Chapter 1

Literature Review

1.1. INTRODUCTION

Plants are autotrophs; they synthesize complex molecules by reducing carbon, nitrogen and sulphur from simple molecules, and have also evolved tissues that specialize in production, utilization and storage of their product.

In plants, sucrose (glucose + fructose) is the major translocatable product of photosynthesis and is simultaneously a source of carbon skeleton and energy for plant organs unable to photosynthesize. The selection of sucrose as a major transport sugar in plants has been related to its metabolically inactive nature (i.e. it's non-reducing) thereby making it a perfect compound for long distance transport or long-term storage without the problem of metabolism easily encountered with glucose (Sauer, 2007). Sucrose is synthesized by the action of sucrose 6-phosphate synthase (SPS; EC 2.3.1.14) which transfers the glucosyl moiety from UDP-glucose to fructose-6-phosphate to produce sucrose-6-phosphate which is in turn dephosphorylated by a sucrose-6-phosphate phosphatase (SPP; EC 3.1.3.00) yielding sucrose as the final product.

Plant organs are generally divided into two categories: "sources and sinks". A source organ such as mature green leaves export photoassimilates, while sink organs such as roots and flowers import photoassimilates. In plants, phloem tissues allow long-distance transport of photoassimilates from source to sink (Sauer, 2007). In sink tissues, sucrose may be used directly for metabolism or may be temporarily stored for use at a later stage of the plant's development.

In plants, sucrose is transported actively over a long distance in the phloem sap and this flow occurs in a specialized network of cells called the sieve elements. Sieve elements lose their nucleus and many organelles during differentiation, but stay connected to companion cells (cells with high metabolic activity). Sieve elements are linked together to form sieve tubes that offer very little resistance to sap flow (Lemoine, 2000). In most crop plants the sieve element/companion cell complex (SE-CC) is symplasmically isolated from the surrounding cells (Gottwarld, et al., 2000). The high solute concentration in phloem sap (i.e. sucrose, amino acid and ions among other compounds) and high osmotic pressure (30 bars; ~ 15 x 10^6 Pa) of the sieve element/ companion cell complex compared to mesophyll cells (13 bars; ~ 13 x 10^5 Pa), has led to the concept of phloem loading (Lemoine, 2000).

According to this idea, the high osmotic pressure in the sieve element/companion cell complex is due to an active loading of solute (mainly sucrose) in those cells. Conversely, this idea may not be general, because in some species, no solute concentration difference exists between the

sieve element/companion cell complex and the surrounding cells (Lalonde et al., 2003). Furthermore, the relatively low viscosity of sucrose may also allow its high translocation rate.

The movement of sap in the phloem occurs through mass flow, driven by the movement of sucrose and water from the sieve tubes in the source organ to the sieve tubes in the sink organs, and this flow is maintained by the continuous unloading of sucrose and water (Lalonde et al., 2003). Transmembrane transport events are, therefore, important for the control of long distance transport of assimilates.

Active accumulation of sucrose in the sieve tubes requires the presence of sucrose transporters to drive it, pointing to the importance of a carrier system for the translocation of solutes from source to sink organs (Figure 1.1). Plasma membrane sucrose transporters allow the cells to fulfill part of their nutritive requirements, and they indirectly participate in the control of long distance transport and in the control of gene expression via the metabolic status of the cell.

The first sucrose carrier system that was specific for sucrose and responsible for its entry into the phloem was postulated in late 1970 (Pazur, 1970; Giaquinta, 1983). The energy for this transport is derived from proton gradient established by an H^+/ ATPase located in the plasma membrane. Unequivocal evidence came from the first identification of a cDNA coding a sucrose carrier (SoSUT1) after several decades of physiological and biochemical experiments (Riesmeier et al., 1992). After these experiments, it was realized that the concept of a single higher plant sucrose transporter gene was incorrect and that plants have at least two sucrose carrier genes. The completion of the *Arabidopsis* and other plants genomes (*Arabidopsis* Genome Initiative, 2000; Yu et al., 2002) subsequently showed a larger number of sucrose transport genes.

In relation to its importance as the prime carbon and energy source in plants, sucrose has acquired important regulatory function that controls cellular metabolism, growth, development and environmental stress resistance (Smeekens, 2000; Sauer 2007). Source activities like photosynthesis and nutrient allocation to cells are usually up-regulated by low sucrose concentration, whereas sink activities like cell growth, development and storage are up-regulated by abundance of sucrose.

Sucrose synthesized in mesophyll cells of leaves is temporarily stored in the vacuole, and its transport is determined by the pool of sucrose available for export. Sucrose leaves the mesophyll cells and from the apoplasm enters the phloem cells. To travel the long distance pathway, several ways are possible as different situations are encountered among plants i.e. sucrose might be loaded into phloem sieve element/companion cell complex symplastically via plasmodesmata and/or

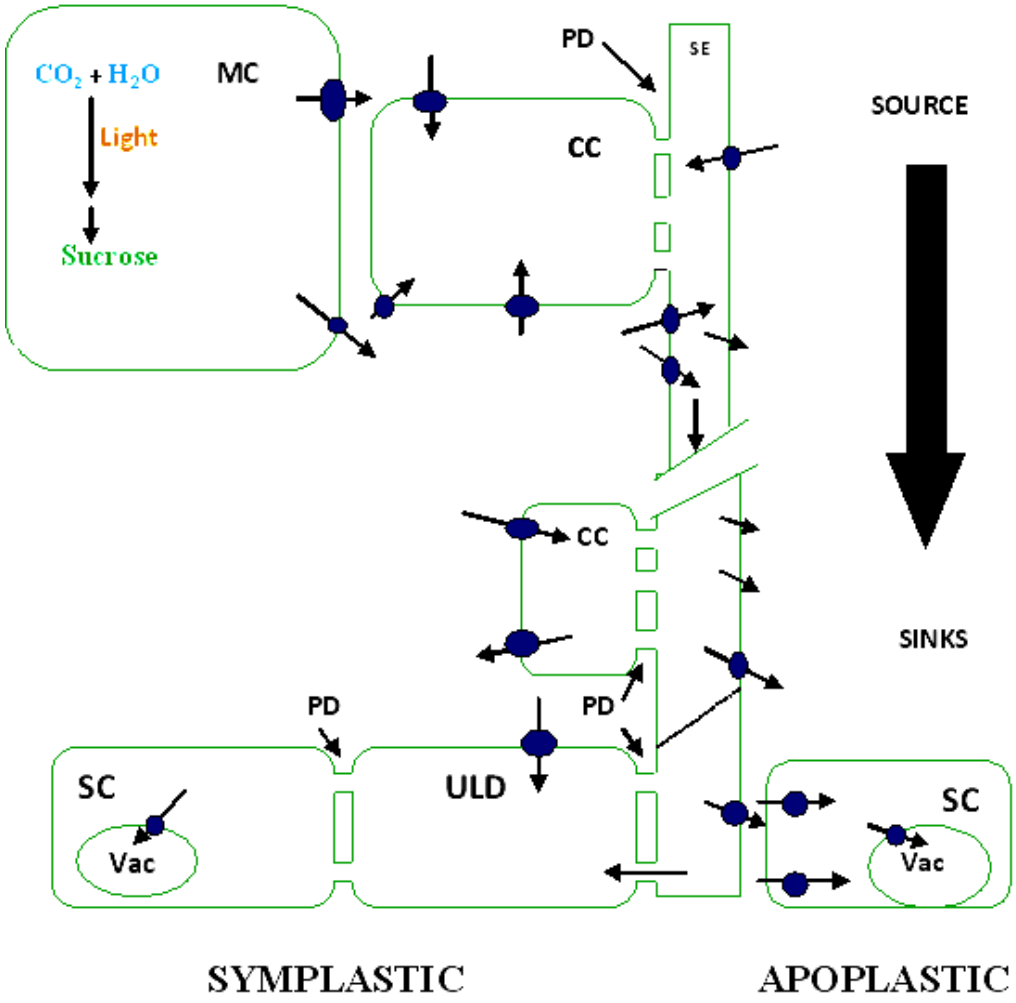


Figure 1.1: The path of sucrose from source to sink.

The upper left image shows a minor vein from a source leaf, the main site of sucrose loading into the phloem.The image on the right side shows the path of sucrose from a mesophyll cell into the sieve element – companion cell (SE-CC) complex and a symplastic and an apoplastic sink. Sucrose transporters of different cells and membranes are shown as closed circles [ ]; arrows indicate the direction of sucrose transport. (Adapted from Sauer, 2007)

apoplastically (Lalonde, 2003). Similarly sucrose unloading from sieve element - companion cell complex into sink cells may occur symplastically via plasmodesmata or apoplastically. All apoplastic loading or unloading of sucrose is postulated to occur via a proton sucrose symporter (Williams, 2000). In sink cells, sucrose can be cleaved by enzyme invertase to hexose and used for sink growth or development (i.e. metabolically active sink) or can be stored as sucrose in vacuoles of sink cells (i.e. storage sink).

Owing to the fact that sucrose has to pass through a considerable number of membranes i.e. from sites of primary assimilation (source tissue) to import dependent tissue and organs (sinks) the

membrane transport of sucrose has been considered as a major determinant of whole plant productivity and yield, and therefore needs to be studied extensively.

1.2. STRUCTURAL ANALYSIS

The number of isolated and cloned sucrose transporters (SUT) also called sucrose carriers (SUC) in some published reports, is rapidly increasing. So far sucrose transporter gene families have been found in major plants analysed, and they belong to the major facilitator super family of transport proteins (Marger and Saier, 1993). Figure 1.2, shows the phylogenic tree calculated for 48 plant sucrose transporters for which full length protein sequences are available in a public data base.

Generally these transporters are encoded by multigenic families that specificially function in sucrose transport; however they may differ by their cellular localization and by the regulation of their expression. As an example, nine putative sucrose transporters were identified in the *Arabidopsis thaliana* genome sequencing, while five sucrose transporters have been identified in rice *Oryza sativa* (Aoki et al., 1999).

GROUP 1

Five proteins have been functionally characterized as sucrose transporters from this group [Hordeum vulgare sucrose transporter 1and 2(HvSUT1, HvSUT2), Oryza sativa sucrose transporter 1(OsSUT1), Saccharum hybridum sucrose transporter 1(ShSUT1), Zea mays sucrose transporter 1 (ZmSUT1) Sauer, 2007]. OsSUT1 has been reported to be expressed in companion cells (CCs) and both OsSUT1 and ZmSUT1 are considered to be involved in phloem loading (Aoki et al., 1999; Scofield, 2007a). Additional function such as sucrose import into developing grains was proposed for group1 members (Aoki et al., 1999). This group thus represents plasma membrane-localized monocot sucrose transporters that catalyze largely the uptake of sucrose into phloem and sink cells.

GROUP 2

They are plasma membrane-localized sucrose transporters from dicots (Sauer, 2007). They are involved in phloem loading or sucrose import into different sink tissues; just as group 1. Gene duplication seems to increase the number of group 2 in several plant species. *Arabidopsis*, common plantain, grapevine, rape (*Brassica napus L.*) and tobacco contain two or more of group 2 genes. This multiple gene copy permits possible adaptation of parameters, such as transcriptional regulation of genes, pH-dependence and K_m values of the proteins to the specific requirements of sucrose transport into phloem of companion cells or specific sink tissues.

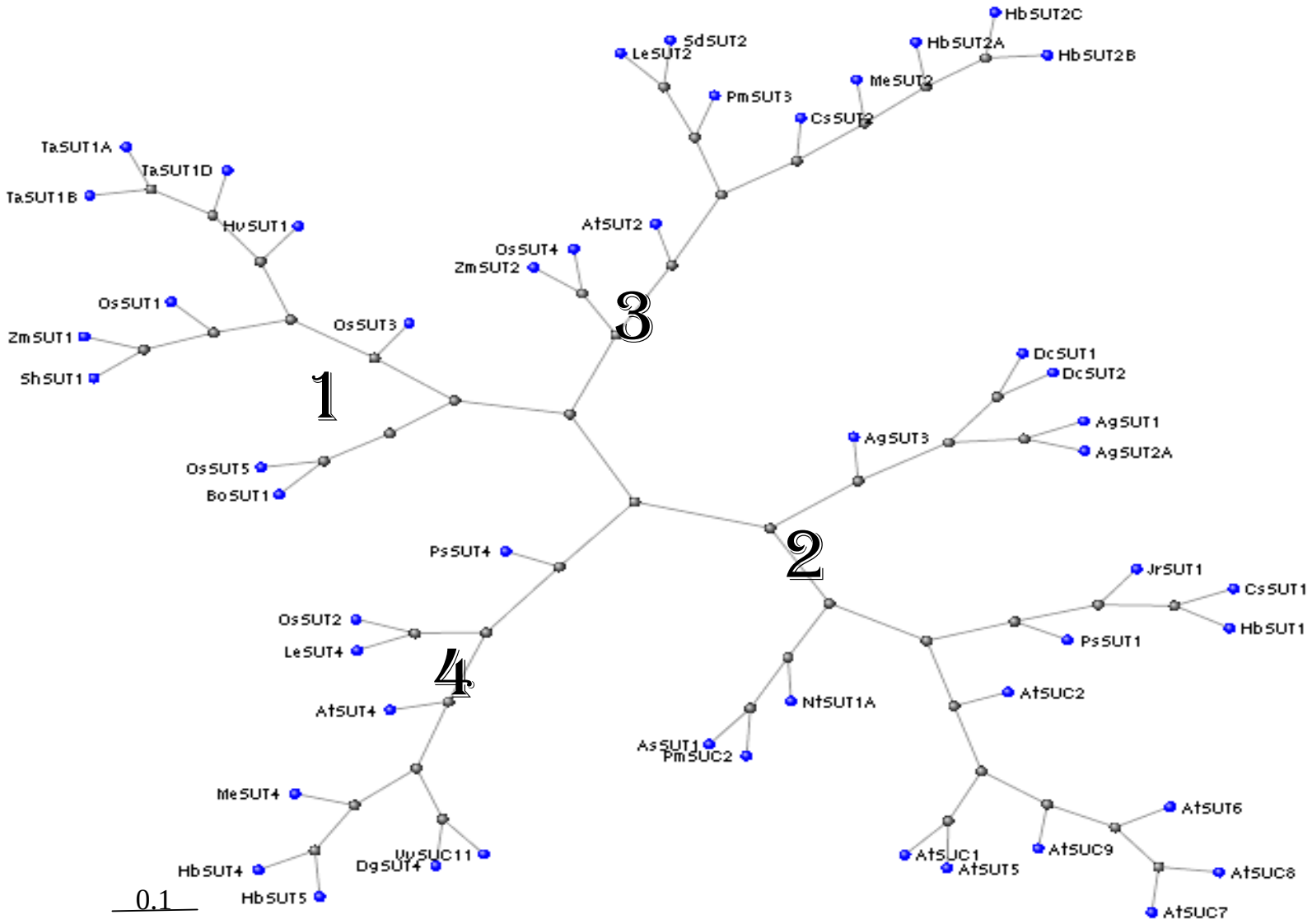


Figure 1.2: Phylogenetic tree for 48 confirmed sucrose transporter protein sequences from publicly accessible databases.

Accession numbers of presented sucrose transporter sequences are: AgSUT1 (Apium graveolens; AAC99332), AgSUT2A (Apium graveolens; AAD45390), AgSUT3 (Apium graveolens; ABB89051), AtSUC1 (*Arabidopsis thaliana*; At1g71880), AtSUC2 (*Arabidopsis thaliana*; At1g22710), AtSUT2 (*Arabidopsis thaliana*; NP178389), AtSUT4 (*Arabidopsis thaliana*; AAG09191), AtSUT5 (*Arabidopsis thaliana*; At1g71890), AtSUC7 (*Arabidopsis thaliana*; At1g66570), AtSUC8 (*Arabidopsis thaliana*; At2g14670), AtSUC9(*Arabidopsis thaliana*; At5g06170), BoSUT1 (*Bambusa oldhamii*; AAY43226), CsSUT1 (*Citrus sinensis*; AAM29150), CsSUT2 (*Citrus sinensis*; AAM29153), DgSUT4 (*Datisca glomerata*; CAG70682),DcSUT1 (*Daucus carota*; BAA89458), DcSUT2 (*Daucus carota*; CAA76369), HbSUT1 (*Hevea brasiliensis*; ABJ51933), HbSUT2A (*Hevea brasiliensis*; ABJ51934), HbSUT2B (*H. brasiliensis*; ABJ51932), HbSUT2C (*H. brasiliensis*; CAM33449), HbSUT4 (*H. brasiliensis*; ABK60191), HbSUT5 (*H. brasiliensis*; ABK60189), HvSUT1 (*Hordeum vulgare*;CAB75882), JrSUT1 (*Juglans regia*; AAU11810), LeSUT2 (*Lycopersicum esculentum*; AAG12987), LeSUT4 (*L. esculentum*;AAG09270), MeSUT2 (*Manihot esculenta*; ABA08445), MeSUT4 (*M. esculenta*; ABA08443), NtSUT1a (*Nicotiana tabacum*; X82276), OsSUT1 (*Oryza sativa*;AAF90181), OsSUT4 (*Oryza sativa*; BAC67164), OsSUT5 (*Oryza sativa*; BAC67165), OsSUT1 (*Oryza sativa*;AAF90181), OsSUT2 (*Oryza sativa*;BAC67163), OsSUT3 (*Oryza sativa*;BAB68368), PmSUC2 (*Plantago major*; X75764), PmSUT3 (*Plantago major*; CAD58887), PsSUT1 (*Pisum sativum*; AAD41024), PsSUT4 (*Pisum sativum*; ABB30162),,SdSUT2 (*Solanum demissum*; AAT40489), ShSUT1 (*Saccharum hybridum*; AAV41028), StSUT1 (*Solanum tuberosum*; CAA48915), TaSUT1A (*Triticum aestivum*; AAM13408), TaSUT1B (*T. aestivum*; AAM13409),TaSUT1D (*T. aestivum*; AAM13410), VvSUC11 (*V. vinifera*; AAF08329), VvSUC12 (*V. vinifera*; AAF08330), ZmSUT1 (*Zea mays*; BAA83501), ZmSUT2 (*Zea mays*; AAS91375).

Protein sequences were aligned and trees were calculated using the program Constrainst Based Protein Multiple Alignment Tool (COBALT; http:www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi?Link_loc=BlastHomeLink). Bars indicate the evolutionary distance. In this phylogenic tree, 48 sequences fall into 4 clearly separable groups: Group 1, Group 2, Group 3, and Group 4 which are discussed in the text.

GROUP 3

These group members are easily distinguishable from other sucrose transporters:

(1) They have a total length of approximately 600 amino acids. All members (frequently named SUC3 or SUT2- type transporters, based on the names of the first characterized transporters

of this group e.g AtSUC3 and LeSUT2) have about 15-20% amino acid more than all other sucrose transporters.

(2) Compared to other sucrose transporter groups they have higher K_m value for sucrose uptake.

(3) In several plant species, they are localized to the sieve element (SE) and in sink tissues; particularly in sink-leaf SE (Sauer, 2007).They are suggested as potential efflux carriers that might be involved in phloem unloading into the apoplast (Barth, 2003). However, they were initially published as sensors with no transport activity (Barker, 2000) but this no longer seems valid as experiments have verify that they are truly transporters (Stefan et al., 2000).

GROUP 4

This group was initially characterized as plasma membrane localized sucrose transporters but lately they have been classified as representing vacuolar sucrose transporters that are primarily expressed in sink tissues (Sauer, 2007).

Sucrose transporter sequences are highly conserved. All sucrose transporter proteins are indicated to be transmembrane proteins with 12 transmembrane helices of which N- and C-terminals are located in the cytoplasmic side of the plasma membrane as determined by hydropathy analyses (Riesmeier et al., 1992; Sauer and Stolz, 1994) and immunolocalization studies with site-specific antibody (Stolz et al., 1999) and are proposed to have a molecular mass of approximately 55kDa. The regions most conserved are the membrane-spanning domains, whereas major differences are located in the N-terminal sequence preceding the first potential membrane-spanning domain and the large hydrophilic loop in the centre of the protein.

The alignment of plant sucrose transporter sequences from the four groups and alignment of the first and second halves of these protein sequences with the sequence of H^+-sugar symporter (a member of “Major Facilitator Super Family”) respectively, supports:

(1) Plant sucrose transporters as a member of “Major Facilitator Super Family”

(2) The hypothesis that the members of this super family evolved by gene duplication and fusion from one or more ancestral transporters with only six transmembrane helices (Marger and Saier, 1993).

This information was used to draw and compare two-dimensional models of sucrose transporters from the four different groups (Figure 1.3). The most obvious differences were detected in the N-terminal and C-terminal domains of the transporters and the loops connecting the predicted transmembrane helices VI and VII (the central cytoplasmic loop) or VII and VIII

(predicted to face the extracellular space in plasma membrane-localized transporters and the vacuolar lumen in transporters of the tonoplast). The 15-20% higher number of amino acids of group 3 transporters can be attributed almost completely to an elongated N-terminus (about 20 amino acids) and to an elongated cytoplasmic loop (about 60 amino acids). Moreover the C-terminal of these group 3 transporters is slightly shorter than those of group 1 or group 2 transporters. The predicted vacuolar transporters of group 4 have the shortest C-terminal ends and a very short linker sequence between the transmembrane helices VII and VIII, therefore they have the shortest peptide sequence of all sucrose transporter family members of a given plant species.Although some motifs are conserved between the N- and C-terminal halves of the protein (e.g., the R–X–G–R–K–R motif between helices 2 and 3, and helices 8 and 9, in the sucrose transporter), the homology found when aligning the N- and C-terminal halves of the transporters are limited, which may be due to gene duplication occurring early during evolution. Furthermore the motif Q–F–G–W–A–L–Q–L was found to be conserved in the 1st helix of most sucrose transporters (Marger and Saier, 1993; Sauer, 2007). Motif R–F–G–R–R (found in the hydrophobic loop 3) and motif K–L–C–K–K (found in the hydrophobic loop 9) are also generally conservered in most sucrose transporters (Marger and Saier, 1993).

Biochemical investigations showed that a diethyl pyrocarbonate (DEPC)-sensitive histidine residue is conformationally linked to the substrate-binding site or translocation site of sucrose transporters. The DEPC-dependent inactivation of sucrose transporters is as a result of accessibility of this inhibitor to the outer surface of the plasma membrane of sucrose transporters, leading to the chemical modification of histidine residues located in the region that is exposed to the extracellular solution (Bush, 1993). Among the proton-sucrose symporters cloned to date, only the histidine residue at position 65 of AtSUC1 is conserved across species of sucrose transporters genes (Lu and Bush, 1998). His-65 is located in the 1st extracellular loop near the second transmembrane domain of the protein. This is consistent with the biochemical analysis that shows that DEPC-sensitive residue is accessible from the extracellular face of the plasma membrane (Bush, 1993).

Lu and Bush, (1998), used site directed mutagenesis to explore the role of His-65 in sucrose transporters. A series of single amino acid substitutions at His-65 were generated which included: Cys, Asp, Gly, Lys, Leu, Asn, Gln, Arg, Ser and Tyr. To investigate the impact of these substitutions on transport activity, each mutant form of SUC1 was ligated into a yeast expression vector and used to transform DBY2615; a yeast mutant deficient in cell wall invertase, which also lacks sucrose transport activity (Kaiser and Botstein, 1986).

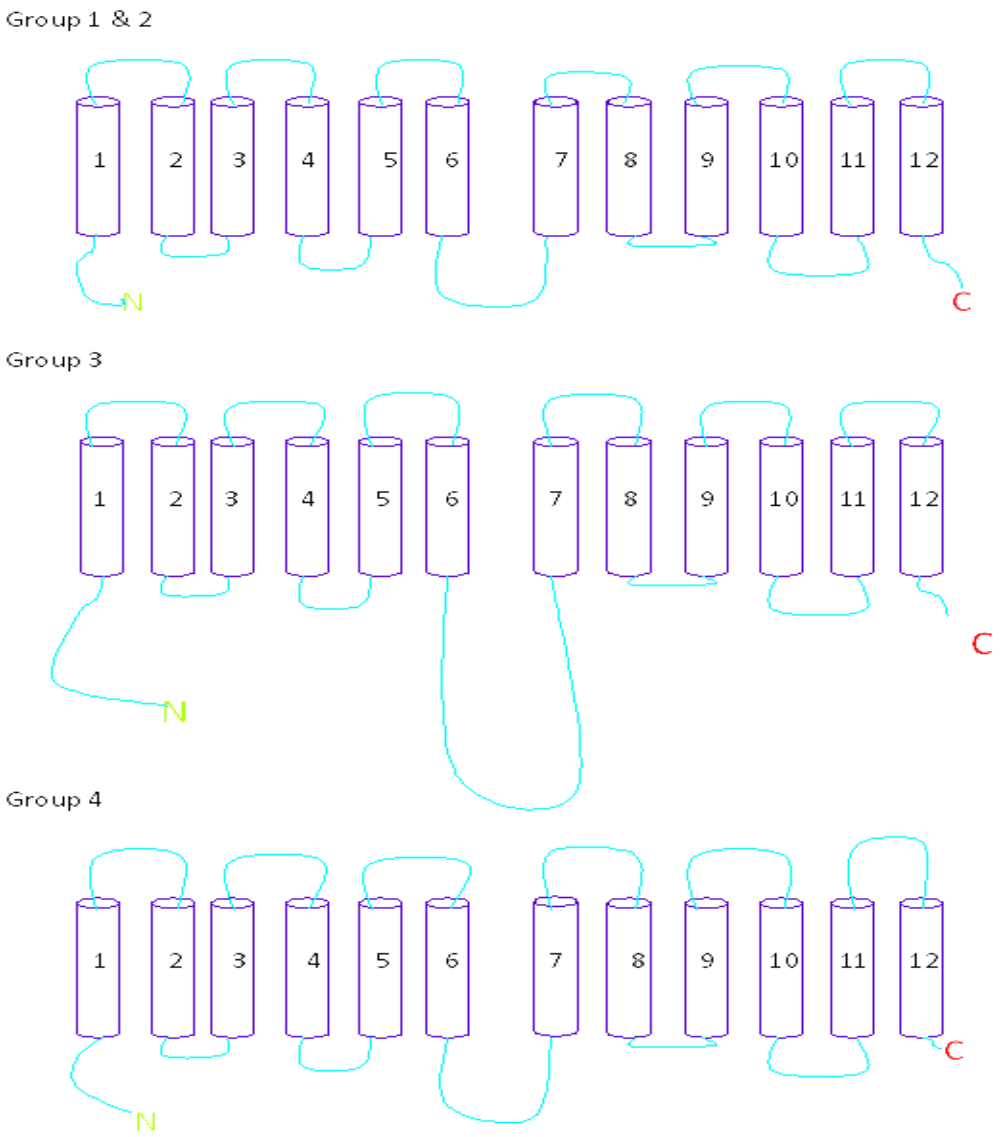


Figure 1.3: Two dimensional models of sucrose transporters for the different subgroups.
The 1st model represents group 1-type (dicot) and group 2-type (monocot) sucrose transporters of plasma membranes.
The 2nd model represents the predicted two dimensional-structures for group 3-type sucrose transporters of plasma membranes.
The 3rd model represents tonoplast sucrose transporters of the group 4-type.
For plasma membrane-localized transporters, N and C-terminals were shown to be on the cytoplasmic side; for tonoplast transporters this is only predicted. The main difference between the sucrose transporters of the different groups are outside the predicted membrane spanning regions. (Adapted from Sauer, 2007)

A sucrose limiting medium (1mM sucrose as the sole carbon source) was established where yeast cells expressing wild-type SUC1 grow slowly, but insert-free vector control does not. Several

patterns of growth were observed in DBY2615 cells transformed with the mutant SUC1. Cells expressing SUC1 mutants with Arg or Lys substitutions grew faster than cells with the wild-type SUC1. Mutants with Asn and Gln substitution allowed growth at about the same rate as wild type; while Gly, Ser and Tyr substituted mutants grow slowly. Leu and Asp substituted mutants gave slower growth than the wild-type, and Cys substituted mutant did not grow at all. These results fully support the proposition that His-65 is an essential residue in sucrose transporters.

Lu and Bush, (1998) also examined the DEPC-dependent inactivation of AtSUC1. Transgenic yeast cells expressing the Gln-65, Arg-65 or Ser-65 form of SUC1 were insensitive to DEPC inactivation as compared to the wild-type transporter. This result shows that His-65 is the inhibitor-sensitive histidine in AtSUC1, and that it is the only histidine residue whose chemical modification affects the sucrose transporter activity. Thus His-65 was suggested to play an important role in defining translocation pathways and participating in the reaction mechanism of the sucrose transporters.

1.3 EXPRESSIONAL ANALYSIS

RNA *in situ* hybridization shows that sucrose transporters are expressed specifically in the phloem, more precisely at high levels in the sieve elements, where the labelling is associated with the branched plasmodesmata connecting the sieve tube and the companion cell and at lower levels in the companion cells [Lalonde, et al., 2003]. An antisense expression of sucrose transporter clone resulted in a stunted plant phenotype that is consistent with a block in assimilate partitioning (Kuhn, 1996).

In solanaceous species localization of SUT1 protein in sieve elements and SUT1 mRNA at the orifices of plasmodesmata interconnecting companion cells and sieve elements indicates that trafficking of mRNA or protein occurs from companion cells into enucleate sieve elements by way of plasmodesmata (Kuhn et al., 1997). Antisense studies in solanaceous species have shown SUT1 is indeed essential for phloem loading and long distance transport (Riesmeier et al., 1994; Kuhn et al., 1997; Burkle et al., 1998). This was confirmed in tobacco, where strong antisense plant had a dwarf phenotype and sugar export from leaves was also drastically impaired. SUT1 was reported to serve as a high-affinity transporter for sucrose ($K_m \approx 1$mM; Riesmeier et al., 1994) whereas SUT4 has a K_m of ≈ 11mM; therefore was reported as a low-affinity sucrose transporter (Weise et al., 2000). Also in solanaceous plants, SUT3 was found to be involved in pollen nutrition, whereas SUT2 was a putative sucrose sensor (Lemoine et al., 1999).

Sucrose transporters were initially expressed in yeast that was engineered to replace invertase with sucrose synthase [Riesmeier et al., 1992]. This avoided extracellular hydrolysis of

sucrose, and allowed the yeast to metabolize sucrose after its uptake. Since yeast normally possesses very low sucrose transport activity, only the cells complemented with a plant sucrose transporter would grow on a sucrose medium. Furthermore, plant sucrose transporters have also been expressed in wild type yeast strains, in the presence of glucose as the sole source of carbon. Under this condition the secretion of invertase was repressed (Gahrtz et al., 1994). Use of protonophores, and the sensitivity of sucrose uptake measured in yeast was taken as an argument to conclude that the *SUT*/*SUC* transporters were proton–sucrose transport systems similar to the ones expected from physiological experiments on plants. Further evidence for proton– sucrose symport was obtained by heterologous expression of the sucrose transporters in *Xenopus* oocytes and the reconstitution of the proton-dependent sucrose transport activity into proteoliposomes containing His-tagged SUC2 (Zhou et al., 1997; Stolz et al., 1995).

1.4 MECHANISM OF ACTION

Among other functions, the plasma membrane of plant cells acts as a selective permeability barrier for extracellular mineral and organic compounds such as: ions, sugars, amino acids, peptides and xenobiotics. The selective permeability results from the properties of the lipidic bilayer and from the activity of many transporters and channels, which are tightly regulated by developmental and environmental factors.

In general active and passive mechanisms have been described for sucrose transporters, although active processes have been characterized in significantly more detail (Bush, 1993). Long distance transport of sucrose in a bulk flow of solution propelled through the plant phloem is catalyzed by series of partial reactions ranging from diffusion between mesophyll cells in the leaf, to active transport of sucrose into the sieve tube-companion cell complex by osmotically generated hydrostatic pressure difference between source (net nutrient exporter) and sink (net nutrient import) ends of phloem path (Lalonde 2003). Evidence for a carrier-mediated step in phloem loading of sucrose came from the use of respiratory inhibitors blocking the process of sucrose transporters (Buckhout, 1994; Sanders, 1986).

The transport mechanism is selective; it only loads sucrose and it allows for apoplastic (i.e from protoplast to wall to protoplast) or symplastic (from protoplast to protoplast via plasmodesmata) transport. In some species sucrose transport is symplastic (from mesophyll protoplast to CC-SE protoplast via plasmodesmata). In others, sucrose loading into the CC-SE complex involves an apoplastic step (mesophyll protoplast to apoplast to CC-SE protoplast) (Lalonde et al., 2003).

Influx of sucrose involves co transport of sucrose with protons, whereas efflux is predicted to involve antiport or facilitated diffusion. Lalonde et al. (2003) provided evidence that apoplastic and symplastic modes of phloem loading occur in minor vein networks and that they may co-exist. For example SE-CC complexes in the same minor vein have been found to exhibit plasmodesmata connectivity consistent with apoplastic and symplastic loading routes (van Bel et al., 1992). A parallel behavior was found to occur in stems, where unloading appears to switch between apoplastic and symplastic routes depending upon the source/sink ratio (Patrick and Offler, 1996).

The mechanism of sucrose transport across the membrane involves a sucrose/proton co-transport system. Sucrose transporters are co-localized to plasma membrane of SE-CC complex with H^+- ATPase that generates the required proton motive force (PMF) to drive the transport of sucrose. According to this model, protons are pumped out of the sieve cells into the apoplast by the membrane-bound H^+- ATPase. Proton concentration increases in apoplast leading to a decrease in the pH, while K^+ is brought into the sieve cells to balance the charge. The proton gradient provides the driving force for transporting sucrose against a gradient, sucrose and protons bind to a carrier protein in the membrane and are released in the sieve tube member. This model was supported by the high pH value of the sieve tubes, moreover when apoplast pH was increased, there was no sucrose uptake. Inhibition by para-chloromercuribenzene sulfonic acid (PCMBS); an inhibitor of membrane proteins further proves that a membrane transporter is involved in sucrose transport into the apoplast (Riesmeier, et al., 1994). Furthermore, uptake studies based on radiolabelled compounds showed that proton fluxes across the plasma membrane were accompanied by sucrose uptake (Marger and Saier, 1993).

This simple pH dependence has been taken as a proof for H^+-co-transport mechanism; however the external pH may also modify the global charge of the membrane. The data obtained often yielded complex kinetics and their interpretation could be clouded by several side effects such as diffusion across the cell wall, metabolism, and intracellular and intercellular compartmentation of the solutes absorbed. However, the use of purified plasma membrane vesicles energized by an artificial proton motive force circumvented most of the problems.

The PMF of any transporter will depend on:

(a) the number and the activity of the H^+-ATPase in the cell, which generates the two components of the PMF that are used by the transporter [i.e. the pH gradient(ΔpH) and electrical gradient($\Delta\psi$)],

(b) the relative part played by these chemical and electrical components (ΔpH and $\Delta\psi$),

(c) the concurrent and partial dissipation of these gradients by other transporters and channels present in the same cell,

(d) the external and internal concentrations of the mineral and organic substrate(s) of the transporter,

Early physiological experiments indicated that sucrose uptake into discs from mature leaves was mediated by at least two saturable carriers superimposed by a linear, non-saturable component (Delrot and Bonnemain, 1981; Maynard and Lucas, 1982). The same situation was described for protoplasts derived from soybean cotyledons (Schmitt et al., 1984). The high affinity component (K_m=2.6 mM) was shown to be a proton–sucrose symporter, while the low affinity component (K_m=35mM) was less dependent on the proton gradient (Delrot, 1981; Delrot and Bonnemain, 1981).

Sucrose transport was also suggested to be through facilitated membrane transport as a result of estimates of sucrose efflux derived from measures of cell surface area and known rates of sucrose export (Giaquinta, 1983). This fact was supported by observation of sucrose efflux from leaf discs, protoplast and plasma membrane vesicles (Giaquinta, 1983).The mechanism of symplastic transport involves intracellular cytoplasmic streaming arranged in series with passive diffusion through interconnecting plasmodesmatas (Lalonde et al, 2003).

Sucrose is unloaded into the apoplast in some tissues (e.g ovules) and into the symplast of others (growing/respiring tissues like young leaves, meristem). Apoplastic unloading can occur via hydrolysis of sucrose by acid invertase to glucose and fructose upon reaching the sink. This helps in maintaining the gradient for the transport, while glucose and fructose are taken up by the sink cells and stored. However for most sinks, phloem unloading follows symplastic routes as illustrated by movement of green fluorescent protein (GFP) (Patrick, 1997). At sinks, photoassimilates move from SE-CC complex to sites of utilization/storage. Thus this process involves transfer across the SE-CC complex boundary (i.e. sieve element unloading) arranged in series with cell-to-cell transport to sites of photoassimilate utilization (post-sieve element transport). Nevertheless, in some cases symplastic transport is interrupted by an apoplastic step in the post-sieve element pathway which occurs where photoassimilate transport takes place in sinks accumulating osmotical high concentration (Patrick, 1997).

1.5 SUBSTRATE SPECIFICITY

All sucrose transporters display an affinity for sucrose in the range 0.3-1.5 mM (Lemoine, 2000), although a little higher affinity for sucrose was noted for AgSUT1 (sucrose transporter in celery). These minor differences in substrate specificity may reflect slight variations in their protein

structures. Nevertheless, all K_m determinations were not made with the same yeast strains and uptake conditions might have been slightly different, therefore one considers that these K_m values are remarkably similar as shown in Table 1.1.

All results in Table 1.1 are obtained by expressing sucrose transporter cDNAs in yeast. They are in perfect agreement with the already described characteristics of sucrose transporters in plants (Hitz, et al., 1986; Hecht, et al., 1992; Lemoine, 2000; Sauer, 2007). They demonstrated that the heterologous expression in yeast cells does not change these major characteristics such as dependence on a proton gradient or sensitivity to inhibitors. They also indicate that the plant sucrose carriers are able to function properly in a different lipid environment (Lemoine, 2000).

Much data have been accumulated on the specificity of the sucrose carrier towards other substrates (Natural / synthetic). These data have been used to construct a model for the interaction of sucrose molecule with its own carrier. According to this model, the substrate recognition by sucrose carriers occurs through hydrophobic interaction with the fructosyl moiety whereas the hydroxyl group at position C-3, C-4 and C-6 of the glucopyranosyl moiety confer the specificity for sucrose recognition (Hitz et al., 1986; Hecht, et al., 1992) This was confirmed from the fact that the apparent K_i for phenyl- and benzyl-α-D-glucopyranoside and phenyl- and benzyl-thioglucopyranosides (analogues for sucrose) were significantly less than the apparent K_m for sucrose itself. Alteration on the glucose portion of phenyl-glucopyranosides resulted in decreased substrate recognition. Variability at C-4, i.e. 4-deoxy and 4-fluoro derivatives of phenyl-α-D-thioglucopyranoside, had K_i values similar to the K_m for sucrose (Hecht et al., 1992). Changing the stereochemistry at C-3 resulted in a molecule that inhibited sucrose uptake but with an apparent K_i significantly greater than the K_m for sucrose. Other stereochemical derivatives at C-1, C-2, or C-5 were without inhibitory activity (Hecht et al., 1992).

Sucrose transporters are much more substrate specific than the hexose transporters and they are encoded by a much smaller number of genes than the hexose transporters (Delrot et al., 2001; Sauer, 2007).The substrate specificity of sucrose transporters, as assessed in leaf discs, is rather narrow, with α-phenyl glucoside, maltose and phlorizin being the only competitors of sucrose uptake. Several dissacharides were tested on the basis of steric constraints out of which only maltose (a dimer of glucose linked in α 1→ 4) inhibited sucrose uptake, whereas its anomer isomaltose had no effect. None of the trisaccharides tested (raffinose and melezitose) were inhibitory to sucrose uptake (Lemoine, 2000). These data are in perfect agreement with the sucrose specificity of sucrose transporters in plant.

Table 1.1: Sucrose transporters affinity for sucrose

Name	Species	Length of Amino Acid	Accession Number	Functional Expression in yeast/K_m	Reference
AtSUC1	*Arabidopsis*	513	481132	Yes/0.45mM	Sauer et al.,1994
AtSUC2	*Arabidopsis*	512	407092	Yes/0.53mM	Sauer et al.,1994
DcSUT1a/b	Carrot	501	2969889, 2969887	Yes/0.5mM	Shakya and Sturm, 1998
DcSUT1a/b	Carrot	515	2969884	Yes/0.7mM	Shakya and Sturm, 1998
NtSUT1	Tobacco	507	575351	No	Burkle et al.,1998
NtSUT3	Tobacco	520	149981	No	Lemoine et al., 1999
OsSUT1	Rice	537	2723471	Yes/ND	Hirose et al., 1997
PmSUC1	*Plantago*	510	1086253	Yes/0.3mM	Gahrtz et al.,1996
PmSUC2	*Plantago*	510	415988	Yes/1mM	Gahrtz et al.,1994
SoSUT1	Spinach	525	549000	Yes/1.5mM	Riesmeier et al., 1992
StSUT1	Potato	516	542087	Yes/1mM	Riesmeier et al., 1993
VfSUT1	*Vicia faba*	523	Z93774	Yes/1.4mM	Weber et al.,1997

Furthermore, the use of inhibitors, such as phloridzin (a specific inhibitor of animal Na^+/glucose co-transport, shown to inhibit sucrose transport in plants (Lemoine, 2000)) and α-phenyl glucoside (a substrate used to demonstrate the hydrophobic interaction between the fructosyl moiety of sucrose with its transporter, a very powerful inhibitor of sucrose uptake) confirm that the properties described for sucrose transport activities in plants are the same as the one of sucrose transporters expressed in yeast. However, as the family of sucrose transporters increases, it will be

very interesting to see whether all carriers are equivalent as far as kinetic properties and affinity for sucrose are concerned.

1.6 REGULATION OF ACTION

Maintainance of nutrient and energy homeostasis within cells and tissues is of vital importance in all multicellular organisms, and requires the constant regulation of metabolic pathways in response to available nutrients. In plants maintenance of energy homeostasis involves refined and flexible regulatory mechanisms to account for the physiological and developmental activities. These mechanisms have provided information as related to nutrient distribution between different plant organs and also source-sink nutrient transition (Smeekens, 2000).

Membrane transport activities are of major importance in multicellular organisms, since they invest about 12% of their genomic information in encoding transport proteins (Tanner and Caspari, 1996). Membrane transporters have a dual function in providing part of the nutrients necessary for cell development and in transducing environmental and endogenous signals. One may, therefore, expect that these activities are controlled in a tight and complex way. Sucrose transport and metabolism in plants is an active process and its concentrations change in relation to development and environmental signals. Soluble sugars, such as sucrose, have been shown to affect timing of flowering, senescence and organ development such as larger or thicker leaves, increased number of tubers and adventitious roots (Paul and Pellny, 2003).

The transcription of sucrose transporters occur the companion cells, thus it will be important to determine the signals and mechanism that are involved in the transfer of their transcripts and/or proteins to their final destination. Moreover, the multiple roles of plant sucrose transporters and especially the central role of sucrose loading into the phloem from source to sink, suggest that sucrose transport may be tightly regulated. Once they have been transcribed, translated and targeted to the membrane, the activity of these transporters will then depend on the thermodynamic environment across the membrane and on post-translational regulation by various processes including phosphorylation/ dephosphorylation, redox regulation and allosteric control.

Transcriptional and post transcriptional regulation

Physiological studies (Lalonde et al., 1999) have shown that plant sugar transport and metabolism are highly regulated by sugar at the transcriptional and post transcriptional level, which include mRNA stability, protein targeting and modification. This implies that various levels of control are implemented that allow plant cells to regulate the fluxes of sugar across the plasma membrane during normal growth and development, as well as when their natural environment is

perturbed. Sugars were reported to repress the expression of photosynthetic genes and to induce the transcription of sink specific enzymes that are involved in sucrose breakdown (Koch, 1996, Godt and Roitsch, 1997). Thus transcriptional regulation of enzymes involved in sucrose and starch biosynthesis may be linked to the expression profile of sucrose transporters (Riesmeier, 1994).

There are many examples of transcriptional regulation of sucrose transporters, such as changes in expression pattern in young leaves undergoing sink to source to transition during seed development or during pollen maturation and germination (Weber et al., 1997). Other evidences has also shown that biotic and abiotic factors such as mechanical wounding/treatment, light, water and salt stresses, phytohormones and pathogen attack have effects on the transcriptional expression of certain sucrose transporters (Sark et al. 1993; Sark et al., 1997; Ritte et al., 1999; Delrot et al., 2000; Williams, 2000; Meyer et al., 2004; Kempema et al., 2007; Sauer, 2007).

Regulation by phosphorylation and variations in sulfur/disulfide bonds

Sucrose transporter activity was shown to be regulated by phosphorylation with the phosphorylated form being less active than the unphosphorylated one (Roblin et al., 1998). Roblin et al. (1998) showed that sucrose uptake in the leaf discs from *Beta vulgaris* can be inhibited by application of okadaic acid (OA); an inhibitor of protein phosphatase 1 and 2. These observations were comfirmed by measuring direct uptake into PMV prepared from leaves infiltrated with OA to exclude possible secondary effects by OA. H^+-ATPase activity was unaffected by OA treatment, and the amount of transporter protein did not decrease as shown by specific antibodies. It was suggested that OA acts directly on the activity of the sucrose transporters by maintaining them in a phosphorylated form, thus inactivating them. Protein kinase activities, which might be responsible for the phosphorylation of sucrose transporters in sieve-elements, have long been detected in the phloem sap (Nakamura et al., 1993). Lalonde et al., (1999), therefore proposed that a proton-coupled sucrose transporter may be regulated in two major ways:-

(1) indirectly by regulating H^+-ATPase activity,

(2) directly by controlling the expression of sucrose transporters at the transcriptional levels and phosphorylation at post- transcriptional levels.

Other factors also known to regulate ATPase activity and /or transcription include: fusicoccin, salicylic acid and anaerobiosis; they probably act at the level of ATP supply for the H^+-ATPase. Sucrose was found to induce accumulation of two plasma membrane ATPases'; LHA4 and LHA2 (Mito et al., 1996). This induction is dependent on sucrose uptake and metabolism, thus the

induction of expression of H^+-ATPase gene by metabolizable sugars may be part of a generalized cellular response to promote cell growth in the presence of abundant carbon source.

Another potential regulation mechanism is via variations in sulfur/disulfide bonds. This type of regulation is generally mentioned for various transporters, and lines of evidence suggest that it may also play a role in the regulation of sucrose transporters (Sakr et al., 1994). In purified plasma membrane vesicles, sucrose transport activity and sensitivity to thiol reagents strongly depends on temperature, which may affect the conformation of proteins (Sakr et al., 1994). Four highly conserved Cys residues are present in various sucrose transporter sequences, including one in the intracellular loop connecting helices 2 and 3, and two in the extracellular loop connecting helices 5 and 6 (Lemoine, 2000).

Sucrose as signaling molecule

Sugars not only stimulate cellular carbon and energy metabolism, but also participate as signaling molecules. Various sugar signals are created by photosynthesis and carbon metabolism in plant source and sink tissues in response to growth, development and environmental stress conditions (Smeekens, 2000). However the use of yeast as a model system has paved the way for the discovery of multiple sugar sensor and signaling pathways. In yeast, it was shown that membrane- bound receptors closely related to glucose transporters (i.e. SNF3 and RGT2), are used to sense external glucose concentrations and activate a signal transduction pathway leading to the regulation of transporter gene expression and insertion, or degradation of proteins at the plasma membrane (Lalonde, et al., 1999). This allows yeast to respond to a rapidly changing external environment. The glucose sensors: SNF3 (senses low glucose levels) and RGT2 (senses high glucose levels) are homologous to yeast and plant glucose transporters, but do not mediate significant glucose transport. Their large C-terminal and membrane-spanning domain are important for glucose recognition and transmission of glucose signal. However, there is no direct evidence for a role of plant glucose/sucrose transporters as a sensor. Nevertheless, the finding that several members of the plant glucose/sucrose transporter families have unusual cytoplasmic extensions, has initiated many studies in relation to the possible role of these proteins in sugar sensing (Sauer, 2007).

Developmental regulation

Expression of sucrose transporters involved in phloem loading was shown to be developmentally regulated by Wright et al. (2003). It was shown that young sink leaves of transgenic tobacco plant expressing green fluorescent protein (GFP) of the AtSUC2 promoter gave little expression of sucrose transporter gene, whereas strong expression was seen in vascular

bundles of mature source leaves (Wright et al., 2003). During this transition from sink to source, expression of sucrose transporter gene was initiated at the tip and proceeds towards the base of the developing leaf. The partial shading of the transgenic tobacco plant leaves resulted in an almost complete lack of sucrose transporter gene expression in the minor veins of shaded areas, although these regions were structurally and fully developed (Wright et al., 2003). Furthermore, light or one of several product of photosynthetic CO_2 fixation was also shown to play an important role for the expression of *Lycopersicum esculentum* SUT1 (LeSUT1) and solanum tuberosum SUT1 (StSUT1), which show reduced expression and reduced protein levels in the dark (Kuhn et al., 1997). It could be said that light-induced increase of SUT1 transcript is based on a circadian regulation of these transporters.

Hormonal regulation

Hormones such as auxins and cytokinin are known to increase the rate of phloem assimilates transport (Lalonde et al., 1999). Fusicoccin and auxin were found to rapidly promote sucrose uptake whereas abscisic acid acts as an inhibitor (Lalonde et al., 1999). Gibberellin was reported to directly promote assimilate export in broad bean (Aloni, et al., 1986), however phloem loading in isolated bundles of celery was directly affected by gibberellins and auxin (Lalonde et al., 1999). The direct connection between hormonal action and sucrose transporter gene expression has not been fully elucidated.

Therefore, in order to comprehend source-sink regulation fully and to be in a position to manipulate the complex interactions for agricultural benefit, it is vital that the underlying membrane transport processes are thoroughly characterized. These include determining precisely the range of sucrose transporters that are involved in sucrose transport from source to sink, their structure-function relationship and defining their cellular and temporal expression pattern.

1.7 REFERENCES

1) Aloni, B., Daie, J. and Wyse, R.E. (1986) Enhancement of (^{14}C) sucrose export from source leaves of *Vicia faba* by gibberellic acid. *Plant Physiology* **82**:962-967
2) Aoki, N., Hirose, T., Takahashi, S., Ono, K., Ishimaru, K. and Ohsugi, R. (1999) Molecular cloning and expression analysis of a gene for a sucrose transporter in maize (zea mays L.) *Plant Cell Physiology*. **40**:1072-1078.
3) Barker, L., Kuhn C., Weise, A., Schulz, A., Gebhardt, C., Hirner, B., Hellmann, H., Schulze, W., Ward, J.M. and Frommer, W.B. (2000) SUT2, a putative sucrose transporters of different affinities colocalized in the same enucleate sieve elements. *Plant Cell* **14**:1567-1577

4) Barth, I., Meyer, S. and Sauer, N. (2003) PmSUC3: Characterization of a SUT2/SUC3-type sucrose transporter from plantago major. *Plant Cell* **15**:1375-1385.

