird vorwiegend in jungen Karyopsen in der Zeit zwischen dem 1. und 4. Tag nach der Bestäubung exprimiert, gefolgt von einen schwächeren Aktivitäts spitze 8-10 Tage nach der Bestäubung. Demgegenüber war die Expression von HvSTP1 in jungen Stadien sehr niedrig, jedoch wird HvSUT1 während der gesamten Karyopsenentwicklung exprimiert mit einem Maximum am 6. Tag nach der Bestäubung. Durch *in situ*-Hybridisierung wurden die Transkripte beider Gene sowohl in maternalen als auch filialen Geweben der Karyopsen lokalisiert. Für beide Hexosetransporter wurden parallele Expressionsmuster in der nucellaren Projektion und den Transferzellen nachgewiesen. Für HvSPT2 kann eine spezifische Funktion in jungen Perikarpzellen abgeleitet werden, während HvSPT1 eher eine Funktion in der frühen Entwicklungsphase des Endosperms zuzukommen scheint.

Abbreviations

ABA	cis, trans-ABscisic Acid
amp	ampicillin
AP	Alkalische Phosphatase
BAC	Bacterial Artificial Chromosome
BCIP	5-Bromo-4-Chloro-3-IndolylPhosphat
ß-ME	ß-MercaptoEthanol
bp	basepairs
BSA	Bovine Serum Albumin
CaMV	Cauliflower Mosaic Virus
cDNA	complementary DeoxyriboNucleic Acid
dCTP	2‘-deoxyCytisine-5‘-TriphisPhate
DAF	Days After Flowering
DDM	DoDecyl-ß-D-Maltoside
DEPC	DiEthylPyroCarbonate
DNA	DeoxyriboNucleic Acid
DNase	DeoxyriboNuclease
dNTP	deoxyriboNucleoside TriphosPhates
DTT	DiThioThreitol
E. coli	*Escherichia coli*
EDTA	EthylenDiaminTetra Acetate
EGTA	Ethylene Glycol-bis(ß-aminoethylether)N,N,N’,N’-Tetraacetic Acid
ELISA	enzyme-linked immunosorbent assay
EM	electron microscope
ER	endoplasmic reticulum
et al.	*et alii*
Fig	figure
g	gram
GUS	ß-glucuronidase
HEPES	N-2-HydroxyEthylPiperazin-N’-2-EthanSulfonacid
His	Histidin
Ig	Immunoglobulin

IPTG	IsoPropyl-ß-D-ThioGalactopyranosid
kD	kilo Dalton
L	Litre
M	Molarity
m	milli
min	minutes
mM	milliMolar
mRNA	messenger RiboNucleic Acid
MS	Murashige Skoog medium
NBT	4-NitroBlau-Tetrazoliumchlorid
OD	Optical Density
ori	origin of replication
P	Promoter
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PFA	ParaFormAldehyd
PMSF	PhenylMethanSulfonylFluorid
poly A	polyadenylAtion signal
PSV	Protein Storage Vacuolar
RACE	Rapid Amplification of cDNA Ends
RNA	RiboNucleic Acid
r.p.m.	rounds per minute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
sec.	seconds
TAE	Tris AcetatE buffer
TBE	Tris BoratE buffer
T-DNA	Transferred DNA
TE	Tris EDTA buffer
TEM	Transmissions Electron Microscope
Tris	Trishydroxymethylaminomethan
Vol.	Volume
w/v	weight/volume
X-gluc	5-bromo-4-chloro-3-indolyl-ß-D-glucuronic acid

1 Introduction

Plants represent the primary source of energy and protein for human life. We are completely dependent upon plants for oxygen as well as food. Besides the oxygen produced in photosynthesis reactions, the carbohydrates produced by this reaction are used just as a symbol for many sugars and carbohydrate-related bio-molecules that plants can make. After synthesis in the vegetative parts of the plant, carbohydrates are translocated to photosynthetically inactive or less active organs, like developing seeds and other storage compartments. Researches on sugar transport into developing and/or storage organs have been carried out. The fluxes of carbohydrates across the plasma membranes of plant cells are catalyzed mainly by hexose and sucrose proton symporters. cDNAs encoding these different transporters have been cloned, the functions and properties of the encoded proteins have been studied extensively in heterologous expression systems, and their location in plants has been shown immuno-histochemically. Higher plants use sugars not only as nutrients but also as signal molecules. The role of metabolites, like sugars, as signal molecules during plant development is attracting much attention in plant science (Sheen *et al*., 1999).

1.1 Sugar transport: loading and unloading the phloem

Although plants are photoautotrophic organisms, they have also heterotrophic tissues and organs. The development and growth of sink tissues depend on carbon import from photo-synthetically active tissues. In plants, sucrose is the major transport form of photo-assimilated carbon. Selection of sucrose as the major transport sugar in plant has been related to its non-reducing nature and relative insensitivity to metabolism. The initial fixation of CO_2 in plants occurs within chloroplasts. Once sucrose has been synthesized in mesophyll cells of source leaves, it has to be translocated to sink organs where it is stored or metabolized. About 80% of the carbon assimilated during photosynthesis are exported from leaves to satisfy the metabolic needs of the non-photosynthetic cells. This fundamental activity allows plants to function as multi-cellular organisms. Sucrose is transported over long distances in solution in the phloem sap. This flow of sap occurs in a

specialized network of cells, called sieve elements. Sieve elements lose their nucleus and many organelles during differentiation, but stay connected to companion cells, cells with a high metabolic activity. Sieve elements are connected to form sieve tubes that oppose very little resistance to the flow of sap. The driving force for this flow occurs through phloem loading and unloading, the entry of sucrose and subsequently water in the sieve tubes in the source organ and, at the other end of the conduit in the sink organs, the continuous unloading of solutes and water. Sugars are synthesized in the mesophyll cells, especially the palisade parenchyma. From there, they have to be trans-located across several cells to reach the veinal network. On their way, several cell types, such as neighboring mesophyll cells, bundle sheath cells, and phloem parenchyma or companion cells, have to be passed before the sugars reach the sieve elements. There is not a uniform mode of sugar export in all plant species. Some plants have a high number of plasmodesmatal connections between mesophyll cells and the sieve element / companion cell complex (SE-CCC). In these species often not sucrose but sugars of the raffinose family are the preferentially exported carbohydrates (Turgeon and Beebe, 1991). In most species, at least crop species, the SE-CCC is simplistically isolated from the surrounding cells. In these plants an apoplastic step in the phloem loading process has been proposed. Direct evidence for the phloem loading of sucrose involving an apoplastic step was obtained by studies using transgenic plants of tobacco (Schaewen *et al.*, 1990), tomato (Dickinson *et al.*, 1991) and potato (Heineke *et al.*, 1992) expressing a yeast-derived invertase, which converts sucrose into hexoses within the apoplastic space. In all these cases, the transgenic plants were retarded in growth.

Because the apoplast is involved in assimilate export, two steps of membrane passage are required: between the cytosol of mesophyll cells and the apoplastic space and between the apoplastic space and the sieve tubes. The characterization of the involved transport mechanisms requires knowledge of the metabolite concentrations within three compartments: the cytosol, the apoplastic space and the phloem sap. The concentration of sucrose is highest in the compartment of synthesis, the cytosol. For barley, the apoplastic concentrations are much lower than those in the cytosol and the phloem sap. Because the sucrose concentration in phloem sap is much higher than in the apoplast, sucrose must be actively transported against the concentration gradient by sucrose transporters. The active loading is assumed to take place at the border between mesophyll and phloem cells. All the sucrose transporters characterized up to now are proton symporters. The driving force of sucrose transport is supplied by a proton ATPase localized in the plasma membrane of the companion cells (Frommer, 1995).

After long distance transport, when sucrose arrives in the sink tissue, it is directly released into the apoplast and may be transported into local sink cells by transporters or it moves out of the phloem and is symplastically transported through plasmodesmata into rapidly growing cells (Tanner and Caspari, 1996). In sink tissues different routes of sucrose utilization exist depending on the mechanism of unloading. In case of apoplastic unloading, sucrose may be hydrolyzed by an apoplastic acid invertase yielding glucose and fructose, which are subsequently transported into the cytosol for further metabolization. In case of symplastic unloading, sucrose could either be utilized via the action of neutral invertase or sucrose synthase. Sink strength is defined as the ability of an organ to attract photoassimilates (Ho, 1988). Sink strength can be estimated by the rates of nutrient uptake measured by incorporation studies using labeled photoassimilates or by the dry matter accumulation during development determined as yield after harvest. Respiratory activity can also contribute to sink strength. During movement from phloem cells into parenchyma cells of sink tissue, sucrose may pass through living cells, cell walls and intercellular spaces. Therefore, the plant cells possess various control mechanisms that allow them to regulate fluxes of the main assimilates across the plasma membrane when their natural environment is directly or indirectly altered.

1.2 Sugar transporters

Sugar transporters are integral membrane proteins responsible for the transport of sugars across the cellular membranes. They play a crucial role in the distribution of sugars throughout the plant. All plant sugar transporters known so far belong to a large superfamily of transmembrane facilitators (MFS, major facilitator superfamily). Members of the MFS have been found in all living organisms and consist of 17 distinct families (Pao, *et al*., 1998). The largest of these families is the sugar porter family, comprising 133 proteins derived from bacteria, eukaryotic protists, yeasts, animals and plants. Higher plants possess two distinct families of sugar transporters: the sucrose transporters that primarily catalyze sucrose transport and the hexose transporters that mediate the transport of a variable range of monosaccharides. The sucrose transporters and the hexose transporters coordinate sugar transport in diverse tissues, at different developmental stages and under varying environmental conditions.

1.2.1 The sucrose transporters

Sucrose synthesized in green leaves is transported via the phloem, the long distance distribution network for assimilates, in order to supply non-photosynthetic organs with energy and carbon skeletons. Physiological analyses of many plants demonstrate that sucrose transporters are essential components of the sucrose translocation pathway. To understand the transport of sucrose across the plasma membrane, two principal routes can be envisaged: 1. Apoplastic: Carrier-mediated transport across the plasma membrane and diffusion through the cell wall, and 2. Symplastic: direct cell-to-cell transport via plasmodesmata. To clarify the actual pathways, molecular approaches are required to direct a better understanding of both, plasmodesma-mediated diffusion and plasma membrane transport processes of sugars. Although the difference in sucrose concentration between the compartments defined by the different membranes is not precisely known, the existence of carriers have been postulated or clearly demonstrated in several membranes of different types of cells (seeFig.1.2.1).

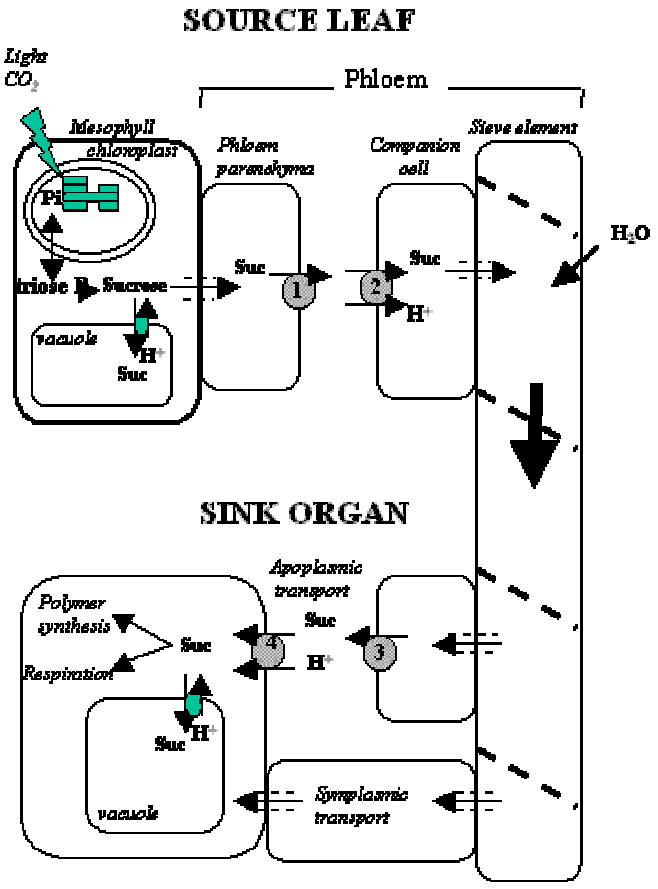


Fig.1.2.1 *Transmembrane-steps mediated by sucrose transporters. The flow of sucrose from the source leaf to the sink organs through the phloem is represented by a big arrow. The numbers in gray circles refer to the different events of membrane transport discussed in the text.*

After synthesis in the mesophyll cell, sucrose may be transported into the vacuole, which determines the pool of sucrose available for export (sucrose is temporarily stored within the vacuole). Intracellular sucrose partitioning primarily concerns sucrose flux between the cytoplasm and vacuolar space and should be mediated by a proton-sucrose anti-porter and /or a facilitated diffusion system (Bush, 1993). The first trans-membrane event between cells exits in mesophyll cell (step 1) and, from the apoplast, sugar enters the phloem cells (step 2). When sucrose is unloaded into the apoplasmic space (step 3), it can be taken up into the sink cells (step 4). There sucrose is used for growth or development of the sink organs or can be stored. According to the different steps identified, sucrose transporters in plants can be of three types: 1. Plasmamembrane influx transporters of the proton-sucrose symporter type responsible for the entry of sucrose into cells; 2. tonoplast transporters proposed to work as sucrose/proton antiporters and 3. plasma membrane efflux transporters responsible for the unloading of sucrose in sink organs or for sucrose exit from the mesophyll cells in close vicinity to the phloem (steps 1 and 3).

Influx transporters

In the past years, significant progress has been made in describing the transport properties and molecular genetics of these critical transport systems. In 1980, the first gene coding for a transport protein, the ß-galactoside transporter of *E. coli*, was cloned (Büchel *et al*., 1980). Only in 1992, the first plant sugar transporter, SoSUT1, was cloned from spinach (Riesmeier *et al*., 1992). This first successful cloning of a plant sucrose transporter was achieved by complementing an engineered yeast mutant with a plant cDNA library. The SoSUT1 cDNA encodes a hydrophobic integral membrane protein with a molecular mass of about 55 kD. Hydropathy analysis suggests that it contains 12 trans-membrane domains. The corresponding gene from potato was obtained by the same procedure. Due to the high level of expression in leaves and the high conservation at the DNA level, heterologous screening has proven to be a useful tool to isolate homologous genes from other species, e.g., tobacco, tomato, arabidopsis and plantago (Gahrtz *et al*., 1994; Sauer and Stolz, 1994; Burkle *et al*., 1997; Weig and Komor, 1996). At present, 29 different cDNAs encoding sucrose transporters have been identified in different plant species, in dicots as well as in monocots (see Table I). The total number is increasing rapidly. All these transporters are sequence related (see fig.1.2.2). The two most distant sequences (OsSUT1 and BvSUT1) still show 37% identity at the protein level.

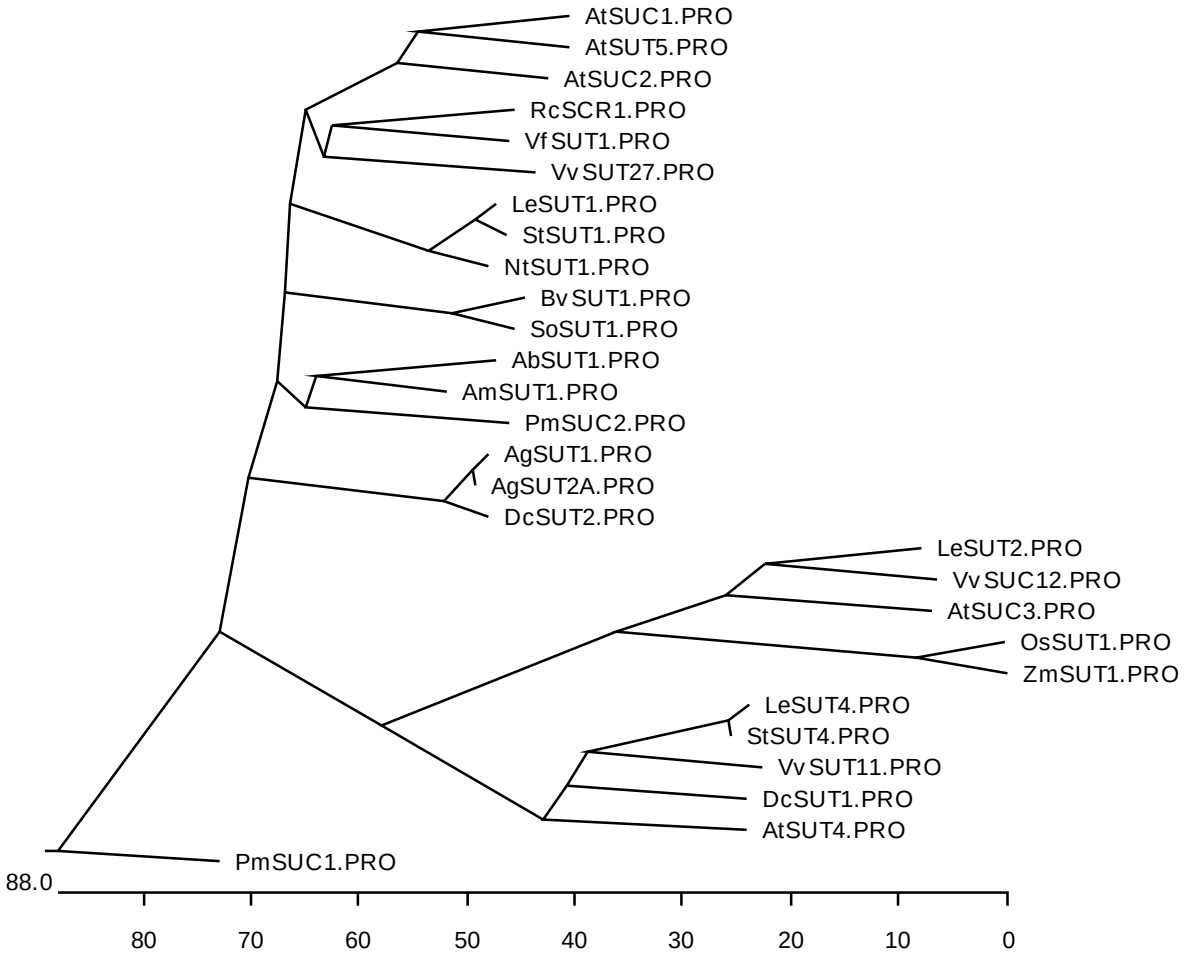


Fig.1.2.2 *Phylogenic tree showing the relatedness of the sucrose transporters listed in Table 1, based on the degree of similarity of their sequence. The image was generated with the clustal method (DNA*MegAlign).*

The sucrose transporter genes encode highly hydrophobic proteins. Most of the corresponding gene products are membrane proteins with 12 putative membrane- spanning segments in the form of α-helices. They have a calculated molecular mass of around 55kD. Extensive sequence analysis and comparison between these different transporter proteins has led to a proposed conserved motif that could be used as a signature for the members of this gene cluster. The initial pattern proposed for the sucrose transporter was R-X-G-R-(KR). It is located in the loop between the second and the third trans-membrane domain. The second pattern, (MSTA)-S-x(2)-(LIVM)-(EYQD)x-(LIMF)-(GCAV)-(RK)-x(3)-(GA),

is located between trans-membrane segment 8 and 9. The second pattern is not heavily conserved in sucrose transporters. Due to the increasing number of plant sucrose transporters available, the consensus patterns will certainly be changed. These conserved sequences have not been related to any particular function, except for the small pattern between the second and the third trans-membrane segments which is predicted to form a ß-turn linking the two a-helices (Baldwin and Henderson, 1989). At present, no three-dimensional structure is available for any sugar transport protein from any species. However, the knowledge of all the sucrose transporter sequences will be of considerable interest for the structure/function studies. Initially, these porters were well-characterized using purified membrane vesicles and imposed proton electrochemical potential differences. This approach allowed a detailed analysis of their transport kinetics, bioenergetics, and substrate specificity. In most cases, trans-membrane transport of sugars has been studied via sugar uptake by unicellular organisms. The yeast expression system has allowed the analysis of the biochemical properties of the transporters because the uptake of radio-labeled sucrose can be measured directly in the presence of competitors and inhibitors. The *Km* value of the sucrose transporters was estimated to be approximately 0.3-1mM (Bush, 1993). Inhibitor studies indicated that a proton gradient is required to allow sucrose transport into yeast cells. All sucrose transporters that have been studied up to now are proton/sucrose symporters. Several possibilities exist to localize the sucrose transporters. Most of the proton/sucrose symporters are localized in the leaf tissue and the phloem, especially in companion cells (Stadler and Sauer, 1996), others are found in sink tissues (Gahrtz *et al*., 1996). It is generally assumed that they are frequently expressed in the sieve element-companion cell complex (SE-CCC) and promote phloem loading. mRNA *in situ* hybridization experiments demonstrated that some of the sucrose transporters, i.e., AtSUC1, AtSUT2, PmSUC1 and StSUT1, are phloem associated. Some members are found to be preferentially expressed in import zones of sink organs where they may catalyze either influx and/or retrieval of sucrose. Sink-specific sucrose transport systems were identified in developing seeds (Weber *et al*., 1997) and anthers (Stadler *et al*., 1999). A rice sucrose transporter, OsSUT1, is expressed in source organs such as leaf blade, leaf sheath and germinating seed, whereas little or no expression was observed in some sink organs as the panicles before heading and the roots. The transcript was observed at high levels in panicles after heading, particularly in the portion containing endosperm and embryo. Sink-specific sucrose transporters have been characterized in seeds of fava bean (Weber *et al*., 1997; Harrington *et al*., 1997). The mRNA of the carrot sucrose

transporter DcSUT2 was also found mainly in taproots. To determine the nature and cellular localization of sucrose transporters in pea seeds, a full-length clone of a proton/sucrose symporter (PsSUT1) was isolated from a cotyledon cDNA library (Tegeder *et al*., 1999). Within developing pea seeds, transcripts of the PsSUT1 gene were detected in all tissues, while transcripts of a sucrose binding protein (GmSBP) were confined to cotyledon epidermal transfer cells. Signal intensities of the PsSUT1 transcripts and protein products were most pronounced in the thin-walled parenchyma cells of the seed coat and epidermal transfer cells of the cotyledons. The resolution of *in situ* hybridization was insufficient to determine the expression at the cellular level (Riesmeier *et al*., 1993). Therefore, immuno-localization studies were required. In *Plantago* and *Arabidopsis*, the transporters PmSUC1 and AtSUC2, respectively, were detected by immuno-fluorescence with specific antibodies in companion cells (Stadler *et al*., 1995; Stadler and Sauer, 1996). On the contrary, immuno-localization using immuno-fluorescence and silver-enhanced immuno-gold staining detected NtSUT1, StSUT1 and LeSUT2 in plasma membranes of enucleate sieve elements of tobacco, potato and tomato, respectively (Kühn *et al*., 1997). This localization coincides with the osmotic gradient observed between sieve elements and companion cells (Lackney and Sjolund, 1991). The differences in sucrose transporter protein localization observed in *Arabidopsis* and *Plantago* compared to tomato, potato and tobacco may be due to differences in the loading mechanisms.

Physiological analyses of plants demonstrate that sucrose transporters are essential components of the sucrose translocation pathway. The best way to study the actual function of a protein is the analysis of mutants. Such an approach was tried by screening T-DNA tagged mutants of *Arabidopsis* by PCR with gene-specific and T-DNA primers (Krysan *et al*., 1996). However, so far no sucrose transport mutants have been described.

Antisense expression of the potato symporter StSUT1 provided convincing evidence that the symporter plays an essential role in phloem loading. To create plants with reduced sucrose transport activity, potato plants were transformed with the StSUT1 gene in antisense orientation (Riesmeier *et al*., 1994; Kühn *et al*., 1996). If sucrose transport mediated by this transporter is essential for phloem loading, a reduction in transport activity should affect carbon partitioning and photosynthesis. As expected, antisense plants with low levels of the symporter message stunted, has reduced root growth, and carbon backed up in the leaves. This phenotype is consistent with the notion that the proton/sucrose symporter mediates active phloem loading (see Table 1).

Tonoplast transporters

As indicated before, vacuolar transporters are supposed to work as proton/sucrose antiporters. An immunological approach by Getz *et al*., (1993) gave some indications that the sucrose transport activity of the red beet tonoplast is associated with polypeptides in the range 55-60 kD when reconstituted in proteoliposomes. However, no further characterization was reported. Only in one case, a protein was shown to be associated with the tonoplast. However, the corresponding cDNA is not closely related to all the other plant sucrose transporters and no sucrose transport function could be attributed to the protein after yeast expression.

Efflux transporters

Several descriptions of sucrose efflux activities have been reported (Laloi *et al.,* 1993). It has been postulated that the influx sucrose transporter could function as an efflux transporter without energetization of the transport, as sucrose would be transported along its concentration gradient. However, no such system has been identified so far.

The next challenge will be to identify more sucrose transporters involved in different transport events, as tonoplast transporters, efflux transporters and sink-specific transporters. No indication exists, for example, that the tonoplast sucrose transporters is related in sequence to other sucrose transporters. New methods will have to be designed and used for these identifications.

Another challenge will be to understand more about the way plants regulate the flow of sucrose at both, the whole plant and the cellular level. This may not only be interesting from a fundamental point of view but also as a possibility to alter the flow of sucrose to sink, or to alter the selectivity of the carrier so that it would accept foreign molecules (xenobiotics or natural) and allow their long distance transport in the plant. Improving the quality of sink is also a positive outcome to be expected. However, there is still much information that needs to be obtained.

Table 1. Currently available information on plant sucrose transporters

Gene name	Refs.	Organism	Length (a.a.)	Functional expression	Site of gene expression
AbSUT1	Knop, C., unpublished	Asarina barclaiana	510	Yes	-
AgSUT1	Noiraud,N., et al., 2000	Apium graveolens	512	Yes	Leaf
AgSUT2a	Noiraud,N., et al., 2000	Apium graveolens	512	Yes	Leaf
AmSUT1	Knop, C., unpublished	Alonsoa meridionalis	502	Yes	Phloem
AtSUC1	Sauer,N., et al., 1994	Thale cress	513	Yes	Phloem
AtSUC2	Sauer,N., et al., 1994	Thale cress	512	Yes	Phloem
AtSUT4	Weise,A., et al., 2000	Thale cress	510	Yes	Enucleate sieve elements
BvSUT1	Westram,A. et al., unpublished	Beet	523	No	Leaf
BvSUT3		Beet	539	Yes	Leaf
DcSUT1	Shakya R., et al., 1998	Carrot	501	Yes	Leaf
DcSUT2	Shakya R., et al., 1998	Carrot	515	Yes	Root
LeSUT1	Barker,L., et al., 2000	Tomato	511	No	Leaf
LeSUT2	Barker,L., et al., 2000	Tomato	604	Yes	Sieve elements
LeSUT4	Weise,A., et al., 2000	Tomato	500	Yes	Enucleate sieve elements
NtSUT1	Buerkle L., et al., 1998	Common tobacco	507	No	Leaf
OsSUT1	Hirose T., et al., 1997	Oryza sativa	538	Yes/ND	Leaf
PmSUC1	Sauer,N., et al., 1996	Common plantain	503	Yes	Phloem
PmSUC2	Gahrtz,M., et al., 1996	Common plantain	510	Yes	Phloem
PsSUT1	Tegeder,M., et al., unpublished	Pea	524	Yes	Seed
RcSCR1	Weig A., et al., 1996	Ricinus	533	Yes	Cotyledon
SoSUT1	Riesmeier,J.W., et al., 1992	Spinach	525	Yes	Leaf
StSUT1	Riesmeier,J.W., et al., 1993	Potato	516	Yes	Phloem
StSUT4	Weise,A., et al., 2000	Potato	488	Yes	Enucleate sieve elements
TaSUT1	Ao Aoki,N., et al., 2002	Bread wheat	523	Yes	Grain
VfSUT1	Weber H., et al., 1997	Faba bean	523	Yes	Cotyledon
VvSUT11	Ageorges,A., et al., 2000	Vitis vinifera.	501	Yes	Grape berry
VvSUC12	Davies,C., et al., 1999	Vitis vinifera	612	No	Grape berry
VvSUT27	Davies,C., et al., 1999	Vitis vinifera.	505	No	Grape berry
ZmSUT1	Aoki,N., et al., 1999	Zea mays	521	Yes	Leaf

1.2.2 The hexose (monosaccharide) transporters

Hexose transport across the membrane plays an important role in plants. In the Calvin cycle and gluconeogenesis, photosynthetically fixed CO_2 is converted into hexoses, such as glucose or fructose, which represent the central units for carbon metabolism, storage and transport. Although sucrose is the principle form of transported carbon in plants, it is hydrolyzed into the component hexoses by sucrose-cleaving enzymes and subsequently transported into the sink. This is especially true in heterotrophic tissues that are symplastically isolated. Sucrose is delivered from the phloem into the apoplast of these cells or tissues. Unloaded sucrose can be taken up by the sink cells either directly via plasma membrane localized sucrose transporters or via hexose transporters after extracellular sucrose hydrolysis. Invertases and sucrose synthases, which are able to metabolize imported sucrose, are found in most sink tissues. Sucrose synthase, reversibly cleaving sucrose, is confined to the cytosolic compartment, whereas invertase, irreversibly cleaving sucrose into fructose and glucose, exists in several isoforms located in the cell wall, the vacuole or the cytosol,. The hexoses are taken up via their specific transporters from the apoplastic space to serve the mitotically active parenchyma. Carrot plants defective in their cell wall-bound invertases exhibit not only a reduced development of their tap root, but also a feed back accumulation of carbohydrates in their leaves resulting in a drastically increased leaf-to-root ratio (Tang *et al.*, 1999). A mutation in an endosperm-specific cell wall invertase from maize causes aberrant endosperm development (Miller and Chourey, 1992). These results underline the important role of extra-cellular sucrose hydrolysis and subsequent hexose transport for plant development.

Over the last 10 years numerous genes encoding hexose transporters have been cloned. The *Chlorella* hexose transporter CkHUP1 was the first proton coupled symporter cloned in an eukaryotic organism (Sauer and Tanner, 1989). Since this is an inducible transport system, a differential screening strategy was used to select potential clones encoding this carrier. They initially identified eight unrelated cDNAs that were uniquely associated with induced cells. The CkHUP1 sequence was then used to screen cDNA- and genomic libraries from other organisms. The first sequences from higher plants with significant homology to known transporters were obtained from *Arabidopsis thaliana*- and tobacco libraries (Sauer *et al.*, 1990). Detailed analyses showed that higher plants possess large families of hexose transporter (STP) genes. *Arabidopsis* contains at least 14 STP genes (Büttner *et al.*, 2000), 8 hexose genes were found in *Ricinus communis* (Weig *et al.*, 1994), and 7 hexose genes in *Chenopodium rubrum* (Roitsch and Tanner, 1994).

