Identification of a Putative SUT1 mRNA Binding Protein Using Yeast Three-Hybrid Analysis

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To my mom Janet who is an excellent teacher and parent

And

To my husband Robert who is my best friend and the love of my life

"I have a plan so cunning, you could put a tail on it and call it a weasel"

"I am anaspeptic, frasmotic and even compunctuous as to have caused you such pericombobulations"

- Edmund Blackadder

ABSTRACT

Sucrose is the major nutrient product of the photosynthesis reactions; its correct distribution via the specialized cells of the phloem is essential to plant survival. The transport of sucrose within solanaceous plants is mediated in part by sucrose transport proteins (SUTs) localized to the plasma membrane of the phloem sieve elements (SEs). SUT1, which is essential for phloem loading of sucrose, shows the predicted localization to the enucleate SE; its mRNA, initially thought to be found exclusively in the closely associated companion cell (CC), is also found in the enucleate SE. The mechanism of arrival and purpose of this mRNA localization in solanaceous plants has become a matter of dispute. The present work deals with the application of an *in vivo* method for the discovery of mRNA binding proteins, and the identification of a putative *LeSUT1* mRNA binding protein.

A high quality cDNA library encoding tomato leaf protein fusions was synthesized and cloned for use in the yeast three-hybrid method (SenGupta *et al.,* 1996). Several RNA hybrid molecules were designed based on the sequence and secondary structure of *LeSUT1* mRNA and used to screen the cDNA library for putative mRNA binding proteins. A single isolated cDNA clone was shown to interact with two structurally dissimilar RNA baits. Northern blot and RACE analysis suggest that the isolated cDNA is a fragment of a larger molecule; sequence analysis suggests the presence of a putative RNA binding domain. The isolated cDNA encodes a novel protein molecule that is able to specifically interact with *LeSUT1* mRNA *in vivo* and may be involved in the localization of *SUT1* mRNA.

While the localization of SUT1 protein was previously established (Kühn *et al.*, 1997), the mechanism of this localization has yet to be identified. In order to examine this process, tobacco sections were exposed to the fungal toxin Brefeldin A (BFA), which inhibits anterograde vesicle transport of proteins. SUT1 protein localization was inhibited by BFA application to fresh sections in preliminary experiments. When fresh tobacco sections were incubated in water, or exposed to no treatment, SUT1 protein was predictably localized to the SE. When fresh sections were exposed to 50 μM BFA for 10 minutes, however, SUT1 protein was sequestered in the CC; was visibly increased upon exposure to 100μM BFA. These initial results contribute to the formation of an emerging model of SUT1 localization in which protein and mRNA are localized in separate but related events. The highly determined secondary structure of *LeSUT1* 3'UTR as well as the existence of multiple polyadenylation states also serves to support the concept of a highly regulated complex system resulting in the localization of SUT1 to the plasma membrane of the enucleate SE.

ZUSAMMENFASSUNG

Saccharose ist das Hauptprodukt der Photosynthese und die richtige Verteilung von Saccharose durch das Phloem ist lebenswichtig für alle Pflanzen. Der Saccharosetransport in Solanaceen wird zum Teil durch Saccharosetransporter (SUT) vermittelt, die in der Plasmamembran der Siebelemente (SE) lokalisiert sind. SUT1, welches wesentlich zur Phloembeladung mit Saccharose beiträgt, ist wie erwartet in den zellkernlosen Siebelementen lokalisiert. Die *SUT1* mRNA, die ursprünglich ausschließlich in den danebenliegenden Geleitzellen (CC) vermutet wurde, wird ebenfalls im zellkernlosen SE gefunden. Die Frage, wie *SUT1* mRNA dorthin gelangt, sowie der Zweck dieser Lokalisierung in SE der Solanaceen ist noch ungeklärt. Die vorliegende Arbeit befasst sich mit der Anwendung einer in vivo Methode zur Suche nach mRNA–bindenden Proteinen sowie mit der Identifizierung eines möglichen *LeSUT1* mRNA-bindenden Proteins.

Eine qualitativ hochwertige cDNA Bibliothek aus Tomatenblättern zur Anwendung in die „Yeast three-hybrid" Methode wurde hergestellt. Basierend auf der Sequenz und der Sekundärstruktur von *LeSUT1* mRNA wurden mehrere RNA Hybridmoleküle entworfen und als „bait" zum „Screening" der cDNA Bibliothek nach möglichen mRNA-bindenden Proteinen verwendet. Ein einziger cDNA Klon interagierte mit zwei in ihrer Struktur unterschiedlichen RNA Hybridmolekülen. Northern Blot und RACE Experimente lassen vermuten, dass die isolierte cDNA ein Fragment eines größeren Moleküls darstellt. Die Sequenzanalyse dieses Fragments zeigt eine mögliche RNA-bindende Domäne. Die isolierte cDNA codiert für ein unbekanntes Protein, welches eine spezifische Interaktion mit der *LeSUT1* mRNA zeigt und möglicherweise an der Lokalisierung der *SUT1* mRNA beteiligt ist.

Obwohl die Lokalisation von SUT1 Protein bereits gezeigt wurde (Kühn *et al*. 1997), ist der Lokalisierungsmechanismus noch nicht geklärt. Um diesen Mechanismus zu untersuchen wurden frische Tabakschnitte mit dem Toxin Brefeldin A (BFA) behandelt, welches den Vesikeltransport von Proteinen hemmt. In vorausgehenden Experimenten inhibierte die BFA-Behandlung die Lokalisierung von SUT1 Protein. Die Inkubation von Tabakschnitten in Wasser sowie gar keine Behandlung der Schnitte führte zur Lokalisierung von SUT1 Protein in SE. Im Gegensatz dazu führte die Behandlung der Tabakschnitte mit BFA (50 μM BFA, 10 min) zur Akkumulation von SUT1 Protein in den Geleitzellen. Dieser Effekt wurde deutlich verstärkt durch die Behandlung mit 100 μM BFA. Diese ersten Ergebnisse tragen zur Erstellung eines sich entwickelnden Modells der SUT1 Lokalisierung bei, in welchem Protein und mRNA in getrennten aber in Beziehung zueinander stehenden Ereignissen lokalisiert werden. Sowohl die klar vorausgesagt Sekundärstruktur von *LeSUT1* 3´UTR als auch das Vorkommen von mehrfachen Polyadenylationstypen unterstützen ebenfalls die Vorstellung eines hochregulierten komplexen Systems, welches in der Lokalisierung von SUT1 an der Plasmamembran des zellkernlosen SE resultiert.

I. Introduction

I.1 Nutrient Transport in Higher Plants

For all species of life on earth, the acquisition of the proper nutrients is essential for existence. Perhaps equally important is the proper distribution of these compounds throughout the organism. In plants, sucrose is the major nutrient product of the photosynthesis reactions. The preference for sucrose use in plants, as opposed to other carbohydrates, may be due to its physiochemical properties. Unlike the hexoses used in the animal kingdom, which do not circulate over long distances, sucrose maintains a low viscosity in solution even at concentrations as high as 200 to 1600 mM, which occur in the phloem (Winzer, *et al.*, 1996). This low viscosity allows for translocation rates in the phloem to remain high (Köckenberger, *et al.*, 1997). The efficiency of sucrose translocation is also high due to the high osmotic potential per carbon atom that is created by the molecule (van Bel, 1996). Sucrose also has the advantage of being a chemically stabile, non-reducing disaccharide.

The reactions that produce sucrose take place, however, in mature leaves; the sucrose must therefore be transferred to various heterotrophic tissues (developing leaves, shoots, roots, flowers, seeds and fruits), which are unable to actively accumulate energy in the form of fixed carbon. A transport system is thus required in order to provide these organs and tissues with sufficient energy to meet daily physiological demands. Plant sucrose transporters play an essential role within this long distance transport pathway, facilitating the proper distribution of energy in response to environmental changes. The investigation of these proteins and their role in the plant is fundamental to an understanding of plant physiology.

I.2 Plant Vasculature: A Long-Distance Distribution Pathway

Higher plants have, unlike their unicellular relatives, developed differentiated tissues to perform specific functions. This high degree of specialization at the tissue level does, however require an increased capacity for nutrient allocation and intercellular communication. Within the plant vascular system, the phloem and xylem elements create a transport network that connects source (net carbon producing) and sink (net carbon using) organs. The phloem delivers fixed carbon to the non-green sink tissues,

while the xylem supplies the photosynthetically active source tissues with water and mineral salts taken up by the root system (Behnke, 1989).

I.2.1 Phloem anatomy

The phloem is comprised of three main cell types; phloem parenchyma cells, the highly specialized, enucleate sieve elements (SEs), and their closely associated companion cells (CCs). These latter two cell populations arise from an unequal cell division of a mother cell during the maturation of the plant (Esau, 1969). The SEs are generally arranged in continuous files of cells, connected at each end by the characteristic sieve plate (Esau and Thorsch, 1985). The SE also maintains close contact with the neighbouring CC via numerous branched plasmodesmata (PD). As plant development progresses, however, the SE becomes increasingly distinct, taking on specific roles. The SE undergoes selective autophagy, progressively losing the majority of its organelles (Esau and Gill, 1971; Thorsch and Esau, 1981; Eleftheriou, 1987) and retaining only a modified endomembrane system, mitochondria, and plastids. The disintegration of the nucleus and ribosomes, especially, renders the mature SE incapable of cell autonomous gene expression; this terminal differentiation has immense consequences on the continued existence of the SE as it becomes dependent upon the CC for a continued supply of numerous proteins. Throughout the life of the plant, the SE and CC remain symplasmically connected via numerous PD, behaving as a functional unit, thus they are often referred to as the SE-CC complex. The CC, which retains both its organelles as well as the ability to divide and differentiate, thus supports the SE by providing many essential components.

As the SE matures into its role as a conduit for nutrient transport, the plasmodesmata in the sieve plate become enlarged from an average diameter of ~33 nm to ~200-400 nm, forming large pores that allow free movement of solutes in the phloem sap (McLean, Hempel et al. 1997; Sjölund 1997). Moreover, organelle disintegration is accompanied by the thickening of the cell wall, perhaps as a prerequisite for the increased turgor pressure that is associated with active translocation of phloem sap.

I.2.2 The Phloem Translocation Stream

The rate of translocation in the phloem is thought to be determined by mass flow, which is caused by water molecules entering the phloem with sucrose loading (Münch, 1930). This creates an osmotic pressure gradient along the transport route and can be quite rapid. Through the generation and maintenance of this pressure gradient, the translocation rate has been estimated to be ~ 110 μm/sec at the cellular level (Fisher, 1990). In addition to sucrose, a variety of solutes, including potassium and amino acids, contribute to the difference in osmotic pressure between phloem and non-phloem tissues. This pressure difference is further augmented by the simultaneous withdrawal of osmolytes and water at the sink tissues.

While the distribution of nutrients via the phloem and xylem are essential to the survival of the plant, this process implies the existence of a crucial prerequisite step, namely the introduction of sucrose (and other nutrients) *into* the translocation stream. How sucrose arrives in the phloem after being produced in the mesophyll cells is of general, monetary and scientific interest.

I.3 Phloem Loading

There are two well-studied forms of phloem loading, namely (i) *Symplasmic* loading via the plasmodesmata and (ii) *Apoplasmic* loading that requires active uptake of solutes. In the symplasmic system, photoassimilates (mainly raffinose and stachyose, but also significant amounts of sucrose) (Zimmermann and Ziegler, 1975; Knop *et al*., 2001) produced in the mesophyll cells are able to pass through the plasmodesmata to the conducting elements of the phloem. In order for this to occur, the transported sucrose must overcome a considerable concentration gradient, and it is still not understood how the sieve element system is able to take up additional solutes in the face of its already high osmotic potential. In plants of this type, the companion cells are also connected to the mesophyll cells via numerous PD and are generally referred to in this case as intermediary cells (ICs) (Gamalei, 1989). Active uptake involving proton-coupled co-transport is believed to be unnecessary between the mesophyll and the phloem cells since assimilates are not required to cross any membranes. One model proposed for symplasmic phloem loading suggests that the size of the sugar molecule plays an

important role in phloem loading (Haritatos *et al.*, 1996, Turgeon, 1996). In this polymer-trapping model, sucrose or galactinose produced in the mesophyll cells is able to diffuse into the ICs through the connecting plasmodesmata due to their small size (Turgeon and Gowan, 1990). Once in the IC, these sugars are converted to larger molecules like stachyose and raffinose by the companion cell localized galactinol synthase (Beebe and Turgeon, 1992). These larger molecules are then able to pass through plasmodesmata to the SEs, but unable to diffuse back to the mesophyll. This is thought to be accomplished due to the presence of the asymmetrically branched plasmodesmata joining the ICs and SEs; the larger molecules are able to pass through the branched PD, but are unable to diffuse back through the narrower PD to the mesophyll. It must be noted, however, that there is no current evidence demonstrating the ability of the plasmodesmata to discriminate between sucrose (0.34 kD) and raffinose (0.5 kD) (Knop *et al.*, 2004). Moreover, a functional sucrose transporter has been identified in a presumably symplasmically loading species, suggesting a cooperation between symplasmic and apoplasmic transport (Knop *et al.*, 2004).

According to Münch's (1930) model of phloem translocation, the SE-CC complex should be absolutely osmotically isolated. In a more realistic version of this model, sucrose is likely lost by diffusion to the apoplast during transport to the sink tissues, requiring active retrieval. In apoplasmic loading, sucrose and other assimilate molecules are actively transported from the apoplast (the intercellular space) across the plasma membrane of the SE or the CC (Komor *et al.*, 1996). Evidence for active transport was shown via immunolocalization studies, demonstrating the presence of a proton pump (H^+-ATPase) and an H^+/sucrose symporter in Arabidopsis CCs (Dewitt and Sussman, 1995; Stadler *et al.*, 1995; Stadler and Sauer, 1996). H^+/sucrose symporters accumulate the osmotically active sucrose to high concentrations in the phloem (typically in the molar range); H^+-ATPases supply the energy necessary for the active transport of solutes (Lalonde *et al.*, 1999). A third class of proteins, the water transporters (possibly aquaporins) take up water from the xylem translocation stream and contribute to the maintenance of the pressure gradient. While these findings clearly implicate the companion cell in apoplasmic loading, an H^+/sucrose symporter has been localized to

the plasma membrane of sieve elements in potato, tomato and tobacco, suggesting that the SE may also be involved in this process (Kühn *et al* 1997).

I.4 Sucrose Transporters

Plant SUcrose Transporters (SUTs) were first isolated via yeast complementation from both potato and spinach (Reismeier *et al,* 1992; 1993). Since these first transporters were identified, genes and cDNAs encoding homologous proteins have been cloned from more than 50 plant species (Lalonde *et al,* 2004). Furthermore, the Arabidopsis and rice (*Oryza sativa)* genome projects have identified medium sized families of sucrose transporter genes in these plants (Lalonde *et al.* 2004). As would be expected for proteins involved in phloem loading, the majority of sucrose transporters studied to date have been shown to be expressed in phloem tissue, although they have also been found in roots (Shakya and Sturm, 1998), seed (Weber *et al.,* 1997) and floral organs (Stadler *et al.,* 1999; Lemoine *et al.,* 1999). One would also expect sucrose transporters to be localized to the plasma membrane of phloem (and other tissue) cells since their main role is to facilitate the crossing of membranes. For several transporters, such precise localization has been achieved, although it must be noted that detailed studies confirming localization have not been carried out for all members of this class of proteins (Williams *et al.,* 2000).

The determination of the kinetic properties, pH optima, inhibitor sensitivity and substrate specificity of the various SUTs was attained via detailed transport studies using radioactive tracers. All sucrose transporters identified to date are energy-dependent and sensitive to protonophores, demonstrating that they function as proton symporters. In all cases studied to date, including the Arabidopsis SUC1 and SUC2 transporters, the K_m for sucrose was found to be in the range of 0.3-2 mM (Reismeier *et al.,* 1992; 1993; Gahrtz *et al.,* 1994; Sauer and Stolz, 1994; Weig and Komor, 1996; Boorer *et al.,* 1996; Hirose *et al.,* 1997; Zhou *et al.,* 1997; Shakya and Sturm, 1998).

The mechanism of sucrose transport has been studied in detail in *Xenopus laevis* oocytes using the two-electrode voltage clamp (StSUT1, Boorer *et al.,* 1996; AtSUC1, Zhou *et al.,* 1997). The stoichiometry of H^+/sucrose cotransport was shown to be 1:1, confirming

previous estimates obtained in plasma membrane vesicles from sugar beet leaves (Bush, 1990; Sloane *et al.,* 1991). Sucrose transporters thus correspond to the high affinity, protonophore sensitive component of sucrose uptake kinetics measured in plants (Maynard and Lucas, 1982). Uptake kinetics appear to have, however, numerous components, including a low affinity protonophore-sensitive system (Delrot and Bonnemain, 1981). Evidence for this postulated low affinity component has recently been presented (Weise *et al,* 2000).

SUT proteins are predicted to be integral membrane proteins with 12 membrane spanning domains and a putative central hydrophilic loop. This 6-loop-6-structure is highly conserved among plant species and is similar to that described for several cation/substrate co-transporters (Henderson, 1990; Kaback, 1992), characteristic of the *major facilitator superfamily* (MFS) (Marger and Saier, 1993).

I.4.1 Sucrose Transporters are Members of a Large Family

Following the isolation of the first sucrose transporters, heterologous screening of cDNA and genomic libraries identified several other sucrose transporters from a variety of plants (Figure 1). Several of these sucrose transporters display specific expression patterns. SUC1 from *Plantago* is specifically expressed in young ovules (Gahrtz *et al.,* 1996) and sucrose transporter transcripts have been detected in transfer cells of cotyledons of *Vicia* seeds (Weber *et al.,* 1997) as well as in epidermal cells of cotyledons in *Ricinus communis* (*RcSUT1*, Bick *et al.,* 1998). Root specific expression has been observed in carrot (*DcSUT2*, Shakya and Sturm, 1998) and pollen specific expression has also been described in some plants (*AtSUC1*, Stadler *et al.,* 1999; *NtSUT3*, Lemoine *et al.,* 1999). To date, however, sucrose transporters involved in efflux from the mesophyll (into phloem), efflux from phloem (to sinks) and transport into vacuoles (storage), have yet to be identified.

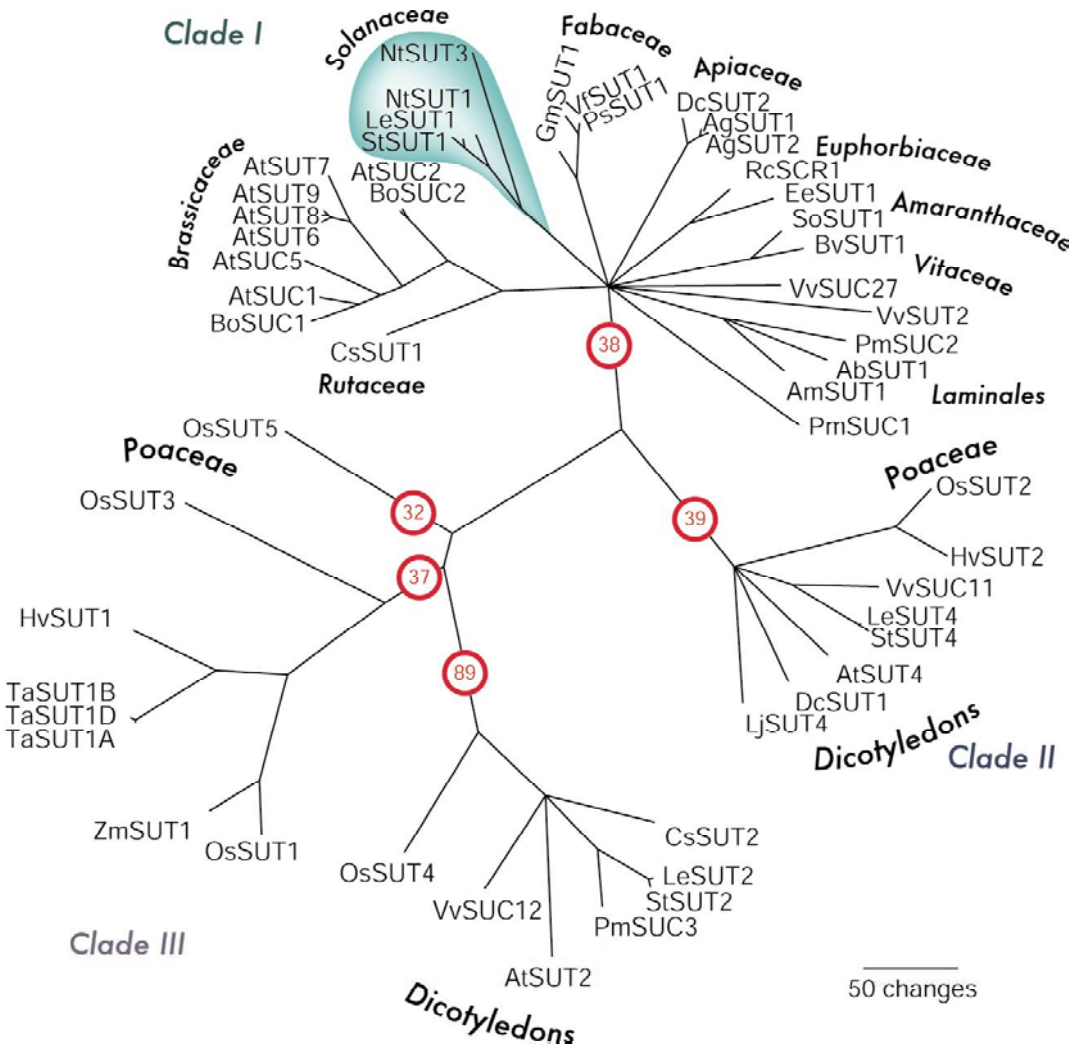

Clade I
Solanaceae
NtSUT3
NtSUT1
LeSUT1
StSUT1
Fabaceae
GmSUT1
VfSUT1
PsSUT1
Apiaceae
DcSUT2
AgSUT1
AgSUT2
Euphorbiaceae
RcSCR1
EeSUT1
Amaranthaceae
SoSUT1
BvSUT1
Vitaceae
VvSUC27
VvSUT2
PmSUC2
AbSUT1
AmSUT1
Laminales
PmSUC1
Brassicaceae
AtSUT7
AtSUT9
AtSUT8
AtSUT6
AtSUC5
AtSUC1
BoSUC1
AtSUC2
BoSUC2
CsSUT1
Rutaceae
38
OsSUT5
Poaceae
OsSUT3
32
37
HvSUT1
TaSUT1B
TaSUT1D
TaSUT1A
ZmSUT1
OsSUT1
89
OsSUT4
VvSUC12
AtSUT2
CsSUT2
LeSUT2
StSUT2
PmSUC3
Dicotyledons
Clade III
39
Poaceae
OsSUT2
HvSUT2
VvSUC11
LeSUT4
StSUT4
AtSUT4
DcSUT1
LjSUT4
Dicotyledons
Clade II
50 changes

Figure 1. Phylogenetic tree of the sucrose transporter (SUT) superfamily. Maximum parsimony analyses were performed using PAUP 4.0b10 (Swofford, 1998), with all DNA characters unweighted and gaps scored as missing characters. Heuristic tree searches were executed using 1000 random sequence additions and the tree bisection-reconnection branch-swapping algorithm with random sequence analysis. The complete alignment was based on 515 sites; 357 were phylogenetically informative. The SUT family can be divided into 3 clades, the solanaceous branch in clade I is highlighted. The red circles give the average length of the central loop of the corresponding clade. All sequences were obtained from NCBI (http://www.ncbi.nlm.nih.gov) or the Aramemnon database (http://aramemnon.botanik.uni-koeln.de/). Sucrose transporters have been found in the following plant species: Ab, *Asarina barclaina* (*AbSUT1*, Knop *et al.,* 2001); Am, *Alonsoa meridionalis* (*AmSUT1,* Knop *et al.,* 2004); Ap, *Apium graveolens* (*AgSUT1,* ac. AF063400; *AgSUT2,* Noiraud *et al.,* 2000); At, *Arabidopsis thaliana* (*AtSUC1, AtSUC2* Sauer and Stolz, 1994; *AtSUT2* Barker *et al.,* 2000); Bo, *Brassica oleracea*; Bv, *Beta vulgaris* (*BvSUT1*, ac. 1076257); Cs, *Citrus sinensis*; Dc, *Daucus carota* (*DcSUT1* and *DcSUT2*, Shakya and Sturm, 1998); Ee, *Euphorbia esula*; Gm, *Glycine max*; Hv, *Hordeum vulgaris*; Le, *Lycopersicon esculentum LeSUT1*, *LeSUT2, LeSUT4*) (Barker *et al.,* 2000; Weise *et al.,* 2000); Lj, *Lotus japonicus*; Nt, *Nicotiana tabacum* (*NtSUT*1, Bürkle *et al.,* 1998) (*NtSUT3*, Lemoine *et al.,* 1999); Os, *Oryza sativa* (*OsSUT1*, Hirose *et al.,* 1997); Ps, *Pisum sativum* (*PsSUT1*, Tegeder *et al.,* 1999); *Plantago major* (*Pm SUC1*, *PmSUC2*, Gahrtz *et al.,* 1994; Rc, *Ricinus communis* (*RcSUT1*, Weig and Komor, 1996); So, *Spinacea oleracea* (*SoSUT1* Reismeier *et al.,*1992); St, *Solanum tuberosum* (*StSUT1,* Reismeier *et al.,* 1993; *StSUT2, StSUT4*); Ta, *Triticum aestivum*; Vf, *Vicia faba* (*VfSUT1*, Weber *et al.,* 1997); Vv, *Vitis vinifera* (Davies *et al.,* 1999); Zm, *Zea mays*. Modified from Lalonde *et al.,* 2004

I.4.2 Solanaceous Sucrose Transporters

Sucrose transporter proteins in solanaceous plants (and in higher plants in general) play a crucial role in sustaining ideal physiological conditions in the organism. Antisense repression of SUT1, leading to reduced transport activity, caused extensive physiological defects in carbon partitioning and photosynthesis in both potato (Riesmeier *et al.,* 1994; Kühn *et al.,* 1996) and tobacco (Bürkle *et al.,* 1998). Leaf sucrose and starch content was increased five to ten-fold, with the hexose content being increased to an even greater degree. Starch grains were a prominent feature of both mesophyll and bundle sheath cells, but were decreased in number in phloem parenchyma and in companion cells (Schulz *et al.,* 1998). The distribution of starch emphasizes that the rate-limiting step in phloem loading occurs at the boundary of the SE-CC complex (Kühn *et al.,* 1999). This data demonstrates that in solanaceous plants, SUT1 is essential for apoplasmic phloem loading of sucrose.