5) Buckhout, T. J. (1994) Kinetic analysis of the plasma membrane sucrose $-H^+$ symporter from sugar beet (*Beta vulgaris L*.) leaves. *Plant Physiology* **106**:991-998

6) Burkle, L., Hibberd, J.M., Quick, W.P., Kuhn, C., Hirner, B. and Frommer, W.B. (1998) The H^+ sucrose co-transporter NtSUT1 is essential for sugar export from tobacco leaves. *Plant Physiology* **118**:59-68

7) Bush, D. R. (1993) proton coupled sucrose transport in plasmalemma vesicles isolated from sugar beet (Beta vulgaris L. cv Great Western) leaves. *Plant Physiology* **89**:1318-1323

8) Chiou, T.J. and Bush, D.R. (1998) Sucrose is a signal molecule in assimilate partitioning. *Proceedings the of National Academic Science of the U.S.A.* **95**:4784-4788

9) Delrot S, Atanassova S, Maurousset L (2000) Regulation of sugar, amino acid and peptide plant membrane transporters. *Biochim. Biophys. Acta* 1465:281-306.

10) Delrot, S. (1981) Proton fluxes associated with sugar uptake in *Vicia faba* leaf tissues. *Plant Physiology* **68**:706–711

11) Delrot, S. and Bonnemain, J.L. (1981) Involvement of protons as a substrate for the sucrose carrier during phloem loading in *Vicia faba* leaves. *Plant Physiology* **67**:560–564.

12) Delrot, S., Atanassova, R., Gomès, E. and Coutos-Thévenot, P. (2001). Plasma membrane transporters: machinery for uptake of organic solutes and stress resistance. *Plant Science* **161**: 391-404.

13) Gahrtz, M., Stolz, J. and Sauer, N. (1994) A phloem-specific sucrose-H^+ symporter from Plantago major L. supports the model of apoplastic phloem loading, *Plant Journal* **9**: 697-706

14) Giaquinta, R.T. (1983) Phloem loading of sucrose. *Annual Review of Plant Physiology* **57**:872-875

15) Gottwarld, J.R., Krysan, P.J., Young, J.C., Evert, R.F. and Sussman M. (2000) Genetic evidence for the inplanta role of phloem-specific plasma membrane sucrose transporters. *Proceedings of the National Academic Science of the U.S.A* **25**:13979-13984

16) Hecht, R., Slone, J.H., Buckhout, T.J., Hitz w, D. and van Der Woode, W.J. (1992) Substrate specificity of H^+-sucrose symporter on the plasma membrane of sugar beet (*Beta vulgaris L*) *Plant Physiology* **99**:439-444

17) Hirose, T., Imaizumi, N., Scofield, G.N., Furbank, R.T. and Ohsugi, R.(1997) cDNA cloning and tissue specific expression of a gene for sucrose transporter from rice (*Oryza sativa* L.) *Plant Cell Physiology* **38**:1389-1396

18) Hitz, W.D., Card, P.J. and Ripp, K.G. (1986) Substrate recognition by a sucrose transporter protein. *Journal of Biological Chemistry* **261**:11986-11991

19) Kaiser, C. and Botstein, D. (1986) Secretion defective mutations in the signal sequence for Saccharomyces cerevisiae Invertase. *Molecular Cell Biology* **6**:2382-2391

20) Kempema, L. A., Cui, X., Holzer, F. M., Walling, L.L. (2007). *Arabidopsis* transcriptome changes in response to phloem-feeding Silverleaf white nymphs. Similarities and distinctions in responses to aphids. *Plant Physiology* **143**: 849-865.

21) Koch, K.E. (1996) Carbohydrate-modulated gene expression in plants. *Annual Review Plant Physiology and Plant Molecular Biology* **47**:509-540

22) Kühn, C., Franceschi, V.R., Schulz, A., Lemoine, R. and Frommer, W.B. (1997) Macromolecular trafficking indicated by localization and turnover of sucrose transporters in enucleate sieve elements. *Science* **275**:1298-1300

23) Kühn, C., Quick, W.P., Schulz, A., Riesmeier, J.W., Sonnewald, U. and Frommer, W.B. (1996) Companion cell-specific inhibition of the potato sucrose transporter SUT1. *Plant Cell and Environment* **19**:1115–1123.

24) Lalonde, S., Boles, E., Hellmann, H., Barker, L., Partrick, J.W. and Frommer, W.B. (1999) The dual function of sugar carriers: Transport and sugar sensing. *Plant Cell* **11**:707-726

25) Lemoine, R. (2000) Sucrose transporters in plants: updates on function and structure. *Biochimica et Biophysica Acta*, **1465**:246-264

26) Lalonde, S., Tegeder, M., Throne-holst, M., Frommer, W.B. and Patrick, J.W. (2003) Phloem loading and unloading of sugars and amino acids. *Plant Cell and Environment* **26**:37-56

27) Lemoine, R., Burkle, L., Barker, L., Sakr, S., Kühn, C., Regnacq, M., Gaillard, C., Delrot, S. and Frommer, W.B. (1999) Identification of a pollen-specific sucrose transporter-like protein NtSUT3 from tobacco. *FEBS Letters* **454**:325-330

28) Lu, J. M.-Y and Bush, D.R. (1998) His-65 in the proton-sucrose symporter is an essential amino acid whose modification with site-directed mutagenesis increases transport activity. *Proceedings of the National Academy of Science of the* U.S.A **95(15):**9025-9030

29) Marger, M.D. and Saier Jr. M.H. (1993) A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. *Trends In Biochemical Science* **18**:3-12

30) Maynard, J. W. and Lucas, W.J. (1982) A re-analysis of the two components phloem system in *Beta vulgaris. Plant Physiology* **69**:734–739.

31) Meyer, S., Lauterbach, C., Niedermeier, M., Barth, I., Sjolund, R.D. and Sauer, N. (2004) Wounding enhances expression of AtSUC3, a sucrose transporter from *Arabidopsis* sieve elements and sink tissue. *Plant Physiology* **134**:684-693

32) Mito, N., Wimmers, L.E. and Benneth, A.B. (1996) Sugar regulates mRNA abundance of H^+-ATPase gene family members in tomato. *Plant Physiology* **112**:1229-1236

33) Nakamura, S., Hayashi, H., Mori, S. and Chino, M. (1993) Protein phosphorylation in the sieve tubes of rice plants. *Plant Cell Physiology* **34**:927-933

34) Patrick, J.W. and Offler, C.E. (1996) Post-sieve element transport of photoassimilate in sink regions. *Journal of Experimental Botany* **27**:1165-1177

35) Patrick, J.W. (1997) Phloem unloading: Sieve element unloading and post-sieve element transport. *Annual Review of Plant Physiology and Plant Molecular Biology* **28**:165-190

36) Paul, M.J. and Pellny, T.K. (2003) Carbon metabolite feedback regulation of leaf photosynthesis and development. *Journal of Experimental Botany* **54**:539-547

37) Pazur, J.H. (1970) Oligosaccharides in the carbohydrates. Vol. 2A 2nd Ed. (R. Pigman and D. Horton Eds.) Pp 69-137. Academic Press New-York.

38) Riesmeier, J.W. Willmitzer, L. and Frommer, W.B. (1992) Isolation and characterization of a sucrose carrier cDNA from spinach by functional expression in yeast. *EMBO Journal*. **11**:4705-4713

39) Riesmeier, J.W. Hirner B. and Frommer, W.B. (1993) Potato sucrose transporter expression in minor veins indicates a role in phloem loading. *Plant Cell* **5**:1591-1598

40) Riesmeier, J.W. Willmitzer, L. and Frommer, W.B (1994) Evidence for an essential role of the sucrose transporter in phloem loading and assimilate partitioning. *EMBO Journal* **13**:1-7

41) Ritte G, Rosenfeld J, Rohig K, Raschke K (1999) Rates of sugar uptake by guard cell protoplast of *Pisum sativum* L. related to the solute requirements for stomatal opening. *Plant Physiology* **121**:647-655

42) Roblin G., Sakr S., Bonmort, J and Delrot S. (1998) Regulation of a plant plasma membrane sucrose transporter by phosphorylation. *FEBS Letters*. **424**:165-168

43) Sakr S., Noubahni M., Bourbouloux A., Riesmeier J., Frommer W. B., Sauer N. and Delrot S. (1997) Cutting, ageing and expression of plant membrane transporters. *Biochimica et Biophysica Acta* **1330**:207-216

44) Sakr, S., Gaillard, C., Roblin, G. and Delrot, S. (1994) Sucrose protectable inhibition of the sucrose transporter by N-ethylmaleimide. *Journal of Experimental Botany* **45**:1091-1096

45) Sakr, S., Lemoine, R., Gaillard, C. and Delrot, S. (1993) Effect of cutting on solute uptake by plasma membrane vesicles from sugar beet (Beta vulgaris L.) leaves. *Plant Physiology* **103:**49-58

46) Sanders, D. (1986) Generalized kinetic analysis of ion-driven co-transport system: II Random ligand binding as a simple explanation for non-michaelian kinetics. *Journal of Membrane Biology* **90**:67-87

47) Sauer, N. (2007) Molecular physiology of higher plants sucrose transporters. *FEBS Letters* **581**:2309-2317

48) Sauer, N. and Stolz, J. (1994) SUC1 and SUC2: two sucrose transporters from *Arabidopsis thaliana*; expression and characterization in baker's yeast and identification of the histidine-tagged protein. *Plant Journal* **6**:67-77.

49) Schmitt, M.R., Hitz, W.D., Lin, W. and Giaquinta, R.T. (1984) Sugar transport into protoplasts isolated from developing soybean cotyledons. II. Sucrose transport kinetics, selectivity and modelling studies. *Plant Physiology* **75**:941–946

50) Scofield, G. N., Hirose, T., Aoki, N. and Furbank, R.T. (2007) Involvement of sucrose transporter, OsSUT1, in the long-distance pathway for assimilates transport in rice. *Journal of Experimental Botany* **58**:3155-3169

51) Shakya, R. and Sturm, A. (1998) Characterization of source- and sink-specific surose/H+ symporters from carrot. *Plant Physiology* **118**:1473-1480

52) Smeekens, S. (2000). Sugar-induced signal transduction in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **51**:49–81

53) Stefan M., Micheal M., Elizabeth T., Carola H., Rainer B., Ruth S. and Norbert S. (2000) AtSUC3, a gene encoding a new *Arabidopsis* sucrose transporter is expressed in cells adjacent to the vascular tissue and in a carpel cell layer. *ThePlant Journal* **24(6)**: 869-882

54) Stolz, J., Ludwig, A., Stadler, R., Biesgen, C., Hagemann, K. and Sauer, N. (1999) Structural analysis of a plant sucrose carrier using monoclonal antibodies and bacteriophage lambda surface display. *FEBS Letters* **453**:375-379

55) Stolz, J., Darnhofer-Demar, B. and Sauer, N. (1995) Rapid purification of a functionally active plant sucrose carrier from transgenic yeast using a bacterial biotin acceptor domain. *FEBS Letters* **377**:167-171

56) Tanner, W. and Caspari, T. (1996) Membrane transport carriers. *Annual Review of Plant Physiology and Plant Molecular Biology* **47**:595-626

57) van Bel, A.J.E. , Gamalei, Y.V., Ammerlaan, A. and Bik, L.P.M. (1992) Dissimilar phloem loading in leaves with symplastic or apoplastic minor-vein configurations . *Planta* **186**:518-526

58) Weber, H., Borisjuk, L., Heim, U., Sauer, N. and Wobus, U. (1997) A role for sugar transporters during seed development: Molecular characterization of a hexose and a sucrose carrier in fava bean seeds. *Plant Cell* **9:**895-908

59) Weise, A., Barker, L., Kuhn, C., Lalonde, S., Buschmann ,H., Frommer, W.B. and Ward, J.M. (2000) A new subfamily of sucrose transporters SUT4 with low affinity/high capacity localized in enucleate sieve elements of plants. *Plant Cell* **12**:2395-2407

60) Williams L.E., Lemoine R. and Sauer N. (2000) Sugar transporter in higher plants – a diversity of roles and complex regulation. *Trends Plant Science* **5**:283-290

61) Wright, K.M., Roberts, A.G., Martens, H.J., Sauer, N. and Oparka, K. (2003) Structural and functional veins maturation in developing tobacco leaves in relation to AtSUC2 promoter activity. *Plant Physiology* **1312**:1555-1565

62) Yu, et al. (2002) A draft sequence of the rice denome (*Oryza sativa* L. ssp. Indica) *Science* **296**:79-92

63) Zhou, J.J., Theodoulou, F.L., Sauer, N., Sanders, D. and Miller, A. J. (1997) A kinetic model with ordered cytoplasmic dissociation for SUC1, an *Arabidopsis* H+/sucrose cotransporter expressed in *Xenopus oocytes. Journal of Membrane Biology* **159**: 113-125

Chapter 2

Research Motivation and Hypothesis

2.1 INTRODUCTION

Plants are constantly exposed to a variety of adverse environmental conditions such as temperature extremes, UV light, salt, insect herbivory and pathogen attacks. The natural resistance of plants to these different environmental stresses therefore depends on the modulation and expression of a large number of inducible but specific genes, for example increase in activity of hydrolytic enzymes (chitinase, protease, e.t.c.); accumulation of cell wall structural proteins (hydroxyproline-rich glycoproteins, glycine-rich proteins, lignin); accumulation of solutes (sucrose, glucose, oligosaccharides, glycine, proline, organic ions) and production of anti-microbial compounds (phytoalexins), to mention a few (Shinozaki and Yamaguchi-Shinozaki, 2000; Chen, 2008).

2.2 PROBLEM STATEMENTS

2.2.1 Drought and Salinity

Drought and salt stress conditions on plants cause hyperionic and hyperosmotic effect resulting in the retardation of plant growth and a decrease in crop productivity. Plants respond to these stresses in a variety of mechanisms at the molecular, cellular and physiological levels in order to regulate the production of and access to sugars for the urgent and increasing metabolic need of stress tissues. Various genes are induced by these stresses and their gene products are thought to function not only in stress tolerance but also in the regulation of gene expression and signal transduction of the response (Hasegawa et al., 2000; Shinozaki and Yamaguchi-Shinozaki, 2000).

Drought and high salt stress adversely affect plant functions by lowering the osmotic water potential of plants, leading to great water loss, resulting to dehydration and ultimately to plant death. Plants tend to overcome these stresses by adjusting the osmotic and ionic potential through the accumulation of organic solutes such as sucrose, glucose, glycine and proline (Rontein et al., 2002). Water moves from high water potential to low water potential, and accumulation of these osmolytes make the water potential lower inside the plant cell thereby preventing the intracellular water loss, helping the cells to maintain their hydrated state thus preventing cellular dehydration.

2.2.2 Aphid Infestation

Aphids, the largest group of phloem feeders (Pollard,1973), penetrate plant tissue by probing intercellullarly through the epidermal and mesophyll cell layer with their stylet, ejecting their saliva into these cells and subsequently feeding on the exuberant supply of photoassimilates translocating in the phloem sieve element(Matsiliza and Botha, 2002, Will and van Bel, 2006).It is

assumed that the saliva is responsible for the onset of responses, and that it contains peroxidases, β-glucosidases and other potential signal-generating enzymes (Miles, 1999). Signals arising from aphid phloem-feeding are also capable of altering the expression of inducible plant physiological gene factors similar to those involved in defense against pathogens (Inbar et al., 1999).

Numerous reports have shown that Aphids generally cause reduction in plant height and shoot weight, longitudinal leaf chlorosis and leaf rolling, and possibly long-term damage to cell and tissues; as a result of callose deposition in the damaged functional phloem, caused by feeding-related pressure loss.

How then can a single cell cope with these sudden changes in the cellular metabolism and additional metabolism task caused by increased energy and carbon requirements that result from enhanced respiratory activities of wounded tissues? In storage organs, this may be accommodated by an increased breakdown of carbohydrate, however in other tissues with little or no carbohydrate reserves (leaves, stem), this additional energy and carbon utilization can only be met by an increased utilization of exogenously supplied carbohydrate in the form of sucrose.

2.3 HYPOTHESES

Sucrose transporters will participate in:

(1) Sucrose transport from source tissues to site of utilization in order to maintain high osmotic potential in plant tissues during drought and salinity stresses,

(2) Sucrose transport from the neighboring apoplasmic cells to meet the metabolic need of the adjacent injured cells during aphid infestation,

(3) A defense action for effective reduction and/or retrieval of leaked photoassimilates in the apoplast back into the phloem.

2.4 AIM

Investigation of the spatial and temporal expression of differentially regulated sucrose transporters in rice (*Orzya sativa* L. cv Nipponbare) cultivar, exposed to drought, salinity and rusty plum aphids (*Hysteroneura setariae*; Thomas).

2.5 SPECIFIC OBJECTIVES

(1) Comparative *in-silico* analysis of *cis*-acting regulatory elements in promoter regions of sucrose transporter gene families in rice and *arabidopsis thaliana.*

(a) Identification of the 5´untranslated (UTR) region of sucrose transporter genes

(b) Analysis of the *cis*-acting regulatory elements present in the 5´untranslated (UTR)

(2) Cultivation of rice (*Orzya sativa* L. cv Nipponbare) at optimum conditions and exposure of plants to drought, salinity and aphid infestation.

(a) Cultivation of rice (*Orzya sativa* L. cv Nipponbare) cultivar at optimum conditions

(b) Maintenance of rusty plum aphids (*Hysteroneura setariae*; Thomas) colonies for plant treatment

(c) Exposure of rice plants to drought, salinity and rusty plum aphids (*Hysteroneura setariae*; Thomas) infestation

(3) Analysis of differentially expressed sucrose transporter during drought, salinity and aphid infestation

(a) Isolation of total RNA from control and treated rice

(b) Amplification of sucrose transporter (SUT) genes by reverse transcriptase polymerase chain reaction (RT-PCR)

(c) Relative quantification of amplified SUT genes by Real-Time PCR

(d) Isolation of total proteins from control and treated rice plants

(e) Monitoring the protein expressional levels of the up-regulated SUT gene by western blot analysis and also the relative quantification of this protein

(4) Determination of spatial location of the up-regulated SUT gene during rusty plum aphids (*Hysteroneura setariae*; Thomas) infestation by β-glucuronidase (*GUS)* activity.

2.6 SIGNIFICANCE OF RESEARCH

Many studies have reported on the regulation of various enzymes and pathogen related proteins (Moran and Thompson, 2001; van de Ven et al., 2000; Kehr, 2006; Thompson and Goggin 2006; Will et al., 2007; Chen, 2008; Walling, 2008), however little is known about the action/role of transporters during environmental or phloem feeding stress responses.

Although research on sucrose transporters is extensively reported the in the literature, data on monocotyledons sucrose transporters are scarce. Monocotyledon seeds of rice, maize, wheat, barley, oat, sorghum, millet etc. are of great agronomic significance, since they represent the main source of carbohydrates for humans. Therefore, understanding the role/function of sucrose

transporter in the distribution of post phloem photoassimilates and the accumulation of carbohydrates would be of major economic importance, especially during abiotic (drought and salinity) and biotic (aphid infestation) stress conditions, which plants are often subjected to during commercial cultivation.

This study putative will help to identify the *cis*-acting regulatory elements and the prediction of the transcription factors involved in the expression and regulation of the rice sucrose transporters; the differential expression and regulation of each sucrose transporter and their localization during environmental abiotic and biotic stresses. Moreover, it will be intriguing to speculate on specific functions that these proteins might exhibit in addition to, or instead of, the simple catalysis of transmembrane sucrose transport. In future this information may aid in breeding programmes for the development of new tolerable rice varieties to drought, salinity and aphid infestation, and other environmental plant stresses.

2.7 REFERENCES

1) Chen, M. S. (2008) Inducible direct plant defense against insect herbivores: A Review. *Insect Science* **15**:101-114.
2) Hasegawa, P.M., Bressan, A.B., Zhu, J.-K., and Bohnert, H.J. (2000). Plant cellular and molecular responses to high salinity. *Annual Review of Plant Physiology and Plant Molecular Biology* **51**: 463–499.
3) Inbar, M., Doostdar, H., Leibee, G.L. and Mayer, R.T. (1999) The role of plant rapidly induced responses in asymmetric interspecific interactions among insect herbivores. *Journal Chemical Ecology* **25**:1961-1979.
4) Kehr, J. (2006) Phloem sap proteins: their identities and potential roles in the interaction between plants and phloem-feeding insects. *Journal of Experimental Botany* **57**:767-774
5) Matsiliza, B. and Botha, C.E.J. (2002) Aphid (*sitobion yakini*) investigation suggests thin walled sieve tubes in barley (*Hordeum vulgare*) to be more functional than thick-walled sieve tubes. *Physiologia Plantarum* **115**:137-143.
6) Miles P.W. (1999) Aphid saliva. *Biological Reviews* **74**:41-85.
7) Moran, P.J. and Thompson, G.A. (2001) Molecular responses to aphid feeding in *Arabidopsis* in relation to plant defense pathways. *Plant Physiology* **125**:1074-1085.
8) Pollard D. G. (1973) Plant penetration by feeding aphids (Hemiptera Aphidoidea) A Review. *Bulletins of Entomological Research* **62**:631-714.
9) Rontein, D., Basset, G. and Hanson A.D. (2002) Metabolic engineering of osmoprotectants accumulation in plants. *Metabolic Engineering* **4**:49-56.

10) Shinozaki, K., and Yamaguchi-Shinozaki, K. (2000). Molecular responses to dehydration and low temperature: Differences and cross-talk between two stress signaling pathways. *Current Opinion Plant Biology* **3**:217–223.

11) Thompson, G.A. and Goggin, F.L. (2006) Transcriptomics and functional genomics of plant defence induction by phloem-feeding insects. *Journal of Experimental Botany* **57**:755-766

12) Van de Ven, W.T.G., LeVasque, C.S., Perring, T.M. and Walling L.L. (2000) Local and systemic changes in squash gene expression in response to silverleaf whitefly feeding. *The Plant Cell* **12**:1409-1423

13) Walling, L.L. (2008) Avoiding effective defenses: strategies employed by phloem-feeding insects. *Plant Physiology* **146**:859-866

14) Will, T., Tjallingii, W.F., Thonnessen, A. and van Bel, A.J.E. (2007) Molecular sabotage of plant defence by aphid saliva. *Proceedings the National Academic of Science of the U.S.A* **104**:10536-10541

15) Will, T. and van Bel, A.J.E. (2006) Physical and chemical interactions between aphids and plants. *Journal of Experimental Botany* **57(4):**729-737

Chapter 3

A comparative *in-silico* Analysis of *Cis*-Acting Regulatory Elements in Promoter Regions of Sucrose Transporter Gene Families in Rice (*Oryza sativa* Japonica) And *Arabidopsis Thaliana*

3.1 INTRODUCTION

The formation of diverse cell types from an unchanging set of genes is governed by biochemical and molecular biological processes that regulate gene activity. The selective expression of this set of genes directs plant development, cellular differentiation and responses to environmental stimuli (Franklin and Cande, 1999). Transcription which is the central step to gene expression is the most widely studied process in cell and molecular biology (Wyeth and Albin, 2004). Gene specific regulation of transcription is of fundamental importance for virtually every aspect of cellular functions.

Much of the regulatory portion of plant genes is located primarily 1000 base pairs upstream of the transcriptional start site and this is generally referred to as the gene promoter region (Dean and Schmidt, 1995). The promoter region consists of specific DNA sequences and response elements that function in the recruitment of protein factors that facilitate transcription of the protein-coding region of the gene. These regulatory sequence elements located on the same strand as the coding region of the gene are called the *cis*-acting regulatory elements or the transcription factor binding sites (TFBs), and they may determine the temporal and spatial expression of the gene. The *cis*-acting regulatory elements are specific short DNA sequence motifs of approximately 5 – 25 base pairs (Rani, 2007). The promoter regions are presented as a proximal promoter to -100 bp upstream of the TATA box and a distal promoter up to 2 kb. The proximal sequence upstream of the gene tends to contain primary regulatory elements, while the distal sequence upstream may contain additional regulatory elements, but often with a weaker influence than the proximal promoter sequence.

Regulation of gene transcription is crucial for the function and development of all organisms. Each cell is the product of complex regulatory gene expression programs involving the transcription of thousands of genes. DNA-binding transcription factors (TFs) are one of the important components of this network. Transcription is shaped by the interaction between transcription factors (TFs) that usually bind to the *cis*-acting regulatory elements in the gene with some additional co-factors to activate or repress gene transcription in response to change in the environment, as well as during development. A single TF seldom controls the expression of a gene; rather evidence has shown that specific combinations of TFs are necessary in order to achieve expression of genes in higher organisms (Lloyd et al., 2001). This occurs by arrangement of multiple TFBs into modules in the promoters of each gene, usually a few hundred base pairs close to the transcription start site [(TSS); Qui, 2003]. Hence, the knowledge of the *cis*-acting regulatory region bound by TFs encoded in a genome can give valuable information essential to construct transcriptional regulatory networks (Qui, 2003).

Computational methods for the identification of *cis*-acting regulatory element sequences associated with genes have long been sought in lieu of the strenuous laboratory procedures required to identify them (Jeffery et al., 2007). Integration of computer science with biology has expedited molecular bioinformatics, processing of large scale data inputs such as microarrays, analysis of genomes, transcriptomes and proteomes (Qui, 2003; Jeffery et al., 2007).

Over the past several years, high-throughput technology has accelerated the sequencing of a large variety of genomes (*Arabidopsis*, rice, human, rat, etc.). Furthermore, gene expression profiling technology such as microarrays and serial analysis of the expression (SAGE) has provided a rapid and parallel analysis of the expression levels of hundreds of thousands of genes. As a consequence, global analysis of a gene regulatory network for understanding cell function has become feasible.

A major challenge of the post-sequencing era is to understand gene regulation on a global scale and to assign function to thousands of genes. Integrative approaches such as computational analysis of gene regulatory regions, their expression profiling and functional characterization are therefore required for gene discovery and pathway elucidation.

Organisms have devoted a significant fraction of their DNA to encrypt *cis*-acting regulatory programmes that control and coordinate gene expression at the level of transcription. The output(s) of the *cis*-acting regulatory programmes depends on the cellular context and extracellular inputs. Typically an external stimulus activates a signal transduction pathway which leads to modification of the activities of several TFs, which in turn target enhancers/regulatory regions of certain genes, effecting their expression.

3.2 PROMOTER PREDICTION

The first step towards the identification of a transcriptional network is to find the promoter region of a gene. Once the promoter region is defined, it will be possible to search for the *cis*-acting regulatory elements by screening the genomic sequence for their presence (Jolly et al., 2005). Although promoter regions are important for gene expression, the progress made so far in identifying them is generally less advanced than in identifying gene coding regions. This is as a result that gene promoter regions are widely diverse, and even highly recognized motifs are not always conserved in all promoters. These problems in promoter prediction occur because the interaction between the TFs proteins and DNA sequences are complex; the various elements involved are highly degenerate, so recognition of characteristic sequences within the promoter element is difficult. Most TFs bind to short degenerate sequence motifs occurring in the genome.

Algorithms that use the occurrence of particular motifs within the sequence and those based on position weight matrix (PWM) is thus frequently used to quantitatively represent the specificity of those sites (Martin et al., 2002). The usefulness of simple examples such as TATA is limited, because it assume independence between adjacent bases and do not allow for the presence of multiple promoter elements, insertions, deletions or variable spacing between elements (Qiu, 2003).

Computational detection of regulatory sites is an intricate task in eukaryotes as the sequence where TFs bind are usually much shorter and generally active in both orientations (van Helden et al., 1998; Caselle et al., 2002).

Many computational methods for promoters' prediction have been developed over the last several years. In general, this can be categorized into two groups. First is the signal-based approach, which depends on the recognition of conserved signals and spacing among patterns such as the TATA-box, CAAT-box, CCAAT-box and TFBs using neural network, genetic algorithm (Promoter 2.0; Knudsen, 1999) or using weight matrix based approach (ProScan; Prestridge, 1995). Second is the context-based approach, which differentiates promoter sequence from non-promoter sequence based on differences in sequence content such as triplet base-pair preferences around transcription start site (TSS) or hexamer frequencies in consecutive 100-bp up-stream region, using linear discriminant function (TSSG, TSSW; Solovyev and Salamov, 1997) or quadratic discriminant analysis (Core promoter; Zhang, 1998). Yet, while those programmes predicted about 13-54% of the promoters correctly, each programme also predicted a number of false positive promoters.

Promoter Inspector system, which was recently developed based on context feature extracted from training sequences by an unsupervised learning technique (Scherf et al., 2000) was shown to greatly reduce level of false positive recognition. The true positive to false positive ratio of 2.3 was obtained, as compared to 0.6 reported for TSSG (Scherf et al., 2000).

With the increase of well-annotated genes, many studies have predicted promoter sequences by aligning full-length mRNA with the corresponding genomic sequences. This approach achieved over 80% accuracy in promoter localization (Trinklein et al., 2003). However, due to the lack of full-length transcripts, a matching approach employed by Liu and States, (2002) was the use of expressed sequence tags (ESTs) to anchor the position of a gene, and then gene prediction software was used to identify the missing 5′ exons of the genes.The 5′ upstream region of the most predicted exon is then searched by another promoter-prediction algorithm for a candidate promoter. They claim that this method (CONPRO) can identify promoters with very high confidence.

3.3 CORE PROMOTER ELEMENTS

Within the proximal promoter region are core elements such as TATA, CAAT, CCAAT and InR and each represent distinct functional entity and are responsible for basal promoter activity. The core promoter encompasses a DNA region of approximately 80 to 100 base pair from position -40 to +40 or -50 to +50, relative to the transcription start site (+1), and can be operationally defined as the minimal stretch of DNA that is sufficient to direct basal levels of accurate transcription initiation in vitro. Core promoter structure has been shown to affect the amplitude by which genes respond to various activators and repressors which appear to play a crucial role in the specific communication between enhancers and their target genes. Computational analysis of the core promoter of plants (Shahmuradov et al., 2003) suggests that core promoters are quite heterogeneous in composition for different conserved *cis*-acting regulatory elements.

The most basic *cis*-acting regulatory element is the TATA-box, which is found in most eukaryotic genes located around position −30 (30 nucleotides upstream from the transcription start site which corresponds to the first nucleotide of the RNA). The TATA-box is often juxtaposed to other elements; the CAAT-box and CCAAT-box. Each element is named on the basis of its nucleotide sequence. The TATA-box is responsible for recruiting the essential RNA polymerase II (RNA pol. II) correctly to the transcription start site (TSS) initiating transcription (Dean and Schmidt, 1995).

Accurate initiation of mRNA synthesis in eukaryotic cells requires basal transcriptional machinery which includes RNA polymerase II and general transcription factors (TF): TFII A, B, D, E, F and H into an initiation-competent nucleoprotein complex (Pre-Initiation Complex; PIC) at the core promoter region (Brunelle and Chua, 1993; Smale and Kadonaga, 2003). TFIID consists of the TATA binding protein (TBP) and TBP-associated factors (TAFs). The universal feature of transcription is binding of TBP to DNA at a specific distance from transcription start site regardless of the presence/absence of the TATA box (Tsai and Sigler, 2000).

In the absence of the TATA box or a promoter with altered TATA-box, TAFs bind to DNA and to other TFs in order to involve TBP in pre-initiation. Consequently, a gene may still be able to recruit RNA polymerase II to a promoter with absence or altered TATA-box, but the efficiency of transcription initiation may be greatly compromised (Goodrich et al., 1996; Tsai and Sigler, 2000). Therefore, it is easy to comprehend why TATA box dominates as a core promoter element having the ability to govern transcription initiation alone, the rest of the core elements usually work in cooperation with others. Examining the effects of mutations on in vitro transcription, a duplicated TATA sequence in the TATA-box was reported to enhance transcription (Mukumoto, et al., 1993).

These studies established the importance of the TATA-box in the correct selection of a transcription initiation site.

The initiator element (InR) is a conserved sequence within the proximal promoter region and function to direct accurate transcription initiation in conjunction with TATA in the absence of other core promoter elements (Smale and Baltimore, 1989). The InR was originally identified as a discrete core promoter element essential for activity of the TATA-less murine terminal deoxynucleotidyl transferase (mTdT) gene promoter (Martinez et al., 1994). TATA and InR function together dramatically to increase the basal promoter activity, as compared with the presence of either TATA or InR alone (Smale and Baltimore, 1989; Martinez, et al., 1994; Zhu, et al., 1995).

Inducible genes always contain a TATA-box and at least two other *cis*-acting elements that play a role in the final stages of environmental signal transduction. Housekeeping genes on the other hand have less diversity in their *cis*-acting regulatory elements and may not even contain a recognizable TATA-box (Dean and Schmidt, 1995).

3.4 IDENTIFICATION OF *CIS*-ACTING REGULATORY ELEMENTS AND COMPOSITE TRANSCRIPTION FACTOR MODULES

After promoter regions are identified, search for *cis*-acting regulatory elements computationally by screening for the presence of TFBs motifs that have already been identified is then possible. Most TFs bind to short (5-25 base pairs) sequence motifs that occur regularly in the genome. A position weight matrix (PWM) is usually used to quantitatively represent the binding specificity of these TFs. Consensus sequence represented by IUPAC string or PWM can be used to search genome for known TF binding-site elements. Programmes have been developed to perform these searches based on PMW and IUPAC, and they include: SIGNAL SCAN, MATRIX SEARCH, Plant CARE, PLACE, Genomatix Matinspector professional, TFSearch etc. Transfac is a data base that collects TFs, TFBs and TFBs sequence profiles (IUPAC or PWM) generated from the published literatures (Matys, 2003).

3.5 STRESS INDUCED/REGULATED GENES

The stress responses of plants are regulated by multiple signaling pathways, and there is significant overlap between the patterns of gene expression that are induced in plants in response to different stresses. For example, the gene expression profiles observed during an incompatible plant fungal interaction overlap with those derived from wounding (Durrant et al., 2000). Stress

associated genes occur primarily at the level of transcription, and regulating the temporal and spatial expression patterns of specific stress genes is an important part of the plant stress response (Rushton and Somssich, 1998). Often, several closely related transcription factors have potential to activate or repress genes through *cis*-acting regulatory sequences that respond to specific stress. Plants have been attested to devote a large portion of their genome capacity to transcription, with the *Arabidopsis* genome coding in excess of 1500 transcription factors (Riechmann et al., 2000).

Plant hormones such as ethylene, salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA) are important regulators of stress responsive pathways. Ethylene and JA are commonly associated with defence pathways activated by pathogen infection and wounding (Zimmerli et al., 2004), while ABA is well known to regulate response to abiotic factors such as salinity and drought (Zhu, 2002). Recently, ABA has been implicated in the establishment of compatible interactions between fungal pathogens and host plants (Mohr and Cahill, 2003). Exogenous applications of ethylene (Jacobs et al., 1999), JA (Zimmerli et al., 2004) and ABA (Finkelstein et al., 2002) have been reported to elicit aspects of the host response to stress stimuli.

Many stress associated *cis*-acting regulatory elements that activate transcription in response to salinity, drought, wounding and pathogen infection have been identified in plants (Higo et al., 1999; Singh et al., 2002; Rani, 2007). Web-based databases such as Plant CARE, PLACE and Genomatix Matinspector professional have provided a convenient way to search for these previously identified promoter motifs in DNA sequences. Plant CARE, PLACE and Genomatix Matinspector professional are databases of plant *cis*-acting regulatory elements and provide access tools for *in silico* analysis of promoter sequences. These databases are a compilation of motifs found in plant *cis*-acting regulatory DNA elements, extracted from earlier published reports, and also from article reviews on the regulatory regions of various plant genes. The original reported motifs, as well as their variations in other genes or other plant species in later reports, are also incorporated (Higo et al., 1999; Lescot et al., 2002; Rani et al., 2007).

3.6 SPECIFIC OBJECTIVE

Sucrose transport is a process highly regulated at the cell level and the plant level. It must respond to variations in the source/sink ratio which in turn depend on many internal and external parameters such as growth, development, photosynthetic activity, light environment, water status, phytopathogen attack, etc. This flexibility is made possible by the presence of multigenic family whose members are expressed in different cells at different times, and under different conditions.

Although various sucrose transporters have been cloned from plant tissues (Sauer, 2007), little is known so far about the expression and the activities of these transporters to environmental

stress. To date, no work has been published on the transcription factors/ *cis*-acting regulatory elements that regulate the expression of sucrose transporter genes during stress.

Therefore the focus of this study was to use available bioinformatic tools to reveal a comprehensive and comparative description of *cis*-acting regulatory elements that are present within the 5´- untranslated region (5´ UTR) of the DNA sequences of sucrose transporter gene families in rice (*Oryza sativa* Japonica cultivar - group) and *Arabidopsis thaliana*; as a source of potential useful promoters for the expression and regulation of these genes. This analysis is the first step towards uncovering transcription regulatory interactions of these genes during development or under environmental stress conditions.

3.7 METHOD

3.7.1 Identification of the 5´Utr of Sucrose Transporter Genes

The complete nucleotide sequence of the sucrose transporter gene family in rice as registered in GenBank by Aoki et al. (2003), under the accession number: OsSUT1 (AF280050), OsSUT2 (AB091672), OsSUT3 (AB071809), OsSUT4 (AB091673), OsSUT5(AB0916674) were obtained from National Center for Biotechnology Information (NCBI) internet database site (www.ncbi.nih.nim.gov). Similarly, the nucleotide sequence of the sucrose transporter gene family in *Arabidopsis thaliana* as registered in Genbank under the accession number: AtSUC1 (At1g71880), AtSUC2 (At1g22710), AtSUC3/AtSUT2 (At2g02860), AtSUC4 (At1g09960), AtSUC5 (At1g71890), AtSUC6 (At5g43610), AtSUC7 (At1g66570), AtSUC8 (At2g14670), AtSUC9 (At5g06170); (Sauer, 2007) were also obtained from National Center for Biotechnology Information (NCBI) internet database site.

Individual sequences of rice and *Arabidopsis thaliana* sucrose transporter gene families were confirmed against the *Oryza sativa* (Japonica cultivar-group) genome and *Arabidopsis thaliana* genome respectively, using the Basic Local Alignment Tool (BLASTN; http://blast.ncbi.nlm.nih.gov/Blast.cgi). Locus link was used to identify a genomic sequence of 1.5 kbp extending 5´ from the translation start site of each sucrose transporter gene to the next gene. These sequences were used for the computational analysis.

3.7.2 *Cis*-Acting Regulatory Elements Analysis

Using database associated search tools, 1.5 kbp of 5′ regulatory region of each sucrose transporter gene in rice and *Arabidopsis thaliana* were scanned for the presence of putative *cis*-acting regulatory elements identical with or similar to the motifs registered in Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/cgi-bin/CallMat_IE55.htpl), PLACE

(http://www.dna.affrc.go.jp/PLACE/signalscan.html) and Genomatix Matinspector professional (http://www.genomatix.de/cgi-bin/matinspector_prof/mat_fam.p1).

The sucrose transporter genes 1.5 kbp of 5′ regulatory region of the coding regions were aligned using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) to search for possible sequence similarities between these regions. Possible sequence similarities between the genes coding sequences and protein sequences of the rice and *Arabidopsis thaliana* sucrose transporter genes; obtained from NCBI were also identified using ClustalW.

3.8 RESULTS

The BLASTN result of the sucrose transporter genes in rice (*Oryza sativa* Japonica cultiva-group) and *Arabidopsis thaliana* genomes are tabulated in Table 3.1. Genomic sequences 1.5kbp up-steam from the translational start site of each gene (as indicated by the start of the coding region, CDS in Table 3.2) were used in the promoter prediction software to identify the presence of several *cis*-acting regulatory elements known to be involved in transcription regulation of plant genes and which could possibly be controlling the expression of the SUT genes in rice (*Oryza sativa* Japonica cultivar-group) and *Arabidopsis thaliana*. This region was used in this study as no introns were indicated upstream of the translational start site of the studied genes, as indicated by the presence of the translational start site within the first exon of all the genes (Table 3. 2), and thus possible regulatory information within the 5′ untranslated region (5′ UTR) could also be identified. Several functional significant *cis*-acting regulatory elements that are associated with plant development, hormonal regulation and stress response were identified upstream of the sucrose transporter gene families in rice (*Oryza sativa* Japonica cultivar- group) and *Arabidopsis thaliana* (Figures 3.1 and 3.2). The names of the identified *cis*-acting elements and their predicted functions are tabulated in Table 3, and their frequencies of occurrence are summarized in Figures 3.3 and 3.4. The selected *cis*-acting regulatory elements were common for the majority of analyzed sucrose transporter gene 5′ regulatory regions .All sequences referred to are numbered from the1st base of the translational start site(ATG).The location of the TATA, CAAT, CCAAT and InR motifs closest to the translational start site (ATG)are also indicated.

Table 3.1: The BLASTN result of rice (*Oryza sativa* Japonica cultiva-group) and *Arabidopsis* sucrose transporter gene families.

GENE	ACCESSION NUMBER	NUMBER OF BASE PAIRS	CDS REGION	5′ UTR BEFORE ATG	LOCATED ON CHROMOSOME	REFERENCE NUMBER	HOMOLOGY TO THE CHROMOSOMAL REGION	CHROMOSOME TOTAL LENGTH	POSITION ON CHROMOSOME
OsSUT1	AF280050	7717	1481 -7358	1 - 1480	3	NC_008396.1	99.8%	36192742	3787231 - 3779481
OsSUT2	AB091672	1869	76 - 1581	1 - 75	12	NC_008405.1	99.6%	27566993	27554242 - 27550273
OsSUT3	AB071809	1713	85 - 1605	1 - 84	10	NC_008403.1	100%	22685906	13263493 - 13269713
OsSUT4	AB091673	2158	40 - 1827	1 - 40	2	NC_008395.1	99.9%	35954743	35567546 - 35571906
OsSUT5	AB091674	1972	12 - 1619	1 - 11	2	NC_008395.1	99.3%	35954743	22169450 - 22165819
AtSUC1	At1g71880	1542	155 -1696	1 - 154	1	NC_003070.9	100%	30427671	27054180 - 27056341
AtSUC2	At1g22710	1956	1 - 1539	None	1	NC_003070.9	100%	30427671	8030641 - 8033117
AtSUC3/ AtSUT2	At2g02860	2138	160 - 1944	1 - 159	2	NC_003071.7	100%	19698290	828352 - 832455
AtSUC4	At1g09960	1974	46 - 1578	1 - 45	1	NC_003070.9	100%	30427671	3244258 - 3247200
AtSUC5	At1g71890	1804	54 - 1592	1 -53	1	NC_003070.9	100%	30427671	27058492 - 27060785
AtSUC6	At5g43610	1479	1 - 1479	None	5	NC_003076.8	100%	26975502	17519079 - 17521008
AtSUC7	At1g66570	1613	81 - 1556	1 -80	1	NC_003070.9	100%	30427671	24835251- 24837322
AtSUC8	At2g14670	1479	1 -1479	None	2	NC_003071.7	100%	19698290	6274606 - 6276317
AtSUC9	At5g06170	1476	1 - 1476	None	5	NC_003076.8	100%	26975502	1869791 - 1871719

Table 3.2: Location of the mRNA and CDS sequences of rice (*Oryza sativa* Japonica cultiva-group) and *Arabidopsis thaliana* sucrose transporter gene families as they appear in the different chromosomes, indicating the positions of all exons and introns. Positional information was obtained from the complete chromosome sequence corresponding to the Reference Number indicated in Table 1. [a] This indicates the position of the transcription start site (TSS); [b] This indicates the position of the translation start site (ATG).

Gene	Chr.	Reference Number	Gene Location	Gene Reference	mRNA Location[a]	CDS Location[b]
OsSUT1	3	NC_008396.1	3779594-3785919	Os03g0170900	Join (3779594..3780012, 3780129..3780172, 3780256..3780319, 3780422..3780655, 3780765..3780908, 3781027..3781221, 3781447..3781522, 3781689..3781788, 3781955..3781988, 3782076..3782160, 3782226..3782290, 3782380..3782478, 3782810..3782875, 3785485..3785919)	Join (3779839..3780012, 3780129..3780172, 3780256..3780319, 3780422..3780655, 3780765..3780908, 3781027..3781221, 3781447..3781522, 3781689..3781788, 3781955..3781988, 3782076..3782160, 3782226..3782290, 3782380..3782478, 3782810..3782875 ,3785485..3785721)
OsSUT2	12	NC_008405.1	27550105-27554222	Os12g0641400	Join (27550105..27550709, 27551473..27551516, 27551588..27551651, 27551733..27552463, 27553663..27553875, 27553895..27554222)	Join (27550548..27550709, 27551473..27551516, 27551588..27551651, 27551733..27552463, 27553663..27553875, 27553895..>27554150)
OsSUT3	10	NC_008403.1	13263493 - 13269713	Os10g0404500	Join (13263493..13263732, 13265391..13265456, 13265607..13265705, 13267020..13267084, 13267210..13267294, 13267389..13267422, 13267539..13267638, 13267811..13267886, 13268085..13268408, 13269090..13269713)	Join (13263577..13263732, 13265391..13265456, 13265607..13265705, 13267020..13267084, 13267210..13267294, 13267389..13267422, 13267539..13267638, 13267811..13267886, 13268085..13268408, 13269090..13269605)
OsSUT4	2	NC_008395.1	35567546 - 35571907	Os02g0827200	Join (35567546..35567848, 35568303..35568368, 35568475..35568573, 35568652..35568716, 35568802..35568886, 35569028..35569061, 35569147..35569246, 35569738..35569813, 35569974..35570315, 35570426..35570569, 35570840..35571073, 35571149..35571212, 35571297..35571340, 35571431..35571907)	Join (35567585..35567848, 35568303..35568368, 35568475..35568573, 35568652..35568716, 35568802..35568886, 35569028..35569061, 35569147..35569246, 35569738..35569813, 35569974..35570315, 35570426..35570569, 35570840..35571073, 35571149..35571212, 35571297..35571340, 35571431..35571601)
OsSUT5	2	NC_008395.1	22165822-22169540	Os02g0576600	Join (22165822..22166318, 22166422..22166465, 22166563..22166626, 22166729..22167109, 22167296..22167475, 22167661..22167736, 22167844..22167946, 22168060..22168093, 22168216..22168300, 22168389..22168453, 22168592..22168690, 22168960..22169025, 22169200..22169540)	Join (22166148..22166318, 22166422..22166465, 22166563..22166626, 22166729..22167109, 22167296..22167475, 22167661..22167736, 22167844..22167946, 22168060..22168093, 22168216..22168300, 22168389..22168453, 22168592..22168690, 22168960..22169025, 22169200..22169439)
AtSUC1	1	NC_003070.9	27054180 - 27056341	AT1G71880	Join (27054180..27055593, 27055680..27055743, 27055883..27056341)	Join (27054334..27055593, 27055680..27055743, 27055883..27056100)

AtSUC2	1	NC_003070.9	8030641 - 8033117	AT1G22700	Join (8030641..8031084, 8031174..8031217, 8031505..8031568, 8031714..8033117)	Join (8030911..8031084, 8031174..8031217, 8031505..8031568, 8031714..8032970)
AtSUC3/ AtSUT2	2	NC_003071.7	828352 - 832455	AT2G02860	Join (828352..828713, 828830..828873, 828970..829033, 829125..829358, 829456..829599, 829728..830066, 830350..830425, 830531..830630, 830740..830773, 830866..830950, 831044..831083, 831210..831308, 831456..831521, 832030..832455)	Join (828546..828713, 828830..828873, 828970..829033, 829125..829358, 829456..829599, 829728..830066, 830350..830425, 830531..830630, 830740..830773, 830866..830950, 831044..831108, 831210..831308, 831456..831521, 832030..832296)
AtSUC4	1	NC_003070.9	3244258 - 3247200	AT1G09960	Join (3244213..3244795, 3245159..3245886, 3246023..3246086, 3246215..3246258, 3246560..3246741, 3246828..3247200)	Join (3244258..3244795, 3245159..3245886, 3246023..3246086, 3246215..3246258, 3246560..3246718)
AtSUC5	1	NC_003070.9	27058439 - 27060785	AT1G71890	Join (27058439..27059748, 27059837..27059900, 27060356..27060785)	Join (27058492..27059748, 27059837..27059900, 27060356..27060573)
AtSUC6	5	NC_003076.8	17519079 - 17521008	AT5G43610	Join (17519079..17520323, 17520426..17520489, 17520839..17521008)	Join (17519079..17520323, 17520426..17520489, 17520839..17521008)
AtSUC7	1	NC_003070.9	24835251-24837322	AT1G66570	Join (24835251..24835478, 24835837..24835900, 24836001..24837322)	Join (24835309..24835478, 24835837..24835900, 24836001..24837242)
AtSUC8	2	NC_003071.7	6274606 - 6276317	AT2G14670	Join (6274606..6274775, 6274908..6274971, 6275073..6276317)	Join (6274606..6274775, 6274908..6274971, 6275073..6276317)
AtSUC9	5	NC_003076.8	1869791 - 1871719	AT5G06170	Join (1869791..1871032, 1871130..1871193, 1871550..1871719)	Join (1869791..1871032, 1871130..1871193, 1871550..1871719)

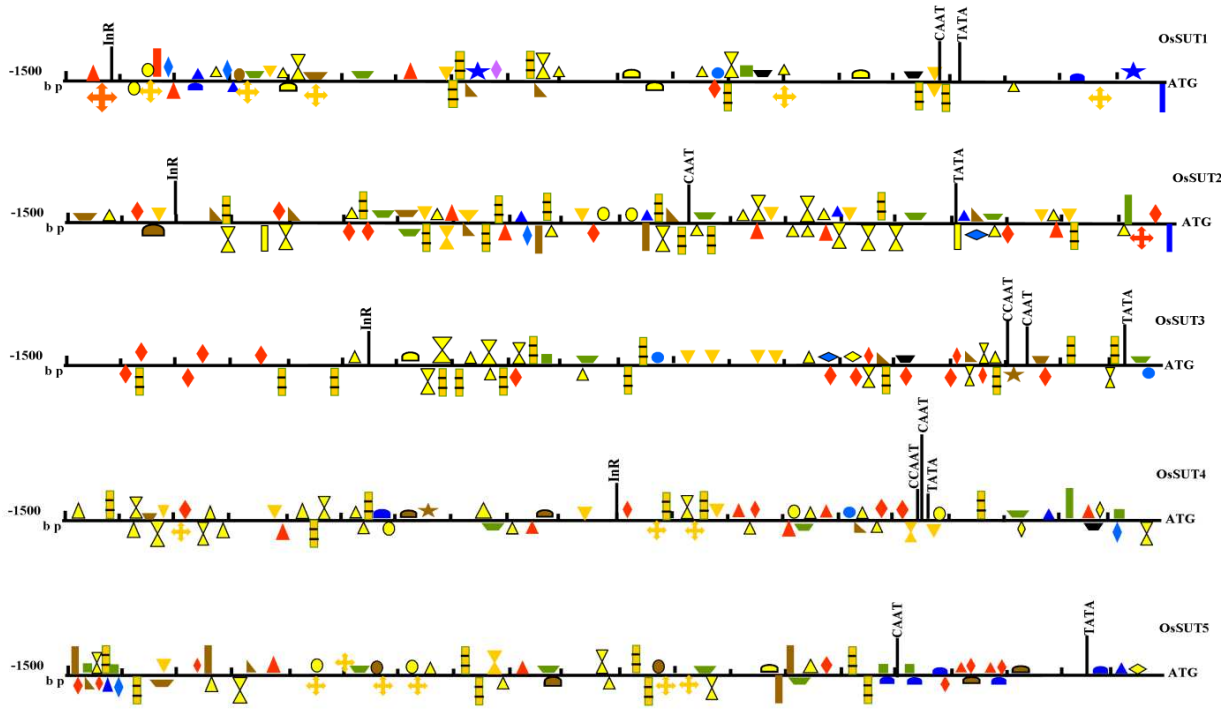


Figure 3.1 Map of the promoter regions of rice (*Oryza sativa* Japonica cultiva-group) sucrose transporter gene family. The 1.5 kb region upstream of the translational start site was analyzed using PLACE, Plant CARE and Genomatix Matinspector professional databases.The consensus sequences corresponding to the various putative *cis*- acting regulatory elements are described in Table 3.3. Positions are with respect to the first base of the translation start site (ATG).

