Dissertation

Functional analysis of the sucrose synthase gene family in *Arabidopsis thaliana*

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Człowiek może znieść bardzo dużo,
lecz pełnia błąd sądząc,
że potrafi znieść wszystko

Fiodor Dostojewski

Abstract

Sucrose synthase (Susy) is a key enzyme of sucrose metabolism, catalysing the reversible conversion of sucrose and UDP to UDP-glucose and fructose. Therefore, its activity, localization and function have been studied in various plant species. It has been shown that Susy can play a role in supplying energy in companion cells for phloem loading (Fu and Park, 1995), provides substrates for starch synthesis (Zrenner *et al*., 1995), and supplies UDP-glucose for cell wall synthesis (Haigler *et al*., 2001).

Analysis of the *Arabidopsis* genome identifies six Susy isoforms. The expression of these isoforms was investigated using promoter-reporter gene constructs (GUS) and real time RT-PCR. Although these isoforms are closely related at the protein level they have radically different spatial and temporal patterns of expression in the plant with no two isoforms showing the same distribution. More than one isoform is expressed in all organs examined. Some of them have high but specific expression in particular organs or developmental stages whilst others are constantly expressed throughout the whole plant and across various stages of development.

The *in planta* function of the six Susy isoforms were explored through analysis of T-DNA insertion mutants and RNA_i lines. Plants without the expression of individual isoforms show no differences in growth and development, and are not significantly different from wild type plants in soluble sugars, starch and cellulose contents under all growth conditions investigated. Analysis of T-DNA insertion mutant lacking *Sus3* isoform that was exclusively expressed in stomata cells only had a minor influence on guard cell osmoregulation and/or bioenergetics.

Although none of the sucrose synthases appear to be essential for normal growth under our standard growth conditions, they may be necessary for growth under stress conditions. Different isoforms of sucrose synthase respond differently to various abiotic stresses. It has been shown that oxygen deprivation up regulates *Sus1* and *Sus4* and increases total Susy activity. However, the analysis of the plants with reduced expression of both *Sus1* and *Sus4* revealed no obvious effects on plant performance under oxygen deprivation. Low temperature up regulates *Sus1* expression but the loss of this isoform has no effect on the freezing tolerance of non acclimated and cold acclimated plants. These data provide a comprehensive overview of the expression of this gene family which supports some of the previously reported roles for Susy and indicates the involvement of specific isoforms in metabolism and/or signalling.

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Abbreviations

°C	degree Celsius
µg	micro gram
µl	micro litre
µM	micro-molar
µmol	micro mol
ACC	acclimated
ATP	adenosine triphosphate
BLAST	basic local alignment tool
bp	base-pairs
cDNA	complementary DNA
CDPK	calcium dependent protein kinase
Col-0	Columbia 0
c_t	threshold cycle
DNA	desoxyribonucleic acid
dNTP	desoxy nucleotides
dsDNA	double stranded DNA
ER	endoplasmic reticulum
EST	expressed sequences tags
et al	and others
ETR	electron transport rate
fmol	femto mol
Fru	fructose
Glc	glucose
Gol	galactinol
GUS	β-glucoronidase gene
h	hour
HPLC	high pressure liquid chromatography
HYG	hygromycine
JIC	John Innes Centre
KAN	kanamycine
kb	kilo-base
KO	knock out
l	litre
LT	electrolyte leakage
m	meter
mg	milligram
ml	millilitre
mM	milli-molar
mmol	milli mol
MPIMP	Max Planck Institute of Molecular Plant Physiology
mRNA	messenger RNA
N_2	nitrogen (gaseous)
NA	non acclimated
NAD(P)H	nicotiamideadeninedinucleotide (phosphate)
NCBI	National Centre for Biotechnology Information
OKA	okadaic acid
PAR	photosynthetically active radiance
PCR	polymerase chain reaction

PEP	phosphoenolpyruvate
PEPC	phosphoenolpyruvate carboxylase
pH	*lat.* pondus hydrogenii
Pi	inorganic phosphate
pI	isoeletric point
PPi	inorganic pyroposphate
ppm	parts per million
Raf	raffinose
RNA	ribonucleic acid
RNAi	interference RNA
RT	room temperature
RT-PCR	reverse transcriptase PCR
s	second
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel-electrophoresis
Ser	serine
SPPase	sucrose phosphate phosphatase
SPS	sucrose phosphate synthase
Suc	sucrose
Susy	sucrose synthase
TCA	tricarboxylic acid cycle
T-DNA	transfer DNA
TGT	target value
TIGR	The Institute for Genomic Research
TP	triose phosphate
UDP	urdine diphosphate
UDPG	UDP-glucose
UGPase	UDPglucose pyrophosphorylase
UK	United Kingdom
UTP	uridine triphosphate
UTR	untranslated region
WGS	whole genome shotgun
WS	Wassilewskija
WT	wild type

1 Introduction

Sucrose plays a central role in the metabolism of higher plants. It functions as a primary product of carbon fixation, a storage compound (e.g., sugar beet, sugar cane, carrot), the principal long-distance transport sugar in many plants and also acts as an important signalling molecule (Koch, 2004).

1.1 Sucrose synthesis

Sucrose is generally synthesized in green leaves, as one of the primary end products of leaf photosynthesis. It is also accepted that, in particular cases, its biosynthesis can occur in some developing sink tissues (e.g., tomato and sugar beet root) and non-photosynthetic source tissues (e.g., germinating seeds).

During the day, the substrate for sucrose biosynthesis is triose phosphate (TP), which is released from the chloroplasts through the triose phosphate translocator in exchange for inorganic phosphate (Pi). TP is converted in the cytoplasm to fructose-1,6-bisphosphate by fructose-1,6-bisphosphate aldolase and then by fructose-1,6-bisphosphatase into fructose-6-phosphate. Fructose-6-phosphate is further converted by phosphoglucoisomerase into glucose-6-phosphate which is then transferred into glucose-1-phosphate by phosphoglucomutase. In the next step UTP is incorporated by UDP-glucose pyrophosphorylase (UGPase) into glucose-1-phosphate to produce UDP-glucose with the release of inorganic pyrophosphate. UDP-glucose (UDPG) is further converted into sucrose-6-phosphate and sucrose by sucrose phosphate synthase (SPS) and sucrose phosphate phosphatase (SPPase), respectively. At night, substrate for sucrose biosynthesis is derived from starch mobilization, possibly by amylolytic breakdown of starch. Degradation of the chloroplast starch by phosphorylase produces glucose-1-phosphate which continues to supply triose phosphate for export and for sucrose synthesis. Glucose and maltose produced during amylolysis also reach the cytoplasm, this can occur directly but is mostly via conversion to triose-3-phosphate.

Besides transcriptional and post-transcriptional regulation of genes encoding for the enzymes of sucrose biosynthesis there are several mechanisms which regulate sucrose synthesis. Accumulation of sucrose in leaves increases the phosphorylation of SPS and its activity decreases (Siegl *et al.*, 1990). The enzyme has also been shown to function as a substrate for SNF-1 related protein kinases (Sugden *et al.*, 1999) which may be important

for modulating its activity. Additionally, the activity of SPS is regulated by glucose-6-phosphate and inorganic phosphate which respectively act as activator and inhibitor (Doehlert and Huber, 1983). Lower hexose phosphate utilization by sucrose synthesis stimulates the synthesis of fructose-2,6-bisphosphate, which inhibits cytosolic fructose-1,6-bisphosphatase activity (Stitt, 1990). This, as well as lower Pi liberation, leads to carbon retention in the chloroplasts for starch synthesis. The importance of fructose-2,6-bisphophate in controlling sucrose synthesis has been shown in transgenic tobacco where elevated concentrations of this metabolite led to decreased fluxes of carbon to soluble sugars, organic acids and amino acids, while enhancing starch accumulation (Scott *et al.*, 1995).

1.2 Transport of sucrose

Many plants use sucrose as the transport metabolite for long distance carbon transport. Generally, two mechanisms of phloem loading are discussed: a symplasmic path via plasmodesmata connecting mesophyll and phloem, a pathway proposed to involve a 'polymer trapping' mechanism of oligosaccharide synthesis to establish a concentration gradient (Turgeon, 1996). Alternatively, or additionally (Turgeon and Medville, 2004), phloem loading can occur via an apoplastic route, in which sucrose is first transported into the apoplast and than is taken up into the sieve element-companion cell complex by an energy-dependent transport system (Van Bel, 1993).

Sucrose transporters play an essential role in apoplastic phloem loading. One of the best characterized sucrose transporters is the potato SUT1 transporter which is highly expressed in the phloem of leaf minor veins (the major site of phloem loading), stem and sink tissues (Riesmeier *et al.*, 1993). Antisense RNA inhibition of SUT1 expression led to strong accumulation of carbohydrates in source leaves and decreased export of sugars to sink organs causing a dramatic reduction in tuber yield (Riesmeier *et al.*, 1994; Lemoine *et al.*, 1996), indicating that SUT1 is essential for carbohydrate partitioning. So far, over twenty different genes for disaccharide transporter have been identified from various plant species (Truernit, 2001).

Phloem unloading can differ between plant species and several tissues, organs and developmental stages (Turgeon, 1989). The efficiency of this process depends on the sink strength of the corresponding tissue. Therefore, different models of phloem unloading can be distinguished. In sink organs, the assimilate concentration is lower than in phloem, thus

unloading by diffusion along a concentration gradient is possible. In order to maintain such a concentration gradient, sucrose is either enzymatically cleaved, stored in subcellular compartments (e.g. vacuole) or converted into osmotically less efficient storage molecules (e. g. starch).

Symplasmic phloem unloading via plasmodesmata has been postulated for sink leaves of tobacco and barley (Kuehn *et al.*, 1999). Several studies on roots have shown that unloading in this tissue can also occur symplasmically (Farrar, 1985). In seeds, the embryonal tissue is symplasmically isolated from the maternal tissue therefore apoplastic solute transport is strongly indicated (Thorne, 1985). The symplasmic connections between the conducting phloem cells and the storage parenchyma cells of potato tubers are numerous thus indicating that symplasmic phloem unloading is possible (Frommer and Sonnewald, 1995).

1.3 Sucrose breakdown

There are two enzymatic paths of sucrose cleavage in plants catalysed either by sucrose synthases (Susy) or invertases. Both of these paths typically degrade sucrose *in vivo* but importantly the products of these reactions significantly differ. Susy (EC 2.4.1.13) is a glycosyl transferase, which catalyses the readily reversible breakdown of sucrose to UDP-glucose (UDPG) and fructose in presence of UDP. Whereas the other enzyme, invertase (EC 3.2.1.26), catalyses the irreversible hydrolysis of sucrose to its component monosaccharides.

Sucrose + UDP = UDP-glucose + fructose
$\Delta G°' = -3.99$ kJ mol^{-1}

Sucrose + H_2O = glucose + fructose
$\Delta G°' = -29.3$ kJ mol^{-1}

Susy is mainly present in the cytoplasm of plant cells but may also be tightly bound to the cellulose synthase complex or actin cytoskeleton (Amor *et al.*, 1995; Sturm and Tang, 1999). Invertase isoforms with different pH optima and biochemical properties are found in the cytoplasm (neutral or alkaline invertases), the vacuole (vacuolar acid invertase), and in

the extracellular space (cell wall acid invertase). The regulation of these enzymes and their importance during plant growth and development will be reviewed in the following sections.

1.3.1 Sucrose synthase

Multiple isoforms of Susy have been identified in many plant and related species. *Physcomitrella,* appears to have four Susy isoforms. In maize, the presence of three genes (*Sus1*, *Sh1* and *Sus3*) was reported (Carlson *et al.*, 2002). In pea, three genes coding Susy isoforms (*Sus1, Sus2* and *Sus3*) have been cloned although Southern blots of genomic DNA revealed at least five genes coding *Sus*-like proteins (Barratt *et al.*, 2001). Sequence analysis of the rice genome revealed six isoforms (Harada *et al.*, 2005). Three Susy isoforms were identified in potato (Fu and Park, 1995) and three in cotton fibers (Haigler *et al.*, 2001).

The release of the complete Arabidopsis genome allowed the identification of six distinct members of the Susy family. Comparison of the deduced amino acid sequences shows that Arabidopsis Susy genes form three classes (Baud *et al.*, 2004). SUS1 and SUS4 are 89% identical to each other, but less than 68% identical to other isoforms. SUS2 and SUS3 are 74% identical to each other but 67% or less identical to other isoforms, and SUS5 and SUS6 are 58% identical to each other but 48% or less identical to other isoforms. SUS5 and SUS6 both have C-terminal extensions of 3 kDa and 14 kDa, respectively, relative to other isoforms. Based on these comparisons *Sus1* (at5g20830) and S*us4* (at3g43190) can be classified among Dicot SUS1 group, *Sus2* (at5g49190) and *Sus3* (at4g02280) belong to the mixed, monocotyledonous and dicotyledonous SUSA group, whereas *Sus5* (at5g37180) and *Sus6* (at1g73370) constitute a separate group on their own (Baud *et al.*, 2004). Comparison of deduced amino acid sequences of different sucrose syntheses is presented on the phylogenetic tree on Figure 3.

1.3.1.1 Cell and tissue specific expression

Tissue specific expression of Susy isoforms has been shown in many systems. The maize *Sh1* promoter was shown to be expressed mainly in the developing endosperm but also in the phloem of leaves, flowers, fruits and roots (Winter and Huber, 2000). The Arabidopsis *Sus1* promoter showed expression in roots and vascular tissue of leaves (Martin *et al.*, 1993). In root nodules of legumes a major nodule-enhanced protein (nodulin-

100) has been identified as a sucrose synthase protein. The level of RNA and protein were higher in nitrogen-fixing nodules than in other tissues and organs of legume plants. Susy activity was shown to increase rapidly during nodule development and to decrease during nodule senescence (Thummler and Verma, 1987). Its enhanced expression suggested that this enzyme plays a key role in maintaining the carbon economy of the nodules and may be involved in the flow of carbon to the bacteroids. The effect of *sus1* mutation revealed *Sus1* to account for 95% and 50% of activities in the embryo and testa respectively, although *Sus2* and *Sus3* are also expressed in these tissues. On the other hand the contribution of *Sus1* in leaves is lower and declines with maturity, whilst *Sus2* and *Sus3* are both expressed in mature leaves (Barratt *et al.*, 2001). It was also reported that higher mRNA levels of Susy and SPS were found in potato epidermal fragments (5.5-fold and 1.4- fold respectively) (Kopka *et al.*, 1997) as well as higher activity of these two enzymes in *Vicia* guard cells (Hite *et al.*, 1993).

1.3.1.2 Environmental influence on expression

Tissue and cell-specific transcription of maize Susy genes is differentially modulated by carbohydrate supply (Koch, 1996), with *Sh1* and *Sus1* being up regulated by low and high carbohydrate, respectively. In detached potato leaves only *Sus4* transcription was induced by sucrose, while the expression of *Sus3* remained unaffected (Fu *et al.*, 1995). Sucrose also affected the expression of the *CitSus1* isoform in detached citrus leaves but had no effect on *CitSusA*, however, the opposite was seen when hexoses were applied (Komatsu *et al.*, 2002). *Sus1* is the only Arabidopsis Susy isoform whose transcript level is up-regulated both in seedlings grown on high sucrose concentration (Martin *et al.*, 1993) as well as in detached mature leaves fed with sucrose (Baud *et al.*, 2004).

Induction of Susy genes by hypoxia or anoxia is a widely described phenomenon in both roots and shoots of monocotyledonous and dicotyledonous species (Marana *et al.*, 1990; Ricard *et al.*, 1998; Zeng *et al.*, 1998). Tolerance of roots to anaerobic stresses includes higher rates of glycolysis and ethanol fermentation to maintain the energy status of the cell (Hole *et al.*, 1992). While the invertase activity is reduced during anoxic germination of rice, Susy activity is enhanced, indicating a role in phloem unloading and for providing substrates for glycolysis (Ricard *et al.*, 1991). Under anaerobic stress, transcription of *Sh1* in maize root was up-regulated, but protein content for this isoform showed only slight increase. *Sus1* transcription was slightly decreased in root but the

protein content increased in the root tip, suggesting that expression of Susy genes in maize under anaerobic conditions is regulated at both the transcriptional and post-transcriptional level (McElfresh and Chourey, 1988; Taliercio and Chourey, 1989). The increased *Sus1* transcription and translation in Arabidopsis roots upon oxygen deprivation reported in Martin *et al*., (1993) was further investigated and showed that also *Sus4* transcription is highly increased upon anoxia (Baud *et al.*, 2004).

Increased transcription of Arabidopsis *Sus1* isoform upon cold stress has been reported in various studies (Fowler and Thomashow, 2002; Baud *et al.*, 2004; Hannah *et al.*, 2005). Recently Susy has been also identified as a cold responsive protein in rice seedlings (Cui *et al.*, 2005).

1.3.1.3 Protein modification

Susy has been reported to undergo post-translational modifications by reverse protein phosphorylation (Winter and Huber, 2000; Hardin *et al.*, 2003), thought to be important for determining its cellular distribution between the cytoplasm, actin cytoskeleton and plasma membrane. The phosphorylation site on maize SS2 protein was identified as Ser15 (Huber *et al.*, 1996) and in soybean nodule-enhanced Susy protein its structural homolog Ser11 (Zhang and Chollet, 1997). Deduced sequences from Susy cDNAs cloned from monocotyledonous and dicotyledonous species show that this phosphorylation site is conserved. However, this is not the case for the Arabidopsis *Sus2* and tomato Susy (accession P49037) proteins. The Ser15 phosphorylation occurs via a calcium-dependent protein-kinase (CDPK). The phosphorylation site shown below appears at the N-terminal end of the protein.

- L – [STA] – R – [LV] – H – S* - [VLQ] - R

The physiological significance of Susy phosphorylation at Ser15 is still not fully understood. Phosphorylation at that serine alters the structure of the amino terminus in a way that may stimulate the catalytic activity of Susy or decrease its association with membranes (Hardin *et al.*, 2004).

Recently, another phosphorylation site *in vitro* and *in vivo* has been identified at Ser170 of the maize Sus1 (Hardin *et al.*, 2003). Phosphorylation again occurred by a CDPK. Utilization of Ser170 as a phosphorylation site is tightly controlled through interaction with

the amino-terminal phosphorylation site. It is postulated that this site is part of a targeting mechanism that promotes Susy protein degradation.

1.3.1.4 Subcellular distribution

Plasma membrane association of Susy was first described by Amor *et al.,* (1995), where the enzyme was found as the most abundant UDPG binding protein on the plasma membrane in developing cotton fiber cells. This suggested a potential role for Susy in channelling UDPG into glucan synthesis directly from cytosolic sucrose. Immunolocalization of Susy in cotton fibers showed a labelling pattern very similar to the orientation of aggregates of cellulose synthase (Haigler *et al.*, 2001). Both endosperm Susy isoenzymes SS1 and SS2 were found to be capable of associating with the plasma membrane (Carlson and Chourey, 1996), which was also found using stem pulvinus and leaf elongation zones of maize plants (Winter *et al.*, 1998). Recently it was also reported tonoplast-association of Susy in red beet (Etxeberria and Gonzalez, 2003).

Membrane association of Susy is relatively strong and can only be released by strong detergents such as digitonin, CHAPS or SDS (Amor *et al.*, 1995) or by phosphorylation of membrane vesicles (Winter *et al.*, 1997). The mechanism of Susy association with membrane is not well understood but it has been shown that protein phosphorylation could be involved (Winter *et al.*, 1997). *In vitro* studies have shown that dephosphorylation of Susy causes increased association with the membrane fraction whereas phosphorylation of membrane proteins caused the release of Susy from the membrane. *In vivo* labelling experiments with [γ^{32}P] ATP showed that in young maize leaves the membrane-associated form of Susy had relatively less radioactivity incorporated than the soluble form of the enzyme.

Possible *in vivo* association of the soluble phosphorylated form of Susy with the actin cytoskeleton has also been postulated (Winter *et al.*, 1998; Azama *et al.*, 2003). Susy was found in crude cytoskeleton fractions and *in vitro* studies showed direct binding to F-actin. Moreover, actin was co-immunoprecipitated using anti-Susy monoclonal antibodies suggesting that some of the Susy may be associated with actin *in situ* (Winter *et al.*, 1998).

1.3.2 Invertases

Invertases, also called β-fructofuranosidase, are hydrolases, which under physiological conditions cleave sucrose irreversibly to glucose and fructose. Three types of invertase are distinguished on the basis of their solubility, localization and pH optima, namely, cytoplasmic neutral invertases and vacuolar and cell-wall acid invertases.

1.3.2.1 Neutral/Alkaline invertases

The neutral/alkaline invertases are found in the cytosol, have pH optima between 7 and 7.8 and are thought to be involved in sucrose degradation when the activities of Susy and acid invertases are very low (Winter and Huber, 2000).
The Arabidopsis genome contains nine genes encoding proteins that show homology with a neutral invertase cloned from carrot (Sturm, 1999). Encoded proteins exhibit high sequence homology with a maximum pair identity of 89% and a minimum of 45% in the amino acid sequences. Neutral and alkaline invertases are not very well characterised as the native enzymes are labile with the activity easily lost after tissue homogenisation (Sturm, 1999).

1.3.2.2 Acid invertases

Acid invertases cleave sucrose most efficiently between pH 4.5 and 5.0 (Sturm, 1999). Generally plants have two classes of acid invertases which differ in their localisation, a difference which can be observed from their pIs. The first class has acidic pI and is localized in the vacuole, whereas the second class has basic pI and is extracellular and bound ionically to the cell wall.

Vacuolar invertases (referred as well as soluble acid invertases) are considered to be important for osmoregulation and cell expansion, control of sugar composition in fruits and storage organs, sucrose import, sugar signalling and they respond to cold (Sturm and Tang, 1999; Koch, 2004).

Vacuolar invertases in many plant species are encoded by small gene families. In maize, Arabidopsis and carrot two isoforms were identified (Winter and Huber, 2000). These isoforms have been shown to differ in their expression pattern e.g. Arabidopsis *Atβfruct3* shows high expression in the cotyledons of one week old plant but was barely detected in latter growth stages, whilst *Atβfruct4* showed high expression in the flowers and

stem of older plants (Tymowska-Lalanne and Kreis, 1998). Vacuolar invertases were found to be differentially regulated in a number of plants. An important finding is the fact that maize *Ivr1* is up-regulated by sugar depletion, whilst *Ivr2* is up-regulated by abundant sugar supply (Koch, 1996).

Recent molecular genetic approaches have shown importance of soluble acid invertases in the regulation of tissue sugar composition in both source and sink tissues (Winter and Huber, 2000). Suppression of vacuolar invertases by antisense RNA in tomato fruit and leaves has been shown to increase the sucrose content in these tissues (Winter and Huber, 2000). The same antisense approach of potato cold-inducible invertase has shown the reduction of hexose sugar accumulation in favour of sucrose accumulation in cold-stored potato tubers (Zrenner *et al.*, 1996) thus confirming the role of vacuole invertases in the control of sugar composition. Antisense inhibition of the main carrot vacuolar invertase caused a reduced tap root size and a marked decrease in soluble sugars, indicating the root size reduction was at least partially the result of a reduction in cellular osmotic potential (Sturm and Tang, 1999). These data also indicated that sucrose cleavage by vacuolar invertase can actively drive phloem unloading, especially when this provides the pathway for sucrose breakdown within the cell (Sturm and Tang, 1999).

Cell wall invertases (also known as insoluble acid invertases) are central to phloem unloading in many sucrose-importing tissues. Their importance is most prominent in sink tissues where an apoplastic step is involved e.g. developing seeds and pollen where lack of plasmodesmatal connection between cells demand an unloading role for cell wall invertase (Koch, 2004). Additionally these enzymes contribute to the development of pollen and localized antisense reduction in cell wall invertases were used to manipulate male fertility (Roitsch *et al.*, 2003). Cell wall invertases also influence unloading in sink organs where plasmodesmatal connections are present and some sucrose moves across the cell wall space. This occurs in developing carrot root (Sturm and Tang, 1999) and in the response to some signals from both biotic (Herbers and Sonnewald, 1998) and abiotic (Sturm and Tang, 1999) stresses.

Cell wall invertases are encoded by small multigene family (Sturm, 1999). In *Arabidopsis* it is predicted that there are six cell wall invertases (Sherson *et al.*, 2003). Two of them have been studied in more detail, showing that *AtcwINV1* is expressed in most tissues of mature plant and *AtcwINV2* is more flower-specific (Tymowska-Lalanne and Kreis, 1998). However recent studies indicate that *AtcwINV3* and *AtcwINV6* should now be

considered as vacuolar fructan exohydrolases with different substrate specificities (De Coninick *et al.*, 2005).

During purification of tobacco cell wall invertases a polypeptide which inhibited enzyme activity, was found and named INH (Weil *et al.*, 1994). It was suggested that INH and cell wall invertases form a complex in the apoplastic space but for complete inhibition of cell wall invertases an additional unknown signal is required (Krausgrill *et al.*, 1998). Expression analysis in tobacco showed that at different stages of development e.g. mature roots INH may act as a regulatory switch for cell wall invertases activity (Krausgrill *et al.*, 1998).

1.4 UGPase, important crossroad of sucrose biosynthesis and breakdown

UGPase (2.7.7.9) represents an important activity in carbohydrate metabolism catalysing the reversible production of UDPG and PPi from glucose-1-phosphate and UTP. Usually UGPase is involved in sucrose synthesis in young and mature leaves, providing substrate for SPS action, whereas in some tissues which depend on imported carbon e.g. apical leaves or potato tubers the enzyme is involved in sucrose cleavage (Winter and Huber, 2000). It seems that UGPase is much more active than other enzymes in the sucrose synthesis pathway indicating that it does not regulate carbon flow to and from sucrose metabolism. This was confirmed by a potato antisense study showing that a 96% decrease of UGPase activity in tubers had no effect on sugar level (Zrenner *et al.*, 1993).

Two highly homologous *Ugp* genes were found in the Arabidopsis genome (At3g03250 and At5g17310), in Poplar tree and rice, and one *Ugp* gene coding for UGPase was found in barley (Kleczkowski *et al.*, 2004). Comparison of deduced amino acids sequences shows that there is 59% to 96% identity among plant UGPases (Kleczkowski *et al.*, 2004).

The expression of Arabidopsis *Ugp* and UGPase activity were up-regulated by phosphate deficiency (Ciereszko *et al.*, 2001), cold temperature, light and sucrose but not glucose (Ciereszko *et al.*, 2001). In Arabidopsis the sucrose effect on UGPase expression was blocked by okadaic acid (OKA), which is an inhibitor of phosphatases 1 and 2A (Ciereszko *et al.*, 2001). This was in contrast to OKA-independent up-regulation of Susy (*Sus1*) (Ciereszko and Kleczkowski, 2002). Thus, sucrose-signalling pathways differentially regulating Susy and UGPase may represent a mechanism where UDPG is

assured to be produced when one of the pathways is inactive or blocked (Kleczkowski *et al.*, 2004).

UGPase is mainly localized in cytosol, although immunolocalization in rice cells has revealed that some protein is located in amyloplasts and Golgi (Kimura *et al.*, 1992). Moreover, fractionation of rice and tobacco cells detected some activity in microsomes (Mikami *et al.*, 2001), whereas in barley a membrane bound fraction of UGPase was found (Becker *et al.*, 1995). A possible role for supplying UDPG by UGPase for cell wall synthesis cannot be excluded especially for source tissues where Susy protein is not very abundant (Dejardin *et al.*, 1999). In sink tissues, on the one hand UGPase could work in conjunction with Susy and/or invertases to break down sucrose, on the other hand UGPase could also remobilize glucose-1-phosphate back to UDPG (Kleczkowski *et al.*, 2004). The involvement of UGPase into cell wall synthesis was demonstrated in cellulose negative mutants of the microorganism *Acetobacter xylinum* that lacked UGPase activity (Valla *et al.*, 1989).

1.5 Importance of UDPG

Uridine diposphate glucose (UDPG) is a substrate in several anabolic reactions, including the synthesis of sucrose, starch and cell wall components (Figure 1). UDPG is generally assumed to be localized only in the cytosol and in the ER. The distribution of UDPG between several metabolic reactions might be regulated by its supply and demand.

The pathway operating between sucrose and hexose phosphate is well established and has been intensively investigated. Glucose-1-phosphate, generated from UDPG, can either enter or leave the hexose phosphate pool through the action of UGPase. Glucose-1-phosphate converted into glucose-6-phosphate by phosphoglucomutase can either be channelled from the cytosol into plastids through a glucose-6-phosphate transporter and take part in starch synthesis or it can remain in cytosol and enter glycolysis (Fernie *et al.*, 2002).

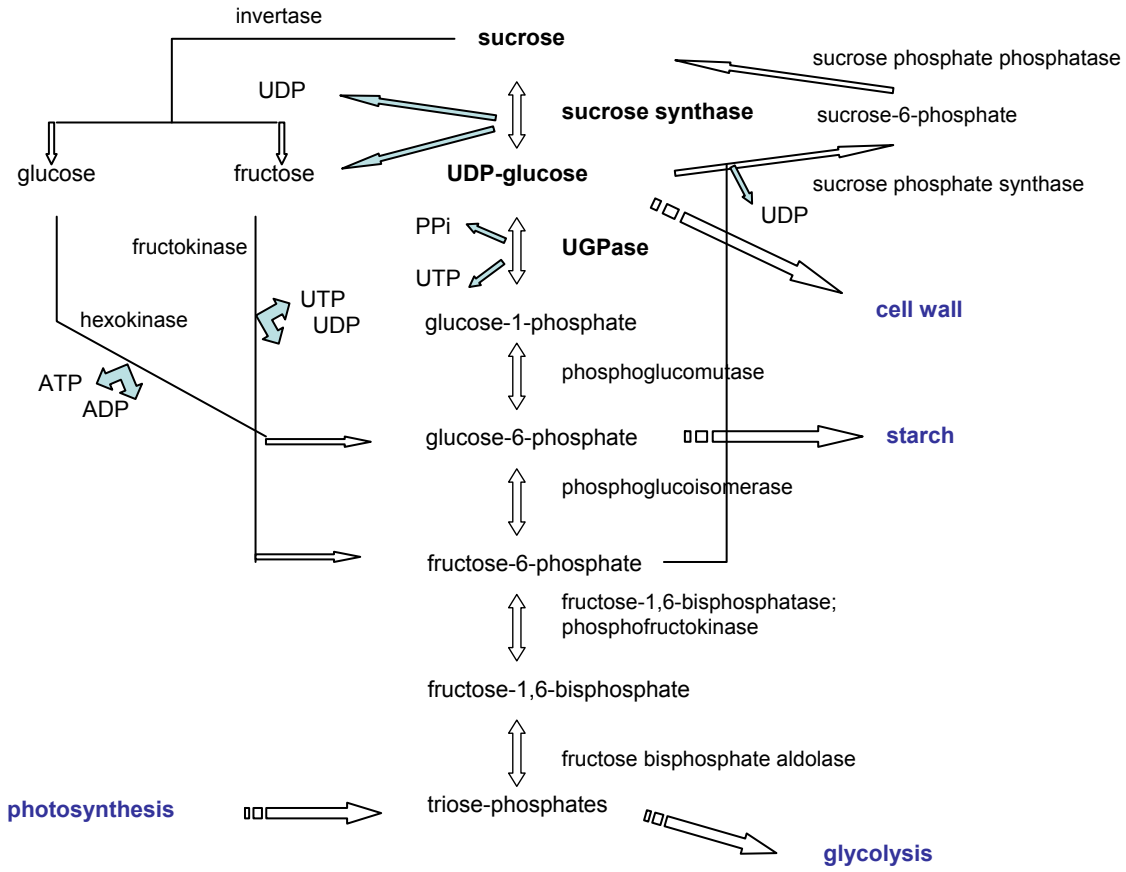


Figure 1: Metabolism of cytosolic carbon.