Morphologically, *SUT1* antisense plants are severely affected, growing at extremely retarded rates. These plants also produce crinkled, chlorotic leaves that also accumulate anthocyanins; this is likely the effect of the change in osmotic conditions due to the accumulation of carbohydrates (Kühn *et al.*, 1996). The observed altered phenotype is dependent upon light intensity and the length of the photoperiod (Kühn *et al.,* 1996) and can also be induced by blocking phloem translocation by heat- or cold-girdling which causes a similar accumulation of carbohydrates (Grusak *et al.,* 1990; Krapp *et al.,* 1993). Interestingly, sucrose uptake by plasma membrane vesicles isolated from constitutively inhibited antisense plants was considerably reduced, whereas uptake by vesicles isolated from phloem inhibited antisense plants remained unchanged as compared to wild type (Lemoine *et al.,* 1996). These observations correlate with data from Western blot analysis in which the quantity of SUT1 protein was not reduced in plants with phloem specific inhibition of SUT1, whereas in constitutive antisense plants, SUT1 protein was barely detectable (Reismeier *et al.,* 1994). While the physiological role of SUT1 was clearly established as crucial to the maintenance of plant health, it had yet to be demonstrated that SUT proteins displayed a physiologically relevant localization pattern.

I.4.3 Localization of LeSUT1

The localization of SUT1 was initially assessed via tissue specific Northern blot revealing that expression occurred primarily in mature leaves and to a lesser extent in sink tissues. The promoter sequence of *Le*SUT1 was able to drive expression of reporter genes in leaf, stem and root phloem (Weise, 2000) suggesting involvement not only in phloem loading, but also in retrieval of sucrose (lost from sieve tubes) along the translocation pathway. These observations were corroborated by *in situ* hybridization of RNA (Riesmeier *et al.*, 1993).

While the general localization of SUT protein and RNA to phloem tissue was an encouraging observation, more precise information about the cellular localization was required. In order to study the cytological expression, immunolocalization studies were undertaken. Unlike the situation found in *Plantago major* and *Arabidopsis* where expression was seen in the CC (Stadler *et al.*, 1995; Stadler and Sauer, 1996), immunofluorescence and immunogold labelling detected SUT1 protein in the plasma membranes of the SEs in tobacco, tomato and potato (Kühn *et al.*, 1997). The protein was not only found in the phloem of minor veins, but also in stems, petioles, and roots. This difference in expression as compared to *Arabidopsis* and *Plantago* may be due to differences in phloem loading mechanisms as described above. There are a number of possible explanations for the presence of SUT1 in the SE: (i) The protein is synthesized within the CC and transported to the SE, (ii) The protein is produced while the SE still has a nucleus and remains throughout the life of the plant.

When observed at the electron microscope level, *SUT1* mRNA (detected via *in situ* hybridization) was localized to both SEs and CCs and preferentially associated with the PD joining the two cells. The presence of *SUT1* mRNA in the SE was a surprising discovery since the CC specific inhibition of SUT1 (as well as mRNA localization to CCs) indicated clearly that transcription of *SUT1* mRNA occurs in the CC. It was assumed, therefore, that translation also occurred in the CC. These results thus inspired several questions: what is mRNA doing in a cell that has no nucleus and more importantly no ribosomes? Is the presence of mRNA in the SE a specifically regulated event? If so, what is the mechanism of this pattern of localization? Furthermore, these results present

a third option (in addition to those outlined above) for explaining the localization of SUT1 protein to the SE: (iii) The mRNA is produced within the CC and transported to the SE via binding to a specific cellular chaperone, with translation occurring at or near the plasmodesmal opening. To date such a cellular chaperone has not been identified, nor has a targeted approach to identifying such a protein been undertaken. The work presented here deals with the questions outlined above and presents insight into the mechanism of RNA localization in solanaceous plants.

I.5 RNA Localization

I.5.1 RNA Localization in Development

In recent years it has become increasingly evident that the role of mRNA in the cell is far more complex than its simple name suggests. The correct distribution of mRNA within and even between cells is a critical event in many developmental systems (Evans Bergsten, *et al.,* 2001). The process of mRNA localization has been extensively studied in a variety of organisms and cell types. In the model systems of *Drosophila*, *Xenopus, S. cerevisiae*, and others, localization is largely mediated by the presence of specific *cis*-acting sequences in the 3'-untranslated region (UTR) of several genes (Bassell *et al.,* 1999; Bashirullah *et al.*, 1998). These sequences are recognized either in their primary form or as specific secondary structure(s) by *trans*-acting RNA binding proteins (RBPs) (Blichenberg, *et al.,* 1999).

The 3'-UTR has been shown to play an important role in polar distribution of mRNAs within or between cells (Palacios and St. Johnston, 2001). In *Drosophila melanogaster*, for example, *bicoid* and *nanos* are transcribed in yolk cells, with the RNAs being subsequently distributed in large complexes to the poles of the embryo (reviewed in Palacios and St. Johnston, 2001) This highly specific localization is mediated by RNA binding proteins, such as *staufen* and *exuperantia*, which bind to specific regions of the UTR of both *nanos* and *bicoid* and, via interactions with cytoskeleton anchoring proteins and motor proteins, are localized to their respective destinations (Palacios and St. Johnston, 2001). Similarly, trafficking of a fusion of human *staufen* with GFP in hippocampal neurons occurs by movement of large ribonucleoprotein (RNP) complexes

with an average velocity of 6.4 μm/min (Köhrmann et al., 1999) with the apparent effect of localizing protein translation. In yeast, both *Ash1* and *Ist2* traffic during cell division between mother and daughter cells (Long et al., 1997). As occurs with *bicoid*, *Ash1* has multiple binding sites for RNA binding proteins. She2p binds to *Ash1* mRNA and together with the interactions of other She proteins the entire complex moves from the mother cell to the daughter cell (Böhl et al., 2000; Long et al., 2000). Localization of the membrane protein *Ist2* uses the same mechanism; moreover, a septin barrier localized at the bud neck blocks the diffusion of Ist2p protein in the daughter cell membrane back to the mother cell (Barral et al., 2000).

I.5.2 Plant viral RNAs and Plasmodesmata

To date much of the knowledge gleaned in area of RNA localization has come from studies of *Drosophila* and *Xenopus* oocytes (reviewed in Etkin and Lipshitz, 1999), however an extensive flow of information has begun to emerge with regard to the plant world. Much of this work has focussed on the mechanisms by which plant viruses infect their hosts; of particular interest has been the study of the tobacco mosaic virus (TMV). TMV is comprised of a single-stranded messenger-sense RNA of approximately 6400 nucleotides that is encapsulated in a helical protein coat of 2160 subunits (Heinlein, 2002). Unlike many viruses that depend upon insects or other vectors for transmission from plant to plant, TMV is transmitted by the physical contact between plant tissue and virus-contaminated surfaces (Shaw, 1999). Infection of the cell proceeds rapidly and involves many steps (Heinlein, 2002), with production of the viral movement protein (MP) being critical to the further spread of the infection.

In order for the infection to spread throughout the plant from the initially infected cell, TMV and other viruses must move across the plant cell wall (Heinlein, 2002). A crucial target of these viruses, therefore, is the plasmodesmata that join plant cells. When studied at the electron microscope level, it can be seen that both the ER and the plasma membrane of adjoining cells are continuous through the pore, with the ER forming an apressed "desmotubule" in the centre (Heinlein 2002). The desmotubule stabilizes the internal structure of the PD and also limits the size of the pore. The size limitation is a result of the fact that both the plasma membrane and the desmotubule are covered in

globular particles, which are linked with spoke-like elements (Ding *et al.,* 1992; Thompson and Platt-Aloia, 1985; Schulz, 1995) that restrict diffusion through the PD.

The size exclusion limit (SEL) of PD is developmentally regulated and decreases when a leaf undergoes the source to sink transition (Imlau *et al.,* 1999; Oparka *et al.,* 1999); changing from ~ 50 kD in sink leaves (Oparka *et al.,* 1999) to ~ 1 kD in mature leaves (Tucker, 1982; Wolf *et al.,* 1989). This is not, however, an irreversible change as has been discovered through study of TMV. Early in the infection cycle, following entry into the first plant cell, the virus takes advantage of the existing protein synthesis machinery and produces the 30 kD movement protein. TMV movement protein then specifically targets PD, increasing the SEL thus allowing the passage of TMV as a viral RNP (vRNP). Consistent with such a role in viral movement, TMV MP was shown to accumulate at PD (Tomenius *et al.,* 1987; Wolf *et al.,* 1989; Atkins *et al.,* 1991; Oparka *et al.,* 1997) and to increase the SEL by at least 20 kD within 3-5 minutes (Waigmann *et al.,* 1994). Furthermore, the estimated diameter of the assembled vRNP is compatible with the diameter of the modified PD (Citovsky *et al.,* 1992).

The implication arising from these observations of the life cycle of TMV is clear; the virus is able to spread itself through the plant by taking advantage of systems that already exist. Thus an endogenous mechanism for both plasmodesmal variation and transport of large molecules must be present. Indeed, a plant MP-related protein, PP16 was discovered in *Cucurbita maxima* (Xoconostle-Cázares *et al.,* 1999). This protein is expressed in the phloem and is able to traffic both itself and RNA through PD. Furthermore, the homeodomain transcription factor *Knotted1* (*Kn1*) from maize has also been shown to traffic in the shoot apical meristem (SAM) (Jackson, 2002; Kim *et al.,* 2002); KNOTTED1 (KN1) protein is detected outside the domain of mRNA expression in the leaf (Jackson *et al.,* 1994). These data contribute to the growing body of knowledge supporting the existence of an endogenous macromolecular trafficking system, the existence of which would be a useful paradigm to explain the localization of SUT1 mRNA in the enucleate SE.

I.5.3 RNA as a Long Distance Signalling Molecule

In recent years it has been shown that RNA has a non cell-autonomous role in mediating control over a number of processes, from embryo development (Lehmann, 1995; Grünert and St. Johnston, 1996; Schnapp *et al.,* 1997) to the activation of defence systems directed against a viral challenge (Jorgensen *et al.,* 1998; Waterhouse *et al.,* 2001). The long distance transport of RNA in plants (and animals) implies a level of continuity between the cells of the organism. In plants, PD allow for cytoplasmic and endomembrane continuity between neighbouring cells (Lucas *et al.,* 1993; Lucas, 1995; Robards and Lucas, 1990). This continuity allows for the proper allocation of nutrients and rapid systemic response to environmental challenges.

Studies performed on a number of plant transcription factors have shown that these endogenous proteins have the ability to interact with and move through PD (Kragler *et al.,* 1998; Lucas *et al.,* 1995; Mezitt and Lucas, 1996). Furthermore, it has been found that phloem sap contains a unique population of transcripts, which can move over long distances (Ruiz-Medrano *et al.,* 1999; Xoconostle-Cázares *et al.,* 1999; Kim *et al.,* 2001). This has led to the idea that there exists an "RNA information superhighway" (Jorgensen *et al.,* 1998) in plants that conveys information in the form of specific RNAs. RNAs bound for this translocation stream are thought to bind specific cellular interactors that in turn bind to PD-specific receptors that mediate the passage into the phloem (reviewed in Lucas *et al.,* 2001; Haywood *et al.,* 2002). Once in the phloem stream, specific proteins would then move to their specific destination with the aid of a phloem-specific protein. This process is highly regulated and involves the selective alteration of the SEL of the PD as well as interactions with numerous components including chaperones and the cytoskeletal network (reviewed in Lucas *et al.,* 2001; Haywood *et al.,* 2002).

When placed in the context of an existing RNA translocation system, the localization of *SUT1I* mRNA to the enucleate SEs in solanaceous plants is perhaps somewhat less perplexing. *SUT1* mRNA could be specifically targeted to the PD for insertion into the PM of the SE, or for transport to distant sites within the plant. In fact it has been suggested that SUT1 may be an endogenous signalling molecule (Haywood *et al.,* 2002).

The proteins that mediate *SUT1* mRNA localization to the SE have yet to be identified, however. Such a step would be an important addition to elucidating the mechanism and relevance of the RNA translocation stream.

I.6 The Role of the *LeSUT1* 3'UTR in mRNA Localization

In order to determine if the *LeSUT1* 3'-UTR plays a role both in the possible trafficking of SUT1 from the CC to SE and in causing reporter proteins to traffic to SE, a 1.2 kb genomic fragment encompassing the 3'-UTR and subsequent downstream sequences was isolated and cloned (Lalonde *et al,* 2003). When expression was assayed, however, the UTR alone was unable to mobilize the reporter gene (GUS or GFP) indicating that translation occurred in the CC. The lack of GUS protein movement between CC and SE is in agreement with previous studies indicating that it has a molecular mass higher than the size exclusion limit of the plasmodesmata joining SEs and CCs (Imlau et al., 1999). In plants transformed with the GFP-UTR constructs, GFP was also localized in the CC, moreover the addition of the 3'-UTR resulted in the formation of particle-like structures and in the loss of the perinuclear localization observed for GFP expressing constructs (Lalonde *et al.,* 2003). These data suggested that the UTR is sufficient to target translation to specific particles and that the resulting protein remains bound to them. These data also show that GFP protein was restricted to CCs and did not seem to traffic to SEs or accumulate in sink tissues, although the GFP protein itself was the same for both constructs (with and without UTR). The CC localization is in contrast to data from Imlau et al. (1999) and perhaps reflects the activity and selectivity of the RolC promoter relative to the *Arabidopsis* SUC2 promoter.

The complete absence of SUT1 from the SEs in these cases suggested that the UTR is insufficient by itself to mobilize reporter activity to the SE. This may be due to the presence of a localization signal in the coding region of the gene as is the case for the yeast *Ash1* gene (Böhl et al., 2000; Long et al., 2000). Similar results to the original 3'-UTR experiments were obtained when the SUT1 coding region was included as part of the construct. While the 3'-UTR was unable to direct localization to the SE, either alone or following a SUT1-reporter gene construct, it is important to note that in each case where the UTR was present SUT1 protein was sequestered into discrete particulate

structures. As these do not occur in the absence of the 3'-UTR, it is interesting to speculate upon their exact nature; they may in fact correspond to RNPs that were able to form, but are missing the necessary signal for transport to the SE. Such a missing signal could be present in the RNA sequence spanning the site of the stop codon, as is the case for *Ash1*; this is an important consideration in the case of the experiments outlined above, since all constructs were made such that the continuity between the coding region and the 3'-UTR was abolished. Thus the possibility remains that a specific signal in the mRNA of SUT1 is present and required for the movement of the mRNA to the SE, while a perhaps separate signal allows the aggregation of the mRNA into an RNP. Such a signal is presumed to exist and is the basis for the search for an interacting partner undertaken in this thesis.

I.7 Research Objectives

The aim of the research presented in this thesis was to investigate, *in vivo*, the mechanism(s) leading to the localization of *Le*SUT1 mRNA to the enucleate sieve elements of the phloem.

Previously, SUT1 protein was shown to be localized to the plasma membrane of the SE in solanaceous plants (Kühn *et al.*, 1997), while the CC was implicated as the site of transcription (Kühn *et al.*, 1996). Immunogold detection of *Le*SUT1 mRNA confirmed the CC localization, but also demonstrated that the mRNA associated preferentially with PD joining the SE and CC and most surprisingly, mRNA was also clearly detected on the SE side of the PD (Kühn *et al.*, 1997). This result was extremely interesting in light of the fact that the SE is devoid of ribosomes, presumably precluding translation in the SE. Thus the aim of this work was to investigate the mechanism and significance of the arrival of SUT1 mRNA in the SE. Early clues were provided from results of SUT1 regulation, which clearly demonstrated the rapid turnover of both protein and mRNA. This ruled out the possibility that the SE was able to retain components from an earlier developmental stage where both nucleus and ribosomes were still present. Furthermore, later studies were able to show that the 3'UTR of SUT1 was able to cause the aggregation of the protein into discrete particles, though it was unable to cause localization to the SE. These early indications led to the postulation that SUT1 was one

of the many endogenous proteins destined to take advantage of the machinery required for entry to the "RNA information superhighway". What remains to be determined, however, is evidence of RNA interacting proteins that mediate the movement of SUT1 mRNA from the CC into the SE as well as the interaction with the PD and insertion of SUT1 protein into the plasma membrane of the SE. The identification and characterization of such putative RNA binding proteins is the central objective of this thesis.

The yeast-three hybrid method (SenGupta *et al.,* 1996) was chosen as the most appropriate technique to use for the elucidation of putative SUT1 mRNA binding proteins. In order to take advantage of this *in vivo* system, production and subsequent cloning of a high quality cDNA library encoding tomato leaf protein fusions was required.

Several RNA hybrid molecules were designed based on the sequence and secondary structure of *Le*SUT1 mRNA and used as "bait" for the three-hybrid method. These RNAs were used to screen the *L. esculentum* leaf cDNA library for putative RNA binding proteins. cDNAs shown to interact with RNA hybrid molecules were isolated, sequenced and tested for specificity of interaction. Biochemical analysis of the interactions will be an essential step in the complete characterization of cDNAs isolated from this screening system. Furthermore, targeted mutagenesis as well as sub-cellular localization studies will be crucial to understanding the actual physiological role of these proteins.

II. Materials

II.1 Abbreviations

SI [Systeme International d'Unités] abbreviations are used throughout this work. Other abbreviations not defined in the text are listed below. Both single and triple letter amino acid codes are used.

3'UTR	3' untranslated region
3-AT	3-amino-triazole
AP	alakaline phosphatase
APS	ammonium persulphate
BFA	brefeldin A
bp	base pairs
BSA	bovine serum albumin
CC	companion cell
CIP	chloroform:isoamylalcohol:phenol (24:1:25)
CLSM	confocal laser scanning microscopy
DAPI	4', 6'-diamidino-2-phenylindole hydrochloride
DEPC	diethylpyrocarbonate
ddH_2O	distilled, deionized water
DTT	dithiothreitol
EDTA	ethylenetriaminetetraaceticacid
EMSA	electrophoretic mobility shift assay
FITC	fluorescein isothiocyanate
IPTG	isopropyl-β-D-thiogalactopyranoside
kD	kilodalton
Le	*Lycopersicon esculentum*
Nt	*Nicotiana tabacum*
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PD	plasmodesmata
PEG	polyethylene glycol

PMSF	phenylmethylsulfonylfluoride
rpm	revolutions per minute
SE	sieve element
SEL	size exclusion limit
SDS	sodium dodecyl sulphate
SSC	saline sodium Citrate
SSPE	saline sodium phosphate, EDTA
St	*Solanum tuberosum*
SUT	sucrose transporter
TEMED	tetramethylethylenediamine
Y3H	yeast three hybrid
v/v	volume for volume
w/v	weight for volume
X-gal	5-bromo-4-chloro-3-indolyl-3-D-galactoside

II.2 Chemicals and Enzymes

Standard chemicals were purchased from Sigma (St. Louis) or Merck (Darmstadt). Those reagents considered essential to the experiments are listed below.

Ambion (Austin, USA): MAXIscript *in vitro* Transcription Kit

Amersham Pharamacia Biotech (Braunschweig): Megaprime™ DNA Labelling System

Deutsche Dynal (Hamburg): Dynabeads® Oligo $(dT)_{25}$

Difco Laboratories (Detroit, USA): Bacto-Agar, Bacto-Tryptone, Bacto-Peptone, Yeast Extract, Yeast Nitrogen Base w/o amino acids.