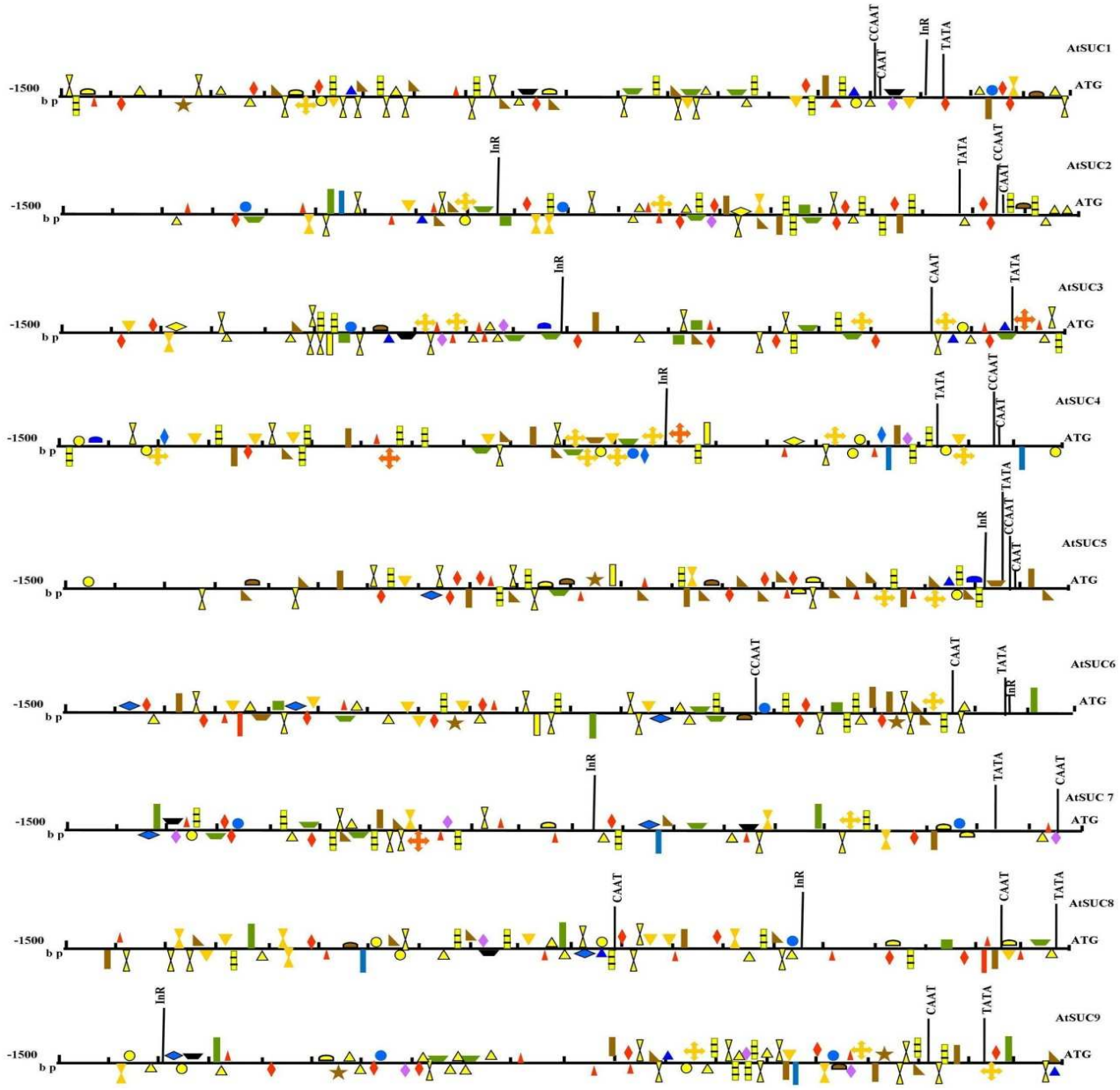
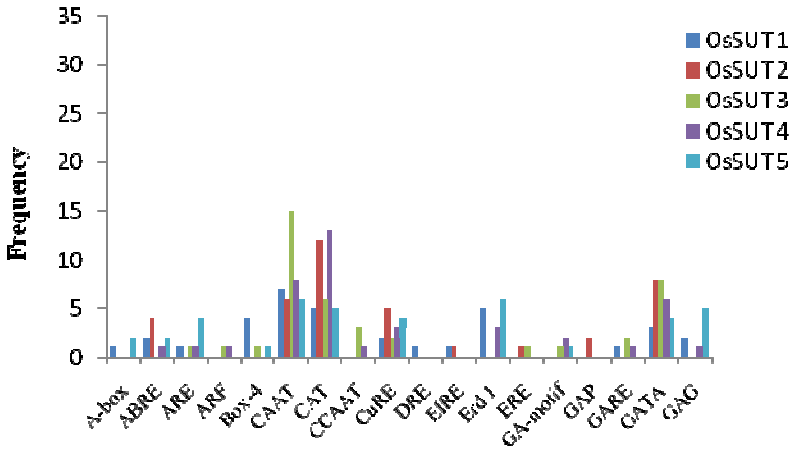


Figure 3.2 Map of the promoter regions of *Arabidopsis thaliana* sucrose transporter gene family. The 1.5 kb region upstream of the translational start site was analyzed using PLACE, Plant CARE and Genomatix Matinspector professional databases. The consensus sequences corresponding to the various putative *cis*-acting regulatory elements are described in Table 3.3. Positions are with respect to the first base of the translation start site (ATG).

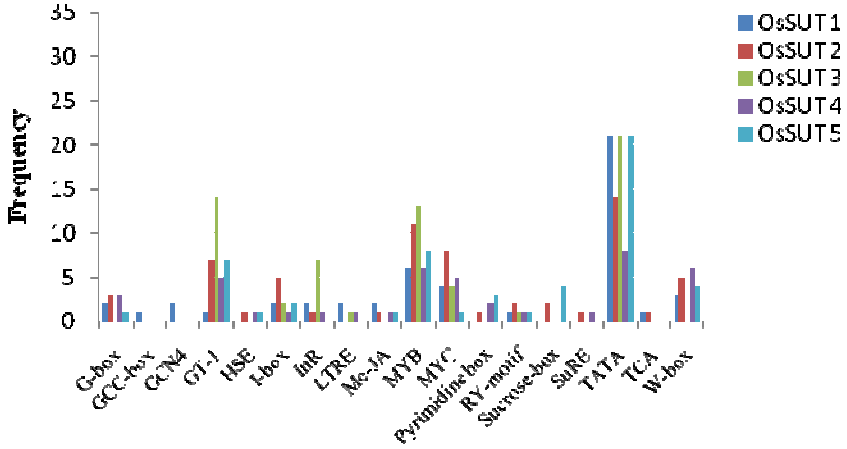
Table 3.3: Putative *cis*-acting regulatory elements identified in the promoter regions of rice (*Oryza sativa* Japonica cultiva-group) and *Arabidopsis thaliana* sucrose transporter gene families. The 1.5 kb region upstream of the translational start site was analyzed using PLACE, Plant CARE and Genomatix Matinspector professional databases.

CIS-ACTING ELEMENTS	SEQUENCE	SYMBOL	FUNCTION	REFERENCE
DRE	A/GCCGAC		Salt/drought responsive element	Dubouzet, et al., 2003
ABRE	(C/A)ACG(T/C)G(T/C/G)		Abscisic Acid Responsive Element; a *cis*-acting regulatory element involved in abscisic acid responsiveness	Kaplan, et al., 2006
A-box (G-motif)	TACGTA		Sugar repression	Toyofuku, et al., 1998
ARE	TGGTTT		Anti-oxidant Responsive Element; a *cis*-acting regulatory element essential for anaerobic induction	Nguyen, et al., 2003
MYC	CATGTG; CACATG; CANNTG		*Cis*-acting regulatory element involved in early response to drought and abscisic acid induction	Shinozaki and Yamaguchi-Shinozaki, 2000; Abe, et al., 2003
GARE	TAACAA(G/A)		Gibberellin Responsive Element; a *cis*-acting regulatory element involved in Gibberellin responsiveness and also partially involved in sugar repression	Gubler and Jacobsen, 1992; Ogawa, et al., 2003
MYB	WAACCA; TAACTG; CNGTTR; YAACKG; GGATA; CAACTG		*Cis*-acting regulatory element involved in regulation of drought inducible gene expression	Shinozaki and Yamaguchi-Shinozaki, 2000; Abe, et al., 2003
TCA	TCAGAAGAGG; TCATCTTCTT		*Cis*-acting regulatory element involved in salicylic acid responsiveness	Kim et al., 1993; Goldsbrough, et al., 1993
Box-4	ATTAAT		Part of a conserved DNA module involved in light responsiveness	Lois, et al., 1989
W-box	TTGAC; TGACT; TGACY; TGAC		*Cis*-acting regulatory element involved in direct fungal elicitor stimulated transcription of defense genes and activation of genes involved in response to wounding	Maleck et al., 2000; Eulgem, 2000; Chen, et al., 2002; Yamamoto, et al.,2004; Zheng et al., 2006; Ross et al., 2007.
Me-JA motif	CGTCA; TGACG		*Cis*-acting regulatory element involved in methyl jasmonates responsiveness	Kim et al., 1993; Rouster, et al., 1997
G-box	CACGTG		*Cis*-acting regulatory element involved in light responsiveness	Sessa , et al., 1995; López-Ochoa, et al., 2007
GAG	GGAGATG		Part of light responsive element	Lois, et al., 1989
RY-motif	CATGCATG		*Cis*-acting regulatory element involved in seed –specific regulation	Ezcurra, et al., 1999; Ezcurra et al., 2000
Erd1	ACGT		*Cis*-acting regulatory element required for early response to dehydration (etiolation –	Simpson, et al., 2003

			induced expression).	
EIRE	TTCGACC		Elicitor Responsive Element; a *cis*-acting regulatory element essential for elicitors responsiveness	Rushton , 1996; Fukuda, 1997
GCC-box	GCCGCC		Core of GCC-box found in many pathogen- responsive genes such as PDF1.2, Thi2.1 and PR4. Also found to function as ethylene-responsive element.	Sessa , et al., 1995; Brown, et al., 2003
GT-1	GAAAAA; GGTTAA		*Cis*-acting regulatory element required for rapid response to pathogen attack, salinity and salicylic acid inducible gene expression	Park, et al., 2004
I-box	GATAA		Light box Element; a *cis*-acting regulatory element conserved in sequence upstream of light-regulated genes	Hiratsuka and Chua, 1997; López-Ochoa, et al., 2007
GATA	GATA		*Cis*-acting regulatory element required for high level light regulated and tissue specific expression	Lam and Chua, 1989;
CAT	GCCACT		*Cis*-acting regulatory element related to meristem (mesophyll) expression	Manevski, et al., 2000
ERE	A(A/T)TTCAAA		Ethylene responsive element	Itzhaki, et al., 1994; Lin, et al., 2007
CuRE	GTAC		Copper responsive element and also involved in oxygen response	Quinn, et al., 2000
Pyrimidine box	TTTTTTCC; CCTTTT		Partially involved in sugar repression. It requires Gibberellin for its induction	Washio, K. ,2003
SuRE	GAGAC		Sulphur responsive element	Maruyama-Nakashita, et al., 2005
GAP	ATGA		Light responsive element	Park, 1996
Sucrose box	NNAATCA		*Cis*-acting regulatory element required for sugar responsive gene expression	Fillion, et al., 1999; Chen, et al., 2002
HSE	CNNGAANNTTCNNG		Heat Stress Element; a *cis*-acting regulatory element involved in Heat Stress responsiveness	Gao, et al., 2008; Larkindale and Vierling, 2008
GA-motif	AAGGAAGA		*Cis*-acting regulatory element involved in light responsiveness	López-Ochoa, et al., 2007
LTRE	ACCGACA; CCGAAA; GTCGAC		Low Temperature Response Element; a *cis*-acting regulatory element required in cold inducible gene expression	Nordin, 1993; Brown, et al.,2001; Dunn, et al., 1998
ARF (AuRE)	GGTCCAT; TGTCTC		*Cis*-acting regulatory element involved in Auxin responsiveness	Ulmasov, et al., 1999, Hagen, G. and Guilfoyle, T. 2002
GCN4	CAAGCCA		*Cis*-acting regulatory element involved in endosperm expression	Wu et al.,1998; Washida, et al., 1999



(A) ***Cis*-acting regulatory elements**



(B) ***Cis*-acting regulatory elements**

Figure 3.3: Illustration of the frequencies of the *cis*-acting regulatory elements identified in the promoter sequences of rice (*Oryza sativa* Japonica cultiva-group) sucrose transporter gene family.

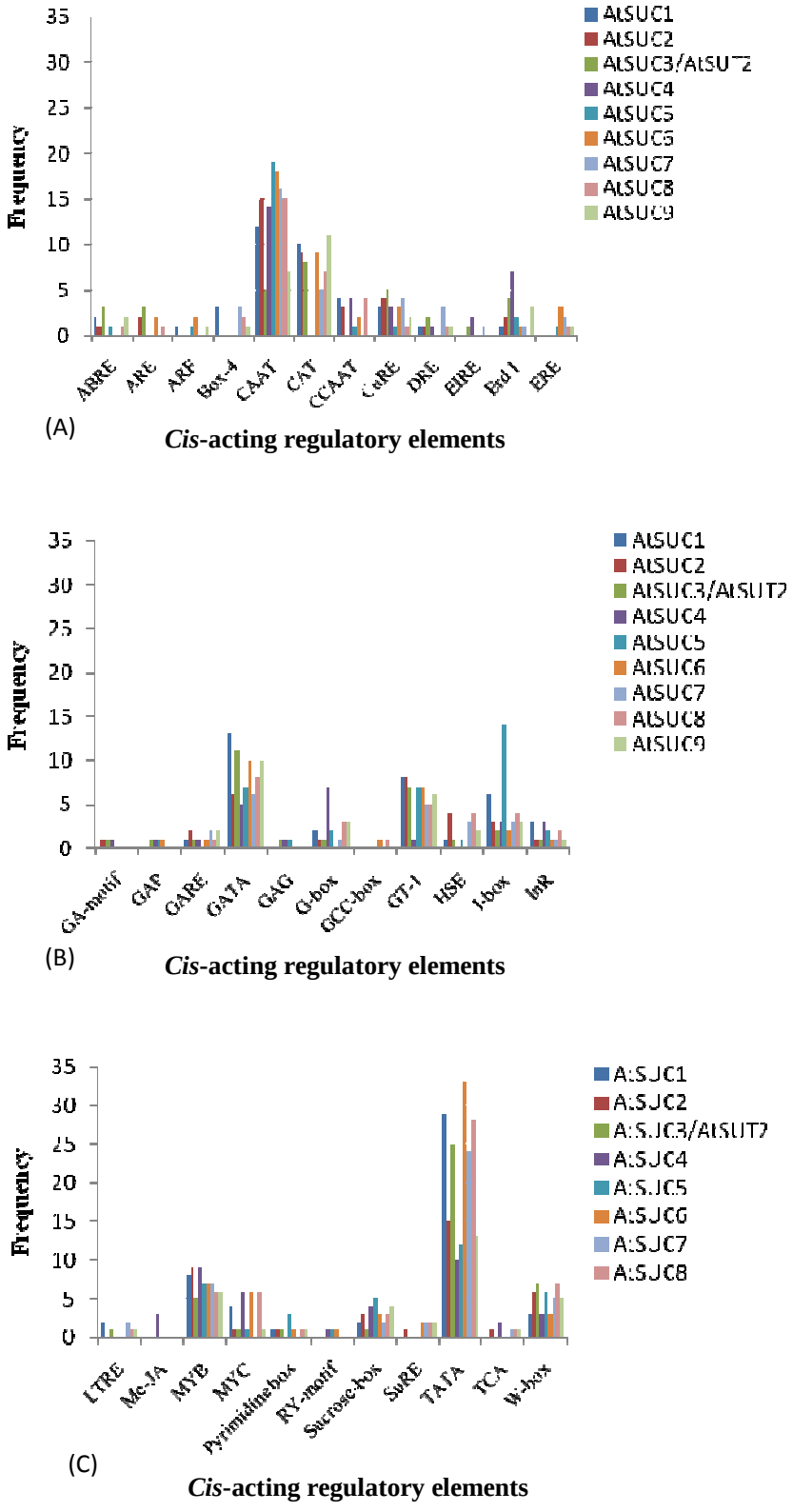


Figure 3.4: Illustration of the frequencies of the *cis*-acting regulatory elements identified in the promoter sequences of *Arabidopsis thaliana* sucrose transporter gene family.

In rice (*Oryza sativa* Japonica cultiva-group) sucrose transporter gene family, the core elements: TATA, CAAT and CCAAT motifs which are closest to the translational start site (ATG) were found to be very close to one another. However InR was found at the distal part of the 5′ regulatory region in all of the genes. Conversely in *Arabidopsis thaliana* sucrose transporter gene family; TATA, CAAT, CCAAT and InR motifs closest to the translational start site (ATG) were found to be very close to one another in several of the genes (AtSUC1, AtSUC5, AtSUC6, AtSUC8), while in the rest (AtSUC2, AtSUC3, AtSUC4, AtSUC7 and AtSUC9); the InR motifs were found at the distal part of the 5′ regulatory region.

Localization of the putative *cis*-acting regulatory elements indicate that; OsSUT1 and OsSUT5 gene expression may be well regulated at the distal region, whilst OsSUT3 gene expression at the proximal region. However OsSUT2 and OsSUT4 have the *cis*-acting regulatory elements evenly spread across the 1.5 kbp 5′ regulatory region. In the case of *Arabidopsis thaliana*; AtSUC2, AtSUC5 and AtSUC9 may be well regulated at the proximal region, whilst the localization of putative *cis*-acting regulatory elements in the rest of the *Arabidopsis* sucrose transporter genes are evenly spread across the 1.5 kbp 5′ regulatory region. Table 3.4 summarises the organization of the *cis*-acting regulatory elements per 300 base pairs upstream of the start codon in each sucrose transporter gene in terms of: cellular development, hormonal regulation and stress response associated *cis*-acting regulatory elements. OsSUT2, OsSUT4, OsSUT5, AtSUC1, AtSUC2, AtSUC6 and AtSUC9 have high number of *cis*-acting regulatory elements that are associated to cellular development as compared to the other sucrose transporters. Low numbers of hormonal regulation associated *cis*-acting regulatory elements were found for the majority of rice and *Arabidopsis* sucrose transporter genes; however significant amounts were found upstream of the coding region for the OsSUT2, AtSUC6 and AtSUC9 genes. Stress associated *cis*-acting regulatory elements were present in appreciable amount in all the rice and *Arabidopsis* sucrose transporters regulatory regions.

CuRE, GATA, GT-1 and MYB motifs were found to be widespread at varying frequencies in all the regulatory regions of both rice and *Arabidopsis* sucrose transporter gene families, whereas others *cis*-acting regulatory elements were found in only some of the regulatory regions of the sucrose transporter families. The W-box motif was widespread at varying frequencies in all the regulatory regions of the sucrose transporter gene families, except OsSUT3 gene, whereas the CAT motif was found in all the regulatory regions of the sucrose transporter gene families at varying frequencies, except AtSUC4 and AtSUC5 genes.

The ABRE motif was found to be distributed within the regulatory regions of all the rice and *Arabidopsis* sucrose transporter genes, except OsSUT3, AtSUC4, AtSUC6 and AtSUC7 genes. The

Me-JA motif was found to be distributed within the regulatory regions of all the rice sucrose transporter genes, except OsSUT3, whereas it was only found in the regulatory region of AtSUC4. GARE was found in OsSUT1, OsSUT3 and OsSUT4 genes, but not in OsSUT2 and OsSUT5 genes, whereas it was found in all the sucrose transporter genes in *Arabidopsis*, except AtSUC5 gene. ERE motif‘s were found in OsSUT2, OsSUT3, AtSUC5, AtSUC6, AtSUC7, AtSUC8 and AtSUC9 genes, whereas the TCA motif was only present in the OsSUT1, OsSUT2, AtSUC2, AtSUC4, AtSUC7, AtSUC8 and AtSUC9 genes and the ARF (AuxRE) motif only in the OsSUT3, OsSUT4, AtSUC1, AtSUC5, AtSUC6 and AtSUC9 genes.

Light responsive *cis*-acting regulatory elements such as Box-4, I-box, GA-motif, GATA, GAP and G-box, were located at various frequencies in all the 5′ regulatory region of the sucrose transporter genes, with the GATA-motif and I-box being the most prominent among them.

Motifs associated with sugar responsive or sugar repression gene expression such as: Sucrose-box, Pyrimidine-box and A-box and those involved in seed-specific regulation such as CAT, GCN4 and RY-motif were located on all the 5′ regulatory regions of the sucrose transporter gene families at different frequencies (Figure 3.3 and Figure 3.4).

The drought associated *cis*-acting regulatory elements Erd 1, MYB and MYC; and the temperature associated *cis*-acting regulatory elements LTRE and HSE; were also present at varying frequencies in all the sucrose transporter gene families (Figure 3.3 and Figure 3.4).

The *cis*-acting regulatory elements associated with wounding, elicitor and pathogen attack; EIRE, GT-1 motifs and GCC-box; were found in the regulatory region of all the sucrose transporter gene families, as were the ARE (Anti-oxidant Responsive Element), CuRE (Copper responsive element) and SuRE (Sulphur responsive element) motifs.

Analysis of the 5′ regulatory regions of rice and *Arabidopsis* sucrose transporter genes, revealed a high divergence in these regions (2 -18% identity, Table 3.5). The coding regions of the rice sucrose transporter genes appear to be poorly conserved (4 - 55% identity; Table 3.5), whereas the coding region for the *Arabidopsis* sucrose transporter genes appear to be more conserved (18-93% identity; Table 3.5). The analysis of the protein sequences of the rice and *Arabidopsis* sucrose transporters revealed higher conservation in the sequences. A 40-71% identity was obtained for the rice protein sequences (Table 3.6) while 42-96% identity was obtained for *Arabidopsis* (Table 3.7).

Table 3.4: Percentage composition of *cis*-acting regulatory elements per 300 base pair up-stream of 5´ UTR of rice (*Oryza Sativa*) and *Arabidopsis thaliana* sucrose transporter genes

	Region +1 to -300				**Region -301 to -600**				**Region -601 to -900**				**Region -901 to -1200**				**Region -1201 to -1500**			
Gene	Total No of *Cis*-element	A (%)	B (%)	C (%)	Total No of *Cis*-element	A (%)	B (%)	C (%)	Total No of *Cis*-element	A (%)	B (%)	C (%)	Total No of *Cis*-element	A (%)	B (%)	C (%)	Total No of *Cis*-element	A (%)	B (%)	C (%)
OsSUT1	5	40	20	40	11	9.1	0	90.9	10	20	10	70	11	18.2	0	81.8	19	5.3	21	73.7
OsSUT2	17	17.7	23.5	58.8	16	25	6.3	68.7	18	22.2	16.7	61.1	18	22.2	0	77.8	11	27.3	0	72.7
OsSUT3	16	18.8	0	81.2	13	15.4	0	84.6	11	9.1	9.1	81.8	11	27.3	0	72.7	7	0	0	100
OsSUT4	11	0	18.2	81.8	17	23.5	11.8	64.7	11	27.3	0	72.7	12	33.3	8.3	58.4	14	35.7	0	64.3
OsSUT5	10	20	10	70	14	21.4	0	78.6	12	25	0	75	13	23.1	0	76.9	19	15.8	10.5	73.7
AtSUC1	14	36	14	50	17	17.6	5.9	76.5	12	8.4	0	91.6	18	5.6	11.1	83.3	11	27.3	9.1	63.6
AtSUC2	13	46.2	0	53.8	21	19	4.8	76.2	13	15.4	7.7	76.9	15	0	13.3	86.7	5	20	20	60
AtSUC3	15	13.3	13.3	73.4	14	0	0	100	12	41.7	0	58.3	21	4.8	9.5	85.7	7	14.3	14.3	71.4
AtSUC4	12	8.4	33.3	58.3	9	0	0	100	18	11.1	11.1	77.8	10	10	0	90	12	8.3	8.3	83.4
AtSUC5	15	20	6.7	73.3	19	10.5	0	89.5	16	12.5	6.3	81.2	11	9.1	9.1	81.8	3	33.3	0	66.7
AtSUC6	12	16.7	8.3	75	16	31.3	6.3	62.4	12	8.3	8.3	83.4	15	13.3	13.3	73.4	11	36.4	9.1	54.5
AtSUC7	9	33.3	11.1	55.6	10	10	0	90	10	10	20	70	19	15.8	5.3	78.9	11	0	18.2	81.8
AtSUC8	12	8.3	0	91.7	10	20	10	70	18	11.1	11.1	77.8	14	14.3	7.1	78.6	11	9.1	0	90.9
AtSUC9	15	13.3	13.3	73.4	24	20.8	20.8	58.4	11	27.3	0	72.7	14	35.7	21.4	42.9	9	22.2	0	77.8

As refer in the table:

A is cellular development associated *cis*-acting regulatory elements (A-box, RY, CAT, Pyrimidine-box, Sucrose-box and GCN4)

B is hormonal regulation associated *cis*-acting regulatory elements (ABRE, ARF, ERE, GARE, TCA, and Me-JA)

C is stress response associated *cis*-acting regulatory elements (ARE, BOX-4, CuRE, DRE, Erd-1, EIRE, GA-motif, GAG, GAP, GATA, G-box, GCC-box, GT-1, HSE, I-box, LTRE, MYB, MYC, SuRE and W-box)

Table 3.5: The clustal-W multiple sequence alignment of rice (*Oryza sativa* Japonica cultiva-group) and *Arabidopsis thaliana* sucrose transporter gene families.

% Homology of Coding Regions

% Homology of Non-Coding Regions

	OsSut1	OsSut2	OsSut3	OsSut4	OsSut5	AtSuc1	AtSuc2	AtSuc3	AtSuc4	AtSuc5	AtSuc6	AtSuc7	AtSuc8	AtSuc9
OsSut1		6	8	4	5	6	5	7	4	5	5	5	5	3
OsSut2	2		35	18	22	53	44	20	48	45	55	51	55	55
OsSut3	8	1		53	55	33	45	47	27	20	50	34	37	36
OsSut4	3	2	1		21	22	16	50	18	18	21	20	22	22
OsSut5	6	2	2	4		48	39	20	16	26	32	28	32	36
AtSuc1	6	3	4	1	3		74	24	53	83	77	73	76	75
AtSuc2	2	2	4	3	1	2		18	40	68	70	65	70	69
AtSuc3	2	3	3	3	3	2	4		42	20	24	22	24	24
AtSuc4	1	2	1	1	1	1	1	1		46	55	50	55	55
AtSuc5	4	2	3	1	5	12	5	5	1		75	69	75	73
AtSuc6	2	7	6	1	3	5	4	7	6	6		93	93	89
AtSuc7	1	2	3	1	4	2	2	9	2	3	9		93	89
AtSuc8	5	7	2	2	4	6	4	3	6	7	5	5		89
AtSuc9	3	1	2	1	1	4	4	4	1	3	18	2	2	

Table 3.6: The clustal-W multiple sequence alignment of rice (*Oryza sativa* Japonica cultiva-group) sucrose transporter protein sequences.

SeqA Name	Length (aa)	SeqB Name	Length (aa)	Identity (%)	SeqA Name	Length (aa)	SeqB Name	Length (aa)	Identity (%)
OsSUT1	538	OsSUT2	501	44	OsSUT2	501	OsSUT4	595	45
OsSUT1	538	OsSUT3	534	71	OsSUT2	501	OsSUT5	534	40
OsSUT1	538	OsSUT4	595	55	OsSUT3	506	OsSUT4	595	57
OsSUT1	538	OsSUT5	534	50	OsSUT3	506	OsSUT5	534	50
OsSUT2	501	OsSUT3	506	42	OsSUT4	595	OsSUT5	534	48

Table 3.7: The clustal-W multiple sequence alignment of *Arabidopsis thaliana* sucrose transporter protein sequences.

SeqA Name	Length (aa)	SeqB Name	Length (aa)	Identity (%)	SeqA Name	Length (aa)	SeqB Name	Length (aa)	Identity (%)
AtSUT1	513	AtSUT2	512	77	AtSUT3	594	AtSUT8	492	43
AtSUT1	513	AtSUT3	594	43	AtSUT3	594	AtSUT9	491	43
AtSUT1	513	AtSUT4	510	49	AtSUT4	510	AtSUT5	509	47
AtSUT1	513	AtSUT5	509	80	AtSUT4	510	AtSUT6	492	50
AtSUT1	513	AtSUT6	492	76	AtSUT4	510	AtSUT7	491	51
AtSUT1	513	AtSUT7	491	75	AtSUT4	510	AtSUT8	492	50
AtSUT1	513	AtSUT8	492	76	AtSUT4	510	AtSUT9	491	50
AtSUT1	513	AtSUT9	491	73	AtSUT5	509	AtSUT6	492	73
AtSUT2	512	AtSUT3	594	43	AtSUT5	509	AtSUT7	491	73
AtSUT2	512	AtSUT4	510	48	AtSUT5	509	AtSUT8	492	74
AtSUT2	512	AtSUT5	509	73	AtSUT5	509	AtSUT9	491	71
AtSUT2	512	AtSUT6	492	72	AtSUT6	492	AtSUT7	491	95
AtSUT2	512	AtSUT7	491	72	AtSUT6	492	AtSUT8	492	95
AtSUT2	512	AtSUT8	492	72	AtSUT6	492	AtSUT9	491	88
AtSUT3	594	AtSUT4	510	46	AtSUT7	491	AtSUT8	492	96
AtSUT3	594	AtSUT5	509	42	AtSUT7	491	AtSUT9	491	87
AtSUT3	594	AtSUT6	492	42	AtSUT8	492	AtSUT9	491	89
AtSUT3	594	AtSUT7	491	42					

3.9 DISCUSSION

Numerous studies have been reported on the expression of SUT genes during development (Aoki et al., 1999; Scofield et al., 2007; Sauer, 2007), but little is still known about the regulation of plant SUT genes during stress conditions (Sark et al., 1997; Meyer et al., 2004). In particular, work regarding the *cis*-acting regulatory elements or transcriptional factors involved in the expression of these genes in plants are very scarce. The publication of the genome sequences for *Arabidopsis* (*Arabidopsis* Genome Initiative, 2000) and rice plant (Ouyang et al., 2007; Yu et al., 2002), and the development of recent bioinformatic tools has opened up the door for studies on the regulation of expression of genes of interest.

Bioinformatics tools available on the World Wide Web were used to identify the putative *cis*-acting regulatory elements present in the divergent region of 1.5 kbp of the 5′ regulatory region starting from translational start site of the sucrose transporter gene families in rice and *Arabidopsis*. Significant variations both in the number and nature of elements identified were observed. This is in accordance with studies where upstream regulatory regions of genes were reported to develop more quickly than the downstream functions of their protein products and that a set of co-regulated genes often share related set of regulatory motifs (Marger and Saier, 1993). The 1.5 kbp of the various SUT genes was found to contain full conserved domains that include the TATA and CAAT boxes as well as other potentially important *cis*-acting regulatory elements that are required for minimal promoter sucrose transporter activity in plants.

Single *cis*-acting regulatory elements were identified in all the SUT sequences studied. Some of these elements are implicated in cell/tissue specific expression while others are involved in regulatory processes, which may contribute to SUT genes expression profiles observed in rice and *Arabidopsis*. However the expression patterns of a gene is not regulated by a single *cis*-acting regulatory element but by a combination of differing regulatory elements conferring different effects at different times or in different cell/tissue (Qui, 2003). It is assumed that genes which have similar expression patterns will have common *cis*-acting regulatory elements in their 5′ regulatory region (Vilo et al., 2000) and therefore a common set of TFs that controls them. Common *cis*-acting regulatory elements are the main factor of co-regulated genes and are often present in the regions where complex protein interaction occur (Wang et al., 2004). However, single motifs can also bind to many TFs thus subjecting the gene to multiple regulatory controls (Yanagisawa, 2002).

It has been established that the sucrose transporter gene family originates from a “Major Facilitator Super Family” and that the members of this super family evolved by gene duplication and fusion from one or more ancestral transporters with only six transmembrane helices (Marger

and Saier, 1993). The high divergence identity witnessed in the 5′ regulatory regions of the sucrose transporter gene families in rice and *Arabidopsis* could be as a result of loss of common *cis*-acting regulatory elements from the ancestral transporter gene. This is in agreement with the degenerative complementation model, which proposes that after gene duplication each daughter gene keeps only a fraction of regulatory elements as compared to the ancestral origin (Lynch and Force, 2000). It may then be inferred that genes having close % identity of *cis*-acting regulatory elements in their regulatory regions with one another may be regulated by similar cellular or environmental factors. Therefore it is presumed that the following pairs of sucrose transporter genes may be regulated by similar cellular or environmental factors: OsSUT1-OsSUT3; OsSUT2-AtSUC6-AtSUC8; AtSUC1-AtSUC5-AtSUC8; AtSUC3-AtSUC6-AtSUC7; AtSUC6-AtSUC7-AtSUC9.

Figure 3.5 illustrates the amount of *cis*-acting regulatory elements present in each rice and *Arabidopsis* sucrose transporter gene families in relation to cellular development, plant hormone, abiotic and biotic stresses. The following sucrose transporter genes; OsSUT2, OsSUT4, OsSUT5, AtSUC1, AtSUC2, AtSUC6, AtSUC8 and AtSUC9 may be well involved in/or regulated by cellular developmental activities due to the high number of cellular developmental associated *cis*-acting regulatory elements present at the 5′ regulatory regions (Table 3.3; Figure 3.5). Plant hormone associated *cis*-acting regulatory elements were found in low number in the 5′ regulatory region of most of the rice and *Arabidopsis* SUT genes. However OsSUT2, AtSUC4, AtSUC6, AtSUC7 and AtSUC9 were identified at a high number as compared to the other sucrose transporters, thus these genes expression may be well regulated by plant hormones (Table 3.3; Figure 3.5). High numbers of abiotic stress associated *cis*-acting regulatory elements were found in all sucrose transporter gene families in rice and *Arabidopsis* (Table 3.3; Figure 3.5), while only relative amounts of biotic stress associated *cis*-acting regulatory elements were found. The highest was found in OsSUT2 and AtSUC3 and the lowest in OsSUT1 and AtSUC7; for rice and *Arabidopsis* respectively. This may infer that the expression of sucrose transporters may be well linked to, and regulated by, environmental stress conditions.

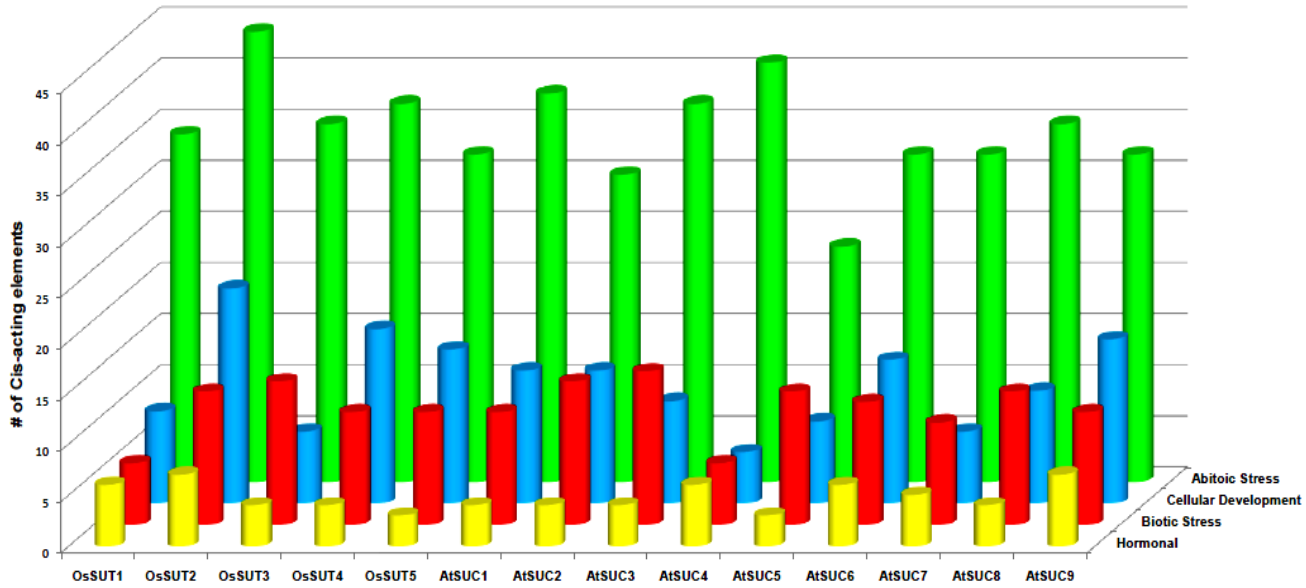


Figure 3.5: Representation of the summation of *cis*-acting regulatory elements associated with cellular development, Plant hormone, abiotic and biotic stresses in each rice (*Oryza sativa* Japonica cultiva-group) and *Arabidopsis thaliana* sucrose transporter gene families.

3.9.1 Cellular Development Associated *Cis*-Acting Regulatory Elements

The 5′ regulatory region of OsSUT1 contains a GCN4 motif; a motif that is conserved in the upstream sequence of many seed storage proteins genes, and has been suggested to act as an essential element that determines endosperm-specific expression (Wu et al., 1998; Washida et al., 1999). Although GCN4 is attributed to seed storage protein genes, its presence in OsSUT1 regulatory region may be related to OsSUT1 transport activity, which would facilitate in transporting sucrose into the germinating seed endosperm resulting in the accumulation of this carbohydrate for subsequent use by germinating seeds as a carbon and energy source. This would explain the observation of Scofield et al. (2007) who found high levels of OsSUT1 transcript expression at the exposed internode, panicle and pedicel of rice plants. They proposed that OsSUT1 may be involved in transporting sucrose through these tissues to the filling grain, or recovering sucrose from the apoplast and maintaining a gradient in other to facilitate a constant loading of sucrose to the filling grain.

The RY-motif was present in all the rice sucrose transporter genes, but present only in AtSUC4, AtSUC5 and AtSUC6 in *Arabidopsis* sucrose transporter genes. The RY-motif is one of

the most studied *cis*-acting regulatory elements involved in seed specific gene expression (Reidt et al., 2000; Ezcurra et al., 2000). It's generally found in the promoter region of seed-specific genes of dicots and monocots, and its removal in this region was found to suppress the seed-specific promoter activity, leading to a low gene expressional level in leaves (Ezcurra et al., 1999; Reidt et al., 2000). This shows the importance of the RY-motif for high-level expression of several seed-specific genes as well as the significance of the motif to function as a negative element in inhibiting expression in non-seed tissues (Ezcurra et al., 1999; Reidt et al., 2000). Rice sucrose transporter genes have been reported to be expressed in sink and source organs; with lower expression in the source than sink organs (Hirose et al., 1997; Furbank et al., 2001; Aoki et al., 2003; Lim et al., 2006; Scofield et al., 2007). AtSUC5 was reported to be expressed in the endosperm and thought to be involved in early seed development (Baud et al., 2005), while AtSUC4 was expressed predominantly in minor (loading) veins in source leaves where efficient sucrose transport is required for phloem loading (Weise et al., 2000). AtSUC6 was reported by Sauer et al. (2004) to encode an unusual protein, and might be a pseudogene.

Sugars have been reported to affect the expression and regulation of many genes involved in plant cellular activities such as photosynthesis, glycolysis, glyoxylate cycle, nitrogen, sucrose and starch metabolism, and cell cycle regulation (Koch, 1996; Smeekens and Rock, 1997; Smeekens, 2000).The existence of Sucrose-box, A-box and Pyrimidine-box in the 5´regulatory region would imply that the expression of these genes may be well regulated by sugar levels. However, the sensitivity of these genes to sugar expression and more generally, the physiological significance of the different boxes identified in the 5´ regulatory region will need to be confirmed by reporter gene experiments. In plants, sugars as an important source of carbon and energy for metabolic intermediates and for structural or storage components play an important role in signaling pathways (Smeekens and Rock, 1997; Smeekens, 2000). Therefore, it is essential that studies designed to understand the mechanisms that regulate the production of and access to sugars in response to various environmental factors (e.g water, light, salt), developmental signals (e.g phytohormones or stage-specific regulators) and metabolic status (carbon:nitrogen balance) are carried out.

The regulatory CAT-box motif has been found in the 5′ regulatory region of a handful of sugar-regulated genes, such as sporamine and amylase genes in sweet potato and rice (Maeo et al., 2001; Morikami et al., 2005). The high frequency of this motif found in the 5′ regulatory region of all the SUT genes further attests to the fact that the synthesis of sucrose transporters is linked with the availability of sugar (sucrose) at the site of utilization or storage in the cell during stress or development.

3.9.2 Hormonal Regulation Associated *Cis*-Acting Regulatory Elements

ABA responsive (ABRE) element has been functionally identified in the 5′ regulatory region of several genes that are induced or regulated by ABA. Most regulatory region of ABA inducible genes contains an ACGTG motif within 300 base pair upstream of the transcription start site (Busk and Pages, 1998). In other genes, multiple ACGT-containing ABREs were found clustered with other elements and appear to function in a complex (Busk et al., 1997). ABRE is also an example of an extended *cis*-acting regulatory element that contains the G-box core sequence [(C/A) ACG (T/C) G (T/C/G)] (Busk and Pages, 1998). Mutational and deletion studies with promoters containing the ABRE show that although the G-box is necessary for the function of ABRE- containing genes, the presence of the G-box is not sufficient for detection of the ABA signal (Busk and Pages, 1998). Rather, the G-box acts in conjunction with other *cis*-acting regulatory element to confer ABA responsiveness. The G-box therefore appears to have a role in the recruitment of a more general transcription factor that functions in conjunction with specific regulatory proteins to activate RNA polymerase II. For example a group of drought and salinity induced trans-acting factors belonging to the bZIP class of proteins interact with ABRE to mediate the ABA-dependent induction of stress response genes (Kang et al., 2002). With the exception of OsSUT3, AtSUC4, AtSUC6 and AtSUC7, all other sucrose transporter genes in rice and *Arabidopsis* may be regulated by ABA, because of the presence of the ABRE motif within their regulatory regions. ABA has been reported to have antagonistic effect on various plant processes such as: seed development, germination and seedling growth (Finkelstein and Gilbson, 2002). Garciarrubio et al. (1997) found that ABA affects the germination of mature seeds and growth of young seedlings of *Arabidopsis* by limiting the availability of nutrients. It could therefore be said that ABA may regulates the expression of sucrose transporters, depriving the plant of the transport of sucrose required for cellular and metabolic activities.

Ethylene is a gaseous hormone that regulates plant growth and development. Ethylene signalling plays an important role in plant responses to a number of biotic (wounding, pathogen infection; Xu et al., 1994; Broekaert et al., 2006) and abiotic stresses (drought, osmotic imbalance; Manavella, 2006; Rosado et al., 2006,) and physiological condition (fruit ripening, senescence; Woltering and van Doorn, 1988; Alexander and Grierson, 2002).The responses of plants to ethylene have been shown to be associated with dramatic changes in gene expression, and ethylene-responsive elements (EREs) conserved in the 5′ regulatory regions of those genes have been identified to bind ethylene-responsive factor (ERF), conferring ethylene-responsiveness (Itzhaki et al., 1994). Each ERE consists of an 8 base pair motif with the sequence A(A/T)TTCAAA and have been identified in the 5′ regulatory region of ethylene-stimulated genes (Itzhaki et al., 1994). The

presence of this motif in the 5′ regulatory regions of OsSUT2, OsSUT3, AtSUC5, AtSUC7, AtSUC8 and AtSUC9 may infer the essence of sugar transport to meet the cellular activities during physiological and/or stress condition(s) regulated by ethylene. The action of ethylene was investigated on its effect on ^{14}C sucrose translocation in growing rice (*Oryza sativa* L. cv Sasanishiki) coleoptiles and leaves (Ishizawa and Esashi, 1988). It was found that ethylene enhances sucrose transport from the scetullum to the coleoptiles by activating sucrose unloading in the growing coleoptiles where imported sucrose is cleaved into fructose and glucose. Tupy (1985) also reported that ethylene treatment of *Hevea brasiliensis* bark rapidly increased the transport of sucrose and water into the latex vessel within a few hours. Recently Dusotoit-Coucaud et al. (2009) showed that HbSUT1A and HbSUT2A gene expressions were distinctly increased in ethylene stimulated *Hevea brasiliensis* bark. It was suggested that HbSUT1A and HbSUT2A are responsible for the import of sucrose into the latex cell leading to the high yield in latex. Exogenously applied ethylene and its precursor 1-aminocyclopropane-1-carboxylic acid (ACC) were also reported to activate sucrose uptake in root discs of sugar beet (*Beta vulgaris* L.), however an inhibitor of ACC synthesis aminiethoxyvinylglycine (AVG) inhibited both endogenous ethylene production and sucrose uptake (Saftner, 1986). Chervin et al. (2006) found that the sucrose transporter SUC11 and SUC12 mRNA expression were down-regulated in cabernet sauvignon berries treated with 1-methylcyclopropane (a specific inhibitor of ethylene receptor) at veraison (when grapes start to ripen and accumulate sugar). Treated plants accumulated less sucrose than the control, thus demonstrating the role of ethylene to sucrose transporters expression and sugar accumulation.

TCA, a *cis*-acting regulatory element involved in salicylic acid responsiveness is known to be present in the non-translated regions of many monocot and dicot plant genes which are induced by one or more forms of stress (Goldsbrough et al., 1993). It is likely that the TCA motif and TCA-1 (a transcriptional factor) through the collaboration with other *cis*-acting regulatory elements and TFs could be involved in regulatory expression of defense genes which are related to different stress conditions. Accumulation of salicylic acid (SA) in plants was found to serve as a systemic signal molecule that induces synthesis of pathogen related protein and other compounds (Metraux et al., 1990). Exogenous applications of SA have been reported to improve plant growth and yield under stress conditions (Singh and Usha, 2003; El-Tayeb, 2005; Arfan et al., 2007; Noreen and Ashraf, 2008). It was assumed that SA assists plants to overcome stress conditions by inducing or enhancing biochemical and physiological processes, most especially the induction of antioxidant activity which protects plants from oxidative damage (Borsani et al., 2001; Khodary, 2004). Thus, the presence of TCA motif in the 5′ regulatory regions of OsSUT1, OsSUT2, AtSUC2, AtSUC3, AtSUC7, AtSUC8 and AtSUC9 genes may point again to the cooperative interaction between these sucrose transporters and stress response proteins. Although sucrose uptake was inhibited by SA in

sugar beet leaf disc, the amount of sucrose transporter mRNA and protein remain unchanged (Bourbouloux et al., 1998). It was assumed that the inhibition of sucrose uptake would have been through the inhibition of H^+- ATPase activity energizing the sucrose transporter, or a general effect of SA inhibition on membrane permeability (Bourbouloux et al., 1998).

Another important stress-signalling molecule in plants is methyl jasmonate (MeJA). The MeJA element has been found in the 5′ regulatory regions of many wound- or pathogen-responsive genes in monocot and dicot plants (Rushton and Somssich, 1998; Rouster et al., 1997) where they confer responsiveness to MeJA . Jasmonates (jasmonic acid and/or its methyl ester, MeJA) have been reported to have a lot of physiological effects on plants such as enhancing leaf senescence, inhibiting seed germination, inducing the increase of vegetative storage proteins and mRNAs encoding late embryogenesis- abundant proteins (Sembdner and Parthier, 1993; Creelman and Mullet, 199; Wasternack and Parthier, 1997). MeJA also induces the expression of proteins/enzymes such as phenylalanine ammonia lyase (Wanner et al., 1995), proteinase inhibitor PI-2 (Farmer et al., 1992), lipoxygenases (Bell and Mullet, 1993; Melan et al., 1993) and proline-rich proteins (Rushton and Somssich, 1998) known to be involved in the chemical defense mechanisms of plants. The presence of the MeJA motif in the 5′ regulatory regions of some of the sucrose transporter genes infers a possible role for SUTs in wound or pathogen stress responses.

Auxin plays an essential role in a wide variety of cellular and developmental processes in plants and these processes are assumed to involve Auxin-induced changes in gene expression (Hagen and Guilfoyle, 2002). Some of these processes include cell division, elongation and differentiation (Nishi et al., 1973), root formation, apical dominance and tropisms (Hagen et al., 1984; Theologis et al., 1985). These processes are believed to involve Auxin-induced changes in gene expression (Hagen and Guilfoyle, 2002). Auxin response factors (ARF) have been functionally identified in regulatory regions of primary/early response genes (genes that are rapidly and transiently expressed by cells within 0.5 - 2 hours in response to environmental stimuli), where they bind to auxin responsive element (AuRE) of auxin-responsive genes, and regulate their expression positively (Paciorek and Friml, 2006). Some of the most thoroughly characterized ARFs are composite elements containing the core sequence TGTCTC or a related sequence (Ulmasov et al., 1995; Ulmasov et al., 1999). The application of auxin to plant cell cultures, seedlings and mature plants have been reported to rapidly alter gene expression. Patrick (1979) reported the increase in the rate of phloem loading in decapitated stems and whole shoots of *Phaseolus vulgaris* L. Also reported was the increase in transcript level of potato sucrose transporter (StSUT1) after the application of auxin to detached leaves by Harms et al. (1994). Conversely auxin was reported to inhibit active sucrose uptake in sugar beet root tissue discs (Saftner and Wyse, 1984).Although

AuRE was found at OsSUT3, OsSUT4, AtSUC1, AtSUC5, AtSUC6 and AtSUC9 at a low frequencies, thus its effects on the expression and/or regulation of these genes could either be to enhance or inhibit the genes transcription, relative to the type of plant tissue in which they are expressed.

