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Post-transcriptional and post-translational regulation of the Sucrose Transporter SUT4 from Solanaceae

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I dedicate
The ink and paper of this research
To my parents,
My younger sister Hongli He
And
My husband Lizhu Li

ABSTRACT

Sucrose Transporters (SUTs, also called Sucrose Carriers-SUCs) are membrane proteins, located in the plasma membrane that function in loading sucrose into phloem (specialized vascular tissue) and in sucrose uptake into sink cells. This study aims to investigate more aspects of physiological functions of sucrose transporters in solanaceae plants. There are three subfamilies of sucrose transporters (SUT1, SUT2 and SUT4) have been identified from *Solanum tuberosum* and *Solanum lycopersicon*. Genetic and biochemical evidences have established that SUT1-type proteins function in phloem loading, but the phloem specific role of SUT2 and SUT4 proteins still needs further elucidation.

A series of detailed analyses of sucrose transporter mRNAs mobility and stability have been performed in this study. Parasitic and graft experiments show that SUT1 mRNAs including (*StSUT1*, *NtSUT1*, *SlSUT1* and *SoSUT1*) are phloem mobile; relative analysis with the help of transcriptional and translational inhibitor show that, SUT1 mRNA stability is mainly regulated at the transcriptional level, whereas SUT2 and SUT4 mRNA stability is mainly regulated at the post-transcriptional level.

The differential regulations show that members of the SUT2 and SUT4 family are obviously subject of a different regulatory network as described for sucrose transporters belonging to the SUT1 subfamily. In order to clarify relative physiological functions of SUT2 and SUT4 subfamilies, *StSUT2* & *StSUT4-RNAi* potato plants were generated in this work. In addition, *SlSUT4* over-expressed tobacco plants were produced and the molecular and phenotypical characters of these transgenic plants illuminated that SUT4 might serve as an inhibitor of SUT1.

Bimolecular fluorescence complementation (BiFC) is used to confirm SUT4 protein-protein interaction partners. Yeast three-hybrid system is employed to identify StSUT4 RNA binding protein(s) from *Solanum tuberosum* cDNA library. Several putative interaction partners are found in two independent screens. Further investigation has showed that only one protein with homologies to the hypoxia-responsive HIG1 protein is confirmed. The StSUT4 mRNA-binding capacity of this HIG1-like protein still needs to be confirmed by an alternative method. New aspects will help to elucidate the coordinated expression of tissue-specificially expressed sucrose transporters.

Key words: sucrose transporter, mRNA mobility, mRNA stability, protein-protein interaction, yeast three-hybrid, RNA-binding proteins

ZUSAMMENFASSUNG

Saccharosetransporter (Sucrose Transporters: SUTs, bzw. Sucrose Carriers: SUCs) sind plasmamembran-ständige Membranproteine, die an der Saccharosebeladung des Phloems, dem spezialisierten Transportgewebe höherer Pflanzen, sowie an der Saccharoseaufnahme in sogenannte *sink* Organe beteiligt sind. Ziel der vorliegenden Arbeit ist eine detaillierte Untersuchung der physiologischen Funktion verschiedener Saccharosetransporter in Nachtschattengewächsen (*Solanaceae*).

Phylogenetisch lassen sich die bekannten Saccharosetransporter der beiden Solanaceen-Arten *Solanum tuberosum* und *Solanum lycopersicon* drei unterschiedlichen Unterfamilien zuordnen: der SUT1-, der SUT2- und der SUT4-Familie. Genetische und biochemische Untersuchungen von transgenen Pflanzen mit reduzierter SUT1-Expression legen eine essentielle Funktion der SUT1-Proteine für die Phloembeladung nahe, während die phloem-spezifische Rolle der SUT2- und SUT4-Proteine noch nicht vollständig geklärt werden konnte.

Im Rahmen der vorliegenden Arbeit gelang die Bestimmung der Halbwertszeit der Transkripte verschiedender Saccharosetransporter, sowie der Nachweis ihrer Mobilität im Phloemsaft. Sowohl durch Pfropfungsexperimente, als auch durch den Nachweis von Wirt-Parasit-Interaktionen konnte eine Phloemmobiliät für StSUT1, NtSUT1, SlSUT1, sowie SoSUT1 gezeigt werden. Hierdurch wurde der Nachweis erbracht, dass SUT-Transkripte tatsächlich im Phloemsaft, d.h. in den Siebelementen des Phloems nachweisbar sind. Inhibitorstudien zeigten zudem, dass für SUT2 und SUT4 eine Regulation der Transkriptstabilität auf post-transkriptionaler Ebene stattfinden muss.Um die Funktion, Regulation und Interaktion der SUT2- und SUT4-Protein aufklären zu können, wurden Doppeltransformanten mit reduzierter SUT2- und SUT4-Expression hergestellt und analysiert. Zudem zeigte die Analyse von SUT4-überexprimierenden Pflanzen, dass Parallelen zu SUT1-inhibierten Pflanzen auftraten. Eine inhibitorische Rolle des SUT4-Proteins auf die SUT1-Beladungsfunktion wird daher diskutiert. Über einen Hefe-3-Hybrid Ansatz wurde daher gezielt nach SUT4 mRNA-bindenden Proteinen unter Verwendung einer Kartoffel-eigenen cDNA Bank gesucht. Mehrere vermeintlich SUT4 RNA-bindende Proteine konnten in unabhängigen Wiederholungen identifiziert werden. Nach weiteren Analysen und dem Ausschluss falsch positiv interagierender Proteine konnte nur für einen der Kandidaten eine RNA-bindende Eigenschaft bestätigt werden: ein durch Protein, das Homoligie zu dem durch Hypoxie induzierbaren HIG1-protein aufweist. Die Bestätitgung der RNA-bindenden Eigenschaft dieses Zinkfingerproteins durch alternative Methoden steht noch aus. Die Identifizierung SUT4-mRNA bindender Proteins könnte bei der Aufklärung der post-transkriptionalen Regulation dieses vermeintlich regulatorischen Saccharosetransporters eine wichtige Rolle spielen.

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Abbreviations and Acronyms

Abbreviation	Complete form
3', 5'	3-prime, 5-prime
3′UTR	3-prime untranslated region
ABA	abscisic acid
ActD	Actinomycin D
AD	Activation domain
Amp^R	Gene encoding ampicillin resistance in *E.coli*
APS	ammonium persulphate
ARF	auxin response factor
A.t.	*Arabidopsis thaliana*
bp	base pairs
BiFC	Bimolecular fluorescence complementation
BSA	bovine serum albumin
ß-ME	ß-Mercaptoethanol
CaMV	cauliflower mosaic virus
CC	companion cell
cDNA	Complementary DNA
CHX	cycloheximide
CLSM	confocal laser scanning microscopy
C.m.	*Cucurbita maxima* (pumpkin)
Cmm	*Cucumis melo* (melon)
C.s.	*Cucumis sativus* (cucumber)
CTAB	Cetyltrimethyl ammonium bromide
cpm	Counts per minute
ddH_2O	distilled, deionized water
DMF	Dimethylformamide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenetriaminetetraaceticacid
EMSA	electrophoresis mobility shift assay
EST	Expressed sequence tag
FT	Flowering Locus T
g	gram
GA	gibberellic acid
GUS	ß-glucuronidase

H_2O_2	Hydrogen peroxide
HEPES	N-2-HydroxyEthylPiperazin-N'-2-EthanSulfonacid
His	Histidine
Hsc70	Heat shock cognate protein 70 kDa
IPTG	isopropyl-β-D-thiogalactopyranoside
JAR1	Jasmonate resistance 1
Km^R	Gene encoding kanamycin resistance in *E.coli*
kDa	Kilo Dalton
LD	Long day
Leu	Leucine
mg	milligram
mM	millimole
ml	millilitre
MeJA	Methyl jasmonate
Met	Methionine
MF	Microsomal fraction
NaOH	sodium hydroxide
ng	nanogram
Nt	*Nicotiana tabacum*
NPT II	Gene encoding kanamycin resistance in plants
NUP A	Nested Universal Primer A
OD	Optical density
ORF	Open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PD	plasmodesmata
pH	Negative decimal log. Of the H+ concentration
phyB line	*Phytochrome B* antisense potato
PEG	polyethylene glycol
PLV	protA-lexA-VP16 peptide
PMV	Plasma membrane vesicles
PMSF	phenylmethylsulfonylfluoride
qRT-PCR	Real-time reverse-transcription PCR
PTGS	Post transcriptional gene silencing
QTL	Quantitative Trait Locus
rpm	revolutions per minute
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	reverse transcription polymerase chain reaction

SE	sieve element
SEL	size exclusion limit
SD medium	Minimal synthetic defined medium for yeast
SD	Short day
S.D.	Standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
Sl	*Solanum lycopersicon*
St	*Solanum tuberosum*
SUT	Sucrose transporter
SoSUT1	Spinach (*Spinacia oleracea L.*) sucrose transporter 1
SUT2	Sucrose transporter 2
SUT4	Sucrose transporter 4
T-DNA	Transfer DNA
TEF	transcription elongation factor
TEMED	tetramethylethylenediamine
Trp	Tryptophan
µg	microgram
µl	microliter
µM	Micromole
Ura	Uracil
UB1	Ubiquitin 1
Y3H	yeast three hybrid
YFP	Yellow Fluorescent Protein
v/v	volume for volume
w/v	weight for volume
WT	Wild-type
X-gal	5-bromo-4-chloro-3-indolyl-3-D-galactopyranoside
ZFP	zinc finger protein

1 Introduction

1.1 Phylogenetic and physiologic analysis of sucrose transporters in plants

1.1.1 Phylogenetic analysis of sucrose transporters in plant kingdom

Sugars are the end products of photosynthesis in plants. They act as substrates for energy metabolism and biosynthesis of complex carbohydrates and provide necessary resources for the growth and development of sink tissues. In addition, sugars also serve as secondary messengers, with the ability to regulate plant growth and development in response to biotic and abiotic stresses (Hammond and White, 2008). The network of sugar signalling has the capacity to regulate the expression of various genes and to interact with other signalling pathways such as phytochrome responses and metabolic pathways (Gibson, 2004).

Sucrose, the major sugar form, is produced mainly in mature leaves (source organs) of plants. To support the growth of various heterotrophic sink tissues (developing leaves, the shoot apex, roots, and reproductive organs), sucrose distribution from source organs to respective tissues is mediated by the vascular system. The enucleate sieve elements (SEs) present in the vascular tissue of the phloem and form the conduits for long distance transport (Weise et al., 2000). Sucrose transport has been studied in a broad variety of plant species and sucrose uptake has often been described as a biphasic transport system. In angiosperms the kinetic of sucrose uptake is comprised of two components with different kinetic properties, one component represents a high affinity-low capacity carrier and the second one represents a low affinity-high capacity transporter (Kühn, 2003).

Plant Sucrose Transporters (SUTs) were first isolated via yeast complementation from both potato and spinach (Riesmeier et al., 1992; Riesmeier et al., 1993). Subsequently, genes and cDNAs encoding homologous proteins have been cloned from more than 60 plant species (Lalonde et al., 2004; Sauer, 2007). SUT proteins are predicted to be integral membrane proteins with 12 membrane spanning domains and a putative central hydrophilic loop. The 6-loop-6-structure of the 12 trans-membrane domain proteins is highly conserved among plant species and is similar to what is characteristic of the major facilitator superfamily (MFS) for several cation/substrate co-transporters (Marger and Saier, 1993). Completion of the rice, Arabidopsis and yeast genomes has allowed the identification of new sucrose transporter-like proteins and the analysis of whole gene families. Phylogenetic analysis has shown that all known sucrose transporters and sucrose transporter-like proteins fall into three clades in dicot plants (Kühn, 2003; Sauer, 2007). Kühn and Grof have carried out a new

phylogenetic analysis of sucrose transporters in monocot and dicot plants (unpublished data). In the new phylogenetic tree shown as follow, all sucrose transporters and sucrose carriers fall into five clades. The SUT1 clade contains only dicot-specific sucrose transporters (Fig. 1), which are mainly expressed in sucrose exporting source leaves (Barker et al., 2000) and are subject to regulation by plant hormones (Harms et al., 1994). Immunolocalization studies have showed that SUT1 is present at high levels in the plasma membrane of sieve elements (Kühn et al., 1997); members of the SUT2 and SUT4 clades exist in both monocot and dicot species; members of the SUT3 and SUT5 clades are monocot-specific sucrose transporters (Fig. 1).

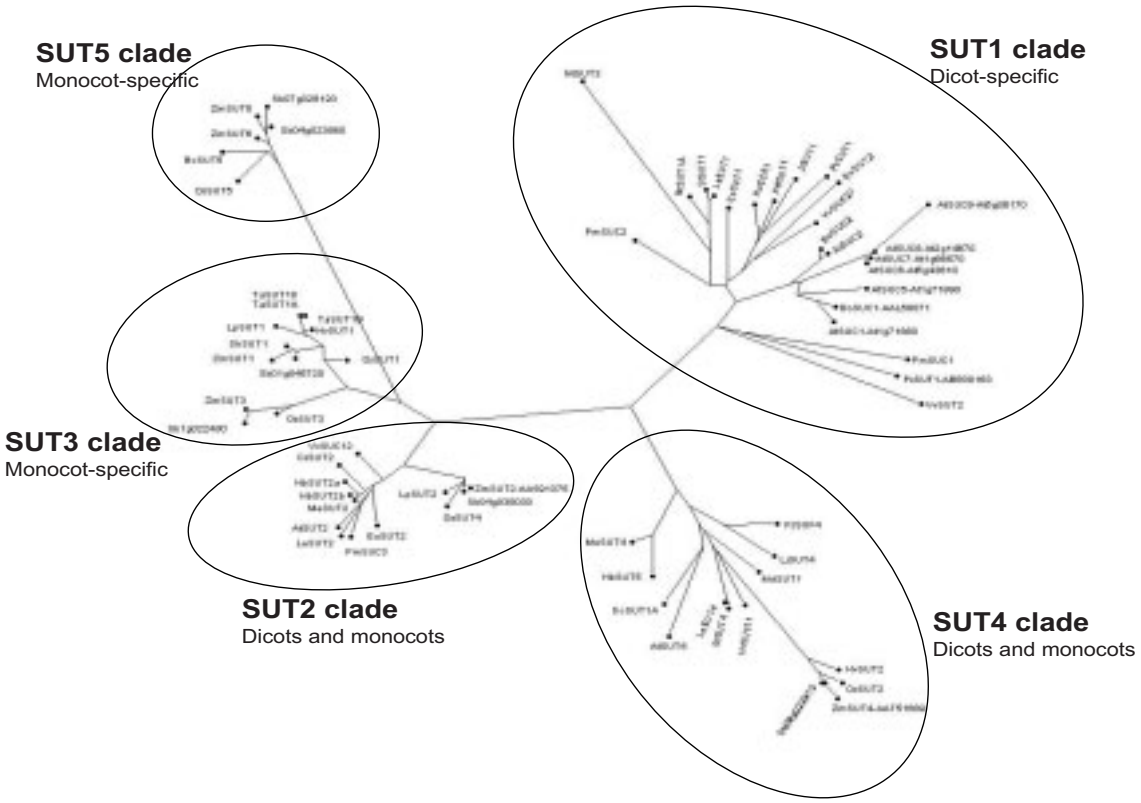


Fig. 1: A phylogenetic tree of sucrose transporters in monocotyledonous and dicotyledonous species base on the similarities of available gene sequences

The phylogenetic tree is constructed using Dendroscope (Huson et al., 2007) and is drawn by Kühn and Grof (unpublished data). Accession numbers of presented sucrose transporter are: *Arabidopsis thaliana*: AtSUC1, At1g71880; AtSUT2, At1g22710; AtSUT2, At2g02860; AtSUT4, At1g09960; AtSUC5, At1g71890; AtSUC6, At5g43610; AtSUC7, At1g66570; AtSUC8, At2g14670; AtSUC9, At5g06170. *Brassica oleracea*: BoSUC1, AAL58071; BoSUC2, AAL58072. *Bambusa oldhami*: BoSUT5, AAY43226. *Citrus sinensis*: CsSUT2, AAM29153. *Daucus carota*: DcSUT1A, CCA76367; DcSUT2, CAA76369. *Eucommia ulmoides*: AAX49396. *Hevea brasiliensis*: HbSUT2a, ABJ51934; HbSUT2b, ABJ51932; HbSUT5, ABK60189. *Hordeum vulgare*: HvSUT1, CAB75882; HvSUT2, CAB75881. Juglans regia: JrSUT1, AAU11810. *Lycopersicum esculentum* renamed *Solanum lycopersicum*: LeSUT1, AAG12987; LeSUT4, AAG09270. *Lotus japonicus*: LjSUT4, CAD61275. *Lolium pernne*: LpSUT1, EU255258; LpSUT2, ACU87542. *Malus x domestica*: MdSUT1, AAR17700. *Manihot esculenta*: MeSUT2, ABA08445; MeSUT4, ABA08443. Nicotian tabacum: NtSUT1A, CAA57727; NtSUT3, AAD34610. *Oryza sativa*: OsSUT1, AAF90181; OsSUT2, BAC67163; OsSUT3, BAB68368; OsSUT4, BAC67164; OsSUT5, BAC67165. *Plantago major*: PmSUC1, CAI59556; PmSUC2, X75764; PmSUC3, CAD58887. *Pisum sativum*: PsSUT1, AAD41024; PsSUF1, ABB30163; PsSUF4, ABB30162. *Populus tremula x Populus tremuloides*: PtSUT1-1, CAJ33718. *Ricinus communis*:

RsSCR1, CAA83436. *Saccharum hybrid*: ShSUT1, AAV41028. *Spinacea oleracea*: SoSUT1, Q03411. *Solanum tuberosum*: StSUT1, CAA48915; StSUT4, AAG25923. *Sorghum bicolor*[a]: SbSUT1, Sb01g045720; SbSUT2, Sb04g038030; SbSUT3, Sb01g022430; SbSUT4, Sb08023310; SbSUT5, Sb04g023860; SbSUT6, Sb07g028120. *Triticum aestivum*: TaSUT1A, AAM13408; TaSUT1B, AAM13409; TaSUT1D, AAM13410. *Vitus Vinifera*: VvSUC11, AAF08329; VvSUC12, AAF08330; VvSUC27, AAF08331; VvSUT2, AAL32020. *Zea mays*[b]: ZmSUT1, BAA83501; ZmSUT2, AAS91375; ZmSUT3, ACF86653; ZmSUT4, AAT51689; ZmSUT5, ACF85284; ZmSUT6, ACF85673. Accession numbers are also used as additional descriptors in the tree in those instances where confusion may arise due to variations in nomenclature.

[a]Sorghum Genome Project : www.phytozome.net

[b]Maize Genome Project: http://www.maizesequence.org/index.html. The Phylogeny website (http://www.phylogeny.fr/version2_cgi/index.cgi) was used extensively for sequence alignment and analysis(Dereeper et al., 2008). The data was converted into Newick format prior to transfer to Dendroscope (http://www-ab.informatik.uni-tuebingen.de/software/dendroscope).

1.1.2 Sucrose transporters in solanaceous plants

The Solanaceae plant family is extensively utilized by human. It is an important source of food (e.g. potato and tomato), spice (e.g. hot pepper) and medicine (e.g. datura, which produces tropane alkaloid). Some crop species such as tobacco, potato and tomato have been studied in detail with respect to carbohydrate partitioning during classical physiological methodology and molecular tools have been used for many genes involved in carbohydrate metabolism (Frommer and Sonnewald, 1995). Sucrose transporters are important for carbohydrate metabolism and are involved in sucrose phloem loading. The sucrose transporter proteins (SUT1, SUT2 and SUT4) have been demonstrated to be co-localized in sieve elements (SEs) of potato source leaves, petioles and stems (Reinders et al., 2002), whereas transcription of SUT1 is shown to take place in companion cells (CCs) (Kühn et al., 1996; Barker et al., 2000; Weise et al., 2000). These three SUT proteins belong to SUT1, SUT2 and SUT4 clades respectively and the RNA levels of them not only follow a diurnal rhythm but also oscillate in constant light (Chincinska et al., 2008). Furthermore, the mode of phloem loading and the mechanisms of SE-specific protein synthesis and protein targeting, which are described for solanaceous plants, resemble those of other plant species such as wheat and opium poppy (Bird et al., 2003; Aoki et al., 2004).

The SUT1 clade

NtSUT1, NtSUT3 (from *Nicotiana tabacum*), SlSUT1 (*Solanum lycopersicum*) and StSUT1 (from *Solanum tuberosum*) belong to the SUT1 clade (Fig. 1). StSUT1 is characterized as a high-affinity sucrose / proton co-transporter and its antisense repression leads to reduced transport activity of the protein causing extensive physiological defects in carbon partitioning and photosynthesis (Riesmeier et al., 1994; Kühn et al., 1996). The role of SUT1 as an essential protein driving sucrose against a concentration gradient into the phloem for long-distance transport is also successfully demonstrated in potato by a transgenic approach (Kühn et al., 1996). Similar effects have been observed in a seed plant (tobacco) confirming the importance of SUT1 for sucrose loading into the phloem via an apoplastic route and possibly for inter-

mesophyll transport (Bürkle et al., 1998). The phenotype of transgenic tomato plants (containing antisense transcripts of *SlSUT1*) (Hackel et al., 2006) is comparable to *SUT1* antisense phenotypes in potato and tobacco (Riesmeier et al., 1994; Kühn et al., 1996; Bürkle et al., 1998) and to the homologous *AtSUC2* insertional mutant phenotype in *Arabidopsis* (Gottwald et al., 2000). The observed phenotype of *SlSUT1* antisense plants is defective fruit development. It is probably due to an undersupply of carbohydrates to sink organs, which accumulate to high amounts in source leaves, leading to severe leaf modifications and osmotic symptoms. The development of sink organs in the *SlSUT1* antisense plants is delayed due to disturbance in supply of sugars to terminal sink organs indicating an essential function of SUT1 in phloem loading and long-distance transport of sugars. Overall, it could be said that in *solanaceous* plants, SUT1 is essential for apoplasmic phloem loading of sucrose.

The SUT2 clade

The SUT2 clade only has three known members in *Solanaceae*, SlSUT2 (from *Solanum lycopersicum*), SdSUT2 (from *Solanum demissum*) and StSUT2 (from *Solanum tuberosum*). The later two are not included in Fig. 1; the protein sequences are highly homologous to SlSUT2. The *SUT2* expression is predominantly detected in sink organs and is identified to colocalize with low and high-affinity sucrose transporters (*SUT4* and *SUT1*) in enucleate sieve elements of tomato and shares features with yeast sugar sensors (Barker et al., 2000). Most importantly, the *SUT2* gene maps on chromosome V of potato and is linked to a major quantitative trait locus (QTL) for tuber starch content and yield (Gebhardt et al., 1991). In contrast to *SlSUT1*, which is induced during the sink-to-source transition of leaves, *SUT2* is more highly expressed in sink than in source leaves and is inducible by sucrose. The SlSUT2 protein is co-localized with the low- and high-affinity sucrose transporters in SEs of tomato petioles. This indicates that multiple *SUT* mRNAs or proteins probably travel from CCs to enucleate SEs and the SUT proteins are translated in SEs (Reinders et al., 2002). Tomato plants transformed with a *SlSUT2* antisense constructs are exclusively affected in tomato fruit and seed development which is explained by three possible explanations: 1) disturbance of pollination attributed to a decreased expression of *SlSUT2* in anthers; 2) *SlSUT2* was directly involved in phloem unloading on the level of the tomato fruits or seeds; or 3) both parameters are involved in the determination of the final fruit size (Hackel et al., 2006).

The SUT4 clade

Sucrose Transporter 4 (SUT4) is originally characterized as plasma membrane localized sucrose transporters (StSUT4 and AtSUC4, HvSUT2) (Weise et al., 2000; Weschke et al., 2000). The transporter activity of SUT4 proteins has been confirmed by sucrose uptake experiments and yeast (*Saccharomyces cerevisiae*) complementation experiments with AtSUT4 (*Arabidopsis thaliana*) and StSUT4

(*Solanum tuberosum*) (Weise et al., 2000), and the orthologous HvSUT2 (*Hordeum vulgare*)(Weschke et al., 2000). The localization of StSUT4 and SlSUT4 in the plasma membrane of phloem sieve elements in potato and tomato is confirmed by immunocytochemical analysis (Weise et al., 2000; Reinders et al., 2002).

In tomato all three principal sucrose transporters, SlSUT1, SlSUT2 and SlSUT4 were described as plasma membrane localized proteins. These transporters may interact physically to adopt the phloem loading activity of solanaceous SEs to different developmental or environmental conditions. The original idea of the hypothesis was that one of these proteins, SUT2, might act as sucrose sensor (Barker et al., 2000) which can interact with a high affinity/low capacity transporter, SUT1 (Riesmeier et al., 1992), and/or with a low affinity/high capacity transporter, SUT4, and regulate the relative activities of these two proteins (Weise et al., 2000). However, Hackel *et al*., (2006) have argued that SlSUT2 might have a dual function in pollen tubes loading and in the phloem specific localization. The authors have assumed SlSUT2 serves as a sucrose transporter rather than sucrose sensor. This idea is consistent with the functional analysis of PmSUC3, which has 75% identity to SlSUT2. PmSUC3 is localized in sieve elements of the *Plantago* phloem and mediates the energy-dependent transport of sucrose and maltose (Barth et al., 2003). In fact, the analyses of potential interactions between these three sucrose transporters with the split-ubiquitin system in yeast cells support the following idea (Reinders et al., 2002; Kühn, 2003) (Fig. 2).

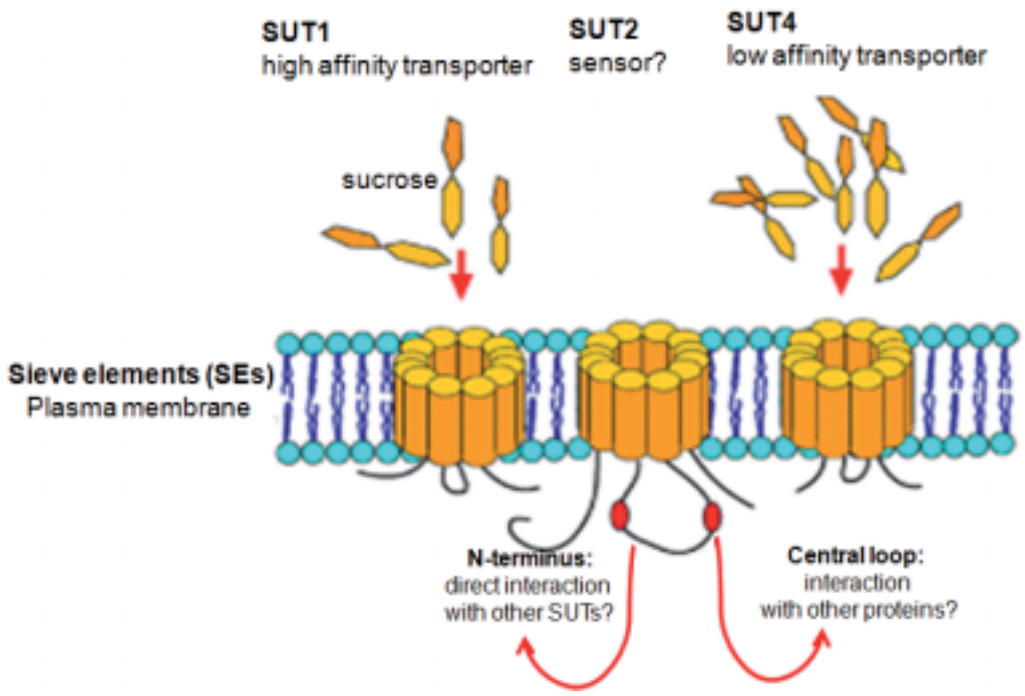


Fig. 2: Potential interaction of SUT1, SUT2 and SUT4 in solanaceous plants (Kühn, 2003)

SUT1, SUT2 and SUT4 are co-localized in the plasma membrane of sieve elements. SUT2 plays a dual function in sucrose transporting in sink organs such as pollens and fruits and a second still unknown

function in the phloem. SUT2 potentially regulates the activity of the high-affinity sucrose transporter SUT1 or the low-affinity sucrose transporter SUT4, either directly or via an indirect signaling mechanism, such as phosphorylation/dephosphorylation. Regulation can occur at the level of protein degradation, internalization of transporter or inactivation. Another possibility is regulation at the transcriptional level. Since *SUT1* and probably also the other sucrose transporter genes are most likely expressed in companion cells, this model would implicate a signal cascade affecting the transcriptional activity of the neighboring companion cells.

1.2 phloem mobile mRNAs and proteins

The phloem of higher plants is a vascular tissue and consists of living cells, enucleate sieve elements (SE) and companion cells (CC). The phloem performs a number of distinct tasks when collecting metabolites and signals from source organs (mainly mature leaves) and transporting these molecules and releasing them into sink organs (sink leaves, flowers, tubers or roots). The metabolites and signals mainly include organic substances such as sucrose and other sugars or sugar alcohols and amino acids, also a diverse range of macromolecules (proteins, RNAs and pathogens). Therefore, the phloem has great potential to facilitate inter-organ coordination and hence to promote plant growth and development (Hir et al., 2008). Plasmodesmata provide a pathway for the trafficking of proteins and RNAs. These phloem mobile signals play a major role in plant nutrition, development and communication. Proteins which move through PD are defined as non-cell-autonomous proteins (NCAPs); RNA is thought to traffic from cell to cell as ribonucleotide-protein complex (RNP). It is now well established that the local movement of NCAPs and RNPs can contribute to the establishment of cell fate and patterning in plant tissues (Lough and Lucas, 2006).

In higher plants the vascular system provides both mechanical strength and long-distance transport capacity. The phloem distributes photo-assimilates from source to sink tissues and is composed of conducting sieve elements, associated with companion cells, and non-conductive phloem parenchyma and fiber cells (Yu et al., 2007). In addition, phloem transports hormones, messenger RNAs (mRNAs), and proteins that may mediate developmental and stress responses (Citovsky and Zambryski, 2000; Lucas et al., 2001; Kehr and Buhtz, 2008). It is not only a nutrient conduit but also functions as an information highway (Lough and Lucas, 2006; Lin et al., 2009). In the previous studies, researchers have established that mRNAs molecules, small RNAs and even transfer RNAs are present in plant vascular tissues. These non-cell autonomous function as plant-unique remote-control systems in gene silencing, pathogen defense, leaf development, tuber formation, phosphate homeostasis and many other physiological processes (Ruiz-Medrano et al., 1999; Lucas et al., 2001; Yoo et al., 2004; Doering-Saad et al., 2006; Omid et al., 2007; Kehr and Buhtz, 2008; Ma et al., 2009).

1.2.1 Phloem mobile mRNAs

A role of RNA as a non-cell-autonomous information macromolecule is emerging as a new model in biology. Studies on higher plants have shown the operation of cell-to-cell and long-distance communication networks that mediate the selective transport of RNA. Mature sieve tubes are probably lack of the ribosomes which are required for a complete translation system. RNA is present, but it is not clear whether it functions as mRNA, tRNA or rRNA (Sjolund, 1997). Since in the mature sieve-tube members without nucleus transcription from nuclear DNA is impossible, short-lived RNA would in any case have to be delivered from the surrounding cells.

It is known that three major types of RNAs can be systemically transported: RNA genomes of viruses, such as *CMV 2b* and *TMV*; endogenous cellular mRNAs for example *SUT1*, *GAI* and *BEL5* mRNAs; and small non-coding RNAs, such as *miR399*, *miR172* and short interfering RNA (Voinnet and Baulcombe, 1997; Citovsky and Zambryski, 2000; Mlotshwa et al., 2002; He et al., 2005; Kehr and Buhtz, 2008; Zhong and Ding, 2008; Lee and Cui, 2009; Martin et al., 2009).

Among the systemically transported RNAs, the presence of endogenous plant mRNAs in the phloem is most surprising, because functional SEs do not contain nuclei or ribosomes that could synthesize proteins from such templates. Notably, endogenous mRNAs have been shown not only to move systemically across the graft junctions but also to function as information molecules (Lucas et al., 2001; Lough and Lucas, 2006). The earliest convincing proof for an endogenous plant mRNA inside SEs comes from the study that the mRNA of *Sucrose Transporter1* (*SUT1*) in Solanaceae is localized in SEs and the PD connecting SEs to CCs (Kühn et al., 1997). Afterwards, mRNAs from *Thioredoxin h*, *Cystatin*, and *Actin* have been detected by RT-PCR in rice phloem samples obtained with the minimal-invasive aphid stylet technique (Sasaki et al., 1998) and, more recently, *thioredoxin h* mRNA was found in phloem exudates of *Brassica napus* (Giavalisco et al., 2006) and several other transcripts, including *SUT1*, *aquaporin*, and a proton *ATPase*, could be amplified from phloem sap of barley (Doering-Saad et al., 2002). There are more comprehensive approaches resulting in the identification of several functionally unrelated mRNAs from *Cucurbita maxima* exudates (Ruiz-Medrano et al., 1999; Ruiz-Medrano et al., 2007). High integral and qualified RNA in potato phloem cells (Yu et al., 2007), a 1326-clone macroarray based cDNA library of celery vasculature (Vilaine et al., 2003), a phloem transcriptome from secondary tissue of the *Arabidopsis* root-hypocotyl (Zhao et al., 2005) and a phloem-enriched cDNA library from *Ricinus communis L.* (Doering-Saad et al., 2006) and so on. These research results are helpful for the identification of phloem transcripts.

Since mature phloem SEs are not equipped for RNA transcription and protein translation, it is likely that endogenous mRNAs function as systemic long-distance signals that can influence gene expression in the target tissues and can thus control diverse processes in plant development and morphogenesis (Ueki and Citovsky, 2001b). Accordingly, the phloem transport of a *CmNACP* mRNA from pumpkin could be directly demonstrated by heterograft experiments between pumpkin and cucumber plants, in which *CmNACP* transcripts could be detected in the cucumber scion phloem and in apical tissues (Ruiz-Medrano et al., 1999). Another grafting study has shown that the transcript of a *KNOTTED1*-like homeobox gene can cross graft junctions and induce phenotypic changes in the scions in tomato, indicating a functional relevance of mRNA long-distance movement (Kim et al., 2001). Additional example comes from potato, where over-expression of a *BEL1-like* transcription factor leads to a marked increase of tubers per plant and this phenomenon is graft-transmissible (Banerjee et al., 2006). Recently, a microRNA *miR172* has been reported as another regulator shared by flowering and tuberization, and this *miR172* affects a long-distance signaling pathway since its effect on tuberization is graft-transmissible (Martin et al., 2009).

The similar observation is found for another gene, *GIBBERELLIC ACID INSENSITIVE (GAI)*, in *Cucurbita maxima* and *Arabidopsis* gain-of-function mutants. The transcripts of *GAI* could move over long distances and cross graft unions. This long-distance translocation leads to a change of the phenotype of scion leaves in grafting experiments (Haywood et al., 2005). The trafficking of *GAI* RNA seems to be selective; since a control *enhanced GFP* (*EGFP*) RNA could not enter the SEs from their expression site in CCs and could thus not enter the long-distance trafficking pathway. Interestingly, sink strength seems not to be the only influence on transport of *GAI* as transgenically expressed pumpkin and Arabidopsis *GAI* RNAs could traffic in non-transgenic scion shoot apex and floral organs but were restricted from developing fruits, pedicles, and peduncles (Haywood et al., 2005). These findings indicate that phloem transport of mRNAs might not be regulated by simple diffusion and the phloem has a capacity to selectively deliver macromolecules to specific plant organs (Ding et al., 2005). Recently, Huang and Yu (Reinders et al., 2002; Huang and Yu, 2009) has made further study on the long-distance movement of *GAI* RNA by Arabidopsis inflorescence grafting and RT-PCR analysis. They demonstrated that long-distance movement of RNA only occurred in specific transcripts and the motifs at coding sequences and 3' untranslated regions of *GAI* RNA play important roles during RNA movement.

The localization of SUT1 protein in sieve elements and the *SUT1* mRNA at the orifices of plasmodesmata interconnecting companion cells and sieve elements, together with the high turnover of both *SUT1* mRNA and protein, indicate that

trafficking of mRNA or protein occurs from companion cells into enucleate sieve elements by way of plasmodesmata (Kühn et al., 1997). *AgSUT2* was detected in a transcriptome analysis of the phloem of *Apium graveolens* (Vilaine et al., 2003). *AtSUC2* transcript is found from phloem sap both by microarray and quantitative RT-PCR in the same experiment. *AtSUT4* transcript is also detectable in the same phloem sap sample by quantitative RT-PCR (Deeken et al., 2008).

1.2.2 Phloem mobile proteins

Phloem specific proteins are involved in metabolism, DNA and RNA binding, protein synthesis and degradation, metal homeostasis and transport, cell wall, structure, signal transduction and stress response, etc. The role of endogenous non-cell-autonomous proteins (NCAPs) in plant development is well established. Insights are also emerging as to the functions of such NCAPs in long-distance signaling through the phloem for development, gene silencing, and pathogen defense. Recently, increasing evidence has suggested that phloem proteins are involved in the trafficking of RNA. RNA binding proteins (RBPs) have been found in the phloem of various plants. These findings provide insight into a novel function for phloem proteins as a component of an RNA-based systemic signaling mechanism. Pumpkin phloem sap contains a set of RBPs including CmPP16-1/-2 (Xoconostle-Cázares et al., 1999; Aoki et al., 2005), Glycine rich RNA-binding protein (Aoki et al., 2005), CmPSRP1 (Yoo et al., 2004), eIF-5A (Giavalisco et al., 2006), CmPP1/2 (Gómez et al., 2005), and CmRBP50 (Ham et al., 2009). Their presence is consistent with the notion that RNA transport in the phloem plays a role in the coordination of developmental and physiological events, at the whole plant level (Ruiz-Medrano et al., 1999; Xoconostle-Cázares et al., 1999; Kim et al., 2001; Haywood et al., 2005; Banerjee et al., 2006). These phloem-mobile RBPs are thought to form ribonucleoprotein complexes with the unique population of mRNA present in the translocation stream (Lough and Lucas, 2006). Furthermore, a significant number of these RBPs are components of the machinery for protein synthesis. However, the function of many of these proteins remains to be established. Interestingly, this RBP category contains ten putative helicases, the function of which may be in mediating the unfolding and refolding of mRNA during its trafficking through the companion cell-sieve element plasmodesmata (summary in table 1).

Table 1: phloem mobile proteins in plants

Protein	Method	Function	Reference(s)
Viral Coat proteins or Movement proteins			
TMV-MP	graft	Change of Plasmodesmata SEL	(Balachandran et al., 1995)
RCNMV MP	infiltration	Systemic infection of	(Xiong et al., 1993)

		RCNMV	
Phloem proteins			
CmPP2 (pumpkin)	Graft, microinjection	Long-distance RNA or viroid trafficking, defense	(Balachandran et al., 1997; Golecki et al., 1998) (Owens et al., 2001)
CsPP2 (cucumber)	Graft	Long-distance RNA trafficking, defense	(Gómez and Pallás, 2004)
CmPP36 (pumpkin)	Graft	A member of the cytochrome b5 reductase family, cell to cell trafficking	(Xoconostle-Cázares et al., 2000)
CmHsc70-1, CmHsc70-2 (pumpkin)	Graft, infiltration	Molecular chaperone, protein cell-to-cell movement	(Aoki et al., 2002)
RNA binding proteins			
CmPP16-1, CmPP16-2 (pumpkin)	Graft, infiltration	RNA binding, increase SEL, phloem mobility	(Xoconostle-Cázares et al., 1999) ; (Aoki et al., 2005)
CmmLec17 (melon)	graft	Phloem mobility of RNA	(Gómez et al., 2005)
eIF-5A	Phloem proteomics	RNA-binding activity and transport	(Thompson et al., 2004) (Giavalisco et al., 2006)
CmPSRP1 (pumpkin)	Heterograft, microinjection	Small RNA-binding, phloem mobility	(Yoo et al., 2004)
CmRBP50 (pumpkin)	Graft	RNA-binding	(Ham et al., 2009)
Signaling pathway			
FT *(Arabidopsis)* Hd3a (rice) SFT(tomato) CmFT (squash)	Under phloem specific promoter control, graft graft	Flower induction	(Corbesier et al., 2007; Jaeger and Wigge, 2007; Notaguchi et al., 2008) (Tamaki et al., 2007) (Lifschitz et al., 2006)
pathogenesis-related protein (PR1, maize)	graft	Induced in response to fungal infection	(Bortolotti et al., 2005)
Florigen and tuberigen	graft	Promotion of flowering or tuberization	(Martínez-García et al., 2002a)

Since all three known sucrose transporters in solanaceous plants are co-localized in the plasma membrane of SE in the phloem (Reinders et al., 2002), the relative mRNAs or homologous mRNAs such as *StSUT1* mRNA, *CmSUT1P*, *AtSUC2* and *AtSUT4* are detected in the phloem (Kühn et al., 1997; Roney et al., 2007; Deeken et al., 2008). The *StSUT4-RNAi* phenotype is graft-transmissible, so phloem specific RNA-binding proteins might be involved in mRNA trafficking between SE and CC, or in long distance transport.

1.3 mRNA stability and RNA-binding proteins

1.3.1 Determinants of mRNA stability in plants

Regulating gene expression from DNA to protein is a complex multistage process with multiple control mechanisms. Transcriptional regulation has been considered to be the major control point of protein production in eukaryotic cells; however, there is growing evidence of pivotal post-transcriptional regulation for many genes. The brief existence of an mRNA molecule begins with transcription and ultimately ends in degradation. Unlike prokaryotic molecules eukaryotic mRNA molecules often require extensive processes such as alternative splicing, RNA editing, capping and poly-adenylation, nuclear transport, mRNA stability and chromatin modification. The regulation of mRNA stability has emerged as a critical control step in determining the cellular mRNA level, with individual mRNAs displaying a wide range of stability that has been linked to discrete sequence elements and specific RNA-protein interactions (Staton et al., 2000). In higher plants, control of mRNA processing, stability and translation are pivotal in a variety of biological and physiological contexts including development, stress response and genome organization.

General mRNA decay pathways are very complicated, several interesting themes are emerging (Belostotsky and Sieburth, 2009). First, macromolecular complexes once viewed as single invariable entities, the exosome and the P-body; appear to be able to assemble with variable subunit composition, and possibly with different downstream functions. Second, functional redundancy appears to be less common than models imply. An mRNA decay pathway could use any of three different deadenylation complexes, and then either 3'- to-5' (exosome) or 5'-to-3' (decapping/XRN4), each of these complexes appears to be essential, and to function on discrete subsets of cytoplasmic mRNA. So, the emerging picture is a complex system with a surprisingly high degree of mRNA substrate specificity. The biggest challenge is to identify the proteins that bind mRNAs and target them to particular decay pathways, and to understand how the activity of these proteins is regulated. These binding proteins may be the key in understanding developmentally regulated general mRNA decay and would shed light on both decay pathways and developmental mechanisms.

Trans-acting factors involved in mRNA stability include both the actual ribonucleases involved in the degradative process and regulatory factors can be supposed to act both positively (to stimulate rapid degradation) and negatively (to protect from degradation). The close relationship between mRNA degradation and translation for at least some mRNAs suggests that some factors may have dual roles in translation and turnover (Jacobson and Peltz, 1996). Three major approaches are used in the identification of new trans-acting factors: 1) cloning of genes containing known

RNA-binding motifs and subsequent assignment of function; 2) identification of proteins that bind to known *cis* determinants of stability by gel mobility shift and /or cross linking assay; 3) biochemical purification of proteins with ribonuclease activity.

1.3.2 The role of stresses in regulating mRNA stability

Although transcriptional activity of the genes in question may be up- or down regulated, transcriptional regulation alone is generally insufficient to achieve rapid changes in gene expression. For instances, the increase in stress protein expression may involve increases in stress protein translation rates or enhanced stress protein mRNA stability. The suppression of normal cellular protein expression may be accomplished by selectively arresting the translation of normal cellular protein mRNAs, sequestering these messages, or selectively destabilizing them. Upon recovery from stress, the suppression of stress protein synthesis may be achieved by post-transcriptional mechanisms. So during stress responses (high light biotic and abiotic stress responses) the modulation of mRNA stability is certainly achieved by selectively destabilizing mRNAs.

Viral infection on host gene expression at the post-transcriptional level is to be investigated. Post-transcriptional mechanisms are important in many transgenic plants exhibiting resistance to viruses. The levels of viral RNAs are frequently reduced in transgenic plants expressing overlapping virus-derived sequences so that these plants show strong resistance to viral infection. It is becoming clear that RNA molecules enter and move within the phloem by means of the long distance delivery system (Lough and Lucas, 2006). The nature of these translocated RNA is diverse, including endogenous mRNAs, pathogenic RNAs, and small RNAs related with gene silencing (summarized in chapter 1.2). In cucurbitaceous, the *PP2* gene family has RNA-binding activity and has potential capacity to increase the plasmodesmata SEL. Interestingly, unlike other phloem lectins, the *CmmLec17* mRNA level in the hypocotyls is relatively stable from the second day after germination to 45 days later. The difference in synthesis and accumulation has been observed between the *CmmLec17* and the previously described phloem lectins suggests a possible alternative function for this RNA-binding protein (Gómez and Pallás, 2004).

Understanding of sucrose transporter expression and regulatory activities involved in distribution of post-phloem photoassimilates and accumulation of carbohydrates appear to be of major economic importance, most importantly during insect herbivore or pathogen attack as a result of wounding. This will have an important role in understanding the individual roles of each sucrose transporter in plant stress physiology. This will also further contribute more information to the poorly understood physiological processes such as: pollen/seed development, root and shoot

growth, stomata opening, plant-pathogen interaction and resistance signaling mechanisms during plant wounding.

1.3.3 RNA-binding proteins in plants

RNA-binding proteins are typically cytoplasmic and nuclear proteins that associate with (bind) double-strand or single-strand RNAs through RNA binding motifs, such as RRMs (RNA recognition motifs), KH (the K homology) motifs, zinc fingers, zinc knuckles, RGG-boxes and DEAD-boxes (RRM). The remarkable diversity of RNA-binding proteins (RBPs), which appears to have increased during evolution in parallel to the increase in the number of introns, allows eukaryotic cells to utilize them in an enormous array of combinations giving rise to a unique RNP for each RNA molecule. RBPs play key roles in post-transcriptional control of RNAs, which along with transcriptional regulation, is a major way to regulate patterns of gene expression during development. Post-transcriptional control can occur at many different steps in RNA metabolism, including splicing, polyadenylation, mRNA stability, mRNA localization and translation (Lee and Schedl, 2006; Glisovic et al., 2008).

In plants, interactions between proteins and RNAs underlie every aspect of plant development and function. Accordingly, plant genomes encode >700 proteins that harbor predicted RNA binding motifs. There are more than 200 putative RNA-binding proteins in *Arabidopsis* genome (Lorkovic and Barta, 2002) but few of these proteins have been identified through the biochemical experiments or the analysis of mutants. To dissect RNA-protein interaction networks in plants, it is necessary to catalogue plant RNA-binding proteins, to identify the RNAs with which they interact, and to determine how those interactions influence the fate of the RNA and downstream processes. The project of PlantRBP develops tools to address each of these issues, and applies them to a set of 40 plant-specific RNA binding proteins that function in the chloroplast. The 40-protein set emphasizes the CRM and PPR protein families, which are largely specific to plants and which together contain ~500 members, most of which are predicted to be targeted to chloroplasts or mitochondria. POGs/PlantRBP (http://plantrbp.uoregon.edu/) is a relational database that integrates data from rice, Arabidopsis, and maize by placing the complete Arabidopsis and rice proteomes and available maize sequences into ‘putative orthologous groups’ (POGs) (Walker et al., 2007).