Invitrogen (Carlsbad, USA): Expressway™ Plus Expression System; Anti-Xpress™ Antibody; One Shot® BL21 (DE3) cells; GeneRacer™ Kit

MBI Fermentas (St. Leon-Rot): Restriction enzymes

Novogen, Inc. (Affiliate of Merck GmbH, Darmstadt, Germany): His-Bind Resin

Perkin Elmer (Foster City, USA): ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit

Stratagene (La Jolla, CA): cDNA synthesis kit; XL2-Blue MRF' ultracompetent cells

II.2.1 Oligonucleotides

Amplifying SUT1 3′UTR for cloning RNAs into pIIIA MS2-1:

RNA-1:

LS1U97/1-100F1: 5′ – GAGCTTCCCGGGAGCAGTTGCAGCTGCC – 3′

LS1U97/1-100R1: 5′ – GAATCCCGGGGAATTCTTAATGGAAACC – 3′

RNA-2:

LS1U97/1-100F1: 5′ – GAGCTTCCCGGGAGCAGTTGCAGCTGCC – 3′

LS1U97/1-180R1: 5′ – GGAACCCGGGGAATTCTACAAAAGAGAAGTA – 3′

RNA-3:

LS1U97/50-149F1: 5′ – GATCCACCCGGGTCACCACCTGCTGATG – 3′

LS1U97/50-149R1: 5′ – GAATCCCGGGGAATTCAGTTTGTTTCTTCTCC – 3′

RNA-4:

LS1U97/86-266F1: 5′ – GCAAGTCCCGGGCGGTTTCCATTAA – 3′

LS1U97/86-266R1: 5′ – GGCTTACCCGGGGAATTCCCACTAAAGAGCT – 3′

RNA-5:

LS1U97/98-200 F1: 5′ – GTTAAACCCGGGGCCATGGGCGGTTTCC – 3′

LS1U97/98-200 R1: 5′ – GGATATCCCGGGGAATTCATGTTTGTTTTC – 3′

Amplifying SUT1 3′UTR for cloning hybrid RNAs into pIIIA MS2-2:

RNA-1:

LS1U97/1-100F2: 5′ – CTAGGTCCGCATGCAGCAGTTGCAGCTGCC– 3′

LS1U97/1-100R1: 5′ – GAATCCCGGGGAATTCTTAATGGAAACC – 3′

RNA-2:

LS1U97/1-100F2: 5′ – CTAGGTCCGCATGCAGCAGTTGCAGCTGCC– 3′

LS1U97/1-180R1: 5′ – GGAACCCGGGGAATTCTACAAAAGAGAAGTA – 3′

RNA-3:

LS1U97/50-149F2: 5′ – CCAGTTACGCATGCTCACCACCTGCTGATG – 3′

LS1U97/50-149R1: 5′ – GAATCCCGGGGAATTCAGTTTGTTTCTTCTCC – 3′

RNA-4:

LS1U97/86-266F2: 5′ – CACCGTAGGCATGCGGCGGTTTCCATTA– 3′

LS1U97/86-266R1: 5′ – GGCTTACCCGGGGAATTCCCACTAAAGAGCT – 3′

RNA-5:

LS1U97/98-200 F2: 5′ – CCTATTGGGCATGCGCCATGGGCGGTTTCC – 3′

LS1U97/98-200 R1: 5′ – GGATATCCCGGGGAATTCATGTTTGTTTTC – 3′

Sequencing hybrid RNAs:

pIIIF: 5′ – TGGATATGGGGGAATTCCGATCCTC – 3′

pIIIR: 5′ – GTTTTACGTTTGAGGCCTCGTGGCG – 3′

Sequencing cDNA inserts:

cDNA F : 5′ – TATCTATTCGATGATGAAGATACCC – 3′

cDNA R: 5′ – CAGTTGAAGTGAACTTGCGGGGTTT – 3′

3' RACE:

c10 extend-1: 5′ – ACGAGGGTGGCATTCTTG – 3′

c10 extend-2: 5′ – GTCCCCAAAGAGCAGATACAC – 3′

c10 extend-4: 5′ – TTACATAAAAGAAGGCAAAACGAT – 3′

5' RACE:

c10 extend-6: 5′ – TAACAGGCAATTCACATCA – 3′

c10 extend-7: 5′ – AGGGTGACTATCCAACAAA – 3′

c10 extend-8: 5′ – GTCCGAACCTCATAACAAC – 3′

Sequences recognized by restriction endonucleases are underlined.

II.3 Bacterial Strains, Yeast Strains, Plant Material and Vectors

II.3.1 Strains

Escherichia coli BL21 (DE3) - gold (Invitrogen Europe, Paisley, UK)

F$^-$, *omp*T, *hsd*SB (r_B^- m_B^-), *gal, dcm* (DE3)

Escherichia coli TOP10F' (Invitrogen Europe, Paisley, UK)

F' {*lac*I^{q}, Tn*10*(Tet^{R})}, *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC), Φ80*lac*ZΔM15, Δ*lac*X74, *rec*A1, *deo*R, *ara*D139, Δ(*ara-leu*)7697, *gal*U, *gal*K, *rps*L (Str^{R}), *end*A1, *nup*G

Escherichia coli 6201 (DMSZ)

F^{-} *pyr*F74::Tn5, *sup*E44, *lac*Y1, *ara*14, *gal*K2, *xyl*5, *mtl*1, *leu*B6, *pro*A2, *hsd*S20, *rec*A13, *rps*L20, *thi*1, lambda^{-}, r_{B}^{-} m_{B}^{-}

Saccharomyces cerevisiae L40 Coat (SenGupta, *et al,* 1996)

Mata; *his*3Δ200,;*trp*1-901; leu2, 3-112; *ade*2; *lys*2-801am; *URA*::(lex-op)$_{8}$-*lac*Z; *LYS2*::(lex-op)$_{4}$-HIS3

Lycopersicon esculentum L. Variety MM (Money Maker)

Nicotiana tabacum cv. Samsun NN

II.3.2 Vectors and Plasmids

For vector maps, refer to Appendix VI.1

pACT2 (Legrain *et al*, 1994)
pIIIA/MS2-1; /MS2-2 (Sengupta *et al,* 1996)
pRSET C (Invitrogen, Carlsbad, USA)

II.4 Media

II.4.1 Media for Escherichia coli

Escherichia coli (DH5α, Top10F') were grown according to Sambrook, *et al.* (1989)

Escherichia coli (BL21 (DE3)) was grown according to Invitrogen recommendations

Escherichia coli (6201) was grown on M9 Minimal medium:

M9 Minimal Medium

5x M9 Salts	200 mL	5x M9 Salts:	
20% Glucose	10 mL		
Uracil (0.2g/100 mL)	10 mL	$Na_2HPO_4 \cdot 7H_2O$	64 g
0.5% Proline	10 mL	KH_2PO_4	15 g
1M $MgSO_4$	1 mL	NaCl	2.5 g
0.5% Thiamine	100 µL	NH_4Cl	5.0 g
Sterile ddH_2O	to 1000 mL	ddH_2O	to 1000 mL

5x M9 salt solution was sterilized via autoclave; all other minimal medium components (except ddH_2O) were separately filter sterilized before assembling medium. Solid M9 medium also contained 2% Agar.

II.4.2 Media for Saccharomyces cerevisiae

Saccharomyces cerevisiae was grown at 28°C in full medium (YPD) or in synthetic deficient medium (SD) supplemented with glucose to a final concentration of 2%. Solid medium also contained 2% Oxoid agar.

III. Methods

III.1 Cloning and Sequencing

Standard methods (plasmid preparation, restriction digests, gel electrophoresis, cloning steps, etc.) were performed as described in Current Protocols (Chanda *et al*, 1999) or by Sambrook *et al* (1989). DNA sequencing was completed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, USA) on an ABI PRISM Sequencer 310 (Perkin Elmer, Warrington, UK).

III.2 Bacterial Transformation

E. coli was transformed via electroporation using the Transporator™ Plus (BTX Inc., USA) according to methods described in the instrument manual.

III.3 Yeast Transformation

Saccharomyces cerevisiae was transformed according to the Lithum acetate/single-stranded carrier DNA/Polyethylene glycol (LiAc/ssDNA/PEG) method (Agatep *et al* ,1998; Gietz and Woods, 2002). Briefly, yeast (2-5 mL culture) is grown under permissive or selective conditions overnight, shaking at 28°C. Following determination of cell titre, the overnight culture is diluted to a final concentration of 5×10^6 cells/mL in media and grown shaking at 28°C until the titre reaches at least 2×10^7 cells/ mL (3-5 hours). Cells are harvested via centrifugation, washed once in ½ volume ddH_2O, and resuspended in 1-10 mL of ddH_2O, depending upon the volume of the starting culture. This homogenate is further divided into aliquots of 50 µL – 3 mL and subjected to another round of centrifugation. The following ingredients are then added to the resulting pellet (3 mL aliquot used as an example) in the order indicated, with gentle mixing after each addition:

2.4 mL	50% PEG
360 µL	1 M LiAc
500 µL	ssDNA (from salmon testis, 2 mg/mL)
x µL	plasmid DNA (1-5 µg)
340-x µL	ddH_2O

The homogenate is vortexed to ensure complete suspension and incubated for 30 minutes at 28°C. The yeast is then subjected to a heat shock for 15-30 minutes at 42°C. The cells are subsequently harvested by centrifugation, resuspended in ddH_2O, and spread onto selective media.

III.4 Yeast Three Hybrid Technique

III.4.1 Yeast Transformation

S. cerevisiae L40coat was transformed as described in section III.3. The yeast was initially transformed with plasmids harbouring a hybrid RNA molecule, plated onto selective media (SDG –ura) and grown for up to 5 days at 28°C. Isolated transformants were selected, grown for up to 5 days in liquid selective (SDG –ura) media and subsequently transformed with 2.5-5.0 μg of amplified, purified *L. esculentum* leaf cDNA library. The entire transformation mixture was plated onto selective media (SDG –leu, -his, + 5 mM 3-AT) and incubated at 28°C for a minimum of 5 days. Transformant colonies were then scored for appearance, harvested and freshly streaked onto selective media (SDG –leu, -ura) over a period of 2-5 days (plates were kept at 4°C during harvesting).

III.4.2 Qualitative β-Galactosidase Activity Assay

Transformed yeast colonies were transferred (via velvet stamp replication) to solid selective media (SDG –leu, -ura or SDG –leu) overlaid with a reinforced nitrocellulose filter. The resultant replica plate was incubated overnight at 28°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 dish was sealed with parafilm and incubated for a maximum of 25 minutes at 37°C. Filters were immediately scored for appearance, scanned and processed and assembled using Adobe® Photoshop® 7.0 and Illustrator® 10.0.

III.4.3 RNA Structure Analysis

RNA secondary structure was predicted using the *mfold* v.3.1 software (Zucker, 2003) http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1-2.3.cgi

III.5 RNA Isolation

Mature leaves or major veins (excised with a sterile scalpel) were harvested from *L. esculentum* directly into liquid nitrogen. Plant material was ground into a fine powder with a pre-cooled mortar and pestle in liquid nitrogen. Ground frozen tissue was then gradually added to a small amount (5-10 mL) of Z6 Buffer (8M Guanidine-hydrochloride, 20 mM MES, 20 mM EDTA pH 7.0). An equal volume of CIP was added to the suspension and the entire solution then shaken for 5-10 minutes. Following centrifugation (7,000g for 10 minutes) the aqueous (top) phase was removed to a fresh tube and CIP extraction was repeated until no particulate matter was visible at the aqueous: non-aqueous interface. 1/20 volumes of 1M acetic acid and 0.7 volumes of 96% EtOH was then added to the extracted aqueous phase and the solution precipitated over night at -20°C. Following centrifugation (6,000g for 25 minutes), the supernatant was discarded and the remaining pellet washed once with 3M sodium acetate then twice with 70% EtOH. The pellet was then lightly dried at room temperature and resuspended in DEPC treated H_2O. RNA was further diluted 1:50 for quantification at OD_{260}.

III.6 mRNA Purification

mRNA was purified from a pool of total RNA using Dynabeads® Oligo $(dT)_{25}$ (Deutsche Dynal GmbH, Hamburg DE). Briefly, pooled total RNA was divided into aliquots and incubated with equal volumes of binding buffer (20 mM Tris-HCl pH 7.5, 1 M LiCl, 2 mM EDTA) at 65°C for 2 minutes to remove RNA secondary structure. Denatured RNA was then added to prepared Dynabeads® (usually 100 µL settled beads) in binding buffer and rocked gently for 3-5 minutes at room temperature to allow annealing. The Dynabeads® were then recovered by placing the reaction tube on a magnet for a minimum of 30 seconds. The supernatant was completely removed, and the Dynabeads® washed in washing buffer (10 mM Tris-HCl pH 7.5, 0.15 M LiCl, 1mM

EDTA). Finally the mRNA was eluted for 2 minutes at 90°C with 5-10 μL 10 mM Tris-HCl and transferred to an RNAse free tube. mRNA was diluted 1:50 for quantification.

III.7 cDNA Library Construction

III.7.1 cDNA Synthesis

cDNA was synthesized from purified *L.* esculentum mRNA using the Stratagene® cDNA synthesis kit according to manufacturer's instructions. cDNA was fractionated on a 1% agarose EtBr gel and bound to DEAE cellulose by inserting the cellulose membrane into the gel, allowing it to contact the cDNA. cDNA was quantified using comparison to prepared lambda DNA standards on EtBr plates (1 μg/mL EtBr in 0.8% agarose). 96 ng of purified, quantified cDNA was then ligated into EcoRI/XhoI digested, dephosphorylated pACT2. The ligation product was then used to transform ultracompetent cells, and the size of the cDNA inserts was determined following digestion with EcoRI and XhoI.

III.7.2 Amplification of cDNA Library

pACT2/cDNA plasmids were used to transform several batches of ultracompetent *E. coli* XL2 MRF'cells via electroporation, which were in turn plated onto LB medium containing ampicillin (0.1 mg/mL) and tetracycline (0.01 mg/mL). Following incubation at 37°C overnight, each plate was flooded with a total of 4 mL of LB medium. Initially 2 mL was added and the bacterial colonies were scraped from the plate (using a bent Pasteur pipette) into the medium. Once the initial suspension was collected, a further 2 mL of LB was added and the scraping repeated. The final suspension was then well mixed and 87% glycerol added to a final concentration of 15%. The amplified cDNA library was then split into aliquots of 500 μL and stored at -70°C.

III.7.3 Titre and Purification of cDNA Library

To establish the titre of the cDNA library, one 500 μL was thawed, serially diluted with LB medium (1/1000 to 1/10,000,000) and 100 μL of each dilution step was plated onto LB +Amp/Tet plates. Following an overnight incubation at 37°C, the colonies were counted. The optimal dilution step yielding approximately 10,000 colony forming units

(cfu) per plate was then plated over 20 LB+Amp/Tet plates. Following a second overnight incubation at 37°C, bacterial colonies were harvested as described for library amplification (section III.7.2, above). The harvested bacterial suspension was centrifuged for 15 minutes at 3,000g, the LB supernatant removed via aspiration and cDNA plasmids were extracted from the remaining pellet using the Qiagen® Maxi prep kit according to kit instructions.

III.8 Yeast Plasmid Extraction

Yeast colonies were grown overnight at 28°C in 2 mL of minimal medium supplemented with the appropriate amino acids. Cells were collected via centrifugation (12,000g for 1 minute) and the pellet resuspended in 200 µL of yeast lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1mM EDTA). 200 µL CIP and approximately 300 µL of acid washed glass beads were added to the homogenate and the entire mixture was vortexed at high speed for 2 minutes. Following centrifugation (5 minutes at 12,000g), the aqueous layer was transferred to a new tube and the DNA precipitated in the presence of 1/10 volumes 3M sodium acetate (pH 5.2) and 2.5 volumes 100% ethanol. Plasmid DNA was collected via centrifugation, washed twice with 70% EtOH and resuspended in ddH_2O containing 2µg/µL RNAse A.

III.9 Northern Blot analysis

Following denaturation of *L. esculentum* RNA in the presence of 1.75% formaldehyde at 65°C for 5 minutes, up to 20 µg of RNA was resolved on 0.22M formaldehyde gels (1.2% agarose). The RNA was allowed to migrate in the gel at 4V/cm (distance between electrodes) for 2-4 hours. Subsequently, the RNA was transferred to nylon membranes via capillary action in 10X SSC and fixed to the membrane via UV cross-linking. Membranes were pre-hybridized for 2-4 hours at 42°C in hybridization buffer (6X SSPE, 50% form amide, 2.5X Reinhardt's reagent, 0.5% SDS, 0.1 mg/mL ssDNA). Following addition of the ^{32}P-labelled probe (labelling performed with the Megaprime™ DNA Labelling Kit), hybridization was performed overnight under the same conditions. The membrane was subsequently subjected to the following wash series:

20 minutes in 2X SSC at room temperature

10 minutes in 2X SSC, 0.1% SDS at 60°C

10 minutes in 1.5X SSC, 0.1% SDS at 60°C

Visualization of radioactive bands was done following exposure of the membrane to a phosphoimaging cassette overnight at room temperature and imaging on a phosphoimager.

III.10 Protein Analysis

III.10.1 Isolation of Soluble Protein from *S. cerevisiae*

Isolated colonies were incubated overnight at 28°C in 2 mL of SD medium supplemented with the appropriate amino acids. Yeast cells were collected by centrifugation (5,000g for 5 minutes at 0°C), the supernatant discarded and the pellet resuspended in 5 volumes (usually 250 μL) yeast lysis buffer (50 mM Tris-HCl pH 8.0, 0.1% Triton X-100, 0.5% SDS). Acid-washed glass beads (0.45-0-50 mm) were added to the suspension to the level of the meniscus and the entire solution vortexed for 5 periods of 20 seconds each (the cell suspension was cooled on ice for 1 minute between each cycle of vortexing). The cell extract was recovered via centrifugation (3,000g for 2 minutes at 4°C) into a fresh tube, centrifuged a second time (12,000g for 5 minutes at 4°C) for clarification, protease inhibitor was added and the extract stored at -20°C until further use. In some cases, the starting cultures, as well as the subsequent steps were scaled up 100X.

III.10.2 Isolation of Soluble Protein from *E. coli*

E. coli BL21 was grown in liquid culture (2-200 mL) overnight at 37°C (2 mL) or for 2-3 days at room temperature (50, 100, 200 mL). Cells were harvested by centrifugation (5,000g for 15 minutes at 4°C), the supernatant discarded and the pellet resuspended in the appropriate volume of ice-cold 20 mM Tris-HCl, pH 7.9 (250μL – 5 mL). The cells were lysed with a probe sonicator (6 x 15 seconds with 15 second breaks; samples kept continuously on ice). The resulting homogenate was transferred to glass tubes, centrifuged for 45 minutes at 6,000g, filtered through a 0.2 μm filter and kept at 4°C.

III.10.3 Purification of Soluble Protein from *E. coli* and *S. cerevisiae*

His-Bind Resin

Protein purifications were performed using HIS-Bind Resin and metal chelation chromatography according to manufacturer's recommendations (Novogen, Inc., Darmstadt, Germany). Briefly, resin was fully suspended and applied to a 5 mL plastic column until the settled resin volume was approximately 1 mL. The resin bed was allowed to drain, and was then subjected to the following wash series. 3 volumes (i.e. 3 mL) sterile ddH_2O, 5 volumes charge buffer (50 mM $NiSO_4$), and 3 volumes binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9). Crude protein extract was added to prepared resin and incubated for 1-4 hours at 4°C. The column was allowed to drain and the resin washed with wash buffer (60 mM imidazole, 0.5 M NaCl, 20mM Tris-HCl pH 7.9). Protein was eluted in elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9). In some cases, modified buffers were used as follows. The column was allowed to drain, the resin was washed (20 mM Tris-HCl, + 20 or 60 mM imidazole) and an elution step carried out (20 mM Tris-HCl, + 100, 200, 300, or 1000 mM imidazole).

Monoclonal Anti-HA Agarose Conjugate

Protein purifications were performed according to manufacturer's recommendations (Sigma, St. Louis, USA). Briefly, the agarose conjugate was suspended in a storage buffer and transferred to a 5 mL plastic column for ease of handling. The agarose bed was allowed to drain, and rinsed with PBS followed by three sequential aliquots of glycine-HCl, pH 2.5 or 3M NaSCN. The column was then blocked and crude protein extract (pH 7-8), was added to the agarose to form a suspension. The homogenate was incubated on a rotary shaker for a minimum of 1 hour at 4°C. The agarose was then allowed to drain, and the column was washed with 10 mL of PBS. Elution was carried out with ten 1 mL aliquots of 0.1M glycine-HCl into vials containing 30-50 µL of 1M Tris buffer, pH 8; or with ten 1 mL aliquots of 3M NaSCN.

III.10.4 Cell Free Protein Synthesis

Protein was expressed from a T7 expression vector according to kit instructions (Invitrogen, Carlsbad, USA). Briefly, the Expressway™ Plus System uses an extract

from the S30 *E. coli* strain to drive the reaction and a transcription/translation mix that achieves optimal protein production in 2-4 hours. The system is designed for T7-based *in vitro* transcription and translation of desired DNA into protein in a single reaction tube. The DNA of interest is sub-cloned into a T7 expression vector (in this case pRSETC), the purified DNA is added to the bacterial extract, buffer and enzyme in the presence of methionine. The reaction is incubated at 37°C for a minimum of 2 hours. Prior to analysis via SDS-PAGE (10-15% acrylamide) and protein gel blot, the protein extract is precipitated with acetone to prevent smearing on the gel. The desired recombinant protein is then detected on a protein gel blot using an antibody against the Xpress™ epitope (see Appendix VI.1 for vector maps).

III.10.5 Western Blot Analysis

Following SDS PAGE, the gel was incubated in transfer buffer for 10-15 minutes before being placed in the transfer apparatus. Proteins were transferred to Nitrocellulose (Protran; Schleicher & Schuell, GmbH, Germany) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Transfer was performed at a constant voltage of 150V for one hour. After completion of transfer, the membrane was washed briefly in water and stained for 1 minute in Ponceau S. The membrane was then incubated in 5% skim milk powder in TBST (SM-TBST; TBST= 1M Tris-HCl pH 8.0, 150 mM NaCl,0.05% Tween 20) for 4 hours at RT. Fresh SM-TBST was added and the membrane incubated overnight at 4°C. Primary antibody was diluted in fresh SM-TBST and added to the membrane; incubation took place at RT for one hour. The membrane was then washed four times with TBST, for a minimum of 10 minutes each time. The secondary antibody (alkaline phosphatase conjugate) was diluted in fresh SM-TBST and added to the membrane; antibody incubation was carried out as already mentioned. The TBST wash was repeated with the addition of a TBS wash step for 10 minutes. Detection was performed using NBT/BCIP stock solution in detection buffer (100 mM Tris, 100 mM NaCl, 10 mM $MgCl_2$,).

III.10.6 Protein Database Search

The in frame translation of cDNA10 was submitted to either the NCBI (www.ncbi.nlm.nih.gov/blast/Blast), PROSITE (www.expasy.org/prosite), or TAIR

(The Arabidopsis Information Resource, www.arabidopsis.org/aboutarabidopsis.html, on www.arabidopsis.org) databases for analysis.

III.11 Brefeldin A Treatment

Two to three centimetre squares were harvested from the fresh leaves of 8 week old tobacco plants and incubated for varying times in water, BFA (50 or 100 μM), or without treatment. Following incubation, sections were fixed in 100 mM PIPES buffer, pH 7.2, 5 mM EDTA, 2 mM $MgCl_2$ with 0.1% (v/v) glutaraldehyde and 4% (w/v) formaldehyde overnight under vacuum, and embedded in agarose. Sections were incubated with SM-TBST as described in section III.10.5 and subsequently with SUT1 primary antibody (rabbit; 1:250). After appropriate wash steps (III.10.5), sections were incubated with FITC conjugated secondary antibodies (anti-rabbit; 1:1000). Sections were subsequently stained with DAPI (2.5 μg/mL in ddH_2O) to reveal nuclei and with aniline blue (0.05%, w/v in potassium phosphate buffer pH 8.5) to reveal sieve plates. Sections were mounted in PBS (including 25% glycerol) and viewed with a microscope (Leica DMR) equipped with a confocal laser scanning head (Leica TCS SP). FITC fluorescence was detected using an Ar/Kr mixed gas laser (exitation line, 488 nm) and emission was recorded between 495-525 nm. Aniline blue and DAPI were excited with a UV laser (50 mW, Coherent, Inc.; exitation 350-365) and emission was recorded in the ranges of 510-540 (Aniline blue) and 435-485 (DAPI). Emissions were recorded sequentially and images were processed and assembled using Adobe® Photoshop® 7.0 and Illustrator® 10.0.