Sucrose degradation triggered by Susy can also generate substrate (UDPG) for cellulose biosynthesis. Cellulose, (1,4) β-linked polyglucan, is synthesized by an enzyme complex associated with the plasma membrane. In one proposed model (Amor *et al.*, 1995; Carlson and Chourey, 1996) Susy can bind to the cellulose synthase complex associated with plasma membrane (Figure 2) and channel UDPG from sucrose catabolism directly towards cellulose synthesis. This model would solve the problem of transferring UDPG across the plasma membrane of the cell.

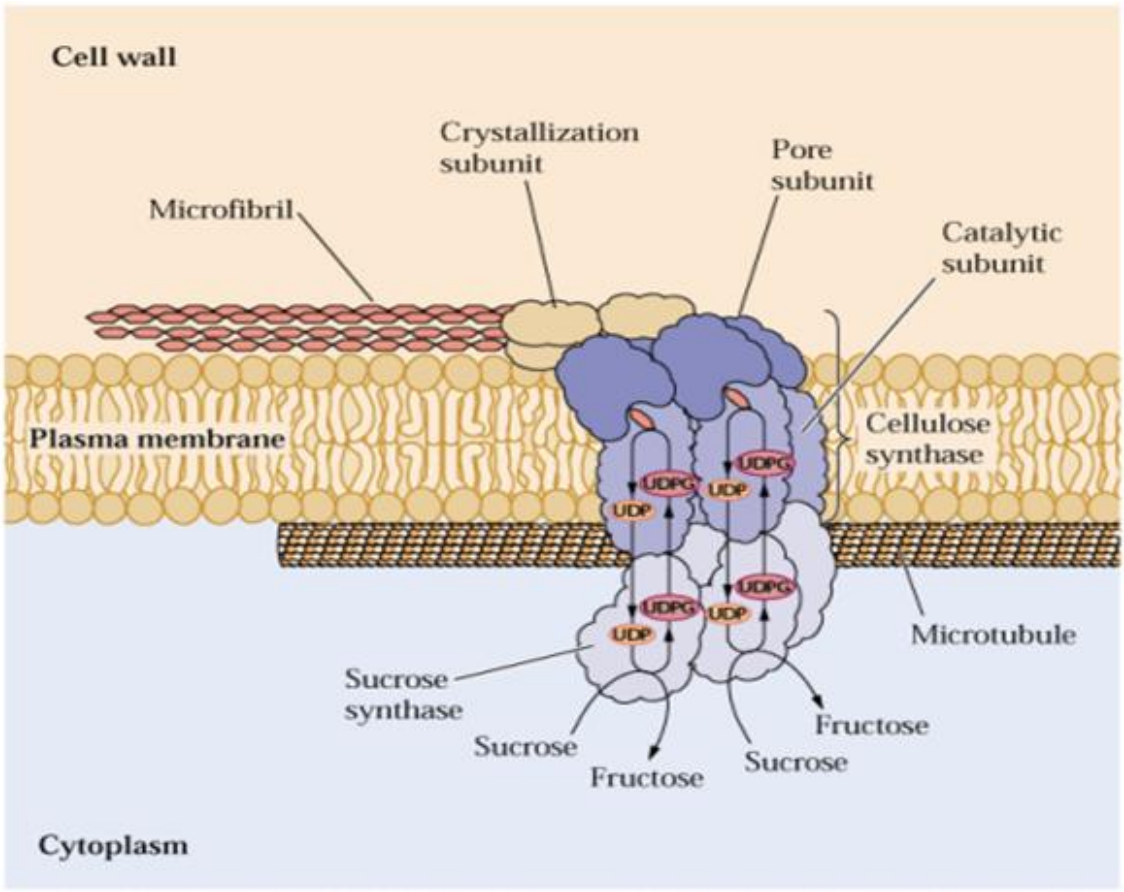


Figure 2: Model of cellulose synthase complex.

Cellulose is formed outside the plasma membrane by incorporation of UDPG into cellulose microfibrils which is derived directly from sucrose cleaved in cytosol by Susy.

The other compounds of cell wall, noncellulose polysaccharides which consist of hexoses, pentoses and uronic acids are also synthesized from UDPG. However synthesis of these compounds occurs in the Golgi apparatus therefore a special transporting system is needed.

1.6 Aims and objectives of the present work

Susy is a key enzyme involved in sucrose metabolism catalysing the reversible conversion of sucrose and UDP to UDP-glucose and fructose. It has been shown in various plant species that Susy can play a role in supplying substrates for starch and cell wall synthesis, as well as energy in companion cells for phloem loading. Analysis of the Arabidopsis genome identifies six different Susy isoforms. In order to characterise this small gene family, the following strategies will be applied:

1. In order to analyse if indeed all Susy genes are expressed in *Arabidopsis thaliana* and additionally to study the spatial and temporal transcript abundance under standard and impaired (various stresses) conditions, real-time RT-PCR, stable transformation and analysis of promoter fusions with a reporter gene (GUS) and the analyses of publicly available data sets of array hybridisation will be performed.
2. In order to study the specific functions of all six isoforms *in planta* available T-DNA insertion mutants or RNA_i lines will be collected and verified prior to further analysis.
3. In all appropriate T-DNA mutant lines plant growth and development as well as carbon partitioning with respect to carbohydrate accumulation and cell wall formation will be investigated.
4. Some of the mutants will be analysed in specific experiments with respect to their individual expression patterns:

- Mutant in the *Sus1* isoform will be subjected to cold acclimation study.
- RNA_i transformants of the *Sus4* isoform will be examined with respect to oxygen deprivation.
- Mutants in the *Sus3* isoform will be investigated for properties of guard cell movement.

2 Materials and Methods

2.1 Chemicals

Restriction enzymes and buffers were obtained either from Fermentas (St. Leon-Rot, Germany) or from Roche (Mannheim, Germany). Biochemical enzymes and substrates were purchased from Roche (Mannheim, Germany) and Sigma (Deisenhofen, Germany), chemicals were obtained from Roche (Mannheim, Germany), Merck (Darmstadt, Germany), Sigma (Deisenhofen, Germany) and Fluka (Taufkirchen, Germany).

2.2 Plant material

Arabidopsis thaliana (L.) ecotype Col-0
Arabidopsis thaliana (L.) ecotype WS

T-DNA mutant collection from Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/) and GABI-Kat (http://www.gabi-kat.de/) (Table 1)

gene	line	identifier for homozygous T-DNA
At5g20830 (*Sus1*)	Salk_014303	S_sus1
At5g49190 (*Sus2*)	Salk_076303 Gabi-Kat_377G03	S_sus2 G_sus2
At4g02280 (*Sus3*)	Salk_019405	S_sus3
At5g37180 (*Sus5*)	Salk_065271 Gabi-Kat_022D04	S_sus5 G_sus5
At1g73370 (*Sus6*)	Salk_019129	S_sus6
At3g03250 (*UGPase1*)	Gabi-Kat_114F04	G_UGPase1
At5g17310 (*UGPase2*)	Salk_015899	S_UGPase2

Table 1: T-DNA insertion mutants from Salk and Gabi-Kat collection used in the project.

T-DNA mutant kindly provided by Alison Smith (John Innes Centre, Norwich, UK)
W_sus3; (at4g02280)
X_sus4; (at3g43190)
G_sus6; (at1g73370)

RNAi transformant from Agrikola (http://www.agrikola.org/index.html)
A_sus4; CATMA3a35550 (at3g43190)

2.3 Seed sterilization and tissue culture

Seeds were surface sterilized (1 min in 95% ethanol, 5 min in 6% sodium hypochloride, washed 5 times with sterile water and suspended in 0.07% agar) and plated on half strength MS medium (Murashige and Skoog, 1962) containing 0.7% agar and 1% sucrose (including 50µg/l kanamycin or 15µl/l hygromycin in the case of transformed plants for promoter analysis), imbibed in the dark for 48h at 4°C and transferred to growth chambers and grown in 16h/8h day/night regime at 120µmol photons m^{-2} s^{-1} and 20°C/18°C. In case of plants grown for gas exchange experiment plated seeds were transferred into growth chambers with conditions: 8h/18h day/night, 120µmol photons m^{-2} s^{-1} and 20°C/18°C

2.4 Plant growth and harvest

After two weeks in tissue culture **plants for promoter analysis** were transferred into soil and grown in 16h/8h day/night regime at 120µmol photons m^{-2} s^{-1} and 20°C/16°C at 75% relative humidity. Arabidopsis tissues and organs were harvested at several time points: imbibed seeds (48h, 4°C, darkness), 5 and 14 days old seedlings (from tissue culture), 5-6 week old (plant in soil): flower buds, flowers, siliques (all stages), stems, colendral and rosette leaves, roots, senescing leaves.
After two weeks in tissue culture **plants for cell wall and carbohydrate analysis**: Col-0, WS, S_sus1, S_sus2, G_sus2, S_sus3, W_sus3, X_sus4, S_sus5, G_sus5, S_sus6, G_sus6, G_UGPase1, S_UGPase2 were transferred into hydroponics culture for additional 3 weeks and grown in 16h/8h day/night regime at 120µmol photons m^{-2} s^{-1} and 20°C/16°C at 60%/75% relative humidity. Liquid medium contained: 1mM KNO_3, 2.5mM $Ca(NO_3)_2$, 0.5mM $MgSO_4$, 0.5mM KH_2PO_4/K_2HPO_4 pH 5.8, 20µM Fe-EDTA, 150µM H_3BO_3, 35µM $MnSO_4$, 2.5µM $ZnSO_4$, 1.5µM $CuSO_4$, 1µM $NiCl_2$, 0.75µM $HMoO_4$, 50nM $CoCl_2$. Air was

applied all the time to the culture using aquarium pump. Medium was changed every 4 days. Harvest was done at the end of the day and night. Whole rosettes and roots were harvested separately and quickly frozen in liquid N_2.

After two weeks in tissue culture **plants for gas exchange analysis**: Col-0, WS, S_sus3 and W_sus3 and were transferred into soil and grown in 8h/16h day/night regime at 120µmol photons m^{-2} s^{-1} and 20°C/16°C at 75% relative humidity. Analysed plants were 6-7 week old.

Seeds of Col-0 for real-time RT-PCR expression analysis were incubated in a 2:1 mixture of GS 90 soil and vermiculite, watered with tap water containing 0.15% Previcur N and covered with a lid. Seeds were kept for one week in a growth chamber in 16h/8h day/night regime at 145µmol photons m^{-2} s^{-1} and 20°C/6°C at 75% relative humidity. After 7 days plants were transferred in a 2:1 mixture of GS 90 soil and vermiculite in pots of 200ml and grown for 42 days. Directly after transfer, plants were watered once with tap water containing 0.15% Previcur N and than watered daily with tap water. Plants were grown in growth chambers in 16h/8h day/night regime at 120µmol photons m^{-2} s^{-1} and 20°C/16°C at 60%/75% relative humidity. After 6-7 weeks all organs were harvested at the same time (flower bud, flower, silique (all stages), stem, colendral and rosette leaf, root) and immediately frozen in liquid nitrogen. Col-0 seeds prior to seed expression analysis were imbibed in darkness for 48h in 4°C on the half strength MS, 0.7% agar, 1% sucrose plates.

Plants for freezing tolerance: Col-0 and the S_sus1 were grown in soil in a greenhouse with supplementary light providing a 16h photoperiod at a minimum of 200 µmol m^{-2} s^{-1}, and at a day/night temperature of 20°C/18°C until bolting (43-46 days after germination). For cold acclimation, plants were transferred to a 4°C growth chamber with a 16h photoperiod at 90µmol m^{-2} s^{-1} for an additional 14 days.

Plants for anoxia experiment: Col-0 and A_sus4 (RNAi transformatns) were grown for 4-5 weeks in terragreen/sand 1:1 mix (mixed with osmocote slow release fertiliser 1:400) at 20˚C, 75% relative humidity in a 16h-light/8h-dark photoperiod with a irradiance of 180-200µmol m^{-2} s^{-}. Anoxic stress treatment was performed by submerging plants into degassed water for 2 days prior to harvest. All roots were immediately frozen in liquid nitrogen after harvesting and stored at -80ºC.

2.5 Plasmids and cloning work

For promoter fragment amplification standard protocols and PfuTurbo DNA polymerase (Stratagene, LaJolla, CA, USA) were used. Fragments of the respective size were amplified using *Arabidopsis thaliana* Col-0 genomic DNA purified with the CTAB method (Sambrook and Russell, 2001) and subcloned using the pPCR-Script® Amp Cloning Kit (Stratagene, Heidelberg, Germany). For promoter analysis each fragment was cloned into the binary vector pGPTV-KAN except for the promoter of Sus4 where pGPTV-HYG was used (Becker *et al.*, 1992). The complete sequences in front of the start codon of the respective sucrose synthase isoform to the adjacent gene in 5´-direction but not more than 2100 base pairs of the intergenic regions were amplified by PCR. Promoter fragments of the following sizes were cloned and the respective sequences for restriction endonucleases were added:

Psus1: - length 1983bp
- cloned with SalI/XbaI sites
- 5g2Pl: 5´-TCGACGAAAAGGTCAAAAAGGAAAACG'3
- 5g2Pr: 5´-TCTAGATGATCCAAAAAAGAGACGCAG'3

Psus2: - length 595bp
- cloned with SalI/XbaI sites
- 5g4Pl, 5´-GTCGACAAAGAATTCTGATTTAATTTTGTG'3
- 5g4Pr, 5´-TCTAGAGATTTTTTCTCAGAGGCAAA'3

Psus3: length 2088bp
-cloned with SalI/SmaI sites
- 4g0Pl: 5´-GTCGACAATTGCGTTGAAAAAGAAGGTT'3
- 4g0Pr, 5´-CCCGGGGAATATTCAGATGATCACTA'3

Psus4: length 1948bp
-cloned with SalI/XbaI sites
- 3g4Pl, 5´-GTCGACTTTCTTCAACAAAGCCCTTCA'3
- 3g4Pr, 5´-TCTAGATTCAAACACAATCACAAAGC'3

Psus5: length 1732bp
- cloned with HindIII/XbaI sites
- 5g3Pl, 5´-AAGCTTAAATGCTCATTGCTCGTAGTT'3
- 5g3Pr, 5´-TCTAGATGTGTTATGTACCTTGAGAC'3

Psus6: length 752bp

-cloned with SalI/XbaI sites

- 1g7Pl, 5´-GTCGACAACACCGTCGTCTGCTTACC'3

- 1g7Pr, 5´-TCTAGAAGAAACAACTGAAGATTCAA'3

2.6 Transformation of bacteria

Transformation of *Escherichia coli* strain DH5α was performed using heat shock method, as described previously (Hanahan, 1983). *Agrobacterium tumefaciens* strain GV3101.pMP90 was transformed by electroporation with a Gene Pulser II, according to manufacture's instruction. *E. coli* strains were grown in LB media while *Agrobacterium tumefaciens* strains were grown in YEB medium (Vervliet *et al.*, 1975). For growth on solid media, 1.5% agar was added. Filter-sterilised antibiotics were added at following concentrations: 50µg/l kanamycin, 125µg/ml gentamycin, 100µg/ml rifampicilin.

2.7 Plant transformation

Arabidopsis thaliana Col-0 plants were transformed with *Agrobacterium tumefaciens* using standard procedures (Bechtold and Pelletier, 1998)

2.8 GUS staining

Expression of the reporter gene was monitored using histochemical staining (Jefferson *et al.*, 1987). Plant material was vacuum-infiltrated and incubated in the dark for several hours up to overnight at 37°C with GUS buffer (50mM sodium phosphate buffer pH 7.2, 10mM EDTA, 0.1% (w/v) Triton X-100, 0.1% (w/v) Tween 20, 210mg/l $K_4[Fe(CN)_6]$ x $3H_2O$, 166mg/l $K_3[Fe(CN)_6)$ and 0.5g/l X-Gluc). Plant material was washed and distained by incubation in 70% (v/v) ethanol and observed using stereo microscope Leica MZ 12.5 or light microscope Olympus BX41.

2.9 RNA extraction and cDNA synthesis

Total RNA was isolated from frozen plant material using NucleoSpin RNA Plant kit (Macherey-Nagel GmbH & Co. KG, Dueren, Germany) according to the manufacture's

instruction. The isolated RNA was additionally purified and precipitated (RNA : 5M LiCl : 100% ethanol in a ratio 1:1:2 (v:v:v) at 4°C for 30 min, centrifuged 10 min at 4°C and 13000rpm, and washed with 70% ethanol). RNA concentration was measured in a SmartSpec Spectrophotometer (BioRad, Hercules, CA, USA) and 2µg of total RNA was used for cDNA synthesis. Absence of genomic DNA contamination was confirmed by real-time RT-PCR, using primers designed on intron sequence of a control gene (At5g65080). RNA integrity was checked on a 2% (w/v) agarose gel.

RT reactions were performed with SuperScript™ III reverse transcriptase (Invitrogen GmbH, Karlsruhe, Germany), according to the manufacturer's instructions. The efficiency of cDNA synthesis was assessed by real-time RT-PCR amplification of control genes encoding ubiquitin 10 (At4g05320f, 5'-CACACTCCACTTGGTCTTGCGT; At4f05320r, 5'-TGGTCTTTCCGGTGAGAGTCTTCA) and elongation factor 1α (At5g60390f, 5'-TGAGCACGCTCTTCTTGCTTTCA; At5g60390r, 5'-GGTGGTGGCATCCATCTTGTTACA).

2.10 Primer designing and real-time RT-PCR reaction

All primers for real-time RT-PCR were design using the programme Primer Express 2.0. Oligonucleotides for real-time RT-PCR:

sus1: sus1f, 5'AGTTCACTGCGGATATTTTCGC'3;
sus1r, 5'CCCAACAGTTTCTTTGCTTCCA'3;

sus2: sus2f, 5'TGCCATGAATAATGCCGATTTC'3;
sus2r, 5'TTGCCCAACATTGTTCTTGCTT'3;

sus3: sus3f, 5'GACCAGACTGATGAGCATGTCG'3;
sus3r, 5'TCTTCACTTTGTCGAGCCTCG'3;

sus4: sus4f, 5'AAGGAATCGTTCGCAAATGG'3;
sus4r, 5'TTTCAGCGGCAACATCCTC'3;

sus5: sus5f, 5'GCAGTGGTAATTCCTCCGAAC;
sus5r, 5'TCCTCTTACTGCGAACGCTACG'3;

sus6: sus6f, 5'CGGAGGCCAGGTTGTTTACAT;
sus6r, 5'AGGCTTGAATCCGAGACCTTGT'3;

ugpase1: ugpase1f, 5'CCAGAGCAAATATCCCCGTGT'3;
ugpase1r, 5'TCCTTGTCGGTCTTTCCCTTG'3;

ugpase2: ugpase2f, 5'GCCCAGCACATTGAATGGA'3;
ugpase2r, 5'TGGTCTCGGAAGCATCTTCAG'3

Polymerase chain reactions were performed in an optical 96-well plate with an ABI PRISM® 5700 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA), using SYBR® Green to monitor dsDNA synthesis. Reactions contained 10µl 2× SYBR® Green Master Mix reagent (Applied Biosystems, Foster City, CA, USA), 1µl cDNA and 125nM of each gene-specific primer in a final volume of 20µl. The following standard thermal profile was used for all PCRs: 50°C for 2min; 95°C for 10min; 40 cycles of 95°C for 15sec and 60°C for 1min. Obtained data of relative transcript amount were analysed like previously described (Czechowski *et al.*, 2004)

2.11 Mutant screening

Genomic DNA prior to mutant screening for homozygotes was isolated either according to alkaline lysis method (Klimyuk *et al.*, 1993) or CTAB method (Sambrook and Russell, 2001). All primers for PCR were design using the programme Primer3. Primers were design to obtain products no longer than 750bp. Oligonucleotides for PCR:

S_sus2; S_sus2F 5'GTTAGGGAATATGTCCGTGTGAA'3
S_sus2R 5'TCCCTGAAGTATGGGGATATTCT'3
G_sus2; G_sus2F 5'CATGTTTCCAGAATGCATAGACTC'3
G_sus2R 5'GAGTCAGCAGACCATCTAATCTCTC'3
S_sus3; S_sus3F 5'CTAAGTTATCCAAACCGAATCGAACCGAATCA'3
S_sus3R 5'ACTCTTCTCTATGGCGAGGCTCGACAAA'3
S_sus5; S_sus5F 5'TTAACACGGCTAGAAAGCTTTGAG'3
S_sus5R 5'CAAACCTAGAACATCGGTTTGAC'3
G_sus5; G_sus5F 5'CGACCCTAGTAATGGGAACATTT'3
G_sus5R 5'CAAACCTAGAACATCGGTTTGAC'3
S_sus6 S_sus6F 5'TCAGATCCTTGTTCCGGTTC'3
S_sus6R 5'TCATGCGAAATCACGGTTTA'3
UGPase1 UGPase1F 5'TTGCAGTTTGTATGCATGGTC'3
UGPase1R 5'CGGCCATTGTTGATTTACAG'3

UGPase2 UGPase2F 5'TCAAGTAAGCCAAACACACTCCT'3
UGPase2R 5'CGAAVAAAAAGAAAACAAGAAGC'3

G_LBb1 5'CCCATTTGGACGTGAATGTAGACAC'3 for Gabi KO mutants
S_ LBa1 5'TGGTTCACGTAGTGGGCCATCG'3 for Salk KO mutants

Two independent PCR reactions were performed to test each line. PCR analyses were carried out using primer pairs specific for the respective Susy or UGPase gene and for T-DNA sequence. To screen for T-DNA insertion following primer combinations were used:

G_sus2;	G_LBa1 and G_sus2R;	expected product: 560 bp
S_sus3;	S_LBa1 and S_sus3R;	expected product 540 bp
S_sus5;	S_LBa1 and S_sus5R;	expected product 660 bp
G_sus5;	G_LBa1 and G_sus5R;	expected product 720 bp
S_sus6;	S_LBa1 and S_sus6R;	expected product 600 bp
UGPase1;	G_LBa1 and UGPase1F;	expected product 740 bp
UGPase2;	S_LBa1 and UGPase2R;	expected product 470 bp

Polymerase chain reactions were performed in 96-well plate with PCR machine (Biometra, Göttingen, Germany). Reactions contained: 1µl of appropriate genomic DNA, 125nM of each primer, 200nM dNTP, 2.5mM $MgCl_2$, 10x polymerase buffer, 0.2U Taq polymerase (Fermentas, St. Leon-Rot, Germany) in a final volume of 20µl. The following standard thermal profile was used for all PCRs: 95°C for 3min; 95°C for 1min; 60°C for 1 min, 34 cycles of 72°C for 20sec and 72°C for 10min. Results were analysed on 1% agarose gel containing ethidium bromide.

2.12 Extraction of soluble sugars and cell wall components

A protocol for cell wall analysis published by Peng *et al.,* (2000) was modified as follows: 200mg of frozen, grind plant material was first subjected to ethanol-water extraction (2 times 20 minutes at 80°C in 80% ethanol with 3mM HEPES pH 7, 20 minutes at 80°C in 3mM HEPES pH 7, combine all supernatants), a subsequent lipid extraction (2 times 60 minutes in chloroform:methanol (1:1; v:v) at 40°C, 30 minutes at 40°C in methanol followed by a wash with water, combine all supernatants) and instead of the DMSO treatment an extra step for

starch granule breakage and removal was added (60 minutes in 0.2M KOH at 95°C, acidify to pH 5.5 with acetic acid, digest starch two times: overnight and additionally for 4 hours in 50mM Na-acetate buffer containing amyloglucosidase and α-amylase at 37°C, followed by a wash with water, combine all supernatants). After pectin (24h in 50mM Na_2CO_3 containing 10mM $NaBH_4$ and shaking at RT, followed by wash with water, combine all supernatants) and hemicellulose extraction (6h and 18h in 1M KOH containing 10mM $NaBH_4$ and shaking at RT, neutralize with acetic acid); followed by additional hemicellulose extraction (6h and 18h in 4M KOH containing 10mM $NaBH_4$ and shaking at RT, followed by a wash with water, combine all supernatants, neutralize with acetic acid) and treatment with Updegraff reagent (30 minutes in Updegraff reagent (conc. HNO_3:80% acetic acid (1:10; v:v) at 95°C, followed by three washes with water and dried completely under vacuum. Such obtained pure cellulose was digested for 1h at RT with 72% H_2SO_4 and glucose was assayed via anthron method (Scott and Melvin, 1953).

2.13 Assay of reducing sugars, sucrose and starch

Sucrose, glucose, fructose (in ethanol extracts) and digested to glucose starch were determined enzymatically (Jelitto *et al.*, 1992) using a microplate spectrophotometer (Bio-Tek-Instruments, Winooski, Vermont). The assay contained a final volume of 210 µl (50 µl of ethanolic extract, 150 µl of 100 mM imidazol buffer with 3 mM $MgCl_2$ (pH 6.9), 5 µl of 36 mg/ml NADP, 5 µl of 60 mg/ml ATP, 0.5U glucose-6-phophate dehydrogenase. The reactions were started by successive addition of 1U hexokinase, 1U phosphoglucoisomerase and 20U invertase (all enzymes were derived from yeast).

2.14 Anthron assay for cellulose determination

Glucose after Seaman hydrolysis was determined using the anthrone method. To 50 µl of dissolved supernatant (filled up to 500µl with water) 1 ml of 0.2% anthrone in conc. H_2SO_4 was added and incubated at 95°C for 5 minutes. Absorbance was measured at 620 nm using a microplate spectrophotometer and compared with standard curve for different glucose concentrations (0-200µM).

2.15 Extraction and assay of Susy

Susy was extracted from roots and leaves at 4°C using 50mM Hepes-NaOH (pH 7.5), 5mM $MgCl_2$, 1mM EDTA, 2mM DTT, 20mg polyvinylpolypyrrolidone and 100µl protease inhibitor (Sigma, Poole, Dorset UK). The homogenate was immediately assayed for the activity of sucrose synthase.

Susy was assayed via assay in the synthesis direction in the direction of detection of UDPG production. The optimised assay contained in 55µl: 100mM 3-[dimethyl(hydroxymethyl)methylamino]-2-hydroxypropanesulphonic acid (Ampso, pH 9.4), 10mM UDPglucose, 10mM fructose (or water in case of blank samples), 10mM UDP[$U^{14}C$]glucose (Amersham plc, Amersham, Buck., UK) at 11.2 GBq.mol^{-1} and 25µl extract. Reaction was run for 20 min in 20°C and stopped by boiling for 2min. Product of reaction was diluted 1:1 with water and 100µl was applied to column stuffed with Dowex (1x8 200-400 MeshCl, Sigma; Pool, Dorset UK), centrifuged for 1min at 300g. Column was washed 2 times with 100µl of water each time and centrifuged as mention above. 1ml of scintillation fluid cocktail (Optihase SuperMix, Wallace) was added and every sample was counted for 5 min using scintillation fluid counter.

Protein concentrations were measured using the Bio-Rad (Hemel Hempstead, Herts, UK) Protein Assay, with bovine serum albumin as the standard.

2.16 Extraction and assay of UGPase

UGPase was extracted and the activity was measured according to protocol described in Zrenner *et al*., (1993).

2.17 Gas exchange measurements

All gas exchange experiments were measured using LI-6400 (LI-COR Bioscience, Lincoln, Nebraska). Stomata conductance, transpiration and assimilation were measured under 4 different light intensities (0, 300, 600, 900 PAR (µmol m^{-2} s^{-1})) 30 min for each light intensity. In all measurements CO_2 concentration was 360 µmol mol^{-1} at constant flow 300 µmol s^{-1}, relative humidity varied between 60-65%. All parameters were calculated by software provided by the manufacturer.

2.18 Chlorophyll fluorescence

All chlorophyll fluorescence measurements were performed using PAM200 fluorometer (Walz, Effeltrich, Germany). Plants pre-adapted in darkness for 20 minutes were submitted into different light intensities (225-1200 PAR). Values for electron transport and photosynthetic yield were automatically calculated by the software provided by the manufacturer.

2.19 Extraction of Susy for protein gels and immunoblotting

Root tissues were extracted at 4°C in 50mM Hepes-NaOH (pH 7.5), 5mM $MgCl_2$, 1mM EDTA, 2mM DTT, 20mg polyvinylpolypyrrolidone and 100µl protease inhibitor (Sigma, Poole, Dorset UK). The homogenate was subjected to centrifugation for 10 min at 10,000g at 4°C and the supernatant precipitated with 10% w/v TCA, washed three times with 80% v/v acetone at –20°C then treated with SDS sample buffer (0.125M 2-amino-2-(hydroxymethyl)-1,3-propanediol(Tris)-HCl (pH6.8), 0.14M dithiothreitol, 4% (w/v) SDS, 20% (w/v) glycerol) before analysis by SDS-PAGE. SDS-PAGE was performed according to (Laemmli, 1970) with a 10% (w/v) acrylamide resolving gel (7cm long, 1mm thick) and 3% (w/v) stacking gel (37.5:1 [w/v] acrylamide:bis-acrylamide) in vertical electrophoresis cell. Gels were run at 4°C. Gels were blotted onto polyvinylidene difluoride (PVDF) membrane according to standard procedure. Membranes after protein transfer were incubated for at least 1 hour at RT in blocking solutions consisting of 5% BSA in 1xTBS (20mM Tris-HCl pH 7.5, 500mM NaCl). Susy antigen was detected for 12h in 4˚C using peptide specific polyclonal affinity purified antibodies designed for sus4 and sus1 isoform (diluted 1:100 in blocking solution). Blots were than washed 6 times with TTBS (1xTBS, 0.1% v/v Tween) and incubated for 2 hours at RT with secondary antibodies (peroxidase-conjugated goat anti-rabbit secondary antibodies, Sigma, Poole, Dorset UK) diluted 1:7000 in 5% BSA and TTBS. The same washing procedure was applied, additionally followed by two washes with TBS and 2 washes with water. The peroxidase was detected using Sigma Fast™ (5-bromo-4-chloro-3-inodyl, phosphate/Nirtoblue tetrazolium) according to manufacturer protocol

2.20 Freezing experiments

Freezing experiments were performed following a method described previously by (Ristic and Ashworth, 1993). Series of consisting of three fully expanded rosette leaves taken from three individual plants were placed in glass tubes containing 200µl of distilled water. The tubes were transferred to a programmable cooling baht set to -1°C; control tubes were left on ice during the entire experiment. After 30 min of temperature equilibration at -1°C ice crystals were added to the tubes in the cooling bath to initiate freezing. After another 30 min, the samples were cooled at a rate of 2°C h^{-1}. Over an appropriate temperature range, samples were taken from the bath at 1 or 1.5°C intervals and thawed slowly on ice. After thawing, leaves were immerse in 7 ml of distilled water and placed on a shaker for 16h at 4°C. Electrolyte leakage was determined as the ratio of conductivity measured in the water before and after boiling the samples, using HI8820N conductivity meter (Hanna Instruments, Kehl, Germany). 6-10 plants were analysed form each genotype (S_sus1 and Col-0) and treatment (non- and cold acclimated). LT_{50} value was calculated as log EC_{50} value of sigmoidal curves fitted to the leakage values using the software GRAPHPAD PRISM3. Statistical analysis of the differences in LT_{50} values were performed using Student t-test with GRAPHPAD INSTAD software. For carbohydrate analysis three leaves from individual plants were frozen in liquid nitrogen immediately after sampling, and homogenized using a ball mill Retsch MM 200 (Retsch, Haan, Germany). The homogenate was extracted twice with 1 ml of 80% ethanol at 80°C. Ethanol extracts were dried and dissolved in 1 ml of water. Afterwards, the samples were de-ionized (AG 501-X8 resin, Bio-Rad, Munich, Germany). Soluble sugars were analysed by HPLC using a CarboPac PA-100 column on a Dionex DX-500 gradient chromatography system coupled with pulsed amperometric detection by a gold electrode (Hincha *et al.*, 2003). 7 plants were analysed from every genotype (Col-0 and S_sus1) and every treatment (non- and cold acclimated). Statistical analysis was performed using t-test with GRAPHPAD INSTAD software.