Gibberellin (GA) is an important phytohormone that controls many areas of plant development, which include seed germination (Bewley, 1997), leaf expansion and stems elongation (Richards et al., 2001), flowering and seed development (Derkx et al., 1994). It has been shown to play a vital role in the stimulation of seed germination by weakened tissues that surround embryo (aleurone and testa), thereby increasing growth rate (Groot and Karssen, 1987; Bewley, 1997; Derkx et al., 1994; Richards et al., 2001). Thus, it was suggested that GA-mediated growth responses are regulated partly by the change in cellular GA concentrations, changing the ability of cells to respond to it (Groot and Karssen, 1987; Bewley, 1997; Derkx et al., 1994; Richards et al., 2001). Gibberellin responsive element (GARE) sequence plays a fundamental function in GA action because changes within this sequence cause a huge reduction in GA-driven gene expression (Gubler and Jacobsen, 1992). GARE have been found in most genes responsible for synthesis, transport and signalling of other hormones, but the role of GARE is yet to be clarified, although uncharacterized crosstalk between GA and other hormones have been proposed (Ogawa et al., 2003). The presence of GARE motif in the 5′ regulatory regions of some of the sucrose transporter genes may be evidence for the significance of these transporters in meeting the carbon and energy demand of the cell during plant cellular development induced by gibberellins. Application of gibberellin to unpollinated ovaries of pea (*Pisum sativum* L.cv Alaska) was found to promote sucrose transport from leaf to the site of hormone application in the unpollinated ovaries (Peretó and Beltrán, 1986). It was suggested that gibberellin activates sucrose transport by increasing sucrose loading into the phloem of leaf (the source organ) and unloading into the ovary (the sink organ). Kuiper et al. (1991) also found that gibberellin activates the uptake of sucrose in flower of Madelon roses, resulting to an increase rate of flower bud opening.

3.9.3 Stress Response Associated *Cis*-Acting Regulatory Elements

GATA-box, G-box, Box-4, GAG, GAP, GA-motif and I-box are known as light responsive *cis*-acting regulatory elements (LREs). They have been found in the regulatory region of light-regulated genes, apparently essential for light-controlled transcriptional activity (Lam and Chua, 1989; Gilmartin et al., 1990). Deletion or mutation of these sequences compromises the ability of the regulatory region to respond to light and ultraviolet radiation. The high frequencies of these sequence elements in all the rice and *Arabidopsis* sucrose transporter genes regulatory regions

demonstrate that sucrose transporter genes expression may be well regulated by light. Potato and tomato sucrose transporter SUT1 was reported to be diurnally regulated by light (Kühn et al., 1997). The expression and activities of transporters may be directly controlled by light through signals that involve some specific light receptors or through photosynthesis, where light may affect the nutrient status of the cells, most especially the cellular sugar level (Krapp et al., 1993). Plant responses to light are complex and affect multiple developmental processes which include chloroplast development, flowering and seed development, circadian rhythms, phototropism, etc. (Natr and Lawlor, 2005).

Although LREs and their binding TFs have been discovered, none of the elements have been identified to solely confer light responsiveness to minimal heterologous promoters (López-Ochoa, et al., 2007). Thus it will be the combination of different *cis*-acting regulatory elements that make up the light responsive unit, rather than an individual element required to confer appropriate photo-responsiveness to a light-sensitive promoter.

The high number of W-boxes found in the sucrose transporter genes may describe them as a wound-responsive protein, and these W-boxes may participate in the regulation of the sucrose transporter genes expression to wounding, be it artificial mechanical damage, or plant wounding by pathogens or insects. It is evident that during this condition there will be a great need for an increase in carbon and energy requirements to meet the cellular metabolism and activation of resistant mechanism. Sucrose transporters may therefore participate in transporting sucrose from the apoplasmic tissues to adjacent injured tissues. This still need to be clarified *in-vitro* so as to identify which of the responses regulates each of the sucrose transporter genes. Only a few experiments have shown an increase in the gene transcript of sucrose transporter genes during wounding. Sakr et al. (1997) reported that cutting and ageing increases the gene transcript of SUT1 in freshly cut or aged tissues of matured sugar beet (*Beta vulgaris* L.) leaves, while silver leaf white fly (SLWF) nymphs feeding on *Arabidopsis* leaves were reported to up-regulate the production of SUT1 (Kempema et al., 2007). Meyer et al. (2004) reported that SUC3 reporter protein was reproducibly increased at all cutting sites of *Arabidopsis* plant. It may be appropriate to attribute this to the high number of W-box found in the regulatory region of AtSUC3 as compared to other sucrose transporter genes analyzed. It should be noted however that other *cis*-acting regulatory elements may have played a vital role in confirming this up-regulation of SUC3. Furthermore, Maleck et al. (2000) used microarray analysis to examine changes in *Arabidopsis* gene expression during systemic acquired resistance (SAR). It was found that W-box sequence was common in the regulatory regions of 26 defense associated and regulated genes. This infers that sucrose transporters may also be up-regulated during SAR as W-box was common in all the regulatory

regions of rice and *Arabidopsis* sucrose transporter genes; with the exception of OsSUT3. W-box or W-box-like sequences are usually clustered together within the small frame of the regulatory region, thus making them respond to a wide range of TFs of pathogens, elicitors and wounding responses (Maleck, 2000; Eulgem, 2000; Chen et al., 2002).The transduction pathways of wounding and biotic stress signals are often complex and may involve common secondary messengers such as the plant hormones jasmonic acid, ethylene and salicyclic acid. A number of possible regulatory elements that were associated with these transduction hormones were also found in the 5′regulatory regions of rice and *Arabidopsis* sucrose transporter genes. Moreover, W-boxes have been found to interact with zinc-fingered WRKY proteins; a family of TFs that are involved in response to pathogen infection and other plant stresses (Ross et al., 2007; Zheng et al., 2006).

It is worth pointing out the presence of GT1- elements in all the rice and *Arabidopsis* sucrose transporter genes. GT1- elements are *cis*-acting regulatory elements that are required for rapid response to pathogen attack, salinity and salicylic acid inducible gene expression. GT1-elements potentially bind the GT- TFs family that act cooperatively with related TFs of other *cis*-acting regulatory elements, modulating cell-type specific transcription (Park et al., 2004). A GT1-element in the regulatory region of a calcium calmodulin gene (SCaM-4) from soybean was identified as being responsible for its induction during pathogen and salinity stresses (Park et al., 2004). The presence of these elements in all rice and *Arabidopsis* sucrose transporter genes may further attest to their rapid induction during these stress conditions, in order to supply carbohydrate to energy demanding tissues.

Furthermore, the GCC-box which is solely found in the 5′ regulatory region of OsSUT1, AtSUC6 and AtSUC8, has been reported in several PR (pathogenesis-related) genes regulatory regions such as PDF1.2, Thi2.1 and PR4, where it also confers ethylene responsiveness. Also Elicitor responsive element (EIRE) was found only in OsSUT1, OsSUT2, AtSUC3, AtSUC4 and AtSUC7. EIRE has been found to occur in the promoter of diverse defense-related genes (Rushton et al., 1996) and has been generally linked with involvment in the induction of plant defense pathways. The presence of these motifs at the regulatory regions of OsSUT1 and OsSUT2 in rice; and AtSUC3, AtSUC4 and AtSUC7 in *Arabidopsis*, may infer that they are required for the import of photoassimilate into an infected cell/tissue to meet the sudden high cellular metabolic energy demand and changes during pathogen infection experienced during pathogen infection (Ibraheem et al., 2008).

The dehydration-responsive element (DRE) was identified in the regulatory region of all *Arabidopsis* sucrose transporter genes, except in AtSUC5. However it was only found in OsSUT1 in rice. This *cis*-acting element is also called DRE/CRT (Dehydration-Responsive Element/C-

repeat) and has been reported in regulating gene expression in response to drought, high salt and cold stress in plants (Dubouzet et al., 2003). Furthermore, several Erd1, MYC and MYB *cis*-acting regulatory elements were identified in the 5′ regulatory regions of all the rice and *Arabidopsis* sucrose transporter genes. These *cis*-acting regulatory elements have been reported to be required for the binding of transcriptional factors that are required for drought inducible gene expression. Drought, salinity and ABA-induced members of NAC-domain transcription factors have been shown to bind at these sequences and promote expression of the downstream genes in *Arabidopsis* and rice (Tran et al., 2004; Gao et al., 2010). Drought and salinity cause lots of structural and physiological damage to plant cells/tissues (Yancey et al., 1982; McCure and Hanson, 1990). Drought causes excessive transpiration water loss through the stomata pore (Lawlor and Cornic, 2002), while salinity causes hyperionic and hyperosmotic potential causing water to flow from plants into the root zone and finally loss of water, resulting in dehydration (Moftah and Michel, 1987; Binzel and Reuveni, 1994). These lead to loss of cell membrane selectivity and integrity, disruption of cellular compartmentalization and loss of activity of membrane bound proteins (Clifford et al., 1998; Chaves et al., 2002; Lawlor and Cornic, 2002). Plants tends to cope with these stress by decreasing cellular osmotic potential through the accumulation of solutes (osmoprotectants) that help in the movement of water into the cell (Morgan 1984). Sucrose is a major osmoprotectant molecule in plants; it helps in maintaining cell hydration and subsequently providing resistance against cellular dehydration (Morgan, 1984; Chaves et al., 2002). Therefore plant development and productivity will depend on a well regulated sucrose transporter expression and activity that will facilitate exchange of sucrose between cells and tissues during drought and salinity stress.

Low temperature responsive element (LTRE), a motif similar to DRE which is responsible for the induction of cold-induced/regulated genes (Brown et al., 2001; Dunn et al., 1998) was identified in the 5′ regulatory region of OsSUT2, OsSUT4, OsSUT5, AtSUC1, AtSUC3, AtSUC7, AtSUC8 and AtSUC9 genes. Jaglo et al. (2001) found in *Arabidopsis* that cold acclimatization involves rapid cold-induced binding of the DRE/CRT binding TFs to the DRE, leading to the expression of cold binding factor (CBF) targeted gene , increasing *Arabidopsis* freezing tolerance. Acclimatization to low temperature will involve the stabilization of cell membranes against freeze-induced damages such as physical cell disruption induced by membrane expansion, cell dehydration, denaturation of proteins and precipitation of macro molecules (Thomashow, 1990; Steponkus and Webb, 1992). Membrane transport activity may be affected by temperature as a result of protein conformational change, change in membrane fluidity and surrounding medium viscosity (Thomashow, 1990). However sucrose was found to protect cell membrane against freeze-induced damages (Anchordoguy et al., 1987; Steponkus and Webb, 1992; Labbe et al., 1997).

Hence its accumulation in plant cells during cold acclimatization was assumed to contribute to the stabilization of cell membranes and protect it from freeze- induced damages (Anchordoguy et al., 1987; Steponkus and Webb, 1992; Labbe et al. 1997). Thus plant acclimatization to low temperature may therefore regulate the expression and activities of sucrose transporters, since they transport sucrose needed for the protection of cell membrane from freeze-induced damages.

Plants are known to adapt quickly to high temperature environmental conditions. During high temperature stress, heat shock protein (HSP; networks of molecular chaperones) help to protect and refold cellular proteins, as well as maintain a balanced cellular homeostasis for plant survival (Vierling, 1991; Hartle, 1996; Larkindale and Vierling, 2008). Genes involved in these mechanisms contain HSE motifs that bind heat shock factors (HSFs), and are up-regulated during heat stress. HSE have been found to be consistently conserved in the regulatory regions of many heat induced genes (Larkindale and Vierling, 2008).The presence of a HSE motif in the 5′ regulatory regions of all rice and *Arabidopsis* sucrose transporter genes, except in OsSUT1, OsSUT3, AtSUC4 and AtSUC6, may imply the ability of the genes to survive high temperature stress conditions or the participation or need of sucrose for energy during high temperature stress conditions. Sucrose has been found to protect and enhance the thermal stability of cell membranes and intracellular proteins (Kilimann et al., 2006). It binds to the cell membrane, lowering its melting temperature and consequently keeping the membrane in its liquid crystalline phase during dehydration (Kilimann et al., 2006; Dhanda and Munjal, 2009). Qui et al. (2008) revealed 22 probe sets of sugar transporters that were involved in heat stress in a heat tolerant wheat (TAM107), thus proposing that sugar signalling will be essential in creating and maintaining plant thermo tolerance.

Found in the cells of aerobic organisms are reactive oxygen intermediates; and they exist in a balance ratio with biochemical antioxidants. Excessive reactive oxygen in cells results from exposure to toxicants, environmental oxidants, and heavy metals that affect the cellular oxidation – reduction (Red-Ox) balance, which interrupts normal biological functions. To overcome the oxidant effects and to restore a state of Red-Ox balance, cells have to reorganize fundamental homeostatic parameters and this often leads to the activation or silencing of transcription of regulatory genes (Adler et al., 1999). The presence of an ARE motif as part of the *cis*-acting regulatory elements of OsSUT1, OsSUT3, OsSUT4, OsSUT5, AtSUC2, AtSUC3, AtSUC6 and AtSUC8 genes could point to the urgent need of carbohydrate in the form of sucrose to meet the sudden change in cellular metabolism required for Red-Ox balance. Thus the rapid synthesis of sucrose transporters may be very imperative to meet the cellular energy demands.

One of the essential micronutrients for plant growth is copper. It is required as a cofactor in proteins that catalyze electron/oxygen transfer reactions (Hänsch and Mendel, 2009). The CuRE

motif has been associated with genes that require copper for their expression, through copper-response elements associated with them (Quinn et al., 2000). These include photosynthetic genes such as plastocyanin; an electron transfer protein in the thylakoid lumen, plastid CuZn-superoxide dismutase, polyphenol oxidase; also in the thylakoid lumen, mitochondrial cytochrome oxidase, extracellular laccase; involved in lignification, and several oxidases (Hänsch and Mendel,2009). Synthesis of sucrose transporters might well be linked to these photosynthetic genes, since the bulk function of these genes is to synthesis glucose which has to be transported from the source to the sink in the form of sucrose by sucrose transporters.

Sulphur responsive element (SuRE) has been reported to be involved in the expression and regulation of gene(s) that is (are) required for environmental sulphur adaptation (Maruyama-Nakashita et al., 2005). Sulphur is one of the basic macro nutrients that is essential for plant growth and productivity (Hopkins, 1999). Its presence in cysteine and methionine contributes to the formation of the thiol group, an essential group in protein tertiary structure formation. Sulphur deficiency was found to reduced photosynthesis through an effect on chloroplast (contains sulphur-rich proteins), resulting to impairment of chlorophyll synthesis (Terry, 1976; Chung, 1982). Thus sulphur stimulation of photosynthetic apparatus will lead to an increase glucose formation and subsequently sucrose formation (Fields and St.Clair, 1984). SuRE may therefore be essential in conferring the expression and regulation of sucrose transporters, which are involve in transporting the sucrose from source to sink tissues.

3.10 CONCLUSION

This computational analysis focuses on non-coding regions of sucrose transporter genes and identified a number of putative motifs at the 5′ regulatory regions. This method provides an efficient way of indicating if a gene may be regulated by a given TF, and a structure for ongoing analysis of the transcriptional gene regulation network. However, transcription is affected by cooperative involvement of many regulators that affect the RNA Polymerase II selectivity and it's binding, in response to various environmental stimuli (Zhu, 1996; Lloyd et al., 2001).

Thus, the predicted *cis*-acting regulatory sequences may not totally correlate with experimental expression data. The question of transcriptional regulation of individual sucrose transporter genes and the biological importance of these sequences would need to be further investigated by using bioinformatics methods with experimental expressional analysis; as it is recognized that bioinformatics methods help in confirming *in vitro* laboratory research. More importantly, clear and full knowledge of the interaction between *cis*-acting regulatory elements and their related TFs would allow a better understanding of transcriptional gene regulation and also

make a foundation for the restructuring of gene regulatory networks, which will help to improve biological research and provide ways to study and model cell response to various stimuli.

The plant RNA polymerase II transcription system shows to be much related to that of other eukaryotes, but little information is known about the functional communication crucial for the selective transcription of plant genes and the way by which *cis*-acting regulatory elements and TFs control the basal transcription machinery. In future, it will be useful to identify the cellular and environmental framework in which these TFs are activated or deactivated, and how gene expression is fashioned to various conditions by combinatorial control of *cis*-acting regulatory elements and TFs.

The studies of the plasma membrane transporters in relation to stress physiology are just emerging, but this should become a rapidly developing areas. To date few works have reported the expression and regulation of sucrose transporter genes (Hirose et al., 1997; Aoki et al., 2003; Scofield et al., 2007; Sauer, 2007). No *cis*-acting regulatory element responsible for or involved in tissue specific expression of sucrose transporters has yet been described. Thus, this analysis reveals the probable *cis*-acting regulatory elements that may be involved in the expression and regulation of sucrose transporter gene families in rice (*Oryza sativa* Japonica cultivar - group) and *Arabidopsis thaliana*, during cellular development and environmental stress conditions.

Further work in this study is therefore aimed at examining the sucrose transporters that are differentially expressed during drought, salinity and aphid infestation in rice (*Oryza sativa* L. cv Nipponbare).

3.11 REFERENCES

1) Abe, H., Urao, T., Ito, T., Seki, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2003) *Arabidopsis* AtMYC2 (bHLH) and AtMYB2 (MYB) Function as Transcriptional Activators in Abscisic Acid Signaling. *The Plant Cell* **15**:63–78.

2) Adler, V., Yin, Z., Tew, K.D. and Ronai, Z. (1999) Role of red-ox potential and reactive oxygen species in stress signaling. *Nature* **18(45):**6104-6111.

3) Alexander, L. and Grierson, D. (2002) Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. *Journal of Experimental Botany* **53 (377):**2039-2055.

4) Anchordoguy, T.J., Rudolph, A.S., Carpenter, J.F. and Crowe, J.H. (1987) Modes of interaction of cryoprotectants with membrane phospholipids during freezing. *Cryobiology* **24:**324–33

5) Aoki, N., Hirose, T., Scofield, G.N., Whitefeld, P.R. and Furbank, R.T. (2003) The sucrose transporter gene family in rice. *Plant Cell Physiology* **44**:223-232.

6) Aoki, N., Hirose, T., Takahashi, S., Ono, K., Ishimaru, K. and Ohsugi, R. (1999) Molecular cloning and expression analysis of a gene for a sucrose transporter in maize (*Zea mays* L.) *Plant Cell Physiology* **40**:1072-1078.

7) *Arabidopsis* Genome Initiative (2000) *Arabidopsis* Information Resources (TAIR), http//www.arabidopsis.org/

8) Arfan. M., Athar, H.R. and Ashraf, M. (2007) Does exogenous application of salicylic acid through the rooting medium modulate growth and photosynthetic capacity in two differently adapted spring wheat cultivars under salt stress? *Journal of Plant Physiology* **6(4):** 685-694.

9) Baud, S., Wuilleme, S., Lemoine, R., Kronenberger, J., Caboche, M., Lepiniec, L. and Rochat C. (2005) The AtSUC5 sucrose transporter specifically expressed in the endosperm is involved in early seed development in *Arabidopsis*. *Plant Journal* **43**:824-836.

10) Bell, E. and Mullet, J.E. (1993) Characterization of an *Arabidopsis* lipoxygenase gene responsive to methyl Jasmonate and wounding. *Plant Physiology* **103**: 1133-1138

11) Bewley, J.D. (1997) Seed germination and dormancy. *Plant Cell* **9**:1055-1066

12) Binzel, M.L. and Reuveni, M. (1994) Cellular mechanisms of salt tolerance in plant cells. *Horticultural Reviews* **16**:33–69.

13) Borsani, O., Valpuesta, V. and Botella, M.A. (2001) Evidence for a role of salicylic acid in the oxidative damage generated by NaCl and osmotic stress in *Arabidopsis* seedlings. *Plant Physiology* **126**:1024-1034.

14) Bourbouloux, A., Raymond, P. and Delrot, S. (1998) Effects of salicylic acid on sugar and amino acid uptake. *Journal of Experimental Botany* **49**:239-247

15) Broekaert, W.F., Delaure, S.L., De Bolle, M.F.C. and Cammue, B.P.A. (2006) The role of ethylene in host-pathogen interactions. *Annual Review of Phytopathology* **44**:393- 416

16) Brown, R.L., Kazan, K., McGrath, K.C., Maclean, D.J. and Manners, J.M. (2003). A role for the GCC-box in jasmonate-mediated activation of the PDF1.2 gene of *Arabidopsis*. *Plant Physiology* **132**:1020–10.

17) Brown, A.P.C., Dunn, M.A., Goddard, N.J. and Hughes, M.A. (2001) Identification of a novel low-temperature-response element in the promoter of the barley (Hordeum vulgare L) gene blt101.1. *Planta* **213**:770-780.

18) Brunelle, A.N., and Chua, N.-H. (1993). Transcription regulatory proteins in higher plants. *Current Opinion in Genetics and Development* **3**: 254–258.

19) Busk, P.K. and Pages, M. (1998) Regulation of abscisic acid induced transcription. *Plant Molecular Biology* **37**:425-435.

20) Busk, P.K., Jensen, A.B. and Pages, M. (1997) Regulatory elements in vivo in the promoter of the abscisic acid responsive gene rab 17 from maize. *Plant Journal* **11**:1285-1295.

21) Caselle, M., Di Cunto, F. and Provero P. (2002) Correlating overrepresented upstream motifs to gene expression: a computational approach to regulatory element discovery in eukaryotes. *BMC Bioinformatics* **3**: 7-12

22) Chen, W., Provart, N.J., Glazebrook, J., Katagiri, F., Chang, H.S., Eulgem, T., Mauch, F., Luan, S., Zou, G., Whitham, S.A. et al (2002) Expression profile matrix of *Arabidopsis* transcription factor genes suggests their putative functions in response to environmental stresses. *Plant Cell* **14**:559-574.

23) Chervin, C., Terrier, N., Ageorges, A., Ribes, F. and Kuapunyakoon, T. (2006) Influence of Ethylene on Sucrose Accumulation in Grape Berry. *American Journal Enology and Viticulture* **57**:4:511-513

24) Chaves, M.M., Pereira, J.S., Maroco, J., Roderigues, M.L., Ricardo, C.P.P., Osorio, M.L., Carvalho, I., Faria, T. and Pinheiro, C. (2002) How plants cope with water stress in the field. Photosynthesis and growth. *Annals Botany* **89**:907–916

25) Chung, H.S. (1982) Effects of sulfur dioxide on pigments, protein content and photosystem II activity of barley and corn leaves. *Korean Journal of Botany* **25**:135–151.

26) Clifford, S.C., Arndt, S.K., Corlett, J.E., Joshi, S., Sankhla, N., Popp, M. and Jones, H.G. (1998) The role of solute accumulation osmotic adjustment and changes in cell wall elasticity in drought tolerance in Ziziphus mauritiana (Lamk.). *Journal of Experimental Botany* **49**:967–977.

27) Creelman, R.A. and Mullet, J.E. (1997) Biosynthesis and action of jasmonates in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**: 355-381.

28) Dean. C. and Schmidt, R. (1995) Plant genomes: a current description. *Annual Review of Plant Physiology Plant Molecular Biology* **46**:395-418.

29) Delrot, S., Atanassova, S. and Maurousset, L. (2000) Regulation of sugar, amino acid and peptide plant membrane transporters. *Biochimica et Biophysica Acta* **1465**:281-306.

30) Derkx, M.P.M., Vermeer, E. and Karssen, C.M. (1994) Gibberellins in seeds of *Arabidopsis thaliana*: Biological activities, identification and effects of light and chilling on endogenous levels. *Plant Growth Regulation***15**:223-234

31) Dhanda, S.S. and Munjal, R. (2009) Cell membrane stability: combining ability and gene effects under heat stress conditions. *Cereal Research Communications* **37(3):**409-419.

32) Dubouzet, J.G., Sakuma, Y., Ito, Y., Kasuga, M., Dubouzet, E.G., Miura, S., Seki, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2003) OsDREB genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. *Plant Journal* **33(4):**751-63.

33) Dunn, M.A., White, A.J., Vural, S. and Hughes, M.A. (1998) Identification of promoter elements in a low-temperature-responsive gene (blt4.9) from barley (*Hordeum vulgare* L.) *Plant Molecular Biology* **38(4):**551-64.

34) Durrant, W.E., Rowland, O., Piedras, P., Hammond-Kosack, K.E. and Jones, J.D.G. (2000) cDNA-AFLP reveals a striking overlap in race-specific resistance and wound response gene expression profiles. *Plant Cell* **12**:963-977.

35) Dusotoit-Coucaud, A., Brunel, N., Kongsawadworakul, P., Viboonjun, U., Lacointe, A., Julien, J.L., Chrestin, H. and Sakr, S. (2009) Sucrose importation into laticifers of Hevea brasiliensis, in relation to ethylene stimulation of latex production. *Annals Botany* **104(4)**:635-47

36) El-Tayeb, M.A. (2005) Response of barley grains to the interactive effect of salinity and salicylic acid. *Plant Growth Regulation* **45**:215-224

37) Eulgem, T., Rushton, P.J., Robatzek, S. and Somssich, I.E. (2000) The WRKY super family of plant transcription factors. *Trends in Plant Science* **5**:199-206.

38) Ezcurra, I., Wycliffe, P., Nehlin, L., Ellerström, M. Rask, L. (2000) Transactivation of the *Brassica napus* napin promoter by ABI3 requires interaction of the conserved B2 and B3 domains of ABI3 with different *cis*-elements: B2 mediates activation through an ABRE, whereas B3 interacts with an RY/G-box. *The Plant Journal* 24(1):57 – 66.

39) Ezcurra, I., Ellerström, M., Wycliffe, P., Stålberg, K. and Rask, L. (1999) Interaction between composite elements in the napA promoter: both the B-box ABA-responsive complex and the RY/G complex are necessary for seed-specific expression. *Plant Molecular Biology* **40(4):**699-709.

40) Farmer, E.E., Johnson, R.R. and Ryan, C.A. (1992) Regulation of expression of proteinase inhibitor genes by methyl jasmonate and jasmonic acid. *Plant Physiology* **98**: 995-1002

41) Fields, R. and Clair, L.L. (1984) The effects of SO2 on photosynthesis and carbohydrate transfer in two lichens: *Collema polycarpon* and *Parmelia chlorochroa. American Journal of Botany* **71**: 986–998.

42) Fillion, L., Ageorges, A., Picaud, S., Coutos-Thévenot, P., Lemoine, R., Romieu, C. and Delrot, S. (1999) Cloning and Expression of a Hexose Transporter Gene Expressed during the Ripening of Grape Berry. *Plant Physiology* **120**:1083-1094.

43) Finkelstein, R.R. and Gibson, S.I. (2002) ABA and sugar interactions regulating development: cross-talk or voices in a crowd? *Current Opinion Plant Biology* **5**:26-32

44) Franklin, A.E. and Cande, W.Z. (1999) Nuclear organization and chromosome segregation. *Plant Cell* **11**:523-534**.**

45) Fukuda, Y. (1997) Interaction of tobacco nuclear protein with an elicitor-responsive element in the promoter of a basic class I chitinase gene. *Plant Molecular Biology* **34(1)**:81-87.

46) Furbank, R.T., Scofield, G., Hirose, T., Wang, X.D., Patrick, J. and Offler, C. (2001) Cellular localization and fuction of a sucrose transporter OsSUT1 in developing rice grains. *Austrailian Journal of Plant Physiology* **28**:1187-1196

47) Gao, H., Brandizzi, F., Benning, C., Larkin, R.M. (2008) A membrane-tethered transcription factor defines a branch of the heat stress response in *Arabidopsis thaliana. Proceedings the of National Academy Science of the U.S.A.* **105(42):** 16398-16402.

48) Gao, F., Xiong, A., Peng, R., Jin, X., Xu, J., Zhu, B., Chen, J. and Yao, Q. (2010) OsNAC52, a rice NAC transcription factor, potentially responds to ABA and confers drought tolerance in transgenic plants. *Plant Cell Tissue Organ Culture* **100**:255–262.

49) Garciarrubio, A., Legaria, J.P. and Covarrubias, A.A. (1997) Abscisic acid inhibits germination of mature *Arabidopsis* seeds by limiting the availability of energy and nutrients. *Planta* **203**:182-187

50) Gilmartin, P.M., Sarokin, L., Memelink, J. and Chua, N-H. (1990) Molecular Light Switches for Plant Genes. *The Plant Cell* **2**: 369-378.

51) Goldsbrough, A.P., Albrecht, H. and Stratford, R. (1993) Salicylic acid-inducible binding of a tobacco nuclear protein to a 10bp sequence which is highly conserved amongst stress-inducible genes. *The Plant Journal* **3(4):**563-571.

52) Goodrich, J.A., Cutler, G. and Tjian, R. (1996) Contacts in context: promoter specificity and macromolecular interactions in transcription. *Cell* **84**:825-830

53) Grooot, S.P.C. and Karssen, C.M. (1987) Gibberellins regulate seed germination in tomato by endosperm weakening: A study with gibberellin-deficient mutants. *Planta* **171**:525-531

54) Gubler, F. and Jacobsen, J.V. (1992) Gibberellin-Responsive Elements in the Promoter of a Barley High-pl a-Amylase Gene. *The Plant Cell* **4**:1435-1441.

55) Hagen, G. and Guilfoyle, T. (2002) Auxin-responsive gene expression: genes, promoters and regulatory factors. *Plant Molecular Biology* **49**: 373–385.

56) Hagen, G., Kleinschmidt, A.J. and Guilfoyle, T.J. (1984) Auxin regulated gene expression in intact soybean hypocotyl and excised hypocotyl sections. *Planta* **162**:147-153.

57) Hänsch, R. and Mendel, R.R. (2009) Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B, Cl). *Current Opinion Plant Biology* **12(3):**259-266

58) Harms, K., Wöhner, R.V., Schulz, B. and Frommer, W.B. (1994) Isolation and characterization of P-type H^+-ATPase genes from potato. *Plant Molecular Biology* **26(3):**979-88.

59) Hartle, F.U. (1996) Molecular chaperones in cellular protein folding. *Nature* **381**:571–579.

60) Higo, K., Ugawa, Y., Iwamoto, M. and Korenaga, T. (1999) Plant *cis*-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acid Research* **27(1):**297-300.

61) Hiratsuka, K. and Chua, N-H. (1997) Light Regulated Transcription in Higher Plants. *Journal of Plant Physiology* **110**: 131-139

62) Hirose, T., Imaizumi, N., Scofield, G.N., Furbank, R.T. and Ohsugi, R. (1997) cDNA cloning and tissue specific expression of a gene for sucrose transporter from rice (*Oryza sativa* L.) *Plant Cell Physiology* **38**:1389-1396

63) Hopkins, W.G. (1999) Plant and inorganic nutrients: In Introduction To Plant Physiology, 2nd edn John Wiley and Sons Inc. USA pp 61-76

64) Ibraheem, O., Hove, R.M. and Bradley, G. (2008) Sucrose assimilation and the role of sucrose transporters in plant wound response. *African Journal of Biotechnology* **7(25):**4850-4855.

65) Itzhaki, H., Maxson, J.M. and Woodson, W.R. (1994) An ethylene-responsive enhancer element is involved in the senescence-related expression of the carnation glutathione-S-transferase (GST1) gene. *Proceedings of the National Academy of Science of the U.S.A.* **91(19):** 8925–8929.

66) Jacobs, A.K., Dry, I.B. and Robinson, S.P. (1999) Induction of different pathogenesis-related cDNAs in grapevine infected with powdery mildew and treated with ethephon. *Plant Pathology* **48**:325-336.

67) Jaglo, K.R., Kleff, S., Amundsen, K.L., Zhang, X., Haake, V., Zhang, J.Z., Deits, T. And Thomashow, M.F. (2001) Components of the *Arabidopsis* C-Repeat/Dehydration-Responsive Element Binding Factor Cold-Response Pathway Are Conserved in *Brassica napus* and Other Plant Species. *Plant Physiology* **127**: 910–917.

68) Jeffery, I.B., Madden, S.F., McGettigan, P.A., Perrière, G., Culhane, A.C. and Higgins, D.G. (2007) Integrating transcription factor binding site information with gene expression datasets. *Bioinformatics* **23**:298-305.

69) Jolly, E.R., Chin C. –S., Herskowitz, I. And Li, H. (2005) Genome-wide identification of the regulatory targets of a transcription factor using biochemical characterization and computational genomic analysis. *BMC Bioinformatics* **6**:275-287.

70) Kang, J., Choi, H., Im, M. and Kim, S. (2002) *Arabidopsis* basic leucine zipper proteins that mediates stress-responsive abscisic acid signalling. *Plant Cell* **14**:343-357.

71) Kaplan, B., Davydov, O., Knight, H., Galon, Y., Knight, M.R., Fluhr, R. and Fromm, H. (2006) Rapid Transcriptome Changes Induced by Cytosolic Ca^{2+} Transients Reveal ABRE-Related Sequences as Ca^{2+}-Responsive *cis* Elements in *Arabidopsis*. *The Plant Cell* **18(10):** 2733-2748.

72) Kempema, L. A., Cui, X., Holzer, F. M., Walling, L.L. (2007). *Arabidopsis* transcriptome changes in response to phloem-feeding Silverleaf white nymphs. Similarities and distinctions in responses to aphids. *Plant Physiology* **143**: 849-865.

73) Khodary, S.E.A. (2004) Effect of salicylic acid on the growth, photosynthesis and carbohydrate metabolism in salt-stressed maize plants. *International Journal of Agriculture and Biology* **6**: 5-8.

74) Kilimann, K.V., Doster, W., Vogel, R.F., Hartmann, C. and Gänzle, M.G. (2006) Protection by sucrose against heat-induced lethal and sublethal injury of *Lactococcus lactis*: An FT-IR study. *Biochimica et Biophysica Acta* **1764(7):**1188-1197.

75) Kim, S.R., Kim, Y. and An, G. (1993) Identification of methyl jasmonate and salicylic acid response elements from the nopaline synthase (nos) promoter. *Plant Physiology* **103**(1): 97-103.

76) Knudsen, S. (1999) Promoter2.0: for the recognition of Pol II promoter sequences. *Bioinformatics* **15**:356–361.

77) Koch, K.E. (1996) Carbohydrate-modulated gene expression in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47**:509-540.

78) Krapp, A., Hofmann, B., Schäfer, C. and Stitt, M. (1993) regulation of the expression of rbcS and other photosynthetic genes by carbohydrates: a mechanism for the sink regulation of photosynthesis. *The Plant Journal* **3**:817-828

79) Kühn, C., Franceschi, V.R., Schulz, A., Lemoine, R. and Frommer, W.B. (1997) Localization and turnover of sucrose transporters in enucleated sieve elements indicate macromolecular trafficking. *Science* **275**:1298-1300

80) Kuiper, D., van Reenen, H.S. and Ribot, S.A. (1991) effect of gibberellic acid on sugar transport into petals of "madelon" rose flowers during bud opening. *Acta Horticulturae* (ISHS) **298**:93-98.

81) Labbe, C., Crowe, L.M. and John, H. (1997) Crowe Stability of the Lipid Component of Trout Sperm Plasma Membrane during Freeze–Thawing. *Cryobiology* **34**:176–182

82) Lam, E., Chua, N–H. (1989) ASF-2: A Factor That Binds to the Cauliflower Mosaic Virus 35s Promoter and a Conserved GATA Motif in *Cab* Promoters. *The Plant Cell* **1**: 1147-1156.

83) Larkindale, J. and Vierling, E. (2008) Core genome responses involved in acclimation to high temperature. *Plant Physiology* **146**:748–761.

84) Lawlor, D.W. and Cornic, G. (2002) Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. *Plant Cell and Environment* **25**:275–294.

85) Lescot, M., Dhais, P., Thijs, G., Marchal, K., Moreau, Y., van de Peer, Y., Rouz, P. and Rombauts, S. (2002) PlantCARE, a database of plant *cis*-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Research* **30(1):**325-327.

86) Lim, J.D., Cho, J-I., Park, Y-I., Hahn, T-R., Choi, S-B. and Jeon, J-S. (2006) Sucrose transport from source to sink seeds in rice. *Physiologia Plantarum* **126**:572-584

87) Lin, R., Zhao, W., Meng, X. and Peng, Y-L. (2007) Molecular cloning and characterization of a rice gene encoding AP2/EREBP-type transcription factor and its expression in response to

infection with blast fungus and abiotic stresses. *Physiological and Molecular Plant Pathology* **70(1-3):**60-68

88) Liu, R. and States, D.J. (2002) Consensus promoter identification in the human genome utilizing expressed gene markers and gene modelling. *Genome Research* **12**:462–469.

89) Lloyd, G., Landini, P. and Bushby, S. (2001) Activation and repression of transcription initiation in bacteria. *Essays in Biochemistry* **37**:17-31.

90) Lois, R., Dietrich, A., Hahlbrock, K. and Schulz, W. (1989) A phenylalanine ammonia-lyase gene from parsley: structure, regulation and identification of elicitor and light responsive *cis*-acting elements. *EMBO Journal* **8(6):**1641-1648.

91) López-Ochoa, L., Acevedo-Hernández, G., Martínez-Hernández, A., Argüello-Astorga, G. and Herrera-Estrella, L. (2007) Structural relationships between diverse *cis*-acting elements are critical for the functional properties of a *rbcS* minimal light regulatory unit. *Journal of Experimental Botany* **58(15-16):**4397-4406.

92) Lynch, M., Force, A. (2000) The probability of duplicate gene preservation by sub-functionalization. *Genetics* **154**: 459–473.

93) Maeo, K., Tomiya, T., Hayashi, K., Akaike, M., Morikami, A., Ishiguro, S. and Nakamura, K. (2001) Sugar-responsible elements in the promoter of a gene for b-amylase of sweet potato. *Plant Molecular Biology* **46**:627–637.

94) Maleck, K., Levine, A., Eulgem, T., Morgan, A., Schmid, J., Lawton, K.A., Dangl, J.L. and Dietrich, R.A. (2000) The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. *Nature Genetics* **26**:403-410.

95) Manavella, P.A., Arce, A.L., Dezar, C.A., Bitton, F., Renou, J.P., Crespi, M. and Chan, R.L. (2006) Cross-talk between ethylene and drought signalling pathways is mediated by the sunflower Hahb-4 transcription factor. *Plant Journal* **48(1):**125-137

96) Manevski, A., Bertoni, G., Bardet, C., Tremousaygue, D. and Lescure, B. (2000) In synergy with various *cis*-acting elements, plant interstitial telomere motifs regulate gene expression in *Arabidopsis* root meristems. *FEBS Letters* **483(1):**43-46.

97) Marger, M.D., Saier, Jr. M.H. (1993) A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. *Trends in Biochemical Science* **18**:3-12.

98) Martin, C. F., John L. S., Ulla, H. And Zhiping W. (2002) Statistical significance of clusters of motifs represented by position specific scoring matrices in nucleotide sequences. *Nucleic Acids Research* **30:**3214-3224

99) Martinez, E., Chiang, C.-M., Ge, H. and Roeder, R.G. (1994) TATA-binding protein-associated factor(s) in TFIID function through the initiator to direct basal transcription from a TATA-less class II promoter. *EMBO Journal* **13**:3115-3126.

100) Maruyama-Nakashita, A., Nakamura, Y., Watanabe-Takahashi, A., Inoue, E., Yamaya, T. and Takahashi, H. (2005) Identification of a novel *cis*-acting element conferring sulfur deficiency response in *Arabidopsis* roots. *The Plant Journal* **42(3):**305-314.

101) Maslov, S., Sneppen, K., Eriksen, K.A., Yan, K.K. (2004) Upstream plasticity and downstream robustness in evolution of molecular networks. *BMC Evolutionary Biology* **4**:9-21.

102) Matys, V., Fricke, E., Geffers, R., Gossling, E., Haubrock, M., Hehl, R., Hornischer, K., Karas, D., Kel, A.E., Kel-Margoulis, O.V. et al., (2003) TRANSFAC: transcriptional regulation, from patterns to profiles. *Nucleic Acids Research* **31**:374–378.

103) Maurousset, L., Lemoine, R., Gallet, O., Delrot, S. and Bonnemain, J.L.(1992) Sulfur dioxide inhibits the sucrose carrier of the plant plasma membrane. *Biochimica et Biophysica Acta* **1105**:230-236.

104) McCure, K.F., Hanson, A.D. (1990) Drought and salt tolerance: Towards understanding and application. *Trends in Biotechnology* **8**:358–362.

105) Melan, M.A., Dong, X., Endara, M.E., Davis, K.R., Ausubel, F.M.and Peterman, T.K. (1993) An *Arabidopsis thaliana* lipoxygenase gene is induced by pathogens, abscisic acid, and methyl jasmonate. *Plant Physiology* **101**: 441450

106) Metraux, J.P., Signer, H., Ryals, J.**,** Ward, E., Wyss-Benz, M., Gaudin, J.**,** Raschdorf, K., Schmid, E., Blum, W. and Inverardi, B. (1990) Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* **250**:1004-1006.

107) Meyer, S., Lauterbach, C., Niedermeier, M., Barth, I., Sjolund, R.D. and Sauer, N. (2004) Wounding enhances expression of AtSUC3, a sucrose transporter from *Arabidopsis* sieve elements and sink tissue. *Plant Physiology* **134**:684-693.

108) Moftah, A.E. and Michel, B.E. (1987) The effect of sodium chloride on solute potential and proline accumulation in soybean leaves.*Plant Physiology* **83**:238–240.

109) Mohr, P.G. and Cahill, D.M. (2003) Abscisic acid influence the susceptibility of *Arabidopsis thaliana* to *Pseudomonas syringae* pv. tomato and *Peronispora parasitica*. *Functional Plant Biology* **30**:461-469.

110) Morgan, J.M. (1984) Osmoregulation and water stress in higher plants. *Annual Review of Plant Physiology* **35**:299–319.

111) Morikami, A., Matsunaga, R., Tanaka, Y., Suzuki, S., Mano, S. and Nakamura, K. (2005) Two *cis*-acting regulatory elements are involved in the sucrose-inducible expression of the sporamin gene promoter from sweet potato in transgenic tobacco. *Molecular Genetics and Genomics* **272**:690–699.

112) Mukumoto, F., Hirose, S., Imaseki, H. and Yamazaki, K.I. (1993) DNA sequence requirement of a TATA element binding protein from *Arabidopsis* for translation in vitro. *Plant Molecular Biology* **23**: 995–1003.

113) Natr, L., Lawlor, D.W. (2005) Photosynthetic plant productivity. In Photosynthesis Handbook (2nd Ed). M. Pessarakli. C.R.C. Press, New York, USA. 501-524.

114) Nguyen, T., Sherratt, P.J., Cecil, B., Pickett, C.B. (2003) Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. *Annual Review of Pharmacology and Toxicology* **43**: 233-260.

115) Nishi, T., Yamada, Y., Takahashi, E. (1973) The role of auxins in differentiation of rice tissues culture in Vitro. *Journal of Plant Research* **86(3)**:183-188.

116) Noreen, S., Ashraf, M. (2008) Alleviation of adverse effects of salt stress on sunflower (H*elianthus annuus* l.) By exogenous application of salicylic acid: growth and photosynthesis. *Pakistan Journal of Botany* **40(4):**1657-1663

117) Nordin, K., Vahala, T., Palva, E.T. (1993) Differential expression of two related, low-temperature-induced genes in *Arabidopsis* thaliana (L.) Heynh. *Plant Molecular Biology* **21(4):**641-653.

118) Ogawa, M., Hanada, A., Yamauchi, Y., Kuwahara, A., Kamiya, Y. and Yamaguchi, S. (2003) Gibberellin Biosynthesis and Response during *Arabidopsis* Seed Germination. *The Plant Cell* **15**:1591-1604.

119) Ouyang, S., Zhu, W., Hamilton, J., Lin H., Campbell, M., Childs, K., Thibaud-Nissen, F., Malek, R.L., Lee, Y., Zheng, L, Orvis, J., Haas, B., Wortman, J. and Buell, C.R. 2007. The TIGR Rice Genome Annotation Resource: improvements and new features. *Nucleic Acids Research* **35**:883-887

120) Paciorek, T. and Friml, J. (2006) Auxin signaling. *Journal of Cell Science* **119**:1199–1202

121) Park, H.C., Kim, M.L., Kang, Y.H., Jeon, J.M., Yoo, J.H., Kim, M.C., Park, C.Y., Jeong, J.C., Moon, B.C., Lee, J.H., Yoon, H.W., Lee, S-H, Chung, W.S., Lim ,C.O., Lee, S.Y., Hong, J.C. and Cho, M.J. (2004) Pathogen and NaCl-induced expression of the SCaM-4 promoter is mediated in part by a GT-1 Box that interacts with a GT-1 – like transcription factor. *Plant Physiology* **135**:2150-2161.

122) Park, S.C., Kwon, H.B. and Shih, M.C. (1996) *Cis*-acting elements essential for light regulation of the nuclear gene encoding the A subunit of chloroplast glyceraldehyde 3-phosphate dehydrogenase in *Arabidopsis thaliana*. *Plant Physiology* **112(4)**: 1563–1571.

123) Patrick, J.W. (1979) An assessment of auxin-promoted transport in decapitated stems and whole shoots of *Phaseolus vulgaris* L. *Planta* **146**: 107-112

124) Peretó, J.G. and Beltrán, J.P. (1986) Hormone directed sucrose transport during fruit set induced by gibberellins in *Pisum sativum.Physiologia Plantarum* **69(2)**:356-360.

125) Prestridge, D.S. (1995) Predicting Pol II promoter sequences using transcription factor binding sites. *Journal of Molecular Biology* **249**:923–932.

126) Qin, D., Wu, H., Peng, H., Yao, Y., Ni, Z., Li, Z., Zhou, C.and Sun, Q. (2008) Heat stress-responsive transcriptome analysis in heat susceptible and tolerant wheat (*Triticum aestivum* L.) by using Wheat Genome Array. *BMC Genomics* **9**:432

127) Qui, P. (2003) Computational approaches for deciphering the transcriptional regulatory network by promoter analysis. *Biosilico* **1(4)**:125-133.

128) Quinn, J.M., Barraco, P., Eriksson, M. and Merchant, S. (2000) Coordinate Copper- and Oxygen-responsive *Cyc6* and *Cpx1*. *Journal of Biological Chemistry* **275(9)**:6080–6089.

129) Rani, V. (2007) Computational methods to dissect *cis* – regulatory transcriptional networks. *Journal of Biological Sciences* **32(7)**: 1325-1330.

130) Reidt, W., Wohlfarth, T., Ellerstrõm, M., Czihal, A., Tewes, A., Ezcurra, I., Rask, L. and Baumlein, H. (2000) Gene regulation during late embryogenesis: the RY-motif of maturation-specific gene promoters is a direct target of the FUS 3 gene product. *The Plant Journal* **21(5)**:401-408.

131) Richards, D.E., King, K.E., Ait-ali, T. and Harberd, N.P. (2001). How gibberellin regulates plant growth and development: A molecular genetic analysis of gibberellin signaling. *Annual Review of Plant Physiology and Plant Molecular Biology* **52**:67–88.

132) Riechmann, J.L., Heard, J., Martin, G., Reuber, L., Jiang, C., Keddie, J., Adam, L., Pineda, O., Ratcliffe, O.J., Samaha, R.R. et al (2000) *Arabidopsis* transcription factors: genome-wide comparative analysis among eukaryotes. *Science* **290**:2105-2110

133) Ross, C.A., Liu, Y., Shen, Q.J. (2007) The WRKY gene family in rice (*Oryza sativa*). *Journal of Integrative Plant Biology* **49**:827-842.

134) Rosado, A., Amaya, I., Valpuesta, V., Cuartero, J., Botella, M.A. and Borsani, O. (2006) ABA- and ethylene-mediated responses in osmotically stressed tomato are regulated by the *TSS2* and *TOS1* loci. *Journal of Experimental Botany* **57(12):**3327-3335

135) Rouster, J., Leah, R., Mundy, J. and Cameron-Mills, V. (1997) Identification of a methyl jasmonate-responsive region in the promoter of a lipoxygenase 1 gene expressed in barley grain. *The Plant Journal* **11(3)**: 513-523.

136) Rushton, P.J.and Somssich, I.E. (1998) Transcriptional control of plant genes responsive to pathogens. *Current Opinion in Plant Biology* **1**:311-315.

137) Rushton, P.J., Torres, T.J., Parniske, M., Wernert, P., Hahlbrock, K. and Somssich, I.E. (1996) Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoter of parsley *PR1* genes. *EMBO Journal* **15:**5690–5700.

138) Saftner, R.A. and Wyse, R.E. (1984) Effect of plant hormones on sucrose uptake by sugar beet root tissue discs. *Plant Physiology* **74**:951-955

139) Saftner, R.A. (1986**)** Effect of Ethylene on Sucrose Uptake in Root Discs of Sugar Beet. *Plant Cell Physiology* **27(5)**: 853-860

140) Sakr, S., Noubahni, M., Bourbouloux, A., Riesmeier, J., Frommer, W. B., Sauer, N. and Delrot S. (1997) Cutting, ageing and expression of plant membrane transporters. *Biochimica et Biophysica Acta* **1330**:207-216.

141) Sauer, N. (2007) Molecular physiology of higher plants sucrose transporters. *FEBS Letters* **581**:2309-2317.

142) Sauer, N., Ludwig, A., Knoblauch, A., Rothe, P., Gahrtz, M. and Klebl, F. (2004) AtSUC8 and AtSUC9 encode functional sucrose transporters, but the closely related AtSUC6 and AtSUC7 genes encode aberrant proteins in different *Arabidopsis* ecotypes. *The Plant Journal* **40**:120-130.

143) Scherf, M., Klingenhoff, A. and Werner, T. (2000) Highly specific localization of promoter regions in large genomic sequences by PromoterInspector: a novel context analysis approach. *Journal of Molecular Biology* **297**:599–606.

144) Scofield, G.N., Hirose, T., Aoki, N. and Furbank, R.T. (2007) Involvement of sucrose transporter, OsSUT1, in the long-distance pathway for assimilates transport in rice. *Journal of Experimental Botany* **58(12):**3155-3169.

145) Sembdner, G. and Parthier, B. (1993) The biochemistry and the physiological and molecular actions of jasmonates. *Annual Review of Plant Physiology and Plant Molecular Bioliogy* **44**: 569-589.

146) Sessa, G., Meller, Y. and Fluhr, R. (1995) A GCC element and a G-box motif participate in ethylene-induced expression of the PRB-1b gene. *Plant Molecular Biology* **28(1):**145-153

147) Shahmuradov, I.A., Gammerman, A.J., Hancock, J.M., Bramley, H.P., Solovyev,Y.V. (2003) Plant Prom: a Database of plant promoter sequences. *Nucleic Acids Research* **31**: 114–117

148) Shinozaki, K. andYamaguchi-Shinozaki, K .(2000). Molecular responses to dehydration and low temperature: Differences and cross-talk between two stress signaling pathways. *Current Opinion in Plant Biology* **3**:217–223.

149) Simpson, S.D., Nakashima, K., Narusaka, Y., Seki, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2003)Two different novel *cis*-acting elements of erd1, a clpA homologous *Arabidopsis* gene function in induction by dehydration stress and dark-induced senescence. *Plant Journal* **33(2):**259-70.

150) Singh, K.B., Foley, R.C. and Oñate – Sánchez, L. (2002) Transcription factors in plant defence and stress responses. *Current Opinion Plant Biology* **5**:430 – 436.

151) Singh, B. and Usha, K. (2003) Salicylic acid induced physiological and biochemical changes in wheat seedlings under water stress. *Plant Growth Regulation* **39**:137-141.