IV. Results

In previous studies, *Le*SUT1 protein was shown to be localized to the plasma membrane of the enucleate sieve element while its mRNA was preferentially associated with plasmodesmata joining the SE-CC complex (Kühn *et al*, 1997). These observations suggested the possibility of mRNA targeting, mediated via the action of mRNA binding proteins interacting specifically with *Le*SUT1 mRNA. The main objective of this work was to identify, using an *in vivo* assay, endogenous proteins that specifically bind the 3'UTR of *Le*SUT1 mRNA. Isolation and characterization of such proteins would contribute greatly to the understanding of the molecular events surrounding the insertion of SUT1 into the plasma membrane of the sieve element.

IV.1 Creation of an *L. esculentum* leaf cDNA library

IV.1.1 *Le*SUT1 RNA is most abundant in whole leaves

Prior to the construction of a cDNA library from tomato tissue, a brief analysis of RNA expression was undertaken. As SUT1 mRNA and protein were shown to be exclusively expressed in the cells of the phloem, an attempt was made to isolate a pool of RNA enriched in SUT1 mRNA. RNA was isolated from whole leaves or from excised major veins; SUT1 RNA was subsequently detected via northern blot analysis using the entire SUT1 coding region as a probe. This analysis distinctly revealed a greater abundance of SUT1 mRNA in the total RNA extracted from whole leaves (Figure 2). Total RNA from tomato leaves was thus used for further experiments.

IV.1.2 Titre and quality of the *L. esculentum* cDNA library

mRNA was purified from total RNA extracted from whole leaves and used for construction of the cDNA library. Following synthesis and quantification of cDNA (section III.7.1), 96 ng of the cDNA was used for insertion into the multiple cloning site of the plasmid pACT2 (EcoRI/XhoI) via a ligation reaction. The quality of the library was then determined by restriction analysis. The average size of the cDNA inserts was 1000 bp, with 79% of tested plasmids (n=48) containing an insert (data not shown). During the amplification phase, the total number of colony forming units (cfu) was estimated by

counting a representative area of the plate (4 cm^2) and using the following equation (based on the area of the entire plate) to approximate the final number:

(*Equation 1*)
$$T_{approx} = \sum_{1}^{p} n_c \left(\frac{A_{plate}}{A_{counted}} \right)$$

Where T_{approx} is the approximate total number of colonies, p is the total number of plates, n_c is the average number of colonies counted over 4 cm^2, A_{plate} is the area of the entire plate and $A_{counted}$ is the area of the representative square.

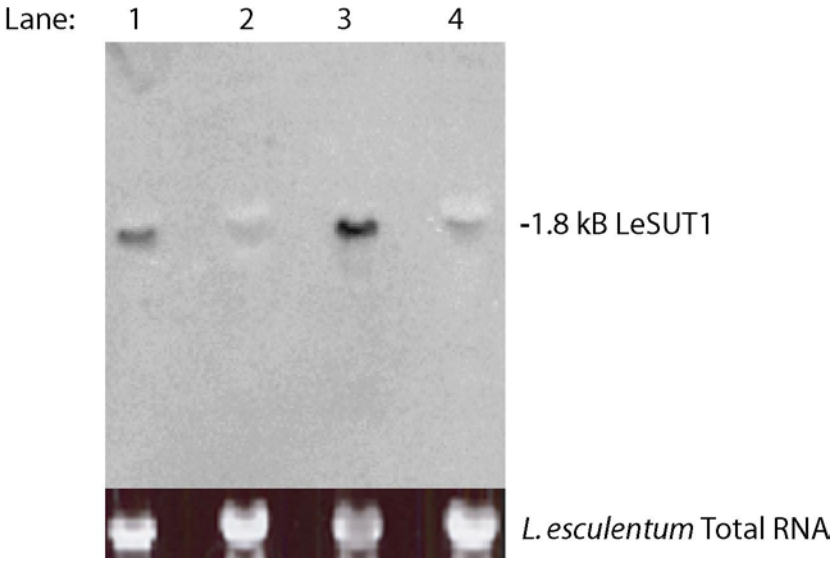


Figure 2. RNA gel-blot analysis of *LeSUT1* expression. Total RNA from whole leaves (Lanes 1 and 3) or isolated veins (Lanes 2 and 4) was resolved on a 0.22 M denaturing formaldehyde gel. Hybridization was performed with ^{32}P-labelled LeSUT1 coding region. For conditions see section III.9. Lanes 1 and 2 contain 26 µg total RNA; Lanes 3 and 4 contain 30 µg total RNA.

Following amplification of the cDNA library to an approximate total of 1.27×10^6 (data not shown), an aliquot was chosen at random and the titre determined. This aliquot is the source of the cDNA referred to throughout the remainder of this work. As described in section II.7.3, serial dilutions of the amplified library were made, the homogenates plated and the resultant colonies counted. Once the ideal dilution step

was identified, the titre was established. The results of the library titre determination are summarized in Table 1.

TABLE 1: Determination of cDNA titre. One aliquot of amplified cDNA library was diluted 1/200,000, and the entire volume spread equally over 20 plates. Two representative squares were counted on each of five randomly selected plates, and the estimated total for each square was calculated using equation 1; the average value was determined.

PLATE	CELLS COUNTED	ESTIMATED TOTAL
1	308	11,350.72
	323	11,903.52
2	305	11,240.17
	341	12,566.87
3	239	8,807.87
	329	12,124.64
4	270	9,950.31
	246	9,065.84
5	217	7,997.10
	345	12,714.29
Average	292.3/4 cm^2	10,772.13/plate
Total over 20 plates		**2.154×10^5**

IV.2 Design of hybrid RNA molecules

IV.2.1 Analysis of SUT1 mRNA Secondary Structure

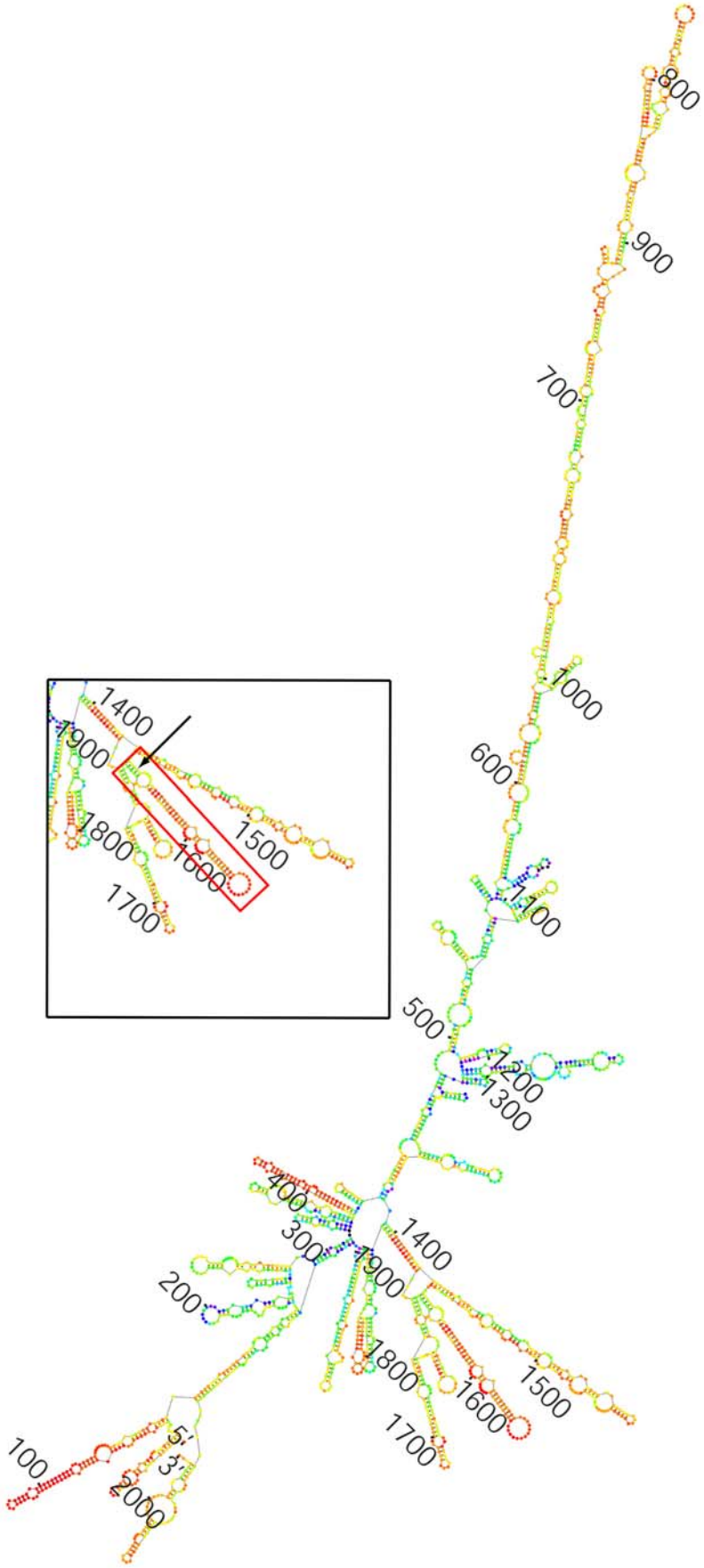
Studies of the RNA viruses of plants suggest the presence of an endogenous RNA transport pathway (Xoconostle-Cazares *et al.*, 1999; Blackman *et al.*, 1998). With respect to the sucrose transporters, localization data has only recently begun to emerge (Kühn *et*

al., 1997), however structural data may help to refine the direction of future experiments. To this end, I have undertaken an analysis of the secondary structure of several known sucrose transporters. The RNA structures of sucrose transporters from tomato (*LeSUT1*, *LeSUT2*, *LeSUT4*) were compared to potato (*StSUT1*) and arabidopsis (*AtSUC1, AtSUC2,* data not shown) using the *mfold* RNA folding software version 3.1 (Zuker, 2003). This program predicts the secondary structure of RNAs based on the free energy associated with a series of base pairs. While several structures are possible, those that are favoured energetically are generally taken as the most likely to occur *in vivo.* Between the sucrose transporters analysed, there is little similarity in full secondary structure, although some regions display similar characteristics. Within each structure, however there are regions of high determination *i.e.,* among the predicted structures they routinely appear in a given state (stem or loop). These regions are depicted as yellow, orange or red dots in the figure (with red being the highest degree of determination, followed by orange, then yellow). Regions that are highly indeterminate (are not routinely predicted to appear in a given state) are depicted with green, blue, and black dots. The regions of high determination are of particular interest when they occur within the 3' UTR, as is the case for LeSUT1 (boxed region Figure 3). They may predict the regions of binding of a putative RBP. Primary sequence homology of the 3'-UTR is highest between *LeSUT1* and *StSUT1* at 58%, while the homology between *LeSUT1* compared to *LeSUT2*, *LeSUT4, AtSUC1* and *AtSUC2* is in the range of 28-45%. Structurally, however, there is not much similarity; LeSUT2 shows not only a moderately determined region comprising the 3'UTR, but also a major region of the first 300bp of the coding region (Figure 4A). LeSUT4, however, shows no such highly determined structure in its 3'UTR, rather, the major conserved region occurs within the coding region (Figure 4B). Such unique structures are understandable in the context of the myriad of protein complexes that may be involved in the spatio-temporal regulation of RNA localization. Each structure may represent a specific code recognized by different associations of RBPs. As would be expected, there is also no apparent similarity between species with both arabidopsis (data not shown) and potato having divergent secondary structures with internal regions of determination either in the coding region, 3' UTR or both (See *StSUT1* mRNA structure, Figure 5). Additionally, the predicted secondary structures of LeSUT1 and

StSUT1 3'-UTRs share some similarities (highly determined stem-loop structures in Figure 3 and 5).

IV.2.2 Predicted Secondary Structure of hybrid RNA molecules

Prior to the cloning of hybrid RNA molecules for use as bait in the Y3H method, several short sequences corresponding to various regions of the *Le*SUT1 3'UTR were analyzed using the *mfold* program (Figure 6). For each analysis, the entire hybrid RNA sequence, including MS2 sequence from the plasmid pACT2 (see Appendix Fig. VI.1 for vector maps), was used. The parameters set for the folding conditions were the default conditions of the program, with the exception of the temperature, which was set at 28°C in order to approximate the experimental environment in yeast; the structural prediction at 20 (normal growing temperature for tomato) and 28°C were, however nearly identical. While the chosen sequences overlap to some degree, the predicted pattern of folding varies widely. Of the five sequences chosen for analysis, one (RNA-5) displayed a conserved structure that is present in the folding prediction of full length *Le*SUT1 (compare to Figure 3, red boxed region). This hybrid molecule was chosen for screening of the cDNA library. The remaining sequences were chosen on the basis of shared secondary characteristics/ sequence with RNA-5, or divergent secondary characteristics as compared to RNA-5. The design of the hybrid RNA molecules and their relative position in SUT1 mRNA is shown in appendix VI.2.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IV.2.3 Cloning of hybrid RNA constructs

Following analysis of the secondary structure, the RNA hybrids were created via insertion into the vector pIIIA MS2-1 or MS2-2. Primers complementary to *Le*SUT1 were designed for the amplification (via PCR) of the pertinent regions of the SUT1 3' UTR. Each primer was designed to introduce restriction sites (*EcoR*I, *Sma*I) flanking the target sequence to facilitate both cloning and the determination of sequence orientation. Following 30 cycles of PCR at the appropriate annealing temperatures, amplified DNA was analyzed by gel electrophoresis, purified from the gel matrix, precipitated and directly digested with *Cfr*9I to eliminate blunt end ligation. The resulting fragment was introduced into *Cfr*9I-digested, dephosphorylated pIIIAMS2-1/2. Following transformation of *E. coli* TOP10F' with the resulting plasmid, DNA was prepared from isolated transformant colonies and the size and orientation of the insert was confirmed both by restriction analysis and sequencing (data not shown).

IV.3 The Yeast Three Hybrid Screening Method

The yeast three-hybrid method (SenGupta *et al*, 1996) is a modification of the classic two-hybrid system (Chien *et al.*, 1991) and relies on the activation of two reporter genes via a hybrid RNA intermediary. The reporter genes, HIS3 and *lac*Z, are both integrated into the genome of the yeast strain L40 coat with expression controlled by the lexA promoter. Also integrated into the genome of L40 coat is the first protein hybrid, which is comprised of the LexA binding protein fused in frame to the MS2 RNA binding protein.

Figure 3: Predicted Secondary Structure of *LeSUT1* mRNA. RNA secondary structure was predicted using the *mfold* version 2.3 computer program. The black-boxed region is a close-up of a portion of the 3'UTR. The arrow indicates the stop codon. Within the black box, the red box indicates an example of a region of high determination within the 3'UTR.

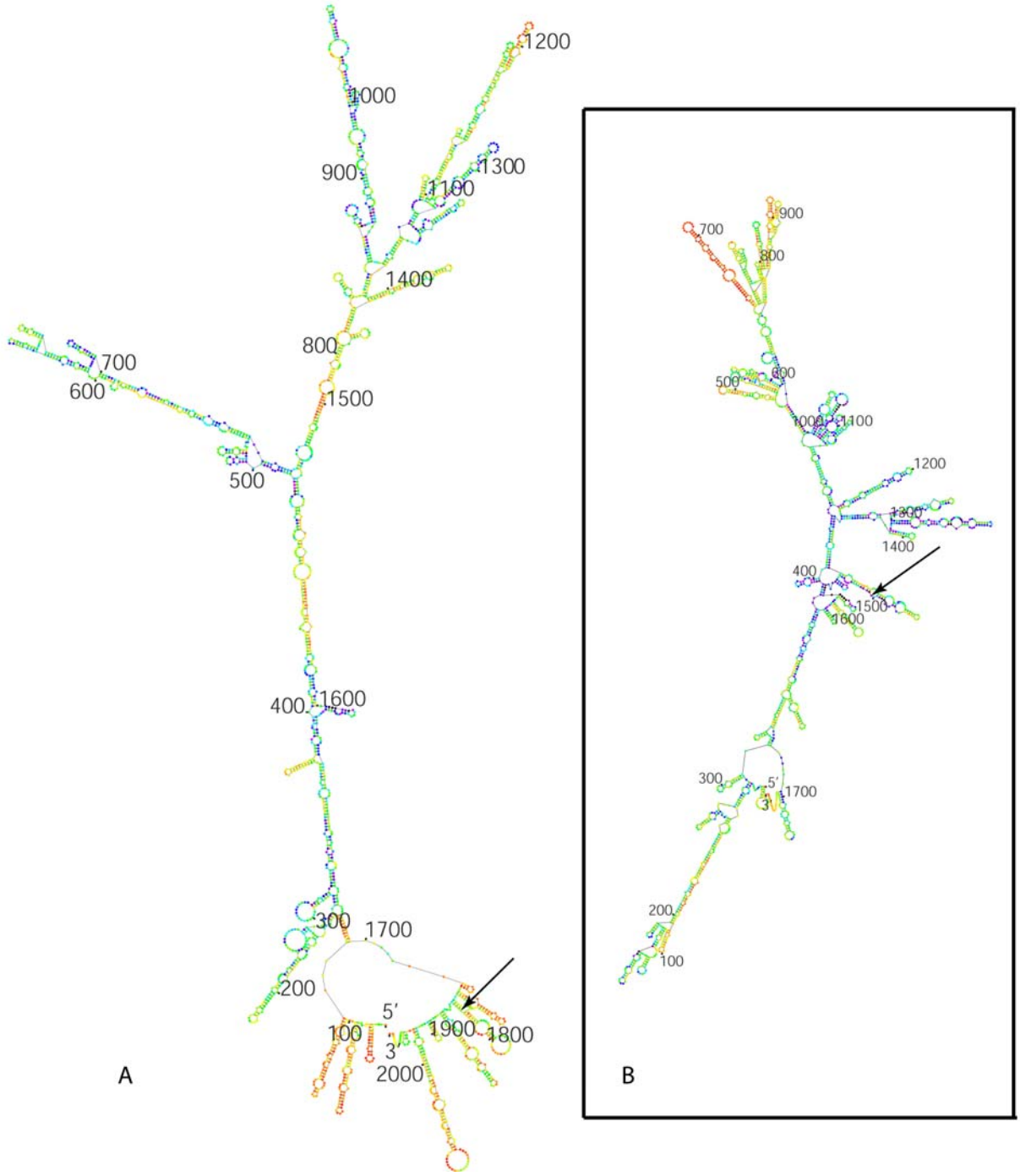
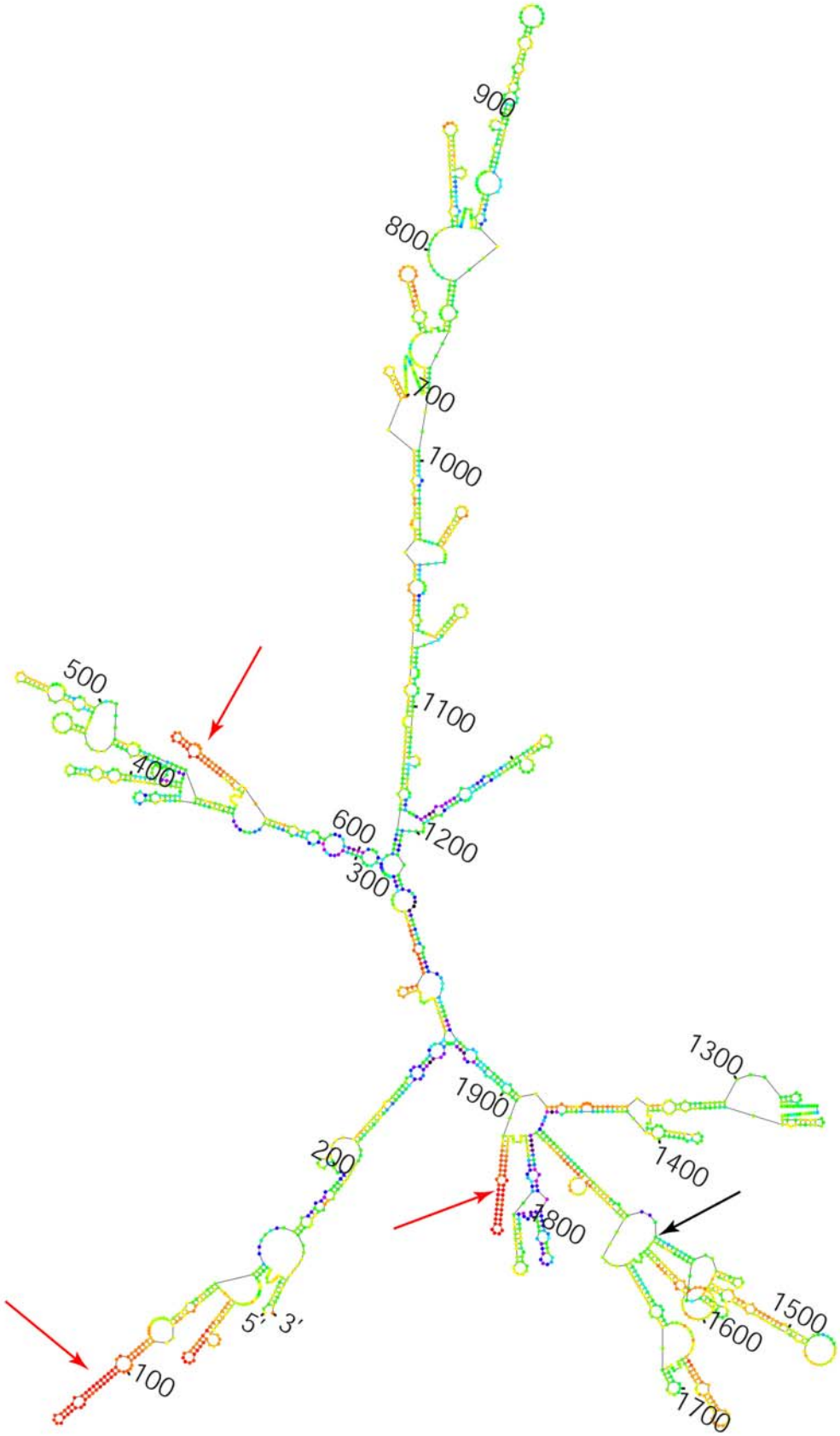


Figure 4: mfold Predictions of *Le*SUT2 and *Le*SUT4 Secondary Structure. A) Predicted secondary structure of *LeSUT2* mRNA. The stop codon is indicated with an arrow (nucleotide 1813). The 3'UTR is less determined in structure than that of *SUT1*. B) Predicted secondary structure of *LeSUT4* mRNA. Arrow indicates stop codon (nucleotide 1501). There are no areas of determination in the 3'UTR, rather, a region of extremely high determination occurs within the coding region (red step loop between 650-750 nt).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In the absence of any introduced plasmids, the LexA binding protein (while constitutively bound to the LexA promoter) is able to effectively prevent transcription of the reporter genes in the absence of an activator (Gal4 AD).