2.21 Data calculation for array hybridisation expression analysis

All GeneChip data in the Genevestigator database was processed using Affymetrix MAS5.0 (Zimmermann *et al.*, 2004). Signal intensity values are arbitrary units and depend on the choice of the target value (TGT: the target value is the 2-98% truncated average signal intensity value of an array processed during normalization). In Genevestigator, all TGT

values were scaled to 1000. To give an idea about how strongly a gene is expressed, signal intensity values were related to absent/present calls and compared to their ranking. In case of analysis of expression pattern whilst applied stresses values were calculated as the ratio between the signal obtained from stressed and unstressed plant and values of more than three were taken as significant change.

2.22 Construction of phylogenetic tree

In case of *Physcomitrella patens* Susy sequences were derived from unannotated whole genome shotgun (WGS) sequences from the DOE Joint Genome Institute (http://www.jgi.doe.gov/index.html). Sequences were identified by Cross-Species Mega BLAST searches of the *Physcomitrella patens* WGS sequences in the NCBI Trace Archive (http://www.ncbi.nlm.nih.gov/Traces/trace.cgi), and manually assembled using GeneDoc (http://www.psc.edu/biomed/genedoc/). The protein coding sequences were deduced by comparison with *Physcomitrella patens* EST sequences where available, and otherwise by comparison of conceptual translations of the genomic sequence with known Susy protein sequences. Protein sequences of *Physcomitrella patens* used in the analysis are included in Appendix.

In case of Maize4 gene genomic sequences at TIGR Maize Database (http://maize.tigr.org/) were aligned with Rice4 protein sequence. TIGR genome survey has revealed with the identification of two contigs AZM5 10048 (3'region) and AZM 17217 (5'region). 3' end of coding region was defined by EST DN219509. Part of the Maize4 sequence was filled with Rice4 (showed in lowercases). The protein sequence for Maize4 gene is included in Appendix.

Poplar tree Susy protein sequences were derived from DOE Joint Genome Institute (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html). The sequences for all other Susy proteins were derived from NCBI (http://www.ncbi.nlm.nih.gov/).

Phylogenetic analysis based on protein sequences was than performed using the GenomeNet CLUSTALW server (http://www.ebi.ac.uk/clustalw/). To display tree TreeView program was used (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html

3 Results

3.1 Phylogenetic analysis of sucrose synthases from Arabidopsis and other plant species

In order to study similarities of Arabidopsis *Sus* genes with that of other plant species phylogenetic comparison was initiated. Phylogenetic analysis based on the predicted amino acid sequences has shown that Arabidopsis *Sus* genes can be classified in three different branches (Figure 3). *Sus1* and *Sus4* the most similar to each other occur on the vast dicotyledonous branch (Dicot SUS1 group). *Sus2* and *Sus3* are closely related as well and appear on the mixed branch where monocotyledonous and other dicotyledonous are present (SUSA group). The third pair *Sus5* and *Sus6* exhibits unique extensions on the 3'end and therefore constitutes an other separate group (New group).

In the phylogenetic tree (Figure 3) an early split of *Sus* genes into two distinct braches can be observed. The new group contains subgroups of monocotyledonous (with rice and maize sucrose synthase isoforms) and dicotyledonous (Arabidopsis and poplar tree *Sus* genes). The second group is divided into the vast monocotyledonous and dicotyledonous group and the group of *Physcomitrella Sus* genes.

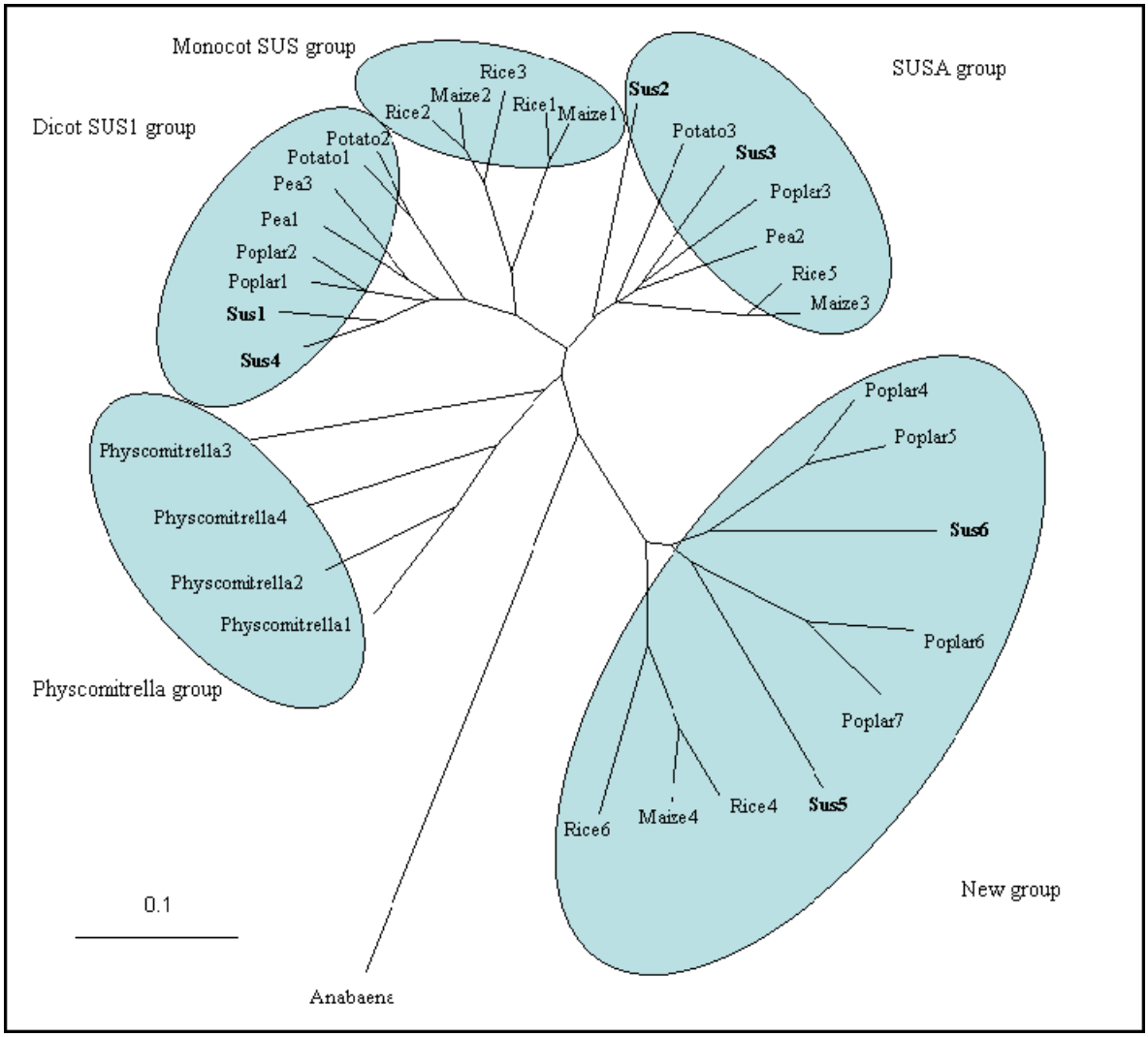


Figure 3: Phylogenetic tree of sucrose synthases from *Arabidopsis thaliana* and other plant species.

Included on unrooted tree are: Arabidobsis sucrose synthases *Sus1* (at5g20830), *Sus2* (at5g49190), *Sus3* (at4g02280), *Sus4* (at3g43190), *Sus5* (at5g37180), *Sus6* (at1g73370), Rice1 (X64770), Rice2, (P31924), Rice3 (Q43009), Rice4 (XP471307), Rice5 (AA118671), Rice6 (XP468546) from *Oryza sativa*; Potato1 (P10691), Potato2 (P49039), Potato3 (AA067719) from *Solanum tuberosum;* Poplar1 (Poptr1:589341), Poplar2 (Poptr1:736740), Poplar3 (Poptr1:692288), Poplar4 (Poptr1:56942), Poplar5 (Poptr1:743479), Poplar6 (Poptr1:556032), Poplar7 (Poptr1:592812) from *Populus trichocarpa;* Pea1 (AAC28107), Pea2 (O24301), Pea3 (P49039) from *Pisum sativum;* Maize1 (P04712), Maize2 (P49036), Maize3 (AAM89473) from *Zea mays;* Maize4 (*Zea mays)* and all Physcomitrella (*Physcomitrella patens)* genes were manually assembled using GeneDoc (http://www.psc.edu/biomed/genedoc/). Phylogenetic analysis based on protein sequences was performed using the GenomeNet CLUSTALW server (http://www.ebi.ac.uk/clustalw/) and TreeView program was used (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) to display results.

3.2 Gene expression studies of the sucrose synthase gene family in Arabidopsis

3.2.1 Quantitative expression analysis of sucrose synthase gene family using real-time RT-PCR

In order to investigate the expression of all sucrose synthases in a quantitative aspect, real-time RT-PCR was performed. A comparative analysis of transcript levels using this method detected expression of all the isoforms in several plant organs and tissues. All transcript levels were normalised to the expression of the ubiquitine 10 (UBQ10) gene and given as mean value of three biological replicates.

For *Sus1* isoform the highest relative transcript level was detected in flower buds, flowers and siliques (1.92, 1.79 and 1.06 respectively), apart from that transcripts for this isoform were detected in other analysed organs on a fairly low level (0.03-0.56). The phylogeneticaly closest to *Sus1*, *Sus4* isoform showed highly tissue specific expression in roots (7.28) and siliques (2.59), whereas in other analysed parts of the plant, expression was at the detection limit (0.001-0.17). Reasonable transcript levels of *Sus2* were only found in siliques (0.38). Highly tissue specific expression was also detected for *Sus3* isoform in seeds (11.07), whereas in flowers transcript levels reached 0.8, and in other organs remained hardly detectable (0.08-0.35). *Sus5* and *Sus6* isoforms were generally expressed on the similar, low and fairly constant level (0.01-0.55) throughout the whole plant. Detailed relative expression of Susy isoforms is visualized in Figure 4.

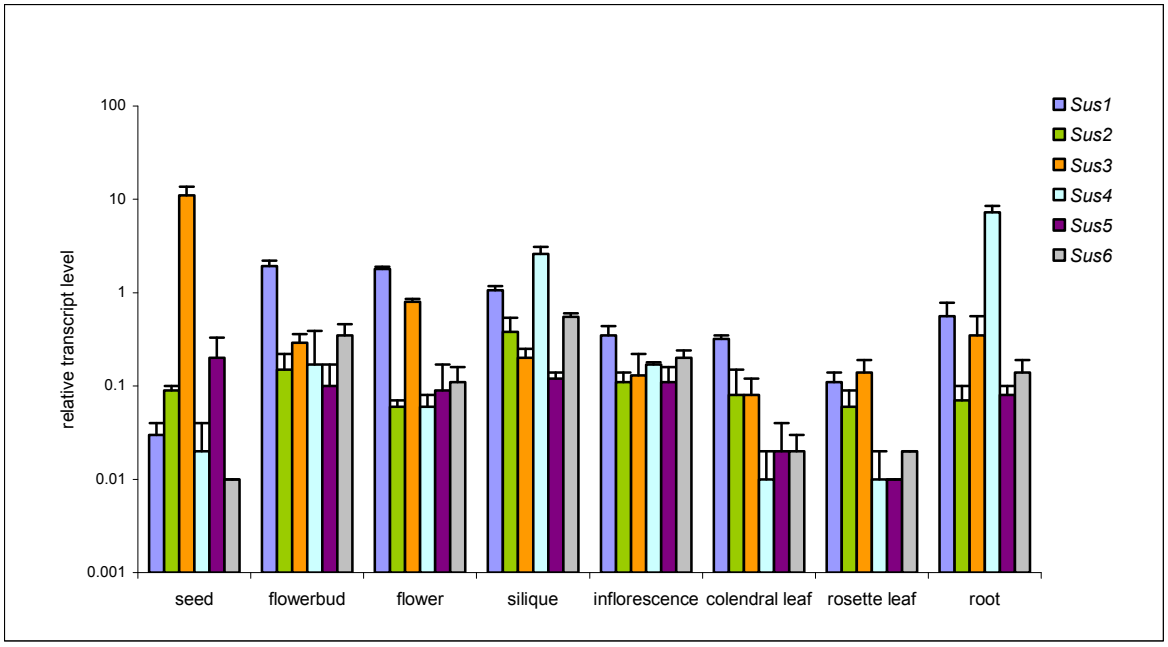


Figure 4: Relative expression profile of the six Arabidopsis sucrose synthase genes.

All values for transcript level were normalized to the expression of UBQ10 gene as the mean and standard deviations of at least three replicates. Due to high variations in the relative transcript levels observed in various plant organs, logarithmic scale was chosen to visualise the results.
Transcript levels of Susy isoforms were analysed from different tissues in 42 day old plants grown in soil in 16h/8h light/dark photoperiod. Col-0 seeds for expression analysis were imbibed in darkness for 48h in 4°C on the half strength MS, 0.7% agar, 1% sucrose plates.

3.2.2 Expression analysis using publicly available data of array hybridisations

In order to gather and investigate all available data about expression patterns of Susy isoforms and further, more detailed, investigate their expression pattern Digital Northern analysis at www.genevestigator.ethz.ch (Zimmermann *et al.*, 2004) was used. The analysis was performed for all isoforms on multiple microarrays concerning two aspects. At first the main interest was to investigate their organ and tissue specific expression and changes during plant development. Another important aspect was the expression analysis under applied stresses, concerning, if existing, up or down regulation of particular isoforms. This sort of studies was also supported by the analysis of microarrays performed in our institute. In particular experiments done by: Joost van Dongen (oxygen deficiency stress, personal communication) and Matthew Hannah (cold stress, (Hannah *et al.*, 2005), Oliver Blaesing (diurnal regulation of gene expression, (Gibon *et al.*, 2004) were further characterized.

3.2.2.1 Expression of sucrose synthase isoforms in different organs and in different developmental stages

Analysis of available data sets showed that among all Susy isoforms, *Sus1* is expressed on the highest, fairly constant level through whole plant (Figure 5) with the exception of siliques and developing seeds (Figure 6). In addition *Sus1* showed also high tissue specific expression in flowers. The closest homolog to *Sus1*, the *Sus4* isoform showed significantly high transcript level only in roots, whereas other plant organs, exhibited only low expression.

Sus2 and *Sus3* showed very strong, highly tissue specific expression in siliques and developing seeds (Figure 6). In these organs *Sus2* is expressed starting from early stages of embryo development in siliques, until the time when embryo has ceased to divide. Reaching that point, it disappears almost completely. *Sus3* expression appears at the end of embryo development and exhibits the strongest transcript level in seeds which are already fully developed. In all other analysed organs, expression of *Sus2* was at the threshold of detection. *Sus3* is additionally expressed in flowers, colendral and senescing leaves.

Sus5 and *Sus6* showed very similar, fairly weak and constant expression through the whole plant and during all developmental stages. None tissue specific expression was observed for both of the isoforms. Qualitative expression of all Susy isoforms is visualised on Figure 5.

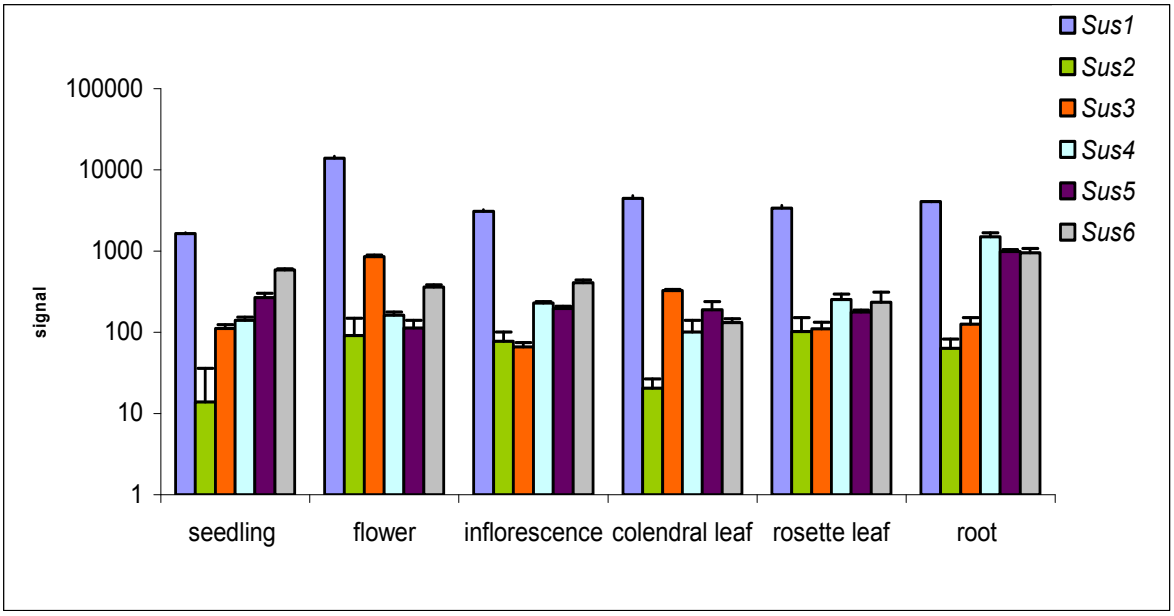


Figure 5: Qualitative expression analysis of sucrose synthase genes using data derived from AtGenExpress project (Schmid *et al.*, 2005).

All signal values for several isoforms are the means and standard deviation of three measurements. Total RNA prior to hybridization with ATH1 chip, were extracted from: 7 day old seedlings, 17 day old roots and rosette leaves, 21 day old flower, inflorescences and colendral leaves. All Arabidopsis plants were grown in soil, at continuous light time regime.

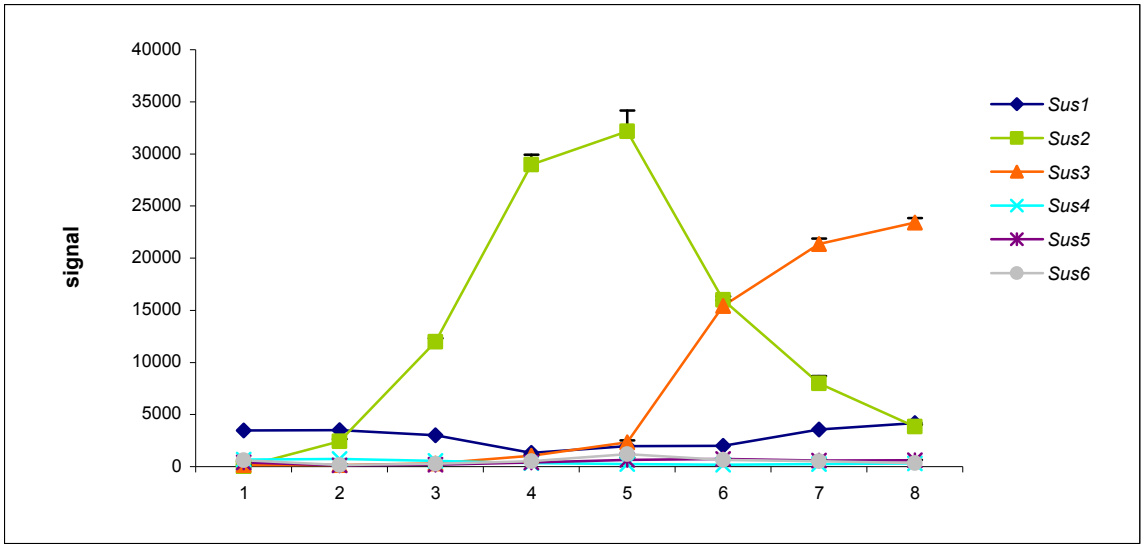


Figure 6: Expression of sucrose synthase isofroms in siliques and developing seeds using data derived from AtGenExpress project.

All signal values for several sucrose synthase genes are the means and standard deviation of three measurements extracted from Genevestigator (Zimmermann *et al.*, 2004). Total RNA was extracted from plants grown in soil, at 16-hours photoperiod for 8 weeks. Numbers 1 to 8 refer to different developmental stages of siliques and seeds (Boyes *et al.*, 2001).

1- stage of embryo development when protoderm is formed;
2- embryonic stage at which the cotyledon primordia have enlarged enough to form two mounds on either side of the apical meristem;
3- a transient stage between the late globular and heart stage when the first cell divisions of the cotyledon primordia begin and cell elongation starts in the procambium;
4- torpedo stage when the embryo elongates into the cellular endosperm and the internal layers of the hypocotyl and radicle differentiate to form the vascular tissue (lipid deposition into the cotyledons begins and organelle differentiation occurs leading to greening of the embryo);
5, 6- stages at which the cotyledons curve down towards the suspensor (protein deposition in the cotyledons begins);
7- stage at which the embryo has ceased to divide;
8- green cotyledons. Stages one, two, three consist of sliques with seeds, stages from 4 to 8 only from seeds.

3.2.2.2 Diurnal expression of sucrose synthase isoforms

Many enzymes, especially involved in plant metabolism are diurnally regulated both at the transcript and protein level (Gibon *et al.*, 2004). The first step of such regulation is accumulation or degradation of transcript, which can later affect enzyme activity at a particular time during a day and night. The sucrose synthase gene family was also subjected to that investigation on a transcript level.

Data analysis revealed that *Sus1* is the only one, among other Susy isoforms, showing diurnal regulation of transcript level (Figure 7). It exhibits the highest transcript accumulation at the end of the day and the lowest at the end of night.

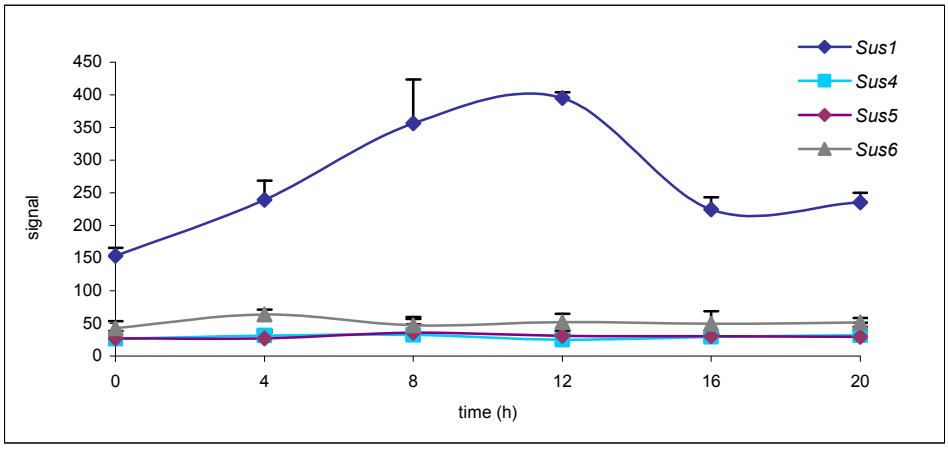


Figure 7: Diurnal regulation of transcript level of sucrose synthase isoforms.

All signal values for several isoforms are the means and standard deviation of three measurements Arabidopsis plants were grown in soil for two weeks in 8h day phytotron and than transferred into a small growth cabinet with 12h day of 160 µE and 20°C for additional three weeks. Harvest of leaf rosettes at a time point was performed sequentially every 4h within a day/night cycle. Sample collection started at the end of night (0); 4, 8, 12 were the samples collected during the day, 16 and 20 were harvested at night. Signal for *Sus2* and *Sus3* was not detected in this experiment (Gibon *et al*., 2004).

3.2.2.3 Response of sucrose synthase isoforms to different stresses

As already pointed out in the introduction, sucrose synthase expression is known to respond to a variety of different environmental treatments. In order to investigate the special involvement of particular isoforms into response to abiotic stresses several experiments were analysed such as: osmocity, cold, oxygen deprivation, drought, wounding, oxidative and salinity stresses.

Response of all Susy isoforms to osmocity stress (Zimmermann *et al.*, 2004) was analysed both in roots and leaves of 18 day old plants treated with 300mM mannitol for 30 min., 1, 3, 6, 12 and 24 hours. Data analysis showed (Figure 8) that in roots only the *Sus3* isoform revealed changes in expression. After treatment for 3, 6, 12 and 24 hours transcript levels for *Sus3* increased respectively: 3.3, 6.2, 6.7, 7.1 fold. In leaves changes for *Sus1* and *Sus3* transcript levels could be found. After 3, 6, 12 and 24 hours of incubation increase in the expression levels for *Sus1* (5.5, 10.9, 6.7, 6.4 times) and *Sus3* (6.2, 54.8, 79.8, 70.8 times) could be observed. However big standard deviations especially for the *Sus3* expression in leaves can lower significantly these ratios.

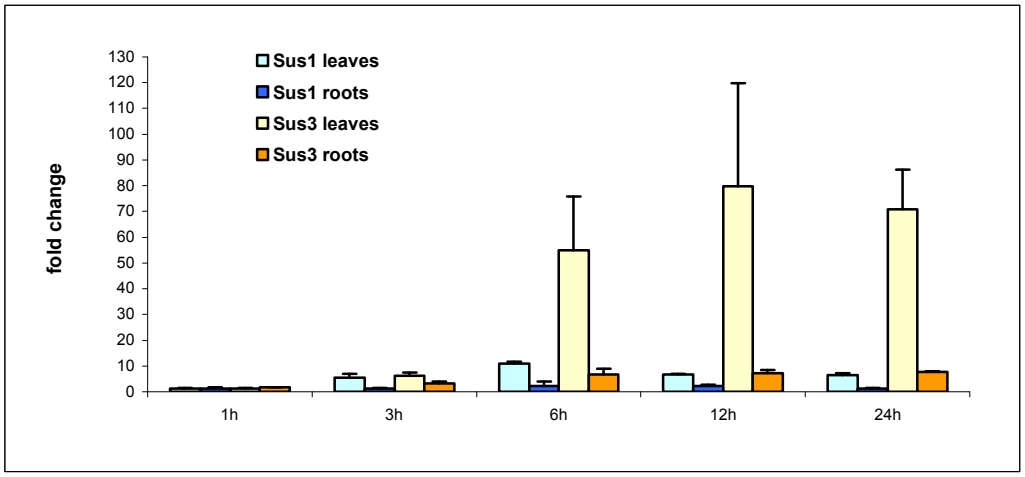


Figure 8: Response of sucrose synthase isoforms in roots and leaves to the osmotic stress.

Arabidopsis seedlings were at first grown for 13 days on half strength MS medium containing agar (5g/l), sucrose (5g/l) and Gamborg's B5 vitamins 2.2 g/l, than transferred for additional five days into liquid medium (half strength MS medium containing Gamborg's B5 vitamins 2.2 g/l). For the first two days plates were kept at continues light at 4°C, for the rest at 16 hour light photoperiod at 25°C. 18 day old plants where treated with 300mM mannitol starting from 30 min. up to 24 hours. Control plants were grown in parallel. Roots and leaves were separated during harvest and influence of mannitol on Susy gene expression was investigated in these two organs. Fold change was calculated as a ratio between signal values of plants treated with mannitol and controls and were the means and standard deviation of two measurements. Data were extracted from AtGenExpress project: osmotic stress time course.

Response of all Susy isoforms to cold treatment (Zimmermann *et al.*, 2004) was analysed in roots and leaves of 18 day old plants treated with cold temperature (4°C) for 30 min, 1, 3, 6, 12 and 24 hours. Only *Sus1* showed significant up regulation: 3.7, 11.7, 28.2 fold in the leaves treated with cold for 6, 12 and 24 hours (Figure 9). In another cold stress experiment (Hannah *et al.*, 2005), where mature plants (40 day old, grown in soil at 16 hour light photoperiod) were treated with cold temperature (4°C) for 14 days, transcript level for *Sus1* showed also significant increase (3.7 fold) in leaves (not shown).

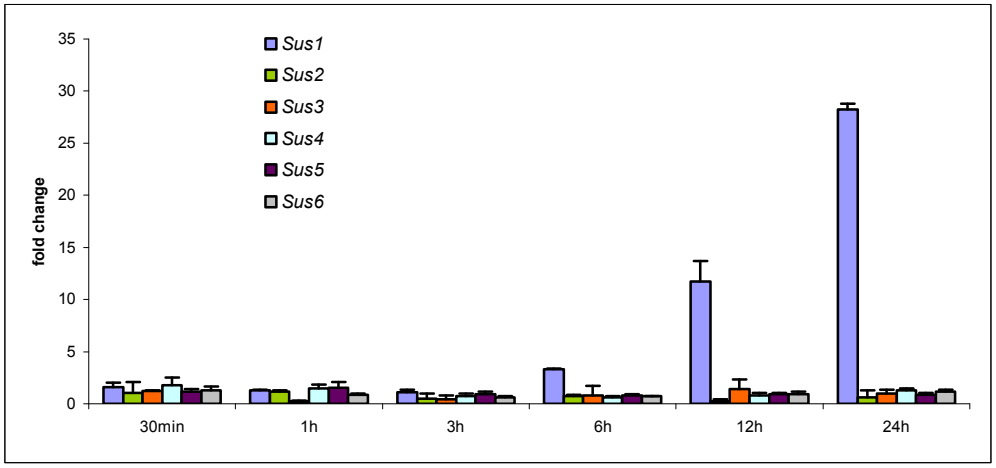


Figure 9: Response of sucrose synthase isoforms in leaves to the cold stress.