Among the RNA binding motifs, the RRMs and KH motifs are most frequently found in Arabidopsis proteins; the RRM can modulate its fold to recognize many RNAs and proteins in order to achieve a multitude of biological functions often associated with post-transcriptional gene function. The two plant-specific RRM-containing proteins of known function, FCA and FPA, have previously been characterized as flowering-time

regulator, were recently found to be required for chromatin modification of the *FLOWERING LOCUS C* (*FLC*) (Bäurle et al., 2007; Bäurle and Dean, 2008). A function has been established for only two KH-domain-RNA binding proteins, *HUA ENHANCER 4* (HEN4) and *FLOWERING LOCUS K* (*FLK)*, with FLK promoting flowering (Balachandran et al., 1995). The abundance of Arabidopsis genes encoding RNA-binding proteins, and the recent identification of developmental and hormone response mutations in Arabidopsis genes that encode RNA-binding proteins, indicate that these will emerge as important players in plant morphogenesis and cellular regulation. It has been shown that *LEAFY HEAD2* encodes a putative RNA-binding protein that could be involved in the regulation of rice shoot development through KNOX (a homeobox gene) and hormone related genes (Xiong et al., 2006). Another Arabidopsis FCA RNA-binding protein, which is homologous to the barley ABA-P1 protein, is found to be an ABA receptor involved in RNA metabolism and in the control of flower time autonomous pathway (Razem et al., 2006).

In fact, plant development requires plant-specific protein functions, as illustrated by the existence of a large number of plant specific transcription factors (Guo et al., 2008). According to Lorkovic Z.J. (2009), many Arabidopsis RBPs are involved in the regulation of plant-specific processes (such as flowering) or are involved in plant responses to changing environmental conditions (stress or hormone induction). In solanaceous plants, the RBPs have analogous functions in floral induction, genome modification or adaptation to various stress conditions, but the relative reports are very limited.

1.4 Light dependent shade avoidance responses in plants

Normally, plants respond to the proximity of neighboring vegetation by elongation to prevent shading (Devlin et al., 2003). Red-depleted light reflected from neighboring vegetation triggers a shade avoidance response leading to a dramatic change in plant architecture. Since transgenic potato plants with reduced expression of *StSUT4* do not response to shading, Chincinska *et al*., (2008) have argued that *StSUT4* accumulation is increased under FR light enrichment (decreased red: far red ratio) in wild-type *Désirée*. This indicates that SUT4 is somehow related to the phytochrome pathway. It is well reported that under persistent FR-rich light conditions, plants undergo a series of morphological changes that include reduced branching, increased plant height, and decreased leaf blade area (Smith, 1992, 1995). These morphological changes are accompanied by changes in physiology including a redistribution of auxin, enhanced ethylene production, modulation of gibberellin action and an acceleration of flowering (Smith and Whitelam, 1997; Morelli and Ruberti, 2000; Vandenbussche et al., 2003; Pierik et al., 2004). Collectively, these traits have been termed the shade avoidance response or the shade avoidance syndrome (SAS) (Smith and Whitelam, 1997;

Ballare, 1999). Shade-avoiding plants have a particular sensitivity to spectral quality of light in red and far red regions of the spectrum. These changes in light quality are detected by the phytochrome family of photoreceptors. Phytochromes are responsible for the SAS of higher plants.

Phytochrome B plays a major role in R: FR ratio perception. The emerging research underlying phytochrome B signal transduction in relation to its role in seedling de-etiolation applies an understanding of the molecular mechanisms (Franklin and Whitelam, 2005).

It is known that potato wild Andean varieties (*Solanum tuberosum* ssp. *andigena*) are adapted to the short-day condition (day length close to 12 h), and are able to tuberize or do so very poor when grown under long-day condition. Another modern cultivated potato varieties derived from Chilean landraces (*Solanum tuberosum* ssp. *tuberosum*) are more adapted to the longer summer days of the temperate regions of Europe and North America. *Désirée* potato belongs to *Solanum tuberosum* ssp. *tuberosum* variety. *StSUT4-RNAi* potato plants are available in both *andigena* and *tuberosum* variety. They show early flowering phenotype. However, *StSUT4-RNAi tuberosum* plants show higher tuber production while *StSUT4-RNAi andigena* potato could produce tubers under non-inductive LD condition (Chincinska et al., 2008). Photoperiod flowering regulation in angiosperms has been investigated in a number of plants revealing the conservation of molecular mechanisms in both dicots and monocots (Yanovsky and Kay, 2003; Greenup et al., 2009). In the photoperiodic pathway, the day length is detected by photoreceptors, and along with entrainment factors, it synchronizes an endogenous circadian clock with the environment. *CONSTANS (CO)* plays a central role by mediating the circadian clock and the floral integrators by positive regulating of *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1)* which promote the expression of genes specifying flowering meristem identity (Suárez-López et al., 2001; Searle and Coupland, 2004).

In tomato plants, *SlSUT1* is mainly expressed in sucrose exporting source leaves and *SlSUT2* has a higher accumulation in sink leaves by Northern blots (Barker et al., 2000). qRT-PCR analysis in mature tomato flowers has showed that *SlSUT2* has a higher expression in anthers compared with petal, sepal or ovaries; while *SlSUT4* is mainly expressed in ovaries (Hackel et al., 2006). *StSUT4-RNAi* potato plants flower and tuberize earlier and do not show shade avoidance responses under shade conditions, suggesting that mediation of the photoreceptor signal transduction correlates with sufficient SUT4 levels (Chincinska et al., 2008). *StSUT4* might act downstream of photoreceptors detecting the light quality in source leaves, and upstream of ethylene and GAs. It is known that photoreceptors, sugar and

phytochromes such as ethylene and GAs are involved in shade avoidance (Pierik et al., 2004; Kozuka et al., 2005). Further analysis about interconnection between these two signaling pathways is still to be done.

1.5 Methods to investigate protein-protein or RNA-protein interactions

1.5.1 The split-ubiquitin membrane yeast two-hybrid assay identify protein interaction partner in a unbiased manner

In order to understand the protein functions, it is useful to know what kind of protein(s) it can bind with. As we know, the yeast two-hybrid system allowed rapid progress in this field, and oligomers of soluble protein have been studied systemically (Fields and Song, 1989), but it is not suitable for the study of membrane proteins interaction. The yeast split-ubiquitin system (SUS) has been developed to detect and report interactions involved in integral membrane proteins by the simple use of a transcriptional reporter (Johnsson and Varshavsky, 1994). The method is based on the ability of the N-terminal “Nub” and C-terminal “Cub” ubiquitin halves to reconstitute a functional protein. NubG is a mutant with severely decreased affinity for Cub; functional ubiquitin can only be reconstituted when NubG and Cub are in close vicinity by fusion with two interaction proteins respectively. Functional ubiquitin is then recognized by ubiquitin-specific proteases (USPs). The selectable SUS, which has used an artificial transcription factor (protein A-LexA-VP 16) to activate the lexA-driven reporter genes HIS3 and lacZ in the nucleus, is successful to detect pairs of interactions among membrane proteins from different organisms such as yeast, plants and animals (Stagljar et al., 1998; Reinders et al., 2002; Thaminy et al., 2003).

Immunolocalization analysis has demonstrated that all three known SUT proteins are localized in the same enucleate sieve element of potato leaves, petioles and stem. The potential of Suc transporters to form homooligomers has already been tested by this yeast-based split-ubiquitin system (Reinders et al., 2002). The results have showed that both SUT1 and SUT2 have the potential to form homooligomers, and all three Suc transporters have potential capability to interact with each other. With the involvement of the group of E. Boles/Frankfurt, this system has been extended on a large-scale basis (Obrdlik et al., 2004). Two-phase partitioning of plant microsomal membranes separating the plasma membrane and endosomal membrane fractions have revealed preferential localization of StSUT4 in the plasma membrane fraction as shown by the use of the StSUT4-specific peptide antibodies (Chincinska et al., 2008). It is worthwhile to use the split-ubiquitin membrane yeast two-hybrid system and screen for SUT protein-protein interaction partners, thereby applying further information about sucrose transporters physiological function in *Solanaceous* plants.

By using the yeast split-ubiquitin system, Jana Reins has identified several putative StSUT1 and StSUT4 interaction partners from potato cDNA libraries which might have potential roles in the regulation of the protein localization, stability, etc. (Diploma thesis, HU, 2006); Undine Krügel has already identified putative SlSUT2 interaction partners from tomato cDNA libraries (PhD dissertation, HU, 2008). Since yeast two-hybrid screens (including split ubiquitin screen) are well known to produce false-positive results, subsequent verification of individual interaction partners by further approaches preferentially in planta is desired, i.e. using co-immunoprecipitation ("pull-down", (Master, 2004)), in vitro association studies (such as gel overlay assay or "far western blots", (Hall, 2004)), surface Plasmon resonance spectroscopy (Cooper, 2003), blue native gel electrophoresis (Eubel et al., 2005), bioluminescence resonance energy transfer (BRET) (Pfleger and Eidne, 2006) and fluorescent protein-based methods (such as FRET, Fluorescence Resonance Energy Transfer, Bhat et al., (2006)).

1.5.2 BiFC system serves as an efficient technique for identification of protein-protein interaction partners

The bimolecular fluorescence complementation (BiFC, also known as "split YFP) assay is based on the observation that N- and C-terminal sub-fragments of GFP (or derivatives, e.g. YFP) do not spontaneously reconstitute a functional fluorophore. However, the two non-functional halves of the fluorophore are brought into tight contact, refold together and generate *de novo* fluorescence if fused to interacting proteins. BiFC serves as a sensor of protein-protein interactions in living cells; it has been extensively used to study protein-protein interactions in a diverse range of organisms and cell types, including yeast, animal and plant cells (Immink et al., 2002; Bhat et al., 2004; Tonaco et al., 2006).

Successful examples of BiFC in plants have been described in three publications of 2004 (Bracha-Drori et al., 2004; Tzfira et al., 2004; Walter et al., 2004). These three articles demonstrate that BiFC can be used to detect expression of different proteins in different subcellular compartments following transient expression in onion epidermis or tobacco (*Nicotiana tabacum)* leaves (Tzfira et al., 2004), infiltration of *Agrobacterium* cells into tobacco (*Nicotiana benthamiana*) or Arabidopsis leaves (Bracha-Drori et al., 2004; Walter et al., 2004). Subsequently, several additional BiFC systems in plants are published (Citovsky et al., 2006; Uhrig et al., 2007), relative information has been reviewed by Ohad et al.(2007). Kim et al., (2008) are able to confirm the interactions between FBL17 (F-box like17, binds to N-terminal of YFP) and the cyclin-dependent kinase inhibitors (KRP6 and KRP7, binds to the C- terminal end of YFP) by BiFC assays in transfected protoplasts. This is one of the alternative methods to confirm the protein interaction partners identified in yeast two-hybrid

assays. At the same time, they also present the co-immunoprecipitation assay between FBL17 and KRP6 or KRP7.

1.5.3 Co-immunoprecipitation and tandem affinity purification techniques

Co-immunoprecipitation (Co-IP) is considered to be a good standard assay for protein-protein interactions especially when it is performed with endogenous (not over-expressed or tagged) proteins. It is conducted to provide independent verification of the yeast two-hybrid screens. These assays can be performed using *in vitro* synthesized versions of bait and prey proteins. The proteins are mixed together and immunoprecipitated using an antibody that is specific to either the bait or prey construct. It is evident that two protein interact with each other if they co-precipitate (Taiz and Zaiger, 2006). Interactions detected by this approach are considered to be true. However, this method can only verify the interactions between suspected interaction partners. Co-IPs coupled with MALDI-TOF MS (Matrix assisted laser desorption ionisation time-of-flight mass spectrometry) are able to identify putative protein interaction candidates. Phee et al., (2006) have found 16 protein candidates which might interact with native holophytochrome either directly or indirectly after Co-IP. Further MALDI-TOF MS analysis has showed that 7 of them are predicted to be putative phytochrome-A interaction proteins and the remaining ones are phytochrome-B interaction partners (Phee et al., 2006).

Tandem affinity purification (TAP) is another technique for studying protein-protein interactions. It involves creating a fusion protein with a designed piece, the TAP tag on the end. This method can be real determination of protein interaction partners quantitatively *in vivo* without prior knowledge of complex composition. TAP offers an efficient and a highly specific mean to purify target protein. It is suitable to detect permanent interactions and allows various degree of investigation by controlling the number of times the protein complex is purified. Leene et al., (2007) have developed a high throughput tandem affinity purification/mass spectrometry platform for cell suspension cultures to analyze the cell-cycle related protein complexes in *Arabidopsis thaliana*.

1.5.4 Yeast three-hybrid assay to identify RNA binding proteins

Yeast Three-Hybrid System is a specific system to detect and analyze interactions between RNA and protein *in vivo*. It is similar to the two-hybrid screen that depends on specific protein-protein interactions (Gietz et al., 1997). The big advantage of the yeast three-hybrid system is to identify RNA-protein interactions in a large array of basic cellular processes. Examples of such processes include: translation, mRNA processing, chromosome replication and crucial decisions during early development (Zhang et al., 1999). Moreover, all RNA viruses regulate their infectivity and

replication via protein-RNA interactions. Clearly, the yeast three-hybrid system is important in understanding the disease process related to RNA viruses from both a basic science and clinical perspective. The method provides a system for studying RNA-protein interactions with the genetic advantages of the two-hybrid system. It may be used to detect specific RNA-binding proteins or target RNAs from a library of cDNAs, or to analyze the structural specificity of identified RNA-protein interactions (Donnini et al., 2004; Piganeau and Schroeder, 2006).

In terms of method, a hybrid RNA binds to each of two hybrid proteins. One part of the hybrid RNA acts in a known interaction, whereas the other part can be used to screen for orphan RNA-binding proteins. The DNA binding and transcription activation domains of the yeast transcriptional activator GAL4 are brought together via the interaction of recombinant fusion proteins with a recombinant RNA. Once this complex is formed, this activates the transcription of reporter gene. When the reporter gene is turned on, its expression can be identified by phenotype or by simple biochemical assay. This technique can be employed if RNA and protein are each known, or can also be used to identify a "partner" when only one component is known (i.e., only the RNA or only the protein is known).

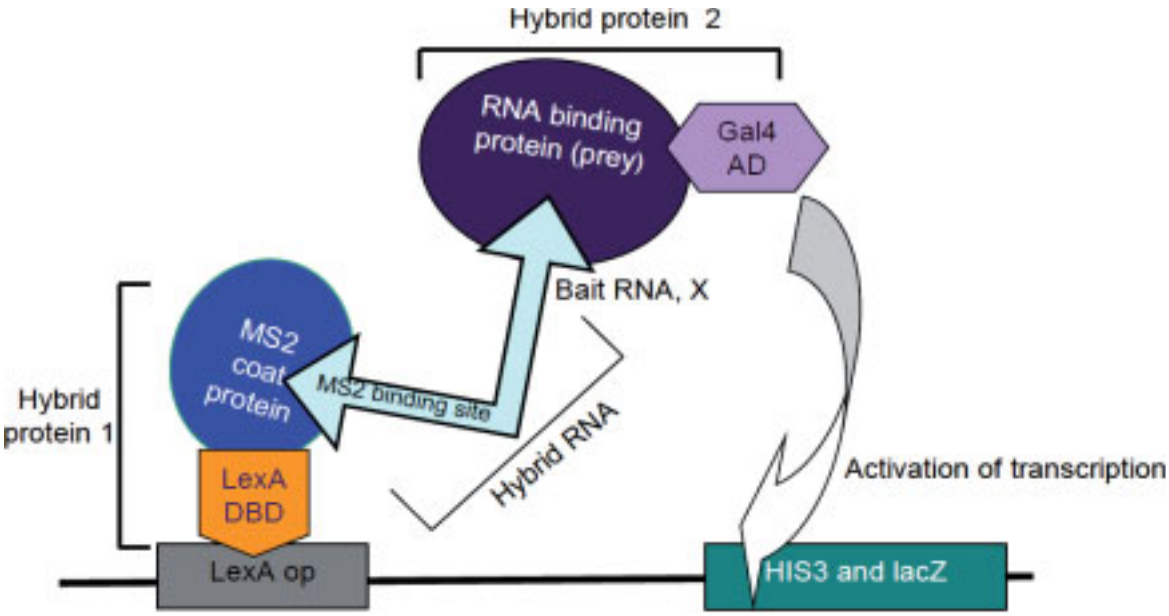


Fig. 3: Specific components of the yeast three-hybrid system to detect and analyze RNA-protein interactions

The system is modified according to the manual of RNA-Protein Hybrid Hunter^{TM} Kit (Invitrogen, version A and D). Commonly used protein and RNA components are depicted. Both LacZ and His3 (green box) are present in strains L40uraMS2 and placed under the control of LexA operators (LexA op, grey box). In addition, it expresses a hybrid protein in which the bacterial LexA DNA-binding domain (LexA DBD, orange) is fused to the coat protein of bacteriophage MS2 (MS2 coat protein, blue circle). The hybrid RNA (light green arrow) contains two MS2-binding sites, one site interacts with MS2 coat protein of the hybrid protein 1, and the other site interacts with the RNA binding site (dark purple, prey cDNA) of the hybrid protein 2. The second hybrid protein is a fusion between the GAL4 or VP16

transcription activation domain (Gal4 AD, light purple) and the RNA binding protein of interest (or "prey"). Only if these three components constitute one unity, the reporter gene is activated.

The constituents of the three-hybrid system typically include: A DNA-binding site that is placed upstream of a reporter gene in the yeast chromosome (e.g. L40uraMS2) (Fig. 3)-usually a 17 nucleotide recognition site for the *E. coli* LexA protein. Hybrid Protein 1 is composed of a LexA DNA-binding domain that is fused to an RNA-binding domain (the coat protein of bacteriophage MS2). The RNA-binding domain interacts with its RNA-binding site in a hybrid RNA molecule (Aside: hybrid denotes a "bifunctional" molecule). The other part of the RNA molecule interacts with a second hybrid protein (Hybrid Protein 2), which consists of another RNA-binding domain. Hybrid protein 2 consists of the transcription activation domain of the yeast Gal4 transcription factor that is linked to an RNA-binding protein. However, this interaction is linked to a transcription activation domain. In essence, the Hybrid RNA is "sandwiched" between two hybrid proteins, which consists of two MS2 coat-protein binding sites linked to the RNA sequence of interest, X. Only when all three components form a complex the reporter gene will be turned on. The product produced indicates an RNA-protein interaction occurred, even transiently.

1.6 The goals of the study

The aim of the project is to analyze the regulation of stability and mobility of plant sucrose transporter mRNAs or proteins. The following aspects are the goals of this work:

1. To clarify whether the mRNA and/or the protein of the sucrose transporter SUT1 shows phloem mobility.
2. To determine the half life of different sucrose transporter transcripts (SUT1, SUT2 and SUT4) regulated at the transcriptional or post-transcriptional level.
3. To investigate more aspects of the physiological function of members of the SUT4 subfamily with the help of transgenic plants over-expressing SUT4 or with inhibited SUT4 expression (combination with former studies in the group).
4. To confirm the SUT protein-protein interaction partners by alternative methods, such as split YFP system.
5. To analyze the StSUT2 and StSUT4 function in potato plants with the help of double transformants with inhibited StSUT2- and StSUT4-expression.
6. To identify StSUT4 RNA-binding protein(s) by yeast three-hybrid system and deduce the regulation mechanism of SUT4 mRNA stability or mobility during cell-to-cell or long distance transport in potato plants.

2 Materials and methods

2.1 Materials

2.1.1 Chemicals and enzymes

2.1.1.1 Chemicals

If not indicated otherwise, chemicals used for experiments described in this thesis were obtained from Sigma (Sigma Aldrich GmbH), Roche (Karlsrule), Applichem GmbH (Darmstadt), GE Healthcare (Freiburg), Fluka-Biochemical (Steinheim), Bioline (Luckenwalde) and radiochemicals Amersham Buchler GmbH & Co KG, J.T. Baker Chemicals, BioRad, Difco Laboratories, Merck AG, Serva Feinbiochemica GmbH & Co,. Parafilm M - American National Can. Hybond N, Amersham Pharmacia Biotech. Whatman 3MM paper, Whatman. Pipette tips and petri dishes, Greiner GmbH. Sterile filtration units – Millipore. Gel extraction kit and plasmid isolation Midi kit, Qiagen. Trisure is used for total RNA extraction from plant tissue, Bioline.

2.1.1.2 Enzymes and antibodies

Enzymes

Restriction enzymes: MBI Fementas (St. Leon-Rot) and New England Biolabs (Schwalbach).

T7 RNA polymerase, RNase Inhibitor, NTP, RNase free DNase I from Fementas

T4 DNA ligase: New England Biolab, Schwalbach

Taq-DNA-polymerase: New England Biolab, Schwalbach

Phusion High Fidelity DNA-polymerase, Finnzymes, Espoo, Finnland; New England Biolabs, Schwalbach

Lysozyme: MBI Fermentas, St. Leon-Rot

Lyticase: Sigma-Aldrich GmbH, Steinheim

BP-Clonase and LR-Clonase Enzyme Mix, Invitrogen, Karlsruhe

First Strand cDNA Synthesis Kit -Bioline

Omniscript Reverse transcriptase, Qiagen, Hilden

Antibodies

SlSUT4 antibody, polyclonal antisera were raised in rabbits by using synthetic peptides corresponding to the sequence within the SlSUT4 central loop (Weise et al., 2000).

C-myc antibody, sigma, recognizes both N- terminal and C-terminal c-Myc tagged proteins.

Goat anti-rabbit IgG-HRP, Sigma, supplied at 200 µg in 0.5 ml volume, horseradish peroxidase conjugated secondary antibody, recommended for use in Western blotting at a dilution of 1:5000 - 1:10000. The starting dilution is 1:5000.

Goat anti-mouse IgG-HRP, sigma, supplied at 200 µg in 0.5 ml volume horseradish peroxidase conjugated secondary antibody recommended for use in Western blotting at a dilution of 1:500 - 1:10000. The starting dilution is 1:2000.

2.1.2 Bacterial Strains

Escherichia coli DH5α, most plasmids used were amplified in this strain

Escherichia coli DB3.0, used for plasmids from Invitrogen with ccd+ amplification

Escherichia coli Rosseta DE3.0, for over expression of recombinant proteins

Agrobacterium tumefaciens strain C58C1, pGV2260, for plant transformations Gene transfer into plants, tobacco and potato (Deblaere et al., 1985)

2.1.3 Yeast strain

Saccharomyces cerevisiae strain L40uraMS2 (Mat a, ura3-52, leu2-3112, his3Δ200, trp1 Δ 1, ade2, LYS2::(LexA op)$_4$-HIS3, ura3::(LexA-op)$_8$-LacZ) carrying pLexA/MS2/Zeo (Zeocin^R) (Invitrogen, RNA-protein hybrid Hunter system) (Matter et al., 2000). This strain is derived from L40 coat described previously (SenGupta et al., 1996).

2.1.4 cDNA library

*Solanum tuberosum m*ixed tissues (sink and source leaf, petiole, stem, flower, root, sink and source tuber), average insert size is 1.3 kb, range from 0.5 to 3.0 kb; complexity: 6× 10^5 primary clones in pENTR^{TM} 1A vector.

2.1.5 Plant materials

Cuscuta reflexa was kindly provided by Kirsten Krause, University of Kiel.

Nicotiana tabacum cv. Samsun NN, *SNN*.

Transgenic tobacco, over-expression of SlSUT1 fusion with GUS report gene (Weise et al., 2000)

Solanum tuberosum ssp. *tuberosum* var. *Désirée*

Solanum tuberosum ssp. *andigena*

Transgenic potato ssp. *tuberosum* var. *Désirée*, over-expression of SoSUT1 fusion with c-myc tag (Leggewie et al., 2003)

StSUT4-RNAi potato ssp. *tuberosum* var. *Désirée* (Chincinska et al., 2008)

Potato *phytochrome B* antisense ssp. *andigena*, anti-PHYB andigena (Jackson et al., 1996)

2.1.6 Vectors

pZero 2 (Invitrogen), pUC ori, Kanamycin resistance (Km^r), Plac (LacZ alpha-ccdB); size 3297bp.

pRH3' (Invitrogen, RNA-protein Hybrid Hunter™ kit version D), parental RNA hybrid cloning vector, Amp^r, pUC ori, RPR1 promoter, RNase P leader sequence, MS2 RNA, URA3 gene, 7907bp.

pRH3'-IRE (Invitrogen, RNA-protein Hybrid Hunter™ kit version D), positive control RNA bait hybrid plasmid, Amp^r, pUC ori, RPR1 promoter, RNase P leader sequence, MS2 RNA, URA3 gene, IRE insertion (iron response element), size 7920bp.

pYESTrp3 (Invitrogen, RNA-protein Hybrid Hunter™ kit version D), parental prey cloning vector, consists of GAL1 promoter, SV40 NLS, VP16 activation domain, CYC1 transcription termination region, pUC origin, bla promoter, Amp^R, TRP1 promoter, TRP1 gene, 5809bp.

pYESTrp3-IRP (Invitrogen, RNA-protein Hybrid Hunter™ kit version D), GAL1 promoter, SV40 NLS, VP16 activation domain, IRP ORF, CYC1 transcription termination region, pUC origin, bla promoter, Amp^R, TRP1 promoter, TRP1 gene, size 8469bp.

pENTR™ 1A (dual selection vector, Invitrogen), pUC ori, Cm^R, Km^R, attP1-ccdB-attP2, size 2700bp.

pGAD10-GW (A, B and C) (Bürkle et al., 2005), parental prey cDNA cloning vector, 8359bp, selection marker: Leu2, functional domain: GAL4 AD, promoter: ADH1 (truncated).

pDONR221 (Invitrogen), is in size of 4762 bp, pUC ori, attP1::ccd gene::attP2, Km^R.

pN-TAP-GW, binary vector for *Agrobacterium* transformation, pN-TAPa (=pYL TAP Nt) (Rubio et al., 2005): 2x35S::TMV; 7799-8647, 2xIgGBD; 8652-9029, 3C cleavage site; 9054-9077, 6xHis; 9084-9101, 9xMYC; 9126-9467, attR1::Cmr::ccdB::attR2; 9501-11375.

pJH212, binary vector for Agrobacterium transformation, is in the length of 9.5kb, pPZP212 derivative, pTiT37 T-DNA border regions, pBR322 origin, and ColE1 and pVS1 plasmid origins for replication in *E. coli* and in *Agrobacterium*, respectively. Bacterial marker gene is spectinomycin resistance; plant marker gene is kanamycin resistance.

pCAMBIA3301 (Cambia, Australia), consists of bacterial kanamycin resistance, CaMV 35S promoter, plant Basta selection, Gus report gene, polyclone sites.

pSPYNE-35S and pSPYCE-35S, plant expression binary vector for agro-infiltration and BiFC assay

2.1.7 Oligonucleotide sequences used in this thesis

The oligonucleotide sequences used for PCR, qRT-PCR, and 3'RACE-PCR are shown in table 2:

Table 2: Oligonucleotide sequences used in the thesis

Primer name	Sequence (5'-3')	Functions
Sl4NTAP att-fw	AAAAAAGCAGGCTATACAAAATGCCGGA GATAGAAAGGCATAG	Forward and reverse primer for subcloning *SlSUT4* into pN-TAP-GW vector
Sl4NTAP att-rev	AGAAAGCTGGGTAACCCAGAGCGTGGAA GAGC	
35S promoter fw	ATCCTTCGCAAGACCCTTCC	Forward and reverse primer for PCR confirmation
SUT4 seq reverse	CTATGCCTTTCTATCTCCGCCAT	
c-myc intern fw	CGAGGAAGACTTGAACGG	Primers for sequence reaction
SlSUT4 intern rev	ACTCTCGGTTTCACCGG	
N-TAPa fw2	ATCCACTAGTGGATCCCCCG	PCR detection of transgenic plants and plasmid DNA
Nos rev	ATCTAGTAACATAGATGACACCGCGCG	
StSUT4 PmeI fw	GGTTTAAACCATGCCGGAGATAGAAAGG CATAG	Forward and reverse primer for subcloning StSUT4 into pRH3'
StSUT4 AatII rev	GAGACGTCTCATGCAAAGATCTTGGGTTT C	
NUP A	AAGCAGTGGTATCAACGCAGAGT	Nest Universal Primer for reverse transcription
StSUT4 3´UTR AatII	GAGAGACGTCACAAAAAGCTAATGCAAA TTGCG	Reverse primer for subcloning *StSUT4* 3'UTR into pRH3'
UB1 fw	CAC CAA GCC AAA GAA GAT CA	Forward and reverse primer of Ubiquitin as inter standard
UB1 rev	TCA GCA TTA GGG CAC TCC TT	
SlSUT1 fw	TTC CAT AGC TGC TGG TGT TC	Forward and reverse primer of *SlSUT1* half life determination
SlSUT1 rev	TAC CAG AAA TGG GTC CAC AA	
SlSUT2 fw	CCT ACA GCG TCC CTT TCT CT	Forward and reverse primer of *SlSUT2* half life determination
SlSUT2 rev	GGA TAC AAC CAT CTG AGG TAC AA	
SlSUT4 fw	TCT CCG CTG ATA TTG GAT GG	Forward and reverse primer of *SlSUT4* half life determination
SlSUT4 rev	GCA ACA TCG AGA AGC CAA AA	
StSUT1 RT-fw	TTCCATAGCTGCTGGTGTTC	Forward and reverse primer of *StSUT1* half life determination
StSUT1 RT-rev	TACCAGAAATGGGTCCACAA	

StSUT2 RT-fw	GCAATGCATTCGGTTCTCAT	Forward and reverse primer of *StSUT2* half life determination
StSUT2 RT-rev	CGGGTCCCCATGATAGACTT	
RT-SUT4 fw	TCTCCGCTGATATTGGATGG	Forward and reverse primer of *SUT4* for quantitative RT-PCR
RT-SUT4 rev	GCAACTACGAGAAGCCAAAA	
StGI RT-fw	GCTTCCTCCACAAGATG	Forward and reverse primer of *StGI* for qRT-PCR
StGI RT-rev	TGGATACCGGTTCCGTATGA	
StFT fw	GTGGATCCTGATGCTCCAAG	Forward and reverse primer of *StFT* for qRT-PCR
StFT rev	TTCCTGTGGTTGCTGGGATA	
StCOL3 fw	CTTCAAACTCCCATCCACGA	Forward and reverse primer of *StCOL3* for qRT-PCR
StCOL3 rev	TTGGAGTAAGCTGGGGAGGT	
SoSUT1-RT-fw	CCC CCT GAA GCT AAA ATT GG	Forward and reverse primer of *SoSUT1-cmyc* fusion for semi-quantitative RT-PCR
c-myc-RT-rev	AGT TGA GGT CTT CTT CGG AGA TTA G	
TEF fw	TGG AAC TGT CCC TGT TGG TC	Forward and reverse primer of *TEF* for quantitative semi-quantitative and Real-time PCR
TEF rev	ACA TTG TCA CCA GGG AGT GC	
NtSUT1 RT-fw	TTG GGG CTG TTG AAC TC	Forward and reverse primer of *NtSUT1* for semi-quantitative RT-PCR
NtSUT1 RT-rev	GCA AAC AGC CAA CAC GAA AT	
GUS RT-fw	CAT GTC GCG CAA GAC TGT AA	Forward and reverse primer of *GUS* for semi-quantitative RT-PCR
GUS RT-rev	GTC CAG TTG CAA CCA CCT GT	
StJAR1 RT-fw	CAGCTGATCCAGGGCACTAC	Forward and reverse primer of *StJAR1* for quantitative RT-PCR
StJAR1 RT-rev	ACCGATGGTTTTGACCTTCC	
NPTII a fw	ACCGGATCTGGATCGTTTCG	Forward and reverse primer for checking Kanamycin resistance gene in transgenic plants
NPTII b rev	TTGGTCCCTCATTTCGAACC	
Bar fw AatII	ATGACGTCCCCAGAACGACGCCCGGCCG	Forward and reverse primer for checking double transformants, amplifying PAT gene
Bar rev BgIII	ACAGATCTTCAAATCTCGGTGACGGGCA G	
StSUT4-RNAi	GAGACTCGAGTGCAAGATCTTGGGTTTCT C	Primer for *StSUT4-RNAi* construct
intron-out-rev:	GATGATTTATGTATATAACAACG	Primer for *StSUT4-RNAi* construct
ADH Prom fw	CAGATGTCGTTGTTCCAGAGCT	Primers for sequence reaction
GAL4 AD	GCGCTTTCACAACCAATTGC	
M13 fw	ACGACGTTGTAAAACGACGGCCAG	
M13 rev	TTCACACAGGAAACAGCTATGAC	

2.2 Media and solutions

2.2.1 Media for bacteria

Media were diluted in one liter of deionized water. For solid media 15 g of agar was added.

LB medium 5 g yeast extract, 10 g tryptone, 10 g NaCl, pH=7.5

LB low salt medium (LB-LS) 5g yeast extract, 10 g tryptone, 5 g NaCl, pH=7.5

SOC medium 5 g yeast extract, 20 g tryptone, 20 mM glucose, 0.5 g NaCl, 2.5 mM $CaCl_2$, pH=7.5

YEP medium (1L)10 g yeast extract, 10 g peptone, 5 g NaCl, 10 g sucrose, pH=7.2
YEB medium (1L) 5 g beef extract, 1 g yeast extract, 5 g tryptone, 5 g sucrose, 2 mM MgSO4-7H2O, pH 7.2

When required, antibiotics were supplemented to the following final concentration: ampicillin 100 mg/l, carbenicillin 100 mg/l, chloramphenicol 25 mg/l (stock solution in 100% ethanol), gentamicin 25 mg/l, rifampicin 100 mg/l (in DMSO), and kanamycin 50 mg/l.

2.2.2 Media for yeast

YPD (YPAD): Yeast Extract Peptone Dextrose Medium (± Adenine) (1 liter): 1% yeast extract, 2% peptone, 2% dextrose (D-glucose), ± 0.1 g adenine. Optionally for making plates, add 17 g Agar-Y (Bio.101 system, Q*BIO gene). The medium was autoclaved for 15 minutes on liquid cycle. Add 100 ml of 20% dextrose (filter sterilized), store medium at room temperature.

SD medium: 1.7 g Yeast Nitrogen base, 5.0 g Ammonium sulphate, 20 g glucose, 0.01% (adenine, arginine, cysteine, leucine, lysine, threonine, tryptophan, uracil), 0.005% (aspartic acid, histidine, isoleucine, methionine, phenylalanine, praline, serine, tyrosine, valine), 17 g yeast agar (for plates), adjust the pH of the solution to 7.0 by adding NaOH pellet, autoclave at 15 pci, 121°C for 15 min.

L40uraMS2: YPAD+Zeocin50mg/L

L40uraMS2 transformed pRH3′, pRH3′/IRE, pRH3′/StSUT4, pRH3’/StSUT4 3’UTR (for the bait): SD-Ura (with Zeocin50 mg/L, optional)

L40uraMS2+pRH3′, L40-ura-MS2+pRH3′/IRE transformed pYESTrp3 and pYESTrp3/IRP: SD-Ura-Trp (with Zeocin 25mg/L, or not), then screening on SD-Ura-Trp-His (with Zeocin25mg/L, or not), LacZ screening in parallel.

L40uraMS2+pRH3′/StSUT4 transformed pGAD10-GW (empty vector): SD-Ura-Leu-His

L40uraMS2+pRH3'/StSUT4 or pRH3'/StSUT4 3UTR transformed pGAD10-cDNA library: SD-Ura-Leu-His+5 mM 3-AT (3-AT, 1 M stock in water, filter sterile, store at -20 ºC)

Solutions for yeast transformation:

10× TE: 100 mM Tris-HCl, pH 7.5; 10 mM EDTA, pH 7.5, and filter sterilized and stored at room temperature (RT). 1X TE, dilute 10× TE 10-folds with sterile water.

10× LiAc: 1 M Lithium Acetate, pH 7.5 (with dilute glacial acetic acid), filter sterilize and store at RT. 1× LiAc 100 mM Lithium Acetate, pH 7.5, dilute 10× LiAc solution 10-fold with sterile, deionized water.

1× LiAc/40% PEG-3350/1× TE: 100 mM Lithium Acetate, pH 7.5, 40% PEG-3350, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. The medium was autoclaved at 121°C, 15 psi for 20 minutes. It was stored at RT.

Z-buffer, pH 7.0: $Na_2HPO_4.7H_2O$ 16.1 g/l, $Na_2HPO_4.H_2O$ 5.5 g/l, KCl 750 mg/l, $MgSO_4.7H_2O$ 246 mg/l, filter-sterilized and store at room temperature.

X-Gal in Dimethylformamide (DMF): a) Prepare X-Gal solution fresh immediately before use; b) To make a 40 mg/ml stock solution, dissolve 40 mg in 1 ml DMF; c). Store at -20°C protected from exposure to light until use.

100 mM Potassium Phosphate, pH 7.0, filter-sterilized and store at room temperature.

2.2.3 Solutions for mini preparation of plasmid DNA

Solution I: 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH8.0), add fresh RNase A (100 μg/ml) before using.

Solution II: NaOH/SDS lysis solution (0.2 M NaOH, 1% SDS)

Solution III: 5 M potassium acetate solution (pH4.8)

SCE-buffer (100 ml): 2M Sorbitol 50 ml, 1M NaCitrate 10 ml, 0.5M EDTA (pH8.0) 2 ml, 38 ml ddH_2O

Lyticase-buffer for isolation of plasmid DNA from yeast cells (15 ml): 1*SCE-buffer 14.85 ml, 1 M DTT 150 μl, lyticase (Fluka, up to 400 U/ ml) 60 μl.

2.2.4 Buffers for plasma membrane fraction isolation

Grinding buffer (1 L)
500 mM sorbitol 91.09 g
50 mM HEPES 11.92 g, adjust pH 7.5 with KOH
5 mM EGTA 2.081 g

Resuspension buffer (10 ml)

1.67 ml sucrose (1.5 M stock solution); 0.25 ml K-phosphate buffer, pH 7.8 (0.2 M stock solution， monopotassiumphosphate 0.2313 g, dipotassiumphosphate 3.1875 g in 100 ml H2O); 15 µl 2 M KCl.

Phase stock solution

40% PEG3350 (200 g PEG+ 300 g H_2O), 20% Dextran T 500 (110 g Dextran+ 400 g H_2O), mix and place in cold room overnight; 0.5 M KCl; 1.5 M Sucrose; 0.2 M K-phosphate buffer, pH 7.0.

26-g Phase (1 x): 11.52 g Dextran, 5.76 g PEG (17.28 g); 0.36 ml KCl; 4.33 ml sucrose; 0.65 ml K-phosphate buffer; add water to 26 g.

36-g Phase (3 x): 11.52 g Dextran, 5.76 g PEG (17.28 g); 0.36 ml KCl; 6 ml sucrose; 0.9 ml K-phosphate buffer; add water to 36 g.

Diluents buffer (250 ml): 0.33 M sucrose; 5 mM Mops-BTP (1 M stock solution); 1 mM EDTA (0.1 M stock solution); 2 mM Dithiothreitol (fresh).

Conservation buffer

5 mM BTP (pH6.5 by dry MES) (250 mM); 20% glycerol (w/v); 250 mM sorbitol; 1 mM DTT

2.2.5 Solutions for Western Blot

6× Laemmli buffer: 1.2 g SDS, 6 mg bromophenol blue, 4.7 ml glycerol, 1.2 ml Tris 0.5 M pH6.8, 2.1 ml dd water, warm it a little bit and shake it till everything is dissolved. It takes time. 0.93 g DTT is added. Wait until it is completely dissolved. Aliquots were kept frozen at -20ºC.

10× running buffer: 30.3 g Tris base, 144.0 g Glycin, 10.0 g SDS. Dissolve in deionized water and fill to 1000 ml. Store at 4°C. Warm the buffer before use to remove possible precipitates. Do not need to adjust pH.

Transfer buffer (semi-dry): 25 mM Tris-HCl, 190 mM Glycin, 20% Methanol, pH8.3.

Blocking buffer: 5% non-fat milk resolved in TBS-TWEEN 20

TBS-TWEEN 20: 25 mM Tris-HCl, 137 mM NaCl, 0.05% TWEEN-20

Home-made ECL solution: Solution A, 100 ml 0.1 M Tris/HCl (pH8.6) + 25 mg luminal (3-aminophthalydrazide, Sigma A 8511); solution B, 11 mg para-hydroxy Coumarin acid (Sigma C9008) in 10 ml DMSO; solution C, 35 % v/v H_2O_2, Mix A:B:C (10:1:0.003) and incubate the blots.

2.2.6 RNase A stock solution

Mix RNase A with 1X TE to yield a 20,000 µg/ml RNase A solution. Boil the solution in a closed container for 10 min to destroy any contaminating DNases. Mix with an equal volume of glycerol to produce a 10,000 µg/ml stock solution. 1 ml aliquots can be stored at -20°C and vortex before using.

2.3 Basic molecular biological methods

2.3.1 Standard methods

Electro-transformation of *E.coli* and Agrobacterium was performed on an ECM 600/630 electroporator (VWR, Darmstadt, Germany) using the manufacturers protocol. Standard molecular biological techniques such as PCR amplification, agarose gel separation, restriction enzyme digestion, DNA or RNA and protein electrophoresis and DNA purification were performed as described by Sambrook and Russell (2001).

2.3.2 Transformation of potato

Potato plants were transformed by Agrobacterium-mediated gene transfer using Agrobacterium tumefaciens strain GV2260. The protocol was modified by Izabela Chincinska according to the reference (Rocha-Sosa et al., 1989). The transgenic plants were regenerated on kanamycin and Basta containing media and multiplied in vitro. After rooting the plants were transferred to soil and further grown in the greenhouse.

Tobacco transformation was performed according to the above protocol with small modifications. Regenerated potatoes were screened by PCR for integration of the construct using Basta, *NPTII* and *StSUT4* primers (*StSUT4-RNAi* and intron-out-rev). Tobacco seeds with pN-TAP *SlSUT4* construct were selected on 2MS+25 mg/l Gentamicin. Plants containing the integrated DNA were amplified in tissue culture and placed in the greenhouse for further analysis.

2.3.3 Plant growth conditions and tissue culture

Potato and tobacco plants in sterile culture were grown on 2MS-medium (Murashige and Skoog, 1962) with 2% sucrose in tissue culture chambers at 24°C, at 50% humidity and 200 µmol photons m-2 sec-1 with a light/dark cycle of 16 h/8 h. Following transformation, leaf discs were put on 2MS with 1 g/l naphtyl acetic acid and 0.1 g/l benzyl amino purine. For the selection of transformant tissue, 3MS with 2 mg/l zeatin and 35 mg/l kanamycin was used. The root induction of plantlets was performed on 2MS with 2 g/l indol butyric acid and 50 mg/l kanamycin. After 2 weeks, plantlets were placed on 2MS containing 50 mg/l kanamycin.

2.3.4 Greenhouse and artificial shade condition

Greenhouse condition: Transgenic plants were regenerated in tissue culture, after rooting the plants were transferred to soil and grown in greenhouse in 60% humidity. The mean photosynthetic photon flux density (PPFD; 400 – 700 nm) was about 150 µmol photons m-2 sec-1 and additional illumination was provided by high-pressure sodium lamps SON-T Green Power and metal halide lamps MASTER LPI-T Plus (Philips Belgium, Brussels). Most of test plants were grown under long day (LD) condition (16h/8h, light/dark cycle, from 6:00 to 22:00 light period, temperature 22°C -25°C). For short day (SD) growth condition (10h/14h, light/dark cycle, from 8:00 to 18:00 light period, temperature 22°C -25°C), tested plants were planted in special phytochamber with artificial growth condition.

Artificial shade condition: plants were grown for 3 weeks in a phytochamber (Heraeus) at 24°C under long day condition with a white-light source (Osram L36W-31) and an additional far-red light source (Chopper light type 730 supplied with a Hama 730-nm filter; Chopper light GmbH). Control plants were exposed to white light alone. PPFD was 200 µmol photons m^{-2} s^{-1} as determined with a (Li-COR). One experiment was performed with *in vitro* leaflet treated with water, transcriptional inhibitor or translational inhibitor. The other experiment was performed with the wild-type and *StSUT4-RNAi*, anti-PHYB potato plants.

Grafting experiments: the plants had 5-6 leaves when they were used for grafting. The experiment was performed as described by Martinez-Garcia et al. (2001). Grafted plants were kept under 100% air humidity condition for 3-4 days until the junction was formed. Humidity was decreased gradually over a period of 3 to 4 days. The capillary tubes were not removed during the further growth of the grafted plants.

2.3.5 Genomic DNA isolation

The genomic DNA isolation was carried out using CTAB method. The CTAB buffer (for 100 ml: 10 ml of 1M Tris, pH 8.0; 28 ml of 5 M NaCl; 4 ml of 0.5 M EDTA, pH 8.0); 2 g of CTAB (Cetyltrimethyl ammonium bromide); 4 g polyvinylpyrrolidone (PVP); 500 µl of β-mercaptoethanol) was prepared 1-2 days before extraction and stored capped. About 50 mg leaf material was harvested into one Eppendorf tube, which was ground into powder using a metal Eppendorf pistil and liquid nitrogen. The tissue powder was next incubated with 500 µl CTAB buffer for 60 min at 55°C. After the samples were cooled to room temperature, 500 µl of chloroform was added and the samples were vortexed and centrifuged 10 min at 14000× g. The upper aqueous phases were transferred into a new reaction tube, 0.08 volumes of cold 7.5 M ammonium acetate and 0.6 volumes of cold isopropanol were added. The genomic

DNA was precipitated in -20°C for at least 15 min. After a short centrifugation (5 min, 14000×g) the pellets were washed twice with 80% ethanol, pour off the liquid and place samples in the Speed Vac for 20 min. DNA pellets were resuspended in 100 µl of TE buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA), 1 µl of DNA template was used for PCR amplification.

2.3.6 RNA isolation and reverse transcription

RNA was isolated from 100 mg of different tissues of greenhouse grown S. tuberosum ssp. *Désirée*, *andigena* and different transgenic lines, with Trisure (Bioline, Luckenwalde, Germany) according to the manufacturer's protocol. In short, the tissue was ground to a fine powder using mortar and pestle or a metal rotor cooled by liquid nitrogen. Then, 100 mg materials were incubated with 1 ml Trisure for 5 min at room temperature, 200 µl of chloroform was added and mixed well by vortexing, incubated for another 5 min at RT, cell debris was removed by centrifugation (12000×g at 4°C for 15 min). The aqueous phase was transferred to a new tube. A 0.6-fold of isopropyl alcohol and 0.1-fold of 3 M sodium acetate were added to the aqueous phase and incubated for 10 min at RT. The precipitated RNA was pelleted by centrifugation (12,000× g at 4°C for 15 min) and after removal of the supernatant the RNA was washed twice with 70% ethanol. After drying, the RNA was resuspended in 30 µl RNase-free water. Reverse transcription was performed with the Qiagen Omniscript RT kit according to the manual. Optimised conditions included using oligo (dT) primer for the reverse transcription reaction on approximately 1 µg of total RNA after digestion with RNase-free DNase (Qiagen, Hilden, Germany).