Hydrophobicity analysis indicates that all these hexose transporters are highly hydrophobic integral membrane proteins, located in the plasma membranes and having the typical 12 trans-membrane structure. The kinetic properties of the encoded proteins have been studied by heterologous expression or by reconstitution into proteoliposomes. This allowed for the first time the analysis of single plant transporter protein without the overlapping activities of other homologous transporters possibly expressed in the same plant cell or in the same tissue. So far, the successful characterization of 13 plant hexose transporters by heterologous expression in yeast and/or oocytes has been reported (see Table 2). In addition, the CkHUP1 hexose transporter from chlorella and the AtSTP1 transporter from *Arabidopsis* have been purified to homogeneity, reconstituted into proteoliposomes and analyzed *in vitro* (Stolz *et al.*, 1994). The substrate specificities of plant hexose transporters are relatively broad, and all of the characterized proteins can transport various hexoses and pentoses. The physiological relevance of this wide substrate specificity is unclear. The expression of hexose transporter genes was analyzed by Northern blot analysis, *in situ* hybridization as well as immuno-histochemical techniques. In the case of AtSTP2-4, tissue-specific expression has been demonstrated using ß-glucuronidase as a reporter gene under the control of the promoters of the three genes. AtSTP2 is expressed in anthers, AtSTP3 in leaves and sepals, and AtSTP4 in anthers and root tips (Truernit *et al.*, 1999). Northern blot analyses suggested that the genes of the tobacco hexose transporter NtMST1 (Sauer and Stadler, 1993) and of the medicago truncatula hexose transporter MtST1 (Harison, 1996) are also most strongly expressed in roots. More detailed analyses of the MtST1 expression by *in situ* hybridization revealed strong expression in the primary phloem fibers and in the region behind the root meristem, most likely the cells of the elongation zone. Most of the so far characterized genes are expressed in cells or tissues that depend on the import of photo-assimilates from green leaves, or their expression is enhanced under conditions of an increased cellular metabolism. Expression of plant hexose transporter genes is also regulated by environmental stimuli, such as pathogen infection or wounding (AtSTP3 and AtSTP4; Truernit *et al.*, 1996). Analyses of AtSTP4 expression in elicitor-treated suspension cultured cells of *Arabidopsis* showed a 50-fold increase compared to untreated control cells. The finding that the expression of a cell-wall invertase is also increased in response

Table 2. *Currently available information on plant sugar (hexose) transporters*

Gene name	**Refs.**	**Functionally characterized in**	**K_M (µM)**	**Transported substrates**	**Site of gene expression**
CkHUP1	Opekarová M. et al., (1994)	S. pombe, Xenopus	Glc: 15/46 Frc: 392 Man: 136 Xyl: 725 Gal: 3000	Glc>Frc>Man > Xyl > Gal	Alga
CkHUP2	Stadler R. et al., (1995)	S. pombe	Gal: 25	Gal>Glc=Xyl >> Man	Alga
CkHUP3	Stadler R. et al., (1995)	S. pombe	Gal: 900	Glc>Frc>Man >Xyl>Gal	Alga
AtSTP1	Stolz J. et al., (1994)	S. pombe, S. cerevisiae, Xenopus, liposomes	Glc: 20	Glc>> Gal>> Frc	guard cells
AtSTP2	Truernit E. et al., (1999)	S. pombe	Gal: 50	Gal>Xyl>Glc =Man	Pollen
AtSTP3	Büttner M. et al., (2000)	S. pombe	Glc: 2000	Glc>Xyl> Man>Gal	green leaves, stress
AtSTP4	Truernit E. et al., (1996)	S. pombe	Glc: 15	Gal>Glc>Xyl =Man	roots, pollen, stress
MtST1	Harrison M.J. (1996)	S. cerevisiae	–	Glc>Frc	roots, leaves, stems, mycorrhiza
NtMST1	Sauer N. and Stadler R. (1993)	S. cerevisiae	–	Glc>>Gal= Xyl	Roots
PhPMT1	Ylstra B. et al., (1998)	–	–	–	Pollen
RcHEX1	Weig A. et al., (1994)	–	–	–	hypocotyl, roots, source leaves
RcHEX3	Weig A. et al., (1994)	S. cerevisiae	Glc: 80	Glc	roots, sink leaves
VfSTP1	Weber H. et al., (1997)	S. pombe	Glc: 30	Glc>Man> Gal>Frc	embryo

to stress suggests a close relationship between apoplastic sucrose hydrolysis and hexose uptake during stress response. A two- to four-fold increased expression has also been found for the hexose transporter gene MtST1 in roots of *Medicago truncatula* after colonization by the mycorrhizal fungi *Glomus versiforme* or *Glomus intraradices* (Harrison, 1996). However, hexose transport activities have also been reported for mesophyll plasma membranes from source leaves (see Table 2). These cells are photo-synthetically active and do certainly not depend on the import of carbohydrates from other tissues. It is generally assumed that these transporters play a role in the retrieval of hexose that have been lost from these cells into the apoplast by passive leakage through the plasma membrane. Hexose transporters are expected to be found also in internal membrane systems, such as in vacuoles or plastids. So far, genes for these transporters have not been identified.

Besides their central role as substrates for carbon metabolism, hexoses may affect the expression of many genes involved in essential processes such as photosynthesis, glycolysis, nitrogen-, sucrose- and starch metabolism and cell cycle regulation. At present, direct evidence for a role of plant hexose transporters in sugar sensing is still lacking. However, in *S. cerevisiae,* two plasma membrane-localized hexose transporters, SNF3P and RGT2P, have been shown to act as glucose sensors (Özcan, 1998). SNF3P senses low glucose concentrations, whereas RGT2P is responsible for the sensing of high glucose concentrations. Both proteins modulate the function of RGT1P, a protein that functions as activator or repressor of transcription depending on the extracellular glucose concentration. SNF3P and RGT2p are localized in the yeast plasma membrane and are homologous to plant hexose transporters but possess unusually long C-terminal extension. Transferring the C-terminal extension of SNF3P to the *S.cerevisiae* hexose transporters, HXT1P and HXT2P converts these proteins into sensors and restores a mutation in SNF3P. Similar modifications may also allow hexose sensing by transport proteins in plant, but no such proteins have been characterized yet.

1.3 Seed development

Plant seeds are typical sink organs dependent on imported assimilates. They provide us with the most important crop products such as starch, storage proteins and oil in different proportions. These products are synthesized in the storage organs, the endosperm or the cotyledons, mainly based on imported sucrose and amino acids. Because of its economic importance, seed metabolism and especially the accumulation of storage products became a subject of intensive investigation. In addition, seeds represent a well-defined system for

analyzing post-phloem assimilate transport, sink metabolism and plant development. Phloem unloading and post-phloem transport of assimilates in maternal and embryonic seed tissues have been the subject of a number of reviews during recent years (Wolswinkel, 1985, 1992; Thorne, 1985; Patrick *et. al*., 1989; Fisher, 1995; Patrick and Offler, 1995; Prioul, 1996; Patrick, 1997; Zamske, 1997; Weber *et. al*., 1997a). Both, the cellular pathway and the physiology of post-phloem assimilate transport within seed tissues were examined in detail for a number of species, especially for wheat (Fisher, 1995), legumes (Patrick and Offler, 1995) and maize (Prioul, 1996).

A seed encloses the embryo proper and accumulates storage products as substrates for early development and seedling growth. Seed devolopment is closely connected with seed metabolism and transport processes. The developmental program only continues normally if a certain metabolic state is sensed at a given time point in a given cell or tissue. Several experimental strategies have provided evidence that certain sugar levels and/or the resulting changes in osmotic values are necessary within defined tissues or cells to maintain a distinct stage of differentiation or to proceed with the developmental program.

Traditionally, flowering plants have been divided into two major groups, the dicots and the monocots. The number of cotyledons found in the embryo is the actual basis for distinguishing the two classes of angiosperms, and is the source of the names Monocotyledonae ("one cotyledon") and Dicotyledonae ("two cotyledons"). The cotyledons are the "seed leaves" produced by the embryo. They serve to absorb nutrients packaged in the seed, until the seedling is able to produce its first true leaf and begin photosynthesis. In the following, I want to concentrate on seed development of one dicot (*Vicia faba*) and one monocot (*Hordeum vulgare*).

1.3.1 **Vicia faba** *seed development*

Developing legume seeds are complex structures containing the embryo and several other tissues including the seed coat, endosperm and suspensor. The development of each organ and differentiation within a single organ occur in a series of steps. The phase of cell division is followed by cell differentiation and storage activities (Borisjuk *et al*., 1995). The large seeds of *Vicia faba* allow to combine physiological, biochemical and molecular approaches with analyses of the underlying developmental processes.

In *Vicia faba* cotyledons, cell differentiation starts at the adaxial region, spreading abaxially with ongoing development. Thus a developmental gradient is generated across the cotyledons, comprising younger mitotically active cells in the abaxial region and older cells in the adaxial region undergoing elongation, endopolyploidization and storage product synthesis. The pattern of starch accumulation correlates with cell expansion and endopolyploidization and is spatially distinct from the pattern of mitotic activity (Borisjuk *et al.*, 1998). The accumulation patterns of legumin and vicilin mRNA as well as of the legumin protein do not follow cell expansion. This suggests that storage protein gene expression is programmed by mechanisms different from that of starch accumulation.

In seeds of *Vicia faba*, sugars are unloaded from the seed coat cells into the seed apoplast to be taken up by the apoplastically-isolated embryo. The early globular embryo is sitting within a solute-filled, endosperm-lined cavity and attached to the maternal seed coat via a four-celled suspensor. The embryo, consisting primarily of two cotyledons and an axis is covered by an endosperm layer, but symplastically isolated. The assimilates transported via the phloem into the seed coat parenchyma cells have to be unloaded into the apoplastic space and taken up by the embryo (Weber *et al.*, 1998). During pre-storage phase, the basic body pattern is established. The subsequent intensive growth of the cotyledons is mainly due to cell expansion and characterized by storage product accumulation. Sugar uptake into the endosperm is carrier-mediated, probably by a proton/sucrose co-transporter (Wang *et al.*, 1995). Transport is energized by the proton motive force generated by a co-localized H^+-ATPase. The sugars have to pass at least two membranes. Two types of sugar transporters have been characterized so far in *Vicia faba*, a sucrose transporter (VfSUT1) and a hexose transporter (VfSTP1). The cDNAs encoding VfSTP1 and VfSUT1 were expressed in yeast in order to confirm transport activity (Weber *et al.*,1997). Sucrose transport by VfSUT1 is energy-dependent and increases with decreasing pH indicating that VfSUT1 belongs to the group of acidic sucrose transporters which were described to play a role in seed development of *Plantago major* (Gahrtz *et al.*, 1996). Like other plant proton/hexose symporters, VfSTP1 has a very low Km for D-glucose of 30 µM (whereas VfSUT1 has a K_m of 3 mM at pH 6), and can transport different hexoses. A major determinant of embryo hexose levels in young legume seeds is an apoplastic invertase preferentially expressed in the inner cell layers of the seed coat. The enzyme cleaves the incoming sucrose into glucose and fructose. During development the tissue harboring the invertase is degraded in a very specific

spatial and temporal pattern as part of the developmental program and is thus creating steep glucose gradients within the cotyledons. These gradients can be measured at nearly cellular resolution and were found to be positively correlated with the cell division rate and negatively with cell differentiation and storage activities.

VfSUT1 as well as VfSTP1 are expressed not only in seeds but also in vegetative tissues. However, in the developing embryo both transporters are expressed in the epidermal cell layer only and can therefore be considered as epidermal markers (Weber ***et al***., 1997).

Seed development is dependent upon import of sucrose which provides both, assimilates for metabolism and soluble sugars as regulators of gene activity (Koch, 1996, for review). A comparative developmental study revealed that the switch between the pre-storage or cell division phase to the storage or differentiation phase is also accompanied by a switch from a higher to a lower hexose (mainly glucose and fructose)/sucrose ratio (Borisjuk ***et al***., 1995).

1.3.2 Barley seed development

Barley is one of the most important crop plants used for animal feeding and for brewing. Because of this importance, barley seed development has been investigated at various levels in a series of studies.

Barley grain development is divided into 3 stages: the cell division phase, the transition phase and the maturation phase (Olsen ***et al***., 1992). After pollination, the fertilized egg cell differentiates into the embryo while the starchy endosperm and the aleurone layers are tissues, which develop after fusion of three cell nuclei. In the mature barley grain, the embryo with its scutellar epithelium and the aleurone tissue consists of living cells while the endosperm is a non-living tissue. Both, the living and the dead cells contain stored compounds of different composition and for different purposes. In the starchy endosperm, starch, protein and cell walls containing the carbohydrate (1-3, 1-4)-ß-glucan are deposited whereas the primary storage materials in embryo, scutellum and aleurone cells are oil and protein.

During germination, the required metabolic energy is created by the oxidation of fatty acids from the oil droplets via the subsequent glycolysis . The energy is used for the synthesis of new carbohydrates and for building the complex cellular machinery which produces the enzymes for mobilization of the stored macromolecules in the endosperm. The embryo sends plant hormones to the aleurone cells to activate the genes which direct the synthesis of the malting enzymes. These are secreted into the endosperm where they

manufacture low molecular weight-sugars and amino acids to be transported via the scutellar epithelium as nutrients to the growing seedling.

The assimilate transport pathway into developing barley seeds has been analyzed in some detail. Photo-assimilate exchange is restricted to a single vascular bundle located at the bottom of the crease, extending across the whole length of the grain. Alternative transport pathways are prevented because cuticulae are formed between both integuments as well as between the inner integument and the nucellar epidermis surrounding the dorsal region of the endosperm except at the crease vein area (Zee and O´Brien, 1970). The crease vein is thought to be the site of phloem unloading (Thorne, 1985). Photo assimilates are symplasmically transported through the maternal tissues and unloaded into the endospermal cavity. The cellular site of efflux are the cells of the nucellar projection located in front of the endospermal transfer cells The cells of the nucellar projection develop wall in-growths to amplify the membrane surface (Wang *et al*., 1994). Tracer movement studies suggested that these specialized transfer cells are involved in solute release. Inhibitor experiments indicated that sugar uptake into the endosperm is carrier-mediated, probably by a proton/sucrose co-transporter (Wang *et al*., 1995). The transport is energized by the proton motive force generated by a co-localized H^+-ATPase. The transfer cells of the starchy endosperm can therefore function as a complex to accumulate sucrose. The subsequent transfer from the transfer cells to the starchy endosperm is thought to be symplasmic (Wang *et al*., 1995).

Aim of this work

At the beginning of my work, only a few seed-specific sugar transporters were known and no sugar transporter was described for monocot seeds. For some plant species, localisation of the transporter mRNA by in situ *hybridisation had been reported. For specific sucrose transporters of* P. major*, the protein had been localised within the vascular tissues by immuno-fluorescence (Gahrtz, et al., 1994). However, nothing was known about the sub-cellular localization of sugar transport proteins.*

Two seed-specific sugar transporter cDNAs, one for hexoses and one for sucrose, as well as a so called sucrose-binding like protein (SBPl-protein), had been described for fava bean (Gahrtz, et al., 1994 and unpublished, respectively something is wrong here!). Furthermore, a polyclonal antibody directed against parts of the SBPl-protein was available in our group. The production of polyclonal antibodies against the two sugar

transporters failed because of general problems in the E. coli*-based expression of membrane proteins. Furthermore, in 1997, work on barley seed development was started in our group. One part of this work aimed at the analysis of sugar transporters specifically expressed in bot,h the maternal and the filial part of the developing barley caryopsis.*

Based on this situation in our group, I started to approach three different tasks:

1. *subcellular localisation of the* V. faba *SBPl-protein using the electron microscopy-based immuno-gold labelling technique;*
2. *generation of antibodies against specific peptides of the* V. faba *hexose and sucrose transporter to be used for Western blot analysis and subcellular localisation of these two proteins;*
3. *isolation and identification of barley-specific sugar transporter cDNAs, generation of antibodies specific for barley sucrose transporters.*

Taken all these three task together, besides of molecular-genetic work resulting from the cDNA-isolation and identification part (task 3), the aim of this promotion work was mainly directed to the subcellular localisation of different sugar-transport related proteins by using of immunological techniques, a part of the transport-protein related analysis work which is difficult to realize but necessary to generate results founding a new quality in the functional interpretation of specific transport-related proteins.

2. Materials and methods

2.1 Materials

2.1.1 Equipment

Protein electrophoresis	Biometra	Göttingen Germany
Electrophoresis equipment	Bio-Rad	Munic Germany
Axioscope fluorescence microscope	Carl Zeiss	Oberkochen Germany
DNA-thermal cycler (Mastercycler 5330)	Eppendorf	Hamburg Germany
Hybridization oven, water bath	GFL	Burgwedel Germany
Centrifuge (Biofuge 13)	Heraeus	Osterode Germany
Photometer	Pharmacia	Freiburg Germany
GeneQuant RNA/DNA calculator	Pharmacia	LKB Biochrom Ltd, UK
Camera	Polaroid	St.Albans, UK
FUJI BAS Imager, Imaging plate	Raytest	Straubenhardt Germany
UV-cross linker 1800	Stratagene	Heidelberg Germany
Vacuum concentrator centrifuge	Uniequip	Martinsried Germany

2.1.2 Enzyme and kits

DNA rapid ligation kit, Taq DNA polymerase, restriction enzymes, T4-DNA ligase, reverse transcriptase	Boehringer	Mannheim, Germany
PCR cloning kit	Clontech	Heidelberg, Germany
Ampli Taq DNA polymerase	Perkin Elmer	New Jersey, USA
Qiagen plasmid mini kit, gel extraction kit and protein expression vectors	Qiagen	Hilden, Germany
NucTrap probe purification kit	Stratagene	Heidelberg, Germany

2.1.3 Chemicals and other consumables

α^{32}P-dCTP, Hybond-N Membran	Amersham Buchler	Braunschweig, Germany
ATP, BSA, dNTPs, SDS	Boehringer	Mannheim, Germany
Unicryl	British BioCell	Cardiff, UK
Bacto-Agar, Bacto-Trypton, yeast extract	Difco	Detroit MI, USA
DEPC, formaldehyde, glutaraldehyde	FluKa	Buchs, Schweiz

Agarose, 1kb-DNA-marker	Gibco-BLR	Gaithersburg MD, USA
ThioFusion expression system kit	Invitrogen	Leek, Holland
Ethanol, Ethidiumbromid, Formamid, HEPES, PEG and Tris	Merck	Darmstadt, Germany
Glycerin, Isopropanol	Roth	Karlsruhe, Germany
Blotting paper	Schleicher&Schüll	Dassel, Germany
DDT, EDTA, x-Gal, Maltose, Tween 20, Natriumcitrat	Serva	Heidelberg, Germany
IPTG, Mineral oil, MOPS	Sigma	Louis MO, USA

2.1.4 Plasmids:

pBK-CMV	Stratagene	Amsterdam Zuidoost, The Netherlands
pBluescribe	Stratagene	Amsterdam Zuidoost, The Netherlands
pUC18	Stratagene	Amsterdam Zuidoost, The Netherlands
pQE30/31/32	QIAGEN	Hilden, Germany
pYES2	Invitrogen	San Diego, USA
pNEV (Erlangen)		

2.1.5 Plant materials

Faba bean (*Vicia faba L* var.minor cv. Fribo, Genbank, Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany) was grown in growth chambers under a light/dark regime of 16h light and 8h dark at 20°C. Seeds were harvested in the middle of the light phase.

Barley (*Hordeum vulgare* cv. Barke) was cultivated in growth chambers. During the generative phase of development, the plants were grown under 16h light, 20°C and 8h dark, 14°C regime. Days after flowering (DAF) were defined by determining anthesis on spikelets in the center one-third of the spikes. Only 5 kernels from each row corresponding to this region were used in all studies presented.

2.1.6 Bacteria strains

Strain	**genotypes**
DH5α	recA1, endA1, gyrA96, thi-1, hsdR17, (rk-mk+), relA1, supE44, u80#lacZ#M15, Tn10, (Tet')]c (Sambrook *et al.*, 1989)
HB101	supE44, hsdS20, (rB-mB-), recA13, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1 (Sambrook *et al.*, 1989)
XL1-Blue	recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac [F'proAB, lacl°Z#M15, Tn10, (Tet')] c (Jerpseth *et al.*, 1992)
XLOLR	#(mcrA)183, #(mcrCB-hsdSMR-mrr)173, recA1, endA1, gyrA96, thi, relA1, lac[F'proAB, lacl°Z#M15, Tn10, (Tet')]c (Stratagene, Amsterdam Zuidoost, The Netherlands)

2.1.7 Yeast strains

DBY2617 (Kaiser and Botstein, 1986)

C13ABYS86 (Bröker *et al.*, 1991)

2.1.8 Oligonucleotides

2.1.8.1 Primers used for amplification of the primary HvSUT1- and HvSUT2 fragment by RT-PCR:

5'-primer:1	T(C/T)CT(C/T)GG(A/G/C/T)(A/G)TCCC(A/G/C/T)CT(A/G/C/T)G
5'-primer:2	AA(C/T)TGGAT(C/T)GCTTGGTT(C/T)CC
5'-primer:3	CA(A/G)TT(C/T)GGTTGGGC(C/T)(C/T)TAC
3'primer:1	CC(A/G/C/T)TTG(A/T)A(G/C/T)GG(A/C/T)CG(C/T)AA(A/G)

2.1.8.2 Primers used for Genome Walking

HvSUT1/G1	AGAGTCTGGACGTAGGGGGTGAGCA
HvSUT1/G2	TAATCCAGCAATAGGGCCGCAGAGC
HvSUT1/G3	ATCACCACCTCGTTCGTTCGCCTATCAAT

HvSUT1/G4 GGAGGCGGAAGAGGAGGAGACGAGGGCGGAAAGTG

HvSUT1/G5 GAGAGGTGTGAGAGGAAGGGAAGGAGCGTGGAGTGAG

HvSUT2/G1 GGACAGCTGCAGCGCCCACCCGAACT

HvSUT2/G2 GCTGGCGAAGGCGTGCGGGATGC

HvSUT2/G3 AGGCACCCACCCGGAGTAGGAGAT

HvSUT2/G4 TAGGAGATCAATCAACCAAGAAGG

HvSUT2/G5 ACAGCTGCAGCGCCCACCCGAACT

HvSUT2/G6 CTGGCGAAGGCGTGCGGGATGC

2.1.8.3 Primers used for sequencing the genomic HvSUT1 gene (BAC sequencing)

HvSUT1/B1 TTTTTACCTTTCCCGTCCTACCTT

HvSUT1/B2 AAACCCCCTTACCTGAAATCTGAC

HvSUT1/B3 TATGTTAGCGCACTTTTCCTG

HvSUT1/B4 CCGCCGACCCCCACCGAGAG

HvSUT1/B5 CCACCTCGTTCGTTCGCCTATCAA

HvSUT1/B6 AACACCCCACATCTTTTATTG

HvSUT1/B7 GCACTGCAACCAAGACG

2.1.8.4 Primers used for sequencing of HvSTP1 and HvSTP2 cDNA

HvSTP1/1 TGCCGCAGCCTGACTTGGAGAATC

HvSTP1/2 CTGGTTTGGGCGTCTCTTGGTG

HvSTP1/3 GACATTAGAGGGCTGCTGAACACG

HvSTP1/4 CTCAATCTCCTTTCCAATCACCTC

HvSTP2/1 GGACGGAGGTGGGGAAAATC

HvSTP2/2 TAACACCTTTTCCTTTCCCAGACC

HvSTP2/3 TTCGAGTAAAGAGCAGGTTGG

HvSTP2/4 GTTTGGCAGTCACCCTTGTCC

HvSTP2/5 GCAACTGCCTCGGATGGATG

HvSTP2/6 GTTCGGTATCGGTTTGGCAGTCAC

2.2 Methods

2.2.1 Expression of transgenic proteins and Western blot analysis

2.2.1.1 Protein preparations from **E.coli***, yeast cells and plant tissues*

E.coli *cells:*

Cells were pelleted from 1ml over night culture (A_{600} ~ 0.7-0.9) by centrifugation at 3,000rpm for 3 minutes and resuspend in 50µl of 1xSDS-PAGE sample buffer (50µM Tris-HCl, 100µM dithiothreitol, 2% SDS, 10% glycerol and 0.1% bromophenol blue). The cells were sonicated briefly to lyse (avoid frothing). Samples were heated at 99°C for 5 minutes, centrifuged at 13,000rpm for 10 minutes and the supernatants were collected in new tubes.

yeast cells:

Yeast cells were pelleted by centrifugation and homogenized in lysis buffer (25mM Tris-HCl and 0.5% SDS, pH 9.0).

plant tissues:

I. Total protein

100mg plant tissue were grinded in 0.5ml ice cold extraction buffer (50mM Tris-HCl, pH 8.0, 5mM EDTA, 5mM Na-diethyldithiocarbamate, 1mM $NaHSO_3$ and 100µg/ml PMSF). The homogenate was centrifuged at 13,000rpm for 10 minutes and the supernatant was stored on ice. Protein concentrations were determined according to Bradford (1976).

II. Extraction of proteins from different subcellular fractions

(modified from Abousalham *et al*., 1995)

1g (net weight) barley protoplasts were harvested and re-suspended in ice-cold buffer (10mM Tris-HCl pH 8.0, 0.33M sucrose, 10mM $CaCl_2$, 5mM $MgCl_2$, 2mM EDTA and 100µM PMSF). The suspension was homogenized in a blender four times for 30s on ice. The homogenate was centrifuged at 8,000g for 10 minutes at 4°C to remove any unbroken cells. The supernatant was centrifuged at 18,000g for 10 minutes at 4°C. The pellet was resuspended in 2ml of ice-cold buffer (10mM Tris-HCl, pH 8.0, 10% glycerol) followed by centrifugation at 100,000g for 60 minutes at 4°C to yield a soluble fraction and a pellet containing the microsomal fraction. The pellet was resuspended in 100µl ice-cold buffer (10mM Tris-HCl pH 8.0, 10% glycerol). The proteins of the soluble fraction were precipitated by ice-cold acetone. The precipitate was dissolved in 100µl ice-cold buffer (10mM Tris-HCl, pH 8.0, 10% glycerol).

III. Isolation of membrane proteins

Membrane proteins were isolated using a procedure according to Larsson *et al.,* 1987. The procedure was modified as follows: 100mg plant tissue were homogenized in 1ml buffer (20mM Tris-HCl, pH 7.5, 0.33M sucrose, 2mM EDTA, 0.5mM EGTA, 50mM ß-ME and 100µM PMSF). The sample was centrifuged at 4°C, 4,000g for 10minutes. The supernatant (S1) was centrifuged at 4°C, 100,000g for 60minutes. Keep the supernatant (S2). The pellet (P2) was re-suspend in buffer H containing 1% Triton X-100. The protein concentration of S2 and P2 were measured according to Bradford (1976).

2.2.1.2 Precipitation of proteins

TCA precipitation: Proteins were precipitated by adding of equal volumes of 10% TCA (W/V) to samples and keeping on ice for 20 minutes, followed by centrifugation at 15,000rpm for 15minutes. The pellet was washed twice with ice-cold ethanol and resuspended in water.

Acetone precipitation: Proteins were precipitated by using equal volumes of –20°C acetone and keeping on ice for 10 minutes, followed by centrifugation at 15,000rpm for 20 minutes. The pellet was washed twice with ice-cold ethanol and resuspended in water.

2.2.1.3 Western blot analysis

Protein concentrations were determined according to Bradford (1976). Equal amounts of protein (50µg) were separated on 12% SDS-polyacrylamide gels (Laemmli, 1970). The proteins were electro-transferred onto Hybond-ECL nitrocellulose membrane by using of transfer buffer (39mM glycine, 48mM Tris, 0.0375% (W/V) SDS and 20% methanol). The immunological detection was performed with specific antisera, using an anti-rabbit IgG peroxidase conjugate in combination with an enhanced chemi-luminescence kit (ECL system, Amersham Buchler GmbH, Braunschweig, Germany).

2.2.2 Expression of the recombinant HvSUT2 protein in* E.coli *and purification

2.2.2.1 Preparation of expression constructs

The type IV pQE expression vector (QIAGEN, GmbH) containing a sequence encoding 6xHis at the 5'-terminus of the inserted gene was used to express the HvSUT2 protein. In-frame ligation of the insert was required because of the 5' location of the 6xHis tag. The pQE type IV vector was linearized using the restriction enzyme HindIII. The BamHI/ XbaI

fragment encoding the sucrose transporter HvSUT2 was blunt ended and ligated into the vector in an insert: vector rate of 10: 1. Ligation and transformation of DH5α cells were done according to the manufacturer's potocol (pQE expression kit; QIAGEN GmbH, Hilden, Germany). The construct was sequenced before use.

2.2.2.2 Expression and purification of the 6xHis tagged HvSUT2 protein

A single colony containing the recombinant pQE vector was picked up from a LB plate and grown in 10ml LB medium containing 100µg/ml ampicillin. The culture was grown overnight with shaking. Different from the manufacturer's protocol, a growing temperature of *30°C,* instead of 37°C, was used for this overnight culture. 100 ml of LB medium containing *2% glucose* and 100µg/ml ampicillin were inoculated with 5ml of this overnight cultures and grown at 37°C with shaking until OD_{600} *~0.4* was reached. At this point, IPTG was added to a final concentration of 0.3mM to induce protein expression*.* After induction, the cultures were incubated for additional *3 hours* at 37°C.

The information written in italics describe the changes of the manufacturer's (QIAGEN) protocol which were made to allow efficient protein expression by using of the transgenic bacterial cultures. The new protocol was developed as part of this promotion work. By using this protocol, relative high amounts of the two membrane proteins were expressed. From the transgenic bacterial cultures, membrane proteins were isolated, identified by Western blot analysis and successfully used to produce recombinant polyclonal antibodies.

The 6xHis-tagged HvSUT2 protein was purified following the QIAexpressionist manufacturer's protocol (QIAGEN GmbH, Hilden, Germany).

2.2.3 Immuno-chemical methods

2.2.3.1 Sub-cellular localization of the sucrose binding protein-like protein (SBPL) in **Vicia faba** *by electron microscopy*

Vicia faba cotyledons of the developmental stages V, VI, VII (Borisjuk *et al*., 1995) were fixed overnight in 3% (W/V) paraformaldehyde and 0.5% glutaraldehyde in 100mM potassium phosphate buffer, pH 7.2 at 4°C. The tissues were dehydrated with an ethanol series (10%-20%-30%-50%-70%-90%-95%-100%-100%). The water-free tissues were low-temperature embedded (0°C) in LRwhite resin, sectioned and immuno-gold labeled with an affinity-purified anti-SBPL polyclonal antibody followed by 15nm gold conjugated secondary antibody (goat anti-rabbit IgG [Amersham]). The sections were evaluated in a CEM 902A transmission electron microscope

(Carl Zeiss, Oberkochen, Germany). Micrographs were taken on Kodak SO163 films (Kodak, Ostfildern, Germany).

2.2.3.2 Immuno-staining of protoplasts and tissue sections

Protoplasts

Protoplasts were isolated from a suspension culture of barley according to Tewes *et al*., (1991). After centrifugation at 700 rpm for 10 minutes, protoplasts ($3x10^5$/ml) were resuspended at 4°C in fixation solution (3.7% paraformaldehyde in sodium phosphate buffer, pH 7.2, 0.4M sorbitol) for 1 hour. The fixed protoplasts were washed 3 times in sodium phosphate buffer, pH 7.2 and spread on polylysin coated slides. The slides were washed over night at 4°C in sodium phosphate buffer within a clamping apparatus used to carry out all staining procedures.

Tissue sections

Barley caryopses (8 DAF, *i.e.* Days After Flowering) were fixed 4 hours at room temperature in 4% paraformaldehyde in sodium phosphate buffer, pH 7.2, dehydrated by an ethanol series (see SBPL-localization in *V. faba* cotyledons), embedded in BMM and sectioned. Sections were fixed on polylysin coated slides. BMM was removed by washing the slides 20min in acetone. Before immuno-staining, the sections were re-hydrated.