The method involves the use of several genetic markers and numerous screening steps to avoid false positive results. While presented in separate sections, the screening steps described for RNA hybrids 3 and 5 were performed simultaneously. The same applies to the screening steps described for RNA hybrids 2 and 4. A schematic drawing of the steps of the method is presented in Figure 7

IV.3.1 Initial Selection: HIS3, ADE2

Screening with RNA-3

As described in section II.3.1, the yeast strain L40 Coat is auxotrophic for the following markers: ADE2, LEU2, URA3, HIS3. The screening process was initiated with the transformation of L40 Coat, first with an RNA hybrid expressed from the plasmid pIIIA-MS2-1, then subsequently with 5 μg of the *L. esculentum* cDNA library as described in section III.3. The entire transformation mixture (usually 10 mL) was spread onto minimal media supplemented with adenine, uracil (ade, ura) and with 5 mM 3AT – a competitive inhibitor of the HIS3 gene product. At this stage, transformant colonies were selected for activation of one reporter gene (growth on his-lacking media) and for maintenance of the cDNA plasmid (growth on leu-lacking media; see Appendix Fig. VI.1 for vector maps). For simplicity, such plates were labelled according to the amino acids absent from the media, namely leucine and histidine; thus, the initial screening plates were SDG –leu, -his, + 5 mM 3AT; media used in the Y3H method will be described thusly for the remainder of this text. Following a minimum of 4 days incubation at 28°C, transformant colonies were counted and scored for appearance. The observed transformation efficiency was quite low, yielding only 1341 cfu/5μg cDNA (data not shown).

Figure 5: mfold Prediction of *StSUT1* mRNA Secondary Structure. Black arrow indicates stop codon (nucleotide 1546), while red arrows display regions of high determination. A highly conserved region appears in the 3'UTR, similar in structure to the stem loop that is predicted to occur in the 3'UTR of *LeSUT1* mRNA.

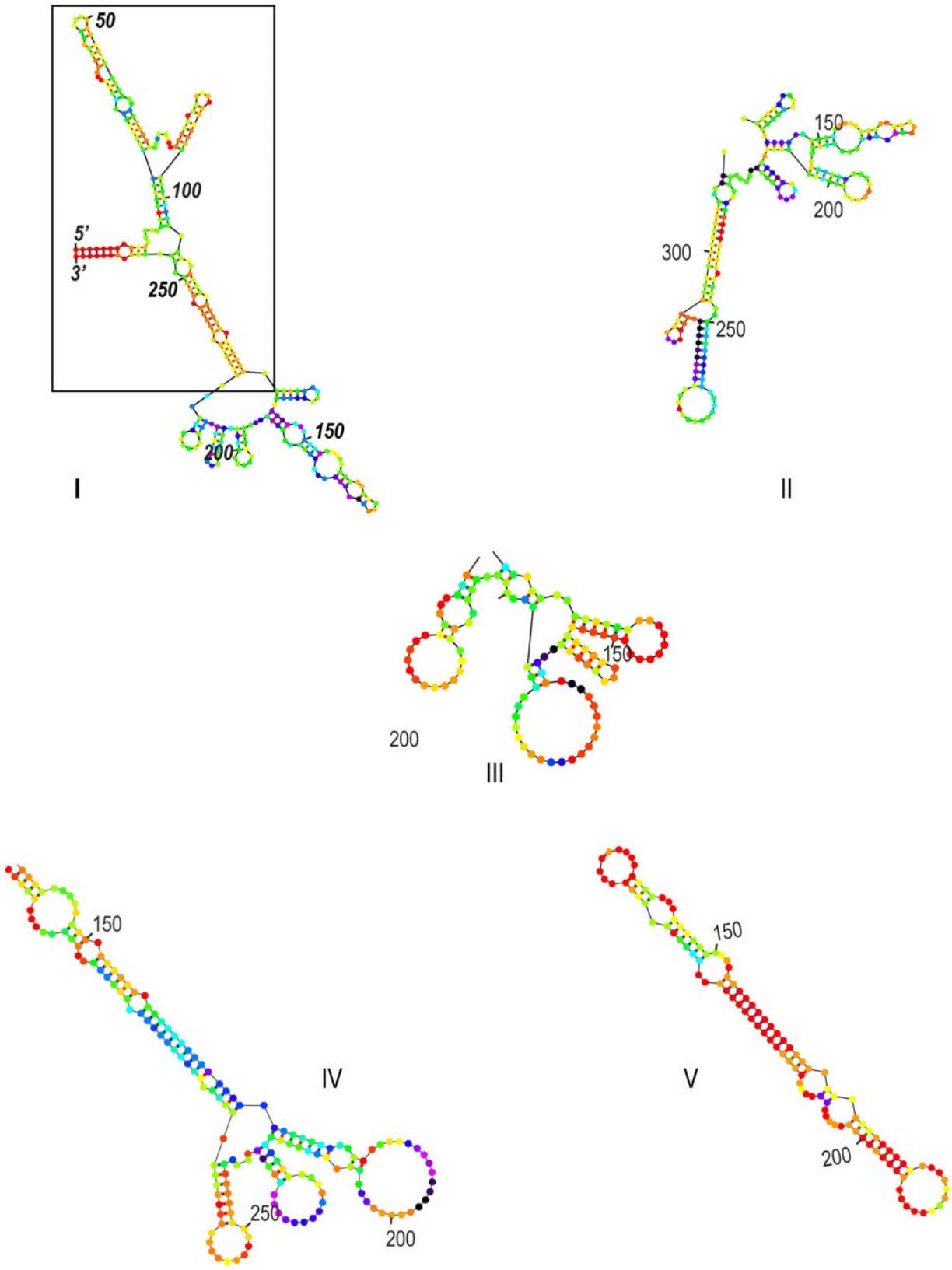


Figure 6: mfold Prediction of Hybrid RNA Secondary structure. The secondary structures of the RNA hybrids used in the Y3H screen were analyzed with the mfold program. The boxed region in RNA-1 is the MS2 RNA, this region is identical for all hybrid RNAs and has been omitted from the remaining drawings. Note : RNA-5 is the same sequence/structure that appears in the SUT1 3'UTR (Fig. 4, red box).

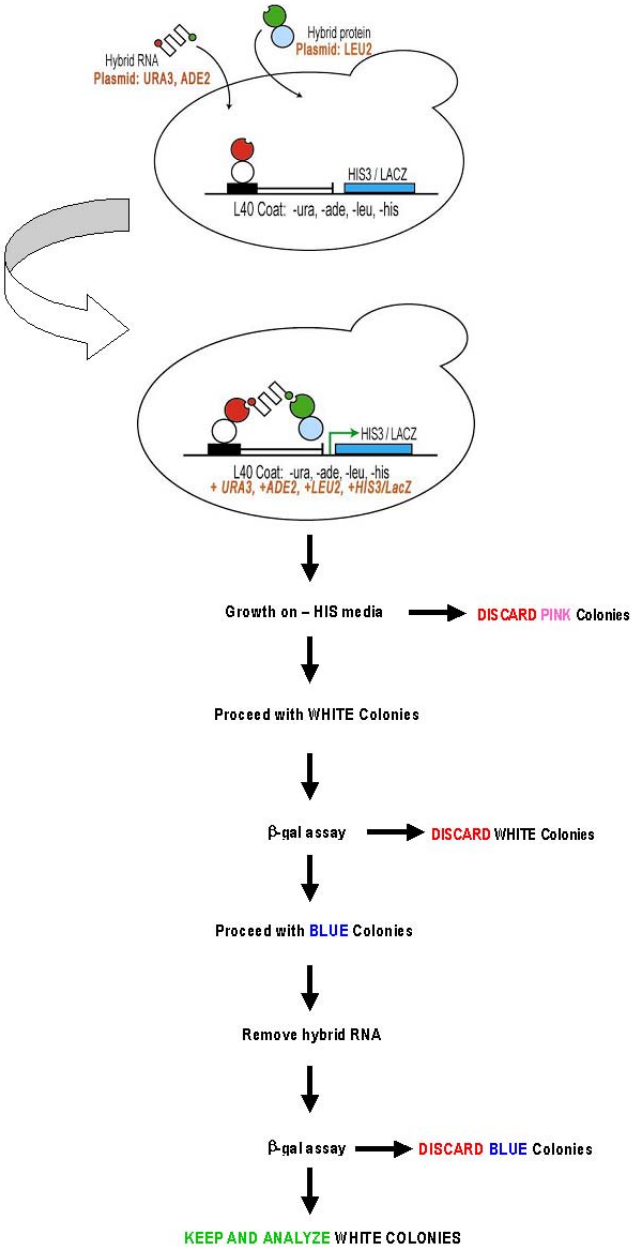


Figure 7: Schematic drawing of Yeast Three Hybrid Method screening steps. General selection based on colour is outlined. For specific steps, see text.

In addition to the selective methods already mentioned, the RNA plasmid also harbours the ADE2 gene, which allows for a further level of control. The initial selection medium contains low levels of adenine at a concentration too low to prevent the initiation of the *de novo* synthesis pathway. Those colonies which lose the RNA plasmid appear pink due to the build up of a red purine metabolite produces via this pathway; colonies maintaining the RNA plasmid, and thus the ADE2 gene, remain white in colour. The loss of the RNA plasmid at this stage is permitted due to the omission of uracil from the medium; thus the first false positives – colonies able to grow in the absence of histidine without the RNA intermediary – are eliminated. Initially, all colonies were individually harvested onto a fresh plate for a further incubation at 28°C (Figure 8A). This enhanced the colour development of *ade2* transformants, but was a time-consuming endeavour. The same result was achieved by incubating transformant colonies 1-2 times overnight at 4°C (data not shown). White transformant colonies were then harvested onto fresh media selective for both the RNA plasmid and the cDNA plasmid (SDG –leu, -ura; Figure 8B).

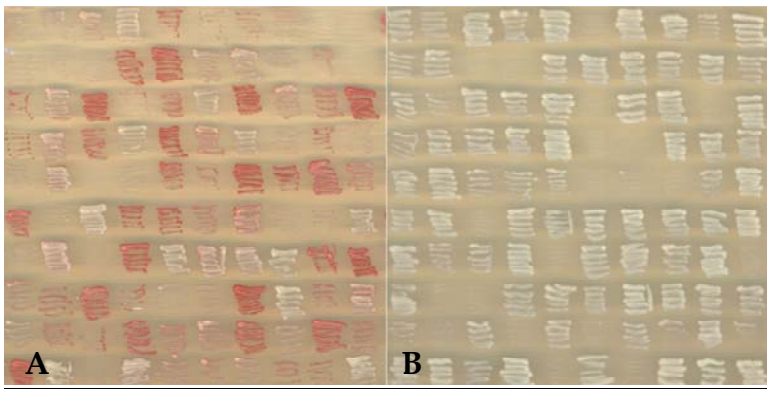


Figure 8: Initial harvesting of Transformant colonies. A) Following a minimum of 4 days incubation at 4°C, all colonies growing on –his medium were transferred to a fresh plate selective only for growth and the presence of the cDNA plasmid (SDG –leu, -his). B) Following incubation, only WHITE colonies were transferred to medium selective for both the RNA plasmid and the cDNA plasmid (SDG –leu, -ura).

Screening with RNA-5

The same procedure was followed for screening the cDNA library with RNA-5. A slightly better transformation efficiency was observed, with 1643 cfu/5µg cDNA (data not shown). Following the requisite incubation at 28°C, transformant colonies were immediately transferred to 4°C for colour enhancement. White colonies were then transferred to fresh media as described above.

Screening with RNA-2 and 4

Again the same procedure was employed for initial screening. The transformation efficiency for RNA-4 was comparable to those previously observed (1127 cfu/5 µg cDNA), however that of RNA-2 was extremely low (41 cfu /5 µg cDNA, data not shown). Moreover, every apparent positive transformant in the case of RNA-2, either rapidly turned pink in colour, or failed to grow (Figure 9).

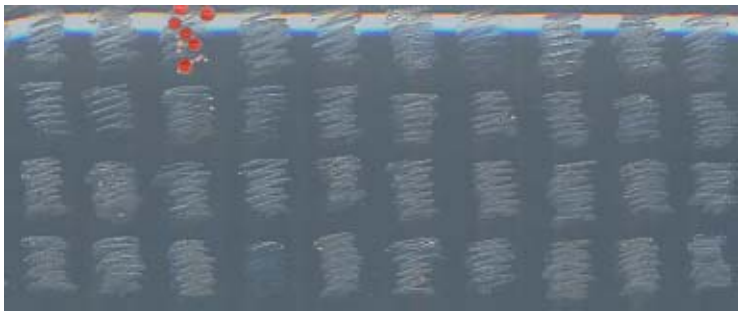


Figure 9: Screening with RNA-2. All initially white colonies that grew on histidine deficient media either failed to grow or turned pink in colour when transferred to fresh plates.

IV.3.2 Subsequent Selection: β-gal Qualitative Assay

The ability of transformant colonies to grow on media lacking histidine suggests that the hybrid components of the Y3H method have assembled in a manner conducive to the activation of the reporter genes. In order to test this hypothesis, a qualitative assay was undertaken in order to determine if the second reporter gene, β-galactosidase (*lacZ*), was also activated. The activity of β-galactosidase is assessed via the conversion of a lactose

analog, X-gal, into a blue chromogenic product. The assay was performed as described in section III.4.2, and a representative result is shown in Figure 10. Those colonies that grew white on histidine lacking media, and also showed a blue colour following the β-gal assay were chosen for further analysis and transferred to fresh selective media.

IV.3.3 Elimination of RNA-independent positive clones

Many of the RNA-independent false positives were eliminated using the previous screening steps. Further steps were required, however, to ensure that the interaction observed is specific. In order to investigate whether the activation of reporter genes was indeed dependent upon the presence of the hybrid RNA molecule, putative positives were replica plated onto medium selective for both the cDNA and RNA plasmids, and supplemented with 5 mM 5-FOA. 5-FOA is converted to the toxic compound 5-fluoro-deoxyuracil (Boeke *et al.*, 1987) by the gene product of *URA3*; yeast cells expressing the *URA3* gene are thus unable to survive on media containing 5-FOA. In order to survive in the presence of 5-FOA, the yeast must eject the RNA plasmid. When transferred to this medium, transformed L40 coat grew slowly and lost the RNA plasmid, which was monitored by observing the colony colour.

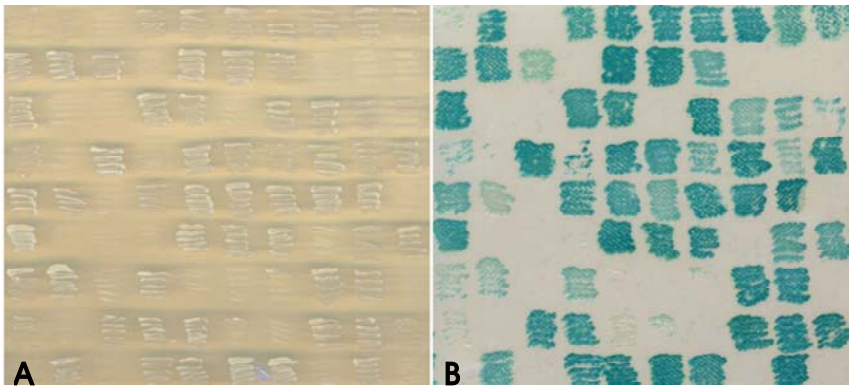


Figure 10: β-galactosidase Qualitative Assay. A). Transformant colonies grown on SDG –leu, -ura. B) Colonies in A transferred via replica to nylon membrane. Filter assay performed as described in section III.4.2.

The RNA plasmid houses not only the *URA3* gene, but also the *ADE2* gene, thus loss of the plasmid causes the colonies to turn bright pink in colour under conditions of low adenine concentration (Figure 11A). The putative positive clones were replica plated twice onto media containing 5-FOA to ensure complete loss of the RNA plasmid. Following this procedure, the β-gal assay was repeated. Only those colonies that remained "white" (actually pink due to the loss of the RNA plasmid) at this stage could be considered for further analysis; activation of the reporter gene was not possible in the absence of the hybrid RNA molecule (Figure 11B).

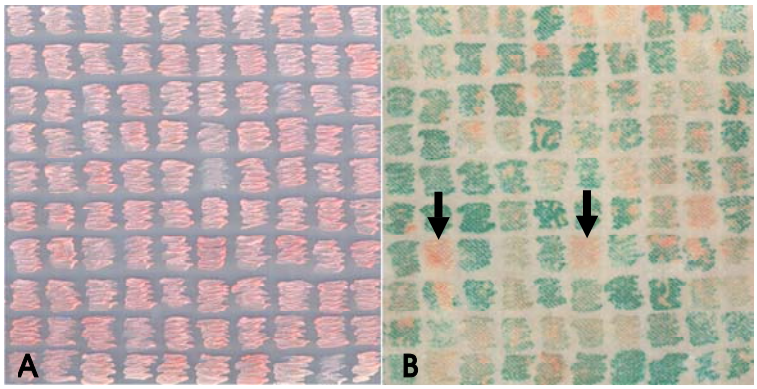


Figure 11: Removal of the RNA plasmid and subsequent β-galactosidase assay. A) The yeast is permitted to lose the RNA plasmid by growth on selective medium supplemented with 5-FOA (SDG –leu, -his + 5mM 5-FOA). Loss of the RNA plasmid is monitored by observation of colony colour; the RNA plasmid houses the *ADE2* gene, loss results in pink colonies. B). Replica plate of A. β-gal assay demonstrates the large number of colonies able to independently activate reporter genes. Arrows: RNA dependent clones.

Of the nearly 3000 original transformants (in the case of the screen involving RNA-3 and 5), only 12 were classified as RNA-dependent at this stage as summarized in Table 2.

TABLE 2: Summary of Y3H screening of the *L. esculentum* leaf cDNA library with LeSUT1 hybrid RNA molecules

	RNA HYBRIDS USED			
TRANSFORMANTS	2	3	4	5
HIS3+ ([3-AT])	41	1341	1127	1643
White: *ADE2* screen	41	509	416	894
LacZ +	n.a.	357	229	317
RNA dependent	n.a.	6	19	6
Specific interaction	n.a.	0	0	1

IV.3.4 Confirmation of interaction

In order to finally confirm the interaction suggested by the screening steps discussed up to this point, cDNA plasmids were extracted from the colonies of interest (as described in section III.8) and used to transform *E. coli* 6201, which is a leub6 mutant; this bacteria cannot grow on media lacking leucine. The plasmid housing the cDNA library (pACT2, see Appendix VI.1 for map) encodes the LEU2 gene, thus its introduction into strain 6201 enables the bacteria to grow on leucine deficient media. Several isolated colonies were picked from each transformation, DNA was subsequently prepared and the DNA preparation was digested with the restriction enzymes *EcoRI* and *XhoI* (insertion sites for cDNA library) to estimate the size of the inserts. In the event of identical digest patterns of DNA prepared from the same transformation, a maximum of two isolates were further tested (data not shown). Plasmid DNA extracted from transformed *E. coli* 6201 was then sequenced in order to rule out the presence of multiple cDNA plasmids in each positive yeast source colony. The isolated cDNA plasmid as well as an RNA hybrid plasmid were then used to "re-transform" L40 coat. In the case of RNA-3 and 5 (which were used to screen the cDNA library concurrently), each isolated cDNA was introduced into L40 coat with (i) the original RNA hybrid with which it was shown to interact and (ii) the second RNA hybrid used for screening. Thus if a cDNA was shown to interact with RNA hybrid 3, it was introduced with either RNA-3 or RNA-5. Of the 12 RNA-dependent clones, only one was confirmed in this manner to interact with *Le*SUT1 RNA (Figure 12). This clone, which will hereafter be referred to as cDNA10,

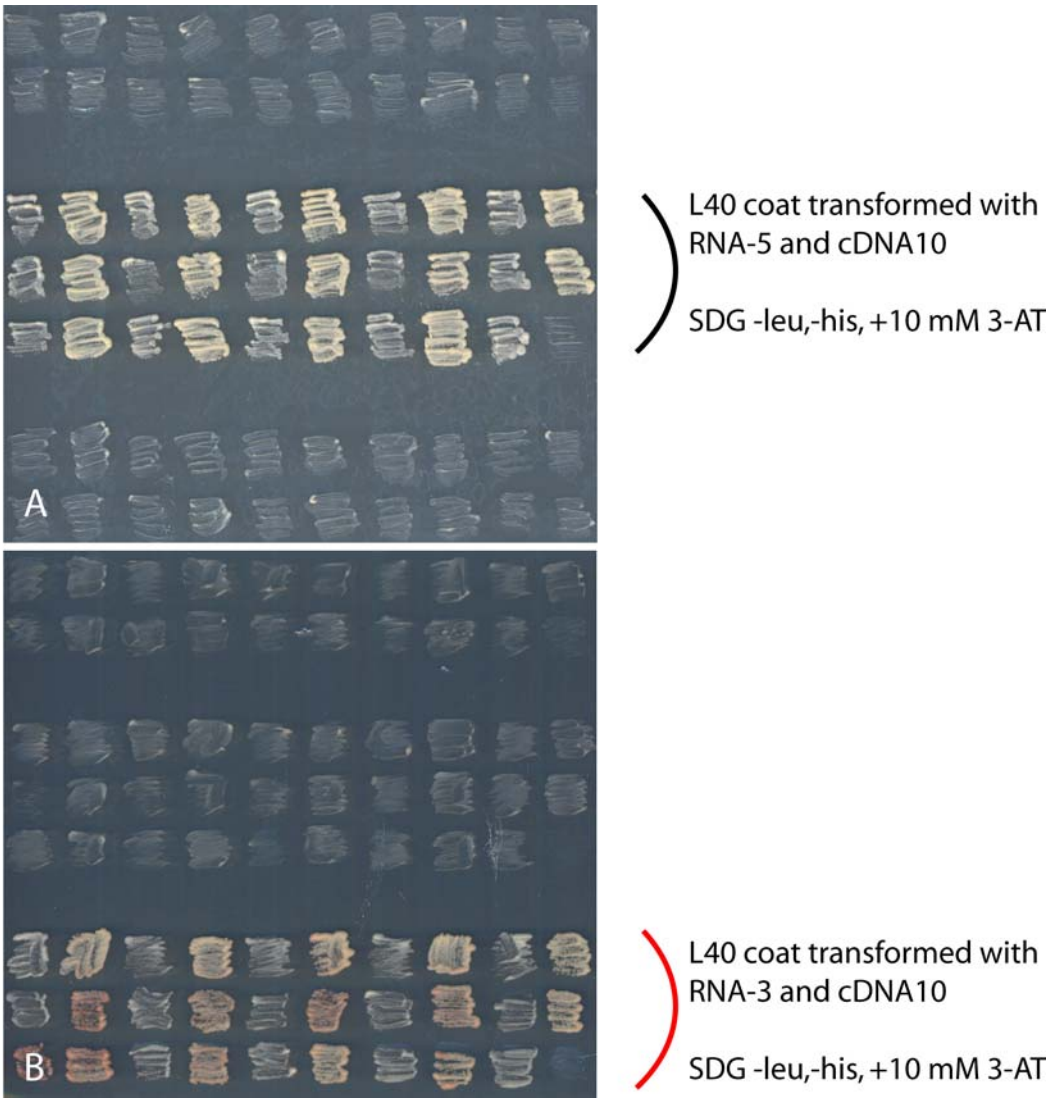


Figure 12: Confirmation of Y3H Interactions. The isolated cDNA10 (or one of the remaining 11 isolated cDNA clones) was introduced into L40 coat that had been previously transformed with either RNA-3 or RNA-5. The yeast was initially plated onto SDG –leu, -ade, -ura to ensure the maintenance of both plasmids. Several small and large colonies were selected from each plate and streaked onto SDG –leu, -his, +10 mM 3-AT. A) A representative section of a plate confirming the interactions observed in the Y3H screen. Colonies containing RNA-5 and cDNA10 are indicated. The patches above and below this section are colonies transformed with RNA-5/cDNA9 and RNA-5/cDNA11, respectively. B). As with A, a representative section is shown, with colonies containing RNA-3 and cDNA10 indicated. The patches above and below this section are colonies transformed with RNA-3/cDNA9 and RNA-3/cDNA11, respectively.

when introduced into L40 coat was able to complement the histidine auxotrophy in the presence of RNA-3 or 5. In the case of RNA-3, however, the colonies became pink in colour after 4 days of incubation at 28°C, suggesting loss of the RNA plasmid. To date cDNA has been isolated from the putative positive clones resulting from the screen using RNA-2 and 4. Of the 19 cDNA plasmids, none were able to complement histidine auxotrophy when reintroduced into L40 coat along with RNA hybrid plasmids (data not shown).