2.3.7 RNA Quantification by real-time RT-PCR

RNA was isolated from different tissues of greenhouse grown or phytochamber grown *Solanum lycopersicon* Moneymaker, *S. tuberosum Désirée*, *Cuscuta reflexa* or *N. tabacum* SNN, *S. tuberosum andigena*, StSUT4-RNAi *Désirée* and anti-PHYB *andigena* as described in 2.1.5 plant material. For grafted tobacco, samples were taken from stem sections with 1, 5, and 10 cm distance from the point of graft union in grafted tobacco plants. Reverse transcription was performed with the Qiagen Omniscript RT kit according to the manual. The cDNAs were diluted in 1:5, 1 µl diluted aliquot was used for the subsequent PCR reaction in the presence of SYBR Green with Hot Goldstar Polymerase (Eurogentec, Seraing, Belgium) in a Corbett RotorGene 6000 Cycler (Qiagen, Hilden, Germany) using the Rotor Gene Software Version 4.6.94. The best products were obtained with the following program: denaturation at 95^{o}C for 30 sec, annealing for 30 seconds at 61^{o}C and elongation for 30 sec at 72^{o}C, in a program of 40 cycles in 20 µl reaction volume. Relative quantification of *SlSUT1*, *StSUT1*, *SlSUT2*, *StSUT4*, *SoSUT1-cmyc*, *GUS* and *StJAR1*

transcript amounts was always calculated in relation to the respective *UBIQUITIN1 (UB1)* transcript level and given in % of ubiquitin for potato (*TEF* for tobacco, Cuscuta and grafted potatoes). Exon-specific primers were designed to obtain a 50-150 bp amplification products. Primer sequences were selected using Primer3 (http://frodo.wi.mit.edu/). In the case of StSUT4, the primers could avoid amplification of genomic DNA and be able to discriminate between sense and antisense transcripts (amplification product outside the region that was used in the antisense construct) (see oligonucleotide list, Table 4).

2.3.8 Northern blot

Total RNAs were isolated from 3 mM ethephon treated tobacco leaves using standard techniques. RNA samples were analyzed by electrophoresis on 2% formaldehyde containing 1.2% agarose gels and blotted onto nylon membrane. DNA probe of *NtSUT4* (91% homologous to *StSUT4*) was labeled with alpha-^{32}P-dCTP by random primer method (Decalabel kit, Fermentas, St.Leon-Rot, Germany) and purified from unincorporated nucleotides using probe purification column. The RNA blots were hybridized as described previously (Taylor and Green, 1995), using the indicated ^{32}P-labeled SUT4 probes. RNA blots were hybridized with a ^{32}P-labeled cDNA probe for the tobacco tubulin for standard control.

2.3.9 Protein extraction and detection

2.3.9.1 Protein purification from SlSUT4 tobacco

Tobacco tissues were homogenized in an extraction buffer containing 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Nonidet P-40, 1 mM DTT, 1 mM PMSF, and 1× complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Protein extracts were centrifuged 12,000×g twice at 4°C for 10 min each, and protein concentration in the supernatant determined by BCA Protein assay (Thermo Scientific, Whaltham, MA, USA). Protein samples (30 µg) were incubated at 37°C for 20 min in 5x sample buffer, run on 12.5% SDS-PAGE gel and blotted onto Nitrocellulose membrane. The blots were probed with different primary antibodies.

2.3.9.2 Microsomal fraction isolation

For microsomal fraction isolation, 100 mg plant material was homogenized in liquid nitrogen, added 600 µl protein extraction buffer containing 30% sucrose, 250 mM Tris-HCl (pH 8.5), 25 mM EDTA, 5 mM DTT, 1×complete protease inhibitor cocktail (Roche) and vortexed for 10 s. Following centrifugation at 7000 rpm for 10 min at 4°C supernatant was transferred to a new 1.5 ml Eppendorf tube, centrifuged at 15000

rpm 20 min at 4°C, pellet was resolved in 30 µl of Laemmli buffer, incubated 20 min at 37°C with shaking, and loaded on SDS-PAGE gel.

2.3.9.3 Plasma membrane fraction isolation

The method used was that developed by Kjellbom and Larsson (1984) for the isolation of plasma membranes from tobacco leaves, but with a modified composition of the two phases. 100 g plant material were harvested and transferred to cold room (4°C), 500 ml grinding buffer was added and grinded in precooled waring blender (10× 5 sec first low, then high speed); filtration through nylon cloth filter (pore 250 µm), 2.5 ml 0.5 mM Penylmethylsulfonyl fluorid (PMSF, highly toxic) was added and then centrifuge 8,500 g for 10 min (Beckman JA16.250 rotor) at 4°C (all centrifugations mentioned below are performed at 4°C); supernatant (take total protein aliquot) transferred to new tubes and centrifuge 50,000 g for 30 min (Beckman JA-30.50 rotor). The membrane pellet was resuspended in 10 ml resuspension buffer with a small brush; the fraction was microsomal fraction (MF). Loading 10 ml MF onto a 26 g two phase system, inverting 20 times and incubating on ice-water bath (2°C) for 2 min, centrifuge 5000 rpm for 5 min; transfer upper phase onto a same volume of 36 g of fresh low phase and reextract old lower phase by another volume of fresh upper phase, centrifuge again, the low phase of first tuber was endomembrane fraction (EF). Reextract 3 times until upper phase became quite clear; the upper phase (about 40 ml) diluted in around 200 ml of diluents buffer, adjusted balance carefully and centrifuged 100,000× g for 60 min in swinging rotor (Beckman, JS-24.38), pellet resuspended in 500 µl of conservation buffer and stored in liquid nitrogen or at -80°C (plasma membrane fraction, PF) .

2.3.9.4 BCA protein assay (Thermo Scientific)

Protein samples were resolved on ice, 30 µl of each protein (total protein, microsomal fraction, endosomal fraction and plasma membrane) was precipitated 10 min using 300 µl 10% TCA on ice, maximal speed centrifuge 10 min, pellet was resuspended in 100 µl 0.1N NaOH, take 50 µl for BCA protein assay.

The protocol of BCA protein assay was according to PIERCE´s protocol, for economical reason, the reaction volume was reduced to 50 µl sample and 1 ml of working reagent (Reagent A contains sodium carbonate, sodium bicarbonate, BCA detergent reagent, and sodium tartrate in 0.1N sodium hydroxide; reagent B contains 4% $CuSO_4.5H_2O$, mixed in 50:1). The samples were incubated at 37°C for 30 min, and cooled to room temperature, then measure OD_{562} on spectrophotometer and calculate the protein concentration according to BSA standard.

2.3.9.5 Western blot

The amount of protein that should be loaded to the gel varies with the experiments; it can be 20 to 100 µg per sample, 20 µg per sample for microsomal fraction and plasma membrane vesicles was taken in this study. The protein concentration was determined using BCA protein assay. Equal quantities of sample buffer were mixed and incubated for 10 min at 70°C. The samples were put in the gel and run until the blue marker to the end of the gel. Proteins were transferred to the nitrocellulose membrane by semi-dry transferring for 60 min at suitable charge condition (according to the size of nitrocellulose membrane, 0.8 mA per cm^2). The membrane was blocked with 1× TBS-T in 5% non-fat milk shaking (slowly) for 2 hours at room temperature or overnight at 4°C.

Primary Antibody: after blocking, without washing, the membrane was put in 5% non-fat milk 1x TBS-T with the anti-primary antibody in a rational dilution and incubated 1-2 hours in the shaker (depending on the sensitivity and specificity of antibody). After the primary antibody incubation wash the blot three times with 1x TBS and then wash 3 times (10 min with 1× TBS + 0.1% TWEEN-20).

Secondary Antibody: incubate for 2 hours in 1× TBS + 0.1% Tween-20 with the secondary antibody (anti-Rabbit or anti-mouse) concentration 1:10 000 (or according to the manufacturer's protocol). After the secondary incubation wash rapidly 1 time with 1× TBS + 0.1% Tween-20 and them wash 3 times (20 min with 1× TBS + 0.1% TWEEN-20).

Develop: before developing the blot, wash 3 times with TBS and scan in the Licor. If there still is some dirtiness and washes an additional 3 times with 1× TBS + 0.1% Tween-20. The detection was performed using the ECL-system: the membrane was put on the wrap film, covered with ECL solution (2 ml solution A, and 200 µl solution B, 0.6 µl H_2O_2) for one minute, then briefly on Whatman paper and sealed with wrap film, immediately developed on CCD camera.

2.3.10 BiFC in living tobacco cells

The first step was making BiFC constructs of the favorite genes using RT-PCR and GATEWAY recombinant techniques (details in results part, Chapter 3.6)

Next step was co-transforming the plasmid set (pSPYNE and pSPYCE) using transient expression assay. This assay was performed by a procedure called agro-infiltration that made use of the bacterial plant pathogen *Agrobacterium tumeficiens.* The *Agrobacterium* stains (GV2260 or EHA105, depending on the antibiotic of

expression vectors) were employed for co-infiltration containing plasmids carrying the sequences in the study. The technique consists in the infiltration of the abaxial surface of a leaf with an *Agrobacterium* suspension using a needle-less syringe. One well suited plant model, *Nicotiana benthamiana,* which has easily infiltrated leaves and produce high levels of protein, is used in the study.

The last step is viewing the image of the living plant cells with YFP-derived fluorescence using fluorescence microscope. After 3-4 days infiltration, the leave discs were collected and observed under Leica TCS-SP2 CLSM.

2.4 Yeast related experiments

2.4.1 Prediction of StSUT4 RNA structures

When designing the strategy to clone our RNA of interest in pRH5′ or pRH3′, consider the following factors. RNAs that interact with RNA-binding proteins generally: 1) Are relatively short in length (Harrington et al., 1997) ; for information about an appropriate size range for your RNA sequence); 2) Form a hairpin loop structure that serves as the recognition site for the RNA binding protein. Since we did not know which part of *StSUT4* RNA interacts with RNA binding protein, the full open reading frame of *StSUT4* was chosen as bait RNA. The *StSUT4* with 3′ untranslated region (3'UTR) was subcloned using 3'RACE RT-PCR, transferred into pRH3′ as bait RNA. To optimize the cloning strategy, Vienna RNAfold web server is used to predict the secondary structures of the RNAs of interest fused to MS2 RNA (see Appendix 6.2). Using the RNA folding program we could decide whether to clone the RNA of interest into pRH5′ or pRH3′ (different position of the multiple cloning site with respect to the MS2 RNA sequence).

2.4.2 The construct of hybrid RNA molecule

The hybrid RNA has the general structures: they consist of the yeast RNase P RNA leader sequence, to tandem MS2 coat protein binding sites, and the RNA sequence of interest, X. the MS2 and RNA X sequences can be in either position relative to one another. Two MS2 coat protein binding sites are used because binding to adjacent sites are cooperative (Bardwell and Wicken, 1990; LeCuyer et al., 1995) . The sites contain a nucleotide change that enhances binding to the coat protein (different position of the multiple cloning sites with respect to the MS2 RNA sequence).

Several RNA hybrid molecules were designed based on the sequence and secondary structure of *StSUT4* mRNA and used as "bait" for the three-hybrid method. These RNAs were used to screen the potato mixed cDNA library for putative RNA binding

proteins. The cDNA insert which shown to interact with RNA hybrid molecules could be isolated, sequenced and tested for specificity of interaction.

Either pRH5′ or pRH3′ can be used to construct an RNA bait to test for our putative RNA-protein interaction. The two vectors differ from one another only in the position of the multiple cloning sites with respect to the MS2 RNA sequence. In the present work, the pRH3′ (upstream of multiple clone sites) vector was chosen for construct of bait RNA.

The RNAs for reverse transcription were extracted from potato sink leaf and flower, PCR reaction was performed with cDNA as template, Husion polymerase (Bioline) is used. The PCR product is with blunt ends using this polymerase, after purification, the PCR products were inserted into blunted pZero 2 vector (from Invitrogen), then restriction by PmeI, AatII (*StSUT4*), the fragments were inserted into pRH3'.

2.4.3 Construct of cDNA library

The cDNA library was from *Solanum tuberosum m*ixed tissues (sink and source leaf, petiole, stem, flower, root, sink and source tuber), had an average insert size of 1.3 kb, range from 0.5 to 3.0 kb, complexity: 6×10^5 primary clones in pENTR™ 1A vector (Undine Krügel, PhD dissertation, 2008), Invitrogen´s Gateway system was used for transferring the cDNA library into pGAD10-GW vector (including LexA-Gal4 chimeric protein, as yeast expression vector)(Bürkle et al., 2005), a mixture of the three vectors each with one Gateway cassette) using LR reaction (10 µl): 3.0 µl pGAD10-GW (A, B, C frame mixed), 0.1 µg/µl (300 ng), 0.8 µl *Solunum tuberosum* cDNA libraries in pENTR™ 1A, 0.4 µg/µl (320 ng) in 8 µl TE buffer, after the addition of 2 µl Clonase mix, the reaction was incubated at 25°C for at least 20 h. Then the mix was treated with Proteinase K for 10 min at 37°C and subsequently deactivated by incubation for 10 min at 80°C, followed by ethanol precipitation: add 1.0 µl 3M NaAc, 2.5 volume ethanol and 1.0 µl Glycogen mixed well, precipitated at -20°C for at least 3 hours, centrifuged at 12,000× g for 15 min, pellet was washed two times with 70% Ethanol, air dried and resolved in 10 µl ddH_2O. The whole LR reaction was used to transform into 100 µl commercial competent cells by electroporation (better use 2 mm electroporation cuvet). 10 ml SOC medium was added immediately, the culture was transferred into 50 ml tube, 225 rpm shaking one hour at 37°C, the cells were then plated on 20 large plates (22×22 cm) of selective solid medium (100 µg/ml ampicillin), 500 µl each one. The plates were completely dry, so single colony can grow on selective medium for good library. The plates were incubated overnight at 30°C; and then transferred to 37°C and incubated for 2-3 hours.

To collect the *E.coli* cells, 10 ml LB medium was put in each plate; 200 ml bacterial cells were centrifuged at 6,000×g for 15 min at 4°C. The plasmid DNA was isolated using a Qiagen Plasmid Mega kit according to the manual. The bacterial pellet was resuspended in 50 ml Solution I (add RNase A 100 µg/ml) and incubated at 37°C for 30 min; then, 50 ml Solution II was added, mixed gently but thoroughly by inverting 4-6 times and incubated in room temperature for 5 min; 50 ml Solution III (chilled on ice before) was added and gently mixed by inverting 4-6 times. The mixture was incubated on ice for 30 min, centrifuged at 20,000×g (4°C) for 30 min. The supernatant containing plasmid DNA promptly was centrifuged again at 20,000×g (4°C) for 15 min. A Qiagen-tip 2500 was equilibrated by applying 35 ml QBT, and the column was allowed to empty by gravity flow. Then the supernatant containing plasmid DNA was applied to the equilibrated Qiagen-tip and entered the resin by gravity flow. The Qiagen-tip was washed with a total of 200 ml QC, plasmid DNA was eluted with 35 ml Buffer QF and precipitated by adding 24.5 ml (0.7-fold) room temperature isopropanol to eluted DNA, mix and centrifuge immediately at 15,000×g for 30 min at 4°C, carefully decant the supernatant; wash DNA pellet with 7 ml of room temperature 70% ethanol, and centrifuge at 15,000× g for 10 min, carefully decant the supernatant without disturbing the pellet, air dry the pellet 30 min, and resolve the DNA in a suitable volume of Buffer C (TE buffer pH 8.0), determination of yield using spectrum photometer. 100 µg aliquots were stored at -20°C.

2.4.4 Outline of working route of yeast three-hybrid assay

The strategy of the Y3H screen as well as selection and analysis of positive clones is shown in Fig. 4.

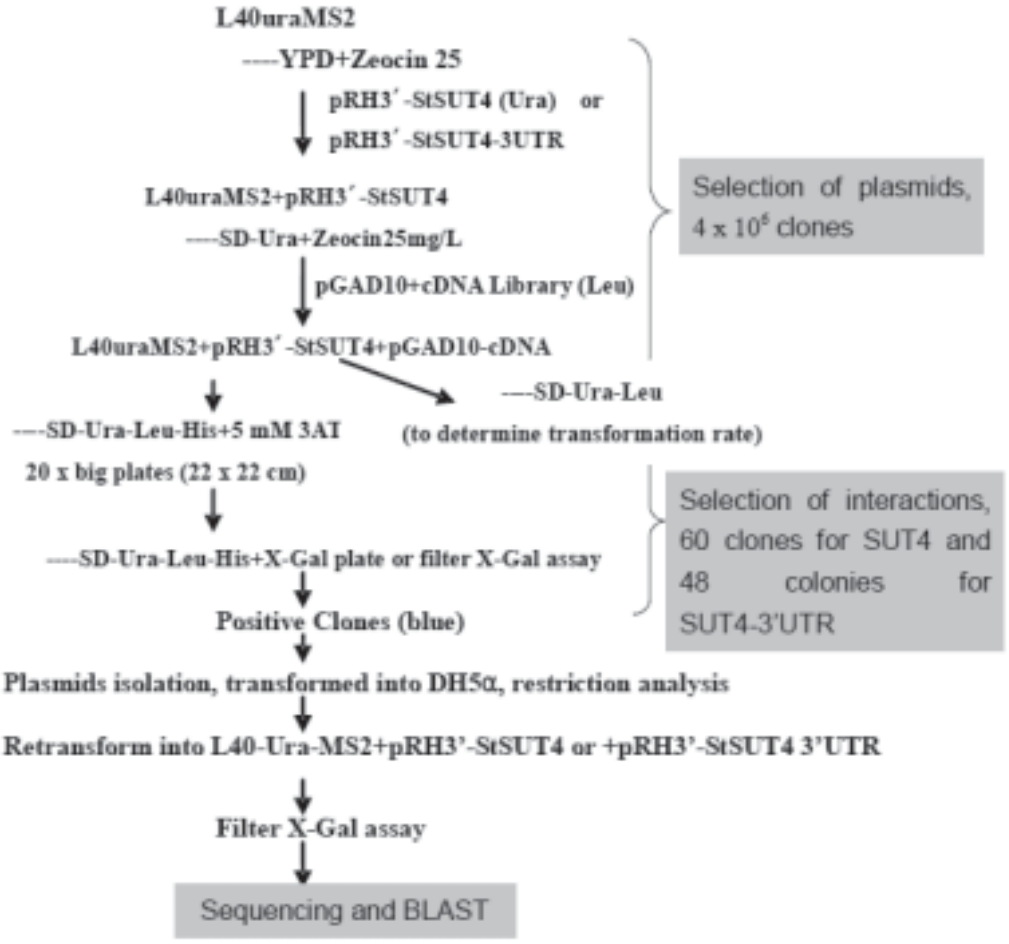


Fig. 4: Flow diagram showing the strategy for the Y3H screen and analysis of positive clones

A yeast strain L40uraMS2 carrying hybrid protein one is transformed with different bait RNA constructs (pRH3'/StSUT4 or pRH3'/StSUT4 3'UTR), the resultant cells are selected on SD-Ura medium; the yeast strain L40uraMS2 with different bait plasmid DNAs are transformed with pGAD10-GW cDNA library, the resultant cells are selected on SD-Ura-Leu-His +5 mM 3-AT for large scale screens. The initial transformation plates are usually incubated at 30°C for a week. When the RNA interacts with the protein produced from a cDNA, the HIS3 gene becomes active and the yeast cells can grow on selective media lacking histidine and/or containing 3-AT. Additional selections were performed using the LacZ reporter and colorimetric assays. Plasmid DNAs were isolated from positive yeast clones, and transformed into *DH5α*, restriction analysis was employed to determine the insert fragments. L40-ura-MS2 cells with bait vector were transformed with putative candidate DNAs, plated on SD-Ura-Leu medium, and then liquid culture was plated on SD-Ura-Leu-His plus 3 mM 3-AT, after X-gal assay confirmation, the putative interaction partners were sent for sequencing and BLAST analysis (Fig. 4).

2.4.5 X-gal analysis of interaction clones

The activity of the LacZ reporter was measured using X-gal, a chromogenic substrate for the LacZ gene product in a filter assay. Using X-gal in a filter assay was more sensitive than using X-gal in agar plates. Transformed yeast colonies were transferred (via velvet stamp replication) to solid selective media (SD-Ura–Leu) overlaid with a reinforced nitrocellulose filter. The resultant replica plate was incubated overnight at 30°C. Following its removal from the surface of the plate, the nitrocellulose filter was immersed for 20-30 seconds in liquid nitrogen, and allowed to thaw on the lab bench for 2-5 minutes. In a separate dish, the filter was overlaid onto a piece of Whatman 3MM paper saturated in modified Z Buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$) supplemented with 300 μg/ml X-gal, the filter colony side is up on the Whatman paper avoiding air bubbles under the filter. The plates are sealed and incubated at 30°C until blue colonies appear (from 30 min to 12 h).

Activation of the lacZ reporter: activation of the transcription of a LexA operator-lacZ is one of the two tests used to detect positive interesting clones in a library screen. Hence, an important preliminary experiment is to determine the baseline activation of the LexA op-lacZ reporter by the bait and library vector.

2.4.6 Reduce non-specific transactivation of the *HIS3* reporter gene

The HIS3 reporter can be scored by plating yeast cells onto plates that lack histidine. Interactions are then identified by growth of colonies on SD minus histidine plates. To continue to work with the specific bait RNA that transactivates the *HIS3* reporter gene in the absence of a partner, include 3-Aminotriazole (3-AT) in the growth medium. 3-AT is a competitive inhibitor of the *HIS3* gene product, His3p. Cells containing more His3p can survive at higher concentrations of 3-AT. Thus the level of resistance to 3-AT monitors the strength of an RNA-protein interaction. The 3-AT resistance assay was performed in this study as following steps (Bernstein et al., 2002): 1) a series of selective medium plates that lack histidine and contain increasing amount of 3-AT (e.g. 1, 3, 5 and 10 mM 3-AT) were prepared, also including plates lacking leucine and uracil; 2) the L40uraMS2 yeast strain was transformed with the appropriate RNA and protein-encoding plasmids plated on SD-Ura-Leu medium, incubated at 30°C for 2 days; 3) multiple average-sized colonies were picked and streaked for single colonies on –Ura-Leu and –His plates; 4) the plates were incubated at 30°C for 3-5 days. Growth should be assessed by the presence of individual colonies and not the smear of yeast from the initial streak.

2.4.7 Small scale yeast transformation

A single yeast colony of L40uraMS2 was incubated in 10 ml of YPAD medium with shaking overnight at 30°C, the OD_{600} of overnight culture was determined, the culture is diluted to an OD_{600} of 0.4 in 50 ml of YPAD medium and grown an addition 2-4 hours, the cells were collected at 3000×g and resuspended in 40 ml 1×TE; the cells were recollected and resuspended in 2 ml of 1×LiAc/0.5×TE; the cells were incubated at 30°C for 10 minutes; for each transformation, mixed cells was prepared with 1 µg plasmid DNA and 100 µg denatured sheared salmon sperm DNA (2 mg/ml, filter sterilized stock solution, incubated at 95°C for 5min then on ice before used) with 100 µl of the yeast suspension from before; 700 µl of 1× LiAc/40% PEG-3350/1× TE was added and mixed well; solution was incubated at 30°C for 30 minutes; then 88 µl DMSO was added for each transformation, mixed well, and heated shock at 42°C for 10 minutes; the cells were collected by centrifuging 3000×g for 2 minutes, resuspended in 1 ml 1× TE and repelleted; the cell pellet was resuspended in 50-100 µl TE and plate on a selective plate.

2.4.8 Large scale yeast transformation

The protocol was slightly modified from The TRAFO PAGE (Agatep et al., 1998). The yeast strain L40uraMS2 (Invitrogen) containing pRH3'-StSUT4 or pRH3'-StSUT4-3′UTR (bait vector) was grown in 100 ml SD medium lacking of uracil (SD-U) liquid media (for 60 times transformation, 60×) at 30°C for 36 h. This starter culture was used to inoculate 300 ml YPAD media to a cell density OD_{600} of 0.3. The following procedure was always performed for 60× transformations. The cell culture was determined and calculated yields (2.5×10^8 cells for each 50 ml of YPAD culture needed, total volume was 300 ml, which needs 1.5×10^9 of cells); the culture was put into an appropriate sterile centrifuge tube, the cells were harvested at 3,000× g for 5 min. The cell pellet was resuspended in the appropriate volume (300 ml) of pre-warmed (30°C) YPAD and transferred to another sterile culture flask; the resulting culture was grown at 30°C on a shaker until titre reaches 2×10^7 cells/ml. The cells were collected by centrifugation at 3,000× g for 5 min, washed via resuspension with 1/2 volume of ddH_2O and collected by centrifugation as above. The pellet was resuspended in the appropriate volume (for 60×, 6 ml) of 100 mM sterile LiAc and transferred to an appropriate centrifuge tube. The mix was incubated for 15 min at 30°C. The cells were collected again by centrifugation. Then 22.6 ml Mix 2 (components shown in table 4) was added to the cells, in the order from top to bottom, and mixed thoroughly by vortex. The transformation mix was incubated at 30°C for 30 min, followed by 1 hour heat shock at 42°C (by inversion for 15 sec after every 5 min to equilibrate the temperature quickly in the larger volume).

Table 3: Components in yeast transformation Mix2

TRAFO SCALE	60×
50% PEG	14.40 ml
1.0 M LiAc	2.16 ml
SS-DNA (2 mg/ml, Sigma)	3.00 ml
Library plasmid DNA	C µl (100 µg)
Sterilized water	2.04 - C ml

The cells were collected by centrifugation, gently resuspended in 10 ml sterile water. For the determination of transformation efficiency, 1 µl of this mix was plated on SD medium lacking uracil and leucine (SD-Ura-Leu) and incubated at 30°C. After 3 days the grown clones were counted and the total number of transformants was calculated. The rest of the cells were plated onto SD medium lacking uracil, leucine, histidine with 5 mM 3-AT (SD-ULH 3-AT) on twenty 22× 22 cm plates and incubated for 7 days at 30°C. To verify putative interactions, positive clones from the screen were restreaked on SD-ULH 3-AT media. Prey plasmids were isolated from yeast culture as described as following (2.4.9). To increase the plasmid DNA quantity, *E.coli DH5a* was transformed with different yeast plasmid DNAs respectively, 24 single clones were picked and isolated plasmid DNAs, subsequently restriction digestion was performed to determine the insert pattern. Then yeast strain L40uraMS2 containing the respective bait plasmid was retransformed with the interesting pray plasmid DNAs, X-Gal filter lift assay confirmed positive clones. Finally, the identity of the positives clones was analyzed by sequencing.

For different bait RNA, a minimum of 1×10^6 clones were screened in the yeast three-hybrid system.

2.4.9 Isolation of plasmid DNA from yeast cells

The protocol which was used for isolation of plasmid DNA from yeast cells in this work as below. The yeast culture was prepared in 4 ml SDG-Leu medium by incubating yeast colonies at 30°C for one week, this step was to eliminate the bait plasmid from yeast cells. The yeast culture was collected by centrifuging 13,000× g, 2 min; 250 µl lyticase-buffer was added to the cell pellet; the cell pellet was resuspended by vortex and incubated 30 min at 37°C; 250 µl, buffer II (same to solution II for *E.coli* plasmid extraction) was added, inverted 5 times to mix well, the solution mix was incubated 5 min at room temperature; 250 µl, buffer III (same to solution III for *E.coli* plasmid extraction) was added, inverted 5 times to mix well, the mix solution was incubated 10 min to 20 min on ice; The cells were collected by

centrifuging 20 min at 14,000× g; the supernatant was transferred into a new tube, 525 µl isopropanol was added, mixed well, 10 min at room temperature; the pellet was collected by centrifuging 20 min at 12,000× g, centrifuge 20 min; the pellet was washed twice with 70% ethanol and air dried, resolved in 5-10 µl ddH_2O.

2.5 Regulation of half life of sucrose transporter mRNAs

2.5.1 Influences on SUT4 under far-red light enrichment

In vitro potato leaves were treated with actinomycin D (ActD, 2 µM in 2.5 mM EDTA, pH6.0), cycloheximide (CHX, 10 µM). The samples were taken at different time point after treatment, frozen by liquid nitrogen. Total RNA was extracted using Trisure followed by DNase I treatment, the cDNA templates from reverse transcription were used for real-time PCR assay, with Ubiquitin1 as inter-standard. ImmoMix^{TM} (Bioline), the reaction volume is 20 µl, 10 µl 2× ImmoMix^{TM} (Bioline), 2 µl Forward primer (10 µM), 2 µl Reverse primer (10 µM), 2 µl $MgCl_2$ (35 mM), 0.4 µl SYBR Green I (50×), ddH_2O up to 20 µl. Program: 95^{o}C, 7 minutes (activation); 95^{o}C 10 sec, 61^{o}C 15 sec, 72^{o}C 15 sec; 45 cycles. The data was analysis using LinRegPCR (for PCR efficiency) and Roter-Gene6 (for Ct value).

2.5.2 Inhibitor studies and feeding experiments

Plants were grown in the greenhouse with a 16 hour light/8 hour dark cycle, and the leaves were harvested from 2 month-old plants. Petioles of detached leaves were recut while submerged in water, 2.5 mM EDTA (pH 6.0) was added to inhibit callose formation, the cut petioles were transferred to inhibitors or phytohormone containing solutions where they were kept for the indicated period of time under greenhouse or phytochamber with or without additional far red light. Ethephon concentration was 3 mM. The concentration of NAA was 50 µM. MeJA concentration was 50 µM. The inhibitors CHX or MG132 were used at a concentration of 10 µM, actinomycin D was used at 2 µM and the final cordycepin concentration was 50 µg per ml. All experiments were repeated two times independently.

2.6 Physiological analysis of transgenic potato and tobacco plants

2.6.1 Real-time monitoring of ethylene (ET) production

Ethylene production was measured in real time using a sensitive laser-based ET detector (type ETD-300; Sensor Sense BV, Nijmegen, the Netherlands) in combination with a gas handling system. The ETD-300 is a state-of-the art ethylene detector based on laser photoacoustic spectroscopy (Harren et al., 2000) that is able to detect on-line c.300 pptv (parts-per-trillion volume, 1:10^{12}) of ethylene within a 5-s

time scale. The gas handling was performed by a value control box (type VC-6, Sensor Sense BV, Nijmegen, the Netherlands), designed for measuring up to six sampling cuvettes per experiment. A detailed description of the method is given by Cristescu et al., (2002).

2.6.2 *In vitro* pollen germination assay

Anthers from potato plants were collected, opened with a razor blade and placed in Eppendorf tubes, vortexed to remove the pollen and incubated in the dark at 26 ^{o}C in 20 mM MES buffer, pH 6.0 containing 15% PEG 4000, 2 or 5% sucrose, 0.07% $Ca(NO_3)_2$ $4H_2O$, 0.02% $MgSO_4.7H_2O$, 0.01% KNO_3 and 0.01% H_3BO_3. Pictures were taken with a Zeiss Axiophot microscope and pollen germination rate was counted with a Fuchs–Rosenthal chamber. Pollen was scored as geminated when the pollen tube was longer than the pollen grain diameter. For each experiment, between 50 and 300 pollen grains were scored (Hackel et al., 2006; Sivitz et al., 2008).

2.6.3 Pollen viability assay

Pollen samples were obtained from 3-day potato flowers under long day condition.

Brewbaker and Kwack (B & K) buffer, 146.1 mM sucrose, 1.6 mM boric acid, 1.2 mM calcium nitrate, 0.8 mM magnesium sulphate heptahydrate, 1.0 mM potassium nitrate, 0.7 µM aniline blue staining (water soluble).

FDA solution, 0.02 g of fluorescein diacetate (FDA) (Sigma-Aldrich GmbH, Munich, Germany), was mixed with 10 ml of acetone. A 20% sucrose solution was made up and 5 ml removed into a separate container. The FDA solution was added drop by drop to these 5 ml of sucrose until persistent turbidity. This solution was used within 30 min of mixing (Pline et al., 2002). One 10 µl drop of the mixture was applied on a clean microscope slide and subsequently pollen was added.

Brewbaker and Kwack (B & K), B & K + aniline blue, and FCR media were made fresh daily before use. After pollen samples were placed on slides in each medium, samples were examined with phase contrast microscopy to enhance visibility of pollen tubes (B & K and B & K + aniline blue), or fluorescence microscopy using a 470 nm excitation filter and a 535 nm emission filter (FCR Method). Each slide containing 50 to 200 pollen grains was visually counted, scoring pollen granules as germinated (pollen tube longer than pollen grain diameter) or nongerminated, for B & K and B & K + aniline blue methods. Using the fluorochromatic reaction test for the transgenic lines' pollen, the viable pollen grains fluoresced in a bright green color, whilst the non-viable grains were of a dull yellow color. A hundred grains visible on the slide were scored and the percentage viability calculated.

2.6.4 Soluble sugar content and starch measurement

Soluble sugars including glucose, fructose and sucrose were extracted from ground tobacco leaf materials in two subsequent steps. 80% (v/v) ethanol (250 µl per approx. 20 mg FW and 150 µl per approx. 20 mg FW) was added, mixed for 20 min at 80°C and centrifuged at max speed for 5 min. The pellet was re-extracted with 50% (v/v) ethanol (250 µl per approx. 20 mg FW) the supernatants were combined and used for immediate assays or stored at -20°C until use.

Sucrose, glucose and fructose were determined enzymatically in extracts following the protocol of Galtier et al., (1993) using a microplate spectrophotometer. The assay contained in a final volume of 800 µl: 790 µl of 100 mM HEPES/KOH with 3 mM $MgCl_2$ (pH 7.0), 10 µl of 12 mg/ml NADP, 10 µl of 20 mg/ml ATP, 0.5 U glucose 6-phophate dehydrogenase and 10 µl of ethanol extract or diluted phloem. The reactions were started by the successive addition of 1 U hexokinase (for glucose), then by 1 U phosphoglucose isomerase (for fructose), at last by 20 U invertase (for sucrose) (all enzymes are extracted from yeast).

For starch staining, the source leaves of living tobacco plants were kept in the dark for a period of 16 hours covering with aluminium foil. The detached leaves were incubated for 30 min in 80% ethanol at 70°C and subsequently stained in Lugol solution (J2/KJ2) for 20 min. Pictures were taken on a luminescent screen.

2.7 DNA sequencing

All DNA sequences reactions were prepared using the Big Dye-terminator, then the reactions were determined by Institute of Genetics in Humboldt University on Abi Prism 377, 3100 or 3730 sequencers (Applied Biosystems, Berlin, Germany) using BigDye-terminator v3.1 chemistry. Premixed reagents were from Applied Biosystems.

2.8 Phylogenetic analysis software

To identify the related sequences in the GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi), nucleic acid-nucleic acid blast and protein–protein BLAST was conducted using the deduced amino acid sequence from putative interaction partners.

ClustalX was used to create multiple alignments (Thompson et al., 1997). The resulting alignment was used to generate the Phylogenetic tree by the neighbor-

joining method with Tree View. Bootstrap analysis was performed to estimate nodal support based on 1000 replicates.

POGs/PlantRBP (http://plantrbp.uoregon.edu/) is a relational database that integrates data from rice, Arabidopsis, and maize by placing the complete Arabidopsis and rice proteomes and available maize sequences into 'putative orthologous groups' (POGs). Annotation efforts will focus on predicted RNA binding proteins (RBPs): i.e. those with known RNA binding domains or otherwise implicated in RNA function.

RNA structures prediction tools: http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi

ExPASy Translate and ProtParam Tool: http://www.expasy.org/tools

InterProScan Sequence Search tool: http://www.ebi.ac.uk/InterProScan/

Real-time PCR primers were picked from Primer 3.0: http://frodo.wi.mit.edu/

Sequences assembling were done using Vector NTI version 9.0 (AlignX) from Invitrogen

Membrane domain prediction: http://aramemnon.botanik.uni-koeln.de/

The Gene Ontology (GO) project is a collaborative effort to address the need for consistent descriptions of gene products in different databases; http://amigo.geneontology.org/

3. Results

The work is described in the following chapters consists mainly of the analysis on sucrose transporters based on the materials and methods mentioned in chapter 2. First, the *Sucrose Transporter1* mRNA phloem mobility was analyzed. The analysis was done with the holoparasite *Cuscuta reflex* grown on wild-type tobacco or wild-type potato, and with intra-specific grafts between wild-type and *SUT1* over-expressing plants (chapter 3.1). The regulation of sucrose transporter mRNAs stability was analyzed by qRT-PCR of sucrose transporter transcript levels in the presence of transcriptional or translational inhibitors (chapter 3.2). Based on these inhibitor studies, a post-transcriptional regulation of the StSUT4 mRNA is assumed. Parts of this work have been published (He et al., 2008). In chapter 3.3, a brief summary of the phenotype of *StSUT4-RNAi* potato plants was given, and the analysis of *StSUT2&StSUT4-RNAi* double transformant plants based on *StSUT2-RNAi* potato was presented. In chapter 3.4, the generation of *SlSUT4* over-expressing tobacco plants was described and the primary phenotypic and molecular analysis was given. In chapter 3.5, far-red light dependent regulation of the *StSUT4* mRNA stability was investigated, and qRT-PCR analysis of genes, which were involved in flower induction and tuberization including *GIGANTEA (GI)*, *StCOL3*, *StSOC1* and *StFT*, helped to elucidate the molecular mechanisms of the *StSUT4* function. In chapter 3.6, the work about the confirmation of SUT4 protein-protein interaction partners which were found by yeast split ubiquitin assays is shown. Based on the GATEWAY cloning system and bimolecular fluorescence complementation (BiFC) the protein-protein interactions of two SUT4-binding candidates are confirmed. Indirect confirmation of the interaction between the StSUT4 protein and a putative ethylene receptor protein is provided by ethylene production measurement of *StSUT4*-inhibited potato plants. In addition, yeast three-hybrid screens were performed to identify the *SUT4* mRNA-binding candidate(s) potentially involved in the post-transcriptional regulation of the *StSUT4* mRNA stability (chapter 3.7).

3.1 Sucrose transporter mRNA's phloem mobility

The phloem mobility of many phloem specific mRNAs and proteins has been analyzed in detail via interspecific graft experiments (Ruiz-Medrano et al., 1999). In this chapter, the phloem mobility of sucrose transporter mRNAs will be discussed. By *Cuscuta reflexa* parasitic experiments and intraspecific graft experiments using transgenic potato and tobacco plants, the phloem mobility of *StSUT1* from potato, *NtSUT1* from tobacco, *SlSUT1* from tomato and *SoSUT1* from spinach was shown as follows.

3.1.1 The phloem mobility of *StSUT1* and *NtSUT1* mRNAs demonstrated in *Cuscuta reflexa*

Cuscuta reflexa (known as dodder) is a holoparasite. It is able to form haustoria to the host plants in order to collect photoassimilates (Kuijt, 1983). The parasitic plant's hyphae build up symplasmic connections to the phloem cells of the host plant allowing the transport of macromolecules as shown by GFP movement from transgenic tobacco plants into *C. reflexa* (Haupt et al., 2001). It is commonly known that *C. reflexa* can use potato as well as tobacco plants as a host. In contrast, tomato plants are resistant against the parasite (Albert et al., 2004). In this study, *C. reflexa* was employed to analyze the movement of *StSUT1* and *NtSUT1* mRNA from host to parasite. Both the full length *StSUT1* transcript and the *NtSUT1* mRNA were detected in RNA of *C. reflexa* which grew on potato or tobacco host plants for 2 weeks using RT-PCR. Fig. 5 showed that *NtSUT1* transcripts were amplified from RNAs of tobacco plants and *C. reflexa* plants grown on *Nicotiana tubacum* ssp. SNN using *NtSUT1* specific primers (lane 1, lane 4 to 7), whereas no amplicon was detected with the negative controls (lane 2 *Pelargonium* and lane 3 *C. reflexa* grown on *Pelargonium*). This was a first hint showing that the *NtSUT1* mRNA moved from host to parasite. Moreover, the full length transcript of *StSUT1* was detected in the parasite *C. reflexa* grown on potato plants (data not shown here). Both experiments were a good argument for mRNA stability during long-distance transport, which potentially takes place in a ribonucleoprotein complex (RNP).

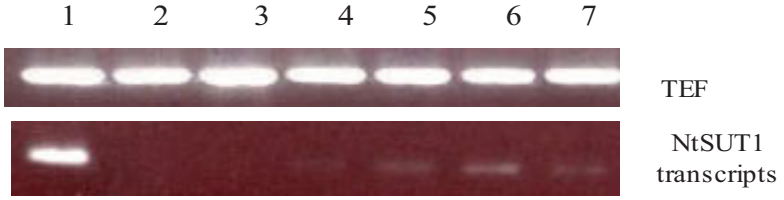


Fig. 5: RT-PCR with RNA from *Cuscuta reflexa* grown for 2 weeks on tobacco or *Pelargonium* plants.

Quality and amount of the reversely transcribed mRNA was tested using standard primers (translation elongation factor TEF). Lane 1: SNN; lane 2: *Pelargonium*; lane 3: Cuscuta grown on *Pelargonium*; lane 4-7: samples from Cuscuta *(Cuscuta reflexa)* grown on tobacco *(Nicotiana tabacum)* plants for at least two weeks before it was harvested. In order to avoid contamination RNA samples from *Cuscuta reflexa* were taken in a 2 cm distance from haustorial interconnection between host and parasite.

3.1.2 *SoSUT1-cmyc* and *SlSUT1-GUS* mRNA phloem mobility in graft experiments

Xoconostle-Cázares et al. (1999) had shown *StSUT1* mRNA cell-to-cell movement through plasmodesmata by microinjection experiments with and without the RNA-binding protein CmPP16 from *Cucurbita maxima*. In 10 out of 10 experiments fluorescently labeled *StSUT1* was able to move from the microinjected cells into

neighboring cells if co-injected with CmPP16, while in one out of 10 experiments *StSUT1* was detected in the neighboring cells when fluorescently labeled *StSUT1* mRNAs were injected without CmPP16. In order to test the *StSUT1* mRNA's phloem mobility, the two intra-specific grafts were performed in this study. In both cases, *SUT1* transcripts were detected in the graft partners by RT-PCR.

Transgenic potato plants used in the first graft experiment have a c-myc tag fusion with the open reading frame of *Spinacia oleracea SUT1* (*SoSUT1*) gene. The *SoSUT1-cmyc* fusion is expressed under a strong constitutive CaMV 35S promoter (Fig. 6A) (Leggewie et al., 2003). Concretely, *SoSUT1-cmyc* over-expressing potato plants line 27 is used to answer the question whether untranslated regions of the *SUT1* transcript are required for phloem mobility. In fact, the *SoSUT1-cmyc* gene does not contain untranslated regions of the mRNA since only the coding region of the cDNA was cloned into the transgenic gene construct. The phloem mobile *SoSUT1-cmyc* which did not contain the 5' UTR or the 3' UTR was detected by RT-PCR. After 25 cycles the tagged *SoSUT1-cmyc* transcripts were detected only in the transgenic *SoSUT1-cmyc*27 potato; however, after 30 cycles the *SoSUT1-cmyc* transcripts were also detected in the WT stock grafted with *SoSUT1-cmyc*27 scion (lane 3 in Fig. 6B); moreover, after 35 cycles the *SoSUT1-cmyc* transcripts were detected in both wild-type stock and wild-type scion (Fig. 6B: lane 2 and lane 3 respectively). Therefore, untranslated regions (UTRs) of *SUT1* transcripts are not required for mRNA movement from one graft partner into the grafted WT potato plants.

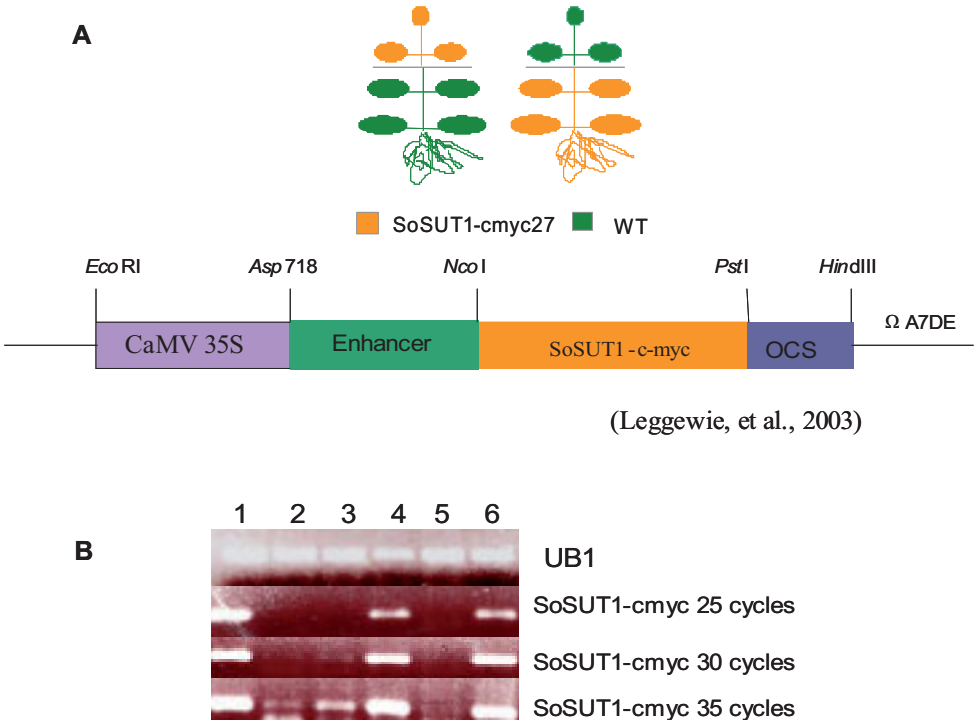


Fig. 6: Phloem mobility of *SoSUT1-cmyc* transcripts in grafting experiment.

A. Schematic representation of the intra-specific graft experiment (above) using transgenic potato plants over-expressing a tagged version of the *SoSUT1* gene of *Spinacia oleracea* under the control of an enhanced CaMV 35S promoter as indicated in the construct (below) used for transformation of *Solanum tuberosum* (Leggewie et al., 2003). **B.** Semi-quantitative RT-PCR of the tagged *SoSUT1* gene in grafted plants. Total RNA was assayed from the following tissues: Lane 1, SoSUT1-myc27 stock; lane 2, wild-type scion; lane 3, wild-type stock; lane 4, SoSUT1-myc27 scion; lane 5, ungrafted wild-type potato; lane 6, SoSUT1-myc27 potato. UB1, ubiquitin 1 was used for internal standard. *SoSUT1* fw and *c-myc* rev primers were used in semi-quantitative RT-PCR.

In another graft experiment, phloem mobility of *SUT1* transcripts was detected when the tomato *SUT1* gene sequence, *SlSUT1*, was expressed under its own promoter in transgenic tobacco plants. A *SlSUT1* promoter::*SlSUT1* gene-GUS fusion construct containing exon as well as intron sequences of the *SlSUT1* gene was transformed into tobacco plants (Fig. 7A) (Lalonde et al., 2003). In this study, the SlSUT1-GUS transgenic tobacco plants (line 38) were used for graft assay. When the SlSUT1-GUS fusion protein was expressed under its own promoter the phloem mobility of the corresponding mRNA was observed in the grafted WT scion by RT-PCR (Fig. 7B). Thus, even the larger mRNA encoding the *SlSUT1-GUS* fusion is able to move in the phloem of grafted tobacco plants.

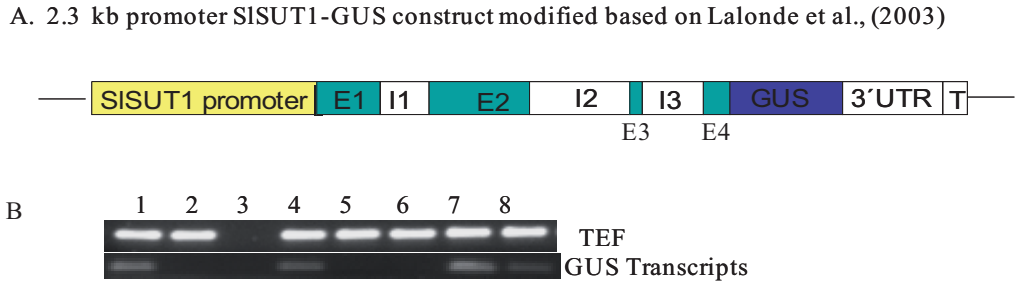


Fig. 7: Phloem mobility of *SlSUT1-GUS* transcript expressed under the endogenous SlSUT1 promoter

A: Scheme of the *SlSUT1-GUS* fusion construct under control of the 2.3 kb promoter fragment of *SlSUT1* (yellow box). The transgenic gene construct consists of four exons (green boxes) and three introns (white boxes) of *SlSUT1* coding region and *GUS* gene (blue box) (Lalonde et al., 2003). Progenies of the transgenic tobacco line 38 expressing *SlSUT1-GUS* were grafted with WT tobacco plants B: *SlSUT1-GUS* transcripts were detected in grafted wild-type tobacco plants via RT-PCR. Lane 1, transgenic SlSUT1-GUS plant line 38; lane 2, tobacco wild-type; lane 3, no-template control; lane 4, transgenic SlSUT1-GUS 38 stock; lane 5, grafted wild-type scion 1 >10 cm from graft union; lane 6, grafted wild-type scion 2 >5 cm from graft union; lane 7, grafted wild-type scion 3 >=1 cm from graft union; lane 8, grafted wild-type scion 4 <1 cm close to graft union. TEF, translational elongation factor was used as internal standard; GUS specific primers were used to amplify the GUS transcripts.