Arabidopsis seedlings were at first grown for 13 days on half strength MS medium containing agar (5g/l), sucrose (5g/l) and Gamborg's B5 vitamins 2.2 g/l, than transferred for additional five days into liquid medium (half strength MS medium containing Gamborg's B5 vitamins 2.2 g/l). For the first two days plates were kept at continues light at 4°C, for the rest at 16 hour light photoperiod at 25°C. 18 day old plants where treated with cold (4°C) temperature starting from 30 min. up to 24 hours. Control plants were grown in parallel. Fold change was calculated as a ratio between signal values of treated plants and controls and were the means and standard deviation of two measurements. Data were extracted from AtGenExpress project: cold stress time course.

Response of Susy isoforms to oxygen deprivation was analysed in roots in the experiment performed by Joost van Dongen (personal communication). In this approach seedlings were grown in different oxygen concentrations: at ambient (21%), 8%, 4% and 1% O_2. Relative transcripts levels when compared with plants at ambient conditions revealed that among all Susy isoforms *Sus1* and *Sus4* exhibit strong induction upon oxygen deprivation. While *Sus1* is induced up to 7.5 times at 1% O_2 *Sus4* isoform increased 60 times in roots (Figure 10).

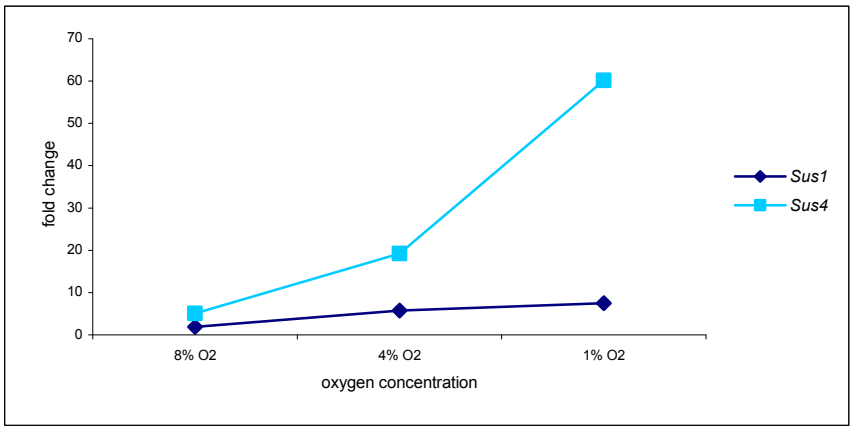


Figure 10: Response of *Sus1* and *Sus4* isoforms to low oxygen level.

Fold change was calculated as a ratio of signal values of Arabidopsis grown in low (8%, 4% or 1%) and ambient (21%) oxygen concentration. Analyzed seedlings were first kept on vertical plates (half strength MS medium, 1% sucrose, 1.5% agar) for 10 days in 16h/8h light/dark photoperiod at 20°C, than transferred into darkness and grown for additional two days in the subsequent oxygen concentration. Low oxygen level was supplemented with nitrogen. CO_2 was at 350 ppm.

None of the Susy isoforms responded with a significant change of transcript level to the applied oxidative, salinity, drought and wounding stress.

3.2.3 Expression of sucrose synthase gene family analysed by stable expression of promoter reporter gene fusion

For more specific analysis and to confirm and further investigate data obtained from microarrays, a promoter reporter gene fusion system was used. Our expectation about that approach was to investigate the occurrence of respective isoform in the aspect of localization in particular cells, tissues and organs under normal growth condition.

3.2.3.1 Preparation of SUS_{1-6}-pGPTV constructs for promoter reporter gene (GUS) studies

The basic strategy was to clone promoter fragments of each Susy isoform into the pGPTV vector. Promoter fragments of the following sizes of the particular Susy isoforms were amplified by PCR, sequenced and cloned at the 5' end of *uid A* gene: *Sus1*-1983 bp; *Sus2*-595 bp; *Sus3*-2088 bp; *Sus4*-1948 bp; *Sus5*-1732 bp; *Sus6*-752 bp. In case of *Sus2* and *Sus6* the compete intergenic region between the putative ATG of the respective Susy coding sequence and the gene in front of it was used. Because the intergenic region between *Sus1*, *Sus3*, *Sus4* and *Sus5* and the next gene upstream was too long putative promoter regions of the sizes indicated were used. Schematic representation of reporter constructs is shown below (Figure 11).

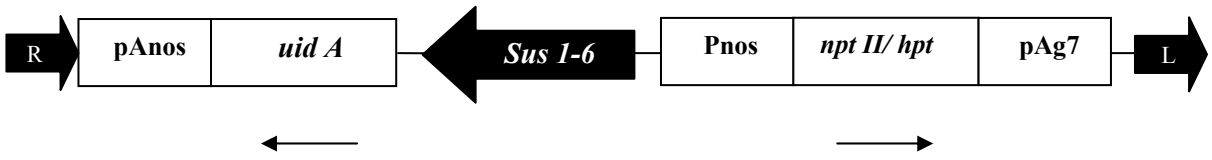


Figure 11: Schematic diagram of the SUS_{1-6}-pGPTV construct (not drawn to scale) used for promoter expression studies.

For five of the six Susy isoforms pGPTV-KAN was used. In case of sus4 cloning was performed with pGPTV-HYG.
Abbreviations: R – right T-DNA border; pAnos – T-DNA nopaline synthase; *uid A* – β-glucuronidase gene; sus 1 – 6 - respective Susy isoform; Pnos – nopaline synthase promoter; *npt II* – neomycin phosphotransferase; *hpt* – hygromycin phosphotransferase; pAg7 – gene 7; L – left T-DNA border.

Arabidopsis thaliana plants were transformed with the six individual constructs and kanamycin or hygromycin plants exhibiting GUS activity were isolated. Resistant plants were kept for seed production. All expression analysis were performed on the transformants from T2 generation of at least four independent transformants.

3.2.3.2 Expression pattern of individual sucrose synthase isoforms

In order to investigate the spatial and temporal expression of all Susy isoforms at least four independent transformant lines per one construct were grown in the long day conditions. From each line five to eight plants were analysed. At specified time points (Table 2) different plant organs were harvested prior to histochemical analysis.

Detailed expression patterns (Table 2) of the individual sucrose synthase isoforms:

Sus1. In 5 and 14 day old seedlings staining was observed in vasculature in cotyledons, and roots. In mature plants staining was present in roots, vasculature of rosette and colendral leaves, stems, petals, sepals, stigma, stamen, siliques, entry point of funiculus into seeds

Sus2. Expression was observed in imbibed seeds. In 5 day old seedlings strong staining was present in cotyledons, hypocotyls and root tips, whereas in 14 day old seedlings staining in the same organs was almost not detectable anymore. In mature plants expression occurred exclusively in seeds.

Sus3. Expression was observed in imbibed seeds. In 5 day old seedlings staining was observed throughout the whole plant, whereas in 14 day seedling it remained only in root. In mature plant weak staining was observed in some lateral roots and root tips. Faint staining was also seen in rosette and colendral leaves. It was expressed in stomatal guard cells and also in senescing leaves. More over expression was observed in petals, stamens and siliques.

Sus4. 5 and 14 day old seedling showed staining in roots. In mature plants expression was observed in roots, vasculature of rosette and colendral leaves, stems, stigmas, stamens, receptacles, pedicels.

Sus5 and ***Sus6***. In 5 and 14 day old seedlings as well as in mature plants staining was always found in vascular tissue throughout whole plant. Expression pattern of these two isoforms is identical but for the *Sus5* isoform the expression is weaker. However these observations were not proven with the quantitative measurements of GUS activity.

Expression of sucrose synthase gene family in selected organs analysed by stable expression of promoter reporter gene constructs is represented on Figure 12.

gene	seeds	seedlings	roots	stem	siliques	flower	colendral leaves	rosette leaves
Sus1	-	vascular tissue of cotyledons, roots	+	+	seed, funiculus entering the seeds	petals, sepals, stigmas, stamens	vascular tissue	vascular tissue
Sus2	+	cotyledons, root tips	-	-	seeds in siliques	-	-	-
Sus3	+	cotyledons, roots	very weak in lateral roots and root tips	-	seeds in siliques	petals, stamens	guard cells, senescing leaves	guard cells, senescing leaves
Sus4	-	roots	+	+	-	stigmas, staments, receptac-les	vascular tissue	vascular tissue
Sus5	-	vascular tissue of cotyledons, roots	vascular tissue	vascular tissue	vascular tissue of silique walls and siliques	vascular tissue of petal, stamen	vascular tissue	vascular tissue
Sus6	-	vascular tissue of cotyledons, roots	vascular tissue	vascular tissue	vascular tissue of silique walls and siliques	vascular tissue of petal, stamen	vascular tissue	vascular tissue

Table 2: Summary of the expression pattern of sucrose synthase gene family analysed by stable expression of promoter reporter gene constructs.

Analysed plants were grown in long day photoperiod (16h/8h light/dark), in soil for approximately eight weeks. Organs were harvested 15 – 20 days after flowering. Col-0 seeds were imbibed in darkness for 48h in 4°C on the half strength MS, 0.7% agar, 1% sucrose plates, after that they were harvested prior to seed expression analysis. Remaining seeds for the seedling expression analysis were transferred for additional five and 14 days into phytotron with 16h light photoperiod.
+ expression was detected in whole organ,
- no expression was observed.

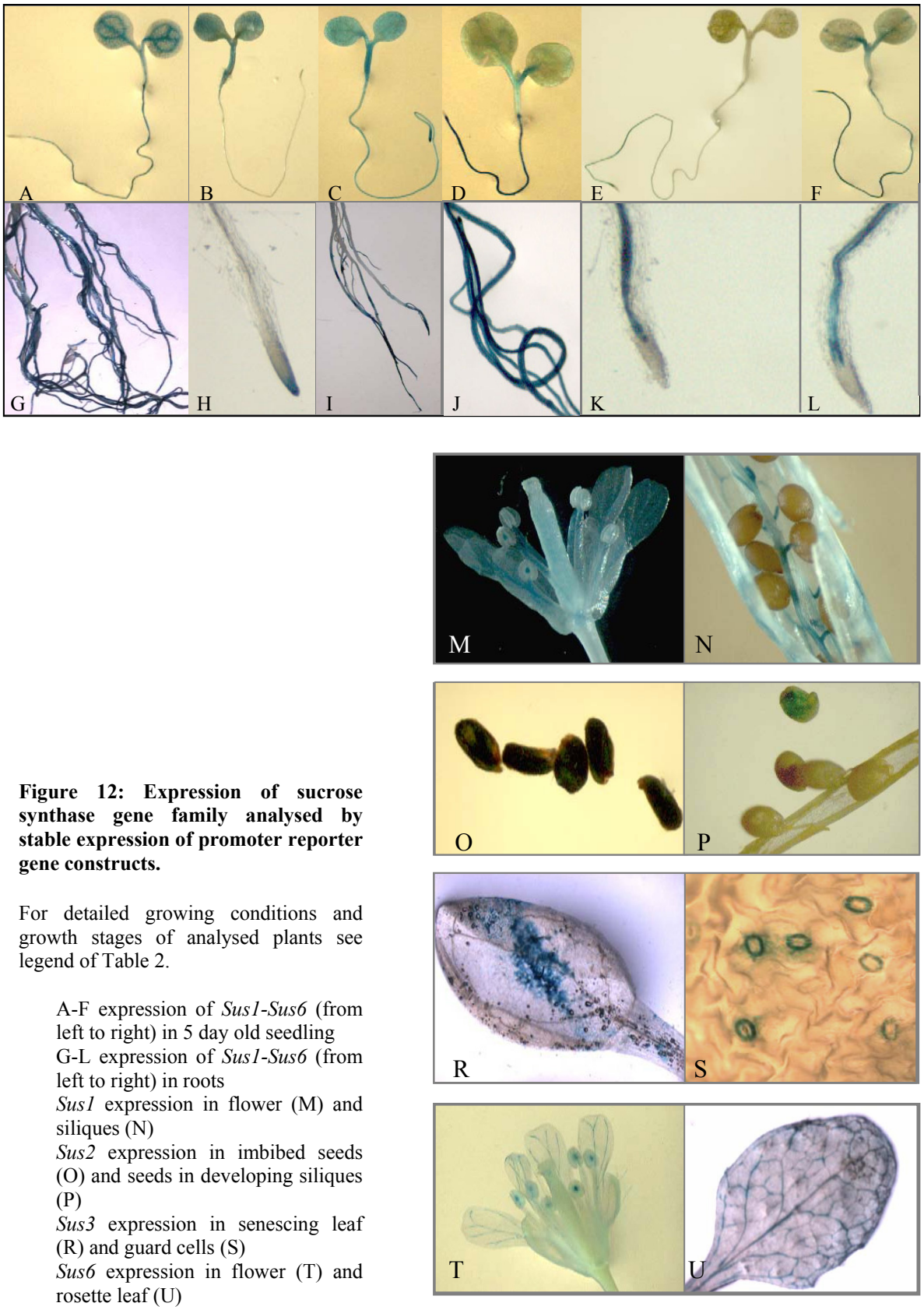


Figure 12: Expression of sucrose synthase gene family analysed by stable expression of promoter reporter gene constructs.

For detailed growing conditions and growth stages of analysed plants see legend of Table 2.

A-F expression of *Sus1*-*Sus6* (from left to right) in 5 day old seedling
G-L expression of *Sus1*-*Sus6* (from left to right) in roots
Sus1 expression in flower (M) and siliques (N)
Sus2 expression in imbibed seeds (O) and seeds in developing siliques (P)
Sus3 expression in senescing leaf (R) and guard cells (S)
Sus6 expression in flower (T) and rosette leaf (U)

3.3 Analysis of the in vivo function of the members of the sucrose synthase and UGPase gene family by functional characterisation of transgenic plants with reduced or completely lacking expression.

In order to analyse if specific Susy and UGPase isoforms have specific functions in sucrose metabolism or cell wall biosynthesis a collection of available T-DNA insertion mutants were analysed in more detail. First of all the T-DNA insertion lines from the SALK or GABI-Kat collection usually available as the heterozygotes for the T-DNA had to be screened to analyse if plants homozygous for the T-DNA insertion are viable. In case where no T-DNA insertion lines were available or no homozygous lines could be obtained RNA_i lines from the AGRIKOLA approach were further analysed.

All gathered T-DNA insertion mutants were in Col-0 background except for W_sus3 and G_sus6 which were in WS background. Both of these mutants have the insertions in exons. Detailed description of all collected mutants in Col-0 ecotype is presented on Figure 13. Mutants kindly provided by Alison Smith (JIC, Norwich, UK) (S_sus1, W_sus3, X_sus4, G_sus6,) were already homozygotes, lacking transcript and protein for the knocked out isoform (personal communication).

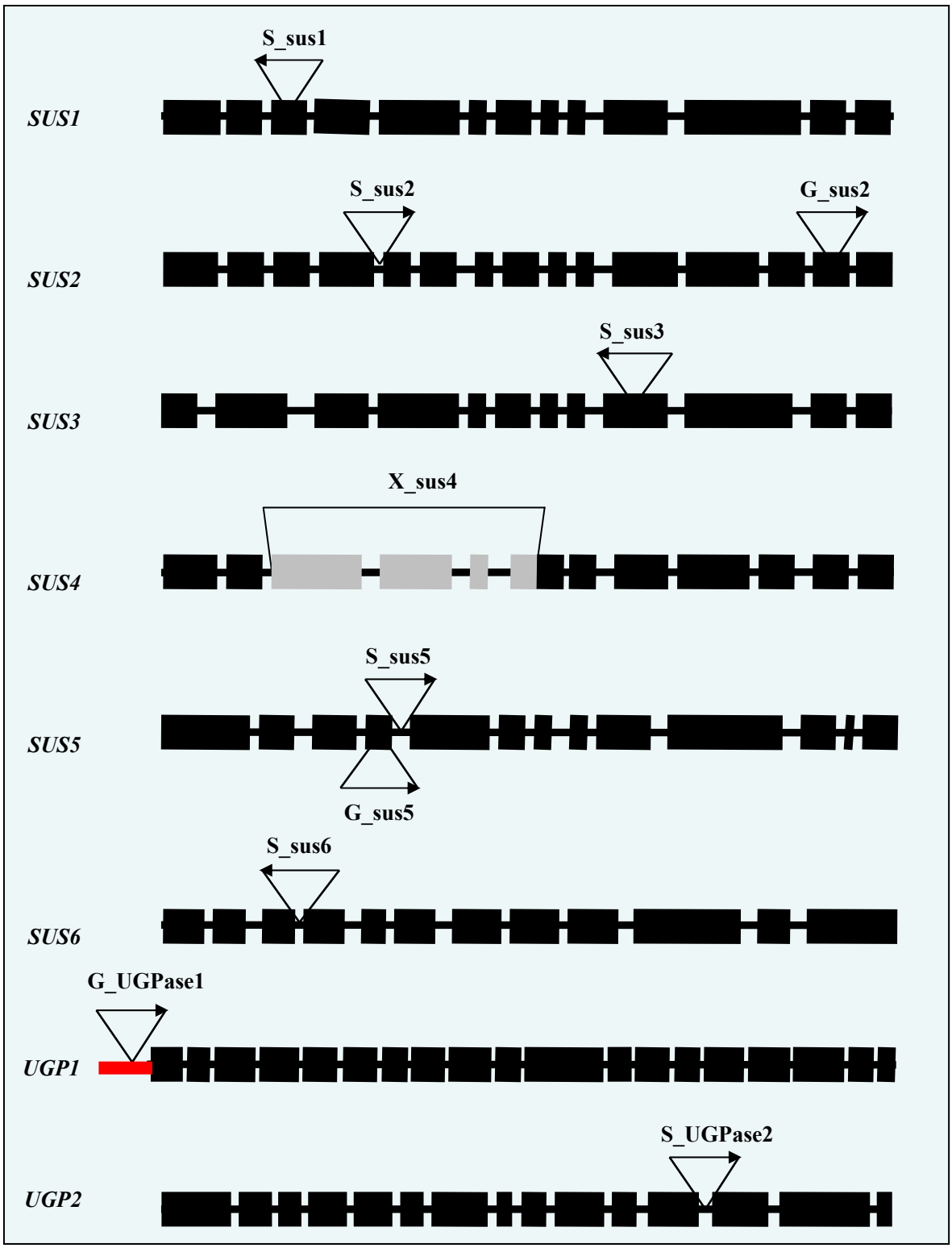


Figure 13: Schematic representation of the *SUS* and *UGP* genes and T-DNA insertions of particular T-DNA knock out mutant in the Col-0 background.

Mutants S_sus2, X_sus4, S_sus5, S_sus6 and S_UGPase2 have the T-DNA insertion in intron, S_sus1, G_sus2, S_sus3, G_sus5 in exon, G_UGPase1 in 5' UTR (red). Mutation in X_sus4 was initially in C24 background but it was backcrossed six times into the Col-0 ecotype selecting each time for the T-DNA insertion in the *SUS4* gene. Part of the *SUS4* sequence (grey) is deleted, from the insertion to mid-way through sixth exon. Direction of arrows indicates the orientation of 35S promoter in T-DNA insertion.

3.3.1 Screening for lines homozygous for the T-DNA insertion

From all T-DNA insertion mutants the offspring was selected by PCR. PCR analyses were carried out using primer pairs specific for the respective Susy and UGPase gene and for T-DNA sequence. Two independent PCR reactions were performed to test each line. Lines were regarded as homozygote when no product occurred with the pair of gene specific primers, while the product with one of the gene specific primer and the primer specific for T-DNA was present (Figure 14). For each line at least three homozygote plants of the particular Susy and UGPase isoform were selected and kept for seed production. All further analysis were performed on T3 or T4 generation.

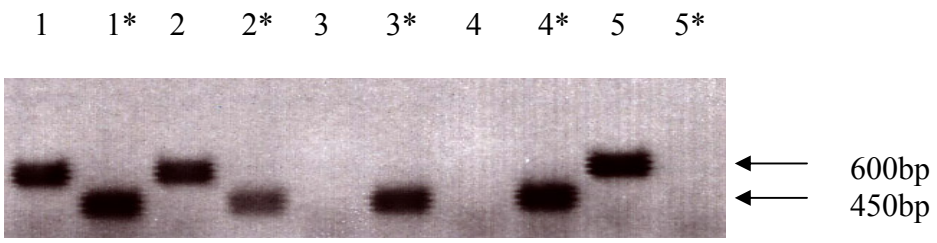


Figure 14: Screening of an example T-DNA mutant (S_019129).

PCR amplified fragments from the T2 generation were resolved on 1% agarose gel. Each mutant plant was tested with two primer pairs. Slots 1-5 represent the DNA of plants 1-5 analysed by PCR reaction with gene specific primers. Slots 1*-5* represent the reaction for detection of T-DNA insertion. If both products appeared (1 and 2) the plant was classified as a heterozygous. When only the product with the gene specific primers appeared (5) plant was classified as a wild type. When only the products with the primers to detect the T-DNA insertion appeared (3 and 4) the plant was classified as a homozygote.

3.3.2 Confirmation of selected homozygote lines by real-time RT-PCR

In order to confirm that the selected homozygote lines lack transcripts of the specific Susy and UGPase isoform, real-time RT-PCR was used. In all analysed lines reduction of transcript level was significant and varied from 81% to 95%. Detailed percentage of reduction of respective mutants and wild type is presented in Table 3.

gene	mutant name	transcript level in Col-0	transcript level in T-DNA insertion mutant	reduction of transcript level
Sus2	G_sus2	0.02 ± 0.002	0.003 ± 0.001	85%
Sus3	S_sus3	0.08 ± 0.01	0.0039 ± 0.002	95%
Sus5	S_sus5	0.007 ± 0.006	0.0011 ± 0.01	84%
Sus5	G_sus5	0.007 ± 0.005	0.0013 ± 0.003	81%
Sus6	S_sus6	0.008 ± 0.005	0.0015 ± 0.01	81%
Ugp1	G_UGPase1	0.045 ± 0.01	0.0085 ± 0.01	81%
Ugp2	S_UGPase2	0.0052 ± 0.01	0.0005 ± 0.01	90%

Table 3: Real-time RT-PCR on selected homozygote Susy and UGPase mutant lines.

From each line three independent plants were analysed. RNA was isolated from rosette leaves and RT rection was performed. For each selected mutant line reaction with gene specific primers (sequences of all primers are the same like for gene expression studies) and standard real time RT-PCR protocol was used. Transcript level is given as the percentage of the expression of UBQ10 gene. Plant material came from the Arabidopsis grown in hydroponics culture for sugar and cell wall analysis harvested at the end of the day.

3.3.3 RNAi transformant in case of *Sus4* isoform

In case of *Sus4* isoform no T-DNA insertion mutant was available at the beginning of the project therefore RNAi transformants were obtained from the Agrikola approach (http://www.agrikola.org/index.html) (Hilson *et al.*, 2004). RNAi lines were analysed using real time RT-PCR in order to find plants lacking transcript for that isoform. As *Sus4* and *Sus1* isoforms are extremely similar (89-95% amino acid identity, Baud *et al.,* 2004) it was expected to see also the reduction in *Sus1* expression. Therefore four RNAi lines (4A, 4B, 4C and 4D) were checked on transcript level for both of the Susy isoforms (Figure 15). 4A, 4B and 4C lines were significantly reduced in the amount of transcript for *sus4* and *sus1*, therefore only they were selected for further analysis.

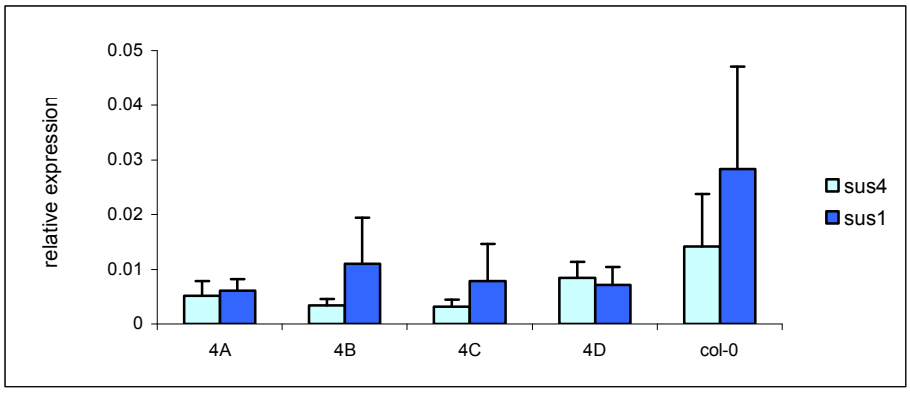


Figure 15: Transcript level of *sus4* and *sus1* isoforms in selected RNAi lines.

RNA was isolated from roots from plants grown in hydroponics culture for sugar and cell wall analysis harvested at the end of the day. All values for transcript level were calculated as a percentage of the expression of UBQ10 as the means and standard deviations of at least four replicates.

3.3.4 No visible phenotype in the individual T-DNA insertion mutant and RNAi lines

From the maize *sh1* mutant (Chourey *et al.*, 1998) and *rug4* mutation in pea (Craig *et al.*, 1999) is it known that the absence of a specific Susy isoform can lead to the shrunken phenotype in seeds. Therefore to investigate if any of the T-DNA insertion mutant and RNAi lines show visible phenotype all plants were grown in soil both in 8h and 16h light photoperiod in phytotron at 120 µmol photons m^{-2} s^{-1} and 20°C/16°C at 60%/75% relative humidity. Under the applied conditions none of the lines showed any visible phenotype in growth and development or a shrunken phenotype in seeds.

3.3.5 Expression of all other sucrose synthases and UGPase isoforms in the T-DNA insertion mutant and RNAi lines was not changed

In all mutant lines, expression of all Susy and UGPase isoforms was analysed. The aim was to investigate if another Susy or UGPase isoform is able to compensate for the loss of Susy or UGPase isoform of the particular T-DNA insertion mutant or RNAi transformant. Therefore the same Arabidopsis plants grown in hydroponics culture (Material and Methods 2.2) were subjected to the measurement of transcript levels of remaining Susy and UGPase isoforms. Data analysis revealed that no significant changes

in the expression of several isoforms in particular T-DNA insertion mutant and RNAi occurred.

3.4 Involvement of sucrose synthases and UGPase isoforms in the synthesis of soluble sugars, starch and cellulose

Previous studies with cotton fibres (Haigler *et al.*, 2001), and maize (Chourey *et al.*, 1998) suggested that sucrose synthase may be involved in channelling UDPG to cellulose synthesis during secondary wall deposition. The same role for UGPase can not be excluded especially in source tissue where Susy protein is not very abundant (Dejardin *et al.*, 1999). Therefore, to investigate the role of each particular isoform of Susy and UGPase on carbohydrate content the previously selected T-DNA insertion mutants were used and the deposition of soluble sugars, starch and cellulose was analysed.

3.4.1 Growing conditions, harvest and extraction protocol

Because of the big differences in tissue specificity it was interesting to see if there are any changes in the content of sugars and cell wall components both in roots and leaves. Therefore hydroponics culture was chosen to grow plants (Figure 16). At least seven wild type and five mutant plants were grown per line. All mutants and respective wild types were harvested at two time points at the end of the day and the end of the night. Whole roots and rosettes were separated during harvest and frozen immediately prior to further biochemical analysis. The protocol used for extraction of soluble sugars, starch and cell wall components published by (Peng *et al.*, 2000) was modified to use bigger amount of initial tissue (200mg). Additionally an extra step for starch removal was added.

Figure 16: Example of hydroponics culture.

Arabidopsis were placed in foam lids with holes (0.5 cm diameter) and immersed in square, black boxes containing approximately five litres of medium. All T-DNA insertion mutant and wild types were randomized in the boxes to avoid position effect. Air was applied to the culture all the time. Medium was changed every four days

3.4.2 Content of soluble sugars, starch and cellulose in roots of sucrose synthase T-DNA insertion mutants.

From the samples collected at the end of the day and night at first amount of glucose, fructose, sucrose and starch were extracted and measured spectrophotometrically according to standard protocol (Jelitto *et al.*, 1992). No significant changes were observed in the concentrations of these carbohydrates in T-DNA insertion mutant lines comparing to respective wild types (Figure 17).

In order to see if there are changes in the sugars accumulation during the day and night between T-DNA insertion mutants and respective wild-types, ratios were built of concentrations of soluble sugars and starch measured at these two time points. No changes were observed in those ratios in plants harvested at the end of day and night (not shown).

Cellulose content was measured spectrophotometrically via anthrone assay only in the samples collected at the end of night. No significant changes were observed in roots comparing to respective wild types (Figure 17).

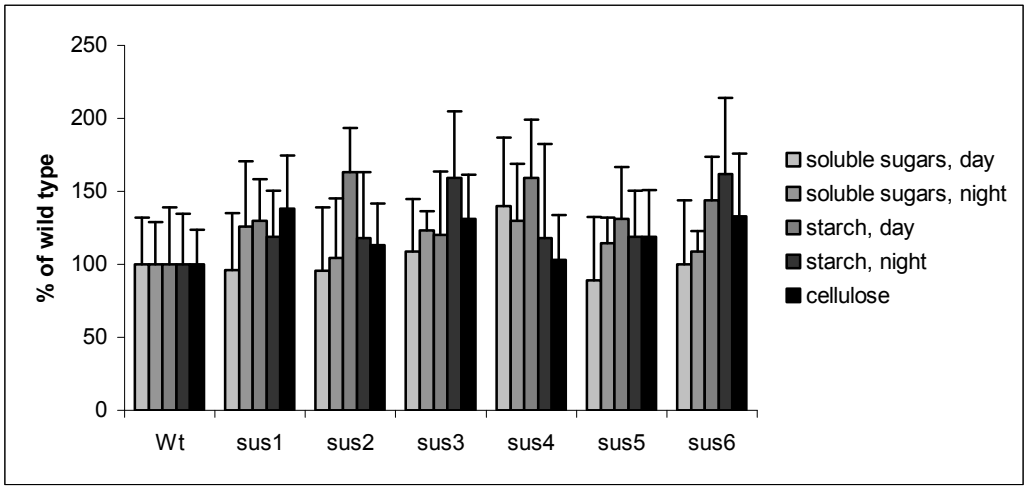


Figure 17: Content of soluble sugars, starch and cellulose in roots of sucrose synthase T-DNA mutants and wild type grown in hydroponics culture, harvested at the end of the day and night.

All results are given in percent of the wild type. The concentration of each carbohydrate of all T-DNA insertion lines was compared with the concentrations of respective wild types: 1.26±0.15 (1.43±0.69), 1.22±0.13 (1.23±0.19), 0.03±0.01 (0.05±0.03), 0.05±0.02 (0.05±0.02), 7.5±2.1 (7.0±1.9) are the concentrations ±SD of at least five independent plants of soluble sugars at the end of the day and night, starch at the end of the day and night, cellulose of Col-0 and WS (values in the brackets), respectively and are expressed in µmol glucose equivalents (g fresh weight)$^{-1}$. In case of sus2, sus3, sus5 and sus6 two independent mutant lines with five independent plants each were used. Statistical analysis of the results was performed using two tailed Student t-test. No significant differences were obtained ($p<0.05$).