152) Smale, S.T. and Kadonaga, J.T. (2003) The RNA Polymerase II Core Promoter. *Annual Review of Biochemistry* **72**:449-479

153) Smale, S. T., and Baltimore, D. (1989) The “initiator” as a transcription control element. *Cell* **57**:103–113

154) Smeekens, S. (2000) Sugar-induced signal transduction in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **51**:49–81.

155) Smeekens, S., Rock, F. (1997) Sugar sensing and sugar-mediated signal transduction in plants. *Plant Physiology* **115**:7-13.

156) Solovyev, V. and Salamov, A. (1997) The Gene-Finder computer tools for analysis of human and model organisms genome sequences. *Proceedings of International Conference of Intelligent Systems for Molecular Biology* **5**:294–302

157) Steponkus, P.L. and Webb, M.S. (1992) Freeze-induced dehydration and membrane destabilization in plants. In G Somero, B Osmond, Eds, Water and Life: In Comparative Analysis of Water Relationships at the Organismic, Cellular and molecular Level. Springer-Verlag, Berlin, pp 338–362

158) Terry, N. (1976) Effects of Sulfur on the Photosynthesis of Intact Leaves and Isolated Chloroplasts of Sugar Beets. *Plant Physiology* **57(4)**:477-479

159) Theologis, A., Huynh, T.V., Davis, R.W. (1985) Rapid induction of specific mRNAs by auxin in pea epicotyls tissue. *Journal of Molecular Biology* **183**:53-68

160) Thomashow, M.F. (1990) Molecular genetics of cold acclimation in higher plants. *Advances in Genetics* **28**: 99–131

161) Toyofuku, K., Umemura, T. and Yamaguchi, J. (1998) Promoter elements required for sugar-repression of the RAmy3D gene for alpha-amylase in rice. *FEBS Letters* **428**:275-280.

162) Tran, L–S.P., Nakashima, K., Sakuma, Y., Simpson, S.D., Fujita, Y., Maruyama, K., Fujita, M., Seki, M., Shinozaki, K. andYamaguchi-Shinozaki, K. (2004) Isolation and functional analysis of *Arabidopsis* stress-inducible NAC transcription factors that bind to a drought-responsive *cis*-element in the early responsive to dehydration stress 1 promoter. *Plant Cell* 16:2481-2498.

163) Trinklein, N.D., Aldred, S.J., Saldanha, A.J. and Myers, R.M. (2003) Identification and functional analysis of human transcriptional promoters. *Genome Research* **1***3*:308–312.

164) Tsai, F.T.F. and Sigler P.B. (2000) Structural basis of preinitiation complex assembly on human Pol II promoters. *EMBO Journal* **19**:25-36

165) Tupý, J. (1985) Some aspects of sucrose transport and utilization in latex producing bark of *Hevea brasiliensis* Muel. Arg. *Biologia Plantarum* **27(1):**51-64.

166) Ulmasov, T., Hagen, G., Guilfoyle, T.J. (1999) Dimerization and DNA binding of auxin response factors. *Plant Journal* **19(3)**:309-19.

167) Ulmasov, T., Liu, Z-B., Hagen, G., Guilfoyle, T.J. (1995) Composite Structure of Auxin Response Elements. *The Plant Cell* **7**: 1611-1623

168) van Helden, J., Andre, B. and Collado-Vides, J. (1998) Extracting regulatory sites from the upstream region of yeast genes by computational analysis of oligonucleotide frequencies. *Journal of Molecular Biology* **281**:827–842

169) Vierling, E. (1991) The roles of heat shock proteins in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **42**:579-620.

170) Vilo, J., Brazma, A., Jonassen, I., Robinson, A. and Ukkonen, E. (2000) Mining for putative regulatory elements in the yeast genome using gene expression data. *Proceedings of International Conference on Intelligent Systems for Molecular Biology* **8**: 384–394.

171) Wang, Z., Dalkilic, M. and Kim, S. (2004) Guiding motif discovery by iterative pattern refinement. ACM Symposium on Applied Computing, Nicosia, Cyprus. March 14–17 pp 162–166.

172) Wanner, L.A., Li, G., Ware, D., Somssich, I.E. and Davis, K.R. (1995) The phenylalanine ammonia-lyase gene family in *Arabidopsis thaliana. Plant Molecular Biology* **27**: 327-338

173) Ward, J.M., Kuhn, C., Tegeder, M. and Frommer, W.B. (1998) Sucrose transport in higher plants. *International Review of Cytology* **178**:41-71.

174) Washida, H., Wu, C–Y., Suzuki, A., Yamanouchi, U., Aklhama, T., Harada, K. and Takaiwa, F. (1999) Identification of *cis*-regulatory elements required for endosperm expression of the rice storage protein glutelin gene Glub-1. *Plant Molecular Biology* **40**:1-12.

175) Washio, K. (2003) Functional Dissections between GAMYB and Dof Transcription Factors Suggest a Role for Protein-Protein Associations in the Gibberellin-Mediated Expression of the *RAmy1A* Gene in the Rice Aleurone. *Plant Physiology* **133**:850-863.

176) Wasternack, C. and Parthier, B. (1997) Jasmonate-signalled plant gene expression. *Trends in Plant Science* **2(8)**:302-307.

177) Weise, A., Barker, L., Kühn, C., Lalonde, S., Buschmann, H., Frommer, W.B. and Ward, J.M. (2000) A new subfamily of sucrose transporters, SUT4, with low affinity/high capacity localized in enucleate sieve elements of plants. *The Plant Cell* **12**:1345-1355

178) Woltering, E.J. and van Doorn, W.J. (1988) Role of Ethylene in Senescence of Petals Morphological and Taxonomical Relationships. *Journal of Experimental Botany* **39(11)**:1605-1616

179) Wu, C-Y., Suzuki, A., Washida, H. and Takaiwa, F. (1998) The GCN4 motif in a rice glutelin gene is essential for endosperm-specific gene expression and is activated by opaque-2 in transgenic rice plants. *Plant Journal* **14**:673-683.

180) Wyeth, W.W. and Albin, S. (2004) Applied bioinformatics for the identification of regulatory elements. *Nature Reviews Genetics* **5**:276-287

181) Xu, Y., Chang, L., Liu, D., Narasimhan, M.L., Raghothama, K.G., Hasegawa, P.M. and Bressan, R.A. (1994) Plant defense genes are synergistically induced by ethylene and methyl jasmonate. *Plant Cell* **6**:1077-1085

182) Yamamoto, S., Nakano, T., Suzuki, K. and Shinshi, H. (2004) Elicitor-induced activation of transcription via W box-related *cis*-acting elements from a basic chitinase gene by WRKY transcription factors in tobacco. *Biochimica et Biophysica Acta* **1679(3)**: 279-87.

183) Yanagisawa, S. (2002). The Dof family of plant transcription factors. *Trends in Plant Science* **7**: 555–560

184) Yancey, P., Lark, M., Hand, S., Bowlus, R. and Somero, G. (1982). Living with water stress: Evolution of osmolyte systems. *Science* **217**:214–1222.

185) Yu et al. (2002) Adraft sequence of the rice denome (*oryza sativa* L. ssp. Indica) *Science* **296**:79-92

186) Zhang, M.Q. (1998) Identification of human gene core promoters in silico. *Genome Research* **8**:319–326.

187) Zheng, Z., Qamar, S.A., Chen, Z. and Mengiste, T. (2006). *Arabidopsis* WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. *Plant Journal* **48**: 592-605.

188) Zhu, J.K. (2002) Salt and drought stress signal transduction in plants. *Annual Review of Plant Biology* **53**:247-273

189) Zhu, Q. (1996). RNA polymerase II-dependent plant *in vitro* transcription systems. *Plant Journal* **10**: 185–188.

190) Zhu, Q., Dabi, T. and Lamb, C. (1995). TATA box and initiator function in the accurate transcription of a plant minimal promoter *in vitro*. *Plant Cell* **7**: 1681–1689.

191) Zimmerli, L., Stein, M., Lipka, V., Schulze-Lefert, P. and Somerville, S. (2004) Host and non-host pathogens elicit different Jasmonate/ethylene response in *Arabidopsis*. *Plant Journal* **40**:633-646.

Chapter 4

Effect of Drought and Salinity on the Expressional Levels of Sucrose Transporters in Rice (*Oryza sativa* L. cv Nipponbare).

4.1 INTRODUCTION

The autotrophic growth of plants depends on their capacity to manufacture carbohydrate photosynthetically. Glucose, sucrose and their derivatives are required by plants as a source of energy and as a carbon-skeleton for cellular and metabolic activities; hence access to sugars is considered as a central factor for plant growth. Thus plant metabolism and productivity will be dependent on the expression and regulation of transporters that facilitate direct exchanges between cells, tissues and organs. These transporters are encoded by multigenic families whose members differ slightly by their expression, substrate specificity, cellular localization and/or regulation.

Previous work has reported observations of expression of sucrose transporters (SUT's) transcripts that are involved in the movement of sucrose into and out of the source and sink tissues, and throughout the long-distance phloem transport via apoplastic pathways that need SUT's to facilitate sucrose movement between cells (Hirose et al., 1997; Hirose et al., 1999; Aoki et al., 2003; Scofield et al., 2007a). Physiological data obtained from plants have shown that long distance transport is controlled by environmental parameters such as light, temperature, osmotic conditions and development (Delrot et al., 2000). The participation of sucrose in guard cells osmotic regulation has been examined in *Commelina* (Reddy and Das, 1986), *Vicia* (Talbott and Zeiger, 1996), *Pisum* (Ritte et al., 1999) and *Arabidopsis* (Meyer et al., 2004), thus connecting a possible role for SUTs in these cells.

Several important experimental breakthroughs carried out during the last decades with yeast have served as a model and the start of a more precise approach in improving the understanding of the role of sucrose transporters in sugar sensing (Smeekeens, 2000; Sauer, 2007). These include the preparation and use of purified plasma membrane and of reconstituted proteoliposomes to study the activity of the transporters in vitro; the cloning of sugar transporters based on yeast complementation; the use of heterologous expression systems to determine the substrate specificity and the thermodynamic aspects of transporter activity; and the preparation of transgenic plants expressing various constructs such as sense and anti-sense transporter cDNA under the control of ectopic or tissue specific promoters. However, understanding of sugar sensing, signalling and regulation with respect to transport of sucrose in plants is still in its infancy due to the complexity revealed by these approaches (Smeekens, 1998; Hellmann et al., 2000; Smeekens, 2000; Sauer, 2007).

Sugars control many plant's metabolic activities at all stages of the plant life cycle, from germination and vegetative growth to reproductive development and seed formation, thus they are considered as plant's sole control growth factor (Sauer, 2007). Sugar may affect the expression of

many genes involved in essential processes such as photosynthesis, glycolysis, glyoxylate cycle, nitrogen, sucrose and starch metabolism and cell cycle regulation (Smeekens, 2000; Sauer, 2007).

Thus the concentration of sugar in the apoplastic cells and sugar transport may affect cellular morphogenesis by control of cell division and metabolism. However, hexose – and/or sucrose – specific signalling mechanisms have recently been shown to participate in gene expression and regulation through a range of proteins which are involved in photosynthesis, carbon metabolism, transport and sugar uptake (Smeekens, 1998). For instance, one or more isoforms of an enzyme may be induced at high hexose/sucrose concentration in a particular tissue, while in other tissues such enzyme or isoform(s) may be induced at low hexose/sucrose concentration (Koch, 1996). Sugars also affect the activity of the enzymes that regulate sugar transporters e.g sucrose was found to activate the plasma membrane H^+- ATPase in tomato (Mito et al., 1996). This effect, which requires the metabolization of the sugar taken up, would promote cell growth when the sugar supply is abundant.

Abiotic stress conditions such as drought and salinity impede plant growth and reduce crop productivity and yield. Abiotic stress in fact is the major reason for crop productivity decline worldwide, reducing average production for essential crops by an excess of 50% (Bray et al., 2000). Abiotic stresses cause losses valued at millions of dollars each year due to a decrease in crop output, which threatens the sustainability of agricultural industry. Plants respond and adapt to these conditions by exhibiting physiological as well as biochemical changes at the molecular and cellular levels, modulating the expression of specific genes (Shinozaki and Yamaguchi-Shinozaki, 2000). Water is acknowledged as the fundamental abiotic determinant of carbon allocation (Davidson, 1969). Hence drought and salinity stresses will be the major factors that will limit plant productivity. Water stress stimulates great changes in source-sink relations by reducting the activities of photosynthetic organs leading to alterations in plant growth. Water deficiency in whole plants reduces photosynthetic capacity resulting in a decrease in export of assimilates which may be responsible for decreased crop productivity (Berman and Dejong, 1996).

4.2 SPECIFIC OBJECTIVE

Irrigation is used exclusively for commercial crops under high demand, resulting in other crops having to be grown under rain-fed or semi-arid land. This trend is increasing rapidly due to global development and climate change, thus crops which are drought tolerant are becoming more widely adopted and grown (Molden et al., 2007). As fresh water supply becomes scarcer, farmers

are more frequently using recycled or brackish water which leads to soil salinization and accumulation of toxic elements. The recent demand of cereals for biofuel production may further encourage irrigation.

Sucrose has been identified as one of the major osmoprotectant molecules, which are collectively involved in regulating osmotic and ionic potential of plants, by helping plant cells to maintain their hydrated state, and consequently offering resistance against drought and high salinity stresses (Rontein et al., 2002). Thus the participatory role of sucrose transport mechanisms in plants is essential to general plant physiology, and most importantly during environmental stress conditions.

This study is therefore aimed at advancing the understanding of sucrose assimilation and the role of sucrose transporters during drought and salinity stresses, with the aim of more effectively exploiting this information to improve and stabilize crop yields under water-limited and/or salinity conditions. Previous experimental work has reported the participation of sucrose transporters in germination, the early phase of plant development and grain filling (Hirose et al., 1997, Aoki et al., 2003, Scofield et al 2007a, Scofield et al 2007b). However data on their role during environmental stress conditions is limited.

4.3 METHOD

4.3.1 Cultivation of Plant Materials and Treatment

4.3.1.1 Cultivation of rice (*Orzya sativa* L. cv Nipponbare) at optimum conditions

Seeds of rice (*Orzya sativa* L. cv Nipponbare) cultivar were obtained as gift from Dr. R. Furbank CSIRO Plant Industry, Canberra, ACT2601, Australia. The rice seeds were germinated according to the method of Scofield et al., 2007a. Rice plants were grown to maturity (3 weeks old at the 4 leaf stage) in flooded paddy tanks in a greenhouse, in 75% compost and 25% perlite. Plant growth was under natural light with a day-time temperature of 28^{o}C and a night-time temperature of 25^{o}C.

4.3.1.2 Treatment of rice (*Orzya sativa* L. cv Nipponbare)

Rice plants cultivated as above were exposed to drought and salinity stress conditions as follows:

4.3.1.2.1 Drought treatment

Twelve rice plants were grouped in triplicate to give four test experimental groups, with similar grouping for the control experiment. Drought was induced by withholding water and allowing the water in the soil to drain out. Treated seedlings' leaves were harvested as grouped at

24hrs (Day 1), 72hrs (Day 3), 120hrs (Day 5) and 240hrs (Day 10) post treatment. Leaves from control plants were harvested the same number of times at each time point without subjecting to drought treatment. Leaves harvested from the test and control experiments were quickly submerged in liquid nitrogen, stored at -80^{o}C and used for total RNA extraction.

Figure 4.1: Greenhouse set-up of rice plants for drought experiment. Rice (*Orzya sativa* L. cv Nipponbare) seeds were grown in a flooded paddy tanks in 75% compost and 25% perlite, day-time temperature of 28^{o}C and night-time temperature of 25^{o}C. The plants were grown to maturity (3 weeks old at 4 leaf stage). Potted plants were taken out of the paddy tanks and allowing the water in the soil to drain out, later placed on a tray. The plants were not watered for the experimental days to introduced drought stress.

4.3.1.2.2 Salinity treatment

Twelve rice plants were grouped in triplicate to give four test experimental groups, with a similar grouping for the control experiment. The experimental rice plants were watered with 100mM NaCl, 200mM NaCl and 300mM respectively. Treated seedling's leaves were harvested as grouped at 24hrs (Day 1), 72hrs (Day 3), 120hrs (Day 5) and 240hrs (Day 10) post treatment. . Leaves from control plants were harvested the same number of times at each time point without watering with NaCl. Leaves harvested from the test and control experiments were quickly submerged in liquid nitrogen, stored at -80^{o}C and used for total RNA extraction.

Figure 4.2: Greenhouse set-up of rice plants for salinity experimentRice (*Orzya sativa* L. cv Nipponbare) seeds were grown in a flooded paddy tanks in 75% compost and 25% perlite, day-time temperature of 28^{o}C and night-time temperature of 25^{o}C. The plants were grown to maturity (3 weeks old at 4 leaf stage). Potted plants were taken out of the paddy tanks and allowing the water in the soil to drain out, later placed on a tray. The plants were watered with 100mM NaCl, 200mM NaCl and 300mM respectively for the experimental days to introduced salinity stress.

4.3.2 Analysis of Differentially Expressed Sucrose Transporter

4.3.2.1 Isolation of total RNA from controls and treated rice plants

All equipment used for the RNA isolation was made RNase free by autoclaving and rinsing thoroughly in RNase away solution. Leaves harvested from the triplicate plants were pooled to minimize inter plant variability and were ground to powder in liquid nitrogen prior to RNA. RNA from the ground tissues was extracted using the SV 96 Total RNA isolation system (Promega, USA), following the manufacturer's instructions. Extracted RNA was quantified using a PerkinElmer Lambda 35 UV/VIS Spectrophotometer at 260nm and electrophoresed on 1% agarose gel to verify its integrity.

4.3.2.2 Amplification of sucrose transporters (SUT) genes by reverse transcriptase polymerase chain reaction (RT-PCR)

The RT-PCR reactions were carried out using an Eppendorf AG 22331, Hamburg Master Cycler, and Qiagen® one step RT-PCR kit (Qiagen, USA), following the manufacturer's instructions. Total RNA template was used at concentrations varying from 50 – 1000ng, in a 50µl RT-PCR reaction volume.

A specific region of each of the rice SUT genes was amplified using primers (Table 4.1) designed by Scofield et al., 2007a, who confirmed the specificity of the primer pairs by sequencing the amplified bands.

Table 4.1: Rice sucrose transporters primer pairs

Gene	Accession No	Primer	Starts At Position	Ends At Position	Length	Sequence	T_m(GC+AT) value
OsSUT1	AF280050	SUT1-f	7323	7341	19	$^{5'}$AGTTCCGGTCGGTCAGCAT$^{3'}$	60
		SUT1-r	7565	7548	18	$^{5'}$ACCGAGGTGGCAACAAAG$^{3'}$	56
OsSUT2	AB091672	SUT2-f	1561	1581	21	$^{5'}$AGGAGGAGAGGTCACCGATAA$^{3'}$	64
		SUT2-r	1800	1778	23	$^{5'}$CCAACATCCAATGTACAACAGCA$^{3'}$	66
OsSUT3	AB071809	SUT3-f	1507	1524	18	$^{5'}$GCCATGGCGTCCGTGTTC$^{3'}$	60
		SUT3-r	1679	1699	21	$^{5'}$GCCTGCTATAGTACCCGCTCT$^{3'}$	66
OsSUT4	AB091673	SUT4-f	1819	1838	20	$^{5'}$TTTGGCTGAGCAGAACACCA$^{3'}$	60
		SUT4-r	2067	2048	20	$^{5'}$ATGTCATTCGGGCAGAGCTT$^{3'}$	60
OsSUT5	AB091674	SUT5-f	1616	1636	21	$^{5'}$CTAGTGCGAAACTCCATCAAA$^{3'}$	60
		SUT5-r	1865	1843	23	$^{5'}$AAAATATTTGGGTTTCCTGAGAT$^{3'}$	60

Where: f is forward primer and r is the reverse primer. See Appendix B for the positions of these primers in the full OsSUTs' sequences.The thermal cycling conditions used are as follow:

Step	Temperature	Time	
Reverse transcription	50^{o}C	30 mins	
Initial PCR activation	95^{o}C	15 mins	
Denaturation	95^{o}C	30 secs	3 step cycling; 40 cycles
Annealing	55^{o}C; 59^{o}C	30 secs	
Extension	72^{o}C	1 mins	
Final extension	72^{o}C	10 mins	

Note: At the annealing step, 59^{o}C was used for OsSUT3, while 55^{o}C was used for SUT1, 2, 4 and 5.

4.3.2.3 Relative quantification of amplified SUT genes by Real-Time PCR

qPCR was carried out using a two step method. Firstly, 1µg of total RNA from each sample was converted to cDNA using the iScriptTM cDNA synthesis kit (BIO-RAD Laboratories, Inc. USA), following the manufacturer's instructions. This kit primes cDNA synthesis using a mixture of oligodT and random hexamers.

Secondly, quantification of the amplified SUT genes was performed using the same gene-specific primers as used for RT-PCR (Table 1). Aliquots of the cDNA corresponding to 50ng of each sample input RNA were used as templates for the Real-Time PCR analysis. Reactions were carried out on a Bio-Rad iCycler Real-Time PCR Detection System with iQ^{TM} SYBR$^{®}$ Green Super mix (BIO-RAD Laboratories, Inc. USA), according to the manufacturer's instructions. The thermal cycling conditions used are as follow:

Step	Temperature	Time	
Initial PCR activation	95^{o}C	15 mins	
Denaturation	95^{o}C	30 secs	3 step cycling; 40 cycles
Annealing	60^{o}C	30 secs	
Extension	72^{o}C	30 secs	
Final extension	72^{o}C	10 mins	
Heat dissociation	65^{o}C - 95^{o}C	30secs	At 1^{o}C increment

PCR products were detected by monitoring the increase in fluorescence of the SYBR Green during the extension phase of each cycle when the SYBR Green bound to double stranded DNA.

The results obtained for the different cDNAs were normalized against the expression levels of two stable reference genes: rice polyubiquitin 1(RubQ1) and rice actin using the qBasePlus programme produced by BioGazelle (version 1.3; http//www.biogazelle.com). The primer sets used are as follows:

Gene	Accession No	Primer	Starts At Position	Ends At Position	Length	Sequence	T_m(GC+AT) value
OsRubQ1	OsU37687	RubQ1-f	1440	1459	20	$^{5'}$GGAGCTGCTGCTGTTCTTGG$^{'3}$	64.5
		RubQ1-r	1539	1520	20	$^{5'}$CACAATGAAACGGGACACGA$^{'3}$	60.4
OsActin	NM_001072362.1	Actin-f	605	626	22	$^{5'}$TGTTGGACTCTGGTGATGGTGT$^{'3}$	62.67
		Actin-r	799	778	22	$^{5'}$CAAGCTTCTCCTTGATGTCCCT$^{'3}$	62.67

The rice polyubiquitin 1(RubQ1) gene is reported to be a highly constitutively expressed gene in rice tissues (Wang et al., 2000), while actin is a generally stable gene in plants. See Appendix B for the position of this primer in the full rice polyubiquitin 1 gene (RubQ1*)* and rice actin sequences.

The relative mean fold expression level and standard error of each of the SUT genes was determined for the treatment and control groups at each time point by reference to the sample with lowest expression for each gene, using qBasePlus (Version 1.3).

4.4 RESULTS

4.4.1. Effect of Treatment on Rice Plants

A gradual and clear significant physical symptom of retardation in the plant growth was observed in rice plants subjected to drought and salinity stresses, as compared to the control. At Day 10 the plant's leaves all appeared withered in both treatments; a phenomenon as experienced in the field (Figure 4.3).

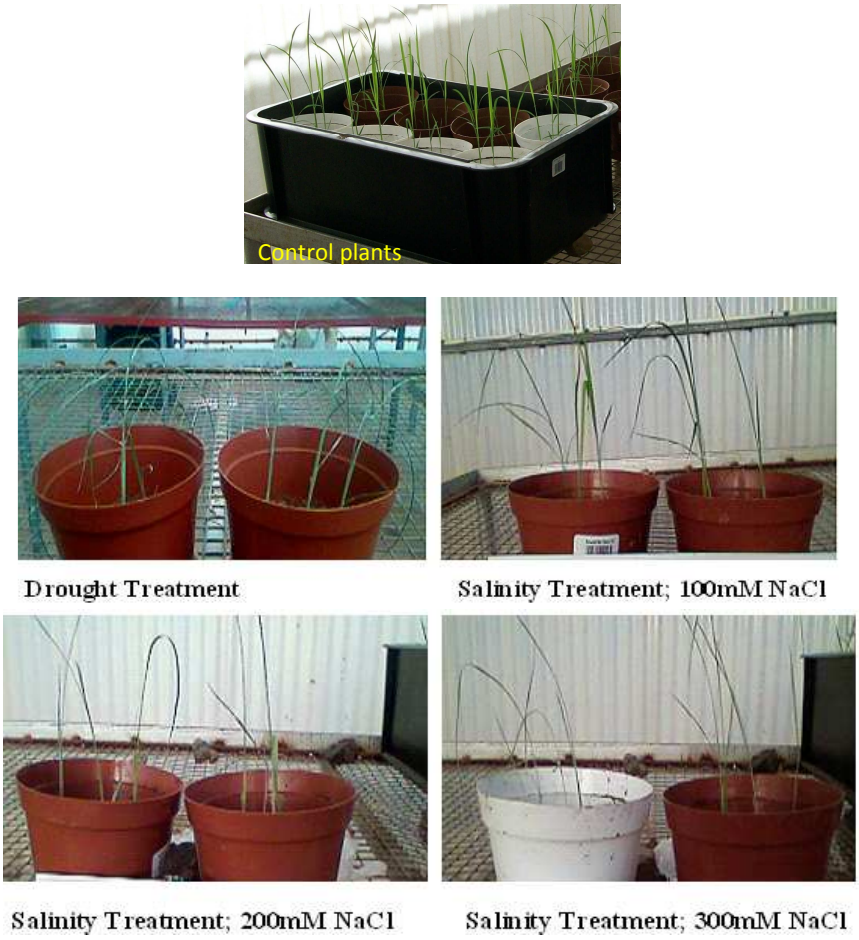


Figure 4.3: Effect of drought and salinity on 3 weeks old rice plants, at 10 days post treatment.All plant leaves appeared weathered in the treated plants as compared to the control.

4.4.2 Total RNA Extraction and Confirmation of OsSUT Primer Pairs

All the total RNA extracts yielded two major bands on 1% agarose gel representing 28S and 18S rRNA. The A_{260nm}/A_{280nm} ratio for each extract was above 2, indicating the high purity of the extracts (Figure 4.4).

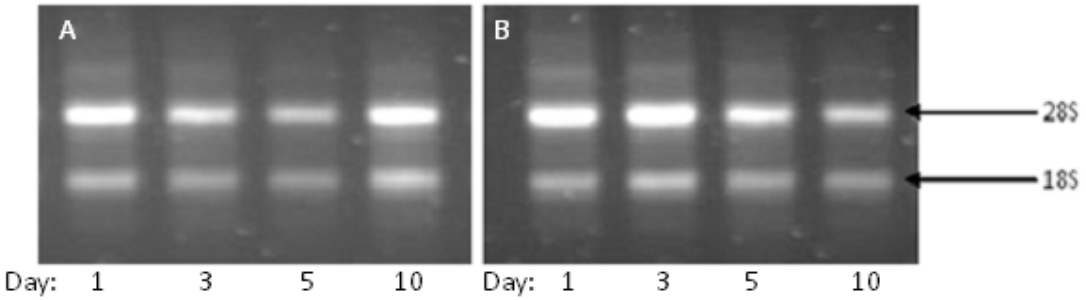


Figure 4.4: Representative sample of 1% agarose gel electrophoresis of total RNA extracts of (A) drought experiment; (B) salinity experiment. See Appendix F for pictures of the rest of the 1% agarose gel electrophoresis of total RNA extracts from control and stressed rice plants.

All primer pairs amplified a single product of the expected size, which was confirmed on 1% agarose gel (Figure 4.5) and analysis of the melt-curves after qPCR (Figure 4.6), which gave a single band or single peak respectively. The exception was OsSUT3 which showed no clear peak in the melt-curve analysis as a result of its overall poor expression during the drought and salinity treatments (See Appendix G). Regardless of this, the RT-PCR reaction was able to amplify the small portion of the OsSUT3 mRNA leading to the observed clear band that gave the expected size.

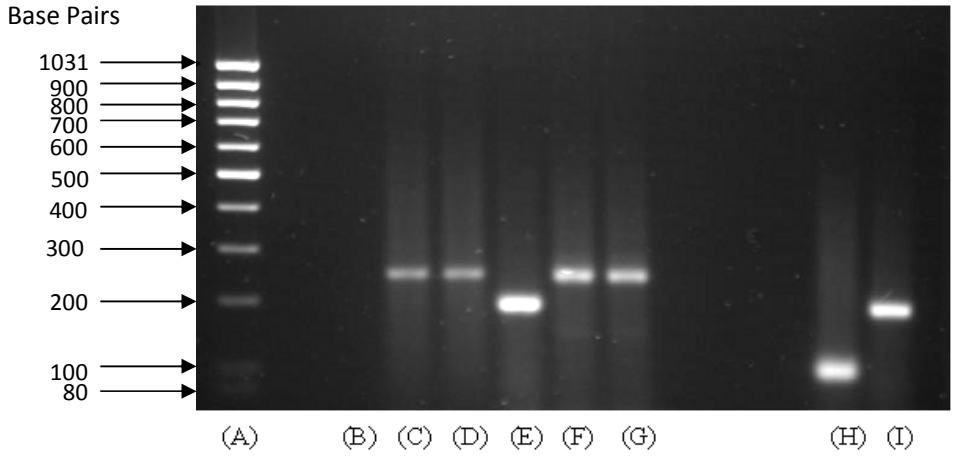


Figure 4.5: Confirmation of RT-PCR amplification by the primer pairs on 1% agarose gel: (A) Fermenta Low Range Mass Ruler DNA Ladder (B) Non-template Control, (C) OsSUT 1; 245bp, (D) OsSUT 2; 243bp, (E) OsSUT 3; 194bp, (F) OsSUT 4; 249bp, (G) OsSUT5; 250bp, (H) RubQ1;100bp, (I) Actin; 195bp.

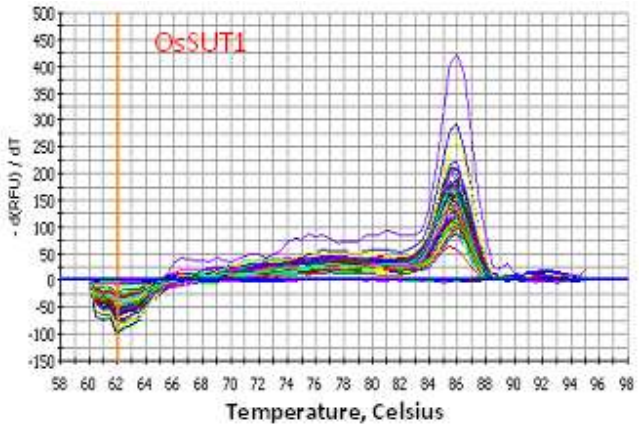


Figure 4.6: OsSUT1 melt curve analysis; representative sample (See Appendix G for the analysis of the rest rice sucrose transporter genes)

4.4.3 Quantitative Real-Time PCR

qPCR analysis of rice sucrose transporter genes revealed differential expression of the genes (Figure 4.7 and Figure 4.8). In all, changes of transcripts were observed in the drought and salinity treated plants when compared with the controls.

Drought treatment:

A gradual increase in SUT2 expression level relative to control was observed in the drought treated plants, with a final relative expression of more than 4-fold observed at day 10, compared to control (Figure 4.7).

A relative increase in expression levels of SUT1 was observed at days 5 and 10 when compared to control, however only 2-fold increase was observed at day 10 (Figure 4.7a). A decrease in SUT5 expression levels relative to control was observed for days 1, 3, 5 and 10, with a final decrease of approximately 3-fold observed at day 10 (Figure 4.7d). Interestingly, a 6-fold decrease in expression level of SUT5 was observed between day 1 and 5.

No observable difference in expression levels relative to the control was observed for SUT4 at day 1, 3 and 5. At day 10 no expression of SUT4 in the drought stressed plants was observed. No clear SUT3 expression was detected in either the control or drought treated plants (data not shown).

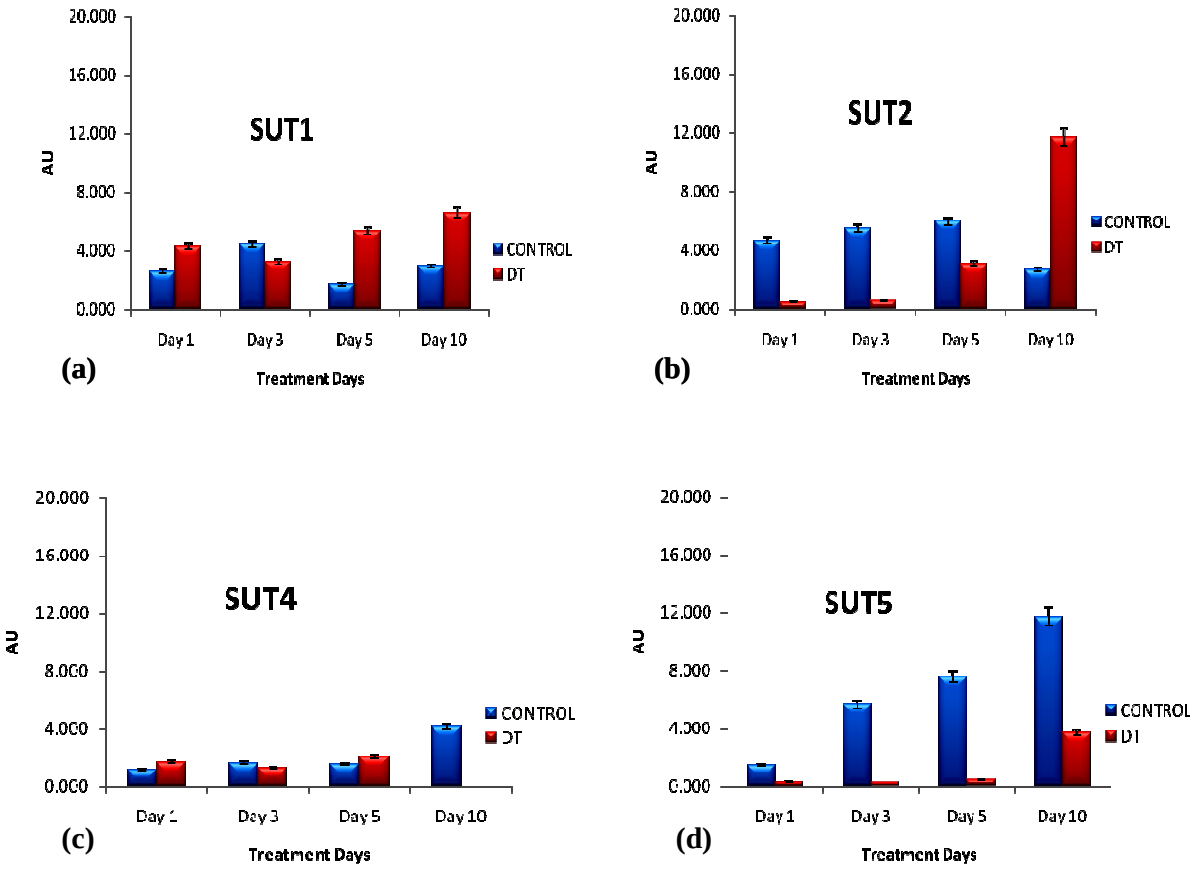


Figure 4.7: Quantitative PCR analysis of OsSUT1, OsSUT2, OsSUT4 and OsSUT5 genes during drought treatment of rice (*Orzya sativa* L. cv Nipponbare) cultivar plants. All values were normalized to the relative expressional levels of rice Polyubiquitin 1 and rice actin genes by analysing the ratios of the mRNA concentrations of individual OsSUT genes to the rice Polyubiquitin 1 and rice actin genes. The average of pooled tissue from triplicate sets of plant material tested in duplicate is shown. Error bars representing the standard error were obtained from the qBase Plus (Version 1.3) analysis, representing 95% confidence. DT represents drought treatment. AU represents the fold change in expression relative to the minimum value obtained for each gene. OsSUT3 showed no detectable transcript expression during this treatment (data not shown).

Salinity treatment:

A 2 to 8-fold increase in SUT2 expression level relative to control was observed in response to 100mM, 200mM and 300mM NaCl treatment of the rice plants (Figure 4.8 b). With all three treatments, there was a progressive increase in gene expression over time, with a more prominent increase with 200mM and 300mM NaCl treatment at day 10. SUT5 only showed a considerable

difference in the expression in treated rice plant as compared to the control in response to 100mM and 300mM NaCl treatment at Day10; evidence of probable up-regulation (Figure 4.8 d).

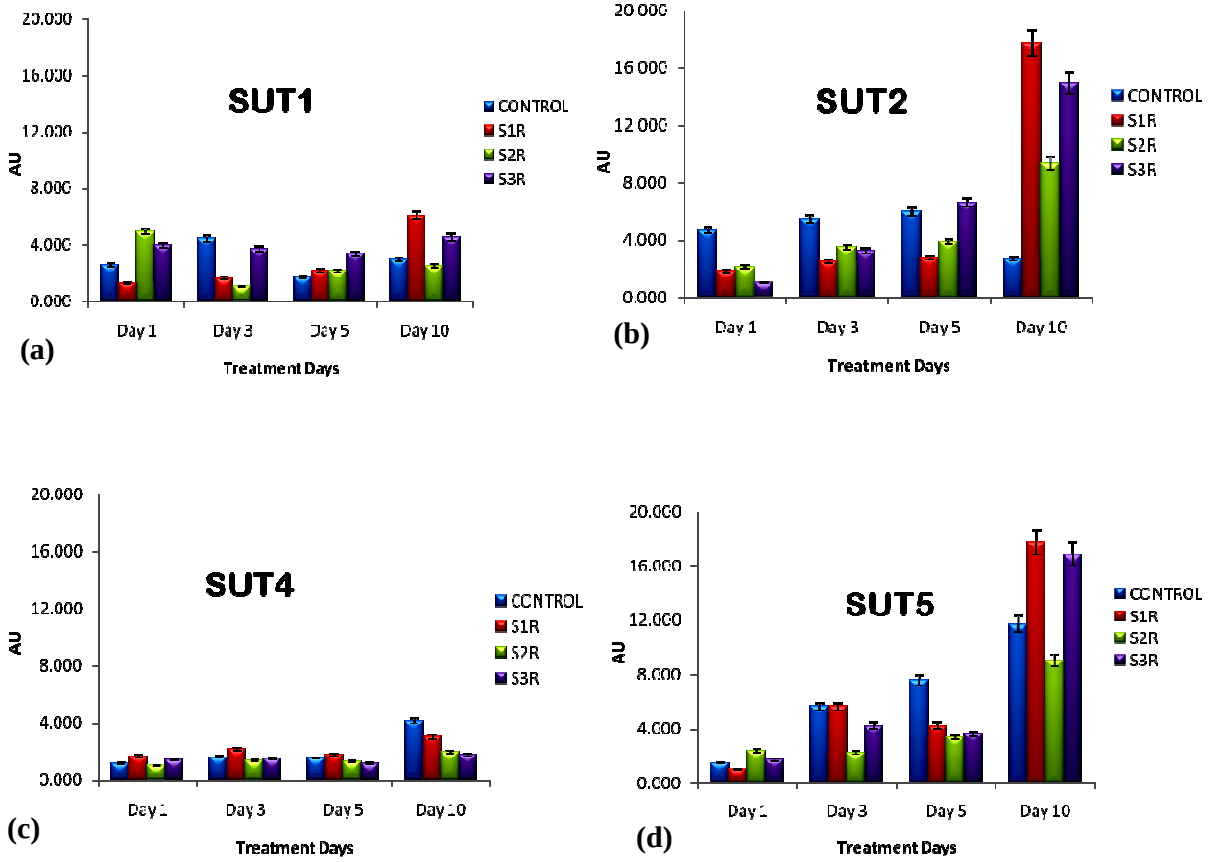


Figure 4.8: Quantitative PCR analysis of OsSUT1, OsSUT2, OsSUT4 and OsSUT5 genes during salinity treatment of rice (Orzya sativa L. cv Nipponbare) cultivar plants. All values were normalized to the relative expressional levels of rice Polyubiquitin 1 and rice actin genes by analysing the ratios of the mRNA concentrations of individual OsSUT genes to the rice Polyubiquitin 1 and rice actin genes. The average of pooled tissue from triplicate sets of plant material tested in duplicate is shown. Error bars representing the standard error were obtained from the qBase Plus (Version 1.3) analysis, representing 95% confidence. S1R, S2R and S3R represent salinity treatment with respect to 100mM, 200mM and 300mM; NaCl respectively. AU represents the fold change in expression relative to the minimum value obtained for each gene. OsSUT3 gave no significant expression during the treatment (data not shown).

SUT1 and SUT4 were expressed at low levels in control and salinity treated rice plants (Figure 4.8 a & b). SUT4 showed no change in expression in the control compared to the treated

plants during the first five days of treatment. However after 10 days, the control plants showed up regulation of SUT4, whilst SUT4 expression in the plants treated with higher concentrations of NaCl was unchanged. There was no clear pattern of change in SUT1 transcript levels in response to salinity treatment. There was no detectable transcript expression of SUT3 in both the control and salinity treated rice plants (data not shown).

4.5 DISCUSSION

Rice, a staple crop for over half of the world's population is sensitive to a variety of abiotic stresses, including salinity, drought, submersion and cold (Chinnusamy et al., 2005).

Drought and salinity stress are environmental conditions in which plants have to cope with both declining water availability and ion toxicity as a result of a decrease in cellular osmotic potential. An excessive amount of salt entering the plant will result in an increase in toxic levels in older transpiring leaves, causing early senescence. This will further reduce the quantity of assimilates produced by the plant and in assimilates transported to the growing tissue, consequently limiting plant growth. This may well explain the poor growth of the plants observed in this study after 10 days of the drought and salinity stress treatments.

Yeo et al. (1991) reported that the growth reduction in rice (*Orzya sativa* L.) after both long term and short term periods of salinity was as a result of a decrease in water supply to the plants, caused by increased salinization. Salt accumulation in soil due to excessive evapotranspiration induced by drought is now a generalized phenomenon as a result of global climate change. This greatly affects plant growth and development. Increased salt concentration has been shown to reduce seed germination in many plant species; it antagonistically affects shoots and roots and also affects the development of plant reproductive organs (Van Hoorn, 1991).

Among the five sucrose transporter genes analyzed in the present study, OsSUT2 was found to be progressively up-regulated during the 10 day course of drought and salinity treatment of three week old rice (*Orzya sativa* L. cv Nipponbare) plants. Additional work needs to be done in order to identify the changes in sucrose metabolism and more precisely to describe the transporters involved at protein level, as well as confirm which respective promoters and transcription factors are involved in their expression. The putative *cis-acting* regulatory elements in the promoter regions of these transporters have been identified *in silico* in Chapter 3. Out of the five OsSUTs identified in rice, OsSUT2 had the highest drought and salinity related *cis*-acting regulatory elements. This suggests the up-regulation of transcription of the SUT2 during drought and salinity stresses reported in this study.

Alternatively, the changes observed may be due to down regulation of sucrose for grain filling, since the major task of the plant will be channelled to overcome dehydration induced during drought and salinity stress. As a result, OsSUTs involved in transport of sucrose to sink organs like flower, anther, and grain may be down regulated. Rice sucrose transporter genes have been reported to be expressed in sink and source organs, with lower expression in the source organs than sink (Hirose et al. 1997; Aoki et al. 2003; Scofield et al. 2007a).

Noiraud et al. (2000) found that AgSUT1 expression was decreased in all organs and distinctly in roots of celery plants when subjected to salt stress (30 days watering with 300mM NaCl). Although AgSUT1 was found to be expressed in matured leaves and in petioles of celery, it is also expressed in sink organs such as roots. Recently Charkazi et al. (2010), found SUT4 and SUT5 to be down regulated in 10 days old salt-sensitive wheat (*Triticum aestivum* L. cv. falat) seedlings, when exposed to different NaCl osmotic potential (-0.25 – 1.5 MPa). Thus the low expression and regulation of OsSUT1, OsSUT3, OsSUT4 and OsSUT5 observed in the present study show that they may not be fully involved in sucrose transport during plant dehydration induced by drought or increase in salinity. However, more comprehensive reporter gene expression studies need to be carried out to fully understand the control of gene expression in different tissues during drought and salinity stresses.

Although increases in mannitol and other polyols were considered to be a response to salt stress (Pardossi et al., 1998; Stoop and Pharr, 1994), our results indicate that sucrose transport is certainly not repressed under these conditions. Mannitol synthesis has been reported to increase at the expense of sucrose (Everard, et al., 1994) during salinity stress in celery plants; however the flux of sugar during these stress conditions still needs to be further studied in a less salt tolerant and economic crop such as rice. Celery plants were reported to grow new leaves when cultivated on a 300mM NaCl solution; a concentration equivalent to half that of sea water (Everard, et al., 1994).

Furthermore, Everard et al. (1994) reported a 4-fold increase in mannitol/sucrose ratio in salt stressed celery plants as compared to controls. Mannitol dehydrogenase was found to be repressed in order to maintain a high level of mannitol, which acts as an osmoprotectant. Sucrose metabolism on the other hand is channelled to meet the energy need of the stressed tissues (Everard et al., 1994). This could therefore indicate that sucrose transport has to be maintained, which conforms to the observed up-regulation of OsSUT2 in source-exporting leaves in order to supply more sucrose for the increased energy for cellular activities and for osmoprotection.

Using clustalW multiple sequence alignment, OsSUT2 (Protein Id: BAC67163) protein was found to have 61% and 83% similarity with AtSUT4 (Protein Id: AAG09191) and HvSUT2 (Protein Id: CAB75881) proteins respectively (See Appendix H). AtSUT4 is a low-affinity/high capacity saturable sucrose transporter (Weise et al., 2000), while HvSUT2 is a vacuolar sucrose

transporter (Endler et al., 2006). AtSUT4 and HvSUT2 were reported to be expressed mainly in mesophyll cells and minor veins of source leaves, where high-capacity sucrose transport is needed for long distance sucrose phloem loading and for vacuolar storage of photosynthetically derived sucrose respectively. Therefore the transport of sucrose facilitated by OsSUT2 may be essential for long distance sucrose transport to meet the osmoprotectant activity of sucrose for the plant and for the cellular energy demands during drought and salinity stresses.

4.6 CONCLUSION

Transport activities of osmolytes are of great significance in plants, because they are only produced in the leaves. Hence, studies should be focused more on the elucidation of the molecular mechanism that links the effect of diverse stress stimuli to source-sink regulation. Coordination of development with the availability of nutrients such as sucrose may aid in ensuring a sufficient supply of nutrient and energy essential in carrying out specific developmental activities and also to withstand environmental stresses.

Attempts to develop environmental stress tolerant plants through genetic transformation have led to several significant successes; but the complex stress response mechanism of plants to enable abiotic stress tolerance hampers progress. Thus, a comprehensive understanding of physiological and molecular processes underlying drought and salt tolerance in the major economic crop plants is needed and more research should be directed towards osmoprotectant metabolism and transport in crop plants and in transgenic plants engineered for salt tolerance.

The differential regulation of sucrose transporter genes during drought and salinity stresses reported here may provide a link to the previous reports of Flowers and Yeo (1981) and Lutts et al. (1995). Sensitivity of rice plants to salinity stress was shown to vary at different growth stages. Plants were more sensitive to salinity stress at the young seedling stage than at the reproductive stage. Therefore improvement of drought and salt tolerance in rice should be targeted at the specific growth stage that is most sensitive to drought and salinity stresses, and which affects plant productivity and yield.

This study reveals the need for and the involvement of OsSUT2 in the adaptation of rice plants to cope with drought and salinity stresses, thus cultivars with a higher expression of this gene may show increased capacity to survive these environmental stresses.

4.7 REFERENCES

1) Aoki, N., Hirose, T., Scofield G. N., Whitefeld P.R. and Furbank R.T. (2003) The sucrose transporter gene family in rice. *Plant Cell Physiology* **44**:223-232.

2) Berman, M. E. and Dojong, T.M. (1996) Water stress and crop load effects on fruit fresh and dry weights in peach (*Prunus persica*) *Tree Physiology* **16**:859-864

3) Bray, E.A., Bailey-Serres, J. and Weretilnyk, E. (2000) Responses to abiotic stresses, In: W. Gruissem, B. Buchannan, R. Jones (Eds.), Biochemistry and Molecular Biology of Plants, American Society of Plant Biologists, Rockville, MD, Pp: 158–1249.

4) Davidson R.L. (1969) Effect of soil nutrients and moisture on root/shoot ratio in *Lolium perenne L and Trifolium repens*. *Annals of Botany* **33**:571-577

5) Delrot, S., Atanassova, S. and Maurousset, L. (2000) Regulation of sugar, amino acid and peptide plant membrane transporters. *Biochimica et Biophysica Acta* **1465**:281-306

6) Endler, A., Meyer, S., Schelbert, S., Schneider, T., Weschke, W., Peters, S.W., Keller, F., Baginsky, S., Martinoia, M. and Schmidt U.G. (2006) Identification of a vacuolar sucrose transporter in *Hordeum vulgare* and *Arabidopsis thaliana* mesophyll cells by a tonoplast proteomic approach. *Plant Physiology* **141**:191-207

7) Everard, J.D., Gucci, R., Kann, S.C., Flore, J.A. and Loescher, W.H. (1994) Gas exchange and carbon partitioning in the leaves of celery (Apium graveolens L.) at various levels of root zone salinity. *Plant Physiology* **106**:281-292

8) Flower, T.J. and Yeo, A.R. (1981) Variability in the resistance of sodium chloride salinity within rice (*Oryza sativa* L.) varieties. *New Phytology* **88**:363-373

9) Hellmann, H., Barker, L., Funck, D. And Frommer, W.B. (2000) The regulation of assimilate allocation and transport. *Australian Journal of Plant Physiology* **27**:489-496

10) Hirose, T., Imaizumi, N., Scofield, G.N., Furbank, R.T. and Ohsugi, R.(1997) cDNA cloning and tissue specific expression of a gene for sucrose transporter from rice (*Oryza sativa* L.) *Plant Cell Physiology* **38**:1389-1396

11) Hirose, T., Endler, A. and Ohsugi, R. (1999) Gene expression of enzymes for starch and sucrose metabolism and transport in leaf sheaths of rice (*Oryza sativa* L.) during the heading period in relation to the sink to source transition. *Plant Production Science* **2**:178-183

12) Koch, K.E. (1996). Carbohydrate-modulated gene expression in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47**: 509-540.

13) Lutts, S., Kinet, J.M. and Bouharmont, J. (1995) Changes in plant response to NaCl during development of rice (*Oryza sativa* L.) varieties differing in salinity resistance. *Journal of Experimental Botany* **46**:1843-1852

14) Meyer, S., Lauterbach, C., Niedermeier, M., Barth, I., Sjolund, R.D. and Sauer, N. (2004) Wounding enhances expression of AtSUC3, a sucrose transporter from *Arabidopsis* sieve elements and sink tissues. *Plant Physiology* **134**: 684-693.

