Examining the role of the barley sucrose transporter *HvSUT1* in increasing grain nutrients in rice (*Oryza sativa*).

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Rice is an important food staple and is the main source of nutrients that sustains a large proportion of the world's population. Rice is rich in carbohydrates but otherwise regarded as a relatively nutrient poor cereal staple, especially after processing by polishing to remove the outer grain layers. Polishing removes a range of nutrients that are concentrated in the bran, such as iron (Fe), zinc (Zn), protein and lipids. In addition, nutrient bioavailability can be compromised by the presence of antinutrients, such as phytate, which binds tightly to mineral ions. Nutrient poor diets can contribute to a range of illnesses that are most severe in vulnerable populations in the developing world.

Sucrose, produced by the plant through photosynthesis, is the major assimilate transported in the phloem of rice plants. The phloem is also the long distance transport pathway for other assimilates. At maturity, sucrose transported into the grain is converted into various storage products as future nutrient reserves for the growing embryo. In the rice endosperm, sucrose is mainly converted to starch, however it is also involved in the biosynthesis of other nutrients such as proteins and fatty acids. In addition, minerals such as Fe and Zn, that are taken up from the soil are also transported via similar pathways as sucrose and deposited along with storage compounds in the grain. Therefore, nutrient loading into the seed is a crucial phase in plant growth and the seed becomes a strong sink competing for available nutrients. Sucrose transport in plants is facilitated by sucrose transporter proteins, SUTs, which are highly expressed in the grain aleurone layer and have important roles in assimilate uploading during grain filling.

This study investigated the hypothesis that increasing assimilate uptake into rice grains through overexpression of the barley sucrose transporter *HvSUT1* could change the nutrients in rice. To test this hypothesis, transgenic rice plants (*Oryza sativa* L. cv. Nipponbare) overexpressing *HvSUT1*, driven either by the constitutive rice *Actin-1* (Act_{pro}) or endosperm specific rice *Globulin-1* (Glb_{pro}) promoter, were generated using *Agrobacterum*-mediated-transformation. These constructs were designated as Act_{pro}::HvSUT1 and Glb_{pro}::HvSUT1 respectively. A number of independent stably transformed plant lines were confirmed by molecular and biochemical assays. Transgene expression was verified by RT-PCR and immunoblotting, which showed that the $Glb1_{pro}$ drove stronger HvSUT1 expression in the grain, compared to Act_{pro}. Transgene function was analysed by [^{14}C]-sucrose uptake assays which suggests that transgenic rice grains driven by both promoters had prolonged active sucrose uptake at later stages of grain filling, compared with non-transgenic (NT) grains. Furthermore, grains expressing

HvSUT1 specifically in the endosperm had increased sucrose uptake significantly at 7 days after anthesis (DAA), a crucial time point in grain development.

Grain nutrient composition and distribution was altered in rice grains expressing HvSUT1, compared to the non-transgenic (NT) line, with more significant differences observed in the composition of grains transformed with Glb_{pro}::HvSUT1 compared to Act_{pro}::HvSUT1. Elemental analysis by Inductively Coupled Plasma Spectrometry (ICP-OES) revealed that transgenic rice grains expressing HvSUT1 had a greater retention of Fe and Zn in the endosperm compared to NT grains. LA-ICP-MS imaging confirmed that elements in the transgenic grains had been redistributed in transgenic rice, with higher counts and greater distribution of micronutrients in the endosperm. Sucrose content increased but uniquely, potassium (K) retention decreased in the endosperm of transgenic grains, which could be explained if the transport processes for these two grain nutrients were linked, possibly as osmotic species involved in cell membrane polarity and/or maintaining cell turgor. A decrease in the soluble starch content, but increases in amino acids and phytate suggested that HvSUT1 overexpression had affected metabolic processes within the grain, leading to changes in the relative proportions of storage products. In addition, the composition of storage proteins and lipids that are also downstream products of sugar metabolism, were modified in rice grains overexpressing HvSUT1.

Overall, the changes in nutrients in the transgenic rice grains support the proposal that increasing sucrose transport can change the nutrient composition of rice and can modify metabolic processes that are responsive to carbon supply. Significantly, in the present study, transgenic grains retained more of the important micronutrients Fe and Zn, in the starchy endosperm. Furthermore, other changes in grain composition suggest that SUT overexpression could be a strategy to improve the nutritional quality of rice. The mechanisms for the changes are yet to be investigated, however, the findings from this study contribute to the understanding of the complex interactions between assimilate partitioning and nutrient deposition in cereal grains. This can aid in future efforts to breed more nutritious staple crops to alleviate nutrient deficiencies in developing countries.

I certify this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text

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Literature Review

1.1 Introduction

Rice, *Oryza sativa* (L.) is a major dietary staple for a large proportion of the world's population. Rice grains mainly contain carbohydrates in the form of sugars and starches, but also minor amounts of proteins, lipids and amino acids. They are also a source of vital micronutrients such as zinc (Zn) and iron (Fe), although these minerals are usually present in very low quantities. Therefore, rice provides dietary calories but is a relatively nutrient poor food source. In poor developing countries where dietary limitations restricts the intake of more nutrient rich foods, rice can comprise the majority of the daily calorie intake. This reliance on rice leads to high rates of disease and illness due to micronutrient malnutrition (Welch and Graham, 2002). The current trends in world food production are not expected to produce enough food for the projected increase in human population over the coming decades (Parry *et al*., 2004) and before long food shortages are predicted to affect a greater number of people worldwide. On top of this, projected outcomes of elevated carbon dioxide levels predict a decline in seed micronutrients (Seneweera and Conroy 1997) and malnutrition will become an issue that cannot be ignored. It is therefore imperative to research novel approaches to 'biofortify' cereal crops, such as rice, to enhance essential nutrients for future food production.

Strategies to increase grain yield and nutrient levels in edible tissues include conventional breeding which is aided by advances in genetic approaches now that the sequencing of the genomes of important crops such as rice and barley have been completed. However, breeding new crop varieties through successive rounds of crossing and selection is both laborious and lengthy. A more targeted and rapid approach for crop improvement is the use of molecular genetic strategies to manipulate and enhance plant processes to promote desirable characteristics in cereals. This will help to alleviate some of the problems associated with limited diets in poorer nations leading to greater food security for these regions.

Rice is important from a human nutrition perspective and it is also a valuable experimental system for investigating the fundamental processes of nutrient movement and uptake into the grain, with these findings also translating to other important cereal crops (i.e. wheat and barley). When considering rice as a staple food, it is important to understand the processes which govern the transport of nutrients into the edible part of the grain. It is well understood that sugars and minerals that enter the seed endosperm are synthesized and/or transported from tissues elsewhere

in the plant. While sucrose is synthesized in photosynthetic tissues of the rice plant, minerals must be taken up from the soil through the roots and subsequently enter seeds through the long-distance vascular pathway (phloem). Therefore, the phloem plays a major role in coordinating the transport of photoassimilates and nutrients from their respective source tissues into growing and developing sinks (i.e. seeds). Better understanding of the regulation of these processes could enable us to increase nutrient content in the edible grain.

Research into sucrose transport in plants has received a great deal of attention in the past decade in light of the discovery and characterization of sucrose transporter proteins (SUTs). Plasma membrane localized SUTs were identified as a fundamental protein facilitating active sucrose transport between tissue compartments for carbon partitioning. Sucrose is the major photoassimilate transported in the phloem and is therefore likely to exert an influence on the movement of a range of other less abundant solutes. Understanding nutrient import into grains is also relevant in terms of human nutrition. Sucrose transported into cereal grains is primarily channelled into starch production, but it is also involved in the metabolic pathways for protein and fatty acid synthesis. Additionally, micronutrients are transported via similar pathways as sucrose and are associated with storage compounds in the grain.

Sucrose transport in plants, especially cereals, and the influence of sucrose on the deposition of other grain nutrients, is the focus of this review. Important components of the transport pathway will be covered such as the other important solutes that are transported in the phloem and the cellular and physical factors that influence the movement of nutrients into seeds. The section on SUTs will encompass their discovery, classification, localization in plant tissues and functional characterisation of their role in sucrose transport, particularly for the important cereal monocot species rice, barley and wheat. Carbon metabolism and assimilate partitioning during grain development will be covered in detail to highlight the importance of sucrose in the production of grain storage products such as starch, proteins and fatty acids. Finally this review will cover the issue of biofortification and the transport and uptake limitations of mineral nutrients into cereal grains. Examples of cereal biofortification strategies through genetic modification of genes for mineral transport will be presented, with an emphasis on Zn and Fe. The literature covered in this review will provide a framework for the aims and hypotheses of this thesis and a context for this research.

1.1.1 Sucrose transport in higher plants

It has been determined that most higher plants use sucrose as their primary assimilate. This preference for sucrose is thought to have evolved because of its low viscosity, stability and non-reactivity under the conditions that are generally found in phloem sap (Ward *et al.*, 1998). Sucrose is transported from where it is synthesized in photosynthetic tissue, to other organs in the plant, where it is used to maintain cellular processes, utilized in cell growth or deposited and converted into storage compounds. This export of carbohydrates from source tissues (where sucrose is synthesized) and import to sink tissues (where sucrose is utilized in growth, maintenance of metabolic processes and storage) is tightly regulated genetically and metabolically. To ensure an efficient and accurate transport and delivery of sucrose along with other assimilates such as amino acids, organic acids, growth regulators, nucleic acids and micronutrients, plants have developed a sophisticated transport pathway through the phloem that encompasses structural components of the plant anatomy as well as various proteins that serve as carriers or transporters to import various molecules into cells.

The mechanism by which phloem sap moves from source to sink was initially proposed to follow the mass flow model, or the Munch Pressure Flow hypothesis (Munch, 1930). In this model, a pressure gradient between the loading of sucrose into sieve elements through companion cells (widely referred to as sieve element companion cell complex SE-CC) at source tissue and the final step of sucrose unloading at sinks drives long distance movement of sucrose between the source (high pressure) and sink (low pressure). This model is good for explaining long distance transport of nutrients in phloem however it implies this is a passive process. In fact, this process is extremely dynamic and involves a range of specific proteins that work as pumps and transporters to maintain the pressure in sieve elements. The action of these proteins facilitates the movement of sucrose and helps supply nutrients to various sink organs in the sucrose transport pathway. Mechanisms of phloem loading at photosynthetic source tissue and long distance transport of sucrose has been discussed thoroughly in many reviews (e.g. Aoki *et al.*, 2012 and references therein) and will only be covered briefly to explain the role of SUTs in this process.

1.1.2 Phloem sap composition

Sucrose constitutes a high proportion of the phloem sap (for example, 25% w/v in rice) in most higher plants including cereals (Fukumorita and Chino, 1982; Winter *et al.*, 1992). The ability to obtain pure phloem sap through aphid stylectomy (Kennedy

and Mittler, 1953; Calatayud *et al.*, 1996) has enabled the components in phloem sap to be measured more accurately leading to a much better understanding of phloem composition. This technique has been especially useful in the analysis of graminaceous species such as rice through the use of laser stylectomy (Kawabe *et al.*, 1980) as other techniques for collecting phloem sap by cutting and bleeding are ineffective, and yields sap with high concentrations of cellular contaminants (Fukumorita and Chino, 1982). Graminaceous phloem sap retrieved through this technique can also be used to examine important mRNA species that are transported within the phloem (Sasaki *et al.*, 1998) including SUT mRNA (Doering-Saad *et al.*, 2002). Furthermore, the ability to analyse "pure phloem" has the potential to improve understanding of the role of compounds that are trafficked through the phloem. Compounds including nutrients, macromolecules such as RNA and proteins are thought to have roles in systemic signalling that may affect the development and function of the whole plant or specific organs (Atkins *et al.*, 2011).

As well as sucrose, other major nutrient species are present in cereal phloem including amino acids (up to 8%) and inorganic ions (Fukumorita and Chino, 1982). Sulphur is incorporated into disulphide bonds and is an essential nutrient integral to the correct conformations of enzymes and proteins, and is also translocated in rice phloem. Sulphur is taken up from the soil in the form of sulphate (SO_4^{2-}) and is converted into organic forms for translocation, such as Glutathione (GSH) and very low concentrations of amino acids such as cysteine and methionine (Kuzuhara *et al*., 1999). GSH in plants is important in reducing oxidative damage and may also have a role in regulating sucrose transporter activity (Krugel *et al.*, 2008 cf. Sun *et al.*, 2010). The dominant inorganic ion in rice phloem sap is potassium (K^+) (150mM) (Fukumorita and Chino, 1982), followed by Chloride (Cl^-) (52mM) (Hayashi and Chino, 1985). Various other ions have been measured in sap at much lower concentrations. These ions buffer the pH of rice sap to approximately 8, allowing a high concentration of organic acids to be transported through the phloem. These organic acids can chelate Ca^{2+}, Fe^{3+} and Zn^{2+}, allowing the translocation of these insoluble metal ions (Hayashi and Chino, 1985). Thus, phloem sap is composed of a mixture of compounds and assimilates produced by the plant and also essential minerals and nutrients taken up into the plant from the soil.

1.1.3 Cereal grain morphology and grain filling

In cereals, the starchy endosperm constitutes the majority of the grain mass (~90%) (Del Rosario *et al*., 1968). The endosperm is heterogeneous and the various cell layers develop in phases after fertilization. Formation of the undifferentiated

endosperm syncytium is followed by cellularisation and differentiation of the main cell types including the aleurone and starchy endosperm (Fig. 1.1) (Sabelli and Larkins, 2009). Intensive research into cereal grain structure has now identified the sites of post-phloem movement of solutes from phloem to grain through the use of histochemical staining and movement of radioactive dyes (Zee 1971; Oparka and Gates, 1981; Wang *et al.*, 1994). While distribution of the cell types varies between cereal grains, a general pattern of sucrose uptake during grain filling appears to be consistent among different species (Patrick and Offler, 1995). Solutes moving into developing grains are known to deposit into tissues of the bran layers, embryo and the endosperm. However, per weight and volume the endosperm constitutes the bulk of the cereal grain and consequently is the main sink for storage products. Hence, this discussion will focus on the morphology and the path of solute uptake into endosperm storage cells during grain filling.

Zee (1971) and Oparka and Gates (1981; 1982 and 1984) have examined the rice spikelet and grain in great detail and a summary of their relevant findings follows. The xylem and phloem in rice are continuous with the vascular bundle (VB) unlike other temperate cereals such as wheat and barley. As shown in Fig. 1.1, this conduit for solutes enters the caryopsis through the base and continues around the longitudinal axis of the grain. The close up of the vascular region of the grain indicates that solutes move symplastically out of the long distance pathway (SE-CC) through the maternal layers of the developing grain (PS-N) until they reach the apoplastically isolated filial tissue layer, the aleurone (Oparka and Gates, 1984). An apoplastic efflux and loading step must then occur to enable the transport of solutes into the aleurone cells (apoplastic loading will be discussed in more detail in section 1.4.3). After being taken up into the outer layers of endosperm cells, solutes can move symplastically through the numerous plasmodesmata (PD) that connect these tissues. This process is thought to be primarily passive as endosperm cells have few associated transporter proteins. However, many nutrient transporters are found associated with storage cell vacuole membranes, therefore active storage and efflux of nutrients into vacuoles may also play a part in driving solute movement into endosperm cells.

It has been shown with solute tracers (both radio- and fluorescently labelled) that in rice the solutes are transported in a circumferential pattern from the VB, through the PS and into the N (Oparka and Gates, 1981). However, the lack of tracer directly below the VB and PS further proves the existence of a discontinuity between these tissues (Oparka and Gates, 1981). However, solutes must pass through the

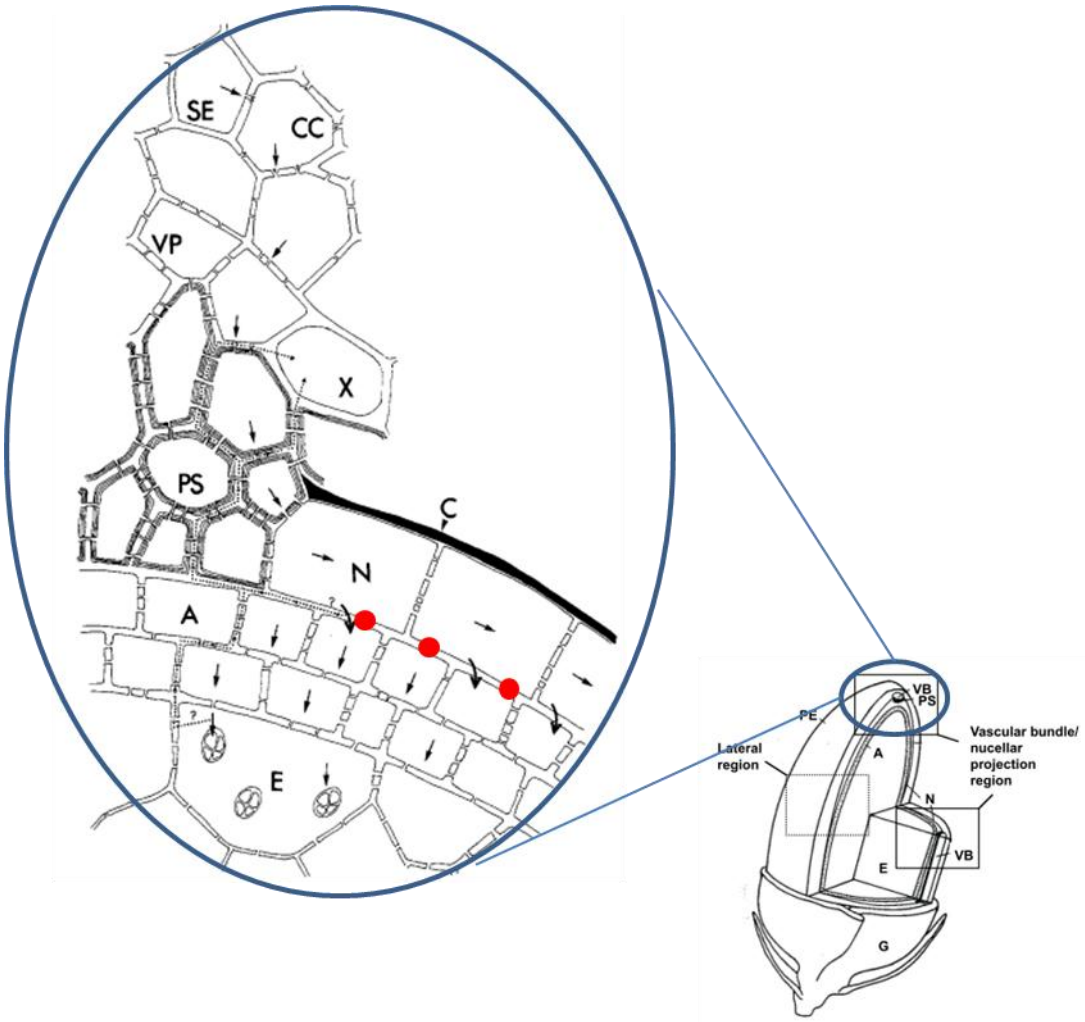


Figure 1.1 Proposed pathway for the movement of nutrients in rice grain

A cut away of the rice grain showing the position of tissue layers in 3 dimensions (Furbank *et al.*, 2001) and a close up of the vascular bundle region where arrows depict the movement of solutes into the endosperm (Oparka and Gates, 1981). Note the multiple connections between adjacent cells in the aleurone and endosperm and between cells in the nucellus, but none between the nucellus and aleurone. Sucrose transporter proteins (SUT) in red actively transports sucrose between the nucellus and aleurone layers. E, endosperm; A, aleurone; N, nucellus; VB, vascular bundle; PS, pigment strand; G, glumes, PE, pericarp; SE, sieve elements, CC, companion cells, VP, vascular parenchyma, X, xylem, C, cuticular layer. The dashed lines and question marks in Oparka and Gates (1981) indicate the proposed pathway for water entering and exiting the grain.

apoplast into rice filial tissue in order to be stored in endosperm cells. Thus the rice aleurone, although lacking typical transfer cell morphology, is significant in controlling the rate of solute translocation during grain filling, and is proposed as the site for sucrose import into storage endosperm tissue.

1.1.4 Cellular transport of nutrients

Plant cells transfer solutes and communicate with neighbouring cells through plasmodesmata (PD). The number of PD varies greatly between species, therefore Gamalei (1989) proposed that plants containing few PD connections are apoplastic loaders and plants that have numerous PD are symplastic loaders. The study of symplastic and apoplastic routes of sucrose movement have mostly been investigated in source leaves due to their accessibility and their importance as photosynthetic organs. The importance of symplastic loading was shown in a maize mutant (*sxd-1*) that displayed leaves with high levels of starch build up due to blockage of PD with callose, a polysaccharide (Botha *et al.*, 2000). Scofield *et al.* (2007a) also found evidence to support the symplastic movement of solutes in rice leaves. However, PD connectivity gives no indication of the rate at which sucrose transport moves symplastically and does not preclude the concurrent movement of sucrose via the apoplastic route (Botha *et al.*, 2008). Sucrose pathways in barley and wheat leaves may differ because they have fewer PD than rice (Botha and Cross, 1997; Aoki *et al.*, 2005). Despite these differences in the ultrastructure of leaf and phloem anatomy among the grasses, evidence now supports that most species utilise both apoplastic and symplastic loading mechanisms for solute transfer between cells (Ayre, 2011).

Apoplastic loading is especially relevant in developing cereal grains where there is a distinct discontinuity between the outer coat and the inner tissues. There are no PD linking these tissues because of the origin of the outer and inner layers from maternal and filial tissues respectively. Therefore the uptake of nutrients into the inner grain layers occurs in two stages: efflux of sucrose into the apoplastic space from the maternal tissue, then energy dependent uptake into filial tissue through transmembrane proteins (Fig. 1.2). Sucrose efflux from cells is thought to be a similar process regardless of where along the long distance transport pathway it occurs, from phloem loading at the source to uploading at the sink tissues (Ayre, 2011).

Sucrose efflux into the apoplast has been demonstrated and measured (Laloi, *et al.*, 1993) and various theories have been put forward as to how cells release sucrose into this space (Patrick and Offler, 1995; Wang *et al.*, 1994), but until recently the candidate for these putative efflux proteins remained a mystery. Some researchers

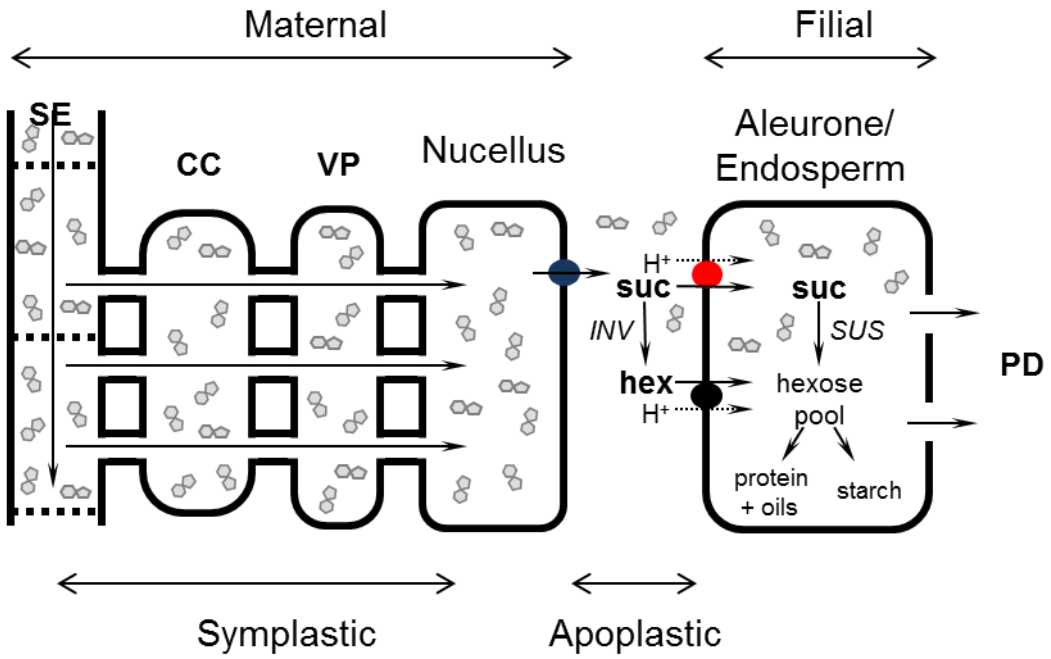


Figure 1.2 Symplastic and apoplastic pathway of sucrose from the phloem into cereal grains

Sucrose is synthesized in photosynthetic tissues then is transported via the phloem (SE, sieve element; CC, companion cells, VP, vascular parenchyma) then flows into the nucellus via a symplastic pathway. The proposed location of the sugar efflux SWEET protein is shown by a blue circle. Evidence supports a uniport mechanism for sucrose efflux into the apoplast (Chen *et al.*, 2012). Sucrose transporter proteins (SUT) in red actively take up 1:1 suc/H^+ from the apoplastic space into the aleurone which is connected to endosperm cells via multiple plasmodesmata (PD). In early grain development, Invertase (INV) breaks down sucrose in the apoplast which is taken up into the filial cells via hexose transporters (black) and utilised mainly for cell growth and elongation. The hexose pool is increased by the breakdown of sucrose by Sucrose synthase (Susy) within aleurone/endosperm. The hexoses formed after the period of cell elongation are utilised in the synthesis of storage products such as starch, protein and oils. This diagram is modified from Aoki *et al*. (2012).

hypothesized that the proteins involved in uptake into cells could reverse their polarity and act as effluxers and this was shown to be possible when the maize ZmSUT1 could reverse its activity from uptake to efflux mode in oocytes when membrane potentials were reversed (Carpaneto *et al.*, 2005). This bi-directional activity of transporters was not demonstrated in any other SUTs. However, in the absence of better evidence, this was an attractive theory to pursue. Chen *et al.* (2012) finally identified novel members from the SWEET family of sugar transporters, characterised as glucose transporters, in Arabidopsis and rice that could mediate sucrose efflux. Specifically AtSWEET11 and 12 and OsSWEET 11 and 14 were shown to transport sucrose in heterologous systems. In addition to normal transport processes in plants, SWEET protein members have also been proposed to play roles in responses to pathogen infection (Baker *et al.*, 2012) along with growth and development (Zhou *et al.*, 2014). Although this identified one of the most crucial missing links in the sucrose transport pathway between plant cells, the localization and function of these SWEET proteins in cereal grains has not been determined. Unlike sucrose efflux, the mechanism for energy dependent sucrose uptake into cells through sucrose transporters (SUTs) has been intensively researched and good progress has been made towards understanding this complex process.

1.1.5 Evidence of sucrose uptake via an active transporter

Giaquinta (1977) proposed a model outlining a pH gradient across the plasma membrane as the driving force for trans-membrane movement of sucrose through a proton coupled symport. Evidence supporting this model of sugar transport was provided by experiments using plasma membrane vesicles (PMV) from sugar beet leaves (Bush, 1989). Further investigations into the biochemical properties of this proton-sucrose symporter determined that the movement of sucrose across the plasma membrane was indeed electrogenic, reliant on the membrane potential that was generated by the 1:1 stoichiometric transport of protons and sucrose into the cell (Bush, 1990).

Movement of sucrose across the plasma membrane involves the coupling of two transport systems. Firstly the active transport of protons through a proton pump that utilizes the energy from the hydrolysis of ATP to ADP to pump H^+ from the cytoplasm to the cytosol. This creates an electrochemical gradient across the membrane which drives sucrose molecules into the cell against a concentration gradient, along with H^+, through a transporter protein (Bush, 1993). The high transport activity of this putative symporter means that sucrose is able to be transported against a considerable concentration gradient into PMV (Bush, 1989). In solutions containing a range of other

plant transport sugars, there is no change to the sucrose transport activity of the PMV, indicating this transporter has a high specificity for sucrose (Bush, 1989).

1.2 Sucrose Transporters (SUTs)

The isolation, cloning and functional analysis of the first plant SUT from spinach *Spinacia oleracea* by Riesmeier *et al*. (1992) opened the floodgates to molecular studies of these important plant proteins. Multigene families of SUTs have been classified in most plant species studied to date. In dicots, SUT genes have been identified and studied in carrot (Shakya and Sturm, 1998), peas (Tegeder *et al.*, 1999), grapevine (Davies *et al.*, 1999), citrus (Li *et al.*, 2003), solanaceous species (Schmitt *et al.*, 2008) such as potato (Kuhn *et al.*, 1996, 2003; Krugel, 2008) and tomato (Hackel *et al.*, 2006) and ornamentals (Knop *et al.*, 2004). SUTs have been most widely studied in the model plant species *Arabidopsis thaliana* (Baud *et al.*, 2005), of which there are nine members of the gene family. The first monocot transporter cDNA was isolated from rice by Hirose *et al.* (1997) and a further four rice SUT genes have since been identified (Aoki *et al.*, 2003). However, the understanding of SUTs in monocots has trailed behind that of dicots despite this group containing all of the cereal crops most widely consumed worldwide.

1.2.1 Plant SUT classification

SUTs are members of the Major Facilitator Superfamily (MFS) of cell membrane proteins that have roles in the transport of a range of solutes essential for cellular function. Members of the MFS are found in all living organisms. For a comprehensive review of this diverse and ubiquitous family of proteins, see Pao *et al.* (1998). Although sugars are essential for all living organisms and different organisms have homologous sugar transport proteins, SUTs of higher plants are unique because unlike other organisms, they utilize sucrose as their main form of transport sugar (Williams *et al*., 2000).

Understanding the relationship between different plant SUTs (also called SUCs or sucrose carriers) is confused by the different naming conventions authors have used. Moreover, SUT genes were named chronologically as they were identified, rather than on their relatedness or similarity in gene or protein structure or function (Braun and Slewinski, 2009). For example, SUT1 from spinach, the first sucrose transporter to be identified in plants has very little sequence similarity to the SUT1 from rice.

With the increasing number of SUT genes being discovered, there has been a confusing evolution of SUT assemblages as the number of group members has

expanded. Phylogenetic trees of current SUT members from plants using gene and protein sequences have been assembled by Williams *et al.* (2000), Sauer (2007), Shiratake (2007), Braun and Slewinski (2009), Kuhn and Grof (2010) and Aoki *et al.* (2012), all of whom used different names for the various groups/types/clades that arise from their analyses. For example, Kuhn and Grof (2010) group the various SUTs into five clades, however their classification does not appear to match the five groups reported in Braun and Slewinski (2009). Clarification of SUT relatedness between plant species is further complicated by the use of different outgroups and the addition or omission of various SUT members from each analysis. For example, while *ZmSUT2* and *LeSUT2* cluster into the same clade in Kuhn and Grof (2010), however in Braun and Slewinski's (2009) analyses, they are only distantly related. These examples demonstrate there is still no consensus on how the groups should be classified. However, to summarise the major findings from these publications, the general phylogenetic grouping reveals five distinct clusters divided into three types. Type I contains only dicot SUTs, whereas types II and III are comprised of both dicots and monocots. It has been speculated that monocot SUTs have resulted from gene duplication events prior to the divergent evolution of dicots and monocots (Aoki *et al*., 2003).

Braun and Slewinski (2009) present an excellent review of grass SUTs, with a discussion of the roles and functions of SUTs in the most agriculturally important cereal species. They attempt to standardise the classification of grass SUTs by renaming and grouping several SUT genes according to their homology to the five rice SUTs. As the focus of this review is on monocot SUTs, from this point onwards the naming convention and classification of the groups proposed by Braun and Slewinski (2009) (Table 1.1) will be employed.

Numerous comprehensive reviews, including those written by Ward *et al.* (1998), Kuhn *et al*. (1997), Lemoine (2000), Williams *et al.* (2000), Truernit (2001), Eckardt (2003), Lalonde *et al.* (2004), Sauer (2007), Shiratake (2007), Braun and Slewinski (2009), Geiger (2011) and Ayre (2011) and Aoki *et al.* (2012) together have covered all aspects of sucrose transport and SUTs in plants. Some focus exclusively on SUTs involved in phloem loading in dicots (Ward *et al.,* 1997; Kuhn *et al*., 1999; Lemoine *et al.,* 2000). Others focus on the phylogenetic classification of the various SUT groups and their physiological roles in phloem loading and carbon partitioning in leaf tissue (Shiratake, 2007; Braun and Slewinski, 2009). The most current reviews combine the growing wealth of knowledge about SUTs into specific discussions regarding processes at plant membranes (Geiger, 2011) or the effects on whole plant

Table 1.1 Classification of SUTs from important plant species

SUT Groups from monocot and dicot species according to phylogenetic relationships based on amino acid sequence alignments by Braun and Slewinski (2009).

Group	Monocot/Dicot	Plant Species	Gene	Reference
1	Monocots	*Oryza sativa* rice	*OsSUT1*	Hirose *et al.* (1997)
			OsSUT3	Aoki *et al.* (2003)
		Hordeum vulgare barley	*HvSUT1*	Weschke *et al.* (2001)
		Zea maize maize	*ZmSUT1*	Aoki *et al.* (1999)
			ZmSUT3	
		Triticum aestivum wheat	*TaSUT1D*	Aoki *et al.* (2002)
2	Dicots	*Arabidopsis thaliana*	*AtSUC1*	
			AtSUC2	
			AtSUC5	
			AtSCU9	
		L*ycopersicum esculentum* tomato	*LeSUT1*	
		Plantago Major potato	*PmSUC1*	Riesmeier *et al.* (1994)
			PmSUC2	
3	Monocots	*Oryza sativa* rice	*OsSUT4*	Aoki *et al.* (2003)
		Zea maize maize	*ZmSUT4*	
	Dicots	*Arabidopsis thaliana*	*AtSUC3 (AtSUT2)*	
		Lycopersicum esculentum tomato	*LeSUT2*	
		Plantago Major potato	*PmSUC3*	
4	Monocots	*Oryza sativa* rice	*OsSUT2*	Aoki *et al.* (2003)
		Hordeum vulgare barley	*HvSUT2*	Weschke *et al.* (2001)
		Zea maize maize	*ZmSUT2*	
	Dicots	*Arabidopsis thaliana*	*AtSUT4 (AtSUC4)*	
5	Monocots	*Oryza sativa* rice	*OsSUT5*	Aoki *et al.* (2003)
		Zea maize maize	*ZmSUT5*	
			ZmSUT6	

assimilate partitioning (Ayre, 2011). The plethora of reviews written in the last few years along with the number of intensive studies into all aspects of how plant sucrose transporters work and function reflect the interest in these transporters

1.2.2 SUT structure, function and activity

Sucrose transporters are trans-membrane proteins composed of 12 conserved helical membrane spanning regions separated by loops that intrude into the cytoplasm and extracellular space. The helices are thought to be arranged so that a pore is formed in the plasma membrane through which sucrose molecules are actively transported along with H^+ (Sauer, 2007). The N and C-termini, as well as the central loop between helices 6 and 7 are thought to be located inside the cell. Transporter activity can be inhibited by the use of several protein modifiers that modify specific amino acid residues, such as *p*-CMBS and DEPC (Bush, 1993b).

The functions of the N and C-termini and the central loop of SUTs have long been debated. A low affinity transporter from *A. thaliana*, AtSUT2, was modified by exchanging its N terminal region with that from a high affinity transporter StSUT1 from *Solanum tuberosum*. This chimeric protein exhibited a higher affinity for sucrose when expressed in a heterologous system (Schulze *et al.*, 2000). Schulze *et al.* (2000) also exchanged the central loop regions between a low and high affinity transporter, however they observed no change in transporter activity. This evidence suggests that the N-termini may be involved in sucrose recognition but based on this data no role for the central loop can be determined.

An alignment of the SUT1 proteins of a number of important monocot species shows the high degree of similarity of amino acid residues between related plants (Fig. 1.3). The most conserved regions appear to be in the putative transmembrane spanning helices, while the loops can be quite variable between plant SUTs. Amongst some of the recognised conserved residues are His-65 from AtSUC1 which was identified by Lu and Bush (1998) as a DEPC sensitive residue and Arg-188 which is 100% conserved in all plant SUTs. Sun and Ward (2012) generated an Arg188LysSUT1 mutant which showed loss of function in yeast and in transgenic *A. thaliana* plants. Sun *et al.* (2012) modelled the 3D structure of OsSUT1, based on its similarities to other extensively studied MFS proteins, such as LacY (lactose:H^+ symporter). Their model shows His-85 in OsSUT1 in the extracellular loop between helices 1 and 2, which agrees with the location of the equivalent His-65 in AtSUC1 modelled by Lu and Bush (1998) (Fig. 1.4 A, B). However, unlike previous models of SUT1, the predicted position of a number of charged residues was within the membrane spanning helices rather than the

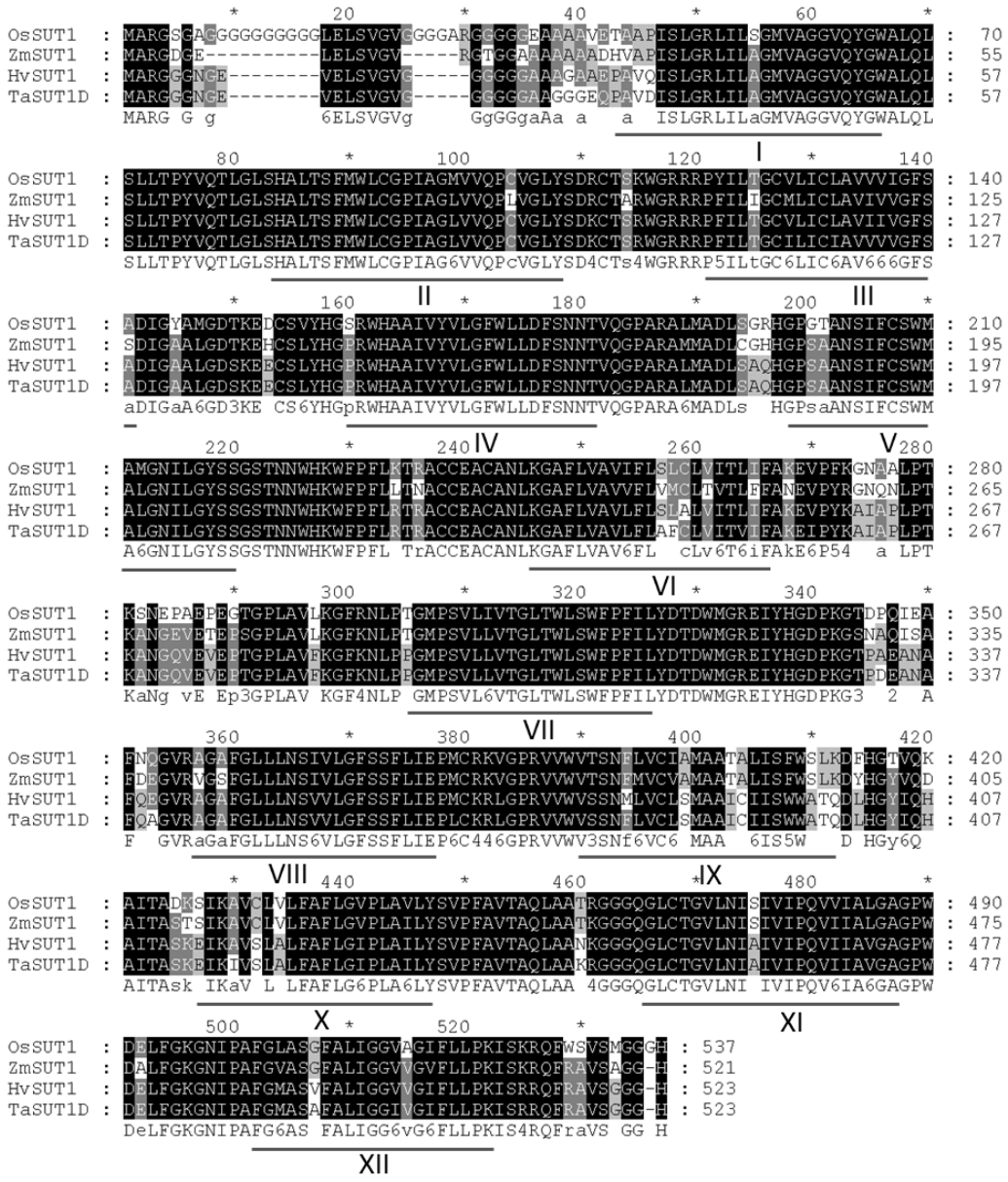


Figure 1.3 Protein sequence alignment of SUT1 from important monocot crop species

A high level of conservation between residues shows shared amino acid identity is 82% in maize ZmSUT1, 80% in barley HvSUT1 and 79% in wheat TaSUT1D compared with rice OsSUT1. Putative transmembrane helices according to Hirose *et al.* (1997) are underlined and numbered (compared with predicted 3D model by Sun *et al.* (2012) which shows a slight change in the putative positions of transmembrane helices Fig 1.4 B). Note the extended N-terminus in OsSUT1 not present in any of the other aligned SUT1 proteins, however there has not been any explanation for the function for this long stretch of mostly G (glycine). Sequences aligned using ClustalX2 (Larkin et al., 2007)..

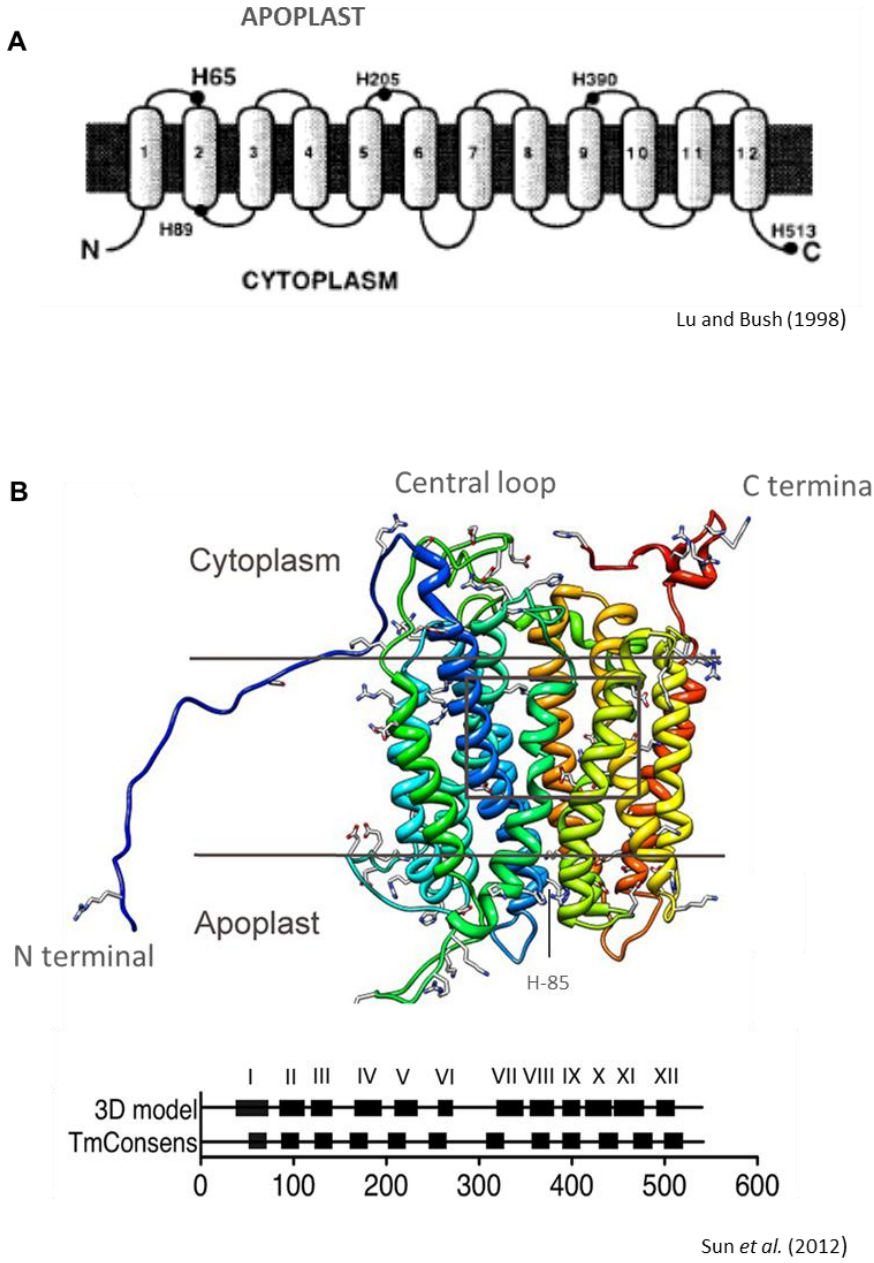


Figure 1.4 Old and new perspectives of the model of SUT structure.

Topological model of AtSUT1 (A) and 3D ribbon model of OsSUT1 (B) showing the position of the transmembrane spanning helices, intra (cytoplasm) and extracellular (apoplast) loops and the position of the N- and C-termini in the cytoplasm. The square outlined in the centre of the barrel structure of the 3D model shows the position of residues mutated in Sun *et al.* (2012). Position of the conserved His-65 (AtSUT1) and His-85 (OsSUT1) are shown on each model. The alignment of transmembrane spanning helices based on the 3D model versus traditional models based on span prediction programs (TmConsens) shows that the 12 transmembrane helices are longer in the 3D model than previously estimated. Note that the N-terminus shown in (B) has been positioned arbitrarily based on stereochemical restraints as there was no template (e.g. LacY) to predict its' placement (Sun *et al*., 2012).

loops. Mutagenesis of five out of six charged residues in these transmembrane spanning regions significantly affected sucrose transport. Results from the mutagenesis of charged residues in the C-termini of OsSUT1 led the authors to hypothesise that this region of the protein may be important for H^+ translocation. Based on the evidence above, roles for the N and C-termini of SUT proteins have been put forward, however no studies have determined the importance, if any, of the central loop. Like previous models, the 3D model by Sun *et al.* (2012) places the central loop in the cytoplasm and it is speculated to have a role in sensing intracellular sugar levels for signal transduction (Lalonde *et al.*, 1999). The sugar sensing role of the central loop is further supported by looking at another member of the rice SUT family, OsSUT4. Like Group 3 dicot SUTs AtSUT2 and PmSUC3, this protein is predicted to have an extended central loop which is structurally similar to yeast sugar sensors (Aoki *et al*., 2003). However Eckardt (2003) references numerous studies that do not support the role of Group 3 proteins as sugar sensors, despite their unique central loop structure. Hence, the function of the central loop in SUT proteins remains obscure.

Functional analysis of SUTs has largely come about from expression of these proteins in heterologous systems. The Group 2 SUTs, made up of only dicots containing many members from *A. thaliana*, have been well characterized in yeast as high affinity sucrose transporters with Km values less than 1.5 mM (Kuhn, 2003). In contrast with Group 2, relevant monocot SUTs have been classified as low to moderate affinity transporters (e.g. OsSUT1 has a $K_{0.5}$ value of 7.5 mM) with a high specificity for sucrose (Hirose *et al.*, 1997; Weschke *et al.*, 2000; Sivitz *et al.*, 2005 cf. Zhang *et al.*, 2007; Sun *et al*., 2010). However, the difficulty encountered by many researchers in expressing monocot transporters in yeast means that functional expression studies have mostly been conducted in oocytes (Aoki *et al*., 2012). Therefore direct comparisons between these two expression systems could explain these differences measured in SUT activity between monocots and dicots or could reflect the different roles that these transporters have within plants.

1.2.3 SUT expression and localization in plant tissues

The potential function of dicot Group 2 SUTs as phloem loaders is to date the only functional classification of any of the plant SUTs identified. Although SUTs have been found to be localized to organs that support this function as phloem loaders, they have also been found to be expressed in a wide variety of tissues in the plant and also in plants that utilize other sugars as their main carbon transport molecule.