3.4.3 Content of soluble sugars, starch and cellulose in leaves of sucrose synthase T-DNA insertion mutants

The same analysis of monitoring carbohydrate concentrations was carried out with leaf samples collected at the end of the day and night. Glucose, fructose, sucrose and starch were extracted and measured like in the case of root samples. Likewise in case of roots no significant changes were observed in the amount of carbohydrates of T-DNA mutants in comparison to respective wild types (Figure 18).

No changes were also observed in the ratios build from the concentrations of soluble sugars and starch in plants harvested at these two time points (not shown).

Leaf samples collected at the end of night were subjected to the measurement of cellulose content. No significant changes were observed in the concentration of cellulose in all analysed mutants in comparison to respective wild types (Figure 18).

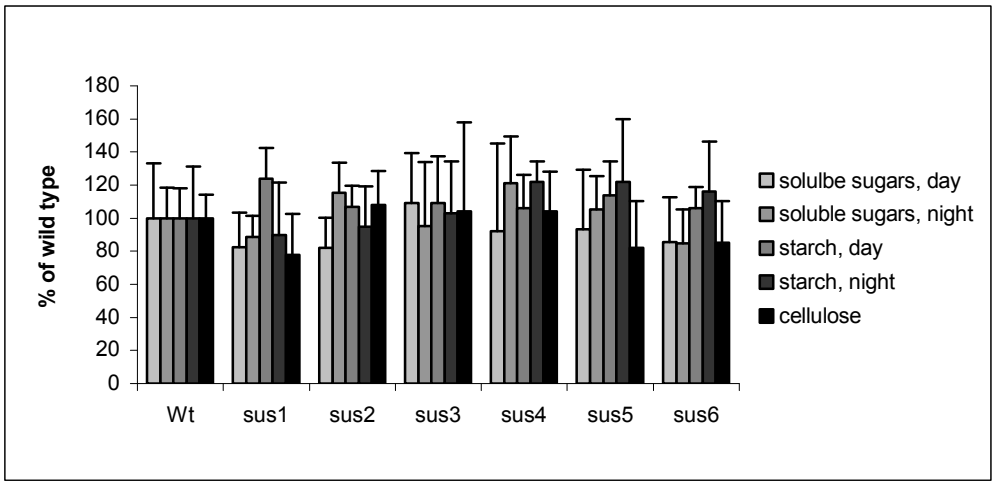


Figure 18: Content of soluble sugars, starch and cellulose in leaves of sucrose synthase T-DNA mutants and wild type grown in hydroponics culture, harvested at the end of the day and night.

All results are given in percent of the wild type. The concentration of each carbohydrate of all T-DNA insertion mutants was compared with the concentrations of respective wild types: 5.59±1.9 (7.11±2.1), 4.13±1.0 (6.99±1.9), 18.5±4.9 (24.4±4.2), 8.5±3.4 (14.2±4.3), 25.3±3.6 (21.4±5.3) are the concentrations ±SD of at least five independent plants of soluble sugars at the end of the day and night, starch at the end of the day and night, cellulose of Col-0 and WS (values in the brackets), respectively and are expressed in µmol glucose equivalents (g fresh weight)$^{-1}$. In case of sus2, sus3, sus5 and sus6 two independent mutant lines with five independent plants each were used. Statistical analysis of the results was performed using two tailed Student t-test. No significant differences were obtained ($p<0.05$).

3.4.4 Sucrose synthase activity was decreased only in S_sus1 mutant

All hydroponically grown mutants and respective wild types were also subjected to the measurement of sucrose synthase activity. Obtained activities (Table 2) were generally very low and comparable with the wild type, except of S_sus1, where the activity calculated per gram fresh weight in roots showed 50% reduction comparing to Col-0.

	roots	**leaves**
plant	activity	activity
S_sus1	0.014 ± 0.001*	0.027 ± 0.010
S_sus2	0.028 ± 0.014	0.022 ± 0.016
G_sus2	0.034 ± 0.012	0.038 ± 0.006
S_sus3	0.023 ± 0.003	0.027 ± 0.008
S_sus5	0.022 ± 0.010	0.022 ± 0.007
G_sus5	0.029 ± 0.020	0.034 ± 0.007
S_sus6	0.023 ± 0.008	0.026 ± 0.009
Col-0	0.030 ± 0.007	0.038 ± 0.012
W_sus3	0.054 ± 0.001	0.018 ± 0.011
G_sus6	0.063 ± 0.024	0.035 ± 0.015
WS	0.041 ± 0.010	0.033 ± 0.015

Table 4: Sucrose synthase activity in T-DNA insertion mutants grown in the hydroponics culture.

Enzyme was assayed in roots and leaves harvested at the end of the day via radioactive assay in the synthesis direction. The results are given as the means and standard deviations of at least three replicas. Activities are given as μmol of incorporated UDPG per min per gram fresh weight. Statistical analysis of the results was performed using two tailed Student t-test, each mutant activity being compared to the activity of respective wild type resulting significant reduction of activity only in case of S_sus1 ($p=0.013$) in roots and is marked with an asterisk.

3.4.5 Content of soluble sugars, starch and cellulose in roots of UGPase T-DNA insertion mutants

Roots collected at the end of the day and night of UGPase T-DNA insertion mutants were subjected to the measurements of soluble sugars, starch and cellulose like in case of Susy mutants. Data has revealed no significant changes between investigated mutants and wild type (Figure 19).

Cellulose content was analysed in root samples collected only at the end of night. No significant changes were observed in the concentration of cellulose in both mutant lines in comparison to wild type (Figure 19).

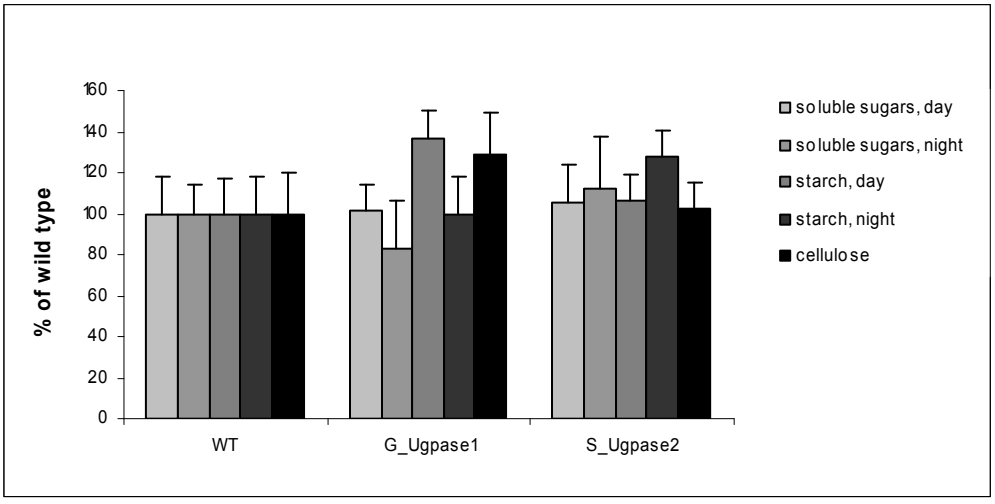


Figure 19: Content of soluble sugars, starch and cellulose in roots of UGPase T-DNA mutants and wild type grown in hydroponics culture, harvested at the end of the day and night.

All results are given in percent of the wild type. The concentration of each carbohydrate of all T-DNA mutants was compared with the concentration of wild type: 1.26±0.15, 1.22±0.13, 0.03±0.01, 0.05±0.02, 7.5±2.1 are the concentrations ±SD of at least five independent plants of soluble sugars at the end of the day and night, starch at the end of the day and night, cellulose of Col-0, respectively and are expressed in µmol glucose equivalents (g fresh weight)$^{-1}$. Statistical analysis of the results was performed using two tailed Student t-test. No significant differences were obtained ($p<0.05$).

3.4.6 Content of soluble sugars, starch and cellulose in leaves of UGPase T-DNA insertion mutants

Soluble sugars and starch were extracted from leaves collected at the end of the day and night of the UGPase T-DNA insertion mutants and the concentrations of glucose, fructose, sucrose and starch were monitored using the same spectophotometric assay like described above (Figure 20). Likewise in the case of Susy mutants, no significant differences between both UGPase lines and control plants were observed.

From the leaf samples collected at the end of night cellulose was extracted and its content was monitored in T-DNA mutants and control plants using anthrone method (Figure 20). Obtained concentrations in case of both UGPase mutants were similar to Col-0 plants.

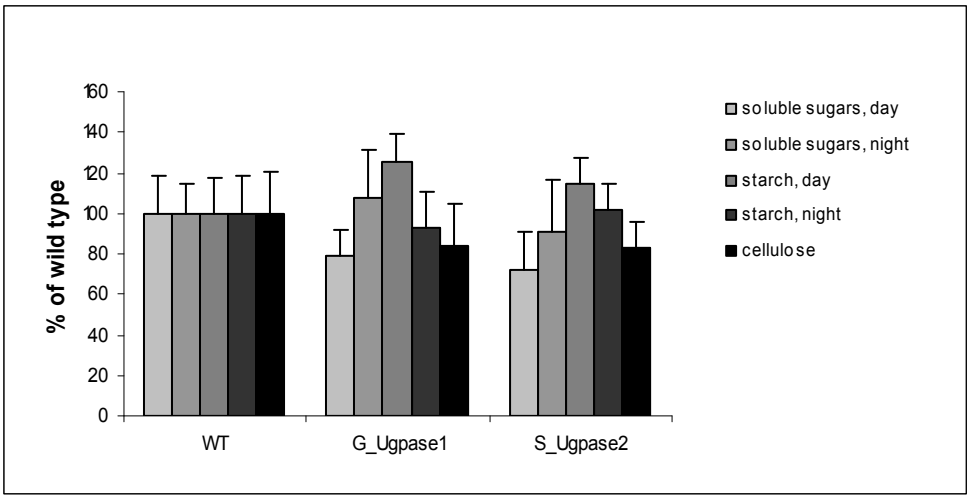


Figure 20: Content of soluble sugars, starch and cellulose in leaves of UGPase T-DNA mutants and wild type grown in hydroponics culture, harvested at the end of the day and night.

All results are given in percent of wild type. Statistical analysis of the results was performed using two tailed Student t-test. The concentration of each carbohydrate of all T-DNA insertion mutants was compared with the concentration of wild type: 5.59±1.9, 4.13±1.0, 18.5±4.9, 8.5±3.4, 25.3±3.6 are the concentrations ±SD of at least five independent plants of soluble sugars at the end of the day and night, starch at the end of the day and night, cellulose of Col-0, respectively and are expressed in µmol glucose equivalents (g fresh weight)$^{-1}$. Statistical analysis of the results was performed using two tailed Student t-test. No significant differences were obtained ($p<0.05$).

3.4.7 UGPase activity in T-DNA insertion mutants was roughly similar to that observed in Col-0

Root and leaf samples harvested at the end of the night of plants grown hydroponically were subjected to the measurement of UGPase activity in order to investigate if the reduction at the transcript level affects also enzyme activity. Obtained results (Table 5) showed no changes in the enzyme activity in roots, in leaves only minor decrease in the activity in G_UGPase1 mutant was seen comparing to the control.

	roots	**leaves**
plant	activity	activity
G_UGPase1	1.7 ± 0.18	4.4 ± 0.06*
S_UGPase2	1.6 ± 0.14	4.5 ± 0.37
Col-0	1.6 ± 0.11	5.8 ± 0.88

Table 5: UGPase activity in T-DNA insertion mutants grown in the hydroponics culture.

Enzyme was assayed in roots and leaves harvested at the end of the night using the method described in Zrenner *et al.,* 1993. Activity is given as μmol per min per gram fresh weight. The results are given as the means and standard deviations of three replicas. Statistical analysis of the results was performed using two tailed Student t-test, each mutant activity being compared to the activity of respective wild type resulting significant reduction of activity only in case of G_UGPase1 (p=0.05) in leaves and is marked with an asterisk.

3.5 Study of freezing tolerance of S_sus1 mutant

Many plants, including Arabidopsis are able to cold-acclimate in response to low non-freezing temperatures a process that involves many biochemical changes. One of these changes is a strong induction of the expression of *Sus1* (Fowler and Thomashow, 2002; Baud *et al.*, 2004), Figure 9). Recently, it was shown that many metabolites, including sugars, increase during cold-acclimation (Cook *et al.*, 2004). Therefore, *Sus1* is a potential candidate gene for metabolising sucrose for use in the synthesis of some of the accumulated metabolites.

Therefore, the freezing tolerance of the S_sus1 mutant was investigated. Non-acclimated (NA) and cold-acclimated (ACC) (2 weeks/4°C) Col-0 and S_sus1 plants were used to measure electrolyte leakage of detached leaves (quantitative measure of freezing tolerance) and a concentration of carbohydrates.

3.5.1 S_sus1 and Col-0 do not differ in non-acclimated and acclimated freezing tolerance

Figure 21 shows that S_sus1 did not significantly differ in freezing tolerance compared to Col-0 under identical experimental conditions, either for NA (Figure 21a) or ACC (Figure 21b) plants leaves.

The LT_{50} values (calculated temperature where 50% electrolyte leakage occurred) were calculated after curve fitting to the electrolyte leakage data. For NA Col-0 and S_sus1 values were -6.7 and -6.26, for ACC Col-0 and S_sus1: -10.95 and -11.28, respectively. Also the cold acclimation capacity which is the ability to increase freezing tolerance under acclimating conditions and is calculated as the difference between LT_{50} of ACC and LT_{50} of NA was not changed.

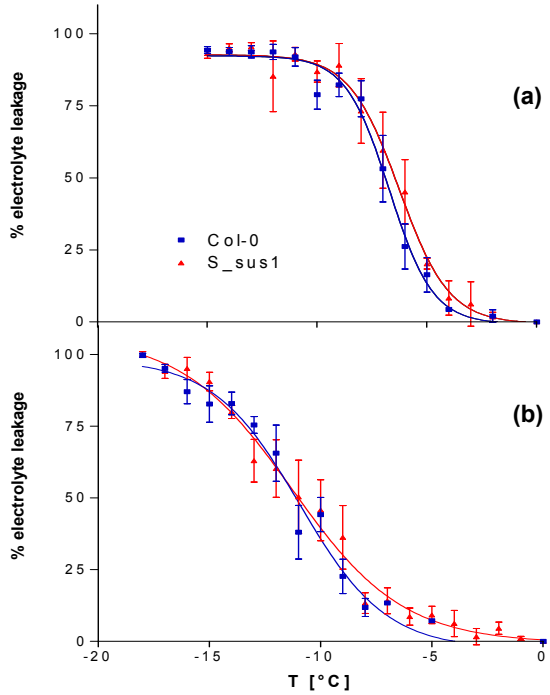


Figure 21: Freezing tolerance of S_sus1 mutant.

Leaves of NA (a) and ACC (b) Col-0 and S_sus1 were frozen to different temperatures. After thawing, electrolyte leakage was measured before and after boiling the samples. Freeze-thaw damage was measured as the percentage of the conductivity of the same samples before boiling and after realising all the electrolytes from the leaf tissue by boiling. Sigmoidal curves were fitted to the leakage data to determine the temperature at which 50% leakage occurred (LT_{50})
NA plants were for 40 days grown in soil in 16h light photoperiod. Cold acclimated plants were moved to 4°C for an additional two weeks.

3.5.2 Content of soluble carbohydrates in S_sus1 and Col-0 leaves

During plant cold acclimation, the content of compatible solutes in leaves increases and these may be important for the development of freezing tolerance (Smallwood and Bowles, 2002). Therefore, the amounts of five sugars glucose (Glc), fructose (Fru), sucrose (Suc), raffinose (Raf) and galactinol (Gol) were measured in leaf samples before and after cold acclimation. The data clearly show (Table 6) that all sugars increase dramatically during cold acclimation in both analysed lines. Although there was a slight increase in sucrose and raffinose in S_sus1 in comparison to Col-0 in both NA and ACC plants these differences were significant only in case of raffinose.

plant line	Glc	Fru	Suc	Raf	Gol
S_sus1 NA	0.32 ± 0.08	0.02 ± 0.01	0.35 ± 0.06	0.03 ± 0.00*	0.05 ± 0.01
S_sus1 ACC	8.00 ± 1.78	2.55 ± 0.77	4.15 ± 0.83	2.58 ± 0.26*	0.59 ± 0.04
Col-0 NA	0.28 ± 0.02	0.01 ± 0.00	0.23 ± 0.03	0.01 ± 0.00	0.08 ± 0.01
Col-0 ACC	5.57 ± 1.46	2.39 ± 0.64	2.36 ± 0.27	1.77 ± 0.18	0.51 ± 0.05

Table 6: Content of soluble carbohydrates in the leaves of S_sus1 mutant and Col-0 control.

Sugar concentrations, given in µmol per gram fresh weight, were assayed in leaves harvested either before (NA) or after (ACC) cold acclimation. All concentrations represent means ±SD of seven replicas. Statistical analysis of the results was performed using two tailed Student t-test and significant differences ($p<0.05$) were marked with the asterisks. The concentration of each carbohydrate of S_sus1 was compared with the respective concentration of Col-0 both in non- and acclimated plants.

3.6 *Sus4* and *Sus1* isoforms strongly response to oxygen deprivation stress

It was reported before (Martin *et al.*, 1993; Baud *et al.*, 2004), Figure 10) that *Sus4* and *Sus1* isoforms, strongly response with the increase of transcript level upon oxygen deprivation. To investigate whether it reflects also protein content and enzyme activity oxygen deprivation stress was performed on RNAi transfromant for *sus4* isoform. Roots were chosen as a potential interesting tissue for analysis because both of the isoforms show one of the highest expressions in that organ.

Therefore, A_sus4 which shows the reduction in expression for both, *sus4* and *sus1* isoforms (Figure 15) was subjected to oxygen deprivation for 48h and than protein content for both of the isoforms as well as the total Susy activity was investigated.

3.6.1 Protein content of sus4 and sus1 isoforms in roots was changed upon oxygen deprivation

Protein level of sus1 and sus4 isoforms in roots was checked using specific antibodies (kindly provided by Daniel Barratt, JIC). Western blot (Figure 22) showed that in all unstressed A_sus4 lines (4A, 4B and 4C) sus4 protein was missing whereas sus1 was significantly reduced. However the same analysis on stressed roots showed that sus4 protein is missing only in two transformant lines 4A and 4C, whereas in 4B it is on comparable level with analysed wild type. Remaining sus1 protein was shown to be increased upon oxygen deprivation.

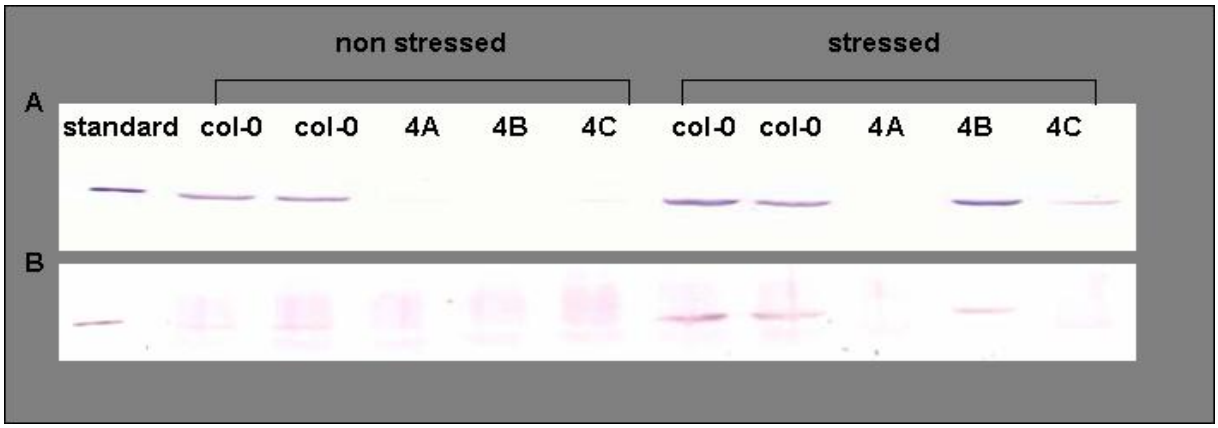


Figure 22: Western blot on A_sus4 transformant showing the effect of the deprivation of oxygen for 48h.

Three lines of A_sus4 transformant (4A, 4B and 4C) and Col-0 were grown for 40 days in terragreen/sand 1:1 mix in 16h light photoperiod. For oxygen deprivation plants were partially immersed in degassed water for 48 hours. After 48h whole roots from stressed plants as well as from non stressed controls (plants grown in parallel but not immersed in degassed water) were harvested. Isolated proteins were blotted and incubated with specific, peptide antibodies for sus1 (A) and sus4 (B) isoform. As standards sus1 (A) and sus4 (B) overexpressed proteins were used. It is important to note that with used antibodies sus4 protein can be only detected in roots upon oxygen deprivation where the protein content for this isoform strongly increases.

3.6.2 Total sucrose synthase activity was decreased in roots upon oxygen deprivation

Protein extracts used for Western blot were also subjected to measurement of total Susy activity in roots (Figure 23). Strong reduction in the enzyme activity in 4A and 4C lines was observed (82% and 73%, respectively) comparing to the wild type.

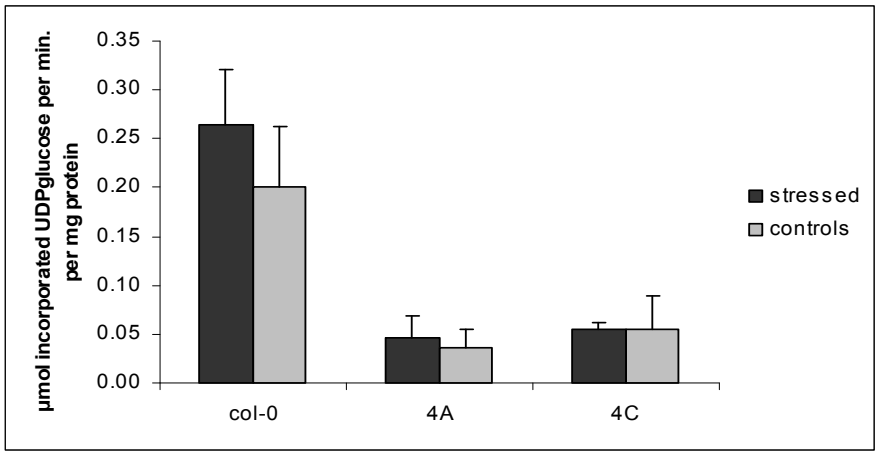


Figure 23: Sucrose synthase activity in roots upon 48h oxygen deprivation.

Enzyme was assayed via radioactive reaction in the synthesis direction as the detection of UDPG production. For more details of used plant material see legend of the Figure 22.

3.7 *Sus3* may be important for the movement of guard cells

Stomatal apertures are regulated by changes in the solutes content of guard cells. Four main solutes are: K^+, Cl^-, $malate^{2-}$ and sucrose. Sucrose together with potassium ions has an influence on stomatal movements. In the morning phase stomatal opening is correlated with the uptake of K^+. In the afternoon phase when sucrose becomes the dominant osmotically active solute, K^+ declines (Talbott and Zeiger, 1998). Not much is known about sugars interconversion within guard cells as well as the fate of sucrose during stomatal closure. It was reported before that higher mRNA levels of Susy and SPS were found in potato epidermal fragments (5.5-fold and 1.4- fold respectively) (Kopka *et al.*, 1997) as well as higher activity of these two enzymes in *Vicia* guard cells (Hite *et al.*, 1993). High SPS and Susy levels indicate that guard cells can have high capacity for sucrose synthesis and degradation. Understanding the regulation of sucrose synthase using T-DNA knockout mutants may be essential for understanding carbohydrate interconversion in guard cells.

Sus3 is the only Susy isoform that is expressed in guard cells. To check its possible role in opening and closing of stomata, chlorophyll fluorescence and gas exchange measurements were performed on two independent T-DNA knockout lines (W_sus3 in WS background and S_sus3 in Col-0).

3.7.1 Chlorophyll fluorescence measurements performed on W_sus3 and S_sus3 showed minor changes in the total photosynthetic capacity

Both T-DNA mutants and respective wild types were subjected to the measurement of chlorophyll fluorescence under different light intensities (225-1200 PAR, photosynthetically active radiance) to investigate whether the mutation in *sus3* isoform has an impact on overall photosynthetic capacity.

Slight changes were observed in electron transport rate (ETR) at high light conditions (Figure 24). S_sus3 showed lower electron transport comparing to Col-0 whereas W_sus3 exhibited higher ETR than WS wild type under high light intensities. But additionally the biggest differences in the ETR were observed between the two ecotypes. Both of the ecotypes reach the maximal ETR at 600 PAR but for WS control plants values were around 20% lower than for Col-0.

Total photosynthetic yield remained unchanged under applied conditions (not shown).

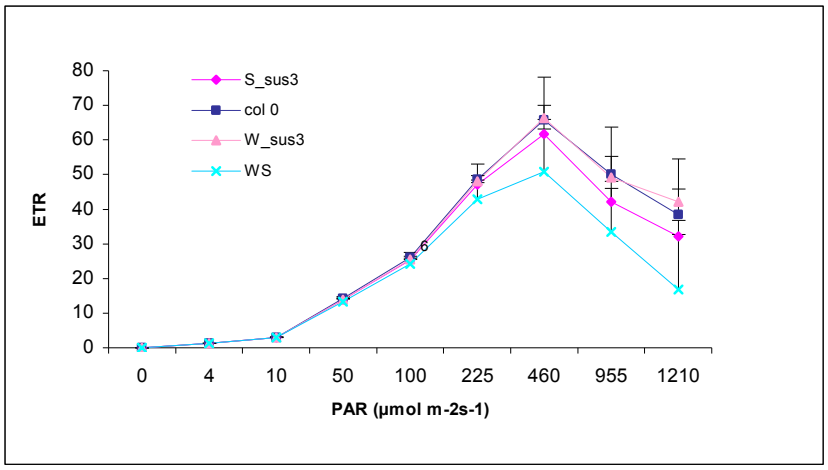


Figure 24: Electron transport rates for S_sus3 and W_sus3 and respective wild types.

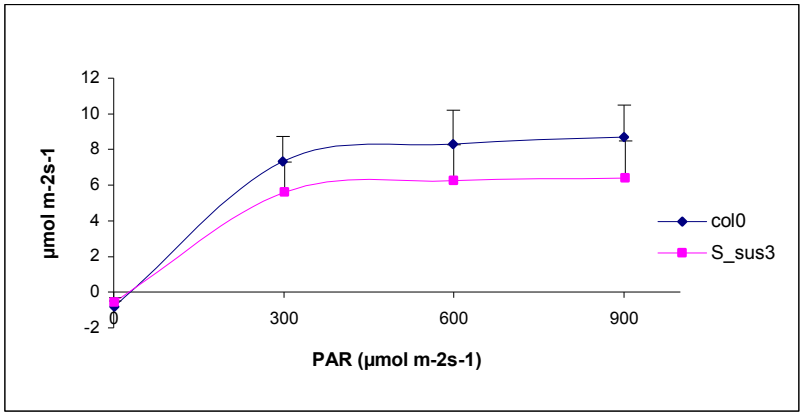
Prior to chlorophyll fluorescence measurement, six week old plants grown in soil in 16 h light photoperiod were pre adapted in dark for 20 – 30 min and than flashed with different light pulses (from 4 till 1200 PAR). At least seven different plants were measured from each line. Statistical analysis of the results was performed using two tailed Student t-test, each mutant parameter being compared to the parameter of respective wild type at the respective light intensity resulting with no significant changes ($p<0.05$).

3.7.2 Only S_sus3 shows changes in the transpiration, conductance and assimilation

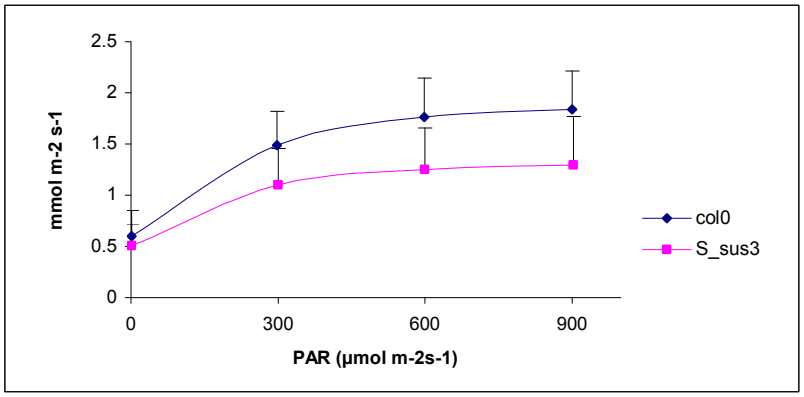
In order to monitor impact of *Sus3* isoform on guard cells movement transpiration rate, assimilation rate and stomatal conductance were analysed for both of the mutants. All these parameters were assigned under four different light intensities (0, 300, 600, 900 μmol $m^{-2}s^{-1}$). In case of W_sus3 mutant no changes in these parameters were observed comparing to WS wild type (data not shown).

In case of S_sus3 lower transpiration rate in the mutant comparing to Col-0 were observed. Additionally rates for stomatal conductance were decreased which can suggest that guard cells are wider open in wild type than mutants. These values correspond also to lower CO_2 uptake for S_sus3 (Figure 25).

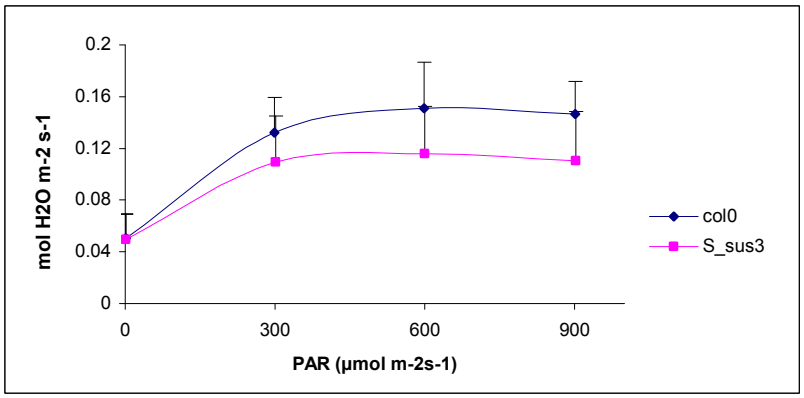
No significant changes in the intracellular CO_2 concentration (C_i) were observed (not shown) in case of S_sus3 mutant when compared to Col-0.



(a)



(b)



(c)

Figure 25: Assimilation (a), transpiration (b) and stomatal conductance for S_sus3 and Col-0.

Eight week old plants, grown in soil in 8h light photoperiod, were subjected to the measurements under four different light intensities. Each parameter was monitored for 30 min under 360 µmol s^{-1} CO_2 concentration and constant flow of 300 µmol s^{-1}. At least six different plants from each line were analysed. Statistical analysis of the results was performed using two tailed Student t-test, each mutant parameter being compared to the parameter of wild type at the respective light intensity resulting with no significant changes ($p<0.05$).