15) Mito, N., Wimmers, L.E. and Benneth, A.B. (1996) Sugar regulates mRNA abundance of H^+-ATPase gene family members in tomato. *Plant Physiology* **112**:1229-1236

16) Molden, D., Schipper, L., de Fraiture, C., Faurès, J., and Vallée, D. (2007). Water for Food, Water for Life: A Comprehensive Assessment of Water Management in Agriculture. Earthscan, London, and International Water Management Institute, Colombo.

17) Noiraud, N., Delrot, S. and Lemoine, R. (2000) The sucrose transporter of celery. Identification and expression during salt stress. *Plant Physiology* **122(4):**1447-1455

18) Pardossi, A., Malorgio, F., Oriolo, D., Gucci, R., Serra, G. and Tognoni, F. (1998) Water relations and osmotic adjustment in Apium graveolens during long-term NaCl stress and subsequent relief. *Physiology Plant* **102**:369-376

19) Reddy, A.R. and Das, V.S.R. (1986) Stomatal movement and sucrose uptake by guard cell protoplasts of *Commelina benghalensis* L. *Plant and Cell Physiology* **27**:1565-1570

20) Ritte, G., Rosenfeld. J., Rohig, K. and Raschke K. (1999) Rates of sugar uptake by guard cell protoplast of *Pisum sativum* L. related to the solute requirements for stomatal opening. *Plant Physiology* **121**:647-655

21) Rontein, D., Basset, G. and Hanson, A.D. (2002) Metabolic engineering of osmoprotectants accumulation in plants. *Metabolic Engineering* **4**:49-56

22) Sauer, N. (2007) Molecular physiology of higher plants sucrose transporters. *FEBS Letters* **581**:2309-2317

23) Scofield, G. N., Hirose, T., Aoki, N. and Furbank, R.T. (2007a) Involvement of sucrose transporter, OsSUT1, in the long-distance pathway for assimilates transport in rice. *Journal of Experimental Botany* **58**(12):3155-3169

24) Scofield, G.N., Aoki, N., Hirose, t., Takano, M., Jenkins, C.L.D. and Furbank R.T. (2007b) The role of the sucrose transporter, OsSUT1, in germination and early seedling growth and development of rice plants. *Journal of Experimental Botany* **58(3)**: 483-495

25) Shinozaki, K. and Yamaguchi-Shinozaki, K. (2000). Molecular responses to dehydration and low temperature: Differences and cross-talk between two stress signaling pathways. *Current Opinion Plant Biology* **3**:217–223

26) Smeekens, S. (1998) Sugar regulation of gene expression in plants. *Current Opinion in Plant Biology* **1**: 230-234.

27) Smeekens, S. (2000) Sugar-induced signal transduction in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **51**:49–81

28) Stoop, J.M.H. and Pharr, D.M. (1994) Mannitol metabolism in celery stressed by excess macronutrients. *Plant Physiology* **106**:503-511

29) Talbott, L. D. and Zeiger, E. (1996) Central roles for potassium and sucrose in guard-cell osmoregulation. *Plant Physiology* **111**:1051-1057

30) Van Hoorn, J.W. (1991) Development of soil salinity during germination and early seedling growth and its effects on several crops. *Agricultural Water Management* **20**:17-28

31) Wang, J., Jiang, J. and Oard, J.H. (2000) Structure, expression and promoter activity of two polyubiquitin genes from rice (Oryza sativa L.). *Plant Science* **156**:201-211

32) Weise, A., Barker, L., Kuhn C., Lalonde, S., Buschmann, H., Frommer ,W.B. and Ward, J.M. (2000) A new subfamily of sucrose transporters SUT4 with low affinity/high capacity localized in enucleate sieve elements of plants. *Plant Cell* **12**:2395-2407

33) Yeo, A.R., Lee, K.S., Izard, P., Boursier, P.J. and Flowers, T.J. (1991) Short and long term effects of salinity on leaf growth in rice (Oryza sativa). *Journal of Experimental Botany* **42**: 881–889

Chapter 5

Effect of Rusty Plum (*Hysteroneura setariae*; Thomas) Aphid Infestation on the Expressional Levels of Sucrose Transporters in Rice (*Oryza sativa* L. cv Nipponbare).

5.1 INTRODUCTION

Plants and insects are continuously interacting together in the ecosystem in a wide range manner. They are close in several advantageous deeds which include shelter, oviposition sites and food supply for insects by plants, while insects help plants in pollination. Nevertheless, depending on the amount of insect attacks, insects might be tremendously harmful to plants resulting in death.

There are two main groups of plant insect pests: the chewing insects and the piercing/sucking insects. The latter pierce cells/tissues with their stylets and subsequently feed on the exuberant amount of plant sap. Some piercing and sucking insects feed on mesophyll cells, epidermal or parenchyma cells; others are phloem feeders (Walling, 2000). The phloem-feeding insects are highly specialized in their mode of feeding and they present a unique stress on plant fitness.

There are various phloem feeding insects that feed on plants. Aphids (*Hemiptera, Aphidoidae*) are the major group, and they are mostly temperate zone insects, with about 4000 species worldwide (Dixon, 1998), of which 250 species are considered pest species (Blackman & Eastop, 2000). Aphids feed on a plant for a prolonged period of time. They suck plant sap from conductive tissue by employing their specialized slender stylet-like mouth parts to probe intercellularly through the epidermal and mesophyll cell layer so as to feed on the photoassimilates contained in the plant's phloem sieve elements and by so doing weaken the plant (Walling, 2000).

Aphids average from about 1/6 cm (Nymph; newly born aphids) to 2/3 cm (adult giant winged aphid). They are pear-shaped and can easily be distinguished from other insects by a pair of tubular appendages of variable length called cornicles that protrudes out from the posterior ends (Figure 5.1). Aphid nymphs are wingless, but wing pads may be visible on those individuals that will mature into winged adults. Adults may be winged or wingless and when wings exist, they are two transparent pairs. They have the same basic characteristic features as nymphs with the exception of wings, if present (Figure 5.1). Winged adults have a hardened and darkened thorax to support their wing structure, but non-winged adults are more uniform in colour and do not have a hardened thorax. Many aphids are green, but there are those that are other colours such as white, yellow, red (pink), and brown, black or speckled. Some aphids have two or multiple colours, such as the green peach aphid where the adults are yellowish green or red form; cabbage aphids and wooly apple aphids where the adults produce a white or gray waxy substance that covers their abdomen; rose aphids where the adults are light green or pink; potato aphids where the adults are pink or green.

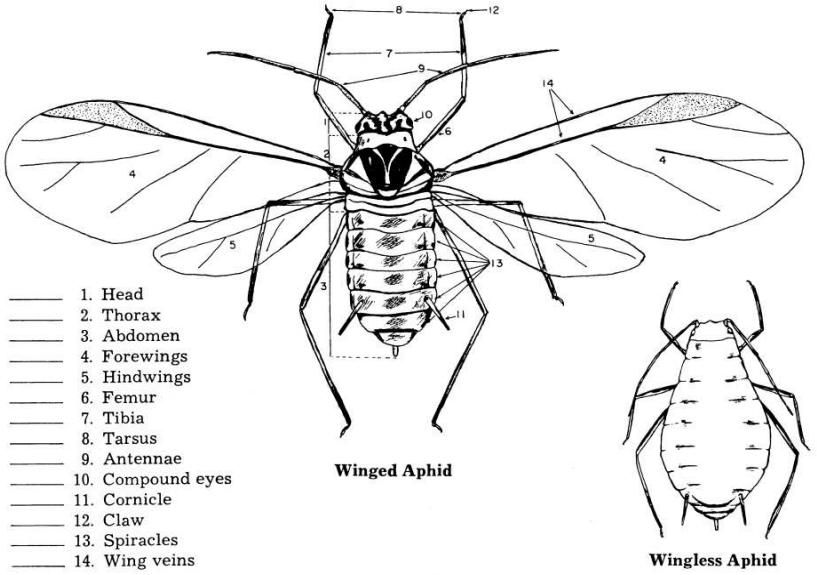


Figure 5.1: Characteristic features of aphids

Source: http://downloads.cas.psu.edu/4H/PartsOfAnInsect.pdf

Detailed aphid description and life cycle are concisely reviewed in Dixon (1987; 1998) and Blackman and Eastop (2000). Using electrical penetration and radiolabelling studies, aphid behavior and the role played by their saliva during feeding have been extensively studied (Tjallingii, 1988; Miles, 1999; Cherqui and Tjallingii, 2000; Tjallingii, 2005; Will et al., 2007). The physiological and biochemical interactions of aphids with plants; and the interaction of other ecological organisms within the aphid-plant co-habitation are reviewed in Ibraheem et al. (In Press, 2011).

Rusty plum aphids (*Hysteroneura setariae*; Thomas) are small rusty brown or dark purple coloured aphids, of about 1.7mm in length. They possess white bands at the base of their antennae, abdomen and appendages; with dark colouration to the upper parts of their appendages (Figure 5.2; Blackman and Eastop, 2000).They are commonly found on cherry, peach and plum trees, but are also pests of a wide genera of grasses such as *Cynodon*, *Eragrotis*, *Cyperaceae*, *Eleusine*, including cereal crops (Blackman and Eastop, 2000). They usually lay their eggs in cracks and crevices over winter, producing their offspring near the flowering period of their host plants, when they start to feed on the abaxial sides of plant leaves, causing significant damage to the leaves. Like other aphids, they produce sticky honey dew that covers foliage leading to sooty mildew infestation and they are also vectors of several plant viruses (Douglas, 2003; Ng and Perry, 2004).

The co-habitation of aphids and plants is very intriguing. Plants have developed an efficient mechanism to protect themselves against aphids, while aphids have found diverse ways of avoiding the negative effects of their host plants defense mechanisms. Plant responses to aphid feeding usually varies from no visible effect to localized discolouring of leaf or stem tissues (chlorosis) to stern reduction of plant vigor, leading to plant death (Walling, 2000). Aphids have been investigated and are found to cause significant damage to valuable crop plants such as wheat, barley, rice, oats etc. due to the following reasons:

(1) they are vectors of numerous pathogens that gain entrance into the plant cells/tissues through aphid infestation on the plants (Matthews, 1991);
(2) they secrete watery saliva that contains several latent signal-generating enzymes such as β-glucosidase, oxidase and many yet unknown proteins/enzymes (Miles, 1999).

Figure 5.2: Rusty plum aphid (*Hysteroneura setariae*; Thomas)

5.2 SPECIFIC OBJECTIVE

With the anticipated rise in the world population, the main concern for agriculture is to attain the highest production of food in a way that is environmentally friendly and cost effective. Losses due to insect herbivory for major economic crops are a significant factor in limiting food production. The wounds caused are potential entry points for opportunistic pathogens, leading to a grave environmental stress for the plant. It is therefore essential for plants to express the product of a range of defence genes at the wound site as a resistance to this stress and also as an obstruction against possible infection by pathogens. During aphid herbivory, it is assumed that insect saliva is

responsible for the triggering of responses because it contains peroxidases, β-glucosidases and other signal-responsive proteins and enzymes (Miles, 1999; Paul et al., 2000). Plant stress responses have been reported to be tightly linked to the up-regulation of sucrose-sink metabolism to satisfy the energy requirements and the activation of these cascades of defence actions (Smeekens, 1998; Rolland et al., 2006; Ibraheem et al., 2008).

It is not known to what extent cells/tissues can cope with unexpected changes in the cellular metabolism that result from increased respiratory activities of wounded tissues. Storage organs can adjust to this change by increasing carbohydrate breakdown, but in other organs with low carbohydrate reserves, carbon and energy requirements are met through carbohydrate exogenously supplied in the form of sucrose. Therefore sucrose transporter activity will be essential to the import of sucrose from the neighboring apoplasmic cells to meet the metabolic needs of the adjacent injured cells. Sucrose transporter activity may also constitute a defense action for effective reduction and/or retrieval of leaked photoassimilates during vascular perturbation by the aphid stylets back into phloem. Mechanisms used by plants to retrieve lost solute back into the apoplast have been postulated by Botha et al. (2008).

This study therefore aims at investigating the differential expression and regulation of rice sucrose transporters during aphid infestation. This approach will aid in understanding the expression and regulatory activities of sucrose transporters involved in the distribution of photoassimilates and the accumulation of carbohydrates during insect herbivory, which will further enhance the knowledge of the sucrose source-sink metabolism.

5.3 METHOD

5.3.1 Cultivation of Plant Materials and Treatment

5.3.1.1 Cultivation of rice (*Orzya sativa* L. cv Nipponbare) at optimum conditions

Seeds of rice (*Orzya sativa* L. cv Nipponbare) cultivar were obtained as a kind gift from Dr. R. Furbank, CSIRO Plant Industry, Canberra, ACT2601, Australia. The rice seeds were germinated according to the method of Scofield et al. (2007a). Rice plants were grown to maturity (3 week old at 4 leaf stage) in flooded paddy tanks (containing 75% compost and 25% perlite), housed in a Conviron EF-10H controlled environment cabinet(Conviron Winnipeg, Manitoba, Canada), and programmed 25^{o}C, 56% (relative humidity), CO_2 (ambient) and at a light intensity of 400 umol m^{-2} sec^{-1}.

5.3.1.2 Maintenance of rusty plum aphids (*Hysteroneura setariae*; Thomas) colonies

Rusty plum aphids colonies were maintained on young rice (*Orzya sativa* L. cv Nipponbare) grown in another Conviron under the same condition as specified above but kept in insect cages (Figure 5.3).

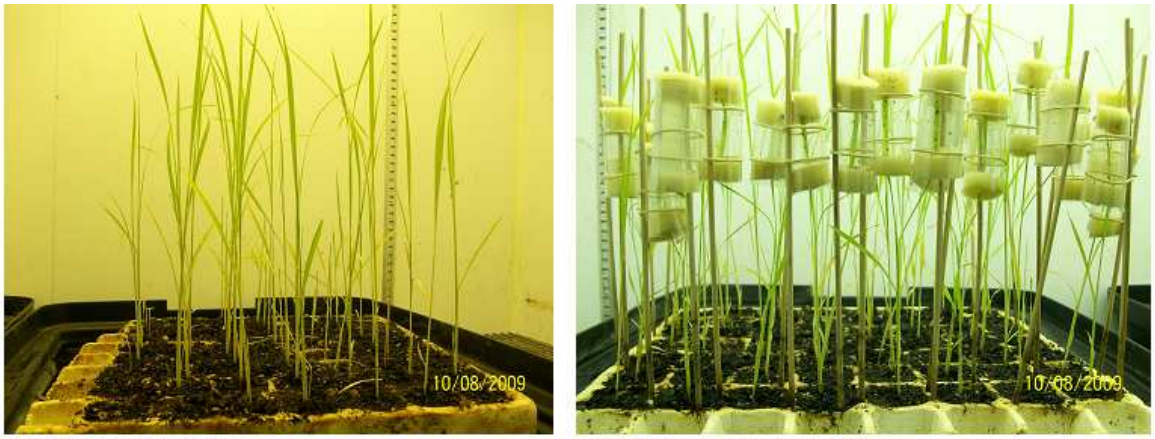
Figure 5.3: (A) Rusty plum aphids (*Hysteroneura setariae*; Thomas) maintained in insect cage

(B) Light microscopy view of Rusty plum aphids (*Hysteroneura setariae*; Thomas) on rice leaf

5.3.1.3 Aphids infestation on rice (*Orzya sativa* L. cv Nipponbare)

Thirty-six rice plants were grouped in triplicate to give six test experimental groups, and six matching control groups. A fine camel hair brush was used to place 10 adult rusty plum aphids on the second or third visible, fully-expanded leaf on experimental plants. An aphid cage was attached to the leaves, enclosing the settled aphids. Test and control plants were maintained in a Conviron under the same condition as specified above, for 10 days (Figure 5.4).

Treated leaves were harvested as grouped at 24hrs (Day 1), 48hrs (Day 2), 72hrs (Day 3), 120hrs (Day 5), 168hrs (Day 7) and 240hrs (Day 10) post treatment. Leaves from control plants were harvested the same number of times at each time point without aphid treatment. Leaves harvested from the test and control experiments were quickly submerged in liquid nitrogen, stored at -80^{o}C for total RNA extraction. In a similar fashion, rice plants were germinated and leaves harvested from groups of triplicate stressed plants and control plants were used for total protein extraction.



CONTROL RICE PLANTS APHID INFESTED RICE PLANTS

Figure 5.4: Conviron set-up of rice plants for Rusty plum aphids (*Hysteroneura setariae*; Thomas) infestation experiment. Rice plants were grown to maturity (3 week old at 4 leaf stage) in flooded paddy tub (containing 75% compost and 25% perlite), housed in a Conviron EF-10H controlled environment cabinet(Conviron Winnipeg, Manitoba, Canada), and programmed 25^{o}C, 56% (relative humidity), CO_2 (ambient) and at a light intensity of 400 umol m^{-2} sec^{-1}.

5.3.2 Analysis of Differentially Expressed Sucrose Transporter Genes

5.3.2.1 Isolation of total RNA from controls and treated rice plants

All equipment used for the RNA isolation was made RNase free by autoclaving and rinsing thoroughly in RNase away solution. Leaves harvested from the triplicate plants were pooled to minimize inter plant variability and were ground to powder in liquid nitrogen prior to RNA extraction. RNA from the ground tissues was extracted using the SV 96 Total RNA isolation system (Promega, USA), following the manufacturer's instructions. Extracted RNA was quantified using a PerkinElmer Lambda 35 UV/VIS Spectrophotometer at 260nm and electrophoresed on 1% agarose gel to verify its integrity.

5.3.2.2 Amplification of sucrose transporters (SUT) genes by reverse transcriptase polymerase chain reaction (RT-PCR)

The RT-PCR reactions were carried out using an Eppendorf AG 22331, Hamburg Master Cycler, and Qiagen® one step RT-PCR kit (Qiagen, USA), following the manufacturer's instructions. Total RNA template was used at concentrations varying from 50 – 1000ng, in a 50µl RT-PCR reaction volume.

Specificity was obtained by using primers for the gene of interest as designed by Scofield et al., 2007. They confirmed the specificity of the primer pairs by sequencing amplified bands, which gave the desired sequences. Thus these primers were used for RT-PCR. (Refer to Chapter 4; Table 4.1 for the rice sucrose transporter primer pairs and RT-PCR thermal cycling conditions).

5.3.2.3 Relative quantification of amplified SUT genes by Real-Time PCR

qPCR was carried out using a two step method. Firstly, 1µg of total RNA from each sample was converted to cDNA using the iScriptTM cDNA synthesis kit (BIO-RAD Laboratories, Inc. USA), following the manufacturer's instructions. Quantification of the SUT cDNA genes was performed using the same gene-specific primers as used for RT-PCR. Aliquots of the cDNA corresponding to 50ng of each input sample RNA were used as templates for the Real-Time PCR analysis. Reactions were carried out on a Bio-Rad iCycler Real-Time PCR Detection System with iQ^{TM} SYBR$^{®}$ Green Super mix (BIO-RAD Laboratories, Inc. USA), according to the manufacturer's instructions. (Refer to Chapter 4; section 4.3.2.3 for the thermal cycling conditions).

PCR products were detected by monitoring the increase in fluorescence of the SYBR Green during the extension phase of each cycle when the SYBR Green was bound to double stranded DNA.The results obtained for the different cDNAs were normalized against the expression levels of two stable reference genes, rice polyubiquitin 1(RubQ1) and rice actin, using the qBasePlus programme produced by BioGazelle (version 1.3; http//www.biogazelle.com). (Refer to Chapter 4; section section 4.3.2.3 for RubQ1 and actin primer pairs).

The relative mean fold expression level and standard error of each of the SUT genes was determined for the treatment and control groups at each time point by reference to the sample with lowest expression for each gene, using qBasePlus programme (Version 1.3).

5.3.2.4 Isolation of total proteins from control and treated rice plants

Leaves harvested from triplicate plants were pooled to minimize interplant variability and ground to powder in liquid nitrogen. Total proteins were extracted using the Bio-Rad total protein extraction kit. 3ml of the kit sample buffer was added to 1g of each ground tissues and placed on ice. The suspensions were distrupted by stirring until samples were homogenous. Samples were centrifuged at 13,000g for 30 minutes and supernatants were collected. The protein content of all samples was determined using the RC/DC assay (Bio-Rad, USA); a modification of the Folin-Lowry assay (Lowry et al., 1951). Protein contents were determined by interpolation from a standard curve using bovine serum albumin Fraction V as a standard.

5.3.2.5 Monitoring the expression levels of OsSUT1 proteins by western blot analysis and its relative quantification.

Polyclonal antibodies raised in a rabbit against a synthetic peptide 'LPTKSNEPAEPEGTGPLAV' were used. It corresponds to a span of amino acid sequence from position 293-311 in the OsSUT1 protein sequence (registered with accession number: AF280050, in the protein data bank of National Center for Biotechnology Information (NCBI; www.ncbi.nih.nim.gov)) produced by Gen Script USA Inc, (Order id number: 101259_1).This peptide is the same as that used by Scofield et al. (2007b). The peptide sequence was used because of the low percent similarity to the protein sequences of the other rice sucrose transporter proteins. The low percent similarity makes it suitable for specific recognition by its antisera. Actin was used as a positive control. Actin (H-300) rabbit polyclonal IgG primary antibody was purchased from Santa Cruz Biotechnology Inc. 2145 Delaware Avenue, Santa Cruz, Califonia, 95060.

SDS-PAGE was performed to separate the protein samples (Laemmli, 1970). Equal protein concentration (5µg) was loaded into each well to ensure the same amount of protein. Protein samples were transferred onto nitrocellulose membrane by a BioRad Protean III system using 48mM Tris pH 9.2, containing 39mM Glycine, 20% Methanol and 0.05% SDS. OsSUT1 and actin proteins were detected by carrying out western blot analysis on two separate Hybond ECL nitrocellulose membranes for the control and the treated protein samples respectively. Anti-OsSUT1 and anti-actin were mixed together in the concentration recommended by manufacturer and were used to probe the Hybond ECL nitrocellulose membranes.

Blotted membranes were blocked overnight in the refrigerator at 2-8^{o}C in ECL blocking and antibody diluent solution, which was composed of 2%(w/v) ECL Advanced Blocking agent dissolved in Tris- Buffer saline, containing 0.1% (v/v) Tween-20 (TBS-T). The blocked membranes were washed with TBS-T at two changes of the buffer and incubated in a 1:5000 and 1:200 dilution of the primary antibodies of OsSUT1 and actin respectively for one hour at room temperature on an orbital shaker (as recommended by the manufacturer). The membranes were washed in a TBS-T buffer volume greater than 4ml/cm^2 of the membrane for 15 minutes at room temperature and also for 3 times 5 minutes with fresh changes of TBS-T buffer.

The goat anti-rabbit IgG-horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology Inc.) was diluted in ECL blocking and antibody diluent solution at 1:5000 (as recommended by the manufacturer). The membrane was incubated with secondary antibody for one hour at room temperature on an orbital shaker, then washed in a TBS-T buffer volume greater than

4ml/cm^2 of the membrane for 15 minutes at room temperature and also for 3 times 5 minutes with fresh changes of TBS-T buffer.

Lumigen™ TMA - 6 A & B detection solutions (GE Healthcare UK Ltd, Buckinghamshire, UK) were mixed in a ratio of 1:1 to give a final volume of 0.1ml/cm^2 of the membrane. The membrane was drained from residual TBS-T buffer; the detection solutions mixture pipette directly onto it and was incubated for 1 minute at room temperature. Excess detection mixture was drained off and the bound detection solution visualized under chemilluminiscent light using the Alliance 4.7 Transilluminator (UVITEC Ltd, Cambridge, UK). The images of the immuno-blots were quantified using the UVI Band software system (UVITEC Ltd, Cambridge, UK). This allowed for quantification of the differential expressed OsSUT1 protein as normalized to the intensities of the respective actin signals in both control and treated plants. To quantify the specific chemilluminiscent signal of OsSUT1 in each lane, the background was subtracted from the obtained values and the result plotted as a fold increase relative to the expression of actin.

5.3.3 Analysis of OsSUT1::*GUS* Activity Localization

5.3.3.1 Construction of OsSUT1::*GUS* gene and transformation into rice plant

The OsSUT1::*GUS* gene was constructed by Scofield et al., 2007b, by amplification of DNA fragment spanning -1675 to +19 nucleotides of the regulatory (promoter) region of OsSUT1 gene, using PCR. The product was ligated into pZH2B transformation vector that carries the hygromicin resistant gene (Figure 5.5) and this construct was transferred into Agrobacterium tumefaciens strain EHA101 (pIG121Hm) by electroporation. Transformation into rice tissue was carried out following the method described by Hiei et al. (1994).

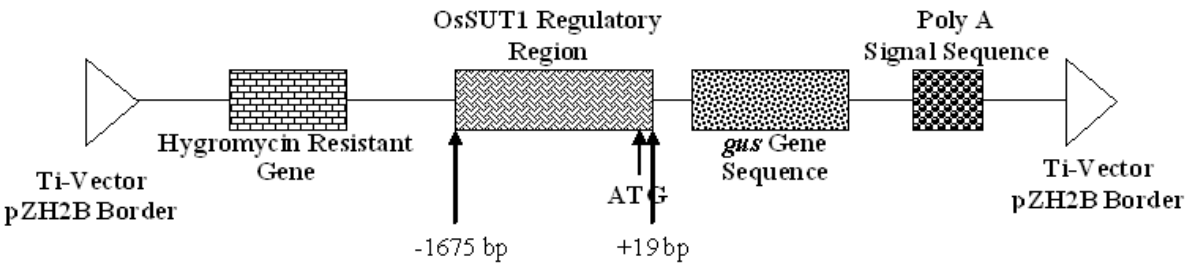


Figure 5.5: Representation of OsSUT1::*GUS* gene fusion construct (Scofield et al., 2007b)

5.3.3.2 Cultivation of OsSUT1::*GUS* transgenic rice plants and aphid treatment

The OsSUT1::*GUS* was constructed and transformed into rice (*Orzya sativa* L. cv Nipponbare) plants by Scofield et al. (2007b). The transgenic rice seeds were obtained as a kind gift from Dr. R. Furbank, CSIRO Plant Industry, Canberra, Australia. Cultivation of these seeds and aphid treatment procedures was the same as described in sections 5.3.1.1 and 5.3.1.3 respectively. After aphid treatment, leaves were stained for OsSUT1::*GUS* expression.

5.3.3.3 Exposure of cut ends of OsSUT1::*GUS* transgenic rice plant to 2% sucrose solution

Cultivation of OsSUT1::*GUS* transgenic rice plants was as described in section 5.3.1.1. A matured leaf was cut at the base and immediately immersed in distilled water, and this was allowed for 10 minutes at room temperature. The leaf was removed and immediately immersed in 2% sucrose solution for 10 minutes at room temperature. Thereafter, the leaf was removed, thoroughly rinsed in distilled water and stained for OsSUT1::*GUS* expression.

5.3.3.4 Staining of transgenic rice for *GUS* expression

Staining of transgenic rice plant tissues for *GUS* expression was carried out using the Imagene Green ^{TM} (C_{12}FDGlcU *GUS* gene expression Kit (I-2908) manufactured by Molecular Probes, USA), following the supplier recommended protocol. Imagene Green is specific for the localization of β-glucuronidase in cells using wide field fluorescence microscopy methods, specifically localizing the fluorescence 5-N-dodecanoyl aminofluorescein; which is formed after enzymatic cleavage of C_{12}FDGlcU substrate by β-glucuronidase, in the cells that expresses the marker gene.

The transgenic rice plant tissues were cut into thin sections (approximately 15µm) using a McILWAIN tissue chopper (Mickle Laboratory Engineering Company Limited, Surrey, England) and collected in 25mM Na-Phosphate buffer (pH 7.2).The unfixed cross-sections were transferred to, and immediately incubated in 100µM of C_{12}FDGlcU at room temperature for 30 minutes.

Post staining treatment involved washing twice in 25mM Na-Phosphate buffer (pH 7.2), and fluorescence visualized using the Olympus BX61 wide-field fluorescence microscope (Olympus, Tokyo, Japan) fitted with U-YFP (UMWIB 2) filter set (10c/Topaz 41028, Chroma Technologies, Battlebro, U.S.A), with absorbance (Abs) / emission (Em) of the reaction product at 390/525 nm. Images were taken and saved using the analySIS$^{(R)}$ Five Olympus Soft Imaging System programme (Olympus GmHb, Germany), and selected images were exported to Corel Draw 12 (Corel Corporation Ottawa, Canada 2003).

5.4 RESULTS

5.4.1 Total RNA Extraction and OsSUTs' Primer Pairs Confirmation

All the total RNA extracts yielded two major bands on 1% agarose gel representing 28S and 18S rRNA(data not shown). The A_{260nm}/A_{280nm} ratio for each extract was above 2, indicating the high purity of the extracts (Figure 5.6).

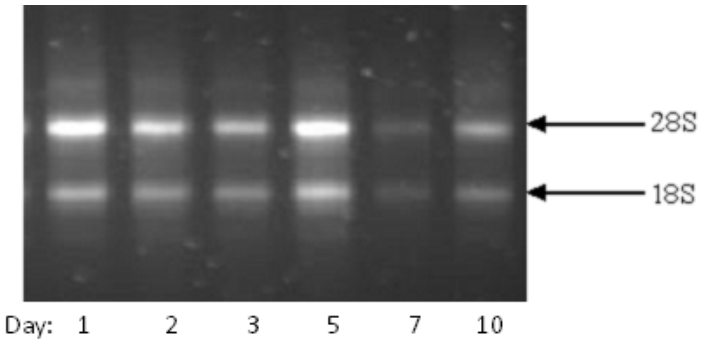


Figure 5.6: Representative sample of 1% agarose gel electrophoresis of total RNA extracts of Aphid infestation experiment (See Appendix F for the pictures of all the 1% agarose gel electrophoresis of total RNA extracts from control and stressed rice plants).

All primer pairs amplified a single product of the expected size, which was confirmed on 1% agarose gel (Figure 5.7) and by analysis of the melt-curves after qPCR (Figure 5.8), which gave a single band or single peak respectively; with the exception of OsSUT3 which showed no clear peak in the melt-curve analysis as a result of its overall poor expression during the aphid treatment (See Appendix G). Despite this, the one step RT-PCR reaction was able to amplify the small portion of the OsSUT3 mRNA leading to the observed clear band of the expected size.

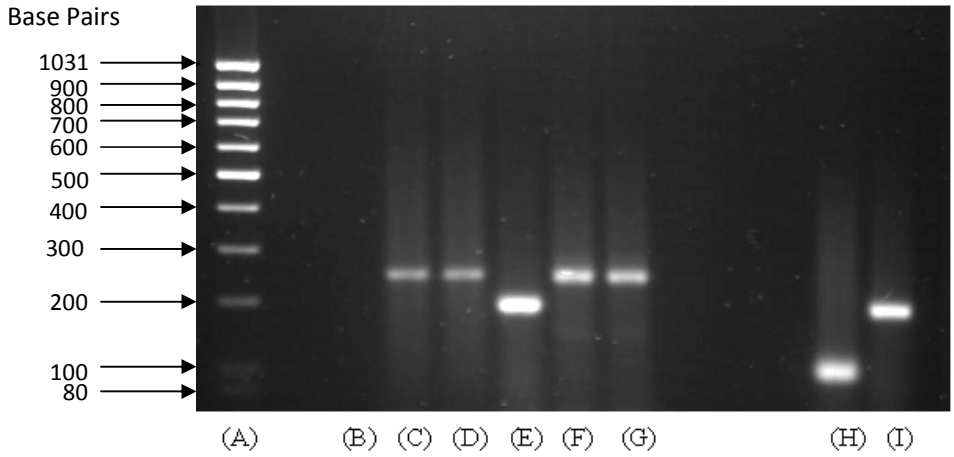


Figure 5.7: Confirmation of RT-PCR amplification by the primer pairs on 1% agarose gel, where:- (A) Fermenta Mass Ruler DNA Ladder; Low Range, (B) Non-template Control, (C) OsSUT 1; 245bp, (D) OsSUT 2; 243bp, (E) OsSUT 3; 194bp, (F) OsSUT 4; 249bp, (G) OsSUT5; 250bp, (H) RubQ1;100bp, (I) Actin; 195bp.

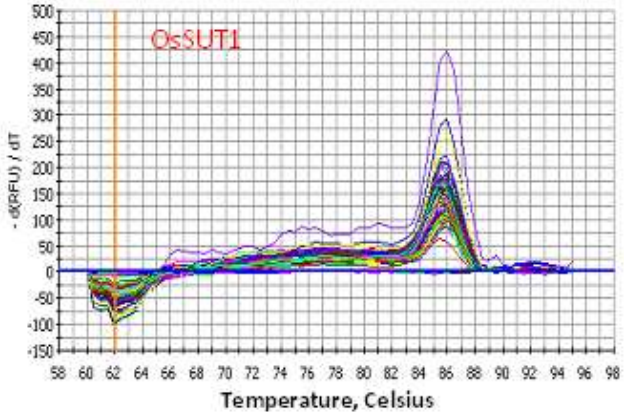


Figure 5.8: OsSUT1 melt curve analysis; a representative sample (See Appendix G for the analysis of the rest rice sucrose transporter genes)

5.4.2 Quantitative Real-Time PCR

qPCR analysis of rice sucrose transporter genes revealed differential expression of the genes (Figure 5.9), with changed transcript amounts observed in the treated plants when compared with the controls.

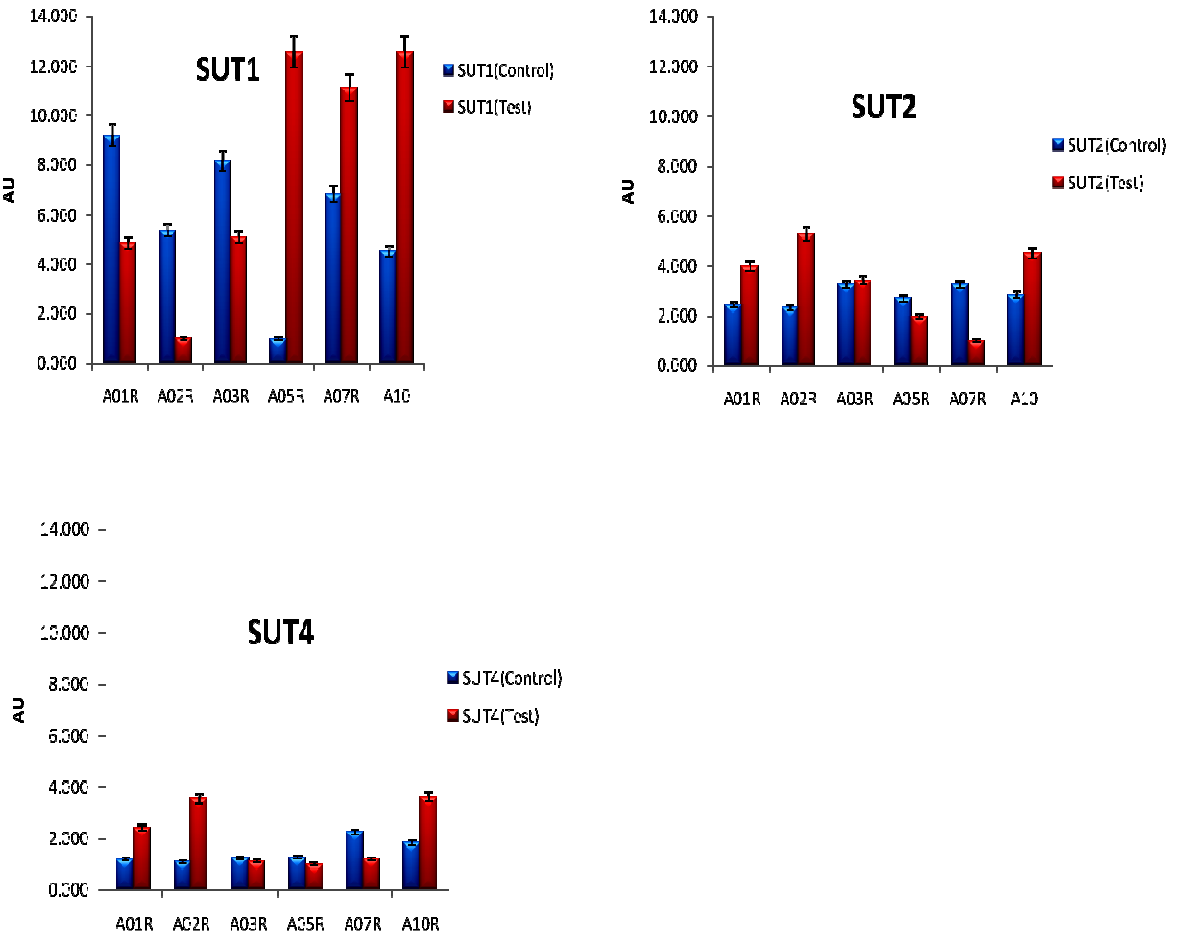


Figure 5.9: Quantitative PCR analysis of OsSUT1, OsSUT2 and OsSUT4 genes during Rusty plum aphid infestation on rice (*Orzya sativa* L. cv Nipponbare) cultivar plants. All values were normalized to the relative expressional levels of rice Polyubiquitin 1 and rice actin genes by analysing the ratios of the mRNA concentrations of individual OsSUT genes to the rice Polyubiquitin 1 and rice actin genes. Each is the average of pooled tissue from triplicate sets of plant material tested in duplicate. Error bars representing the standard error were obtained from the qBase Plus (Version 1.3) analysis, representing 95% confidence. A01R, A02R, A03R, A05R, A07R and A10R represent samples from aphid treatments on day 1, 2, 3, 5, 7 and 10 days after infestation. AU represents the fold change in expression relative to the minimum value obtained for each gene. SUT5 showed no clear difference in transcript expression levels, while no detectable transcript expression of SUT3 was found (data not shown).

There was an observable progressive up-regulation of SUT1 gene when the transcript levels in the treated plants were compared with the controls. At first, the transcript level of SUT1 in the treated plant was reduced to approximately half of the control at day 1. It further diminished on day 2, but gradually showed an up-regulation at day 5 through to day 10 when the difference between

the transcripts levels of the control plants from the treated was approximately 12-fold, 1.5-fold and 3-fold; with respect to days 5, 7 and 10.

The expressional levels of SUT2 and SUT4 were relatively low in both control and treated plants, and a transient upward trend followed by down regulation was observed during the period of infestation. Expression was elevated to approximately 2.5-fold and 3-fold with respect of SUT2 and SUT4 in treated plants compared to control after 48hrs (day 2) of infestation and then declined back to control transcript levels until day 5, and much lower at day 7. The transcript level in the treated plants later rose again to approximately 1.5-fold and 2-fold with respect to SUT2 and SUT4 in treated plants compared to controls at day10.

SUT5 showed no clear difference in transcript expression in the control and treated rice plants, while there was no detectable SUT3 transcript expression in the control and treated rice plants (data not shown).

5.4.3 Western Blot Analysis and Relative Quantification of OsSUT1 Protein

SDS-PAGE separation of 5µg of protein from each pooled triplicate set of control and treated plant samples is shown in Figures 10a and 10b, with the western blot analysis (Figures 10c and 10d) revealing differential expression of OsSUT1 protein. The chemiluminiscent OsSUT1signals normalized to the intensities of respective actin signals in the control and treated plant are shown as a percentage fold increase of OsSUT1 relative to actin (Figure 10e).

This result indicates that OsSUT1 protein expression increases as the days of aphid infestation progress. A similar change was noticeable in control plants; however, at a lower expression level. A significant increase in the protein levels in the treated plants to approximately 2.5-fold of the control plants was observed on day 1. Appreciable increases were also found on days 2, 3, 5 and 10, when the treated plants had approximately 1.5 times the level of OsSUT1 protein when compared to the control plants (day 7 showed an approximately 1.2-fold increase in the treated plants relative to the expression in the control plants). These changes demonstrate that cellular expression of OsSUT1is regulated during development as well as in response to physiological changes occurring during phloem feeding.

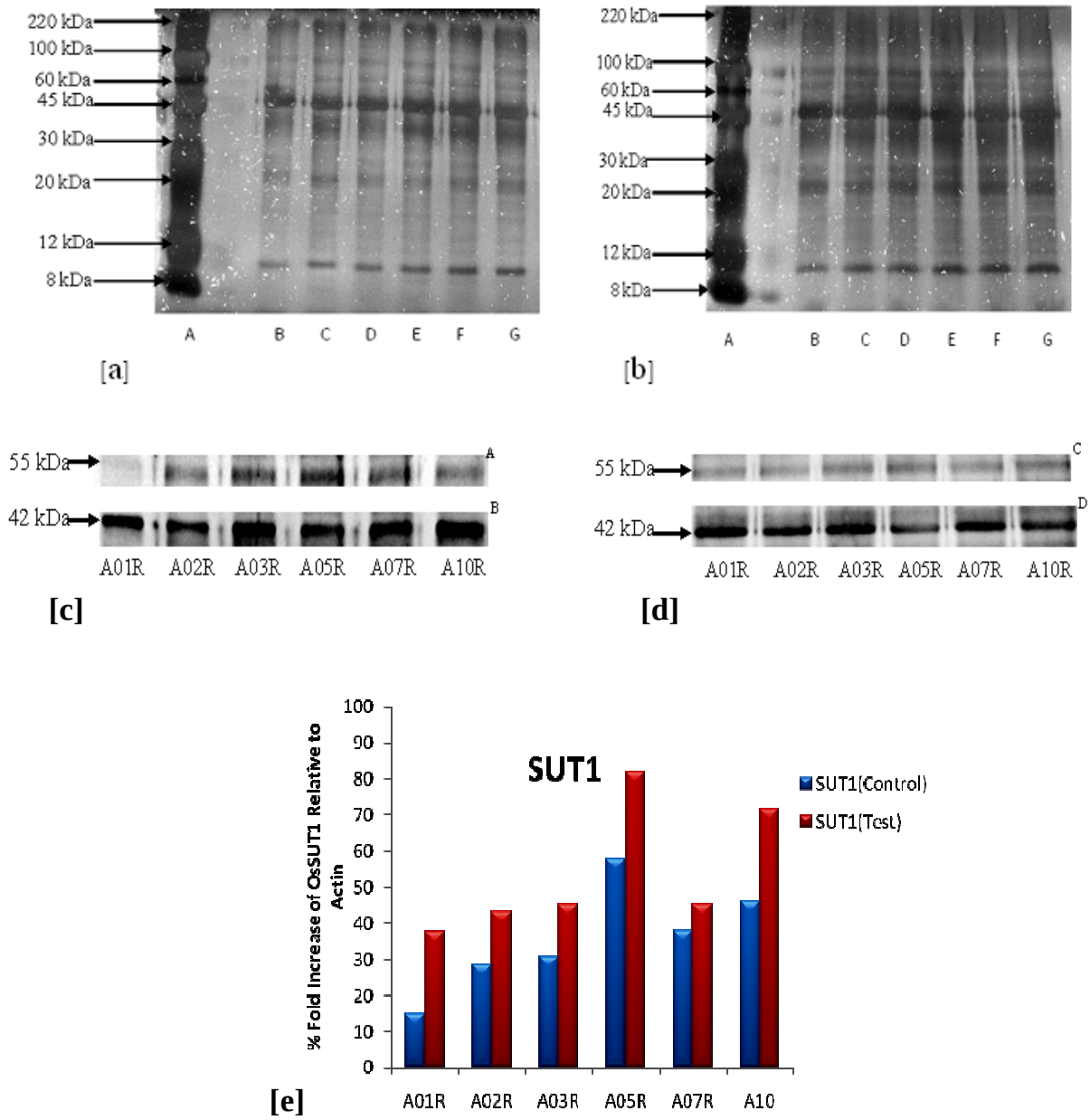


Figure 5.10: Western blot analysis and relative quantification of OsSUT1protein in rice (*Orzya sativa* L. cv Nipponbare) cultivar plants. [a] and [b] are 12% SDS-PAGE of plants protein samples. [a] is control while[b] is aphid infested samples. Each sample is an average of pooled tissue from triplicate sets of plant material. 5µg of protein was loaded into each lane. In [a] and [b]: A, Sigma Color Burst™ electrophoresis marker; B, day 1; C, day 2; D, day 3; E, day 5; F, day 7; G, day 10. [c] and [d] are western blotting analysis is as described in section **5.3.2.5**. In [c]: A and B are OsSUT1 and Actin proteins in control plant samples. In [d]: C and D are OsSUT1 and Actin proteins in aphid infested plant samples. A01R, A02R, A03R, A05R, A07R and A10R represent samples from aphid treatments on day 1, 2, 3, 5, 7 and 10 days after

infestation. [e] OsSUT1 chemilluminescent signals were quantitated using the UVI Band software package from UVITEC and result normalized to the intensities of respective Actin signals in both control and treated rice plants. Results are shown as a percentage fold increase of OsSUT1 relative to Actin.

5.4.4 OsSUT1::*GUS* Activity Localization

Cross-sections of the infested leaf tissues of OsSUT1::*GUS* transgenic rice (*Oryza sativa* cv Nipponbare) plants harvested at 12hrs and 1, 2, 3, 5, 7 and 10 days after rusty plum aphids infestation(DAI) were examined in order to establish the localization of OsSUT1 promoter::*GUS* activity at the cellular level. *GUS* activity was as indicated by the yellowish-green 5-dodecanoylaminofluorescein formed after enzymatic cleavage of C_{12}FDGlcU substrate by β-glucuronidase, in cells which are expressing OsSUT1 promoter::*GUS* activity (Figure 5.11). Figures 5.11 A-I show the expression of the *GUS* activity in cross-sections of leaves. Sections from the control plants (no aphid, see Figure 5.11A for example) generally contained autofluorescence associated with the lignified tissues. Here low levels of OsSUT1 promoter::*GUS* expression (darts) are limited to isolated parenchyma cells in proximity to the large metaxylem vessels. Importantly, none was evident associated with the phloem pole of the vascular bundles. This result is similar to that described by Scofield et al. (2007a).

In the plants that had been infested with aphids, OsSUT1 promoter::*GUS* activities were also found in the phloem and xylem parenchyma cells, fluorescence signal intensity from the OsSUT1 promoter::*GUS* increased with the length of time of aphid infestation. Figures 5.11 B-I illustrate the progression of the OsSUT1 promoter::*GUS* expression with increasing time of aphid infestation. By day three, some evidence of activity within the phloem tissues became evident, suggesting that leakage from damaged phloem tissue had commenced stimulation of the OsSUT1 protein, as more solute was leaked from the symplast into the apoplast (leakage to intercellular spaces). After seven days (Figure 5.11G) damage to the phloem tissue appears to be severe and high levels of OsSUT1 promoter::*GUS* were evident in all sections in repeated experiments. OsSUT1 promoter::*GUS* expression was clearly enhanced with increasing aphid feeding time.

Occasionally, OsSUT1 promoter::*GUS* expression was seen within the large metaxylem vessels, with strongest fluorescence intensity associated with the pit membrane regions bordering the vessels and their xylem parenchyma cells (Figure 5.11 I). Likewise, during exposure of the transgenic rice plants to 2% sucrose solution, OsSUT1 promoter::*GUS* expression was seen within

the protoxylem as well as in xylem parenchyma between the two large metaxylem vessels (Figure 5.12 A, B). Some expression was also associated with the phloem parenchyma (Figure 5.12 B).

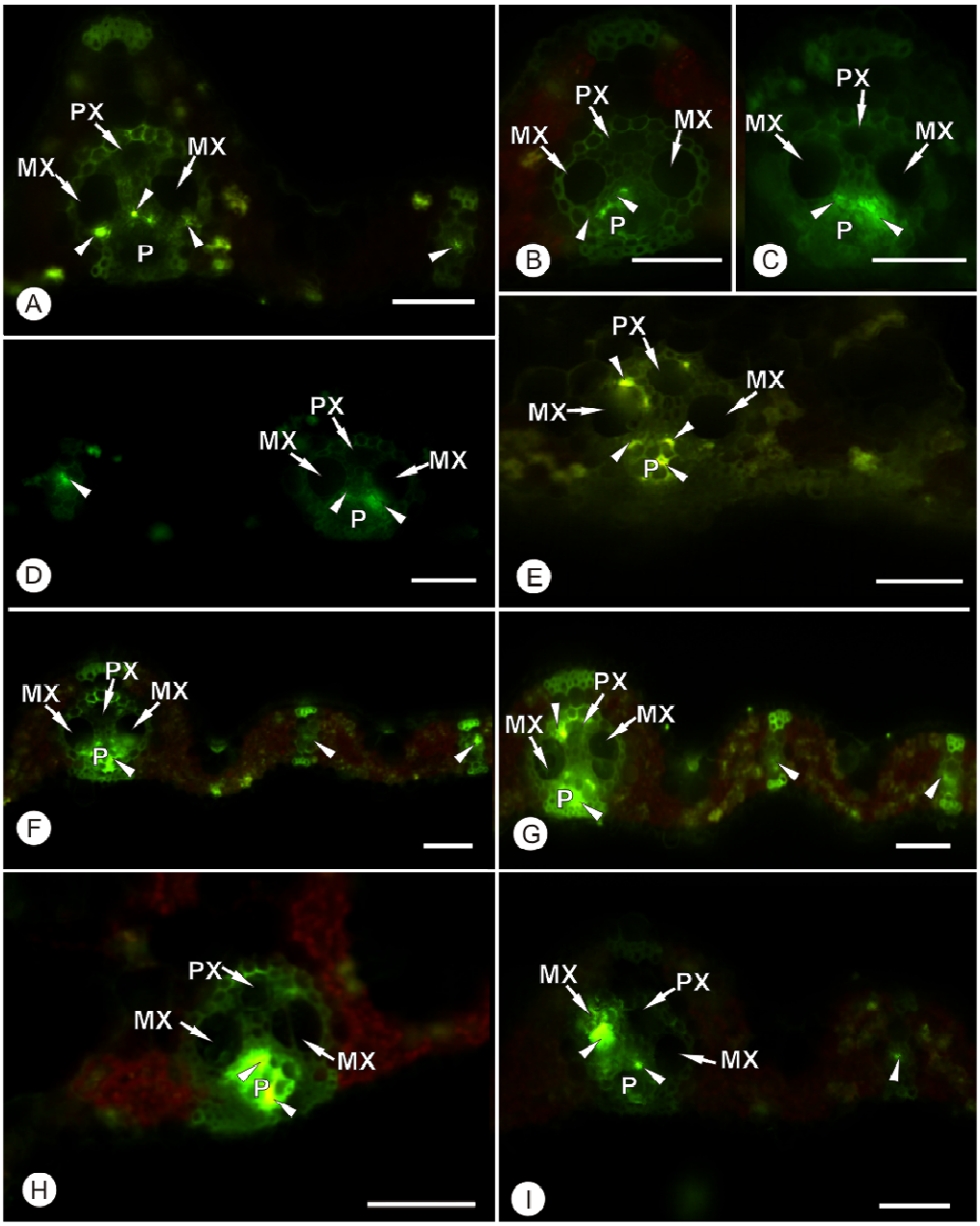


Figure 5.11: (A – I) Cross-sections through the mid portions of mature of rice leaf blade tissues, counterstained with ImaGene Green$^{(TM)}$ for the cellular localization of OsSUT1-promoter::*GUS* expression during aphid infestation. Arrowheads show the localization of the bright yellow green OsSUT1-promoter::*GUS* expression in the vascular parenchyma associated with the xylem as well as with phloem parenchyma and companion cell/sieve element complexes. (A) Cross-sections of large showing a large (left) and small intermediate (right) vascular bundle in control (uninfested) leaf blade.