In *A. thaliana*, SUT genes are expressed in rosette leaves specifically in phloem and surrounding cells as well as in floral tissue (Sauer and Stolz, 1994; Truernit, 2001). In carrot, sucrose transporter transcripts have also been detected in storage cells in the tap root (Shakya and Sturm, 1998). The high expression of SUTs in germinating seeds of cereals also suggests they play an important role in remobilization of sucrose from starch reserves in the endosperm to feed the growing embryo, although the mechanisms for this remains unclear (Aoki *et al.*, 2012). More recently, a number of SUTs have been found to be localized to the tonoplast in cereals and their function as potential vacuolar transporters has been implicated (Endler *et al.* 2006; Eom *et al.*, 2011). The wide variety of plant SUT expression patterns in diverse plant tissues indicates that they are functionally specific in tissue types amongst plant species.

1.2.4 Role of monocot SUTs

The antisense knockdown of *SUT1* in tomato (Hackel *et al.*, 2006) and potato (Riesmeier *et al.*, 1994; Kuhn *et al.*, 2003) has demonstrated an essential role for this transporter in phloem loading, however to date there have been no similar conclusions drawn from similar experiments with monocot SUTs (Ishimaru *et al.,* 2001; Scofield *et al*., 2002).

The role of SUTs in phloem loading in monocots is not clear and results derived from various cereal species give conflicting clues as to what role these proteins play in this process. Interestingly, none of the monocot SUTs align with any Group 2 dicot SUT. This could indicate that functional conservation is not paralleled by structural conservation, probably due to the long evolutionary divergence between these two plant classes. However, another explanation could be that monocots may not employ SUTs for phloem loading or a member from one of the other sugar transporter groups takes up this role. As monocots are thought to employ an apoplastic step in phloem loading thus requiring a SUT for sucrose loading, it is likely that structurally distinct SUT members exist between monocots and dicots with similar functions, but are yet to be identified or fully characterised.

While the presence of *SUT* mRNA has been recorded in the leaves of several agriculturally important species of monocots, such as rice, maize, barley and wheat (Hirose *et al.*, 1997; Aoki *et al.*, 2002; Aoki *et al.*, 2003; Weschke *et al.*, 2000), their function in this tissue has not yet been elucidated. Slewinski *et al.* (2009) showed that a stunted phenotype displayed by *sut1* mutant maize plants, accumulated high levels of carbohydrate in leaves similar to plants that were cold girdled to inhibit sucrose transport from leaves. These mutant plants were also not able to transport labelled

sucrose as readily as control plants. These findings suggest that *ZmSUT1* plays an important role in sucrose loading in maize. However, this is the only evidence in the literature to date to suggest that monocot SUTs are involved in phloem loading in leaves.

The wheat genes, *TaSUT1A*, *1B* and *1D* are expressed in many tissues including developing grain, stems and leaves (Aoki *et al.*, 2002). However it is proposed that *TaSUT1* may also be involved in phloem loading as SUT1 protein localizes to sieve elements in flag leaf blades and sheath before and after heading (Aoki *et al.*, 2004). Presence of *SUT1* in wheat leaf sheaths and internodes also points to a possible role in sucrose uptake into these tissues for temporary storage as soluble carbohydrates (e.g. fructans) (Aoki *et al.*, 2004). Similarly in barley, temporal and spatial expression of the two barley *SUT* genes led Weschke *et al.* (2000) to suggest that *HvSUT1* may be involved in grain development while *HvSUT2* is likely to have a more general housekeeping role. Rapid induction of *HvSUT1* expression in the grain coincided with an increase in sucrose and sucrose synthase mRNA levels, immediately before starch accumulation in grain. Moreover, *HvSUT1* was detected in only very small amounts in source and sink leaves and in the roots. In contrast, *HvSUT2* was expressed constitutively in all organs (Weschke *et al.*, 2000) and is now thought to be active in the tonoplast to remobilize sugars from the vacuole (Endler *et al.*, 2006). The high degree of homology between *HvSUT1* and the much better characterised and studied *OsSUT1* (82.9%) lends more support for an essential role for *HvSUT1* in barley grain development and grain filling.

OsSUT1 is the first cloned monocot sucrose transporter to be identified and functionally expressed in yeast (Hirose *et al.*, 1997) and is the most well studied rice SUT. The transport activity of OsSUT1 has also been well characterized with this transporter showing similar activity to other cloned monocot SUT1 transporters demonstrating a low affinity, but high specificity for sucrose (Sun *et al.*, 2010). Even though four more SUT genes from the rice genome have since been identified, classified and functionally expressed by Aoki *et al.* (2003), there has been very little progress made towards understanding their different roles in rice plants. The different but overlapping temporal and spatial expression patterns of the genes suggest diverse roles in source and sink tissue.

Northern blots and expression studies of *OsSUT1* mRNA found highest transcripts in the filling grain, leaf sheath and stem (Furbank *et al.*, 2001) especially after heading (Hirose *et al.*, 1997). In situ hybridization of a *OsSUT1* probe (Matsukura *et al.*, 2000) and *GUS* expression of *OsSUT1* promoter::GUS expressing plant lines

(Scofield *et al*., 2007) indicates that *OsSUT1* expresses specifically in the plasma membranes of companion cells (CC) in phloem. In addition, OsSUT1 protein was found to localize to the aleurone, sub aleurone and nucellus of developing rice grains (Furbank *et al.,* 2001). Unexpectedly, given the patterns of *OsSUT1* expression, constitutive knockdown of *SUT1* in rice plants did not affect leaf sucrose, starch or photosynthesis (Ishimaru *et al.*, 2001). However, grain filling and grain weight decreased in anti-sense plants where *SUT1* transcripts in the panicle were significantly reduced compared to WT (Scofield *et al.*, 2002). These findings point to the involvement of *OsSUT1* in active energy dependent transport of sucrose into filial tissues in rice grain.

SUT1 is also implicated as an important transporter in germinating rice seed. *OsSUT1* transcription was detected in the scutellar vascular bundle, 2 -3 days after imbibition (Matsukura *et al.*, 2000; Scofield *et al.*, 2007b). This is not surprising as this conduit supplies remobilized sugars from the endosperm to the embryo. *SUT1* mRNA levels in germinating seeds were increased by endogenous sucrose and light, but repressed when starved of sucrose (Matsukura *et al.*, 2000). From results of glucose feeding experiments on seeds, Chen *et al.* (2010) suggested that various signalling pathways involving sugars, ABA and SUT1 regulate processes that promote or delay germination. These studies suggest that SUT1 is also involved in retrieval of apoplastic sugars to the embryo in germinating seed. Like seeds, pollen grains, are symplastically isolated from maternal tissue. *OsSUT1* and *OsSUT3* are preferentially expressed in pollen during flowering however their temporal patterns of expression are distinct (Hirose *et al.*, 2010). SUT1 mutants produce pollen that matures normally with normal accumulation of starch, but are impaired in germination possibly due to another role for SUTs in pollen tube elongation. Unfortunately, SUT3 mutants have not been successfully generated, therefore significance of SUT3 in pollen cannot be determined. It is possible that these two transporters act in tandem with differing roles for normal pollen development.

There is scant evidence for the role or function of the other four rice SUTs compared to what is known about OsSUT1. Various studies have found that *OsSUT2* and *OsSUT3* transcripts are localized to the internode tissue (Scofield *et al.*, 2007a) and to developing pollen grains and panicles before pollination (Takeda *et al.*, 2001). However, work by Eom *et al.* (2011) has characterized OsSUT2 as a tonoplast symporter through localization studies and functional expression in yeast. This overturns previous hypotheses that suggested sucrose transporters in the vacuolar membrane are antiporters. Eom *et al.* (2011) studied rice plants where a mutation

resulted in complete knockout of *SUT2* transcripts. They found that plants were retarded in all growth measurements for both vegetative and reproductive parameters. This research was the first to highlight the crucial role of OsSUT2 in rice. *OsSUT5* is expressed more highly in sink and source leaves rather than the panicle (Aoki *et al.*, 2003). Scofield *et al.* (2007a) also detected SUT protein in guard cells in the pedicel through epitope labelling, however they were not able to identify exactly which SUT was present. Therefore, despite numerous studies undertaken to understand the importance of SUTs in rice sucrose transport, there are many gaps in our knowledge that remain to be explored.

All the evidence presented above, specifically the similarities in protein sequence and high expression of *OsSUT1*, *HvSUT1* and all the *TaSUT1* genes in grains of rice, barley and wheat respectively, indicates that they play similar roles in sucrose transport into the grain. This does not however discount the role of other *SUT* genes in grain filling or additional roles of SUT1 in long distance sucrose transport in the phloem and carbon partitioning.

1.2.5 SUT overexpression in sink tissues

To date, there have been few reports on the effect of overexpressing sucrose transporters to investigate the effects of increased sucrose transport in sink tissues. A study by Rosche *et al.* (2002) introduced *StSUT1* from potato into the developing cotyledons of pea seeds to enhance the activity of the endogenous *SUT*s. They found that selective expression of *StSUT1* in cells that normally do not show high levels of *SUT* expression allowed a greater capacity for sucrose transport from the apoplast than would normally occur, thereby producing a stronger sink for sucrose influx. Sun *et al.* (2011) transformed potato plants by overexpressing two SUTs from rice under the control of the tuber specific patatin 1 promoter. Interestingly, plants overexpressing *OsSUT5Z* gave higher average tuber yields and had significantly higher levels of sugars and starch, while tuber yield of plants overexpressing *OsSUT2M* were lower than controls and tubers did not show significantly different levels in sugars or starch content. This unexpected result might be explained by the different functions that these proteins normally have in rice. For example these two rice genes have been previously identified and studied *in planta* by Aoki *et al.* (2003) and show differences in their temporal and spatial expression patterns, which implies that they may have different functions in sucrose transport.

The only study that has applied this strategy of overexpressing SUT protein to upregulate sucrose import into cereal grains was conducted by Weichert *et al.* (2010).

They fused the HvSUT1 cDNA to a Hordein B1 promoter, both from barley, and transformed wheat with *Agrobacterium* to direct expression of the sucrose transporter specifically in endosperm cells. This strategy was designed to increase the capacity of wheat grains to uptake sucrose. Similar to the study by Rosche *et al.* (2002), they chose to express this protein in cells that were not normally involved in sucrose uptake to create a driving force to pull more sucrose into the targeted cells, thereby creating a stronger sink for assimilate uptake. Interestingly, no difference in sucrose levels was observed in transgenic grains compared to controls during the entire monitoring period of grain development. However, there was a significant increase in both dry and fresh weight accumulation in the transgenic plants towards the end of grain maturation. Their findings indicated that the increase in sugar supply stimulated expression of genes for storage proteins and other genes that controlled metabolic processes, hormones and other signals. Their results were a substantial breakthrough to our understanding of how sucrose uptake could affect assimilate partitioning in cereals. Most significantly they appeared to be able to overcome the persistent drawback of a negative correlation between grain protein content and yield. More extensive field tests of these transgenic lines over several years have moderated this conclusion. Despite consistent significant increase in yield, lower sucrose and a trend towards lower protein and starch was observed (Saalbac *et al.*, 2014). Interestingly, there were also higher concentrations of micronutrients iron and zinc in the grain. The contradictory results between the two studies could partly be explained by the different environmental conditions the plants were exposed to. The variability in the data for different generations of the same plant line grown between different years supports this idea. Therefore further studies of these plants in the field would be required to unambiguously determine the effect of overexpressing HvSUT1 in wheat endosperm. These studies also raise obvious questions as to whether these findings are applicable to other cereal grains. Given the similarities in *SUT1* genes and SUT1 proteins amongst barley, wheat and rice yet the differences in nutrient pathways into the grain between rice and the other two cereals, it is somewhat unpredictable how rice will respond to SUT1 overexpression.

1.3 Carbon and nutrient partitioning

Carbon in the form of sugars is produced in photosynthetic tissues then transported to growing tissues such as roots and grains. The network of nutrient flow from source to sink tissues is complex and changes with the different stages in plant growth. Nutrient partitioning to the various sink organs reflects the changing needs of

the plant for sucrose and other assimilates as the plant develops. At the grain filling stage in cereal crops, the developing grain becomes a strong sink for nutrients, competing with other organs to deposit and store enough nutrients in the caryopsis to sustain the growth of the next generation. Therefore, carbon production and partitioning of the available nutrients is controlled by various networks within the plant which regulate this process, including through changing the expression of various proteins central to carbon partitioning, such as SUTs.

1.3.1 Sink organs and cereal grains as strong storage sinks

Plant organs that take up and utilize nutrients are defined as sinks. Examples include young developing leaves, roots and seeds. The measure of the ability for a sink to import nutrients has been described as sink strength. Doehlert (1993) defines sink strength as the rate of utilization of nutrients (such as sugars and amino acids) supplied to a sink (tissues that receive nutrients) from a source (where nutrients are formed) and measured by growth rate or dry matter accumulation. However, according to Ho (1988) this is a measure of the 'apparent sink strength' and not the most accurate measure of 'actual sink strength' because it does not take into account the net carbon lost through respiration. An alternative definition of; sink strength is the competitive ability of an organ to import assimilates (Herbers and Sonnewald, 1998). Sink strength is therefore limited by the size and the activity of the sink which are the physical and physiological constraints of the organ. These intrinsic features are a better measure of an organ's 'potential sink strength', according to Ho (1988), and are genetically determined and not affected by supply. Geiger and Shieh (1993) suggest that sink strength measurements provide an indication of important changes during different developmental stages of plant growth, therefore they present the idea that sink strength needs to be measured over a time course to understand specific processes.

Doehlert (1993) suggested that sinks were primarily limited by the supply of nutrients from source tissue, using an example of maize kernels grown *in vitro* that accumulated higher levels of proteins and starch due to the higher levels of nitrogen supplied in the media. However, this example *in vitro* does not fully examine the complexity of nutrient flow and competition between organs in the plant, as well as the metabolic processes that occur in the sink tissue. Developing fruits and seeds are particularly strong sinks that compete with each other for available assimilates, and partitioning of carbohydrates and other nutrients are a priority during this important stage of plant development (Ho, 1988). A review of the available literature on activities

of plant sources and sinks led Marcelis (1996) to conclude that there does not appear to be a direct cause and effect supporting the contribution of source strength to dry matter partitioning.

In wheat, Jenner and Rathjen (1977) found that sucrose levels continued to increase in developing grain, although the supply to the grain remained stable over the period of grain filling, which indicates that sink strength for sucrose rather than supply is a limiting factor of assimilate partitioning. To determine whether certain rice cultivars were source or sink limited, Asli *et al.* (2011) artificially thinned tillers and reduced the sizes of flag leaves in rice plants during grain filling. Their artificial manipulation studies showed that in some rice varieties, removing flag leaves or reducing the numbers of grain per panicle only resulted in a very modest increase in yield, suggesting that there is sink limitation to increasing grain yield. However, their study was conducted only over one season in the field and others have shown that nutrient levels in rice can vary enormously between years (Samonte *et al.*, 2006). Rice panicles typically contain a combination of spikelets that flower early and have superior cell division and grain filling rates compared to later flowering spikelets and therefore grains from these have been labelled superior and inferior respectively (Yang *et al*., 2006a). Artificially manipulating sink competition by removing grains on the same spikelet or supply by cutting source leaves did not affect the sucrose uptake in superior rice grains (Tang *et al.*, 2009). However, the same treatments significantly increased and decreased sucrose uptake and final grain weight in inferior grains, respectively. Thus the intrinsic qualities in seed that are genetically determined can also be a factor in determining sink strength.

Mechanisms of sucrose uptake are similar in developing storage sinks, although the structural morphology is significantly different between dicot seeds and monocot grains. Different developmental stages of a plant's growth determines the temporal and spatial allocation of nutrients to sinks that are often synthesized elsewhere in source tissue. The complexity of source sink relationships in plants can be highlighted with the example that plant organs can be both a source and sink for nutrients, depending on the stage of plant growth. For example, cereal stems are reversible storage sinks that remobilize stored nutrients back to the plant to be supplied to developing grain. This switching of source sink relationships is a complex one that is highly regulated by complex metabolic processes.

Grains undergo various stages of development during grain filling. After fertilization cell division then expansion commences, the latter overlapping with the storage phase when most of assimilates are accumulated. At this stage, the imperative

for nutrient storage in grains and seeds becomes irreversible (Patrick and Offler, 2001). The rate of nutrient loading is highest during this storage phase, although this rate reaches a steady state which is a factor of both the physical size of the sink and its physiological activity. During this phase, there is a switch from maternal to filial control of metabolism in the developing seed as the carbon and other nutrients taken up into the grain are converted into storage products (Borisjuk *et al.*, 2004; Zhang *et al.*, 2007). In rice, pollination occurs approximately 6 days after heading (DAH) (Furbank *et al.*, 2001). After fertilization, the developing caryopsis undergoes a short stage of elongation through cell differentiation (four days) followed by 12 days of expansion after which the maximum grain length has been attained (Hirose *et al.*, 2002b). This second stage of caryopsis development is also when maximum grain filling occurs. Solutes are supplied not only from photosynthesizing leaves but also from starch reserves remobilized from the rice stem (Yang *et al.*, 2001). Therefore, the critical stage determining the sink strength of rice grains appears to be from about 5 to 17 days after flowering which overlaps with maximum expression of OsSUT1 (Furbank *et al.,* 2001) and sucrose uptake in caryopses (Aoki *et al*., 2003).

1.3.2 Evidence for endosperm regulation of nutrient uptake

The mechanism by which sucrose unloads from the sieve elements into developing seeds has been comprehensively reviewed by Thorne (1985), Jenner *et al.*, (1991), Patrick and Offler (1995; 2001) and Zhang *et al.* (2007). A consideration of the evidence points towards the filial tissue as the main control centre that determines the seed's sucrose uptake capacity.

Subsequent transport of sucrose into the endosperm after uptake from the apoplasm is through PD, to be deposited within the endosperm and other storage tissues (Fig. 1.1). Endosperm cells are not usually associated with sucrose uptake, and this coincides with the lack of SUT expression in this tissue (Bagnall *et al.*, 2000; Furbank *et al.*, 2001). Rather, sucrose deposition appears to be a passive process where a high density of PD facilitates the movement of sucrose into storage cells in the endosperm (Patrick and Offler, 2001). Consequently, it appears that the rate limiting step is most likely to be at the aleurone or grain boundary where there is very high expression of SUTs (Bagnall *et al.*, 2000; Weschke *et al.*, 2000; Furbank *et al.*, 2001). In grains, the endosperm constitutes the majority of the tissue in the developing caryopsis and is thus a strong sink for sucrose. Most photosynthate loaded into endosperm are eventually converted into storage products (e.g. starch), to be released through

enzymatic processes to the embryo during germination. Hence, this sink has a major influence on the compartmentation of nutrients within the plant. Filial demand for nutrients and the influence on nutrient transport through metabolic, hormonal or signalling channels is not well understood. Moreover, the complexity of each mechanism and the strong likelihood that all of these are finely integrated to contribute to grain filling means that understanding or controlling the grain filling process is an extremely challenging endeavour.

1.3.3 Apoplastic regulation of sucrose uptake

Although the apoplastic fluid pool outside each cell is small (Ho and Gifford, 1984; Patrick and Offler, 2001), the sugar turnover rate is high (Fisher and Gifford, 1986). Therefore coordination of nutrient transport events involving the apoplast appears to exert a large influence on sucrose transport into the grain. The rate of removal of sucrose from the cytosolic free space appears to affect the import rate of sucrose into the cytoplasm (Gifford and Evans, 1981). At grain filling, increased sucrose metabolism by enzymes converting sucrose into storage products, increases sucrose flux into storage cells so that the apoplast is constantly undergoing changes in sucrose levels. This is thought to have an upstream signalling effect from maternal tissues to transport pathways and ultimately to source tissue to increase sucrose flow.

Feedback and feed-forward mechanisms that control grain development are highly sensitive to apoplast solute concentration and composition, so the apoplast is thought to provide some buffering function (Patrick and Offler, 2001). Additionally, solute concentration differences between the cytosol and apoplasm are relatively small (Patrick, 1997). Therefore minute changes in sugar concentration can either control local solute uptake into the cell, or may activate long distance signals back to the source tissues to indicate sucrose sufficiency or deficiency (see Lalonde *et al.*, 1999).

It has been suggested that low sink sucrose concentration can modify the sucrose transport activity by triggering a 'famine' response, resulting in increased sucrose uptake when sucrose concentrations were decreased *in vitro* (Patrick and Offler, 1995). Conversely, subsequent transport activity decreased in a concentration dependent fashion when detached beet leaves were exposed to high levels of sucrose (Chiou and Bush, 1998). Exogenous sucrose supply to rice embryos was also found to stimulate transport activity in germinating rice seeds (Matsukura *et al.*, 2000). However, the endogenous response to sucrose levels during grain filling has not been investigated in developing cereal grains. Also, whether this response is due to the effects on biosynthetic processes in storage tissues (Jenner *et al.*, 1991), or related to

modifications in expression of genes involved in carbon metabolism (Weber *et al.*, 1997) is difficult to examine due to the complex interplay of factors affecting sucrose transport into developing grains.

As well as sucrose concentration, other properties of the apoplast can also potentially influence sucrose uptake into cells. Several studies have examined the regulation of SUT activity by changes in pH and found that decreases in extracellular pH can increase substrate affinity of SUTs in most species including barley (Sivitz *et al*., 2005) and rice (Sun *et al*., 2010). Presumably, the extracellular pH can be controlled by the activity of the membrane bound proton pump associated with the SUT symporter. In addition, solute osmolality has been shown to exert a large influence on solute uptake in seeds (reviewed in Patrick and Offler, 1995). Although there is considerable evidence for this control in dicots, especially legumes, the same control may not exist in cereals. The transport pathway in cereal species is complicated by apoplastic barriers at the maternal/filial interface. To date, the influence of sucrose in the apoplastic space on sucrose uptake in grains is not well understood.

1.3.4 Sucrose signalling and gene expression

Vaughn *et al.* (2002) showed that SUT transcription was highly regulated in the phloem in response to sucrose levels, which suggests that increased sink demand for sucrose will increase photosynthetic activity at source tissue due to sucrose signalling, and vice versa. Shimada *et al.* (2004) generated transgenic rice plants with grains defective in starch synthesis, hence weaker sinks, however it was determined that sucrose concentrations in phloem sap were the same in both transgenic and control plants. This result was surprising because according to Vaughn *et al.* (2002) there should have been a corresponding decrease in phloem sap sucrose due to lower carbon demand in the sink. This implies that source and sink regulation through sugar signalling is probably not constitutive, and that signal transduction may have more local effects in sink uploading. Paul and Driscoll (1997) suggest that photosynthetic activity could be more responsive to the C:N balance, rather than directly from sugar signalling. Murchie *et al.* (2002) also suggest that N remobilized from starch reserves had a greater influence on carbon assimilation during grain filling than photosynthetic activity, which did not positively correlate with increased sink demand.

With the rice genome now sequenced, numerous studies have looked at gene expression profiles in rice during grain filling. The complex expression patterns observed suggest that grain filling is a highly coordinated process and that differences between low and high grain filling varieties can be due to differentially expressed genes

involved in sugar metabolism (Zhu *et al*, 2003; Liu *et al*, 2010; Teo *et al*, 2011). The understanding of source/sink relationships is further complicated by the idea that assimilate partitioning in some rice varieties may be source- while others are sink-limited (Asli *et al.*, 2011). Many genes covering a diverse range of functions are known to be affected by the availability of sucrose (reviewed in Thomas and Rodriguez, 1994; Koch, 1996 and Loreti *et al.*, 2001). In general, under low sucrose conditions, genes for photosynthesis and transport are activated, whereas high levels of sucrose induce genes for storage and metabolism.

Cells respond to internal and external sucrose concentrations, strongly suggesting there is some mechanism for sensing cytoplasmic and apoplasmic levels of sucrose. Several authors have suggested that SUTs may play a role in sugar sensing (Chiou and Bush, 1998; Lalonde *et al.*, 1999; Schulze *et al.*, 2000), however there has been no strong evidence presented to support this hypothesis. While it is not known exactly how plant cells 'sense' sugars, it is likely that responses to high or low sucrose levels are transduced through different pathways to alter gene expression (Herbers and Sonnewald, 1998). This is presumably through both a membrane bound and an internal sensor to detect external and internal sugar levels, respectively (Lalonde *et al.*, 1999). Therefore, rapid responses in adjacent plant cells in response to sugar supply is possible, whereas modification of activity at source tissue in response to cellular changes in sucrose concentration at sinks is likely to be delayed due to the time required for the source to respond to sink demand.

Roitsch (1999) suggests that gene expression is coordinated by sugars and stresses that stimulate cell responses through different regulatory channels. Wounding response to sap sucking insects such as aphids has been shown to increase *SUT1* expression in rice xylem in order to retrieve excess sucrose leaking out of the phloem (Ibraheem *et al.*, 2013). Regulation of *SUT* genes appear to differ in response to stress through interactions with transcription factors or hormones and regulatory regions in the 5' end of the genes (Ibraheem *et al.*, 2010). Salt stressed rice plants with lowered photosynthesis however, did not affect grain filling (Sultana *et al.*, 1999). Similarly, salt stressed soybean did not show any differences in seed development (Egli and Bruening, 2004). These findings support Roitsch's (1999) model and suggests that plants have mechanisms to divert sucrose to sink tissues when exposed to external stresses. In addition, Fernandes *et al.* (2008) found that although different environmental stresses elicit different transcriptional responses in maize, there is considerable 'cross talk' between metabolic signalling pathways that result in similar regulatory responses.

Rice, however, is sensitive to temperature stress and grain C and N content is adversely affected under high temperatures (Tashiro and Wardlaw, 1991).

Plants have a complex signalling network to respond not only to changes in their physical environment but also to the changes happening within the plant throughout its development. Sucrose, being the major component of the long distance transport pathway can exert a big influence on switching particular genes either on or off. As well as long distance communication to source tissue, there is also ample evidence to suggest that localised changes in sugar supply can affect the regulation of genes for sugar transport and metabolism. Adverse changes in sugar supply, particularly during grain filling can affect final nutrient content of grains. While plants can respond relatively quickly to overcome environmental stresses, under extreme environmental changes such as increased global warming, changes in the nutritional quality of grains can become a major issue in the future (Seneweera and Conroy 1997).

1.3.5 Starch and storage product synthesis in cereal grains

Coordinated control of grain filling through numerous pathways means that various storage products are controlled in a similar manner and could potentially be regulated by sucrose supply (Zhu *et al.*, 2003). Uptake of sugars into storage cells in the stems of sugarcane or into maize kernels is primarily in the form of hexoses formed by hydrolysis of sucrose by Invertases (INV), specifically Cell Wall Invertases (CWI) that are localized extracellularly in the apoplast. However, in other cereal monocots, sucrose is the primary sugar taken up into filial tissues in wheat (Jenner and Rathjen, 1977), barley (Thorne, 1985) and rice (Nakamura, 1989). Hirose *et al.* (2002b) showed that both sucrose and hexose (glucose and fructose) contents in rice grain were highest at approximately 3 DAA, coinciding with cell elongation but before grain filling begins. A similar pattern for CWI is seen in barley, with greatest activity of sucrose cleaving enzymes measured at early stages of grain development (Weschke *et al.*, 2003). The apoplastic sucrose/hexose ratio appears to change temporally, with a marked increase in sucrose as the grain begins the storage phase (Patrick and Offler, 2001; Weschke *et al.*, 2003) caused by a decline in extracellular INV levels (Weschke *et al.*, 2003). In rice, the activity of CWI in the maternal tissue is coordinated with sucrose transporter *OsSUT1* expression in aleurone and endosperm during early grain filling (Hirose *et al.*, 2002). Lim *et al.* (2006) reviewed the current literature on CWI and SUTs in cereals to emphasize the many associations between this enzyme and SUT activity which supports its role in regulating carbon metabolism.

Although a small pool of soluble sugars is present at maturity, a majority of the sucrose is converted to starch as the main storage product in the endosperm. The highest rate of starch biosynthesis is between 1 and 12 DAA, although only the starch synthesized after 4 days is thought to contribute to storage in the endosperm (Perez *et al.*, 1975). Sucrose synthase (Susy), ADP-Glucose Pyrophosphorylase (AGPase) and soluble starch synthase (Sss) catalyse the three main steps in the sucrose to starch pathway in cereal endosperm. Susy cleaves sucrose into hexoses which are used in the production of ADP-glucose by AGPase in the cytoplasm. From there, the ADP-glucose is transported into amyloplasts for conversion into starch by one of three Sss isoforms (SsI. SsIIa and SsIIIa). The starch complex is made up of linear chains of glucose molecules (amylose) while the activity of starch branching enzymes (SBE) hydrolyse 1,4-glycosidic bonds, creating 1,6 bonds which form branched molecules (amylopectin). The proportion of these two types of starch is an important determinant of the physical, nutritional and cooking qualities in rice and other starches.

Activities of Susy and AGPase were shown to be higher and also to peak earlier in superior versus inferior rice grains (Liang *et al.*, 2001). Increasing sucrose supply up to a point in detached rice ear cultures resulted in increased activities of Susy, AGPase and Sss (Sasaki *et al.*, 2005). In addition, a number of other studies also found that Susy activity was positively correlated with grain filling and final grain weight in rice (Zhao *et al.*, 2006; Tang *et al.*, 2009). Constitutive Susy expression in maize increased ADP-gluc and starch content significantly in endosperm (Li *et al.*, 2013). A barley mutant deficient in Sss, made by disrupting the *SsIIa* gene showed severely reduced starch content and shrivelled grains (Clarke *et al.*, 2008). In contrast, SsI deficient mutant rice lines showed no noticeable phenotype (Fujita *et al.*, 2006). On the other hand, upregulating cytosolic AGPase in rice seed did not increase the starch content as significantly as the intermediate metabolites in the starch synthesis pathway (e.g. ADP-gluc) (Nagai *et al.*, 2009). These results indicate that coordination of the various enzymes are important for proper starch biosynthesis and suggest that the processes within the amyloplast in endosperm cells may be the rate limiting step for starch synthesis in cereal grains.

A proportion of the carbon supplied to the rice seed is channelled into production of protein. Protein composition largely determines the amino acid profile of the grain. For example, the main storage protein in rice is globulin while wheat and barley are prolamin rich (Kawakatsu *et al.*, 2010a). Within the grain, hexoses derived from metabolism of sucrose are also channelled into lipid production. Lipids play a role in rice quality and have significant health benefits to humans. In the grain, lipids are

components of organelles and storage lipids are broken down and mobilised to the germinating rice seed (He *et al*., 2011). A more detailed discussion of the various classes of storage proteins, amino acids and lipids in cereals grains can be found in the introduction to Chapter 5.

1.4 Plant mineral nutrition and biofortification

Plants require a range of mineral nutrients to grow and thrive. Similarly humans require a range of comparable nutrients, which are acquired through consuming a balanced diet including edible crop plants. A deficiency of these essential minerals is especially prevalent in the diets of people living in developing countries that have a high dependency on low nutrient staples such as rice. The resulting human health effects from mineral malnutrition or 'hidden hunger' have been well documented (Welch and Graham, 2002).

The range of strategies utilised to increase the quantity and/or quality of mineral elements in edible food crops has been grouped under the term 'biofortification'. Traditional methods of plant breeding by selecting lines with higher grain micronutrient content has been moderately successful in producing more nutritious crops (Welch and Graham, 2002). Exploiting the genetic plasticity of crop plants through more targeted means using molecular marker-assisted breeding and plant tissue culture techniques is another approach. More direct strategies for biofortification such as induction of mutations through chemicals or radiation have been used in developing new crop varieties with beneficial characteristics for human nutrition (Clarke *et al.*, 2008). Surprisingly, there does not seem to be any public resistance to accepting mutation breeding of food crops, however, use of genetic modification (GM) of crops is still opposed vehemently by the public at large. This is despite the wide range of benefits afforded by the use of this technology that has now been available for decades (Bouis *et al.*, 2003). GM is a targeted means to manipulate specific plant characteristics. Quantitative strategies aim to increase the concentration of an important limiting nutrient, such as zinc, iron or β-carotene. Qualitative strategies include knockdown of undesirable anti-nutrients, such as phytate and polyphenols or to increase promotional substances that aid in the absorption of minerals, such as some amino acids, inulin and prebiotics.

Thorough reviews covering the range of biofortification approaches include White and Broadley (2009), Waters and Sankaran (2011), Murgia *et al.* (2012) and Bashir *et al.* (2013). In biofortification strategies, the relationship between anti-nutrients and micronutrients is a complex issue which encompasses plant and human

physiology as well as cultural aspects and is too complicated to be comprehensively covered in the scope of this review. For detailed discussions on this topic, see House (1999), Brinch-Pederson *et al.* (2007) and Borg *et al.* (2009).

1.4.1 Importance of minerals in human health

Iron (Fe) and zinc (Zn) are amongst the most deficient elements in a staple human diet and are also important in plant nutrition. However, even the more abundant minerals such as Calcium (Ca), Magnesium (Mg) and Potassium (K) can be limiting in human nutrition but are also critical components in plant processes.

Human Fe deficiency is prevalent worldwide, and can lead to anaemia, morbidity and death. The World Health Organisation (WHO) report on the incidence of anaemia between 1993 and 2005 estimated that more than 1.62 billion people were affected (Benoist *et al.*, 2008). Zn is particularly important in a range of cellular processes in humans, so Zn deficiencies can have negative consequences for adults and children including lowered immune systems, premature delivery in pregnant women, childhood diarrhoea and stunting in infants and children. Ca deficiency and its importance for bone health is well recognised in more developed parts of the world however Ca has important roles in many other processes in the body that are less publicised and can contribute to various health issues in underdeveloped countries (FAO & WHO, 2005). Mg and K are the two most abundant intracellular cations in the human body, with Mg being a critical component in many enzymatic cellular processes. Mg also regulates the Sodium (Na)-K pump in animal cells which is impaired under Mg deficiency, which can lead to an imbalance of both Mg and K in humans (Rude, 1989).

Correction of inadequate nutritional intake of micronutrients through increasing consumption of higher nutrient dense foods such as meat in the diets of those affected by micronutrient malnutrition is not an option as those that are most affected do not have access to these types of food. Therefore, increasing micronutrients, such as Fe and Zn in staple crops is a more practical endeavour and is of paramount importance.

1.4.2 Mineral transport in plants and distribution in cereal grain

For effective biofortification of cereal grains, minerals must be taken up from the soil and then transported to the grain, either directly or sequestered into vegetative tissue for later partitioning into the grain under nutrient limitation (Waters and Sankaran, 2011). Mineral ions have different mobility in plant transport pathways and

the route these nutrients take into the grain differs. Although both the xylem and phloem contribute to long distance movement of Fe (Guerinot, 2010; Thomine & Lanquar, 2011) and Zn (Rengel, 2002), uptake into grains is largely phloem dependent. The following discussion will therefore focus on the phloem pathway of micronutrients.

To understand how minerals can be increased in plant tissues, the transport and movement of these micronutrients through the phloem and the barriers that stop their accumulation in edible tissues must first be understood. Grain architecture and structure are completely different between rice and barley/wheat, hence there are major differences seen in the ability of these grains to accumulate different nutrients. For example, Zn content in rice endosperm can be quite high when compared to that found in wheat and barley endosperm (Choi *et al*., 2007). Whether this is related to the pathway of solutes into the grain (circumferential flow in rice versus through the crease in wheat/barley) or the xylem discontinuity that exists in wheat/barley, is unclear. The significance of these differences and the correlation with mineral deposition between the cereals has not been elucidated but it can be hypothesized to play an important role in the distribution of minerals in the grain.

Micronutrients can be transported as chelated compounds or as components of other phloem mobile species that are transported in plant sap. Fe is highly reactive and insoluble under the alkaline conditions of the phloem, and therefore it must be bound to carrier compounds for transport through the plant (Haydon and Cobbett, 2007). In addition, once Fe is deposited into sink tissues, it must be bound or sequestered to maintain homeostasis in the cells. This is in contrast to Zn which is relatively phloem mobile (Kobayashi and Nishizawa, 2008) and is an essential component of many enzymes and transcription factors, hence its movement and transport is likely to be closely linked to the expression and movement of these proteins. Could the reactivity and the ion complexes affect their transport into the grain? Can we hypothesize that because Zn is less reactive than Fe, the smaller complexes or free Zn are able to be transported much more easily, hence the higher levels of Zn than Fe found in endosperm? However, Zn and Fe are both present in relatively low concentrations compared to macronutrients and sugars. Furthermore, Zn and Fe concentrations in cereal grains are positively correlated (Zhao *et al.,* 2009), which implies that their levels are similarly regulated, a trait that has been exploited in traditional plant breeding (Ghandilyan *et al*, 2006). Since sucrose makes up the bulk of phloem sap, it can be hypothesized that the movement of this transport sugar could potentially have a great influence on the movement of these micronutrients into sink tissues.

It is thought that transporters such as Mugineic acids (MA), Nicotianamine (NA), oligopeptides and amino acids bind to and carry reactive ion species in the phloem. Transmembrane transporters such as yellow stripe-like (YSL) (Curie *et al.*, 2001) and ZIP-like (Hell and Stephan, 2003) proteins then mobilise them into cells or move them across cell layers. These transporters are involved in the entire transport pathway that takes up micronutrients from the soil, delivering them from the roots to the shoots, and finally depositing them into sink tissues (Rengel, 2002; Kobayashi and Nishizawa, 2008).

Borg *et al.* (2009) and Tauris *et al.* (2009) have generated thorough models of Zn and Fe trafficking from phloem to grain which involve a number of transporters and proteins (Fig 1.5). The hypothesized pathways show striking similarities and involve some of the same transport proteins. In brief, vacuolar sequestration and release of both Fe/Zn is mediated by VIT1 and NRAMP proteins respectively, and once released into the cell cytosol, MA/NA chelate Fe/Zn. These chelated complexes are taken up into the endosperm and developing embryo via YSL and ZIP's located in the plasma membrane of the modified aleurone. Within the filial tissue, Zn and Fe is transported into storage vacuoles and sequestered as phytate complexes or with ferritin in the starchy endosperm (Brinch-Pederson *et al.*, 2007) or may be bound to proteins (Ozturk *et al.*, 2006).

Of the other mineral nutrients, phosphorus (P) is mainly translocated in the phloem as inorganic phosphate (Pi) (Bieleski, 1969), taken up into cells through Pi transporters (Smith *et al.*, 2003) and stored in grains primarily as phytate. Sulphur (S) is transported as sulphate (SO_4^{2-}), before undergoing enzymatic processes that results in the assimilation of S into amino acids such as cysteine or methionine (Saito, 2000). A high concentration of Mn is found in the phloem, however it had previously been thought that Mn was transported primarily via the xylem (Pearson *et al*., 1994; Rengel, 2002). However, knockdown of *OsYSL2*, a Fe-NA and Mn-NA long distance phloem transporter in rice resulted in decreased grain Mn which suggests that it is phloem mobile, at least in rice (Ishimaru *et al.* 2010).

Distribution of metal ions in grain tissue layers has been examined using histological and imaging techniques. For visualization of Fe in rice, Choi *et al.* (2007) stained grains with Perl's Prussian blue (PPB) which revealed that the blue staining correlated with Fe density of different rice genotypes, and was mostly localized to the embryo. The red colour from staining wheat grains with Diphenyl thiocarbazone (DTZ) howed that the distribution of Zn was mainly in the embryo and aleurone, and was most intense during early grain filling (Ozturk *et al.*, 2006). It was also possible to use

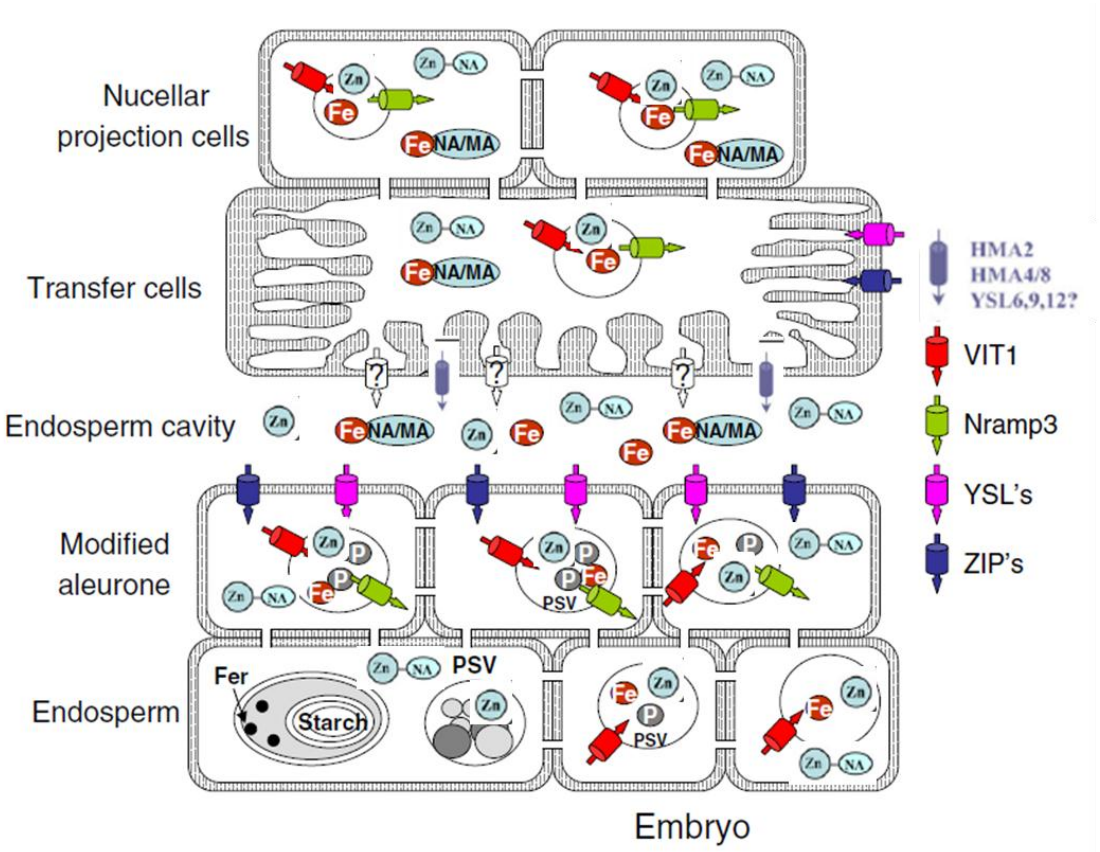


Figure 1.5 Combined models for Fe and Zn transport into barley grains

This diagram shows the some of the putative transporters and chelators involved in moving Fe and Zn ions from the outer layers of the grain into the endosperm for storage. NA, nicotianimine, MA, mugineic acid; PSV, protein storage vacuole, Fer, ferritin, VIT1, vacuolar ion transporter; Nramp3, natural resistance associated macrophage protein; YSL's, yellow stripe like proteins; ZIP, zinc/iron regulated transporter protein. Fe/Zn have been put into the same PSV in the diagram for convenience, however may not be associated in the same storage vacuoles as depicted. Diagram is modified from Borg *et al.* (2009) and Tauris *et al.* (2009).

this method for semi-quantitative determination of Zn dense wheat genotypes (Choi *et al.*, 2007). Although these staining techniques are useful for high throughput and require little specialized equipment, they are not very sensitive. For example, wheat grains with less than 15 mg kg^{-1} Zn show no colour development with DTZ (Ozturk *et al.*, 2006). Also, there is a lack of fine detail in the various tissue layers, other important elements are omitted and it is difficult to examine the distribution of different elements in the same grain.

Imaging techniques are now available that can detect low concentrations of multiple elements in sectioned cereal grains *in situ*. Digital images can be superimposed to generate fine image maps of the distribution of multiple elements in the various grain layers. Techniques for imaging mineral distribution in cereal grains include Synchrotron x-ray fluorescence spectroscopy/microscopy (μ-XRF/S-XRF or XFM), secondary ion mass spectrometry (NanoSIMS), Laser ablation inductively coupled mass spectrometry (LA ICP-MS) (reviewed in Zhao *et al.*, 2014) and Particle induced X-ray emission (micro-PIXE). In barley and rice, XRF images showed that Fe is found mostly in the embryo, scutellum and outer aleurone with no distribution in the starchy endosperm in mature (Lombi *et al.*, 2009, 2011; Kyriacou *et al.*, 2014) and germinating (Takahashi *et al.*, 2009) grains. NanoSIMS imaging of Fe in wheat found Fe distributed in the aleurone and that it had subcellular associations with phytate (Moore *et al.*, 2012). Zn is found throughout the endosperm of rice as well as associating with Fe and Mn in the scutellum and embryo (Lombi *et al.*, 2009; Johnson *et al.*, 2011; Kyriacou *et al.*, 2014). In contrast, barley grains have the most Zn in the embryo and crease tissue (seen longitudinally and in cross section), with very little penetrating into the inner endosperm (Lombi *et al.*, 2011). Other nutrient elements such as Mn, K, P and S were imaged in wheat grain cross sections using LA-ICP-MS (Wu *et al.*, 2013) and have similar patterns of elemental distribution in outer aleurone compared to longitudinal sections of rice (Lombi *et al.*, 2009; Kyricaou *et al.*, 2014). Micro-PIXE analysis of rice showed uniform distribution of Zn and Cu. This was confirmed, with ICP-OES analysis showing very little decreased in concentration even after 20% of the outer layers had been removed by milling. Mn and Fe, however, were more concentrated on the surface of the grain (<200 μM thick) and as a consequence, 20% milling resulted in loss of most of these two micronutrients (Ogiyama *et al.*, 2008).

The strikingly different distribution of Zn and Fe, especially in rice, implies that these ions are potentially regulated by different transport process and that one of the barriers for Zn transport could be located in the crease which is absent in rice. The distribution of mineral nutrients in cereal grains correlates with the distribution of

phytate (Iwai *et al.*, 2012) and protein bodies (Prom-u-thai *et al.*, 2008) primarily in the embryo and aleurone. Low levels of micronutrients in endosperm could also be related to the low abundance of transcripts for genes in mineral transport and storage in this tissue (Tauris *et al.*, 2009). The sensitivity of these techniques has allowed mapping of even less abundant trace nutrients such as Selenium (Se) (Moore *et al.*, 2010) and toxic metals that are contaminants in crop species, such as Arsenic (As) and Cadmium (Cd) (Lombi *et al.*, 2009; Moore *et al.*, 2010). In addition, μ-XRF can be used in tandem with μ-Xray absorption near edge spectroscopy (μ-XANES) to show distribution and speciation of As in rice grains *in situ* (Meharg, *et al.* 2008; Lombi *et al.*, 2009).

Despite similarities in the transport processes that move mineral nutrients from the plant vasculature into grain, it is clear that minerals are not distributed uniformly in grain tissues. The ability to visualize the location in the grain where each mineral is found can provide some clues as to the important barriers or limitations to increasing grain mineral nutrient content.

1.4.3 Remobilization from source to sink

Zhang *et al.* (2012a) examined the effect of source sink manipulation on mineral nutrients in wheat and found that increasing sucrose supply through solution culture actually decreased the mineral concentration in the grains. They also used source removal through defoliation to examine this relationship. More current techniques, however, using genetic manipulation and targeting of genes from the source or sink can examine the relationship between sucrose flux and nutrient deposition more directly in sink tissues. In Zhang *et al.*'s (2010) experiments, they manipulated the source while the sink capacity remained the same; this resulted in a negative feedback and a decrease in the uptake of minerals. This indicates that sink capacity may be a better target for manipulation.