3.2 Sucrose transporter mRNA stability

The mobility of *SUT1* mRNA molecules requires protection against nucleolytic degradation during transport through plasmodesmata and during long distance

trafficking in the phloem. It is known that the StSUT1 protein is decreased within 4 h of cycloheximide treatment (Kühn et al., 1997) and the accumulation of *StSUT1*, *StSUT2* and *StSUT4* mRNAs follow a diurnal rhythm in potato (Chincinska et al., 2008). This indicates that the half life of sucrose transporter mRNAs and proteins is short. In the following chapter, the half life of sucrose transporter mRNAs in solanaceous plants is determined by qRT-PCR with the help of transcriptional and translational inhibitors, and the relative regulation mechanism will be discussed.

3.2.1 Short half life of sucrose transporter mRNAs

It is commonly known that messenger RNA (mRNA) is transcribed as a complementary copy of DNA which is eventually translated into an amino acid chain. The mRNA travels from nucleus to cytoplasm for translation. The synthesis of mRNA is at a much higher rate to maintain a steady state because mRNA is constantly degraded in the cytoplasm.

Measuring the half life of mRNA is difficult to determine experimentally due to its short life. However, knowledge of the half life of mRNA is important to find out the stability of different types of mRNA. Leclerc et al., (2002) have described an alternative method to determine mRNA half life ($t_{1/2}$) based on the qRT-PCR procedure. Due to the qRT-PCR's sensitivity, half life of mRNAs expressed at very low level can be determined even if Northern blots weren't sensitive enough. This method is regarded as a reliable way to measure mRNA half life. The transcript accumulation of all three known sucrose transporters in potato follows circadian rhythm according to the qRT-PCR analysis on wild-type potato plants (Chincinska et al., 2008).

In this study the half life of sucrose transporter mRNAs was determined by qRT-PCR with the help of actinomycin D as an inhibitor of transcription and of cordycepin as an inhibitor of polyadenylation. Most of sense mRNA degradations follow first-order kinetics (Lam et al., 2001), and sucrose transporter transcripts degradation can be fitted with first-order kinetics (R-squared greater than 0.9). Treating the detached potato leaves with cordycepin the half life of *SlSUT1* mRNA was calculated as 88.1 ± 13 min. The *StSUT1´*s half life was measured to be 69.3 ± 3 min by using actinomycin D or cordycepin (Fig. 8A). These results correlated with previous work of Vaughn et al., (2002). The author demonstrated that the half life of *SUT1* mRNA from *Beta vulgaris*, *BvSUT1,* was 108 min. The half life of *SUT2* and *SUT4* from potato and tomato showed a marginally longer half life of 130 min as shown by transcriptional inhibition using actinomycin D or cordycepin (Table 4). Since transcript accumulation of all three known sucrose transporters in potato follows a circadian rhythm

(Chincinska et al., 2008), the transcript amounts have been related to the corresponding water control after the indicated periods of treatment.

Table 4: Half life of sucrose transporter mRNAs

The half life of different sucrose transporter transcripts was determined via qRT-PCR on mRNA isolated from detached plant leaves after feeding experiments with appropriate inhibitor. RNA quantification was performed in relation to the amount of *UBIQUITIN1* transcripts as internal standard. Water treated plant material was taken as a reference. The standard deviation was given.

gene	mRNA half life	Inhibitors	Reference
SlSUT1	88.1 ± 13 min	cordycepin	(He et al., 2008)*
StSUT1	69.3 ± 3 min	cordycepin; act D	(He et al., 2008)*
BvSUT1	108 min	cordycepin	(Vaughn et al., 2002)
SlSUT2	131.6 min	cordycepin	(He et al., 2008)*
SlSUT4	131 min	cordycepin	(He et al., 2008)*
StSUT4	131 min	Act D	(He et al., 2008)*

* This work has been published on the Open Plant Science Journal (2008), 2: 1-14.

3.2.2 SUT1 expression is regulated at the transcriptional level

The accumulation of sucrose transporter1 protein is rapidly affected by cycloheximide (CHX) treatment shown by Western blot analysis (Kühn et al., 1997). Sucrose transporter transcript accumulation was investigated in the presence of CHX (cyclo) and quantified by qRT-PCR analysis. The mRNA levels of both *StSUT1* from potato and *SlSUT1* from tomato were rapidly reduced within 4 h upon CHX (cyclo) application (Fig. 8A and B). Taking together both SUT1 protein and *SUT1* transcript amounts are affected by inhibition of translation.

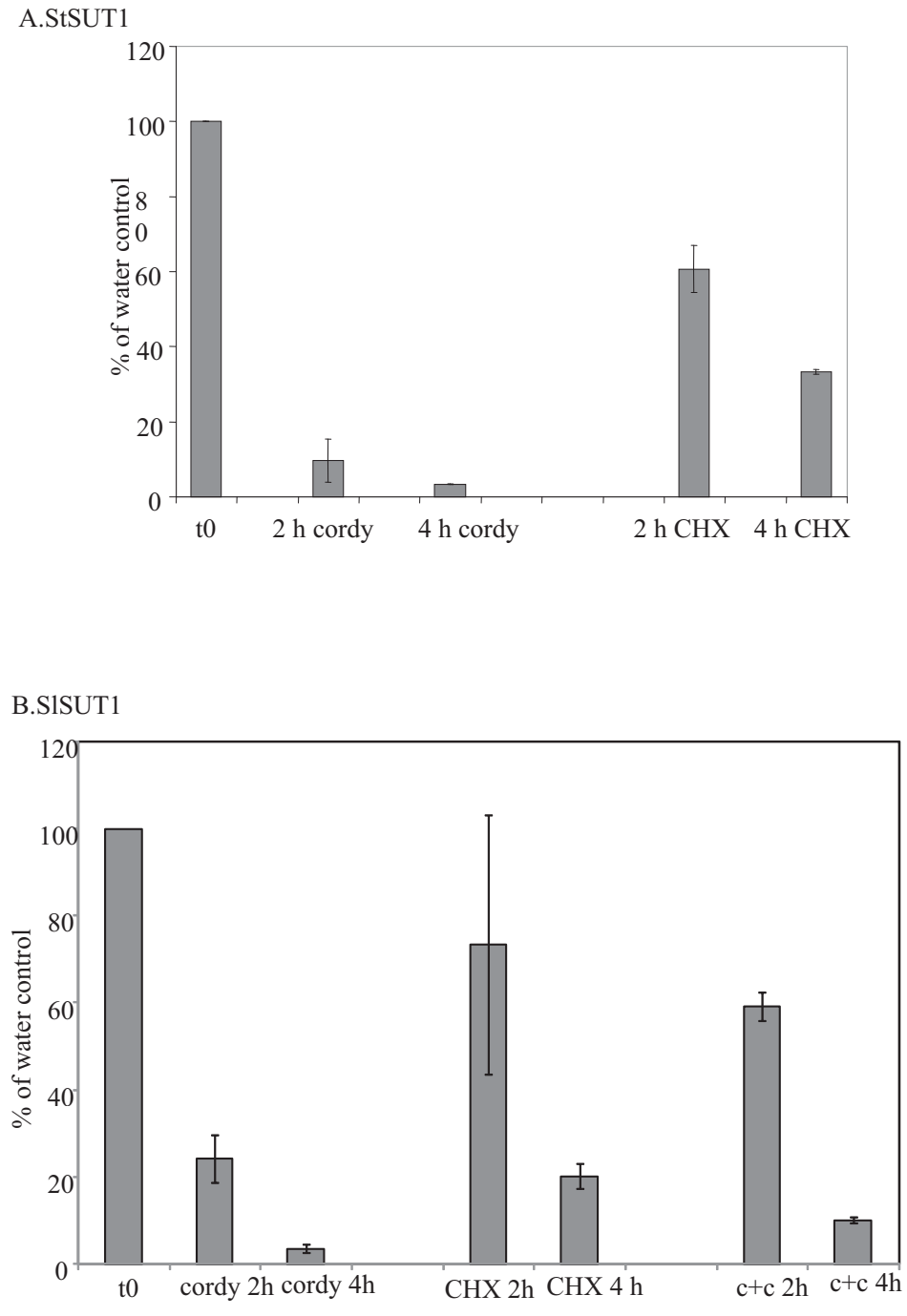


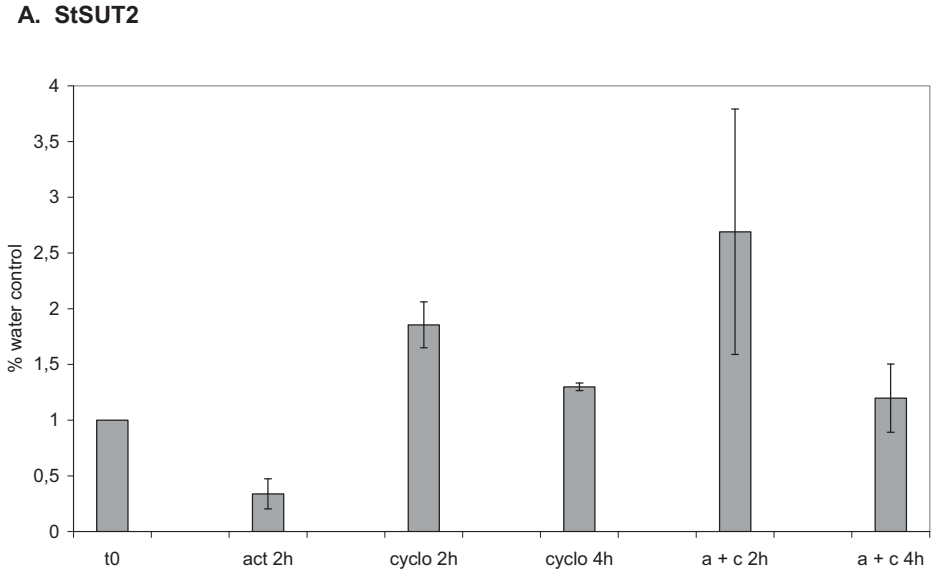
Fig. 8: Real-time PCR analysis of sucrose transporter SUT1 mRNA accumulation in the presence of transcriptional and translational inhibitors

A. Determination of *StSUT1* mRNA accumulation by cordycepin (cordy) and cycloheximide (cyclo or CHX). Half life of *StSUT1* mRNA was calculated based on real-time quantification and presented in Table 4. B. Half life of *SlSUT1* mRNA as determined by cordy treatment. 6 h after cordycepin supply no mRNA is detectable (not shown). *StSUT1* and *SlSUT1* mRNA amount were decreased in the presence of the translational inhibitor CHX. Simultaneous incubation with cordy and cyclo (c+c) does not lead to significant prolongation of *SlSUT1* mRNA half life. Quantification of transcripts is relative to the amount of ubiquitin transcripts. In order to exclude diurnal changes of transcript levels, the values are given in % of the corresponding water control after the indicated period of time.

Translational inhibition by CHX can affect the accumulation of sucrose transporter transcripts by two different processes. One possible process could be carried out by reducing amounts of short-lived transcriptional regulators that modify transcriptional activity. The other alternative could be due to the RNA-binding factors affecting the stability of transcribed messenger RNA. These two processes could specifically stabilize *SUT1* mRNA or destabilize *SUT2* and *SUT4* mRNA. In order to distinguish between transcriptional and post-transcriptional effectors, experiments with translational and transcriptional inhibitors treating detached potato or tomato leaves were performed simultaneously (Fig. 8). In the presence of both transcriptional and translational inhibitors the half life of *SlSUT1* mRNA was decreased compared to the untreated control (Fig. 8B), as it was observed by using both inhibitors alone. Consequently (Fig. 8A and B), SUT1 mRNA stability was mainly controlled at the transcriptional level.

3.2.3 *StSUT2* and *StSUT4* expression is regulated post-transcriptionally

In addition the accumulation of *SUT2* and *SUT4* transcripts from potato and tomato was analyzed in the presence of CHX and Act D, the translational inhibitor had the opposite effect on transcript amounts as previously observed for *SUT1* mRNA. In contrast to *SUT1* mRNA, the levels of the *SUT2* and *SUT4* mRNAs from potato and tomato were increased in the presence of CHX: the mRNA amounts increased strongly within the short period of only 2 h upon CHX treatment (Fig. 9). The half life of *StSUT2* and *StSUT4* mRNA was significantly prolonged in the presence of both inhibitors in comparison to the treatment with only the transcriptional inhibitor ActD. The amount of *StSUT2* and *StSUT4* transcripts was increased compared with the untreated control (Fig. 9A and B). Thus, the half life of transcripts was prolonged by CHX even when the transcription was blocked.



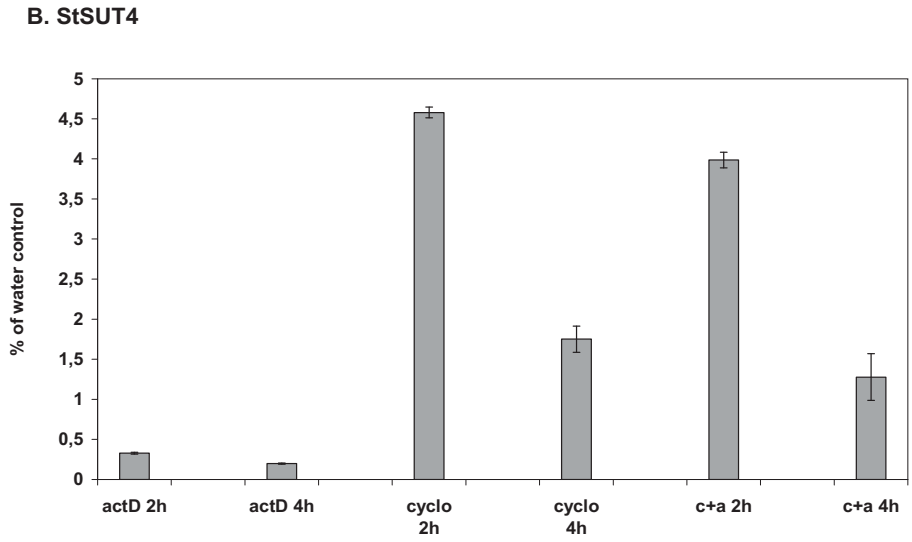


Fig. 9: Real-time RT-PCR analysis of sucrose transporter *StSUT2* and *StSUT4* mRNA stability in the presence of transcriptional and translational inhibitors

A. *StSUT2* mRNA quantification was determined in the presence of actinomycin D for 4 hours (ActD 4h), cycloheximide (cyclo 4h) and both inhibitors (a+c 4h), t0 represented samples before treatment. Half life of *StSUT2* mRNA was calculated based on real-time quantification and presented in table 4. *StSUT2* mRNA accumulated upon 4 h cyclo treatment (cyclo 4h). *StSUT2* mRNA half life was prolonged by cyclo even if transcription was blocked (a+c 4h). **B.** *StSUT4* mRNA half life was calculated by ActD treatment and presented in table 4. Inhibition of translation increased *StSUT4* transcript accumulation 4.5 fold within 2 h (cyclo 2h). *StSUT4* mRNA half life was prolonged by simultaneous treatment with translational and transcriptional inhibition (c+a 2h and c+a 4h). Quantification of transcripts was related to the amounts of the ubiquitin transcripts. In order to exclude diurnal changes of transcript levels, values were given in % of the corresponding water control after the indicated period of time.

In order to test whether SUT transcription depended on short-lived protein which was degraded via the 26S proteasome pathway, the transcript quantification was performed in the presence of the inhibitor of the 26S proteasome, MG132. The inhibitor of the 26S proteasome MG132 did not affect transcript stability or accumulation, whereas cycloheximide (CHX) led to a decreased *StSUT1* transcript amount (Fig. 10A). Simultaneous treatment with Cyclo and MG132 led to low *StSUT1* transcript amounts as with CHX alone. Therefore, the inhibitor of the 26S proteasome is not able to stabilize *StSUT1* mRNA in the presence of Cyclo. It is concluded that proteins responsible for high *StSUT1* transcript levels under normal condition (in the absence of CHX) are not degraded by the 26S proteasome since *StSUT1* transcripts disappear efficiently within 4 h of incubation with both inhibitors.

CHX had the opposite effect on *SUT2* and *SUT4* transcripts accumulation, the *StSUT2* and *StSUT4* transcripts were increased within only 2 h of treatment (Fig. 9A and B; Fig. 10B). The inhibitor of the 26S proteasome, MG132 had no significant effect on *StSUT2* transcript amounts, and it was able to prevent the stabilizing CHX effect on *StSUT2* transcripts leading to similar transcript amounts as seen in the untreated

control (Fig. 10B). Therefore, negative effectors on *StSUT2* transcript accumulation were most likely degraded via the ubiquitin-mediated 26S proteasome pathway since inhibition of their degradation abolished the effect of the inhibition of their synthesis.

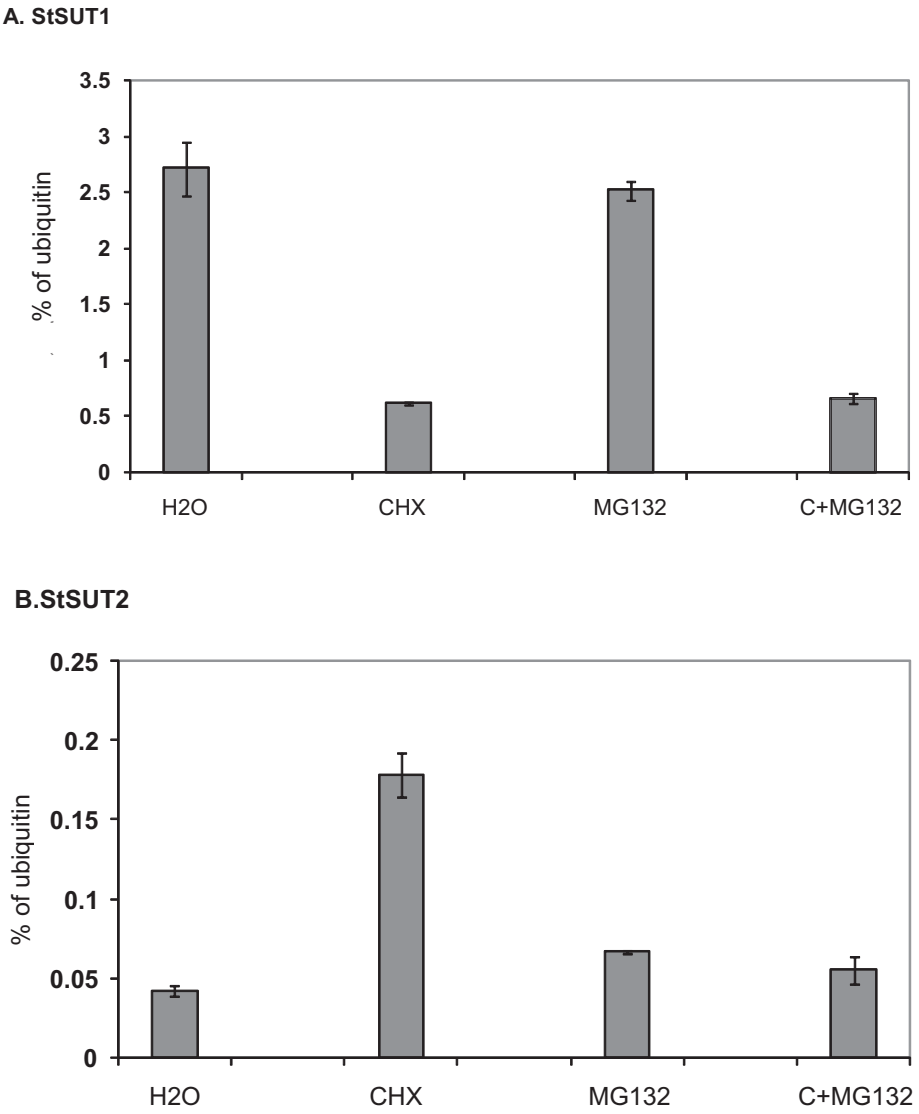


Fig. 10: Effects of the 26S proteasome inhibitor MG132 on sucrose transporter mRNA half life

A. 4 h cycloheximide (CHX) treatment affects *StSUT1* transcript stability. This effect is not decreased in the presence of the 26S proteasome inhibitor MG132. B. *StSUT2* mRNA level was increased upon 2 h Cyclo treatment due to increased mRNA stability. This stabilizing effect was abolished by the 26S proteasome inhibitor MG132. The qRT-PCR result was based on duplicate measurements for each sample. Y-axis: % of ubiquitin transcript in each sample, 1 equaled to 100%. Values were calculated by % of *ubiquitin* level in the same samples. 1 equals to 100%.

3.3 Analysis of *StSUT2&StSUT4-RNAi* potato plants

Post-transcriptional regulation of *StSUT2* and *StSUT4* mRNA stability can correlate with other physiological functions of SUT2 and SUT4 proteins. In this chapter, the RNA interference technique was employed to generate the *StSUT2&StSUT4-RNAi*

double transformants. The phenotypic and molecular characteristics were analyzed in the *StSUT2&StSUT4-RNAi* potato plants.

3.3.1 Confirmation of transformation

Several transgenic lines of *StSUT2-RNAi* potato expressing a kanamycin resistance gene as a selective marker were accessible for this study. *StSUT2* expression levels from sink leaves of different transgenic lines were analyzed by qRT-PCR. The result revealed that only line 28.1 had significantly reduced *StSUT2* expression compared to the wild-type control (Fig. 11). There was no significant phenotypic modification on these *StSUT2-RNAi* lines compared to wild-type *Désirée* regarding flowering and tuberization.

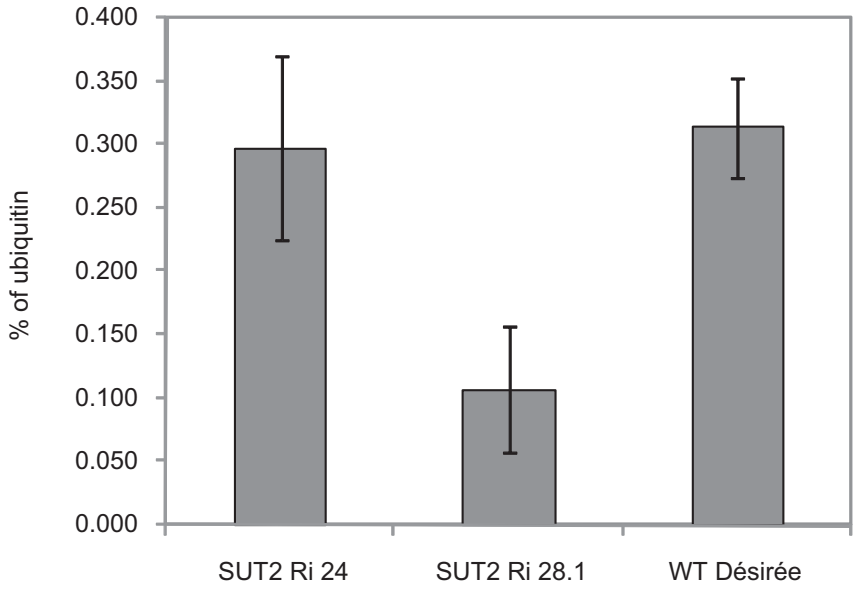


Fig. 11: The expression level of *StSUT2* gene in sink leaves of different *StSUT2-RNAi* lines

The RNA samples were taken from sink leaves of *StSUT2-RNAi* line 24, line 28.1 and wild-type *Désirée*. The qRT-PCR was performed using *StSUT2* specific primers. *UBIQUITIN1* (*UB1*) was used as an internal standard. The qRT-PCR result was based on duplicate measurements for each sample. Y-axis: % of ubiquitin transcript in each sample, 1 equaled to 100%. The error bars represented ± S.D.

The *StSUT4-RNAi* construct, which was already used for inhibition of *StSUT4* expression in transgenic potato plants, was originally cloned into the PstI site of pJH212, a pPZP212 derivative with a neomycin phosphotransferase gene (*NPTII*) as plant selectable marker (Chincinska et al., 2008). In order to clone the fragment in a binary vector with a different selectable marker gene, the *SUT4-RNAi* fragment was inserted into the PstI site of pCambia3301 (binary vector from Cambia, Australia) carrying a Basta resistance gene for plant selection (Fig. 12).

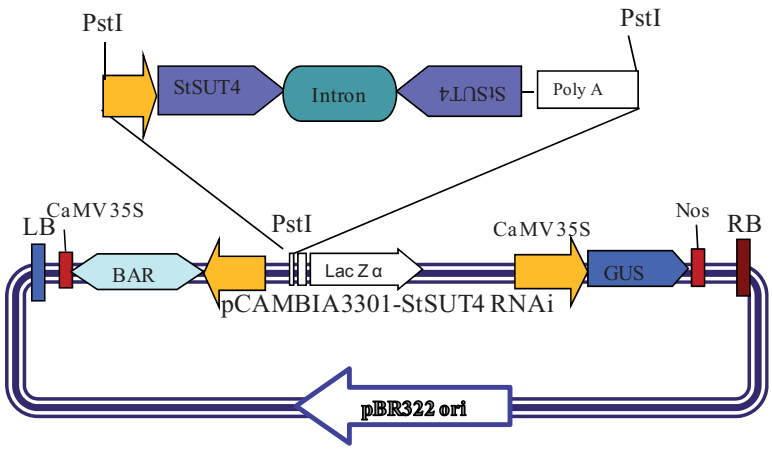


Fig. 12: pCAMBIA3301 containing a *StSUT4-RNAi* construct and an herbicide Basta resistance gene

The *StSUT4-RNAi* fragment from pJH212-SUT4 RNAi (Chincinska et al., 2008) was transferred into pCambia3301 by ligation into the PstI restriction site. There is the BAR gene (light blue hexagon) under CaMV 35S promoter (yellow arrow) control in pCambia3301 vector and followed by the CaMV 35S terminator (red), which encodes for the enzyme phosphinothricin-acetyltransferase and leads to increased tolerance towards the herbicide Basta.

According to the qRT-PCR results, transgenic *StSUT2-RNAi* line 28.1 had lower *StSUT2* level (Fig. 11). Subsequent gene transfer based on this transgenic line was performed with *Agrobacterium tumefaciens* (strain C58C1, pGV2260). Transgenic potato plants were generated by *Agrobacterium*-mediated transformation using both the Basta resistance (*PAT* gene encoding the phosphinothricin-acetyl transferase) and kanamycin resistance (*NPTII*) genes as the selective markers. There were no other phenotypic effects associated with transgenic expression during vegetative growth except tolerance towards kanamycin and the herbicide Basta. A number of putative transgenic lines were recovered on an antibiotic selection. Plants did not display any obvious morphological phenotype while cultivated on a selection medium under tissue culture conditions. After transfer of the regenerated plants to the greenhouse 14 of 16 transformants showed a similar phenotype like *StSUT4-RNAi* potatoes regarding flowering (Chincinska et al., 2008): the *StSUT2&StSUT4-RNAi* potato flowered 5 to 30 days earlier than wild-type *Désirée* (data not shown). The double transformants were confirmed by PCR amplification of BAR genes from the genomic DNA (Fig. 13, lane 1-10).

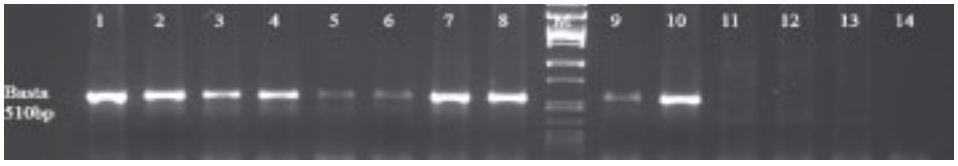


Fig. 13: PCR analysis of *BAR* gene from transgenic lines

Genomic DNAs were isolated from selected *StSUT2&StSUT4-RNAi* potato lines. PCR reactions were performed using BAR gene specific primers. No. 1-10 (double transformation lines): line 1-1, line 1-2, line 2-2, line 2-3, line 3-2, line 3-4, line 4-1, line 4-3, line 5-2, line 8-1, respectively. M, lambda-DNA PstI digested. 11: wild-type *Désirée*; 12: line 24.1 (*StSUT2-RNAi* potato); 13: line 38 (*StSUT4-RNAi* potato); 14: water control.

3.3.2 The expression level of *StSUT4* and *StSUT2* in *StSUT2&StSUT4-RNAi* potato lines

Previous study showed that *StSUT2* expression was reduced in the transgenic *StSUT2-RNAi* line 28.1 (Fig. 11), but these plants did not show significant phenotypical modification regarding flowering time, tuber formation etc. For further characterization of the double transformants, qRT-PCR results showed that *StSUT2* expression had no significant reduction in the *StSUT2&StSUT4-RNAi* line 3-4 (based on *StSUT2-RNAi* line 28.1). Nonetheless, *StSUT2* level was increased at several time points of the day compared to wild-type *Désirée* (at 6:00, 9:00, 12:00 and 21:00 in Fig. 14); a similar result was obtained in another double RNAi line 4-3 (data not shown).

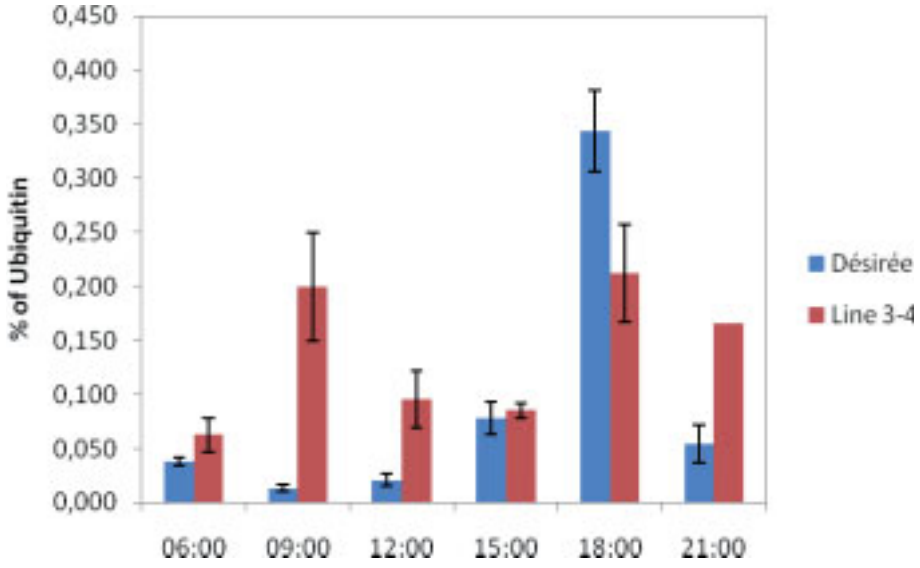


Fig. 14: Relative quantification of *StSUT2* expression in sink leaves of wild-type *Désirée* and *StSUT2&StSUT4-RNAi* line 3-4 via qRT-PCR

Only at 18:00, *StSUT2* was reduced compared to wild-type *Désirée*. At other times of the day, *StSUT2* transcripts were rather increased. *StSUT2* specific primers were used; the potato ubiquitin was used as an internal control. The qRT-PCR result was based on duplicate measurements for each sample. Y-axis: % of ubiquitin transcript in each sample, 1 equaled to 100%. The error bars represented ± S.D.

The *StSUT4* transcription level was lower in *StSUT2&StSUT4-RNAi* plants compared to wild-type *Désirée* plants at all detected time points. The plants were grown under LD condition, samples were collected from different time points, and qRT-PCR results showed that the *StSUT4* expression was repressed in double transformants, line

3-4 and line 4-3 compared to wild-type *Désirée* (Fig. 15). The diurnal oscillation of *StSUT4* in wild-type *Désirée* was different to published data which shown *StSUT4* peak accumulation in source leaves at 15:00 (Chincinska et al., 2008). These results indicate a different expression pattern in sink and source leaves.

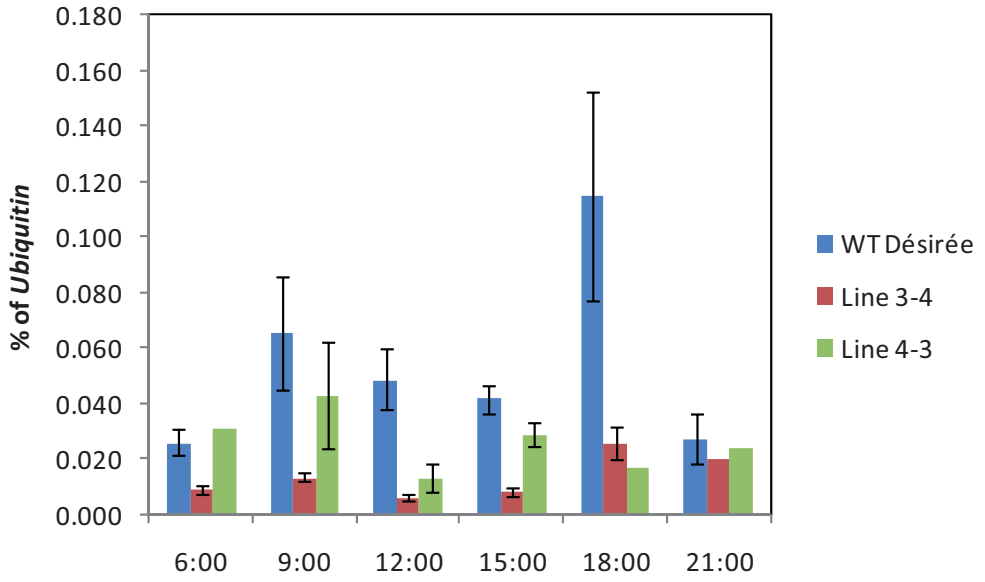


Fig. 15: qRT-PCR assay of *StSUT4* expression in sink leaves of *StSUT2&StSUT4-RNAi* potato plants under long day condition

Wild-type *Désirée*, *StSUT2&StSUT4-RNAi* line 3-4 and line 4-3 were grown under long day condition. The RNA samples were taken from sink leaves of the plants at different time points. The qRT-PCR result showed that up to 50% reduction of *StSUT4* expression level in both transgenic potatoes compared to relative time point wild-type *Désirée*. *StSUT4* specific primers were used. The potato ubiquitin was used as an internal control. The qRT-PCR result was based on duplicate measurements for each sample. Y-axis: % of ubiquitin transcript in each sample, 1 equaled to 100%. The error bars represented ± S.D.

Real-time RT-PCR analysis was performed using the sink leaves of another transgenic line 3-2; the results showed that both *StSUT2* expression and *StSUT4* expression were reduced compared to wild-type *Désirée* (Fig. 16). This transgenic line showed stronger phenotypes in the flower buds. Their flower buds were generated earlier than wild-type *Désirée*, and some of them were dead after 3-4 days.

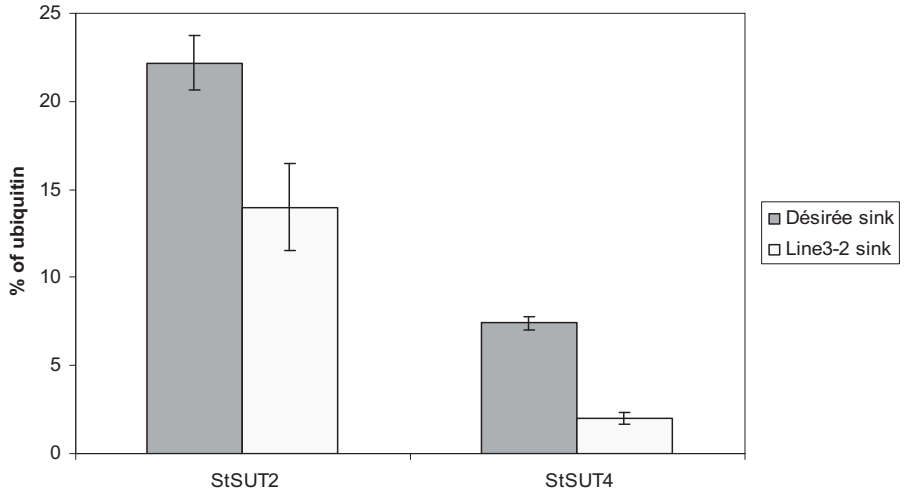


Fig. 16: qRT-PCR analysis of *StSUT2* and *StSUT4* expression in *StSUT2&StSUT4-RNAi* line 3-2 under LD condition.

Wild-type plants and transgenic line 3-2 were grown under long day condition. Plant samples were taken from sink leaves at 15:00. In sink leaves, the *StSUT2* expression is reduced by 30%, *StSUT4* expression is reduced by 70%. *StSUT2* and *StSUT4* specific primers were used in the qRT-PCR. The potato ubiquitin was used as an internal control. The qRT-PCR result was based on duplicate measurements for each sample. Values were given in % of ubiquitin transcript in each sample. Y-axis: 25 equals to 25% of ubiquitin expression. The error bars represent ± S.D.

3.3.3 Analysis of *StSUT2* and *StSUT4-RNAi* potato plants

3.3.3.1 Flowering time

Flower formation represents a transition from the vegetative to the reproductive phase of development. Flowering is a phase in the life of a plant when the shoot meristem is induced to develop sepals, petals, stamens, and carpels instead of leaves. In this work, the *StSUT2&StSUT4-RNAi* potato plants, in which *StSUT4* expression was significantly reduced, showed a similar phenotype like *StSUT4-RNAi* potatoes regarding flower induction and internode elongation. They flowered earlier than wild-type *Désirée* (Fig. 17A and D). Transgenic potato line 3-2 had different flower buds and flower petal color (B and E) compared to wild-type *Désirée* (C and F). When double transformants started to flower, it was noticed that they had less source leaves and were smaller than wild-type *Désirée* plants (Fig. 17A and B).

Fig. 17: Phenotype of *StSUT2&StSUT4-RNAi* plants

Picture A: *StSUT2&StSUT4-RNAi* line 4-3 was shorter than wild-type *Désirée* after 4 weeks growth in the greenhouse; picture B: *StSUT2&StSUT4-RNAi* line 3-2 had smooth flower buds, and differed from wild-type *Désirée*'s flower buds (picture C); picture D: line 4-3 flowered 5 days earlier than wild-type; the plant height of line 4-3 was shorter than wild-type *Désirée* when the plants started to flower; picture E: line 3-2 flower showed light color whilst wild-type *Désirée* flower petals were pink colored (picture F).

The source leaves from *StSUT2&StSUT4-RNAi* potato plants (10 different lines tested) at the beginning of flowering were significantly less than wild-type *Désirée* (13.8 source leaves instead of wild-type with 15.6 source leaves, Fig. 18A). The internode elongation of *StSUT2&StSUT4-RNAi* lines was slower, and the plant height of *StSUT2&StSUT4-RNAi* lines was shorter than wild-type *Désirée* when they started flowering (Fig. 18B).

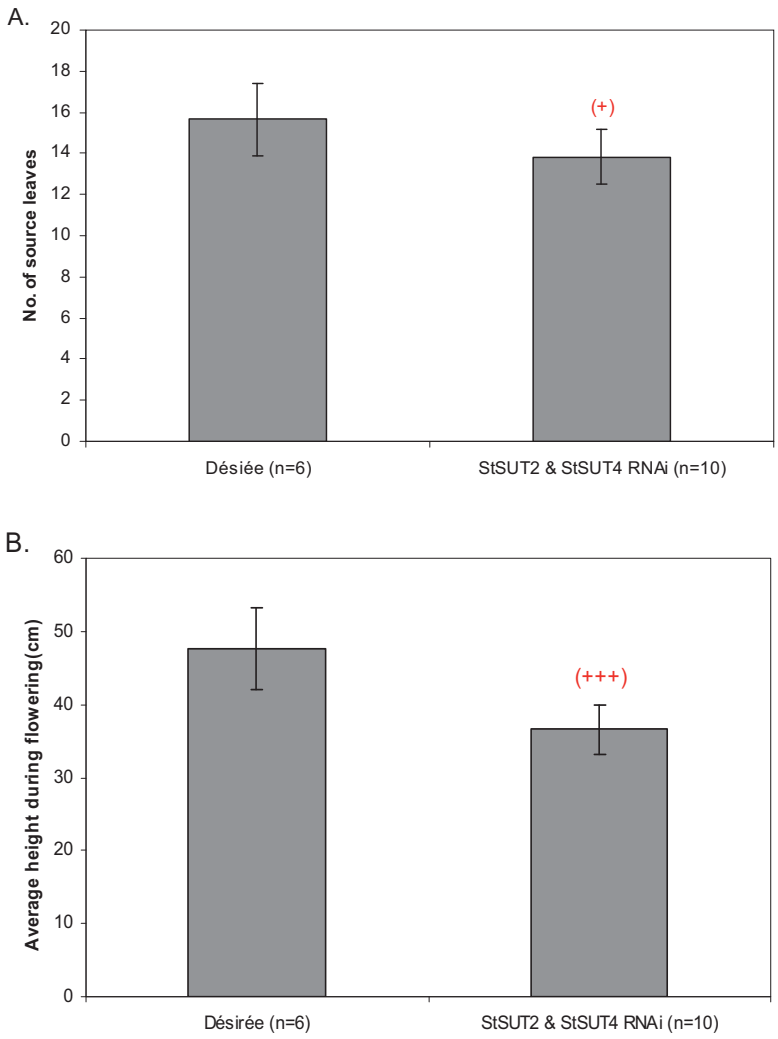


Fig. 18: The total height and number of source leaves of wild-type and *StSUT2&StSUT4-RNAi* potato plants starting to flower.

Graph A: the number of source leaves was counted when potato plants started to flower; graph B: the total height of *StSUT2&StSUT4-RNAi* potato was significantly lower than wild-type *Désirée*; the error bars represent ± S.D. The plus signs represented significant differences between members of a pair [unpaired t-test: (+) $P < 0.05$; (++) $P < 0.01$; (+++) $P < 0.001$)].

3.3.3.2 Pollen germination and viability

Pollen germination, along with pollen tube growth, is an essential process for the reproduction of flowering plants. Studies on pollen germination in potato are mostly aimed at developing a reliable test to access pollen fertility for breeding purposes. It is reported that SlSUT2 plays an important role during pollen germination and pollen tube growth (Hackel et al., 2006). Pollen sterility is a major obstacle to sexual recombination of potato (*Solanum tuberosum*. L) dihaploids. Pollen viability assays are useful to identify characters that correlate with observed fertility (Trognitz, 1991). Viability of pollen produced by *StSUT2&StSUT4-RNAi* lines was investigated in order to define whether StSUT2 had effects on pollen germination and fertility. Pollen grains were stained with FDA (fluorescein diacetate). FDA is a substrate processed by

an esterase in a living cell; released fluorescein results in a bright fluorescence of a viable pollen grain and proves the intactness of the plasma membrane. The pollen viability and pollen germination rate were analyzed in different potato lines. The viability of pollen from double transformants was lower than in wild-type and *StSUT4-RNAi* potato flowers according to the number of bright fluorescent pollen. Obviously, the *StSUT2-RNAi* line 28.1 and *StSUT2&StSUT4-RNAi* line 4-3 plants showed lower pollen viability than in wild-type *Désirée* and *StSUT4-RNAi* line 38 (Fig. 19).

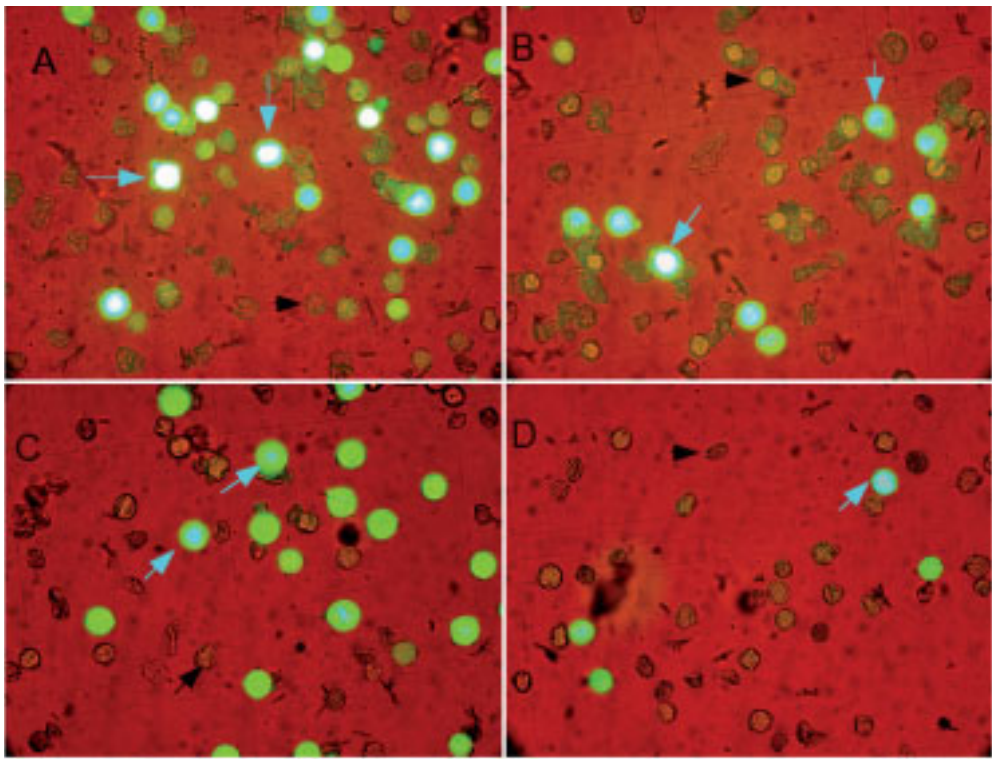


Fig. 19: Pollen viability tested by the FDA method

The pollens were collected from fresh flowers and were incubated in FDA medium for 30 min. Picture A: pollen from wild-type *Désirée*; picture B: pollen from *StSUT2-RNAi* line 28.1; picture C: pollen from *StSUT4-RNAi* line 38; picture D: pollen from *StSUT2&StSUT4-RNAi* line 4-3. The viable pollen grains fluoresced in a yellow green color, whilst the non-viable grains were not stained. Light blue arrows showed viable pollens, black arrows showed unviable or damaged pollens.

By qRT-PCR analysis, the reduced expression of *StSUT2* and *StSUT4* was discovered in the apex of *StSUT2&StSUT4-RNAi* potato line 8-1 and line 5-2 (Fig. 20A). An alternative method was performed in this investigation to confirm the above observation in these two transgenic lines. Therefore, pollen from different transgenic potato plants were incubated for 30 min in B&K medium, and pollen viability was calculated by counting the stained pollen granules. Low pollen viability was observed in other transgenic lines. Further statistical analysis showed that the double transformants line 5-2 had lowest pollen viability (18%) whereas *StSUT2-RNAi* line 28.1 had a slightly higher pollen viability of 30%. The pollen of double transformant

line 5-2 and the *StSUT2-RNAi* line 28.1 presented a significant lower viability than WT pollen (Fig. 20B).