Immuno-staining procedures

Slides were incubated for 1 hour with 3% BSA in sodium phosphate buffer at room temperature to prevent non-specific immuno-staining. After incubation with the primary antibody in sodium phosphate buffer for 1 hour at 37°C, slides were washed three times in sodium phosphate buffer and incubated with the second antibody (FITC-rabbit IgG) for 1 hour at 37°C. The slides were washed three times in sodium phosphate buffer and distilled water, embedded in Vectashield mounting medium and analyzed with the fluorescence microscope Optiphot-2. Micrographs were taken on Kodak Elite 400 films (Kodak, England).

2.2.3.3 Immuno-fluorescence staining of biotinylated cell surface proteins

Freshly isolated barley protoplasts were washed twice with 25mM HEPES buffer containing 0.4M sorbitol and 2.5% sucrose (HSS, pH 6.0) and slowly cooled to 4°C. Prior to labeling, protoplasts were washed once with ice-cold HEPES buffer, pH 8.0. Sulfosuccinimidyl 2-

biotinamidoethyl-1,3-dithiopropionate (NHS-SS-Biotin, Pierce) was dissolved in ice-cold HEPES buffer, pH 8.0 and immediately added to the protoplasts in a ratio of 2:1 (v/v) to give a final concentration of 1mM. Biotinylation of the protoplast suspension was preformed on ice for 25 minutes with gentle agitation. Excess biotin was removed by washing with ice-cold HEPES buffer, pH 6.0. Biotinylated cells were washed with 0.1M Pipes buffer, pH 6.9 for 5 minutes and fixed with 4% paraformaldehyde (w/v) in 0.1M Pipes for 1 hour. After washing, cells were permeabilized with 0.5% TritonX-100 for 15 minutes and were then spread on polylysin-coated slides. Slides were washed in PBS at 4°C over night within a clamping apparatus. All staining procedures were carried out in that equipment. 3% BSA was used for blocking for 1 hour at room temperature. Afterwards, slides were incubated with mouse anti-biotin IgG diluted 1:100 in PBS containing 1% BSA. Labeling was performed at room temperature for 40 minutes by incubation with FITC-conjugated goat anti-mouse IgG secondary antiserum diluted 1:80 in PBS. Control experiments were carried out as described above, using non-biotinylated protoplasts.

2.2.4 DNA isolation and Southern blot analysis

Genomic DNA was isolated from young leaves. Fresh tissue was frozen and ground in 3 vol. 500mM NaCl, 50mM Tris-HCl (pH 8.0), 50mM EDTA, 1% (v/v) ß-mercaptoethanol, followed by addition of polyvinylpyrrolidone (PVP, 25kDa) to 6% and SDS to 2% final concentration before incubation of the mixture for 10 minutes at 65°C. After addition of 5M potassium acetate to a final concentration of 0.5M, the DNA was precipitated with 0.6 vol. isopropanol. The pellet was dissolved and purified by phenol-chloroform-isoamyl alcohol extraction. The DNA concentration was measured by photometer, and DNA quality was checked on a 0.7% agarose gel. 10µg of DNA were digested with restriction enzymes, separated on a 0.8% agarose gel, blotted in 0.4M NaOH on Hybond N^+ nylon membrane (Amersham, UK), UV-crosslinked and hybridized with ^{32}P dCTP-labeled probes. A 984 bp DNA fragment (nucleotides 1788 to 2722), specific for HvSTP1 and a 630 bp cDNA fragment (nucleotides 11 to 641) specific for HvSTP2 were used for labeling. The membranes were washed three times at 62°C under low-stringency conditions (1x SSC / 0.1% SDS / 0.1% sodium pyrophosphate) and exposed to Kodak X-Omat AR films.

2.2.5 RNA isolation and Northern blot analysis

Total RNA was isolated from anthers and the female part of the flower immediately before anthesis, as well as from developing caryopses, growing and mature leaves and roots. Tissues were sampled and stored at –80°C. To isolate the RNA, 0.2g frozen material was ground in 0.7 ml extraction buffer (1M Tris-HCl, pH 9.0; 10mM EDTA, 1% SDS) and 0.7ml of 10mM Tris-saturated phenol / chloroform / isoamylalcohol (25:24:1, v/v/v), pH 8.0. After centrifugation the aqueous phase was extracted with phenol / chloroform and precipitated with 1/10 vol. 3M NaAc (pH 5.2) and 2.5 vol. ethanol. Nucleic acids were collected by centrifugation and dissolved in 200µl water. The RNA was precipitated by addition of 1 vol. 200µl of 4M LiCl at 0°C overnight. RNA was collected by centrifugation, carefully washed with 2M LiCl and 70% ethanol and stored at –80°C. Immediately before Northern blot analysis, the RNA pellet was dried and dissolved in 25µl RNase-free water. The concentration of total RNA was measured by photometer. From each probe, 15µg of RNA were denatured, loaded on a RNA gel and blotted on Hybond N^+ nylon membrane (Amersham, UK) following Amersham's protocol for Northern blot analysis. Membranes were sequentially hybridized according to Church and Gilbert (1984) using the probes specific for HvSTP1 and HvSTP2 (see "Southern blot analysis" for description). Additionally, the membrane was hybridized to a 26S-rDNA fragment to estimate the amount of total RNA bound on each lane. The probes were labeled with ^{32}PdCTP by using the Megaprime labeling Kit (Amersham, UK). Pre-hybridization as well as hybridization was done at 65°C in 0.5M Na-phosphate, pH 7.2, containing 7% SDS, 1% bovine serum albumin, 2mM EDTA in the presence of 100µg/ml of denatured salmon sperm DNA for competition. After hybridization, blots were washed three times at 65°C in 40mM Na-phosphate buffer, pH 7.2, containing 1% SDS and 2mM EDTA. The hybridization signals were quantified by using a FUJI BSA 2000 Bio-Imaging-Analyzer (Raytest, Straubenhardt, Germany).

2.2.6 Cloning of barley sugar transporter genes

2.2.6.1 Reverse transcriptase polymerase chain reaction (RT-PCR) of total RNA from barley caryopses

First strand cDNA was synthesized from 2µg total RNA isolated from barley caryopses (8 days after flowering) by using of 10ng oligo $(dT)_{16}$ with 200 units of Superscript reverse transcriptase (Gibco BRL). The single strand cDNA was used as template for PCR. For amplification of specific products, primers described in 2.1.8.1 were used. For each reaction, 2µl of denatured cDNA mixture was added to 5µl 10xTaq buffer, along with magnesium

chloride to a final concentration of 1.5mM, 1.0mM dNTPs and 50 pmol of each of the two primers. Sterile water was added to 50 µl together with 1 unit of Taq polymerase enzyme (Gibco BRL). The PCR reaction was carried out in a thermal cycler (Eppendorf, Mastercycler 5330) for 40 cycles with a primer annealing temperature of 50°C and an extension temperature of 72°C. The RT-PCR cDNA product was cloned in pUC18 and sequenced.

2.2.6.2 Isolation of cDNAs by cDNA library screening

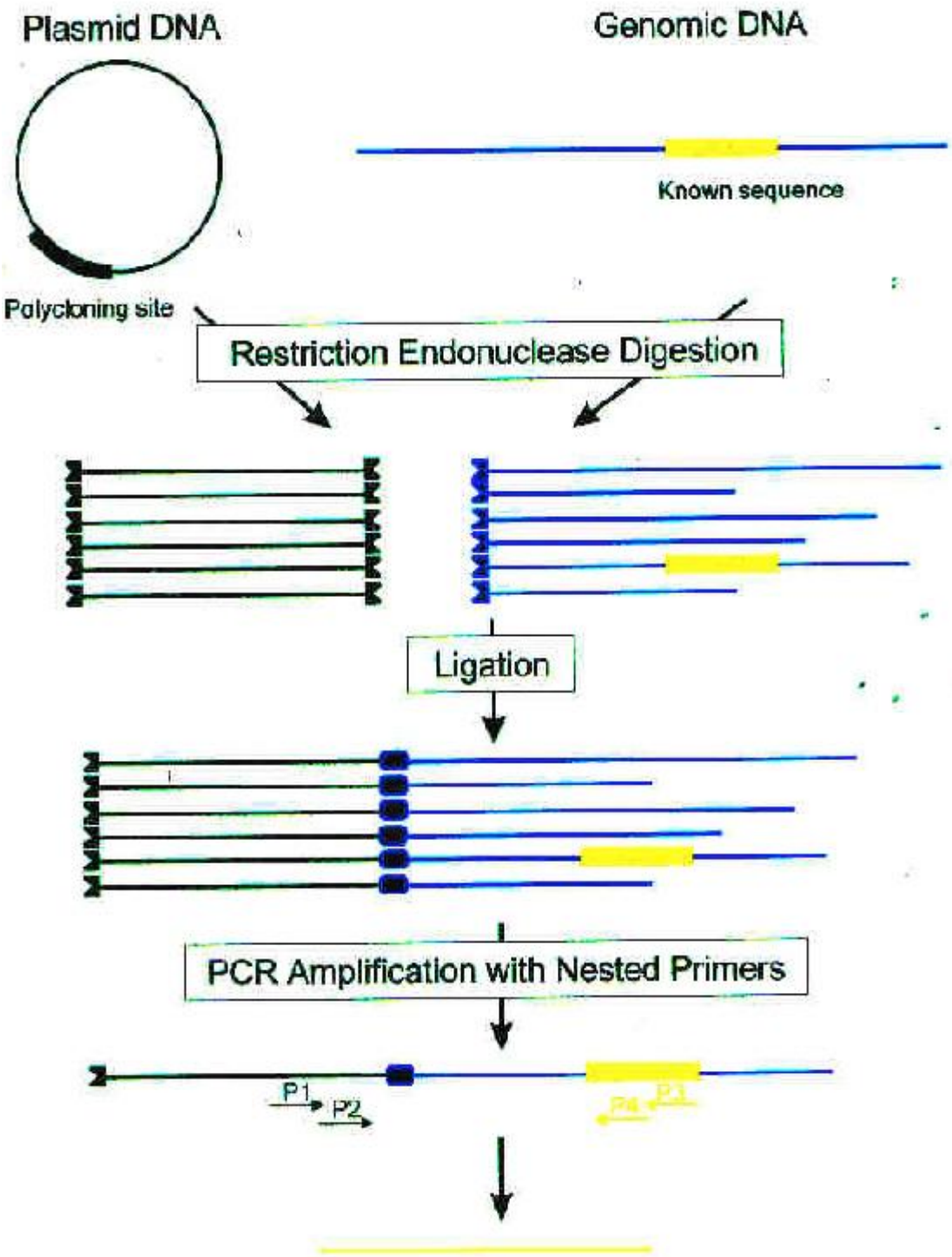
An aliquot of a barley caryopses cDNA library (about 100,000 plaques) was plated out on 12 petri dishes 8cm in diameter. The plaque-specific DNA was transferred to Hybond C nitrocellulose membranes (Amersham Life Sciences, UK). The filters were pre-hybridized in 6xSSC containing 5xDenhardt's solution, 0.4% SDS, and 100µg/ml salmon sperm DNA for 4 hour at 65°C. The two DNA fragments specific for HvSTP1 and HvSTP2 used as probes were labeled with α-[^{32}P]-dCTP by using the Mega prime labeling kit (Amersham, UK). The filters were hybridized at 65°C for 16 hours under the conditions described above for pre-hybridization. Membranes were washed progressively down to 1% SSC, 0.1% SDS. All positive phages were selected and purified following Amersham's protocol for cDNA library screening. After *in vivo* excision, the cDNA inserts of positive clones were sequenced. However, no sugar transporter sequence was found. Alternative of *in vivo* excision, the cDNA inserts of positive phages were amplified by PCR and sub-cloned into plasmid pUC18. All positive clones were sequenced. The HvSTP2 full-length gene was found. To get the HvSTP1 cDNA sequence, the positive phages were sequenced directly.

2.2.6.3 Cloning of the barley sucrose transporter cDNAs into the yeast expression vector NEV

The full-length cDNA of HvSUT1 (SmaI/Not1 fragment) and HvSUT2 (BamHI/XbaI fragment) were blunt-end ligated into the unique EcoRI restriction site (blunt ended) of NEV-E. The resulting plasmids NEV-s-HvSUT1 and NEV-s-HvSUT2 carry the inserts in sense orientation; NEV-a-HvSUT1 and NEV-a-HvSUT2 carry the inserts in antisense orientation. The plasmids were transformed into the *S. cerevisiae* strain RS453 (Mata, ade2-1, trp1-1, can1-100, leu2-3, 112, his3-1, ura3-52). Transformed yeast cells were designated as HVY-S1, HVY-S2 and HVY-A1, HVY-A2, for sense and antisense orientation of the fragments, respectively.

2.2.7 Structure of the genomic HvSUT1 gene and promoter study

2.2.7.1 Genome walking



An efficient modification of the genome walking method (shown above) was created by using the whole pUC18 plasmid as adapter. Highly purified genomic DNA was isolated

from young barley leaves and than digested with various restriction enzymes: 1. TagI, 2. EcoRI, 3. HindIII, 4. KpnI, 5. PstI, 6. SalI, 7. SacI, 8. SmaI, 9. SphI, 10. XbaI, 11. BamHI. The same enzymes were used to digest the cloning vector. The digested genomic DNA and the adapter (pUC18) were ligated. The resulting fragments were used as templates in a nested PCR reactions by using two vector specific primers (P1, P2) and two primers specific for the known HvSUT1 gene sequence (P3, P4). The amplified PCR products were sequenced.

2.2.7.2 Screening of the barley BAC library

The BAC (Bacterial Artificial Chromosome) system is based on *E. coli* and its single-copy plasmid F factor. It is capable for maintaining genomic DNA fragments of > 300 kilobase pairs. For cloning, genomic barley DNA was isolated and partial digested with the restriction enzyme NotI.. DNA fragments ranging from 50 to 200 kilobase pairs were ligated into the BAC plasmid pBAC108L in a molar ratio of 10:1. The BAC clones were spotted onto membranes. The BAC library represents 6 copies of the barley genome (17 filters). It was kindly provided by Dr. Dagmar Schmidt (Ag Gen- und Genomkartierung, IPK).

Barley BAC library filters were stripped in 0.5% SDS at 95°C for 20 minutes and pre-hybridized in buffer (0.5M Na_2HPO_4, 0.5M NaH_2PO_4, pH 7.2, 7% SDS and 1% BSA) containing 100µg/ml salmon sperm DNA, for 4 hours at 65°C. 100ng ^{32}PdCTP-labeled probes were used for hybridization at 65°C overnight. Filters were washed 3 times in 2xSSC (0.3M NaCl and 0.5M Natriumcitrat), 2 times in 1xSSC and 1 time in 0.5xSSC, followed by exposing to Kodak X-Omat AR films. Positive BAC colonies addresses were identified and picked up. The large BAC DNA inserts were isolated and directly sequenced using the primers described in 2.1.8.3.

3. *Results*

3.1 Hexose transporters

3.1.1 The hexose transporter VfSTP1

The full length cDNA of the hexose transporter VfSTP1 was cloned from a *Vicia faba* seed specific cDNA library. VfSTP1 mRNA expression was analyzed by Northern blotting and *in situ* hybridization (Weber *et al.*, 1997). Functional analysis of the VfSTP1 protein was done in yeast cells in the group of Prof. Norbert Sauer (University Erlangen). The hexose uptake behavior of the transgenic yeast cells indicate that VfSTP1 encodes a functional hexose transporter.

3.1.1.1 Generation of the VfSTP1 protein-specific antibody

My work was focussed on the study of the temporal and spatial transport protein expression patterns by using Western blot analysis and immunocytochemistry. As a prerequisite for such studies, production of protein specific antibody was required. To generate the VfSTP1-specific antibody, either the recombinant protein used for antigen had to be synthesized or the antibody had to be custom-made against synthetic peptides representing hydrophilic domains of the VfSTP1 protein.

About 0.5-1.0 mg VfSTP1 protein are required for the production of the specific antibody. To prepare the corresponding antigen amounts, overexpression of the VfSTP1 protein in *E.coli* was tried. However, no *E.coli* strain, transformed by the expression vector containing the VfSTP1 gene in sense orientation, could survive. After that, it was tried to express the VfSTP1 protein in a yeast expression system. Unfortunately, the expression level of this protein in the yeast cells was too low to generate the required antigen amounts. Therefore, we decided to produce antibodies directed against hydrophilic peptide domains of the VfSTP1 protein.

Based on computer protein sequence analysis as well as comparison with other known hexose transporter protein sequences, 3 peptides were selected for antibody production. One, *PE0279*, is within the big middle loop and the others, *PE0280* and *PE0281*, are localized near the C-terminal region of the VfSTP1 protein (see Figure 3.1.1.1).

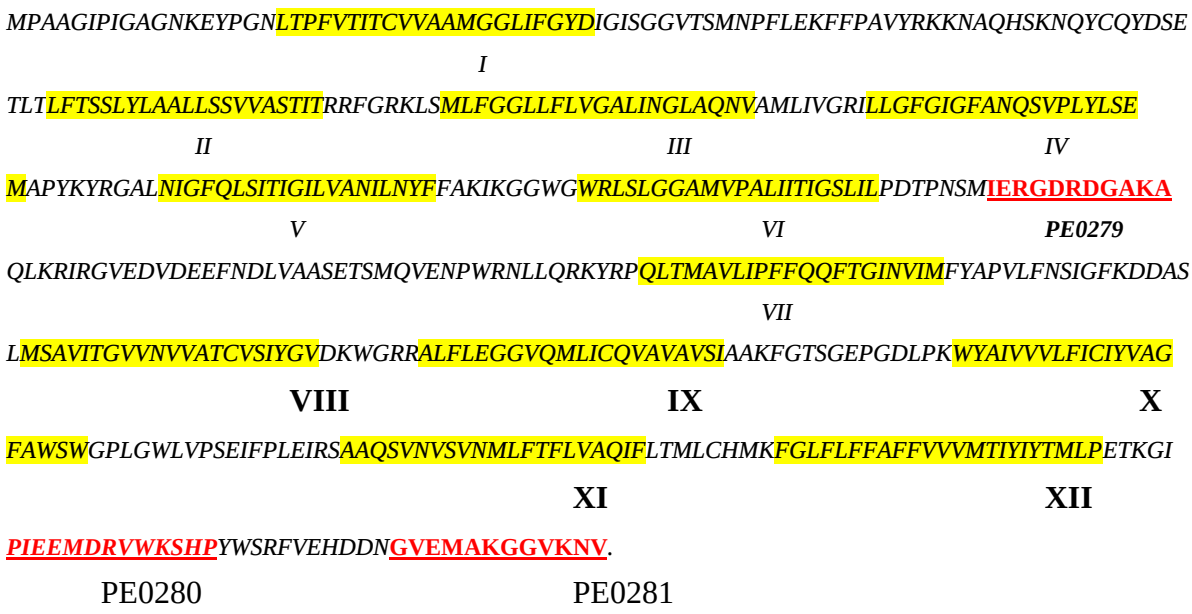


Fig. 3.1.1.1 Putative protein sequence of VfSTP1. The 12 transmembrane domains are marked in yellow. The peptide sequences selected for antibody production are shown in red letters.

Anti-peptide antisera against these three peptides were generated by the pepScan *GmbH* company in three steps: 1. peptide synthesis; 2. conjugation of each peptide with a carrier protein; 3. immunization of rabbits.

From 6 immunized rabbits, 6 anti-peptide antisera and pre-immun sera were provided. To check the specificity of these antisera, ELISA assay have been carried out by pepScan *GmbH* company. The ELISA reports show that all 6 anti-peptide antisera from rabbits K32-K37 recognize the corresponding antigen peptides *(data not shown).*

3.1.1.2 Specificity of the peptide antisera for the whole VfSTP1 transport protein

- ***Cloning VfSPT1 cDNA in the yeast expression vector pYES2***

To study the specificity of the 6 peptide antisera, the VfSTP1 gene was cloned into the yeast expression vector pYES2 (kindly provided by Prof. G. Kunze, IPK-Gatersleben) in both sense and anti-sense orientation and expressed in Baker's yeast cells.

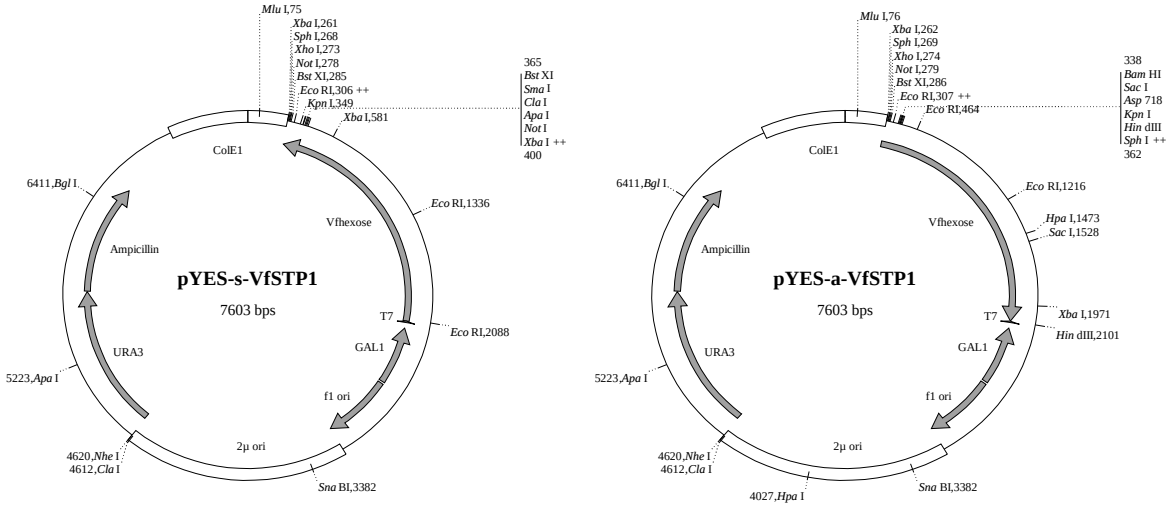


Fig. 3.1.1.2 Cloning of VfSPT1 cDNA into the yeast expression vector pYES2 in sense (pYES-s-VfSTP1) and anti-sense (pYES-a-VfSTP1) orientation.

- ***Western blot analysis of proteins extracted from transgenic yeast cells***

The transformed yeast cells were grown in liquid SD medium containing 2% glucose (I) or inoculated in SD medium containing 2% galactose (II). Total protein was isolated from yeast cells transformed with the vector pYES2 including the VfSTP1 gene in both sense and anti-sense orientation. A certain amount of the total yeast protein was loaded onto a 12% SDS-PAGE gel. In Western blot analysis, only the antiserum directed against peptide antigen PE0280 produced by rabbit K34 was able to recognize a polypeptide of 55 kDa in total protein extracts from the yeast strain transformed with the VfSTP1 gene in sense orientation. No signal was detected in the total protein extracts from the yeast strain transformed with the VfSTP1 gene in antisense orientation (Figure 3.1.1.3). The antiserum against PE0280 from rabbit K34, called as HEX-34, was affinity-purified. The purified IgG fraction was used for further experiments.

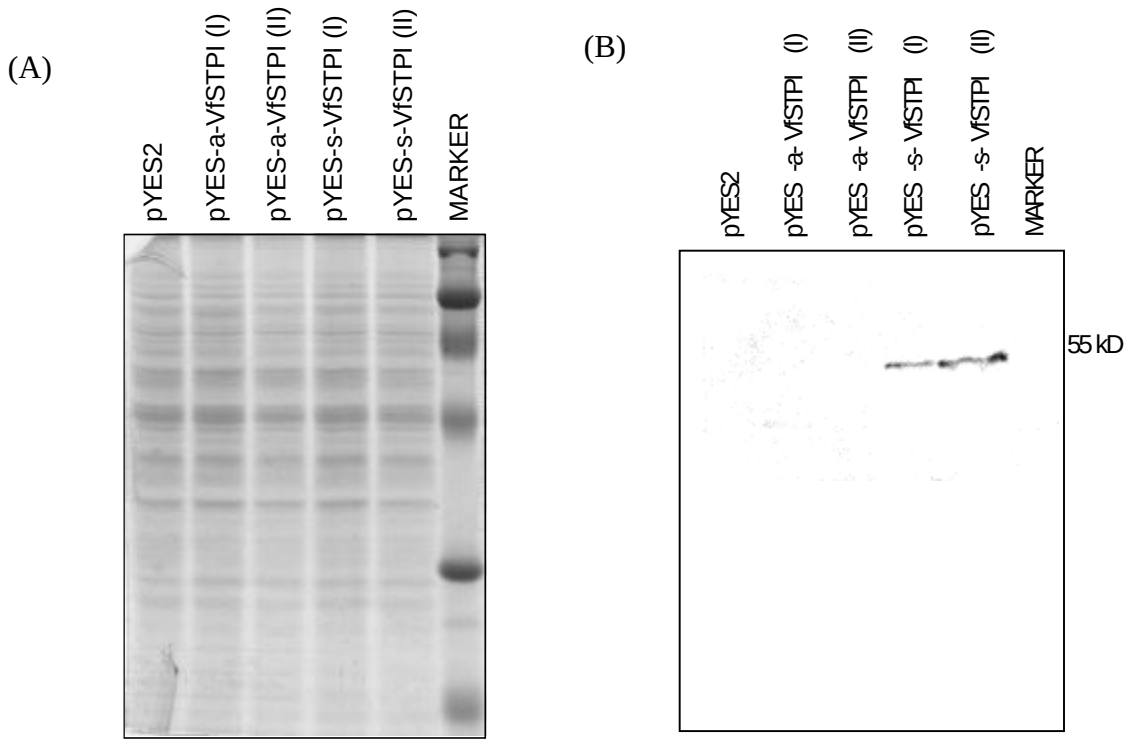


Fig. 3.1.1.3 Cross reactivity of peptide antiserum HEX-34 with the VfSTP1 protein produced in transgenic yeast. (A) Coomassie stained gel showing the pattern of the total protein isolated from yeast cells. (B) Western blot analysis of the VfSTP1 protein expressed in yeast cells transformed by pYES-a-VfSTP1 or pYES-s-VfSTP1 using HEX-34 as primary antibody.

- ***Immuno-cytochemical detection of the VfSTP1 protein expressed in transgenic yeast cells***

  In order to check the specifity of HEX-34 antibody for subcellular localization of the VfSTP1 protein, immuno-cytochemical experiments were preferred. Transformed yeast cells expressing the VfSTP1 protein were incubated in SD medium containing 2% galactose for one day. Then, yeast cells were collected and prefixed before embedding. Immuno-gold labeling was carried out by using of sections of resin-embedded cell material. Gold particles were only found in yeast cells transformed with pYES-S-VfSTP1 but not in yeast cells transformed with the gene in antisense orientation (Figure 3.1.1.4).

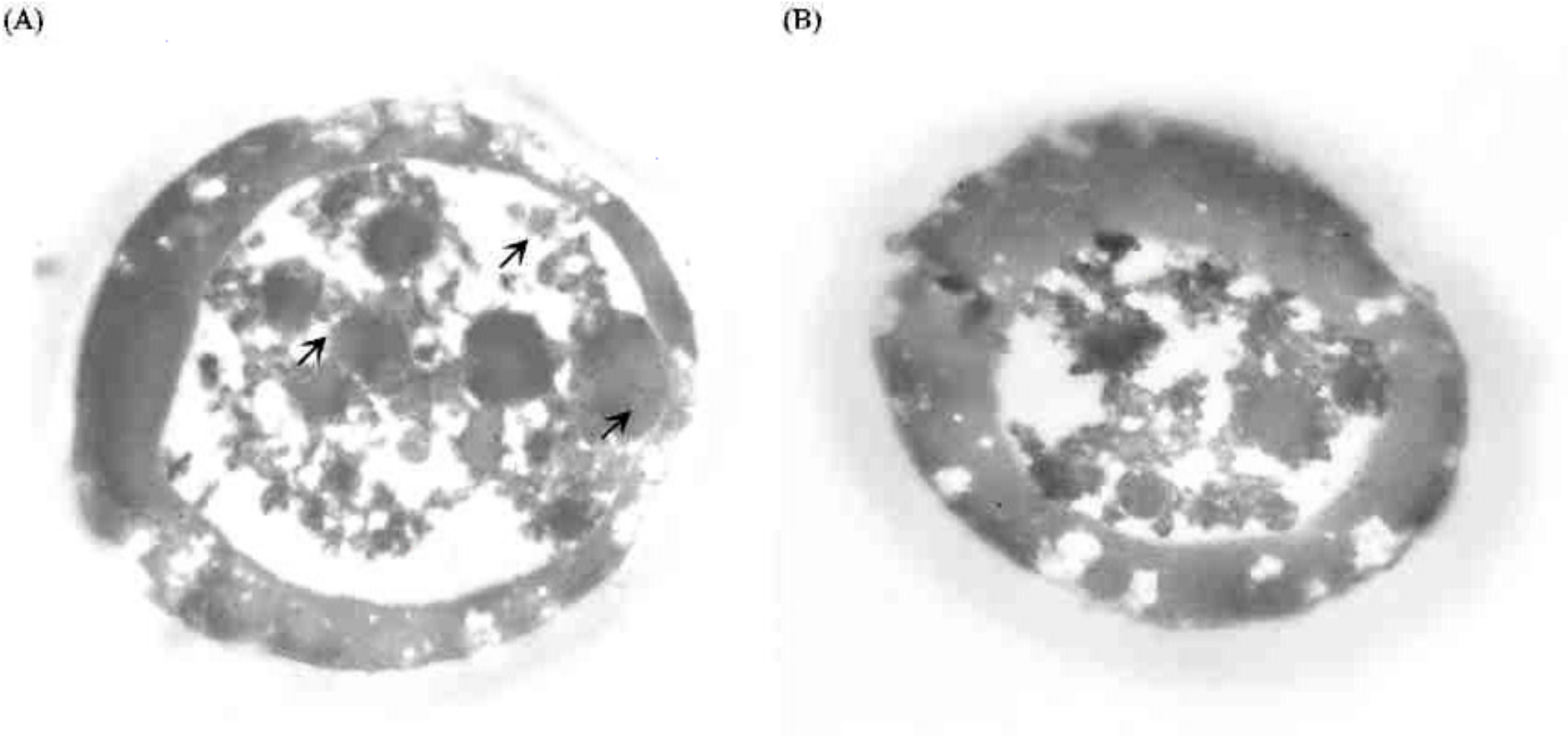


Fig 3.1.1.4. Immunogold detection of the VfSTP1 protein in transgenic yeast cells. (A) Yeast cells transformed with plasmid pYES-S-VfSTP1. (B) Yeast cells transformed with plasmid pYES-A-VfSTP1. HEX-34 was used as primary antibody. The arrows point to gold grains.

3.1.1.3 Western blot analysis of the VfSTP1 protein in Vicia faba *plants*

- ***VfSTP1 protein expression pattern during seed development of* Vicia faba**

Seed development of *Vicia faba* is divided into seven stages based on morphological and histochemical characteristics (Borisjuk *et al*., 1995). The expression of the hexose transporter protein in different developmental stages (stage V, VI and VII) of *Vicia faba* cotyledons were examined by Western blot analysis.