Assimilate remobilization from flag leaf to grain during senescence is tightly controlled through various signalling pathways that are under hormonal control (Biswas *et al.*, 1986). Removal of rice panicles resulted in delayed senescence and increased accumulation of sugars and starch in the flag leaf (Nakano *et al.*, 1995). Sugars that are not stored or utilized in growth, decline in levels in rice culms and sheaths between 7 and 21 DAA (Yang *et al.*, 2002). Whether remobilization of mineral nutrients, like sugars, from vegetative tissues during maturity and senescence to developing grains contributes to final mineral concentration is contradictory between studies. Wu *et al.* (2010) found that decreases in leaf, stem and root Zn correlated with increases in grain Zn during important stages in rice development. Yoneyama *et al.*

(2010) also found that Zn decreased significantly in flag and upper leaves in later plant maturity but Fe concentrations remained relatively high with little remobilization to the grain. However, Sperotto (2013) found that removing the flag leaf did not affect Fe, Zn or Mn in rice grains. Based on this evidence, other tissues must also contribute to the temporary storage and remobilization of mineral nutrients to the grain. For example, Pearson and Rengel (1994) found that Zn and Mn accumulated in the glumes (palea and lemma) of wheat for later retranslocation into the grain. In addition, continuing supply from roots could contribute to grain mineral nutrient concentration, although Wu *et al.* (2010) found that of the zinc supplied to grain came from other plant parts and not directly from the roots.

Numerous studies have shown that Zn is transported from source to sink tissues in the phloem (Pearson and Rengel, 1994; Pearson *et al.*, 1996a). Although sucrose and Zn mainly enter wheat grains through the phloem, the endosperm seems to be a stronger sink for sucrose compared to Zn (Pearson *et al.*, 1996b). It has been theorized that sulphur containing compounds that bind Zn may be responsible for increased levels of Zn in grain, however Stomph *et al.* (2011) found no correlation between presence of S and Zn in wheat grains, which implies that sulphur containing compounds have negligible effect on total Zn content. This correlates with the low levels of sulphur containing amino acids in rice phloem sap, which together with Stomph's (2011) findings suggest that grain zinc levels are likely affected by other sink related factors. Levels of micronutrients in endosperm are generally low compared with their levels in the embryo and surrounding maternal layers such as the pericarp. Additionally, during grain filling there is an excess accumulation of other micronutrients such as Fe and Mn as well as Zn in the crease as a result of their slow rate of unloading from the phloem (Stomph *et al.*, 2011). Clearly, there is some barrier to the movement of more micronutrients into the endosperm, which further supports the hypothesis that there is a sink limitation affecting the rate of Zn unloading.

Many membrane transporters are coupled to proton pumps, such as H^+ATPases that supply the protons required for proton coupled co-transport, symport or antiport, of diverse molecules such as sugars (Hirose *et al.*, 1997), sulphates (Saito, 2000) and phosphates (Smith *et al.*, 2003). The coordinated action of these membrane proteins would be expected to be tightly controlled and excess of a particular nutrient could potentially have wide ranging effects on the transport of other nutrient species.

1.4.4 Effect of nitrogen status on mineral transport

Traditional methods of supplying N to cereal crops through the roots with or without foliar application of Zn have been used to examine the effectiveness of increasing N to biofortify cereal grain iron and zinc content. Under experimental conditions, increasing N supply to plants through the roots has been shown to increase micronutrient uptake and transport in vegetative tissues of wheat (Erenoglu *et al.*, 2011). In combination with Zn foliar application this can give favourable increases in Fe and Zn in whole grains and in the edible fractions of wheat grain (Kutman *et al.*, 2011). This increase in grain micronutrient level under high N fertilization, and Zn foliar application has also been observed under field conditions (Cakmak *et al.*, 2010). However, these observations for wheat were not mirrored in rice, where increasing N application did not consistently increase micronutrients in the edible portion of the grain (Zhang *et al.*, 2008). Fe levels, in particular, remained low in brown rice although over the whole plant the Fe levels were observed to increase. Because Fe is present in such low concentrations in phloem sap, Fe must therefore rely on the movement of other assimilates, such as sucrose, to be carried and deposited in the sink tissues. Therefore, modification of the assimilation pathway, especially during critical grain filling stages for sucrose could very likely enhance the deposition of micronutrients specifically in grain tissue.

As well as increasing the overall nutrient status of the plant, N application can also result in increasing the concentration of seed storage proteins (SSP) in the grain. There appears to be a direct relationship between N supply to the plant and grain protein content (Jenner *et al.*, 1991). Although the processes of starch and protein deposition in grain are independent of each other, they are invariably linked as the size and physiology of the grain limits the proportion of these products. Traditionally, plant breeding has not been able to overcome the negative correlation between yield and protein content in cereal grains, despite there being no obvious negative correlations between the genetic regions that determine these traits, at least in bread wheat (Groos *et al.*, 2002). Proteins make up only a small percentage of the storage product in cereal grains however proportions of the different protein classes are integral in grain quality characteristics and are an important source of plant protein in human nutrition. Although protein concentration is quite variable between cereal genotypes, in general rice contains much less total protein compared to wheat or barley. In addition, the composition of SSP, hence the abundance of various essential amino acids, in rice is quite different from these other cereals.

Disruption of N remobilization from flag leaves to wheat grains significantly reduced grain protein and Zn and Fe deposition (Uauy *et al.*, 2006b). These traits appear to be linked which suggests that manipulation of grain protein can potentially give a corresponding increase or decrease in micronutrient content. Under drought conditions, Peleg *et al.* (2008) found protein content was highly correlated with Zn and Fe for field grown wheat populations. Post-flowering foliar Zn application to wheat increased both protein and Zn, which led Cakmak *et al.* (2010) to speculate that increased protein synthesis through N fertilization at grain filling can create a stronger sink for Zn.

1.4.5 General strategies for GM biofortification of cereals

Rice is processed for consumption by polishing off the outer bran layers to produce white rice. This increases the storage period of rice, and there is a cultural and historic preference for white rice. Removing the bran, however, significantly lowers the nutritional content of rice. Therefore, biofortification of rice is only effective if there can be an increase in nutritional properties of the endosperm, rather than in the aleurone or embryo of the grain. Untargeted expression of transgenes to drive increase of micronutrients in rice grains may not be successful if the redistribution of minerals is not penetrating to the inner endosperm of the grain. Zhang *et al* (2012) disrupted the genes VIT1 and VIT2, vacuolar metal transporters, in rice and found reallocation of Fe to the embryo resulted from reduced accumulation in leaves. This experiment shows that manipulation of sink capacity can cause a reallocation of nutrients in the plant, however as the embryo is removed during polishing, this particular strategy cannot be applied for biofortification. Numerous strategies to increase micronutrient levels in cereal grains have attempted to use the idea that increasing sink strength can be used to 'pull' more of a particular micronutrient into the grain by creating a stronger sink for nutrients. These 'pulling' strategies have relied on the overexpression of various proteins to bind or transport more micronutrients in the grain.

Ferritin, an iron-binding protein, has been investigated in several studies as a means to increase cereal iron content by overexpressing ferritin to sequester more iron. Constitutive expression of soybean ferritin resulted in higher levels of iron only in the vegetative tissues, at the expense of the rice and wheat grain (Drakakaki *et al.*, 2005). Targeted overexpression of ferritin in rice endosperm gave more promising results with an increase in ferritin levels corresponding to an increase in iron content in the grain (Goto *et al.*, 1999; Vasconcelos *et al.*, 2003; Qu *et al.*, 2005). In addition, in some cases, lines with higher Fe had corresponding increases in grain Zn content

(Vasconcelos *et al.*, 2003; Qu *et al.*, 2005). However, there were only modest increases in micronutrient levels that did not correspond with much higher ferritin levels, with the added drawback that plants with higher grain Fe showed more than 50% less iron in the leaves, compared to controls (Qu *et al.*, 2005). These studies indicate that although increased ferritin in the grain provided a stronger sink to bind more iron, transport of iron into the cells may be the limiting step. This has led other researchers to investigate iron and zinc uptake and transport into grains by manipulating other proteins that have been identified as transporters for micronutrients.

OsNAS1, *2* and *3*, a family of genes for proteins involved in the production of nicotianimine (NA), a metal chelator in rice, is induced in roots and upregulated under soil Fe deficiency (Lee *et al.*, 2009). Activation of *OsNAS3* increased levels of NA, Fe and Zn in rice grains (Lee *et al.*, 2009). Similarly, barley *HvNAS1* driven by a constitutive rice *Actin* promoter gave moderate increases in Fe and Zn in polished rice of T_1 and T_2 plants (Masuda *et al.*, 2009). However, PPB staining showed that although some Fe was visible in the endosperm, most of the staining was observed only around the periphery of the grain. Johnson *et al.* (2011) generated three populations of transgenic rice overexpressing the three *OsNAS* genes with a *double 35S* promoter. They found that lines overexpressing *OsNAS2* gave the highest increases in NA, which correlated with increased Fe and Zn concentrations. However, μ-XRF elemental maps showed that Fe was still only distributed mostly in the aleurone and subaleurone. Constitutive overexpression of *ZIP4*, a putative Fe and Zn translocator, in rice increased Zn in roots but decreased Zn concentration in both shoots and seeds, which suggests that instead of increasing the overall micronutrient content of the plant, the available Zn was redistributed unevenly to specific plant parts (Ishimaru *et al.*, 2007a). Expressing multiple genes in tandem has been an approach employed by various research groups to increase metal uptake and storage in the plant while also introducing genes that can enhance nutrient absorption. Lucca *et al.* (2001) overexpressed a chimeric construct with genes for Fe storage (*ferritin*), break-down of anti-nutrients (phytase) and cysteine for increasing the absorption of non-haem iron in rice, however they did not report whether the Fe was more bioavailable. Wirth *et al.* (2009), and Wang *et al.* (2013) created rice expressing multiple genes involved in storage (*ferritin*), transport (*NAS*) and break-down of anti-nutrients (phytase) to increase Fe concentration by up to six times in rice grain. Corresponding increases in Fe resulted in increases of non-target endogenous expression of other genes critical in mineral nutrient transport (Wang *et al.*, 2013). Masuda *et al.* (2012) tried a similar gene stacking approach with soybean *ferritin*, *HvNAS1* and *OsYSL2*, a Yellow stripe-like (YSL) metal transporter, to

increase Fe and Zn in field grown rice by 4.4 and 1.6 fold respectively. In another study by Ishimaru *et al.* (2010) overexpression of *OsYSL2* driven by the *OsSUT1* promoter for high expression in phloem cells resulted in Fe and Mn content in polished rice that was approximately 4 and 1.5 times higher than WT, respectively. VIT1 and VIT2, vacuolar metal transporters have also been investigated as a targets for manipulation as they can drive greater uptake of metals into the storage vacuole. Unexpectedly, Zhang *et al.* (2012) measured elevated levels of cadmium (Cd) of up to 60% more in rice grains overexpressing VIT1 and VIT2 although these proteins did not transport Cd *in vitro* in yeast cells. NRAMP5, another metal transporter has been reported to transport not only Fe and Mn but also Cd (Ishimaru *et al.*, 2012). S-XRF studies of the distribution of As, Cd and Nickel (Ni) in rice from various countries revealed that while As had a similar distribution in grain as Fe and Zn, Cd and Ni were found throughout the endosperm (Meharg *et al.*, 2008). If plants engineered with enhanced metal transporters for Fe and Zn were to be grown on contaminated soils, there could be a risk of increasing the toxic heavy metal load in edible tissues.

The examples above highlight that caution needs to be taken when manipulating metal transporters as we do not know enough about the specificity of many metal transporters *in planta*. Proteins in heterologous systems may not behave the same or show the same specificity as *in planta*, hence results may be unexpected when proteins are overexpressed in plants. However, it can be argued that the benefits of this technology outweigh the risks. Despite all the benefits of using GM for biofortification and having systems in place to ensure the transparent reporting of results from such research, it will be a long time before biofortified rice will be accepted widely by the public. Until public opinion can be changed, there is little likelihood of the widespread acceptance and commercialization of such crops, although there is no doubt amongst plant scientists that there is an urgent need for this research.

1.5 Aims of this work

Weichert *et al.* (2010) demonstrated that overexpressing *HvSUT1* in wheat driven by an endosperm specific promoter could increase sucrose uptake capacity. This change in sucrose supply to the grain resulted in a cascade of effects that were primarily changes in grain metabolism. As a result, transgenic wheat grains overexpressing *HvSUT1* showed changes in their protein profiles and increased protein content without a yield penalty. Increased sink strength as well as higher protein in grains has been shown to correlate positively with micronutrient levels. Preliminary ICP-OES analysis on these wheat grains indicated that they were indeed higher in

micronutrients (James Stangoulis, pers. comm.). Based on that study's findings, we hypothesised that the strategy of increasing sucrose uptake through SUT1 manipulation could be used to increase micronutrients in rice, another important crop species. Further work on T_3 to T_5 generations of field grown *HvSUT1* overexpressing wheat found higher zinc and iron in those grains compared to controls (Saalbac *et al.*, 2014). This study lends more support to this project's primary objective which is to explore the effect of increasing the uptake capacity of the rice grain through ectopic SUT1 expression on the deposition of micronutrients.

In this study, rice plants will be transformed using *Agrobacterium* to integrate a construct encoding the barley *HvSUT1* cDNA driven by the rice globulin endosperm specific promoter, *Glb-1*, targeting expression of *SUT1* in the endosperm. Null segregants (NT) from these transgenic lines and plants transformed with constructs to constitutively drive *HvSUT1* with the rice *Act-1* promoter will be used as controls. Transgenic rice lines will be characterised for transgene copy number, *HvSUT1* gene and protein expression and sucrose uptake using *in vitro* assays. In contrast to the study with wheat, measurable changes will be analysed in polished rice rather than only in the whole seed, as this has implications from a human nutrition perspective. In order to test the hypothesis, polished and unpolished rice grains from selected plant lines will be analysed for protein and micronutrients. Timing of nutrient transport may play a part in final grain micronutrient concentrations, therefore grains at different time points during development will also be analysed. To visualize the distribution of minerals in the grain, elemental maps will be generated from mature control and transgenic grains and using Laser ablation ICP-MS.

The studies by Weichert *et al.* (2011) and Saalbac *et al.* (2014) on *SUT1* overexpression in wheat provide a platform to explore the loading and storage of other nutrients in cereal grains. Metabolic changes within the rice grain affected by sugar supply can also be investigated by measuring the concentration and composition of other metabolites and nutrients. In addition to the main hypothesis, another objective of this thesis is to examine whether endosperm specific expression of *SUT1* could alter partitioning of carbon from the primary storage product starch, into other storage products such as proteins or lipids, thereby changing the nutritional composition of rice. As well as measuring growth and yield parameters of transgenic plants, rice grains will also be analysed for nutritional changes in sugars and starch, storage proteins, amino acids and fatty acids. The overall aim of this thesis is to investigate increasing sucrose transport in an important cereal crop, rice, as a potential biofortification strategy. This project attempts to do this using a transgenic approach

and the results from this study will provide points for comparison with previous work conducted on wheat by Weichert *et al.* (2010) and Saalbac *et al.* (2014).

The following work will be broken into chapters describing distinct yet related aspects of the research (i) the generation and characterisation of the transgenic plants, (ii) the analysis of micronutrient deposition and localisation the transgenic rice grains, (iii) the analysis of growth and yield characteristics in transgenic plants as well as lipid and protein changes in transgenic rice grains.

Transformation and characterisation of rice plants overexpressing the *HvSUT1* transgene

2.1 Introduction

Over the last few decades there have been many advances in the transformation and regeneration of transgenic monocot cereal species for the purposes of research. Rice in particular has received a great deal of attention as a model plant for transgenic studies of cereals due to its relatively small genome size (e.g. approximately 3.89 x 10^8 bp compared to barley's 5.3 x 10^9 bp) and the completion of the International Rice Genome Sequencing Project in 2004 (IRGSP, 2005). Research into transformation systems for rice has resulted in protocols for efficient and reliable regeneration of transgenic rice plants however transgenic rice has previously never been developed successfully at Flinders University. Therefore the final protocol for this study was based on trial and error and consideration of many factors investigated in published studies on rice transformation, rather than relying on empirical evidence from previous successes on site.

A number of factors need to be taken into account when transforming rice ensure success in integrating a desired transgene. In addition to the important factors in cereal transformation as reviewed in Cheng *et al.* (2004), the study presented here required additional considerations: (i) the choice of promoters for the desired expression of the transgene, (ii) the method of gene delivery, (iii) the choice of cloning vectors, (iv) the tissue culture conditions for growth and regeneration of transgenic plants and, (v) the ideal growing conditions to ensure strong vegetative growth and optimal seed setting for multigenerational studies of stably transformed plants. The aim was to produce populations of rice plants expressing the barley sucrose transporter *HvSUT1,* constitutively and specifically in the endosperm. Therefore, careful choice of promoters was the first consideration.

For constitutive expression of transgenes in cereals, dicot and monocot promoters have previously been used in rice. The widely used Cauliflower Mosaic Virus 35S (*CaMV-35S*) promoter has been shown to give low expression in rice (McElroy *et al.*, 1990, 1991; Cao *et al.*, 1992), although other studies have contradicted these results by developing rice with high expression of transgenes driven by *CaMV-35S* (Battraw and Hall, 1990; Terada and Shimamoto, 1990; Datta *et al.*, 1998). Compared with other strong constitutive promoters such as maize ubiquitin 1 (*ZmUbi1*) which has patchy expression (Cornejo *et al.*, 1993), the rice actin 1 promoter (*Act1*) (McElroy *et al.*, 1990) containing the functionally important rice actin *intron-1* (McElroy *et al.*, 1991; 1994)

has been shown to direct high-level expression in all the rice tissues tested. Interestingly, *ZmUbi1* promoter is stress inducible (Cornejo *et al.*, 1993; Christensen and Quail, 1996) and when driving *uidA* expression gave heavier GUS staining in tissues that had been heat shocked (Takimoto *et al.*, 1994). Given the aim of the transformation study presented here, the *ZmUbi1* promoter was discarded as it could potentially give inconsistent result. Another advantage of the *Act-1* promoter is that it drives consistently strong expression during various stages of rice plant development (Zhang *et al.*, 1991). Hence, $Act1_{pro}$ was chosen to drive constitutive expression of the *HvSUT1* transgene in this study.

Careful choice of a suitable promoter is necessary for targeted temporal and spatial gene expression. Grain specific transgene expression is a goal for bio-fortification studies because the grain is the edible portion of the plant. Promoters to direct endosperm specific expression in rice have been isolated from cereal species, mostly having homologous regulatory sequences in their corresponding 5' flanking regions (Nakase *et al.*, 1996) although some promoters require both 5' and 3' gene regions for endosperm specific expression (Qu and Takaiwa, 2004). Various studies have compared the tissue specificity and temporal expression patterns of grain specific promoters in rice by fusing them to reporter genes such as β-glucuronidase (*GUS*) and green-fluorescent protein (*gfp*). Not all endosperm specific cereal promoters are suitable for targeted expression in rice. For example, the wheat high-molecular-weight glutenin (*HMW-Glu*) promoter drives expression of *gfp* in many other tissues in rice (Furtado *et al.*, 2008). While the barley *Hor-B1* promoter drives grain specific *GUS* and *gfp* expression and is stably inherited up to the T_9 generation in barley (Choi *et al.*, 2003), in rice it drives expression in rice grains as well as other tissues (Furtado *et al.*, 2008). These studies indicate that regulation of endogenous promoters may differ from introduced promoter sequences from other species. It was therefore decided to use a native promoter to drive transgene expression.

The rice α-globulin (*Glb-1*) promoter, which starts -1007 bp upstream from the *Glb-1* start codon (Nakase *et al.,* 1996), drives strong and highly endosperm specific gene expression in rice (Wu *et al.*, 1998a; Furtado *et al.*, 2008). When shortened to -980 bp (Wu *et al.*, 1998a) and -896 bp (Hwang *et al.*, 2002) upstream, the *Glb-1* promoter retained strong and specific activity. Hwang *et al.* (2002) found that by deleting progressively longer fragments from the 5' end of the sequence, lower *GUS* expression was observed. Compared with other grain specific promoters from rice (Wu *et al.*, 1998a; Qu and Takaiwa, 2004), or other cereals (Hwang *et al.*, 2001; Furtado *et al.*, 2008), *Glb-1* consistently showed strong expression of reporter genes that was

highly endosperm specific. This promoter has also been used successfully to drive transgenes in a number of studies. A high concentration of human lysozyme has been produced in rice endosperm, which confirms its usefulness in expressing highly species-divergent recombinant proteins (Hwang *et al.*, 2002). Moreover, plants transformed with a construct containing the *Glb-1* promoter, stably maintains promoter specificity in subsequent generations of rice (Qu *et al.* 2005; Furtado *et al.*, 2008). Homologous transgene silencing can also be an issue if a T-DNA construct carries the same promoter that drives both the transgene and the selection marker (Matzke and Matzke, 1995). Therefore careful consideration of combinations of promoters used can minimise the chances of seeing widely variable phenotypes in a transgenic population (Butaye *et al.*, 2005).

In rice, the tissue most amenable to transformation has been found to be callus derived from scutellum tissue (Hiei *et al.*, 1997). Foreign DNA is introduced into this tissue either through infection with *Agrobacterium* or biolistic bombardment. The advantages of using *Agrobacterium* over a gene gun for transformation, is that fewer copies of the transgene are delivered to the cells (Dai *et al.* 2001). This makes breeding of single copy homozygous plants a more simple process. Transformation success is also very dependent on the *Agrobacterium* strain. A number of strains have been used successfully to transform monocots, however the most successful for recalcitrant species have been the 'hyper-virulent' EHA101, EHA105 and AGL0 and AGL1 (Hensel *et al.*, 2009). The 'Normal' strain LBA4404, combined with 'super-binary' plasmids gives similar transformation efficiencies in rice as these hyper-virulent *Agrobacterium* strains (Hiei *et al.*, 1994) but has varying degrees of success in transformation of many other cereals (see Cheng *et al.*, 2004). AGL1 was originally developed by Lazo *et al.* (1991) for the purpose of being able to host *A. thaliana* genomic DNA libraries that could be easily transformed into plants. This strain carries a resident disarmed plasmid pTiBo542 that contains hyper-virulent *vir* genes for T-DNA transfer and an engineered mutation in its genomic *RecA* gene that aids in stabilizing recombinant plasmids, thereby increasing transformation efficiency. AGL1 has been used successfully to transform various cereals including indica rice (Khanna and Raina, 1999), elite japonica rice cultivars (Upadhyaya *et al.*, 2000), barley (Tingay *et al.*, 1997) and wheat (Wu *et al.*, 2008a). The japonica rice 'Nipponbare' was used as the model rice for sequencing by the IRGSP, is amenable to transformation (see Abe and Futsuhara, 1986) and has been chosen for this study.

Traditional binary vectors that can replicate in *E. coli* and *Agrobacterium* and are used to introduce recombinant T-DNA into plants are generally large with limited

restriction sites for the introduction of transgenes (Bevan, 1984). Therefore cloning into these vectors can be difficult. The development of Gateway™ (Invitrogen) compatible transformation vectors that can be used with the Gateway™ cloning system (Invitrogen) has streamlined and simplified this for plant transformation (for an overview of plant Gateway™ cloning and vectors see Earley *et al.* (2005) and Karimi *et al.*, (2007). Himmelbach *et al.* (2007) developed a range of overexpression (OE) and knockdown (RNAi) Gateway™ binary vectors specifically for the efficient cloning and transfer of T-DNA into cereals. These convenient modular vectors have a pVS1 *ori* for plasmid stability in *Agrobacterium*, Hpt^r driven by *ZmUbi1* for selection of positive transformants with hygromycin and a strong cereal specific promoter or MCS (to introduce a tissue specific promoter) to drive the transgene expression. These binary vectors were used successfully to transform barley (Himmelbach *et al.*, 2007). This system in combination with the *Glb-1* and *Act-1* promoters was chosen for driving endosperm and constitutive *HvSUT1* expression in transgenic rice in this study.

The rice transformation protocol used in this study has been modified from Hiei *et al.* (1994), Aldemita and Hodges (1996), Toki *et al.* (1997), Toki *et al.* (2006) and Shrawat and Good (2011). These studies standardised the use of an osmotic mix of sugars such as sucrose and glucose/maltose and acetosyringone in the co-cultivation media for plasmolysis of the callus cells and *vir* gene induction respectively, thereby promoting infection by *Agrobacterium*. They also investigated the optimum tissue culture conditions to ensure effective transgene integration and plantlet regeneration, including temperature, light intensity (also in Liu *et al.*, 2001), choice of starting material and media composition. Hence the protocol described in the following materials and methods section includes the relevant and practical elements from these studies. Plantlet regeneration from infected callus tissue can be a limiting step in the development of transgenic rice plants. Therefore, other factors were adjusted to optimise the conditions for successful regeneration such as callus age at the time of infection (Bajaj and Rajam, 1995), proportions of auxin/cytokinin in regeneration media (Abe and Futsuhara, 1986; Rueb *et al.*, 1994) and dehydration of callus at various stages of infection and cultivation (Tsukahara and Hirosawa, 1992).

To ensure adequate vegetative growth and good seed set, growth conditions must be optimised for specific species or cultivars. Rice is a short-day plant (i.e. it requires less than 12 hours of daylight to flower) however recommended growing conditions for the cultivar Nipponbare varies widely between studies. Conditions include 30^0C day and 27^0C night temperatures with a 12 hour day length (Aldemita and Hodges, 1996); 28^0C day and night with 14 and 10 hours of light and dark (Shrawat and

Good, 2011); 30^{0}C day time and 25^{0}C night time temperatures with 11 hours of light and 13 of dark in a Biotron (Ohnishi *et al.*, 2011) and 25^{0}C day and 20^{0}C night time temperatures (Yamakawa *et al.*, 2007). From these widely varying conditions for growing Nipponbare it appears that this cultivar is not very photoperiod sensitive because it has been grown successfully under all these conditions. In addition, large fields of Nipponbare are grown successfully in Yanco, New South Wales, where the maximum daylight exceeds 12 hours throughout summer (Russell Reinke (IRRI), pers comm. 2012). Due to the strict conditions for research into GMOs in South Australia, work on transgenic rice plants must be conducted inside an Office of the Gene Technology Regulator (OGTR)-licensed contained plant growth facility, such as a PC2 greenhouse. In this system, space limitations are an issue and the results from pot trials cannot be extrapolated to the field. However optimum temperatures can be sustained in a greenhouse under a semi-controlled environment. The optimum temperature for Nipponbare is between 20 to 30 degrees and therefore greenhouse temperatures maintained at these temperatures allow optimum growth and grain fill.

Specific expression of reporter genes in rice endosperm by the *Glb-1* promoter has been comprehensively demonstrated in numerous studies (see Section 2.1) however in this investigation the cloned *Glb-1* sequence was tested by GUS staining of *GUS* transformed rice tissues to prove the function of the cloned promoter sequence from rice gDNA. GUS has also been used as a control to compare the transformation efficiency of the protocol for rice transformation using the pIPKb Gateway binary vectors.

Transgenic plants generated for hypothesis testing must be accurately assessed for copy number and transgene expression to determine that the transgene is functional. In most cases, Southern blotting has been the most reliable method for copy number determination as well as segregation analysis of the resulting progeny of the T_0 transgenic lines. It is also advantageous to determine the location of the transgene position integrated into the genome of the plant. Methods that utilise different variations on PCR have been developed for this purpose. For example, Adapter Ligation PCR (AL-PCR) and Inverse PCR (I-PCR) have been used to assess gene locations in Arabidopsis (O'Malley *et al.*, 2007) and rice (Sallaud *et al.*, 2003). These methods have the advantage of requiring less DNA than in Southern blotting (ng vs µg concentrations) to determine copy number and have the additional advantage of locating the position of gene insertion in the genome by sequencing across the junctions of the integration sites.

In most cases, stable transformation of transgenes are best assessed in single copy transformants as it is easiest to examine segregation patterns in seed to determine copy number and zygosity and to select homozygous lines for further analysis . Typically in *Agrobacterium* transformation of rice, one to a few copies of the transgene are inserted into the genome. Transgene dosage (number of copies) does not always correlate with gene expression and can result in unexpectedly low activity of the target gene, which can be easily determined when working with reporter genes such as GUS (Hobbs *et al.*, 1993). However, this situation is further complicated when multiple copies of a transgene homologous to an endogenous gene is introduced into a plant, especially when the gene is conserved for a critical cellular function, as for the *SUT1* gene. Tight control of mRNA or protein levels in plant cells could be part of the plant's natural defence for dealing with abiotic or biotic stresses that regulates gene expression through various mechanisms (Matzke and Matzke, 1995). Epigenetic factors such as DNA methylation and/or histone modification could prevent transcription, or there may be post-transcriptional processes that quickly turnover excess mRNA, thereby resulting in silencing of the transgene and potentially the endogenous gene.

In transgenic plant studies, RNA transcript and protein are measured to determine that a transgene is being expressed. In this study, transcripts for the transgenic and endogenous versions of *SUT1* will be assessed by RT-PCR. However, gene transcription does not necessarily reflect the amount of functional protein that is being expressed. In particular, there is a low correlation between levels of *SUT* transcript and SUT proteins from various plant species. SUT protein is either not detected in the same tissues as the transcript, or the concentrations are not as high as would be expected from the amount of transcript that is present. In wheat, Bagnall *et al.* (2000) measured transcripts of *SUT1* in wheat caryopses, however SUT1 protein did not localize to the same tissues. Rosche *et al.* (2002) observed similar discrepancies between SUT transcript and protein in peas. These results imply that there is some post transcriptional regulation of SUTs, therefore detecting both transcript and protein would be necessary to comprehensively demonstrate stable inheritance of the *HvSUT1* transgene. No commercial antibody for HvSUT1 protein was available. Therefore, in this study, a peptide antigen was designed and an antibody produced and purified enabling the detection of HvSUT1 protein by immuno-blot analysis.

The work in this chapter describes the molecular cloning of plasmid constructs and the subsequent plant tissue culture and *Agrobacterium* transformation of rice to generate transgenic rice plants. The specificity of promoter expression was examined

by histochemical analysis of plants expressing GUS driven by either the endosperm specific (*Glb-1*) or constitutive (*Act-1*) promoter. Plants containing the barley sucrose transporter *HvSUT1* coding sequence driven either by *Glb-1* or *Act-1* were characterised using molecular and biochemical methods to provide evidence for the stable inheritance of this transgene through T_1 and T_2 progeny. From these analyses, suitable sucrose transporter overexpressing (SUT-OE) transgenic rice lines were chosen for further investigation.

2.2 Materials and Methods

2.2.1 Preparation of overexpression plasmids

Binary Gateway® destination vectors for cereal transformation developed by Himmelbach *et al.* (2007) were kindly provided by Dr. Jochen Kumlehn from IPK Gatersleben, Germany. pIPKb vectors (pIPKb001 Acc. EU161567; pIPKb003 Acc. EU161569) in *E. coli* strain DB3.1 were streaked onto LB media-agar plates with spectinomycin (spec)(25 µg ml^{-1}) and incubated 16 h at 37 ^{0}C. One colony from each culture was inoculated into 10 ml LB media with spec (25 µg ml^{-1}) for overnight culture in an orbital shaker at 37 ^{0}C. Cells were pelleted by centrifugation at 3000 xg for 15 min and plasmid DNA isolated using the Wizard® *Plus* SV Minipreps kit (Promega) according to the manufacturer's instructions. Plasmid DNA was eluted in 200 µl of ultrapure laboratory grade water (Milli-Q® water) and concentration was estimated by spectrophotometric analysis (NanoDrop 2000, Thermo Scientific). Plasmid identities were verified by restriction digests with *Pst*I run on a 1% agarose gel.

2.2.2 Amplification of *Glb-1* promoter from rice gDNA and ligation into pIPKb001

Genomic DNA (gDNA) was extracted from rice seedlings. Freshly harvested tissue was ground in liquid nitrogen (LN2) and gDNA was extracted using the plant DNeasy minikit (Qiagen) according to the manufacturer's protocols. gDNA was further purified and concentrated by the addition of 10 µl of Sodium acetate (NaAc) pH 5.2 to a 90 µl aliquot of gDNA and precipitated with 2.5 x volumes of ice cold 99% ethanol (EtOH) at -20 °C for 2 h. The gDNA pellet obtained after centrifuging at 14000 xg for 15 min was washed with 1 ml of 80% EtOH, dried at room temperature (RT) for 10 min and resuspended in 50 µl of Milli-Q® water.

The 981 bp *Glb-1* (*Glb-1*$_{pro}$) promoter sequence (Acc. AY427575.1) was amplified from rice gDNA using PCR (Appendix A, PCR 1) with primers containing

restriction sites *Spe*I and *Asc*I at the 5' and 3' ends for ligation into the MCS site in pIPKb001. The specific band at ~1000 bp was cut out following agarose gel electrophoresis and further purified using the Wizard® SV gel and PCR clean up kit (Promega). The PCR product was further concentrated and purified by EtOH precipitation and checked on a 1% agarose gel.

Separate double digests of pIPKb001 and the *Glb-1*$_{pro}$ PCR product with *Spe*I and *Asc*I (10 U μl^{-1}), 1x NEB buffer 4, BSA (0.2 ug μl^{-1}) plus 400 ng of pIPKb001 template or 50 ng of Glb1 template were incubated at 37 °C for 1 h. Digests were heat inactivated at 80 °C for 20 min and reactions cleaned up with the Wizard® SV Gel and PCR Clean-Up System (Promega). 10 µl ligation reactions with T4 ligase in ligation buffer (NEB) with an insert: vector ratio of 1:3 was conducted at RT for 16 h. Ligase was heat inactivated at 65 °C for 10 min and the entire 10 µl used to transform 50 µl of chemically competent DB3.1 *E. coli* by heat shock. Transformed cells were plated onto LB agar plates containing spec (25 µg ml^{-1}) and chloramphenicol (chlor) (34 µg ml^{-1}) at 30 °C for 16 h. One colony growing on the selection plate was inoculated into LB broth culture and plasmid DNA was prepared with a Miniprep kit (Promega). An analytical restriction digest with *Pme*I and *Hind*III that cuts on either side of the MCS was carried out on the isolated plasmid DNA. This destination vector is designated pIPKb001:Glb$_{pro.}$

2.2.3 Amplification of *HvSUT1* cDNA and Gateway® cloning

The p6U:HPT:HOSUT (HOSUT) plasmid (Weichert *et al.,* 2009) containing the *HvSUT1* coding sequence (cds) (Acc. AJ272309.1) (Weschke *et al.*, 2000) was obtained from Dr. Hans Weber (IPK, Germany). Extra plasmid DNA was isolated and purified after electroporation into DH10B *E. coli* cells and selection on LB with spec (25 µg ml^{-1}). Plasmids were verified by PCR amplification (Appendix A, PCR 2) and sequencing (AGRF Sequencing Facility, Adelaide) using sequencing primers (Appendix A).

The 1967 bp *HvSUT1* cds was amplified from the HOSUT plasmid with primers containing attB sites (final product length 2028 bp) for Gateway® recombination (Appendix A, PCR 3). The PCR product was polyethylene glycol (PEG) purified and cloned into donor vector pDONR221 with BP Clonase® II according to the protocols in the Gateway® Technology with Clonase® II User Guide (Invitrogen by Life Technologies). DH10B *E. coli c*olonies electroporated with BP reactions growing on kanamycin (kan) (50 µg ml^{-1}) were confirmed by PCR (Appendix A, PCR 2). A single colony containing the recombinant pDONR221 was grown in LB medium containing kan (50 µg $ml^{-1)}$ with shaking at 37°C for 16 h. Entry vector DNA (designated

pENTR221:HvSUT) (conc. 205.1 ng μl^{-1}; 260/280=1.91) was prepared for cloning into Gateway® destination (pIPKb) vectors.

Gateway® reactions between entry vector pENTR221:HvSUT and destination vectors pIPKb001:Glb_{pro} or pIPKb003 were carried out using LR Clonase® II according to the Invitrogen User Guide (see above). Following the reaction, 1 µl was electroporated into DH10B *E. coli* and positive colonies were selected on LB plates with spec (25 µg ml^{-1}). Colonies growing on spec (25 µg ml^{-1}) were confirmed by PCR (Appendix A, PCR 4). A single colony containing the recombined plasmid with the construct Glb_{pro}:HvSUT1 was inoculated into LB with spec (25 µg ml^{-1}) to prepare plasmid DNA for transformation of *Agrobacterium* strain AGL1. The same procedure was carried out to isolate plasmids containing the construct Act_{pro}:HvSUT1.

To determine the specificity of the *Act-1* and *Glb-1* promoters, the control plasmid pENTR™-GUS was recombined with entry vectors pIPKb003 and pIPKb001:Glb_{pro} in separate LR reactions. Selection of colonies and isolation of plasmids containing the constructs Act_{pro}:GUS and Glb_{pro}:GUS was carried out as previously described.

2.2.4 *Agrobacterium* rice transformation

Electrocompetent *Agrobacterium* (AGL1) cells were prepared using standard procedures (Wise et al., 2006). Four transformations were conducted with the following destination plasmids containing the constructs i) Glb_{pro}:HvSUT, ii) Act_{pro}:HvSUT, iii) Glb_{pro}:GUS and iv) Act_{pro}:GUS. Aliquots of AGL1 (20 µl) were electroporated with 1 µl of plasmid DNA (~150-300 ng). Cells were plated onto YM media plates (Appendix B) containing spec (25 µg ml^{-1}) and rifampicin (rif) (20 µg ml^{-1}) and incubated at 28 ^{0}C until large well separated colonies were observed, approximately 3 days (d). Colony PCR (Appendix A, PCR 4; PCR 5) was used to confirm transformation of AGL1 with the destination plasmids. Additional verification of plasmid identity was undertaken by extracting plasmids from *Agrobacterium* cells, as described previously for *E. coli,* and electroporating them back into DH10B *E. coli*. After amplification in DH10B, plasmid DNA was extracted, purified and analysed by cutting with restriction enzyme *Eco*RI. These digested products were compared by agarose gel electrophoresis with the *Eco*RI digested progenitor DNA that was electroporated into AGL1.

For virulence gene induction a single AGL1 colony harbouring the correct destination plasmid was inoculated into 2 ml of LB broth with rif (20 µg ml^{-1}) and spec (25 µg ml^{-1}) and incubated overnight with shaking at 28 ^{0}C. The culture was then

streaked onto yeast extract-mannitol (YM) media plates (Appendix B) with antibiotics and the plates incubated for 2-3 d at 28 ^{0}C in the dark. Confluent growth of AGL1 grown on YM media was scraped off and resuspended in 30 ml of 2N6-AS liquid media (Appendix B), which contains acetosyringine (AS) (20 µg ml^{-1}), to yield an OD600 of 0.1/0.2. This suspension was used for infection of rice callus.

2.2.5 Rice callus induction, infection and selection

The protocols for callus induction and rice transformation were adapted primarily from Hiei *et al* (1997) and Toki *et al.* (2006). Nipponbare rice seeds (40-50) were dehulled and sterilized with 70% ethanol for 1 min and washed in Milli-Q® water. Seeds were sterilized again with a 50% commercial bleach solution containing a drop of Tween20, and gently agitated on a rotary mixer for 20 min. After rinsing thoroughly with sterile Milli-Q® water (5x) the seeds were dried briefly on sterilized filter paper before inoculating 9-12 seeds per plate onto N6D medium solidified with 0.4% gelrite (Appendix B). Dishes were sealed with Micropore™ surgical tape (3M™) and cultured under continuous light (20-25 µmol m^{-2} s-1) at 28 ^{0}C for 3-4 weeks. Healthy proliferating callus were re-cultured onto fresh N6D media plates every 7 d.

Pieces of healthy calli were immersed and gently washed in the AGL1 suspension for 2 min. After blotting dry on sterile Kimwipes®, the infected callus was transferred onto sterilized filter paper moistened with 2N6-AS solution and placed onto plates containing 2N6-AS media solidified with 0.4% gelrite. The plates were co-cultivated at 25 ^{0}C in the dark for 3 d then washed 5x with sterile Milli-Q® water. After a final wash in sterile Milli-Q® water containing timentin (tim) (150 µg ml^{-1}) the callus was dried rapidly on filter paper before culturing on solid N6D media (Appendix B) with hygromycin (hyg) (50 µg ml^{-1}) and tim (150 µg ml^{-1}) at 32 ^{0}C in the dark for two weeks. After two weeks, surviving healthy callus tissue was transferred to fresh N6D plates with further antibiotic selection.

2.2.6 Plantlet regeneration and growth conditions

Calli that continued proliferating under selection were transferred to tissue culture plates containing solid REIII media (Appendix B) with the hormones naphthalene acetic acid (NAA) (0.05 µg ml^{-1}) and kinetin (2.5 µg ml^{-1}) and antibiotics, under light (~50 µmol m^{-2} s^{-1}) for shoot induction. Callus with healthy shoots were transferred to hormone free HF media (Appendix B) containing hyg (50 µg ml^{-1}) and tim (150 µg ml^{-1}) to maintain selection, in tissue culture tubes, under a 12 h light/dark regime. Plantlets with healthy root and shoot systems were transferred to soil.

All media was washed thoroughly from the roots before plantlets were transferred to small 350 ml pots filled with Green Wizard® potting mix (Debco Pty Ltd, Victoria) in water filled trays where seedlings were 'hardened off' for 7 d. After 14 d, the seedlings were transferred to larger pots (2.8 L) with the pot base constantly submerged in water from transplant until plant maturity. The mix contained sufficient fertilizer for 4 months hence no additional fertilizer was added. Plants were grown in the PC2 greenhouse facility at Flinders University, South Australia under natural light between the months of August and January. Day length varied between 11 to 14 hours with min/max temperatures of 25 ^{0}C night and 29 ^{0}C day.

2.2.7 Histochemical GUS staining of transformed rice tissue

Callus transformed with Glb_{pro}:GUS and Act_{pro}:GUS constructs were stained with X-gluc solution using a standard protocol for detecting colorimetric β-glucuronidase expression in plant tissues (Jefferson, 1987). The staining solution was made up of 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) in dimethyl formamide (1 mg ml^{-1}), Potassium ferricyanide (50 μM) and Potassium ferrocyanide (50 μM) in 0.1 M Sodium phosphate buffer with a drop of Triton X. Callus was immersed in the staining solution and vacuum infiltrated in a speed vac chamber for 2 min at 0.4 bar before incubating at 37 ^{0}C in the dark overnight. Plants regenerated from callus tissue transformed with *GUS* were grown until seed set and grain filling. Grains at ~15 DAA were cut longitudinally with a clean razor blade and anthers and leaf samples were collected and stained as described above for callus tissue.

2.2.8 Southern blotting

For isolation of clean gDNA at high concentrations for Southern blotting (Southern, 1975), rice leaves were ground to a powder in a pre-chilled mortar and pestle under nitrogen and extracted with Cetyl trimethylammonium bromide (CTAB) (Ausubel, *et al.*, 1995). The finely ground tissue was added to preheated CTAB buffer (2% CTAB, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, 1.4 M NaCl) with the addition of fresh β-mercaptoethanol (βME) (0.2%) just before use. Samples were vortexed then incubated at 60 °C for 45-60 min. After cooling, an equal volume of phenol/chloroform/isoamyl alcohol (P:C:I) (Sigma) was added and the sample mixed on a rotary shaker at RT for 20 min. Plant debris was pelleted by centrifuging at 3000 xg for 15 min. The supernatant was mixed with 0.6 volume of Isopropanol and gDNA precipitated overnight at -20 °C. After pelleting the gDNA at 3000 xg for 10 min at 4 °C,

pellets were washed with 80% EtOH and resuspended in TE buffer. RNA contamination was removed by incubating the sample with RNAse A (40 µg ml^{-1}) at 37 °C for 30 min. gDNA was re-precipitated using NaAc and EtOH as described previously. Samples of gDNA (10 µg) were cut with either *Hind*III or *Eco*RI. Fragments were separated by electrophoresis on 1% agarose gels, transferred to a Hybond N+ membrane (Amersham) by capillary transfer in neutral 10x SSC buffer overnight then fixed to the membrane by UV irradiation (Gene-linker , Biorad). Transfer was checked by incubating the gel post-transfer with GelRed (Biotum) and viewing under UV light. A 498 bp probe template for DIG labelling was amplified from Glb:HvSUT plasmid DNA by PCR (Appendix A, PCR 6). The probe DNA was labelled using random primed labelling with DIG-11-dUTP (DIG-High Prime labelling starter kit manual, Rosche). Optimum probe yield and concentration was determined by a dot blot comparing the probe to DIG labelled control DNA in a dilution series from 0.01 pg $µl^{-1}$ to 1 ng $µl^{-1}$. Pre-hybridization (50 ^{0}C, 2 h) and hybridization (68 ^{0}C, overnight) in DIG Easy Hyb buffer was followed by high stringency washes (final wash 0.1x SSC at 68 ^{0}C). Following the wash steps, the membrane was blocked with blotto (Maleic acid buffer; 0.1 M Maleic acid, 0.15 M NaCl adjusted to pH 7.5 with NaOH plus 5% skim milk powder) for 3 h at RT, incubated with DIG antibody (blotto plus 1:10,000 anti-digoxigenin-AP (75 mU ml^{-1}) for 30 min and washed 2x for 15 min each with washing buffer (Maleic acid buffer plus 0.3% v/v Tween 20). After equilibrating the membrane in detection buffer (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl) for 5 min the membrane was developed in CSPD ready–to-use solution for chemiluminescent detection. Bands were imaged with a CCD camera (Chemidoc, Biorad).

Attempts to determine the copy number and integration site of the transgene copies in the rice genome using adapter ligation PCR (O' Malley *et al.*, 2007) gave unreliable and inconsistent results. Hence, only estimations of copy number are included in Table 2.3 while a description of the method, representative gel pictures and sequencing data have been summarized in Appendix C.

2.2.9 Segregation analysis and leaf assay

Mature seeds from regenerated T_0 rice plants were germinated on wet filter paper for one week, before being transferred to selection plates with hyg (50 µg ml^{-1}) to determine the number of resistant T_1 progeny of each line. Seeds were dehusked and sterilized as described in section 2.2.5. After two weeks on selection media, seedlings were scored as resistant if their shoots were green and the roots had continued to grow, or sensitive if growth was stunted and the seedlings had failed to thrive under

selection. A segregation ratio of 3 resistant: 1 sensitive was expected for plants containing 1 copy of the transgene. Any plants displaying any other ratios were scored as containing >1 transgene copy. A number of T_1 plants were grown to maturity and the seeds collected for further segregation analysis to determine zygosity of putative T_1 single copy, homozygous lines (100% survival of T_2 on hyg = homozygous T_1; 75% survival on hyg = heterozygous, 0% survival on hyg = null).

To support the segregation results, further segregation of T_1 plants not grown on selection were analysed by leaf assays of plants in the late vegetative stage, according to the protocol outlined in Wang and Waterhouse (1997). Plates containing media made of MS salts pH 5.7, 0.5 mg l^{-1} 6-benzyaminopurine (BAP) and hyg (100 µg ml^{-1}) solidified with 4% gelrite were prepared. Three leaves from each plant were cut 2cm from the tip and placed with the cut side embedded in the media. Each plate was divided into four sectors with each sector containing three leaves from three transformed plants, plus one sector containing three leaves from one control wild type (WT) plant. After 7 d, the leaf tips were scored as resistant if they were green with no sign of necrosis, while hyg sensitive leaf tips were necrotic with black or bleached stripes. Seeds from T_1 plants, including those identified as null segregants, that were used in the leaf assay were germinated on hyg (100 µg ml^{-1}) to determine zygosity.