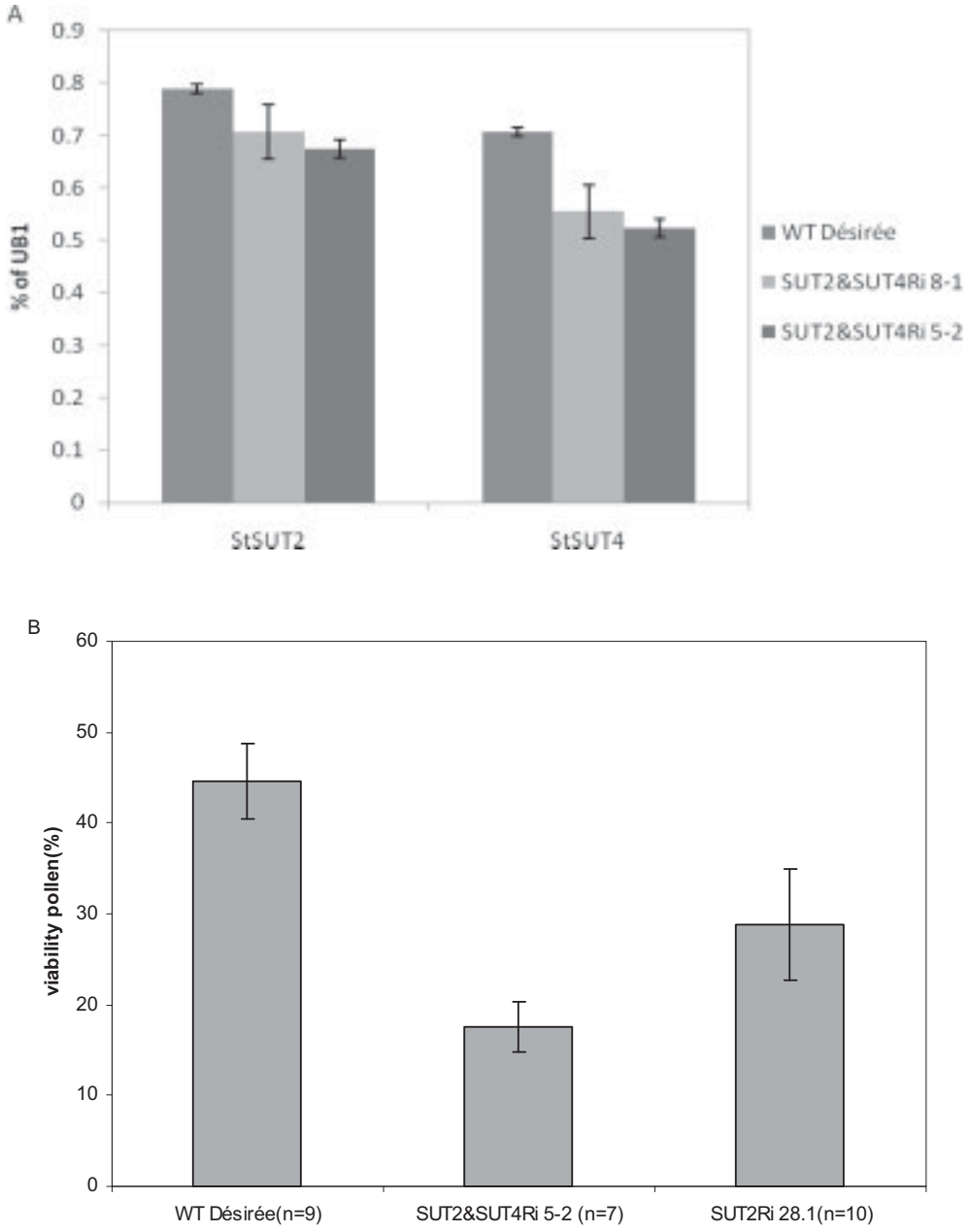


Fig. 20: The pollen viability of *StSUT2&StSUT4-RNAi* potato in B&K medium.

Graph A, the wild-type *Désirée*, *StSUT2&StSUT4-RNAi* line 8-1 and line 5-2 were transferred to the greenhouse from sterile room at the same time. The RNA samples were taken from 6 week old potato apex. qRT-PCR analysis was performed using cDNAs as templates. *StSUT2* and *StSUT4* specific primers were chosen, and ubiquitin 1 primers were used for internal standard control. The qRT-PCR result was based on duplicate measurements for each sample. Values were calculated by % of ubiquitin expression level. 1 equaled to 100%. Graph B, pollen viability of *StSUT2&StSUT4-RNAi* potato (Line 5-2), *StSUT2-RNAi* potato (Line 28.1) was compared to wild-type pollen. The viability was calculated by % viable pollen out of total counted pollen and determined three times independently. For each experiment between 50 and 200 pollen grains were counted. The error bars indicated ± S.D.

To investigate the effect of StSUT2 inhibition on pollen germination, the pollen germination experiments were performed. Pollen taken from the above plants was incubated in B&K medium at 26°C overnight. The pollen germination rate from tested potato plants was very low, even in wild-type *Désirée* plants (12%±2%); the double transformants had more abnormal pollen and the pollen germination rate was lower than wild-type *Désirée* (Fig. 21).

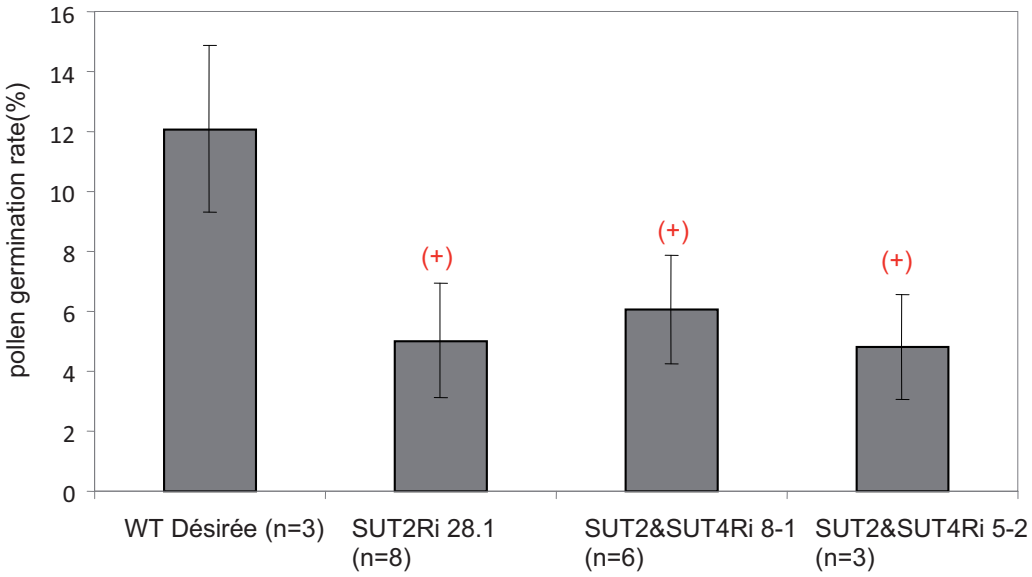


Fig. 21: The pollen germination rate of different transformants and wild-type plants.

Graph B, the pollen germination rate of about transgenic plants was calculated. *StSUT2&StSUT4-RNAi* (Line 8-1 and Line 5-2) and *StSUT2-RNAi* (SUT2Ri 28.1) showed lower germination rate than wild-type *Désirée*. The error bars represented ± S.D. Plus signs (+) represented significant differences between members of a pair (unpaired t test: (+) $P < 0.05$).

3.3.3.3 Tuber yield of transgenic *StSUT2&StSUT4-RNAi* potato plants

StSUT4-RNAi potato plants are described to produce a higher tuber yields compared to wild-type potato plants (Chincinska et al., 2008). In this study, two generations of *StSUT2&StSUT4-RNAi* plants (10 different lines) were planted in the greenhouse together with wild-type *Désirée* to calculate the tuber production of transgenic plants. After 10 weeks under LD conditions, watering was stopped and tuber yield of each pot was measured after two more weeks. Most of the tested transgenic lines showed reduced tuber yield compared to the average tuber yield of wild-type (Fig. 22). The line 3-2, which showed apparent phenotypes, had a significant reduction of tuber yield: only 15-25% of the wild-type tuber yield.

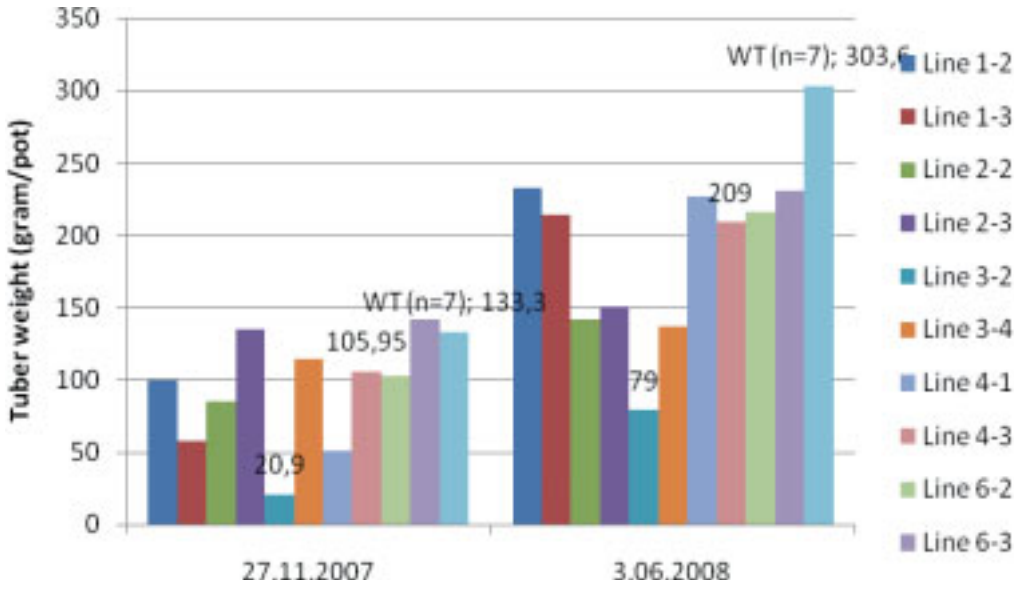


Fig. 22: Comparison of tuber yield in different *StSUT2&StSUT4-RNAi* lines

Two generations of WT *Désirée* (n=7) and different *StSUT2&StSUT4-RNAi* potato plants were put into the greenhouse at the same day, watering was stopped after 10 weeks. The tuber yield from individual pot was measured at 27.11.2007 and 03.06.2008 respectively.

Another observation regards the changed number of branches in *StSUT2&StSUT4-RNAi* potato plants. The transformant line 8-1 and line 5-2 contained slightly more branches compared to wild-type *Désirée* and *StSUT4-RNAi* line 81 (Fig. 23A). Unlike the *StSUT4-RNAi* potato plants, double transformants (line 8-1 and line 5-2) showed a slightly reduced or wild-type like tuber yield in comparison to wild-type potatoes (Fig. 23B). This was consistent with reduced tuber yield in other 10 transgenic lines (Fig. 22). Nonetheless, differences in tuber yield were not significant.

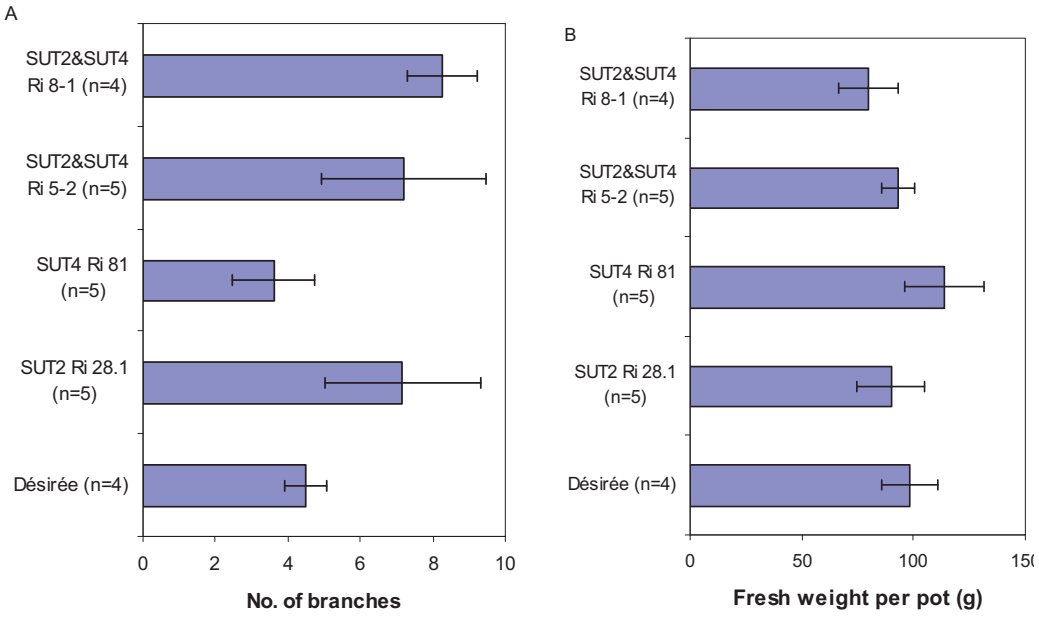


Fig. 23: Branch number and tuber yield of different potato lines.

Graph A, average branch number per pot of different potato lines: SUT2&SUT4 Ri 8-1 (n=4): four individual pots of double transgenic line 8-1; SUT2&SUT4 Ri 5-2 (n=5): five individual pots of double transgenic line 5-2; SUT4 Ri 81 (n=5), five individual pots of *StSUT4* RNAi line 81; SUT2 Ri 28.1 (n=5): five individual pots of *StSUT2-RNAi* line 28.1; WT *Désirée* (n=4): four individual pots of wild-type *Désirée*. Graph B, the average tuber yield of different potato lines, the line names are the same as graph A, tuber yield is given in gram (fresh weight); X-axis, unit of measurement is gram. The error bars represent ± S.D., the number of measured pots was indicated in the brackets (n).

3.3.3.4 Tuber dormancy and sprouting

It is known that potato tubers may exhibit all three types of dormancy: immediately after harvest the meristems (eyes) are in a state of endodormancy, not sprouting even when temperature and moisture are at favorable conditions. The other two types are ecodormancy (due to environmental factors) and paradormancy (due to external physiological factors) (Vreugdenhil, 2007). Previous investigations on tuber dormancy are mainly referred to endodormancy. It has been suggested that many factors are involved in tuber dormancy, such as hormones. They are defined as low-molecular weight (mobile) compounds which influence growth and development in low concentrations and have been often suggested to play a crucial role for tuber dormancy regulation. It is described that both ABA and ethylene are required for dormancy induction. Cytokinins are involved in the breaking of dormancy whereas gibberellins and auxins are more likely involved in subsequent sprout growth (Vreugdenhil, 2007).

In this study tubers of *StSUT2-RNAi* and *StSUT2&StSUT4-RNAi* potato plants sprouted 10 to 35 days later than potato wild-type tubers (data not shown). After 2 months storage in a cold room the wild-type tubers started to sprout, whereas tubers from transgenic potato plants needed a longer dormancy. It was observed that the sprouts of wild-type tubers were longer then those of *StSUT2-RNAi* line 28.1 and double transformants line 8-1 and line 5-2 (Fig. 24A). Since the tuber dormancy and sprout state were unequal, the potato plants showed different growth levels 40 days after the transfer into soil (Fig. 24B).

Fig. 24: Tuber sprouting of different transgenic potato plants.

Photo A, tubers start sprouting after three months stored in cool room, wild-type tubers (top left) had longer sprouts than transgenic *StSUT2-RNAi* line 28.1 (top right) and *StSUT2&StSUT4-RNAi* (line 8-1 (bottom, left) and line 5-2 (bottom, right). Photo B, potato tubers were put into soil at the same time, *StSUT2&StSUT4-RNAi* line 3-2, line 3-4 and line 4-3 were sprouting 35 days, 20 days and 10 days later than wild-type *Désirée* respectively.

3.4 SUT4 over-expression in tobacco plants

The tandem affinity purification (TAP) strategy is an efficient approach for protein complex purification from many different organisms such as bacteria (Gully et al., 2003), mammalian cells (Knuesel et al., 2003), insect cells (Forler et al., 2003), tobacco leaves (Rohila et al., 2004), rice (Rohila et al., 2006) and Arabidopsis (Rubio et al., 2005; Leene et al., 2007). In addition, the TAP system allows protein isolation under native conditions, which contributed to the functional studies in which the activity or post-translational modification studies on one protein or protein complex can be examined (Puig et al., 2001). The original idea of this study is to identify SUT4 protein-protein interaction partners using TAP techniques and co-immunoprecipitation (Co-IP). The transgenic tobacco plants containing N-terminal

TAP fused with SlSUT4 protein were generated in this study in order to get more information about the physiological function of SUT4 proteins in *Solanaceae* and to obtain the opportunity to isolate SUT4-interacting proteins by tandem affinity purification.

3.4.1 Confirmation of tobacco transformation

3.4.1.1 Recombinant pN-TAPa SlSUT4 construct

According to BLAST analysis based on EST databases there is 91% similarity between *SlSUT4* and *NtSUT4* (Altschul et al., 1990). Therefore, *SUT4* over-expressing tobacco plants were generated in order to analyze the physiological function of SUT4 in solanaceous plants in this study. For this purpose, the *SlSUT4* gene from tomato was cloned into the binary vector pN-TAPa allowing tandem affinity purification of the tagged protein. In short, the *SlSUT4* open reading frame from tomato (*Solanum lycopersicon ssp. Moneymaker*) flower cDNA was PCR-amplified using attB sequence containing primers and cloned into the pDONR 221 plasmid using the BP reaction (GATEWAY; Invitrogen). At this stage, DNA sequence analysis was performed. The transfer of genes from the pDONR 221 plasmid to the corresponding TAPa vector was performed using LR reaction (GATEWAY; Invitrogen). The pN-TAPa SlSUT4 construct was used for subsequent tobacco transformation (Fig. 25).

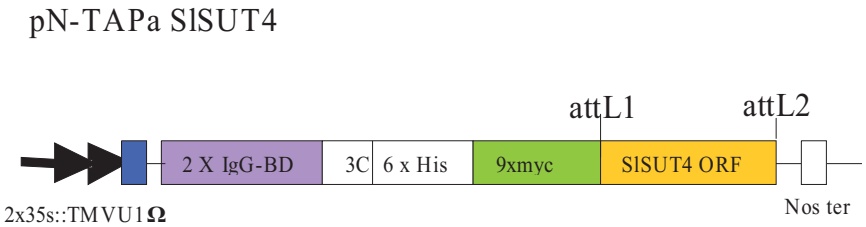


Fig. 25: Diagrammatic representation of the expression cassette in N-TAPa SlSUT4 vector

pN-TAPa SlSUT4 vector allows translational fusion of the N-terminus of proteins of interest to the TAPa tag. The expression is driven by two copies of the cauliflower mosaic virus 35S promoter (2x35S, black arrows) and a tobacco mosaic virus (TMV) U1 Ω translational enhancer (blue box). The TAPa tag consists of two copies of the protein A IgG binding domain (2× IgG-BD, purple box), an eight amino acid sequence corresponding to the 3C protease cleavage site (3C, white), a six histidine stretch (6× His, white), and nine repeats of the myc epitope (9× myc, green box). pN-TAPa vector contains GATEWAY cloning sites (*att*R1::Cm^r::ccdB::*att*R2). A Nos terminator (Nos ter) sequence is located downstream of each expression cassette (Rubio et al., 2005), the open reading frame of *SlSUT4* from potato are subcloned into GATEWAY cloning sites of N-TAPa by BP and LR reaction (yellow box).

3.4.1.2 Confirmation of successful transformation

Tobacco plants (*Nicotiana tabacum* cv. Samsun NN) were transformed by the leaf co-cultivation method. Transgenic tobacco plants with over-expressed *SlSUT4* under the

constitutive CaMV 35S promoter showed gentamicin resistance since the construct for transformation contained the gentamicin acetyltransferase resistance gene. The regenerated tobacco plants were screened by PCR amplification on genomic DNA. Two out of fourteen tested plants had 1.5 kb amplicon using *SlSUT4* gene specific primers (data not shown). Moreover, these two transgenic plants developed visible symptoms compared to wild-type SNN. The seeds collected from line 02 and line 05 then were selected on MS medium contained 30 mg/L of gentamicin. The resistant seedlings were transferred to the greenhouse. Using a CaMV 35S promoter forward primer and *SlSUT4* reverse primer for PCR amplification of genomic DNA of several transgenic lines, a 1 kb DNA-band was detected which was absent in the wild-type SNN (Fig. 26).

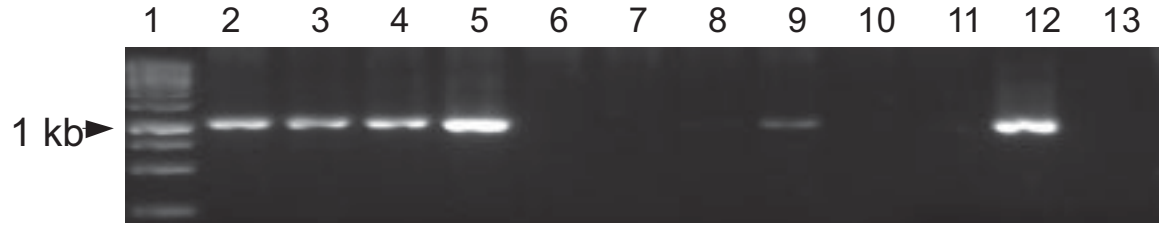


Fig. 26: PCR confirmation of N-TAPa SlSUT4 transformed tobacco plants

The genomic DNA was isolated from several transgenic lines and wild-type using the CTAB method. PCR amplification was performed with CaMV 35S promoter forward primer and *SlSUT4* reverse primer. Lane 1: 1kb molecular ladder (Fermentas); lane 2-lane 9: line 02 T1-1, line 02 T1-2, line 02 T1-3, line 05 T1-1, line 05 T1-2, line 05 T1-3, line 02 T2-1, line 02 T2-2, line02 T2-3; lane 10 and line 11, two wild-type SNN; lane 12, positive control pN-TAPa SlSUT4 plasmid DNA; lane 13, water as a negative control.

3.4.2 *SlSUT4* mRNA accumulation in N-TAPa *SlSUT4* 02 tobacco

Quantitative RT-PCR was performed with cDNA of source leaves taken from wild-type *SNN* and N-TAPa $SlSUT4_{02}$ tobacco at 9:00, 11:00, 13:00, 15:00, and 17:00. The results showed that *SlSUT4* expression was significantly induced in the transgenic line 02 at the beginning of the light period (Fig. 27, 9:00 and 11:00), the increased amounts of SUT4 transcripts were not detected in the afternoon (13:00, 15:00 and 17:00). It cannot be excluded that WT levels of *SUT4* mRNA were due to amplification of the endogenous *NtSUT4* mRNA because of the high homology between *NtSUT4* and *SlSUT4* (91%).

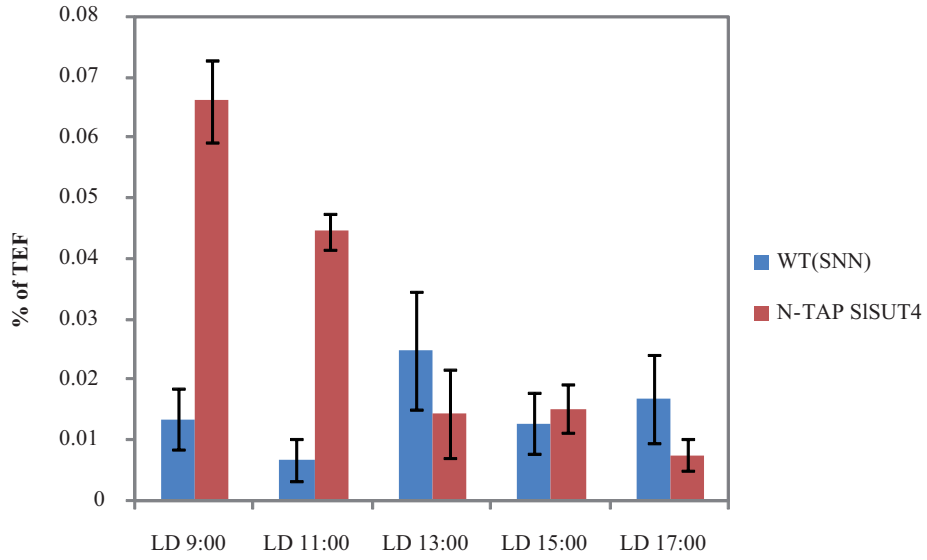


Fig. 27: *SUT4* accumulation in wild-type SNN and N-TAPa SlSUT4$_{02}$ over-expressing tobacco plants

The samples were taken from source leaves of 5 weeks old tobacco plants grown under long day conditions (LD). SUT4 accumulation is up-regulated in the morning between 9:00 and 11:00, down-regulated in the afternoon 13:00 and 17:00. The error bars represent ± S.D. Due to the high sequence similarities between *StSUT4* and *SlSUT4*, *StSUT4*-specific primers were used for qRT-PCR. Internal standard control is the *TRANSLATION ELONGATION FACTOR* (TEF). The qRT-PCR result was based on duplicate measurements for each sample. Values were given in % of the level of TEF transcripts in each sample, 1 equals to 100%.

3.4.3 SlSUT4 protein level in N-TAPa SlSUT4 tobacco

The transgenic plants were also analyzed at the protein level. The N-TAPa tag is 293 amino acids in length; the encoded protein is around 32 kDa (Rubio et al., 2005). To determine the expression level of the TAP-tagged protein, total proteins were isolated from leaves of T0 transgenic tobacco and WT SNN plants. Immunoblotting with monoclonal anti-Histidine antibody (Sigma) showed that additional bands (around 55 kDa, 46 kDa and 30 kDa) were detected in N-TAPa SlSUT4 line 02 and line 05 compared to wild-type SNN (Fig. 28). None of these additional bands was in the expected size of the full length N-TAPa SlSUT4 fusion protein.

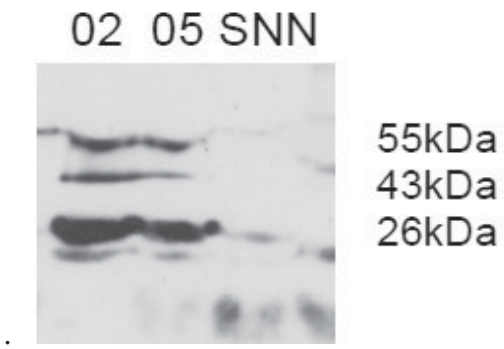


Fig. 28: Immunoblot confirmation of T0 N-TAPa SlSUT4 transgenic tobacco lines

Total protein samples (30 μg) extracted from T0 generation of N-TAPa SlSUT4 line 02, line 05 and wild-type SNN were loaded onto 12.5% SDS-PAGE. Immunoblotting was performed using monoclonal anti-histidine serum. 02: N-TAPa SlSUT4 02; 05: N-TAP SlSUT4 05; SNN, wild-type SNN.

In order to identify the SUT4 protein level an affinity-purified polyclonal antibody, which was raised against a SlSUT4 peptide, was used for immunoblot assays. The SlSUT4 protein has a calculated molecular weight of 54.29 kDa. The apparent molecular mass of the protein recognized by the anti-SUT4 polyclonal antiserum is 46 kDa corresponding to the apparent molecular mass of the hydrophobic protein in SDS-PAGE (Weise et al., 2000). Two bands (46 kDa and 55 kD) were detected in N-TAPa SlSUT4 tobacco line 02-2, line 02-3, and line 02-4 (Fig. 29, lane 2, 3 and 4), while only one very weak band was detected in wild-type SNN (Fig. 29, lane 1). The calculated molecular mass of the tagged fusion protein was expected to be in the range of 80 kDa (N-TAPa tag is 293 amino acids and its molecular weight is around 32 kDa). However, only bands of a truncated protein bands were detected on the Western blots.

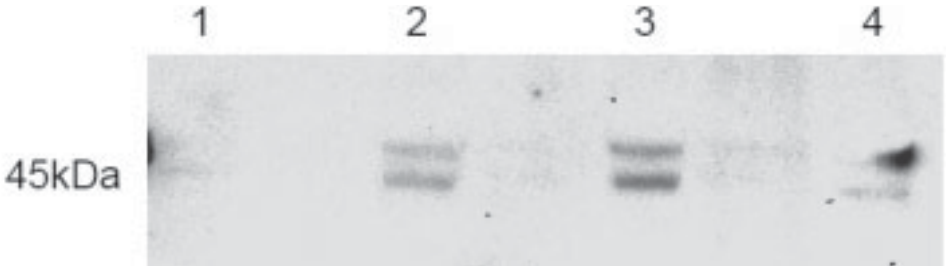


Fig. 29: Immunoblot confirmation of SlSUT4 protein level in N-TAP SlSUT4 line02 transgenic lines

Total protein samples (30 μg) extracted from T1 generation of N-TAPa SlSUT4 line02 were loaded onto 12.5% SDS-PAGE. Immunoblotting was performed using specific SlSUT4 antibodies. Lane 1, total protein from WT SNN; lane 2, total protein from N-TAPa SlSUT4 line 02-2; lane 3, total protein from N-TAPa SlSUT4 line 02-3; lane 4, total protein from N-TAPa SlSUT4 line 02-4.

Obviously, the N-TAPa SlSUT4 fusion protein was not integral according to the above immunoblot results (Fig. 28 and 29). Thus, it was impossible to use tandem

affinity purification techniques with commercial TAG antibody (anti-Histidine or anti-myc) on these transgenic tobacco plants. Nonetheless, the SlSUT4 protein was over-expressed in the transgenic plants (Fig. 29). Some phenotypic observations were performed on them (shown as follow).

3.4.4 Phenotypes of N-TAPa SlSUT4 tobacco plants

Internodes of the N-TAPa SlSUT4 tobacco plants were slightly shorter but thicker. Plant growth was retarded before flowering and the shape of source leaves were asymmetric. The source leaves were abnormally shaped compared to wild-type *SNN* (Fig. 30A) and this phenotype was observed in transgenic line 02, line 05 and their next two generations. The transgenic plants showed a semi dwarf phenotype before flowering. Towards the end of the vegetative period (before flowering), these differences decreased substantially. The transgenic tobacco plants had a longer vegetative period resulting in late flowering compared to wild-type *SNN*. They had more internodes when starting flowering (Fig. 30B and C).

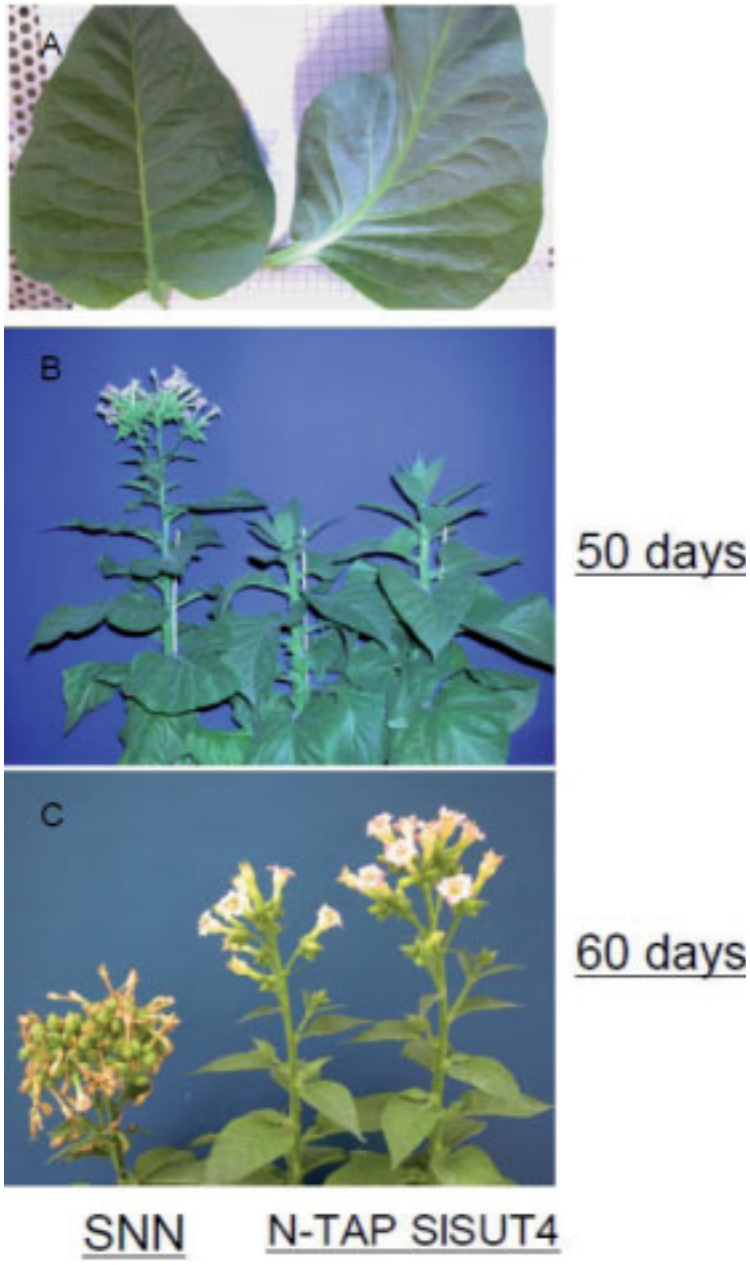


Fig. 30: Phenotype of N-TAPa SlSUT4$_{02}$ tobacco lines

Photo A, source leaves of wild-type SNN and N-TAPa SlSUT4 tobacco line 02-T1 generation; photo B, wild-type tobacco started to flower 50 days after transfer into the green house, while transgenic tobacco still maintained vegetative growth; photo C, N-TAPa SlSUT4 line02-T1 tobacco are taller than SNN 60 days after transfer into the greenhouse, they started flowering 10 days later than wild-type SNN tobacco plants.

Since *SlSUT4* transcripts showed a maximal accumulation at 9:00 (Fig. 27) the SUT4 protein might reach a highest level two hours later. Therefore, samples for determination of the accumulation of soluble sugars were taken at 11:00 when SlSUT4 protein was supposed to be expressed at higher levels. Sugar concentrations were measured in wild-type SNN and transgenic N-TAPa SlSUT4 line02-T1 tobacco leaves. The amounts of glucose, fructose and sucrose were significantly increased in transgenic plants at 11:00 in the morning: the glucose content in N-TAPa SlSUT4

tobacco plants was 4.5 fold higher than in WT plants, the fructose content was around 2 fold and the sucrose content was increased by 20% of wild-type (Fig. 31). Thus, SUT4 over-expression led to increased levels of soluble sugars in source leaves.

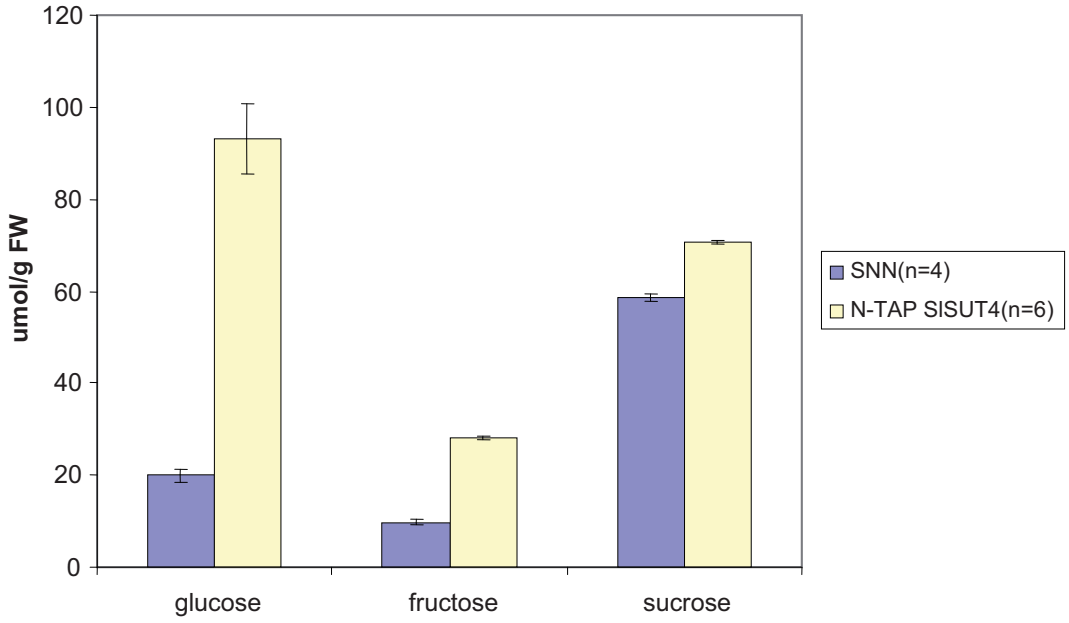


Fig. 31: Concentrations of soluble sugars in leaves from N-TAPa SlSUT4 02-T1 plants

Samples were taken from source leaves of 6 week-old tobacco plants at 11:00 (at the beginning of photoperiod). Soluble sugars were extracted using 80% ethanol and determined using enzymatic assay. The error bars represent ± S.D. of number of tested plants in the brackets.

Overexpression of *SUT4* leading to higher amounts of soluble sugars in leaves might be explained by an inhibitory effect of SUT4 on SUT1 activity. Starch was accumulated in transgenic *SlSUT4* over-expressing tobacco source leaves due to the increased amount of sugars (Fig. 32). The wild-type and N-TAPa SlSUT4 line 02-T1 tobacco leaves were kept in darkness for 16 hours. Wild-type plants had only low levels of starch after prolonged darkness, whereas N-TAPa SlSUT4 line 02-T1 tobacco showed strong iodine staining. This indicates that they were unable to mobilize excess carbohydrate accumulated during the previous day (Fig. 32: left, WT SNN; right, transgenic N-TAPa SlSUT4 line 02-T1).

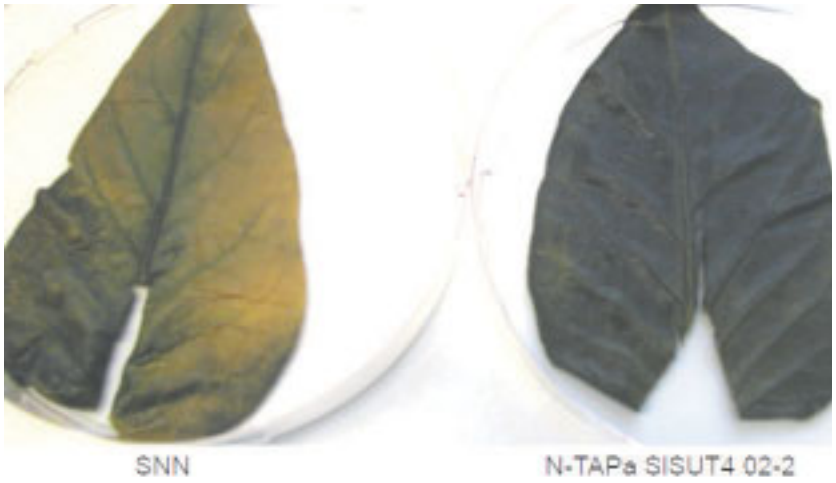


Fig. 32: Starch accumulation in N-TAPa SlSUT4 over-expressed tobacco source leaf

The tobacco source leaves, both SNN (left) and N-TAPa SlSUT4 over-expressed line 02-2 (T1 generation) (right), were covered with foil for 16 hours. After destaining for one hour incubation at 70°C with 80% ethanol the leaves were stained with Lugol's solution.

The growth speed of transgenic plant line 02-T1 was slow before flowering (Fig. 33) especially at stage 2 (from day 37 to day 45 after transferred into greenhouse). The wild-type tobacco plants grew 2.5 cm per day during this stage while the transgenic plants only grew 1.25 cm per day (Fig. 33). The wild-type started flowering at stage 3 (from day 45 to day 52 after transfer to greenhouse) while transgenic plants still stayed at the vegetative phase. After flower the wild-type tobacco plants almost stopped growth, transgenic plants started growing faster, reaching higher size than wild-type plants at the period of flowering phase.

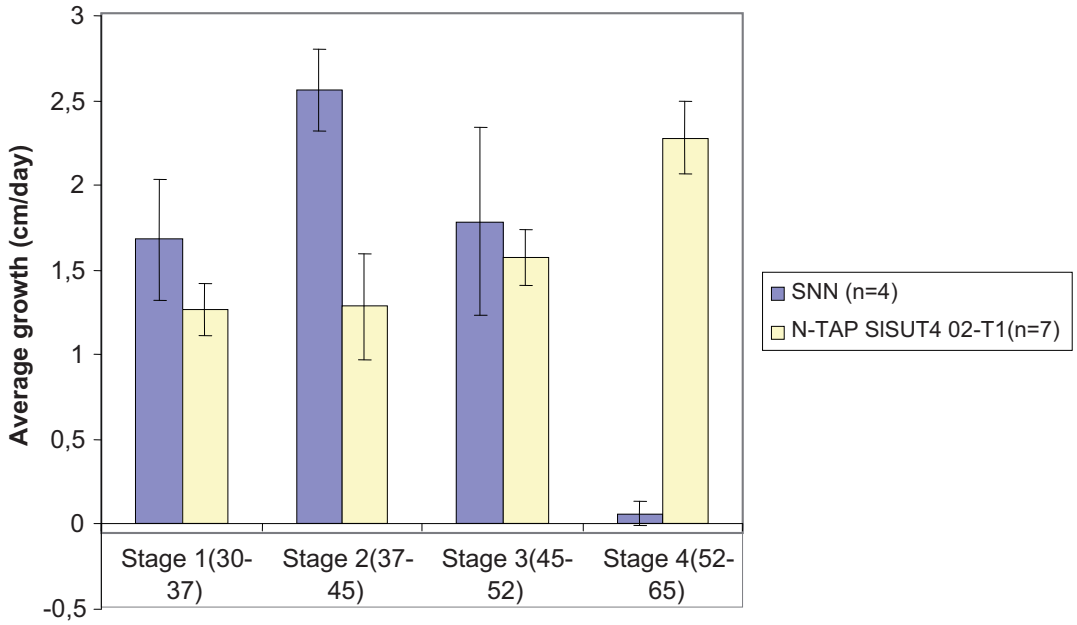


Fig. 33: The average growth speed of transgenic N-TAPa SlSUT4 line 02-T1 tobacco plants

The wild-type SNN (n=4) and transgenic N-TAPa SlSUT4 line 02-T1 generation (n=7) tobacco plants were transferred from tissue culture to the greenhouse at the same time. The plant height was

measured after 30 days, 37 days, 45 days, 52 days and 65 days. Afterwards, average growth speed was calculated by cm per day (cm/day).

The stamens of transgenic line 02 and line 05 looked abnormal, and the anther produced less pollen. The ovary of transgenic line 02 and line 05 was not developed very well and it was dark brown colored. Some of the transgenic plants were sterile. Although, some of them were fertilized, the seed yield of transgenic tobacco was significantly reduced compared to wild-type SNN (transferred to the greenhouse from tissue culture at the same time). T0 generation of these transgenic lines only produced a few seeds. The T1 generation of line 02 showed a significant reduction of the seed yield compared to wild-type SNN (Fig. 34).

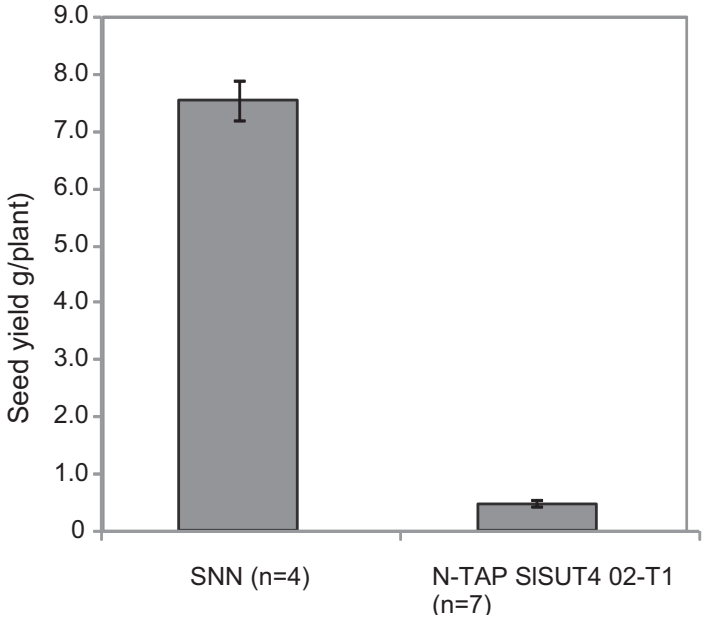


Fig. 34: Seed yield of transgenic N-TAPa SlSUT4 02-T1 tobacco plants

Wild-type SNN (n=4) and transgenic N-TAPa SlSUT4 tobacco line 02 plants (T1 generation, n=7) were kept in tissue culture before transferring to greenhouse. They were put into pots at same time. The seeds of individual plant were collected after maturation, and the weight of seeds was measured. Average seed yield was calculated in gram per plants.

3.4.5 SUT4 in solanaceous plants is a plasma membrane protein

It has already been shown that StSUT4 is a plasma membrane protein in potato by infiltration tobacco and potato leaves with *StSUT4-GFP* fusion construct and Western-Blot using StSUT4 specific antibody (Chincinska et al., 2008). Here, the plasma membrane vesicles (PMV) of tobacco plants were isolated using two-phase partitioning system. The resultant protein samples contained the followings: total protein (from first supernatant), microsomal fraction (pellet after second centrifugation), and endomembrane fraction (low phase of the beginning two-phase purification) and plasma membrane vesicles (last pellet after 3-4 two-phase purification and ultra centrifugation). The protein concentration was measured by

BCA assay, 20 µg of protein was loaded, and then western blot was performed using SlSUT4 specific antibodies raised against a 22 amino acid synthetic peptide of the SlSUT4 central loop region. The corresponding region of NtSUT4 (EST sequences are available) shares 86% identity with SlSUT4 at the protein level (19 out of 22 amino acids are identical), so it is assumed that the anti-SlSUT4 antiserum can cross-react with the tobacco NtSUT4 protein. The SUT4 protein with a molecular mass of about 46 kDa could be detected in plasma membrane vesicles (PMV) only, of both wild-type and transgenic *SlSUT4* tobacco plants. There was a slightly increased SUT4 protein amount in transgenic SlSUT4 line 02-T1 compared to wild-type tobacco plants (Fig. 35, lane 4: SNN PMV and lane 7: SlSUT4 line 02-T1 PMV). In the endomembrane fraction no SlSUT4 protein of the correct size was detectable (lane 3 and lane 6). The smaller band of around 35 kDa (in MF and EF) was supposed to be a degradation product of the SUT4 protein.

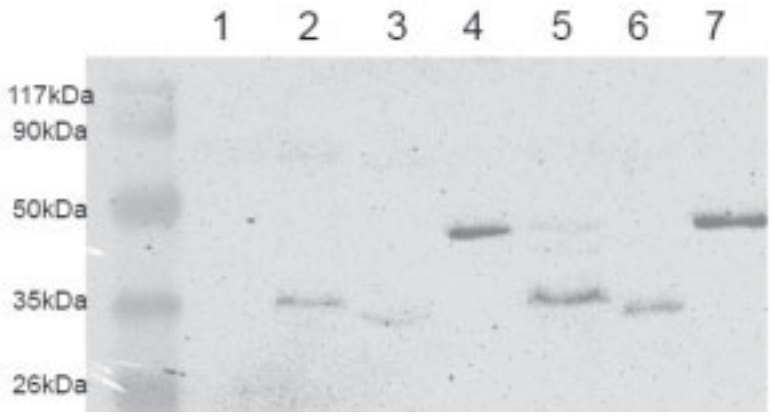


Fig. 35: Immunoblot of SlSUT4 in different tissues

Plasma membrane vesicles (PMV) were isolated using two-phase partitioning from wild-type SNN and N-TAPa SlSUT4 02-T1 tobacco plants, while total protein from wild-type SNN and microsomal fraction (MF), endomembrane fraction (EF) from wild-type SNN and N-TAPa SlSUT4 02-T1 were also isolated. 20 µg of protein was loaded to 12.5% SDS-PAGE, immunoblotting with SlSUT4 specific antibody. Sample order: Lane 1, SNN total protein; lane 2, SNN MF; lane 3, SNN EF; lane 4, SNN PMV; lane 5, SlSUT4 02-T1 MF; lane 6, SlSUT4 02-T1 EF; lane 7, SlSUT4 02-T1 PMV.