(B – I) illustrate localization of the OsSUT1-promoter::*GUS*, which is progressively more intense, with increasing aphid feeding time. After 12 and 24h (Figure 5.11B, 11C) reaction product is clearly strongly associated with the vascular parenchyma adjacent to the large metaxylem vessels. This remained relatively unchanged through day two (Figures 11D). By three days, (Figure 5.11E) stronger expression of OsSUT1-promoter::*GUS* was evident. Here, expression was evident in xylem parenchyma cells between the metaxylem and the protoxylem lacunae, as well as on the phloem face of vascular bundles. Longer feeding (5, 7 and 10 days; Figures 5.11 F, G and H-I respectively), increasing OsSUT1-promoter::*GUS* was expressed in the phloem tissue. Abbreviations: MX, metaxylem; P, phloem tissues; PX, protoxylem. Scale bar: A – I = 200µm. [Note: for a high photographic resolution image see Appendix J (a)].

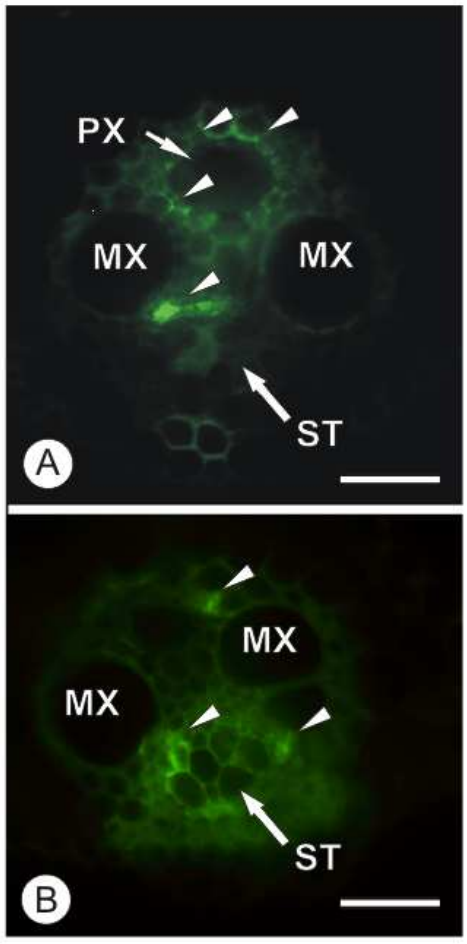


Figure 5.12 :(A-B) Show the effect of exposure of the cut ends of mature leaves to a 2% sucrose solution. (A) Shows strong OsSUT1-promoter::*GUS* expression in parenchyma cells associated with the protoxylem (PX, darts) as well as in xylem parenchyma between the two large metaxylem vessels (MX). Some expression is associated with the phloem

tissue as well. (B) Cross-section, 5 cm above the base of the cut leaf. OsSUT1-promoter::*GUS* is associated with the protoxylem lacuna (arrowhead) in this large vascular bundle, as well as some with the xylem parenchyma associated with the metaxylem vessels. Some phloem parenchyma associated with the phloem is fluorescing. Bars = 200 µm. [Note: for a high photographic resolution image see Appendix J (b)].

5.5 DISCUSSION

Molecular techniques have revealed that wounding and pathogen infection elevate the expression of sugar transporters (Sakr et al. 1993; Truernit et al., 1996; Sakr et al., 1997; Fotopoulos et al., 2003; Meyer et al., 2004; Kempema et al., 2007). Wounding will result in the leaking of solutes, including sucrose, from the phloem and therefore the differential expression and regulation of these sucrose transporters may be needed to facilitate the readsorption of the sugars.

In plants, responses to abiotic and biotic stresses may be spontaneous or may occur a day or two after the initial environmental stress, as a result of changes in gene activity leading to the production of proteins which are not normally produced in plants or normally made in small quantities in particular organs (Inbar et al., 1999; Paul et al., 2000; Shinozaki and Yamaguchi-Shinozaki, 2000; Chen, 2008).

Expression of the majority of the characterized sucrose transporter genes is confined largely to the vascular tissue; in particular the phloem, where the gene products are involved in phloem loading (Reismeier et al., 1994; Truernit and Sauer, 1995; Stadler et al., 1996). High expression of sucrose transporter genes is found mostly in source leaf tissues and the suppression of their expression using antisense technology clearly supports the concept of their phloem loading capability (Riesmeier, 1994; Lemoine et al., 1996).

In this study OsSUT1gene expression was up-regulated during the 10 day aphid treatment of three week old rice (*Orzya sativa* L. cv Nipponbare) plants. The suggests that OsSUT2 & 4 are not involved in sucrose transport in three week old rice, but may be required during later stages of plant development (e.g. sinks, flowering and grain filling; Hirose et al., 1997; Furbank et al., 2001; Aoki et al., 2003; Lim et al., 2006; Scofield et al., 2007a; Scofield et al., 2007b). OsSUT3 and OsSUT5, which showed no clear difference in transcript expression in the control and experimental plants during the 10 day aphid treatment, have been reported elsewhere to be highly expressed in the

source leaf blade tissues where they may be involved in the transport of sucrose from the apoplasm to sink organs (Hirose et al., 1999; Aoki et al., 2003).

Under control conditions, SUT1 gene expression has been reported to be mainly localized in companion cells in rice leaves (Scofield et al., 2007b). Other immunolocalization studies revealed the presence of SUT1 protein in the enucleated sieve elements (SEs) and associated with the plasma membrane of tobacco, potato, tomato and also in wheat and rice; indicating that phloem loading occurs at the plasma membrane of SEs and thus supporting the proposition that the SUT1 protein may have been imported to the sieve element from the adjacent companion cells via pore-plasmodesmatal connections (Riesmeier et al., 1994; Kühn et al., 1996; Kühn et al., 1997; Lalonde et al., 2003; Botha, 2005; Aoki et al., 2006; Scofield et al., 2007a). Previous work has also shown OsSUT1-promoter::*GUS* expression at the xylem parenchyma cells surrounding the xylem vessels (Fukuda et al., 2005; Scofield et al., 2007b). SUT1 thus has different patterns of localization of its gene transcript compared to the functional SUT1 protein.

Wounding is known to increase the expression of sucrose transporters (Sakr et al. 1993; Sakr et al., 1997; Meyer et al., 2004; Kempema et al., 2007). The *in-silico* promoter analysis of the OsSUT genes, as reported in Chapter 3, revealed differential amounts of W-box, GT1-elements and GCC-box. However, they were found more in OsSUT1 than in the other OsSUT genes. These *cis*-acting regulatory elements are required for response to wounding; be it artificial mechanical damage, insect herbivory or pathogen infection. OsSUT1 gene expression is therefore likely to be regulated by wounding.

The expression of OsSUT1-promoter::*GUS* within the vascular parenchyma cells and companion cell/sieve element complexes, is due to injury inflicted by aphids probing and penetrating the phloem sieve tubes and the xylem tissues, resulting in the puncture of epidermal and mesophyll cells. Injection of salivary material (which contains numerous enzymes and proteins that degrade plant material) consequently triggered plant responses which were evident as bright, localized fluorescence regions in these tissue regions (see bright spots in Figure 5.11). As a result of aphid feeding and probing, a large number of vascular cells would be damaged. Not surprisingly, the resultant leakage of sugars from the phloem tissue would stimulate the expression of SUT1 as is evident in the images in Figure 5.11 (B – I), where a large proportion of the fluorescence is associated with vascular parenchyma and companion cells. The appearance of fluorescence associated with xylem parenchyma (Figure 5.11) is a result of the aphids probing xylem (drinking water) thus causing additional leakage of sugars to the xylem apoplast and a consequential recovery of the sugar by the OsSUT1 located in the xylem parenchyma cells (Figure 5.12). Thus it is not surprising to observe as the days of infestation progress to observe the

appearance of OsSUT1-promoter::*GUS* expression localized within the vascular parenchyma and/or companion cells associated with phloem. However, it should be noted that it is also possible that the *GUS* reaction fluorescent product could have leaked into these cells following damage, giving rise to the observe appearance of fluorescence in the cells.

OsSUT1 expression is thus similar to STP4 gene (sugar transport protein 4) in *Arabidopsis* which showed changes in transcript levels both at different developmental stages and under the environmental stress of mechanical wounding, elicitor treatment and pathogen infection (Truernit et al., 1996). Further reports have also shown the regulation of sucrose transporters during wounding. Sakr et al. (1997), when studying the effects of mechanical stress on membrane transport of sugar in aged and freshly cut tissues of matured sugar beet (*Beta Vugaris* L.) leaves, reported that there was a large increase in the gene transcript of SUT1. Meyer et al. (2004) showed that SUC3 reporter protein was reproducibly increased at all cutting sites of *Arabidopsis* leaves. AtSUT1 was reported to be up-regulated in *Arabidopsis* during silver leaf white fly (SLWF) nymphs feeding (Kempema et al., 2007). Like aphids, SLWF nymphs are known phloem-feeders. However, SLWF nymphs feed carefully intercellularly on the phloem and they rarely damage the epidermal or mesophyll cells prior to puncturing of the phloem cells (Freeman et al., 2001).

There are bound to be sudden increases in cellular metabolism as a result of enhanced respiratory activities of wounded tissues during this aphid infestation. This may however, not always be adequately energized by endogenous resources of the particular wounded or infested cells. Thus, sugars need to be loaded into the affected cells to prevent the risk of energy constraint; this can be achieved by the rapid and simultaneous induction of genes involved in sucrose uploading. Although symplasmic sucrose import into the affected cells through plasmodesmata could be possible through simple diffusion (Botha, 2005), this might not be quick enough to permit a speedy wound defense response; therefore it is likely that the apoplastic sucrose transport will need to be up-regulated.

The data reported here lend strong support to the proposal by Botha et al., (2008) for an efficient retrieval pathway of solutes from the xylem to the xylem parenchyma cells in the rice leaf blade. The pathway involves transfer from the apoplast to the symplast via the pit membrane between metaxylem vessels and xylem parenchyma, across the vascular parenchyma cell plasma membrane. Their transmission electron microscopy (TEM) and dye (5,6-Carboxyfluorescein diacetate; 5,6-CFDA) studies provided evidences that this interface is essential in retrieval of large solutes, possibly via endocytosis. It was suggested that the pit membrane in association with the xylem parenchyma cells are potentially involved in retrieval, regulation of resorption and/or movement of solute leaked into the apoplast by their ability to take up the membrane- impermeant

fluorophores. This clearly supports the findings of this study, which showed high induction of OsSUT1- Promoter::*GUS* expression in the vascular parenchyma associated with the xylem as well as with phloem parenchyma and companion cell/sieve element complexes in rice leaf blades during the aphid infestation, than in control.The high expression of OsSUT1 in the xylem parenchyma cells is evidence for a specific function: the retrieval of sucrose leaked into the xylem apoplast.

5.6 CONCLUSION

The aim of biotechnologists and plant breeders is to engineer durable and multi-mechanistic resistance to insect pests. To achieve this, knowledge of inducible defense mechanisms and anunderstanding of the diversity of plant response to insect attack is needed.

Attempts to control aphids by spraying vulnerable crops with insecticides has limited success due to the development of increased aphid resistance to insecticides, as well as the fact that aphids protect themselves in unusually curled leaves, preventing insecticides from reaching them (Du Toit, 1989; Quick et al., 1991). Thus the development of resistant cultivars is seen as the most realistic means of crop protection in terms of efficiency, cost value and environmental sustainability. In spite of the extensive studies on plant resistance, the exact molecular defense mechanism of resistant plants is still not clear. This lack of knowledge slows the process of effective resistant cultivar development, as aphids are able to develop the capacity to infest resistant crops (Quick et al., 1991).

The abundance and diversity of these insect pests creates a challenge to the understanding of molecular and physiological plant responses and resistance mechanisms against them. Aphid feeding on *Arabidopsis* leaves has been linked to responses that are generally associated with virulent pathogens, in contrast with the induction of pathogen related (PR) genes associated with chewing insects (Truernit et al., 1996). Thus the extent of plant responses to aphid feeding implies that many vital genes and mechanisms used by plants for perception, tolerance and resistance to phloem feeding are still unknown or poorly understood.

Plants and other higher organisms have multi-gene families whose gene products are highly related. Therefore, reporter gene fusion techniques to tag individual members of a gene family, along with transformation of these fusion genes into the genome, has enabled the expression of the individual gene to be studied separately from the other members, thus overcoming the background expression of other members of the family that can mask patterns of individual gene expression.

The regulatory parameters often studied in transgenic plants are those involved in spatial and temporal expression of gene activities. These reporter gene techniques also enable highly important genes with low amounts of product to be studied.

The differential expression and regulation of sucrose transporters in rice (*Orzya sativa* L. cv Nipponbare) during aphid infestation reported here may be a valuable information to be explore in breeding programmes to improve rice cultivars to tolerate insect herbivory or pathogen attack as a result of wounding.

5.7 REFERENCES

1) Aoki, N., Scofield, G.N., Wang, X-D., Patrick, J.W., Offler, C.E. and Furbank, R.T. (2006) Expression studies indicate sugar transporters play key roles in germinating wheat seeds. *Plant Physiology* **141**:1255-1263
2) Aoki, N., Hirose, T., Scofield, G.N., Whitefeld, P.R. and Furbank, R.T. (2003) The sucrose transporter gene family in rice. *Plant Cell Physiology* **44**:223-232
3) Blackman, R.L. and Eastop, V.F. (2000) Aphids on the World's Crops. An Identification and information guide. John Wiley and Sons Ltd, Chichester, UK.
4) Botha, C. E. J., Aoki, N., Scofield, G. N., Liu, L., Furbank, R. T. and White, R. G. (2008) A xylem sap retrieval pathway in rice leaf blades: evidence of a role for endocytosis? *Journal of Experimental Botany* **59(11)**: 2945–2954
5) Botha, C.E.J. (2005) Interaction of phloem and xylem during phloem loading: functional symplasmic roles for thin- and thick-walled sieve tubes in monocotyledons. In: Holbrook NM, Zwieniecki MA, eds. Vascular transport in plants. Amsterdam: Elsevier Academic Press, Pp 115–130.
6) Brewer, M. J. and Donahue, J.D. (1998) Leaf and stem feeding Aphids. Cooperative extension service, University of Wyoming, College Of Agriculture, B-1050.4 Feb., Pp 1-4
7) Burd, J.D. and Burton, R.L. (1992). Characterization of plant damage caused by Russian wheat aphid (Homoptera: Aphididae). *Journal of Economic Entomology* **85**:2017-2022
8) Chen, M. S. (2008) Inducible direct plant defense against insect herbivores: A review. *Insect Science* **15**:101-114
9) Cherqui, A. and Tjallingii, W.F. 2000. Salivary proteins of aphids; a pilot study on identification, separation and Immunolocalization. *Journal of Insect Physiology* **46**:1177-1186
10) Dixon, A.F.G. (1998) Aphid Ecology: An Optimization approach Ed2. Chapman and Hall, New York.

11) Dixon, A.F.G. (1987) Parthenogenetic reproduction and the rate of increase in aphids. *In Aphids, Their Biology, Natural Enemies and Control. Edited by Minks AK, Harrewijn P. Elsevier.* Pp 269-287

12) Douglas, A.E. (2003) Nutritional physiology of aphids. *Advances in Insect Physiology* **31**:73-140.

13) Du, Toit, F. (1989) Inheritance of resistance in two *Triticum aestivum* lines to Russian wheat aphid (Homoptera: Aphididae) in South Africa. *Journal of Economic Entomology* **82**:1251-1253

14) Fotopoulos, V., Gilbert, M.J., Pittman, J.K., Marvier, A.C., Buchanan, A.J., Sauer, N., Hall, J.L. and Williams, L.E. (2003). The monosaccharide transporter gene, *AtSTP4* and the cell-wall invertase, *Atβfruct1*, are induced in *Arabidopsis* during infection with the fungal biotroph *Erysiphe cichoracearum. Plant Physiology* **132**:821 – 829

15) Freeman T.P., Buckner J.S., Nelson D.R., Chu C.C., Henneberry T.J. (2001) Stylet penetration by Bemisia argentifolli (Homoptera: Aleyrodidae) into host leaf tissue. *Annals Entomological Society of America* **94**:761-768

16) Fukuda A, Fujimaki S, Mori T, Suzui N, Ishiyama T, Yamaya T, Fuiwara T, Yoneyama T, Hayashi H. (2005) Differential distribution of proteins expressed in companion cells in the sieve element-companion cell complex of rice plants. *Plant and Cell Physiology* **46**:1779-1786.

17) Furbank, R.T., Scofield, G., Hirose, T., Wang, X.D., Patrick, J. and Offler, C. (2001) Cellular localization and fuction of a sucrose transporter OsSUT1 in developing rice grains. *Australian Journal of Plant Physiology* **28**:1187-1196

18) Hiei, Y., Ohta, S., Komari, T., Kumashiro, T. (1994) Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. *The Plant Journal* **6**:271-282

19) Hirose, T., Endler, A. and Ohsugi, R. (1999) Gene expression of enzymes for starch and sucrose metabolism and transport in leaf sheaths of rice (*Oryza sativa* L.) during the heading period in relation to the sink to source transition. *Plant Production Science* **2**:178-183

20) Hirose, T., Imaizumi, N., Scofield, G.N., Furbank, R.T. and Ohsugi, R. (1997) cDNA cloning and tissue specific expression of a gene for sucrose transporter from rice (*Oryza sativa* L.) *Plant Cell Physiology* **38**:1389-1396

21) Ibraheem, O., Hove, R.M. and Bradley, G. (2008) Sucrose assimilation and the role of sucrose transporters in plant wound response. *African Journal of Biotechnology* **7(25)**:4850-4855.

22) Ibraheem, O., Botha, C.E.J. and Bradley, G. (2010) *In-silico* analysis of *cis*-acting regulatory elements in 5′ regulatory regions of sucrose transporter gene families in rice (*Oryza sativa*

Japonica) and *Arabidopsis thaliana. Journal of Computational Biology and Chemistry* **34 (5-6)**:268-283

23) Ibraheem, O., Hove, R.M. and Bradley, G. (In Press, 2011) Understanding Aphid–Plant Physiological Interaction. *Fort Hare Papers.* Accepted for publication

24) Inbar M., doostdar H., Leibee G.L. and Mayer R.T. (1999) The role of plant rapidly induced responses in asymmetric interspecific interactions among insect herbivores. *Journal of Chemical Ecology* **25**:1961-1979

25) Kempema, L. A., Cui, X., Holzer, F. M., Walling, L.L. (2007). *Arabidopsis* transcriptome changes in response to phloem-feeding Silverleaf white nymphs. Similarities and distinctions in responses to aphids. *Plant Physiology* **143**: 849-865.

26) Kühn, C., Quick, W.P., Schulz, A., Lemoine, R. and Frommer, W.B. (1997) Localization and turnover of sucrose transporters in enucleated sieve elements indicate macromolecular trafficking. *Science* **275**:1298-1300

27) Kühn, C., Quick, W.P., Schulz, A., Riesmeier, J.W., Sonnewald, U. and Frommer, W.B. (1996) Companion cell-specific inhibition of the potato sucrose transporter SUT1. *Plant Cell and Environment* **19**:1115–1123.

28) Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680-685.

29) Lalonde, S., Tegeder, M., Throne-holst, M., Frommer, W.B. and Patrick, J.W. (2003) Phloem loading and unloading of sugars and amino acids. *Plant Cell and Environment* **26**:37-56

30) Lemoine, R., Kühn, C., Thiele, N., Delrot, S. and Frommer, W.B. (1996) Antisense inhibition of the sucrose transporter in potato: effects on amount and activity. *Plant Cell and Environment* **19**:1124–1131.

31) Lim, J.D., Cho, J-I., Park, Y-I., Hahn, T-R., Choi, S-B. and Jeon, J-S. (2006) Sucrose transport from source to sink seeds in rice. *Physiologia Plantarum* **126**:572-584

32) Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with Folin phenol reagent. *Journal of Biological Chemistry* **193**:265-275

33) Matthews, R.E.F. (1991) Relationship between plant viruses and invertebrates. In: R.E.F. Matthews Ed. Plant Virology, Ed3, Academic press, New York. Pp 520-561.

34) Matsiliza B. and Botha C.E.J. (2002) Aphid (*sitobion yakini*) investigation suggests thin walled sieve tubes in barley (*Hordeum vulgare*) to be more functional than thick-walled sieve tubes. *Physiologia Plantarum* **115**:137-143

35) Meyer, S., Lauterbach, C., Niedermeier, M., Barth, I., Sjolund, R.D. and Sauer, N. (2004) Wounding enhances expression of AtSUC3, a sucrose transporter from *Arabidopsis* sieve elements and sink tissue. *Plant Physiology* **134**:684-693

36) Miles, P.W. (1999) Aphid saliva. *Biological Reviews* **74**:41-85

37) Miller, H., Porter, D.R., Burd, J.D., Mornhingweg, D.W. and Burton, R.L. (1994). Physiological effects of the Russian wheat aphid (Homoptera: Aphididae) on resistant and susceptible barley. *Journal of Economic Entomology* **87**:493-499

38) Ng, J.C. K. and Perry, K. L. (2004) Transmission of plant viruses by aphid vectors. *Molecular Plant Pathology* **5(5):**505–511

39) Paul, N.D., Hatcher, P.E and Taylor, J.E. (2000) Coping with multiple enemies: an integration of molecular and ecological perspectives. *Trends Plant Science* **5**: 220-225.

40) Pollard D. G. (1973) Plant penetration by feeding aphids (Hemiptera Aphidoidea) a review. *Bulletins of Entomological Research* **62**:631-714

41) Quick, J.S., Nkongolo, K.K., Meyer, W., Peirs, F.B. and Weaver, B. (1991). Russian wheat aphid reaction and agronomic and quality traits of resistant wheat. *Crop Science* **31**:50-53.

42) Riesmeier, J.W., Willmitzer, L. and Frommer, W.B. (1994) Evidence for an essential role of the sucrose transporter in phloem loading and assimilate partitioning. *EMBO Journal* 13:1–7

43) Rolland, F., Baena-Gonzalez, E. and Sheen, J. (2006). Sugar sensing and signaling in plants: conserved and novel mechanisms. *Annual Review of Plant Biology* **57**: 675-709.

44) Sakr S., Noubahni M., Bourbouloux A., Riesmeier J., Frommer W. B., Sauer N. and Delrot S. (1997) Cutting, ageing and expression of plant membrane transporters. *Biochimica et Biophysica Acta* **1330**:207-216

45) Sakr, S., Lemoine, R., Gaillard, C. and Delrot, S. (1993) Effect of cutting on solute uptake by plasma membrane vesicles from sugar beet (Beta vulgaris L.) leaves. *Plant Physiology* **103(1)**:49-58

46) Scofield G. N., Hirose, T., Aoki, N. and Furbank R.T. (2007a) Involvement of sucrose transporter, OsSUT1, in the long-distance pathway for assimilates transport in rice. *Journal of Experimental Botany* **58(12)**:3155-3169

47) Scofield, G.N., Aoki, N., Hirose, t., Takano, M., Jenkins, C.L.D. and Furbank R.T. (2007b) The role of the sucrose transporter, OsSUT1, in germination and early seedling growth and development of rice plants. *Journal Experimental Botany* **58(3)**: 483-495

48) Shinozaki, K., and Yamaguchi-Shinozaki, K. (2000). Molecular responses to dehydration and low temperature: Differences and cross-talk between two stress signaling pathways. *Current Opinion in Plant Biology* **3**:217–223.

49) Smeekens, S. (1998) Sugar regulation of gene expression in plants. *Current Opinion in Plant Biology* 1: 230-234.

50) Stadler, R. and Sauer, N. (1996) The *Arabidopsis thaliana* AtSUC2 gene is specifically expressed in companion cells. *Botanica Acta* **109**:299–306.

51) Tjallingii, W.F. (2005) Salivary secretions by aphids interacting with proteins of phloem wound responses. *Journal of Experimental Botany* **57**:739-745

52) Tjallingii, W.F. (1988). Electrical recording of stylet penetration activities. In: A. K. Minks and P. Harrewijn (eds), Aphids. Their Biology, Natural Enemies and Control. Vol. B. Elsevier Science Publishers B.V. Amsterdam, Pp. 95-108

53) Truernit, E., Schmid, J., Epple, P., Illig, J. and Sauer, N. (1996) The sink-specific and stress-regulated *Arabidopsis* STP4 gene; enhanced expression of a gene encoding a monosaccharide transporter by wounding, elicitors and pathogen challenge. *Plant Cell* **8**:2169-2182

54) Truernit, E. and Sauer, N. (1995) The promoter of the *Arabidopsis thaliana* SUC2 sucrose-H^+ symporter gene directs expression of β-glucuronidase to the phloem: evidence for phloem loading and unloading by SUC2. *Planta* **196**: 564–570.

55) Wang J., Jiang J. and Oard J.H. (2000) Structure, expression and promoter activity of two polyubiquitin genes from rice (*Oryza sativa* L.). *Plant Science* **156**:201-211

56) Will, T., Tjallingii, W.F., Thonnessen, A. and van Bel, A.J.E. (2007) Molecular sabotage of plant defence by aphid saliva. *Proceedings of the National Academic of Science of the U.S.A* **104**:10536-10541

57) Will, T. and van Bel, A.J.E. (2006) Physical and chemical interactions between aphids and plants. *Journal of Experimental Botany* **57(44)**:729-737

58) Walling, L.L. (2000). The myriad plant responses to herbivores. *Journal of Plant Growth and Regulation* **19**:195-216

Chapter 6

Future Prospect

FUTURE PROSPECT

Photosynthesis allows plants to reduce inorganic carbon, nitrogen and sulphur, generating sugars, amino acids and peptides that play major physiological roles for plants themselves, and also for heterotrophic organisms feeding on plants. Sugar production through photosynthesis is the most fundamental activity in plant life. The processes of sugar synthesis, transport, utilization and storage are active and tightly linked to cell physiology, plant growth and development and environmental status. Sugar effects on gene expression have been characterized to its metabolism for energy production (Roitsch, 1999).

Current research has yielded a rapid increase in exciting information on proton-coupled co-transporters of the plasma membrane. The work has revealed the high degree of involvement of these transporters at the subcellular, cellular, tissue and organ levels. It has draw attention to the discovery and description of new transporter clones. However the physiological and biochemical work in terms of expression and regulation, especially under the environmental stress conditions to which plants are often subjected, are scarce. These environmental stress conditions are of great threat to plants, preventing them from attaining their complete genetic potential, and consequently limiting crop productivity. In response to these stress conditions, various genes are expressed and regulated, helping to alleviate their pronounced effects, and resulting in plant tolerance.

Sucrose transporters are essential proteins in carbon partitioning. They are localized at strategic positions and are therefore a central key for regulation. Physiological studies have shown that plant sucrose transporters and metabolism are highly regulated at the transcriptional and post-transcriptional levels. Plant cells are able to take up sucrose for energy production and for cellular carbon requirements through the sucrose transporter system that is coupled with an H^+-ATPase, which facilitates its activity.

Recently there have been considerable advances in understanding the role of sucrose transporters in plants, and that specific transporters play an essential role in apoplastic phloem loading and unloading. However, as more genes encoding plant sucrose transporters are identified and cloned, it is becoming evident that they are likely to have a variety of very specific functions in plants. Efforts towards revealing the function of sucrose transporters in higher plants was made when the first gene encoding sucrose transporters were cloned from spinach and potato (Riesmeier et al., 1992; Riesmeier et al., 1993).

Thus, the study of sucrose transporters in relation to different abiotic and biotic stress physiologies should become a rapidly evolving area. Major attention should be focused on the

elucidation of the molecular mechanisms that link the effect of diverse stress stimuli to source-sink sucrose regulation. To date, only a few comprehensive functional analyses of sucrose transporters in a small number of plant species have been documented. It will be a major challenge in the next few years to decipher the expression, localization, function, regulation and mechanism of action of these proteins encoded by putative membrane transporter sequences present in the rice genome at the subcellular and tissue levels, most importantly during the environmental stress conditions to which they are often exposed.

Identification of new transporters needs to be continued in plant species not yet investigated, most particularly in economic plants (cereals, fruit species). Understanding of the role of these transporters in individual species will provide further evidence of membrane sucrose transport in plants and their role towards plant development and stress tolerance. Immunolocalization studies of sucrose transporters have been restricted to only a few plants (*Arabidopsis, Solanaceae* and *Plantago*), but this should be carried out on a larger number of plant species. This will provide more knowledge of sucrose transporter regulation and sugar sensing.

Many sets of transporters are still unidentified, such as proton/solute antiporters and/or transporters mediating efflux across the tonoplast and the plasma membrane. There are the possibilities of sucrose efflux carriers that carry sucrose from the mesophyll cells to the apoplastic cells or from the conducting cell complex to the receiving cells in the sink, in addition to the normal plasma membrane sucrose transporters, but this still has to be investigated. To date no sucrose efflux carrier has been isolated. Experimental topological analysis of the transporters should be undertaken to check the structures predicted by the hydropathy algorithms. More precise information regarding the nature of the signals that trigger different sucrose transporter responses to abiotic and biotic stress will also need to be investigated in future.

As some for the investigated rice sucrose transporters contain hormonal associated *cis*-acting regulatory elements that could be involved in their expression and regulation, the expression patterns of sucrose transporters in relation to plant hormones therefore need to be investigated in future in other to give insight into the role of hormones in sucrose transporters functions.

In future quantitative determination sucrose and *GUS* activity will be expedient in order to further strengthen the evidence of sucrose import by the transporters and the increasing GUS expression respectively.

It will also be important to study the expression of sucrose transporters in the wild-type versus relevant OsSUT- mutant cultivars. This will give more insight into the relevance or role of the up-regulated sucrose transporters during the various stress treatment on the rice plants.

REFERENCES

1) Riesmeier, J.W., Willmitzer, L. and Frommer, W.B. (1992) Isolation and characterization of a sucrose carrier cDNA from spinach by functional expression in yeast. *EMBO Journal* **11**: 4705–4713.

2) Riesmeier, J.W., Hirner, B. and Frommer, W.B. (1993) Potato sucrose transporter expression in minor veins indicates a role in phloem loading. *Plant Cell* **5** : 1591–1598.

3) Roitsch T (1999) Source-sink reguklation by sugar and stress. *Current Opinion Plant Biology* **2**:198-206

4) Singh, R.B. (1993) Research development strategies for intensification of rice production in the Asia-Pacific region. In: Muralidharan K, Siddiq EA, Editors. New Frontiers in rice research. Hyderabad, India: Directorate of Rice Research, Pp 25-44

5) Xiao, J., Grandillo, S., Ahn, S.N., McCouch, S.R., Tanksley, S.D., Li, J. and Youn, L. (1996) Genes from wild rice improve yield. *Nature* **384**:223-224

LIST OF ARTICLES EXTRACTED FROM THESIS

1) Ibraheem, O., Hove, R.M. and Bradley, G. (2008)

   Sucrose Assimilation and the Role of Sucrose Transporters in Plant Wound Response. *African Journal of Biotechnology* **7 (25):** 4850-4855.

2) Ibraheem, O., Botha, C.E.J. and Bradley, G. (2010)

   In-silico Analysis of *Cis*-Acting Regulatory Elements in 5′ Regulatory Regions of Sucrose Transporter Gene Families in Rice (*Oryza sativa* Japonica) and *Arabidopsis Thaliana*. *Journal of Computational Biology and Chemistry* **34 (5-6)**: 268 - 283

3) Ibraheem, O., Hove, R.M. and Bradley, G. (2011)

   Understanding Aphid–Plant Physiological Interactions. *Fort Hare Papers.* Accepted for publication

4) Ibraheem, O., Dealtry, G., Roux, S. and Bradley, G. (2011)

   Effect of Drought and Salinity on the Expressional Levels of Sucrose Transporters in Rice (*Oryza sativa* Nipponbare) Cultivar Plants. *Plant Omics Journal*: Accepted for publication.

5) Ibraheem, O., Dealtry, G., Roux, S., Botha, C.E.J. and Bradley, G. (2011)

   Up regulation of the rice sucrose transporter, OsSUT1, is enhanced by Rusty Plum (*Hysteroneura setariae*; Thomas) aphid infestation of mature rice (*Oryza sativa* Nipponbare) leaves. *The Plant Cell*: Under Review.

APPENDICES

APPENDIX A

RNA Quantification Protocol

1 A_{260} Unit of ssRNA = 40µg/ml of H_2O

The value is based on the extinction coefficient of RNA in H_2O.

Calculation:-

1) Assuming volume of ssRNA sample = 100µl (0.1ml)
2) Dilute 25 µl of sample + 475 µl of H_2O (1/20 dilution).
3) Assume A_{260} of this dilution = 0.56
4) Concentration of ssRNA in sample = 0.56 x 40µg/ml x 20 = 448µg/ml
5) Amount ssRNA in sample = 448µg/ml x 0.1ml = 44.8µg

APPENDIX B

OsSUTs cDNA Sequences

Note: Arrow indicates the primer. The primer sequence is underlined and italized

OsSUT1 (Accession No: AF280050)

```
   1 gatcagggta aaatgtgagg ccgacatgtg tattactggt caatggcaga actgaaattc
  61 gaaaatgtat tgatgtatta ttcctctttt tttttgttca aattaagatg acacgtaggc
 121 tccagcaacc atccacgtgg cggcaggttg cgcacgtcac tccatctcca acacatcagc
 181 attttcagca aagaaagaaa aaactgtagt ggactttgca gaaaacgctg acgacgagta
 241 cgtacgaatt aatgccttaa tcatatgata tctccaaaga ttaattaata agtggcttgc
 301 ttaattttgc tactaatcgc tatctcgacg tagccagaga gccatgcatg catcacagcg
 361 agcccacggc cgcacgagca ctcgccaacg aattcgctag gcgtacaccg tgaatgattt
 421 gatgcgttga ttatgggtat tcatattcct ttatgaaagg ttattgtcag acttttttta
 481 ttccacaaga tcgatcatac tacaaagtta ttctacaata gtttagaaca cttatccagt
 541 tgtgttagaa tataataatg atggatggat atgtatgcca tattaaacaa tctaaattcc
 601 ccacaaaaca tataaaagaa cactataata aactatggtt tatccaacat ggacatatat
 661 ttaaatgaag tgcgatctcc ggtgctcttt ctggtaggat gaatgatgat agagataaaa
 721 gcgtttacaa atatggcctc aagcaaaatt cattatatta attaaatcaa tgaaacattt
 781 actggattaa taaaactcca tgctactcca ttataaatga acgcacacct atatatagca
 841 aaattcctat ttgccagtag gtccaatact tcggatctgt ttttttctt ttaaatatcc
 901 aaaattgatt ttggataact actcgacggt acaaacgaat taaaccagct atttcaacgt
 961 cgagtggatt taaaacactc ctctattaaa ttcacctaca gaaagtcgtt cccgttgaaa
1021 taatcgcacc gtctagaagc tcggcaagcg tgtcgctaat ccgatactaa ctccattaat
1081 tccattttca tttcaataat tgttgaagtt attactgaac tggaaataat aaaggcaggg
1141 gggtgtacac tgggtgtgta caaagtgttg gtgagcatag cagttggcag gtgcgccacc
1201 ctttattata ttcctccttt ctctctctct ctctcccccc ctccctccct ttaaatgctt
1261 cgcctctctc gctcgtctct ccaaacacaa acccaccacc tcctcctcct cctcccatcc
1321 agcacgcgcc tcctctctcg cgcggctttc catttccatc tccccctcct cctcctacgt
1381 ctccgccgct cctcacttcc tccactcgat ttcctttctt ggcctctcct cctctgacac
```

1441 aggggtgtgc aggtttgtgt ttgtgcgtgg cgcgtccgcc atggctcgcg gcagcggggc
1501 cggaggaggc ggcggcggtg gtggtggggg gctggagctg tcggtcgggg tcggcggcgg
1561 cggcggcgcg cgtggtgggg gaggcggtga ggcggcggcg gcggtggaga cggcggcgcc
1621 gatcagcctc gggaggctca tcctctccgg gatggtcgcc ggcggcgtgc agtacgggtg
1681 ggcgctccag ctctccctcc tcacccccta cgtccaggta tatatatata tactgtactc
1741 ctgtgtattt tgcacactct catgcatgca tctgaaactc tgaataatcc tgcaaaaaat
1801 atatacaagt atctcacgtg tggatttgat tttgttcgcg agtttttgct cgtgctagct
1861 gttctaatct gcaaggtttc atggtagtag attatatatg tcctggttac aatcttttca
1921 aggttgtaat cttgtaattt ttttctgctt tgtcaatatt aacttcgcac tgtctggtgt
1981 gagttgttaa aaaaatatat atataagatt ggggaatttt tcttcatgtg tggggagaga
2041 tttatttttt tttgtttgaa cggtagattt gactagtgtc taaaagtacc gtaataaatt
2101 caatctgcat gccttttgta attttttttt gctcatgtgt ttatatacat gtggccaaat
2161 atgcatttga tgcactagct atcatttttg tcattttgta gttattcctt ttcctttcct
2221 ttttttttcct ttttcttttt gttgtcacta gaggattcat atcctctcga ttcctctatg
2281 aaacgtcggc gttttctgcc tcttttctga agaatctgta tacctatata agaatcaaaa
2341 tcttttataa gttctttttg tgcactttgg atcaatcagg ctagccagca tgacacttag
2401 catcagtacc ggtctactgt accgagattc attttacgct tgacacgttt ctcaactcct
2461 tgtttgattc cattttacct caattagact gtttgattcc gttttatctc agatatgaca
2521 ctccttttttt taaaattcca ttttacctca gatgctgtac agttccataa aaaaatatac
2581 atctgctcca actcacctta tccttcttgt aataaccacg tattaggtta atagctacta
2641 atagctagag atgtaaacaa aaataagcag acaagggcaa aagtgagcgg taataatggg
2701 agaaataaac tgtcggtcct ccgcgcgcag ttaaaggtag gcagatgacg ggtccctccc
2761 ctctattcca tactaaccgc aagatgaaac taatattctt actgctagat atataaatca
2821 tccttcattt agcttcatct ccctccttaa gatgtgttct tttcttccat ttcctaactt
2881 ctactccctt cgtttgacgt acacatattt cctaaactgc caaacgatgt gtttctacca
2941 aatatttata tggtttaaaa aacatattaa tatatttttt gtctttttag ctaatattta
3001 attaattatg tgctaaacta ccgctcaatt ttgtgtaccg ggagaaaggg ttctcaatca
3061 ttacaaaaga acacagccta gtctccatga aaaacatcac ttttatttta agacagagca
3121 agtggagcga ttttataact ttactatagt acaaaattaa tggtcagaaa tttaaaattt
3181 tgaccgaatt ttatcgtaag catcaaatat ttattaccgg agagatagag tattggttat
3241 ttgcctctct gtcgtcaaat attctaaaca aagacgatct tcttagtggt taaacttaaa
3301 tctcagtcct tccaggcgtt tatttgaagg atttatagcg gagataaaca gctccttttt
3361 tttcggaaat acgtaaggag cgcacttttg tattaagggg aataaaaagg gtctgtacag
3421 ggaaaaccaa aaaacagcag gggacaggaa taaacagctc ctcacttcct ctttgtatca
3481 gtccatacta attaacaaag tgttccaaaa tgttctgtac tttttctgta gaactttata
3541 tgcagttcat cacccctgat ggtccatgca tcaggtctag ctacctactg ttgtattgaa
3601 gtatttctga cgaagtttta tccttacctt agccttctcg agccaagtgc cataaagatg
3661 gttgctgtag aggcaactcc aacccacttt aaccgcgcac atgaaaaata aaggggtaaa
3721 caaggaaatt ctaggtcaga tatatatgtc ctgtggactg tggcacagtt gcaacagagc
3781 aattgcatag gcaaatgagg gcagatcatc atgtacccat agcctgttgg tgctatgtta
3841 gcatccaagc attgccattt taactattaa gattcttctt cttatatttg acaagtgaaa
3901 taaatttagt gactgaacat atatagaacg atgaacagtc tttgtatgtt tattcatgcc
3961 atgggggaaa gagaagtgtg atctgaagtt attggataga tagctgttag ttgtagcatg
4021 caaggcaatg catcctggaa ccaacagacc aacagagctg gggatatttc atctgctaaa
4081 tttctgggaa attttatttt gtggtaatca atatgtgttc actctttgca actggtacac
4141 gctctctgat gtttctgaat atcttccaca gtgatgcaga tcttgttgac acagcaaact
4201 caacaaacat agtcattttt ttaaggaatg aagcatgatc atctattgtc agagtagatt
4261 accagtcaaa gtttttttat aagccaaaac aactgatttt ctaccttctg ttatttacag
4321 acactcggac tgtcgcatgc tcttacttca ttcatgtggc tctgtggtcc tattgctggc
4381 atggtggtaa gtaccaatac caaattatct gctctatcga tatatacgag acaaagcttt
4441 tgccctcttt taaaaaaaaa taaatcaaat taattcatgt tatggaatct gtactttctg
4501 caatgtaaat tcaagttact ttagtgtctt cactacaaca aaaacttggg tatcctgaac
4561 tcctgtatct atcagagttt gaagctgcat tttctgtagc ttctgatatg tgtaatgtag
4621 atcatactgt cataattcat cagctttcat aaaaaatgtt tgtgtacaag tcatattttt
4681 tttaattctt gatccattat catctcccaa tcctgcaggt tcaaccatgc gtgggtctat
4741 acagtgatag gtgcacctca aaatggggaa gacggaggcc atatattctt acaggatgtg
4801 tgttgatatg cttggctgta agaaccactg aaccttgaac tcttattcct taagttgaag
4861 tccccaaatt ttctctcata ttgacattgc actttatgta tgtaggtcgt agttattgga
4921 ttttcagctg acattggata cgccatgggt gatacaaagg aagactgcag gtaactgaac
4981 attttgcgta tcataattag cagataacag ttatctgata caccatgtaa tgcagtgtct
5041 accatggatc acggtggcat gctgctattg tatatgtcct gggattctgg cttcttgatt
5101 tttccaacaa cactgtgcag gttagtgcct actatataat aatcatgtta aagaaatttg
5161 tacaaagaat cataacaaaa atcttaattt tgcaatgcaa tttctagggt ccagcacgtg
5221 ctctaatggc tgatttatca ggtaattttt catagccttg tctttcagag ttaactacca

```
5281 aatttttatt ggtttccaca gttcactaac acttgattga ttccagaaaa aaaaaagttg
5341 cccttgtgtt ctgggcttct ggtatcagtg ggattttgga tttgatctcc taattttcgt
5401 tctgtaggac gtcatggacc tggcacagct aactccatct tctgttcttg gatggcaatg
5461 ggaaacatcc taggatactc ctctggttcc acaaacaatt ggcacaagta agttactctt
5521 atgtatcagt ttcatacaga ttttctgttt agcacagtct attcagttgc attcttttct
5581 gaaagaaggc aagtgaaatt catattacat tagtagagaa ggtgaaattt gtttacaagt
5641 attgagttgc attctcccta attaattgcg caggtggttt ccattcctta aaacaagagc
5701 ttgctgtgaa gcttgtgcaa atttgaaagg tgcatttctg gtggctgtgg taagttctgt
5761 tattacctca taaactgcct gctgataata ctttaacaat gtgctaatat tagtctttgt
5821 aataagatag tactatactg aaaatatttt agcgagtatg agtaatttaa cttacatatt
5881 gtattgctgt tcctcttttt tcaaccctgt catattggtt gctttttttc acagcctaac
5941 atgctcttgt ttggtcattt tcccctgttt tcagattttc ctgtccctgt gcttggttat
6001 aactctgata tttgccaagg aagttccatt caaaggaaat gcagccctcc caacaaaatc
6061 aaacgagcca gctgagcctg aaggcactgg tccactagca gtgttgaaag gcttcaggaa
6121 cttacctact gggatgcctt ctgtgcttat tgtgactggc cttacctggg tatgcaatat
6181 acatcagaaa aagaactttt cagattactg aaaaaaatgc tactcgatca tatcttagtt
6241 atatcttata gtaccttacc ttttctatga tatgattgtc cttgtagttg tcttggttcc
6301 cattcatcct ctacgacact gactggatgg gccgtgagat ctaccacggt gacccaaagg
6361 gaactgatcc tcagatcgag gctttcaacc agggtgtcag ggcaggtgca tttggcctgc
6421 tactgaattc ggtaagatct gattggtttc tgaattctga ttgccacaac tactgtcaaa
6481 tctttaacac tcgagccatg ttctttggca gcaactaacc atgggcttct gatcgtgtag
6541 attgtgctag gattcagctc attcttgatt gaaccaatgt gccggaaagt cgggccaagg
6601 gtggtgtggg tgacgagcaa tttcctcgtc tgcatcgcca tggctgcaac tgcactgatc
6661 agcttctggt cactcaagga cttccatgga actgttcaga aagccatcac tgcagacaag
6721 agcatcaagg ctgtctgcct cgtcctcttt gcgtttcttg gagtccctct cgccgtaaga
6781 atcactgaca cttcaactta aacacattgc taaaaaaaat tatttcctca agaatgcctt
6841 atcctgatga actgtctgaa aatttgcgcg atgcaggttc tgtacagtgt tcccttcgca
6901 gtgacggcgc aattagcggc cactagaggg ggtggccaag gtatgaatcc caacattctt
6961 gcagcttttc ttgacataat tcggtgttgt ttggatgctg gtctaatcca tggcgatctg
7021 cagggctctg caccggcgtc ctcaacatct ccatcgtcat ccctcaggtt aattagctgc
7081 caaattctaa tccgtttccc ttgaaaccga atcagatata cttcatggat tattagaatt
7141 tgaatgctca tggattcatg gttgcgtggt gttcttccct caggtggtca tcgcgctggg
7201 agccgggccg tgggacgagc tgttcgggaa ggggaacatc ccggcgttcg gcctcgcctc
7261 gggattcgcg ctcatcggcg gcgtcgccgg aatattcctg ctgcccaaga tctccaagcg
                 OsSUT1-f
7321 ccagttccgg tcggtcagca tgggcggcgg tcactgaccg ccccgctctc gccgccggct
7381 gcggcggcgg cggcggccgg agcagcgccg ccggaagtgg tgagctttgt agttctcgtt
7441 tgcaatggat caaaggcgcc ccctcttttt ttttttacat agggtaatta attaattaag
                                                          OsSUT1-r
7501 gctgttaatt aggaagaggt gaggtggccc tgcagctggg gcccacgctt tgttgccacc
     ____
7561 tcggtagaag agaataagca gatcatgcag ataatatgga actgtaaaac tgttttgtgt
7621 tgtgtgtgtg tgcagtgtgc ttggtaatta gtgtgtaatg aatggtcagg agaagtgcag
7681 tgaactaagt gaaagtgagc ttccatcatc atcatca
```

OsSUT2 (Accession No: AB091672)

```
   1 ggcaagagac aacccactcc tcttctgaac taacccaaag atcaggtcgg aggcggaggc
  61 aaacacgaga agaagatgcc gcggcggcct agcggcggcg gcggcggcgc tggtcccgcg
 121 gcggccgcgg tgcgcaaggt ccccctgcgg aagcttctgc gtgcggcgtc ggtggcgtgc
 181 ggcgtgcagt tcgggtgggc gccgcagctc tccctgctga ccccctacgt ccaggagctc
 241 ggcatcccgc acgccttcgc cagcctcgtc tggctctgcg gccccctctc cggcctcctc
 301 gtccagcccc tcgtcggcca cctctccgac cgcatcgccc ccgccgcctc ccccctcggc
 361 cgccgccgcc ccttcatcgc cgccggcgcc gcttccatcg ctgccgcagt tctcaccgtc
 421 agattctccg ccgacctcgg ccgaatcttc ggcgattcca tcaccccggg ctccacccgc
 481 ctcggcgcca tcaccgccta cctcgtcggc ttctggctcc tcgacgtcgg caacaacgct
 541 acacagggac cctgcagggc cttccccgcc gacctcaccg agaatgaccc aaagaggact
 601 cggatagcta atgcttactt ctcattgttc atggccctgg gaaacatact tggatatgcc
 661 actggggcat acagtggctg gtacaagata ttcccgttca ccgttactcc atcatgtagc
 721 atcagctgtg ccaacctcaa gtcggccttt ctacttgata ttatcatttt ggtggtcact
 781 acatgcatca ctgtagcatc agtgcaagag cctcaatcct ttggaagtga tgaagcagat
 841 caccctagca cagaacagga agctttcctc tgggaacttt ttggatcatt ccggtacttt
```

```
 901 acattaccgg tttggatggt tttgattgtt actgccctca catggattgg atggtttcca
 961 tttatcctct ttgataccga ttggatgggt cgagagatct atcgtggaag tccagatgat
1021 ccaagtataa ctcagagcta tcatgatggt gtgagaatgg gttcttttgg tctgatgctg
1081 aactcggtcc ttcttggatt cacttctatt gtactagaga agttatgtcg gaagtgggga
1141 gctggactgg tgtggggtgt ctccaatatc ctaatggcat tgtgctttgt ggcaatgctt
1201 gtaataacat atgtggcaaa gaatatggat tatccaccta gtggagtacc accaaccggc
1261 attgtcattg cttccctggt agtttttaca attttaggag cgcccctggc gatcacgtac
1321 agtataccat atgcaatggc tgctagtcgg gttgaaaatc tgggacttgg ccaaggtcta
1381 gcaatgggca ttcttaattt ggctattgtc ataccacagg ttattgtgtc actgggtagc
1441 gggccctggg accaactgtt tggtggtggc aatgcaccag cctttgcagt ggctgctgct
1501 gcatctttta tcggtgggct ggtggctatt ctgggccttc cacgagcccg cattgcatca
                OsSUT2-f
1561 aggaggagag gtcaccgata agaatattgc tacatataaa ttgtcggcca ttctttgcaa
1621 ttcgactcat aagaggcact cggaacgcta tgcagtgcat gggggaattg tatattatct
1681 ccgaatcaag aaggggataa tgcttgcttt ctccatgagc tatttttgcc ttttcatgc
                                                      OsSUT2-r
1741 cggatcatca tatgctgtcg tacattggat gatcttatgc tgttgtacat tggatgttgg
1801 tcatttgtag agatactagt gaataaaagt tgcaggagtt ggttcactcg agaaaaaaaa
1861 aaaaaaaaa
```