2.2.10 Semi quantitative RT-PCR

The housekeeping gene, elongation factor 1 alpha (*eEf1α*), is expressed stably across different tissues and at different developmental stages in rice, and was determined by Jain *et al.* (2006) to be one of the most reliable internal standard for use in RT-PCR. To standardise expression, *eEf1α* was amplified to compare relative expression of endogenous *OsSUT1* and transgenic *HvSUT1* transcripts. A pilot study for each gene (*eEf1α*, *OsSUT1* and *HvSUT1*) was performed to determine the linear range for the amplification of each gene fragment (Appendix D). Total RNA was extracted from 5 developing rice grains per plant, from the same position in each panicle of three biological replicates using a plant RNA extraction kit (Bioline). Samples were treated with DNase to remove DNA contamination and RNA quality was checked by agarose gel electrophoresis. RNA concentration was estimated using a spectrophotometer (Nanodrop 2000, Thermo Scientific) and equal amounts of RNA per sample was reverse transcribed with oligo(dT) primers using the iscript™ cDNA synthesis kit (Biorad). RT-PCR using gene specific primers amplified a 103 bp fragment of *eEf1α* (Appendix A, PCR 7), a 481 bp fragment of *OsSUT1* (Appendix A, PCR 8) and a 325 bp fragment of the transgene *HvSUT1* (Appendix A, PCR 9). The choice of intron spanning primers in *eEf1α*

and *OsSUT1,* plus a minus RT control was used to control for genomic DNA contamination in the cDNA samples. For comparison, 10 µl of each PCR reaction was run on the same 1% agarose gel for each gene. Densitometry using Image Lab software (Biorad) was used to quantify the intensity (arbitrary units) of *OsSUT1* and *HvSUT1* bands relative to *eEf1α*.

2.2.11 Production of anti-HvSUT1 antibody and immunoblotting

A peptide targeting the putative solvent exposed loop region between the sixth and seventh membrane spanning helices of HvSUT1, AIAPLPTKANGQVEVEP was synthesized and conjugated to keyhole limpet haemocyanin (KLH) via the addition of a cysteine residue at the C-terminus (Auspep pty ltd). The choice of antigen for antibody production was limited due to the overall hydrophobicity of the protein and high homology (81.3%) with the endogenous rice SUT1 (Weschke *et al.*, 2000) (Fig. 1.3). Polyclonal antibody serum was produced in rabbits immunised subcutaneously following a standard 10.5 week dosing schedule (SAHMRI).

To partially purify the antibodies from rabbit serum, 0.5 volume of saturated NH_4SO_4 was added, incubated on ice then centrifuged for 10 min at 14,000 xg at 4 ^{0}C. The protein pellet was dissolved in Sodium phosphate (Na_2HPO_4) buffer pH 7.0 to the same original volume as the serum.

To improve the specificity of the antibody, the antiserum was further purified using affinity purification. To do this, Toyopearl Epoxy 650M (TOSOH) activated resin (0.25 g rehydrated in 4 ml of Milli-Q® water to give a 1 ml resin volume) was resuspended to give a total volume of 4 ml in Na_2HPO_4 buffer. The peptide antigen (3 mg synthesized with an additional N-terminal cysteine residue) was dissolved in 1 ml of Na_2HPO_4 buffer and added to the resin to immobilize the peptide antigen to the resin via conjugation to the cysteine at the N terminus. After an overnight incubation on a rotating platform at RT, the resin was washed sequentially 3x with Milli-Q® water, 3x with 1 M NaCl, 3x with Milli-Q® water then mixed with 5 ml of 1 M ethanolamine to block free active sites. This mixture was then incubated with rocking overnight at RT then washed sequentially with 3x Milli-Q® water, 3x 1 M NaCl then finally 3x with Na_2HPO_4 buffer. The prepared resin was settled into a 1 cm diameter econocolumn (Biorad) and stored in Na_2HPO_4 buffer at 4 ^{0}C until use.

The partially purified antiserum was passed through the column 3x then the column was washed 3x with Na_2HPO_4 buffer. The bound IgGs were eluted with 5x 1 ml volumes of 0.15 M Glycine pH 2.3 then each fraction immediately neutralised with 100

µl of 1 M Tris pH 10.2. The presence of IgGs in the eluted fractions was tested and the concentration of HvSUT1 specific antibody was titrated against dilutions of the unconjugated peptide antigen by dot blot analysis to determine antibody dilution for use in immunoblotting (Appendix E).

Ten rice grains harvested from the tip of panicles at varying days after anthesis (DAA) were placed into 2 ml microtubes with a 5 mm stainless steel ball, frozen in liquid nitrogen (LN2) and ground for 30 sec using a Retsch mixer mill (MM400) at maximum vibrational frequency (30 Hz). These steps were repeated until the plant tissue was powdered and no large pieces were distinguishable. Crude total membrane proteins were extracted using the method of Furbank *et al.* (2001) as follows. A 1:4 volume of plant material to extraction buffer was added to each tube and vortexed vigorously. The extraction buffer was made up of 250 mM Tris-HCl pH 8, 25 mM EDTA, 30% w/v sucrose and fresh 5 mM DTT with the addition of protease inhibitors (1 mM benzamidine, 5 mM ε-aminocaproic acid, 2 mM leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF)). After centrifuging at 3000 xg for 10 min at 4 ^{0}C, the supernatant was transferred to a fresh tube and centrifuged at 15,800 xg for 120 min at 4 ^{0}C to collect total membrane proteins. The protein pellet was resuspended in sample buffer made up of 50 mM HEPES buffer pH 7.5, 5 mM EDTA and 2 mM DTT with the addition of the same protease inhibitors as the extraction buffer.

Equal volumes of protein sample were loaded and separated by 12% SDS-PAGE before transfer onto nitrocellulose (GE Healthcare Life Sciences) by electro-blotting. Protein loading was determined either by incubating blots in Ponceau red or running duplicate samples on gels that were stained with either Coomassie blue or SYPRO® Ruby gel stain. After rinsing in Milli-Q® water, blots were blocked overnight at 4 ^{0}C in blotto (made with 1x TBS-T; 50 mM Tris pH 7.5, 150 mM NaCl, 0.05% v/v Tween 20 and 5% skim milk powder), incubated with HvSUT1 affinity purified primary antibody at a 1:500 dilution in blotto for 3 h at room temperature, washed 2x for 5 min each in TBS-T followed by incubation for 1 h with Goat anti-rabbit IgG-horseradish peroxidase (HP) conjugated secondary antibody (Rockland Immunochemicals Inc.) diluted 1:5000 in blotto. After washing 3x for 5 min each, blots were developed in Western Star Chemiluminescent substrate (Biorad) and imaged with the Chemidoc CCD camera (Biorad).

Duplicate gels and blots were run to investigate protein expression of H^+-ATPase proton pumps associated with SUT proteins. Blots were incubated with a commercial plasma membrane (PM) specific H^{+}-ATPase polyclonal antibody (Agrisera) at a dilution of 1:1000. This antibody is reactive to a protein with a molecular weight of

approximately 100 kDa in many plant species, including rice. This protein also serves as a marker to confirm the presence of PM proteins in total membrane protein samples.

2.3 Results

2.3.1 Construction and verification of SUT-OE vectors for rice transformation

Amplification of rice gDNA with *Glb-1*$_{pro}$ specific primers resulted in four major bands (Fig. 2.1 A). Despite trying numerous PCR conditions, there were consistently three extra bands, two of higher and one of lower molecular weight, apart from the expected band at approximately 1000 bp. Therefore, the annealing temperature was chosen to give the highest concentration of the band at ~1 kb (Putative *Glb-1*$_{pro}$), four aliquots run on the gel and the gel bands cut out under UV. The gel slices were combined and the DNA purified. The extracted DNA was run on an agarose gel to check for the presence of one band of the expected size (Fig. 2.1 B). The putative *Glb-1*$_{pro}$ PCR product was digested with *Spe*I and *Asc*I and cloned into the MCS of pIPKb001 and the plasmid sequenced. Analysis of the position of the cut sites of *Spe*I and *Asc*I in the MCS of pIPKb001 was examined by entering the MCS sequence into NEB Cutter V2.0 (http://tools.neb.com/NEBcutter2/ index.php). Sequencing across the junction of the ligation sites confirmed the correct orientation of the *Glb-1* promoter sequence into pIPKb001.

An analytical digest of pIPKb001:Glb$_{pro}$ with enzymes that cut either side of the MCS showed a distinct band at 1 kb, while this band was not present in the empty pIPKb001 plasmid, indicating the successful cloning of the *Glb-1* sequence into the MCS (Fig. 2.2 A). Another digest of binary plasmids containing the endosperm specific promoter (*Glb-1*) or the constitutive promoter (*Act-1*) with *Pst*I yielded 5 and 6 fragments, respectively, that ran at the correct sizes as predicted (NEBcutter V2.0) (Fig. 2.2 B).

To investigate the identities of the non-specific bands at approximately 750, 1500 and 3000 bp, a BLAST (Altschul *et al.*, 1990) of the primer sequences was performed against the rice genome. However, due to the short sequence, the number of hits were too numerous to draw any conclusions. However, a BLAST of the 981 bp

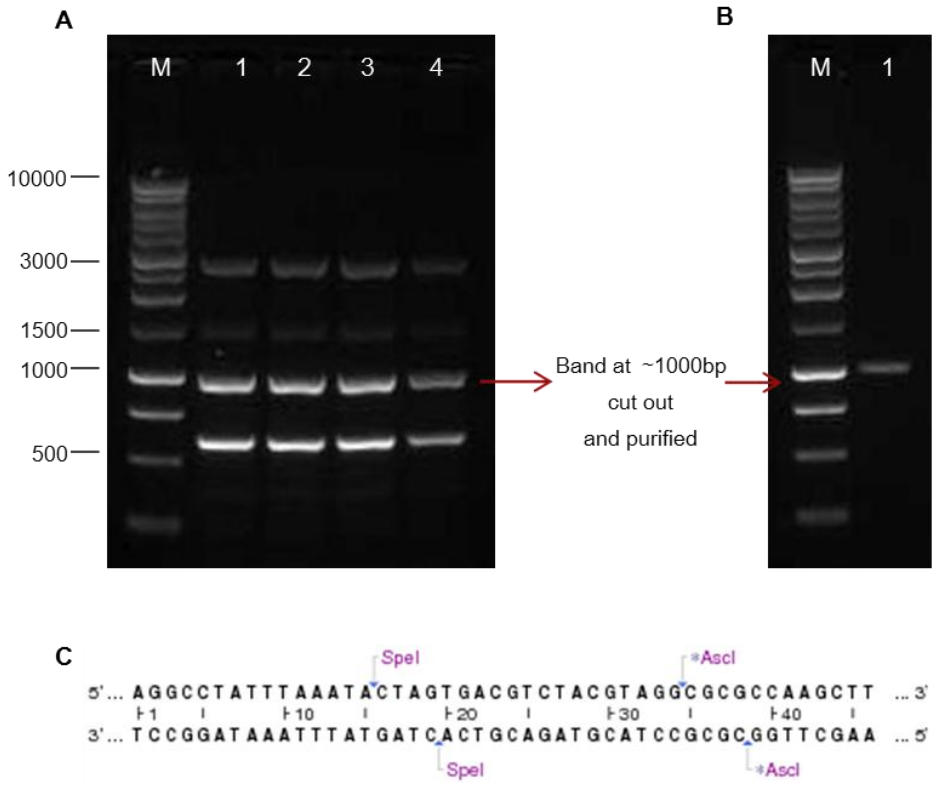


Figure 2.1 Amplification and gel purification of *Glb-1* promoter sequence from rice gDNA.

(A) Rice gDNA extracted from seedlings was used as a template for amplification of the *Glb-1* promoter with primers specific to the region from -981 upstream of the rice globulin gene and containing *Spe*I and *Asc*I restriction sites. Lane 1 to 4 represent aliquots of the same PCR reaction run on a preparative gel showing multiple non-specific bands. (B) The bands at ~1000bp were cut out of the gel and combined to isolate the putative *Glb-1* promoter sequence. (C) The MCS site into which the 981 bp PCR fragment was ligated (sequence and restriction sites from NEB Cutter V2.0) of the pIPKb001 vector and plasmids were confirmed by sequencing. MW marker is in bp.

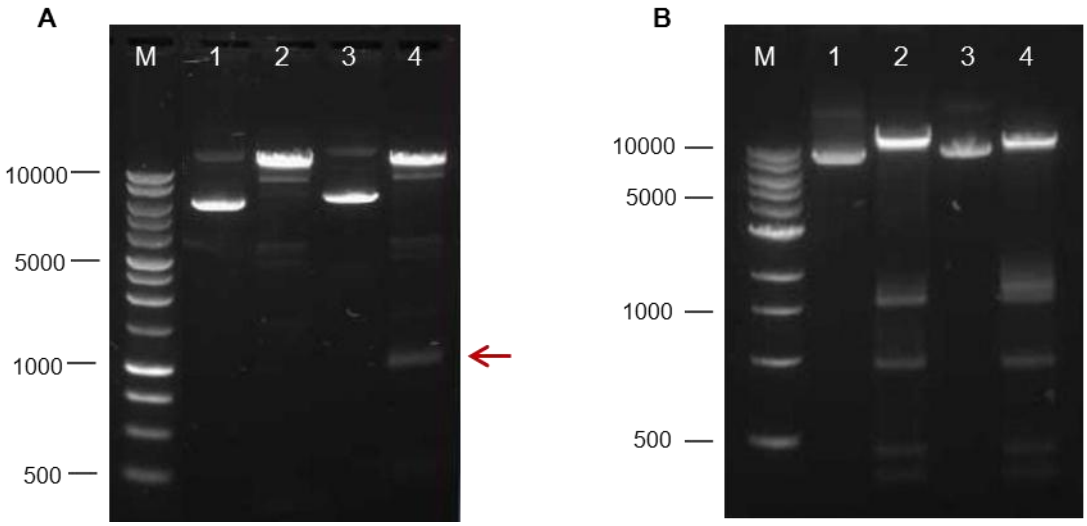


Figure 2.2 Analytical restriction digests of destination plasmids used in *Agrobacterium* transformation of rice.

Cut sites and length of expected DNA fragments were determined using NEB Cutter V2.0 (http://tools.neb.com/NEBcutter2/). Glb-1$_{pro}$ fragment released after digestion indicated by a red arrow. MW markers in bp.

M	1 kb DNA Ladder
Lane 1	pIPKb001
Lane 2	pIPKb001 cut with *Hind*III and *Pme*I
Lane 3	pIPKb001:Glb$_{pro}$
Lane 4	pIPKb001:Glb$_{pro}$ cut with *Hind*III and *Pme*I

M	1 kb DNA Ladder
Lane 1	pIPKb001:Glb$_{pro}$
Lane 2	pIPKb001:Glb$_{pro}$ cut with *Pst*I
Lane 3	pIPKb003
Lane 4	pIPKb003 cut with *Pst*I

promoter sequence against the rice (Oryza sativa {japonica; taxid: 39947}) genome collection in the NCBI database (http://www.ncbi.nlm.nih.gov) revealed multiple matching sequences. While the ~600 bp region directly upstream of the gene was unique to the *Glb-1* promoter, ~380 bp of the 5' region at the start of the promoter sequence showed high homology to various sequences in all 12 of the rice chromosomes (Table 2.1). The target *Glb-1* promoter region of the gene for 26 kDa globulin in chromosome 5 was the only sequence that showed almost 100% identity to the 981 bp cloned *Glb-1*$_{pro}$ fragment. When the start position (24625415) was located on chromosome 5 (NC_008405.2) the repeat region was located upstream of Os05g0499100, which was identified as the expected gene for 26 kDa globulin. An identity score of <100% was due to a one-base gap in the *Glb-1*$_{pro}$ sequence compared with chromosome 5 (24625721). This gap is not located in any *cis* regulatory sites in the promoter (-893 bp) therefore regardless of whether the gap was due to a publication error or polymorphism between japonica cultivars, promoter activity should not be affected. The presence of this highly homologous region in other chromosomes, such as 9 and 12 (Table 2.1), could explain the multiple non-specific bands that were consistently amplified with the *Glb-1* promoter specific primers in PCR of rice gDNA (Fig. 2.1 A).

2.3.2 Gateway® cloning of *HvSUT1* coding sequence into pIPKb expression vectors

Sequencing and PCR confirmed that the *HvSUT1* sequence in the HOSUT plasmid obtained from IPK, Germany was error free (data not shown). Due to the length and high Tm of the sequence-specific regions of attB primers used to amplify the barley *HvSUT1* cds from HOSUT plasmid DNA, PCR amplification required a period of optimisation. Final conditions included the modification of cycling conditions into a two-step PCR with both annealing and extension at the same temperature (72 °C). In addition, plasmid template concentration appeared to be crucial for PCR success (Fig. 2.3).

All colonies analysed after recombining the attB tagged HvSUT1 PCR product into pDONR221 contained the correct insert (Fig 2.4 A). Plasmid DNA extracted from a culture from one colony (pENTR221:HvSUT-6) was recombined with either the pIPKb001:Glb$_{pro}$ (Fig. 2.4 B) or pIPKb003 (Fig. 2.4 C) destination plasmids. Cloning appeared to be more efficient with the endosperm specific plasmid as all colonies that were screened by colony PCR were positive (Fig 2.4 B) while only four of the ten colonies screened from recombination with pIPKb003 were positive for *HvSUT1* (Fig

Table 2.1 Analysis of sequence matches between *Glb-1* gene promoter and the rice genome (*Oryza sativa* L. ssp. japonica)

Summary of BLAST results of the 380bp start of the 5' UTR of the 26kDa alpha globulin gene promoter with the rice genome.

Rice Chromosome	No. of matches	Maximum % Identity	Accession
5	30	99	NC_008398.2
9	7	96	NC_008402.2
12	18	94	NC_008405.2
7	23	87	NC_008400.2
1	38	87	NC_008394.4
4	19	87	NC_008397.2
6	23	86	NC_008399.2
3	32	87	NC_008396.2
2	31	98	NC_008395.2
11	20	85	NC_008404.2
10	18	86	NC_008403.2
8	21	87	NC_008401.2

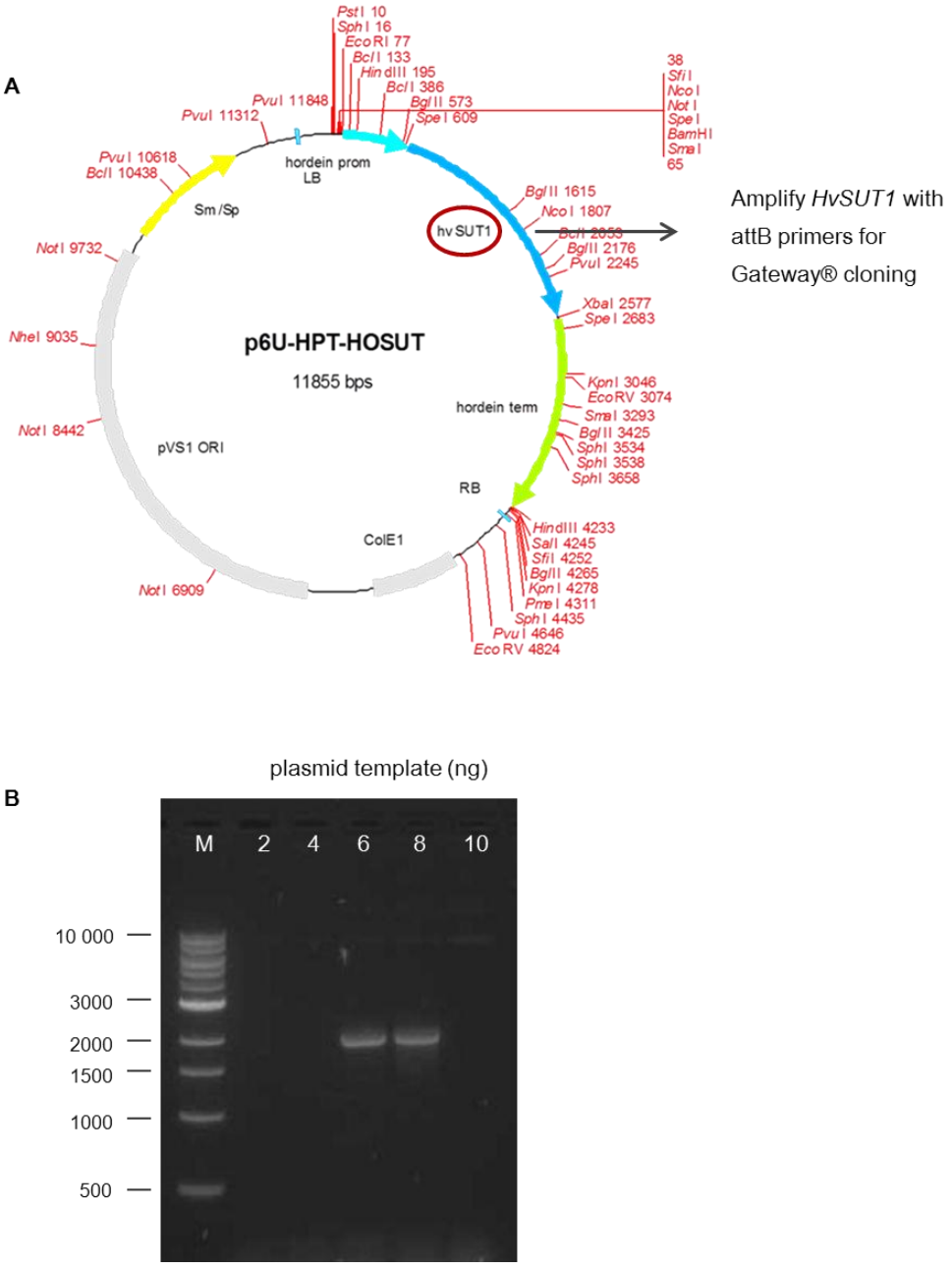


Figure 2.3 Plasmid template and PCR optimisation to amplify *HvSUT1* coding sequence.

(A) Plasmid map of HOSUT plasmid (Weichert *et al.*, 2011) provided courtesy of Hans Weber, IPK Germany. The barley *HvSUT1* sequence circled in red (1967bp) was amplified by two-step PCR with primers containing the attB sites required for Gateway cloning. (B) Titrations of plasmid template in PCR gave a specific band of the correct size with an optimum 6 or 8 ng of plasmid DNA. MW markers in bp.

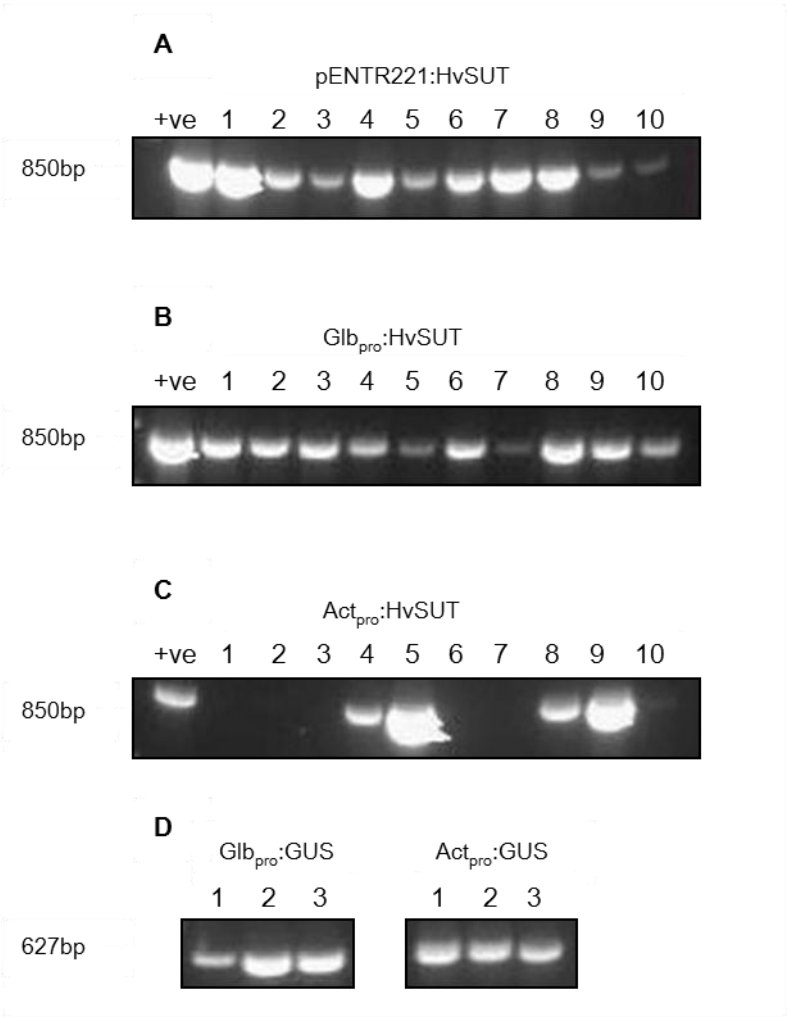


Figure 2.4 PCR analyses of *E. coli* colonies growing on selection media after electroporation with Gateway® cloning products

Ten bacterial colonies growing on selection after transformation with Gateway® reactions between: (A) attB tagged HvSUT1 PCR products and pDONR221 BP reaction to produce entry plasmid pENTR221:HvSUT, (B) pENTR221:HvSUT and pIPKb001:Glb$_{pro}$ LR reaction to produce Glb$_{pro}$:HvSUT and (C) pENTR221:HvSUT and pIPKb003 LR reaction to produce Act$_{pro}$:HvSUT. The 850bp PCR product confirms the presence of the *HvSUT1* gene. The original HOSUT plasmid was used as a positive control. (D) PCR of colonies growing on selection after LR reactions with control plasmid pENTR:GUS and destination plasmids. The 627bp PCR product confirms the presence of the *GUS* gene.

2.4 C). A small number of colonies generated from LR reactions with the destination plasmids and pENTR-GUS were also screened by PCR (Fig 2.4 D). Plasmids from a single colony on each plate that passed the PCR screening step (designated Glb_{pro}:HvSUT-1, Act_{pro}:HvSUT-4, Glb_{pro}:GUS-3 and Act_{pro}:GUS-3) were electroporated into *Agrobacterium* strain AGL1.

Plasmid copy number in *Agrobacterium* is low, therefore DNA yield was too low to be able to analyse the plasmids by digests. Hence, plasmids were electroporated back into *E. coli* (DH10B) and cells were grown to harvest DNA for analysis by PCR and restriction digest. PCR with gene specific primers on controls (empty destination vectors) yielded a number of non-specific bands while the expected band at 1000bp was amplified in Glb_{pro}:HvSUT-1 and Act_{pro}:HvSUT-4 (Fig. 2.5, A). Figure 2.5 A and B shows analytical digests of plasmids obtained from transforming cells after LR reactions (lane 1) alongside digests of plasmids extracted from *Agrobacterium* and passaged through *E. coli* (lane 2), that are identical for Glb_{pro}:HvSUT-1 (Fig. 2.5 B) and Act_{pro}:HvSUT-4 (Fig 2.5 C). Empty destination vectors cut with the same enzyme (lane 3) and no enzyme negative controls (lane 4) also gave the expected band sizes on the gel. These steps provided final proof of the identity of the SUT-OE plasmids that were used in plant transformation. AGL1 harbouring GUS containing constructs were used directly for rice transformation and the tissue specificity of expression was confirmed by a colorimetric GUS staining of rice tissues (section 2.3.4).

2.3.3 Transgenic plants regenerated with SUT-OE constructs

The callus used for *GUS* transformation was 2-3 weeks old, however due to the extra time required to clone *HvSUT1* successfully, callus that was finally used for transformation with *HvSUT1* was approximately 2 months old. After co-cultivation of callus tissue with AGL1 harbouring SUT-OE plasmids on hyg selection plates, very few calli survived and proliferated (Fig. 2.6 A). Healthy white callus continued to proliferate on shoot regeneration media, and healthy shoots and small roots were visible after a few weeks (Fig. 2.6 B). Rapid root growth was observed when plantlets were transferred to hormone free media (Fig. 2.6 C) and plants continued to grow without any obvious phenotype compared with WT plant seeds germinated and grown under the same conditions (Fig. 2.6 D). The time period for each phase of shoot and root regeneration was determined by when shoots or roots were observed, and varied between callus pieces. The total time on shoot regeneration media was approximately 1 month for all constructs. After transfer to hormone free media, roots grew rapidly

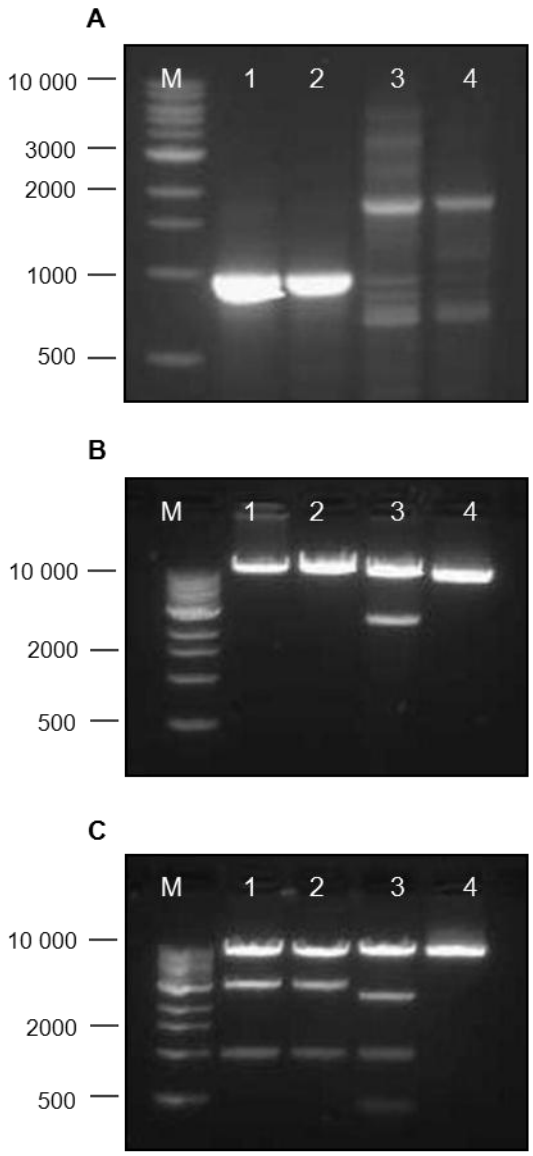


Figure 2.5 PCR and digests to confirm identity of cloned destination plasmids used in rice transformation.

HvSUT1 specific PCR products derived from plasmids extracted from AGL1 transformed with constructs Glb_{pro}:HvSUT (A, Lane 1), Act_{pro}:HvSUT (A, Lane 2) and negative controls pIPKb001:Glb_{pro} (A, Lane 3) and pIPKb003 (A, Lane 4). Plasmid DNA, extracted from AGL1 and re-transformed back in to *E. coli,* was extracted for characterisation by digestion with *Eco*RI. Patterns of bands after restriction digestion were identical to that of plasmid DNA first cloned into E.coli confirmed the identities of Glb_{pro}:HvSUT (B) and Act_{pro}:HvSUT (C) in AGL1. Gels B and C show in Lane 1) digest of the original LR cloned plasmid with *Eco*RI, Lane 2) digest of the plasmid extracted from AGL1 and passaged through *E. coli* with *Eco*RI, Lane 3) digest of empty pIPKb plasmids and Lane 4) corresponding plasmids incubated with no enzyme (negative controls).

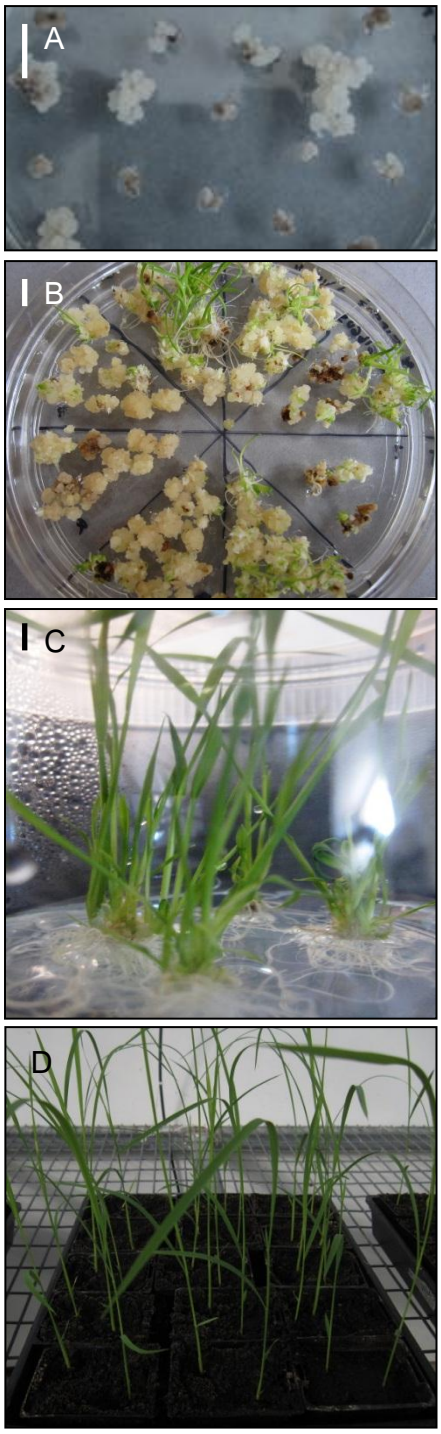


Figure 2.6 Steps in rice callus induction, tissue culture and regeneration of transgenic plants.

A) Rice callus growing on hyg that had been successfully transformed after infection with AGL1 harbouring SUT-OE plasmids proliferated and were white while untransformed callus became necrotic, turned brown and failed to grow. B) Callus from each transformation event (line) were separated and shoots regenerated on media with hormones and considered clones of each line. C) Although small roots appeared on the plantlets during shoot induction, roots grew prolifically on hormone free media and only plants with healthy root systems were transferred to soil. D) Plantlets transferred to soil were 'hardened off', to grow in glasshouse conditions in small pots submerged in water trays. Plants were transplanted to large 2.8L pots after two weeks and grown to maturity.

Scale bar represents 5mm.

and most plantlets were ready to be transferred to soil after 2 weeks. Although fewer calli were used for *GUS* transformation, transformation efficiencies for plants regenerated with *GUS* were much higher compared to plants transformed with *HvSUT1* (Table 2.2). Moreover, more plants were regenerated after transformation with constructs containing the endosperm specific *Glb-1*$_{pro}$ compared with the constitutive *Act-1*$_{pro}$. To confirm that all plants that regenerated contained the transgene, gDNA was extracted from immature leaf material and checked by PCR (Appendix A, PCR 4). Positive bands of the correct size indicated at least one copy of the transgene was present in each T_0 plant (data not shown). Each T_0 plant transformed with *HvSUT1* is identified by a code designated to describe the promoter, putative independent plant line and clone number. For example, the first plantlet derived from a callus transformed with the construct Glb$_{pro}$:HvSUT was designated G1.1. Further plantlets that regenerated from that same piece of callus were designated G1.2, hence plant line 1, clone 2, and so on. A plant regenerated from a second piece of callus derived from a different seed was assigned G2.1. The same numbering convention was given to plants transformed with Act$_{pro}$:HvSUT, however those plants were designated A1.1 etc.

2.3.4 Specificity of promoters examined by GUS expression

Plants transformed with constructs containing Glb$_{pro}$:GUS and Act$_{pro}$:GUS fusions were assayed for tissue specificity by incubating various tissues in X-gluc solution which is expected to produce a blue coloured product if the *uidA* gene is being expressed. The *Glb-1* promoter was expected to drive spatially specific expression of GUS only in the endosperm, while the *Act-1* promoter should drive GUS expression in all tissues constitutively. As expected, there was no GUS activity detected in untransformed callus (Fig. 2.7 A) and callus transformed with Glb$_{pro}$:GUS (Fig. 2.7 B). However, GUS activity was detected in all tissues that were tested of rice plants transformed with Act$_{pro}$:GUS, with GUS staining seen as early as the callus stage (Fig. 2.7 C). Segregation of the Act$_{pro}$:GUS transgene could be seen in the anthers of T_0 transgenic plants (Fig. 2.7 D), as individual pollen grains were differentially stained various shades of blue, indicative of segregation of the transgene following meiosis. No GUS staining was evident in pollen grains from plants transformed with Glb$_{pro}$:GUS (Fig. 2.7 E). Rice grains from non-transgenic Nipponbare plants that were incubated with X-gluc as controls did not produce any blue product (Fig. 2.7 F). Rice grains from plants

Table 2.2 Transformation efficiency of rice regenerated after *Agrobacterium* transformation with overexpression constructs.

Summary of results for transformation and regeneration of rice plants containing constructs for SUT-OE and GUS expression driven by the endosperm specific *Glb-1* or constitutive *Act-1* promoters from rice. Transformation efficiency is calculated as the no of regenerated T_0 plants recovered and grown to maturity divided by the original no. of callus infected with AGL1 * 100 (%).

Construct	**Glb_{pro}:HvSUT**	**Act_{pro}:HvSUT**	**Glb_{pro}:GUS**	**Act_{pro}:GUS**
Number of calli	135	147	50	50
Hyg^r calli	78	89	35	29
Hyg^s calli	57	58	15	21
Callus survival %	58	61	70	58
Total T_0 plants	20	12	28	15
Transformation efficiency %	14.8	8.2	56	30
Putative T_0 lines	6	6	7	7

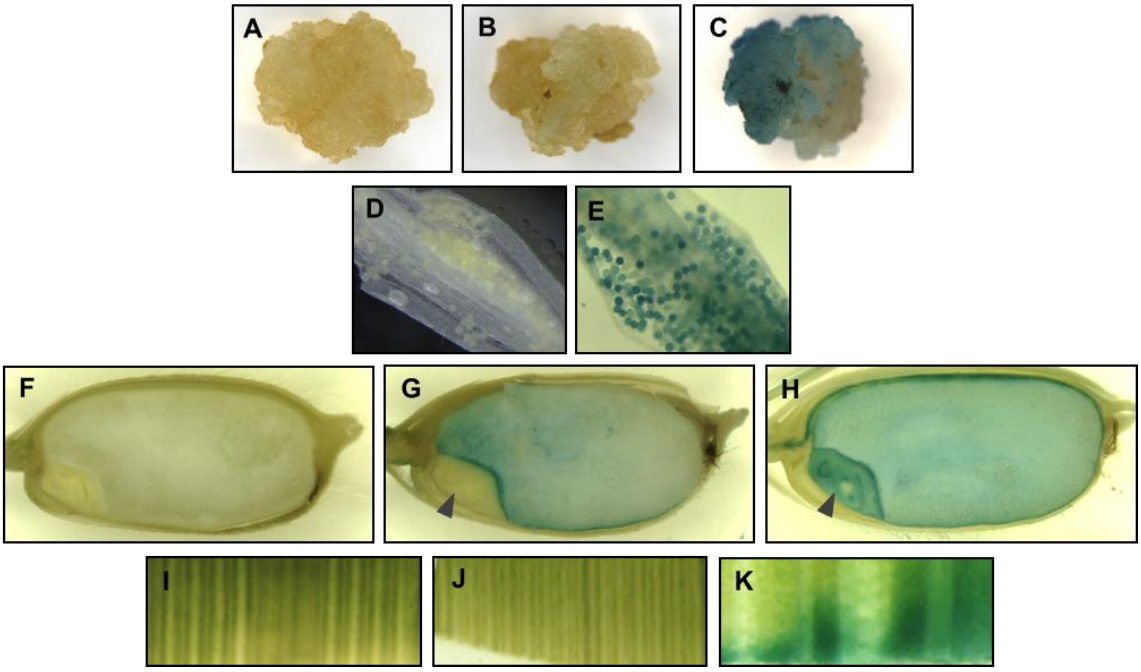


Figure 2.7 GUS expression in various rice tissues driven by the *Glb-1* endosperm specific and *Act-1* constitutive promoters.

Representative images of tissues from non-transgenic control (WT) plants or from rice transformed with Glb_{pro}:GUS or Act_{pro}:GUS constructs after GUS staining showing GUS activity (blue stained tissue). The expression pattern across all tissues studied confirmed the specificity of the *Glb-1* promoter that drives endosperm specific gene expression in rice grain (G) but not in the callus (B), anthers (D), or leaves (J), while the constitutive *Act-1* promoter drives expression of GUS in callus (C), anthers (E), grain (H) and leaves (K). No GUS activity was detected in control WT callus (A), grain (F) and leaf (I).

transformed with either construct were stained blue, however the pattern of staining in the grain tissues differed between Glb_{pro}:GUS (Fig. 2.7 G) and Act_{pro}:GUS (Fig. 2.7 H). There was no staining in the embryo of rice grains transformed with Glb_{pro}:GUS (Fig. 2.7 G, grey arrow) while the embryo of rice grains transformed with Act_{pro}:GUS (Fig. 2.7 H, grey arrow) was stained a deeper blue than the endosperm. Small pieces of leaf tissue from non-transgenic and Glb_{pro}:GUS transformed plants produced no blue colour, but there was visible blue staining, especially along the cut edges of the leaf. Slightly stronger blue staining in the scutellum of Glb_{pro}:GUS grains indicated either expression of GUS in this tissue or leakage of blue pigment into this tissue layer. GUS intensity as well as distribution in the endosperm was also quite variable between different grains from different putative Glb_{pro}:GUS transformed plants (Fig. 2.8). The scutellum and aleurone layers of some grains stained an intense blue (Fig. 2.8, A) while GUS activity was confined to the endosperm in most of the other rice grains (Fig. 2.8, B, C and D). However, none of the grains transformed with Glb_{pro}:GUS showed any staining of the embryo or husks. *GUS* transformed plants were made and grown solely to test tissue specificity of the *Glb-1*$_{pro}$ and *Act-1*$_{pro}$ promoters in rice tissue in T_0, hence no further characterization of these transformed plants was undertaken.

2.3.5 *HvSUT1* transgene inheritance and copy number of plant lines

Stable inheritance and copy number of the *HvSUT1* transgene in the T_1 generation of transformed plants was examined by Southern blotting and hybridisation analysis. gDNA was extracted from representative hyg resistant plants from each putative line. In addition, putative clones of some lines were also investigated to determine whether individual plants regenerated from a single callus were identical. Southern blots of gDNA from immature T_1 plants from putative *Glb-1* and *Act-1* SUT-OE lines hybridised with a *HvSUT1* gene specific probe, showed different patterns of hybridization (Fig. 2.9). This verified that one or more copies of the T-DNA had integrated into the genome of the transgenic plants and that it had been stably inherited in T_1. The hybridisation patterns also indicated that that the putative plant lines were independent transformants. However, not all the putative clones were found to have the same hybridisation patterns when analysed by Southern blot. The plants G2.2 and G2.3 were regenerated from the same piece of callus, but gave differing banding patterns after hybridisation (Fig. 2.9 A). This indicates that these two plants

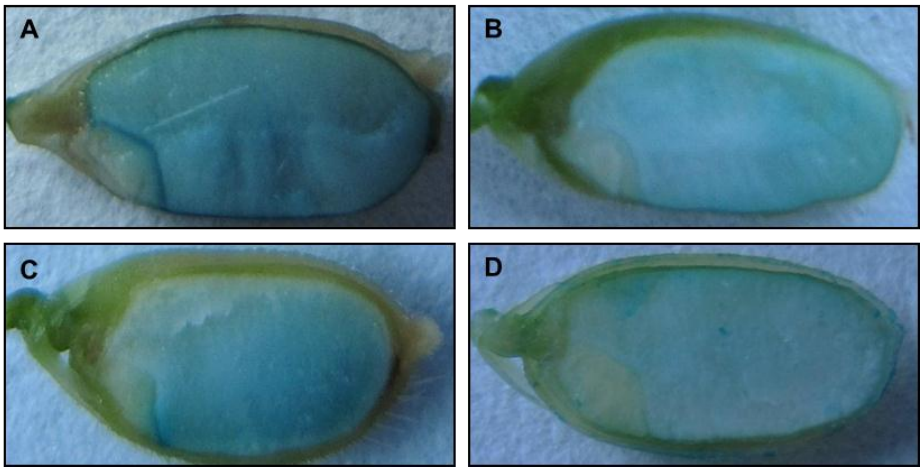


Figure 2.8 GUS expression directed by *Glb-1* in developing rice grains from putative independently transformed plants.

Developing rice caryopses from plants transformed with Glb_{pro}:GUS constructs were harvested at approximately 15 DAA, cut in half longitudinally and incubated in X-gluc solution to analyse GUS activity and localization. Images A – D are four representative grains from four putative T_0 plant lines. Note the differential staining of endosperm tissue in different rice grains.

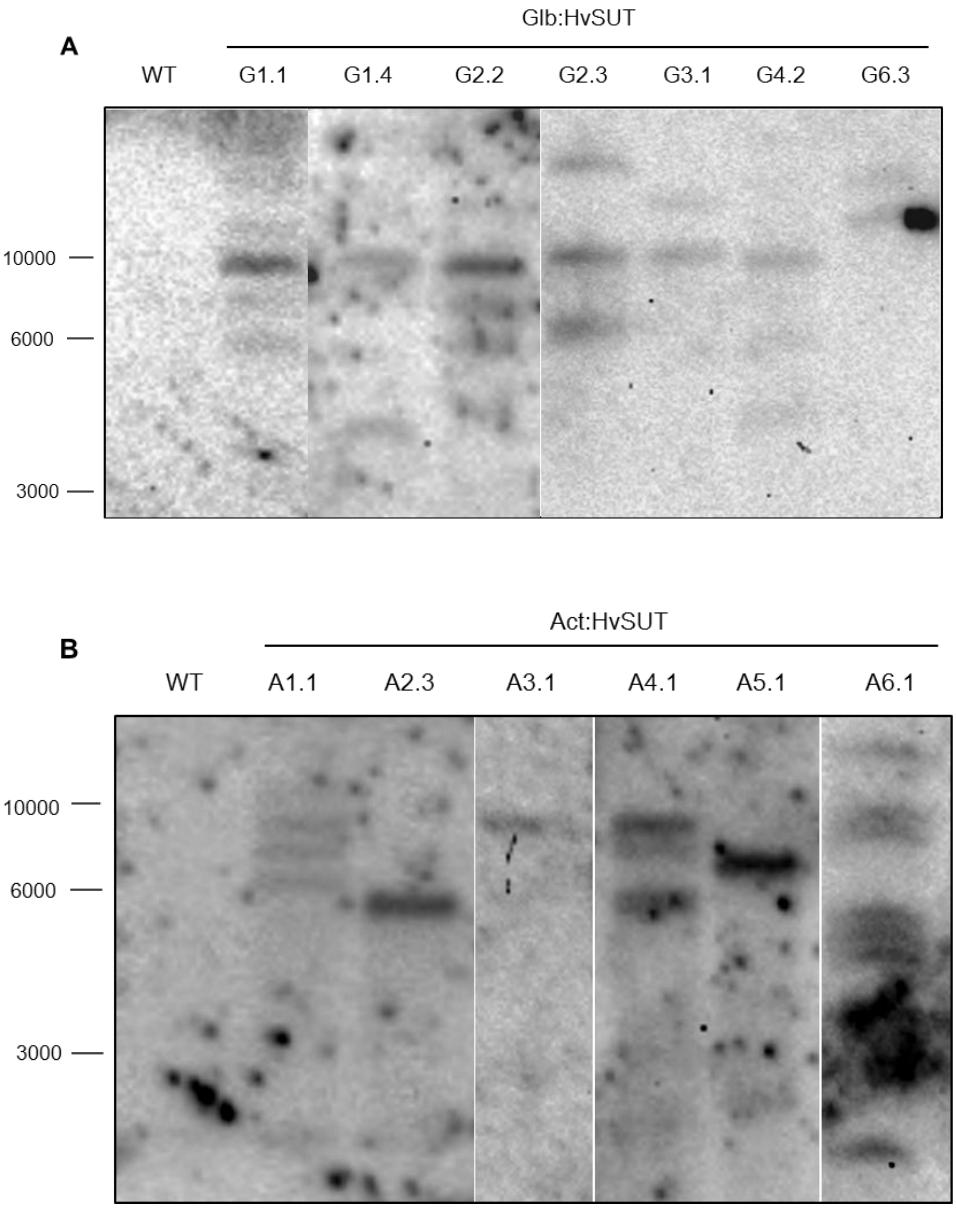


Figure 2.9 Southern blots to determine *HvSUT1* transgene copy number in independent transgenic lines and putative clones.