4 Discussion

4.1 Flowering plants contain multiple isoforms of sucrose synthase

The sequencing of the *Arabidopsis thaliana* genome revealed the presence of six sucrose synthase genes (Baud *et al.*, 2004). Comparison of the predicted amino acid sequences, supported by analysis of intron/exon structure, places the Arabidopsis genes in three different groups: *Sus1* and *Sus4,* which are the most similar to each other, lie on the vast dicot SUS1 branch, *Sus2* and *Sus3* appear on the mixed branch that includes both monocotyledonous and dicotyledonous plants (SUSA), while *Sus5* and *Sus6* belong to a newly identified separate group. A fourth group contains sucrose synthases only from monocotyledonous plants (monocot SUS) (Komatsu *et al.*, 2002; Baud *et al.*, 2004; Harada *et al.*, 2005).

The recently sequenced genome of *Populus trichocarpa* shows that this dicot species contains seven Susy genes, which are orthologons with the three pairs of Susy genes in Arabidopsis. Analysis of the rice genome and maize genomic and cDNA sequences showed that these two monocot plants have orthologs from the mixed SUSA group and the *Sus5*/*Sus6* in addition to those belonging to the monocot specific SUS group. These findings suggest that probably all Angiosperms have multiple sucrose synthase genes, and that at least two of the Susy gene families arose before monocot and dicot plants diverged about 200 million years ago (Bremer, 2000). The primitive bryophyte (moss) *Physcomitrella patens* also contains at least four sucrose synthase genes, but these cluster in a separate family from these in Angiosperms (John Lunn, personal communication).

4.2 Arabidopsis sucrose synthase genes show distinct but also partially overlapping expression patterns.

In addition to the real time RT-PCR data presented in chapter 3.2.1 quantitative data on transcript levels of the six isoforms of sucrose synthase in Arabidopsis were also available from real-time RT-PCR experiments (Baud *et al.*, 2004), and from microarray experiments, accessible via the Digital Northern server at www.genevestigator.ethz.ch (Zimmermann *et al.*, 2004). The published real time RT-PCR data were normalized to the EF1A4ά gene whereas my data were normalised to the UBQ10 gene, therefore, the two

data sets, are not directly comparable at a quantitative level. Nevertheless, qualitative comparisons were possible, but these did not always agree with each other, or with the microarray data. For example, (Baud *et al.*, 2004) reported for roots that *Sus4* transcript levels are almost 100-times higher than those of *Sus1*, whereas the microarray data (Figure 5) indicated that *Sus4* is expressed at a level about 25% lower than *Sus1.* The real-time RT-PCR data from my experiments (Figure 4) showed that *Sus4* is expressed at a level 10 times higher than *Sus1.* It has been shown in Baud *et al*., 2004 that expression level of *Sus1* in flower is comparable with *Sus2* and *Sus3* but 10 times lower than of *Sus5* and *Sus6*. Digital Northern reported that actually the transcript abundance for *Sus1* is 100 and 20 times higher that for *Sus6* and *Sus5* respectively. Quantitative measurement of transcript level (Figure 4) has shown 10 times higher expression of *Sus1* in comparison to *Sus5* and *Sus6.* Baud *et al*., 2004 reported that *Sus5* and *Sus6* transcript levels are comparable with or higher than those of *Sus1* in roots, stems, leaves and flowers, but Digital Northern reports that *Sus1* transcript levels are four or more times higher than those of either *Sus5* or *Sus6* in all these organs. However real-time RT-PCR data presented here showed that expression of *Sus5* and *Sus6* is approximately 10 times lower than *Sus1* in roots, leaves and flowers and it only can be compared with stem.

There are several possible reasons that could account for the different results from these real time RT-PCR and microarray experiments. First, each set of experiments used plants at different developmental stages and grown under different conditions. For example, Baud *et al*., (2004) took roots from 30-day old grown on MS medium plants, the microarray experiment were done on 17-day old, soil-grown plants, while the data presented in chapter 3.2.1 were obtained from 40-day old plants grown in a mixture of soil and vermiculite. Another important difference between the two real-time RT-PCR experiments is that different ecotypes of Arabidopsis were used. In our laboratory *Arabidopsis thaliana* ecotype Col-0 was used whereas Baud *et al*., (2004) analysed the WS ecotype. Differences between these two sets of experiments could also arise from the different control genes that were used to compare the expression of individual Susy isoforms (Czechowski *et al.*, 2005). Discrepancies between microarrays and real time RT-PCR experiments have been observed previously, especially for low abundance transcripts (Czechowski *et al.*, 2004).

Although the three expression datasets do not give identical results, several important and consistent conclusions can be made. Clearly each of the Susy isoforms shows a different expression pattern. Some of them are highly expressed in particular

organs and developmental stages, but barely detectable or not expressed in other parts of the plant. In most experiments, *Sus2* and *Sus3* were shown to be expressed at a very high level during seed development (Figure 6, Baud *et al.,* 2004); *Sus2* rises to a peak at the beginning of seed development and then falls dramatically, whereas *Sus3* first appears in the mid phase of development and its expression rises continuously throughout seed maturation with transcript still present in germinating seeds. Some of the Susy isoforms exhibit fairly constant transcript abundance throughout the whole plant, and across developmental stages e.g. *Sus1, Sus5* and *Sus6* are expressed in most of the organs. Final general conclusion is that more than one isoform is expressed in all of the tissues examined but at different levels depending on the tissue and stage of development.

For more specific investigation, the expression patterns of the sucrose synthase genes were investigated further using promoter-reporter gene analysis. In general, expression of the GUS reporter gene confirmed the results previously obtained by real-time RT-PCR analysis and provided greater detail of the expression patterns. As shown by the real-time RT-PCR and microarray results all of the isoforms were detected in roots (Figure 12 G-L) but each gene clearly showed different expression patterns: *Sus1* and *Sus4* were detected throughout the roots, whereas *Sus2* was found only in root tips at the seedling stage, *Sus*3 in lateral roots, and *Sus5* and *Sus6* in the vasculature of roots. In addition, *Sus3* was found to be specifically expressed in guard cells (Figure 12S) whereas *Sus1* was detected at the entry point of funiculus into the seed (Figure 12N).

This comprehensive gene expression analysis indicated that, although the Arabidopsis sucrose synthases are closely related at the protein level, they show different spatial and temporal expression patterns in the plant under standard growth conditions. However, different isoforms respond differently to various abiotic stresses. In Arabidopsis transcript level for *Sus1* rises in response to osmotic (Figure 8, Baud *et al.,* 2004), cold (Figure 9, Martin *et al*., 1993, Baud *et al.,* 2004), water (Baud *et al.,* 2004) and oxygen deprivation stresses (Figure 10, Martin *et al*., 1993, Baud *et al.,* 2004), whereas *Sus3* is up regulated only in response to drought (Baud *et al.,* 2004) and osmotic stress (Figure 8, Baud *et al*., 2004), and *Sus4* responds only to oxygen deprivation stress (Figure 10, Baud *et al.,* 2004). The effects of carbohydrate supply on the expression of the *Sus1* isoform (Dejardin *et al*., 1999, Baud *et al*., 2004) are not clear because possible up regulation of the isoform by sugar-induced osmotic stress can not be excluded. Interestingly, *Sus2*, *Sus5* and *Sus6* did not respond to any of those stresses.

4.3 RNAi lines and T-DNA insertion mutants are an appropriate tool to study *in planta* the functions of individual isoforms

The roles of the various sucrose synthase and UGPase isoforms were investigated further using T-DNA insertion mutants and RNAi lines. The authenticity of the T-DNA insertion mutants and RNAi lines was checked in two different ways. First, if possible more than one independent T-DNA insertion line was used for each of the isoforms. Second, transcript levels were measured in all of the putative T-DNA insertion mutants and RNAi lines to confirm the expected reduction in the level of the respective transcript (Table 3, Figure 15). Surprisingly, none of the mutants showed complete loss of expression. This might be due to the very high sensitivity of real-time RT-PCR, contamination of real time RT-PCR reactions and the fact that very low levels of expression often results with high standard deviation whilst measurements (Table 3). Another possible explanation for that would be that either cryptic promoter or 35S promoter which is included in the T-DNA insertion could drive expression of a partial transcript of respective Susy isoform. In fact, it has been shown on Figure 13, which represents the orientation of T-DNA insertion that can be true for most of the analysed mutants. This hypothesis in the future could be tested by using the multiply primer pairs whilst analysing transcript abundance. From real time RT-PCR measurements I found plants with ≤ 20% of the level of transcript in wild type plants. Because of the potential problems with real time RT-PCR analysis as described above I refer to these plants as knockdown rather than knock out mutants even though the expression in fact have been zero. For all of the sucrose synthase and UGPase isoforms appropriate mutants or RNA_i lines with substantial reduction in expression of the individual isoforms were obtained.

4.4 None of the sucrose synthase isoform seems to be essential for carbon partitioning under standard growth conditions at any stage in plant development

None of the Susy or UGPase T-DNA insertion mutant lines showed either impaired growth or differences in morphological development when compared to wild type plants. This result shows that in Arabidopsis none of the isoforms has a specific and indispensable function. All of the T-DNA insertion mutants were also analysed for metabolic phenotypes affecting carbon partitioning and accumulation during the day-night cycle or cell wall formation in different organs of the plants. No significant differences were observed in the

content of soluble sugars, starch and cellulose in roots (Figure 17) or leaves (Figure 18) harvested at the end of the day or at the end of the night. These results confirm that none of the Susy isoforms has a specific or indispensable function in the analysed organs of Arabidopsis. Therefore, it can be concluded that more than one isoform is involved in supplying UDPG for cellulose synthesis, and in providing substrates for producing the energy needed for phloem loading. Despite the different spatial and temporal expression pattern of the sucrose synthase isoforms in Arabidopsis, the results obtained from mutants suggest that the isoforms do not have highly specialised functions. However, it is still possible that more detailed studies on individual organs at different stages of development could reveal specific functions for some isoforms. Another possibility is that the missing Susy isoforms could be replaced by invertases. If sucrose were broken down via a hydrolytic route, then UGPG and hexose phosphates would be derived via the action of hexokinase, phosphoglucomutase and UGPase. It is also unlikely that just one isoform of UGPase is directly involved in supplying UDPG for cellulose synthesis, as neither of the T-DNA insertion mutants revealed any obvious phenotype (Figure 19, Figure 20). The involvement of UGPase in supplying substrate for the cellulose synthesis need to be tested by analysis of a double knock out mutant, and generation of such a mutant is now in progress in our laboratory.

Sucrose synthases may have multiple functions within a cell: supplying substrates for glycolysis, as well as callose synthesis on the inner part of the plasmalemma and xyloglucan synthesis in the Golgi apparatus, and transporting sucrose across the tonoplast (Etxeberria and Gonzalez, 2003) These multiple functions may also be influenced by post-translational changes to the protein e.g. phosphorylation has been proposed to decrease enzyme association with membranes. In several species, such as maize or pea loss of function Susy mutant show clear morphological and biochemical phenotypes (Chourey *et al.*, 1998; Craig *et al.*, 1999). Therefore, the apparent lack of any phenotypes in Arabidopsis T-DNA insertion mutants was unexpected. In the past, the involvement of sucrose synthase isoforms in metabolism has been extensively studied in maize, pea, potato and cotton. Developing maize seeds lacking one sucrose synthase isoform (*sh1* mutant) showed reduced starch content and a shrunken phenotype, which was due to a reduction in cell wall integrity, whereas the lack of another sucrose synthase isoform (*sus1* mutant) only decreased starch content (Chourey *et al.*, 1998). The *rug4* mutation in pea sucrose synthase caused reduction in starch content which was followed by reduced seed mass and failure to fix N_2 in *Rhizobium*-containing root nodules (Craig *et al.*, 1999). Tubers of transgenic

potato plants with reduced sucrose synthase activity accumulated less starch than wild type (Zrenner *et al.*, 1995), and transgenic cotton plants with decreased sucrose synthase activity showed repression of fibre cell initiation and elongation, and seed development (Ruan *et al.*, 2003). It has been shown that in high-yielding sink organs in crop plants, one particular isoform of Susy accounts for most of the enzyme activity in any one cell types at certain stages of development. Consequently, the loss of this isoform results in a significant reduction in flux from sucrose to storage product synthesis. In non-domesticated species such as Arabidopsis, loss of a single isoform, except for *Sus1*, does not affect enzyme activity. That could be due to compensatory increases in the activity of one or more of the other isoforms. However, at least at the transcript level, there did not appear to be any compensation for the loss of one isoform by increase in expression of the other isoforms, but post-transcriptional up regulation of the remaining sucrose synthases can not be excluded. Other possibility is that in a plant such as Arabidopsis that has relatively small sink organs, residual Susy activity from the other isoforms is sufficient to meet the cell's metabolic needs.

4.5 Loss of Sus1, the cold inducible isoform, has no effect on plant freezing tolerance

Many plants have the ability to sense low temperature and respond by activating mechanisms that lead to an increase in freezing tolerance. This process is known as cold acclimation. A prominent role has been demonstrated for the *C-repeat binding factors (CBF) 1, -2* and *-3* (Gilmour *et al.*, 1998) which are also known as *dehydration responsive element binding1 (DREB1) b, -c* and *-a*, respectively (Liu *et al.*, 1998). The CBF/DREB transcription factors specifically bind the dehydration-responsive element (DRE)/C-repeat cis-acting element that is present in the promoter regions of many cold responsive genes (Stockinger *et al.*, 1997; Liu *et al.*, 1998). The DRE core motif is A/GCCGAC, although recently the consensus motif for the binding of CBF3/DREB1A has been more precisely characterized as A/GCCGACNT (Maruyama *et al.*, 2004). From 8000 Arabidopsis genes, 4% were found to respond to low temperature (Fowler and Thomashow, 2002). However, of these only 12% could be assigned to the CBF regulon and at least 28%, including 15 genes encoding known or putative transcription factors, were not affected by CBF over-expression, indicating the existence of alternative low-temperature regulons (Fowler and Thomashow, 2002). Increased freezing tolerance involves the action of multiple

mechanisms including changes in lipid composition, and the accumulation of compatible solutes with cryoprotective properties such as sucrose, raffinose, proline and hydrophilic LEA or LEA-like polypeptides that are thought to function in dehydration tolerance (Thomashow, 1999).

Induction of *Sus1* by cold stress has been widely reported at the transcript (Baud *et al.*, 2004; Cook *et al.*, 2004; Hannah *et al.*, 2005), Figure 9) and protein (Cui *et al.*, 2005) levels. *Sus1* has been shown to be cold-regulated but was reported not to be a CBF target gene (Cook *et al.*, 2004). This suggests that its higher expression is regulated by other mechanisms. However, in a more recent study *Sus1* was shown to be up-regulated by CBF2 overexpression (Vogel *et al.*, 2005), and to contain the CRT/DRE element A/GCCGACNT 500bp upstream of the transcription start site in the promoter region (Hannah *et al.*, 2005). The absence of any obvious effect on S_sus1 in freezing tolerance and sugar accumulation, in both acclimated and non-acclimated plants, was surprising in light of these findings. One possible explanation is that other Susy isoforms compensated when *Sus1* was absent, but real-time RT-PCR performed on acclimated and non-acclimated S_sus1 plants did not reveal changes in transcript abundance of any other Susy isoform (data not shown). Therefore, although the previously discussed post-transcriptional regulation of sucrose synthase proteins cannot be ruled out, it seems unlikely there is a compensation by other isoforms.

The extensive accumulation of various metabolites in response to cold stress has been reported previously (Cook *et al.*, 2004; Kaplan *et al.*, 2004). Sugars, in particular raffinose, galactinol, hexoses and sucrose, as well as the pools of several amino acids (e. g. proline, arginine, cysteine) were increased in response to cold. Glucose, fructose and sucrose were accumulated within one hour of low temperature treatment, whereas galactinol and raffinose content only increased after 24 hours (Kaplan *et al.*, 2004). *Sus1* shows up regulation at the transcript level after six hours of cold (Figure 9, (Cook *et al.*, 2004). This could indicate *Sus1* being involved in sucrose breakdown to produce UDPG that would then be used as the substrate for the production of galactinol and raffinose. The possibility that mobilization of sucrose occurs via neutral invertase is rather unlikely as this enzyme has not been that broadly reported to be induced by low temperature.

Another possible explanation is that up regulation of *Sus1* under low temperature conditions is the secondary effect resulting from the massive accumulation of carbohydrates that is known to occur during cold stress. Sugars are known to induce expression of some genes but repress some others (Koch, 1996; Smeekens, 2000). Glucose

and sucrose were shown to modulate maize *Sh1* and *Sus1* expression (Koch *et al.*, 1992). The lack of any obvious phenotype in the S_sus1 mutant in terms of both freezing tolerance and sugar accumulation, in both acclimated and non-acclimated plants, is consistent with this view. However, it has also been reported that decreased osmotic potential rather than increased sugar concentration is really responsible for up regulation of *Sus1* expression (Dejardin *et al.*, 1999; Baud *et al.*, 2004). This observation together with the fact that *Sus1* also responds to osmotic and dehydration stress (Figure 8, Baud *et al.*, 2004), makes it possible to conclude that the regulation of *Sus1* is likely to involve two or even more signal transduction pathways.

4.6 Reduced sucrose synthase induction upon oxygen deprivation has no obvious effect on plant performance

Induction of Susy genes by anoxia or hypoxia has been widely described before, in both monocotyledonous and dicotyledonous species (Ricard *et al.*, 1998; Zeng *et al.*, 1998). It is generaly accepted that sucrose breakdown under limited oxygen supply mainly occurs via Susy and UGPase, rather than via the invertase pathway, which is thought to be inhibited under these conditions (Zeng *et al.*, 1999). Sucrose cleavage and provision of hexose phosphates for glycolysis via sucrose synthase costs less energy (1 mol PPi mol^{-1} Suc) compared with degradation via invertase and hexokinase (2 mol ATP mol^{-1} Suc) (van Dongen *et al.*, 2003). Investigation of the effects of hypoxia on antisense Susy potato plants showed cessation of root elongation and ultimately death of the meristematic cells due to lack of oxygen in the organ. This study indicates that the main physiological role of sucrose synthase is to channell carbohydrates into cell wall polymers rather than to fuel glycolysis (Biemelt *et al.*, 1999). Studies on maize roots showed that the *Sus1* transcript level rises together with sucrose synthase activity as a response to hypoxia but not to anoxia, however the totally opposite behaviour of transcript abundance and activity was observed for the *Sh1* isoform (Zeng *et al.*, 1998). The expression of Arabidopsis *Sus1* and *Sus4* isoforms has been shown to respond strongly to oxygen deprivation both in roots and leaves (Figure 10, Baud *et al.*, 2004). This increase in expression was followed by the increase of sucrose synthase 1 and 4 proteins (Figure 22), as well as total Susy activity (Figure 23). Analysis of the A_sus4 transformant under oxygen deprivation conditions revealed no visible phenotype compared to wild type. This lack of any phenotype might be due to remaining activity of Susy (Figure 23). Therefore, to further investigate the role of

Susy in the metabolism under conditions of reduced oxygen supply, double mutants for the *Sus1* and *Sus4* isoforms need to be investigated. Complete loss of *Sus1* and *Sus4* could than reveal the importance and involvement of other specific sucrose synthase isoforms under oxygen deprivation.

4.7 Sus3 isoform might be involved in guard cell osmoregulation and/or bioenergetics

The reporter gene experiments presented in chapter 3.2.3 showed that *Sus3* is the only sucrose synthase isoform expressed in guard cells under standard growth conditions (Figure 12S). In previous studies, a high level of expression of one *Sus* isoform was observed in potato epidermal fragments (Kopka *et al.*, 1997), as well as high activity of sucrose synthase in *Vicia* guard cells (Hite *et al.*, 1993). Hite and *et al*. (1993) reported high levels of SPS and Susy in the guard cells, and they also detected acid invertase activity which, is known to degrade sucrose in the vacuole. All these findings show that guard cells have a high capacity for sucrose synthesis and degradation. This is consistent with the changes that occur during stomatal movement. Stomatal opening and closure is a result of changing solute accumulation in the guard cell pair. During the morning phase, stomatal opening is correlated with the uptake of K^+, but in the afternoon phase, when sucrose becomes the dominant osmotically active solute, K^+ declines and stomatal closing is correlated with a decrease in sucrose. It was concluded that changes in other counterions such as malate^{2-} and other organic anions are also involved in stomatal movements, and mainly stabilize cytosolic pH during H^+ extrusion when stomata are opening. Malate synthesis is highly dependent on phosphoenolpyruvate carboxylase (PEPC), which is regulated by cytoplasmic pH, glucose-6-phosphate, triose phosphate (activator) and L-malate (feed-back inhibitor) (Tarczynski and Outlaw, 1993). During stomatal opening starch degradation in guard cell chloroplasts can provide some of the carbon (maltose and glucose) for malate synthesis (Ritte and Raschke, 2003), but recent studies also underline the importance of carbon import from the apoplast through sugar transporters (Ritte *et al.*, 1999; Stadler *et al.*, 2003). Carbohydrates and organic ions could either be stored in the vacuole to maintain guard cell turgor, or consumed in the TCA cycle to provide ATP to drive the activity of the proton pump. During stomatal closure, malate would be delivered from the vacuole to the cytoplasm and than to the guard cell apoplast through anion channels, or be consumed in the TCA cycle in the mitochondria.

Given the specific expression pattern of the *Sus3* in the guard cells, the S_sus3 T-DNA insertion mutant was investigated for any effect on photosynthetic capacity and stomatal function. It was found that lack of the *Sus3* isoform did not change overall photosynthetic performance, analysed by chlorophyll fluorescence, as the rates of electron transport and photosynthetic yield were not changed (Figure 24). This suggests that the photochemical reactions and photosynthetic metabolism were not affected. However, there are indications that transpiration, stomatal conductance and CO_2 assimilation were impaired in the S_sus3 T-DNA insertion mutant (Figure 25). Although the differences in these parameters were not significantly different as judged by the Student's t-test, the same trend was consistently observed in two separate experiments on large groups of plants grown six months apart. In general the S_sus3 mutant tended to show lower transpiration and assimilation rates, as well as lower stomatal conductance, indicating that the guard cells do not open as wide as in wild type. This might be the result of impaired sucrose metabolism in the guard cells. Both the opening and closing movements of the stomatal guard cells are active, energy consuming steps. The generally accepted view is that guard cells posses a high respiratory rate together with limited photosynthetic capacity. The abundance of mitochondria together with high respiration rates, suggest that oxidative phosphorylation is an important source of ATP. In contrast, guard cells contain few chloroplasts-about one third of those found in mesophyll cells (Willmer and Fricker, 1996), which are smaller, with limited thylakoid structure and reduced chlorophyll content (Vavasseur and Raghavendra, 2005). Biochemical studies have detected all the enzymes needed for operation of the Calvin cycle, but very low Rubisco activity. These observations, together with chlorophyll fluorescence images, suggest that the photosynthetic carbon reduction pathway is active in guard cells but only at a low level (Vavasseur and Raghavendra, 2005). Thus, it is rather unlikely that guard cell photosynthesis alone can supply solutes or energy for stomatal movement. Another fact which is worth to consider is that in sink tissues imported sucrose can undergo a futile cycle of degradation and resynthesis (Geigenberger and Stitt, 1993; Nguyen-Quoc *et al.*, 1999). Geigenberger and Stitt (1993) suggested that sucrose resynthesis in sink tissues is catalysed by both Susy and SPS. In guard cells which are also depended on the sucrose transported from apoplast (Stadler *et al.*, 2003) sucrose synthase, SPS and invertase might operate a futile cycle of sucrose synthesis and breakdown which would then allow very sensitive control of the net sucrose degradation when stomata are closed and net sucrose accumulation when stomata are opened.

The sucrose concentration plays a crucial role in guard cell osmoregulation (Talbott and Zeiger, 1998). In intact *Vicia faba* leaves, sucrose content varied from 400 up to 1200 fmol per guard cell pair, which correlated with an increase in aperture size from 5 to 18 μm (Talbott and Zeiger, 1996). Increased sucrose concentration during stomatal opening could be due to the uptake of the sucrose and/or the synthesis of sucrose by SPS. Not much is also known about the fate of sucrose during stomatal closure. Sucrose may be exported or metabolised within the guard cell, possibly being converted to starch or entering glycolysis (Willmer and Fricker, 1996; Vavasseur and Raghavendra, 2005), and so Susy activity in guard cells might play a role in cleavage of sucrose during stomatal closure. Consequently, guard cells are likely to be at least partially dependent on sucrose delivered from the apoplast, which would be used as both an osmotically active solute and an energy source. The high activities of sucrose degrading enzymes in guard cells is consistent with the idea that sucrose is utilized by guard cells in a variety of different ways: transported and stored in the vacuole, used as an osmoticum in the cytosol and supplying the substrates for starch or malate synthesis.

5 Conclusions and Outlook

1. Analysis of the *Arabidopsis thaliana* genome identifies six sucrose synthase isoforms. The expression of these isoforms was investigated using promoter-reporter gene investigation (GUS), real-time RT-PCR and analyses of publicly available data sets and revealed that all sucrose synthase isoforms show different tissue and organ specific expression throughout the plant development. Only *Sus1*, *Sus3* and *Sus4* were shown to respond differently to various abiotic stresses.
2. For the functional analysis of sucrose synthase isoforms in Arabidopsis T-DNA insertion mutants and RNA_i lines were collected and verified prior to further analysis.
3. Analysis of all T-DNA insertion mutants showed no differences in growth and development under applied growing conditions. Analysis with respect to carbon partitioning and cell wall formation revealed that none of the mutants was significantly different from the wild type in its content and accumulation of soluble sugars, starch and cellulose in the analysed organs.
4. Loss of *Sus1,* the cold inducible isoform, showed no effect on the plant freezing tolerance and accumulation of various carbohydrates of non acclimated and cold acclimated plants.
5. Analysis of plants with reduced expression of both *Sus1* and *Sus4* isoforms that are up regulated by oxygen deficiency, revealed no obvious effects on plant performance under oxygen deprivation.
6. Loss of *Sus3* isoform, that is exclusively expressed in guard cells, revealed only minor influence on guard cell osmoregulation and/or bioenergetics.

The data of this thesis provide a comprehensive overview of the expression of sucrose synthase gene family in Arabidopsis. Some questions concerning the functional characteristics of all sucrose synthase isoforms could be answered however some new have been raised.

As none of the Arabidopsis sucrose synthase isoform seems to have a specific and indispensable function within the plant further analysis of double mutants should be employed. Therefore, double knock out mutants of the phylogeneticaly closest *Sus* isoforms (*sus1*/*sus4*, *sus2*/*sus3* and *sus5*/*sus6*) and both *Ugp* isoforms should be analysed with respect to carbon partitioning and cell wall formation. In these mutants content of soluble sugars, starch, xyloglucans and cellulose should be verified.

More specifically the double mutant of *sus1/sus4* isoforms have to be employed to study the importance and involvement of sucrose synthase isoforms into metabolism under oxygen deprivation.

In case of further investigation of influence of *Sus3* isoform on guard cell movements stomatal kinetic in S_sus3 mutant should be analysed in order to see possible changes in guard cell bioenergetics. Possibly the stomatal apertures should be measured as well.

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8 Appendix

Protein sequences of sucrose synthases from various plant species used for the construction of phylogenetical tree.