3.5 The effects on genes involved in flower induction and tuberization in *StSUT4-RNAi* potato plants

Observations of *StSUT4-RNAi* potato plants revealed early flowering, a higher tuber yield, and reduced sensitivity to normal shade and artificial shade (with enriched far-red light) conditions (Chincinska et al., 2008). Potato tuberization is one of multiple outputs of the single-input phytochrome B sensory system. Several genes are involved in the phytochrome B sensory system, such as *GIGANTEA* (*StGI*), *CONSTANS*-like (*StCOL3*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*StSOC1*) and *FLOWERING LOCUS T* (*StFT*) (Rodríguez-Falcón et al., 2006; Sarkar, 2007;

Rutitzky et al., 2009). Phytochromes specifically phytochrome B also play a role in photoperiodic control of flowering in both SD- and LD-plants (Martínez-García et al., 2002b). There are good evidences to indicate that the flowering and tuberizing signals might be similar. Regulatory pathways involved in day-length control of tuberization appear to be closely related to those controlling flowering time in *Arabidopsis* (a LD plant) or rice (a SD plant) (Jackson, 1999; Martínez-García et al., 2002a; Rodríguez-Falcón et al., 2006).

The SUT4-mediated flower and tuber induction was graft-transmissible, wild-type potato plants from both *ssp. andigena* and *ssp. tuberosum* grafted with *StSUT4-RNAi* plants that included their source leaves, showed a similar phenotype as transgenic *StSUT4-RNAi* plants: they displayed early flowering and produced higher amounts of tubers compared to grafted control plants (Chincinska et al., 2008). It is suggested that a phloem mobile molecule may be involved in triggering flowering time and tuberization in a *SUT4*-dependent manner. In this chapter, the expression of homologous genes involved in the photoperiod-dependent flowering and tuberization pathway, such as *GI*, *COL3*, *SOC1* and *FT* was investigated in *StSUT4-RNAi* potato plants.

3.5.1 StSUT4 accumulation was induced under FR light enrichment

Here, the accumulation of *StSUT4* in detached WT potato leaves was calculated by qRT-PCR. The detached potato leaves were put under white light or under white light enriched in far-red wavelengths. The results showed that the *StSUT4* expression level was increased under far-red light induction especially after four hours induction. The expression level of *StSUT4* was significantly induced compared with white light condition (Fig. 36). These results applied further arguments for the assumption that *StSUT4* expression might be controlled by of phytochrome B.

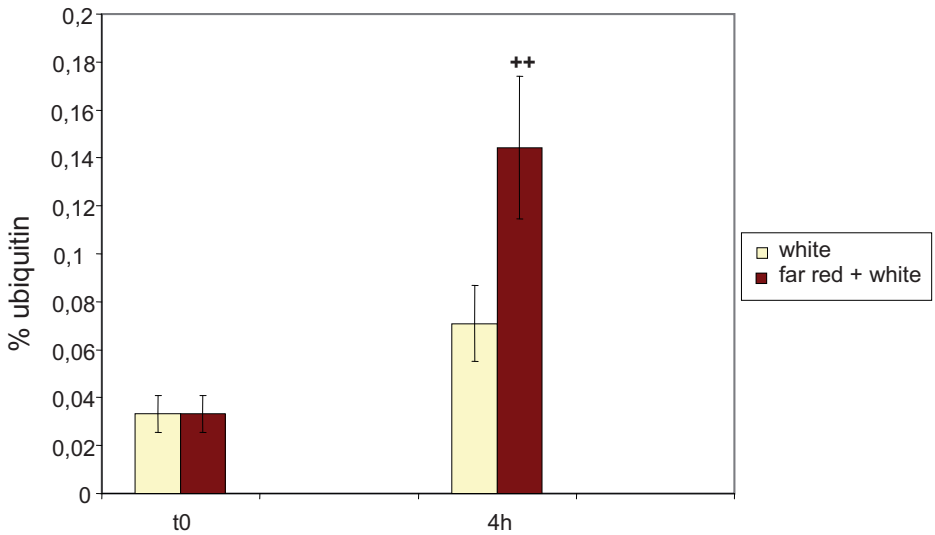


Fig. 36: StSUT4 mRNA accumulation under far-red light enrichment

The detached potato leaves (WT *Désirée*) were put into EDTA buffer (2.5 mM, pH 6.0) under white light or white light together with additional far red light. Samples were taken before far-red light induction (t0) and after 4 hours induction (4h). Under far red enrichment StSUT4 expression was significantly induced. After 4 hours its expression level was twice as high as under white light condition. *StSUT4* specific primers were used in qRT-PCR analysis, the potato *ubiquitin* mRNA level was used as an internal control. The qRT-PCR result was based on duplicate measurements for each sample. Values were given in % of the level of ubiquitin transcripts in each sample, 1 equals to 100%. The error bars represent ±S.D.

3.5.2 Increased StSUT4 mRNA accumulation was caused by prolonged half life of transcripts under FR enrichment

With the help of the transcriptional inhibitor ActD the half life of *StSUT4* transcripts under additional far red light was determined by qRT-PCR. After 30 min treatment the *StSUT4* mRNA amount was twice as high as under white light conditions. However after 90 min treatment SUT4 transcript level was reduced under both conditions: white light condition and far-red enrichment, compared to the water control. The SUT4 mRNA accumulation was reduced slower under far-red light enrichment. The half life of *StSUT4* transcripts was prolonged significantly compared to its half life under white light (Fig. 37). These results argued for a post-transcriptional control of SUT4 transcript stability. It was assumed that the prolonged SUT4 half life time under far-red light enrichment involved RNA-binding proteins protecting the SUT4 mRNA for sequence-specific mRNA decay by ribonucleases.

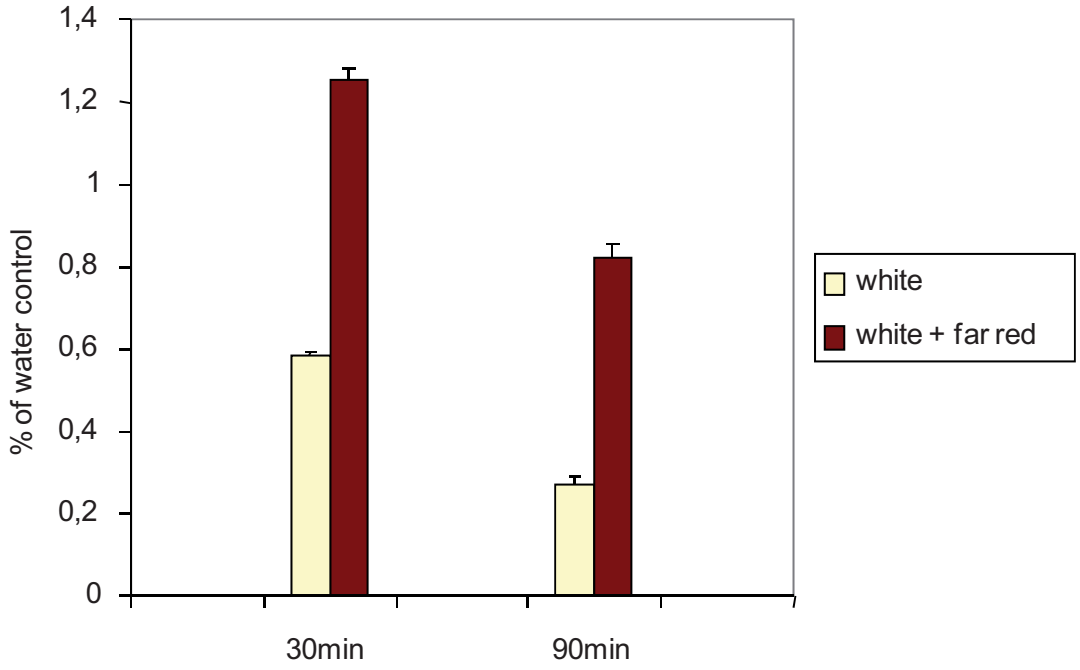


Fig. 37: The prolongation of SUT4 half life under far red light enrichment.

Detached potato leaves (WT *Désirée*) were treated with 10 µM of ActD under white light or white light together with additional far-red light. Samples were taken after 30 minutes (30min) and 90 minutes induction (90min). The SUT4 transcripts accumulated two fold after 30 min of additional far red light

induction compared with the white light control. After 90 minutes induction, the SUT4 transcript level was reduced, while under far-red enrichment, disintegration was slower than under white light condition. StSUT4 specific primers were used in qRT-PCR analysis. The potato ubiquitin transcripts levels were used as an internal control. The SUT4 expression in the ActD treated sample was compared with relative water control. Y-axis: 1 equals to 100% of water control. The error bars represent + standard deviation (S.D).

3.5.3 Far-red light affects *StSUT4* and *StJAR1* accumulation

Many potato species, such as *Solanum demissum* and *Solanum tuberosum* ssp. *andigena*, require short days for the induction of tuberization and will not tuberize either if maintained in photoperiods longer than their critical day-length, or if the dark period of a short day regime is interrupted by a light treatment (what is termed a night-break, NB) (Ewing and Struik, 1992). Phytochrome is known to be involved in photoperiodic responses such as tuberization and flowering because the effects of a red light pulse during the night can be reversed by a far-red light treatment given immediately afterwards - a well-documented characteristic of phytochrome responses (Jackson et al., 1996).

Transgenic *Solanum tuberosum* ssp. *andigena* plants with a lower level of phytochrome B were produced using antisense technology (Jackson et al., 1996). The anti-PHYB plants were insensitive under far-red light enrichment and they became able to tuberize in both long and short days. Thus, the reduction of phytochrome B levels led to the loss of the photoperiodic control of tuberization in these plants. Jackson (1999) stated that phytochrome B plays a major role in photoperiodic response.

Jasmonic acid and related cyclopentanones are fundamental plant signaling molecules. In Arabidopsis, jasmonate response 1 (*JAR1*) gene is similar to the auxin-induced soybean *GH3* gene. *JAR1* is reported to be involved in phytochrome signaling pathway in Arabidopsis and rice (Staswick et al., 2002; Tanaka et al., 2002). JAR1 is able to form jasmonic acid conjugates with the ethylene precursor-aminocyclopopane-1-carboxylic acid (ACC) (Halitschke and Baldwin, 2005). This evidence indicates a role of JAR1 in the cross-talk between jasmonate and ethylene signaling. In this study a high homologous gene of jasmonate-resistant4 in tobacco (*NtJAR4*) was found in potato plants and was named *StJAR1*. The full length of the cDNA assembled with available ESTs in *Solanum tuberosum* has a length of 1912bp. The encoded protein has a predicted molecular weight of 64.6 kDa (data not shown). In order to determine whether StJAR1 expression is methyl jasmonate (MeJA) inducible, qRT-PCR analysis was performed in MeJA treated detached potato leaves. The results indicated that *StJAR1* was induced by MeJA after 60 min treatment with 50 μM MeJA (Fig. 38).

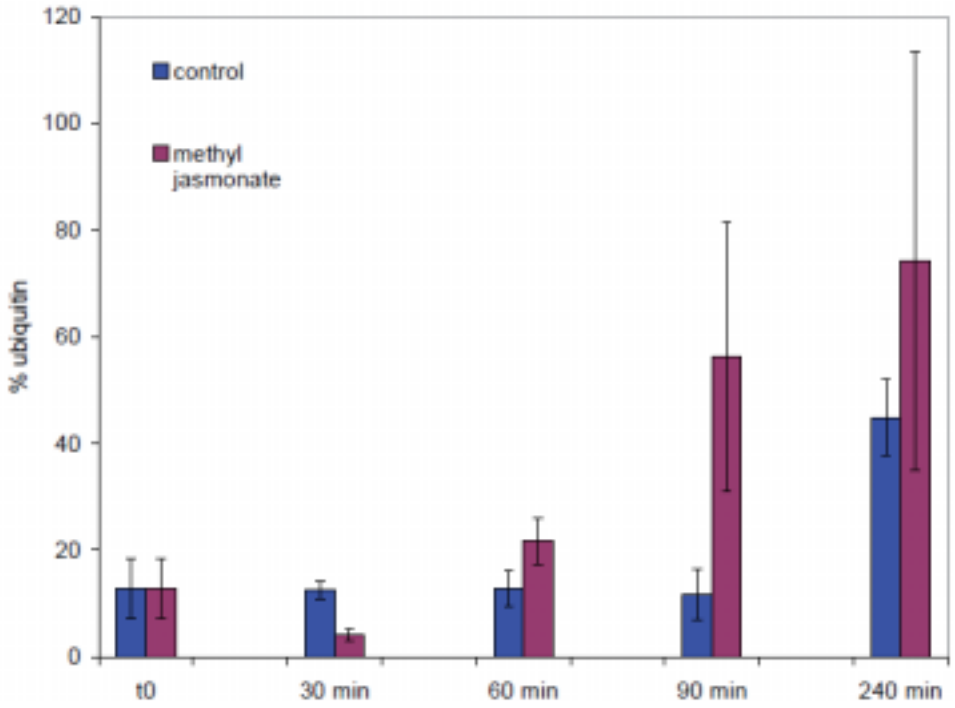


Fig. 38: Real-time RT-PCR of StJAR1 expression in potato leaves treated with 50 µM methyl jasmonate (MeJA)

Detached potato leaves were treated with 50 µM MeJA and water solution (for control). Samples for RNA isolation were collected before treatment (t0), after 30 min, 60 min, 90 min and 240 min treatment. *StJAR1* specific primers were used in qRT-PCR of *StJAR1* expression, potato *ubiquitin* RNA contents were used as internal standard. The qRT-PCR result was based on duplicate measurements for each sample. Values were given in % of *ubiquitin* expression of each sample. The error bars represent ± standard deviation (S.D.).

Moreno et al., (2009) demonstrated that FR light effects on jasmonate signaling were functional significant, since FR light failed to increase tissue quality in jar1 (a mutant impaired in jasmonate response). In this work, wild-type potato ssp. *andigena* and anti-PHYB *andigena* together with *StSUT4-RNAi* potato plants were grown under white light or white light together with FR light at least for 6 days. The *StSUT4* and *StJAR1* expression in these plants were analyzed by qRT-PCR. Results showed that the *StSUT4* expression was induced under far-red light enriched conditions in wild-type *andigena*. While in anti-PHYB *andigena* plants and *StSUT4-RNAi* line 81 plants, the *StSUT4* accumulation was almost no change under far-red light enrichment, and the *StJAR1* accumulation was reduced under far-red light compared to white light condition (Fig. 39). Since anti-PHYB potato plants showed a constitutive shade avoidance syndrome, it could be speculated that high levels of *StJAR1* and *StSUT4* were needed for the development of SAS in response to shading.

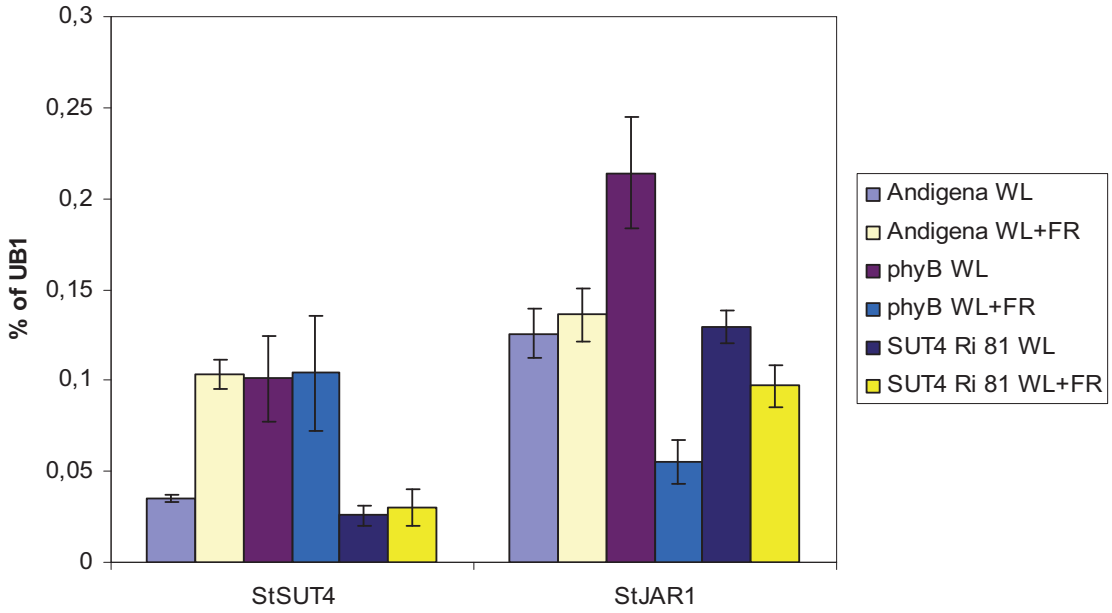


Fig. 39: The expression of *StSUT4* and *StJAR1* in wild-type *andigena, StSUT4-RNAi* and anti-PHYB line under different light conditions

The expression of *StSUT4* and *StJAR1* in potato *WT andigena, StSUT4-RNAi* line 81 and *phytochrome B* antisense plants under different light conditions was determined by real-time PCR. Potato wild-type and anti-PHYB plants were kept for 6 days under white light or under white light complemented with additional far-red light to mimic artificial shade conditions by decreasing the red: far-red ratio. The gene specific primers of *StSUT4* and *StJAR1* were used in qRT-PCR reaction. The qRT-PCR result was based on duplicate measurements for each sample. Values were calculated in % of *ubiquitin* expression of each sample. Y-axis: 1 equals to 100%. The error bars represent ± standard deviation (S.D.).

3.5.4 The expression of *GIGANTEA (GI)* in *StSUT4-RNAi* potato plants

The *GIGANTEA* (*GI)* gene in *Arabidopsis* was isolated by Fowler et al. (1999). It encodes a novel, putative membrane protein and the *GI* expression is regulated by the circadian clock with a peak in transcript levels 8–10 h after dawn. *gi* mutant was independently identified on the basis of a defect in inhibition of hypocotyls elongation in red but not in far-red light, which implicates GI in PHYB signaling (Huq et al., 2000). GI promotes flowering under LD in *Arabidopsis thaliana* (a LD plant) and represses flowering under SD in rice (a SD plant) (Yanovsky and Kay, 2003). The expression of a *GI* homolog gene in potato is higher under LD compared to SD. The gene is also higher in wild-type *Andigena* plants compared to transgenic plants with reduced *PHYB* level under LD condition. These results indicate that GI could be responsible for the repression of tuberization under LD in potato (Rutitzky et al., 2009). To determine the relationship between *StSUT4* and the phytochrome B-dependent pathway, real-time PCR analysis of *StGI* and *StJAR1* in the apex of different transgenic potatoes was performed. It was noticed that *StJAR1* transcripts were increased in *StSUT4-RNAi* (line 81), while the expression of *StJAR1* in *StSUT2-RNAi* (line 28.1) was not changed (Fig. 40). These findings begged the question

whether StJAR1 might be involved in the post-transcriptional regulation of StSUT4. *StGI* expression was seen to be reduced in *StSUT4-RNAi* potato plants (line 81). This was probably due to the inhibition of StSUT4 (Fig. 40). In addition, StGI is known to be involved in the tuberization (and flower inducing) pathway in potato plants (Rodríguez-Falcón et al., 2006). Therefore, this study begged also the question whether *StSUT4* and *StGI* are members of the same tuber and flower inducing pathway.

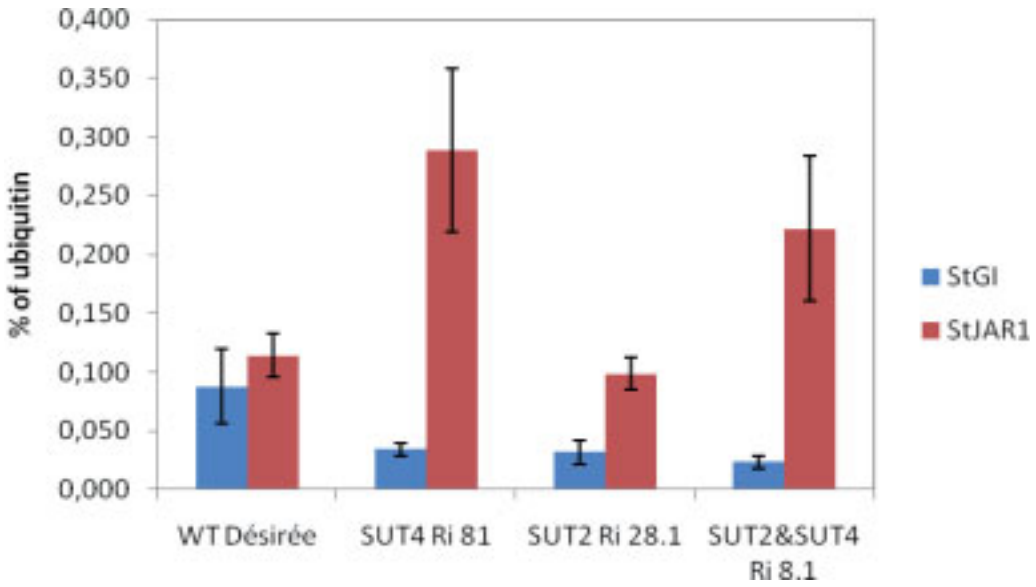


Fig. 40: The gene expression of *StJAR1* and *StGI* in different potato lines

The *StJAR1* expression was increased 1.5 fold and 0.8 fold in *StSUT4-RNAi* line 81 and double RNAi line 8.1 compared to wild-type potato plants, but it was not increased in *StSUT2-RNAi* line 28.1; the expression level of *StGI* was reduced in all three transgenic lines. Gene specific primers of *StGI* and *StJAR1* were used in qRT-PCR. Ubiquitin was used as an internal control. The values were given in % of the level of ubiquitin transcripts in each sample. The qRT-PCR result was based on duplicate measurements for each sample. The error bars represent ± S.D.

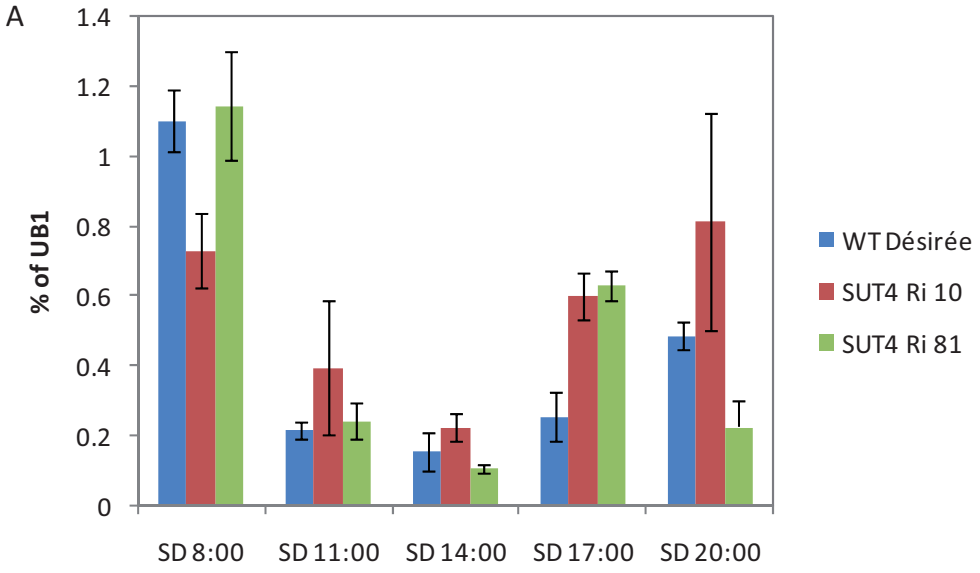
3.5.5 The expression of different flowering genes in *StSUT4-RNAi* potato plants under SD condition

In order to elucidate the molecular mechanism of the early flowering phenotype of *StSUT4-RNAi* potato plants, the expression of *StCOL3* (potato *CONSTANS*-like 3), *StSOC1* (potato *suppressor of CONSTANS1*) and *StFT* (potato Flower Locus T) genes in these plants had been analyzed by I. Chincinska (PhD dissertation, HU, 2009). *StSUT4-RNAi* line 10, line 81 and wild-type potato plants were placed under LD conditions (16h/8h, light/dark cycle: from 6 o'clock to 22 o'clock in the light period), and RNA samples were taken from source leaves at different time point. Real-time RT-PCR analysis was performed using gene specific primers. The *StCOL3* mRNA level was reduced in *StSUT4-RNAi* line 10 and line 81 compared to wild-type control at most of the tested time points. The relative *StSOC1* expression level in transgenic

plants was also reduced, so the inhibition of flowering by *StSOC1* was missing. *StFT* mRNA level was induced in *StSUT4-RNAi* potato plants. The accumulation of *StFT* mRNA was able to promote the synthesis of StFT protein. The resultant protein promoted flower induction under LD condition in *StSUT4-RNAi* potato plants.

For further investigation, the *StSUT4-RNAi* line 10 and line 81 together with wild-type potato plants were grown under SD condition (10h/14h, light/dark cycle: from 8:00 to 18:00 light period). The samples were taken every three hours (shown as SD 8:00, SD 11:00, SD 14:00, SD 17:00 and SD 20:00 in Fig. 41); qRT-PCR analysis was performed with the cDNA of the samples. The following results confirmed that the expression of flowering induction genes was affected in *StSUT4-RNAi* transgenic lines under SD condition as well. Increased accumulation of *StCOL3* transcripts in *StSUT4-RNAi* potato plants had been observed at the end of the light period (Fig. 41A); the level of *StSOC1* transcripts was increased as well (Fig. 41B). It is known that *StCOL3* has a promoting effect on FT expression under SD conditions (Rodríguez-Falcón et al., 2006). Thus, it is not surprising to detect higher levels of *FT* transcripts in StSUT4 RNAi plants as a consequence of *StCOL3* mRNA accumulation (Fig. 41C).

These results were analyzed with two biological replicates.



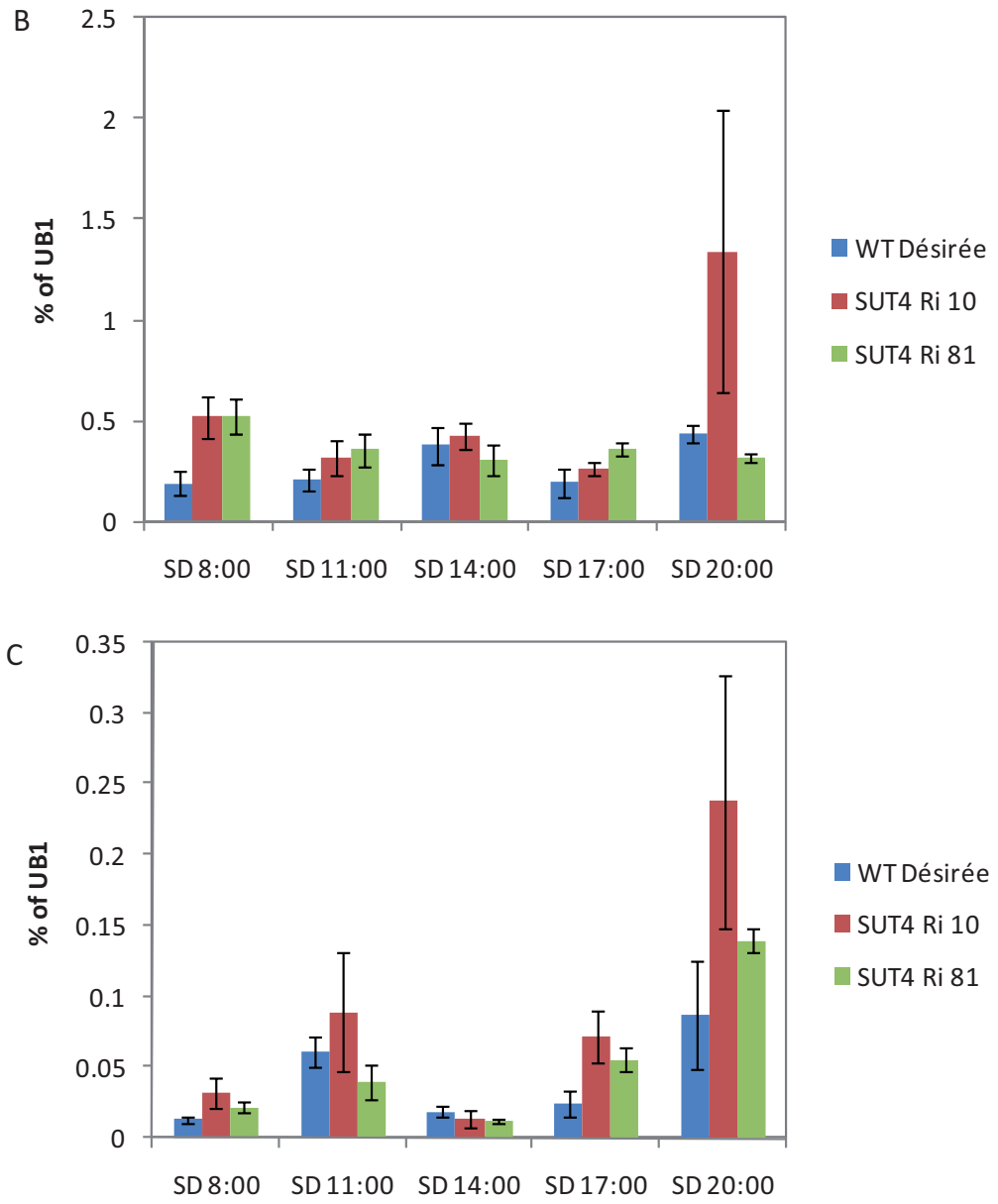


Fig. 41: The expression levels of *StCOL3, StSOC1 and StFT* in StSUT4-RNAi potato plants under short day (SD) conditions

The 5 week old wild-type *Désirée* and *StSUT4-RNAi* potato (line 10 and line 81) were grown under SD condition. RNA samples were taken at 8:00, 11:00, 14:00, 17:00 and 20:00. The expression level of *StCOL3* (A), *StSOC1* (B) and *StFT* (C) was analyzed using qRT-PCR. The relative gene specific primers were used. The qRT-PCR result was based on duplicate measurements for each sample. Y-axis: % of ubiquitin transcript in each sample, 1 equaled to 100%. Values were calculated in % of ubiquitin level (as an internal standard). The error bars represented ± S.D.

3.6 Confirmation of SUT4 protein-protein interaction partners

Previous studies have shown the important physiological function of SUT4 in solanaceous plants. To get more intensive knowledge about StSUT4 protein, it is helpful to investigate the protein interaction partners of SUT4 protein. The split-ubiquitin membrane yeast two-hybrid screen using StSUT4 as bait protein had been performed by Jana Reins (Reins, 2006). Several putative StSUT4 interaction partners

were identified. In this study, an alternative method (BiFC) was employed to confirm the interactions between StSUT4 and two of the putative interaction partners: a protein disulfide isomerase and a putative ethylene receptor in plant cells.

3.6.1 The confirmation of protein disulfide isomerase (PDI) interacting with SUT4 protein

Among the interaction partners identified by Jana Reins and Undine Krügel using the yeast split-ubiquitin assay, a protein disulfide isomerase (PDI) was found in all three screens (StSUT1, SlSUT2 and StSUT4 as bait respectively). PDIs are oxidoreductases that catalyze the formation, reduction, and isomerisation of disulfide bonds in newly synthesized secretory proteins, which are in the lumen of the endoplasmic reticulum, ER (Freedman et al., 1995). PDI-mediated disulfide bond formation in the target proteins is necessary for their proper folding, stability, catalytic activity and interaction with other proteins (Aslund and Beckwith, 1999). In plants, PDIs have been shown to assist the folding of storage proteins during the biogenesis of protein bodies in the seed endosperm (Li and Larkins, 1996). In Arabidopsis, the expression of some PDI genes is up-regulated in response to the unfolded proteins (Martinez and Chrispeels, 2003).

Undine Krügel has already confirmed the interaction between SUT4 and PDI by GST pull-down assay (PhD dissertation, HU 2008). We aimed at visualization of this protein-protein interaction in plant cells using Bimolecular Fluorescence Complementation (BiFC). The open-reading frame of PDI (accession number EST707280) was fused to the C-terminal half of YFP (pSPYCE) using GATEWAY technology (StPDI-SPYCE). BiFC vectors (Walter et al., 2004) contain the enhanced YFP (E-YFP). The StSUT4 was fused to the N-terminal half of YFP (pSPYNE) in the same experiment (StSUT4-SPYNE). The reconstitution of YFP fluorescence was examined by transient co-expression of StSUT4-SPYNE and StPDI-SPYCE in tobacco (ssp. *Nicotiana benthamiana*) leaves. 4 days after infiltration, the leaves were harvested for observation of the living plant cells with YFP-derived fluorescence using Leica Confocal Laser Scanning microscope (Leica TCS SP2, CLSM). StSUT4 and StPDI complex was localized in the endoplasmic reticulum (ER) (Fig. 42A).

The open reading frame of StPDI was subcloned into pK7YWG2.0 (Karimi et al., 2005), which contained the enhanced YFP, to investigate the subcellular localization of StPDI. Tobacco leaves were co-infiltrated with Agrobacterium containing pK7YWG2.0-StPDI and an ER marker protein (HDEL) fused to mCherry. StPDI-YFP fluorescence was detected in the ER (Fig. 42B). The StPDI was co-localized with the ER marker protein (HDEL) in the ER. The StPDI was not only localized in the ER, the StPDI-YFP fluorescence could be detected in the plasma membrane rafts and in the vesicles (data not shown).

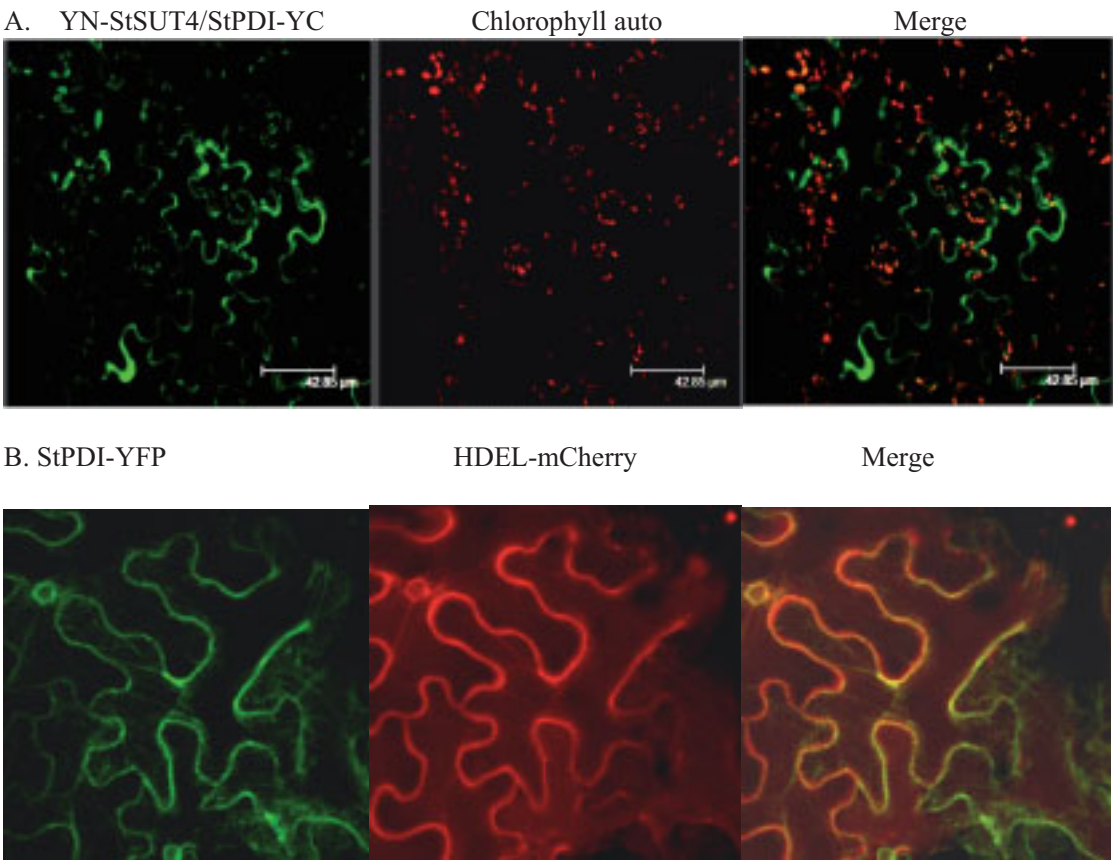


Fig. 42: Co-localization of StSUT4 and StPDI by BiFC

Agrobacterium strain EHA105 was transformed with different plasmid DNAs. Transient expression was performed by co-infiltration of *N. benthamiana* leaves with StSUT4-SPYNE and StPDI-SPYCE construct. Four days after infiltration, the leaves were observed under CLSM. The YFP fluorescence as shown in green was excited with 514 nm excitation and detected in the range of 530-575 nm. Chlorophyll autofluorescence is displayed in red and was detected in the range of 600-700 nm. The mCherry was excited with 587 nm excitation and detected in 610 nm. A: StSUT4-SPYNE +PDI (EST707280)-SPYCE; B: colocalization of a StPDI-YFP construct under control of the CaMV 35S promoter with an ER marker (HDEL-mCherry after infiltration of N.b: the merged picture showed that StPDI was targeted to the ER.

3.6.2 Physiological evidence for the interaction between the sucrose transporter StSUT4 with a putative ethylene receptor protein

It is well known that the plant hormone ethylene plays an important role during vegetable growth and development. Ethylene is not only involved in basic cellular processes such as cell elongation, cell division, and cell death, but also physiological processes such as seed germination, leaf senescence and fruit ripening (Abeles et al., 1992). It is worth mentioning the unpublished work of Jana Reins. She performed yeast split-ubiquitin assay in order to identify StSUT4 protein interaction partners from a potato cDNA library. One of the putative interaction partners (EST423729) shows 95% homology to the ethylene receptor ETR2 from tomato (Reins, 2006), named StETR1. Ethylene receptors are, at least in some cases, ethylene inducible. It has been demonstrated that increased levels of receptor reduces ethylene sensitivity.

This supports the negative regulation of ethylene receptors models (Ciardi et al., 2000). Therefore further analyses were performed to confirm the interaction between SUT4 and the ETR1 protein and to investigate the physiological functions of this interaction.

3.6.2.1 Co-localization of ETR1 and SUT4 in the ER

The open reading frame of the ETR homolog (EST423729) was amplified from potato cDNA by RT-PCR. For BiFC assay, the StETR1 was subcloned into pSPYCE (StETR1-SPYCE) and was co-infiltrated with StSUT4-SPYNE. The results confirmed the interaction between StSUT4 and StETR1 and the complex was also localized in the ER (data not shown). The binary vector pK7WG2.0 was employed for the localization of StETR1. The tobacco leaves were co-infiltrated with pK7WG2.0-StETR1 and an ER marker (HDEL) fused with mCherry. 4 days after infiltration, the leaves were observed under CLSM. The results showed that StETR1 was co-localized with HDEL-mCherry in the ER (Fig. 43).

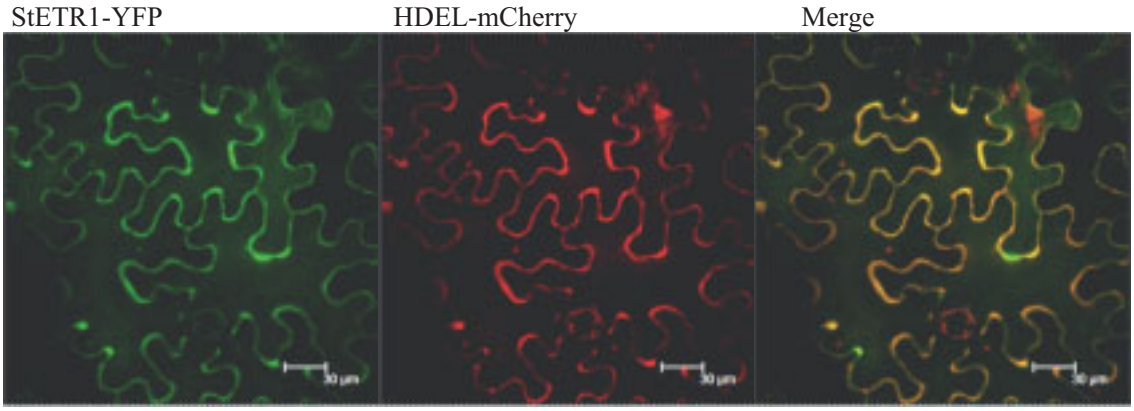


Fig. 43: Localization of StETR1

Agrobacterium strain EHA105 cells were transformed with different plasmid DNAs. Transient expression was performed by co-infiltration of *N.b* leaves with ETR1 (EST423729) in pK7YWG2.0 and an ER marker protein HDEL fused with mCherry. Four days after infiltration, the leaves were observed under CLSM. The YFP fluorescence as shown in green was excited with 514 nm excitation and detected in the range of 530-575 nm. The mCherry was excited with 587 nm excitation and detected in 610 nm.

3.6.2.2 SUT4 expression in tobacco is inducible by ethylene

StSUT4 is known to be ethephon inducible as previously shown by qRT-PCR (Chincinska et al., 2008). This is one hint for StSUT4 to be involved in ethylene signaling. Northern blot analysis with tobacco plants treated with 3 mM ethephon confirmed SUT4-induction by ethylene in tobacco plants as well. The *SUT4* mRNA started to accumulate one hour after ethephon treatment and 24 hours after induction the SUT4 mRNA accumulation was highest (Fig. 44).

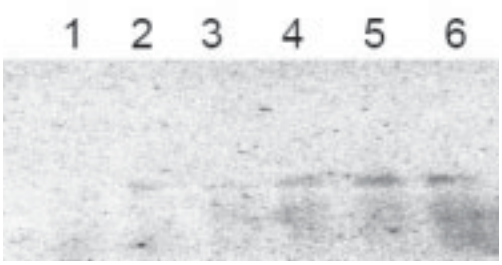


Fig. 44: Northern blot of tobacco leaves treated with 3 mM ethephon solution

Tobacco WT plants were treated with 3mM ethephon. Samples were taken before treatment (lane 1), and after treatment 1 h, 3 h, 6 h, 12 h, and 24 h after treatment (lane 2-6). RNA samples were separated in a formamid- containing agarose gel and transferred onto a nitrocellulose membrane.

3.6.2.3 The impact of StSUT4 inhibition on ethylene production

For the detection of plant hormone ethylene, a CO_2 laser-based photoacoustic detector combined with a flow-through system is applied because of its sensitivity of the device and its time-resolved analysis in comparison to the traditional methods (such as gas chromatography which is currently the standard technique most widely used) (Cristescu et al., 2008).

The production of ethylene was measured in wild-type potato plants and *StSUT4-RNAi* potatoes using a CO_2 laser-based photoacoustic detector. Briefly, the detector consists of a line-tunable CO_2 laser emitting radiation in the 9- to 11-μm infrared wavelength region and a photoacoustic cell, in which the gas is detected. The laser-based ethylene detector is able to distinguish between different gases by making use of their wavelength-dependent fingerprint absorption characteristics (Cristescu et al., 2002). Trace gases released by the biological samples (WT or StSUT4 RNAi potato lines) were transportted to the photoacoustic cell through a flow system by using air as the carrier gas. Ethylene gas mixtures were sensitively measured by the laser-based ethylene detector due to distinct fingerprint-like spectrum of ethylene in the CO_2 laser wavelength range (Brewer et al., 1982). The differences of ethylene production between the *StSUT4-RNAi* lines and wild-type plants are summarized in Fig. 45: graph A shows reduced ethylene production of *StSUT4-RNAi* line 38 and line 10 in comparison to potato wild-type plants; graph B shows reproducible results of ethylene production by *StSUT4-RNAi* line 81 (line 811 and line 812 were two individual plants). So the StSUT4 has a positive effect on ethylene production. These results apply an indirect hint for the putative interaction between SUT4 protein and the ethylene receptor protein.

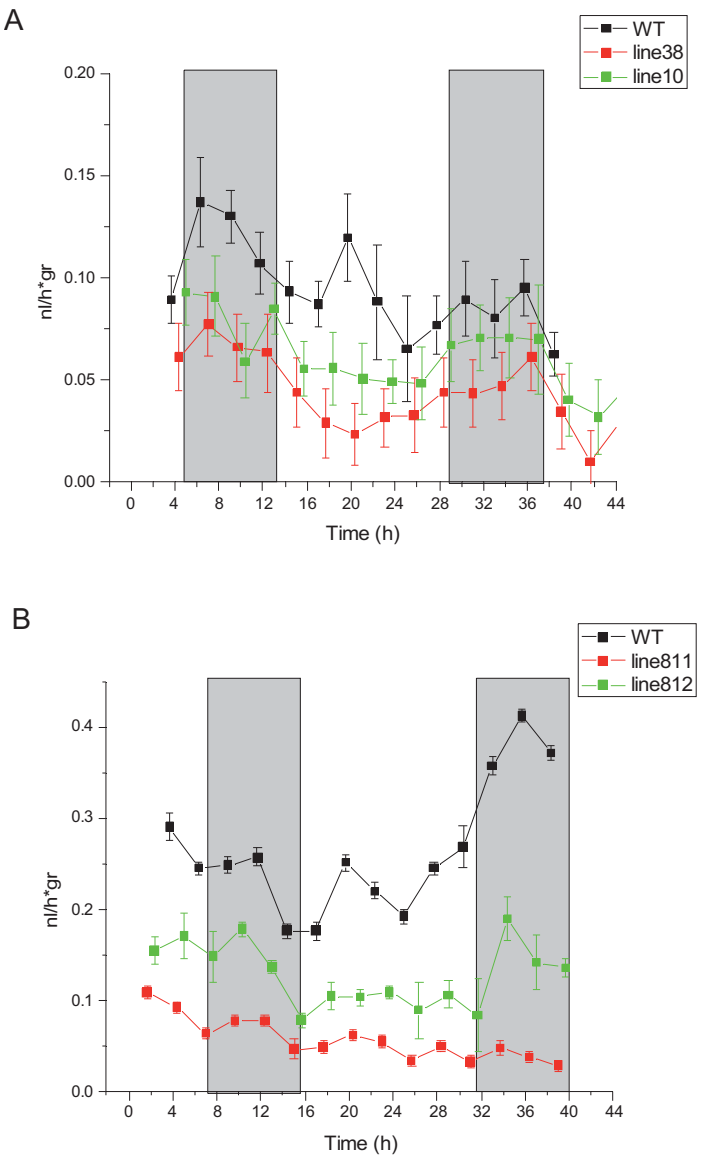


Fig. 45: Ethylene production from wild-type and *StSUT4-RNAi* potato plants

The tissue cultured potato plants of wild-type *Désirée* and *StSUT4-RNAi* potato plants were employed in the experiments. X-axis: time is in hours and the grey areas are dark period (LD 16h, white light 270 umol/m^2s). Y-axis: the rate of ethylene production was expressed in nanoliters per hour per gram (fresh weight) (nl/h*gr). The tested plants were kept in 2MS medium for 3-4 weeks before ethylene measurement. The fresh weight (FW) of the plants: A, WT *Désirée* 2.49 g; *StSUT4-RNAi* line 38 1.86 g; *StSUT4-RNAi* line 10 1.84 g. B, WT *Désirée* 3.86 g; *StSUT4-RNAi* line 811 5.44 g; *StSUT4-RNAi* line 812 4.17 g.