Blots are composite images of lanes with the lowest background, cut from different Southern blots to give a representative sample of the plant lines that were analysed for copy number. Results presented show gDNA that was digested with either EcoRI HF (Glb:HvSUT) or HindIII (Act:HvSUT), hybridized to a DIG labelled *HvSUT1* specific probe conjugated to Alkaline phosphatase and developed with a chemiluminescent substrate to detect bands. The number of copies of the *HvSUT1* transgene in each plant line is represented by the number of bands in each lane. WT gDNA was used as a negative control. MW marker is in bp.

arose from independent transformation events. Despite numerous attempts to optimise the hybridisation protocol by using varying amounts of DNA (10 to 20μg), varying the digestion conditions, optimising the transfer conditions, varying the hybridisation temperature and increasing or decreasing the probe concentration, blots either gave no bands, high background which sometimes obscured bands or indistinct bands making it difficult to interpret results. Therefore the copy number of transgenic lines was estimated from the best lanes from each blot (results summarised in Fig. 2.9) and further analysis of transgene copy number was estimated following hyg resistance in T_1 plants.

Growth of T_1 progeny under hyg selection (Fig 2.10, A) gave a range of segregation ratios. Selection plates with seeds from WT (left) and two Glb_{pro}:HvSUT transgenic lines (centre and right) show distinct segregation patterns after 7 days. In addition, the plate on the right contains some albino plants which indicates that there were potentially somaclonal changes in plants from this line. Segregation results were further supported by the leaf assays for some of the plant lines (Fig 2.10, B). Leaf tips incubated on selection media (left image) developed distinct necrotic black streaks if sensitive to hyg compared with the healthy green tissue of plants resistant to the antibiotic (middle image). Typical results from one sensitive control plant and two resistant and one sensitive plant line can be seen on the right panel of Fig. 2.10.

A number of unfavourable phenotypes were observed at the seedling stage and during grain filling. Both plants designated G5 did not produce enough viable seed for further analysis. A proportion of the seedlings from lines G2 and G6 were albino (Fig. 2.10 A), while some A2.1 seedlings were variegated (Fig. 2.11 A). During grain filling, the seeds from plant A1.1 were observed to have an unusual phenotype. The palea and lemma did not fuse properly, so that the grain extruded out at the tip but the individual grains were large and appeared to mature normally although fewer grains on the panicle were filled compared to WT Nipponbare plants (Fig 2.11 B). All other plants grew and developed normally and were indistinguishable from WT plants grown under the same conditions (Fig. 2.11 C).

A summary of the copy number estimates from Southern blotting, seed germination and leaf assay segregation analyses for particular plant lines chosen for further analysis are presented in Table 2.3. Segregation ratios determined either by seed germination on hyg or leaf assays gave consistent results where there was data for those plant lines, however the bands in Southern blots did not always complement these results. For example, G3.1 appeared to have two bands in the Southern blot but segregated in a Mendelian fashion. A number of other lines also gave contradictory

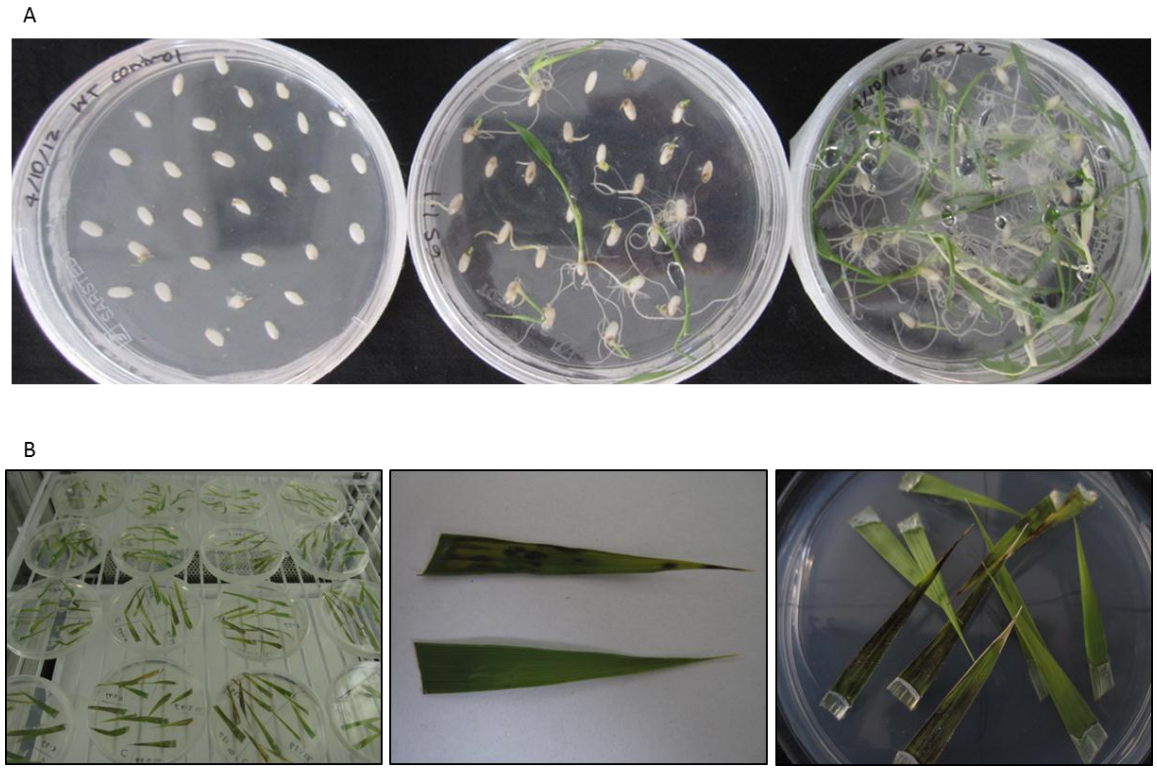


Figure 2.10 Segregation of rice seedlings on hyg selection media and leaf assays of T_1 transgenic plants.

(A) For seed segregation analysis, dehusked seeds were surface sterilized then placed on selection for 7 days before scoring ratios of resistant to sensitive plants. (B) For the leaf segregation analysis, leaves were incubated under constant light for 7 days on MS media with hyg. The middle panel of B shows a comparison of a leaf tip sensitive (top) and resistant (bottom) to hyg. The right panel in B shows a plate divided into four sections, each with replicate leaves from each plant (bottom left quarter with hyg sensitive control plants). Leaves sensitive to hyg showed distinct necrotic black streaks while resistant leaves remained green.

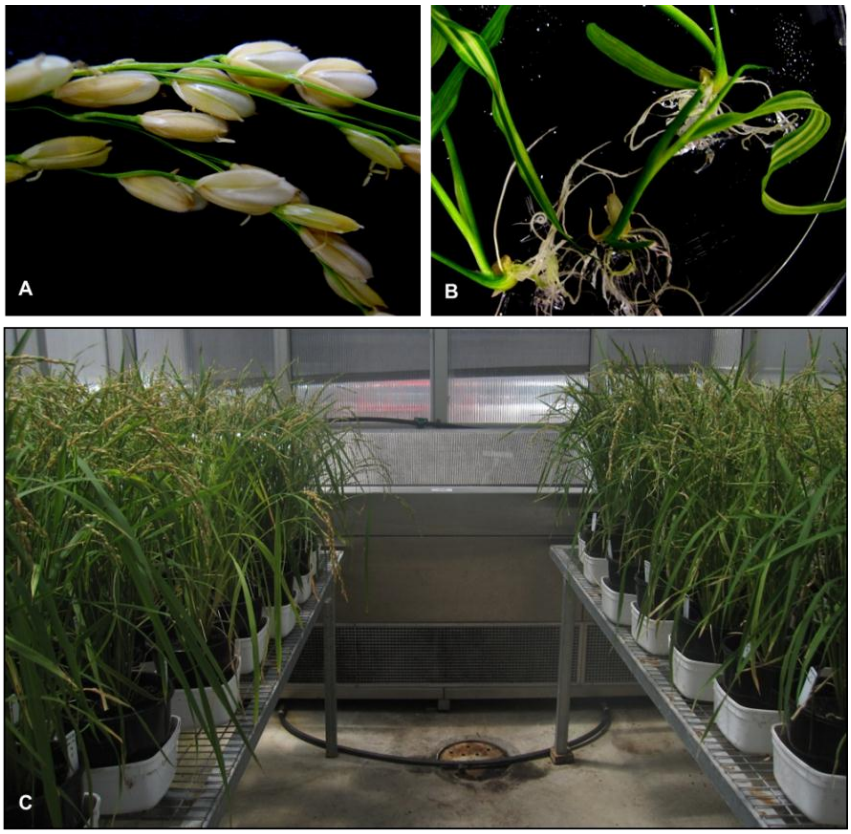


Figure 2.11 Phenotypes of rice plants transformed with SUT-OE vectors.

The range of phenotypes observed from transforming rice with the *HvSUT1* transgene included A) developing rice grains at late grain filling from plant A1.1 showing unusual phenotype, B) variegation in some seedlings from line A2.1. However, the majority of plants from the remaining regenerated lines grew without any apparent phenotypic differences to WT Nipponbare under greenhouse conditions (C) and most produced viable seed for further analysis.

Table 2.3 Summary of copy number in rice lines transformed with SUT-OE constructs chosen for analysis

Transgene copy number estimated by segregation of seedlings on hyg, segregation of plants by rice leaf assay and Southern blot of *HvSUT1* in T_1 progeny.

Plant line. (T_0)	T_1 segregation on Hyg			X^2 value	T_1 Hyg leaf assay			X^2 value	Southern Blot	Copy No.
	Total	Hyg^R	Hyg^S		Total	Hyg^R	Hyg^S			
G1.4	29	25	4	1.94*	30	25	5	1.11*	1	**1**
G3.1	32	24	8	0*	26	16	10	2.51*	2	**2**
G6.2	29	22+	7	0.01*	17	9	8	4.41	2	**2**
A2.3	29	21	8	0.10*	4	4	0	nd	1	**1^**
A5.1	29	19	10	1.39*	4	3	1	nd	1	**1^**

\+ 17 seedlings normal healthy green, 5 albino

^ T_2 plants grown for analysis were heterozygous and still segregating

nd not determined because sample size too small

* accept the null hypothesis, data fits 3:1 segregation ratio, 1 d.f., $p<0.05$

results between these two methods of copy number analysis (Appendix F). Moreover, putative clones for some of the plant lines did not have the same patterns of bands in Southern blotting (e.g. G1.1 and G1.4; G2.2 and G2.3) and segregation results were not consistent between clones.

Based on the copy number results and overall phenotype, a number of lines were chosen for analysis. Given the indistinct Southern blot images, especially for the Glb_{pro}:HvSUT lines, plant lines that behaved as single copy lines as determined by segregation analysis were grown on to obtain further generations of plants. The limited number of plant lines estimated to have one copy of the *HvSUT1* transgene constrained the choice of plants for further study. Lines G1.4 and G3.1 were estimated to have one or two copies by Southern blot, but segregated in a Mendelian fashion. Hence populations of homozygous plants derived from T_2 seeds that gave 100% germination on hyg were grown for analysis. All A2.3 and A5.1 T_1 SUT-OE plants grown to maturity were estimated to be heterozygous by leaf assay, therefore segregating A2.3 and A5.1 T_2 populations grown for further analyses were either hetero- or homozygous. Non-transgenic greenhouse grown Nipponbare (WT), or in later generations null segregants (NT) from selfed transgenic plants were grown as controls.

2.3.6 *HvSUT1* transcript and protein expression in transgenic plants

Semi quantitative RT-PCR derived from RNA extracted from developing caryopses of lines G1.4, G3.1, A2.3 and A5.1 T_2 plants at 8 DAA showed that *HvSUT1* was expressed in both leaf and grain tissue when driven by the constitutive *Act-1* promoter, but also with the endosperm specific *Glb-1* promoter (Fig. 2.12). In grain tissue, endogenous *OsSUT1* expression was consistent amongst all plant lines. In contrast, transgenic *HvSUT1* transcript expression was significantly higher than the *eEf1α* control transcript; lowest in line G1.4 (1.5 fold) and the highest in G3.1 (6.5 fold). Both *Act-1*$_{pro}$ driven constitutive lines had approximately the same level of *HvSUT1* expression (4 fold compared to *eEf1α*). In general, the *HvSUT1* transgene was expressed more highly than the endogenous *SUT1* at 8 DAA.

In leaf tissue, the presence of *OsSUT1* transcript was expected, however *HvSUT1* transcript was also amplified at approximately the same level (~ 1 fold) in the *Glb-1* driven lines. This was unexpected as this endosperm specific promoter appeared to drive GUS specifically in the grain (section 2.3.4) and previous studies had

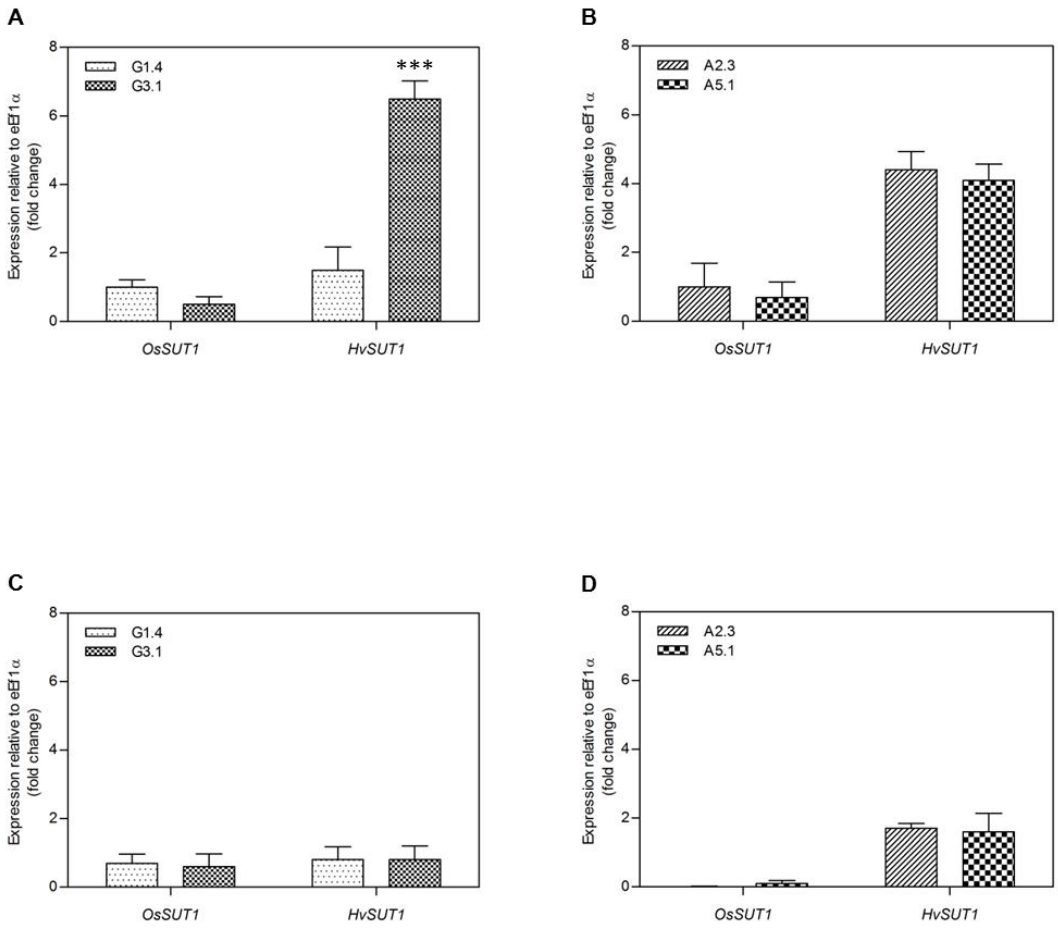


Figure 2.12 Endogenous and transgenic *SUT1* transcript expression in grain and leaf

Expression levels of endogenous *OsSUT1* and transgenic *HvSUT1* genes expressed as fold change relative to housekeeping gene *eEF1α*. RNA was extracted from developing grains (8 DAA) from plants transformed with *HvSUT1* driven by endosperm specific *Glb-1* (A) or constitutive *Act-1* (B) promoters and leaf samples from the same plants (C and D respectively). Values are means of three replicates + SD. Significant difference according to t-test between comparative plant lines *** $p<0.001$

demonstrated its specificity in rice tissues. In contrast, the *Act-1* driven lines had very low endogenous *SUT1* expression in leaf tissue but 2 fold change in *HvSUT1* transcript relative to the housekeeping internal standard *eEf1α* which could indicate some interaction between endogenous and transgenic *SUT1* expression.

Immunoblots of crude total membrane protein preparations from rice grains at grain filling probed with the anti-HvSUT1 antibody gave a band at the predicted molecular weight of HvSUT1 (~55 kDa) in G1.4 and A2.3 lines (Fig. 2.13, A). The 55 kDa band was absent in the non-transgenic control protein samples, but was more prominent in the G1.4 protein samples compared with membrane proteins from line A2.3. However another prominent non-specific high molecular weight band (~68 kDa) also cross reacted with the antibody. The band at 68 kDa persisted in all immunoblots on rice membrane proteins probed with anti-HvSUT1, including proteins prepared from non-transgenic plants. This unidentified protein appeared to be expressed consistently between replicates and plant lines.

Leaf protein samples probed with the anti-HvSUT1 antibody showed high cross reactivity to many non-specific proteins in the sample (Fig 2.13 B). No clear band was detected at 55 kDa for either the *Glb-1*$_{pro}$ or the *Act-1*$_{pro}$ driven lines. Instead, approximately 6 bands of varying molecular weight (from 30 to 100 kDa) were present for each sample, including the negative controls.

Immunoblots with the commercial anti-H^+-ATPase antibody detected a band at the correct size (100 kDa) but also cross reacted with lower molecular weight bands between 58 to 68 kDa in both grain (Fig. 2.14 A).and leaf samples (Fig. 2.14 B). The non-specific bands did not seem to interfere with detection of the target protein, hence further immunoblot images are presented showing only the bands of interest. Expression of the proton pump H^+-ATPase did not change relative to HvSUT1 expression in T_2 plants (Fig. 2.14 C). At 10-12 DAA, there was strong expression of HvSUT1 protein in G1.4 compared with much lower protein expression in A2.3 rice grains. Protein samples from NT controls showed no band cross reacting with anti-HvSUT1 at 55 kDa. The same protein samples probed with anti-H^+-ATPase antibody showed consistent H^+-ATPase protein expression in all biological replicates of NT and G1.4 plants. Although increased levels of HvSUT1 protein were found in the transgenic line, there was no effect on H^+-ATPase protein accumulation.

Total membrane proteins were extracted from a number of other *Glb-1* SUT-OE transgenic lines to determine whether copy number of the *HvSUT1* transgene correlated with higher protein expression (Fig. 2.15 A). Immunoblots of developing

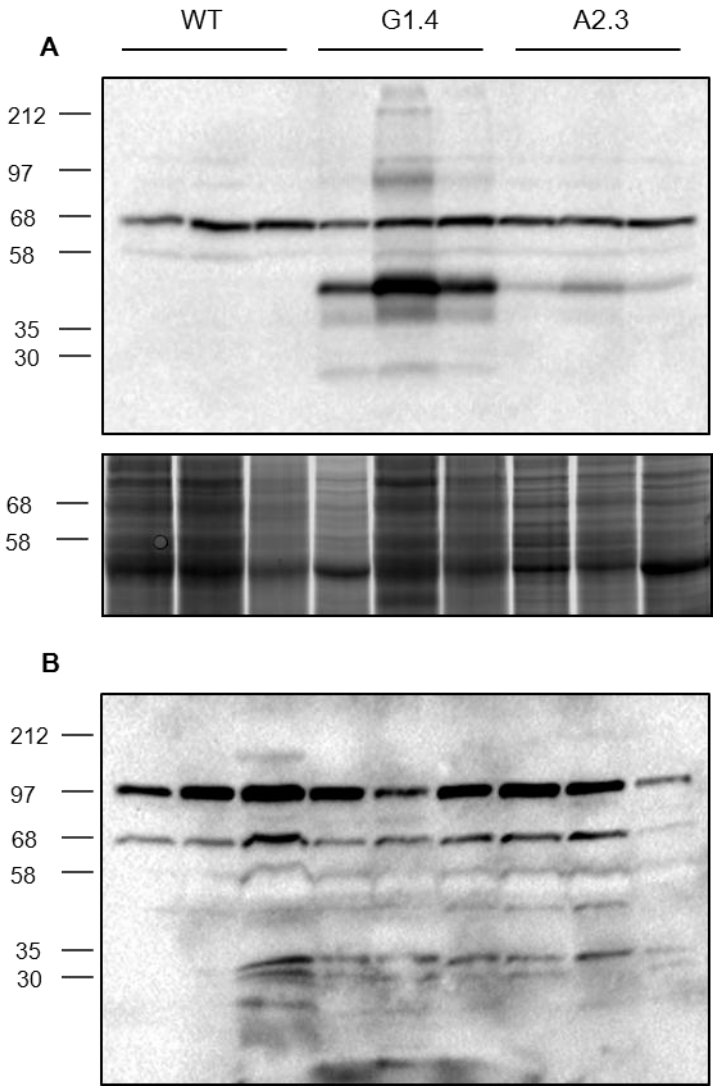


Figure 2.13 Immunoblots of grain and leaf proteins probed with anti-HvSUT1 antibody.

Total crude plasma membrane protein samples from grain (A) and leaf (B) of 3 biological replicates from each line were run on 12% SDS PAGE gels, transferred to nitrocellulose and probed with affinity purified anti-HvSUT1 (1:500), followed by Goat anti-rabbit IgG-horseradish peroxidase HP conjugated secondary antibody (1:5000). Blots were visualized using a chemiluminescent substrate and 1 sec exposures captured with a CCD camera. Pre-stained markers were imaged with white light and aligned with chemiluminescent images to determine molecular weights of bands. Duplicate grain protein samples were run on a gel and stained with SYPRO® Ruby (Life Technologies) to indicate protein loading. MW of proteins in kDa.

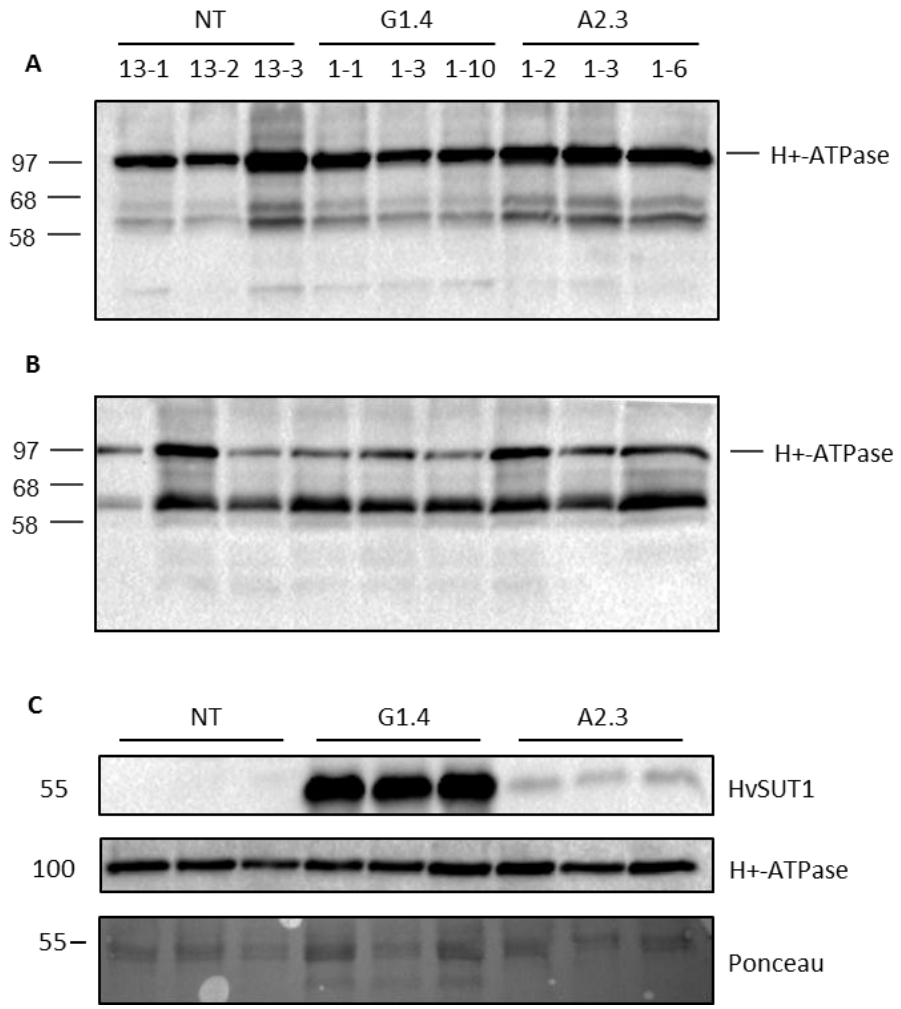


Figure 2.14 Immunoblots of grain and leaf proteins probed with H^+-ATPase commercial antibody.

H^+-ATPase protein expression in grain (A) and leaf (B) samples and compared with expression of HvSUT1 protein in grain (C). 3 biological replicates from each line were run on 12% SDS PAGE gels, transferred to nitrocellulose and probed with commercial anti-H^+-ATPase(1:5000) or HvSUT1 as previously described, followed by Goat anti-rabbit IgG-horseradish peroxidase HP conjugated secondary antibody (1:5000). Blots were visualized using a chemiluminescent substrate and 1 sec exposures captured with a CCD camera. Pre-stained markers were imaged with white light and aligned with chemiluminescent images to determine molecular weights of bands. MW of proteins in kDa.

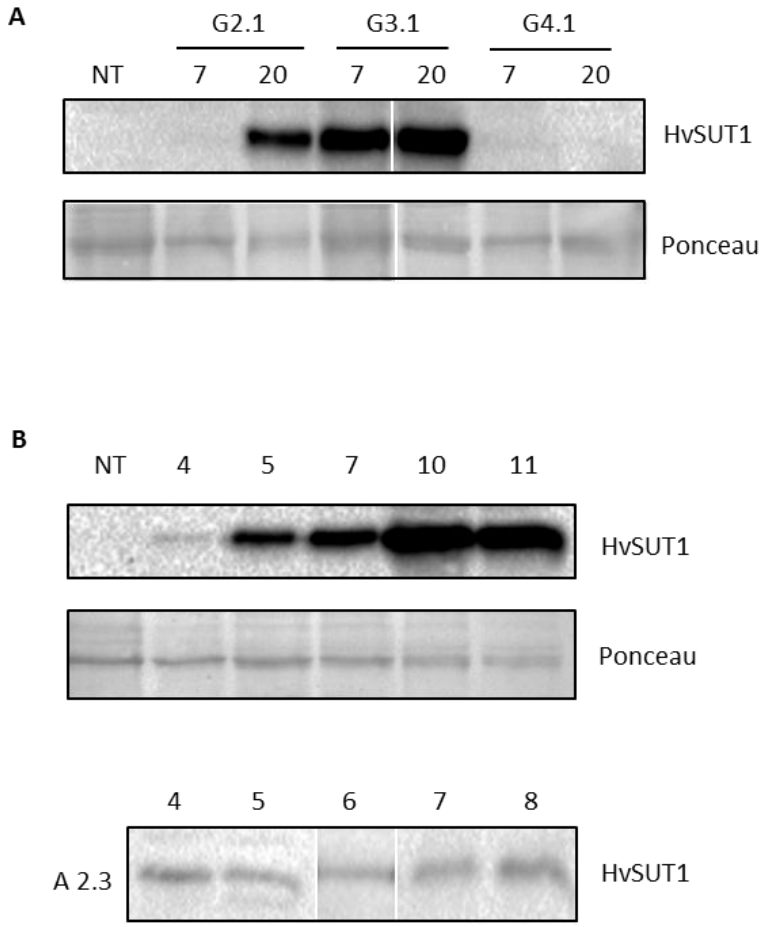


Figure 2.15 HvSUT1 expression in independent Glb_{pro} SUT-OE lines and changes in protein expression during grain filling

Immunoblot images showing only the band of interest at 55kDa (HvSUT1) with Ponceau staining of total protein as loading controls. (A) A number of different transgenic lines carrying various copies of Glb_{pro}:HvSUT transgene were analysed for HvSUT1 protein expression at early and late grain filling. (B) An immunoblot of membrane proteins prepared from immature grains of line G1.4 from 4, 5, 7, 10 and 11 days after anthesis shows changes in HvSUT1 protein expression compared with membrane proteins extracted from line A2.3.

grains of lines G2.1, G3.1 and G4.1 containing potentially 4, 2 and >5 copies respectively, probed with anti-HvSUT1 at early (7 DAA) and late (20 DAA) grain filling suggested that higher copy number did not equate with greater levels of HvSUT1 protein. While G3.1 (estimated to have 2 transgene copies) had high expression of HvSUT1 protein at both time points, detectable amounts of HvSUT1 was only seen at the later time point for G2.1 (4 copies). Despite the higher predicted number of copies of HvSUT1 in G4.1 (>5), no protein was detectable by immunoblot at either 7 or 20 DAA.

The *Glb-1* SUT-OE line G1.4 was investigated further for variable HvSUT1 protein expression at crucial time points in early grain development (Fig 2.15 B). There was low but detectable HvSUT1 expression at 4 DAA. This was followed by increased protein expression at 5 and 7 DAA. Expression is higher again at 10 DAA and remains high at 11 DAA. In comparison, HvSUT1 protein expression in line A2.3 remained consistently low between 4 and 8 DAA.

2.4 Discussion

A large number of factors were taken into account to achieve maximum success in transformation, however biological systems do not always behave predictably in each situation. Despite the commonplace protocols that have been developed for rice transformation, the number of independent transgenic lines regenerated in this study was quite low relative to transformation efficiencies of 50 and 85% reported in Aldemita and Hodges (1996) and Hiei *et al*. (1997) respectively. The higher efficiency of transformation with GUS compared to HvSUT1 in the present study could be related to the age of the callus used for transformation. Callus at two weeks would have much higher active cell division than callus maintained for two months on media (Toki *et al*., 2006). Therefore, the earlier infection of the callus with the GUS constructs could be the reason for the higher percentage of plants transformed with GUS compared with SUT-OE vectors. Furthermore, a gene such as *HvSUT1* that is central to carbon metabolism could potentially exert a strong selective pressure if expressed or leaky expression occurs in callus tissue. Generally, the lower efficiencies of transformations with *Act-1*$_{pro}$ compared with *Glb-1*$_{pro}$ fusion constructs support this supposition. Hygromycin resistance from the *hpt* gene driven by the constitutive *ZmUbi* promoter can also be a cause of strong negative selection. The damaging effects of transgene expression in rice cells could thus account for the modest number of transformants obtained in the present study, and could explain the differences in transformation

efficiencies observed between the constructs containing *Act-1*$_{pro}$ and the *Glb-1*$_{pro}$ fusion constructs.

Histochemical GUS staining of plant tissues from rice transformed with the Glb$_{pro}$:GUS construct demonstrated that the cloned promoter sequence was functional and drove transgene expression in the endosperm. This promoter sequence did not drive GUS expression in other rice tissues, therefore we can conclude that it was grain specific, although the assay was not sensitive enough to determine if expression was only in the endosperm. Detailed analysis and sequencing of the amplified and cloned *Glb-1* promoter sequence used in this rice transformation unequivocally confirmed that it contained all the regulatory regions required for strong endosperm specific expression in rice. In comparison, plants transformed with the Act$_{pro}$:GUS construct expressed GUS in all tissues, as expected for a constitutive promoter. The 26 kDa globulin gene is encoded by a single copy gene and expressed specifically in endosperm (Qu and Takaiwa, 2004; Wakasa *et al.*, 2011). However, analysis of the 981 bp promoter region revealed homologous conserved regions in multiple positions in all chromosomes of the rice. Deletions of the promoter approximately 300 bp upstream of the gene in some cereal storage protein sequences caused a loss of specificity in gene expression (Takaiwa *et al.*, 1996; Wu *et al.*, 1998). However, in this case the homology is in the first 380 bp of the promoter region and includes repeat AT-rich regions that are thought to contain potential cis-elements for transcriptional activation of genes. Indeed, this 5' flanking region in rice *Glb-1* shares consensus sequences with Albumin, another seed storage protein (Nakase *et al.*, 1996). GUS expression driven by the rice promoter *GluB-1* was decreased when the AT-rich region in the distal 5' flanking region was deleted (Takaiwa *et al.*, 1996). It is thought that the deletions caused the loss of the AT-rich regions necessary for transcriptional activation of gene expression. These homologous regions are also present in other promoters of the multigenic cereal storage protein families (Nakase *et al.*, 1996). This indicates that these regions are essential for functional activity of these promoters. It was therefore unavoidable that other non-specific regions were amplified in this attempt to isolate the entire *Glb-1* promoter sequence from rice genomic DNA.

Exhaustive analysis of the cloned gene sequences by PCR, analytical restriction digests and sequencing in all the Gateway cloning steps confirmed the amplification and recombination of the *HvSUT1* transgene into the pENTR and pDEST plasmids was complete and error free. Transgenic plant lines were successfully generated from the molecular cloning and transformation of rice callus tissue with constructs containing *HvSUT1* driven by the endosperm specific rice *Glb-1* or rice *Act-1* constitutive promoter.

Selection on antibiotic selection media ensured the presence of at least one copy of the construct. Molecular analysis and segregation of the rice lines determined putative transgene copy number in each transgenic line. Copy number estimates using each analysis method were not always comparable. A number of explanations for this disparity in copy number can be proposed. Southern blotting, normally the most reliable in copy number analysis for transgenic plants, did not give as clear results as expected. The high background and absence of clear bands made copy number difficult to estimate in some plant lines. Although the blots were washed under high stringency conditions, overestimation of copy number due to non-specific hybridisation of the DIG-labelled probe cannot be ruled out. Segregation analysis, the other commonly used method for copy number determination, can also give unreliable estimates in transgenic plant lines. This could be due to a number of reasons including integration patterns of transgenes in the host genome and the instability transgenes can introduce into a plant genome, particularly in the early generations. T-DNA insertions through *Agrobacterium* transformation arise through illegitimate recombination (Mayerhofer *et al.*, 1991) and can result in unintended rearrangements of the plant genome as well as recombination in the actual T-DNA strand upon integration (Gheysen *et al.*, 1987; 1991). Complicated arrangements of tandem repeats of transgenes can give unexpected segregation patterns on selection (Stahl *et al*., 2002). Although segregation analysis relies on the assumption that transgenes are inherited according to Mendelian laws, complex recombination events within transformed plants over generations could add confusion to the observed ratios of plant progeny. Nevertheless, segregation analysis is widely used in most transgenic plant studies to estimate copy number, hence plants that behaved as single copy lines on selection in the present study were grown for further analysis, although the Southern blots may indicate more than one copy. Seeds from transgenic plants were germinated on hyg, therefore ensuring that plants raised for analysis contained at least one copy of the transgene.

High expression of *HvSUT1* relative to the endogenous *OsSUT1* transcript was measured in grain tissues in each of the two *Glb-1* and *Act-1* lines, establishing that the *HvSUT1* transgene was active in that tissue. *HvSUT1* transcript was also detected in *Act-1* driven leaf tissue. Although the *Glb-1* promoter is supposed to be endosperm specific, *HvSUT1* transcript was also unexpectedly detected at the same levels as *OsSUT1* in leaf tissue of plants transformed with the Glb_{pro}:HvSUT construct. Amplification of *eEf1α* and *OsSUT1* with intron spanning primers did not indicate gDNA contamination therefore this result was unexpected. Qu and Takaiwa (2004) reported that *GUS*, driven by the endosperm specific 10 kDa prolamin promoter, was also

detected in leaf tissue. However, when the Nos terminator (*Nos*T) was replaced with a short non coding sequence from the native gene after the stop codon, promoter specificity was restored. The gene construct used in this study fused the *Glb-1* promoter upstream of the *HvSUT1* coding sequence but was terminated by *Nos*T from *A. tumefacians*. Likewise, Wu *et al.* (1998a) and Qu and Takaiwa (2004) reported some weak GUS activity in rice leaf and stem tissue of plants transformed with constructs containing the *Glb-1* promoter and *Nos*T. There have been no reports that the tissue specificity of the *Glb-1* promoter is affected by the terminator sequence, however it is possible that termination by a foreign sequence could result in leaky expression. Furtado *et al.* (2008) however, also used *Nos*T in their rice transformation constructs, but did not report any non-specific gfp expression in other tissues when *gfp* was driven by the *Glb-1* promoter. Given that the same promoter and terminator sequences were used in all these studies, the difference between them could possibly be explained by the differences in mobility and background signals inherent in the reporter proteins GUS and gfp (de Ruijter *et al.*, 2003) rather than the specificity of the *Glb-1* promoter.

A number of non-specific bands were always evident in immunoblots using the synthesized anti-HvSUT1 antibody. However, these non-specific bands were present in non-transgenic as well as transgenic protein samples. It was very clear in rice grain protein samples that transgenic rice transformed with *HvSUT1* was expressing HvSUT1 protein. The possibility exists that anti-HvSUT1 could be cross reacting with the endogenous OsSUT1 but given the following argument, that seems unlikely. The 17 amino acid peptide sequence synthesized for antibody production was chosen as it showed low similarity with OsSUT1 in a sequence alignment. Moreover, there was consistently no signal at ~55 kDa for any of the non-transgenic samples compared with the transgenic samples, which indicates the antibody was selective for HvSUT1 at 55 kDa. Non-specific bands were also detected in blots probed with the commercial anti-H^+-ATPase antibody. A possible explanation for the non-specificity of both antibodies could be related to the quality of the protein samples. Grain samples prepared for immunoblotting included the lemma and palea as well as the grain pedicels. Also, other membrane proteins from the mitochondria and vacuoles were not excluded using this technique. Crude rice leaf membrane protein samples are likely to contain a diverse range of proteins (Islam *et al.*, 2004) therefore signal from the relatively low expression of HvSUT1 in leaves could be masked by the higher abundance of cross reactive proteins in the sample or by contaminating compounds, such as phenolics. Unfortunately no HvSUT1 protein was able to be detected in leaf tissue using the anti-HvSUT1 antibody. One of the most abundant proteins likely to be found in

photosynthetic tissue is ribulose 1.5-bisphosphate carboxylase/oxygenase (RuBisCo). Rice RuBisCo large subunit (rbcL) (P0C512, UniProt) is estimated to be ~53 kDa, which is very close in size to HvSUT1 protein (55kDa). The brief spin prior to loading the SDS-Page gels was not enough to remove highly abundant RuBisCo from leaf protein samples, therefore it is likely rbcL was masking the detection of the much less abundant HvSUT1 protein. Hence it could not be determined whether the *Act-1* SUT-OE lines were expressing HvSUT1 protein constitutively. Given more time, optimising the purification of plasma membranes through methods such as dextran-polyethylene glycol two-phase partitioning (Santoni, 2007) could remove contaminating proteins, allowing the detection of HvSUT1 in leaf tissue immunoblots to clarify this issue.

Tentative copy number of transgenes in independent *Glb-1* driven lines did not correlate with levels of protein expression in rice grains. Introduction of a transgene similar to an endogenous gene may result in co-suppression of both genes (Hobbs *et al.*, 1993). This could explain the transgenic plant line G4.1, estimated to have 4 or 5 transgene copies, but with undetectable levels of HvSUT1 protein in developing grains at either 7 or 20 DAA. Another plant line, G2.1 with an estimated 4 copies of *HvSUT1*, had detectable levels of HvSUT1 protein at 20 DAA but not at 7 DAA which suggests that the *Glb-1* promoter in this line was active at a later stage of grain filling. In contrast, HvSUT1 protein expression in lines G3.1 and G1.4 were detected at all the time points investigated. There was also no clear relationship between transgene expression and protein levels in the transgenic lines studied at early grain filling. Higher levels of *HvSUT1* transcript in G3.1 and both *Act-1* driven lines compared to G1.4 did not equate to higher signals for SUT1 protein detected in immunoblots for those lines. Instead, there was more abundant HvSUT1 protein in G1.4 compared to A2.3. These findings are consistent with other studies in wheat (Bagnall *et al.*, 2000) and peas (Rosche *et al.*, 2002) where presence of *SUT1* transcript did not match with SUT1 protein. Like those studies, there must be some translational control in regulation of SUTs in these rice plants, although the mechanism has not been elucidated. Unfortunately the position of transgene integration into the genome of each of the plant lines could not be determined, however, this could also contribute to the variability in transgene expression between lines.

Plasma membrane H^+-ATPase activity provides crucial energy for secondary transport of many compounds and ions in plant cells (Morsomme and Boutry, 2000). There is evidence that activity of PM H^+-ATPase is regulated by changes in the apoplast such as varying sugar concentration and pH (Portillo *et al.*, 2000). Activation of the PM H^+-ATPase is also thought to be involved in cellular expansion in plants (Morsomme

and Boutry, 2000). Experimental evidence from *A. thaliana* indicates that phosphopeptides of the two most abundant H^+-ATPases increased with increasing sugar supply and plasma membrane vesicles supplied with sucrose showed greater proton pumping activity (Niittyla *et al.*, 2007). For these reasons, we might have anticipated changes in H^+-ATPase protein levels in the transgenic lines overexpressing *HvSUT1*. However, compared to the NT plants, G1.4 plants with high expression of HvSUT1 protein did not have any greater abundance of H^+-ATPase. Other studies suggest that the abundance of H^+-ATPase protein isoforms in plants is relatively constant despite changes in cytosolic conditions and it is the activity of proton pumps changed through signal activation rather than bulk changes in protein abundance affecting H^+-ATPase activity (Gaxiola *et al.*, 2007). In addition, changes in the pump's activation state through enzyme activation of regulatory regions in the N- or C-terminus and high turnover of H^+-ATPase protein can affect activity (Portillo *et al.*, 2000). Indeed, *AHA1* and *AHA2*, members of the Arabidopsis PM H^+-ATPase family are identified as ion homeostasis housekeeping genes and are expressed in all tissues. This could explain the lack of any noticeable difference in abundance of H^+-ATPase protein in immunoblots comparing control and transgenic lines. Instead, functional assays would need to be carried out to detect any measurable differences in proton pump activity.

Some transgenic rice plants in this study showed interesting phenotypes after regeneration with the *HvSUT1* transgene. Somaclonal variation has been widely reported in tissue culture and can occur through epigenetic changes that may be induced by steps in culturing plant tissues (Kaeppler *et al.* 2000; Miyao *et al.*, 2012). A detailed study by Sun *et al.* (1983) of somaclonal variations in populations of rice regenerated from callus culture identified a wide range inherited traits such as multiploidy, dwarfism, awned grains and chloroplast mutations resulting in albinism or variegation. Some of the lines generated in this study showed potential evidence of somaclonal variations, such as albinism and variegation. The frequency of certain mutations in rice plants regenerated from callus is apparently quite high and can be transmitted through to a number of generations (Oono, 1985). DNA from sibling plants regenerated from the one callus can also show totally different Restriction Fragment Length Polymorphisms (RFLPs) therefore Muller *et al.* (1990) have described calli as being a "genetic mosaic". This supports the findings in this study that some of the putative 'clones' regenerated from single callus pieces may have arisen from heterogeneous calli as they did not contain the same pattern of transgene integration.

This study did not investigate the effect of overexpressing *HvSUT1* on the other four rice *SUT* genes. All five *SUT*s are expressed in the developing grain, although their expression patterns differ dramatically (Aoki *et al.*, 2003). All genes other than *SUT1* express from 1 DAA, while *SUT1* is not detected until 3 DAA, with peaks in expression at 5 and 7 DAA. Since the functional roles of the other SUT proteins in rice have not been well studied, they could very well show significantly changed expression patterns in these transgenic plants. We cannot rule out that there have been unintended effects on the expression of the other SUT genes in these plants. Further work is required on the effect of overexpressing *HvSUT1* on the other four endogenous SUT genes in these transgenic rice lines.

This chapter has described the generation and characterisation of numerous rice lines overexpressing the barley *HvSUT1* gene driven either by the *Glb-1* endosperm specific or *Act-1* constitutive promoter. RT-PCR and immunoblots have shown that these plants express transgenic *HvSUT1* RNA and HvSUT1 protein. Putative homozygous *Glb-1* SUT-OE and sibling NT lines and heterozygous *Act-1* SUT-OE plant lines have been chosen for further analysis of the effects of *HvSUT1* overexpression on nutrition, growth and yield of rice plants. The results from these analyses will address the hypotheses of this study.

Temporal and spatial changes in mineral nutrients in rice grains overexpressing *HvSUT1*

3.1 Introduction

Micronutrient deficiencies in humans are responsible for high rates of disease and illness worldwide, particularly in developing countries where dietary limitations due to poverty limits the intake of more nutrient rich foods (Welch and Graham, 2002). Cereal staples, and rice in particular, are very low in essential mineral nutrients such as zinc (Zn) and iron (Fe). Moreover, processing of rice by polishing can remove 60 to 80% of the nutrients which are concentrated in the outer bran layers of the grain (Hansen *et al.*, 2012). Conventional means of micronutrient biofortification through plant breeding and nutrient fertilizer application has had varying success in cereal crop species (Gregorio, 2002; Ghandilyan *et al.*, 2006). The natural genetic potential of cereals is limited and breeding new crop varieties through successive rounds of crossing and trait selection is laborious and lengthy. Nutrient fertilization can increase nutrients in cereals however this strategy is not sustainable and negative effects from fertilizer overuse has been well documented (Rengel, 1999). It is therefore imperative to research novel approaches to biofortify cereal crops, such as rice, with enhanced nutrients for future food production.

Molecular genetic strategies can be used to manipulate and enhance plant processes to promote desirable characteristics in cereals. Attempts to increase Zn and Fe in rice endosperm through transgenic means have focused on overexpressing genes for metal chelators and transporters. For example, numerous researchers have introduced ferritin, a strong Fe chelator, into grains to create a stronger storage sink (Goto *et al.*, 1999; Drakakaki *et al.*, 2000; Vasconcelos *et al.*, 2003; Qu *et al.*, 2005; Sivaprakash *et al.*, 2006). Genes for metal transporters such as nicotianamine synthase (*NAS*) and yellow stripe-like (*YSL*) proteins have been overexpressed in rice to increase Zn and Fe two to three fold in the grain (Lee *et al.*, 2009; Masuda *et al.*, 2009; Johnson *et al.*, 2011). Wirth *et al.* (2009) and Masuda *et al.* (2012) stacked multiple genes involved in storage, transport and break-down of anti-nutrients (phytate) to increase Fe in the endosperm by up to six times more than that found in wild type (WT). Corresponding increases in Fe can result in increasing the expression of non-target endogenous genes critical in mineral nutrient transport (Wang *et al.*, 2013). Unfortunately, many metal transporters are not specific but can also have a high affinity for heavy metal contaminants such as cadmium (Cd) (Ishimaru *et al.*, 2012). Some metal chelators for Zn and Fe, such as NRAMP5, have been shown to transport Cd in the phloem where

they are transported and then loaded into the rice grain (Yoneyama *et al.*, 2010; Ishimaru *et al.*, 2012). The heavy metal transporters HMA2 and HMA4, which are localized to the plasma membrane, are known to transport both Zn and Cd (Colangelo and Guerinot, 2006). Increases in toxic metals in edible portions of transgenic grains could be an undesirable side effect of specifically increasing metal transport proteins to increase grain micronutrients. Therefore, despite success in strategies leading to increased mineral nutrient concentration in cereal grains, alternative approaches for transgenic biofortification of cereals should also be explored.