OsSUT3 (Accession No: AB071809)

```
   1 cttcgatctc ttgggatata actagcttag ctatagctct agctaaatag gttcgtacgt
  61 ataggatatt tgatccattg atcgatggcc gtcgacatgg agctcgacgg cggtggcgac
 121 ggcaagggca aagccccgcc gcagataagc ctgtcggggc tattccttgc gtgcatggtc
 181 gccggtggcg tgcagtacgg ctgggcgctg cagctctctc tcctcacccc gtacgttcag
 241 acattgggaa ttcctcatgc actcacttca gttatgtggc tctgtggccc tattgctggc
 301 ttaattgttc agccatgcgt cggcctgtac agtgacaagt gcacatccag ccttggaaga
 361 cgtagaccgt tcatcctcac tggatgcata atcatctgca tatctgtgat cgtcatcggg
 421 ttctcctcgg acatcggcta cgctctcggc gacacgactg aggactgcaa ggtttacagg
 481 ggtcctcgtt accatgcagc ggcggcattc atcctcggat tctggctgct cgacttctcc
 541 aacaacaccg tgcagggtcc agctcgcgct ctgatggccg atttgtcagg tcggcatggc
 601 ccaagcgcgg ccaacgcgat cttctgttct tggatggcat taggcaacat ccttggctac
 661 tcctccggat ccaccaatga ctggcacaag tggtttccct ttctcatgac cagggcttgc
 721 tgcgaggcct gcgcgaatct caaagcggcc ttcttggttg cagtggtgtt tttggggttg
 781 tcgacggcgg tgacgatggt gtttgcgagg gaggtggcgc tggacccagt ggcggcggcg
 841 aagcggaacg agggtgaggc gtcggggctc ctcgccgtgt tcaaggggat gaagaacctc
 901 cccgtcggga tgccgtcggt gcttatcgtc accggcctca cctggctctc gtggttcccc
 961 ttcatcctct tcgacaccga ctggatgggc cgcgagatct accatggccg cccggacggc
1021 tcccccgccg aggtcaccgc cttccaagag ggtgtccgcc aaggcgcctt cggcctcctc
1081 cttaactcga tcgtgctggg catcagctcg ttcctgatcg agccgatgtg ccgtcggctg
1141 ggcgcccgcg cggtgtgggt gatgagcagc gccgtcgtgt gcgtcgccat ggcggccgtc
1201 tccgtgctca gcgcgtggtc gctcggcgac ttcggcggct cggtgcagga cgcagcgcgg
1261 gcgccggcgg aggagggcgg cgtcagggcg tcggcgctgg cgctcttcgt cttcctcggc
1321 ctccccttcg ccgtcctctg cagcgtcccg ttcgccgtca cggcgcagct cgcggcgagc
1381 cgcggcggcg ggcagggcct ctgcaccggc gtcctcaaca tctccatcgt cgtgccgcag
1441 atggccatcg ccctcggcgc cgggccctgg gacgagctgt tcggggaggg gaacatcccg
                OsSUT3-f
1501 gcgttcgcca tggcgtccgt gttcgccgcc gcggccgccg ccgccggcgt cgtcttgctg
1561 cccaaggtct ccgtccgctc cgtcagcatg gccggcggcc actgatcact actagtctat
                                                                    ←
1621 cacccttttt ttttccgtgg ttttaaatct cccgctatag ctgctgctat ctccagttag
            OsSUT3-r
1681 agcgggtact atagcaggct ataagccagc tgt
```

OsSUT4 (Accession No: AB091673)

```
   1 cgcagatctc accaaaccct accagatcta cgccccgcca tggactccgc cgccggcggt
  61 ggcggcctca cggccatccg cctgccctac cgccacctcc gcgacgccga gatggagctc
 121 gtcagcctca acggcggcac cccccgcgga ggctccccca aggaccccga cgccacgcac
```

```
 181 cagcaggggc cccccgccgc ccgtaccacc accaccagga agctcgtcct cgcctgcatg
 241 gtcgccgccg gcgtgcagtt cggctgggcg cttcagctct cgctcctcac gccctacatc
 301 cagaccctag gaatagacca tgccatggca tcattcattt ggctttgtgg acctattact
 361 ggttttgtgg ttcaaccatg tgttggtgtc tggagtgaca aatgccgttc aaagtatgga
 421 agaaggagac cgttcatttt ggctggatgc ttgatgatat gctttgctgt aactttaatc
 481 ggattttctg cagaccttgg ttacatttta ggagatacca ctgagcactg cagtacatat
 541 aaaggttcaa gatttcgagc cgctattatt ttcgttcttg ggttctggat gttggatctc
 601 gcaaaccata cagttcaagg tcctgctcgt gccctttag ctgacctttc aggtcctgat
 661 cagtgtaatt ctgcaaatgc aatttttgc acatggatgg ctgttggaaa cgttcttggt
 721 ttttcatctg gtgctagtgg gaattggcac aagtggtttc cttttctaat gacaagagca
 781 tgctgtgaag cttgtagtaa tttgaaagcc gcttttctgg ttgcagttgt attccttttg
 841 ttttgtatgt ctgttaccct gtactttgct gaagagatcc cgctggaacc aacagatgca
 901 caacgattat ctgattctgc gcctctcctg aatggttcta gagatgataa caatgcatca
 961 aatgaacctc gtaatggagc acttcctaat ggtcatacag atggaagcaa tgtcccagct
1021 aactccaacg ctgaggactc caattcaaac agagagaatg tcgaagtttt caatgatgga
1081 ccaggagcag ttttggtgaa tattttgact agcatgaggc atctacctcc tggaatgtac
1141 tctgttcttc tagttatggc tctaacatgg ttgtcgtggt ttccctttt ccttttgat
1201 actgactgga tgggacgtga ggtttaccat ggggacccaa atggcaactt gagtgaaagg
1261 aaagcttatg acaacggtgt ccgagaaggt gcatttggtt tgctattgaa ttcagttgtc
1321 cttggatttg ggtccttcct tgttgatcca ctatgccggc tgatgggtgc tagactggtt
1381 tgggcaatca gcaacttcac agtgtttatc tgcatgctgg ctacagcaat attaagttgg
1441 atctcttttg atttgtactc aagtaaactt caccacatca ttggagcaaa taaaacagtg
1501 aagaattcag ccttgattgt tttctcccta cttggactgc cactctcgat cacatatggc
1561 gttccttttt ctgtgactgc tgagctgact gctggaacag gaagtggaca aggtctggca
1621 acaggagtcc tgaaccttgc aatcgttgtt ccgcagatag tagtgtcact aggagcaggt
1681 ccatgggatg ctctctttgg gggagggaac gtccctgctt tcgccttggc ttccgttttc
1741 tcactaggag ctggtgtcct cgcggtcctt aagctaccca agctgccaaa ctcttacaga
                                  OsSUT4-f
1801 tctgctgggt tccatgga***tt tggctgagca gaacacca***gc cgcatggtgt gtaacattga
1861 gaaatgcaac tccattttgc cattcgttta cagtgaaatg attcttttta cctactacta
1921 caacagaata agctgaaaag atagagatta ggatagagag ctaggtaact agtccagtta
1981 ggttgatgtg catacaaggc aattggaagg tgtaagagct gtatctactt ttttgacaga
                  OsSUT4-r
2041 aaaatgt***aag ctctgcccga atgacat***ggc ggatagattt tacaatggat gtaatcatgt
2101 actatatata acacgttttg gtcacagctt gccaaaaaaa aaaaaaaaaa aaaaaaaa
```

OsSUT5 (Accession No: AB091674)

```
   1 gtacgacggc aatggaggaa ggccgccgtg gcgaccgcga ggggaagagc gcggccgggt
  61 ggaccgccct gtcgacgacg aagacgatgc tggaggagaa gcggcggctg caggccaacg
 121 ggagcgtcgg cggcgacgcc gggacgagtg gcttccggag gatcgtgcgg ctgttcttcg
 181 cctgcatggt ggccggcggc atccagtacg gctgggcgct gcagctgtcc ctcctctcgc
 241 cctactctca gactctcggg atttcccact cgtacgtttc cctgacatgg atctgcggac
 301 caatcgctgg atttgtggtg caacccatcg tcggttacta cagtgacaga tgcaccatga
 361 agatgggccg caggaggcct ttcatccttg ttggctgcct catcatctgc atctccgtca
 421 tgatcatcgg tttctcggcg gacattggac gtcacctcgg tgacaccaaa gagcactgca
 481 gcacgtacac gggtcctcgc tggtcggccg ccatggtgta cattgttggc ttctggttcc
 541 ttgacttcgc taacaacact gtccaggggc cggctcgtgc catgatggct gatctgtcag
 601 ctggacacca tggccccaac gtcggccagt cgatcttctc cctgtggatg gctatcggca
 661 gcgtcctcgg ctacttgtcc ggcgccgacg ggaaatggca cgaatggttc ccatggctga
 721 aaaccgcggc gtgctgcgac gcctgcgcga acctcaaggg cgccttcttc accgccgtgc
 781 tactcatcgt cgtcagcatg acggtgacga tgtacctcgc cgacgagatg ccgctggaca
 841 agcaggacgt cgacacctcc ggcggcggcg gttgcgccgt gttcgtcgac ctcttcaaga
 901 gcctcaggaa cctgcccccc gccatgttca aagtgctcgc cgtcacggcc gtcacctggc
 961 tgtcgtggtt ccccttcatc cagtacaaca ccgactggat gggacgggag atctaccacg
1021 gcgagccgca gggcacggcg gccaaggccg acgtgtacga cgccggcgtc agggagggcg
1081 ccatgggcct gctcttctgc tccgtcgcgc tcggcgtcac ctccttcgtg atccccaagc
1141 tctgccgcag gctgacgtcc aaggtggtct ggtccatcag caacttcctc gtgttcgcgc
1201 tcatggcggt gatggtcgcc gtcggcatgg tgtccatgag agggtaccgg ccgtcgctcg
1261 ccgccggact caccgggccg gacccgacgc tcaaggccgt cgcgctcgtc gtcttcgcgc
1321 tcatcggcat cccccaggcc gtgttgttca gtgttccatg ggccgtcgct tctgaggtta
1381 ctgctgagga gggtggcggc caaggtcttg ccattggtgt cctcaacatt gccattgttg
1441 tcccacagct ggtgatcgcg ctcaccgccg gcccgatcga cggcgccttc aacaagggga
```

```
1501 acacgccggc gttcggcatc ggcggcgcct tcgccttcat ctgtggagtc cttgcgctca

1561 tctggctacc aaagacaagg ggtgtgtcca acgccgccgt cgtcgccggc ggtcactagt

                 OsSUT5-f
1621 gcgaaactcc atcaaaggca ccacccggcc ggcctatcgc cgatcgatgt gccatgaatt
1681 gggagtgcat gagactctga aaattttcgc tacaaactca atgttgattc gttcgctgtg
1741 ttgtcgttag ttttttttt ccctactcgt tcatgtccat gtgagagtgt taagccgtag
                                                    OsSUT5-r
1801 aggacaaaca ggaattttcc tggctgcctt gtttattggc agatctcagg aaacccaaat

1861 atttaagat gaagaattgt tctccctgct ttagagtggt agcagcttct gaacaacgag
1921 aaaaaaaaaa agaagacatt tgcatgacaa aaaaaaaaaa aaaaaaaaaa aa
```

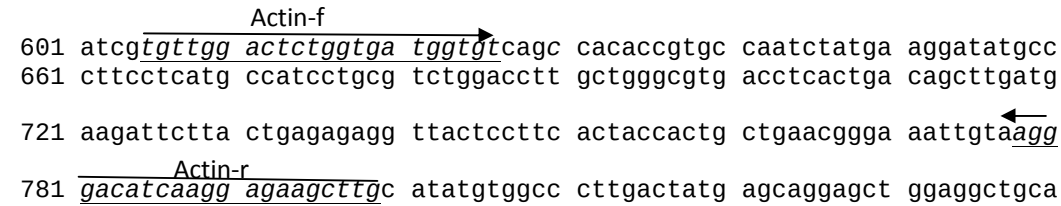
Rice polyubiquitin (RubQ1) (Accession No: OsU37687)

```
   1 cgcagagaaa aaaaaatctc ccctcgaagc gaactgcgaa tcgccttctc aagatgcaga
  61 tctttgtgaa gaccctcacc ggcaagacca tcaccctcga ggttgagtcc tcggacacca
 121 ttgacaatgt caaggccaag atccaggaca aggagggcat ccccccggac cagcagcgtc
 181 tcatcttcgc tggcaagcag cttgaggatg gccgcaccct ggccgactac aacatccaga
 241 aggagtccac cctccacctt gtcctcaggc tcaggggagg catgcagatc ttcgtcaaga
 301 ccttgactgg caagaccatc acccttgagg tcgagtcgtc tgacaccatt gacaatgtca
 361 aggccaagat ccaggacaag gagggcatcc ccccggacca gcagcgtctc atcttcgctg
 421 gcaagcagct tgaggatggc cgcaccctgg ctgactacaa catccagaag gagtccaccc
 481 tccaccttgt gctcaggctc aggggaggta tgcagatctt cgtcaagacc ttgactggca
 541 agaccatcac cctcgaggtc gagtcgtctg acaccattga caatgtcaag gccaagatcc
 601 aggacaagga gggcatcccc ccagaccagc agcgtctcat cttcgccggc aagcagctgg
 661 aggatggccg cacccttgct gactacaaca tccagaagga gtccaccctc cacttgtgc
 721 tcaggctcag gggaggtatg cagatcttcg tcaagaccct gaccggcaag accatcaccc
 781 tcgaggtcga gtcctcggac acgatcgaca atgtgaaagc caagatccag gacaaggagg
 841 gtatcccccc ggaccagcag cgtctcatct tcgccggcaa gcagctggag gatggccgca
 901 ccttggctga ctacaacatc cagaaggagt ccacccttca cctggtactc aggctcaggg
 961 gtgggatgca gatcttcgtg aagaccctga ctggcaagac cattaccctt gaggttgagt
1021 cgtccgacac tattgacaac gtgaaggcga agatccagga caaggagggc atccccccgg
1081 accagcagcg tctcatcttc gccggcaagc agctggagga tggccgcacc ttggctgact
1141 acaacatcca gaaggagtcc acccttcacc tggttctcag gctcaggggt gggatgcaga
1201 tcttcgtgaa gaccctgact ggcaagacca ttacccttga ggttgagtcg tccgacacta
1261 ttgacaacgt gaaggcgaag atccaggaca aggagggcat ccccccggac cagcagcgtc
1321 tgatctttgc tggtaagcag cttgaggatg gccgcacctt ggcggattac aacatccaga

1381 aggagtccac actccacctg gtcctccgcc tccgtggtgg ccagtaagtc ctcagccatg
          RubQ1-f
1441 gagctgctgc tgttcttggg ttcacaagtc tgccattgtc ttccccaatg gagctatggt
                                  RubQ1-r
1501 tgtctggtct ggtccttggt cgtgtcccgt ttcattgtgt actatttacc tgtaatgtgt
1561 atccttaagt ctggtttgat ggtgtctgaa acgttttgct gtggtagagc agcatggaag
1621 aactataatg aataagtgat ccctaatcat tgtgtccaaa aaaaaaaaaa aaaaaaaaa
```

Rice (*Oryza sativa* Japonica cultivar-group) Actin mRNA (Accession No:NM_001072362.1)

```
   1 gcgtcgactc cactctcgcc tcctcgcgac tcggagttca ccgccgccac cgcctccgcc
  61 gccgatctcc ccgtcccgcc gcgccgccgc caccgtcctc cctcctccgg ccgcatcccc
 121 ggaattcaga accagtagga ggaaatggct gacggcgagg acatccagcc ccttgtgtgt
 181 gacaatggaa ctggcatggt caaggctggg tttgctgggg acgatgcgcc cagggctgtt
 241 ttccctagta tcgtggggcg cccccgtcac accggtgtga tggttggtat ggggcagaag
 301 gatgcctatg ttggtgatga ggcgcagtcc aagagaggta tcctcacctt gaagtacccg
 361 atcgagcatg gtattgttag caactgggat gacatggaga agatctggca tcacaccttc
 421 tacaacgagc tccgtgtcgc gcccgaggag caccctgtgt tgctgactga ggccccgctc
 481 aaccccaagg ctaacaggga gaagatgacc cagatcatgt ttgagacttt caatgtgcca
 541 gctatgtatg tcgccatcca ggccgtgctc tccctgtatg ccagtggacg tacaactggt
```




```
601 atcgtgttgg actctggtga tggtgtcagc cacaccgtgc caatctatga aggatatgcc
661 cttcctcatg ccatcctgcg tctggacctt gctgggcgtg acctcactga cagcttgatg

721 aagattctta ctgagagagg ttactccttc actaccactg ctgaacggga aattgtaagg

781 gacatcaagg agaagcttgc atatgtggcc cttgactatg agcaggagct ggaggctgca
841 aagagcagct catctgtgga gaagagctat gagctgcctg atggacaggt gatcaccatt
901 ggggcagaga ggttccgatg ccctgaggtc ctcttccagc cctctttcat cggtatggaa
961 gctcctggaa tccatgagac cacttacaat tccatcatga agtgtgatgt ggatatcagg
1021 aaggacttgt atggtaacat tgttctcagt ggtggatcaa ccatgttccc tggtattgct
1081 gaccgtatga gcaaggagat cactgccctc gcaccaagca gcatgaagat caaggtggtg
1141 gcaccgcctg agaggaaata cagtgtctgg ataggagggt ccatccttgc ctcccttagc
1201 accttccaac agatgtggat ctcaaaggga gagtatgatg agtcgggtcc agcaattgtt
1261 caccggaagt gcttctaagc tctggctttt taatcgctta tctacaaggc agtttctttt
1321 cagttttcac aagcccttgt catgtaagct actctgtttg ggattgttgg tgtcctatca
1381 gaatgttgtt ggatttgttt catggctgaa tattatgcac cttctgaact cttggtatgc
1441 atttgtccgg tggctgctgt attatacatg gcatgcatgg tccaactttt gtcatgctaa
1501 gtttgtggtt agtggtagga ccaaaagaaa aatgggagat gtacccacta cctaggatcg
1561 tctattgtaa tcgtacttgt gcgagtcatg ttatcattta tcttgtacat tcatctccaa
1621 gataagtgat ctggttcttt gatcgtttg
```

APPENDIX C

RC/DC Protein Assay Protocol

The protein concentrations of plants extracts were determined using the following protocol:

1). Reagent A was prepared by adding 20µl of DC reagent to 1ml of DC Reagent A

2). A standard curve was obtained by using bovine serum Albumin (BSA) as a standard protein. 1.5mg/ml of stock solution of BSA was prepared.

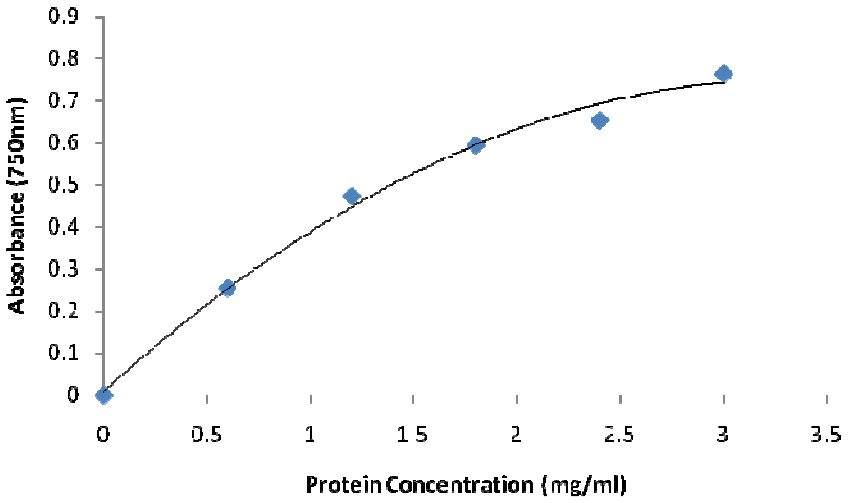
The procedure is as follows:

Steps	Tube1	Tube2	Tube3	Tube4	Tube5	Tube6
BSA (1.5mg/ml) Sterile H_2O Protein Concentration	0µl 200µl 0	40µl 160µl 0.12mg	80µl 120µl 0.24mg	120µl 80µl 0.36mg	160µl 40µl 0.48mg	200µl 0µl 0.60mg
RC Reagent I(vortex after addition and incubated for 1 min.)	500µl	500µl	500µl	500µl	500µl	500µl
RC Reagent II (Centrifuge at 15,000g for 3-5 mins)	500µl	500µl	500µl	500µl	500µl	500µl
Discard the supernatant by inverting the tubes on clean absorbent tissue. Allow the liquid to drain completely from the tubes.						
Reagent A (Tubes incubated at room temperature for 5mins until precipitate was completely dissolved.	510µl	510µl	510µl	510µl	510µl	510µl

This was vortexed before proceeding to the next step)						
Reagent B (This was added and solution vortex immediately. Solutiona were incubated at room temp for 15mins	4ml	4ml	4ml	4ml	4ml	4ml
Absorbance was taken at 750nm	0.0	0.255	0.473	0.594	0.653	0.764
Protein Conc. (mg/ml)	0.0	0.60	1.20	1.80	2.40	3.00

Absorbance (750nm) was plotted against the protein concentration to give a proteinstandard curve.

Standard Curve for Protein Determination



APPENDIX D

OsSUTs Protein Sequences

Note: The peptide sequence used for the production of synthetic peptide is the sequence that is underlined below in the OsSUT1 protein sequence.

OsSUT1 (Accession No: AF280050)

MARGSGAGGGGGGGGGLELSVGVGGGGGARGGGGGEAAAAVET
AAPISLGRLILSGMVAGGVQYGWALQLSLLTPYVQTLGLSHALTSFMWLCGPIAGMVV
QPCVGLYSDRCTSKWGRRRPYILTGCVLICLAVVVIGFSADIGYAMGDTKEDCSVYHG
SRWHAAIVYVLGFWLLDFSNNTVQGPARALMADLSGRHGPGTANSIFCSWMAMGNILG
YSSGSTNNWHKWFPFLKTRACCEACANLKGAFLVAVIFLSLCLVITLIFAKEVPFKGN
AA**LPTKSNEPAEPEGTGPLAV**LKGFRNLPTGMPSVLIVTGLTWLSWFPFILYDTDWMG

REIYHGDPKGTDPQIEAFNQGVRAGAFGLLLNSIVLGFSSFLIEPMCRKVGPRVVWVT
SNFLVCIAMAATALISFWSLKDFHGTVQKAITADKSIKAVCLVLFAFLGVPLAVLYSV
PFAVTAQLAATRGGGQGLCTGVLNISIVIPQVVIALGAGPWDELFGKGNIPAFGLASG
FALIGGVAGIFLLPKISKRQFRSVSMGGGH"

OsSUT1 Synthetic Peptide Sequence % Similarity With Other OsSUT Proteins

Sucrose Transporter Protein	% Similarity to OsSUT1
OsSUT1	**100**
OsSUT2	**11**
OsSUT3	**27**
OsSUT4	**5**
OsSUT5	**5**

OsSUT2 (Accession No: AB091672)

MPRRPSGGGGGAGPAAAAVRKVPLRKLLRAASVACGVQFGWAPQ
LSLLTPYVQELGIPHAFASLVWLCGPLSGLLVQPLVGHLSDRIAPAASPLGRRRPFIA
AGAASIAAAVLTVRFSADLGRIFGDSITPGSTRLGAITAYLVGFWLLDVGNNATQGPC
RAFPADLTENDPKRTRIANAYFSLFMALGNILGYATGAYSGWYKIFPFTVTPSCSISC
ANLKSAFLLDIIILVVTTCITVASVQEPQSFGSDEADHPSTEQEAFLWELFGSFRYFT
LPVWMVLIVTALTWIGWFPFILFDTDWMGREIYRGSPDDPSITQSYHDGVRMGSFGLM
LNSVLLGFTSIVLEKLCRKWGAGLVWGVSNILMALCFVAMLVITYVAKNMDYPPSGVP
PTGIVIASLVVFTILGAPLAITYSIPYAMAASRVENLGLGQGLAMGILNLAIVIPQVI
VSLGSGPWDQLFGGGNAPAFAVAAAASFIGGLVAILGLPRARIASRRRGHR

OsSUT3 (Accession No: AB071809)

MAVDMELDGGGDGKGKAPPQISLSGLFLACMVAGGVQYGWALQL
SLLTPYVQTLGIPHALTSVMWLCGPIAGLIVQPCVGLYSDKCTSSLGRRRPFILTGCI
IICISVIVIGFSSDIGYALGDTTEDCKVYRGPRYHAAAAFILGFWLLDFSNNTVQGPA
RALMADLSGRHGPSAANAIFCSWMALGNILGYSSGSTNDWHKWFPFLMTRACCEACAN
LKAAFLVAVVFLGLSTAVTMVFAREVALDPVAAAKRNEGEASGLLAVFKGMKNLPVGM
PSVLIVTGLTWLSWFPFILFDTDWMGREIYHGRPDGSPAEVTAFQEGVRQGAFGLLLN
SIVLGISSFLIEPMCRRLGARAVWVMSSAVVCVAMAAVSVLSAWSLGDFGGSVQDAAR
APAEEGGVRASALALFVFLGLPFAVLCSVPFAVTAQLAASRGGGQGLCTGVLNISIVV
PQMAIALGAGPWDELFGEGNIPAFAMASVFAAAAAAAGVVLLPKVSVRSVSMAGGH

OsSUT4 (Accession No: AB091673)

MDSAAGGGGLTAIRLPYRHLRDAEMELVSLNGGTPRGGSPKDPD
ATHQQGPPAARTTTTRKLVLACMVAAGVQFGWALQLSLLTPYIQTLGIDHAMASFIWL
CGPITGFVVQPCVGVWSDKCRSKYGRRRPFILAGCLMICFAVTLIGFSADLGYILGDT
TEHCSTYKGSRFRAAIIFVLGFWMLDLANHTVQGPARALLADLSGPDQCNSANAIFCT
WMAVGNVLGFSSGASGNWHKWFPFLMTRACCEACSNLKAAFLVAVVFLLFCMSVTLYF
AEEIPLEPTDAQRLSDSAPLLNGSRDDNNASNEPRNGALPNGHTDGSNVPANSNAEDS
NSNRENVEVFNDGPGAVLVNILTSMRHLPPGMYSVLLVMALTWLSWFPFFLFDTDWMG
REVYHGDPNGNLSERKAYDNGVREGAFGLLLNSVVLGFGSFLVDPLCRLMGARLVWAI
SNFTVFICMLATAILSWISFDLYSSKLHHIIGANKTVKNSALIVFSLLGLPLSITYGV
PFSVTAELTAGTGSGQGLATGVLNLAIVVPQIVVSLGAGPWDALFGGGNVPAFALASV
FSLGAGVLAVLKLPKLPNSYRSAGFHGFG

OsSUT5 (Accession No: AB091674)

MEEGRRGDREGKSAAGWTALSTTKTMLEEKRRLQANGSVGGDAG

TSGFRRIVRLFFACMVAGGIQYGWALQLSLLSPYSQTLGISHSYVSLTWICGPIAGFV
VQPIVGYYSDRCTMKMGRRRPFILVGCLIICISVMIIGFSADIGRHLGDTKEHCSTYT
PRWSAAMVYIVGFWFLDFANNTVQGPARAMMADLSAGHHGPNVGQSIFSLWMAIGSV
LGYLSGADGKWHEWFPWLKTAACCDACANLKGAFFTAVLLIVVSMTVTMYLADEMPLD
KQDVDTSGGGGCAVFVDLFKSLRNLPPAMFKVLAVTAVTWLSWFPFIQYNTDWMGREI
YHGEPQGTAAKADVYDAGVREGAMGLLFCSVALGVTSFVIPKLCRRLTSKVVWSISNF
LVFALMAVMVAVGMVSMRGYRPSLAAGLTGPDPTLKAVALVVFALIGIPQAVLFSVPW
AVASEVTAEEGGGQGLAIGVLNIAIVVPQLVIALTAGPIDGAFNKGNTPAFGIGGAFA
FICGVLALIWLPKTRGVSNAAVVAGGH

APPENDIX E

Sodium Docecyl Sulphate Polyacrylamide Gel Electrophoresis (SDSPAGE) Analysis. The Mini-Protean® 3 Cell Instruction (modified protocol of the method according to Laemmli, 1970)

10 x Running buffer (pH 8.3)

30.3 g Tris Base

144.0 g Glycine

10.0 g SDS

Make up to 1 L with distilled water and do not adjust pH. Store at 4°C. if precipitation occurs, warm up to room temperature prior to use. Dilute 50 ml stock in 450 ml distilled water prior to use.

Sample buffer (SDS reducing buffer)

7.10 ml deionized water

2.50 ml Tris-HCl buffer (0.5 M, pH 6.8)

5.0 ml glycerol

4.0 ml SDS solution (10 % w / v)

0.4 ml bromophenol blue (0.5 % w / v)

Store at room temperature. Add 50 µl β-mercaptoethanol to 950 µl sample buffer prior to use. Dilute protein samples at least 1:2 with sample buffer and boil for 4 minutes prior to loading onto SDS-PAGE gel.

10 % Ammonium persulphate (APS)

Add 100 mg (0.1 g) APS to 1 ml deionised water. Prepare fresh daily.

Stacking gel buffer (0.5 M Tris-HCl, pH 6.8)

Dissolve 6 g Tris (Hydroxymethyl) Aminomethane (Merck) in 100 ml distilled water. Adjust the pH to 6.8 with Hydrochloric acid (HCl).

Resolving gel buffer (1.5 M Tris-HCl, pH 8.8)

Dissolve 18.15 g Tris (Hydroxymethyl) Aminomethane in 100 ml distilled water. Adjust the pH to 8.8 with Hydrochloric acid (HCl).

Gel formulations:

SDS-PAGE stacking gel (4 %)

Reagent	**Volume (ml)**
Distilled water	6.1
30 % Degassed Acrylamide / Bis Acrylamide	1.3
0.5 M Tris-HCl, pH 6.8	2.5
10 % w / v SDS	0.1
10 % APS	0.05
TEMED	0.01

SDS-PAGE resolving gel (12 %)

Reagent	**Volume (ml)**
Distilled water	3.4
30 % Degassed Acrylamide / Bis Acrylamide	4.0
0.5 M Tris-HCl, pH 6.8	2.5
10 % w / v SDS	0.1
10 % APS	0.05
TEMED	0.01

Electrophoresis was run at a constant voltage of 200Volts.

Gel staining and de-staining:

Coomassie Stain:

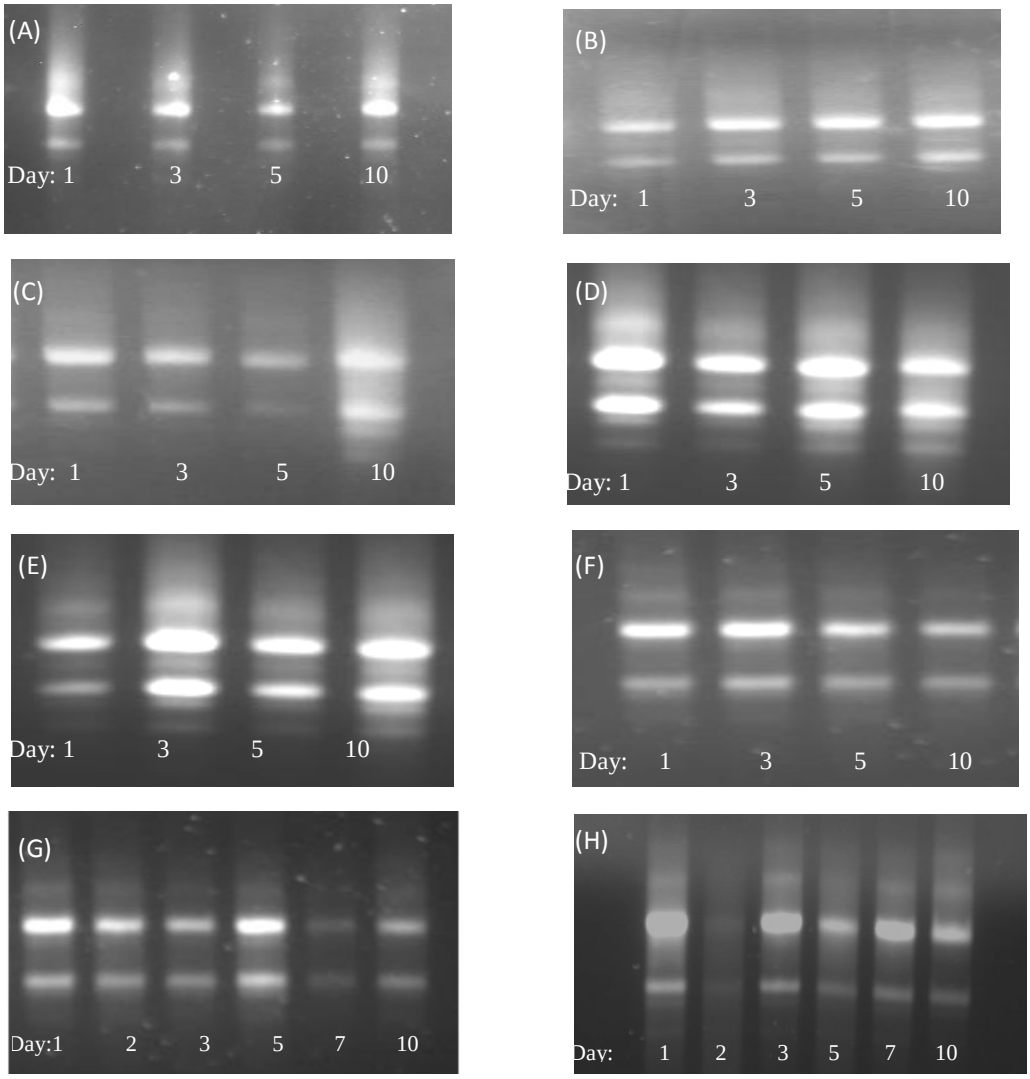
Staining contains 1g of Coomassie brilliant blue R250 in 40 % methanol and 10 % glacial acetic acid and 50% H_2O. It's stored in a dark bottle. Gels were stained for one hour on a shaker.

Coomassie Destaining:

Destaining solution Contains 40% methanol, 10% glacial acetic acid and 50% H_2O. Gels were destained until destaining solution is no longer dark purple and a clear background is observable on the gel.

APPENDIX F

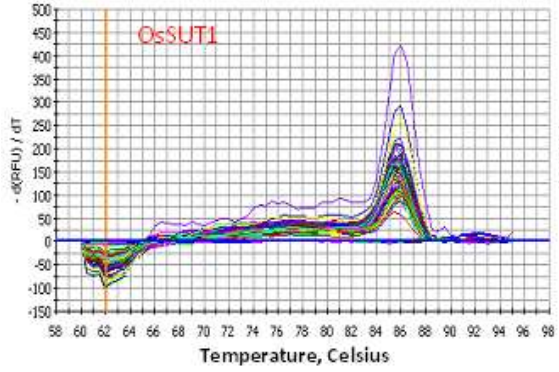
1% Agarose Gel Electrophoresis of Total RNA Extracts from Control and Stressed Rice Plants

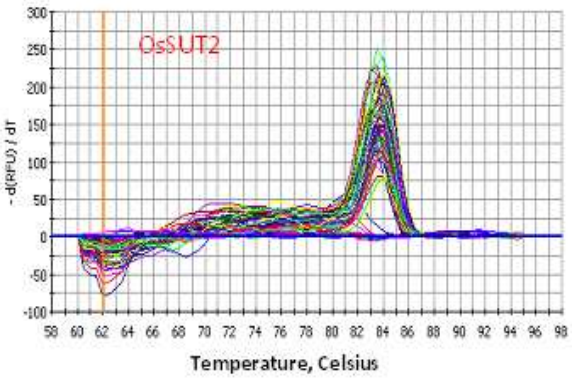


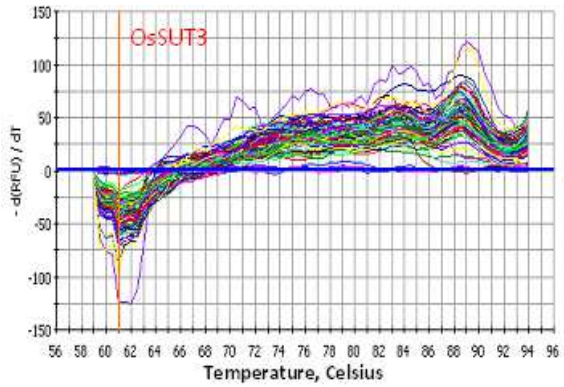
(A)Control Drought Experiment, (B) Drought Experiment, (C) Control Salinity Experiment (D) Salinity Experiment; 100mM NaCl, (E) Salinity Experiment; 200mM NaCl (F)Salinity Experiment; 300mM NaCl (G) Control Aphid infestation Experiment (H) Aphid infestation Experiment

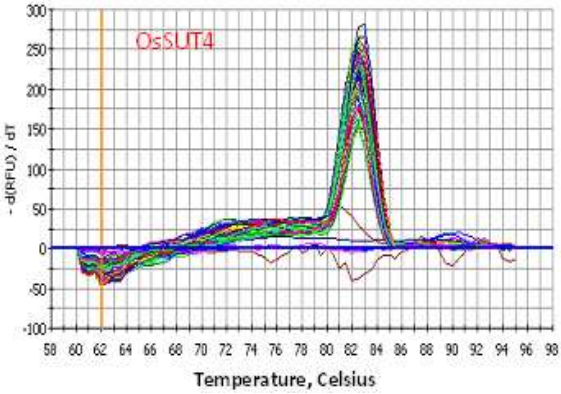
APPENDIX G

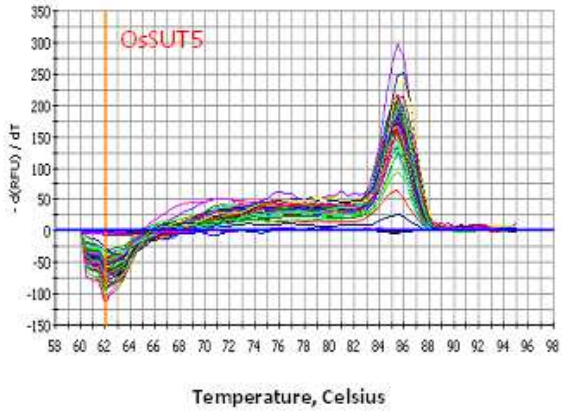
Melt Curve Analysis of OsSUTs' Genes

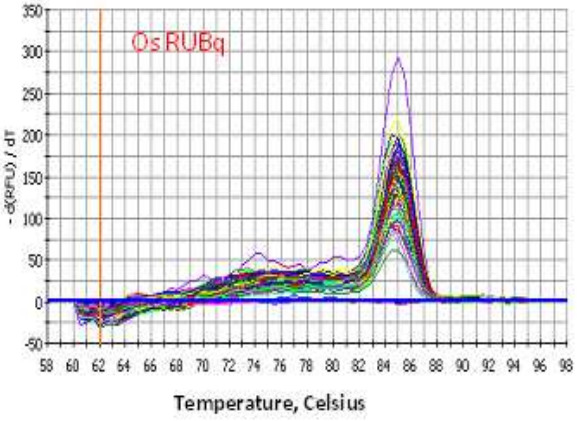


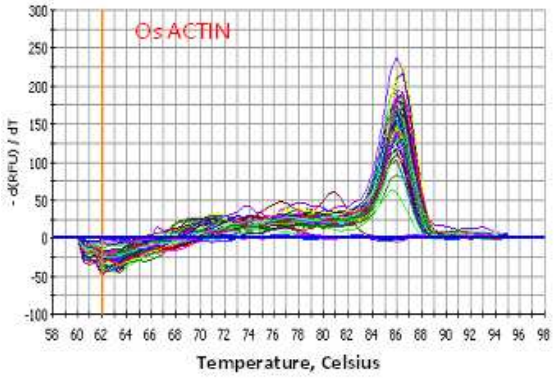












APPENDIX H

Clustal W Multiple Sequence Alignment of OsSUT2, AtSUT4 and HvSUT2

```
>OsSUT2(BAC67163.1)
MPRRPSGGGGGAGPAAAAVRKVPLRKLLRAASVACGVQFGWAPQ
LSLLTPYVQELGIPHAFASLVWLCGPLSGLLVQPLVGHLSDRIAPAASPLGRRRPFIA
AGAASIAAAVLTVRFSADLGRIFGDSITPGSTRLGAITAYLVGFWLLDVGNNATQGPC
RAFPADLTENDPKRTRIANAYFSLFMALGNILGYATGAYSGWYKIFPFTVTPSCSISC
ANLKSAFLLDIIILVVTTCITVASVQEPQSFGSDEADHPSTEQEAFLWELFGSFRYFT
LPVWMVLIVTALTWIGWFPFILFDTDWMGREIYRGSPDDPSITQSYHDGVRMGSFGLM
LNSVLLGFTSIVLEKLCRKWGAGLVWGVSNILMALCFVAMLVITYVAKNMDYPPSGVP
PTGIVIASLVVFTILGAPLAITYSIPYAMAASRVENLGLGQGLAMGILNLAIVIPQVI
VSLGSGPWDQLFGGGNAPAFAVAAAASFIGGLVAILGLPRARIASRRRGHR

>AtSUT4(AAG09191)
MATSDQDRRHRATRNRPPIPRPSNSSSRPVVPPPRSKVSKRVLLRVASVACGIQFGWALQLSLLTPYVQE
LGIPHAWASVIWLCGPLSGLFVQPLVGHSSDRCTSKYGRRRPFIVAGAVAISISVMVIGHAADIGWAFGD
REGKIKPRAIVAFVLGFWILDVANNMTQGPCRALLADLTENDNRRTRVANGYFSLFMAVGNVLGYATGSY
NGWYKIFTFTKTVACNVECANLKSAFYIDVVFIAITTILSVSAAHEVPLASLTSEAHGQTSGTDEAFLSE
IFGTFRYFPGNVWIILLVTALTWIGWFPFILFDTDWMGREIYGGEPNIGTSYSAGVSMGALGLMLNSVFL
GITSVLMEKLCRKWGAGFVWGISNILMAICFLGMIITSFVASHLGYIGHEQPPASIVFAAVLIFTILGIP
LAITYSVPYALISIRIESLGLGQGLSLGVLNLAIVIPQVIVSVGSGPWDQLFGGGNSPALAVGAATGFIG
GIVAILALPRTRIQKPIPLP

>HvSUT2(CAB75881)
MPPRRPNTGG GGGTSSSAAP APRKVPLRSL LRAASVACGV QFGWALQLSL LTPYVQELGI
PHAFASLVWL CGPLSGLLVQ PLVGHLSDRI TPANSPLGRR RPFIAAGAAS IAFAVLTVGF
SADLGRLFGD NVVPGSTRIG AIIVYLVGFW LLDVGNNATQ GPCRAFLADL TENDPRRTRI
ANAYFSLFMA LGNILGYATG AYNGWYKIFP FTITGSCGVS CANLNSAFLL DIIILAITTY
ISVATVQDNP TFGSDEAAPP SSHEEEAFLF ELFGSFKYFT MPVWMVLIVT SLTWVGWFLF
ILFDTDWMGR EIYRGSPEIV ADTQKYHDGV RMGSFGLMLN SVVLGITSIG MEKLCRKWGA
GLVWGVSNII MALCFVAMLI ITYVAQNLDY GPSGAPPTGI VAASLIVFTI LGAPLSITYS
IPYAMAASRV ENLGLGQGLA MGILNLSIVI PQIIVSLGSG PWDQLFGGGN APSFWVAAAA
SFVGGLVAIL GLPRARLGPK KKTTQR
```

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	OsSUT2_BAC67163	501	2	AtSUT4_AAG09191	510	61
1	OsSUT2_BAC67163	501	3	HvSUT2_CAB75881	506	83
2	AtSUT4_AAG09191	510	3	HvSUT2_CAB75881	506	60

```
OsSUT2_BAC67163.1_      ------------MP-RRPSGGGGG--AGPAAAAVR-KVPLRKLLRAASVACGVQFGWAPQ 44
HvSUT2_CAB75881_        ------------MPPRRPNTGGGGGTSSSAAPAPR-KVPLRSLLRAASVACGVQFGWALQ 47
AtSUT4_AAG09191_        MATSDQDRRHRATRNRPPIPRPSNSSSRPVVPPPRSKVSKRVLLRVASVACGIQFGWALQ 60
                                 * *    ..  : ..... * **. * ***.******:***** *

OsSUT2_BAC67163.1_      LSLLTPYVQELGIPHAFASLVWLCGPLSGLLVQPLVGHLSDRIAPAASPLGRRRPFIAAG 104
HvSUT2_CAB75881_        LSLLTPYVQELGIPHAFASLVWLCGPLSGLLVQPLVGHLSDRITPANSPLGRRRPFIAAG 107
AtSUT4_AAG09191_        LSLLTPYVQELGIPHAWASVIWLCGPLSGLFVQPLVGHSSDRCT---SKYGRRRPFIVAG 117
                        ****************:**::*********:******* *** :   *  *******.**

OsSUT2_BAC67163.1_      AASIAAAVLTVRFSADLGRIFGDSITPGSTRLGAITAYLVGFWLLDVGNNATQGPCRAFP 164
HvSUT2_CAB75881_        AASIAFAVLTVGFSADLGRLFGDNVVPGSTRIGAIIVYLVGFWLLDVGNNATQGPCRAFL 167
AtSUT4_AAG09191_        AVAISISVMVIGHAADIGWAFGD--REGKIKPRAIVAFVLGFWILDVANNMTQGPCRALL 175
                        *.:*: :*:.: .:**:*  ***    *. :  ** .:::***:***.** *******:

OsSUT2_BAC67163.1_      ADLTENDPKRTRIANAYFSLFMALGNILGYATGAYSGWYKIFPFTVTPSCSISCANLKSA 224
HvSUT2_CAB75881_        ADLTENDPRRTRIANAYFSLFMALGNILGYATGAYNGWYKIFPFTITGSCGVSCANLNSA 227
AtSUT4_AAG09191_        ADLTENDNRRTRVANGYFSLFMAVGNVLGYATGSYNGWYKIFTFTKTVACNVECANLKSA 235
                        ******* :***:**.*******:**:******:*.******.** * :*.:.****:**
```

```
OsSUT2_BAC67163.1_      FLLDIIILVVTTCITVASVQEPQSFG-SDEADHP-STEQEAFLWELFGSFRYFTLPVWMV 282
HvSUT2_CAB75881_        FLLDIIILAITTYISVATVQDNPTFG-SDEAAPPSSHEEEAFLFELFGSFKYFTMPVWMV 286
AtSUT4_AAG09191_        FYIDVVFIAITTILSVSAAHEVPLASLTSEAHGQTSGTDEAFLSEIFGTFRYFPGNVWII 295
                        * :*::::.:** ::*::.::    . :.**    *  :**** *:**:*:**.  **::

OsSUT2_BAC67163.1_      LIVTALTWIGWFPFILFDTDWMGREIYRGSPDDPSITQSYHDGVRMGSFGLMLNSVLLGF 342
HvSUT2_CAB75881_        LIVTSLTWVGWFLFILFDTDWMGREIYRGSPEIVADTQKYHDGVRMGSFGLMLNSVVLGI 346
AtSUT4_AAG09191_        LLVTALTWIGWFPFILFDTDWMGREIYGGEPNIG---TSYSAGVSMGALGLMLNSVFLGI 352
                        *:**:***:*** ************* *.*:      .*  ** **::*******.**:

OsSUT2_BAC67163.1_      TSIVLEKLCRKWGAGLVWGVSNILMALCFVAMLVITYVAKNMDYPPSGVPPTGIVIASLV 402
HvSUT2_CAB75881_        TSIGMEKLCRKWGAGLVWGVSNIIMALCFVAMLIITYVAQNLDYGPSGAPPTGIVAASLI 406
AtSUT4_AAG09191_        TSVLMEKLCRKWGAGFVWGISNILMAICFLGMIITSFVASHLGYIGHEQPPASIVFAAVL 412
                        **: :**********:***:***:**:**.*:: ::**.::.*     **:.** *:::

OsSUT2_BAC67163.1_      VFTILGAPLAITYSIPYAMAASRVENLGLGQGLAMGILNLAIVIPQVIVSLGSGPWDQLF 462
HvSUT2_CAB75881_        VFTILGAPLSITYSIPYAMAASRVENLGLGQGLAMGILNLSIVIPQIIVSLGSGPWDQLF 466
AtSUT4_AAG09191_        IFTILGIPLAITYSVPYALISIRIESLGLGQGLSLGVLNLAIVIPQVIVSVGSGPWDQLF 472
                        :***** **:****:***: : *:*.*******::*:***:*****:***:*********

OsSUT2_BAC67163.1_      GGGNAPAFAVAAAASFIGGLVAILGLPRARIASRRRGHR- 501
HvSUT2_CAB75881_        GGGNAPSFWVAAAASFVGGLVAILGLPRARLGPKKKTTQR 506
AtSUT4_AAG09191_        GGGNSPALAVGAATGFIGGIVAILALPRTRIQKPIPLP-- 510
                        ****:*:: *.**:.*:**:****.***:*:
```

Where: (*) means identical amino acid

(:) means similar amino acid

(.) means partially similar amino acid

APPENDIX I
Copyright of Aphid Diagram Used in Thesis

Re: FW: Comment To Webmaster - agsci.psu.edu

Wednesday, May 12, 2010 7:14 AM

From:

"Steve Jacobs" <sbj2@email.psu.edu>

Add sender to Contacts

To:

modelsss2001@yahoo.com

Ibraheem,

You may use the figure providing you give proper credit to the author and department.

Please credit: Lloyd E. Adams, Professor Emeritus (deceased), Department of Entomology, The Pennsylvania State University.

Steve

Steve,
We received a request to use an illustration from a fact sheet currently on the college Web site. This fact sheet is not an Ag Communications product, so I was hoping you might take a look at the illustration and respond to the query below.
Thanks,
Pete

Peter Kauffman
Art Editor
Agricultural Communications and Marketing
College of Agricultural Sciences,Penn State
University Park, PA 16802
Ph: 814-863-2700
Fax: 814-863-2704
E-mail: pak6@psu.edu

From: do.not.reply@ag.psu.edu [mailto:do.not.reply@ag.psu.edu]
Sent: Wednesday, May 12, 2010 4:37 AM
To: WebServices
Cc: Laurie Ann Yearick; Mary Joan Wodecki; Rhonda Rae Demchak
Subject: Comment To Webmaster - agsci.psu.edu

Name: IBRAHEEM Omodele
E-Mail Address: modelsss2001@yahoo.com
Phone Number: +27721571094
Address: Department of Biochemistry and Microbiology, University of Fort Hare, P.Bag X1314, Alice 5700 South Africa
County (If PA resident): Not a PA resident
How would you rate the organization of the pages in our Web site?: Well Organized - easy to find information

Comments:
Good day.
I'm a PhD student of the Department of Biochemistry and Microbiology, University of Fort Hare,South Africa.
I'm seeking for a permission to use the aphid diagram which I got from the internet from your link :
http://downloads.cas.psu.edu/4H/PartsOfAnInsect.pdf
for my research thesis write-up.
I will be happy if this permission can be granted.
Thanks for your good will.
IBRAHEEM Omodele
------ End of Forwarded Message

- -
Steven B. Jacobs, Urban Entomologist

Penn State Univ., Department of Entomology

513 ASI Building University Park, PA 16802,Voice: 814-863-3963, FAX: 814-865-3048

APPENDIX J
(a) High Photographic Resolution Image of Figure 5.11

(b) High Photographic Resolution Image of Figure 5.12