3.7 Screen for StSUT4 RNA-binding proteins using yeast three-hybrid system

The inhibitor studies indicate that StSUT4 mRNA stability is regulated at the post-transcriptional level. It is assumed that the prolonged SUT4 half life time under far-red light enrichment involves RNA-binding proteins protecting the SUT4 mRNA for sequence-specific mRNA decay by ribonucleases. To investigate the putative StSUT4 mRNA-binding protein(s), the yeast three-hybrid (Y3H) technique was employed.

The Y3H assay allows identification of RNA and protein binding partners as well as the dissections of higher-order RNA/protein complexes (Bernstein et al., 2002). The system is based on the yeast two-hybrid system and consists of three components: two hybrid proteins and one hybrid RNA (see introduction Chapter 1.5.4). The strength of this approach is the detection of RNA/protein interactions *in vivo*. A protein homologous to the hypoxia-induced HIG1 protein has been identified as a putative *StSUT4* RNA binding protein.

3.7.1 Establishment of the yeast three-hybrid system

3.7.1.1 Construction of bait vectors

The secondary structure of *SlSUT1* mRNA was predicted using the mfold version 2.3 computer program (Panford-Walsh, 2004). A region of high determination (more possibility for RNA-binding) within the 3'UTR of *SlSUT1* was found. Since the predicted secondary structure of *SlSUT4* mRNA showed no areas of determination in the 3'UTR, a region of extremely high determination might occur within the coding region (red stem loop between 650-750nt). *StSUT4* in potato was highly homologous to *SlSUT4* in tomato, it was reasonable that the high determination region also existed within the coding region.

The secondary structures of *StSUT4* and *StSUT4+3'UTR* were predicted by using http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi (Appendix 6.2). The predicted structures appeared in a given state (stem or loop). These regions are depicted as yellow, orange or red dots in the figures (with red representing the highest degree of determination, followed by orange, then yellow). The highly indeterminate regions (are not routinely predicted to appear in a given state) are depicted with green, blue, and black dots.

3.7.1.2 Subcloning of *StSUT4* and *StSUT4 3´UTR*

Using the total RNA of potato flowers, reverse transcription reaction was performed using oligo (-dT) primer for *StSUT4* and NUPA primer for *StSUT4* 3'UTR. Primer pairs (*StSUT4* PmeI fw and *StSUT4* AatII rev for amplification of *StSUT4*; *StSUT4* PmeI fw and *StSUT4* 3'UTR AatII rev for amplification of *StSUT4 3'UTR*) were used in the following PCR amplification. Subsequently the PCR products were subcloned into pZero 2 with kanamycin resistance in bacteria. The complete cDNA sequence of *StSUT4* was deposited in Genebank with the updated accession number AF237780.

3.7.1.3 Control experiments

To make sure that the yeast three-hybrid system can be used to detect the RNA-protein interactions, a series of experiments were performed to combine yeast strain, bait plasmid and prey cDNA library. From Invitrogen's RNA-protein Hunter Kit (Version D), positive and negative control plasmids (pRH3', pRH3'/IRE, pYESTrp3 and pYESTrp3/IRP) were supplied, and the yeast strain L40uraMS2 was tested before large-scale screen.

A modified yeast three-hybrid analysis based on SenGupta (1996) was carried out in this study combining version A and version D of the RNA–protein Hybrid Hunter Kit (Invitrogen) to screen for SUT4 RNA binding proteins. The introduction of plasmids expressing the bait and prey constructs into the yeast L40uraMS2 host strain (Invitrogen) were carried out in two successive steps. The employed yeast strain, L40uraMS2, contains a *HIS3* gene and a *LacZ* gene under the control of LexA-operator. In addition, the first hybrid protein was expressed in L40uraMS2 which the bacterial LexA DNA-binding domain was fused to the coat protein of the bacteriophage MS2. The MS2 coat protein recognized an RNA-stem loop structure in MS2 RNA (Matter et al., 2000). The bait-encoding plasmid was first selected on yeast synthetic dextrose medium. In separate transformations, the L40uraMS2 was transformed with the parent bait vector pRH3' and plasmid pRH3'/IRE (Invitrogen; encodes the iron responsive element (IRE) RNA) for the control experiments. Cells that contained the appropriate bait-encoding plasmid or parent vector were then transformed with plasmid expressing prey plasmid pYESTrp3 or pYESTrp3/IRP respectively (Invitrogen; IRP encodes the IRE binding protein) and were selected on yeast SD medium (Ura-, Trp-, His-). In addition, 5 mM 3-AT was included to suppress background growth due to autoactivation of the *his3* gene (tested before large scale transformation). X-Gal filter assay was performed with different combinations to test whether the host strain L40uraMS2 strain was adequate to perform a systematic yeast three-hybrid screen. The results showed that the system was suitable for a systematic screen for RNA-binding proteins (Fig. 46).

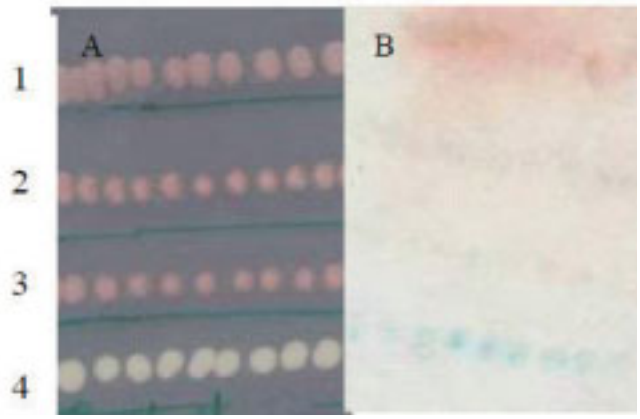


Fig. 46: The control experiments with positive and negative control constructs.

A, the yeast cells were grown on SD-Ura-Trp without additional adenine medium. Line 1, pRH3′+pYESTrp3; line2, pRH3′/IRE+pYESTrp3; line 3, pRH3′+pYESTrp3/IRP; line 4, pRH3′/IRE+pYESTrp3/IRP, only in line 4 yeast clones showed white color when they grew on SD-Ura-Trp medium without additional adenine. B, yeast colonies were transferred via replica to filter membrane. Filter assay was performed as described in materials and methods, blue color indicated the IRE/IRP interaction.

3.7.2 Large scale screening

Two independent large scale screens were performed using different bait RNAs: the first one contained the *StSUT4* open reading frame as bait RNA, the second one contained the StSUT4 open reading frame together with the 3′untranslated region (3'UTR) as bait RNA. General information about the screens was summarized in Table 5. The yeast strain L40uraMS2 contained pRH3'/StSUT4 carrying the GAL4 activation domain (AD) was successively transformed with a potato cDNA library in pGAD10-GW. The number of primary transformants was determined by selection on SD-Ura-Leu. 4.4×10^6 primary transformants were then pooled for HIS3 plus 5 mM 3-AT selective medium. *HIS3* and *lacZ* reporter gene expression could be confirmed for all 60 randomly selected histidine prototrophs. In a second screen performed with pRH3'/StSUT4 3'UTR as bait RNA, 48 randomly selected histidine prototrophs were positive in the X-Gal filter assay. After transformation of *E.coli DH5α* with isolated yeast plasmid DNAs, restriction analysis of the identified prey plasmids revealed more than 30 different restriction patterns (the insert length was calculated from restriction analysis and BLAST results). Repeated transformation of the yeast strain L40uraMS2+pRH3'/StSUT4 or L40uraMS2+pRH3'/StSUT4 3'UTR with the identified prey candidates confirmed yeast growth mediated by 7 putative interaction partners from each screen respectively. Six of them were identified independently in both screens for several times (The accession numbers with underlines, Table 5). DV625645 was found only in the screen of StSUT4 as bait RNA, while EF062357 only in the screen of StSUT4+3'UTR as bait RNA. In total, eight candidates were confirmed by reintroducing into yeast (Table 5), the yeast cells containing bait (pRH3'+StSUT4) and prey plasmid DNAs were grown on SD-ULH medium and turned blue after X-Gal filter assay (details shown in chapter 3.7.3). Raw sequence data was passed through several steps, such as cloning vector or pray vector removal, quality evaluation, BLASTN or BLASTX (P) and alignment. Reading frames and the existence of 5'-untranslated regions were calculated from the BLAST reports.

Table 5: General information about large scale transformation

Bait mRNA	Transformants	No. of positive clones	Numbers and EST of putative interaction partners in *Solanum tuberosum*
StSUT4	4.4×10^6	60	7 (CV472316, DV625645, CV494117, DE340953, DQ222490, DN743291,ES463778)
StSUT4+3'UTR	1.2×10^6	48	7 (CV472316, CV494117,DE340953, DQ222490, DN743291, ES463778, EF062357)

3.7.3 Confirmation of interactions

The yeast three-hybrid system possesses an inducible promoter for the conditional expression of the third partner. This allows performing an additional control of interaction specificity especially in one screening experiment for a RNA-binding partner from cDNA library. In this study the positive clones were identified firstly by appearance of the histidine prototroph and secondly by their ß-gal activity. The L40uraMS2 yeast cells with pRH3'-StSUT4 were transformed with three prey plasmid DNAs showing different restriction patterns, and filter lift assays were performed to test the X-gal activity. Results showed that 1-8(7) (DV525645), 3-18u(6) (ES463778) and 1-10(4) (CV472316) were positive (Fig. 47, line 2-4) whereas the transformation with the empty pGAD10-GW did not show blue color development in X-gal filter assay (Fig. 47, line 1).

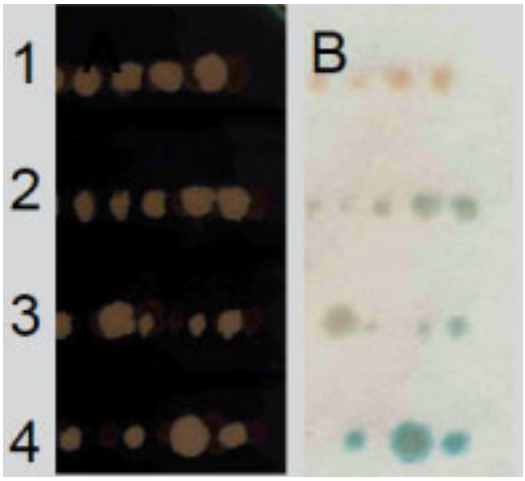


Fig. 47: Confirmation assays by retransformation of L40uraMS2 cells

L40uraMS2 cells containing pRH3`/StSUT4 bait constructs, were transformed with different prey vectors. Line 1, pRH3´/StSUT4+pGAD10-GW; line 2, pRH3´StSUT4+ 1-8(7); line 3, pRH3´/StSUT4+ 3-18u(6); line 4, pRH3´/StSUT4+ 1-10(14). Picture A: yeast colonies grown on SD-Ura-Leu-His medium, Picture B: filter lift X-gal assay of the same plate.

3.7.4 Elimination of false positives from the screen

Two types of positives were obtained from the initial transformations. The first type of transformants, termed as "RNA-dependent", required the hybrid RNA to activate

HIS3. The second type of positive transformants, termed "RNA-independent", activated HIS3 either with or without the hybrid RNA. The RNA-independent positives were able to carry proteins, which might bind to the promoter regions of the reporter genes or proteins. It must be noted that either reporter genes or proteins interact directly with the LexA-MS2 coat protein fusion. In the yeast hybrid assays, "prey" plasmids need to be tested in order to determine whether they activate the reporter genes in the absence of the "bait RNA" plasmid. This was achieved by transforming each "prey" plasmid back into L40uraMS2 without bait vector. This strain could be assayed for the expression of either the HIS3 reporter or the ß-galactosidase reporter. The filter X-Gal assay showed 1-6u(5) was RNA-independent false positive (Fig. 48, line 3). Sequencing and BlastN analysis (Altschul et al., 1990) showed that the prey plasmid 1-6u(5) (EF062357) encoded the LEAFY transcription factor of *Solanum tuberosum*. The transcription factor itself can bind to the LexA operon, thereby allowing activation of the reporter genes via the GAL4 activation domain.

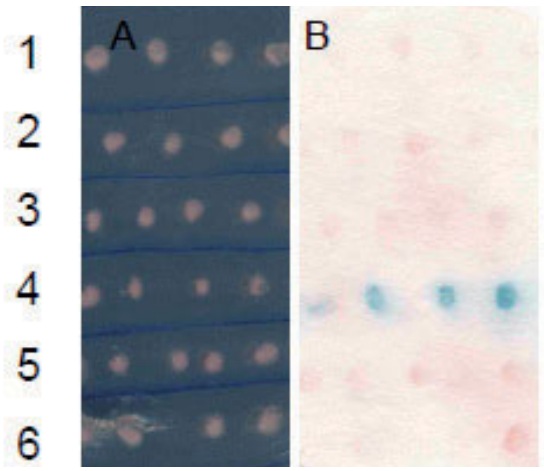


Fig. 48: the elimination of RNA-independent false positive clones in yeast three-hybrid assay

L40uraMS2 cells were transformed with prey plasmid DNAs. A, resultant cells were grown on SD-Leu medium; B, filter X-gal assay of the yeast cells. Line 1, 9-11(2) (DE340953); line 2, 3-18u(6) (ES463778); line 3, 1-10(4) (CV472316); line 4, 1-6u(5) (EF062357); line 5, 3-6u(4) (DQ222490); line 6, 1-8(7) (DV625645).

3.7.5 Further analyses to eliminate false positives

After elimination of one RNA-independent false candidate, the remaining seven putative interaction partners were isolated and sequenced. The sequence results were analyzed using NCBI BLASTN (for nucleotide to nucleotide) or BLASTX (for nucleotide to protein) to remove the vector parts. ExPASy proteomics server was employed to translate DNA sequence into protein.

Among the seven putative interaction partners, five were reported as potential RNA-binding proteins based on recent publications (Zimmer et al., 2001; Park et al., 2004;

Walker et al., 2007). Six of the seven putative interaction partners excluding DN74329 were detectable in the plant phloem (Aoki et al., 2002; Omid et al., 2007; Lin et al., 2009). Further analysis showed that only one of the putative interaction partners was inserted in the same reading frame as the Gal4 transcription activation domain. This positive interaction partner (CV494117) was 77.9% homologous to a hypoxia responsive C3HC4-type RING Zinc finger protein in Arabidopsis (accession number: NP_190386).

3.7.5.1 The putative RNA-binding protein

The cDNA clone 10-5(6) identified in 4 independent yeast colonies, was 100% identity to CV494117 (EST sequence in *Solanum tuberosum*). The open reading frame of CV494117 has 246 bp and the predicted length is 82 amino acids (8.863 kDa). According to ARAMEMNON database prediction, the protein contains 2 transmembrane domains and belongs to the secretory pathway. It does not contain a zinc finger motif but has homologies to the N-terminal HIG1 domain of C3HC4 RING zinc finger proteins (carrying the zinc finger motif at their C-terminus). The homologous C3HC4-type RING finger protein in Arabidopsis contains a hypoxia-induced protein conserved domain and a RING finger domain. This protein family belongs to RING finger-containing ubiquitin ligase. The RING domain is originally named after the acronym for the first RING finger gene (*Really Interesting New Gene*), which is a small protein motif and consists of four pairs of ligands binding to two ions (Freemont et al., 1991; Freemont, 1993). COP1 (CONSTITUTIVE PHOTOMORPHOGENIC1) is one of the well studied C3HC4-type RING finger proteins, and is regarded as a component of the signal transduction chain linking light signals to plant development (von Arnim and Deng, 1993; Raghuvanshi et al., 2001). COP1 has also a role as an E3 ubiquitin ligase, which targets photomorphogenesis-promoting transcription factors for ubiquitinylation and degradation in Arabidopsis and rice (Mazzucotelli et al., 2006; Ma et al., 2009).

3.7.5.2 Confirmation of the RNA-binding activity of putative interaction partner in yeast

The putative RNA-binding protein (CV494117) was subcloned from potato flower cDNA by using a RACE-based PCR approach. The coding sequence was transferred into pGAD10-GW using Gateway recombinant system (Invitrogen). The sequencing analysis showed that the putative interaction partners were under Gal4 activation domain regulation. Further confirmation test was done with pGAD10-StHIG1.

The yeast strain L40uraMS2 with pRH3'/StSUT4 was transformed by putative interaction partners: 10-5(6) and 3-10(7) (independent pray constructs with

CV494117 insert were detected); pGAD10-StHIG1, and empty vector controls and some other pray constructs respectively. Subsequently, the product cells were plated on SD-Ura-Leu medium for plasmids selection. The yeast cells of three independent clones and empty control were incubated at 30°C shaker in SD-Ura-Leu medium for 1-2 days until OD_{600} around 1.0. Then the OD600 value was measured and the cell concentration was calculated. All the cell cultures were adjusted to the same concentration using SD-Ura-Leu medium, 10 µl of the cells were taken and put on selective medium (SD-UHL, SD-UHL+2 mM 3-AT). After 5 days, the yeast cells with HIG (prey) and SUT4 3'UTR started to grow on SD-UHL medium (Fig. 49A). The yeast cells contained StSUT4 bait and 10-5(6) and 3-10(7) prey grew on SD-UHL+2 mM 3-AT medium, and X-gal assay showed positive blue color (Fig. 49B: No.3 and 5) which could not detect on empty prey vector control (No. 16). Nonetheless, yeast cells with pRH3'/StSUT4 and other pray vectors (No.1, 2, 4, 6 and 7) did not turn blue, so only StHIG1 was one putative interaction partner of StSUT4 and the rest of tested pray plasmid DNAs were classed as false positive clone.

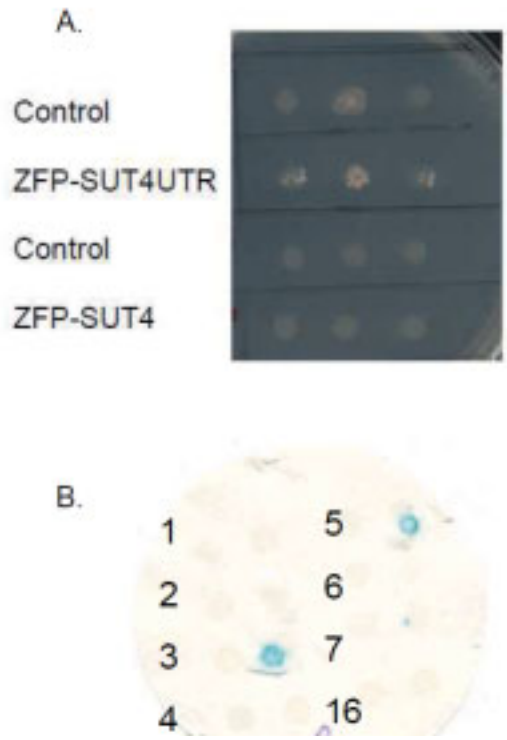


Fig. 49: The retransformation of HIG1-like protein into L40uraMS2 containing bait vector

A. L40uraMS2 cells containing pRH3'/StSUT4 3'UTR (line 1 and line 2) or pRH3'/StSUT4 (line 3 and line 4) were transformed with pGAD10-GW (A, B and C version mixed) (line 1 and line 3) or pGAD10-StHIG1 (line 2 and line 4) respectively. The yeast culture (10 µl) was put on SD-ura-leu-his medium for 5 days.

B. L40uraMS2 cells containing pRH3'/StSUT4 were transformed with different pray constructs. The yeast culture (10 µl) was plated on SD-ura-leu-his+2 mM 3AT, after 5 days, only 3 and 5 had some clones. Pray constructs order as:1, 8-1sp; 2, 11-6sp; 3, 10-5(6)-CV494117; 4, 3-6u(1); 5, 3-10(7)-CV494117; 6, 3-18u(6); 7, 3-10(2); 16, control (GAD10-GW). sp means plasmid DNA isolated from the screen with a StSUT4 fragment as bait; 3-6u(1) means plasmid DNA isolated

from the screen with StSUT4 3'UTR as bait. The other plasmid DNAs are from the screen with StSUT4 as bait.

4 Discussion

The work of this thesis was focused on sucrose transporters mRNA or protein interaction partners to clarify the physiological and molecular functions of them, mainly about SUT4. This chapter critically discusses the above results. The following contents are included in the discussion: the *SUT1* mRNAs are phloem mobile in solanaceous plants (chapter 4.1); the regulation mechanisms of sucrose transporter mRNAs' stability (chapter 4.2); then physiological functions of SUT4 and the involvement of *StSUT4* in the flower and tuber induction pathway (chapter 4.3); sink specific *StSUT2*'s physiological function (chapter 4.4); the regulation of SUT protein stability and localization (chapter 4.5). Finally, this discussion seeks to identify *StSUT4* RNA binding protein using yeast three-hybrid systems (chapter 4.6).

4.1 The *SUT1* mRNAs are phloem mobile in solanaceous plants

This chapter critically discusses the methodology to achieve the above results. In this study the phloem mobility of *StSUT1*, *NtSUT1*, *SlSUT1-GUS* and *SoSUT1* transcripts were detected through parasite and graft experiments (Fig. 5, 6 and 7). The detection of *NtSUT1* from *Cuscuta reflexa* grown on *Nicotiana tabacum* (Fig. 5) correlated with previous investigations regarding the phloem mobility of sucrose transporter mRNA (*CmSUTP1*), *Cucurbita maxima,* from the host into the dodder plant *Cuscuta pentagona Engelm* (Roney et al., 2007). In addition, the detection of the full length *StSUT1* transcripts in *Cuscuta reflexa* grown on potato provided a good evidence for mRNA stabilization during transport potentially in a ribonucleoprotein (RNP) complex (He et al., 2008). *StSUT1* mRNA cell-to-cell mobility was already shown by microinjection experiments either with or without RNA-binding protein CmPP16 from *Cucurbita maxima* (Xoconostle-Cázares et al., 1999). The previous studies of the apparent symplastic isolation of host sieve elements and absorbing hyphae suggested that a symplastic link occurred at some point in the host–*Cuscuta* interface to allow the passage of pathogenic RNAs or RNA–protein complexes from the phloem of one species to another one (Dörr, 1990; Haupt et al., 2001). The above results showed that phloem-mobile *Sucrose Transporter1* mRNAs were transferred from tobacco, potato and squash to *Cuscuta.* Therefore, this study argues for a symplastic pathway that is probably utilized for the transport of macromolecules in this system.

Cell-to-cell and long-distance trafficking of macromolecules probably relies on endogenous mechanisms to select and dock RNAs on the transport pathway. However, detection of RNAs in phloem cells is not trivial due to the difficulty of access to the phloem cell content and the abundance of enzymes which degrade RNA. Several

specific mRNA molecules are capable of long distance trafficking, but this capability is not common for all phloem transcripts. Therefore, grafting is an appropriate technique to investigate the phloem mobility of macromolecules because it keeps the connection between rootstock and scion through the vascular tissues, phloem and xylem. According to Omid et al., (2007) performing heterograft experiments to detect long distance transported mRNAs, six out of 43 examined transcripts are capable of long-distance trafficking from melon stocks to pumpkin scions. Thus, the combination of graft techniques and transgenic plants applied more evidence of specific SUT1 mRNAs' mobility such as *SoSUT1* (Fig. 6) and *SlSUT1* (Fig. 7). In the case of *SlSUT1* transcripts, the short distance mobility of the GUS transcripts fused with *SlSUT1*, which was only detected in the wild-type tobacco scion close to the graft union (Fig. 7, lane 7 and lane 8). These results raise some questions whether the sucrose transporter mRNAs were degraded during trafficking and what kind of mechanism regulated the mRNA stability. Jae-Yean Kim (2005) has represented a RNP relay model: the non-cell- autonomous proteins (NCAPs) including KN1 can bind to its own RNA and facilitate RNA trafficking. NCAP, expressed source cells, binds to its RNA and moves to neighboring cells as RNPs. In target cells with low levels of RNP, the RNP dissociates and initiates translation. Increased NCAP protein concentration stimulates reassociation with its RNA, which then moves to neighboring cells.

Since the *SUT1* mRNAs have short half life (*SlSUT1*, 88.1 ± 13min; *StSUT1*, 69.3 ± 3 min) (Table 4), it is reasonable to have some specific RNA-binding proteins (RBPs) which are involved in the *SUT1* mRNAs stabilization and transport. In other words, RBPs which exist in the phloem could protect the RNA from cleavage; RBPs are also required for the import of RNAs from CC to SE through the connecting pore plasmodesmal units (PPUs). Panford-Walsh (2004) has identified a putative *SUT1* mRNA binding protein using yeast three-hybrid analysis in her dissertation but the physiological function of the protein are subjected to further investigation.

The *StSUT4-RNAi* phenotype in potato plants is graft-transmissible (Chincinska et al., 2008) and SUT4 is supposed to play a role as an inhibitor of SUT1 (Izabela Chincinska, PhD dissertation, HU 2009); the subsequent question deals with the real mobile signal. There are at least four possibilities:

1. the *SUT1* mRNA as mobile signal while its expression is down regulated by *SUT4*, and the *SUT4* expression is regulated by short lived RNA-binding protein(s).

2. *SUT4* mRNA serves as a mobile signal with the help of phloem specific RNA-binding protein such as Heat shock cognate 70 (Hsc70), which contains a short viable region (SVR) motif in the C-terminal. This allows Hsc70 chaperones to

engage the plasmodesmal non-cell-autonomous translocation machinery (Aoki et al., 2002).

3. *SUT4* expression influences the genes such as *CO*, *SOC1* and *FT,* which are involved in the flowering pathway that will be discussed below.

4. SUT4 protein influences SUT1 protein activity by post-translational regulation of SUT1 protein.

4.2 Regulation mechanisms of sucrose transporter mRNA stability

4.2.1 The determination of half-life of sucrose transporter mRNAs is important

The emerging fate of mRNAs is an important point of regulation of gene expression. In general, mRNA localization and stability, and protein translation are based on the cooperation between *cis*- and *trans-acting* elements. The half-life of individual mRNA plays a central role in controlling gene expression (Feng and Niu, 2007). Determination of *SUT* mRNA half-life is important to the understanding of *SUT* expression and mechanisms involved in the regulation of the level of transcripts in response to the environmental changes or developmental cues. In addition, the stability of mRNA may determine how rapidly the synthesis of the encoded protein can be shut down after transcription ceases. In this study, the real-time reverse-transcription PCR (qRT-PCR) is employed to determine the half life of sucrose transporter mRNAs (chapter 3.2). It is well known that qRT-PCR allows precise and reproducible determination of the number of mRNA transcripts quantitatively (Gibson et al., 1996; Heid et al., 1996). Real-Time RT-PCR is a reliable method for measuring mRNA half-life due to its sensitivity. The half-life of mRNAs expressed at very low level can be determined in cases in which Northern blots may not be sensitive enough (Leclerc et al., 2002).

The half-life of mRNA molecules can vary over a wide range. Typical plant mRNA molecules appear to exhibit half lives of several hours (Taylor and Green, 1995). The mRNA half-lives varied greatly across the Arabidopsis genome ranging from 0.2 to >24 h with a mean of 5.9 h and median of 3.8 h (Narsai et al., 2007). The half-lives of sucrose transporter mRNAs are ranging from 68 min to 131 min (Table 4), which belong to unstable mRNAs. The unstable mRNAs are particularly interesting because they often encode key regulators such as transcription and growth factors. The short half lives (i.e. < 2 hours) of these transcripts facilitate rapid changes in mRNA abundance which can be critical for normal cellular function. The presence of AU-rich elements in the 3'UTR of these transcripts is associated with highly unstable characters (Schobert et al., 1995). The *StSUT2* mRNA contains one of the AUUUA elements in the 3'UTR. The *StSUT1* mRNA contains one of this AUUUA elements

and *StSUT4* mRNA contains two elements within the coding region. Probably, this is one reason for short half life of *StSUT4* mRNA (He et al., 2008). Binding of an AU rich element binding factor to these elements might affect *SUT4* RNA stability.

4.2.2 Different regulations of sucrose transporter mRNAs stability

Sucrose transporter mRNAs have a very short half life and their accumulation is tightly controlled at various levels. Whereas, transcriptional control of the diurnally expressed *StSUT1* gene seems to have a high impact on its accumulation (Fig. 8), *StSUT2* and *StSUT4* transcript accumulation is highly dependent on *de novo* biosynthesis of negative acting regulators indicated by the enhanced stability in the presence of CHX within only 2 h (Fig. 9). The mRNA half life is prolonged through inhibition of transcription and translation. This requires a tight post-transcriptional control of these two sucrose transporter related genes in a sequence-specific manner.

As it was shown previously, the *SlSUT1* mRNA follows a diurnal expression pattern (Kühn et al., 1997). *DcSUT1* and *DcSUT2* of carrot are diurnally regulated in leaves but expressed at a constantly low level in tap roots (Shakya and Sturm, 1998). The diurnal rhythm of sucrose transporter expression in potato plants is also detected in sink organs such as flowers and sink leaves (Chincinska, Kühn, unpublished). Sucrose transporter mRNA oscillation is detected not only under dark/light cycles but also under constant light conditions arguing for a circadian regulation (Chincinska et al., 2008).

For several circadian genes a specific post-transcriptional mRNA decay mechanism has been postulated (Lidder et al., 2005; McClung, 2006). The circadian control of messenger stability was found to be associated with a sequence specific decay pathway (Lidder et al., 2005). Instability determinants of clock controlled mRNAs might be located within untranslated regions, introns or within the coding region as shown for mRNA of *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) (Yakir et al., 2007). In Fig. 9A and 9B the *StSUT2* and the *StSUT4* mRNA undergoes sequence-specific post-transcriptional mRNA decay as previously shown in other circadian genes such as *CCR-LIKE (CCL)* and *SENESCENCE ASSOCIATED GENE 1* (Lidder et al., 2005). The half life of *StSUT2* and *StSUT4* is prolonged by CHX even in the presence of transcriptional inhibitor actinomycin D.

The following model summarizes the positive effect of auxin on the transcriptional activity of the *SUT1* genes via transcriptional activators, whereas *SUT2* and *SUT4* genes are obviously negatively affected by short-lived proteins either at the transcriptional level potentially *via* AUX/IAA proteins or at the post-transcriptional level via RNA-binding proteins (Fig. 50). The important regulatory function of

phloem specific RNA-binding proteins and sequence-specific mRNA degradation is point out based on the post-transcriptional regulation of *SUT2* and *SUT4* mRNA stability (as shown in Fig. 9 and 10)

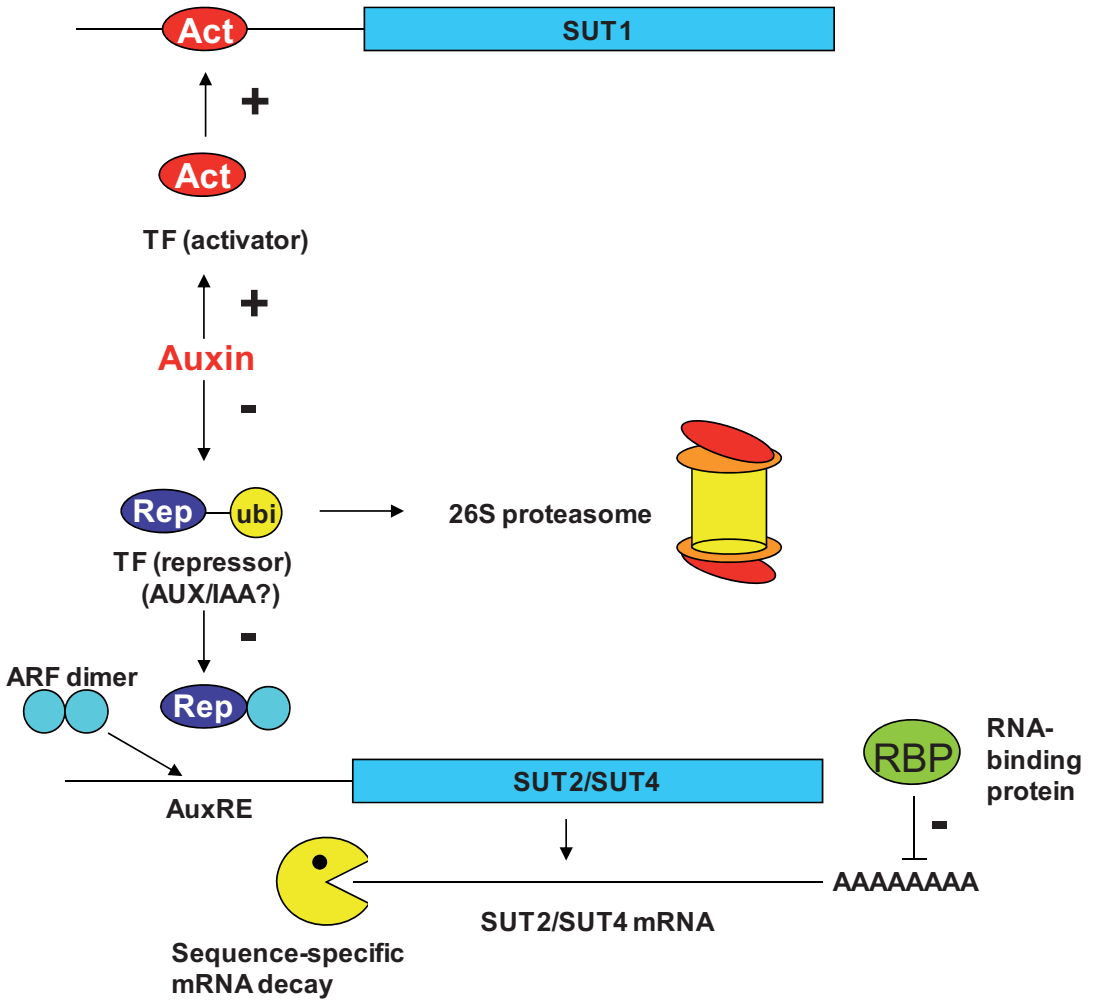


Fig. 50: Transcriptional and post-transcriptional regulation of sucrose transporter mRNAs

According to He et al., (2008) the transcriptional control of *SUT1* mRNAs in response to auxin is predominant. The model was adapted to the relative results of the inhibitor studies. The negative transcription factors (potentially short lived Aux/IAA proteins) are ubiquitinated and degraded by the 26S proteasomal pathway. Heteromerisation of this repressor with the auxin response factor ARF potentially prevents homodimerisation and thereby binding to the auxin responsive element AuxRE identified in the *LeSUT2* promoter region.

Sucrose transporter expression might also be regulated by microRNAs since in Arabidopsis microRNAs targeted against seven out of nine different sucrose transporter genes have been identified (Lu et al., 2005). In the case of *AtSUT2* and *AtSUT4* no corresponding microRNAs could be found, indicating that the regulation of these transporter genes might be different from all others, which are members of the phylogenetic SUT1 clade.

4.3 The physiological functions of SUT4

4.3.1 Localization of SUT4 in solanaceous plant cells

The results of immunoblot show that the SUT4 protein of the expected size is mainly expressed in plasma-membrane fractions (Fig. 35). This correlates with previous studies in potato and tomato plants (Weise et al., 2000; Chincinska et al., 2008). However, the detection of a smaller band in the microsomal fractions and the endomembrane fraction indicates that the degraded or spliced form of SUT4 might exist in the endoplasm. The localization of group 4-type proteins in SEs is unpredictable due to the SE cells, which do not possess vacuoles. AtSUC4 and HvSUT2 are localized to the tonoplast by Endler et al. (2006), and high levels of *SlSUT4* mRNA are detected in sink tissues (Weise et al., 2000). Therefore, the above group 4-type transporters may represent vacuolar sucrose transporters that are primarily expressed in sink tissues. Localization of StSUT4-GFP in plant cells reveals a very similar distribution of the fusion protein both at the plasma membrane and in internal membranes surrounding the nucleus (Chincinska et al., 2008). It is suggested that SUT4 from solanaceous plants could be localized in both plasma membrane and the endomembrane system and undergoes dual targeting.

4.3.2 SUT4 affects sucrose efflux from source leaves

The investigation of *SlSUT4* over-expressing tobacco plants shows that the sucrose efflux was reduced in source leaves because of the accumulation of soluble sugars (Fig. 31). This result, to some extent, correlates with the investigation of *StSUT4-RNAi* potato plants. The *StSUT4-RNAi* potato plants showed an enhanced sucrose efflux at the end of the light period, and the Suc content was increased in *in-vitro* grown tubers and in the shoot apical meristem at earlier stages of *StSUT4-RNAi* plants (Chincinska et al., 2008). The sufficient carbohydrate supply to sink organs stimulates the development of sink organs. Therefore, the *StSUT4-RNAi* potato plants developed an early-flowering phenotype. It is well known that SUT1 is the most important sucrose (Suc) transporter for the efflux of sucrose from mature leaves because it is highly expressed in source leaves (Bürkle et al., 1998; Kühn, 2003; Hackel et al., 2006; Slewinski et al., 2009). Over-expression of the *SUT1* gene in transgenic tobacco plants leads to a similar early-flowering phenotype (Riesmeier et al., 1994) as observed in *StSUT4* inhibited potato plants (Chincinska et al., 2008). However, SUT4 might play a role as an inhibitor of SUT1. The inhibition of sucrose efflux in source leaves results in the shortage of carbohydrates in sink organs, so the *SlSUT4* over-expressed tobacco plants show a significant retarded growth and a long vegetative growth phase (Fig. 30 and Fig. 33). Similar phenotypes are observed on *NtSUT1* antisense tobacco plants (Bürkle et al., 1998).

SUT2 and *SUT4* expression is more prominent in sink tissues: *SlSUT2* in pollen and *SlSUT4* in ovary while *StSUT4* mainly in flowers and tubers (Hackel et al., 2006; Chincinska et al., 2008). Phenotypical analysis of *StSUT4-RNAi* potato plants reveals that the inhibition of *StSUT4* expression leads to early flowering, increased tuber yield and reduced sensitivity of the plants towards enriched far-red light (i.e. in canopy shade).

4.3.3 StSUT4 is involved in shade avoidance responses

Under canopy shade condition, the ratio of red: far-red light is low and far-red light is somehow enriched. The plants show shade avoidance responses such as a rapid and pronounced increase in the elongation growth rate of the stems and petioles. *StSUT4* reduced potatoes do not show shade avoidance responses under shade conditions (Chincinska et al., 2008). Under artificial shade condition, the *SUT4* accumulation is induced in detached wild-type potato leaves (Fig. 36). Further analysis shows that the increased *SUT4* level is caused by prolonged half life of *SUT4* under far-red light enrichment (Fig. 37).

Two phytochrome-encoding genes (*PHYA* and *PHYB*) have been characterized in potato (Heyer and Gatz, 1992, 1992b). Among these only *PHYB* stably accumulates in green leaves, and is thus likely involved in sensing day-length duration. By transforming *andigena* potatoes with an antisense construct for *PHYB* gene, Jackson et al., (1996) argue that *PHYB* does play a crucial role in day-length perception and in the regulation of the SD pathway of tuberization. The anti-PHYB potato plants, which show down regulation of phytochrome B, tuberized earlier under LD condition (Jackson et al., 1998). A similar phenotype has been found in *StSUT4-RNAi* potato plants (Chincinska et al., 2008). It has been shown that *PHYB* at least in LD plants affects flowering negatively via inhibition of *CONSTANS* and *FT* (Cerdan and Chory, 2003; Endo et al., 2005). So PHYB inhibits tuberization and flowering in LD conditions and is relevant for the induction of a shade avoidance response under a far-red enriched light regime.

The *phyB* antisense potato plants show constitutive shade avoidance responses, whereas *StSUT4*-RNAi plants do not show SAS even under shade conditions. Quantification of the *SUT4* transcript levels indicates that *SUT4* mRNA accumulation increases upon shading (Fig. 39). In *phyB* plants the *SUT4* mRNA level is already increased under illuminated conditions, whereas in *SUT4*-inhibited plants no such increase of the *SUT4* mRNA level could be observed (Fig. 39). Therefore, it is concluded that these increased *SUT4* mRNA levels are essential for the development of the shade avoidance syndrome. SUT4 is potentially negatively regulated by PHYB under white light conditions and is responsible for the PHYB response.

The *StSUT2&StSUT4-RNAi* potato plants show early flowering (Fig. 17) and short internodes (Fig. 18). This is consistent with the results from *StSUT4-RNAi* potato plants (Chincinska et al., 2008). The analysis of *StSUT4-RNAi* plants reveals a potential role of the SUT4 protein in the interconnection between photoreceptor mediated responses and the gibberellin signal transduction pathway. This is because GA biosynthetic enzymes, as well as shade avoidance response including internodes elongation and early flowering, are affected in these plants (Chincinska et al., 2008).

The SlSUT4 over-expressing tobacco plants keep staying green for a longer period (Fig. 30 and Fig. 33), and a similar phenotypes has been obtained from *phyB* over-expressing tobacco plants and heterologous *phyB* over-expressing potato plants (Thiele et al., 1999). The retarded growth phenotype (statistical analysis in Fig. 33) is similar to: *NtSUT1* antisense tobacco (Bürkle et al., 1998), *StSUT1* antisense potato (Kühn et al., 2003) and *SlSUT1* antisense tomato plants (Hackel et al., 2006). These results supply more hints for the hypothesis that SUT4 might be an inhibitor of SUT1.

Jasmonates, particularly phytohormone jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA), regulate many developmental processes such as embryogenesis, pollen, seed development and root growth (Liechti et al., 2006). The *StJAR1* protein sequence shows high homology to the *JASMONIC ACID RESISTANT1* (*JAR1*) protein in *Arabidopsis*. The JAR1 gene encodes a JA-amino acid synthetase involved in conjugating JA to isoleucine (Staswick and Tiryaki, 2004). The expression of *StJAR1* is inducible by MeJA (Fig. 39). The *jar1* mutant Arabidopsis plants exhibit a decreased sensitivity to exogenous JA, are susceptible to certain pathogens, and are unable to exhibit rhizobacteria-induced systemic resistance (ISR) (Pieterse et al., 1998; Staswick et al., 1998). These plants also have altered response to ozone (Overmyer et al., 2000). However, the *jar1* plants do not show male sterile phenotype, this suggests that the activity of JAR1 is required for an optimal JA signaling in some but not all responses in *Arabidopsis* (Staswick and Tiryaki, 2004). Tanaka et al.,(2002) have analyzed the *AtGH3a* expression in wild-type, the *phyB* mutant under constant white light or FR condition, the results indicated that expression of *AtGH3a* gene is under control of the light stable phytochrome namely phytochrome B (PHYB). The analysis of *StJAR1* expression in *phyB* antisense potato plants under FR shows *StJAR1* expression is under control of PHYB (Fig. 39), this is consistent with the above study, so StJAR1 is supposed to be downstream of PHYB in the potato flowering pathway.

Flowering of Arabidopsis is promoted by long days and delayed by short days. Mutations in the *GIGANTEA* (*GI*) gene delay flowering under long days but have only small or no effect at all under short days (Fowler et al., 1999). The mutation *gigantea* (*gi*) is recessive and belongs to the late-flowering mutations in *Arabidopsis*

thaliana. The late-flowering mutations result in a pronounced delay in flowering due to a prolonged phase of vegetative growth, which is manifested by an increased number of primary foliage leaves in the rosette (i.e. vegetative nodes). Araki and Komeda (1993) have examined the nature of the gi mutation, the results indicate the *GI* locus is involved in the promotion of floral initiation (entrance of the meristem into the transitional stage) by long-day photoperiods. According to the real-time PCR analysis of *StSUT4-RNAi* potato, the *GI* expression is reduced in the transgenic lines compared to wild-type *Désirée* (Fig. 40), so SUT4 functions might be involved in the regulation of *GI* expression. Whereas, *StJAR1* expression is increased in *StSUT4-RNAi* and double RNAi potato plants under normal light conditions, there is no change in *StSUT2-RNAi* potato plants (Fig. 40). Therefore, it is assumed that *StJAR1* act upstream of *StSUT4* without affecting *StSUT2* expression.

Since *StSUT4* expression in *StSUT2&StSUT4-RNAi* potato plants are down-regulated (Fig. 15 and Fig. 16); the plants show early flowering and shorter internodes like *StSUT4-RNAi* potato plants (Fig. 17 and Fig. 18). This is an evidence to demonstrate that *SUT4* is involved in flower inducing pathway.

4.3.4 SUT4 serves as an component in potato flowering and tuberization pathways

For decades, there are attempts to identify and discuss whether the “florigen” and the “tuberigen” are identical or not. The mRNA or the protein of the FLOWERING LOCUS T (FT) has been believed to play a role in flower induction as a phloem mobile signal (Wigge et al., 2005). The flower and tuber inducing signal in *StSUT4-RNAi* plants is shown to be transmitted in grafted plants (Chincinska et al., 2008). Flower induction in *StSUT4-RNAi* plants is obviously light dependent. It is not known how the light perception by photoreceptors in source leaves is transmitted toward the shoot apical meristem to induce flowering. The relationship between *StSUT4* and other components such as S*tGI, StCOL3, StSOC1 and StFT* is speculated (Fig. 51). This is based on the expression of different genes in *StSUT4-RNAi* potato plants under LD or SD conditions. Under SD conditions, *StCOL3* expression is induced due to the missing down-regulation of *StSUT4* in *StSUT4-RNAi* potato plants; *StSOC1* is induced and the *StFT* expression is up regulated and promotes the flowering induction (Fig. 41). While under LD conditions, *StCOL3* is down regulated in *StSUT4* inhibited potato plants; the *StSOC1* is reduced and the flower inhibition by *StSOC1* is absent; the *StFT* expression level is still up-regulated (Izabela Chincinska, PhD dissertation, 2009). Therefore, the transgenic *StSUT4-RNAi* potato plants show early flowering and tuberization even under LD condition (Fig. 51).

Previous observations in potato plants with reduced expression of *StSUT4* revealed lower levels of soluble sugars at the beginning of the light period and an increased sucrose efflux from leaves (Chincinska et al., 2008). In transgenic potato, tobacco and tomato plants with reduced *SUT1* expression, the sucrose efflux from leaves is reduced and a high level of soluble sugars is accumulated in the leaves (Bürkle et al., 1998; Kühn et al., 2003; Hackel et al., 2006). SUT1 is therefore assumed to be the main phloem loading sucrose transporter in leaves. *SlSUT4* over-expressing tobacco show a similar phenotype like SUT1 down-regulated potato, tobacco and tomato plants, which are late flowering and show high accumulation of soluble sugars in source leaves, retarded growth, lower seed yield and so on (Fig. 30-34). All these observations indicate that SUT4 might serve as an inhibitor of SUT1 during phloem loading.

Induced expression of *StSUT4* in response to gibberellins application in wild-type plants led to even more pronounced differences between wild-type and *StSUT4-RNAi* plants regarding tuber yield and internode elongation, indicating a reciprocal regulation of *StSUT4* and gibberellins (Chincinska et al., 2008).