(A) (B)

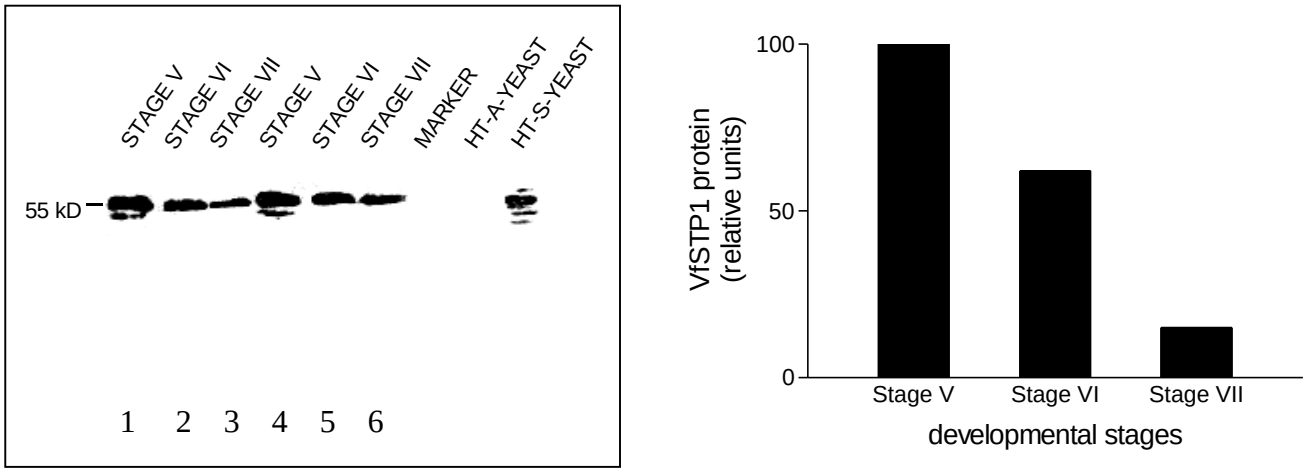


Fig. 3.1.1.5 Immuno-chemical detection of the VfSTP1 protein expression level in developing seeds of* Vicia faba*. (A) Western blot analysis of cotyledons during different developmental stages. Total protein was extracted from* Vicia faba *cotyledons of the developmental stages V, VI and VII. The identical amounts of protein (lines 4 -6) as well as the total protein extracted from the same seed fresh weight in each developmental stage (lines 1 -3) were loaded onto a SDS-PAGE gel. Immuno-detection was performed with the affinity-purified HEX-34 anti-peptide antibody.

(B) Quantification of the Western blot result shown in (A), based on lines 1-3. The Western blot data were analyzed by the software Tina ver.2.08 beta (Raytest Sprockhövek, Germany). The bars show relative units of VfSTP1 protein expression.

The profile of VfSTP1 protein expression in developmental stages V, VI and VII is comparable to the VfSTP1 mRNA profile shown before for the developmental stages IV-VII. (Weber *et al*., 1997).

- ***Tissue-specific expression of the VfSTP1 protein***

To analyze the tissue specific expression level of the VfSTP1 protein in *Vicia faba* plants, total protein was extracted from different tissues as stem, leaf, root, pod, seed coat and cotyledons (developmental stage V), loaded onto SDS-PAGE gel and examined by Western blot analysis. Fig.3.1.1.6 shows the tissue specific expression of the VfSTP1 protein.

(A) (B)

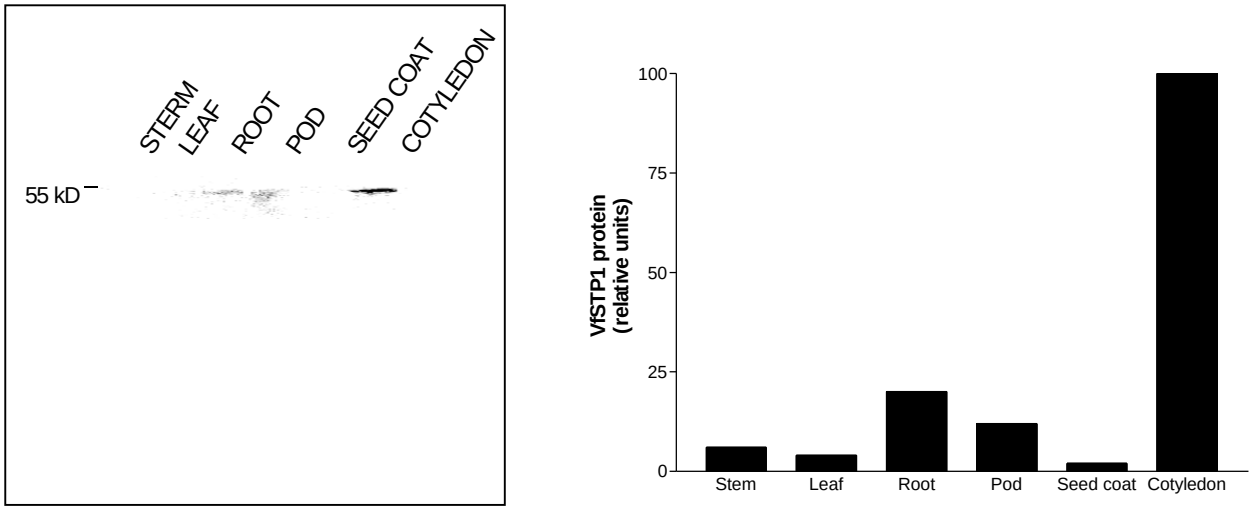


Fig. 3.1.1.6 Expression of the VfSTP1 protein in different plant tissues. (A) Western blot analysis of different tissues. Total protein was extracted from different tissues of Vicia faba *plants. 50µg total protein extracted from each tissue were loaded onto a SDS-PAGE gel. Immuno-detection was performed with the affinity purified HEX-34 anti-peptide antibody. (B) Quantification of the Western blot analysis result shown in (A). The bars show relative units of the expressed protein using the highest expression level for 100%.*

The highest expression of VfSTP1 was found in cotyledons. VfSTP1 expression was also found in roots. Only a very low VfSTP1 protein level has been detected in green tissues as leaves and in the pod.

- ***Levels of VfSTP1 protein expressed in the membrane and the cytoplasm fraction of cotyledonary cell***

In order to study the intercellular distribution of VfSTP1 protein, the membrane as well as the cytoplasm fractions were isolated from *Vicia faba* cotyledonary cells and separated on a SDS-PAGE gel.

(A) (B)

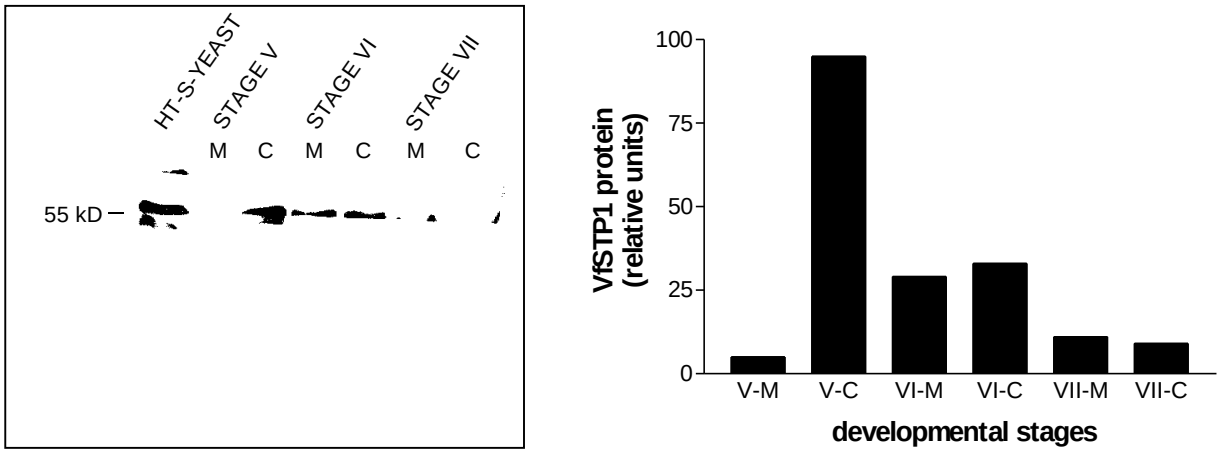


Fig. 3.1.1.7 Sub-cellular distribution of protein VfSPT1 in developing cotyledons of Vicia faba*. The VfSTP1 protein expression was detected by Western blotting (A). The Western blot data were analyzed by the software Tina ver.2.08 beta (Raytest Spockhövel, Germany). Quantification of the Western blot result is shown in (B). Membrane (M) and cytoplasm (C) fractions were extracted from* Vicia faba *cotyledons in developmental stage V, VI and VII (i.e., V-M: membrane fractions extacted from cotyledons of developmental stage V). The bars show relative units of protein expression.*

In developmental stage V (early development), theVfSPT1 protein was found only in the cytoplasm fraction, whereas in the later developmental stages VI and VII, VfSTP1 protein was found not only in the cytoplasm but also in the membrane fraction. As shown before (Fig.3.1.1.5), the VfSTP1 protein amount drops down during seed development.

3.1.2 Putative hexose transporter cDNAs from H.vulgare seeds

In order to identify seed specific hexose transporter cDNAs from barley, a partial EST library consisting of sequences of about 7,000 cDNA clones from a caryopses specific library was checked. Using the BLASTX analysis computer program, two EST clones, HY05O16T3 and HY08B01VR, were identified as hexose transporter cDNAs by their homology to known plant hexose transporters at the amino acid level.

3.1.2.1 Characterization of two different hexose transporter clones by cDNA sequence analysis

The two clones preliminary characterized by the ESTs HY05O16 and HY08B01, were subjected to further DNA sequences analysis. The HY05O16 sequence was homologous to the 5' sequence of known hexose transporters of other higher plant species. To the contrary, the HY08B01 sequence was homologous to 3' end sequences. Neither of these two clones contains the full-length gene. To isolate full-length genes, a barley caryopses phage cDNA library was screened at first with a 600 bp BamHI-/ Asp718 fragment isolated from HY05O16. After stripping of the filters, a 900 bp HindIII-/ PstI fragment isolated from HY08B01 was used in a second round of hybridization. Two full-length putative hexose transporter cDNA sequences were identified. These cDNA sequences were designated as HvSTP1 (HY05O16) and HvSTP2 (HY08B01).

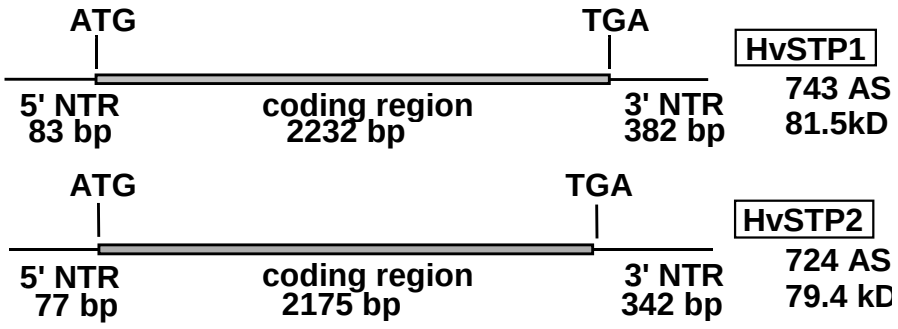


Fig. 3.1.2.1 cDNA organization of genes HvSTP1 and HvSTP2.

The putative amino acid sequences of both, HvSTP1 and HvSTP2 were aligned with the deduced protein sequence of 14 known hexose transporters (Figure 3.1.2.2).

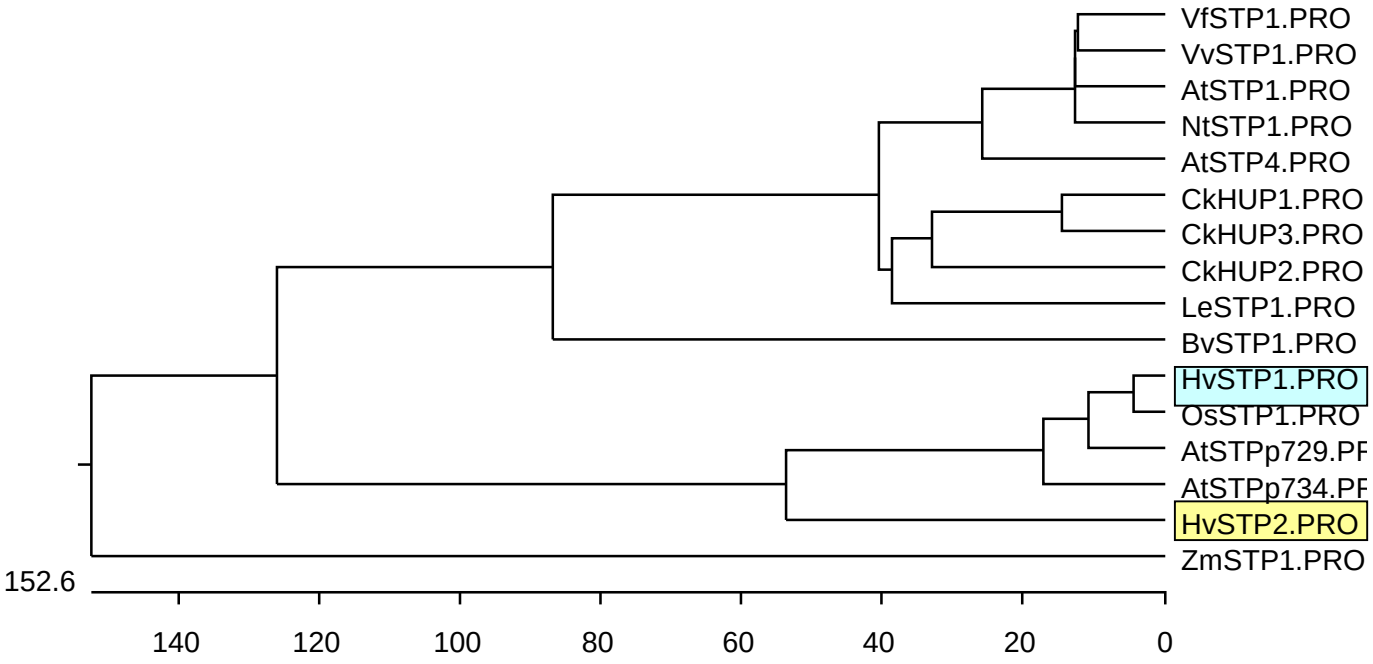


Fig. 3.1.2.2 Alignment of monocot and dicot hexose transporter amino acid sequences.
The order of branching within the dendrogram indicates statistical similarities between hexose transporters from different plant species. The amino acid sequences of HvSTP1 and HvSTP2 were aligned with the corresponding hexose transporters as follows: A. thaliana (AtSTP1, No. 134976; AtSTP4, No.99758; AtSTPp729, No. 7488211; ATSTPp734, No. 4836905); B. vulgaris BvSTP1, No. 7484647); C. kessleri (CkHUP1, No. 2851499; CkHUP2, No. 3024001; CkHUP3, No. 3024002); L. esculentum (LeSTP1, No. 7446737); N. tabacum (NtSTP1, No. 100347); O. sativa (OsSTP1, No. 11386295); V. faba (VfSTP1, No. 1935020); V. vinifera (VvSTP1, No. 4138724); Z. mays (ZmSTP1, No. 8347248).

HvSTP1 is highly homologous (84% identity) to OsSTP1. HvSTP1 and HvSTP2 show 63% similarity. Both, HvSTP1 and HvSTP2 cluster with “big” transporters more than 700 AS in length.

3.1.2.2 Genomic organization of genes HvSTP1 and HvSTP2

The 600 bp BamHI/Asp718 fragment isolated from HY05O16 containing the 5' end of gene HvSTP1 and the 900 bp HindIII-/PstI fragment isolated from HY08B01 containing the 3' end of the gene HvSTP2 were used for Northern, Southern and *in situ* hybridization analyses.

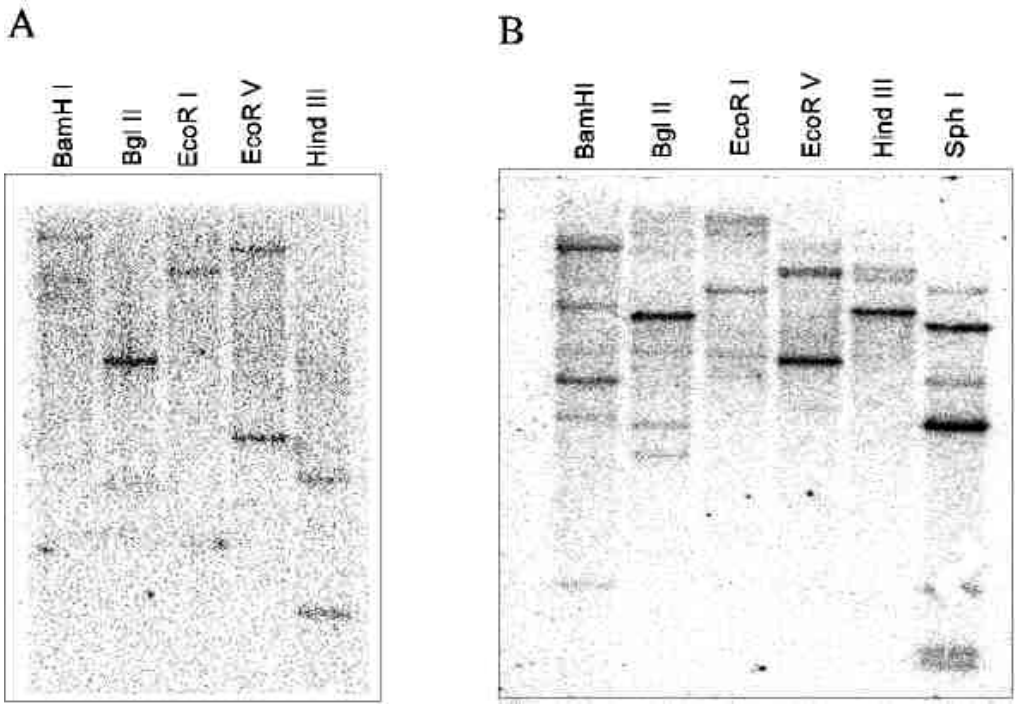


Fig. 3.1.2.3 Genomic organization of genes HvSTP1 and HvSTP2.

Genomic DNA was isolated from barley leaves, and 10µg were digested by the restriction enzymes BamHI, BglII, EcoRI, EcoRV and HindIII. The fragments were separated by agarose gel electrophoresis, blotted and hybridized with HvSTP1 (A) and HvSTP2 (B) specific probes labelled by ^{32}P.

Whereas only one copy of HvSTP2 was identified within the barley genome (Figure 3.1.2.3B), HvSTP1 seems to be member of a small gene family. In every lane of the HvSTP1-specific blot (Figure 3.1.2.3A), one or two bands of higher intensity were found representing sequences highly homologous to the HvSTP1 fragment used for probe. Bands of lower signal intensity hint to other members of the HvSTP1 gene family showing a lower degree of homology.

3.1.2.3 Expression analyses of hexose transporter mRNA in developing caryopses

mRNA expression of the genes HvSTP1 and HvSTP2 were studied by Northern blot analysis using of the same fragments mentioned in 3.1.2.2.

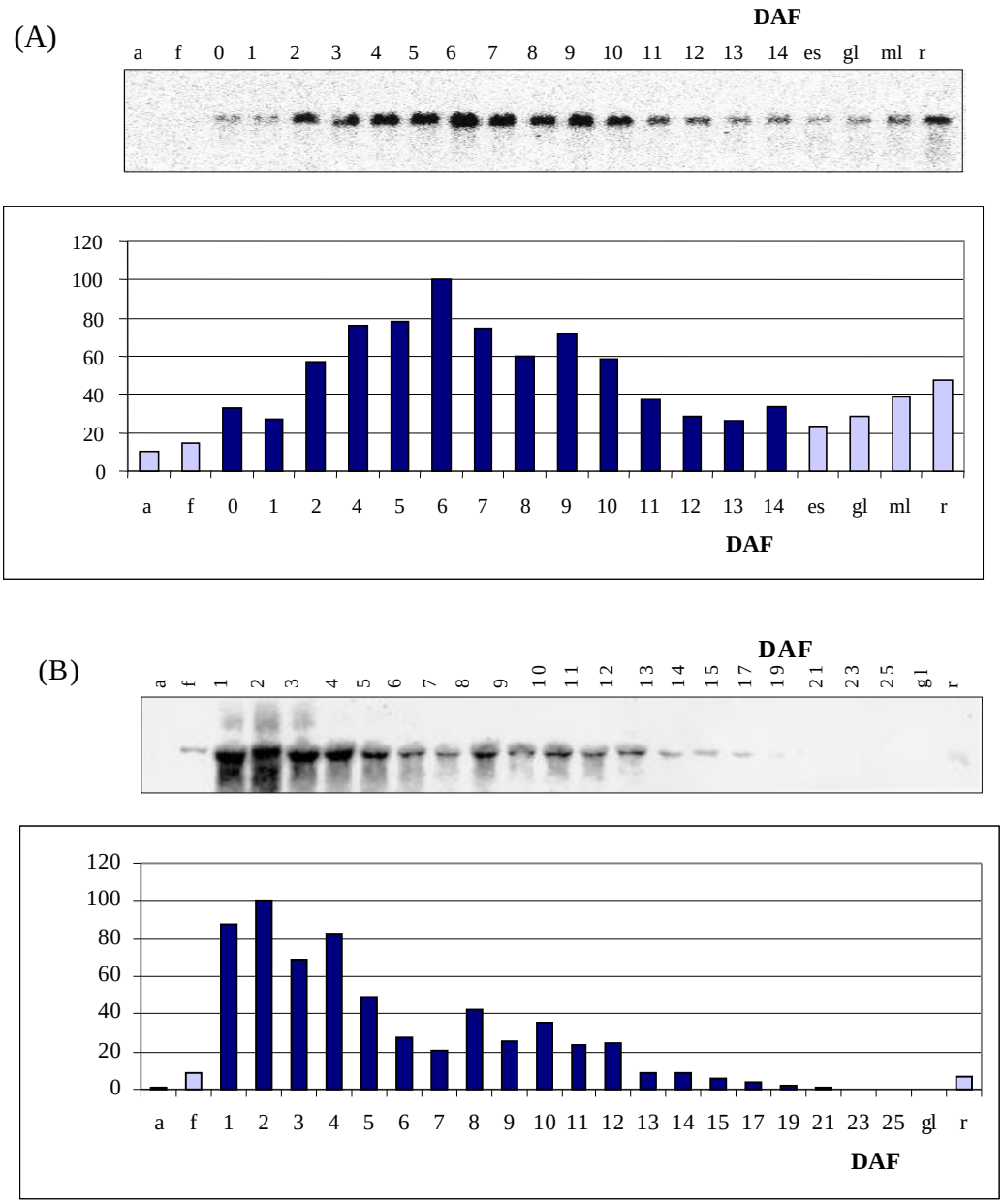


Fig. 3.1.2.4 Analysis of HvSTP1 (A) and HvSTP2 (B) transcripts in developing caryopses (0-25 DAF, 0-14 DAF, respectively). a, anthers; f, female part of the flower before pollination; gl, growing leaf; r, root; ml, mature leaf; es, etiolated seedling. For quantification of the Northern analysis data, the filters were exposed to a Fuji Imaging plate type BASIII, and analyzed using a Fuji Bio Imaging Analyzer and BAS 2000 software package (Fuji Photo Film C.Tokyo, Japan), and by Tina ver. 2.08 beta software (Raytest Sprockhövel, Germany). The bars show relative units of mRNA expression. DAF, day after flowering.

A single band of 2.6 and 2.7 kb was detected using the HvSTP1 and HvSTP2 specific probes, respectively. The level of HvSUT1 mRNA was highest around 6 days after flowering, but the transcript is also present in comparable amounts in later developmental stages as well as in other plant organs analyzed. HvSTP2 mRNA levels were very high in young caryopses (1-4 days after flowering) and much lower in mature tissues.

The following table shows the appearance of the HvSTP1 and HvSTP2 transporter mRNAs in developing tissues of the young caryopses by in situ hybridization.

HvSTP1

Days after flowering	2	3	4	5	6	7	8
Pericarp	+	+	+	++	+++	+++	+++
Integument	-	+++	+++	+++	+++	+++	+++
Nucellar projection	-	-	++	+++	+++	+++	+++
Endospermal Transfer cell	-	-	++	+++	+++	+++	+++
Endosperm	-	+++	+++	+++	+++	+++	+++

HvSTP2

Days after flowering	2	3	4	5	6	7	8
Pericarp	+++	+	+	+	+	+	+
Integument	-	++	+++	-	-	-	-
Nucellar projection	-	-	++	+++	+++	+++	+++
Endospermal Transfer cell	-	-	++	+++	+++	+++	+++
Endosperm	-	-	-	-	-	-	+++

3.2 Sucrose transporters

3.2.1 The sucrose transporter VfSUT1

A sucrose transporter full-length cDNA clone (VfSUT1) was isolated from a *V. faba* seed-specific cDNA library. Gene expression was analyzed by Northern blot and *in situ* hybridization. The transport function of the VfSUT1 protein was studied in yeast cells in cooperation with Prof. Norbert Sauer, University Erlangen. The sucrose uptake behavior of the transgenic yeast cells indicated that the VfSUT1 clone is encoding a functional sucrose transporter (Weber *et al.*, 1997).

3.2.1.1 Generation of the VfSUT1 protein-specific antibody

To compare the mRNA and protein expression level of VfSUT1, and further on, to localize the VfSUT1 protein by immuno-cytochemical methods, a VfSUT1 specific antibody was required.

To get enough antigen, it was tried at first to over-expression of the VfSUT1 protein in E.coli. However, no colony transformed by the VfSUT1 gene in sense orientation survived. Then, a yeast system was used to express the VfSUT1 protein. However, the VfSUT1 protein expression level was too low to generate enough amount of the antigen. Therefore, we decided to produce antibodies directed against peptide domains of the VfSUT1 protein. Based on computer protein sequence analysis as well as by comparison with other known sucrose transporter protein sequences, 2 peptides, *PE0326* and *PE0327*, were selected for antibody production.

MEPLSSTKQINNNNNLAKPSSLHVETQPLEPSPLRKIMVVASIAAGVQFGWALQLSLLTPYVQLLGIHHTWAAYIWLCGP

I

ISGMLVQPIVGYHSDRCTSRFGRRRPFIAAGSIAVAIAVFLIGYAADLGHSFGDSLDQKVRPRAIGIFVVGFWILDVANN

II *III* *IV*

MLQGPCRALLGDLCAGNQRKTRNANAFFSFFMAVGNVLGYAAGAYSKLYHVFPFTKTKACNVYCANLKSCFFLSIALLTV

PE0326 *V* *VI*

LATSALIYVKETALTPEKTVVTTEDGGSSGGMPCFGQLSGAFKELKRPMWILLLVTCLNWIAWFPFLLFDTDWMGKEVYG

PE0327 *VII*

GTVGEGHAYDMGVREGALGLMLNSVVLGATSLGVDILARGVGGVKRLWGIVNFLLAICLGLTVLVTKLAQHSRQYAPGTG

VIII *IX*

ALGDPLPPSEGIKAGALTLFSVLGVPLAITYSIPFALASIFSSTSGAGQGLSLGVLNLAIVIPQMFVSVLSGPWDALFGG

- ***X*** ***XI***

GNLPAFVVGAVAALASGILSIILLPSPPPDMAKSVSATGGGFH.

XII

Fig. 3.2.1.1 Putative protein sequence of VfSUT1. The 12 transmembrane domains (Weber et al., 1997) are shown in yellow. Selected peptide sequences are designated by red letters.

Antisera against these two peptides were generated by immunization of 4 rabbits by the pepScan *GmbH* company (see procedure described in 3.1.1).

From all 4 immunized rabbits, anti-peptide antisera and pre-immune sera were provided. To check the specificity of these antisera, ELISA assay has been carried out by pepScan *GmbH* company. The ELISA reports show that all 4 anti-peptide antisera from rabbits KIX-KXII recognize the corresponding antigen peptides (data not shown).

3.2.1.2 Specificity of the peptide antisera for the whole VfSUT1 transport protein

Cloning of the VfSUT1 cDNA into the yeast expression vector pYES2

To study the specificity of all 4 anti-peptide antisera, the VfSUT1 gene was cloned into yeast expression vector pYES2 in both, sense and antisense orientation and expressed in yeast cells.

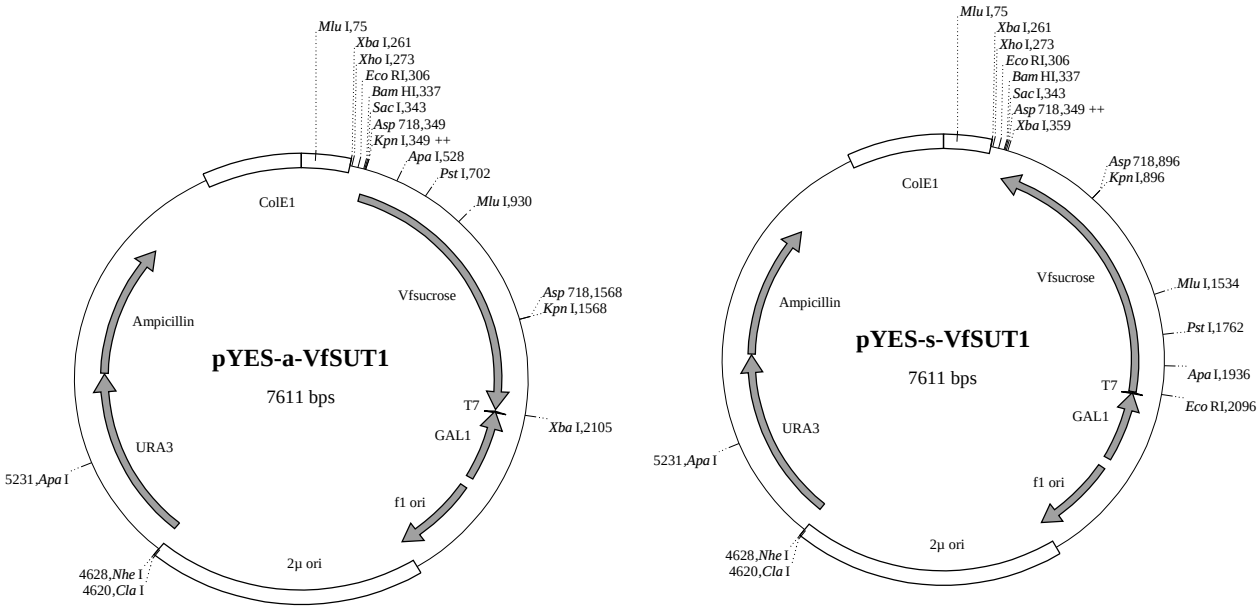


Fig. 3.2.1.3 Cloning of the VfSUT1 cDNA in the yeast expression *vector pYES2 in sense (pYES-s-VfSUT1) and anti-sense (pYES-a-VfSUT1) orientation.*

Western blot analysis of transgenic yeast cells

Total protein was isolated from yeast cells transformed with the vector pYES-a-VfSUT1 or pYES-s-VfSUT1. The same amount of the total protein was loaded onto a 12% SDS-PAGE gel, and the cross-reactivities of the produced antisera were checked by Western blot analysis. Only the antiserum directed against peptide antigen PE0327 from rabbit KXI was able to recognize a single polypeptide at 55kDa in total protein extracts of that yeast strain transformed with the VfSUT1 gene in sense orientation. By using of the same antiserum, no signal was detected in the total protein extracts isolated from the yeast strain transformed with the VfSUT1 gene in antisense orientation. The antiserum against PE0327 from rabbit KXI was affinity-purified and designated as SUT-XI. The purified SUT-XI IgG fraction was used for further experiments.