IV.4 Sequence Analysis of a putative *Le*SUT1 mRNA binding protein

The sequence of the original cDNA10, which was isolated using the Y3H screening method, was relatively short (Figure 14A). This initially isolated sequence was compared to a database of known DNA and protein sequences. No significant homologies were observed, as indicated by E-value (Table 3). The predicted protein (prediction based on the translational reading frame of the plasmid pACT2; a fusion protein is produced, but only the portion encoded by cDNA10 was used for database searches) produced from this initial sequence was also analyzed for putative motifs; the sequence possessed no identifiable nucleic acid binding domains, transmembrane motifs or other signal sequences, although a region somewhat similar to an RNA recognition motif present in many plant G-rich RNA binding proteins (14B, Lorkovic and Barta, 2002) was observed. As efforts to identify conserved sequences were fruitless, an attempt to attain the entire sequence of the cDNA was undertaken.

IV.4.1 3'RACE and 5' RACE

Primers for amplifying the 3' and 5' ends of cDNA10 were designed as described in section II.2 (a schematic drawing of the relative positions of the RACE primers appears in Appendix VI.4). The RACE (Rapid Amplification of cDNA Ends) reaction was carried out in accordance with manufacturers instructions. Briefly, total RNA or mRNA was dephosphorylated, de-capped and ligated to a specified RNA oligonucleotide (supplied in kit). Ligated RNA was then transcribed using the Superscript™ III reverse transcriptase and an Oligo dT primer (supplied in kit). 5' ends were amplified using cDNA10 specific primers and kit supplied primers homologous to the ligated RNA oligo. 3' ends were amplified using cDNA10 specific primers and kit supplied Oligo dT

primers. Initial results are seen in figure 15; suggesting that cDNA10 is in fact a portion of a longer sequence.

A

3′- gaattcGGCACGAGGGTGGCATTCTTGATGTACTATATTACCTGTGCTAT
GTAAACACAAAACTGTATAAGCTATGTAGCTTCAACTAGTTGTGTAGTTTC
TTTGTACAATTTGTTGGATAGTCACCCTAAAAGCCTTTTATTGCTGACATCA
GTTGTCCCCAAAGAGCAGATACACTGATACTATTTGTGCTCAACTTCTTTAA
GCTTTGGACTCTTGAAACATTTTGATGTGAATATGCCTGTTATTTCAATGCTA
AAGAGTGTTGCTCTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAActcgag-
5′

B

LGGHMAMEAPGIRIRHEGGILDVLYYLCYVNTKLYKLCSFN

Figure 14: DNA and protein sequence of cDNA10. A) The consensus sequence of three independent clones isolated from transformed bacteria. The restriction sites (*EcoRI* and *XhoI*) are underlined. B) In frame translation of the cDNA sequence. The shaded region is similar to a conserved domain from plant G-rich RNA binding proteins.

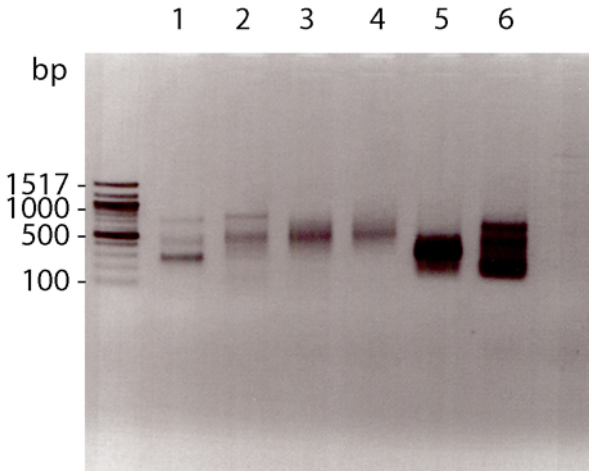


Figure 15: Amplification of 5′ and 3′RACE Products. Lane 1) Amplification using Gene Racer (kit) 5′ primer and c10extend-6, followed by a nested PCR reaction with the Gene Racer 5′ nested primer and c10extend-7. Lane 2) Amplification using Gene Racer (kit) 5′ primer and c10extend-6. Lane 3) Amplification using Gene Racer (kit) 3′ primer and c10extend-1, followed by nested PCR reaction with the Gene Racer 3′ nested primer and c10extend-2. Lane 4) Gene Racer (kit) 3′ primer and c10extend-1. Lanes 5 and 6) Gene Racer 3′ and 5′ control reactions, respectively.

Table 3: BLASTp Results for In-frame Translation of cDNA10. The in frame translation of cDNA10 was submitted to the BLASTp database for comparison. The first three matches are shown; the E values, are quite high, however indicating a low degree of homology

Unknown [Spodoptera litura nucleopolyhedrovirus]	E value=2.1
[Oryza sativa (japonica cultivar-group)]	E value=3.7
Ornithine decarboxylase [Drosophila melanogaster]	E value=5.0

IV.5 Expression Analysis of a putative *Le*SUT1 mRNA binding protein

IV.5.1 Northern blot analysis of cDNA10

Total RNA was isolated from *L. esculentum* leaves and mRNA purified as described in sections III.5 and III.6. RNA and expression was analyzed by northern analysis, using ^{32}P-labelled cDNA10 (entire sequence) as a probe. As seen in Figure 16, a band much larger than the length of the isolated cDNA is observed. There remained, however, a high degree of background in all cases tested.

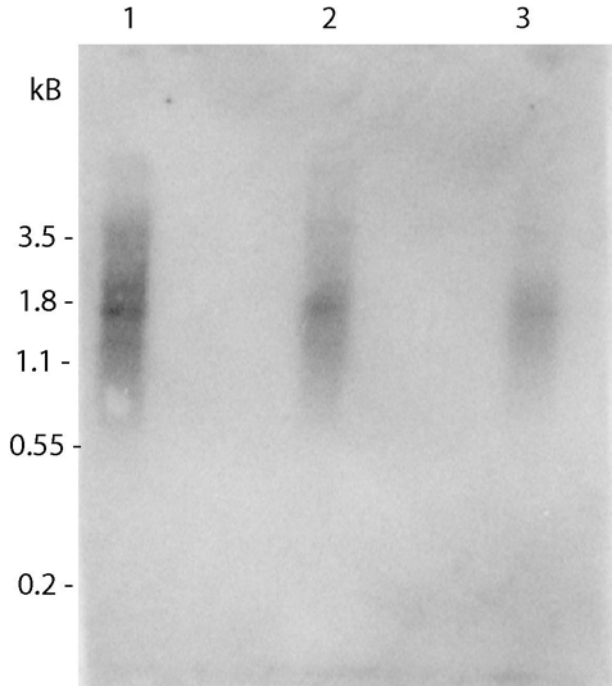


Figure 16: Northern Analysis of cDNA10 Expression in Mature Leaves. RNA gel blot analysis was carried out as described in section II.9. In lane 1, 5 μg total RNA was loaded on the gel; 3 μg was used in lane 2; 1.5 μg used in lane 3

IV.6 Expression and Purification of a putative *Le*SUT1 mRNA binding protein

IV.6.1 Purification of protein from using monoclonal anti-HA agarose

Once the cDNA was confirmed to interact with hybrid RNA *in vivo*, followed by an analysis of expression pattern, an attempt was made to purify the putative binding protein. The expected size of the fusion protein produced by the plasmid pACT2 was estimated to be 21 kD, including the Gal4AD and N-terminal HA (hemagluttinin; allows subsequent purification of recombinant protein) tag. The expression of the fusion protein is controlled by the constitutive alcohol dehydrogenase (ADH) promoter. As an initial step, a pilot expression experiment was set up in order to determine the ideal time for bacterial induction. Isolated cDNA was used to transform *E. coli* TOP10 F', a single transformant colony was selected and incubated in 3 mL liquid medium overnight at 37°C. Aliquots (500 μL) were taken from this primary culture and used to inoculate

fresh 20 mL cultures (six in total). Each sub culture was then grown for 5 hours at 37°C and treatment conditions set up according to Table 4.

TABLE 4: Treatment conditions for pilot expression. The expression of cDNA10 in the plasmid pACT2 is controlled by the yeast alcohol dehydrogenase (ADH) promoter. While constitutively active, this promoter is inhibited to a degree by the presence of glucose (but not maltose), and presumably inducible by the addition of EtOH. The effect of all three compounds on protein expression was tested.

	Treatment Number						
	t_0	1	2	3	4	5	6
Treatment	n.a.	No treatment	0.5% EtOH	0.5% EtOH, 2% glucose	0.5% EtOH, 2% maltose	2% glucose	2% maltose

Following treatment, each subculture, with the exception of t_0, was grown for an additional 4 hours at 37°C. The sample t_0 was used as a baseline for expression, bacteria was harvested from this sample via centrifugation and stored at -20°C until further analysis. Aliquots of 1 mL were taken every hour from each of the remaining sub cultures, cells harvested via centrifugation and stored at -20°C prior to gel analysis. Once all aliquots were taken, protein was extracted from yeast cells according to methods outlined for mechanical lysis of yeast in section III.10.1. Extracted protein samples were then applied to a 10% SDS-poly acrylamide gel (10% stack), and subjected to electrophoresis. As seen in Figure 17, there was no indication of induction. A similar result was obtained when larger starting cultures of 10 mL were used (data not shown). While no pattern of induction was observed, the results from the Y3H screening indicated that recombinant protein was in fact produced. Thus instead of inducing transient over expression, yeast was grown on a large scale to allow normal expression, followed by purification of the fusion protein from a large crude extract. Protein was purified as described in section III.10.1, and incubated with monoclonal anti-HA agarose conjugate. Protein extracts were subsequently analysed by SDS-PAGE, both 10 and 15%. Despite numerous endeavours in this area, no purified protein was ever observed

or recovered (data not shown). A similar set of experiments was undertaken with large cultures of yeast with the same result.

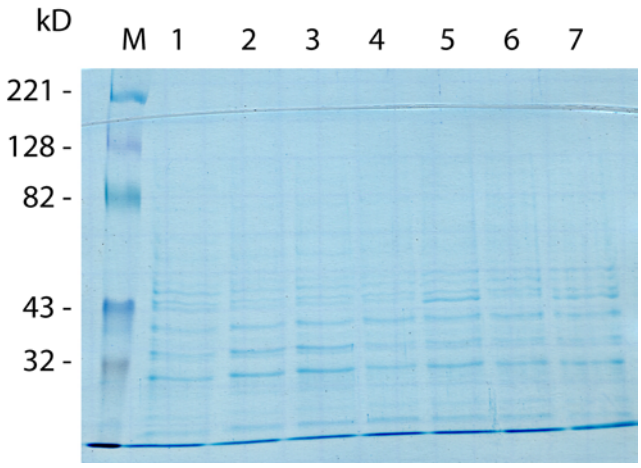


Figure 17: Induction of cDNA10 Over expression in *S. cerevisiae*. Cultures grown overnight and induced for 3 hours. Lane1, t_0 (sample taken before induction); Lane 2, no treatment, but culture incubated for 3h; Lane 3, 0.5% EtOH; Lane 4, 0.5% EtOH, 2% glucose; Lane 5, 0.5% EtOH, 2% maltose; Lane 6, 2% glucose; Lane 7, 2% maltose. M= protein marker

To facilitate protein production, the coding sequence (without any pACT2 sequence) for cDNA10 was sub-cloned, in frame (confirmed by sequencing), into the expression vector pRSETC (see Appendix VI.1 for map). This vector had the advantage of a poly histidine region for affinity purification, an epitope sequence recognized by a readily available antibody, a ribosomal binding site, and an inducible promoter. The expected size of the fusion protein (including N-terminal tag) produced from pRSETC was estimated to be 10 kD.

IV.6.2 Purification of protein using HIS-BIND RESIN

Once the DNA sequence for cDNA 10 had been cloned into pRSETC, a large scale induction experiment, as described in Table 5, was undertaken with some alterations. For expression from pRSETC, *E. coli* BL21 was used as a host strain and in place of EtOH as an induction agent, IPTG was used. Cultures, typically 250 or 500 mL, were grown at

room temperature for 3-4 days prior to induction with IPTG. 50 mL aliquots were taken every hour for a minimum of 4 hours, and crude protein extracted as described in section III.10.2. Again no pattern of induction was observed (Figure 18), and an attempt to purify the recombinant protein from large scale crude extracts was undertaken. Crude extract was applied to the HIS-Bind resin as described in section III.10.3. In all cases, no recombinant protein was ever observed following SDS-PAGE analysis (Figure 19).

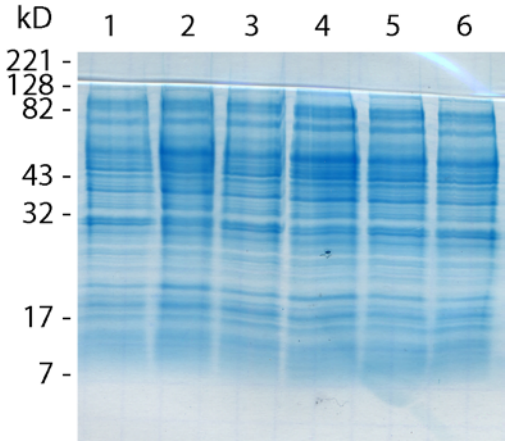


Figure 18: Over expression of cDNA Fusion Protein in Bacteria. IPTG was used to induce over expression of cDNA10 fusion protein in bacteria. *E. coli* BL21 was grown as described in methods. Lane 1) Sample harvested prior to addition of IPTG. Lanes 2-6) Samples harvested 1, 2, 3, 4, and 16 hours after the addition of IPTG. No pattern of induction was observed.

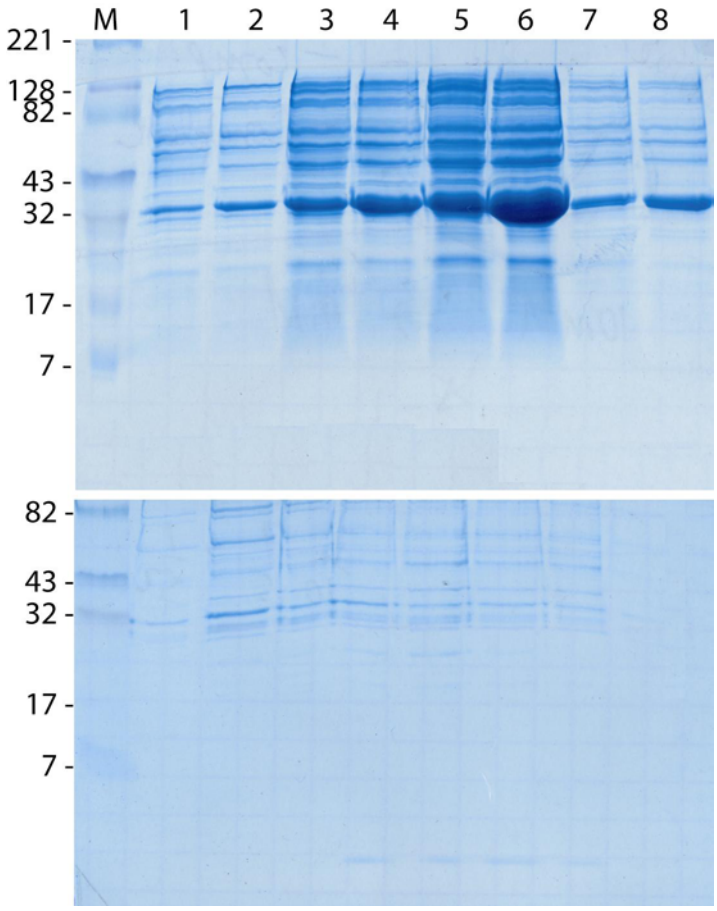


Figure 19: HIS-Bind Purification of cDNA10. Following incubation with HIS-BIND resin, the wash (upper panel) and elution (lower panel) fractions were collected and applied to a 15% SDS-acrylamide gel (10% stack). The expected size of the fusion protein is 10 kD.

In order to determine if recombinant protein was being produced from the pRSET vector, the induction experiment was repeated and replica SDS-PAGE analysis was carried out. One gel was stained immediately with coomasie blue to visualize protein bands, while the second gel was used in a western blot (see section III.10.5). The blot was analysed using the Anti-Xpress™ Antibody as a probe, an alkaline phosphatase conjugated secondary antibody and chromogenic detection,. As seen in Figure 20, there was no detectable recombinant protein present in any of the samples tested.

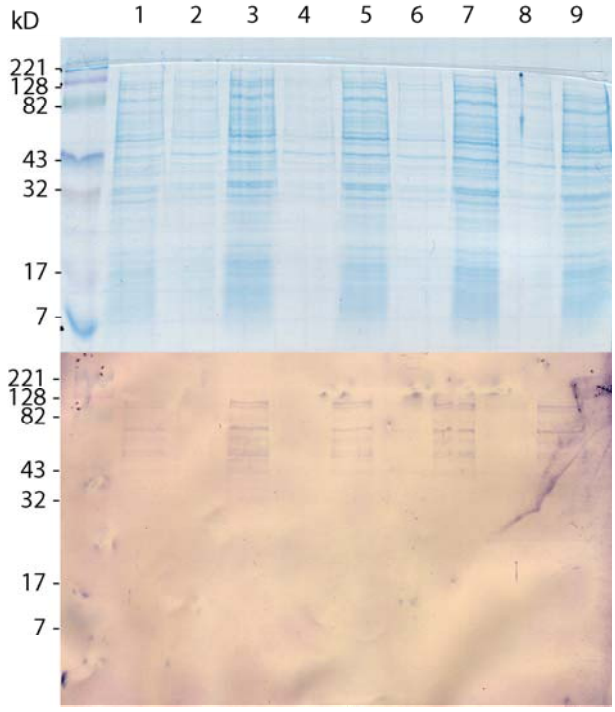


Figure 20: Detection of Recombinant Protein via Western blot. A small scale IPTG induction experiment was carried out over four hours. For each time point protein was twice extracted, i.e., protein was extracted from the initial bacterial pellet, the supernatant taken and a second extraction performed on the remaining pellet. Upper portion: Lanes 1, 2) Samples taken prior to addition of IPTG. Lanes 3, 4) Samples taken 1 hour after addition of IPTG. Lanes 5, 6) Samples taken after 2 hours. Lanes 7, 8) Samples taken after 3 hours. Lane 9) Initial supernatant of sample taken 4 hours after addition of IPTG. Lower portion: Corresponding western blot; no fusion protein is detected.

IV.6.3 Cell free protein synthesis

As a final attempt to produce pure recombinant protein, a cell free protein synthesis system was used. Using the Expressway™ Plus Expression System (Invitrogen, Carlsbad, USA) according to manufacturer's instructions, full length recombinant protein was produced from the sequence of cDNA 10 (Figure 21). Recombinant protein was detected using the Anti-Xpress™ antibody (alkaline phosphatase conjugate) and

colour development. The detected protein (lane 1) corresponds to the predicted size of the recombinant protein, as predicted by amino acid sequence.

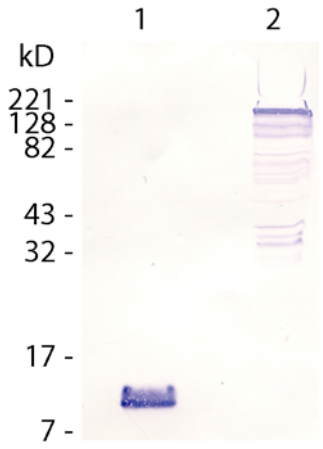


Figure 21: Cell Free Expression of cDNA10 fusion protein. The cell free transcription/translation reaction was performed as described in methods. Proteins were detected via Western blot, using the Anti-Xpress™ Antibody (alkaline phosphatase conjugate). Lane 1) cDNA10 fusion protein at the expected size of ~10 kD. Lane 2) LacZ fusion protein expressed from control vector.

IV.7 Effect of Brefeldin A on SUT1 Localization

BFA is a fungal toxin that rapidly blocks anterograde transport by inhibiting vesicle formation at the Golgi apparatus. (Fujiwara *et al.,* 1988; Satiat-Jeunemaitre *et al.,* 1996). In order to determine if the localization of SUT1 protein was affected by application of BFA, fresh sections were incubated in 50 or 100 μM BFA as described in section III.11. Incubation with BFA for as little as 10 minutes resulted in the localization of SUT1 being transferred from the SE to the CC. At lower BFA concentrations the localization is diffuse, while it become more closely associated with the CC nucleus at higher concentrations (Figure 22). While these results are preliminary, BFA does in fact appear to affect SUT1 localization. This effect is visible at concentrations of 50 μM and significantly increased at 100 μM as seen in Figure 22.