Arabidopsis thaliana protein sequences included in phylogenetical analysis

AtSus1 (at5g20830)
MANAERMITRVHSQRERLNETLVSERNEVLALLSRVEAKGKGILQQNQIIAEFEALPEQTRKKLEGGPFF
DLLKSTQEAIVLPPWVALAVRPRPGVWEYLRVNLHALVVEELQPAEFLHFKEELVDGVKNGNFTLELDFE
PFNASIPRPTLHKYIGNGVDFLNRHLSAKLFHDKESLLPLLKFLRLHSHQGKNLMLSEKIQNLNTLQHTL
RKAEEYLAELKSETLYEEFEAKFEEIGLERGWGDNAERVLDMIRLLLDLLEAPDPCTLETFLGRVPMVFN
VVILSPHGYFAQDNVLGYPDTGGQVVYILDQVRALEIEMLQRIKQQGLNIKPRILILTRLLPDAVGTTCG
ERLERVYDSEYCDILRVPFRTEKGIVRKWISRFEVWPYLETYTEDAAVELSKELNGKPDLIIGNYSDGNL
VASLLAHKLGVTQCTIAHALEKTKYPDSDIYWKKLDDKYHFSCQFTADIFAMNHTDFIITSTFQEIAGSK
ETVGQYESHTAFTLPGLYRVVHGIDVFDPKFNIVSPGADMSIYFPYTEEKRRLTKFHSEIEELLYSDVEN
KEHLCVLKDKKKPILFTMARLDRVKNLSGLVEWYGKNTRLRELANLVVVGGDRRKESKDNEEKAEMKKMY
DLIEEYKLNGQFRWISSQMDRVRNGELYRYICDTKGAFVQPALYEAFGLTVVEAMTCGLPTFATCKGGPA
EIIVHGKSGFHIDPYHGDQAADTLADFFTKCKEDPSHWDEISKGGLQRIEEKYTWQIYSQRLLTLTGVYG
FWKHVSNLDRLEARRYLEMFYALKYRPLAQAVPLAQDD

AtSus2 (at5g49190)
MPTGRFETMREWVYDAISAQRNELLSLFSRYVAQGKGILQSHQLIDEFLKTVKVDGTLEDLNKSPFMKVL
QSAEEAIVLPPFVALAIRPRPGVREYVRVNVYELSVDHLTVSEYLRFKEELVNGHANGDYLLELDFEPFN
ATLPRPTRSSSIGNGVQFLNRHLSSIMFRNKESMEPLLEFLRTHKHDGRPMMLNDRIQNIPILQGALARA
EEFLSKLPLATPYSEFEFELQGMGFERGWGDTAQKVSEMVHLLLDILQAPDPSVLETFLGRIPMVFNVVI
LSPHGYFGQANVLGLPDTGGQVVYILDQVRALENEMLLRIQKQGLEVIPKILIVTRLLPEAKGTTCNQRL
ERVSGTEHAHILRIPFRTEKGILRKWISRFDVWPYLETFAEDASNEISAELQGVPNLIIGNYSDGNLVAS
LLASKLGVIQCNIAHALEKTKYPESDIYWRNHEDKYHFSSQFTADLIAMNNADFIITSTYQEIAGSKNNV
GQYESHTAFTMPGLYRVVHGIDVFDPKFNIVSPGADMTIYFPYSDKERRLTALHESIEELLFSAEQNDEH
VGLLSDQSKPIIFSMARLDRVKNLTGLVECYAKNSKLRELANLVIVGGYIDENQSRDREEMAEIQKMHSL
IEQYDLHGEFRWIAAQMNRARNGELYRYIADTKGVFVQPAFYEAFGLTVVESMTCALPTFATCHGGPAEI
IENGVSGFHIDPYHPDQVAATLVSFFETCNTNPNHWVKISEGGLKRIYERYTWKKYSERLLTLAGVYAFW
KHVSKLERRETRRYLEMFYSLKFRDLANSIPLATDEN

AtSus3 (at4g02280)
MANPKLTRVLSTRDRVQDTLSAHRNELVALLSRYVDQGKGILQPHNLIDELESVIGDDETKKSLSDGPFG
EILKSAMEAIVVPPFVALAVRPRPGVWEYVRVNVFELSVEQLTVSEYLRFKEELVDGPNSDPFCLELDFE
PFNANVPRPSRSSSIGNGVQFLNRHLSSVMFRNKDCLEPLLDFLRVHKYKGHPLMLNDRIQSISRLQIQL
SKAEDHISKLSQETPFSEFEYALQGMGFEKGWGDTAGRVLEMMHLLSDILQAPDPSSLEKFLGMVPMVFN
VVILSPHGYFGQANVLGLPDTGGQVVYILDQVRALETEMLLRIKRQGLDISPSILIVTRLIPDAKGTTCN
QRLERVSGTEHTHILRVPFRSEKGILRKWISRFDVWPYLENYAQDAASEIVGELQGVPDFIIGNYSDGNL
VASLMAHRMGVTQCTIAHALEKTKYPDSDIYWKDFDNKYHFSCQFTADLIAMNNADFIITSTYQEIAGTK
NTVGQYESHGAFTLPGLYRVVHGIDVFDPKFNIVSPGADMTIYFPYSEETRRLTALHGSIEEMLYSPDQT
DEHVGTLSDRSKPILFSMARLDKVKNISGLVEMYSKNTKLRELVNLVVIAGNIDVNKSKDREEIVEIEKM
HNLMKNYKLDGQFRWITAQTNRARNGELYRYIADTRGAFAQPAFYEAFGLTVVEAMTCGLPTFATCHGGP
AEIIEHGLSGFHIDPYHPEQAGNIMADFFERCKEDPNHWKKVSDAGLQRIYERYTWKIYSERLMTLAGVY
GFWKYVSKLERRETRRYLEMFYILKFRDLVKTVPSTADD

AtSus4 (at3g43190)
MANAERVITRVHSQRERLDATLVAQKNEVFALLSRVEAKGKGILQHHQIIAEFEAMPLETQKKLKGGAFF
EFLRSAQEAIVLPPFVALAVRPRPGVWEYVRVNLHDLVVEELQASEYLQFKEELVDGIKNGNFTLELDFE
PFNAAFPRPTLNKYIGDGVEFLNRHLSAKLFHDKESLHPLLKFLRLHSHEGKTLMLNNRIQNLNTLQHNL
RKAEEYLMELKPETLYSEFEHKFQEIGLERGWGDTAERVLNMIRLLLDLLEAPDPCTLENFLGRIPMVFN
VVILSPHGYFAQDNVLGYPDTGGQVVYILDQVRALETEMLQRIKQQGLNITPRILIITRLLPDAAGTTCG
QRLEKVYGSQYCDILRVPFRTEKGIVRKWISRFEVWPYLETFTEDVAAEISKELQGKPDLIIGNYSDGNL
VASLLAHKLGVTQCTIAHALEKTKYPDSDIYWKKLDEKYHFSCQFTADLIAMNHTDFIITSTFQEIAGSK
DTVGQYESHRSFTLPGLYRVVHGIDVFDPKFNIVSPGADMSIYFAYTEEKRRLTAFHLEIEELLYSDVEN
EEHLCVLKDKKKPIIFTMARLDRVKNLSGLVEWYGKNTRLRELVNLVVVGGDRRKESQDNEEKAEMKKMY
ELIEEYKLNGQFRWISSQMNRVRNGELYRYICDTKGAFVQPALYEAFGLTVVEAMTCGLPTFATCNGGPA
EIIVHGKSGFHIDPYHGDKAAESLADFFTKCKHDPSHWDQISLGGLERIQEKYTWQIYSQRLLTLTGVYG
FWKHVSNLDRLESRRYLEMFYALKYRPLAQAVPLAHEE

AtSus5 (at5g37180)
MEMTSGSLGNGIPEAMGQNRGNIKRCLEKYIENGRRVMKLNELMDEMEIVINDVTQRRRVMEGDLGKILC
FTQAVVIPPNVAFAVRGTPGNWQYVKVNSSNLSVEALSSTQYLKLKEFLFDENWANDENALEVDFGALDF
TLPWLSLSSSSIGNGLSFVSSKLGGRLNDNPQSLVDYLLSLEHQGEKLMMNETLNTARKLEMSLILADVFL
SELPKDTPFQAFELRFKECGFEKGWGESAGRVKETMRILSEILQAPDPQNIDRFFARVPRIFNVVIFSVH
GYFGQTDVLGLPDTGGQVVYILDQVKALEDELLQRINSQGLNFKPQILVVTRLIPDAKKTKCNQELEPIF
GTKYSNILRIPFVTENGILRRWVSRFDIYPYLERFTKVKSYIRMDATTKILDILEGKPDLIIGNYTDGNL
VASLMANKLGITQATIAHALEKTKYEDSDIKWKEFDPKYHFSSQFTADLISMNSADFIIASTYQEIAGSK
ERAGQYESHMSFTVPGLYRVVSGINVFDPRFNIAAPGADDSIYFPFTAQDRRFTKFYTSIDELLYSQSEN
DEHIGYLVDKKKPIIFSMARLDVVKNLTGLTEWYAKNKRLRDLVNLVIVGGFFDASKSKDREEISEIKKM
HSLIEKYQLKGQFRWITAQTDRTRNGELYRSIADTRGAFVQPAHYEAFGLTVIEAMSCGLVTFATNQGGP
AEIIVDGVSGFHIDPSNGEESSDKIADFFEKSGMDPDYWNMFSNEGLQRINECYTWKIYANKVINMGSTY
SYWRHLNKDQKLAKQRYIHSFYNLQYRNLVKTIPILSDIPEPPPLPPKPLVKPSASKGSKRTQPRLSFRL
FGA

AtSus6 (at1g73370)
MSSSSQAMLQKSDSIAEKMPDALKQSRYHMKRCFASFVGGGKKLMKREHLMNEIEKCIEDSRERSKILEG
LFGYILTCTQEAAVVPPFVALAARPNPGFWEYVKVNSGDLTVDEITATDYLKLKESVFDESWSKDENALE
IDFGAIDFTSPRLSLSSSIGKGADYISKFISSKLGGKSDKLEPLLNYLLRLNHHGENLMINDDLNTVAKL
QKSLMLAVIVVSTYSKHTPYETFAQRLKEMGFEKGWGDTAERVKETMIILSEVLEAPDNGKLDLLFSRLP
TVFNVVIFSVHGYFGQQDVLGLPDTGGQVVYILDQVRALEEELLIRINQQGLGFKPQILVVTRLIPEARG
TKCDQELEAIEGTKHSHILRVPFVTNKGVLRQWVSRFDIYPYLERFTQDATSKILQRLDCKPDLIIGNYT
DGNLVASLMATKLGVTQGTIAHALEKTKYEDSDAKWKELDPKYHFSCQFTADLIAMNVTDFIITSTYQEI
AGSKDRPGQYESHTAFTMPGLCRVVSGIDVFDPKFNIAAPGADQSVYFPYTEKDKRFTKFHPSIQELLYN
EKDNAEHMGYLADREKPIIFSMARLDTVKNITGLVEWYGKDKRLREMANLVVVAGFFDMSKSNDREEKAE
IKKMHDLIEKYKLKGKFRWIAAQTDRYRNSELYRCIADTKGVFVQPALYEAFGLTVIEAMNCGLPTFATN
QGGPAEIIVDGVSGFHIDPNNGDESVTKIGDFFSKCRSDGLYWDNISKGGLKRIYECYTWKIYAEKLLKM
GSLYGFWRQVNEDQKKAKKRYIEMLYNLQFKQLTKKVTIPEDKPLPLRLASLRNLLPKKTTNLGAGSKQK
EVTETEKTKQKSKDGQEQHDVKVGEREVREGLLAADASERVKKVLESSEEKQKLEKMKIAYGQQHSQGAS
PVRNLFWSVVVCLYICYILKQRFFGANSAQEY

Physcomitrella (*Physcomitrella patens)* protein sequences

Physcomitrella1
MATQPALRRLNSIQERVQKVVQSNRNLILDLLSRYVKQGRTILQPHHLLDELNNLGDADQVAEIKDSAFGNLLQNCQEAMV
LPPWVGFAVRPRPGIWEYVRINVEELTLEELSVSEYLSFKEQLANGTDGEDPFVLELDFAPFNANFPHMTRPSSIGHGVQF
LNRHLSSKLFHTPDSMEPLFEFLRMHTYRGQTLMLNDRIASLVRLRPQLVKAEEALSKLPEKTPFADFAHQLQGLGLEKGW
GNSAGRALETIKMLQDLLQAPDPDTLEKFLARILMVFSVVIVSPHGYFGQEGVLGLPDTGGQVVYILDQVRALENEMLENL
QLQGLDIIPQIVILTRLIPNAIGTTCNQRIEKVTGSRFSHILRIPFRHDGKVLNNWISRFDVYPYLETYAQEAAREISTDL
AGPPDLIIGNYSDGNLVATLMCQQLGVTQCTIAHALEKTKYPDSDIYWKKFEEKYHFSCQFTADLIAMNHADFIITSTYQE
IAGSAKTVGQYESHQAFTMPGLYRVVNGVNVFDPKFNIVSPGADMDVYFPYTDKERRLTKLHPTIEDLLFGTEQSDEHIGV
IDKSKPILFTMARLDKVKNLTGLVELYGKNNKLKELTNLVIVGGEINPAKSKDREEVKEIAKMHDFIKEYNLHNSFRWIRS
QTNRVQNGELYRYIAEAGGVFVQPALYEGFGLTVVEAMTCGLPTFATLHGGPAEIIEHGISGFHIDPYHPDEVADELVTFF
EKVKSDSSFWTKISEAALQRIYSSFTWKLYAERLMTLTRVYGFWKYVSNLHRREARRYLEMFYTLKFRELVKTVPLSKDDE
GPEEKTETKARLGPGQAAIVGTPASA*

Physcomitrella2
MAANGVAPKKPVLQRLNSIQERVQSAVQEHRNVIIDLLSRYVKQGRTHLQPHHIVDELNSLTEADRVTEIKDSAFGLLLLN
CQEAIVLPPWLGLAVRPRPGIWEYLRINVEELILEELSVSEYLGFKEQLANSTDVRDPFLLELDMAPFNSNFPRMTRPSSI
GHGVEFLNRHLSLKLFQTADGIEPLFQFLRMHTYRGQTLMLNDRITSLRRLRPQLVKADDILSKLPEDTPFTDFAHKLQEL
GLEKGWGNTAGRVVETIKLLEDLLQAPDPDTLEKFLARIPMVFSVVIVTPHGYFGQDGVLGLPDTGGQVVYILDQVRALEN
EMLENLQLQGLDIVPKIVILTRLIPNAFGTTCNQRIEKVHGSRFSHILRIPFRNDGQILKNWISRFDVYPYLENYAQEAAS
EICADLSGPPDLIIGNYTDGNLVATLLCQHLGVTQCTIAHALEKTKYPDSDIYWKNFEEKYHFSCQFTADLIAMNHADFII
TSTYQEIAGSAKTVGQYESHQAFTMPSLYRVVNGIDVFDPKFNIVSPGADMTVYYPFTDKQHRLTKLHPAIEKLLFSSDQT
DEHVGIIDKDKPILFTMARLDRVKNLTGLVELYGKNEKLREMTNLVIVGGEIDPAKSKDREEVKEIEKMHSFIKQYNLHNH
FRWIRSQTNRVQNGELYRYIADAGGVFVQPALYEGFGLTVVEAMTCGLPTFATMHGGPAEIIVNGISGFHIDPYHPEGVAE
VLVSFFEKVKTDPGVWTRISEAALQRIYSNFTWKLYAERLMTLTHVYGFWKYVSNLQRRESKRYLEMFYTLKYRELVKTVP
LSSDNESPEEKTDKKAHMGPPTDAALVGVPLAAGEITKAILESTAIH*

Physcomitrella3
MDGIATQAGALPRMTSMNKKIQGSLDDHRNENLRILSKLTAKRKALMQPHEVIDELNKAAEESGSLKIMDGPLARVFSLCQ
EAIVLAPWVGLALRPRPGLWEYMRINVEEMIVEELTTSEYLSFKECLADENRCNDLYVLELDIEPFNVGFPRMTRPQSIGN
GVQFLNRHLSSRLFRDADSMEPLVEFMRVHKYKDQTLLLNESITNVVRLRPALIKAEEYLIKLPNDQPLKDFYSKLQELGL
ERGWGDTAGRVLEMIHLLLDLLQAPDPDILEKFLARIPIVFSVAIISPHGYFGQSNVLGMPDTGGQVVYILDQVRAMEKEM
LKNIKLQGLDIEPQIVVVTRLIPNANGTTCNQRIEQIEGTKHSRILRVPFRNENGILHNWISRFDVYPFLENFVYDVAQEL
TVELPGKPDFIIGNYTDGNLVASLLCHQLGVTQCNIAHALEKTKYPDSDIYWKKFEEKYHFSCQFTADLIAMNQADFIITS
TYQEIAGSEDTVGQYESHVAFSLPGLYRVVNGIDVFDPKFNIVSPGADTIVYFSFTEKDRRLTDLHDKIEKLLYDPEQTAE
HIGSLKDRNKPILFSMARLDKVKNISGLVEMFAKNPRLRELVNLVVVAGNIQKEKSKDREEMAEIDKMHNLMKEYELDGDF

RWLCAQTDRVLNGELYRYIADSHGAFVQPALYEGFGLTVIEAMTCGLPTFATCHGGPKEIVVSDVSGFHIDPFHPESASKI
IVDFFERCTKEKDYWTKLSDGGLERIRTKYTWEIYAERLLTLSRVYGFWKFVSKLGRRETRRYLEMFYILKFRELVKTVPV
ASDDKSYLKEQEKKV*

Physcomitrella4
MSQPRPTLRRLTSLKERVESSLQEHRNELLHLLQGYVAQGRSILQPHHLQDQLAAVHDAAHIQDTAIGKLLQNCQEAMVSP
PWVGFAVRPRPGIWEYVRINVEELIVEELSVSEYLGFKEQLSLGSDSIDLYVLELDFEPFNAHFPRMTRPSSIGHGVQFLN
RHLSSKLFQNPESMEPLFQFLRLHTYRGETLMLNERIATFSRFRPQLVRAEEALSKLPEDTPFSSFAHRLQELGLEKGWGN
TAGRVLQTLKLLLDLLQAPDPDTLEKFLARIPMIFTVCIVSPHGYFGQAGVLGLPDTGGQVVYILDQVRALENQMLENLQL
QGLDFKPQIVILTRLIPNANGTTVNQRIEKVSGTQHSRILRVPFQHEGNILKNWISRFDVYPYLENYAQDAAREVLGELQG
RPDLIIGNYSDGNLVATLLSHYLDVTQCIIAHALEKTKYPDSDIYWKDFEEKYHFSCQFTADLIAMNSADFIITSTYQEIA
GSADTVGQYESHQAFTMPGLYRVVNGIDVFDPKFNIVSPGADMNIYYPFADKERRLTSLQESIEELLYSPEQTDEHIGLID
KEKPILFSMARLDRVKNLTGLVEMYGKNQKLKEFVHLVIVGGEINPSKSKDREEVREIEKMHNLIKRYKLENNFRWIRSQT
NRIRNGELYRYIADSQGAFVQPALYEGFGLTVVEAMTSGLPTFATSHGGPAEIIEHGISGYHIDPYYPDEAAEQIVAFFEK
CKNEPGLWNKVSEAGLQRIYSSYTWKIYAERLMTLSAVYGFWKYVSKLHRQEARRYLEMFYILKFRELARTVPLSKDDEDV
LEKVEKKAQLGPGVGAIVGEAATAVEARKAVTGHT*

Poplar tree (*Populus trichocarpa)* protein sequences

Poplar1
MAERALTRVHSIRERVDETLKAHRNEIVALLTRIEGKGKGILQHHQIVAEFEAIPEDTRKTLAGGAFAEVLRSTQEAIVV
PPWIALALRPRPGVWEYIRLNVQALVVEELRVAEYLHFKEELVDGGCNGNFVLELDFEPFNASFPRPTLSKYIGNGVEFL
NRHLSAKLFHDKESLHPLLAFLKVHCHKGKNMMLNDRIRNLDSLQYVLRKAEEFLSTLKPDTPYSQFEHKFQEIGLERGW
GDTAERVLEMIRLLLDLLEAPDPCTLETFLGRIPMVFNVVIMSPHGYFAQDNVLGYPDTGGQVVYILDQVRALENEMLQR
IKKQGLDIIPRILIITRLLPDAVGTTCGQRLERVYGSEHCDILRVPFRDGKGMVRKWISRFEVWPYLETFTEDVAAEIAK
ELQGKPDLIIGNYSDGNIVASLLAHKLGVTECTIAHALEKTKYPDSDIYWKKFDEKYHFSCQFTADLFAMNHTDFIITST
FQEIAGSKDTVGQYESHTAFTLPGLYRVVHGIDVFDPKFNIVSPGADESIYFPYTDEKRRLTSFHPEIDELLYSPVENEE
HLCVLKDRNKPILFTMARLDRVKNLSGLVEWYGKNTKLRELVNLVVVGGDRRKESKDLEEQAEMKKMYSHIEKYNLNGQF
RWISSQMNRVRNGELYRYICDTKGAFVQPALYEAFGLTVVEAMTCGLPTFATCNGGPAEIIVNGKSGFHIDPYHGEKAAE
LLVDFFEKCKVDPAHWDKISHGGLQRIQEKYTWQIYSQRLLTLTGVYGFWKHVSNLDRLESRRYMEMFYALKYRKLAESV
PLTKE*

Poplar2
MSVLTRVQSIRERLDETLKTHRNEIVALLTRIEGKGKGILQHHQIIAEFEAIPEEIRKILAGGAFSEVLRSTQEAIVLPP
WVALAVRPRPGVWEYVRVNVQALVVEELRVAEYLHFKEELVDGGSNGNFVLELDFEPFSASFPRPTLSKYIGNGVEFLNR
HLSAKLFHDKESLHPLLAFLKVHCHKGKNMMLNDRIHNLDSLQYVLRKAEEYLSSLKPETPYSQFEHKFQEIGLERGWGN
TAERVLQMIQLLLDLLEAPDPCTLETFLGRIPMVFNVVIMSPHGYFAQDNVLGYPDTGGQVVYILDQVRALESEMLLRIK
QQGLDITPRILIITRLLPDAVGTTCGQRLEKVYGSEHCDILRVPFRDEKGMVRKWISRFEVWPYLETYTEDVAAEIAKEL
QGKPDLIIGNYSDGNVVASLLAHKLGVTECTIAHALEKTKYPDSDIYWKKFDEKYHFSCQFTADLFAMNHTDFIITSTFQ
EIAGSKDTVGQYESHTAFTLPGLYRVVHGIDVFDPKFNIVSPGADESIYFPYTEKKLRLTSFHEEIEELLYSSVENDEHL
CVLKDRNKPILFTMARLDRVKNLTGLVEWYGKNTKLRELANLVVVGGDRRKESKDIEEQAEMKKMYSHIEKYKLNGQFRW
ISSQMNRVRNGELYRYICDTKGAFVQPALYEAFGLTVVEAMTCGLPTFATCNGGPAEIIVHGKSGFHIDPYHGVQAAELL
VDFFEKCKADPSYWDKISQGGLQRIQEKYTWKIYSQRLLTLTGVYGFWKHVSNLDHRESRRYLEMFYALKYRKLADSVPL
TIE*

Poplar3
MANPKLERIPSMRERVQDTLSANRNVLVSLLSRYVEQGKGILHPNNLIDELDNIVCDDAARLSLKDGPFSEVLKAAQEAI
VLPPFVAVSIRPRPGVWEYVRVDVSQLNVEELTVSQYLRFKEELVDGPSNDPYVLELDFEPFNAAFPRPTRSSSIGNGVQ
YLNRHLSSNMFRNKDTLEPLLDFLRVHKYKGHALMLNDRIKSVSRLQSALLKAEEYISKLPSETLYTEFEYTFQGMGFER
GWGDTAARVLEMMHLLLDILQAPDPSTLETFLGRVPMVFNVVILSPHGYFGQANVLGLPDTGGQIVYILDQVRALENEML
LRIQQQGLDFKPKILIVTRLIPDSKGTSCNQRLERVSGTEHTHILRVPFRSEHGILRKWISRFDVWPYLETFAEDAASEI
VAELQGIPDFIIGNYSDGNLVASLLAYKMGVTQCTIAHALEKTKYPDSDIYWKKFDDKYHFSCQFTADVLAMNNADFIIT
STYQEIAGTKTTVGQYESHTAFTLPGLYRVVHGINVFDTKFNIVSPGADMDIYFPYSDKQKRLTTLHGSIEKMLYDSEQT
DDWIGTLTDKSKPIIFSMARLDRVKNISGLVECYGKNARLRELVNLVVVAGYIDVKKSNDREEILEIEKMHELMKKYKLD
GQFRWLTAQTNRARNGELYRYIADTKGAFVQPAFYEAFGLTVVEAMTCGLPTFATCHGGPAEIIEHGVSGFHMDPYYPDQ
AAEFMADFFEKCKDDPSYWKKISDAGLQRIYERYTWKIYSERLMTLAGVYGFWKYVSKLERRETRRYLEMFYILKFRDLV
KTVPLSIEDWH*

Poplar4
MASQTALQRSETITESMPEALRQSRYHMKKCFSRFVAPGKRLMKRQHLMDEVDESIQDKNERQKVLEGLLGYILSCTQEA
AVIPPFVAFAVRPNPGFWEYVKVNAEDLSVEGISVSEYLQLKEMVFDEKWANNENALELDFGAMDFSTPRLTLSSSIGNG
VNYMSKFMSSKLSGSSEAAKPLLDYLLALNHQGENLMINQTLDTVAKLQEALIVAEVVVSAFPKDTPYQDFQQRLRELGF
ETGWGDTAERVKETMRLLSESLQAPYPMKLQLLFSRIPNMFNIVIFSPHGYFGQSDVLGLPDTGGQVVYILDQVRALEEE
LLLKIKHQGLGVKPRILVVTRLIPNAGGTKCNQEVEPIFGTQHSHIVRVPFKTEKGVLPQWVSRFDDAADKVLEHMDSKP
DLIIGNYSDGNLVASLMARKLSITLGTIAHALEKTKYEDSDVKWKELDAKYHFSCQFTADMIAMNSADFIITSTYQEIAG
SNVRPGQYESHTAFTMPGLCRVVSGINVFDPKFNIASPGADQSVYFPYTEKQKRLTSFHPAIEELLYSNEDNHEHIGYLA
DRKKPIIFSMARLDTVKNITGLTEWFGKNTKLRNLVNLVVVAGFFDPSKSNDREEIAEIKKMHALIEKYQLKGQFRWIAA
QTDRYRNGELYRCIADTKGAFVQPALYEAFGLTVIEAMNCGLPTFATNQGGPAEILVDGISGFHIDPNNGDESSNKIADF

FEKCKTDAEYWNKMSAAGLQRIYECYTWKIYANKVLNMGSVYGFWRQTNKEQKLAKQRYIEAFYNLQFNNLVGYCGQLVL
*

Poplar5

MASAPVLKRSETIAESMPDALRQSRYHMRICFSRYMSASIRLMKRQHIMDEVDKSIQDKNERQKVLEGLLGYILSSTQEA
AVVPPFVAFAVRPNPGFWEYVKVNAEDLSVDGISVSEYLQFKEMIFDEKWASNENALEVDFGAMDFSTPRLTLSSSIGNG
LNYMSKFMSSKLRGNSDAAKPLLDYLLALDHQGENLMINQALDSVSKLQAALIVAEVVVSAFPKDAPYQDFQQSLKRLGF
EKGWGDTAERVKETMRMLSESLQAPEPVKLELLFSRIPNVFNIVIFSPHGYFGQSDVLGLPDTGGQIVYILDQVRALEEE
LLLKIRQQGLSVKPQILVITRLIPHAGGTKCNQEVEPIFGTKHSHIVRVPFKTEKGVLPQWVSRFDVYPYLERFAQDAAD
KVREHMDCKPDLLIGNYSDGNLVASLMAQKLGTTLGTIAHALEKTKYEDSDAKWKELDPKYHFSCQFTADMIAMNTADFI
ITSTYQEIAGSKNRPGQYESHVAFTMPGLCRVVSGINVFDPKFNIASPGADQTVYFPYTEKQKRLTSFHPAIEELLYNNE
DNNEHIGYLADKKKPIIFSMARLDTVKNITGLTEWYGKNAKLRNLVNLVVVAGFFDPSKSNDREEIAEIKKMHSLIEKYQ
LKGQFRWIAAQSDRYRNGELYRCIADTKGAFIQPALYEAFGLTVIEAMNCGLPTFATNQGGPAEIIVDGISGFHIDPNNG
DESSNKIADFVEKCKTDAEYWNKMSATGLQRIYECYTWKIYANKVLNMGSVYGFWRQMNKEQKLLKQRYIEAFYNLQFRN
L

Poplar6

MATLKRSDSIADNMPEALKQSRYHMKRCFAKYIEKGRRTMKLQQLLDEMENVIDDQVERTRVLQGLLGDIWFSIQEAVVN
PPYVALSIRPSPGFWEFVKVNSADLSVEGITATDYLKFKEMIYDENWAKDANALEVDFGAFDFSVPHLTLSSSIGNGLGF
VSKFATSKLSGRLESAQPLVDYLLSLNHEGEKLMINETLSSVRKLRMALIVAEAYLSGLPKDTQYQNFETSFKAWGFEKG
WGNTAERVKETMRCLSEVLQAPDPLNMENFFSRLPTVFNVVIFSPHGYFGQADVLGLPDTGGQVVYILDQVKALEDELLL
RIEQQGLNIKPQIVVVTRLIPEARGTKCNQELESINGTKHSNILRVPFSIENKVLRQWVSRFDDVITKLLDLMQRKPDLI
IGNYTDGNLAATLMASKLGITQATIAHALEKTKYENSDVKWKELDPKYHFSCQFMADTIAMNATDFIIASTYQEIAGSKD
RPGQYESHASFTLPGLCRVVSGIDVFDPKFNIAAPGADQSVYFPYTEKQSRFTKFHPAIEELLYSKVVNDEHIGYLEDKK
KPIIFSMARLDTVKNLTGLTEWYGKNKRLRGLVNLVIVGGFFDPNKSKDREEMAEITKMHGLIKKYRLNGQFRWIAAQTD
RNRNGELYRCIADTKGAFVQPALYEAFGLTVIEAMNCGLPTFATNQGGPAEIIVDGISGFHIDPQNGDESSNIIADFFEK
CKVDPGYWNKFAAEGLKRINECYTWKIYAKKLLNMGNMYSFWRQLNKEQKLAKQRYIQMLYNLQFRRLILNRAVQDELSR
DCRGTTTNLNSKFVWIPQ*

Poplar7

MATLKRSDSIADNMPEALKQSRYHMKKCFAKYIEKGRRTMKLQQLLDEMENVIDDQVERTRVLEGLLGDIWFSIQEAVVN
PPYVAFSIRPSPGFWEYVKVNSANLSVEGITVTDYLKFKEMIYDENWAKDANALEVDFGAFDFSVPHLTLSSSIGNGLGF
VSKFVTSKLSGRLENAQPLVDYLLSLNRQGEKLMINETLGTVGKLQMALIVAEVYLSGLAKDTPYQNFEISFKEWGFEKG
WGDTAERVKETMRCLSEVLQAPDPMNMEKFLSRLPTVFNVVIFSPHGYFGQADVLGLPDTGGQVVYILDQVKALEEELLL
RIKQQGLNVKPQIVVATRLIPDARGTTCNLEFEAIDGTKYSNILRVPFRVENRVLRQWVSRFDEVTTKILDLMEGKPDLI
IGNYTDGNFAATLMAGKLGITQATIAHALEKTKYENSDVKWKELESKYHFPCQFMADIVAMNATDFIIASTYQEIAGSKD
RTGQYESHAAFTLPGLCRVVSGVNVFDPKFNIAAPGADQSVYFPHTEKQSRFTQFNPDIEELLYSKVVNDEHIGYLEDKK
KPIIFSMARLDTVKNLTGLTEWYGKNKRLRGLVNLVIVGGFFDPNKSKDREEMAEIKKMHELIEKYQLKGQIRWIAAQTD
RKRNGELYRCIADTKGAFVQPALYEAFGLTVIEAMNCGLPTFATNQGGPSEIIVDGISGFHIDPKNGDESSNIIADFFEK
CKVDPGHWNKYSLEGLKRINECYTWKIYANKLLNMGNVYSFWRQLNKEQKLAKQRYIQLFFNLKFRELVQSVPIPTEEAQ
TPASEPTARTQSSAR*

Maize (*Zea mays*) protein sequences

Maize1

MAAKLTRLHSLRERLGATFSSHPNELIALFSRYVHQGKGMLQRHQLLAEFDALFDSDKEKYAPFEDILRA
AQEAIVLPPWVALAIRPRPGVWDYIRVNVSELAVEELSVSEYLAFKEQLVDGQSNSNFVLELDFEPFNAS
FPRPSMSKSIGNGVQFLNRHLSSKLFQDKESLYPLLNFLKAHNYKGTTMMLNDRIQSLRGLQSSLRKAEE
YLLSVPQDTPYSEFNHRFQELGLEKGWGDTAKRVLDTLHLLLDLLEAPDPANLEKFLGTIPMMFNVVILS
PHGYFAQSNVLGYPDTGGQVVYILDQVRALENEMLLRIKQQGLDITPKILIVTRLLPDAAGTTCGQRLEK
VIGTEHTDIIRVPFRNENGILRKWISRFDVWPYLETYTEDVSSEIMKEMQAKPDLIIGNYSDGNLVATLL
AHKLGVTQCTIAHALEKTKYPNSDIYLDKFDSQYHFSCQFTADLIAMNHTDFIITSTFQEIAGSKDTVGQ
YESHIAFTLPGLYRVVHGIDVFDPKFNIVSPGADMSVYYPYTETDKRLTAFHPEIEELIYSDVENSEHKF
VLKDKKKPIIFSMARLDRVKNMTGLVEMYGKNARLRELANLVIVAGDHGKESKDREEQAEFKKMYSLIDE
YKLKGHIRWISAQMNRVRNGELYRYICDTKGAFVQPAFYEAFGLTVIESMTCGLPTIATCHGGPAEIIVD
GVSGLHIDPYHSDKAADILVNFFDKCKADPSYWDEISQGGLQRIYEKYTWKLYSERLMTLTGVYGFWKYV
SNLERRETRRYIEMFYALKYRSLASQVPLSFD