As well as nutrient concentration, the spatial distribution of mineral nutrients in cereal grains has been an area of great interest especially with the development of sensitive techniques that can visualize elements *in situ*. Staining grains with metal specific dyes has previously shown that Zn and Fe are distributed mainly in the bran (Ozturk *et al.*, 2006; Choi *et al.*, 2007) however these techniques do not allow visualization of tissue specific localization of multiple elements. New techniques such as Synchotron XFM, NanoSIMs and LA ICP-MS have now been used to generate more detailed images of mineral distribution in grain tissues. These techniques confirm that the outer bran layers and the embryo is especially rich in mineral nutrients with very little penetrating into the inner endosperm (Lombi *et al.*, 2009; Moore *et al.*, 2010; 2011; Wu *et al.*, 2013). The distribution of minerals correlates with the higher amount of gene transcript for mineral transport and storage in the aleurone compared with the endosperm (Tauris *et al.*, 2009). The paucity of transcripts for metal transporters in the endosperm also explains why endosperm cells either do not have or have very little metal transport activity (Tauris *et al.*, 2009). Higher levels of phytate (Iwai *et al.*, 2012) and protein bodies (Prom-u-thai *et al.*, 2008) primarily in the embryo and aleurone are also associated with the tissue localization of mineral elements. Nutrient flow within the endosperm is limited to the relatively slow and inefficient diffusion of solutes through the plasmodesmata connecting adjacent endosperm cells while the aleurone is a site of active transport processes. This could account for the decreasing gradient of sucrose (Smyth *et al.*, 1988; Pearson *et al.*, 1996), storage proteins (Del Rosario *et al.*, 1968; Ellis, *et al.*, 1987) and minerals (Lombi *et al.*, 2009; Lombi *et al.*, 2011) from the periphery of the grain aleurone towards the starchy endosperm in the grain centre. This decreasing nutrient gradient towards the grain centre is especially critical in determining nutrient availability in rice as the outer aleurone, and hence most of the nutrition, is removed with polishing.

Sucrose accounts for 17-25% w/v of the solutes transported in rice phloem (Fukumorita and Chino, 1982), therefore the strength of the grain sink for sucrose

could influence the transport of minor assimilates such as minerals and amino acids. Sucrose uptake into plant cells is controlled through Sucrose-H^+ symporter (SUT) proteins located in the plasma membrane (PM) that drive active uptake of sucrose from the apoplastic space into the cell. Most recently SWEET proteins have been identified as the likely candidate protein for sucrose efflux into the apoplasm (Chen *et al.*, 2012) although the role of these proteins in the grain has yet to be confirmed. The energy to drive sucrose uptake is provided by a proton pump H^+-ATPase which hydrolyzes ATP to ADP, generating a proton gradient across the PM and driving the uptake of a 1:1 ratio of H^+ and sucrose into the cell against a sucrose concentration gradient. Families of SUTs have now been identified in the most important cereal crops including rice (Hirose *et al.*, 1997), maize (Aoki *et al.*, 1999) barley (Weshke *et al.*, 2000) and wheat (Aoki *et al.*, 2002). Based on alignments of amino acid sequences, the monocot SUT1 proteins cluster together into one group based on their homology (Braun and Slewinski, 2009; Sun *et al.*, 2010) with 82%, 80% and 79% homology of rice *OsSUT1* with maize *ZmSUT1*, barley *HvSUT1* and wheat *TaSUT1D*. When studied in heterologous systems, *HvSUT1* (Sivitz *et al.*, 2005) and *OsSUT1* (Sun *et al.*, 2010) both showed high specificities for sucrose however *HvSUT1* had a higher affinity for sucrose than *OsSUT1* at similar membrane potential and external pH. Expression and localization of *SUT1* transcript and protein in vascular tissue in stems and leaves as well as in grain tissue in cereals point to important roles in both phloem loading (Weschke *et al.*, 2000; Aoki *et al.*, 2004; Scofield *et al.*, 2007) and grain development (Weschke *et al.*, 2000; Furbank *et al.*, 2001; Aoki *et al.*, 2002). However, much higher expression of SUT1 in developing grains suggests that their primary role may be in grain filling. Constitutive antisense knockdown of *OsSUT1* in rice did not affect the loading of sucrose into the phloem but produced plants with a shrivelled grain phenotype (Scofield *et al.*, 2002). *SUT1* transcript levels measured during grain development in both rice and barley grains follow a very similar pattern which in barley correlates with the levels of sucrose measured in the developing grain during early grain filling (Weschke *et al.*, 2000). Taken together, this evidence points to a similar and crucial role for SUT1 proteins in assimilate partitioning in rice and barley grains.

Increasing the sucrose transporter activity in peas by overexpressing the potato *StSUT1* driven by a seed specific *vicilin* promoter resulted in increased sucrose uptake and growth of pea cotyledons (Rosche *et al.,* 2002). In addition, these transgenic peas displayed an earlier onset of protein accumulation, a changed protein profile and increased total protein content during development and at maturity (Rosche *et al.*,

2005). In wheat grains overexpressing the barley *HvSUT1* there was increased total protein which was attributed to the preferential increase in prolamins, with no decrease in yield (Weichert *et al.*, 2010). In further field studies of these transgenic *HvSUT1* overexpressing lines, it was found that yield was also significantly increased over several seasons (Saalbac, *et al.*, 2014) although the promising increase in protein concentration was not evident under these field conditions. Most surprisingly, a corresponding increase in the micronutrients Zn and Fe was measured in *HvSUT1* overexpressing wheat grains compared to WT lines (Saalbac *et al.*, 2014). There is strong evidence that increasing sucrose uptake can increase seed sinks for other nutrients and that sucrose plays an important role in the processes of assimilate uptake and storage protein formation (Rosche *et al.*, 2005; Weichert *et al.*, 2010). Moreover, reducing sucrose supply to cereal sinks has been shown to decrease Mn and Zn transport *in vitro* (Pearson *et al.*, 1996). Sucrose can also act as a signal molecule regulating gene expression (Loreti *et al.*, 2001) and enzymes that control metabolic changes during rice grain development (Sasaki *et al.*, 2005; Tang *et al.*, 2009). SUT1 activity is heavily dependent on plasma membrane electrophysiology (Sun *et al.,* 2010) and sucrose uptake is potentially tightly controlled by minute changes in the maternal filial boundary in the grain (Bagnall *et al.*, 2000). However transport processes in starchy endosperm are less likely to exert an influence on nutrient uptake as these cells do not express SUT1 transcripts (Furbank *et al.,* 2001) and have low expression of metal transporters (Tauris *et al.,* 2009).

The aims of this chapter were to analyse transgenic rice plants overexpressing the *HvSUT1* transgene to determine if: 1) they have higher Zn and Fe concentrations in the rice grain and whether 2) a higher proportion of Fe and Zn was distributed in the grain endosperm. Rice plants expressing *HvSUT1* RNA and HvSUT1 protein from Chapter 2 were analysed to determine transgene function and mineral nutrient concentration and distribution. This research investigates a novel method for exploring nutrient changes in rice due to changes in sucrose transport and provides a contrast with the findings of Weichert *et al.* (2011) and Saalbac *et al.* (2013) in wheat.

3.2 Materials and methods

3.2.1 Plant material and growth conditions

Non-transgenic (*Oryza sativa* L cv. Nipponbare) and characterised transgenic rice seed were sterilized then germinated on wet filter paper for 7 days before being transferred to 350 ml pots filled with Green Wizard® potting mix (Debco Pty Ltd,

Victoria) in water filled trays. Plants were 'hardened off' to acclimatise to greenhouse conditions and then transferred to larger pots (2.8 L) submerged in water. The potting mix contained sufficient fertilizer for four months of plant growth hence no additional fertilizer was added. Growth was in a greenhouse (Flinders University, South Australia) under natural light between the months of August and January (day-length between 11 and 14 hr) with daytime and night-time temperatures of 29 ^{0}C and 25 ^{0}C. Panicles were tagged on the first day of flowering to identify grains at different developmental stages at days after anthesis (DAA).

3.2.2 [^{14}C]-sucrose uptake in grain slices and detached rice grains

At mid to late grain filling, grains from the tips of panicles at 15 and 20 DAA were selected for analysis. Five transverse grain slices, cut with a razor blade from separate grains (approx. 0.5mm thick) were combined and incubated in [^{14}C]-sucrose to determine uptake rate of cells in the caryopsis (Rijven and Gifford, 1983). The reaction buffer used in this experiment was adapted from Bagnall *et al.* (2000) and contained 10 mM sucrose, 32 mM KCl, 180 mM sorbitol and 10 mM (MES)-Tris pH 6.4. Immediately after cutting, slices were placed into unlabelled reaction buffer at 4 ^{0}C and gently washed twice for 15 min to remove cell debris. After a pre-incubation in the unlabelled buffer at 25 ^{0}C for 20 min, labelled reaction buffer containing 2.22 MBq mmol^{-1} sucrose was added (Wardlaw *et al.*, 1995) and the slices were incubated for 20 min (Furbank *et al*., 2001). After removing the labelled sucrose, ice cold reaction buffer was added and slices were washed twice for 15 min to remove residual label. Replicate treatments were carried out in parallel with the sucrose uptake assays, but prior to addition of [^{14}C]-sucrose label, the slices were pre-incubated in unlabelled reaction buffer containing 1 mM diethyl pyrocarbonate (DEPC) for 20 min. Grain slices were washed before incubating with labelled reaction buffer as described. The slices were ground fresh using a Retsch mixer mill (MM400) and extracted in 80% boiling v/v EtOH for 60 min. Radioactivity in the samples was measured by adding the sucrose extract to 10x volume of scintillation cocktail (Ultima Gold™ Perkin Elmer) and assayed in a liquid scintillation counter (Perkin Elmer LSA Tri Carb 2810TR) with automatic quench correction and background subtraction. The sucrose uptake rate was calculated from the specific activity of the [^{14}C]-sucrose labelled incubation buffer and expressed on a fresh weight basis.

Detached grains were used to assay sucrose uptake at early grain filling from 5 to 10 DAA. Intact whole grains were removed from the top of developing panicles by

cutting below the base of the grain, twice under water, until 1 mm of the pedicel remained. The detached grain was then transferred to a closed 0.5 ml tube with the pedicel immersed in labelled reaction buffer and incubated at 25 ^{0}C for 4 hr. Uptake was terminated by removing the reaction buffer and washing twice for 15 min in ice cold sterile water. Sample preparation and scintillation counting was the same as for the grain slices. Individual grains were ground using a Retsch mixer mill (MM400) and radioactivity was measured as for the grain slices. At least four grains from each of four plants (n=16) were assayed for each time point in grain filling.

3.2.3 Micronutrient and crude protein analysis

Elemental analysis of grains was carried out using ICP-OES (Waite Analytical Services, Adelaide) according to the methods outlined in Wheal *et al.* (2011). At maturity, panicles were dried at 37 ^{0}C for three days and grains dehusked using a laboratory bench-top rice husker and mill (Model JGMJ8098, Miltec). T_1 grains from T_0 plants were pooled from all panicles and three technical replicates of 15 to 30 grains were subsampled for elemental analysis. For T_3 grains, unpolished (brown) and polished (white) rice samples were analysed for mineral nutrient concentration. For analysis of T_3 polished rice grains, 100 grains were subsampled from each plant and bran was removed by polishing in a modified laboratory grain polisher (Kett PEARLEST) then ground to flour in a bench top mill (Mini-Mill, Fritsch PULVERISETTE 23).

For analysis of mineral accumulation in immature rice grains, panicles were harvested at 5, 10, 15, 20 and 25 DAA. Entire panicles were dried for 24 hr at 85 ^{0}C before dissecting, counting and weighing caryopses from panicles. 10 similar sized grains per sample were combined and analysed by ICP-OES. Results are means and standard deviations of four biological replicates for each time point.

Total Nitrogen (%) in polished rice flour samples was determined by a Dumas Total Combustion Method using an Elementar instrument (WAS). Crude total protein was calculated by Nitrogen x 5.26 (Fujihara *et al.*, 2008).

3.2.4 Sugar and starch analysis of polished rice

Approximately 250 mg of polished rice flour was weighed into 15 ml tubes and 1 ml of 80% v/v EtOH was added. The tube was inverted to thoroughly wet the sample and then an extra 1 ml of 80% EtOH was used to wash down the residue on the sides of the tube, before sugars were extracted on a shaking hot block for 1 hr at 85 ºC. Tubes were centrifuged at 3000 xg for 10 min before the supernatant was removed and the

residue was re-extracted with another 3 ml of 80% EtOH. Both extracts were combined and made up to exactly 5 ml. Aliquots of the sugar extract were diluted 4x in ultrapure laboratory grade water (Milli-Q® water) and sugars were separated by high performance anion-exchange chromatography (HPAEC). Samples were passed through a Carbo Pac PA-20 column at a flow rate of 0.35 ml min^{-1} and 30 ^{0}C using a Dionex 600 chromatography system (Thermo Scientific) with integrated pulsed amperometric detection (PAD) of sugars. Settings used were according to the manufacturers recommended settings in the Dionex Chromeleon software. The system was prepared by flushing with 100 mM KOH for 5 min followed by pre-equilibration with 50 mM KOH until samples were loaded into the auto sampler (set to 4 °C to minimize evaporation). Sugars were eluted using an isocratic system and peak identification was based on mixed sugar standards with final concentrations of 0.001 mg ml^{-1} to 0.1 mg ml^{-1} sucrose and 0.0001 mg ml^{-1} to 0.01 mg ml^{-1} glucose and fructose. Rhamnose (0.01 mg ml^{-1}) was added as an internal standard to all samples.

To estimate starch, enzymatic breakdown of starch into glucose was performed according to the methods described in McCleary and Monaghan (2002). Glucose was measured using HPAEC-PAD as described above, and the percentage of starch per sample was estimated from these values. To measure soluble starch (SS), 10-12 mg of milled rice flour was weighed into tubes and soluble sugars were extracted as described above. The flour pellet was dried and then wetted with 20 µl of 50% v/v EtOH. 300 µl of diluted α amylase (Sigma) solution (200 U ml^{-1} in 100 mM NaAc buffer pH 5.0) was added and mixed immediately. The tube was incubated at 100 °C for 10 min, with vortexing every 2 min. Tubes were incubated in a 50 °C water bath for 30 min after adding 20 µl of amyloglucosidase (AMG, 300 U ml^{-1}) (Sigma). Tube contents were added to Milli-Q® water to give a total volume of 10 ml and then spun at 3000 xg for 10 min. An aliquot of the supernatant was diluted 1:1000 with Milli-Q® water and glucose was quantified as above. The pellet remaining from the removal of soluble starch was deemed the resistant starch (RS). The RS pellet was resuspended and washed 2x with 50% EtOH and spun at 3000 xg for 10 min to collect the washed starch pellet. The pellet was dissolved in 200 µl of 2 M KOH by vigorously shaking in an ice bath for 30 min, then neutralized with 800 µl of 1.2 M NaAc buffer (pH 3.8). 20 µl of AMG was added and the tube incubated at 50 °C for 30 min with intermittent mixing. Samples were diluted 1:50 before measuring glucose.

3.2.5 LA-ICP-MS imaging of elemental distribution

Mature rice grains were embedded in 100% epoxy resin and polymerised overnight at 70 °C without prior fixing or dehydrating. Processing and handling of the sample was kept to a minimum to minimise disturbance of the elemental distribution in the grain. The sample surface was prepared for ablation by cutting with a glass knife on an ultramicrotome (Ultracut S, Leica) (Adelaide Microscopy, South Australia).

Elemental distribution was analysed by Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) using a Resonetics M-50-LR 193 nm Excimer laser (Laurin Technic Pty) coupled to a quadrupole ICP-MS (Agilent 7700s) at Adelaide Microscopy, SA. The unique dual-volume small volume ablation cell (Laurin Technic Pty) used with the M50 gives high sample uniformity and rapid wash out thereby enhancing detection of signal sensitivity. Ablation was performed under Nitrogen (0.7 L min^{-1}), with ablated material transported by Argon (0.93 L min^{-1}) for ionization by the ICP.

Embryo samples were ablated with a beam size of 8 µm and a scan speed of 12 µm s^{-1} while transverse and longitudinal scans of whole rice grains were ablated with a 24 µm beam size at 32 µm s^{-1}. All imaging was conducted in parallel raster lines across the sample with the distance between lines the same as the beam spot size. The energy density of the laser at each ablation point was ~6 J cm^2 and the predicted depth of material ablated from the sample was 5-10 µm. The dwell time for each element was 0.01 sec, hence with the 9 elements analysed per sample, the total sweep time was ~0.1 sec. A 30 sec background acquisition period was employed at the beginning of each raster with a 15 sec delay at the end of each line programmed to allow for sample processing. Each run was calibrated and assessed for drift correction by measuring glass standards NIST610 and NIST612 (US National Institute of Standards and Technology) at the start and end of the run.

ICP-MS data processing and image compilation was conducted using the open source software program Iolite (http://www.iolite.org.au/Iolite.html) developed by Melbourne University; as plug-in for the data analysis program Igor Pro (WaveMetrics). The data from each element was corrected for drift using NIST standards and images were compiled after subtracting background intensity to give images displaying a heat map of the signal intensity of each element measured as counts per second (cps). Overlapping elemental distribution was investigated for some groups of elements by overlaying the distribution maps to produce composite tricolour 2-D images.

3.3 Results

3.3.1 Analysis of mineral nutrients in *HvSUT1* overexpression lines

Segregating T_1 grains from T_0 parent lines were analysed for macro and micronutrient concentrations and compared to wild type (WT) Nipponbare plants grown at the same time under the same conditions as the transgenic lines. Unpolished and polished rice grains from *Glb-1* (G1.4, G3.1 and G6.2) and *Act-1* (A5.1 and A2.3) SUT-OE plants containing one to two copies of the transgene were analysed. Macro- (Fig 3.1) and micronutrient (Fig. 3.2) concentrations analysed by ICP-OES are summarised for each line as a percentage of WT concentration (set at 100%). Unpolished rice from all five transgenic lines had either the same or slightly increased concentrations of Mg, K, P and S. This increase in macronutrient concentration was more striking in polished rice samples. Mg and K showed the highest increase in concentration compared to WT in most of the plant lines. The three *Glb-1* SUT-OE lines in particular showed markedly different patterns of macronutrient accumulation in the polished rice. While line G1.4 had modest increases in all four elements, line G3.1 consistently had a greater increase in Mg and P when compared with all the other SUT-OE plants. In contrast, Fe, Zn and Ca concentration decreased in the transgenic plant lines in unpolished rice, while Mn was almost double the concentration in WT. This trend for decreased Zn and Ca was also observed in polished rice. However, the Fe concentration in polished grains was higher than in the WT. The concentration of Mn in polished rice ranged from 75 to 125% more than in WT. As only polished white rice is consumed, this was a promising result that warranted further investigation.

When germinated on selection media, a number of T_1 seedlings of G6.2 were albino, even though the T_0 plants had developed normally. Although G6.2 showed more of an increase in nutrient concentration than G1.4, the segregation results along with the presence of albino seedlings posed difficulties in interpreting results. Therefore analysis of this line was not continued due to the unlikely survival of the albino seedlings and the possibility of somaclonal variation leading to unknown changes in other traits. Seeds from T_1 plants that were identified as containing the transgene in the leaf assay from G1.4 and G3.1 were germinated on hyg to determine zygosity. Homozygous SUT-OE lines G1.4 and G3.1 and segregating A2.3 and A5.1 were grown for analysis of sucrose transporter function.

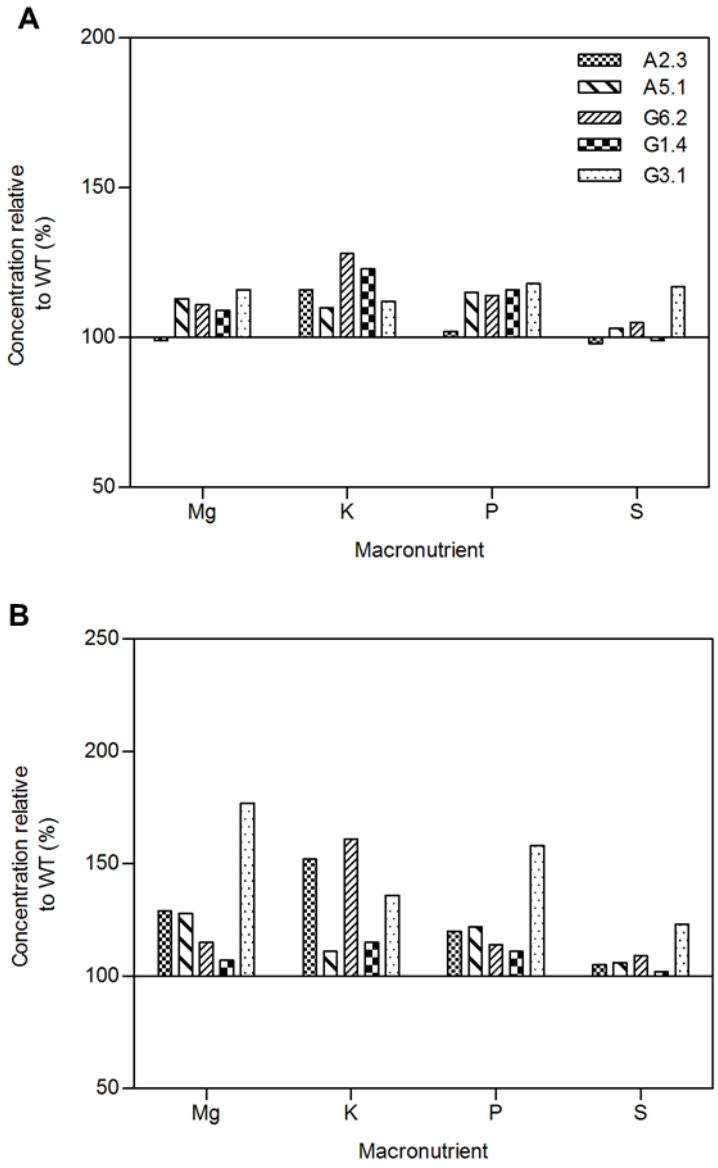


Figure 3.1 Macronutrient concentration of T_1 grains from SUT-OE T_0 lines compared with WT

Comparison of the macronutrient concentration of unpolished (A) and polished (B) rice grains from T_0 transformed rice lines containing the *HvSUT1* transgene driven by the endosperm specific (G1.4, G3.1, G6.2) and constitutive promoters (A2.3, A5.1) relative to the concentration in WT (100%).

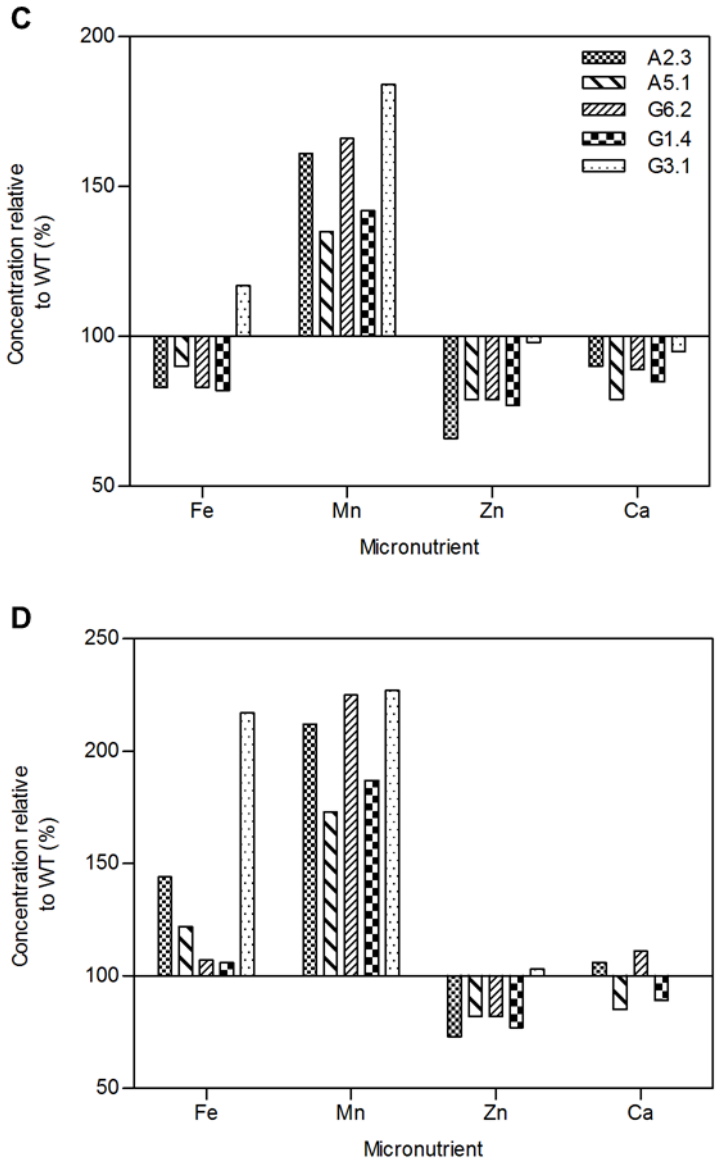


Figure 3.2 Micronutrient concentration of T_1 grains from SUT-OE T_0 lines compared with WT

Comparison of the micronutrient concentration of unpolished (A) and polished (B) rice grains from T_0 transformed rice lines containing the *HvSUT1* transgene driven by the endosperm specific (G1.4, G3.1, G6.2) and constitutive promoters (A2.3, A5.1) relative to the concentration in WT (100%).

3.3.2 [^{14}C]-sucrose uptake in grain slices

To demonstrate *HvSUT1* function, [^{14}C]-sucrose uptake was measured at 15 and 20 DAA in immature grain slices of T_2 grain from T_1 plants from *Glb-1* SUT-OE lines G1.4, G3.1, and *Act-1* SUT-OE lines A2.3 and A5.1 and compared with sucrose uptake of a control NT line (Table 3.1). Compared with 15 DAA, sucrose uptake was lower at 20 DAA for all lines indicating sucrose transport decreased between 15 and 20 DAA. There was also no change in sucrose uptake between any of the lines at 20 DAA. At 15 DAA, sucrose uptake in caryopses from transgenic lines G3.1, G1.4 and A5.1 were the same as NT controls. In contrast, sucrose transport was significantly higher in A2.3 at 15 DAA. Pre-incubation with DEPC inhibited sucrose uptake in NT, G3.1 and G1.4 grain slices by approximately 20 to 40 % at 15 DAA which verified that there was a component of active sucrose transport in these rice grains. In the *Act-1* SUT-OE lines, A2.3 and A5.1, however, sucrose uptake was relatively insensitive to DEPC treatment. Inhibition of sucrose uptake by DEPC in the control line was highest at 15 DAA (40%) however at 20 DAA sucrose transport was only inhibited by 15%. In contrast, there was a similar response to DEPC treatment in G1.4 or G3.1 between 15 and 20 DAA.

[^{14}C]-sucrose uptake rates in T_3 grain slices from all plant lines were approximately double that seen in T_2 (Table 3.2). Sucrose uptake was highest at 13 DAA, then declined from 15 DAA to 20 DAA, as was observed in T_2 grains. There was no significant difference in rates of sucrose uptake between NT and G1.4 at 13, 15 and 20 DAA. A2.3 T_3 grain slices took up significantly less sucrose than NT at 13 and 15 DAA. Treatment with DEPC reduced sucrose uptake by between 30 and 40% at 13 DAA in the three lines (Fig. 3.3). At 15 DAA, the percentage of active sucrose uptake inhibited by DEPC was similar in NT, G1.4 and A2.3. However, at 20 DAA, sucrose uptake inhibited by DEPC in NT and A2.3 was less than 20% but in G1.4 it remained similar to that seen at 13 DAA. In NT and A2.3, the change in DEPC inhibition was highly significant between 13 and 20 DAA (Fig 3.3). The decline in DEPC inhibition was significant for A2.3 between 13 and 15 DAA, and between 15 and 20 DAA for NT. The percentage of DEPC inhibition in G1.4, however, did not differ between the three time points.

Table 3.1 Comparison of sucrose uptake rate in T_2 grain slices from **T_1 rice measured by [^{14}C]-sucrose label incorporation and scintillation counting**

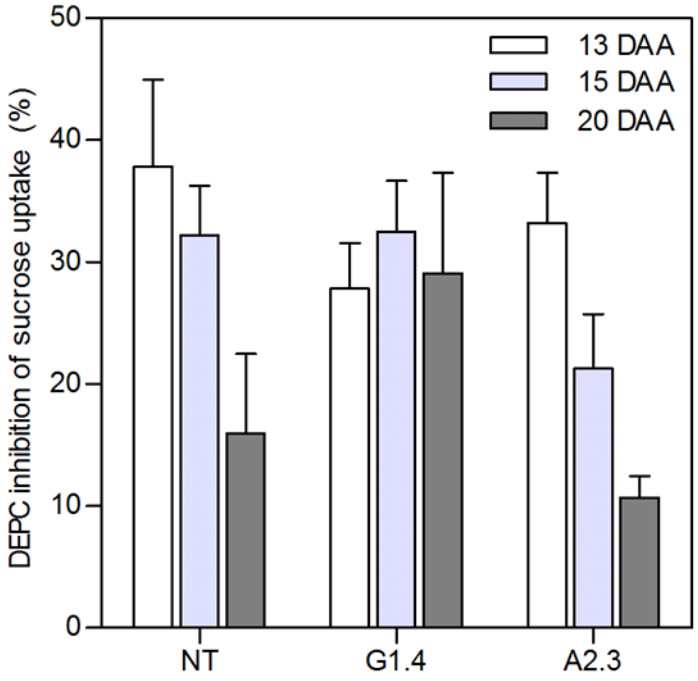
Slices of immature rice grains collected at 15 and 20 DAA were prepared and incubated in 10mM sucrose solution. Pre-treatment with DEPC to inhibit proton coupled transport before [^{14}C]-sucrose uptake was conducted in parallel experiments. Values are mean ± SD from 4 replicates of separate grains taken from the tip of rice panicles. Significant differences according to one way ANOVAs are *p<0.05, ***p<0.001. NT, non-transgenic controls.

	Seed treatment at no. of DAA					
	15 DAA			20 DAA		
Plant	[^{14}C]-sucrose uptake	+ DEPC	% inhibition	[^{14}C]-sucrose uptake	+ DEPC	% inhibition
	nmol sucrose g^{-1} FW min^{-1}			nmol sucrose g^{-1} FW min^{-1}		
NT	6.5 ± 1.4	3.9 ± 0.2	40.0	5.8 ± 1.4	4.9 ± 1.0	15.5
G1.4	6.7 ± 1.8	5.3 ± 0.3	20.9	5.7 ± 2.2	4.2 ± 1.5	26.3
G3.1	6.1 ± 0.4	4.3 ± 0.9	29.5	5.0 ± 0.7	3.7 ± 1.2	26.0
A2.3	8.7 ± 1.5*	8.3 ± 2.1***	4.6	7.0 ± 3.4	6.4 ± 2.2	8.6
A5.1	7.4 ± 2.1	6.4 ± 2.7*	13.5	5.2 ± 1.2	4.2 ± 2.1	12.2

Table 3.2 Comparison of sucrose uptake rate in T_3 grain slices from **T_2 rice measured by [^{14}C]-sucrose label incorporation and scintillation counting**

Immature grain slices were incubated with [^{14}C]-sucrose and measured for label incorporation alongside samples pre-incubated with DEPC to inhibit proton coupled sucrose transport. Changes in the amount of sucrose uptake inhibited by DEPC compared between NT, G1.4 and A2.3 over three time points after anthesis. Values are mean ± SD from 3 replicates of separate grains taken from the tip of rice panicles. Significant difference according to one way ANOVAs are; ** p<0.01, *** p<0.001

	Seed treatment at no. of DAA					
	13 DAA		15 DAA		20 DAA	
	nmol sucrose g^{-1} FW min^{-1}					
Plant	[^{14}C]-sucrose	+ DEPC	[^{14}C]-sucrose	+ DEPC	[^{14}C]-sucrose	+ DEPC
NT	15.6 ± 1.4	9.6 ± 0.5	12.8 ± 0.4	8.7 ± 0.5	9.3 ± 0.9	7.8 ± 0.8
G1.4	16.5 ± 0.6	11.9 ± 0.2**	11.7 ± 0.7	7.9 ± 0.9	9.6 ± 1.6c	6.9 ± 1.7
A2.3	13.4 ± 0.4**	8.9 ± 0.3	9.7 ± 0.7***	7.6 ± 0.2	7.2 ± 0.4	7.0 ± 1.4



13 vs 20	***	ns	***
13 vs 15	ns	ns	*
15 vs 20	**	ns	ns

Figure 3.3 Comparison of sucrose uptake response to DEPC in NT, G1.4 and A2.3 plant lines at 13, 15 and 20 DAA

Grain slices pre-incubated with DEPC to inhibit sucrose uptake in T_3 rice grains at 13, 15 and 20 DAA and percentage of inhibition compared between plant lines for each time point. Results in the table are 2-way ANOVA tests for statistical significance with significance values as follows; ns, not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

3.3.3 Grain composition and mineral nutrient retention in mature rice grain

Soluble sugars and starch measured in flour prepared from polished rice grains showed some striking differences (Fig. 3.4). Although glucose and fructose was slightly elevated in G1.4 compared to NT and A2.3, the differences were not significant. Sucrose however was significantly higher in G1.4 compared to both NT (2.8 fold) and A2.3 (1.4 fold). In contrast, soluble starch in G1.4 was significantly lower. Resistant starch and protein contents in polished rice, however, varied little across all three lines.

In T_3 polished rice grains, there were few differences in mineral nutrient concentration between the three plant lines (Fig. 3.5). In line G1.4, Fe, Mn and Zn concentrations were not significantly different to NT controls or line A2.3. In the macronutrients analysed in unpolished grains, K was increased significantly in G1.4 compared to either NT or A2.3. In polished rice, micronutrients did not differ significantly between G1.4, A2.3 and NT. Interestingly, K was significantly lower in both G1.4 and A2.3 in polished rice compared to NT. P was also higher in A2.3, but P concentration in G1.4 and NT was similar. To determine the proportion of minerals retained in the endosperm after milling, the percentage of mineral nutrient concentration in the polished grain relative to the unpolished was calculated and is presented in Fig. 3.6. These results reveal that transgenic rice grains from both the *Glb-1* and *Act-1* SUT-OE lines have significantly higher retention of Mg, P, and Zn in the endosperm after milling compared with the control NT. However, increased Fe retention is only significantly different in grains expressing HvSUT1 in the endosperm. In contrast, there is a highly significant reduction in K retention in endosperm in both transgenic lines.

3.3.4 [^{14}C]-sucrose uptake in detached rice grains

An investigation to determine the contribution of increased HvSUT1 protein expression on sucrose transport between 5 to 10 DAA was performed by measuring [^{14}C]-sucrose uptake in detached immature grains of line G1.4 (Fig 3.7). There were consistently more detached grains with higher rates of sucrose uptake at 7 DAA in G1.4 compared with NT (Fig. 3.7 A, B). Although there was a range of uptake rates measured from each plant line, there was a clear proportion of grains which showed a much higher uptake in G1.4 (Fig. 3.7 B) compared to NT (Fig. 3.7 A). Sucrose uptake either before or after 7 DAA was not significantly different between NT and G1.4 (Fig 3.7 C).

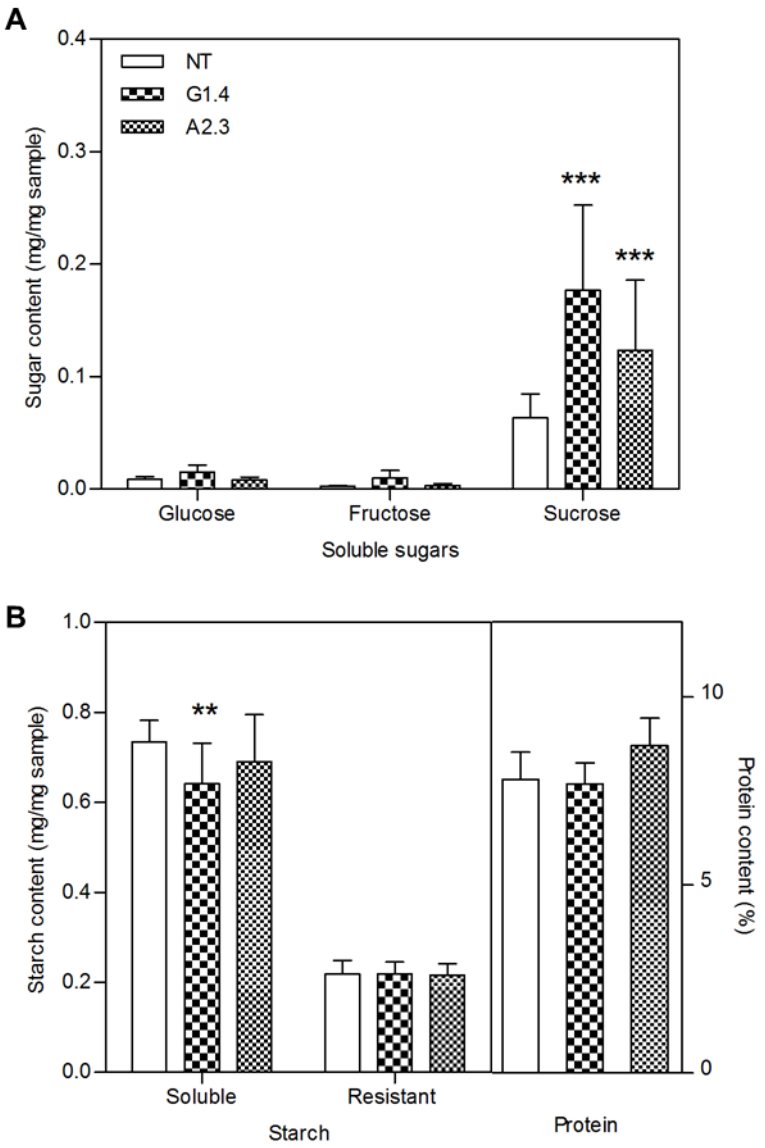


Figure 3.4 **Sugar, starch and protein content in mature T_3 polished rice**

(A) Soluble sugar concentrations of glucose, fructose and sucrose in milled rice from NT and representative transgenic SUT-OE lines measured using HPAEC-PAD. (B) Starch content estimated by solubilisation of polished rice flour and enzymatic digestion to glucose, before (soluble) and after (resistant) treatment with KOH. Glucose was quantified as for the soluble sugars. Protein content was estimated from Nitrogen content, measured using a combustion method, and multiplied by 5.26. Statistical significance according to 2 way ANOVAs are ** $p<0.01$, *** $p<0.001$ for n=9.

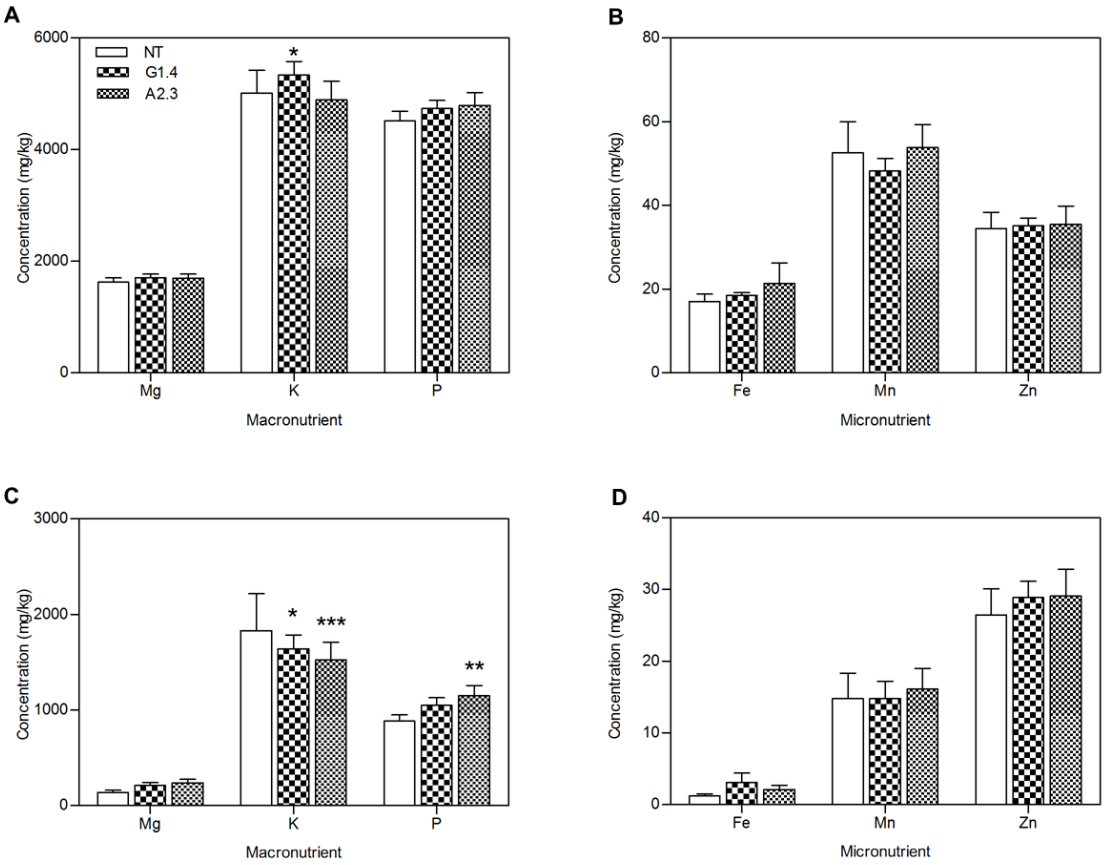


Figure 3.5 Mineral nutrient concentration in mature T_3 rice grains analysed by ICP-OES

Unpolished rice macronutrient (A) and micronutrient (B) concentrations showed little change between plant lines; control NT and transgenic G1.4 and A2.3 except with higher K in G1.4. In polished rice, macronutrients (A) showed significant changes in K and P in some lines while micronutrient (D) concentration in did not change between plant lines. Statistical significance according to 2 way ANOVAs are;*p<0.05, **p<0.01, ***p<0.001 for n=9

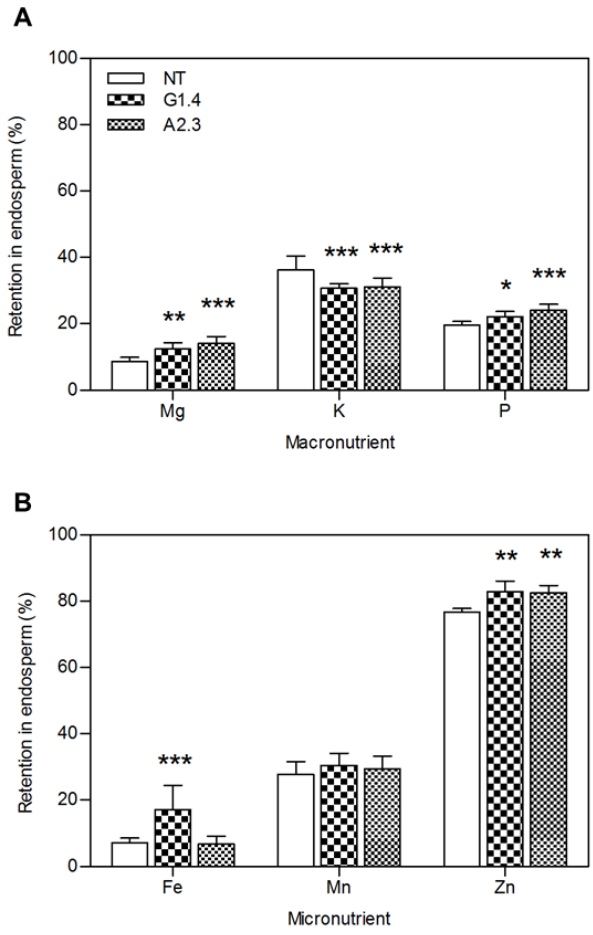


Figure 3.6 Mineral nutrient retention in mature T_3 rice grains

The percentage of macronutrients (A) and micronutrients (B) retained in the endosperm after polishing was calculated from the ratio of mineral concentrations analysed by ICP-OES in polished to unpolished rice grains * 100. Statistical significance according to 2 way ANOVAs are;*p<0.05, **p<0.01, ***p<0.001 for n=9

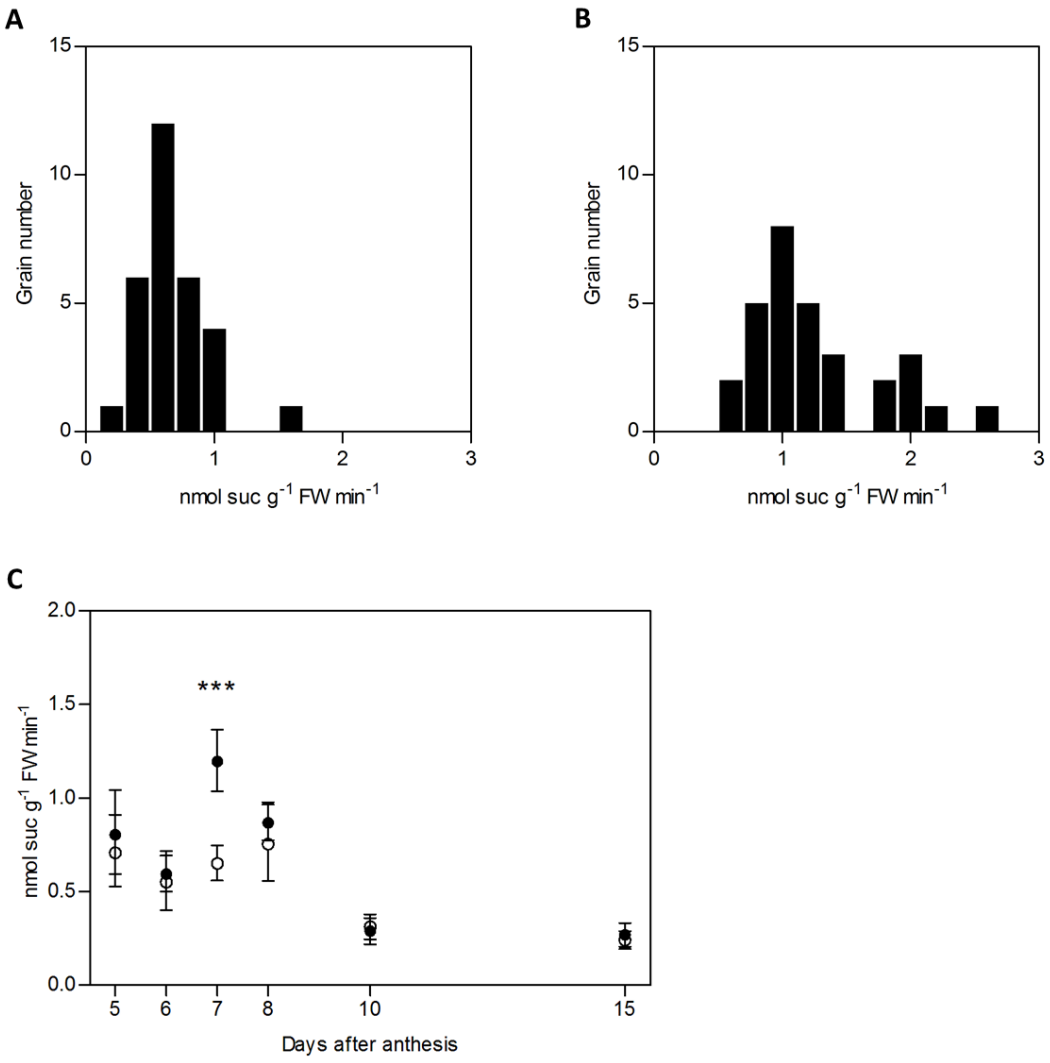


Figure 3.7 Sucrose uptake in immature detached rice grains from 5 to 15 DAA

Total sucrose uptake into (A) NT and (B) G1.4 lines detached rice grains at 7 DAA depicted as histograms of number of individual grains graphed along the range of total sucrose uptake measurements for each plant line. Due to the right skewed data, t-tests were conducted on transformed data (log Y+1) and data points plotted on the graph are mathematical means calculated by back transforming the data with lower and upper 95% confidence intervals (CI). (C) Sucrose uptake compared between transgenic G1.4 (black circles) and control NT (white circles) in immature rice grains from 5 to 15 DAA. Statistical significance according to t-test is *** $p<0.001$

Patterns of sucrose uptake over time was similar between the two lines with highest rates of [^{14}C]-sucrose transport into grains measured at 7 or 8 DAA in NT and G1.4 respectively. There was a transient decrease in labelled sucrose uptake at 6 DAA, but this was observed in both NT and G1.4. Sucrose uptake was lowest at 10 and 15 DAA but similar in both NT and G1.4. Overexpression of *HvSUT1* with the endosperm specific *Glb-1* promoter from rice appears to promote increased uptake of sucrose specifically at 7 DAA, however, at later time points, sucrose uptake in G1.4 was slightly increased but not significantly different to NT.