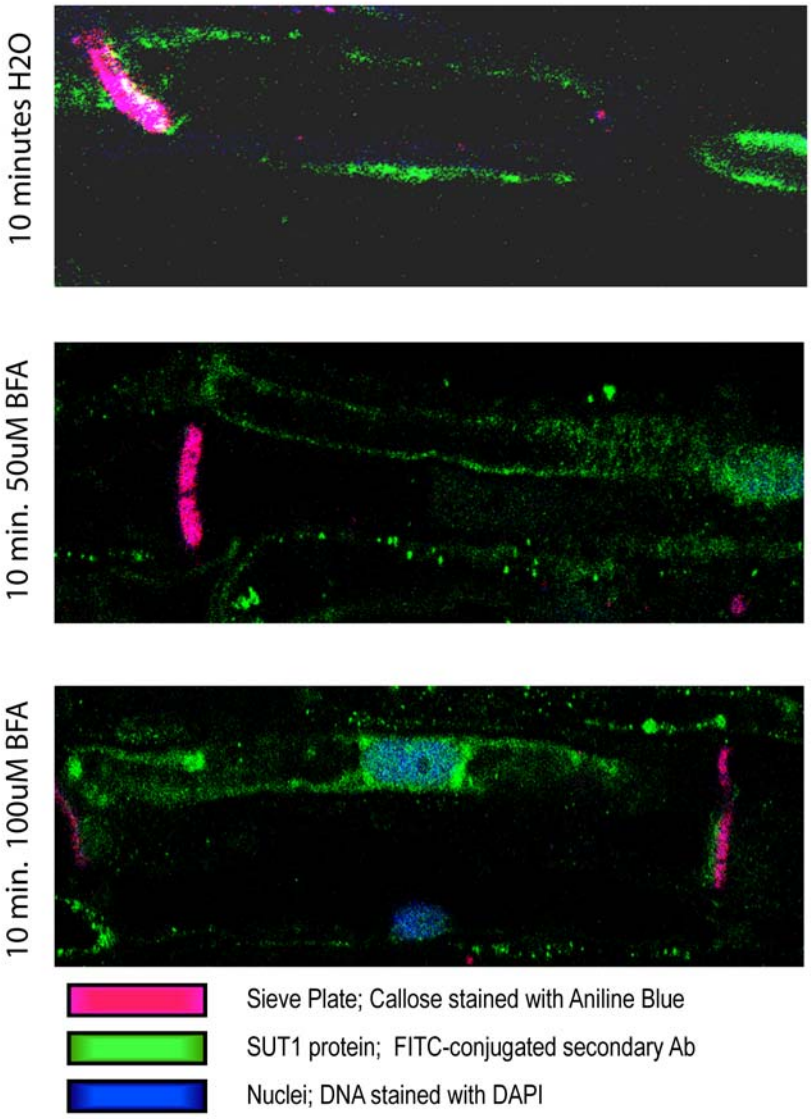


Figure 22: Effect of BFA on SUT1 Localization. Fresh sections of tobacco were prepared as described in section III.11. When incubated in ddH_2O (or left untreated, data not shown) SUT1 is localized to the SE, which is identified by the characteristic sieve plate (upper panel). Application of BFA rapidly sequesters SUT1 in nucleated adjacent to the SE and presumed to be CCs. This effect is seen at 50 μM (middle panel), and is significantly increased at 100 μM (lower panel).

V. Discussion

In recent years, the universality of biological systems has become increasingly clear. The genetic mechanisms in place in bacteria, fungi, and animals also exist in plants to a large extent. Thus the tools used to study these organisms are widely interchangeable. In the present study, an *in vivo* genetic screen was applied to solanaceous plants for the first time to investigate the mechanism of SUT localization from the CC to the SE. Currently, there are two models for this localization (i) mRNA localization with subsequent translation at or near the PD, or (ii) Classical anterograde transport of translated protein bound for export. While these two models are by no means mutually exclusive, this thesis deals mainly with the first model as a hypothesis for initial experimentation.

For use in the screening system discussed here, a high quality cDNA library from tomato leaves was created and used, yielding a putative *Le*SUT1 mRNA binding protein. This protein is able to bind to SUT1 mRNA *in vivo* and is unique at both the nucleic acid and protein level. The complete analysis of the nature of this protein will be crucial to the understanding of mRNA transport within and between plant cells.

V.1 RNA Protein Interactions

V.1.1 Traditional Assays for Identifying RNA-protein Interactions.

RNA-protein interactions are fundamental to many cellular processes including RNA localization. Interactions between RNA and proteins have traditionally been studied using extensive biochemical assays such as RNA band shifts, and RNA-protein cross-linking. While these are useful analytical tools, there are several disadvantages associated with them (Putz *et al.,* 1996). For instance, interacting proteins often exist in low abundance and are consequently difficult to detect. Furthermore, these biochemical techniques do not easily allow the identification of target RNAs recognized by a known RNA binding protein. Finally, these methods do not allow direct identification of the genes encoding the proteins or RNAs of interest (Putz *et al.,* 1996; Martin *et al.,* 2000).

V.1.2 Y3H: An *in vivo* Method

The yeast three-hybrid system, developed independently by Putz *et al* (1996) and SenGupta *et al* (1996), addresses many of these difficulties and provides an *in vivo* method of detecting RNA-protein interactions. This method relies on the physical, as opposed to the biological, properties of RNA and protein molecules allowing a wide variety of interactions to be detected and analysed (Zhang *et al.,* 1998). It has been used to successfully recover several RNA binding proteins that specifically interact with a known target RNA sequence, including histone mRNA binding proteins and protein regulators of mRNA translation (Martin *et al.,* 1997; Zhang *et al.,* 1997; Jan *et al.,* 1999). To date however, this method has been applied only to genes from the animal kingdom. In this system, the expression of selectable marker genes depends upon a specific RNA-protein interaction. It also takes advantage of a transactivator protein (*e.g.* Gal4p) whose DNA binding and activation domains are functionally independent allowing for separation of each domain with retention of activity (Chien *et al.,* 1991).

In the yeast three-hybrid system, the DNA binding domain (LexA BD in the case of the system used in this thesis) is fused to a known RNA binding protein (MS2-coat protein). This first protein hybrid is bound to the upstream activating sequence of the reporter genes (*HIS3* and *lacZ*) that will be transcribed in yeast in the presence of an activator. The second fusion protein consists of the Gal4 transcription activation domain fused to an unknown putative RNA binding protein (encoded by the cDNA library). The plasmid which houses the Gal4 activation domain allows cloning of the cDNA library such that a nuclear localization sequence (NLS) is introduced as part of the fusion protein. Thus these two fusion proteins can be brought into close proximity in the nucleus via interaction with the third hybrid molecule, the RNA hybrid. This molecule consists of an RNA (MS2) known to bind to the afore mentioned RNA binding protein (MS2 coat protein), and fused to the cloned RNA of interest. This initial complex of the first protein hybrid bound to the RNA hybrid acts in this case as "bait" for the second protein hybrid. The binding of a putative interacting protein ("prey") and the RNA of interest will reconstitute a fully functional trans-activating protein at the upstream activating sequence, allowing transcription of the reporter genes. The principle of the method is depicted in Figure 23.

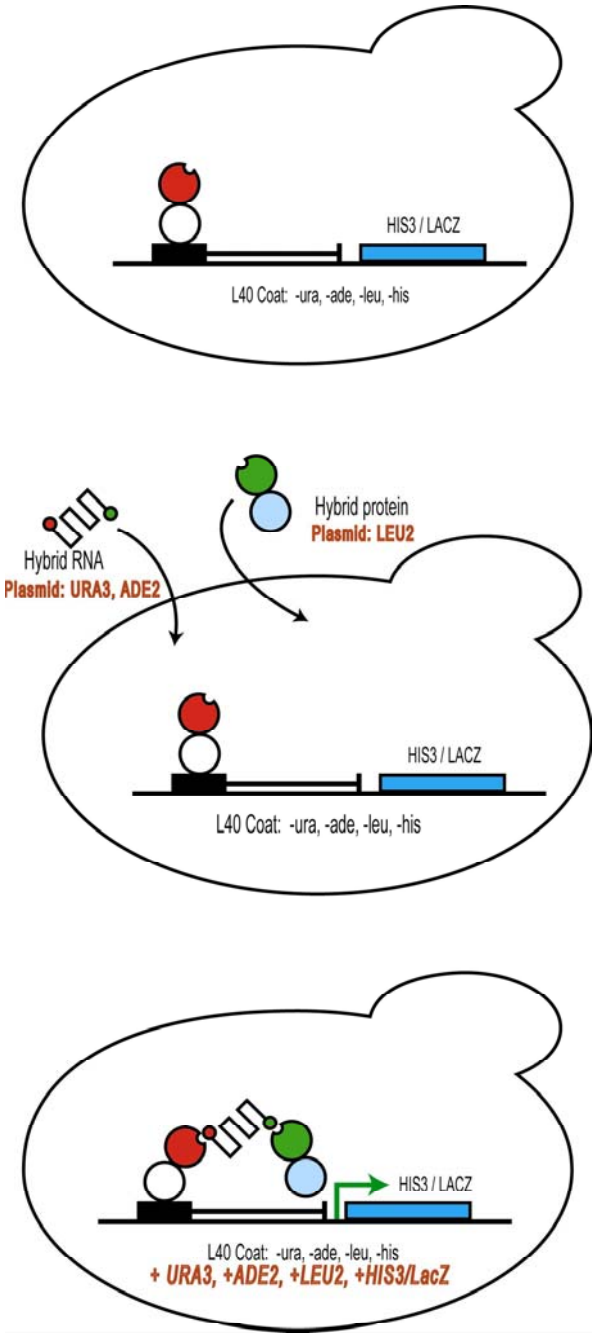


Figure 23: Yeast Three Hybrid Method. The upper portion depicts L40 coat prior to transformation. The LexA promoter region (black box) is bound by the LexA binding protein (white circle) as part of the first protein hybrid, preventing transcription of reporter genes (blue box). Auxotrophic markers are indicated. Following introduction of the RNA hybrid and the second protein hybrid (middle portion), the hybrid RNA is bound by the MS2 binding protein (red circle, first hybrid protein) and the putative RNA binding protein (green circle, second hybrid protein), tethering the Gal4 AD (blue circle) to the promoter region resulting in transcription (green arrow, bottom portion).

The expression of the reporter genes is monitored through several screening steps in order to confirm that all three-hybrid molecules are indeed interacting. The expression of *lacZ* was measured here by the *in vivo* visualization of colonies exposed to X-gal (See Figure 10). The expression of *HIS3* (which produces the His3p, imidazoleglycerol-phosphate dehydratase) confers the ability to grow on medium lacking histidine; the level of *HIS3* expression can be assessed by the inclusion of 3-AT in the selection medium. 3-AT is a competitive inhibitor of the *HIS3* gene product and therefore, cells containing more His3p can survive at higher concentrations of 3-AT. Thus the degree of resistance of the yeast reflects the *HIS3* expression level and consequently the strength of the interaction. Moreover, the stringency of the screening process can be adjusted to test the degree of interaction. In the screens undertaken in this work, 3-AT was included in the selection medium to a final concentration of 5 mM. This was based on an initial test experiment using varied concentrations of 3-AT. Yeast was transformed with the appropriate plasmids and spread onto selective medium supplemented with 1, 2, 5, 10, 15, 20, or 25 mM 3AT (data not shown). Based on colony number and appearance, the concentration of 5 mM was chosen as the most appropriate for yielding a wide range of both high and low strength interactions. Furthermore, the interaction between cDNA 10 and RNA-5 appears to be quite strong since the yeast is able to grow on selective media containing concentrations of 3-AT as high as 25 mM (data not shown).

cDNA10 was still able to complement histidine auxotrophy in L40 coat transformed with RNA-3 when grown on media containing 5 mM 3-AT. The colonies turned pink, however, when grown on 10 mM 3-AT, suggesting the loss of the RNA plasmid under these conditions and a relatively weak interaction. This observation argues strongly in favour of a stable interaction between a particular region of the 3'UTR of *LeSUT1* (see boxed region in figure 3 and RNA-5 in figure 5) and cDNA10; the same concentration of 3-AT (25 mM) was used to recover the protein SLBP1 that specifically interacts with histone SL RNA (Martin *et al.*, 1997). The weak interaction between cDNA10 and RNA-3 suggests that sequence, as well as secondary structure, may play a role in the recognition of a putative RNA binding protein; the sequence of RNA-3 and RNA-5 overlap to a degree as seen in appendix VI.2, while the secondary structures are quite different (Figure 5).

There are, however a number of limitations associated with this method of detecting RNA-protein interactions. Due to the chimeric nature of the RNA and protein molecules involved, the folding or accessibility of the bait or prey might be affected by the other moiety of the molecule (Jaeger, *et al.*, 2004). Another disadvantage is that many proteins require cofactors to bind to their RNA partner and these cofactors may not be present in the yeast nucleus, precluding interaction with the RNA in question. Furthermore, some proteins bind to their target RNA with a low affinity or in a transient manner, thus the three-hybrid system may miss such an interaction due to a lack of sensitivity. Indeed, the inclusion of 3-AT in the medium guarantees that the screening will be relatively stringent; the loss of observed transient interactions is the price paid for a reduction in background noise. Finally, despite the advantage of observing an *in vivo* interaction, the very nature of the method means that the interaction takes place in an environment influenced by many cellular parameters. This is likely to be one of the primary causes of the false-positive interactions that occur. The screening steps outlined in this thesis were used to eliminate these clones.

Despite a number of disadvantages, the Y3H method remains a viable tool for analysing RNA-protein interactions. It has been widely used to select unknown proteins and RNAs and also for studying RNA-protein interactions of previously assumed interactions (Jaeger *et al.*, 2004). The method has been used successfully to identify protein partners for RNAs ranging in size from 21-1600 nt in length (Rho *et al.*, 2000; 2002). The protein interactors themselves have varied in size from 70 – 963 amino acids long. The short length of cDNA10 (~20 aa) may thus correspond to a new class of small interacting partners, but more likely it represents a protein domain. The initial results from the 3' and 5' RACE reactions suggests that cDNA10 isolated from the Y3H screen is actually part of a larger sequence (Figure 15). These results are, however quite preliminary and must be confirmed via Southern blot to ensure that sequence corresponding to cDNA10 is actually a product of the RACE reactions. To date, the sequence of cDNA10 appears to be unique at both the protein and DNA level. This may represent a new class of plant mRNA binding protein and may also be the first step in identifying the mechanism of SUT1 mRNA localization. It is important however, to

keep the limitations of the three-hybrid system in mind; the interactions presented here must be further complemented with other tests including traditional functional assays, *in vitro* binding studies and immunoprecipitation. This would show conclusively the *in vivo* relationship between SUT1 mRNA and cDNA10.

V.2 A Novel Protein Interacts with *LeSUT1* mRNA

V.2.1 Common Features of RNA Binding Proteins

The sub-cellular localization of mRNA has proven to be a highly conserved mechanism for the spatial restriction of protein synthesis. This level of posttranscriptional control is thus able to regulate the distribution of proteins within the cell (reviewed in Bashirullah *et al.,* 1998). In some cases, translational control is coupled to RNA localization, preventing premature or even ectopic protein synthesis. The *cis*-acting sequences present in RNA molecules are varied and have been identified in numerous localized mRNAs; these sequences occur primarily in the 3'UTR (Bashirulla *et al.,* 1998). As would be expected, the array of proteins (*trans*-acting factors) that are able to bind these sequences is also varied; these proteins do, however have a number of conserved characteristics. Some of these characteristics include zinc fingers, zinc knuckles, RGG (arginine rich) boxes and DEAD boxes, but these particular features alone cannot be used to exclusively predict RNA-binding function (Lorkovic and Barta, 2002).

Many of the protein motifs involved in RNA binding were discovered through studies of the heterogeneous nuclear ribonuclear proteins (hnRNPs) from human cells (Burd and Dreyfuss, 1994; Swanson, 1995). hnRNPs were first described (Dreyfeuss *et al.,* 1993; Swanson, 1995) as a large family of chromatin associated RNA-binding proteins but later investigations have shown a wide variety of roles in mammalian cells (reviewed in Krecic and Swanson, 1999). The most common motif is the consensus sequence RNA-binding domain (CS-RBD), also referred to as the RNA recognition motif (RRM). The RRM contains two short consensus sequences, the RNP1 octamer and the RNP2 hexamer. These sequences, which are present not only in hnRNPs, but also in a large number of other RNA-binding proteins, occur in a structurally, but not sequence conserved region of approximately 80 amino acids (Burd and Dreyfuss, 1994; Swanson,

1995). The second most common protein domain (Burd and Dreyfuss, 1994) is the K homology (KH) motif, which was first identified in the human hnRNP K protein (Siomi *et al.*, 1993). The KH domain is approximately 60 amino acids long with a characteristic arrangement of hydrophobic residues and a conserved consensus sequence, VIGXXGXXI, mapping to the middle of the domain (Burd and Dreyfuss, 1994). Both the RRM and the KH domains appear to be highly conserved protein structures as they have been found in a number of organisms ranging from bacteria to humans (Burd and Dreyfuss, 1994). These motifs are both necessary and sufficient for the binding of the protein in which they occur to the target RNA. Many of the proteins are involved in RNA processing in the nucleus, but also in RNA localization (Bashirulla *et al.*, 1998).

In plants, there exist a large number of RNA-binding proteins, as defined by the presence of conserved sequences (Albà and Pagès, 1998; Lorkovic and Barta, 2002). The actual function of these proteins is however, largely unknown. The plant RNA binding proteins identified to date all contain an RRM as well as a number of auxiliary motifs (mentioned above). In *Arabidopsis* alone, there are 196 RRM containing proteins encoded by the genome (Lokovic and Barta, 2002). The elucidation of specific functional data for these and other plant RNA-binding proteins will contribute greatly to the understanding of the processes underlying not only RNA localization, but also RNA stability and processing.

V.2.2 cDNA10 is a Unique, Putative *LeSUT1* mRNA Binding Protein

The yeast three hybrid screening process described in this work has yielded a unique protein that has the ability to bind *LeSUT1* RNA *in vivo*. This was demonstrated by the ability to reconstitute a functional transcriptional activator by binding SUT1 mRNA tethered to the promoter region of two reporter genes. Furthermore, when the isolated cDNA was used to transform the yeast strain L40 coat (containing the either hybrid RNA-3 or -5) it was able to fully complement histidine auxotrophy. All other cDNAs isolated in similar screens were unable to complement auxotrophy upon reintroduction into L40 coat.

A number of methods were undertaken in order to establish the identity of the isolated clone. The DNA and protein sequences were used as the query sequences against several databases of genetic information. These searches yielded no significant homologies to any of the sequences in the compiled databases. One likely reason for this is the relatively short length of the cDNA sequence. While there are many small proteins that exist in the cell, the complete lack of homology to any known proteins suggests two possibilities. First, the protein encoded by cDNA 10 is a completely new protein, with no as yet discovered relatives in any organism, or second, this protein is actually a fragment of a larger protein. The latter proposition is much more likely to be the case. The RNA sequence may have been truncated during the any of the steps required for the construction of the cDNA library, but most particularly during the fractionation of the cDNA. This is one of the many pitfalls associated with this procedure; during which cDNA may be lost. A second indication that the isolated clone is not actually full length is the preliminary results obtained from 5' and 3' RACE reactions as well as northern analysis. In both cases there is the indication that a larger transcript exists. There was, however a high degree of background in all cases and a Southern analysis is required to confirm the presence of the isolated cDNA sequence. That said, a truncated cDNA clone is still useful in that it may represent a particular protein motif that is required for the binding of RNA.

The conserved sequences of RNA binding proteins are often sufficient and necessary for the binding of RNA. Of particular interest is the RNP2 (a sub element of RRMs) consensus sequence found as part of the RRM in the plant glycine-rich RNA-binding proteins (a subclass of RRM-containing proteins whose function is largely unknown, Lorkovic and Barta, 2002). This consensus sequence is BxFJGGJ, where B= H, K, R and J=I, L, M, V (Lorkovic and Barta, 2002); thus the sequence could read KxFIGGL, or HxFIGGI for example. This last potential sequence is not that dissimilar from the sequence HEGGIL, which occurs in the first portion of the protein fragment encoded by cDNA10 (see Figure 14). This short motif could be responsible for the interaction observed via the Y3H screening system. While it is not identical to the consensus sequence, it retains similar elements and could exhibit a similar mechanism of RNA binding activity. The isolation of the full sequence of this clone will be crucial to

confirming the physiological relevance of cDNA10. It must be noted, however, that the interaction of this protein with the mRNA of SUT1 does not necessarily imply that it is in fact involved in RNA localization. There are a number of reasons why protein should be bound to a nascent RNA chain and these include, polyadenylation, export from the nucleus, translational repression and stabilization. Given, however, the nature of the SUT1 sequences chosen and the resultant RNA secondary structures, it is tempting to speculate upon the role this cDNA might play in the localization of SUT1 mRNA. This cDNA might be involved in the recruitment of other localization factors or even binding to cytoskeletal elements. This can only be demonstrated by determining whether cDNA10 is also able to interact with other proteins. The majority of identified RNA-binding proteins are also able to interact with numerous cofactors and often assemble into ribonucleoprotein particles as described in section I. An in-depth study of the full-length protein, including determination of the protein interactors, will be essential to distinguishing the extent to which this protein interacts with SUT1 mRNA. To this end, the yeast three hybrid system has also been used to dissect some RNP complexes (Sonoda and Wharton, 1999; 2001); thus a clearer concept of the events surrounding RNA processing and localization is beginning to emerge.

V.3 An Emerging Model of *LeSUT1* mRNA Localization

V.3.1 *LeSUT1* mRNA Interacts with cDNA10

While the exact nature of the interaction between *LeSUT1* mRNA and cDNA10 has yet to be determined, the fact that the interaction exists is of much interest. Using the *in vivo* yeast three-hybrid method for detecting RNA-protein interactions, I was able to demonstrate that a highly determined (in terms of RNA secondary structure) portion of the 3'UTR of *LeSUT1* mRNA is bound by a unique cDNA encoded protein. This specific isolate was able to activate two yeast reporter genes in a manner dependent upon the presence of SUT1 mRNA "bait". Through the various rounds of screening to eliminate false positive interactions, this clone demonstrated a clear ability to interact with the hybrid RNA. Furthermore, the reintroduction of the isolated clone into the yeast strain L40 coat separated it from other clones isolated in the same round of screening; only cDNA10 was confirmed at this point as being able to interact with the RNA. These

results point strongly to a specific interaction between these two molecules. A critical step in affirming this hypothesis, however, will be to perform *in vitro* assays. One of the most widely used assays for studying RNA-protein interactions *in vitro* is the electrophoretic mobility shift assay (EMSA). In this assay, the RNA of interest is labelled, either with radioactivity, or fluorescence and subsequently incubated with purified protein extract. This mixture is then assessed using standard gel electrophoresis techniques and the extent of migration of the RNA is determined (either by exposure of the radioactive samples to film, or excitation of the fluorescent molecule). Based on its size, the RNA will migrate a particular distance in the gel; if the protein binds to the RNA, the RNA will be retarded in the gel due to the larger size of the complex and the corresponding band will not migrate to the same distance as the RNA alone. Combined with results from yeast three-hybrid analysis, this method has been used to demonstrate conclusively, the RNA-binding ability of a number of proteins (Long *et al.*,2001; Surjit *et al.*, 2004).

One of the prerequisites for the use of the EMSA method, is the purification (or at minimum, over expression) of the protein of interest. To this end, numerous attempts were undertaken in order to induce maximal expression of cDNA10 in both bacteria and yeast (see section IV.6). In the case of yeast expression, the expression of cDNA10 was under the control of the constitutive ADH promoter (see Appendix A1 for vector maps). Despite varying conditions of induction, no indication of over expression was ever observed on coomasie stained gels. This is possibly due to the small size of the expected fusion protein; detection via dye staining may not be the most appropriate method to detect a small protein from a crude extract. Thus the next step was to attempt purification of the fusion protein from yeast. As can be seen from the vector map, the fusion protein contains an HA (hemagluttinin) tag. Crude protein extracts from yeast were applied to a column packed with Anti-HA conjugated to agarose. Following incubation and elution, there still appeared no detectable amounts of over expressed protein. This may have been due to the relatively harsh elution conditions (see section III.10.2), which are liable to render the antibody, conjugate inactive. More likely, however is that the fusion protein was simply not being produced to detectable levels. Furthermore, since the ADH promoter did not seem readily inducible, the entire

sequence of cDNA10 was sub-cloned (in frame) into the vector pRSETC under the control of the inducible T7 promoter. Similar induction experiments were undertaken, with similar results. No protein was detectable on coomasie stained gels under any of the conditions tested; again the small size of the protein may be a factor since the fusion protein created from the pRSETC plasmid was predicted to be shorter than the one produced from the plasmid pACT2. Purification of the fusion protein was again attempted, taking advantage of the presence of a poly histidine tag incorporated into the protein fusion. This his-tag permits the purification of fusion proteins via metal chelation chromatography as described in section III. As seen with the anti-HA purification method, no detectable amounts of purified protein were recovered (confirmed via western blot using Anti-Xpress antibody, Figure 18). This lead to the speculation that there was an error in the sequence or the reading frame, but this proved not to be the case. DNA was isolated from bacteria transformed with cDNA10 in pRSETC and analysed both via restriction digest and sequencing. In both cases, the in frame sequence of cDNA10 was found (data not shown).