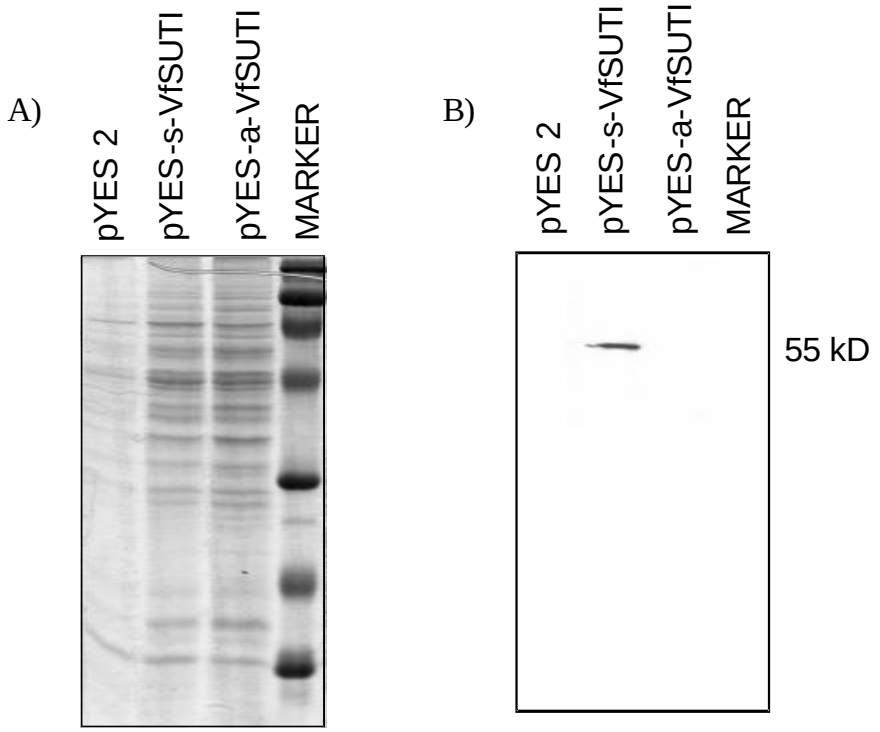


Fig. 3.2.1.4 Cross-reactivity of the peptide antiserum SUT-XI with the VfSUT1 protein produced in transgenic yeast cells. (A) Coomassie stained gel showing the pattern of the total protein isolated from yeast cells. (B) Western blot analysis of VfSUT1 protein expression in yeast cells transformed by pYES-A-VfSUT1 or pYES-S-VfSUT1 using SUT-XI as primary antibody.

pYES 2, yeast cells transformed by the empty pYES 2 plasmid; pYES-S-VfSUT1, yeast cells transformed by pYES-S-VfSUT1; pYES-A-VfSUT1, yeast cells transformed by pYES-A-VfSUT1.

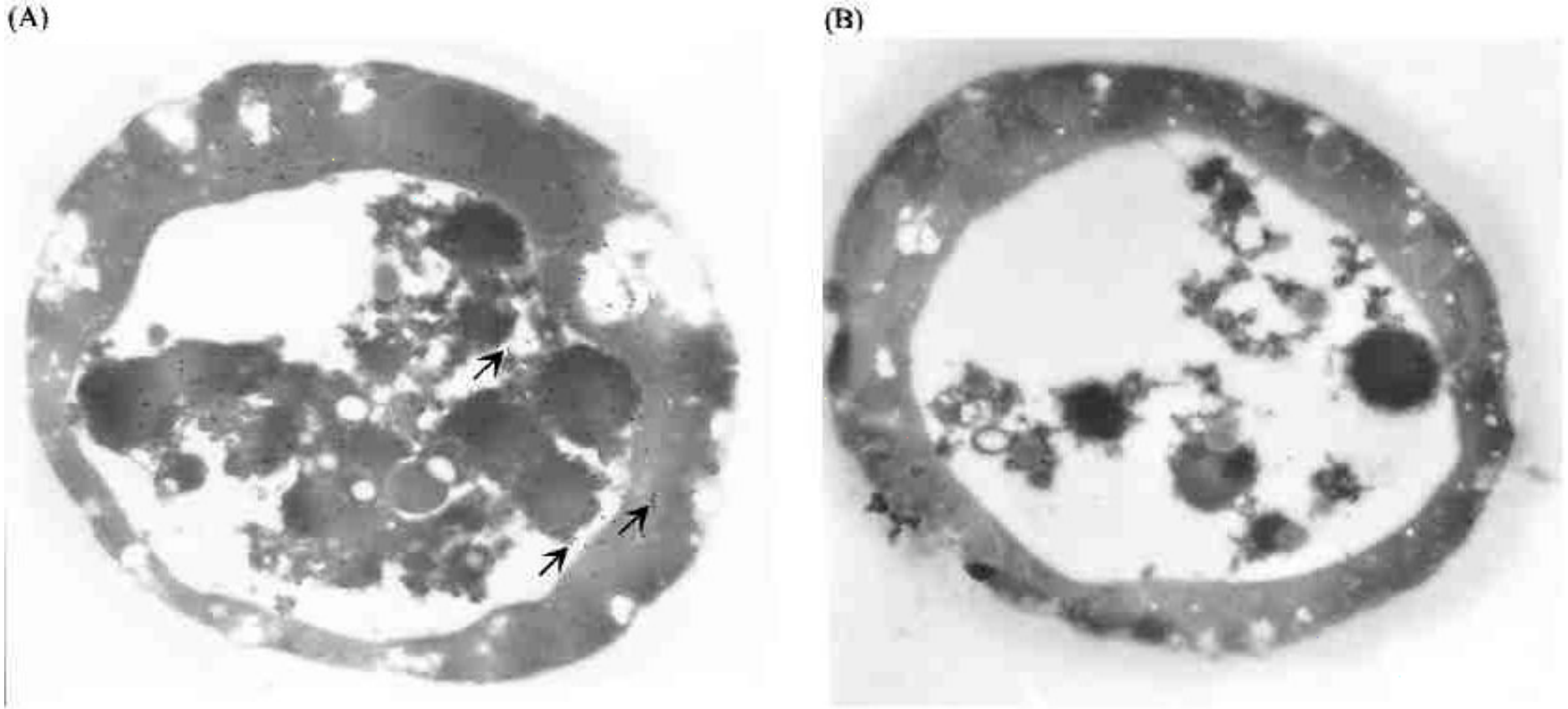


Fig.3.2.1.5. Immunogold detection of the VfSUT1 protein in transgenic yeast cells using the SUT-XI antibody. (A) Yeast cells transformed with pYES-s-VfSUT1. (B) Yeast cells transformed with pYES-a-VfSUT1. The arrows point to immuno-gold labels.

Immuno-localization of VfSUT1 protein expressed in yeast cells (immuno-gold labeling)

To check the usefulness of the SUT-XI antibody for the subcellular localization of the VfSUT1 protein, we have tried to localize the protein within pYES-s-VfSUT1 yeast cells. The cells were incubated in SD medium containing 2% galactose for one day. After that, cells were collected and prefixed before embedded. Immuno-gold labeling was carried out on sections of resin embedded cell material. Gold particles were found in pYES-s-VfSUT1 yeast cells whereas no gold particles were detected in yeast cells transformed with vector pYES-a-VfSUT1 (Fig. 3.2.1.5).

3.2.1.3 Western blot analysis of VfSUT1 protein extracts from Vicia faba

VfSUT1 protein expression pattern during seed development

The sucrose transporter protein expression levels at different developmental stages (stage V, VI and VII) of *Vicia faba* cotyledons were examined by Western blotting.

(A) (B)

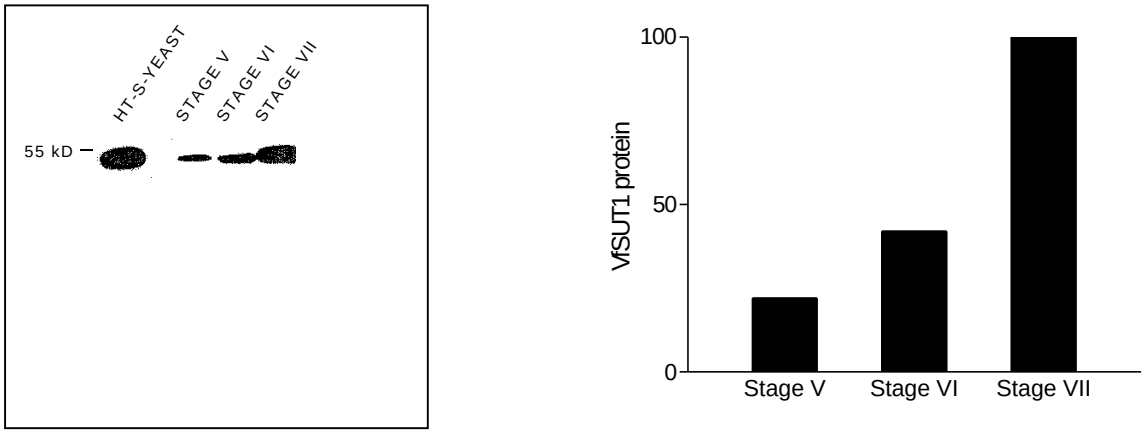


Fig. 3.2.1.6 Immunochemical detection of the VfSUT1 protein expression level in developing cotyledons of Vicia faba*. (A) Western blot analysis of the VfSUT1 protein. Immuno-detection was performed with the affinity purified SUT-XI anti-peptide antibody. Total proteins were extracted from* Vicia faba *cotyledons of the developmental stages V, VI and VII. The same amount of total protein (50µg) was loaded on each lane of a SDS-PAGE gel. HT-S-YEST, total protein isolated from yeast cells transformed with pYES-s-VfSUT1 (positive control). (B) Quantification of the Western blot analysis data. The Western blot data were analyzed by Tina ver. 2.08 beta software (Raytest Spockhövel, Germany). The bars show relative units of VfSUT1 protein expression using the highest expression level for 100%.*

The VfSUT1 protein expression in developmental stages V, VI and VII is comparable to the VfSUT1 mRNA profile measured before by Weber *et al*.,(1997).

Tissue specificity of VfSUT1 protein expression

To analyze the tissue specific expression of the VfSUT1 protein in different organs of the *Vicia faba* plant, total protein was isolated from different tissues as stem, leaf, root, pod, seed coat and cotyledons of developmental stage V and analyzed by Western blotting. As shown in Fig.3.2.1.7, VfSUT1 protein is mainly expressed in cotyledons. Furthermore, weak VfSUT1 expression was detected in the total protein isolated from pods.

(A) (B)

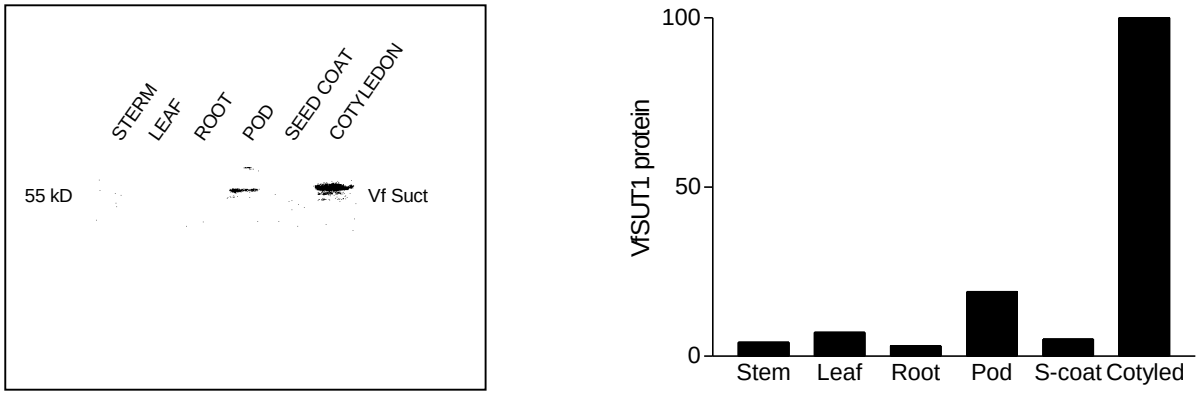


Fig. 3.2.1.7 Tissue-specificity of VfSUT1 protein expression in different plant organs. (A) Result of the Western blot analysis. (B) Quantification of Western blot result. The bars show relative units of protein expression using the highest expression level for 100%.

Expression of the VfSUT1 protein in membrane- and cytoplasmic fractions of* Vicia faba *cotyledons

To analyze the intercellular distribution of the VfSUT1 protein, the membrane- and the cytoplasmic fractions of *Vicia faba* cotyledon cells were isolated, and the proteins isolated from these fractions were separated on SDS-PAGE gel.

(A) (B)

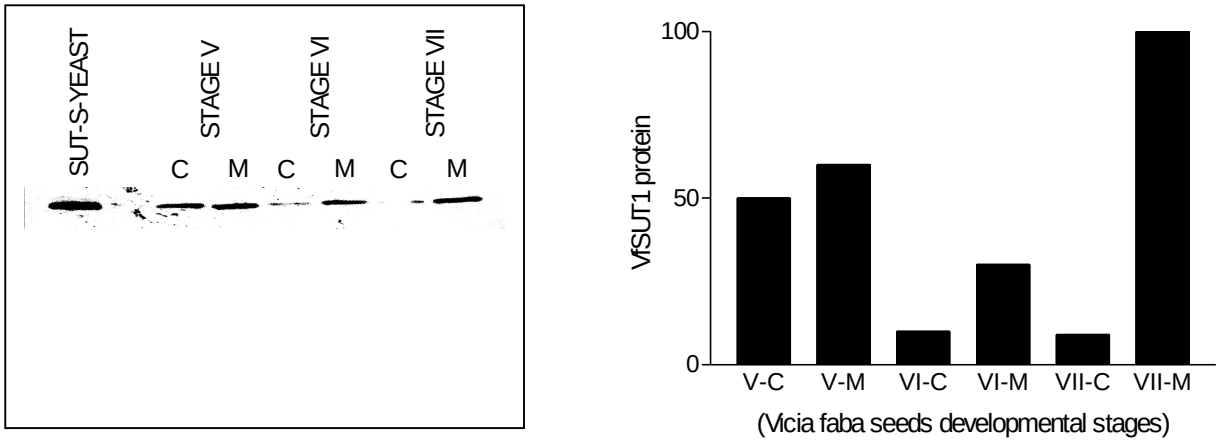


Fig. 3.2.1.8 Subcellular distribution of VfSUT1 in developing cotyledons of Vicia faba*. Membranes (M) and cytoplasmic fractions (C) were extracted from* Vicia faba *cotyledons at developmental stage V, VI and VII. The VfSUT1 protein expression was detected by Western blotting (A) and quantified (B). The bars in (B) show relative units of protein expression. For further explanation see legend of Fig. 3.2.1.6.*

In developmental stage VI and VII, the VfSUT1 protein was detected mainly in the membrane fraction, whereas similar VfSUT1 protein amounts were found in both the membrane and the cytoplasmic fraction isolated from cells of the developmental stage V.

3.2.2 *Study of sucrose transporters in* Hordeum vulgare

3.2.2.1 cDNA cloning revealed two sucrose transporter genes expressed in the developing barley caryopses

To isolate seed-specific sucrose transporter cDNAs from barley grains, two degenerated primers (see below) for RT-PCR were designed based on the alignment of known sucrose transporter cDNA sequences. Additionally, two rice EST's showing homology to sucrose transporter cDNAs from dicot species were included.

5'-primer: **T(C/T)CT(C/T)GG(A/G/C/T)(A/G)TCCC(A/G/C/T)CT(A/G/C/T)G**

3'primer: **CC(A/G/C/T)TTG(A/T)A(G/C/T)GG(A/C/T)CG(C/T)AA(A/G)**

Total RNA from developing barley caryopses (11-18 DAF) was used as template for RT-PCR. A 193bp cDNA fragment has been amplified, sub-cloned into pUC18 and sequenced. The sequence is shown in Figure 3.2.2.1.

TCCTCGGAATCCCTCTGGCCATTCTGTACAGTGTCCCTTTCGCGGTGACTGCGCASTGGCGGCGAACAAAGGCGGTGG
ACAAGGGCTGTGCACGGGCGTGCTGAACATCGCCATCGTGATACCGCAGTGAATCATCGCGGTGGGTGCCGGGGCCGT
GGGACGAGCTGTTCGGCAAGGGCAACATCCGGCGTTC

Fig. 3.2.2.1 Sequence of the 193bp cDNA fragment amplified from total caryopses RNA by RT-PCR.

The BLAST search showed that the sequence of this cDNA fragment is homologous to known sucrose transporter cDNA sequences.

This fragment 193bp in length was used to probe a *H. vulgare* phage cDNA library generated from developing caryopses. After two independent screening procedures, two different types of sucrose transporters were identified and designated as HvSUT1 and HvSUT2. The HvSUT1 and HvSUT2 cDNAs possess a single open reading frame of 1,694 and 1,521 bp encoding 523 and 507 amino acids, respectively. The putative amino acid sequences of both, HvSUT1 and HvSUT2 have the typical 12 membrane spanning domain structure (Figures 3.2.2.2A and B).

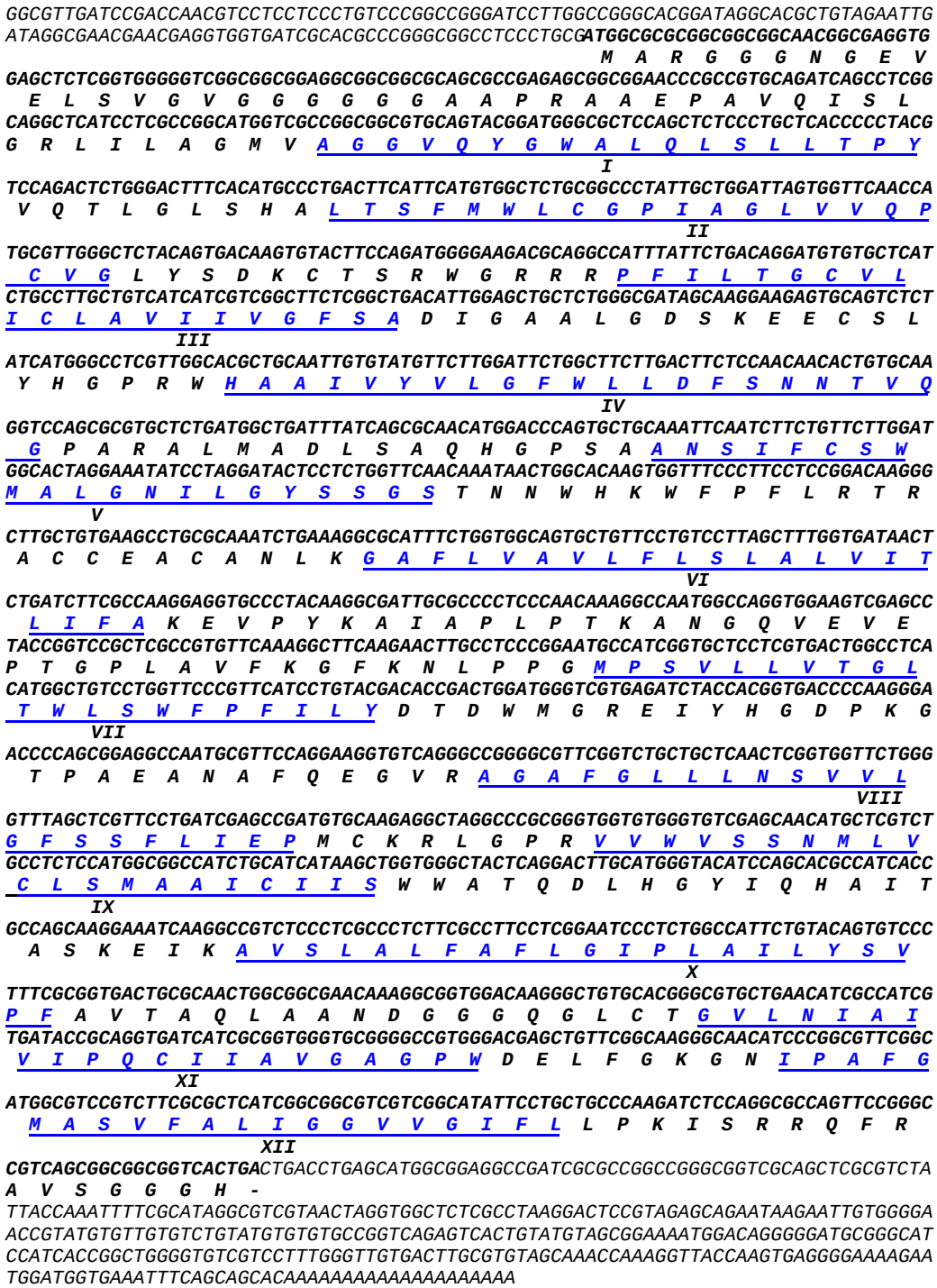


Fig. 3.2.2.2 A HvSUT1 cDNA and derived amino acid sequence.

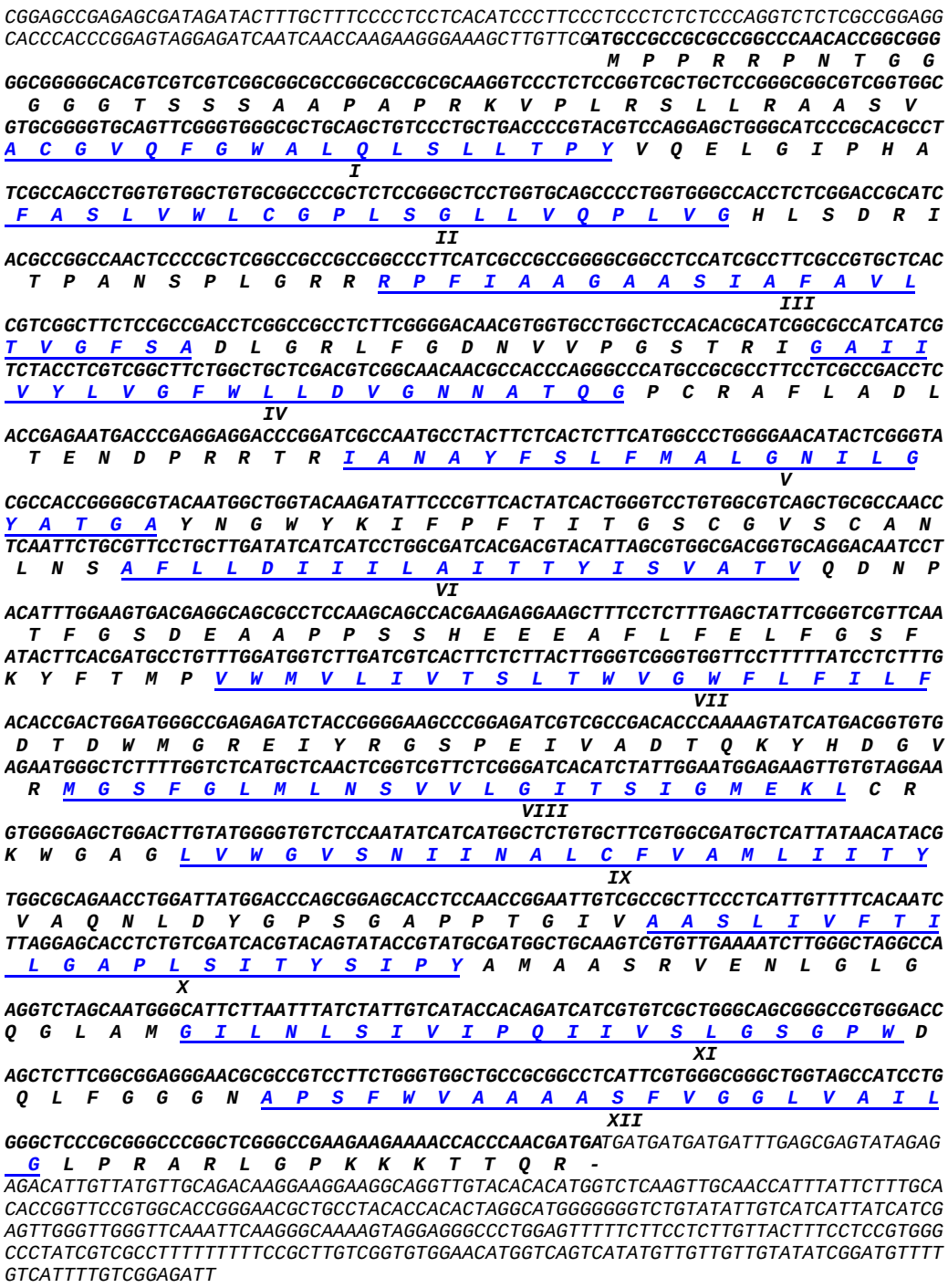


Fig. 3.2.2.2 B HvSUT2 cDNA and derived amino acid sequence.

The putative amino acid sequences of both sucrose transporters were aligned with the deduced protein sequences of 23 sucrose transporters known up to now (Figure 3.2.2.3).

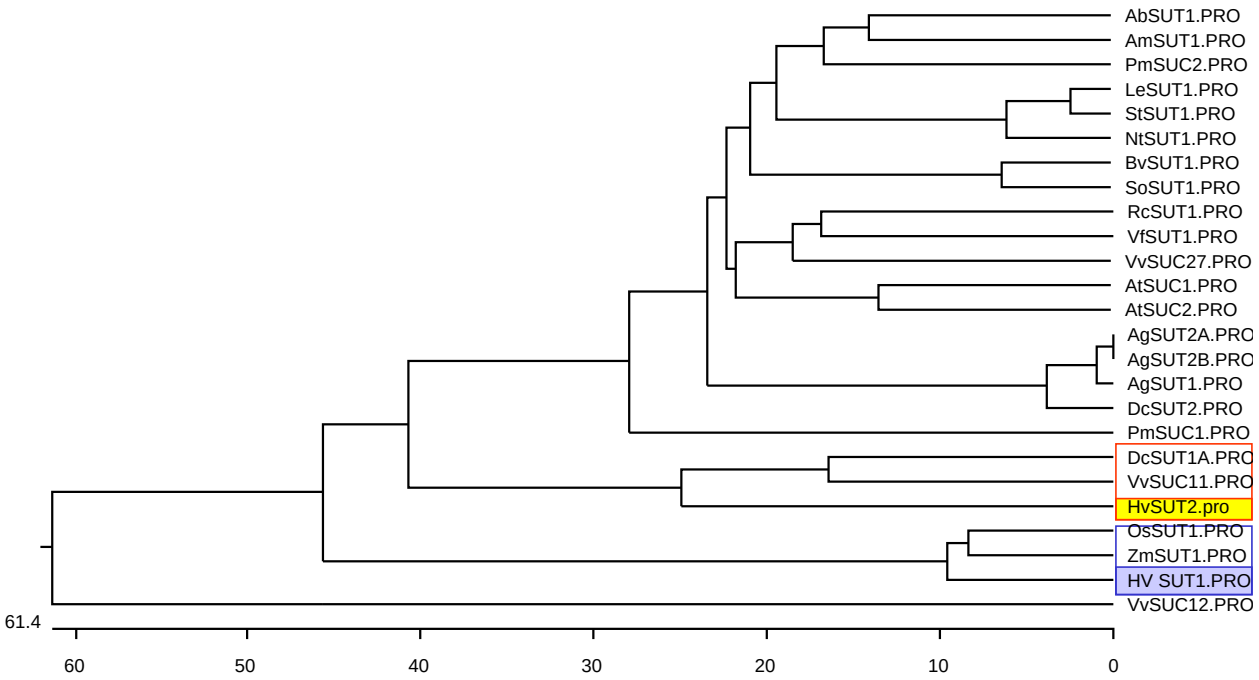


Fig. 3.2.2.3 Comparison of monocot and dicot sucrose transporters based on the similarities of their amino acid sequences.

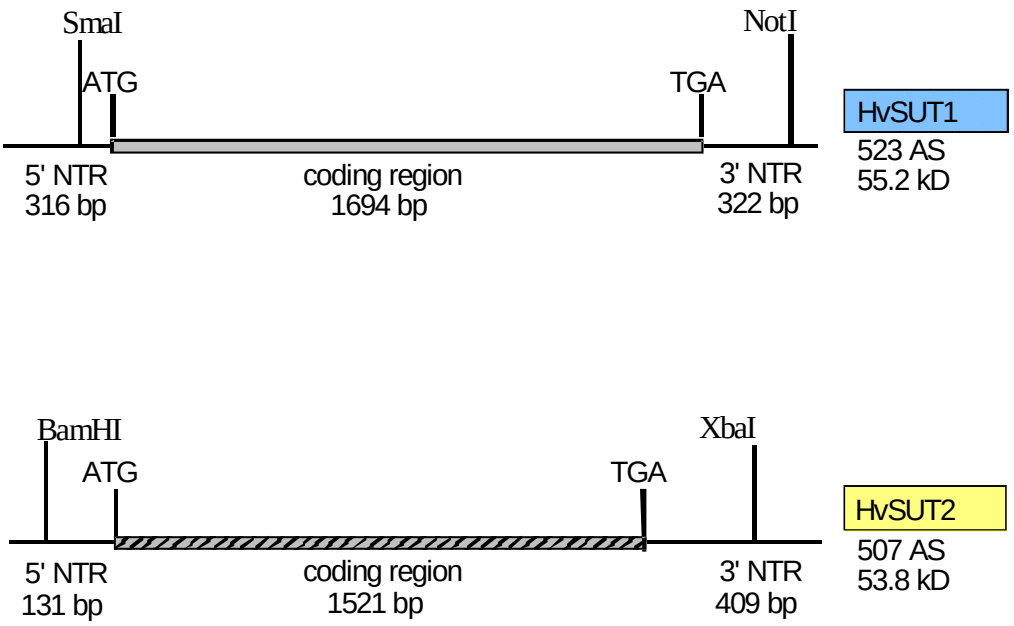
The order of branching within the dendrogram indicates statistical similarities between sucrose transporters from different plant species. The amino acid sequence of HvSUT1 was aligned with those of the corresponding sucrose transporters as follows: A. barclaiana (AbSUT1, No. 6120115); A. graveolens (AgSUT1, No. 4091891; AgSUT2A, No. 5566434; AgSUT2B, No. 5566437); A. meridionalis (AmSUT1, No. 6120117); A. thaliana (AtSUC1, No. X75365; AtSUC2, No. X75382); B. vulgaris (BvSUT1, No. 1076257); D. carota (DcSUT1A, No. 2969887; DcSUT2, No. 2969884); H. vulgare (HvSUT2); L. esulentum (LeSUT1, No. 1076602); N. tabacum (NtSUT1, No. 575351); O. sativa (OsSUT1, No. 2723471); P. major (PmSUC1, No. 1086250; PmSUC2, No. 1086253); R.communis (RcSUT2, No. 468262); S. oleracea (SoSUT1, No. 549000); S. tuberosum (StSUT1, No. 542087); V. faba (VfSUT1, No. 1935019); V. vinifera (VvSUC11, No. 6434829; VvSUC12, No. 6434831; VvSUC27, No. 6434833); Z. mays (ZmSUT1, No. 5771354.)