3.3.5 Accumulation of mineral elements in developing grains

Given the differing patterns of sucrose uptake during grain filling between non-transgenic NT and G1.4 lines, the accumulation of macro- (Fig. 3.8) and micronutrients (Fig. 3.9) was examined in immature rice grains collected from G1.4 and NT plants every five days from 5 to 25 DAA. Mineral nutrient accumulation from early to late grain filling, expressed as elemental content per grain, was not different between the two plant lines. Nutrient accumulation was similar in line A2.3 for all the mineral elements examined from 10 to 25 DAA (data not shown). Mg and P content in the grain at 5 DAA was relatively low compared with K. The pattern of accumulation from 5 to 25 DAA for Mg and P was similar, and again differed from that seen in K. Incremental increases in Mg and P content in the rice grain per time point resulted in a smooth curve representing steady Mg and P accumulation during grain filling. In contrast, K content increased sharply from 5 to 10 DAA, after which there was little further K accumulated in the grain. Indeed, in the NT control, there was a slight decrease in K content at 20 DAA that was significantly lower than G1.4, but only at this time point. Micronutrient accumulation in NT and G1.4 rice grains was very similar from 5 to 25 DAA. Very little Fe had accumulated in the rice grain at 5 DAA, and any further uptake was modest, with most of the Fe content accumulated by 15 DAA. Zn showed a more steady accumulation curve from 5 to 25 DAA, although the highest accumulation was early in grain filling, between 5 and 10 DAA. Mn accumulation, on the other hand, was greatest between 5 and 10 DAA which was the peak in Mn content, after which Mn continued to decline until 25 DAA.

3.3.6 Distribution of elements analysed by LA-ICP-MS

Localization of macronutrients Mg, K and P and micronutrients Fe, Mn and Zn in rice grain was determined by generating maps of elemental distribution using

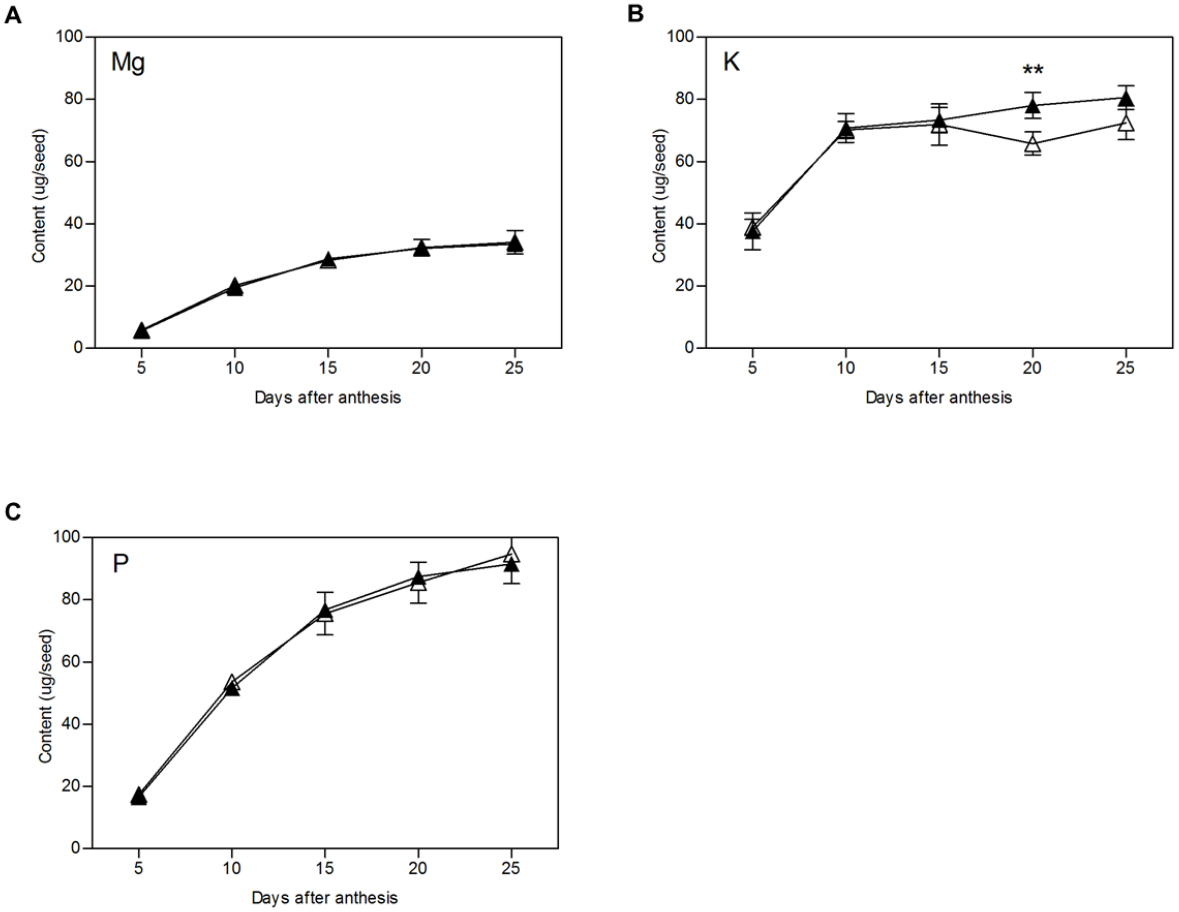


Figure 3.8 Macronutrient content in developing rice grains between 5 and 25 DAA

Change in macronutrient concentration in developing rice grains of NT (white triangles) and G1.4 (black triangles) transgenic plants from 5 to 25 DAA. Values are means ± SD (n=4). Significant difference according to t-test is ** $p<0.01$

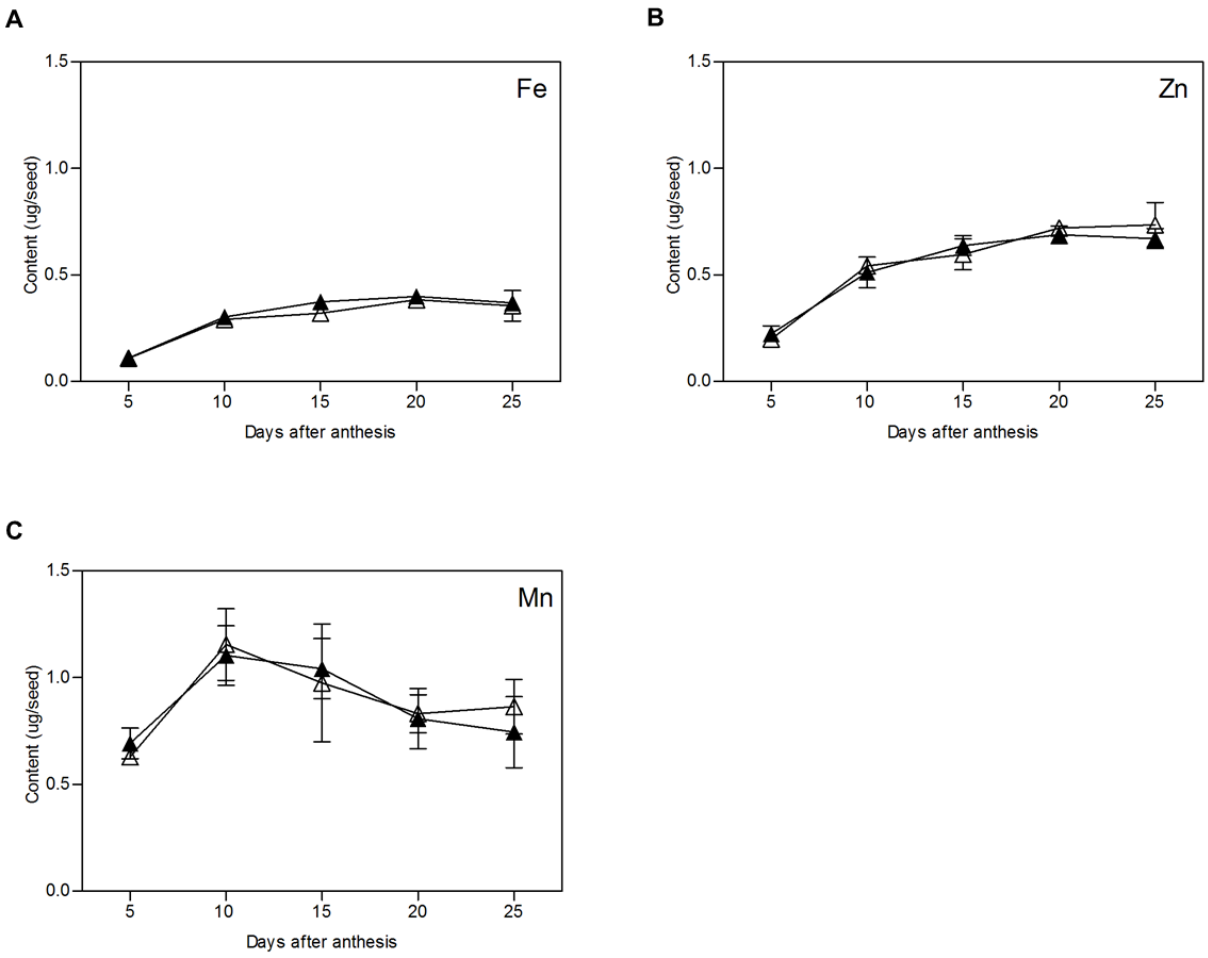


Figure 3.9 Micronutrient content in developing rice grains between 5 and 25 DAA

Change in micronutrient concentration in developing rice grains of NT (white triangles) and G1.4 (black triangles) transgenic plants from 5 to 25 DAA. Values are means ± SD (n=4).

LA-ICP-MS. The counts for each element have been scaled identically therefore this method allows comparison of nutrient distribution between the lines. A number of similar observations can be made for all three plant lines. Transverse maps of representative grains from control NT, *Glb-1* and *Act-1* SUT-OE lines show the accumulation of most of the elements in the aleurone (Fig. 3.10 & 3.11). In contrast, the elemental counts in the endosperm were much lower, and the transition zone between these two tissues was very distinct. Mg appears to be localized to the dorsal region (identified by an arrow in Fig. 3.10) of the aleurone, with less in the lateral section and almost none in the endosperm (Fig 3.10). In contrast, K and P are distributed circumferentially around the entire aleurone, but are similarly low in the endosperm. Compared with NT controls and line A2.3, G1.4 grains show a more even distribution of Mg, K and P around the aleurone instead of localizing mainly to the dorsal region. Like the macronutrients, there was a tendency for the micronutrients to be distributed across the dorsal portion of the grain, although Mn and Zn penetrated into the endosperm more than Mg, K and P. While only small changes in macronutrient distribution was observed between lines, changes in the micronutrients Fe, Mn and Zn in the endosperm of transgenic line G1.4 compared to NT and A2.3 was more evident (Fig 3.11). In NT controls, Fe and Mn are localized to the aleurone while Zn has a more even distribution across the whole grain. In stark contrast, Fe, Mn and Zn had higher counts in the middle of the endosperm in G1.4 grains with the counts for Mn and Zn in starchy endosperm as high as counts from the outer aleurone. There was also patchy Fe distribution in the inner endosperm of G1.4 grains, but to a lesser extent than Mn and Zn. Fe distribution in line A2.3 was similar to NT controls, however, there was more Mn in the lateral region and more even Zn distributed across the grain, with aleurone and endosperm Zn indistinguishable. Longitudinal sections of grain were also analysed by LA-ICP-MS and support the spatial distribution of elements seen in transverse sections (Appendix G).

Tricolour images generated by overlaying the longitudinal distribution maps of multiple elements give further detail about the co-localization of mineral elements within the different grain tissues (Fig. 3.12). Distribution of macronutrients was similar in both transgenic lines and in the NT control (Fig 3.12 A, B & C). The three macronutrients Mg, K and P colocalize together in the aleurone, contrasting with the very low distribution of these nutrients in the starchy endosperm. Fine detail could not be distinguished in the embryo however Mg, K and P generally also occurred together in this tissue. The distribution of micronutrients and P, Fe and Zn in endosperm of grains from *Glb-1* and *Act-1* SUT-OE plants and the grains from NT controls were

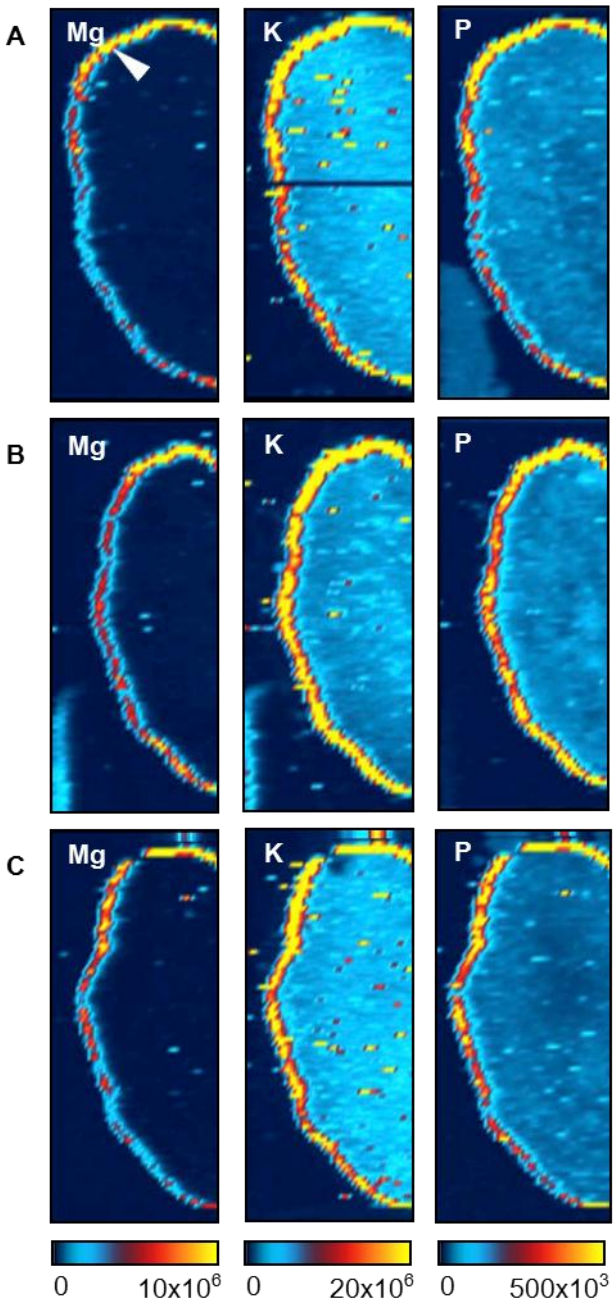


Figure 3.10 LA-ICP-MS elemental maps of macronutrients in transverse sections of rice grain

Comparison of Mg, K and P distribution between NT control (A) and transgenic G1.4 (B) and A2.3 (C) in transverse sections of mature rice grain. Normalized counts per second for each element are scaled from dark blue (lowest) to highest (yellow). All grains are in the same orientation with the dorsal and ventral sides of the grain at the top and bottom of the images respectively. The aleurone is marked by a white arrow.

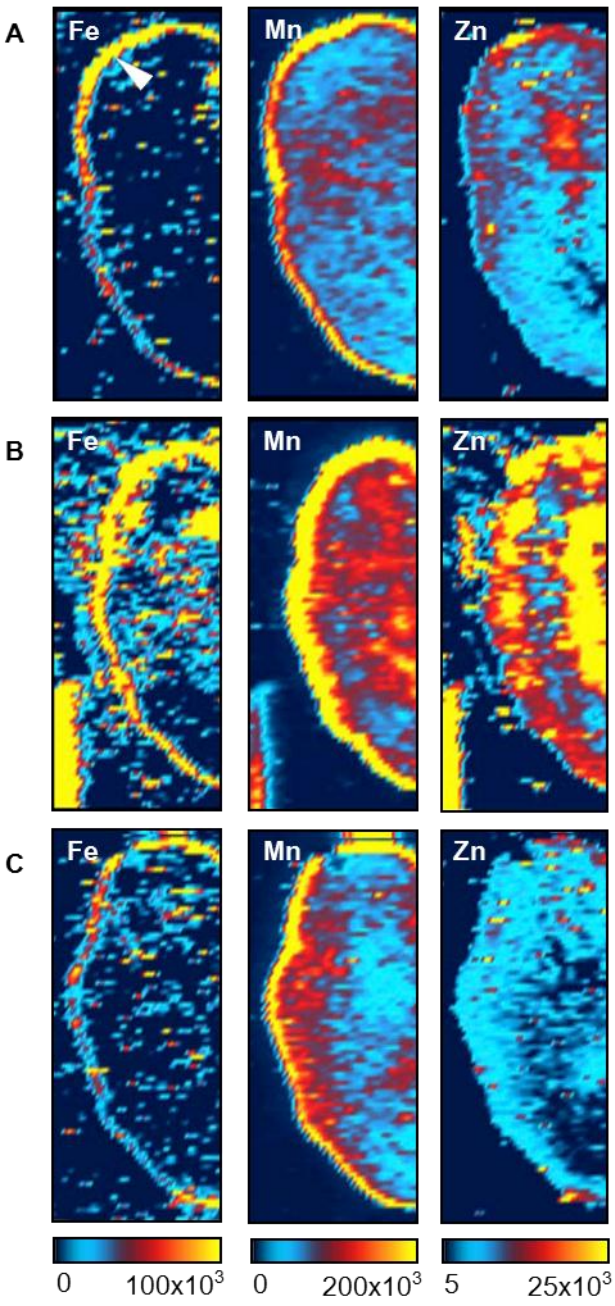


Figure 3.11 LA-ICP-MS elemental maps of micronutrients in transverse sections of rice grain

Comparison of Fe,Mn and Zn distribution between NT control (A) and transgenic G1.4 (B) and A2.3 (C) transverse sections of mature rice grain. Normalized counts per second for each element are scaled from dark blue (lowest) to highest (yellow). All grains are in the same orientation with the dorsal and ventral sides of the grain at the top and bottom of the images respectively. The aleurone is marked by a white arrow.

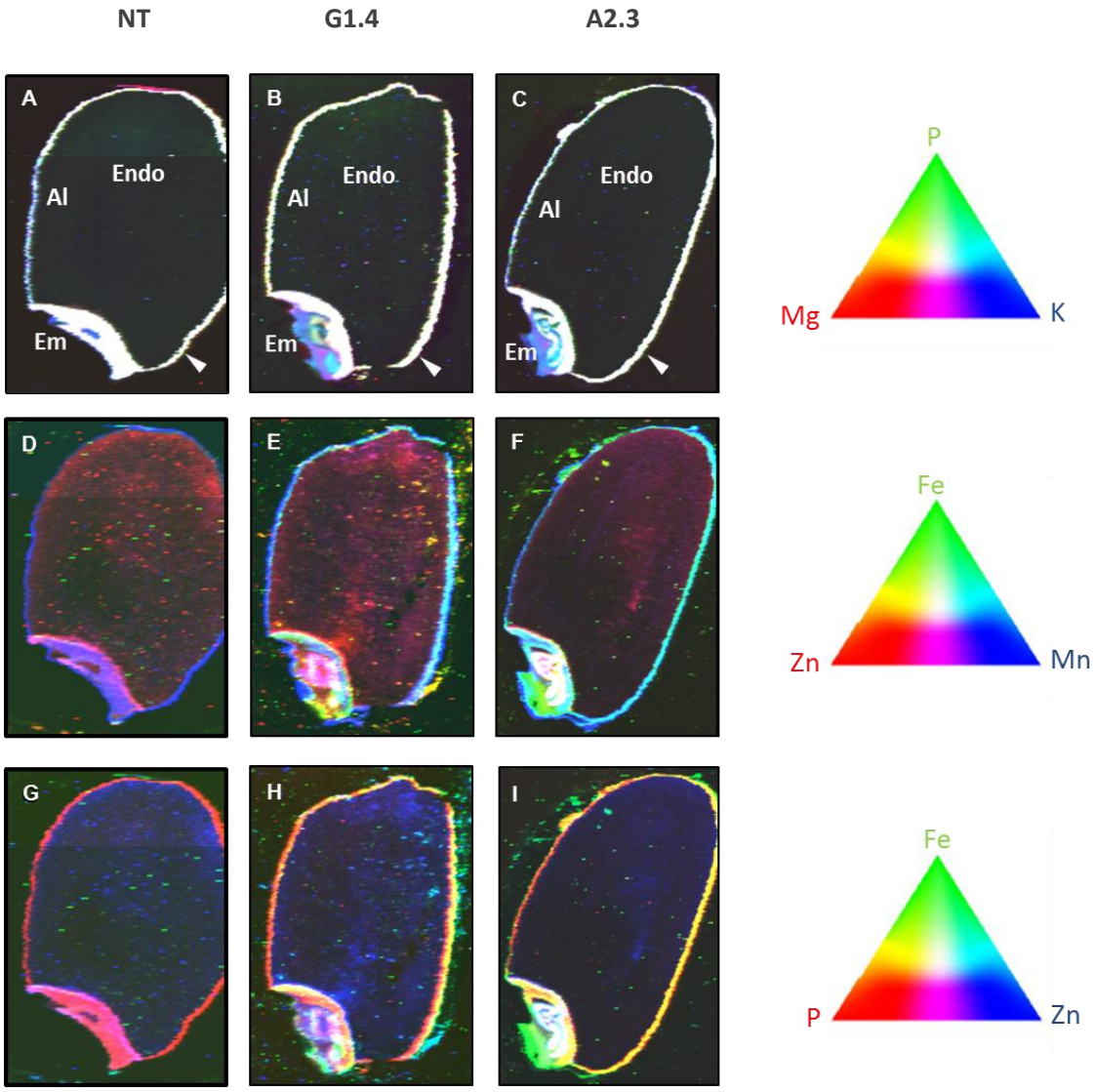


Figure 3.12 Tri-colour elemental maps of longitudinal sections of rice grain

Colocalization of macronutrients Mg, P and K in NT (A), G1.4 (B) and A2.3 (C); micronutrients Zn, Fe and Mn in NT (D), G1.4 (E) and A2.3 (F) and P, Fe and Zn in NT (G), G1.4 (H) and A2.3 (I) in longitudinal grain sections. Rice grains are oriented with the embryo at the bottom of the image with the ventral and dorsal sides of the grain on the left and right respectively. Em, embryo, Al, aleurone, Endo, endosperm and the arrow shows the ovular vascular trace (OVT). NT images are a composite of two grains due to distortions in the image of the end of the grain furthest from the embryo after data processing.

similar, however colocalization in the aleurone was strikingly different. The tricolour map for micronutrients indicates that Zn and Mn are distributed in the endosperm of all three lines (Fig 3.12 D, E and F). However, while the aleurone in the NT grain contains only Mn out of the three micronutrients, Fe and Mn occur together in the aleurone layer of G1.4 and A2.3 grains. There are also distinct differences in mineral colocalization patterns in the embryos of all three lines.

P is primarily stored as phytate in cereal grains and is a strong sink for metal ions. Therefore a tricolour image of P, Zn and Fe was generated to investigate the relationship between the distributions of these three elements (Fig 3.12 G, H & I). Zn is distributed in the starchy endosperm of rice grains from all three plant lines. Zn in G1.4 is unevenly distributed with higher Zn adjacent to the scutellum, while in NT and A2.3, Zn is concentrated in the distal section of the grain (i.e. the area furthest away from the embryo). The distribution of P, Fe and Zn is dramatically different in the aleurone of the transgenic lines compared to NT. In non-transgenic rice grains, P and Zn colocalize in the aleurone and embryo. In comparison, P and Fe are distributed together in the aleurone of transgenic lines G1.4 and A2.3. The ovular vascular trace (OVT), which is highlighted by arrows in Fig. 3.12 A, B and C, is thicker in G1.4 compared to the control NT and A2.3. There are also changes in colocalization of P, Fe and Zn particularly in this region of G1.4 grains, and the aleurone of G1.4 appears to be less homogeneous. The outer aleurone, like A2.3, is yellow which indicates localization of P and Fe, however the pink layer underneath suggests that P and Zn are enriched in this zone. This dual layered aleurone is absent in both NT and A2.3. Distribution of micronutrients in the embryo also appeared distinctly different between all three lines. Hence colocalization of micronutrients was examined with higher resolution scans of the embryo to examine micronutrient distribution in specific embryo tissues.

Elemental images of the embryo confirm that nutrient distribution of some elements in the embryo of transgenic grains was changed compared to NT grains (Fig 3.13 & Fig 3.14). Overall, the counts for Mg, K and P were higher in the embryo of line G1.4 compared to both NT and A2.3. All three macronutrients in the embryo of NT were distributed mainly in the scutellum region, with very little in the coleoptile, epiblast or radicle (Fig. 3.13 A, D & G). In G1.4, the scutellum was clearly where most of the Mg was distributed (Fig. 3.13 A). K and P were distributed together in the scutellum, however K was also distributed across all the tissues in the embryo, while P was also distributed in the coleoptile and radicle of G1.4 (Fig. 3.13 E & H). There were similar counts for Mg, K and P in the embryo of A2.3 compared to NT, however the distribution of macronutrients in A2.3 was more similar to G1.4, with distribution of K

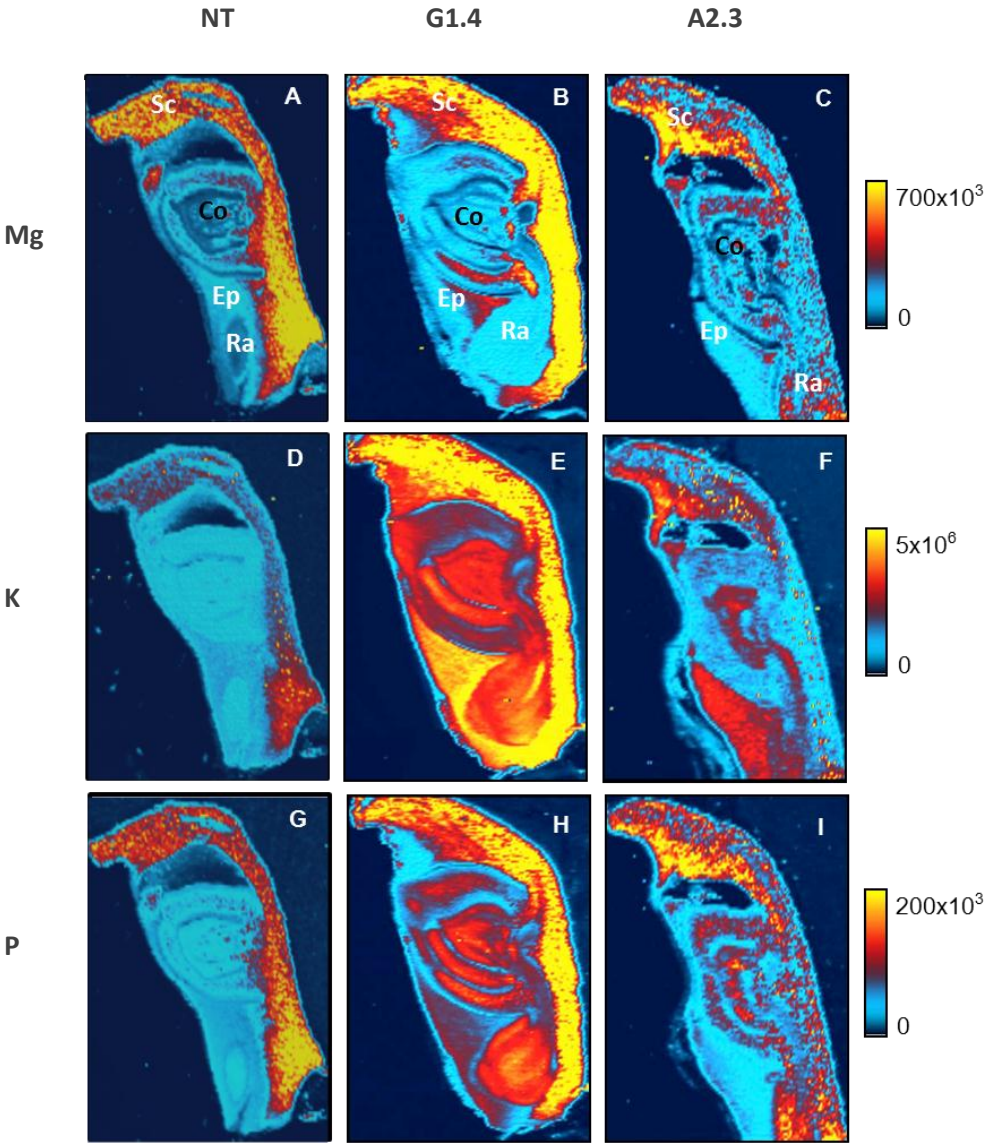


Figure 3.13 LA-ICP-MS elemental maps of macronutrients in rice embryos

Distribution of Mg in NT (A), G1.4 (B) and A2.3 (C), K in NT (D), G1.4 (E) and A2.3 (F), and P in NT (G), G1.4 (H) and A2.3 (I) in rice embryo derived from longitudinal sections of rice grain. Normalized counts per second for each element are scaled from dark blue (lowest) to highest (yellow). Embryos are oriented with the ventral and dorsal side of the grain on the left and right respectively. Sc, scutellum; Co, coleoptile; Ra, radicle; Ep, epiblast.

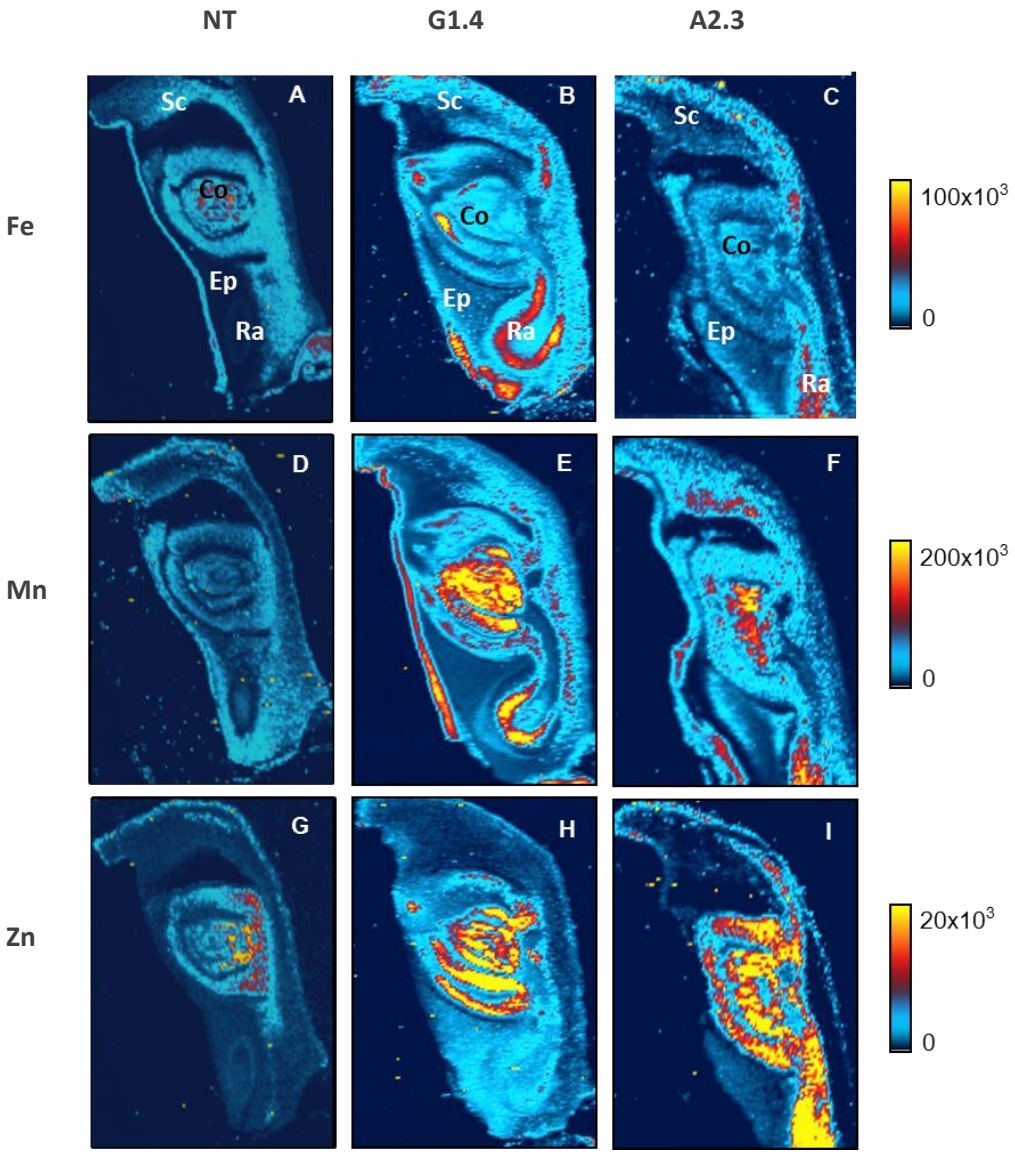


Figure 3.14 LA-ICP-MS elemental maps of micronutrients in rice embryos

Distribution of Fe in NT (A), G1.4 (B) and A2.3 (C), Mn in NT (D), G1.4 (E) and A2.3 (F), and Zn in NT (G), G1.4 (H) and A2.3 (I) in rice embryo derived from longitudinal sections of rice grain. Normalized counts per second for each element are scaled from dark blue (lowest) to highest (yellow). Embryos are oriented with the ventral and dorsal side of the grain on the left and right respectively. Sc, scutellum; Co, coleoptile; Ra, radicle; Ep, epiblast.

and P also in the epiblast and coleoptile (Fig. 3.13 C, F & I). There were low counts for Fe, Mn and Zn in the embryo tissues of NT, with slightly more Fe and Zn distributed in the coleoptile than in the surrounding tissues (Fig. 3.14 A, D & G). In contrast, the coleoptile and radicle of line G1.4 had higher counts for micronutrients. Fe was generally distributed in the radicle, Zn was distributed in the coleoptile and increased counts for Mn was found in both these tissues in G1.4 (Fig. 3.14 B, E & H). In A2.3, patterns of micronutrient distribution were similar to G1.4, although the counts were slightly lower and Zn was also distributed in the radicle (Fig. 3.14 C, F & I).

Tricolour images confirm the distinctly different distribution of elements in the embryo of each plant line (Fig. 3.15). Mg, K and P colocalized together in the scutellum of NT and G1.4 (Fig. 3.15 A & B). However, only Mg and P occurred together in the scutellum of A2.3 (Fig. 3.15 C). P and K generally colocalized in the coleoptile and radicle while Mg and K occurred together in the epiblast of all three lines. Fe was generally not found to be distributed with Zn in any of the embryo tissues (Fig. 3.15 D, E & F). In contrast, Zn and Mn distribution overlapped in the coleoptile of NT and G1.4, and Fe and Mn were found together in the radicle of A2.3. Apart from these associations, Mn was uniquely distributed across the embryo of all three lines and Fe was localized to certain tissues in G1.4 and A2.3. Colocalization of P with Zn occurred in the coleoptile of NT and G1.4 but not in A2.3 (Fig. 3.15 G, H & I). The scutellum and radicle of A2.3, in contrast, showed that P and Fe were distributed together in these tissues. Overall, colocalization of minerals in the embryo of A2.3 was uniquely different to both NT and G1.4.

3.4 Discussion

Sucrose transporters (SUTs) are an important component of carbon partitioning in cereals, particularly during grain filling. SUT activity and their association with increasing micro- and macro-nutrients has not been studied extensively. Evidence from studies by Weichert *et al.* (2010) and Saalbac *et al.* (2014) suggest that manipulating sucrose uptake in cereals could modify carbon and nitrogen partitioning, resulting in changes in protein and minerals in the grain In the present study, transformed rice plants containing barley *HvSUT1* driven either by the endosperm specific promoter *Glb-1* or the constitutive promoter *Act-1* were examined to determine whether potential changes in carbon flux could affect nutrient concentration and deposition of mineral nutrients within the rice grain.

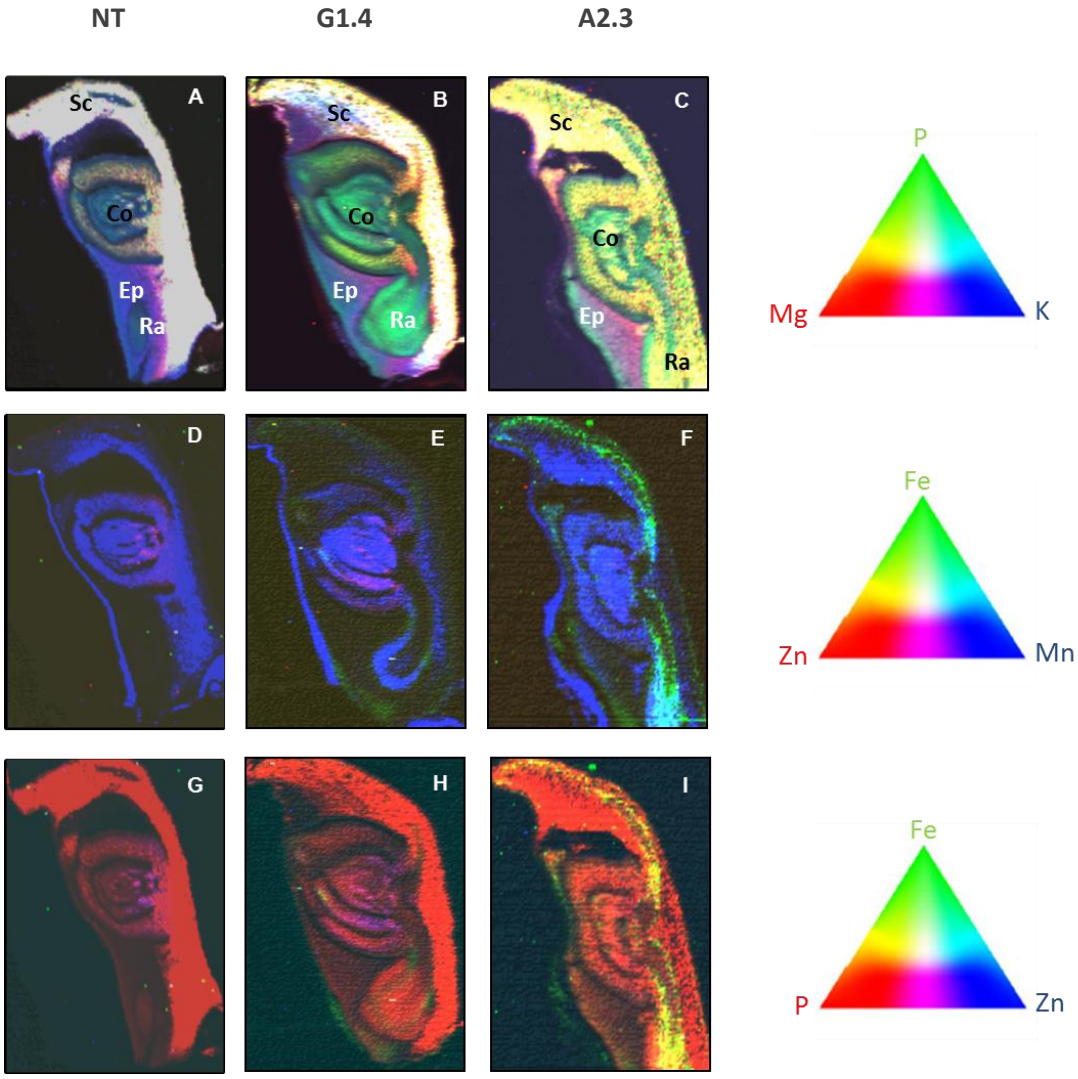


Figure 3.15 Tri-colour elemental maps of rice embryos

Colocalization of macronutrients Mg, P and K in NT (A), G1.4 (B) and A2.3 (C); micronutrients Zn, Fe and Mn in NT (D), G1.4 (E) and A2.3 (F) and P, Fe and Zn in NT (G), G1.4 (H) and A2.3 (I) in rice embryos. Embryos are oriented with the ventral and dorsal side of the grain on the left and right respectively. Sc, scutellum; Co, coleoptile; Ra, radicle; Ep, epiblast.

3.4.1 Changes in grain composition in rice grains overexpressing HvSUT1

Rice plants regenerated after tissue culture and infection with *Agrobacterium* harbouring SUT-OE constructs, were characterised (see Chapter 2) and grown to maturity. Conspicuous differences between *HvSUT1* overexpressing wheat reported by Saalbac *et al.* (2014) and the *Glb-1* SUT-OE rice in the present study are related to the sugar, starch and protein contents in transgenic wheat and rice. In the present study, sucrose was significantly increased, soluble starch decreased and there was no change in protein content in transgenic SUT-OE rice line G1.4. Wheat grains overexpressing *HvSUT1* driven by the barley *B-Hor* promoter had consistently lower sucrose and higher protein content while there were no significant changes in starch (Weichert *et al.*, 2010; Saalbac *et al.* 2014). The promoter used in those studies was different to the *Glb-1* promoter used in this research. Although endosperm specific expression of the *Glb-1* promoter in rice has been well established (Wu *et al.*, 1998; Furtado *et al.*, 2008) and the *B-Hor* promoter drives strong gene expression in barley endosperm (Cho *et al*., 2002; Furtado *et al*., 2009), the *B-Hor* promoter's endosperm specificity in wheat has not been tested. Hence we cannot rule out that differences in sucrose uptake and grain composition between the two studies could be due to differences in promoter activity.

Some of the differences observed between rice and wheat transformed with the *HvSUT1* transgene could also be explained by the differences in grain phenotype and developmental differences between wheat and rice. Rice grain size is almost constant for a variety and is physically limited by hull size (Yoshida, 1976). Final grain sizes of modern wheat and barley cultivars on the other hand can vary in grain size according to yearly environmental changes such as temperature (Yoshida, 1976) and many other factors. Grain size can also increase at later stages of grain maturity in wheat according to the sink capacity of the grain (Borras *et al.*, 2004) and/or the amount of previously stored stem carbon (Blum, 1998). This has been demonstrated in detached panicle cultures of rice and wheat, where optimum sucrose supply to rice panicles did not increase individual grain weight but only the weight of the panicle (Villareal *et al.*, 1987). However, wheat panicles cultured in a similar system had increased single grain weight when competing sinks were removed (Zhang *et al.*, 2012). It is possible that continued sucrose transport into rice grains at later grain maturity cannot result in further grain expansion hence the excess sucrose that continues to be loaded into the grain due to expression of HvSUT1 accumulates in the mature grain. Coordination of the various enzymes involved in starch synthesis can also be regulated by photosynthate supply (Wang *et al.*, 2007) therefore excess sucrose could result in a

deregulation of starch synthesis, thereby affecting conversion of sucrose into starch and resulting in the decrease in soluble starch observed in *Glb-1* SUT-OE rice grains. This proposed explanation for the differences between rice and wheat is further supported by the fact that Saalbac *et al.* (2014) did not observe changes in starch content of *HvSUT1* overexpressing wheat grains, although the wheat grains were larger, which suggests that in wheat the excess sucrose is utilized in other processes, for example in growth and synthesizing other storage products. Although, they only measured total starch content, and in the present study, only soluble starch was significantly decreased while total starch was the same between controls and transgenic plants. Therefore, widely different sink responses to *HvSUT1* overexpression between rice and wheat provides a reasonable explanation for the disparity observed in carbohydrate and starch contents in rice measured in this study and wheat in Weichert *et al.* (2010) and Saalbac *et al.* (2014).

3.4.2 HvSUT1 overexpression modifies grain filling behaviour in rice grains

Despite similar protocols used to measure sucrose uptake in grain slices, rates of sucrose uptake in NT grains measured in this study (9.3 nmol g^{-1} FW min^{-1}) were much lower than values from comparable assays conducted by Furbank *et al.* (2001) (~30 nmol g^{-1} FW min^{-1}). The linear range of sucrose uptake reported by Furbank *et al.* (2001) was over 30 min, however even slight changes in assay conditions, including room temperature, could have an impact on sucrose transport rates, and could explain the disparate values between the two studies. It is also possible that the linear range of sucrose uptake in the different transgenic lines have been affected unequally, therefore clearer conclusions may be drawn from future experiments by comparing a range of incubation times. Interestingly additional HvSUT1 expression in rice did not result in noticeable increases in sucrose uptake between 13 and 20 DAA. In contrast, wheat kernels transformed with *HvSUT1* had increased sucrose uptake compared with controls at peak grain filling (Weichert *et al.*, 2010). Given the contrasting transformation protocols, the different promoters used and species differences, the dissimilarities in sucrose uptake between these two cereals were not unexpected.

Rice grain filling is very sensitive to small changes in environmental conditions (Chen *et al.*, 2012b) therefore the much higher sucrose uptake rates in assays of T_3 compared to T_2 grains could be attributed to seasonal changes. T_3 grains were harvested from plants grown over the same months, but one year later than T_2 under more controlled conditions. In the growth facilities available, it was not possible to

control for small changes in the growing environment such as light intensity, humidity and slight temperature fluctuations during grain filling. However, in this study, control plants and transgenic lines from the same generation were grown simultaneously in the greenhouse under exactly the same conditions, and hence are comparable.

Sucrose uptake rates between grain from transgenic plants and controls were not significantly different in T_3 grain slices. To determine that sucrose accumulation was due to active sucrose uptake, treatments were run in parallel with a 20 min pre-incubation of the grain slices in DEPC (as in Weichert *et al.* 2011) which inhibits protein coupled symport through covalent modification of a conserved histidine residue in the transporter protein (Lu and Bush, 1998). Although there was increased sucrose taken up by *Act-1* SUT-OE T_1 grains, the transport mechanism was insensitive to DEPC and so it appears that uptake was probably not due to SUT activity which could be due to the lack of inhibitor access to modify the transporter protein. This lack of response to DEPC was intriguing and suggests that in these lines sucrose uptake was not mediated by an active sucrose transporter but rather some unidentified mode of non-active transport or other adsorption was at work.