Maize2

MGEGAGDRVLSRLHSVRERIGDSLSAHPNELVAVFTRLKNLGKGMLQPHQIIAEYNNAIPEAEREKLKDG
AFEDVLRAAQEAIVIPPWVALAIRPRPGVWEYVRVNVSELAVEELRVPEYLQFKEQLVEEGPNNNFVLEL
DFEPFNASFPRPSLSKSIGNGVQFLNRHLSSKLFHDKESMYPLLNFLRAHNYKGMTMMLNDRIRSLSALQ
GALRKAEEHLSTLQADTPYSEFHHRFQELGLEKGWGDCAKRAQETIHLLLDLLEAPDPSTLEKFLGTIPM
VFNVVILSPHGYFAQANVLGYPDTGGQVVYILDQVRAMENEMLLRIKQCGLDITPKILIVTRLLPDATGT
TCGQRLEKVLGTEHCHILRVPFRTENGIVRKWISRFEVWPYLETYTDDVAHEIAGELQANPDLIIGNYSD
GNLVACLLAHKMGVTHCTIAHALEKTKYPNSDLYWKKFEDHYHFSCQFTTDLIAMNHADFIITSTFQEIA
GNKDTVGQYESHMAFTMPGLYRVVHGIDVFDPKFNIVSPGADLSIYFPYTESHKRLTSLHPEIEELLYSQ
TENTEHKFVLNDRNKPIIFSMARLDRVKNLTGLVELYGRNKRLQELVNLVVVCGDHGNPSKDKEEQAEFK
KMFDLIEQYNLNGHIRWISAQMNRVRNGELYRYICDTKGAFVQPAFYEAFGLTVVEAMTCGLPTFATAYG

GPAEIIVHGVSGYHIDPYQGDKASALLVDFFDKCQAEPSHWSKISQGGLQRIEEKYTWKLYSERLMTLTG
VYGFWKYVSNLERRETRRYLEMLYALKYRTMASTVPLAVEGEPSSK

Maize3

MSAPKLDRNPSIRDRVEDTLHAHRNELVALLSKYVNKGKGILQPHHILDALDEVQGSGGRALAEGPFLDV
LRSAQEAIVLPPFVAIAVRPRPGVWEYVRVNVHELSVEQLTVSEYLRFKEELVDGQHNDPYVLELDFEPF
NVSVPRPNRSSSIGNGVQFLNRHLSSIMFRNRDCLEPLLDFLRGHRHKGHVMMLNDRIQSLGRLQSVLTK
AEEHLSKLPADTPYSQFAYKFQEWGLEKGWGDTAGHVLEMIHLLLDIIQAPDPSTLEKFLGRIPMIFNVV
VVSPHGYFGQANVLGLPDTGGQIVYILDQVRALENEMVLRLKKQGLDVSPKILIVTRLIPDAKGTSCNQR
LERISGTQHTYILRVPFRNENGILKKWISRFDVWPYLETFAEDAAGEIAAELQGTPDFIIGNYSDGNLVA
SLLSYKMGITQCNIAHALEKTKYPDSDIFWKNFDEKYHFSCQFTADIIAMNNADFIITSTYQEIAGSKNT
VGQYESHTAFTLPGLYRVVHGIDVFDPKFNIVSPGADMSIYFPHTEKAKRLTSLHGSIENLIYDPEQNDE
HIGHLDDRSKPILFSMARLDRVKNITGLVEAFAKCAKLRELVNLVVVAGYNDVNKSKDREEIAEIEKMHE
LIKTHNLFGQFRWISAQTNRARNGELYRYIADTHGAFVQPAFYEAFGLTVVEAMTCGLPTFATLHGGPAE
IIEHGVSGFHIDPYHPEQAANLMADFFDRCKQDPDHWVNISGAGLQRIYEKYTWKIYSERLMTLAGVYGF
WKYVSKLERLETRRYLEMFYILKFRELAKTVPLAIDQPQ

Maize4 sucrose synthase 4 coding sequence (used lowercase indicate parts derived from rice4 sequence)

ATGGCCTCCAAGCTGAGTTTCAAGAGGGCAGACAGCATCGCGGAAAGCATGCCCGATGCG
CTGAGGCAGAGCCGGTACCAGATGAAGAgATGCTTCCATAGGTATGTTTCCAAGGGAAGG
AGGCTCTTGAAGAACCAGCAGCTCATAGAGGAGCTGGACAAATCACTGGATGACAAAGTC
GAGAGGGAAAAGCTTGTTGAAGGCTTCCTGGGTTACATTATTTGTTCCACGCAGGAAGCA
GTGGTGCTACCGCCCTATGTCGCATTTGCTGTCAGGATGAATCCTGGCATCTGGGAGTAT
GTCAAAGTTCATTCTGATGACCTGTCGGTCGAAGGAATCACACCCTCTGAGTACCTCAAG
TTCAAGGAGACATTATATGACGAGAACTGGGCCAAGGATGACAACTCACTGGAAGTCGAT
TTCGGTGCTCTTGACCTCTCAACACCCCATCTGACACTGCCATCGTCCATAGGAAACGGG
CTCCAGTTTGTGTCCAAATTCATGTCCTCCAAGCTGGGCGACAAGCCTGAAATTAGCATG
AAGCCGTTGCTGGACTACTTGCTTTCGCTAAACTACCGTGGCGAGAAGCTGATGGTTAAC
GACACCATCGATACTGTGAACAAGCTTCAGACAGCGCTGCTACTTGCGGAGGTATTTGTT
AGCGGGTTGCCAAGATACACCCCATTCCTGAAGTTTGAGCAAAGGTTTCAAGAGTGGGGG
TTGGAGAAGGGGTGGGGTGACAATGCTGAAAGGTGCAAAGAGACGCTGAATTGCCTCTCT
GAAGTGCTACAGGCGCCAGACCCTATCAACATGGAGAAGTTCTTCAGCAGAGTTCCATCC
ATATTCAACATAGTTGTCTTCTCCATCCACGGCTACTTTGGCCAAGAGAAGGTTCTTGGC
TTGCCAGACACCGGTGGCCAGGTGGTCTACATCCTGGACCAAGTCAGGGCCCTTGAAGAG
GAGTTGCTGCAAAGAATCAAGCTGCAGGGTCTGAACGTGACACCAAAGATTCTTGTGCTG
ACTAGGCTGATACCAGATGCCAAGGGTACAAAATGCAATGTGGAGCTCGAGCCAGTTGAA
AATACAAAACATTCCCACATACTTCGTGTGCCATTCAAGACTGAAAACGGCAAGGAGTTG
CGCCAGTGGGTGTCCCGGTTTGACATCTACCCTTACCTAGAGAGATATGCCCAGaactct
tgtgccaaaATTCTTGACATTTTGGAGGGCAAGCCGGACCTGATCATCGGCAACTACACT
GATGGCAACTTAGTGGCGTCCCTCATGTCAAGCAAACTAGGGGTCACTCAGGGGACAATC
GCGCACGCTCTAGAGAAGACAAAGTATGAAGATTCAGATGTGAAGTGGAGAGATCTGGAT
CAGAAGTACCATTTCTCCTGCCAATTCACTGCAGATATGATTGCCATGAACACTAGTGAC
TTTATCATCACTAGCACATACCAAGAAATCGCTGGAAGCAAGGAGAAGCCTGGGCAGTAC
GAGCACCACTACGCATTCACAATGCCGGGGCTCTGTCGCTACGCCACGGGCATCAATGTC
TTCGATCCAAAGTTCAACATCGCCGCACCCGGTGCAGACCAGTCCATCTACTTCCCCTTC
ACGCAGAAGCAGAAGCGGCTGACAGATTTGCACCCACAGATTGAGGAGCTGCTCTACAGC
AAGCAGGACACCGGTGAACACAGAGGGTATCTGGCGGACAGAAACAAGCCTATCATCTTC
TCGATGGCAAGGCTGGACAAGGTGAAGAATATCACCGGGCTAGTGGAGTGGTACGGCCAG
AACAAGAAGCTGAGGGACCTGGTAAACCTTGTCGTCGTCGCGGGCCTGCTGGAAGCGTCG
CAGTCCAAGGACCGGGAGGAGATTGAAGAGATCAACAGGATGCACAGCCTGATCGACAAG
TATCAGCTGAAAGGACAGATTCGCTGGATCAAGGCACAGACTGACCGTGTCCGCAACGGT
GAGCTGTACCGTTGCATTGCAGACACCAGGGGTGCATTTGTTCAGCCTGCACTCTATGAA
GCGTTCGGGCTGACGGTCATTGAGGCGATGAACTGCGGGCTGACAACCTTTGCGACGAAC
CAGGGAGGGCCAGCGGAGATCATCGTGGACGGTGTCTCCGGTTTCCACATAAACCCAACG
AATGGCAGGGAGGCAAGCAACAAGATCGCCGAGTTCTTCCAGAAGTGCAAGGAAGACCCA
AGCTACTGGAACAAGGTGTCCACTGCTGGGCTCCAGCGCATCTACGAGTGCTACACATGG
AAGATCTATGCAACTAAAGTCCTGAACATGGGCTCGACGTATGGCTTCTGGAAGACTCTG
AACAAGGAGGAGAGAGTGGCCAAGCAGCGCTACCTGCAGATGTTCTACAACCTCCAGTTC
AGGAACCTGGCAAAGACTGTCCCAAGGCTGTTTGAACATCCTCCTCCGCAAGCCCCAGCA
GGCGCAGGGCCTAGTACGATGACGGTAACGAGGCCGAAAGAAAGAAAGACACAGACTAGG
ATCCAGAGGATCATGACCAGCTTGATGGGGCAGAAGCCTGCTACTTCTGAATAA

Maize4 protein sequence (used lowercase indicate parts derived from rice4 sequence)

MASKLSFKRADSIAESMPDALRQSRYQMKrCFHRYVSKGRRLLKNQQLIEELDKSLDDKV
EREKLVEGFLGYIICSTQEAVVLPPYVAFAVRMNPGIWEYVKVHSDDLSVEGITPSEYLK
FKETLYDENWAKDDNSLEVDFGALDLSTPHLTLPSSIGNGLQFVSKFMSSKLGDKPEISM
KPLLDYLLSLNYRGEKLMVNDTIDTVNKLQTALLLAEVFVSGLPRYTPFLKFEQRFQEWG

LEKGWGDNAERCKETLNCLSEVLQAPDPINMEKFFSRVPSIFNIVVFSIHGYFGQEKVLG
LPDTGGQVVYILDQVRALEEELLQRIKLQGLNVTPKILVLTRLIPDAKGTKCNVELEPVE
NTKHSHILRVPFKTENGKELRQWVSRFDIYPYLERYAQnscakILDILEGKPDLIIGNYT
DGNLVASLMSSKLGVTQGTIAHALEKTKYEDSDVKWRDLDQKYHFSCQFTADMIAMNTSD
FIITSTYQEIAGSKEKPGQYEHHYAFTMPGLCRYATGINVFDPKFNIAAPGADQSIYFPF
TQKQKRLTDLHPQIEELLYSKQDTGEHRGYLADRNKPIIFSMARLDKVKNITGLVEWYGQ
NKKLRDLVNLVVVAGLLEASQSKDREEIEEINRMHSLIDKYQLKGQIRWIKAQTDRVRNG
ELYRCIADTRGAFVQPALYEAFGLTVIEAMNCGLTTFATNQGGPAEIIVDGVSGFHINPT
NGREASNKIAEFFQKCKEDPSYWNKVSTAGLQRIYECYTWKIYATKVLNMGSTYGFWKTL
NKEERVAKQRYLQMFYNLQFRNLAKTVPRLFEHPPPQAPAGAGPSTMTVTRPKERKTQTR
IQRIMTSLMGQKPATSE*

Rice (*Oryza sativa*) protein sequences

Rice1
MAAKLARLHSLRERLGATFSSHPNELIALFSRYVNQGKGMLQRHQLLAEFDALIEADKEKYAPFEDILRA
AQEAIVLPPWVALAIRPRPGVWDYIRVNVSELAVEELSVSEYLAFKEQLVDGHTNSNFVLELDFEPFNAS
FPRPSMSKSIGNGVQFLNRHLSSKLFQDKESLYPLLNFLKAHNHKGTTMMLNDRIQSLRGLQSSLRKAEE
YLMGIPQDTPYSEFNHRFQELGLEKGWGDCAKRVLDTIHLLLDLLEAPDPANLEKFLGTIPMMFNVVILS
PHGYFAQSNVLGYPDTGGQVVYILDQVRALENEMLLRIKQQGLDITPKILIVTRLLPDAVGTTCGQRVEK
VIGTEHTDILRVPFRSENGILRKWISRFDVWPFLETYTEDVANEIMREMQAKPDLIIGNYSDGNLVATLL
AHKLGVTQCTIAHALEKTKYPNSDIYLDKFDSQYHFSCQFTADLIAMNHTDFIITSTFQEIAGSKDTVGQ
YESHIAFTLPGLYRVVHGIDVFDPKFNIVSPGADMSVYFPYTEADKRLTAFHPEIEELLYSEVENDEHKF
VLKDKNKPIIFSMARLDRVKNMTGLVEMYGKNAHLRDLANLVIVCGDHGNQSKDREEQAEFKKMYGLIDQ
YKLKGHIRWISAQMNRVRNGELYRYICDTKGVFVQPAFYEAFGLTVIEAMTCGLPTIATCHGGPAEIIVD
GVSGLHIDPYHSDKAADILVNFFEKCKQDSTYWDNISQGGLQRIYEKYTWKLYSERLMTLTGVYGFWKYV
SNLERRETRRYIEMFYALKYRSLASAVPLAVDGESTSK

Rice2
MGEAAGDRVLSRLHSVRERIGDSLSAHPNELVAVFTRLVNLGKGMLQAHQIIAEYNNAISEADREKLKDG
AFEDVLRSAQEGIVISPWVALAIRPRPGVWEYVRVNVSELAVELLTVPEYLQFKEQLVEEGTNNNFVLEL
DFEPFNASFPRPSLSKSIGNGVQFLNRHLSSKLFHDKESMYPLLNFLRAHNYKGMTMMLNDRIRSLSALQ
GALRKAEEHLSGLSADTPYSEFHHRFQELGLEKGWGDCAKRSQETIHLLLDLLEAPDPSTLEKFLGTIPM
VFNVVIMSPHGYFAQANVLGYPDTGGQVVYILDQVRAMENEMLLRIKQQGLNITPRILIVTRLLPDATGT
TCGQRLEKVLGTEHTHILRVPFRTENGIVRKWISRFEVWPYLETFTDDVAHEIAGELQANPDLIIGNYSD
GNLVACLLAHKMGVTHCTIAHALEKTKSPNSDLYWKKFEDHYHFSCQFTTDLIAMNHADFIITSTFQEIA
GNKDTVGQYESHMAFTMPGLYRVVHGIDVFDPKFNIVSPGADMSIYFPYSESRKRLTSLHPEIEELLYSE
VDNNEHKFMLKDRNKPIIFSMARLDRVKNLTGLVELYGRNPRLQELVNLVVVCGDHGNPSKDKEEQAEFK
KMFDLIEQYNLNGHIRWISAQMNRVRNGELYRYICDTKGAFVQPAFYEAFGLTVVESMTCGLPTFATAYG
GPAEIIVNGVSGFHIDPYQGDKASALLVEFFEKCQEDPSHWTKISQGGLQRIEEKYTWKLYSERLMTLTG
VYGFWKYVSNLERRETRRYLEMLYALKYRTMASTVPLAVEGEPSNK

Rice3
MGETTGERALTRLHSMRERIGDSLSAHTNELVAVFSRLVNQGKGMLQPHQIIAEYNAAIPEGEREKLKDS
ALEDVLRGAQEAIVIPPWIALAIRPRPGVWEYLRINVSQLGVEELSVPEYLQFKEQLVDGSTQNNFVLEL
DFEPFNASFPRPSLSKSIGNGVQFLNRHLSSKLFHDKESMYPLLNFLRAHNYKGMTMMLNDRIRSLDALQ
GALRKAEKHLAGITADTPYSEFHHRFQELGLEKGWGDCAQRVRETIHLLLDLLEAPEPSALEKFLGTIPM
VFNVVILSPHGYFAQANVLGYPDTGGQVVYILDQVRAMENEMLLRIKQQGLNITPRILIVTRLLPDAHGT
TCGQRLEKVLGTEHTHILRVPFRTENGTVRKWISRFEVWPYLETYTDDVAHEISGELQATPDLIIGNYSD
GNLVACLLAHKLGVTHCTIAHALEKTKYPNSDLYWKKFEDHYHFSCQFTADLIAMNHADFIITSTFQEIA
GNKETVGQYESHMAFTMPGLYRVVHGIDVFDPKFNIVSPGADMSIYFPFTESQKRLTSLHLEIEELLFSD
VENTEHKFVLKDKKKPIIFSMARLDHVKNLTGLVELYGRNPRLQELVNLVVVCGDHGKESKDKEEQAEFK
KMFNLIEQYNLNGHIRWISAQMNRVRNGELYRYICDMRGAFVQPALYEAFGLTVIEAMTCGLPTFATAYG
GPAEIIVHGVSGYHIDPYQNDKASALLVEFFEKCQEDPNHWIKISQGGLQRIEEKYTWKLYSERLMTLSG
VYGFWKYVTNLDRRETRRYLEMLYALKYRKMATTVPLAIEGEASTK

Rice4
MASKLSFKRMDSIAETMPDALRQSRYQMKRCFQRYVSKGKRLLKNQQLMEELE
KSLDD-KVENEKLVEGFLGYIICSTQEAVVLPPFVAFAVRMNPGIWEYVKVHSDDLSVEG
ITPSEYLKFKETLYDEKW--AKDDNSLEVDFGALDLSTPHLTLPSSIGNGLQFVSKFMSS
KLG-GKPESMKPLLDYLLTLNYRGEKLMINDTIDTVSKLQTALLLAEVFVSGLPKYTPYL
KFEQRFQEWGLEKGWGDTAERCKETLNCLSEVLQAPDPTNMEKFFSRVPSIFNIVIFSIH
GYFGQEKVLGLPDTGGQVVYILDQVRAMEEEL---LQRIKQQGLHVTPKILVLTRLIPDA
KGTKCNVELEPVENTKYSHILRVPFKTEDGKDLRQWVSRFDIYPYLERYAQ-------NS
CAKILDILEGKPDLIIGNYTDGNLVASLLSNKLCVTQGTIAHALEKTKYEDSDVKWREMD
QKYHFSCQFTADMISMNTSDFIITSTYQEIAGSKEKPGQYEHHYAFTMPGLCRYATGINV
FDPKFNIAAPGADQSIYFPFTQKQKRLTDLHPQIDELLYSKDDTDEHIGYLADRNKPIIF
SMARLDKVKNITGLVEWYGQNKKLRDLVNLVVVAGLLDASQSKDREEIEEINKMHNLMDR

YQLKGQIRWIKAQTDRVRNGELYRCIADTKGAFVQPALYEAFGLTVIEAMNCGLPTFATN
QGGPAEIIIDGVSGFHVNPINGREAGIKIADFFQKCKEDPSYWNKVSTAGLQRIYECYTW
KIYATRVLNMGSTYSFWKTLNKEERQAKQRYLQIFYNVQYRNLAKAVARAGDQQARQTTT
GVAPSEIVVRPKERKPQTRMQRILTRLAGQKPPVSE

Rice5

MSGPKLDRTPSIRDRVEDTLHAHRNELVALLSKYVSQGKGILQPHHILDALD
EVQSS-GG--RALVEGPFLDVLRSAQEAIVLPPFVAIAVRPRPGVWEYVRVNVHELSVEQ
LTVSEYLRFKEELVDGQ---YNDPYILELDFEPFNASVPRPNRSSSIGNGVQFLNRHLSS
IMF-RNKDCLEPLLDFLRGHRHKGHVMMLNDRIQSLGRLQSVLTKAEEHLSKLPADTPYS
QFAYKFQEWGLEKGWGDTAGYVLEMIHLLLDVLQAPDPSTLETFLGRIPMIFNVVVVSPH
GYFGQANVLGLPDTGGQIVYILDQVRALENEM---VLRLKKQGLDFTPKILIVTRLIPEA
KGTSCNQRLERISGTQHTYILRVPFRNENG-ILRKWISRFDVWPYLEKFAE-------DA
AGEIAAELQGTPDFIIGNYSDGNLVASLLSYKMGITQCNIAHALEKTKYPDSDIYWTKYD
EKYHFSCQFTADIIAMNNADFIITSTYQEIAGSKNTVGQYESHTAFTLPGLYRIVHGIDV
FDPKFNIVSPGADMSIYFPYTEKAKRLTSLHGSLENLISDPEQNDEHIGHLDDRSKPILF
SMARLDRVKNITGLVEAYAKNARLRELVNLVVVAGYNDVKKSKDREEIAEIEKMHELIKT
YNLFGQFRWISAQTNRARNGELYRYIADTHGAFVQPAFYEAFGLTVVEAMTCGLPTFATV
HGGPAEIIEHGISGFHIDPYHPDQAANLIADFFEQCKQDPNHWVEVSNRGLQRIYEKYTW
KIYSERLMTLAGVYGFWKYVSKLERRETRRYLEMFYILKFRELAKTVPLAVDEAH

Rice6

MAVGLRRSDSIADMMPEALRQSRYQMKRCFQRYVSQGKRLMKRQQLLDELD
KSVDD-KADKDQLLQGFLGYVISSTQEAAVLPPFVAFAVRMNPGIWEFVKVHSANLSVEQ
MTPSDYLKNKEALVDDKWGAYDDDSQLEVDFGALDLSTPHLTLPSSIGKGAHLVSRFMSS
KLT-DNKK---PLLDYLLALSHRGDKLMINDILDTVDKLQTALLLAEVYVAGLHPDTNYS
EFEQKFQEWGLEKGWGDTAETCKETLSSLSEVLQAPDPINMEKFFSTVPCVFTVVIFSIH
GYFGQEKVLGMPDTGGQVVYILDQVRALEDEL---LQRIKQQGLNATPKILVLTRLIPEA
KGTKCNVELEPIENTKHSNILRVPFKTEDGKVLPQWVSRFDIYPYLERYAQ-------DS
SVKILEILEGKPDLVIGNYTDGNLVASLLTSKLGVTQGTIAHALEKTKYEDSDIKWRELD
HKYHFSCQFTADMIAMNTSDFIIASTYQEIAGSKEKPGQYESHYAFTMPGLCRYATGINV
FDPKFNIAAPGADQSVYFPFTQKQKRLTDLHPQIEELLYSKEDNNEHIGHLADRSKPIIF
SMARLDKIKNITGLVEWYGQNKRLRDLVNLVIVGGLLDPSQSKDREEIEEINKMHSLINK
YQLVGQIRWIKGQTDRVRNGELYRCIADTKGAFVQPALYEAFGLTVIEAMNCGLPTFATN
QGGPAEIIVDEVSGFHINPLNGKEASDKIADFFQKCKEDLIYWSKMSTAGLQRIYECYTW
QIYATKVLNMASIYGFWRTLDKEERQAKQHYLHMFYNLQFRKLAKNVPTLGEQPA-QPTE
SAEPNRIIPRPKERQVCPFLRNLLKKETGNN

Pea (*Pisum sativum*) protein sequences

Pea1

MATDRLTRVHSLRERLDETLTANRNEILALLSRIEAKGKGILQHHQVIAEFEEIPEENRQKLTDGAFGEV
LRSTQEAIVLPPWVALAVRPRPGVWEYLRVNVHALVVENLQPAEFLKFKEELVDGSANGNFVLELDFEPF
TASFPRPTLNKSIGNGVQFLNRHLSAKLFHDKESLHPLLEFLRLHSYKGKTLMLNDRIQNPDSLQHVLRK
AEEYLGTVAPDTPYSEFEHRFQEIGLERGWGDTAERVLESIQLLLDLLEAPDPCTLETFLDRIPMVFNVV
ILSPHGYFAQDDVLGYPDTGGQVVYILDQVRALESEMLNRIKKQGLDIVPRILIITRLLPDAVGTTCGQR
LEKVYGTEHCHILRVPFRDQKGIVRKWISRFEVWPYLETYTEDVAHELAKELQGKPDLIVGNYSDGNIVA
SLLAHKLGVTQCTIAHALEKTKYPESDIYWKKFEEKYHFSCQFTADLFAMNHTDFIITSTFQEIAGSKDT
VGQYESHTAFTLPGLYRVVHGIDVFDPKFNIVSPGADQTIYFPYTETSRRLTSFYPEIEELLYSTVENEE
HICVLKDRSKPIIFTMARLDRVKNITGLVEWYGKNAKLRELVNLVVVAGDRRKESKDLEEKAEMKKMYEH
IETYKLNGQFRWISSQMNRVRNGELYRVICDTKGAFVQPAVYEAFGLTVVEAMATGLPTFATLNGGPAEI
IVHGKSGFHIDPYHGDRAADLLVEFFEKVKTDPSHWDKISQGGLQRIEEKYTWQIYSQRLLTLTGVYGFW
KHVSNLDRLESRRYLEMFYALKYRKLAESVPLAVEE

Pea2

MSTHPKFTRVPSIRDRVQDTLSAHRNELISLLSRYVAQGKGILQPHNLIDELDNILGEDHATLDLKNGPF
GQIINSAQEAIVLPPFVAIAVRPRPGVWEYVRVNVFELSVEQLSVSEYLSFKEELVEGKSNDNIILELDL
EPFNASFPRPTRSSSIGNGVQFLNRHLSSNMFRNKDCLEPLLDFLRVHTYKGHALMLNDRIQSISKLQSA
LVKAEDHLSKLAPDTLYSEFEYELQGTGFERGWGDTAARVLEMMHLLLDILQAPDPSTLETFLGRVPMVF
NVVILSPHGFFGQANVLGLPDTGGQVVYILDQVRALESEMLVRIKKQGLDFTPRILIVTRLIPDAKGTTC
NQRLERVSGTEYTHILRVPFRSEKGILRKWISRFDVWPFLETFAEDVASEIAAELQCYPDFIIGNYSDGN
LVASLLAYKMGVTQCTIAHALEKTKYPDSDIYWKKFEDKYHFSCQFTADLIAMNNADFIITSTYQEIAGT
KNTIGQYESHTAFTLPGLYRVVHGIDVFDPKFNIVSPGADMTIYFPYSDKEKRLTALHSSIEKLLYGTEQ
TDEYIGSLTDRSKPIIFSMARLDRVKNITGLVESYAKNSKLRELVNLVVVAGYIDVKKSSDREEIEEIEK
MHDLMKQYNLNGEFRWITAQTNRARNGELYRYIADTKGAFVQPAFYEAFGLTVVEAMTCGLPTFATNHGG
PAEIIEHGVSGFHIDPYHPDQASELLVDFFQRCKEDPNHWNKVSDGGLQRIYERYTWKIYSERLMTLAGV
YSFWKYVSKLERRETRRYLEMFYILKFRDLANSVPIAKG

Pea3
MASLTHSTSLRQRFDETLTAHRNEILSLLSRIEAKGKGILQHHQIIAEFEEIPEENRQKLVNGVFGEVLR
STQEAIVLVPFVALAVRPRPGVWEYLRVDVHGLVVDELSAAEYLKFKEELVEGSSNENFVLELDFEPFNA
SIPKPTQNKSIGNGVEFLNRHLSAKLFHGKESLQPLLEFLRLHNHNGKTIMVNDRIQNLNSLQHVLRKAE
DYLIKIAPETPYSEFEHKFQEIGLERGWGDTAERVVETIQLLLLDLLDGPDPGTLETFLGRIPMVFNVVIL
SPHGYFAQDNVLGYPDTGGQIVYILDQVRALEEEMLKRIKQQGLDITPRILIITRLLPDAVGTTCGQRLE
KVYNTEHCHILRVPFRTEKGIVRKWISRFEVWPYLETFSEDVANELAKELQGKPDLIVGNYSDGNIVASL
LAHKLGVTQCTIAHALEKTKYPESDIYWKKFDDKYHFSSQFTADLFAMNHTDFIITSTFQEIAGSKDTVG
QYESHTAFTLPGLYRVVHGIDVFDPKFNIVSPGADLSIYFPYTETERRLTSFHPDIEELLYSTVENEEHI
CVLKDRSKPIIFTMARLDRVKNITGLVECYGKNARLRELVNLVVVAGDRRKESKDLEEIAEMKKMYGLIE
TYKLNGQFRWISAQMDRIRNGELYRVICDTKGAFVQPAIYEAFGLTVIEAMSCGLPTFATCNGGPAEIIV
HGKSGYHIDPYHGDRAAETLVEFFEKSKADPTYWDKISHGGLKRIHEKYTWQIYSDRLLTLTGVYGFWKH
VTNLERRESKRYLEMFYALKYSKLAESVPLAVEE

Potato (Solanum tuberosum) protein sequences

Potato1
MAERVLTRVHSLRERVDATLAAHRNEILLFLSRIESHGKGILKPHELLAEFDAIRQDDKNKLNEHAFEEL
LKSTQEAIVLPPWVALAIRLRPGVWEYIRVNVNALVVEELSVPEYLQFKEELVDGASNGNFVLELDFEPF
TASFPKPTLTKSIGNGVEFLNRHLSAKMFHDKESMTPLLEFLRAHHYKGKTMMLNDRIQNSNTLQNVLRK
AEEYLIMLPPETPYFEFEHKFQEIGLEKGWGDTAERVLEMVCMLLDLLEAPDSCTLEKFLGRIPMVFNVV
ILSPHGYFAQENVLGYPDTGGQVVYILDQVPALEREMLKRIKEQGLDIIPRILIVTRLLPDAVGTTCGQR
IEKVYGAEHSHILRVPFRTEKGIVRKWISRFEVWPYMETFIEDVAKEISAELQAKPDLIIGNYSEGNLAA
SLLAHKLGVTQCTIAHALEKTKYPDSDIYWKKFDEKYHFSSQFTADLIAMNHTDFIITSTFQEIAGSKDT
VGQYESHMAFTMPGLYRVVHGINVFDPKFNIVSPGADINLYFSYSETEKRLTAFHPEIDELLYSDVENDE
HLCVLKDRTKPILFTMARLDRVKNLTGLVEWYAKNPRLRGLVNLVVVGGDRRKESKDLEEQAEMKKMYEL
IETHNLNGQFRWISSQMNRVRNGELYRYIADTKGAFVQPAFYEAFGLTVVEAMTCGLPTFATNHGGPAEI
IVHGKSGFHIDPYHGEQAADLLADFFEKCKKDPSHWETISMGGLKRIEEKYTWQIYSESLLTLAAVYGFW
KHVSKLDRLEIRRYLEMFYALKYRKMAEAVPLAAE

Potato2
MAERVLTRVHSLRERLDATLAAHRNEILLFLSRIESHGKGILKPHQLLAEFESIHKEDKDKLNDHAFEEV
LKSTQEAIVLPPWVALAIRLRPGVWEYVRVNVNALIVEELTVPEFLQFKEELVNGTSNDNFVLELDFEPF
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Poato3
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HALMKEHNLDGQFRWISAQMNRARNGELYRYIADKRGIFVQPAFYEAFGLTVVEAMTCGLPTFATCHGGP
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