HvSUT1 is highly homologous to ZmSUT1 (82.9% identity) from maize and OsSUT1 (81.3% identity) from rice, the only two sucrose transporters published so far from monocot species (Aoki, N. *et al.* 1999; Hirose *et al.*, 1997). However, HvSUT1 and HvSUT2 were only 39.2% identical. HvSUT2 neither clustered together with the monocot transporters HvSUT1, ZmSUT1 and OsSUT1 nor with most of the dicot transporter families. It has highest homology to DcSUT1A (58.7 % identity) from carrot and VvSUC11 (61.3% identity) from grapevine. These three transporters seem to form an independent group.

3.2.2.2. Cloning the HvSUT1 and HvSUT2 cDNAs into the yeast expression vector pNEV

To study whether the HvSUT1 and HvSUT2 cDNAs encode functional sucrose transporter proteins, sucrose uptake experiments were done by expression of the HvSUT1 and the HvSUT2 proteins in yeast cells. The HvSUT1 cDNA SmaI/NotI fragment and the HvSUT2 cDNA BamHI/XbaI fragment (Figure 3.2.2.4) were cloned into the yeast expression vector pNEV in both, sense and antisense orientation. The resulting recombinant plasmids were named pNEV-S/A-HvSUT1 and pNEV-S/A-HvSUT2 (Figure 3.2.2.5).

The sucrose uptake experiments were done in cooperation with the group of Prof. Norbert Sauer, Erlangen University. The results indicate that both, HvSUT1 and HvSUT2 encode functional sucrose transporters (data not shown).



- ***Fig. 3.2.2.4 The HvSUT1 and HvSUT2 cDNA fragments used for cloning into the yeast expression vector pNEV.***

3.2.2.3 Structure of the genomic HvSUT1 gene including 1,445 bp of the promoter region

In situ hybridization data indicate that HvSUT1 displays a specific expression pattern within the developing caryopses. It is mainly expressed in endospermal transfer cells which represent the putative site of assimilate exchange between the maternal and the filial part of the caryopses. Therefore, isolation and functional analysis of the HvSUT1 promoter could gain more insight into the role of HvSUT1 in barley and would provide the possibility to express specific genes nearly exclusively within the transfer cells. To isolate the promoter of HvSUT1, a modified genome walking procedure was used (see Material and Method, 2.2.7.1). The HvSUT1-specific fragment isolated by this procedure was 1,100 bp in length. The nucleotide sequence of this genomic DNA fragment is shown in Figure 3.2.2.6. Because of the big first intron (783 bp) of the HvSUT1 gene, only a short real promoter fragment (165 bp) could be identified by genome walking.

TCTTCCGCCTCCCGTCAGCCCCCCTCTTCTTCCCCGGCGTTGATCCGACCAACGTCCTCCTCCCTGTCCCGGCCGGGATC
CTTGGCCGGGCACGGATAGGCACGCTGTAGAATTGATAGGCGAACGAACGAGGTGGTGATCGCACGCCCGGGCGGCCTCC
CTGCG***ATG****GCGCGCGGCGGCGGCAACGGCGAGGTGGAGCTCTCGGTGGGGGTCGGCGGCGGAGGCGGCGGCGCAGCGCCG*
AGAGCGGCGGAACCCGCCGTGCAGATCAGCCTCGGCAGGCTCATCCTCGCCGGCATGGTCGCCGGCGGCGTGCAGTACGG
*ATGGGCGCTCCAGCTCTCCCTGCTCACCCCCTACGTCCAG*GTATACCACACGTACATATACATGCAACCCGTGTAGAGCT

Intron I

GAGGTTCACGCGCGCGCCGCGAGGCCATGTACGTCCATGCACTGCGTCTGCGTGCGCGGCGGTGCTGTGTCTCTGTCTTC
GTCCTGGCCGTGCATGCGCGCCTTCGGGACGACCAAAGCTACTCCGTCCGTAGTTTTTAGTGCCTTGCTTTCTAAAAATA
AATCCGCAAGGTGTGAAGAGAAGCATGAAAGATACTCTACCCTGCAACTTTTTTTGTCCTTGATCGTTTTTCATTTCTCA
AGTTTTGTGCAGCTTTCAATTTCTTTTTTTCGAACGGTAGATTCAAGCTCAACACAACAAGTTTTTTAGTATTCAGAAGA
AACGTGAGGTTTGACCCGAGAAAGTCAAGATTAGTTTCTTGTTCTTTTGCACTGGGTTTTGTTCTTTTTTTTCGAAACAA
GGGCACAGTTGCAACAAGAAGAAGTTGGCCTGGGCAAGTAAGGGCATGTGTGGGTTGATAAGCTGAGGGTGCGCTTTACT
TTTCAAAATATCTGAACTGTGAAAACCTTCTATATGTTTGTGTGGAACAGCTACAGCTTTGGGGTGAAAGAGTTCAAAGT
TTTTTAAACCATTTGCTACTGCTAGATAACAGAAGAATACTGTAAAATTCAGTACTAACAACTTACTGGTGGAGTTGTTT
CCTTTTTGCGCCAAGAAATTTGATGGAACTTAGAGTTGCTGATACTGCAAATGCTACTACTTGTTCTGTCCACTGACTAT
CTGAACTTTGCCATATTTGTGCAG*ACTCTGGGACTTTCACATGCCCTGACTTCATTCATGTGGCTCTGCGGCCCTATTGC*
TGGATTAGTGGTTCAACCATGCGTTGGGCTCTACAGTGACAAGTGTACTTCCAGATGGGGAAGACGCAGGCCATTTATTC
TGACAGGATGTGTGCTCATCTGCCTTGCT

Fig. 3.2.2.6 Sequence of the HvSUT1 DNA fragment isolated by genome walking. The sequences of the first two exons are underlined.

We have used a set of filters containing a barley BAC library representing 6-fold the barley genome, which was kindly provided by Dr. Dagmar Schmidt (research group “Gen und Genomkartierung”, IPK-Gatersleben) to analyse further the 5’-flanking region of HvSUT1. The DNA fragment isolated by genome walking (shown in Figure 3.2.2.6) was used to screen this

barley BAC library at high stringency. 6 independent positive BAC clones were isolated, representing a single genomic DNA species. This result indicates that only one copy of the HvSUT1 gene exists within the barley genome. About 1.4 kb of the HvSUT1 gene promoter region, together with the coding region of the gene, which include 9 exons divided by 8 introns and a 322 bp 3´ non-translated region was sequenced and designated G-HvSUT1. The total length of the G-HvSUT1 gene is 4862 bp. The putative amino acid sequence as well as the promoter region and the exon (red) /intron (black) structure of G-HvSUT1 are shown in Figure 3.2.2.7.

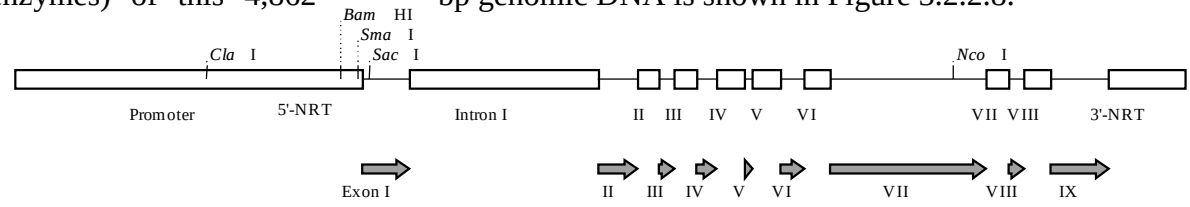
```
-1445   ACATCTAGTTATATCCCATTCCTATGATAAGTATTCTCGGACAGAGGAAGTACTACCTAAGTAACTTTCACTATTTCCTC
-1365   TGTTTTGGTTTAGGCTGGCCATAGTGGGAGTAACTTAGGTAGTATCATGTACTTGGGAATAGCAATCATGCTGATGTGGC
-1285   GGACATTTAAAGAAGAGAAAGAGGGTTAGAGTAACATAGGAGTATGTGTCATGCATGTCAATAAATGAGGTCATCCCATG
-1205   ATACTAGTTTATGATACTATGCATTATGAAGATAGTATCATACTATAGTATTATATGCATGATACTACTATATGATACTC
-1125   TTCACTATGACCAGCCTTATAAGCATCTTACGAAAACCAAATAATTCCAAAAACAATAAGGCACGATGCATTAACTTTCA
-1045   CCTCGTTTCTTGTTTTTTGACTTGCATAAAGGAATCAACCAATAAAAGATGTGGGGTGTTTATATTTGTAATGGCTTGAG
 -965   ACTAACTAGCACGACATGCAGTGATCAATTCATTGCATGCAATGATATAAATTAGCAAGTTAACATTAATTTCTTTTGTT
 -885   TTCCTCTTCGTCTTGGTTGCAGTGCACAACCTAAGATGACTTATAAACCGAGACGGAGGGGGTATGACCAGTCAAGCCCT
 -805   TTACCCTTGGACCATGATTTGACCATAATAATCAGACTTCAGTAGACGGAAGTAAAAAACACTCTCTAATCCATATCAAT
 -725   TATCGCTGATTTAATAGCTAATTCATATTAAATGTCGTCGATTCAGTAGGGGTTCTAAATCTGCGACAATTAATATGGAT
 -645   CGATAAATGCAAGCCCCAAAGGCCGAATCCAAATCACAACCAAGTGGAAATAGATAAATCTTATCAGGAAAAGTGCGCTA
 -565   CATAGATAAACCAAGTGTACACGGATATATATAAATCTTTTATATCAAGAAAAGCGTAGTACTACAAAGATAGATAAAGG
 -485   GCAGGGGAAGaGaATAGAGGCTCATTAAAAACAGAGCAAGAATAAATCCACGTTAGAACCTAGTACCACATACAAGTGCA
 -405   GCAGGGCCGGGGAAGGGTGATGCAGTAGATAGTAGCATGGACGGCCGAGAGGCCGCATCCTGGGTAATTTAGGGCGGCCC
 -325   GCGTCGCGCTCGATCGGCACACACACGCCGCCTTTAAATGCCCCGCGTTCCATCGCTCCCAACACAAACCCACACCACCA
 -245   CCCCTCCTCTCCTCACTCCACGCTCCTTCCCTTCCTCACACCTCTCTCCCCCACTCGCACTTTCCGCCCTCGTCTCCTCC
 -165   TCTTCCGCCTCCCGTCAGCCCCCCTCTTCTTCCCCGGCGTTGATCCGACCAACGTCCTCCTCCCTGTCCCGGCCGGGATC
  -85   CTTGGCCGGGCACGGATAGGCACGCTGTAGAATTGATAGGCGAACGAACGAGGTGGTGATCGCACGCCCGGGCGGCCTCC
   -5   CTGCG
    1   ATGGCGCGCGGCGGCGGCAACGGCGAGGTGGAGCTCTCGGTGGGGGTCGGCGGCGGAGGCGGCGGCGCAGCGCCGAGAGC
          M  A  R  G  G  G  N  G  E  V  E  L  S  V  G  V  G  G  G  G  G  G  A  A  P  R
   81   GGCGGAACCCGCCGTGCAGATCAGCCTCGGCAGGCTCATCCTCGCCGGCATGGTCGCCGGCGGCGTGCAGTACGGATGGG
        A  A  E  P  A  V  Q  I  S  L  G  R  L  I  L  A  G  M  V  A  G  G  V  Q  Y  G  W
  161   CGCTCCAGCTCTCCCTGCTCACCCCCTACGTCCAGGTATACCACACGTACATATACATGCAACCCGTGTAGAGCTGAGGT
         A  L  Q  L  S  L  L  T  P  Y  V  Q
  241   TCACGCGCGCGCCGCGAGGCCATGTACGTCCATGCACTGCGTCTGCGTGCGCGGCGGTGCTGTGTCTCTGTCTTCGTCCT
  321   GGCCGTGCATGCGCGCCTTCGGGACGACCAAAGCTACTCCGTCCGTAGTTTTTAGTGCCTTGCTTTCTAAAAATAAATCC
  401   GCAAGGTGTGAAGAGAAGCATGAAAGATACTCTACCCTGCAACTTTTTTTGTCCTTGATCGTTTTTCATTTCTCAAGTTT
  481   TGTGCAGCTTTCAATTTCTTTTTTTCGAACGGTAGATTCAAGCTCAACACAACAAGTTTTTTAGTATTCAGAAGAAACGT
  561   GAGGTTTGACCCGAGAAAGTCAAGATTAGTTTCTTGTTCTTTTGCACTGGGTTTTGTTCTTTTTTTTCGAAACAAGGGCA
  641   CAGTTGCAACAAGAAGAAGTTGGCCTGGGCAAGTAAGGGCATGTGTGGGTTGATAAGCTGAGGGTGCGCTTTACTTTTCA
  721   AAATATCTGAACTGTGAAAACCTTCTATATGTTTGTGTGGAACAGCTACAGCTTTGGGGTGAAAGAGTTCAAAGTTTTTT
  801   AAACCATTTGCTACTGCTAGATAACAGAAGAATACTGTAAAATTCAGTACTAACAACTTACTGGTGGAGTTGTTTCCTTT
  881   TTGCGCCAAGAAATTTGATGGAACTTAGAGTTGCTGATACTGCAAATGCTACTACTTGTTCTGTCCACTGACTATCTGAA
```

961 CTTTGCCATATTTGTGCAGACTCTGGGACTTTCACATGCCCTGACTTCATTCATGTGGCTCTGCGGCCCTATTGCTGGAT
T L G L S H A L T S F M W L C G P I A G
1041 TAGTGGTTCAACCATGCGTTGGGCTCTACAGTGACAAGTGTACTTCCAGATGGGGAAGACGCAGGCCATTTATTCTGACA
L V V Q P C V G L Y S D K C T S R W G R R R P F I L T
1121 GGATGTGTGCTCATCTGCCTTGCTGTGAGTACTACTGTTCCTTTGTTATCTTCTTACGGTTCATAACTTTTTATTATGTT
G C V L I C L A
1201 TTTTCCTAATAAAAGTTCGATTTACTCGCAGGTCATCATCGTCGGCTTCTCGGCTGACATTGGAGCTGCTCTGGGCGATA
V I I V G F S A D I G A A L G D
1281 GCAAGGAAGAGTGCAGGTGATGTGCTCGTTGCCTGACTTTGGCTTTGCATTTTCCATAGCGACTGACGGTTGCAAATTGG
S K E E C
1361 TCTGACATTTTGACGTTGTAATTACAGTCTCTATCATGGGCCTCGTTGGCACGCTGCAATTGTGTATGTTCTTGGATTCT
S L Y H G P R W H A A I V Y V L G F
1441 GGCTTCTTGACTTCTCCAACAACACTGTGCAAGTAAGTGCTTTTATATGAGTAACAGTCATGTTCAAGAATTTCGACGCG
W L L D F F N N T V Q
1521 ACTAACCTGACTTGATCGGTTAAAAAAAGAGTACTAACCTGACTTGATTTTGGCATGGAATTTGTAGGGTCCAGCGCGTG
G P A R
1601 CTCTGATGGCTGATTTATCAGGTAATTTTTCATGACATGGTTGTGATGCTAGTTGGTTCAGGCAGACTAATTAAACTAGT
A L M A D L S
1681 GGCTGCTACGAAAATCAATGTGATTTGGGATCTGATCTCCATTCTTTTGTTCAACAGCGCAACATGGACCCAGTGCTGCA
A Q H G P S A A
1761 AATTCAATCTTCTGTTCTTGGATGGCACTAGGAAATATCCTAGGATACTCCTCTGGTTCAACAAATAACTGGCACAAGTA
N S I F C S W M A L G N I L G Y S S G S T N N W H
1841 AGCCAACGCTTCTGTACACACTGCATGCGTCTCTTATGTTCTGTTCTTCACACAAGTAGTATGTATGCTGACAAGTTCCA
1921 TTTTGCTTGGCTGATTTCAATAGGTGGTTTCCCTTCCTCCGGACAAGGGCTTGCTGTGAAGCCTGCGCAAATCTGAAAGG
K W F P F L R T R A C C E A C A N L K
2001 CGCATTTCTGGTGGCAGTGCTGTTCCTGTCCTTAGCTTTGGTGATAACTCTGATCTTCGCCAAGGAGGTGCCCTACAAGG
G A F L V A V L F L S L A L V I T L I F A K E V P Y K
2081 CGATTGCGCCCCTCCCAACAAAGGCCAATGGCCAGGTGGAAGTCGAGCCTACCGGTCCGCTCGCCGTGTTCAAAGGCTTC
A I A P L P T K A N G Q V E V E P T G P L A V F K G F
2161 AAGAACTTGCCTCCCGGAATGCCATCGGTGCTCCTCGTGACTGGCCTCACATGGCTGTCCTGGTTCCCGTTCATCCTGTA
K N L P P G M P S V L L V T G L T W L S W F P F I L
2241 CGACACCGACTGGATGGGTCGTGAGATCTACCACGGTGACCCCAAGGGAACCCCAGCGGAGGCCAATGCGTTCCAGGAAG
Y D T D W M G R E I Y H G D P K G T P A E A N A F Q E
2321 GTGTCAGGGCCGGGGCGTTCGGTCTGCTGCTCAACTCGGTGGTTCTGGGGTTTAGCTCGTTCCTGATCGAGCCGATGTGC
G V R A G A F G L L L N S V V L G F S S F L I E P M C
2401 AAGAGGCTAGGCCCGCGGGTGGTGTGGGTGTCGAGCAACATGCTCGTCTGCCTCTCCATGGCGGCCATCTGCATCATAAG
K R L G P R V V W V S S N M L V C L S M A A I C I I
2481 CTGGTGGGCTACTCAGGACTTGCATGGGTACATCCAGCACGCCATCACCGCCAGCAAGGAAATCAAGGCCGTCTCCCTCG
S W W A T Q D L H G Y I Q H A I T A S K E I K A V S L
2561 CCCTCTTCGCCTTCCTCGGAATCCCTCTGGCCGTAAGCAATCAAGCAATCCCTTTCTGCCACTTGATTTGGCTCCCTTTC
A L F A F L G I P L A
2641 TTTTTTCATTTCTGACGAAGCGAGTGCTCCTGTTGCACCGTAGCAGATTCTGTACAGTGTCCCTTTCGCGGTGACTGCGC
I L Y S V P P A V T A
2721 AACTGGCGGCGAACAAAGGCGGTGGACAAGGTGCGTGGACGAATGCTCCTGAGCTTTATTCTTCGGTGTTGCCCACTTAA
Q L A A N K G G G Q
2801 TTTTTGTTTTTGTTCTGGTGTTGACGGGTAACTGATTGATTATCTGTTTGGATGGTGCAGGGCTGTGCACGGGCGTGCTG

```
                                                                G  L  C  T  G  V  L
2881    AACATCGCCATCGTGATACCGCAGGTGATCATCGCGGTGGGTGCGGGGCCGTGGGACGAGCTGTTCGGCAAGGGCAACAT
          N  I  A  I  V  I  P  Q  V  I  I  A  V  G  A  Q  P  W  D  E  L  F  G  K  G  N
2961    CCCGGCGTTCGGCATGGCGTCCGTCTTCGCGCTCATCGGCGGCGTCGTCGGCATATTCCTGCTGCCCAAGATCTCCAGGC
          I  P  A  F  G  M  A  S  V  F  A  L  I  G  G  V  V  G  I  F  L  L  P  K  I  S  R
3041    GCCAGTTCCGGGCCGTCAGCGGCGGCGGTCACTGACTGACCTGAGCATGGCGGAGGCCGATCGCGCCGGCCGGGCGGTCG
          R  Q  F  R  A  V  S  G  G  G  H.
3121    CAGCTCGCGTCTATTACCAAATTTTCGCATAGGCGTCGTAACTAGGTGGCTCTCGCCTAAGGACTCCGTAGAGCAGAATA
3201    AGAATTGTGGGGAACCGTATGTGTTGTGTCTGTATGTGTGTGCCGGTCAGAGTCACTGTATGTAGCGGAAAATGGACAGG
3281    GGGATGCGGGCATCCATCACCGGCTGGGGTGTCGTCCTTTGGGTTGTGACTTGCGTGTAGCAAACCAAAGGTTACCAAGT
3361    GAGGGGAAAAGAATGGATGGTGAAATTTCAGCAGCACAAAAAAAAAAAAAAAAAAAA
```

Fig.3.2.2.7 Sequence of the promoter region and exon/intron structure of G-HvSUT1.

The exon/intron boundaries of G-HvSUT1 follow the canonical 'GT...AG' rule (Hanley and Schuler, 1988). The exon/intron structure and a restriction map (showing only most common enzymes) of this 4,862 bp genomic DNA is shown in Figure 3.2.2.8.



Untranslated regions		***Translated regions***		
Promoter region	*-1445----1*			
5' NTR	*-1--- 315*	*Exon I*	*1---195*	*65aa*
Intron I	*196--- 979*	*Exon II*	*980---1144*	*55aa*
Intron II	*1145---1231*	*Exon III*	*1232---1296*	*21aa*
Intron III	*1297---1387*	*Exon IV*	*1388---1472*	*29aa*
Intron IV	*1473---1587*	*Exon V*	*1588---1621*	*11aa*
Intron V	*1622---1737*	*Exon VI*	*1738---1837*	*33aa*
Intron VI	*1838---1943*	*Exon VII*	*1944---2592*	*217aa*
Intron VII	*2593---2686*	*Exon VIII*	*2687---2750*	*21aa*
Intron VIII	*2751---2860*	*Exon IX*	*2861---3103*	*81aa*
3'-NTR	*3104---3417*			

Fig. 3.2.2.8 Exon/intron structure and restriction map of the genomic HvSUT1 gene. The open boxes represent untranslated regions and include the promoter region (the first open box), 8 introns and the 3'-untranslated region. The filled arrows indicate the 9 exons.

3.2.2.4 Functional studies of the HvSUT1 promoter

To check the function of the HvSUT1 promoter, a 1,400 bp DNA fragment (the sequenced promoter region) was fused to the GUS reporter gene to express the resulting construct transiently in protoplasts.

The promoter fragment of G-HvSUT1 was generated by PCR

Because there was no enzyme suitable for cutting out the HvSUT1 promoter region from the BAC clone, specific primers were used for amplification. PCR primer sequences are shown below. DNA isolated from the HvSUT1 BAC clone was used as a template for PCR.

5'primer: **ACTTTCACTATTTCCTCTGTTTTG**

3'primer: **GATCACCACCTCGTTCGTT**

A 1,400bp DNA fragment has been amplified sub-cloned into pUC18 and sequenced for control. The fragment was designated P-1400 .

Cloning of the P-1400 DNA fragment into the vector pRT103GUS

To analyze of the HvSUT1 promoter, the P-1400 fragment was cloned into the pRT103GUS plasmid. In the transient assay, the plasmid pRT103GUS containing the bacterial GUS gene driven by the CaMV35S promoter (Figure 3.2.2.9B) was used as positive control. A negative control plasmid, designated as pRTGUS-pro(-) (Figure 3.2.2.9C) was constructed by cutting off the CaMV35S promoter sequence. The HvSUT1 promoter fragment P-1400 was ligated into the pRTGUS-pro(-) vector, which was opened by the enzyme NcoI. The resulting construct containing the promoter fragment in sense orientation was designated as GUSp14s (Figure 3.2.2.9A).

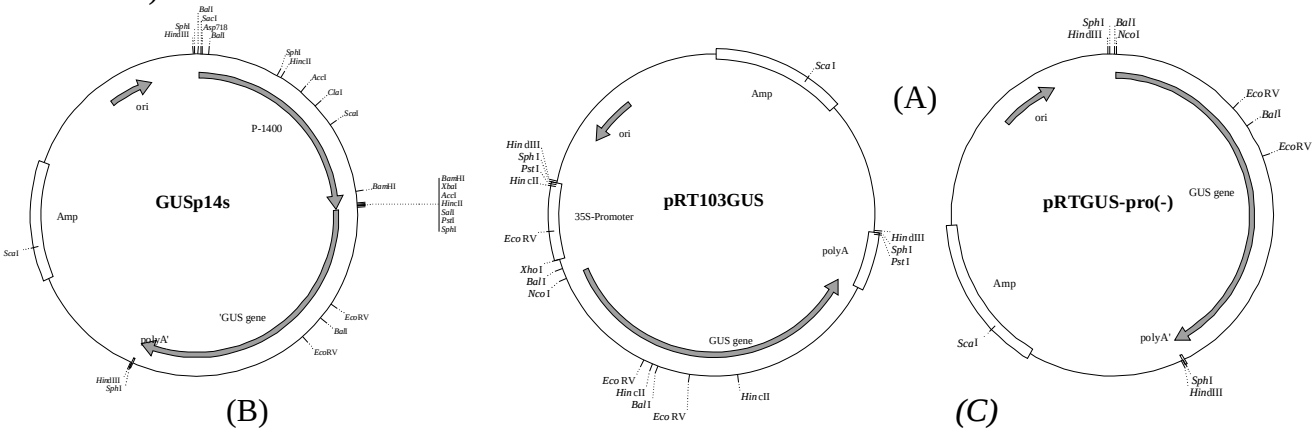


Fig. 3.2.2.9 Constructs used to analyze HvSUT1 promoter activity.

(A) Map of plasmid GUSp14s used in the transient expression assay to study the HvSUT1 promoter function. (B) Map of plasmid pRT103GUS, used for positive control. (C) Cloning map of the plasmid pRTGUS-pro(-), used for negative control.

Analysis of HvSUT1 promoter activity in a transient expression system

A suspension culture of *Nicotiana plumbaginifolia* was used for protoplast isolation. Protoplasts were transformed with the GUSp14s plasmid and, additionally, with the construct pRT103GUS (positive control), pRTGUS-pro(-) (negative control 1) and pRTGUSp14a (negative control 2). Transient GUS activity was determined 48 hours after transformation using the GUS-Light kit (Tropix).

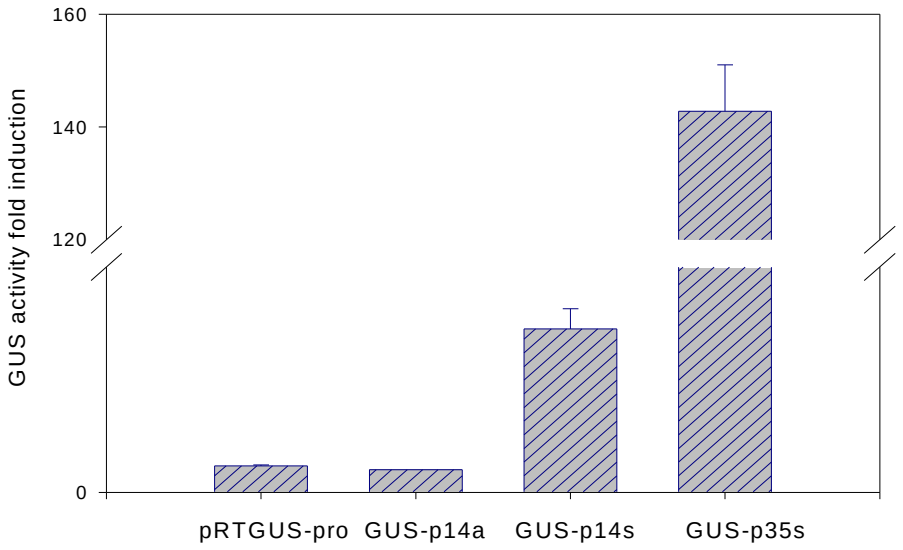


Fig. 3.2.2.10 Transient expression analysis of the HvSUT1 gene promoter fragment p-1400 in N. plumbaginifolia *protoplasts. The columns represent the median value of three independent experiments. Standard deviation was calculated by using of the SigmaPlot program. The HvSUT1 promoter driven expression (GUS-p14s) is shown as 5-fold induction as compared to the activity measured for negative control pRTGUS-pro(-) and GUS-p14a.*

As shown in Fig 3.2.2.10, activity of the HvSUT1 promoter was confirmed. Because dicot protoplasts were used to analyze the activity of a monocot promoter, only a relatively low GUS activity (5% of the positive control, GUS-p35s) was detected.

3.2.2.5 Expression and localization of the HvSUT2 protein

The HvSUT2 protein has sucrose transport function, but it has low homology (only about 40%) to most of the sucrose transporters analyzed up to now. The comparison of sucrose transporters amino acid sequences (Figure 3.2.2.3) indicats higher homology to transporters involved in sweetening of grapevine (61.3%, Davies *et al.,* 1999) and in sugar accumulation in carrot tap roots (58.7%, Sturm *et al.*, 1998). Especially from speculations made by Davies (1999), a specific function of HvSUT2 in loading of sucrose into the vacuole of young endospermal cells was postulated. By immuno-cytochemical methods, intracellular localization of the HvSUT2 protein should be possible to get additional and guiding information to explain the specific function of this highly interesting sucrose transport protein.

To localize the HvSUT2 protein in barley grains, a specific antibody was required. To raise the HvSUT2 specific antibody, production of high amount of HvSUT2 protein was necessary.

***Overexpression of the HvSUT2 protein in* E.coli**

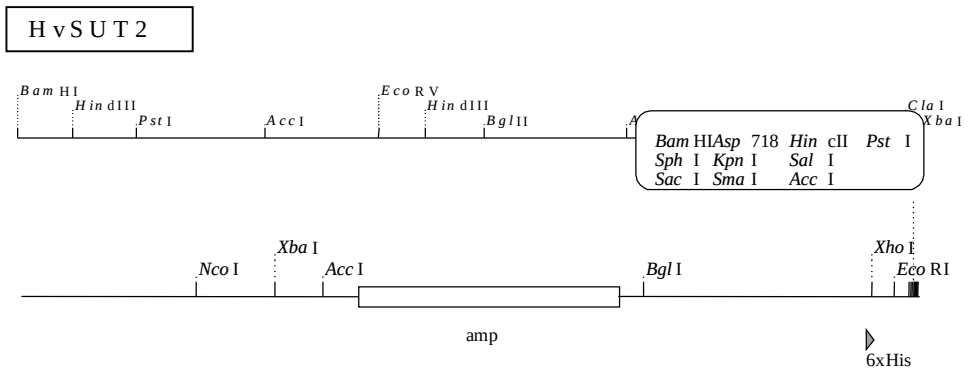
A fragment containing the complete HvSUT2 cDNA (Figure 3.2.2.11A) was ligated into the *E.coli* expression vector pQE (QIAGEN GmbH) in both, sense and antisense orientation. The resulting plasmids are designed as pQE-S/A-SUT2 (Figure 3.2.2.11B). pQE-S-SUT2 was used for overexpression of the transporter protein, pQE-A-SUT2 was used for negative control.

Both plasmids were transformed into the *E.coli* strain DH5α. To overcome the problems known for expression of heterologous transporter proteins in *E.coli*, the common protocol for protein over expression was modified (see Material and Method, 2.2.2.2). By using the modified protocol, the HvSUT2 protein was successfully over expressed and purified (Figure 3.2.2.12A).

The purified HvSUT2 protein was used to prepare a polyclonal antibody (work was done in co-operation with Dr. R. Manteuffel, IPK-Gatersleben). The specificity of the antibody was confirmed by dot blot (data not shown) and Western blot experiments (Figure 3.2.2.12B).