The results of the three-hybrid screening, however, show that the protein must indeed be present in the yeast cells. Moreover, the reintroduction of the isolated cDNA confirmed the interaction between the new protein and the SUT1 mRNA. Thus the inability to express the protein in bacteria may be caused by an unforeseen cellular event. It is possible that the short RNA encoded by the isolated cDNA is somehow unstable and is degraded before it can be fully translated. The difficulties encountered up to this point, thus lead to the consideration of new methods of over expression.

The Expressway™ Plus Expression System, designed for cell-free, T7 based *in vitro* transcription and translation of target DNA into protein was chosen as the most suitable method to overcome the challenges associated with over expression of cDNA10. This system uses extract from an *E. coli* strain to drive the transcription/translation reaction. As seen in Figure 21 (section IV.6.3), the use of this method finally yielded the desired result. Full length fusion protein was produced in high amounts, as detected by western blot. Production of fusion protein is the crucial first step required prior to undertaking the RNA EMSA. Purification of this fusion protein from this crude extract does,

however remain an issue. While a pure protein extract is desirable, this may not be a necessary prerequisite for the EMSA assay, and preliminary experiments using the crude extract are a natural next step in the *in vitro* characterization of this interaction.

V.3.2 SUT1 Protein Localization: An Alternate Hypothesis

Up to this point, I have only dealt with the localization of SUT1 mRNA, which is the basis of this work; but what of the localization of SUT1 protein? In 1997, Kühn *et al* demonstrated convincingly both the localization of SUT1 protein to the plasma membrane of the enucleate SE as well as the localization of SUT1 mRNA near the PD joining SE and CC. Prior to these data, the CC was implicated as the site of *SUT1* transcription (Kühn *et al.,* 1996). The question remains, how does the highly hydrophobic SUT1 protein arrive in the plasma membrane of the SE? The cell is a very ordered environment with all proteins and nucleic acids having a defined purpose and destination. After translocation into the endoplasmic reticulum (ER), most proteins destined for secretion must pass through the Golgi apparatus before reaching the exterior of the cell. Integral membrane proteins, especially, are modified post translationally and protected from the aqueous environment of the cytoplasm until their insertion in their destined membrane. They are transported in an anterograde manner (*i.e.,* from ER to Golgi to membrane) in coatomer protein (COPII) coated vesicles (Barlowe *et al.,* 1994; Rothman and Wieland, 1996; Robinson *et al.,* 1998). If this fundamental tenet of cell biology holds true, then it is likely that some portion of *SUT1* mRNA is translated into protein in the CC and undergoes the routine pathway of anterograde transport. If this is the case, then the transport of SUT1 protein to the SE should be affected by treatment with brefeldin A (BFA).

BFA is a fungal toxin that has widely been used to block protein excretion in a variety of eukaryotic cells (Fujiwara *et al.,* 1988; Satiat-Jeunemaitre *et al.,* 1996). BFA rapidly blocks anterograde transport by inhibiting vesicle formation at the Golgi apparatus. If SUT1 protein is actually translated in the companion cell prior to transport to the SE, then this process should be prevented upon exposure to BFA. To test this hypothesis, wild type tobacco plants were chosen as a model solanaceous plant instead of tomato. This was done because there was no functional antibody against SUT1 available; the same is true

for tobacco, but the antibody against potato SUT1 cross reacts strongly with tobacco SUT1 due to the high degree of similarity. Furthermore, tobacco develops relatively large leaves which improves the microscopic analysis of the phloem. 2-3 cm squares were harvested from the fresh leaves of 8 week old tobacco plants and incubated for varying times in water, BFA (50 or 100 μM), or without treatment. Following incubation, fixation, and agarose embedding, sections were incubated with SUT1 primary and CY-3 or FITC conjugated secondary antibodies. Sections were subsequently stained to reveal nuclei and sieve plates and viewed with a confocal scanning laser microscope. While these are preliminary results, BFA does appear to affect the localization of SUT1 (Figure 22, section IV.7). Incubation with BFA seems to sequester SUT1 in the CC, while control sections showed localization in the SE. These results must however be repeated to confirm initial observations and rule out artefacts. If, however, SUT1 is translated in the CC and transported to the SE via the anterograde pathway, it is clear that two distinct localization events are taking place; SUT1 mRNA is targeted to the PD joining SE and CC, while SUT1 protein is targeted to the plasma membrane of the SE. Thus the question is, are these two events mutually exclusive?

V.3.3 SUT1 in the SE: A Convergence of Hypotheses

In the work presented by Kühn *et al* in 1997, the full localization pattern of SUT1 in solanaceous plants was demonstrated. With these results, new questions were raised concerning the role of RNA localization in plant cells, the ability of plasmodesmata to regulate passage, and the mechanism of nutrient partitioning in plants. The localization of SUT1 protein to the plasma membrane of the SE was relatively easy to reconcile; the protein was likely to be produced in the CC and transported to the SE for insertion into the membrane. The discovery of SUT1 mRNA within the SE, however, has caused a rethinking of this simple paradigm. Why is the mRNA in SE? Why is it preferentially localized to the PD joining SE and CC?

As discussed earlier, there is clearly a mechanism for RNA transport via plasmodesmata; the study of plant RNA viruses suggests the presence of a high-jacked endogenous pathway (Heinlein, 2002). The clustered localization pattern of SUT1 mRNA around the PD (Kühn *et al.*,1997) suggests that a targeted concentration of these

molecules must occur; a random leaking of RNA between cells would be unlikely to produce such a pattern. But what is the purpose of specifically targeting SUT1 mRNA to the SE, or at the very least to the PD? Unfortunately, there is insufficient data in this field to go beyond pure speculation at this point. Perhaps, SUT1 mRNA associates in a RNP with specified cofactors in the CC. The formation of this RNP may be dependent upon the presence of SUT1 mRNA. This entire complex would then function in a similar manner to the movement proteins of plant RNA viruses and specifically target the PD for an increase in the SEL. This action could be separate, but related to the translation of SUT1 in the CC. This could be achieved by having different pools of SUT1 mRNA in the CC. For example, one pool could be targeted to the PD via association with a particular set of chaperones, while another set could be destined for immediate translation and export via the anterograde pathway. The difficulty with this hypothesis is explaining how the cell could distinguish the two pools of mRNA. Perhaps the answer lies with the initial synthesis of the mRNA. SUT1 is known to have three different polyadenylation states (Lalonde *et al.*, 2003) resulting in three transcripts with the same coding region, but 3'UTRs of varying length. This is an interesting point, since differing polyadenylation has been linked to developmental control of translational timing (reviewed in Ewalds-Gilbert *et al.*, 1997). A scenario might exist where the three different transcripts are synthesized, and depending upon the length (or even structure) of the UTR, interact with a different subset of interacting proteins (Figure 24). One subset of proteins might immediately direct the mRNA to the PD, another target it to the ER and Golgi, while the third may signal movement to distant sites via the phloem translocation stream. If this were the case, it might finally reconcile the seemingly disparate results observed thus far. The mRNA pool targeted to the PD might act as a

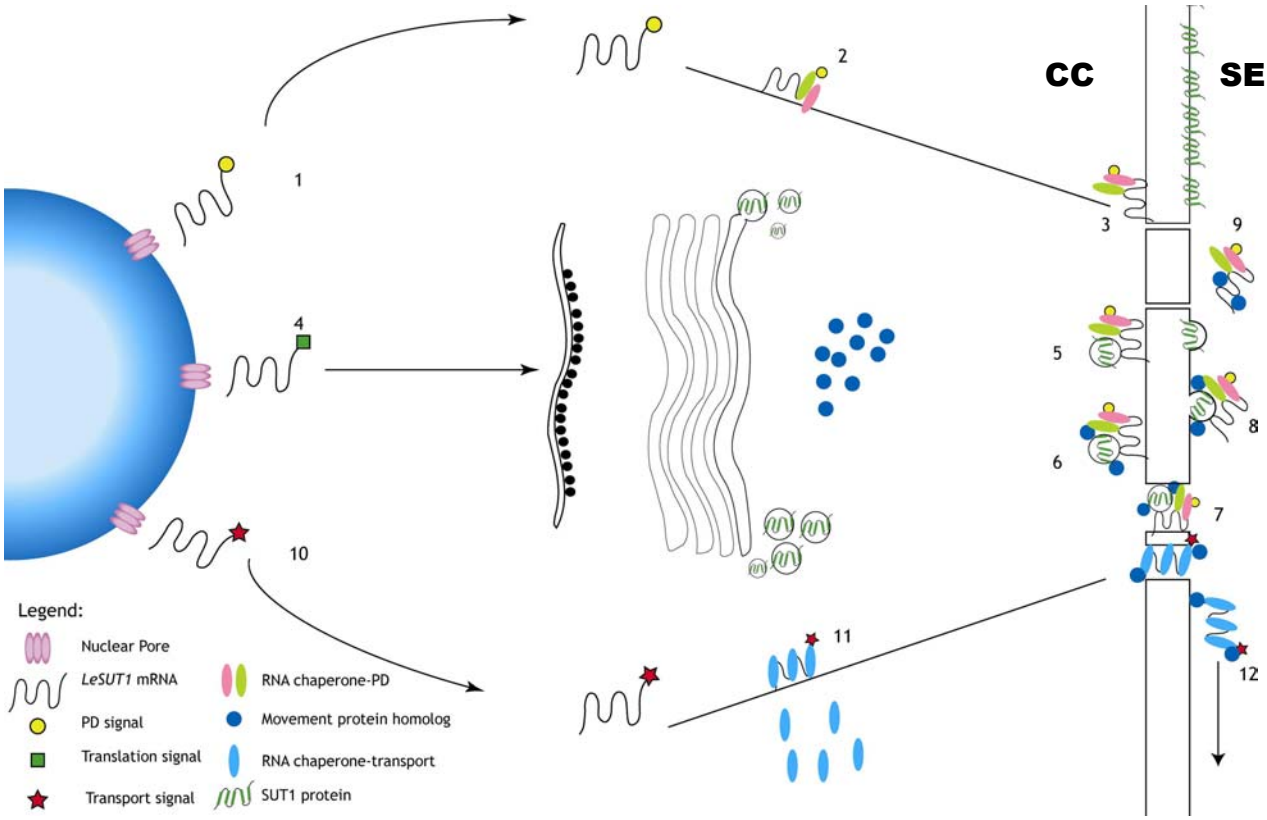


Figure 24: Localization of SUT1 protein and mRNA. Both SUT1 mRNA and protein have been localized to the SE. These localization events may be separate, but not necessarily mutually exclusive. Three different transcripts are synthesized in the CC, differing in length of 3' UTR (1, 4, 10), possibly containing different localization signals. One transcript is targeted to the PD via interaction with specific chaperones and the cytoskeletal machinery (2). This complex reaches the PD, but is unable to change the SEL (3), acting as a "gatekeeper". A second transcript (4) is destined for immediate translation and export in Golgi vesicles; these vesicles may interact with the previously assembled RNA-protein complex (5), allowing the recruitment of plant movement protein homologs (6). This functional complex is able to alter the SEL and the entire complex passes through the PD (7) to the SE. The vesicle is then able to fuse with the SE plasma membrane allowing insertion of SUT1 protein (8). The RNA protein complex is recovered and recycled (9). The last transcript (10) is destined for long distance transport and interacts with a specific set of chaperones (11); this complex is directly able to recruit movement protein homologs, and passes into the phloem translocation stream (12) for transport to distant sites within the plant.

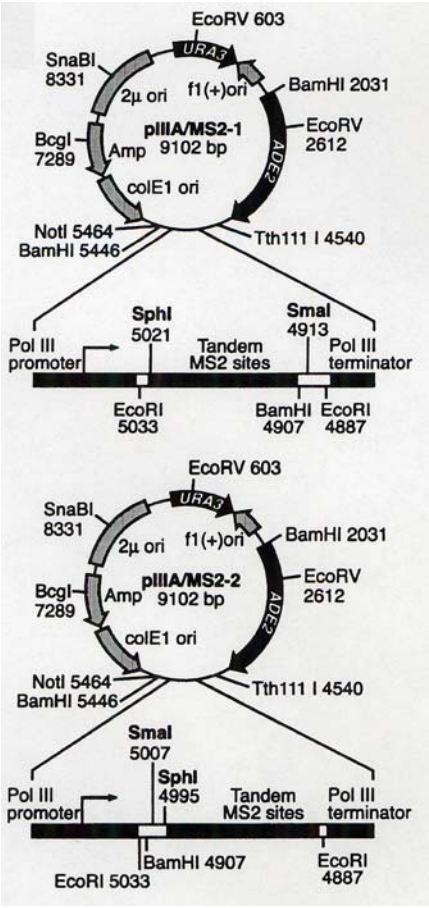
sort of "gate keeper", increasing the SEL of the PD only when a vesicle containing SUT1 protein arrives at the PD. This could involve the specific recruitment of an endogenous movement protein-like chaperone like CmPP16, discovered in cucurbits (Xoconostle-Cázares *et al.,* 1999). The two complexes may then pass to the SE where the vesicle would fuse with the PM and the coat components would then presumably be redirected to the CC. The "gate keeper" complex might then also be recruited back to the CC side to await further action. This is of course, highly speculative and extensive work must be done in order to conclusively describe the events taking place during localization of SUT1 to the SE. Such a system must surely rely upon cytoskeletal elements in the cell and it will be interesting in the future to determine what interactions SUT1 might have with these proteins. Furthermore, a continued study of the proteins that interact both with SUT1 protein and mRNA will be essential to the understanding of this complex system.

In this work, I have presented one of the initial steps to elucidating the mechanism of SUT1 mRNA localization in solanaceous plants. A unique protein has been isolated and shown to interact *in vivo* with SUT1 mRNA in a specific manner. Further biochemical tests will be required to corroborate this finding and localization experiments will be necessary to confirm a physiological role for this protein. The isolation and characterization of the full-length sequence of this cDNA clone, however, is essential for a better understanding of its role in the cell. It will also be interesting to determine what protein partners interact with cDNA10; cytoskeletal elements, PD associated proteins, or even ribosomal proteins. As a long-term goal, it will be intriguing to observe this interaction in plants via the generation of GFP reporter genes, for example. The refining of the BFA experiments will also be a valuable tool for dissecting this complex question, perhaps lending credence to the concept of two separate but related pathways involved in the localization of SUT1 to the plasma membrane of the sieve element. These steps will contribute greatly to the molecular characterization of the events surrounding nutrient allocation.

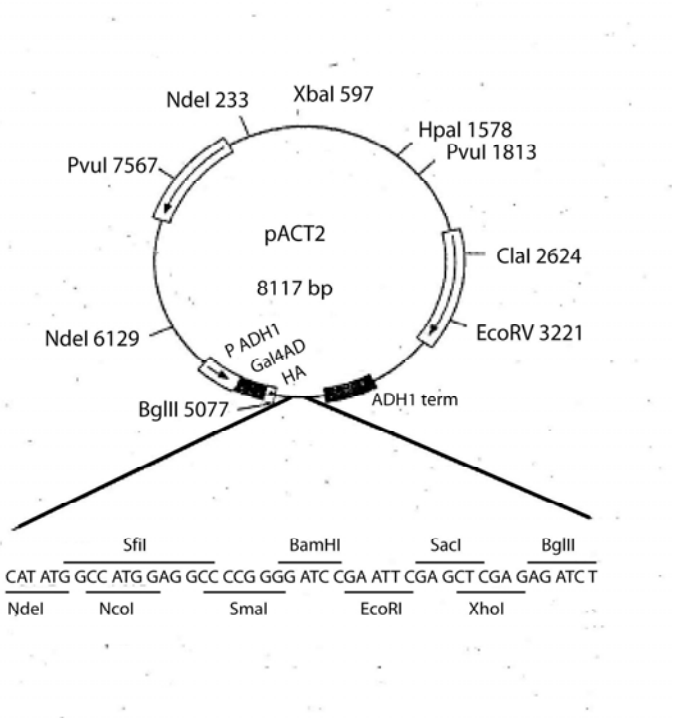
VI. Appendices

VI.1 Vector Maps

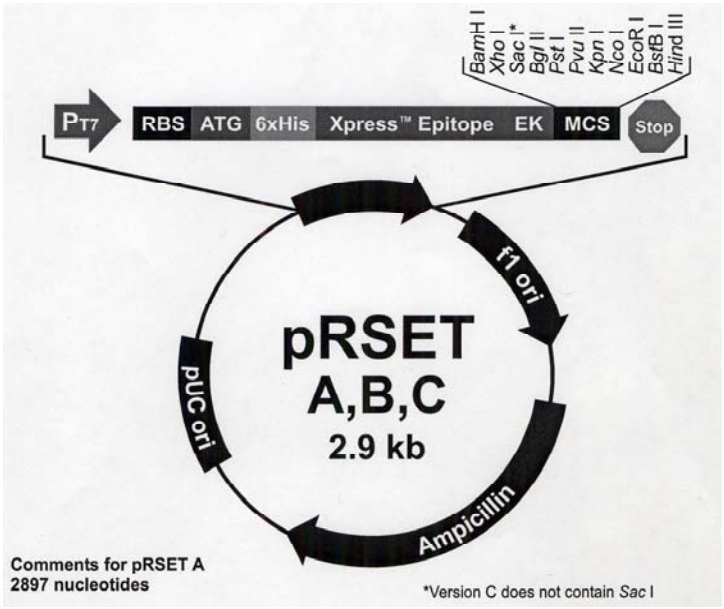
VI.1.1 Vectors for Cloning RNA Hybrid Molecules



VI.1.2 pACT2 for cDNA Cloning

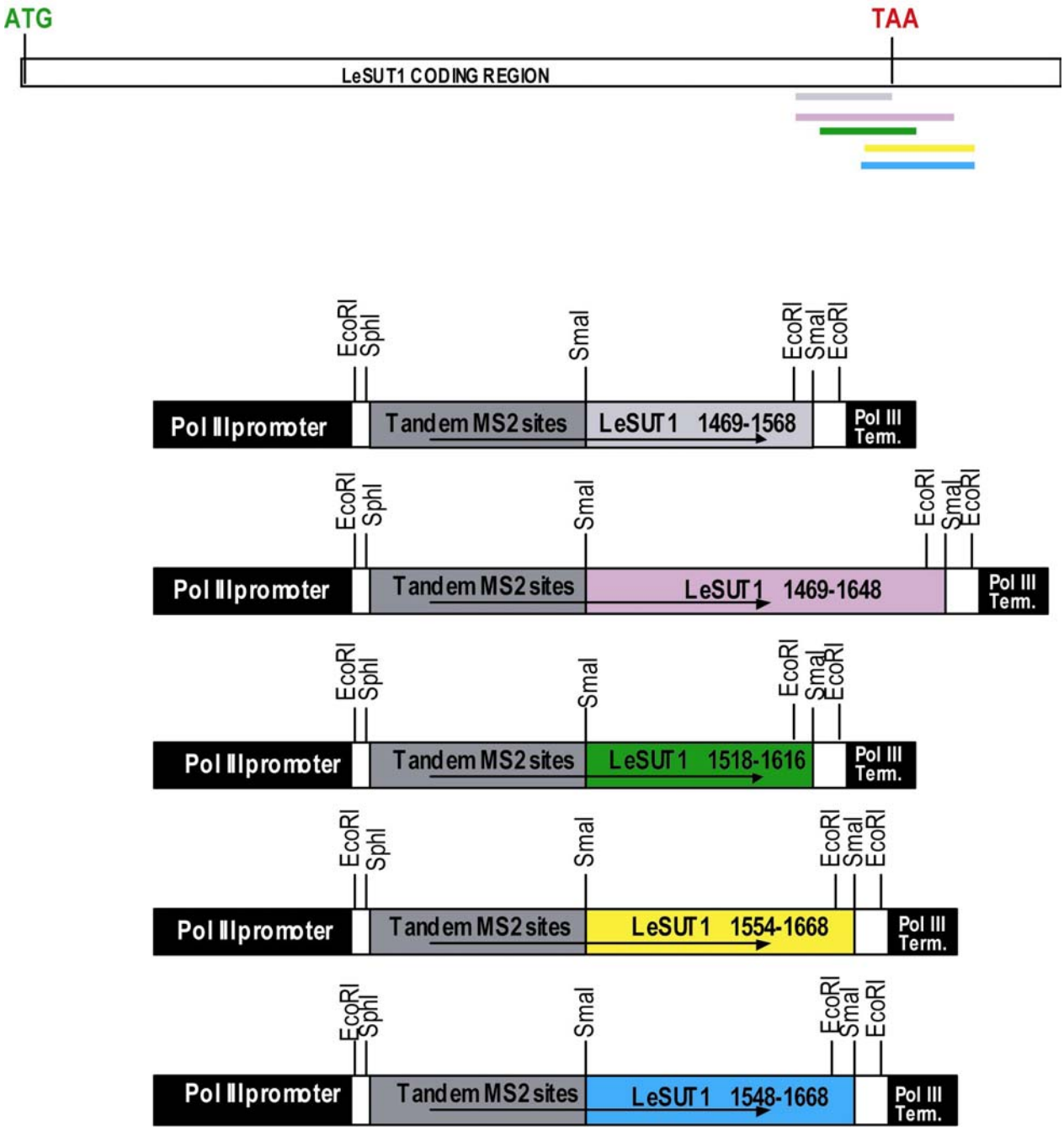


VI.1.3 pRSETC for Protein Expression



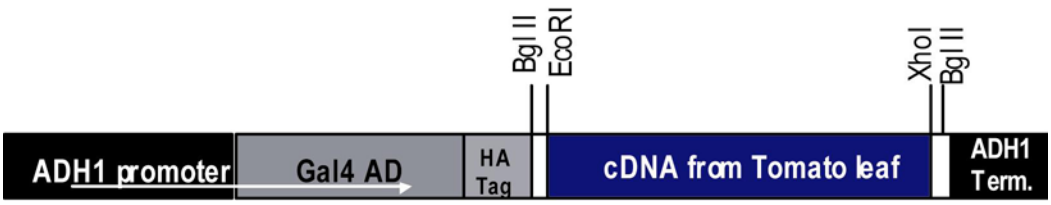
VI.2 Cloning Strategy

VI.2.1 Cloning of RNA Hybrid molecules



Various regions of SUT1 mRNA were sub-cloned into the vectors pIIIA-MS2-1/-2 for use in the Y3H screens. The design of the clones for introduction into pIIIA-MS2-1 is shown here; regions of SUT1 were amplified with gene specific primers with the addition of an *EcoRI* site at the 3' end to facilitate determination of orientation

VI.2.2 Cloning of cDNA Library



Synthesized cDNA was engineered with an 5' *EcoRI* site and a 3' *XhoI* site for efficient cloning into the vector pACT2.

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IX. Curiculum Vitae

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