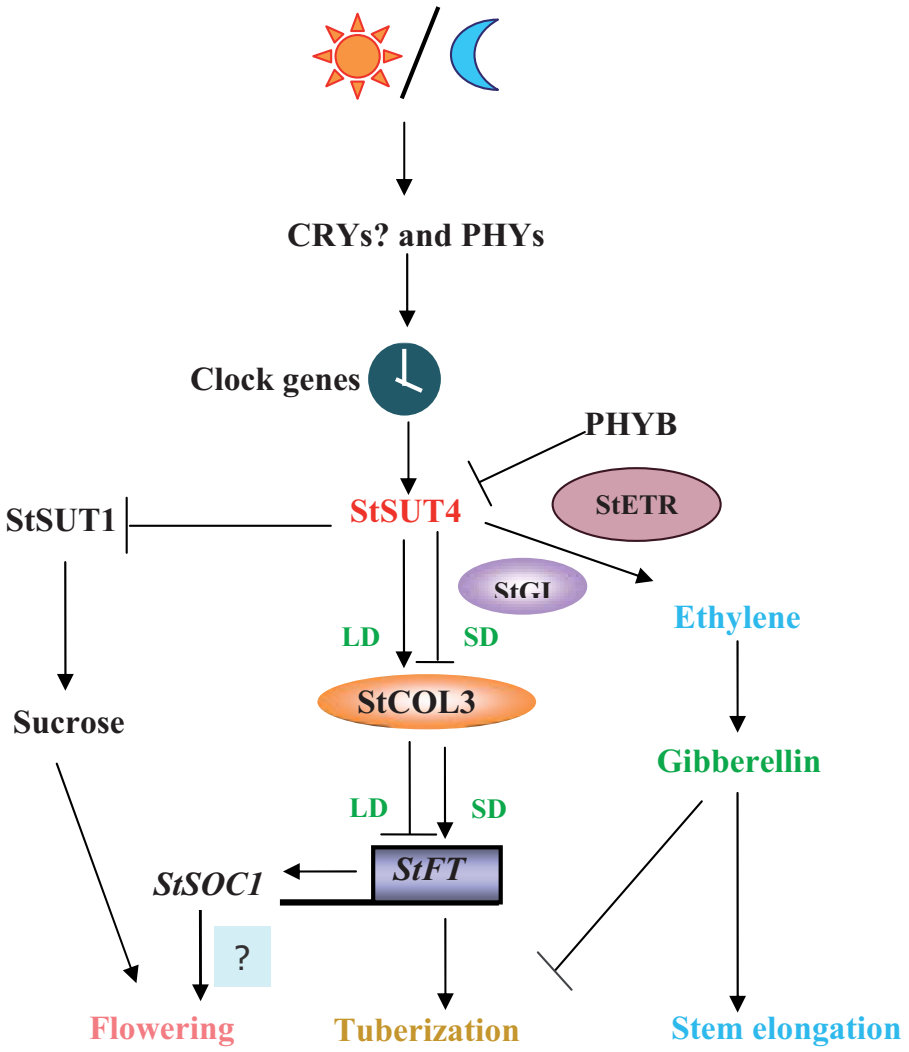


Fig. 51: The Long-day (LD) and Short-day (SD) pathway controlling flowering and tuberization in potato

The pathway is based on Rodriguez-Falcom et al. (2006). Some modification of the above illustration based on the recent results of the working group at Humboldt University is completed by Christina Kühn (unpublished). The analysis under LD condition is performed by Izabela Chincinska (PhD dissertation, 2009). The circadian clock is reset by phytochromes and cryptochromes and is the central component of the day-length measuring mechanism.

4.4 The role of StSUT2 in potato plants

The inhibitor study shows that *StSUT2* mRNA stability is regulated at the post-transcriptional level (Fig. 9 and 10). The pollen of some *StSUT2&StSUT4-RNAi* potato plants (line 5-2, line 8-1, line 4-3) has a lower viability and a reduced germination rate (Fig. 19-21), and the *StSUT2* expression level is reduced in sink organs of line 3-2, line 5-2 and line 8-1 (Fig. 16 and Fig. 21A). It is already shown that *SlSUT2* protein in tomato contributes functionally as a sucrose transporter and is essential for pollen tube growth and thereby for efficient pollination in tomato flowers (Hackel et al., 2006). So it is not accidental that *StSUT2-RNAi* potato and *StSUT2&StSUT4-RNAi* potato plants showed lower pollen viability and a lower pollen germination rate. This confirms that *StSUT2* is involved in pollen tube growth and pollination in potato plants as well.

The *StSUT2&StSUT4 RNAi* potato plants have a slightly reduced tuber yield (Fig. 22 and Fig. 23), which are not consistent with *StSUT4-RNAi* potato plants that show an increased tuber production (Chincinska et al., 2008). It must be indicated that *StSUT2* might be a regulator of tuber production. Sucrose transport from source leaves to sink tubers is known to be essential for starch accumulation in developing tubers (Kühn et al., 2003). Genetic variability of the genes controlling sucrose transport may have a quantitative effect on the starch content of mature tubers. The QTLs for tuber starch content and tuber yield have been positioned on the potato RFLP map and they are linked to some factors controlling tuber starch content which have pleiotropic effects on tuber yield (Schäfer-Pregl et al., 1998). Genes controlling sucrose transport are candidates for QTL controlling tuber starch content due to their essential physiological functions.

Three unlinked *SUT* loci are identified in the potato molecular map: *SUT4* on linkage group IV, *SUT1* on linkage group XI, and *SUT2* on linkage group V (Barker et al., 2000). On linkage group IV, QTLs for tuber starch content are located in the interval flanked by RFLP loci *GP180(a)* and *GP261(a)* and are closely linked to the branching enzyme locus *BE* (Schäfer-Pregl et al., 1998).

The *StSUT2&StSUT4 RNAi* potato plants seem to be more sensitive to *Phytophthora infestans* (data not shown here) and have more branches (Fig. 23A). The tubers of the transgenic plants have a longer dormancy period (Fig. 24). Promoter analysis by the

Web Signal Scan Program (http: //www.dna.affrc.go.jp/sigscan/signal1.pl) has been performed with the genomic *SlSUT1* and *SlSUT2* sequences. Analysis of the genomic *SlSUT2* sequence reveals *cis*-elements necessary for induction by ABA or SA, regulation by GA, ethylene, light, wounding, salt, water stress and for pollen-specific expression. In addition, two auxin response elements (AuxRE) are located in the *SUT2* promoter region and in the 10th intron arguing for a regulation via the auxin response factor (ARF) (He et al., 2008). This might be a reason why the *StSUT2-RNAi* and *StSUT2&StSUT4-RNAi* potato plants have more branches. The increased branches need more vegetative growth, so the carbohydrates from photosynthesis have to supply to branch growth. The tuberization might be inhibited due to the deficient nutrient supply from source organs. The tuber yield of StSUT2&StSUT4 is reduced due to the shortage of carbohydrates in sink organs (Fig. 22). Potato tuberization is a complex developmental process involving interactions between several environmental, biochemical and genetic factors. Further investigation about the involvement of StSUT2 in tuberization is highly recommended.

StSUT2-RNAi and *StSUT2&StSUT4-RNAi* potato plants show a lower pollen germination rate (Fig. 21) and the pollen viability is significantly reduced (Fig. 19 and Fig. 20). These results suggest that *StSUT2* is properly involved in sucrose uptake during pollen development and germination in potato plants. It is consistent with SlSUT2 function in tomato, AtSUC1 function and AtSUC3/ AtSUT2 in Arabidopsis (Meyer et al., 2004; Hackel et al., 2006; Sivitz et al., 2008).

In summary, *StSUT2* plays a role during pollen germination as previously shown for *SlSUT2,* and in tuber development. The expression of *StSUT2* might be regulated by auxin. The molecular mechanism of StSUT2 function needs to be elucidated.

4.5 Regulation of sucrose transporter protein stability and localization

4.5.1 PDI might be involved in protein folding and localization of sucrose transporters

Using the BiFC assay, the interaction between StSUT4 and StPDI is confirmed (Fig. 42A). The protein complexes are localized in the endoplasmatic reticulum (ER). Additional localization experiments show that StPDI was co-localized with an ER marker protein (Fig. 42B). It is consistent with most PDI localization experiments, e.g. Shorrosh et al., (1993) have confirmed the localization of PDI to the ER in soybean root nodules by immunogold electron microscopy. It is known that the ER is a part of endomembrane system and is responsible for the synthesis, processing, and sorting of proteins and lipids and for the regulation of cytosolic calcium levels (Staehelin, 1997;

Voeltz et al., 2002). The ER is moreover an integral component of plasmodesmata in higher plants and forms the desmotubule linking the ER of neighboring cells (Roberts, 2005). Thus, plasmodesmata offers two potential pathways for transport between cells: the cytoplasmic sleeve for cytosolic solutes and the desmotubule for ER-associated molecules respectively (Schulz, 1999). Martens et al., (2006) show that the ER membranes of CC and SE are intimately connected, and provide a membrane pathway available for signal transduction and posttranslational membrane protein transport. The ER is an important site of PDI function, in part due to the oxidizing environment that favors disulfide bonds (Houston et al., 2005).

The PDI families are molecular chaperones that contain thioredoxin (TRX) domains and aid in the formation of proper disulfide bonds during protein folding. Houston et al., (2005) have carried out a genome-wide search of Arabidopsis and produced a comprehensive list of 104 genes encoding protein with TRX domains. There are 10 classes of PDI-like proteins in plants with the various functions. Eps1, a PDI-like protein of yeast, has a transmembrane domain and is involved in ER-associated protein degradation. The co-localization of StSUT4-StPDI complex in the ER might be related to the degradation of StSUT4, and this argument is consistent with the detection of a truncated SUT4 protein in the endomembrane fraction of tobacco leaves on Western blots (Fig. 35). The protein sequence of StPDI identified in this study contains two TRX domains, belongs to the orthologs of AtPDI11. It has the domain organization of a-a'-ERp29 without b or b' domains. The KDEL ER retention signal is missing in C-terminal of StPDI. It is possible that PDIs without the ER retention signal have a relatively broad subcellular localization and different functions.

PDI proteins are also found in plasma membrane rafts, at the cell surface, in the nucleus, and some of them can be secreted (Turano et al., 2002; Gruber et al., 2009). PDI can act as secretion factor or escort proteins. The YFP localization of StPDI (EST 707280) shows that the PDI is also detectable at the PM, potentially in rafts-like microdomains. These results indicate that PDI is probably involved in the vesicle trafficking of SUT4 protein from ER to PM.

4.5.2 SUT4 is involved in ethylene signaling pathway through interaction with ETR, an ethylene receptor

The interaction between StSUT4 and StETR1 is confirmed by BiFC. StETR1 is co-localized with an ER marker protein (Fig. 43) which is consistent with previous studies in Arabidopsis (Chen et al., 2002). SUT4 is ethylene inducible in both tobacco and potato plants. In wild-type tobacco, *SUT4* mRNA levels increased after 1 hour induction with 3 mM ethephon on Northern-Blot (Fig. 44), and the induction keeps at least 24 hours. This correlates with SUT4 induction detected in wild-type potato

leaves treated with 350 µM ethephon by qRT-PCR (Chincinska et al., 2008). On the other hand, the ethylene production in *StSUT4-RNAi* potato plants is restricted (Fig. 45). It is one reason to explain that *StSUT4-RNAi* potato plants show reduced shade avoidance responses under shade condition. This is a hint for StSUT4 to be a positive regulator of the ethylene signaling pathway.

It has been shown in tobacco plants that ethylene and GA interact with phytochrome-mediated SAS (Pierik et al., 2004). The ethylene insensitive tobacco plants have reduced responses to neighbours. This research shows that the gaseous plant hormone, ethylene, plays an important role in shade avoidance. On the other hand, low red to far red enhanced ethylene production in wild-type tobacco plants, resulting in shade avoidance. The ETR1 could play a role in ethylene sensing and SUT4 expression is inducible by ethylene. Since the ethylene-sensing stimulates R:FR-mediated shade avoidance responses it is argued that SUT4 has a positive effect on shade avoidance responses of plants. Further investigations regarding the StSUT4 involvement in the ethylene and GA pathway are still required.

4.6 Yeast three-hybrid system for identification of RNA-protein interactions

The yeast three-hybrid (Y3H) assay is a genetic method to detect RNA-protein interactions in yeast. The interaction can be monitored by cell growth, colony colour, or the levels of a specific enzyme. In this method, a wide variety of interactions could be accessible since the interesting interaction is analyzed independent of its normal biological function (Bernstein et al., 2002). Performing a three-hybrid experiment is a daunting task involving many different steps like two-hybrid assay. There are a number of potential problems ranging from merely aggravating to potentially insurmountable. Some technical problems appeared during cloning genes of interest into properly designed plasmids and while introducing those plasmids into yeast. Once the bait plasmid and prey cDNA library are introduced into the cells, a variety of *in vivo* criteria must be met.

4.6.1 Hybrid RNA (Bait vectors)

The secondary and tertiary structures of RNAs often are critical in their interactions with proteins. Most RNA inserts of suitable sizes tested in the three-hybrid system could form relatively stable structures *in vitro* (Zhang et al., 1999). The producing RNAs should be comparable and high abundant since the RNA abundance may be important in the determination of the level of signal produced from the reporter gene (Bernstein et al., 2002). The host yeast strain is normally transformed with the RNA plasmid first. Cells containing the RNA plasmids are then transformed with a cDNA

library fused to the activation domain (AD) of a transcription factor. Hybrid RNAs usually are not toxic to the host cell. When they are toxic, the RNA plasmid and the cDNA can be cotransformed into the host cells, so the detection of RNA-protein interaction would be possible. In this study, the host strain L40uraMS2 is transformed with the bait plasmid pRH3′/StSUT4 or pRH3'/StSUT4+3'UTR. The StSUT4 RNA is generated rather than SUT4 protein. According to the growth condition of cells, the bait plasmid pRH3′/StSUT4 or pRH3'/StSUT4+3'UTR is not toxic to L40uraMS2.

Two bait RNAs, 1503 nt and 1709 nt respectively are inserted into pRH3' and the resultant constructs are used as bait RNA during large scale transformation. The size of the RNA insert appears to be an important determinant of the three-hybrid activity. The sensitivity of detection of RNA-protein interactions is reduced by the large inserts. These two bait RNAs seem to be too large to get genuine interaction partners and several false positives are detectable during large scale screens.

4.6.2 Differences of VP16 and Gal4 activation domain

The first criterion of activation domains is that the protein fusions have to be expressed from plasmids at appropriate high level to generate an interaction signal but not so high in order to prevent toxicity. Yeast cells containing the RNA plasmid were transformed with cDNA library fused to a transcription activation domain (AD). The potato cDNA library used in this study was originally in pENTR^{TM} 1A, so it is convenient to transfer the cDNA library into pGAD10-GW AD vector using GATEWAY BP and LR clonase. The great advantage of the GATEWAY^{TM} cloning system is the possibility to transfer the gene sequences of an entire library quickly and easily from the original vector to a destination vector. In this study the entire potato cDNA library was transferred to the pGAD10 expression vector by *in vitro* recombination in a single tube reaction. The pGAD10-GW vector contains Gal4 transcription activation domain and the full length ADH promoter. This family of transcription AD could reduce the toxicity problem caused by pYESTrp3 vector, which is designed for the LexA system using the activation domain of the VP16 transcription factor. The VP16 transcription AD is a strong AD that may cause toxicity when it is over-expressed. So the utilize of pGAD10 which has a GAL4 AD somehow could reduce the toxicity problem using VP16 transcription AD. In this study, the positive and negative control experiments are performed using the supplied control constructs and yeast strain L40uraMS2. L40uraMS2 is transformed with pYESTrp3 or pYESTrp3/IRP and pRH3' or pRH3'/IRE. X-gal assay and histidine selection results show that the system might efficiently be used to screen for SUT4 RNA-binding protein (Fig. 46). Nonetheless, the missing positive control carrying a Gal4 activation domain is still an open problem.

4.6.3 Identification of sequence specific RNA binding proteins

The Y3H system allows the identification of naturally occurring RNA and protein partners, and dissection of higher-order RNA-protein complexes. Like other genetic strategies, Y3H has one attractive feature that a clone encoding the protein of interest could be obtained directly in the screen. The system offers the possibility of connecting RNAs and proteins on a broad and genome-wide scale. Human stem-loop binding protein (SLBP) (or hairpin binding factor, HBF) is the first RNA-binding protein cloned using the three-hybrid system (Martin et al., 1997). Three categories of false positives are common in the Y3H system. One category of false positives is partial cDNA fragments coding for artificial short peptides mimicking false positive interaction, or cDNAs which are not in frame of the Gal4 activation domain. Five out of seven initial identified putative interaction partners belong to these false positives since the sequence results show that they are not fused to the Gal4 activation domain. A second is to define a false positive as a protein which binds non-specifically to the RNA while some others activate the reporter independently of the RNA ("RNA-independent positives"). The RNA-independent positives are common in performing screens for RNA-binding proteins using cDNA libraries. Corresponding control experiments have been performed in this study, and the LEAFY protein was identified and discarded as RNA-independent false positive (Fig. 48). A third point is to consider clones that reproducibly interact by yeast three-hybrid as plausible partners but whose interaction cannot be readily confirmed by other means (EMSA, UV-cross linking or RNA-protein immunoprecipitation and so on). Although, the SUT4 RNA-binding activity of StJAR1 (ES463778) and Hsc70 (DV525645) was shown by UV-cross linking (data not shown), it is not certain whether the binding activity is due to the ATP-binding capacity of both proteins.

The cDNA (CV494117) carrying hypoxia-induced conserved domain was identified in 4 independent yeast colonies. It is 77.9% identical to a C3HC4 RING-finger protein in *Arabidopsis thaliana*. It has been reported that the protein is involved in various biological processes. In plants, several RING (*Really Interesting New Genes*) proteins have been identified and characterized, for example, PEX10, a C3HC4 zinc-RING finger peroxisomal membrane protein which is required for photorespiration and leaf peroxisome contact with chloroplasts (Schumann et al., 2007). In addition, CaKR1, an ankyrin-repeat domain C3H1 zinc finger protein in *Capsicum annuum* is involved in responses to cold and salinity stress (Seong et al., 2007). And *Capsicum annuum* RING Zinc Finger Protein 1 (CaRZFP1) was isolated from a cDNA library from hot pepper plants treated by heat-shock and was inducible by diverse environmental stresses (Zeba et al., 2006). Transgenic tobacco lines expressing the *CaRZFP1* gene show enhanced growth until flowering (Zeba et al., 2009). Microarray analysis shows up-regulation of a gibberellin-responsive gene in P35S:CaRZFP1

seedlings. The gibberellin-responsive gene has been found to be involved in hypocotyl and stem elongation (Peng et al., 1999). It is suggested that StHIG1 might play a role in connecting StSUT4 with the gibberellin pathway.

Classical zinc finger proteins often employ multiple zinc fingers to bind to nucleic acids. Aromatic residues within the zinc fingers typically interact with the nucleic acid to impart sequence specificity (Matthews and Sunde, 2002). In addition to binding to nucleic acids, zinc fingers can also bind to proteins. Furthermore, there are a number of proteins that contain multiple zinc fingers that mediate distinct binding interactions. For example, the transcription factor TFIIIA, a dual RNA/DNA binding protein, contains nine zinc fingers, and individual zinc fingers are used to recognize both RNA and DNA targets as discussed by Apponi and colleagues (Apponi et al., 2007). They argue that Nab2 (an essential, shuttling hnRNA which is required for both mRNA poly(A) tail length control and poly(A) mRNA transport), a CCCH-type of zinc finger proteins could interact with another RNA binding protein (Pub1) to modulate mRNA stability and strengthen a model where nuclear events are coupled to the control of mRNA turnover in the cytoplasm (Apponi et al., 2007). Since the putative interaction partner StHIG1 only contains the N-terminal HIG1 domain of the C3HC4 RING-finger ubiquitin ligase and no RING-finger domain, the interaction of StSUT4 RNA and StHIG1 probably has other regulatory mechanism. This is still to be investigated.

4.6.4 Limitations of yeast three-hybrid assay

For yeast three-hybrid screening, the analyzed RNA sequences are restricted in two respects. First, runs of four or more U's in succession can terminate the transcription by RNA polymerase III. Second, the length of the RNA inserts should be less than 150-200 nucleotides (nt) to yield higher signals: longer inserts commonly reduce the level of activation of the reporter. In principle, both of these limitations might be overcome by using a different polymerase such as bacteriophage RNA polymerase. A system using RNA polymerase II had been described (Putz et al., 1996). The RNA-protein Hunter system from Invitrogen is using a bacteriophage RNA polymerase. The size of the RNA insert appears to be an important determinant of three hybrid activity. In reconstruction experiments using known RNA-protein partners, RNA sequences that are less than 150 nt in length (e.g., IRE 51 nt, U1-70K 45 nt) typically yield substantial and specific reporter activation (Bernstein et al., 2002). Nevertheless, substantial signals can be detected from longer RNAs, for example, Rho and Martinis used a 1600 nt RNA containing the yeast cobI intron to detect its interaction with two different proteins. Their results were consistent with a novel model for a ternary splicing complex (two proteins: one RNA) in which both protein splicing partners bind directly to the bI4 intron and facilitate its self-splicing activity (Rho and Martinis, 2000). In this study, the inserted RNAs, StSUT4 ORF and StSUT4+3'UTR

are 1503 nt and 1709 nt respectively; there are at least 30 possible deduced secondary structures for one bait RNA (the appendix only shows one). This might reduce the specificity of RNA-protein binding, only one interaction partner was detected, and the specific binding activity of this interaction partner is still to be determined. It is possible that some other interaction partners, having only weak binding activity to StSUT4, are not found yet.

The three-hybrid system has been applied to known protein–RNA interactions for a variety of purposes, using proteins from many different families and species (Bernstein et al., 2002; Riley and MaherIII, 2007). In addition, the three-hybrid assay can reveal aspects of a RNA–protein interaction that are important *in vivo* even when these are problematic to be detected *in vitro*. In this investigation, Y3H is used to identify protein partners of a known RNA sequence (StSUT4). The review of literature shows that several screens published to date reveal that few positive clones satisfying selection criteria were isolated, but the "correct" cDNA should be among them (Bernstein et al., 2002). In order to perform such experiment, it is required to arrange the stringency of the screen to yield a reasonable number of initial positives; 5 mM 3-AT concentration has been used to decrease the yeast background growth in the screens. More than two hundred clones were grown on selective medium and 108 positive clones were stained blue in LacZ assays. The detected seven RNA-dependent cDNAs have potential RNA-binding activities except JAR1; five of them were frequently appearing in both screens. Further sequencing analysis revealed that only the StH1G1 is in the frame of the Gal4 activation domain. All other putative interaction partners showed frame shifts or one or more stop codons before the start codon of the open reading frame. The alignment of the predicted amino acid sequences showed that some of putative interaction partners encode a short unspecific peptide: SRGSELSNKFVQKSRL (data not shown), encoded by the attachment site of the GATEWAY vector pGAD10 that has been used for the expression of the potato cDNA library.

It has long been known that the cucurbit phloem sap contains ubiquitin (Schobert et al., 1995; Schobert et al., 1998), and the investigation of Bill Lucas´s lab reveals that pumpkin phloem sap also contains various ubiquitin activating (E1), ubiquitin-conjugating (E2) and ubiquitin ligase enzymes (E3). With respect to the E3 type ubiquitin ligase systems, it is clear that a number of components remain to be identified within the phloem sap. Irrespective of the fact that some of these components have yet to be identified by co-immunoprecipitation, the presence of the detected 116 phloem proteins associated with ubiquitin-mediated proteolysis offers strong support for the hypothesis, which states that the functional enucleate sieve tube system has retained the capacity for proteolysis (Lin et al., 2009). The fundamental questions posed by these results relate to the process by which the 26S proteasome is

assembled in sieve elements and whether these complexes are anchored, in some way, to existing cytoskeleton or to the plasma membrane to limit their mobility within the phloem translocation stream. The putative interaction partner StHIG1, which has homologies to an ubiquitin ligase enzyme (E3), has potential to interact with ubiquitin-conjugating enzyme (E2). Both E2 and E3 might exist in the phloem sap and play a role for the regulation of the SUT4 mRNA stability or transport.

Taking together, the Y3H assay is suitable to screen for StSUT4 RNA binding proteins using a potato cDNA library. The sensitivity of this method was probably impaired due to the large inserts that had been used as bait constructs. Further investigations are still needed to confirm the interaction between StSUT4 mRNA and the putative RNA-binding partners.

5 Conclusion

This study attempts to investigate the post-transcriptional and post-translational regulation of sucrose transporters in Solanaceous plants. For this purpose, a series of experiments were performed, including parasitic and graft experiments, transgenic techniques combined with RNA interference, qRT-PCR, BiFC and yeast three-hybrid assays. These approaches were aimed to apply new information about the phloem mobility of sucrose transporter transcripts and the regulation mechanism of sucrose transporters (SUT1, SUT2 and SUT4). In other words, this work attempted to answer the following questions:

Are sucrose transporter mRNAs able to move through plasmodesmata in solanaceous plants?

What is the half life of circadian oscillating sucrose transporter transcripts and what kind of regulation mechanism of sucrose transporters exist?

What is the sink-specific physiological function of the weakly expressed sucrose transporters StSUT2 and StSUT4 in potato plants?

What kinds of proteins are involved in the regulation of *SUT4* mRNA stability and protein function and/or localization?

To sum up, the *NtSUT1* transcripts were detected in the holoparasitic *Cuscuta reflexa* grown on tobacco plants. Through intra-species graft experiments between two sets of transgenic plants and wild-type plants, CaMV 35S-SoSUT1-cmyc transgenic potato and SlSUT1p-SlSUT1-GUS transgenic tobacco plants, the transgenic *SUT1* mRNAs were detected in relative wild-type graft partners in both cases. In addition, studies on the stability and half life of the phloem-mobile sucrose transporter mRNAs showed that all previously known sucrose transporter from *Solanum tuberosum* are regulated in a circadian rhythm and that their transcripts showed an extremely short half life of 60 to max. 131 min. Moreover, inhibitor studies show that both *SUT2* and *SUT4* mRNA accumulation is controlled at the post-transcriptional level while *SUT1* mRNA stability is regulated at the transcriptional level. More aspects of SUT4 physiological functions are clarified with the help of *SlSUT4* over-expressing tobacco. It is assumed that SUT4 plays a role in the inhibition of SUT1 phloem uploading. The interactions between StSUT4 and a protein disulfide isomerase (PDI) or StSUT4 and a putative ethylene receptor protein have been confirmed by bimolecular fluorescence complementation (BiFC). Subcellular localization of the two interacting proteins by YFP-fusion reveals their presence in the ER. The corresponding YFP fusion construct can now be used for further confirmative experiments using the FRET technique.

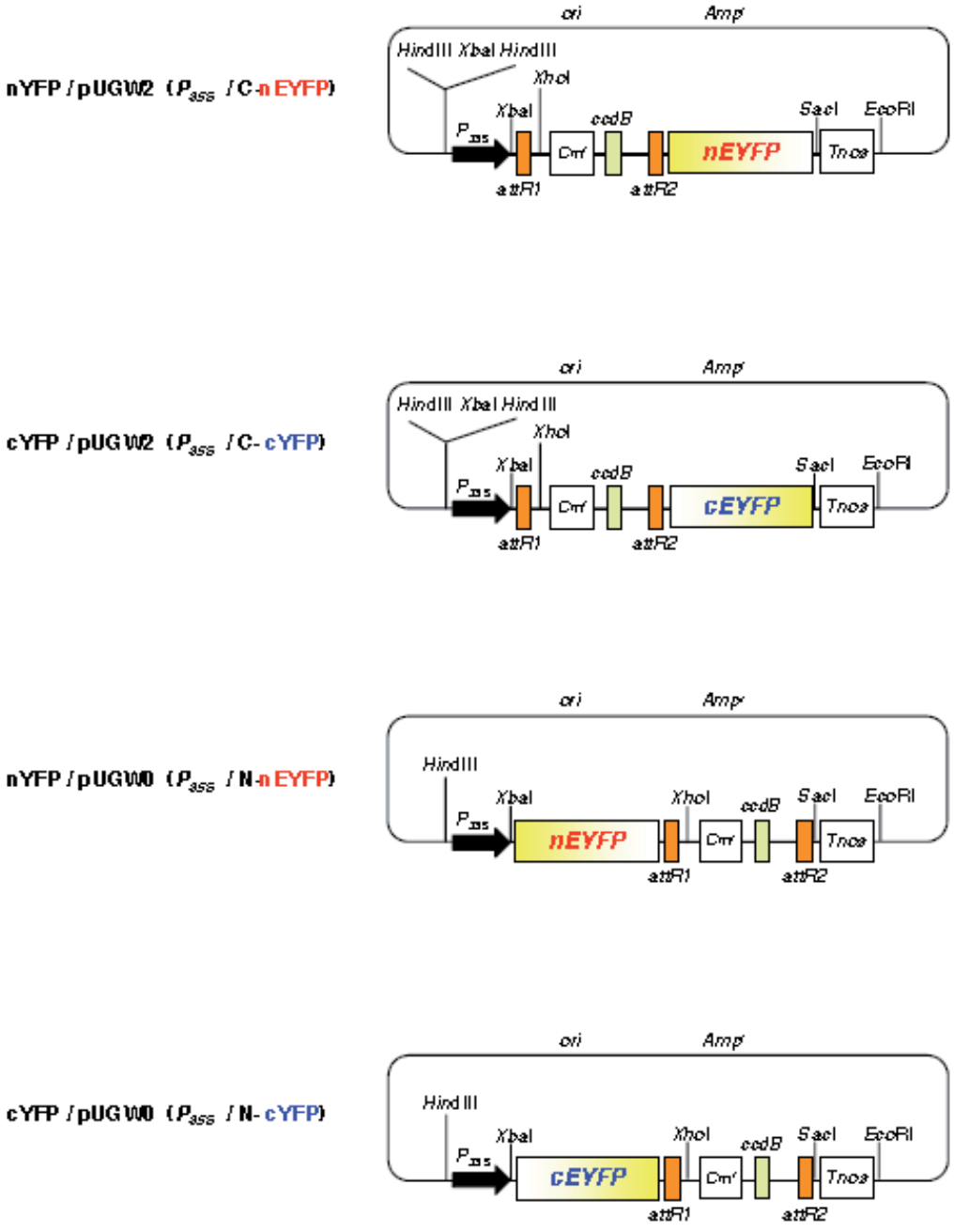
With the help of the RNA interference method, *StSUT2&StSUT4-RNAi* potato plants were generated. The relative analysis on several transgenic lines indicates that StSUT2 in potato plants is involved in pollen development like SlSUT2 in tomato plants. The reduced tuber yield of *StSUT2&StSUT4-RNAi* potato plants indicates that SUT2 might have a positive effect on tuber production. The influences on the genes, which are involved in the photoperiodic-dependent flowering and tuberization pathway such as *PHYB*, *GI*, *CO*, *SOC1*, and *FT* in *StSUT4-RNAi* potato plants under LD and SD conditions provide good evidences that SUT4 is involved in this pathway. The yeast three-hybrid approach has been established to identify RNA-protein interaction partners of the SUT4 mRNA molecule. Only one of the putative SUT4 mRNA-binding candidates could be confirmed by repeated yeast transformation: a protein containing a highly conserved hypoxia induced HIG1 domain found in many C3HC4 RING zinc finger proteins is supposed to bind to SUT4 mRNA.

Hopefully, this study would challenge to further thoughts regarding the physiological function of sucrose transporters, their mRNA phloem mobility, and the regulation of mRNA and protein stability. Further investigations about the regulation mechanism of SUT4 mRNA stability and protein transport are required.

6 Appendix

6.1 Map of vectors used in this study

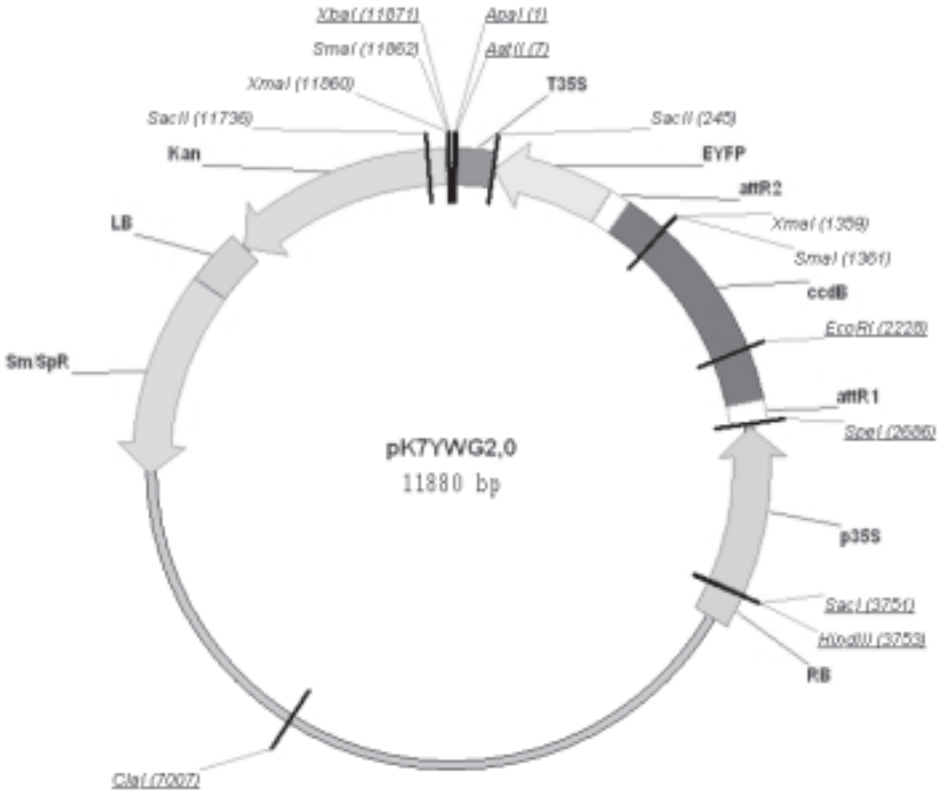
6.1.1 Schematic representation of plant-compatible gateway BiFC vectors (Invitrogen)



Supplement 1: The Structure of the BiFC gateway vectors

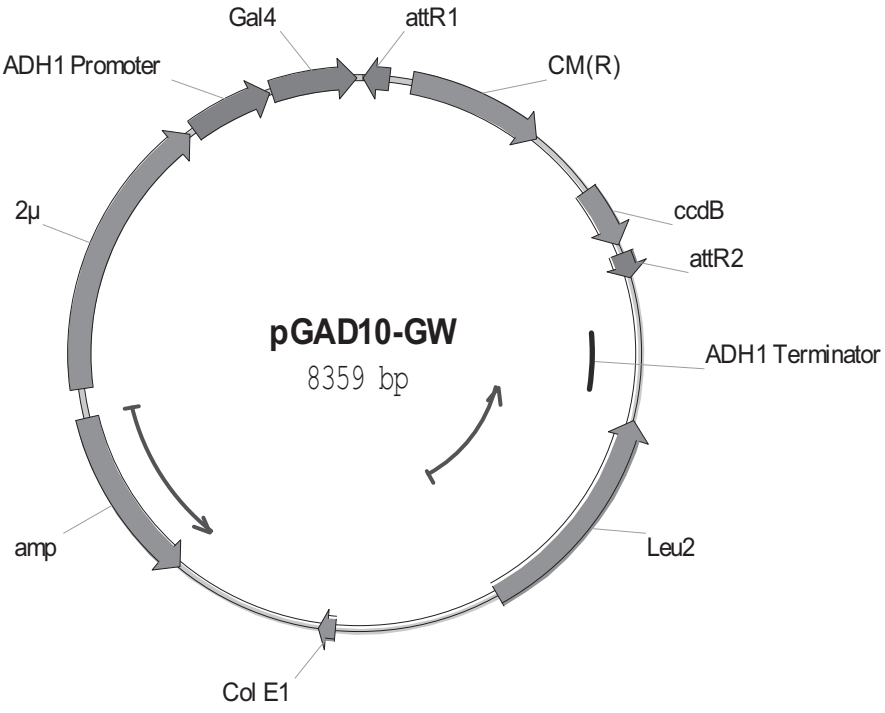
The vector used as the starting plasmid was pUGW2 and pUGW0 (Nakagawa et al., 2007) which were constructed from pUC119. The EYFP (239 amino acids) were separated into nEYFP (1-174) and cEYFP (175-239), and then introduced into pUGW2 and pUGW0, respectively, to make four types of vectors. Ori, ColE1 origin; Ampr, ampicillin resistant marker; Cmr, chloramphenicol resistant marker. The fusion gene is expressed by CaMV 35S promoter.

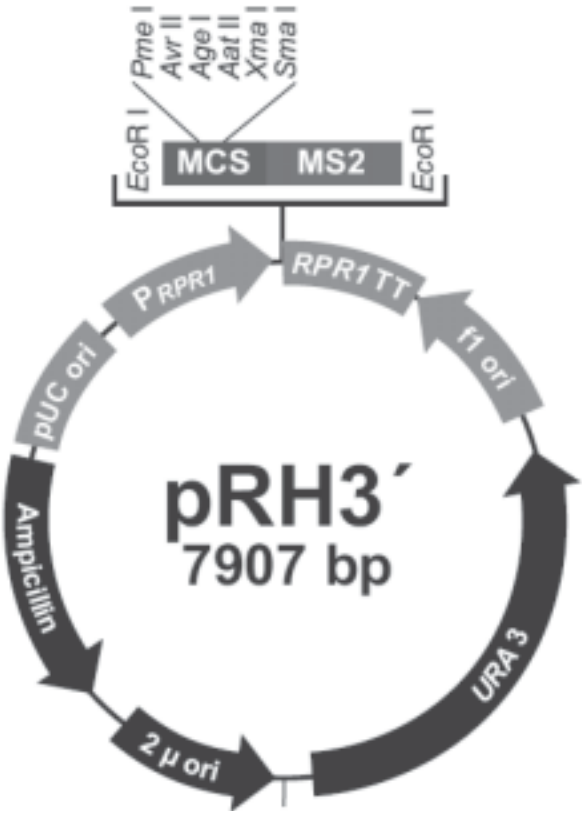
6.1.2 Binary vector for protein localization in plant cells



Supplement 2: Map of binary vector pK7YWG2,0 (Karimi et al., 2005)

6.1.3 pGAD10-GW and pRH3' for yeast-three hybrid assay



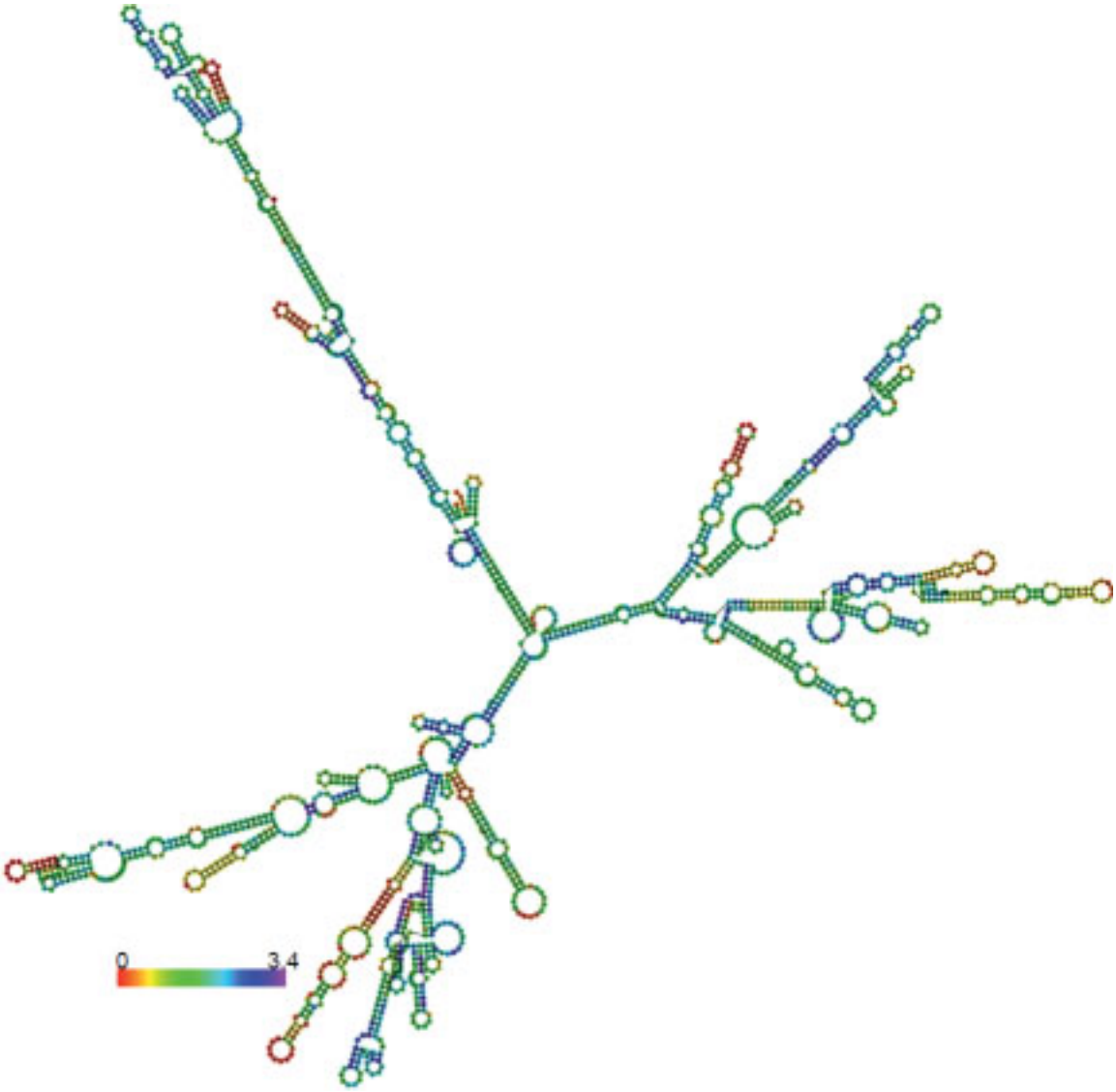


Supplement 3: The vectors for yeast three-hybrid assay

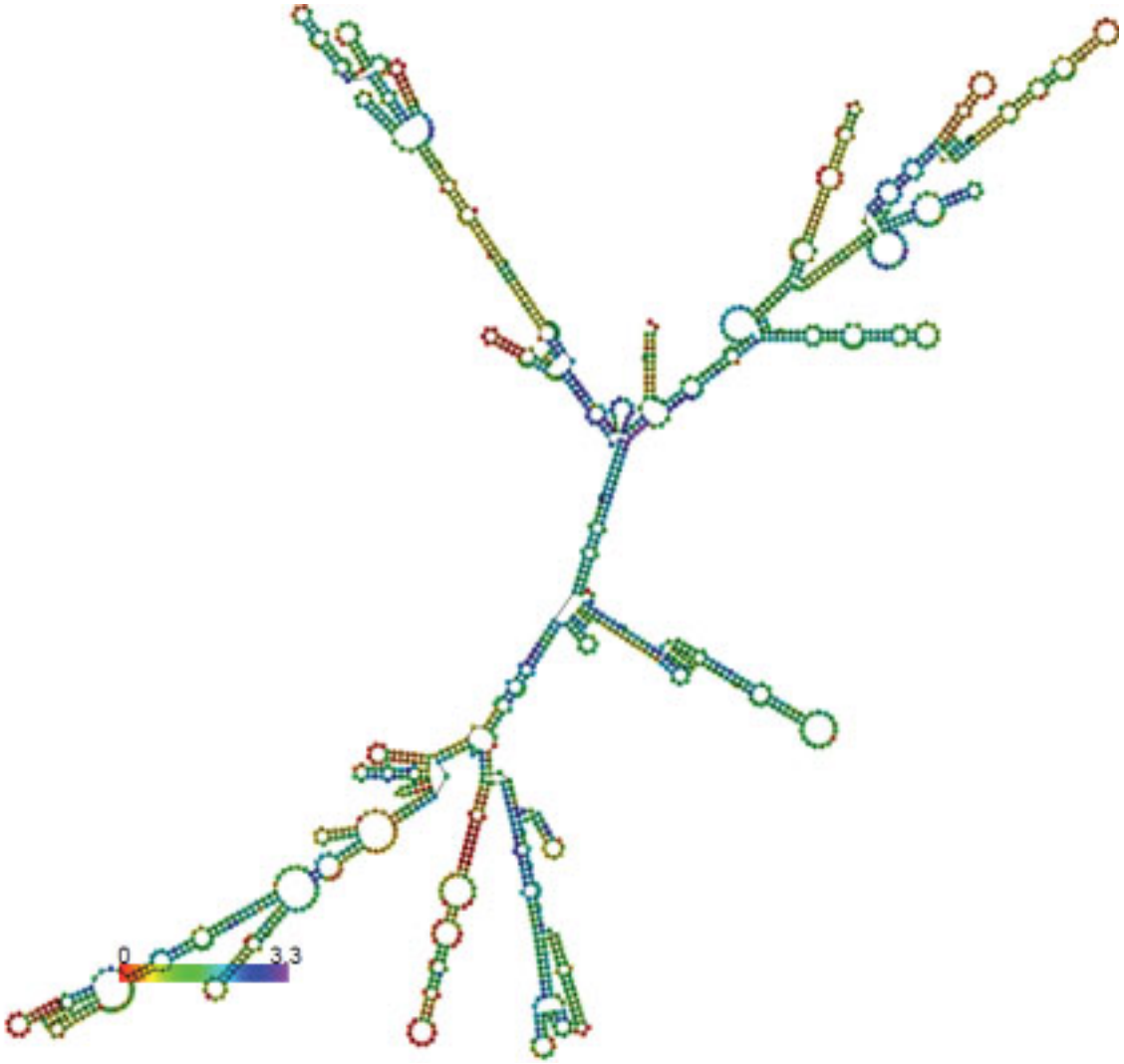
The maps of pray vector pGAD10-GW (Bürkle et al., 2005) and bait vector pRH3´ (Invitrogen).

6.2 Secondary structure of different length of StSUT4 mRNAs

The secondary structures of *StSUT4* (Supplement 1, 1503 nt)*, StSUT4 3'UTR* (Supplement 2, 1709 nt) were predicted by using the Vienna RNA secondary structure server.



Supplement 4: secondary structure of 1709nt of StSUT4 +3´UTR



Supplement 5: Secondary structure of 1503 nt of StSUT4 RNA

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Publication

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1) Hongxia He, Izabela Chincinska, Christina Kühn* (2010) Post-transcrtiptional control of sucrose transporter mRNAs in the phloem. Plant Biology, submitted.

2) Undine Krügel, **Hongxia He**, Jana Reins, Schulze WX, Johannes Liesche, Bernhard Grimm, Christina Kühn* (2010) Identification of sucrose transporter StSUT1 interacting proteins by split ubiquitin and mass spectrometry revealed SUT1 association to lipid rafts in plants. Plant Physiology, submitted.

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Oral presentations

1) Hongxia He. Sucrose transporter mRNA mobility. Dec.8 (2006) Spree-Havel colloquium, Potsdam, Germany

2) Hongxia He (speaker). Izabela Chincinska, Bernhard Grimm, Christina Kuehn. May 19-21st (2008) Final meeting of SSP1108, Hirschberg castle, Germany

3) Hongxia He (speaker), Undine Krügel, Christina Kühn. Oct. 12-16th (2008) The fifth international workshop of Solanacuous Genome (SOL2008), Cologne, Germany

Posters

1) XV FESPB Congress Federation of European Societies of Plant Biology, Lyon Frankreich 2006 "StSUT4-RNAi plants do not show shade avoidance response"

Izabela Domanska, **Hongxia He**, Bernhard Grimm, Christina Kühn

2) XIV International Workshop Plant Membrane Biology, Valencia, Spanien, 2007: "Homodimerization of the sucrose transporter StSUT1 induces targeting to lipid raft-like structures"

Undine Krügel, **Hongxia He**, Izabela Chincinska, Bernhard Grimm, Christina Kühn

3) No. 125 Botanikertagung Hamburg 2007: "Identification of SUT4-mRNA binding proteins".

Hongxia He, Undine Krügel, Izabela Chincinska, Bernhard Grimm, Christina Kühn

4) 21. Tagung Molekularbiologie der Pflanzen Dabringhausen 2008: "The sucrose transporter StSUT4 interconnects phytochrome and ethylene signaling"

Izabela Chincinska, Johannes Liesche, **Hongxia He**, Undine Krügel, Antje Rövert, Christina Kühn

ERKLÄRUNG

Hiermit erkläre ich, Hongxia He, dass ich die vorliegenden Arbeit selbstständig verfasst, und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.
Berlin, den ....
Hongxia He