Beside of HvSUT2, a second sucrose transporter (HvSUT1) exists in barley caryopses. Although the degree of homology between the two transporters is relatively low (42% identity), the possibility of cross-reactions exists. Therefore, the HvSUT1 protein expressed in transgenic yeast cells was used to check the specificity of the HvSUT2 antibody. Total protein was isolated from yeast cells transformed with the plasmids NEV-S-SUT1 and NEV-S-SUT2 (cloning maps are shown in Figure 3.2.2.5). 50µg total protein per lane were loaded onto a 12% SDS-PAGE gel and analyzed by Western blotting. The HvSUT2-specific antibody recognized a polypeptide at 55kD only in the total protein isolated from the NEV-S-HvSUT2 transformed yeast cells. No signal

(A)



pQE(Hind III)

(B)

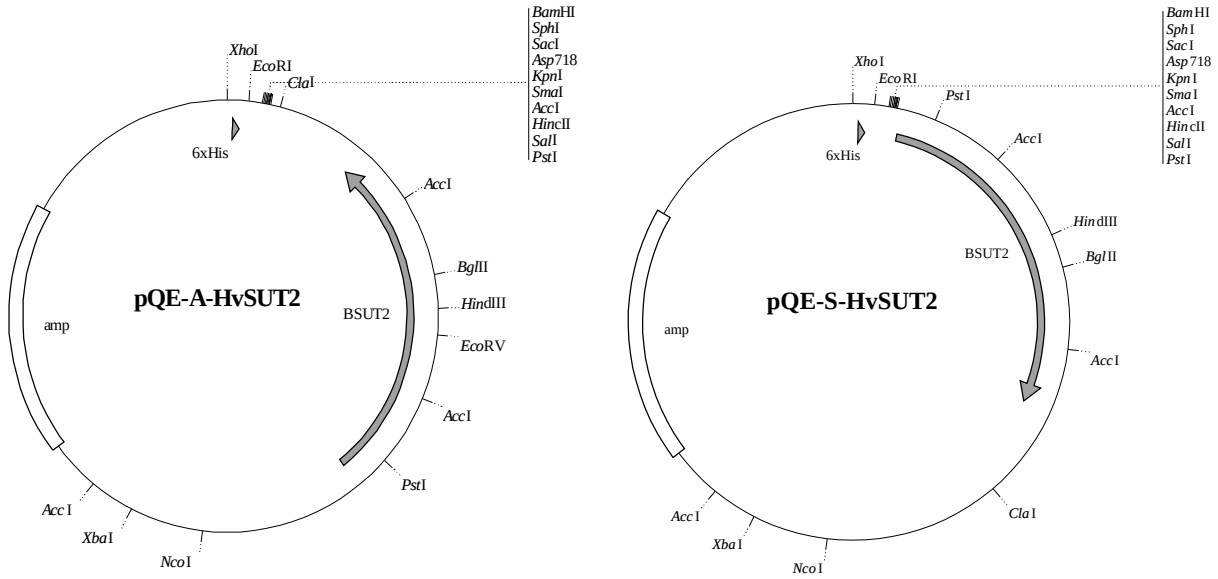


Fig. 3.2.2.11 Cloning maps of pQE-S/A-HvSUT2.

(A) Restriction map of the HvSUT2 gene (BamHI/XbaI fragment, above) and plasmid pQE opened by HindIII (below). The blunt-ended HvSUT2 cDNA was ligated into the pQE plasmid opened by HindIII and blunt ended. (B) Maps of the plasmids pQE-S/A-HvSUT2 containing the HvSUT2 cDNA fragment linked to the 6xHis tag of pQE in both sense and antisense orientations.

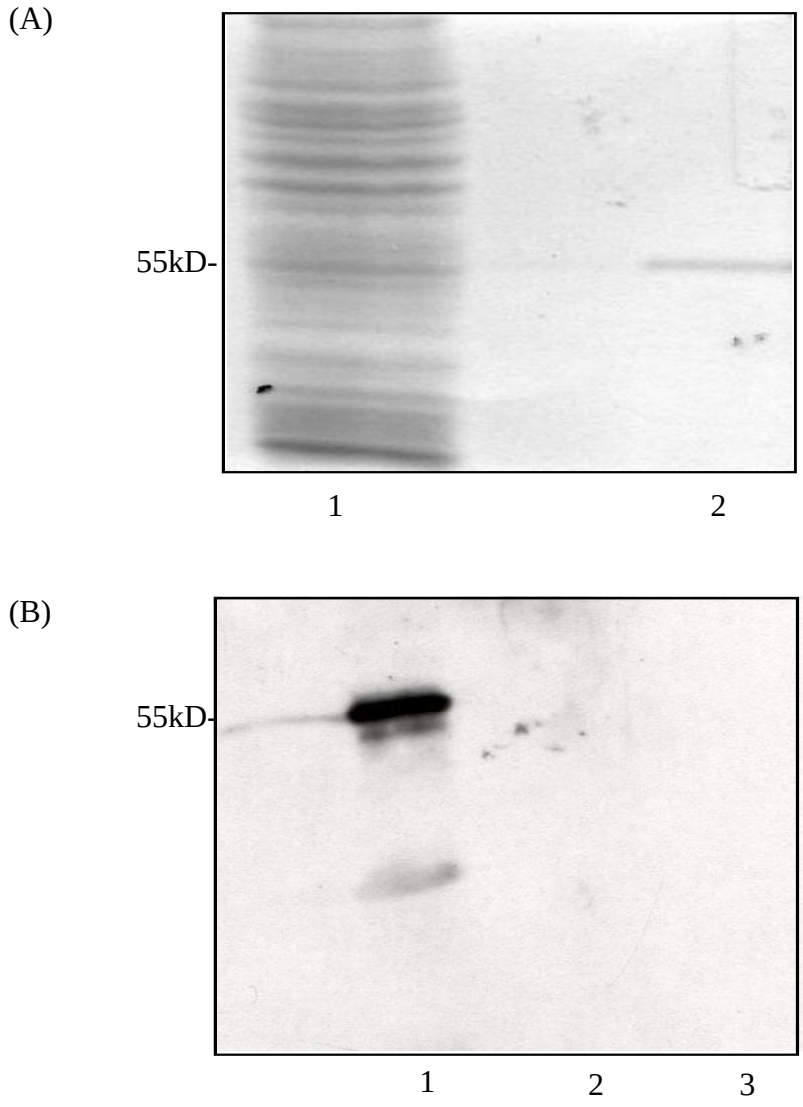


Fig.3.2.2.12 Specificity of the HvSUT2 antibody checked by Western blotting.

(A) Coomassie staining of a SDS gel used for separation of the proteins extracted from the over expression pQE-S-HvSUT2 E.coli. strain (lane 1) and the purified HvSUT2 protein used for preparation of the antibody (lane 2).

(B) Western blot analysis of total proteins from yeast cells transformed with plasmids NEV-S-HvSUT2 (lane 1), NEV-S-HvSUT1 (lane 2) and empty vector pNEV (lane 3). The HvSUT2 antibody was used for detection of the HvSUT2 protein.

was detected in the total protein isolated from the yeast cells transformed with NEV-S-HvSUT1 used for negative control (Figure 3.2.2.12B). The result indicates that the anti-HvSUT2 antibody recognizes specifically the HvSUT2 protein and does not cross-react with the HvSUT1 protein. The antiserum against the HvSUT2 protein was purified on Protein A-Sepharose and used for further experiments.

Immuno-fluorescence staining of protoplasts using the HvSUT2 specific antibody

Indirect immuno-fluorescence is known to be a sensitive assay for the evaluation of the polyclonal antibody. To avoid the auto-fluorescence caused by plant cell wall coupons, barley protoplasts were used. Barley protoplasts were rendered highly fluorescent when incubated with rabbit anti-HvSUT2 antibody followed by FITC labeled goat anti-rabbit IgG. When using adsorbed antibody (antigen-antibody complex) as primary antibody to control the specificity of immuno-labelling, only very weak fluorescence was evident (Figure 3.2.2.13). To quantify the intensity of the immuno-fluorescence, all protoplasts were photographed and converted into black and white pictures. The quantified fluorescence activities were scored by Tina ver. 2.08 beta software (see figure 3.2.2.14).

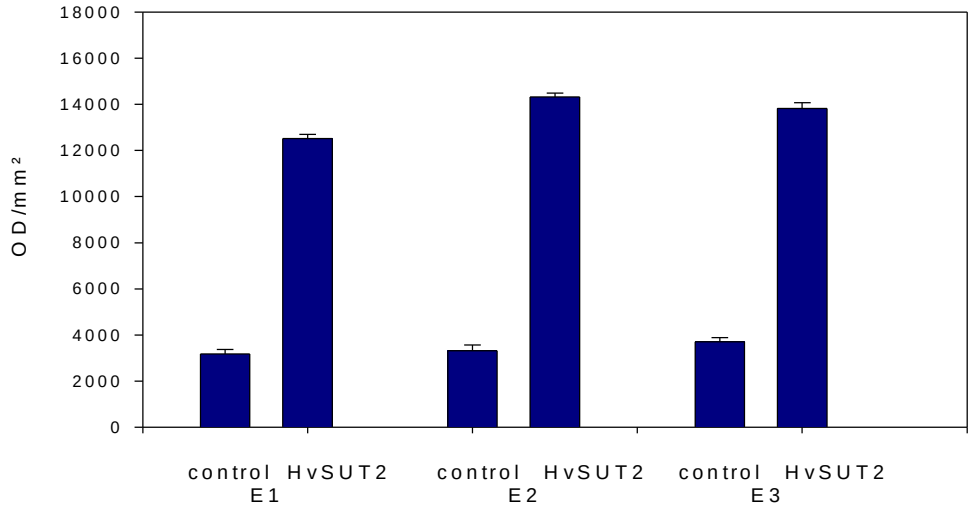


Fig. 3.2.2.14 Immunofluorescence intensity of barley protoplasts labeled by using the HvSUT2 specific antibody. Three independent experiments (E1, E2 and E3) have been done for quantitative analysis. Within each experiment more than 100 protoplasts were counted. Statistics has been carried out following the SigmaPlot program.

The data show that HvSUT2-FITC labeled protoplasts give an around 5-fold stronger fluorescence than controls labeled by the antigen-antibody complex. This result indicated that HvSUT2 is expressed in barley protoplasts.

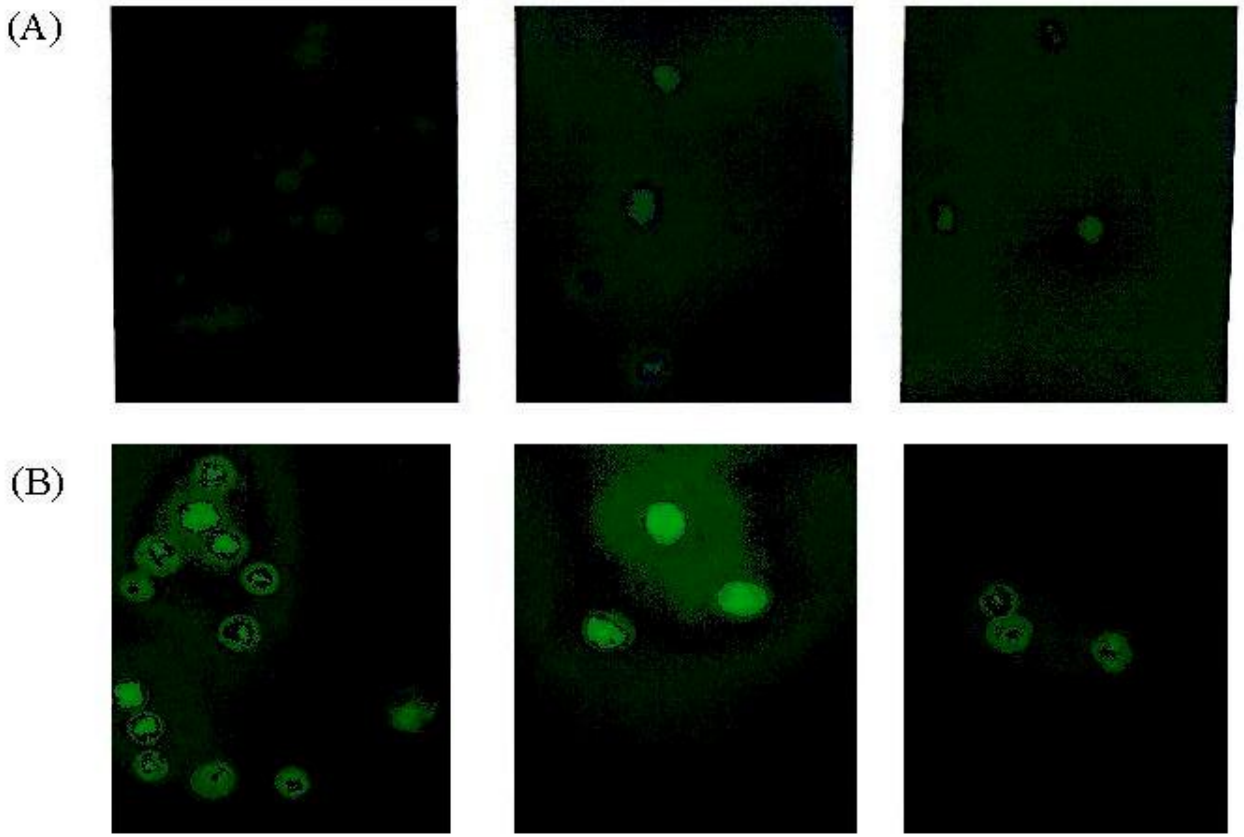


Fig. 3.2.2.12 Immunolabelling of barley protoplasts using the HvSUT2 specific antibody. (A) Adsorbed anti-HvSUT2 antibody was used as primary antibody (negative control). (B) Anti-HvSUT2 antibody was used as primary antibody.

Localization of the HvSUT2 protein

Histological organization of a barley caryopses

Figure 3.2.2.15A shows a transverse section through the middle region of caryopses 6 days after flowering (DAF). In this developmental stage, the well developed pericarp (P) represents the main part of the caryopses. Three different tissue layers, the outer (OI) and inner integument (II), each composed of two cell layers and the mono-layered nucellar epidermis (NE) surround the filial part of the caryopses. At this developmental stage the endosperm has already differentiated into the starchy endosperm (SE) and the endospermal transfer cells (ET). In front of the endospermal transfer cells, the nucellar epidermis is changed into the so-called nucellar projections (NP). Further conspicuous elements of the grain are the large main vascular bundle and three smaller veins, two at the lateral sides and one at the dorsal side (VT). Figures showing the histological organization of a barley caryopses are kindly provided by Dr. R. Panitz.

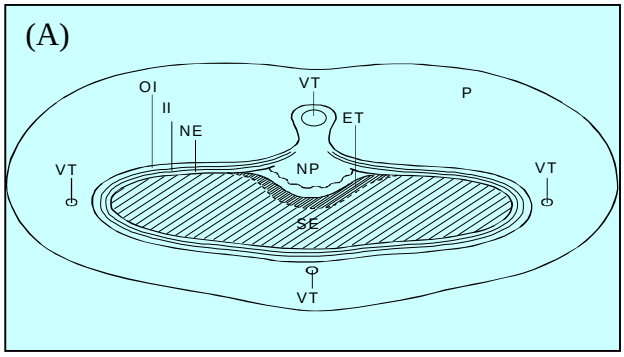
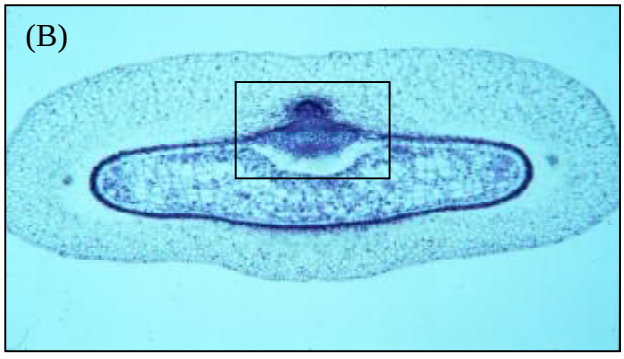
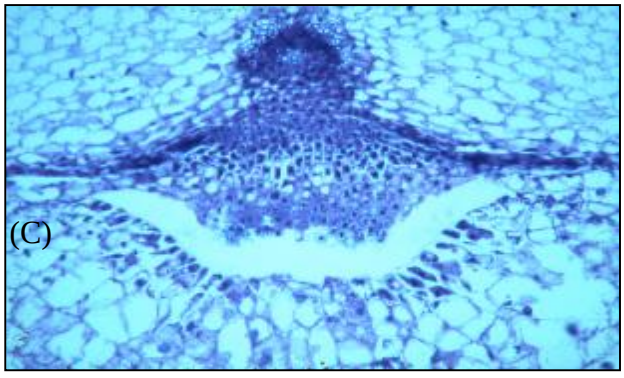


Fig. 3.2.2.15 (A) Histological organization of a barley caryopses at 6 DAF. ET, endospermal transfer cells; II, inner integument; NE, nucellar epidermis; NP, nucellar projection cells; OI, outer integument; P, pericary; SE, starchy endosperm; VT, vascular tissue.



(B) Transverse section from the middle part of a caryopses (8DAF). The barley caryopses was cross sectioned in 5µm and stained by toluidene blue.



(C) Central part of a caryopses with nucellar projection cells and endospermal transfer cells; transverse section, toluidine blue staining.

In situ hybridization showed that HvSUT2 mRNA is mainly expressed in the endospermal transfer cell layer (Weschke *et al*., 2000).

Localization of the HvSUT2 protein in membranes

To check the intracellular distribution of the HvSUT2 protein, a biotinylation internalization assay was used which enables examination of the cell surface proteins by immuno-chemical and immuno-cytological techniques (Crook *et al*., 1998). The cell surface proteins of protoplasts freshly isolated from a barley suspension culture were labeled by biotin. Afterwards, the membrane proteins and cytoplasmic proteins were fractionated (see Materials and methods 2.2.3.3). Both, cytoplasm- and plasma membrane fractions were detected by anti-biotin antibody (A) as well as the HvSUT2 specific antibody (B) after sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoreses (PAGE) (Figure 3.2.2.16).

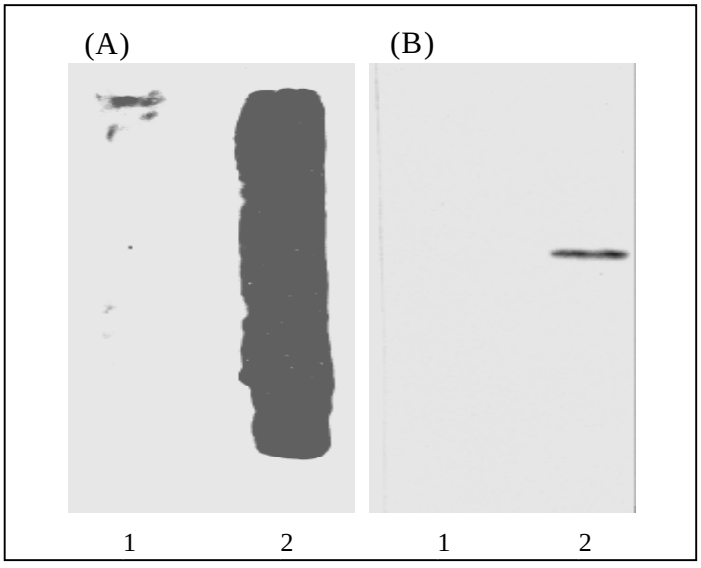


Fig. 3.2.2.16 Immunochemical detection of biotinylated membrane proteins (A) and the HvSUT2 protein (B) in cytoplasm (lane 1) and within the plasma membrane fraction (lane 2) of biotinylated barley protoplasts.

The HvSUT2 protein was only detected in the plasma membrane fraction.

Localization of the HvSUT2 protein on the sub-cellular level

To investigate the subcellular distribution of the HvSUT2 protein further and more precise, various subcellular fractions were isolated. Western blot analysis of the different cell fractions was carried out by using the HvSUT2 protein-specific antibody.

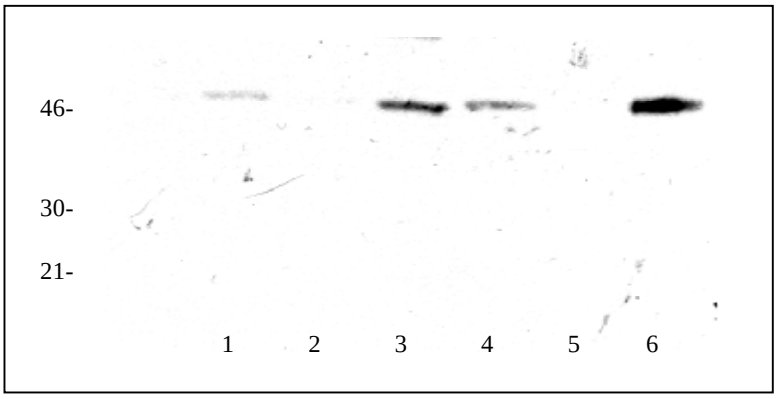


Fig. 3.2.2.17 Detection of the HvSUT2 protein by Western blot analysis of different cell fractions of barley protoplasts. Proteins isolated from three subcellular fractions, lane 1, microsomal fraction (100,000g pellet), lane 2, soluble fraction (100,000g supernatant), lane 3, plasma membrane fraction (18,000g pellet) as well as lane 4, total protein isolated from protoplasts, and lane 6, total protein isolated from barley caryopses (positive control) were separated by SDS-PAGE gel electrophoresis and analyzed by Western blotting. The molecular mass of marker proteins (lane 5) is indicated at the left.

The HvSUT2 protein was found mainly in the plasma membrane fraction. Only a very weak signal was seen in the microsomal fraction. Also vacuoles from barley protoplasts were isolated. By Western blot analysis, the HvSUT2 protein was not found within total proteins isolated from vacuole membranes (data not show). This result indicates that HvSUT2 protein is mainly localized in the cell plasma membrane, but not in tonoplast membrane.

3.3 Study of a sucrose binding protein-like protein (VfSPBL) of V. faba

The cDNA of a 52kD soybean SBP-homologue of *Vicia faba*, VfSBPL, was isolated and characterised. The VfSPBL gene (1,595bps, 482aa) was isolated from a cotyledon-specific cDNA library of *Vicia faba*. cDNA sequence analysis shows that this gene has high homology to the psp54 gene which was found in pea (Castillo *et al.*, 2000) and to the sucrose binding protein gene of soybean (Grimes *et al.*, 1992) (on the amino acid level, 85.3% to the psp54 gene and 51.5% to the SBP of soybean).

3.3.1 Protein expression pattern during seed development

To characterize the VfSBPL protein, a polyclonal anti-SBPL antibody was produced in rabbits using the whole VfSBPL protein as antigen. The specificity of the Anti-SBPL antibody was studied by adsorption tests (data not shown). It was shown that this antibody binds only to the VfSBPL protein which was over expressed in *E. coli.*

By using the anti-SBPL antibody, VfSBPL protein expression patterns during seed developmental and germination were studied by Western blot analysis and immuno-cytochemistry.

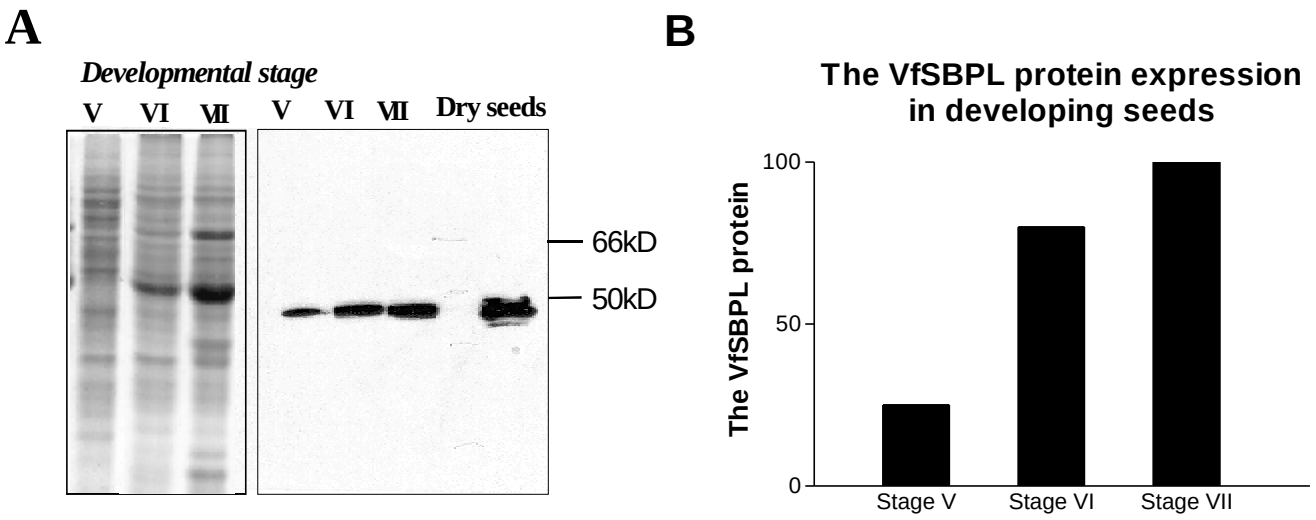


Fig. 3.3.1 VfSBPL (Sucrose Binding Protein Like) protein expression level during seed development. 50µg of total protein, isolated from dry seeds, developing cotyledons of stage VII (lane 3), stage VI (lane 4) and stage V (lane 5), were loaded onto a 12% SDS-PAGE gel. VfSBPL was detected by the anti-SBPL antibody.

Increasing amounts of the VfSBPL protein were detected during development, reflecting roughly the mRNA pattern (Heim *et al.*, 2001). In dry seeds, the protein accumulates to appreciable

amounts. Under the same experimental conditions no cross-reaction of the antibody with vicilin storage protein (the most abundant 50kD protein) was detectable.

3.3.2 Immuno-localization of the VfSPBL protein

To understand more about the function of the VfSBPL protein, it has been immuno-localized on the sub-cellular level by using electron microscopy.

During early seed development (stage V), the VfSBPL protein was mainly detected in the vacuole, weak labeling of the plasma membrane and the endoplasmic reticulum (ER) was found as well.

In seed development stages VI and VII, the VfSPBL protein was mainly detected in protein bodies. No membrane association was visible (see fig.3.3.2).

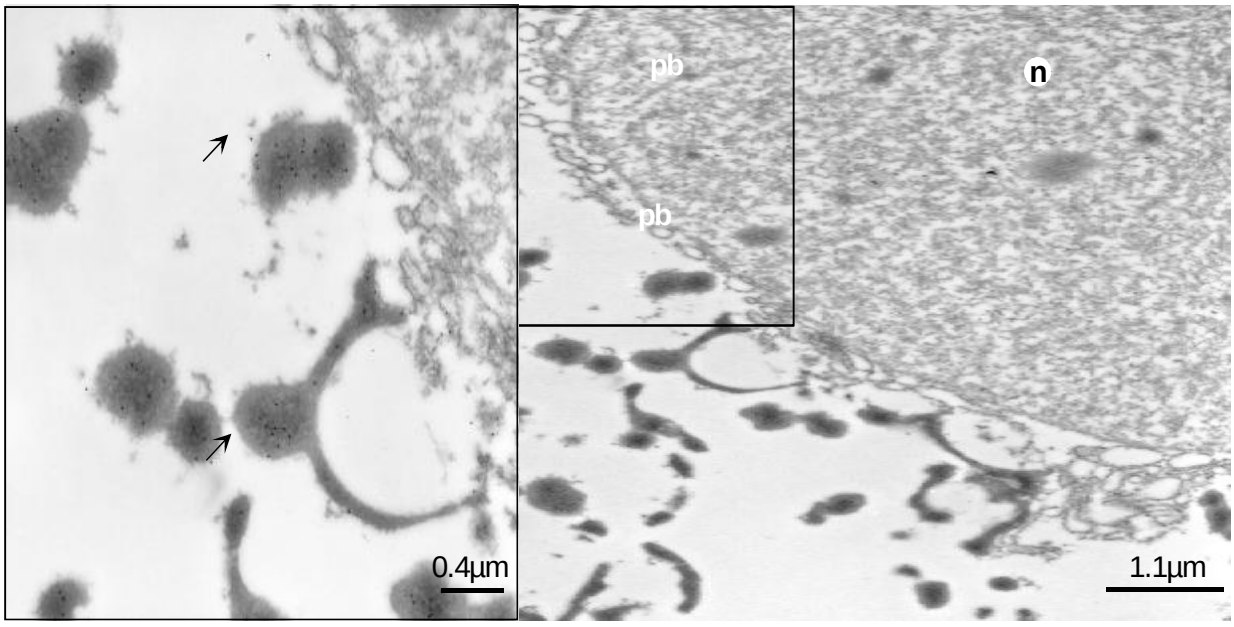


Fig. 3.3.2 Electron micrographs of a V. faba *cotyledon cell at stage VII showing immunogold-localization of VfSBPL in protein bodies. n = nucleus, pb = protein bodies. Left part of the figure shows an enlarged portion of the right micrograph. The arrows point to immuno-gold label.*

3.3.3 VfSBPL protein degradation during seed germinating

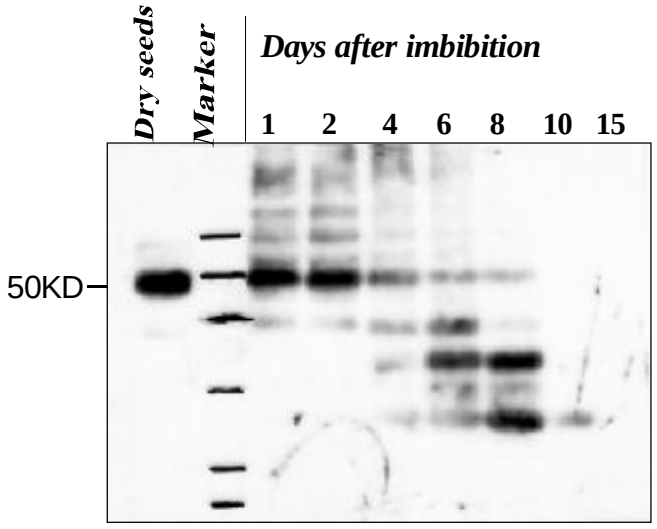


Fig. 3.3.3 Detection of the VfSPBL protein degradation during seed germination
Total protein was isolated from dry seeds and germinating seeds 1, 2, 4, 6, 8, 10 and 15 DAI (Days After Imbibition). In each lane, 50µg extracted protein was separated by SDS-PAGE gel. The blot was immuno-probed with affinity purified anti-VfSBPL antibody.
Western blot analysis results show that VfSBPL protein degradation begins at about 4 DAI.

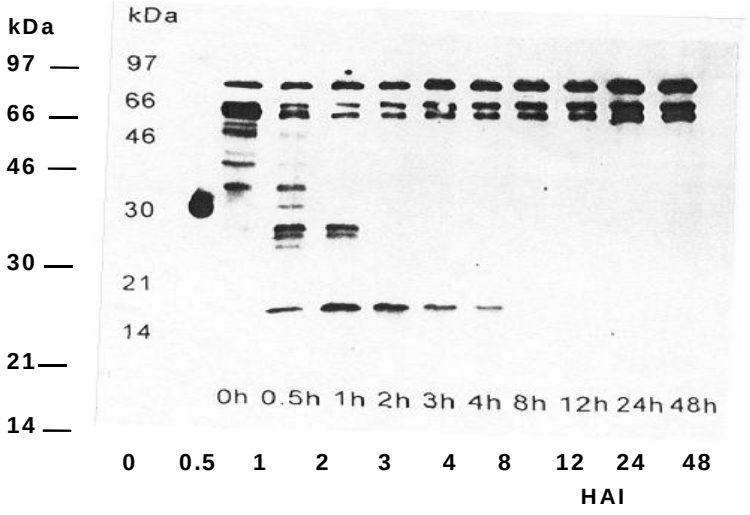


Fig. 3.3.4 Detection of V. faba *vicilin protein degradation during seed germination*
50µg of total protein isolated from seeds after 0, 0.5, 1, 2, 3, 4, 8, 12, 24 and 48 HAI (Hour After Imbibition), were loaded onto a 12% SDS-PAGE gel, and separated by protein electrophoresis. Vicilin was detected by an anti-vicilin-specific antibody.

Vicilin degradation begins at about 1 hour after imbibition.

Like a storage protein, VfSBPL was localised in protein bodies. To test the degradation pattern of VfSBPL in comparison to the 50kD storage protein vicilin, we determined the proteolytic degradation pattern during seed germination. As seen in Fig. 3.3.3, degradation of the VfSBPL protein starts later than that of vicilin (for vicilin degradation during germination of *Vicia sativa* seeds see Müntz *et al*., 1998) at about 4 days after imbibition.

4. Discussion

Plant seeds are typical sink organs. They are characterised by their storage function for mainly carbohydrates, proteins and/or oils. The carbon skeletons necessary for production of these compounds are predominantly derived from sucrose. Sucrose is mainly destinated by the source organs of the plants and transported via the vascular bundles to maternal tissues surrounding the embryosac which harbours the filial tissues. Young filial tissues are characterised by a high glucose to sucrose ratio resulting from high invertase activities especially found in maternal tissues (Weber et al., 1997). Later in development, invertase activity drops and sucrose concentration increases rapidly together with the mRNA level of sucrose transporters localized specifically in those cells which supply the filial tissues with assimilates (transfer cells). In parallel, specific enzymes of the carbohydrate metabolism, such as sucrose synthase and sucrose phosphate synthase are induced at both, the mRNA and enzyme level. The increasing activity of these enzymes points to the beginning of starch accumulation in the storage organs of different seeds, for instance in seeds of the dicot Vicia faba and the monocot Hordeum vulgare (Weber et al., 1997, Weschke et al., 2000).

Because the place of sucrose entrance, cleavage and symplastic transport, i.e. the maternal tissue is apoplastically isolated from the filial tissues, transporters are necessary to accumulate both types of sugars (monosacharide and disaccharide) against a concentration gradient into the growing part of the seed (filial tissue). As explained before, two types of sugars, the monosaccharides glucose and fructose, the disaccharide sucrose characterize the two different phases in the early development of the filial tissue:

1. the phase of cell division and elongation characterized by high monosaccharide concentrations (pre-storage phase);
2. the beginning of accumulation processes labelled by a rapid increase of disaccharide concentration (storage phase).

Therefore, hexose transport activities should be mainly expected very early during seed development whereas sucrose transporter(s) should be active later on.

4.1 Intracellular localization of the hexose- and the sucrose transport protein of **Vicia faba**

As shown before for VfSTP1 transcript levels (Weber et al., 1997), amount of the VfSTP1 protein is highest in cotyledons early in development (stage V). Decreasing protein amounts were found at later developmental stages (VI and VII). Parallels between mRNA- and protein levels were also registered regarding the tissue specificity of VfSTP1 expression. Next to cotyledons, VfSTP1 mRNA and protein is highest in roots followed by pods (see Fig. 3.1.1.5). However, VfSUT1 mRNA- and protein expression profiles during development are completely different. VfSUT1 mRNA is highest at stage V and decreases during later development, whereas VfSUT1 protein amount increases from stage V onwards and reaches its highest level at stage VII (compare Fig. 3B in Weber et al., 1997 and Fig. 3.2.1.6). Additional experiments are necessary to explain this difference.

12 membrane-spanning domains were identified in both proteins, VfSTP1 and SUT1. Therefore, membrane association of the two proteins should be expected. However, subcellular localization of both proteins shows distribution between the cytosol and the membrane system with changing ratios of the protein amount between the two compartments. For both transporters, the highest relative amount of membrane-bound protein was found during the storage phase (stage VII). Earlier in development, increasing relative amounts of the protein were found in the cytosol. This is especially true for VfSTP1 (compare Fig. 3.1.1.7 and 3.2.1.8). We conjecture that remarkable parts of the two proteins are only loosely or not bound to membranes. Especially for the hexose transporter, affinity to membranes seems to be very low early in development, i.e. during the phase of highest hexose concentration in the cotyledonary tissues and, therefore, highest expected transport activity. Better methods for determination of the developmental stages and separation of membrane protein are required to draw the conclusion. Again, this discrepancy can be explained only based on additional experiments.

Unfortunately, anti-peptide antibodies against both transport proteins recognize the antigene only on Western blot membranes but not in sections prepared for electron microscopy. Therefore, data regarding sub-cellular localization of the two transporters based on immuno-gold labelling are missing. The failure of EM-based sub-cellular localization experiments may be caused by the anti-peptide antibody itself. When the

peptide used for antibody preparation is not explored to the surface of the respective protein, it cannot be recognized. Additional problems may result from the preparation of slides for immuno-gold labelling, i.e. possibly, the techniques used for fixation, embedding and hybridization of the antibody to the tissue probes are not compatible to that type of antibodies used.

4.2 Sucrose and hexose transporters in developing barley caryopses

4.2.1 Two different sucrose transporters, HvSUT1 and HvSUT2, are expressed in developing caryopses.

The 198bp-fragment described in the result part of this work (3.2.2.1.) was the first isolated cDNA sequence coding for a monocot-specific sucrose transporter from plants. The amplified fragment contains a region common for all sucrose transporters known up to this moment. Besides of isolation of the more common sucrose transporter HvSUT1, using of this over-all homologous region in cDNA-library screening has given the possibility to isolate and identify a second member of the sucrose transporter gene family, HvSUT2, showing a very low degree of homology to all other known transporters specific for plant seeds. However, functional expression in yeast cells and sensitivity against protonophores indicates that both, HvSUT1 and HvSUT2 can mediate sucrose uptake and thus probably represent sucrose H^+ co-transporters. Compared with other carries' *K*m values (1 mM), HvSUT1 and HvSUT2 have a clearly higher *K*m of 7.5 and 5 mM, respectively. However, because no kinetic data are available from sucrose transporters of monocot species, these differences could also be simply ascribed to specific properties of transporters from monocots.

The difference between the two barley-grain specific sucrose transporters shown at the sequence level (Fig. 3.2.2.3) was functionally demonstrated by the analysis of their behaviour against glucose. The sucrose transport mediated by HvSUT1 in transgenic yeast cells is ***stimulated by glucose***, whereas HvSUT2-specific sucrose transport in the same system is ***glucose-repressed***. Furthermore, HvSUT2, as compared to HvSUT1, transports very low amounts of sucrose (N. Sauer, personal communications). Both, the outstanding glucose-repression of the HvSUT2-mediated sucrose transport, and the very low sucrose transport level, can be eventually explained by a sensor function of this protein (for speculations about the possible connection between the low level of sucrose transport and a putative sensor function of the appropriate transporter see

Barker *et al*., 2000). However, HvSUT2 does not contain the big cytoplasmic loop found within a new type of sugar transporters/sensors described for Arabidopsis, tomato and potato recently (Barker *et al*., 2000; Weise *et al*., 2000). Instead, HvSUT2 contains a very short motif (three amino acids in length), which is found in none of the sucrose transporters analysed up to now.

Different approaches can be pursued to get evidence for a possible HvSUT2 sensor function:

1. subcellular localisation of the HvSUT2 protein;
2. analysis of the *in vitro*-sugar regulation of both transporter mRNAs by using a barley suspension culture-system;
3. mutation analysis of the specific „three amino acid motif“ found only in HvSUT2;
4. antisense-approaches to suppress the HvSUT2-function in transgenic barley plants.

Subcellular localization of the HvSUT2 protein as one point of these possible approaches was part of this work (see result part of this work and below for discussion).

4.2.1.1 The HvSUT2 protein is present in endospermal transfer cells

The HvSUT2 protein, characterized by it’s amino acid sequence, is grouping rather to dicot sucrose transporters, identified as being active in the sweetening of grape berries (Davies et al., 1999) and sugar accumulation in carrot tape roots (Sturm et al., 1998), than to sucrose transporters from monocots (see Fig.3.2.2.3). A specific function of HvSUT2 in loading of sucrose into the vacuole of elongating cells was postulated especially from speculations made by Davies et al. (1999) as well as from the high HvSUT2 mRNA expression level found in the elongating part of young barley seedlings (W. Weschke, unpublished). The result of in situ hybridization data shows accumulation of the HvSUT2 mRNA in the caryopses tissue 6DAF (Weschke et al., 2000). This observation is also in accordance with results reported by Tegeder et al. (2000) showing the localization of a sucrose transport protein in the transfer cell layer of pea cotyledons. To verify this speculation by subcellular localization of the transporter protein, a polyclonal antibody against the complete HvSUT2 sequence was produced.

However, localization of the HvSUT2 protein in pericarp and in the nucellar epidermis as expected from the results of *in situ* hybridization failed because the HvSUT2

antibody shows cross-reactivities especially to starch-containing cells located especially in the pericarp and starchy endosperm (data not shown).

As apprehended from these results, it was not possible to use the HvSUT2 antibody successfully in immuno-gold labeling experiments. First of all, a very strong and non-compatible labeling of starch granules was visible as it was seen before on sections after immuno-flourescence staining. Two possibilities exist to interpret these two identical results performed by using of independent methods: 1. The antibody reacts in an unspecific way. This is rather unlikely because the protein used for antigen production was purified to a high extent and nearly no cross-reactivity was found by using the antibody in analysis of the HvSUT2 protein produced in yeast (see Fig. 3.2.2.12). 2. The antibody reaction is specific. In the moment, we are not able to find any convincing argument to explain the presence of a sucrose transporter in starch granule. Nevertheless, a relative high HvSUT2 mRNA expression level was found in the pericarp and relatively late in caryopses development, and the mRNA expression correlates in both cases to only moderate levels of sucrose (Weschke *et al.*, 2000). Possibly, a second HvSUT2-function in sink tissues is the transport of sucrose mobilized from starch granules into the cytoplasm. Because of the strong cross-reactivity of the antibody to starch granules, only a relatively low concentration of the antibody was used in the immuno-gold labeling procedure. The low antibody concentration and, furthermore, the expected localization of the protein in membranes (see the 12 membrane-spanning structure of HvSUT2, fig. 3.2.2.2 B) eventually preventing some epitopes against recognition by the antibody may be reasons for the disappointing results of subcellular HvSUT2-localisation by electron microscopy.

4.2.1.2 The HvSUT2 protein is localized in plasma membranes of barley protoplasts.

From our results, it is clear now, that the HvSUT2 protein is localized mainly in plasma membranes. The first positive result was coming from a specific immuno-fluorescence staining procedure using freshly isolated protoplasts from a barley suspension culture as a target. Using the HvSUT2-specific antibody, a strong fluorescence of the outer protoplast membrane was visible. Using the antibody saturated before by the antigen for negative control only about 20% of the fluorescence measured before were provable (see Fig. 3.2.2.14). However, from the technique we have used for immuno-staining of the protoplasts it was unclear whether any pass of the antibody through the plasma membrane can be expected. Therefore, we have used a biotinylation internalization

assay (Crook *et al.*, 1998) to label at first the protoplast membranes by biotin. Afterwards, the membranes were fractionated, and the proteins isolated from the membrane fractions were analyzed by Western blotting. As shown in Fig. 3.2.2.18, HvSUT2 protein was found mainly in the plasma membrane fraction. Additionally, a less intensive but clearly visible signal was found in the microsomal fraction.

Besides of these two biotinylation assays, we have tried to isolate vacuoles from barley protoplasts. Western blot analyses were performed, but no HvSUT2-specific signal was found in the vacuolar membrane fraction (data not shown).

Taking in account especially the result of the last experiment, localization of the HvSUT2 protein in tonoplast membranes as speculated before, is unlikely. Because it is localized in plasma membranes, the HvSUT2 protein may be part of a sensor / transporter complex built up together with HvSUT1 at least in the transfer cells of the starchy endosperm during filling phase of the barley grain. Similar complexes are discussed for mammalians, and their presence is also speculated for plant transfer tissues (Reinders et al., 2002)

However, HvSUT2 function within the maternal pericarp remains unclear. It is possibly related to a function for sucrose distribution in maturing tissues as discussed for the grape berry transporter VvSUT1 by Ageorges et al., (2000).

4.2.1.3 The structure of the genomic HvSUT1 gene as compared to known genomic sucrose transporter sequences

The only genomic sucrose transporter sequences published up to now are reported by Barker et al. (2000) for two members of the SUT2 gene family described as being putative sucrose sensors in tomato (LeSUT2) and Arabidopsis (AtSUT2). The two transporter genes show a high conservation of the number of exons/introns. Thus, 14 exons/13 introns have been identified for the AtSUT2 transporter whereas 13 exons/13 introns were found in the incomplete genomic sequence of LeSUT2. Comparing these results to the exon/intron structure of HvSUT1 (9 exons, 10 introns), a clear difference is visible. However, similarities can also be found. The first intron is the longest one in all three transporters sequences. Furthermore, the middle part is characterized by a stretch of very short exons/introns but the number of exons is different (6 for these two SUT2 transporters, 5 for HvSUT1). Possibly, the additional exon sequence codes for the big cytoplasmic loop, which characterizes the two putative sensors. On the other hand, the 3' part of the genomic sequences is completely different between the two members of the

SUT2 group and HvSUT1. Here, specific functions can be encoded separating the putative sensors from the barley gene coding for a protein, which shows all characteristics of a quite normal sucrose transporter (Weschke et al., 2000). It is mainly expressed in the endospermal transfer cells which represent the putative site of assimilate exchange between the maternal and the filial part of the caryopses. Further more, HvSUT1 expression correlates with the rapid increase of sucrose concentration in the filial part of the caryopses immediately before the beginning of the grain filling period.

4.2.2 The maternal and the filial tissues of developing caryopses are served by two different hexose transporter isoforms

Contrary to *V. faba* where only one hexose transporter isoform was identified (Weber ***et al.,*** 1997), two isoforms are expressed in barley seeds. Quantitatively, accumulating barley grains are mostly built up by the endosperm, the storage organ for starch as well as protein. Storage and reproductive function are separated in barley seeds and realised by two genetically different tissues, the triploid endosperm and the diploid embryo, respectively. Legume seeds accumulate storage compounds in cotyledons, which are part of the embryo proper. The legume endosperm is a non-persisting tissue and fulfils only a transient storage function. Because storage processes are realised in both, the endosperm of barley and the cotyledons of *V. faba* seeds, some parallels in the development and of the two tissues should be expected.

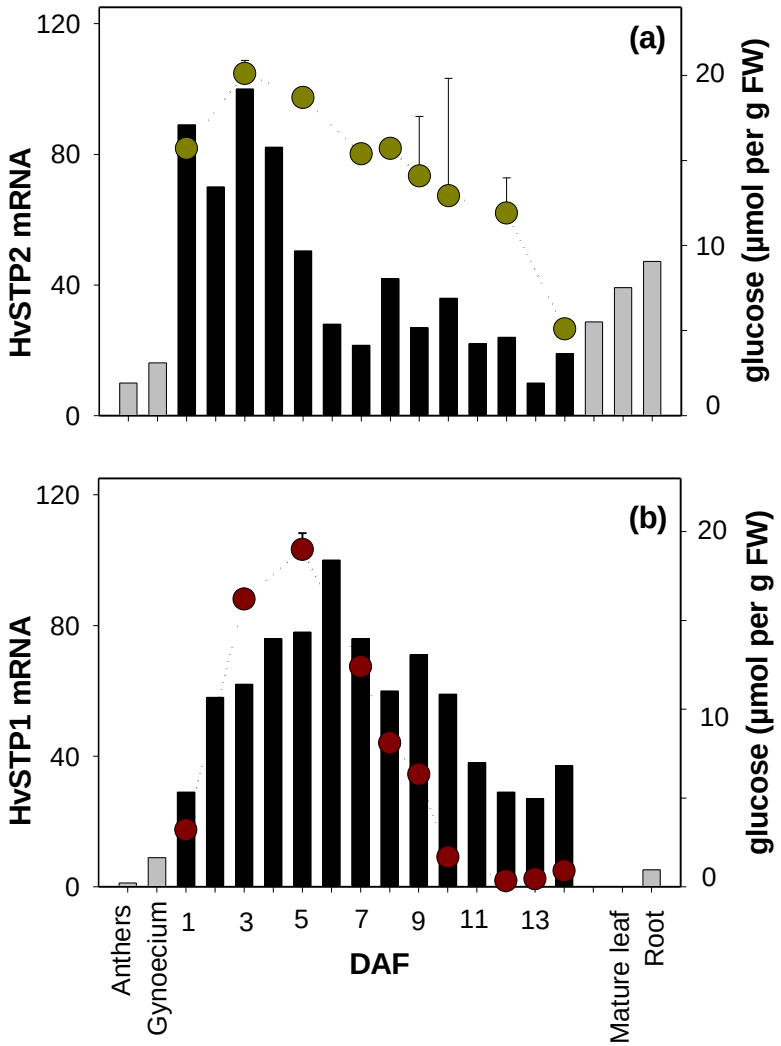


Fig. 4.1.2.1 HvSTP2 *and* HvSTP1 *transcript level (black bars) estimated from whole developing caryopses and compared to the glucose amount in maternal [(a), yellow circles] and filial tissues [(b), red cycles].*

High hexose concentrations and, additionally, high hexose/sucrose ratios are typically found in developing legume cotyledons during pre-storage phase characterized by cell division and -elongation processes (Weber *et al.* 1997, Wobus and Weber, 1999). The high hexose concentration results from the activity of a cell wall-bound invertase localized in the thin-walled parenchyma of the seed coat (Weber *et al.*, 1995). mRNA of the *V. faba*-specific hexose transporter VfSTP1 is localised mainly in the young cotyledons (develop stage V) and the endosperm of developing *V. faba* seeds. A

function in supporting the young dividing cotyledon tissue with hexoses is discussed (Weber *et al.*, 1997).

Hexose levels within the maternal fraction of young barley caryopses are high at 1 DAF, remained constant at about 30 µmol g^{-1} throughout development and decreased only at 12 DAF when the maternal tissue degrades (Fig. 4.1.2.1; the hexose levels were taken from Weschke *et al.*, 2002). In the filial fraction, levels increased from low values at 1 DAF, reach highest levels at 5 DAF and decreased to very low levels after 10 DAF. As shown in Fig. 4.1.2.1, hexose levels estimated in both, the maternal and the filial fraction of developing barley grains correlate to the level of *HvSTP2*-and *HvSTP1*-mRNA, respectively. To prove the thesis that HvSTP2 specifically serves the maternal tissues whereas HvSTP1 delivers hexoses to feed the young fast dividing cells of the starchy endosperm, transcripts of the two hexose transporter were localised by *in situ* hybridization (Fig. 4.1.2.2, taken from Weschke *et al*., 2002).

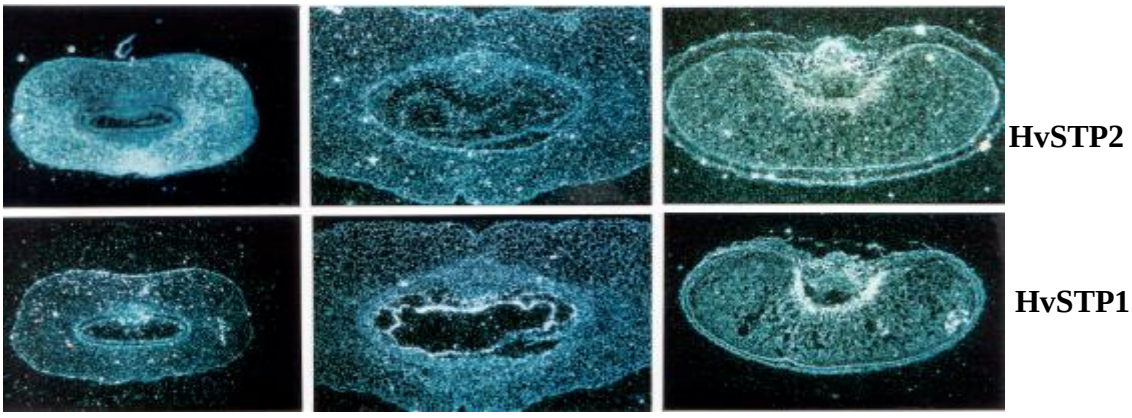


Fig. 4.1.2.2 Tissue-specific accumulation of hexose transporter HvSTP1- *und* HvSTP2-*mRNA in transverse sections of young developing caryopses as shown by* in situ *hybridization.*

HvSTP2-specific signals were present in most of the pericarp cells at 2 DAF with strongest labelling of the dorsal region and around the lateral veins. At 3-4 DAF, labelling was generally weak. At 7-8 AF, *HvSTP2*-specific signals were present mainly within endospermal transfer cells and in cells surrounding the crease vein. On the contrary, label specific for *HvSTP1*-mRNA was very low in pericarp at 2 DAF. Later on, at 3-4 DAF a strong signal could be detected in the syncytial endosperm layer. At

7-8 DAF, a strong signal occurred in the endospermal transfer cells similar to but much stronger than that of the *HvSTP2*-mRNA. *In situ* hybridization results show that HvSTP1 is nearly endosperm-specific with preference to the syncytial stage and, later in development, fully differentiated transfer cells. On the contrary, HvSTP2 shows a broader range of expression and is mainly active in the young pericarp.

As shown by further experiments (Weschke *et al.*, 2002), expression of the hexose transporter HvSTP2 correlates with the spatial expression pattern of cell wall-bound invertase HvCWINV2, whereas invertase HvCWINV1 and hexose transporter HvSTP1 are co-ordinately expressed, during early development (3 DAF) in the first cell rows of the growing starchy endosperm and later in development in endospermal transfer cells. We conclude that the maternal-filial boundary of the barley caryopses is most probably involved in the transfer of glucose already at early stages. Accordingly, hexose levels within the embryosac are highest at this stage. Together with cell wall-bound invertases, especially HvCWINV1, HvSTP1 is responsible for providing fast dividing endosperm cells with hexoses. On the other hand, hexoses generated within the maternal pericarp mainly by HvCWINV2 were immediately transported into the respective cells by the hexose transporter HvSTP2. As shown before for sucrose (Weschke *et al.*, 2000), maternal as well as filial-specific producing and transporting systems exist for hexoses, too.

*4.2.3 Similarities and differences of sugar transport activities in developing dicot (***V. faba***) and monocot (***H. vulgare***) seeds*

In dicot and monocot plants, genetically different parts of the seed are used to deposit storage compounds. In monocots like barley, mostly starch is deposited in the triploid starchy endosperm whereas legumes like the faba bean store starch and protein in comparable amounts in the cotyledons being part of the diploid embryo. Further more, organization of the maternal tissues is different. V. faba seeds are delivered by the funiculus connecting the pod to the seed coat. The maternal tissues of the barley caryopses are not divided into two parts. The pericarp harbouring the main vascular bundle and, during early development, additionally three small veins is directly connected to the integuments and the nucellus tissue. Nevertheless, gradients of hexoses and sucrose measured in the filial seed part of the two species are comparable regarding the two main developmental stages (pre-storage and storage phase), and the change in

the sucrose-to-hexose ratio described as being a type of marker labelling the transition from the pre-storage to the storage phase of V. faba seeds was found in barley, too (Weber et al., 1996 and Weschke et al., 2002). The described similarity may result from the activity of cell wall-bound invertases (CWINV) found to be specifically expressed in the maternal and the filial seed part of the two species. However, whereas a maternal- and a filial-specific CWINV isoform was identified in both species, only one hexose- and one sucrose transporter were described for V. faba. Both transporters are mainly expressed in the transfer cell layer of the cotyledons (Weber et al., 1997). For barley, two seed-specific isoforms of hexose- as well as sucrose transporters have been identified, one of them mainly expressed in the maternal pericarp, the other one in endospermal transfer cells (Weschke et al., 2002 and 2000, respectively). The two hexose transporters are co-ordinately expressed together with that CWINV isoform specific for the respective part of the seed. The question whether only one isoform of hexose- and sucrose transporters serves all parts of the V. faba seed cannot be answered yet. However, the existence of maternal-specific isoforms of the two sugar transporters not identified up to now can be supposed derived from the results found in barley.

*4.3 The sucrose binding protein-like gene from V. faba (***VfSBPL***)*

4.3.1 VfSBPL exhibits structural similarities to SBP genes from soybean and pea.

A V. faba cDNA fragment showing 70% homology to the soybean sucrose-binding protein (GmSBP) described by Grimes et al. (1992) was amplified from double-stranded cotyledon-specific cDNA by PCR. The full-length cDNA clone isolated after hybridisation of the VfSBPL-specific fragment to a phage cDNA library displays a single open reading frame encoding 482 amino acid residues. The resulting VfSBPL protein has a predicted molecular mass of 54.6 kDa (Heim et al., 2001).

Several members of the SBP family have been described only from legumes at either the cDNA and/or the protein level. Besides of the 62 kDa GmSBP (Grimes et al., 1992), a second soybean SBP with a molecular mass of 64 kDa and 91% homology to GmSBP at the nucleotide level was found (Pedra et al., 2000). Further more, a pea SBP with a deduced molecular mass of 54 kDa was described by Castillo et al. (2000). On the amino acid level, 85% homology was found between the SBP protein from pea and the V. faba SBPL described here.

Different software packages were used to predict the tertiary structure of the VfSBPL protein. A typical trans-membrane domain was identified showing in addition signal peptide character. By sequencing, the first 11 amino acids of the mature SBP protein of V. narbonensis were identified. The sequenced residues were found to be identical with the respective residues of the mature VfSBPL (Wüstenhagen and Müntz, personal communication) indicating that the potential signal sequence is cleaved off at the predicted site by processing enzymes.

4.3.2 The VfSBPL protein has no sucrose transport activity but its accumulation and degradation behaviour is comparable to that of storage proteins.

The VfSBPL cDNA was cloned into the vector NEV-E and transformed into a specific yeast strain, which allows the detection of sucrose transport through the yeast cell membrane. Contradictory to results of Overvoorde et al. (1996) for GmSBP, not such transport could be detected for VfSBPL. Nevertheless, this result does not exclude the principle ability of VfSBPL to bind sucrose.

A polyclonal antibody raised against a recombinant fusion protein was produced in rabbits, and accumulation of VfSBPL was analysed in developing and germinating faba bean seeds. In developing seeds, VfSBPL accumulates from stage V onwards to high amounts measured in dry seeds, i.e. like a storage protein during seed maturation. In germinating seeds, degradation of VfSBPL as measured by SDS gel electrophoresis starts at about 4 days after imbibition. As visible by comparison of Fig. 3.3.3 and 3.3.4, the time scale of degradation is not comparable between VfSBPL and vicilin, that type of *V. faba* storage proteins showing homology and some evolutionary relations to SBPL (Braun *et al.*, 1996, Grimes and Overvoorde, 1996). However, VfSBPL is stably accumulated in dry seeds and mobilized immediately after imbibition, i.e. it shows properties comparable in general to those of storage proteins.

To analyse the SBPL accumulation pattern at subcellular level, the SBPL-specific antibody was used in electron microscopic immuno-localisation studies. At the beginning of the storage phase, gold grains were detected mainly in vacuoles but at lower level also at the plasma membrane and the endoplasmic reticulum (data not shown). Later in development (stage VII) label was found exclusively at protein clumps (Fig. 3.3.2) formed at this developmental stage within storage vacuoles. The same pattern of labelling was described for legumin and vicilin (Fischer et al., 2000).

4.3.3 The sucrose binding proteins of legumes – structurally related to each other but different in function?

The functional characterisation of the first isolated 62 kDa protein from soybean seeds (GmSBP) as sucrose binding protein was based on several lines of evidence as the tight plasma membrane association and the immuno-localisation in phloem companion cells and cotyledon transfer cells (see Grimes *et al.*, 1992). The data published on functional characterization in yeast (Overoode *et al.*, 1996) suggest very limited but provable sucrose transport across yeast cell membranes. However, these data are not corroborated by structural features because the 62 kDa protein does not contain any membrane spanning domain and should, therefore, form no pores for direct transport. Recently, Pedra *et al.* (2000) reported that a soybean cDNA coding for a 64 kDa GmSBP, closely related to the 62 kDa GmSBP, affects plant growth and carbohydrate partitioning in leaves of transgenic tobacco plants when expressed in sense and antisense orientation under control of the 35S promoter suggesting a SBP function in sucrose partitioning. These data are clearly different from the results reported about over-expression of the VfSBPL in transgenic potato plants (Heim *et al.*, 2001).

Another function of SBP proteins with respect to desiccation was suggested by the work of Castillo *et al.* (2000). These authors isolated a 16 kDa protein from nuclei of pea embryo axis before germination. The amino acid sequence was found to be homologue to the 3'-terminal part of a pea cDNA encoding a 54.4 kDa protein 85% homologous to the VfSBPL described in this work. No indications referring to the presence of a protein homologous to the pea 16 kDa sequence were found in *V. faba*. Neither we have seen a 16 kDa band in our Western blot analyses nor any labelling was found over nuclei in the immuno-localisation studies.

The 52 kDA VfSBPL shows several characteristics of a storage protein. It accumulates in protein storage vacuoles and is mobilised during early stages of germination. Further more, it is structurally related to vicilin storage proteins (Heim *et al.*, 2001). However, because the gene has been separated from typical vicilin storage proteins early in evolution, preserved functions or functions in addition can be suggested. Based on the data available for different sucrose binding- or sucrose binding-like proteins from different legume species we suggest that these proteins may not only be different in size but also in function. Further clarifying experiments especially for the VfSBPL are under way.

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Publikationen

Aus den, in dieser Arbeit dargestellten, Ergebnissen sind bislang folgende Publikationen hervorgegangen, die im Text nicht zitiert werden.

1 Ute Heim, Qing Wang, Thorsten Kurz, Ljudmilla Borisjuk, Sabine Golombek, Birgit Neubohn, Klaus Adler, Norbert Sauer, Hans Weber, Ulrich Wobus. (2001) A 52-kD sucrose binding protein homologue of *Vicia faba*, VFSBPLL-vicilin, has functional characteristics of a storage protein. *Plant Mol Biol* 47(4), 461-474

2 Winfriede Weschke, Reinhard Panitz, Norbert Sauer, Qing Wang, Birgit Neubohn, Hans Weber and Ulrich Wobus. (2000) Sucrose transport into barley seeds: molecular characterization of two transporters and implications for seed development and starch accumulation. *The Plant Journal* 21(5), 455-467.

3 Winfriede Weschke, Reinhard Panitz, Sabine Gubatz, Qing Wang, Ruslana Radchuk, Hans Weber and Ulrich Wobus (2002). The role of invertases and hexose transporters in controlling sugar ratios in maternal and filial tissues of barley caryopses during early development. *The Plant Journal* 32, 1-17.

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Erklärungen gemäß §5 Abs.2 der Promotionsordnung vom 17.09.1998

Ich habe mich vor Einleitung dieses Promotionsverfahrens noch nie anderweitig um einen Doktorgrad beworben.

Ich habe diese Arbeit selbständig und ohne fremde Hilfe verfaßt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet. Verwendete Quellen und Hilfsmittel sind an den entsprechenden Stellen kenntlich gemacht.

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Gatersleben, March 2003

Declaration

I hereby declare that, all the work presented in this manuscript is my own, carried out solely with the help of the literature and aid cited.

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Gatersleben, March 2003