Another component of sucrose transport into rice grains that is insensitive to DEPC treatment is a non-active, non-saturable mechanism that is theorised to be involved in sucrose uptake into filial tissues (Zhang *et al.*, 2007). A theoretical mechanism for facilitated diffusion of sucrose into plant cells that is characterised by a low affinity/high capacity phase of sucrose transport has been described by Ayre (2011). This unknown mechanism could possibly explain the DEPC insensitive uptake of sucrose stimulated by overexpression of *SUT1*, which interestingly also seems to be stimulated by decreased expression of *SUT1.* In anti-sense RNAi lines of *OsSUT1* suppressed plants, Scofield *et al.* (2002) demonstrated decreased *OsSUT1* expression, yet these transgenic rice seeds continued to take up sucrose. This uptake was via a mechanism that was insensitive to the sulfhydryl modifier *p*-CMBS, hence Scofield *et al.* (2002) postulated that their transgenic RNAi rice seeds must be taking up sucrose by diffusion rather than active uptake. Similarly, there is further evidence from dicots of this 'diffusional' mechanism of sucrose transport. Lanfermeijer *et al.* (1991) identified a non-saturable component of sucrose uptake in pea cotyledons at various stages of development. Overvoorde *et al.* (1997) described a soybean sucrose binding protein (SBP) that was not sensitive to pH or sulfhydryl modifiers which suggested that H^+ did not mediate transport in the soybean SBP. Zhou *et al.* (2007) go on to characterise these proteins as 'sucrose facilitators' or SUFs that act as diffusers of sucrose and are not affected by DEPC treatment. They highlighted these proteins as having possible

roles in sucrose efflux. Currently, no information is available on potential candidates for this mechanism of sucrose diffusion in cereal grains, although given the recent discovery of the family of SWEET efflux proteins, it is tempting to speculate they could play a role in this process.

Rice grains from A2.3 T_3 did not display the same sucrose uptake behaviour as T_2 grains. Compared to NT, A2.3 sucrose uptake was comparatively lower at all the time points measured. This inconsistent behaviour between T_2 and T_3 indicates that the transgene or the metabolic effect in this line was possibly less stable over multiple generations over different growing periods or possibly that *Act-1* driven *HvSUT1* was potentially more sensitive to environmental changes. In addition, continued segregation of the two sets of seed from A2.3 T_2 and T_3 generations could have confounded the results. Ideally further generations of this line should be grown to confirm homozygous plants and to measure seasonal effects on sucrose uptake.

While active sucrose transport, measured as the proportion of sucrose uptake inhibited by DEPC, decreased as NT grains matured, DEPC similarly inhibited sucrose uptake in G1.4 over the three time points. There was a similar result for A2.3 grains, but only between 15 and 20 DAA. Although total sucrose uptake declined in all the lines between 13 and 20 DAA, the continued strong inhibition by DEPC treatment even at 20 DAA, suggests that endosperm specific, and also constitutive expression of *HvSUT1* may drive prolonged active grain filling compared to non-transgenic rice. Transgenic wheat transformed with *HvSUT1* also displayed increased fresh and dry weight accumulation, but only at later grain development, which could be attributed to altered grain filling behaviour (Weichert *et al.*, 2011). The period between 15 and 19 DAA in rice grain development is classified as the late storage phase (Tang *et al.*, 2009) when peaks in sucrose metabolizing enzymes and abscisic acid (ABA) that are involved in storage product synthesis and grain maturation decline (Tang *et al.*, 2009). Usually, assimilate uptake slows and finally ceases as the grain reaches physiological maturity at 20 DAA. G1.4 grains diverged from this normal phase of grain development by prolonged active sucrose uptake at 20 DAA, indicating that the late storage phase was delayed or modified due to the overexpression of *HvSUT1* in rice endosperm.

To mimic *in vivo* sugar supply at early grain development, labelled sucrose uptake was measured in detached rice grains, analagous to methods employed in studies with whole pea cotyledons (Rosche *et al.*, 2005; Zhou *et al.*, 2009). The lid of the tube was kept closed to increase the humidity around the grain to minimise transpiration (Zee, 1971; Oparka and Gates, 1984). In this way, a crude estimate of the transport activity of the cells in the caryopsis could be measured and compared

between plant lines. As well as expressing high levels of HvSUT1 protein, transgenic rice grains of G1.4 took up significantly more sucrose at 7 DAA. Although the contribution of xylem to solute uptake in this assay cannot be ruled out, the conditions of high humidity and low light were maintained to minimise this occurring. Additionally, all control and transgenic grains were assayed simultaneously. With such a dramatic increase in assimilate flux in G1.4 at this critical time point in grain development, when crucial changes in grain development are occurring, processes that lead to the accumulation of storage products and nutrients could be modified, thereby influencing nutrient deposition in mature grain.

3.4.3 Significance of K in plasma membrane transport processes to explain lowered retention in endosperm

Mineral elements in T_1 rice grains derived from T_0 transgenic plant lines showed contrasting changes in mineral nutrients when analysed by ICP-OES, although there were some unexpected decreases in micronutrient concentration. The effect of the transgene in this generation was not expected to be significant due to the gene dilution from the segregating seeds and controls were WT as no NT lines were yet available. However, if there had been an obvious phenotypic effect from transformation with *HvSUT1*, these results could have highlighted particular plant lines for further analyses. The results were indeed worthy of further investigation despite the mineral nutrient concentrations for Fe and Zn in transgenic rice grains being unexpectedly lower, rather than higher as may have been expected from previous reports of *HvSUT1* overexpression in wheat (Saalbac *et al.,* 2014).

Analysis of nutrients in unpolished T_3 grain confirmed that although total mineral concentrations were similar between the transgenic lines and controls, a significant and novel finding of this study is that there were changes in nutrient retention in rice grains from *Glb-1* and *Act-1* SUT-OE plants. Notably, a higher proportion of the essential micronutrients Fe and Zn were retained in the endosperm, even after more than 20% of the outer bran layers had been removed. Additionally, the proportion of Mg and P remaining in the grain after polishing was significantly increased. It is intriguing that K concentration increased in unpolished, yet decreased in polished rice of G1.4. Likewise, K concentration was decreased in polished grains of A2.3 and compared with the NT controls, both G1.4 and A2.3 had significantly less K retained in the endosperm. To explain these findings, the roles of K in sucrose transport and ion homeostasis need to be considered.

K is the most abundant cation in rice phloem and high concentrations of K are found in seeds. However there is little data available to explain the mechanisms of K^+ uptake into sink cells and specifically into rice endosperm. Models for the interactions between sucrose and K has been formulated from studies in legume roots cells (Zhang *et al.*, 2002) and seed coats (Zhang *et al.*, 1997), Arabidopsis phloem (Deeken *et al.*, 2002) and protoplasts from maize vascular tissue (Bauer *et al.*, 2000). In light of the similarity between the processes of sucrose loading into phloem and sucrose unloading into sinks, the findings from these studies can be extrapolated to explain some of the trends observed in *HvSUT1* expressing rice.

PM H^+-ATPases are involved in providing energy and protons for K and sucrose transport activities (Briskin and Gawienowski, 1996; Britto and Kronzucker, 2008) and uptake into the cell can be a highly coordinated process between the activities of non-selective cation channels and SUTs (Zhang *et al.*, 2007). Out of the large number of families of K+ transporters and channels that have been identified, potassium channels such as the Arabidopsis AKT2/3 can be postulated to be involved in modifying sucrose and K in *HvSUT1* overexpressing rice. The coupling of sucrose and K transport was investigated in an *akt2-1* Arabidopsis loss of function mutant where sucrose loading into phloem was severely affected by this mutation (Deeken *et al.*, 2000). From these results, it was determined that AKT2/3 plays a role in maintaining the electrical potential across the plasma membrane by preventing sucrose induced membrane depolarization (Deeken *et al.*, 2000; Britto and Kronzucker, 2008). This is further supported by experimental data that shows *OsSUT1* expressed in oocytes responded to increasing sucrose concentration by increasing membrane depolarization (Sun *et al.*, 2010).

Gajdanowicz *et al.* (2011) conducted computer modelling that simulated post-translational modification of AKT2 from an inward into a non-rectifying channel, which is thought to occur *in vivo*, and observed a rapid increase on sucrose uptake. In parallel, H^+-ATPase activity was reduced through hyperpolarization caused by the switch, so instead of H^+ driving sucrose flux, K^+ ions were channelled out of the cell through AKT2, generating an electrochemical gradient across the membrane for increased sucrose transport. This model could provide an explanation for the increased sucrose, decreased K and unchanged H^+-ATPase protein expression observed in both *Glb-1* and *Act-1* SUT-OE rice. Furthermore, it was found that a member of the AKT3 subfamily in maize, ZMK2 colocalized with the sucrose transporter ZmSUT1, and its activity was voltage-independent (Bauer *et al.*, 2000). This K channel in maize, a monocot, therefore

behaves like the non-rectifying channel proposed in Gajdanowicz *et al.* (2011) and hence provides evidence that K channels can have similar roles in monocot species.

Sucrose and K are also coordinated in regulating cell turgor pressure. There is evidence that in sugar beet sink cells, sucrose uptake is regulated by internal turgor pressure, with excessive turgor inhibiting further uptake (Wyse *et al.*, 1986). This increase in turgor is associated with an increase in K efflux from cells in order to osmoregulate the inner cell pressure (Bell and Leigh, 1996). A large proportion of the K in plant cells is found in the vacuole, which takes up most of the space within the cell and helps to maintain cytosolic K concentrations (Leigh and Wyn Jones, 1984). Thus, vacuolar storage of K is thought to be critical in controlling turgor pressure of the plant cell. There is evidence to suggest that when K is limiting, vacuoles can also use other ions such Na, Mg and Ca but also sucrose to compensate as the main osmoticum to maintain turgor pressure (Leigh, 1984).

The increased sucrose content and Mg retention, combined with the significantly lower K concentration observed in the endosperm of *HvSUT1* overexpressing rice grains in this study, could also be related to vacuolar processes in maintaining turgor pressure of cells under high sucrose levels. Furthermore, increased vacuolar sequestration of minerals in an attempt to control ion homeostasis in endosperm cells can also be speculated to explain the increased retention of Fe and Zn observed in the endosperm of *HvSUT1* overexpressing rice grains.

3.4.4 Mineral distribution in rice grains in response to endosperm overexpression of *HvSUT1*

In contrast to Saalbac *et al.* (2013) who recorded elevated Fe and Zn concentrations in wheat overexpressing *HvSUT1* grown in the field, pot grown rice overexpressing *HvSUT1* in the present study did not have significantly increased concentrations of Fe or Zn in either polished or unpolished rice compared to controls. The range of Fe/Zn in T_3 polished and unpolished rice in the present study is in agreement with the values for non-transgenic rice grains reported in pot grown plants (Masuda et al., 2009; Johnson *et al*., 2011). To minimize issues with toxicity in this closed system and because it was estimated that the potting mix contained enough nutrients for the entire growing period of the rice plant, pot grown rice was not provided with excess fertilizer. Although the mix contained abundant macronutrients, such as P and N, micronutrients have to be maintained at lower concentrations, and perhaps could have become limiting at the later stages of plant development. There is evidence that application of mineral fertilizers at late grain development can enhance

mineral nutrient accumulation in cereal grains (Cakmak et al. 2010). Therefore, we can only speculate how the transgenic rice lines would respond to fertilization as there were significant increases in nutrient retention in polished grain of *HvSUT1* overexpressing rice even though there was almost no change in nutrient concentration in unpolished or polished rice grains.

The most notable changes in rice grains transformed with *HvSUT1* were the significant retention of Mg, P and Zn in the endosperm of SUT-OE grains and also Fe in *Glb-1* SUT-OE grains. In addition, there were substantial differences in elemental distribution mapped by LA-ICP-MS between the NT and both transgenic lines. Mineral accumulation patterns in developing transgenic rice grains between 5 and 25 DAA was the same as in non-transgenic rice, and closely mirrored the patterns for P, Fe and Zn reported in Iwai *et al*. (2012). Hence, the changes in mineral retention and distribution in mature transgenic grains did not appear to be due to increased accumulation of minerals during grain development but could result from a redistribution of available minerals in the grain. Mature rice grains from T_2 plants were over milled (~20% of outer layers removed) to investigate the content and proportion of nutrients penetrating into the inner endosperm of grains from transgenic G1.4 and A2.3 plants and NT controls. Usually commercial milling of rice removes <10% of the outer bran layers and results in the removal of 60-80% of important mineral nutrients (Hansen *et al.*, 2012). Ogiyama *et al.* (2008) found that removing between 10-20% of the outer rice grain layers through polishing did not further affect the ratio of minerals in polished rice compared with brown rice. Hence over milling and grinding polished grains into flour aided in standardising and homogenizing samples before all analyses.

Elemental maps of Fe, Mn, Mg, K and P in rice grain showed that these elements were concentrated in the aleurone, relative to the endosperm. This is in agreement with elemental maps of WT rice by Johnson *et al.* (2011), Iwai *et al.* (2012) and Kyriacou *et al.* (2014). Distribution of elements in barley grain (Lombi *et al.*, 2011) using XRF, and in wheat using LA-ICP-MS (Wu *et al.*, 2013) also show this preferential distribution of elements to the aleurone. However, the presence of the crease vascular tissue in barley and wheat, which is composed of only phloem, made these images less comparable structurally to the elemental maps in the present study. Like Johnson *et al.* (2011), the maps presented here show that Zn is evenly distributed across the starchy endosperm of rice and has no preferential allocation to the aleurone. The allocation of Zn to the bran and embryo in wheat (Lombi *et al.*, 2011) and barley (Wu *et al.*, 2013), with very little in endosperm is in stark contrast to rice. There has been no theory put forward to explain the reason for this divergence in Zn distribution between these

cereal crop species. Others have observed that with increasing plant Zn supply, there is not an increased allocation of Zn to the endosperm in either wheat (Stomph et al., 2011) or rice (Jiang et al., 2008). Hence the differences in Zn transport pathways within the grain are likely to contain the limiting process that affects Zn distribution in the endosperm.

The localised distribution of elements in the rice embryo was variable depending on the element and between plant lines. Compared to either NT or A2.3 grains, there were high counts for Mg, K and P, especially in the aleurone but also K and P in the coleoptile and radicle of G1.4. Mg, K and P were also distributed mainly to the scutellum of A2.3, however there were generally much lower counts for all these nutrients. In contrast with the macronutrients, the tissues with the highest counts were the coleoptile, in the case of Mn and Zn, and the radicle in the case of Fe in G1.4. Contradicting a number of other studies (Takahashi *et al.*, 2009; Johnson *et al.*, 2011; Kyriacou *et al.*, 2014), there was no particular localization of Fe, Mn or Zn in the scutellum in preference to the other embryo tissues. Endosperm specific and constitutive expression of *HvSUT1* in rice grains in this study did not appear to change the distribution of Zn in the embryo. The distribution of Zn in the plumule and radicle in this study aligns more with Lu *et al.* (2013), who observed similar localization of Zn using μ-XRF.

The differences in spatial distribution of minerals in rice grains between plant lines could be explained by taking into account the potential differences in rice grain development due to increased sucrose supply. Ishimaru *et al.* (2003) observed that the ventral nucellus was almost completely compressed by 9 DAA as storage products in the endosperm filled the cells, causing the expansion and crushing of the nucellus against the hull. The dorsal vascular region however, the only pathway for assimilates to enter the grain from the long distance pathway, remains uncompressed until later grain maturity. This is associated with the greater abundance of storage proteins in the dorsal region of the grain (Ellis *et al.*, 1987) and could also explain the localization of nutrients particularly in the dorsal region of the rice grains in this study. The expansion of endosperm cells with starch and storage products could also potentially constrain transport activity as the cell plasma membranes are compressed, thereby hindering the activity of membrane bound proteins. The ovular vascular trace (OVT) evident particularly in the longitudinal grain images, appear thicker in G1.4 compared to the control NT and A2.3 and there were also changes in colocalization of mineral elements in this region in G1.4 grains. The inability of the grain to expand with increasing assimilates due to the rigid hull, could explain the redistribution and

concentration of mineral nutrients distinctly around the OVT and penetrating into the endosperm. The transverse images also indicate that some elements, such as Mg, K and P were distributed more evenly in the lateral regions rather than only in the dorsal region of the aleurone, compared with the NT grains. In addition, there is an obvious redistribution of micronutrients in the middle of G1.4 grains. Thicker vasculature, more even circumferential distribution of nutrients and the mapping of Fe, Mn and Zn in inner endosperm are in line with the increased retention of mineral nutrients measured in polished G1.4 rice grains compared to NT and A2.3.

Phytic acid (PA) which accounts for more than 70% of the P in rice grain (Ogawa *et al*., 1979), is distributed primarily in the aleurone (Iwai *et al*., 2012) and binds strongly with minerals to form phytate. In transgenic rice grains expressing *HvSUT1*, similar distribution of P and Fe in the aleurone is made apparent in tricolour maps overlaying the distribution of P, Fe and Zn. PA and Zn have been shown to have different distributions in rice grain (Liang *et al*., 2008) and speciation of Zn with P containing compounds was not found in barley grain (Perrson *et al.,* 2009). However in this study, Zn and P were found to colocalize together in the aleurone of both NT and G1.4, and to a lesser extent in A2.3. Furthermore, mineral nutrients appeared to be homogeneously distributed in the aleurone and embryo of NT grains, while in rice grains overexpressing *HvSUT1*, tricolour elemental maps revealed changed patterns in elemental distribution. LA-ICP-MS imaging therefore provides further evidence for the changes in mineral nutrient distribution and retention in rice grains with potentially increased sucrose uptake.

Ogawa *et al.* (1979) speculated that K had important roles in early endosperm development because at grain maturity they observed that K was redistributed from the endosperm to the aleurone for storage. The steep accumulation of K up to15 DAA measured in NT rice grains in this study followed by a slight decrease in content, is mirrored in WT rice grains analysed in Iwai et al. (2012), and lends support to Ogawa et al.'s (1979) theory. Hence, the transient increase in K accumulation in G1.4 rice grains at 20 DAA compared to NT was substantiated and could contribute to the the differences observed in K distribution in G1.4 grains. Increased K in unpolished mature G1.4 could be due to increased transport of K from the endosperm into the embryo and aleurone of the rice grain when additional K was available for assimilation. Removal of the embryo and aleurone during polishing, therefore removed a large proportion of the K in the grain. The decreased concentration and retention of K in the endosperm of mature G1.4 grains supports this theory. As the embryo images for rice grain show, K was widely distributed in the embryo, which suggests crucial roles for this ion in plant

growth. Indeed, Lu *et al.* (2013) visualized rapid remobilization of K into the shoot and root of germinating seedlings from 12 to 48 hours after imbibition. The high mobility to, and preferential storage of K in tissues that can directly supply the embryo could explain the K distribution observed in grains from the transgenic lines that were overexpressing *HvSUT1*.

It was interesting that Mn, out of all the elements measured did not show greater retention in endosperm of transgenic grains, although elemental maps showed that it is distributed with Zn in the endosperm. Although Mn and Zn are both phloem mobile, it is generally agreed that Mn is transported to cereal grains via the xylem (Rengel *et al.*, 2002). However, reduction of sucrose transport into wheat grains *in vitro* resulted in Zn and Mn reduction (Pearson *et al.*, 1996). Pearson *et al.* (1996) suggested that while Zn was affected by the mass flow of sucrose, reduction in Mn loading into the phloem at the xylem discontinuity was the reason for the decrease in Mn transport. However, in rice grains there is no discontinuity and the xylem and phloem are continuous but there is scarcity of data to explain the contribution of the xylem in grain nutrient loading in rice. Oparka and Gates (1984) suggest that the xylem can function as a conduit for the removal of water in opposition to the phloem rather than in loading water into the grain. This theory is supported by observations that, unlike wheat and barley, rice lemma and palea are fused tightly to prevent water loss and do not have stomata (Oparka and Gates, 1984). This could also explain the pattern of Mn accumulation measured in immature rice grains that, contrary to the other mineral nutrients measured, showed a decline in Mn content as the grain matured. In addition, Mn should not be affected by decreases or increases in sucrose transport. Indeed, Mn retention did not change in transgenic rice grains expressing *HvSUT1,* and so appeared to be insensitive to potentially increased sucrose flux.

3.5 Conclusions

Rice plants transformed with *HvSUT1* transgene and expressing HvSUT1 protein had changed patterns of sucrose uptake rather than displaying net increases in sucrose uptake rate. *HvSUT1* expression changed sucrose and starch contents, but not total protein content in mature rice grains. The most significant finding was increased mineral nutrient retention in the endosperm of the transgenic grains. In addition, elemental maps of rice grains analysed by LA-ICP-MS showed that there were differences in the distribution of micronutrients Fe and Zn compared to control non-transgenic grains.

Evidence from this study supports the hypothesis that increasing sucrose uptake can increase distribution of micronutrients in the rice endosperm. However, total mineral nutrient concentrations did not change between transgenic and control grains. Stomph *et al.* (2011) suggested that like sucrose, grain sink strength could be a strong determinant for the ability of grains to take up Zn. Sink strength can be affected by assimilate uptake capacity and the size and metabolic activity of the storage sink, but also source tissues. In the present work, sink strength was manipulated but the source (soil nutrient supply) may have been the limiting factor. By necessity these transformed plants were grown only under greenhouse conditions. Ideally, more single copy transgenic lines should be evaluated under field conditions, as transgenic plants may respond very differently to controlled or semi-controlled greenhouse conditions (Schuh *et al.*, 1993; Anand *et al.*, 2003; Weichert *et al.*, 2010). Further analyses of these transgenic plant lines under field conditions and over several seasons is required to conclusively determine *HvSUT1* transgene effects on rice grain mineral nutrients.

Changes in nutritional quality, growth and yield impact of *HvSUT1* overexpressing rice

4.1 Introduction

Sucrose transported into cereal grains during grain development is converted into a range of storage products that can be utilised by the growing embryo at germination. The remobilization of these reserves of starch, protein and lipids that have been stored in the endosperm and aleurone is crucial to feed the growing embryo, especially before the growing rice seedling can synthesise its own nutrients. Hence, the quantity, quality and distribution of these storage products in the mature rice grain are important for rice seed and seedling vigour. In addition, nutrient composition of rice grains, particularly in the endosperm, has implications for grain quality and human health.

Starch is the major storage product in rice grain that is broken down into sugars to supply the developing embryo. However a proportion of the carbon and nitrogen supplied to the rice seed is channelled into the production of seed storage proteins (SSP). Extensive research on the different solubility fractions of SSP from rice gives estimates of the contribution of each fraction to the total in rice endosperm. Albumins make up the smallest proportion at 2% (Shyur *et al.*, 1988) to 5% (Ju *et al.*, 2001) of total SSP, followed by globulins which make up approximately 10% (Shyur *et al.*, 1988). The remaining SSP are prolamins (20 to 30%) and 60 to 80% glutelins (also referred to as 11S-type globulins) which, like in oats *Avena sativa* L., make up the majority of rice SSP (Juliano and Boulter, 1976; Kawakatsu *et al.*, 2010). Storage protein synthesis begins early in grain development, with the major polypeptides of glutelin proteins visible on SDS-PAGE gels at 5 to 6 DAA (Yamagata *et al.*, 1982). Globulin synthesis also commences at 5 to 6 DAA, while protein bands identified as prolamin do not appear until the mid-stage of grain filling, at 10 DAA. By this time, all SSP's found in the mature grain will have been synthesized in the milky endosperm of rice (Yamagata *et al.*, 1982). Storage proteins are sorted into two distinct protein bodies distributed throughout the endosperm. Glutelins and globulins are compartmentalized in protein storage vacuoles (PSV) (sometimes referred to as Protein Body II (PB-II) and described as large and irregularly shaped) (Yamagata *et al.*, 1982; Krishnan *et al.*, 1992) while prolamins are stored in small spherical protein bodies, sometimes referred to as PB-I (Yamagata *et al.*, 1982; Nagamine *et al.*, 2011). Albumin, on the other hand, appears to associate with starch within the rice endosperm (Yamagata *et al.*, 1982). Differential accumulation of prolamin was observed in wheat overexpressing *HvSUT1* (Weichert *et al.,* 2010), but SSP composition between wheat

and rice is very different (Shewry and Halford, 2002), hence the effects of increased sucrose uptake capacity on storage protein synthesis in rice cannot be inferred from analogous studies in wheat and requires investigation.

Some amino acids (AA) that are considered essential as they must be consumed because they cannot be synthesised *de novo* in the human body, occur in very low concentrations in cereal products. These essential AA include lysine, methionine and tryptophan. Other AA, although non-essential, also contribute to human health. The predominant storage proteins in wheat and barley are prolamins which are high in glycine and glutamine and deficient in lysine, threonine and tryptophan (Yamagata *et al.*, 1982; Shewry and Halford, 2002). Prolamins are further separated into sulphur-poor (13 kDa) and sulphur rich (10 and 16 KDa) groups, that have low or high cysteine content, respectively (Hagan *et al*., 2003; Shewry, 2007; Kawakatsu *et al.*, 2010). In contrast, rice has comparatively more lysine than either wheat or barley which reflects the higher proportion of glutelins in the grain (Shewry, 2007). Globulin, which is rich in sulphur, is the other major storage protein in rice endosperm (Krishnan *et al.*, 1992). There is also a small free amino acid pool in cereal grains that can provide information about the total amino acids bound in storage proteins (Schaeffer and Sharpe, 1997). Attempts to increase essential AAs in rice have included knockdown of 13kDa prolamin indigestible protein by RNAi (Kim *et al.*, 2013). The resulting rice grains contained slightly increased glutelin protein, resulting in 28% more lysine. Hagan *et al.* (2003) introduced a sunflower seed albumin, high in methionine and cysteine, into rice but found that there was little increase in these AA. Instead, they observed that the transgenic rice grains had a different storage protein profile and an increase of non-storage proteins in the endosperm. Further analysis of these plants grown under sulphur limitation by Islam *et al.* (2005) showed that, in particular, sulphur rich glutelins and globulin protein were reduced. These examples highlight that storage protein composition is plastic and any changes observed in storage protein profiles are likely to be reflected in amino acid composition, thereby changing the nutritional profile of the rice grain.

Rice lipids are another important component of the rice grain and are mostly localized to the embryo and aleurone layers, with very little in the endosperm. Lipids are an important storage form for nutrients that can be quickly mobilised to the embryo during germination. The three main classes of lipids in rice are triacylglycerols (TAG) (neutral), phospholipids (PL) (polar) and free fatty acids (FFA). PL and FFA are thought to be the main lipids associated with starch in rice endosperm and complexed with amylose while TAG are associated more with lipid bodies in the bran layers, including the sub-aleurone (Choudhury and Juliano, 1980). TAG in unpolished and

polished rice does not vary significantly, however there are distinct changes in FFA composition, especially with higher oleic and lower linoleic acids in unpolished rice (Yasumatsu *et al.*, 1964). There also appears to be very little natural variability in lipids between rice genotypes, as amongst five Japanese rice varieties there were no significant differences in FA composition in the bran (Yoshida *et al.*, 2011). Carbon partitioning into lipids in a waxy rice mutant with low amylose was the same as controls, hence excess carbon not converted into starch was not diverted into production of more rice lipids (Zhang *et al.*, 2012). However, this study only measured total lipids and did not examine any changes in lipid composition. An interesting study by Ekman *et al.* (2008) used oat as a model to examine partitioning of carbon supplied in the form of [^{14}C] radiolabelled sucrose, into lipid synthesis. Oats, unlike other cereals, stores a relatively large percentage of oils in endosperm cells, alongside starch. Oil biosynthesis occurs early in grain development but does not interfere with starch synthesis which starts at the same time but continues throughout grain ripening. Ekman *et al.* (2008) found that detached panicles from a high oil oat genotype fed with [^{14}C]-sucrose, accumulated more ^{14}C into seeds and partitioned a higher proportion of the ^{14}C into lipids in the endosperm, compared with a medium oil oat genotype, with no change in starch concentration between genotypes. Regulation of sucrose partitioning is thus important in determining lipid composition in cereal grains. Although rice does not normally accumulate lipids in the endosperm, storage products in rice are more closely aligned with oats rather than barley or wheat (Shewry and Halford, 2002). Since lipid synthesis is also affected by sucrose partitioning, lipid composition in rice grains under increased sucrose uptake also needs to be explored.

Phytate, the main storage form of P in grain tissues, is present in cereal staples and complexes tightly to mineral ions, preventing them from being properly absorbed in the human intestine (Hunt, 2003). The presence of phytate inside PSVs in aleurone and embryo cells has been demonstrated by staining and transmission electron microscopy (TEM) and correlated with high grain Fe (Prom-u-thai *et al.*, 2008). Endosperm does not contain visible amounts of phytate, however Prom-u-thai *et al.* (2008) measured up to 16 μmol g^{-1} in a rice genotype with low Fe concentration. Diets containing only plant staples as opposed to diets including meat, have much less bioavailable Zn for absorption mainly due to the presence of plant anti-nutrients such as phytate. Wessells *et al.* (2012) found a negative correlation between adequate Zn intake and national diets lacking in food from animals sources. They also found strong positive correlations between diets with a high P:Zn ratio and inadequate zinc intake. Predicted P:Zn, phytate:Zn (Ma *et al.*, 2005; Norhaizan & Nor Faizadatul Ain, 2009) and phytate x Ca/Zn (Fordyce *et al.*, 1987) molar ratios derived from food content estimates

have been used as indicators of bioavailability of mineral nutrients in human diets. Suggested values above which mineral absorption is compromised are <18 for phytate:Zn, <1 for phytate:Fe, <0.17 or <0.24 for phyate:Ca and <200 for phytate x Ca:Zn (Fordyce *et al*., 1987; Ma *et al*., 2005; Gibson *et al*., 2010). However, there is little consensus on whether these predicted numbers can have any useful application in human nutritional studies. Nevertheless, these predictive arithmetic models for bioavailability have advantages over *in vivo* methods using animal or human subjects as they are non-invasive, require no animal/human ethics approval and can be an effective alternative in predicting the impacts of inhibitory compounds in foodstuffs on nutrient absorption (Wienk *et al.*, 1999).

Seed nutrient content not only has implications for human health, but also in seed viability and establishment of the growing seedling. It has been reported that increased Zn content in wheat seeds gives young seedlings a growth advantage, especially in Zn deficient soils (Rengel and Graham, 1995). Seed size and protein content is also positively correlated to the growth of more vigorous wheat seedlings (Ries and Everson, 1973; Evans and Bhatt, 1977). Increase in seedling vigour was also observed in spring barley and oats with higher protein content (Welch, 1977). However, there does not appear to be any yield advantage of mature plants grown from seeds with higher protein content (Welch, 1977). There is a paucity of information on the relationship between nutrient content in rice grains and the effects on seedling growth and yield of mature plants in following generations. It is particularly important to investigate potential advantages or disadvantages of manipulating nutrient contents on future generations of transgenic plant lines to resolve any unintended effects arising from plant transformation.

Previous chapters have described the creation and characterisation of transgenic rice lines overexpressing the barley *HvSUT1* gene. In addition, detailed analysis of the changes in micronutrient levels and distribution of minerals in SUT-OE plants was undertaken. In this chapter, rice grains from transgenic plants were also analysed for changes in storage products that are potentially affected by increased sugar supply to rice grains. It is hypothesized that changing sucrose flux into the grains not only affects the distribution of mineral elements, but also stimulates changes in concentrations and compositions of other grain storage products. The nutrient content and composition of mature rice grains could also exert effects on the growth and yield of the future generations of plants, hence the same plant lines were analysed for growth and yield parameters at the seedling stage and at maturity.

4.2 Materials and methods

4.2.1 Storage protein fractions

Storage proteins were extracted sequentially from polished rice flour using protocols and buffers that were modified from Osborne solubility fractionation used in Yamagata *et al*. (1982) and Wakasa *et al*. (2009). Samples of polished rice flour (25 mg) were shaken vigorously with 500 µl of the following extraction solvents for 2 hr at RT for 2x. In between each protein fraction, the flour pellet was spun down and the pellet was washed with ultrapure laboratory grade water (Milli-Q® water) before changing buffers. Albumins and globulins (Alb/Glob) were extracted together with 0.5 M NaCl, 10 mM Tric-HCl pH 6.8, followed by prolamin (Pro) extraction with 55% n-propanol. In addition, albumins/globulins and prolamins were precipitated in 100% acetone before resuspending the protein pellet in sample buffer (50 mM Tris-HCl pH 6.8, 8 M Urea, 4% SDS, 20% glycerol, 5% βME, 0.01% bromophenol blue). The flour pellet remaining after Alb/Glob and Pro extraction was shaken with sample buffer to extract the remaining glutelins (Glut). Protein samples were boiled for 5 min, and centrifuged at maximum before running on 15% SDS-PAGE gels. Gels were stained with Coomassie brilliant blue (CBB) (0.1% Coomassie R-250, 40% v/v EtOH, 10% acetic acid), destained overnight in Milli-Q® water and bands imaged in an EZ Doc Imager (Biorad). Replicate samples (three per line) were run on the same gel to enable relative quantification of storage proteins by densitometry. Relative quantity of protein bands of plant lines was analysed using ImageLab software (Biorad) with manual band detection and automatic background subtraction.

Protein bands were cut out from CBB stained gels and analysed by Matrix assisted laser desorption/ionization time of flight (MALDI-TOF/TOF) mass spectrometry (Proteomics International, Perth). In brief, protein bands were digested with trypsin then peptides extracted for analysis using a 5800 Proteomics Analyzer (AB Sciex). Protein identification was determined using Mascot software searching on the Ludwig NR database for matches to the peptide sequences for *Oryza sativa* subsp. japonica.

4.2.2 Amino acid quantification by LC-MS

Quantification of free amino acids was conducted by LC-MS (Metabolomics Australia, Melbourne University). Polished rice flour (25 mg) was extracted with 50% aqueous MeOH (with the addition of 2% internal standard of 0.5 mg mL^{-1} each of ^{13}C-Sorbitol and $^{13}C5$-^{15}N Valine and 0.25 mg mL^{-1} each of 2-aminoanthracene and

pentafluorobenzoic acid) with 20 mM EDTA as a metal chelator (total 200 µl volume). After mixing and centrifuging at 13000 xg for 10 min, the supernatant was removed to a fresh tube and a 10 µl aliquot was added to 70 µl of 200 mM borate buffer (1 mM ascorbate, 10 mM TCEP, 25 µM 2-amino butyric acid) and mixed. The tubes were centrifuged for 2 min at 13000 xg then 20 µl of 6-aminoquinolyl-*N*-hydroxysccinimidyl carbamate (AQC) reagent was added and incubated with shaking for 10 min at 55°C. After centrifuging at 13000 xg for 1 min, the supernatant was added to HPLC vials for analysis. Calibration standards were run at 0.1 to 150 µM concentration. Final concentrations of amines were normalized to the internal standards. Amino acid concentrations are expressed as milligram of amino acid per gram of flour sample on a dry weight basis.

4.2.3 Lipid and fatty acid composition

Fatty acid analysis was carried out by Waite analytical services (WAS). In brief, lipids were extracted from rice flour samples using a Bligh and Dyer method with salt modification as described in Makrides *et al*. (1996). Lipid classes were separated using Thin Layer Chromatography (TLC). To determine proportions of fatty acid methyl esters (FAMEs) in each fraction, they were extracted and separated then analysed by gas chromatography (GC) (Hewlett Packard 6890 Gas Chromatograph with a SGE BPX70 column and a Flame Ionisation Detector (FID).

4.2.4 Phytate extraction and quantification

Samples of polished rice flour (250 mg) and 10 ml of 1.25% sulphuric acid were mixed in 15 ml tubes and incubated on a rotary shaker at RT for 2 hr. After spinning the tube down at 3000 xg for 10 min, a 1 ml aliquot of supernatant was transferred to 1.5 ml tube and spun at 13000 xg for 10 min. The supernatant was diluted 1:100 with Milli-Q® water before measuring using HPAEC-PAD.

Phytate was quantified by comparing samples to calibration standards from a stock solution of D-myo inositol 1,2,3,4,5,6-Hexakisphosphate, Dodecasodium salt from *Zea mays* (407125, Calbiochem). 10 µl injections of sample were passed through a Dionex IonPac AS 11 2x250mm analytical column with 2x50mm guard column at a flow rate of 0.5 ml min^{-1} and eluted with increasing eluent concentrations of 20 mM to 43.5 mM KOH generated using the Dionex RFIC-eluent generator.

4.2.5 Growth and grain yield

Seeds from each line were germinated on wet filter paper and seedling height was first recorded at transplant to soil (1 week after germination) and daily until 30 days after transplant (DAT) for 16 plants from each plant line. At 7, 15 and 21 DAT, four plants of each line were gently removed from the pot, soil washed from the roots, the plant divided into roots and shoots and the samples weighed. The samples were then dried at 85 °C overnight and roots and shoots were reweighed.

Remaining plants were monitored daily for changes in development. At the ends of the vegetative stage (approximately 8 weeks after transplant) plant height was measured. At anthesis, panicles were tagged and the date recorded. Days to anthesis and days to maturity (number of days to 50% flowering) were calculated from the date of transplant (7 days after sowing). When the majority of the grains on a plant had turned straw coloured, panicles were harvested and dried in paper bags at 37 °C for three days. The remaining straw was collected and dried at 85 °C for three days and weighed. Panicle length and the number of filled grains per panicle were estimated from at least four comparable panicles per plant. Yield and grain weight was estimated from subsamples of grains from each plant. Means and standard deviations were calculated from 16 plants for each line.

4.3 Results

4.3.1 Storage protein fractions in polished rice

Storage proteins extracted sequentially from polished rice flour and separated by SDS-PAGE were stained with CBB to visualize the abundant protein bands for each protein fraction (Fig. 4.1). The protein profile for the albumin/globulin fraction showed a very prominent band between 20 and 30 kDa which is most likely the 26 kDa globulin (Fig. 4.1 A). Low molecular weight (LMW) bands below 20 kDa are likely to be a number of polypeptides that are unresolved. A number of HMW bands at 55 kDa and above are also apparent, however there appeared to be a higher abundance of a protein resolving at 55 kDa in line G1.4. Hence this band was chosen for identification by MALDI-TOF/TOF. In the prolamin protein fraction (Fig. 4.1 B), there were two prominent bands at approximately 14 and 16 kDa, although these thick bands could be masking a number of unresolved polypeptides. A low abundance protein in the prolamin fraction resolved as a faint band at ~18 kDa, however it was noted that it was consistently more abundant in G1.4 samples. Hence, this band was also cut out and

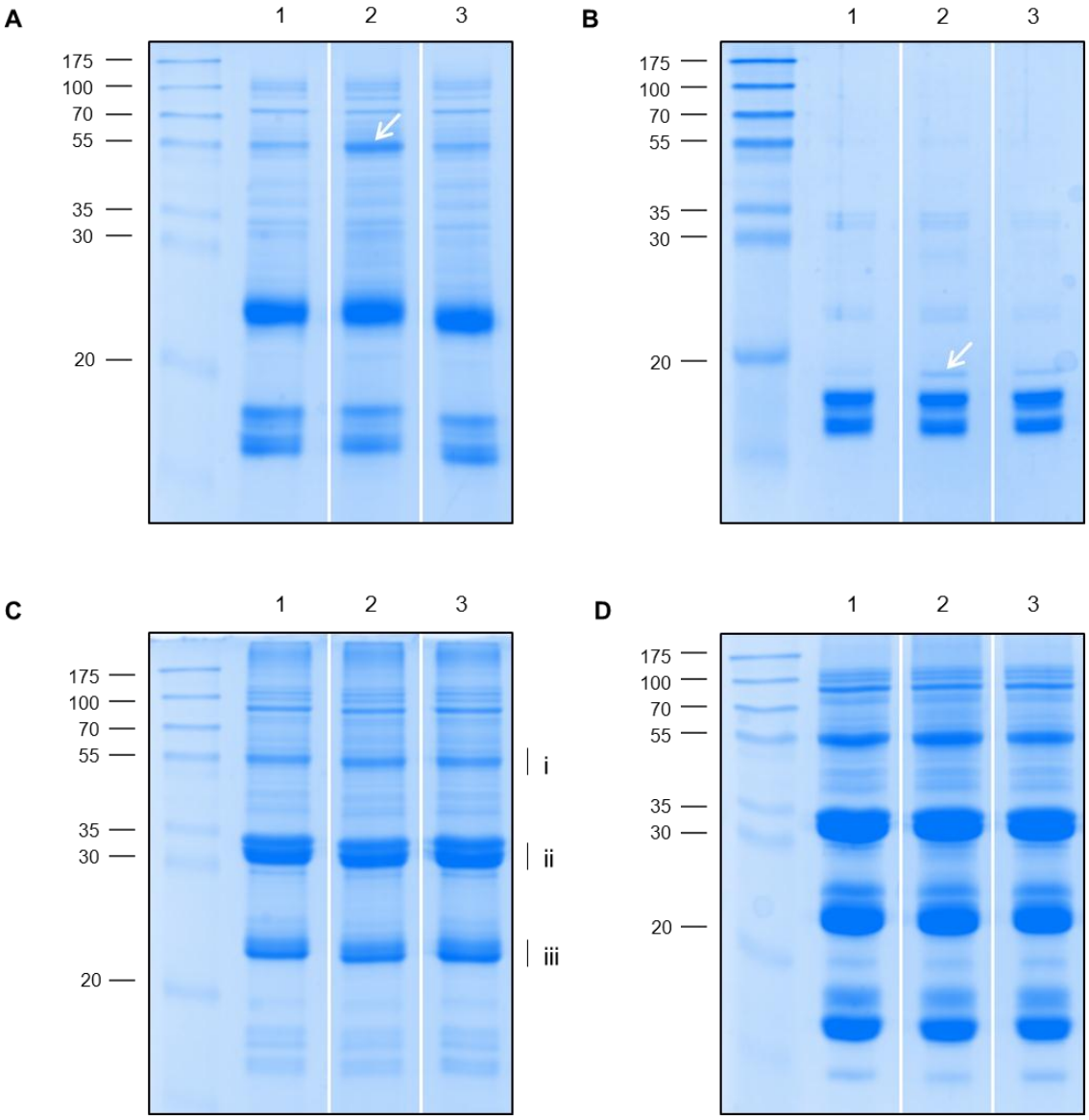


Figure 4.1 Storage proteins in rice endosperm

Storage protein classes albumins/globulins (A), prolamins (B) and glutelins (C) were extracted from polished rice flour sequentially using Osborne fractionation. Equal amounts of protein samples were separated on 15% SDS-PAGE gels. Total storage protein (D) was also extracted with sample buffer to extract all storage proteins for comparison. Original gel pictures contained three lanes each of representative storage protein profiles for NT (Lane 1), G1.4 (Lane 2) and A2.3 (Lane 3). However, due to the consistency of protein profiles between replicates, gels are presented with replicate lanes removed. White arrows highlight the bands for a ~55kDa protein in the albumin/globulin fraction and a <20kDa band in the prolamin fraction that were excised and identified by MALDI-TOF/TOF. Prominent bands in the glutelin fraction (C) are (i) proglutelin, a glutelin precursor, (ii) glutelin acidic subunits and (iii) glutelin basic subunits.

identified by MALDI-TOF/TOF. Bands that resolved at 55 kDa, 30-35 kDa and ~23 kDa in the glutelin protein fraction (Fig. 4.1 C) are likely to be the precursor, acidic and basic subunits respectively of rice glutelin. There does not appear to be any difference in the abundance of glutelin protein polypeptides discernable by CBB staining between the plant lines. Likewise for the bands in the total storage protein CBB stained gel (Fig 4.1 D), that are similar between lines.

Relative quantification of storage protein polypeptides was analysed by densitometry for comparison between plant lines as it was acknowledged that some differences in protein abundance were indistinguishable by eye. As expected, the density of the 55 kDa protein band from the albumin/globulin fraction and the 18 kDa protein band from the prolamin fraction were both significantly higher, relative to total protein and expressed as arbitrary units, in line G1.4 compared to the NT control (Fig. 4.2 A). There was also significantly more of the 18 kDa protein in line A2.3. The difference in abundance of glutelin polypeptide bands in Fig 4.1 C do not appear to differ between plant lines, however when analysed by densitometry, the acidic subunits between 30 and 35 kDa in line G1.4 were significantly less than in NT controls (Fig. 4.2 B). There was no change in proglutelin or the glutelin basic subunits between the three plant lines in the glutelin fraction. No discernable difference was seen between the ~14 and 16 kDa bands in the prolamin fraction CBB stained gel, however increased abundance of the 16 kDa band relative to total protein was measured in line G1.4 (Fig. 4.3 A). There was no difference in band density of the ~14 kDa prolamin polypeptide in any of the samples. The 26 kDa protein band (Fig. 4.1 A) was slightly less abundant in both G1.4 and A2.3 compared to NT in the globulin protein fraction (Fig. 4.3 B).

4.3.2 Identification of protein bands by MALDI-TOF/TOF

A number of peptides were identified after tryptic digest and MALDI-TOF/TOF of the 55 kDa band from the albumin/globulin fraction (Table 4.1). Rather than resolving as one protein, the band appears to be a group of polypeptides resolving at the same molecular weight. Four putative proteins with molecular masses between 53-56 kDa and whose sequences matched the same peptides have been excluded from this analysis as they have not been characterised and there is no published information on their identities. The remaining proteins that were identified from the sample by having at least one unique peptide per match, were all members of the glutelin multigene family. Two hits were for glutelin proteins with different accession numbers and with different molecular weights (56011 and 56211 Da). Three members of the GluA family of glutelin proteins, GluA1, GluA2 and GluA3 were identified in the sample as well as

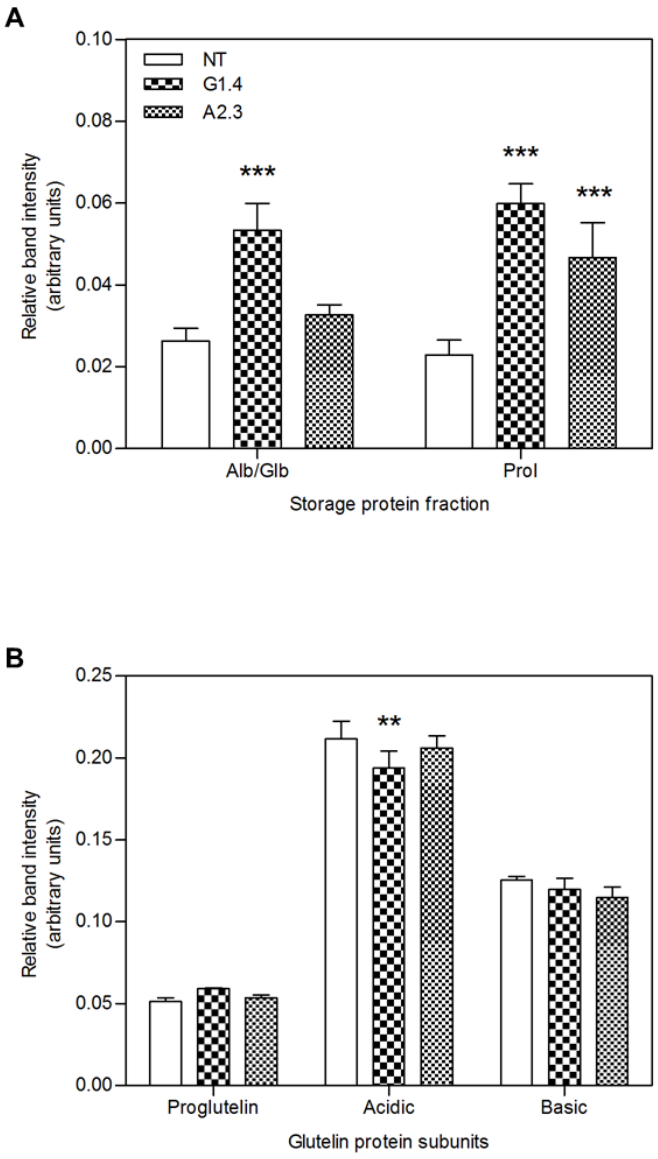


Figure 4.2 Relative quantification of unidentified protein bands and glutelin subunits

Densitometry to determine relative quantities of two proteins bands from CBB stained gels from the Alb/Glob and Pro storage protein fractions sent for MALDI-TOF/TOF protein identification (A) and relative quantities of Glut subunits in the Glut storage protein fraction (B). Densitometry using Image Lab software (Biorad) was used to quantify the intensity of each band relative to total protein loaded. Values are means + SD (n=3). Significant differences according to One way ANOVAs are **p<0.01, ***p<0.001.

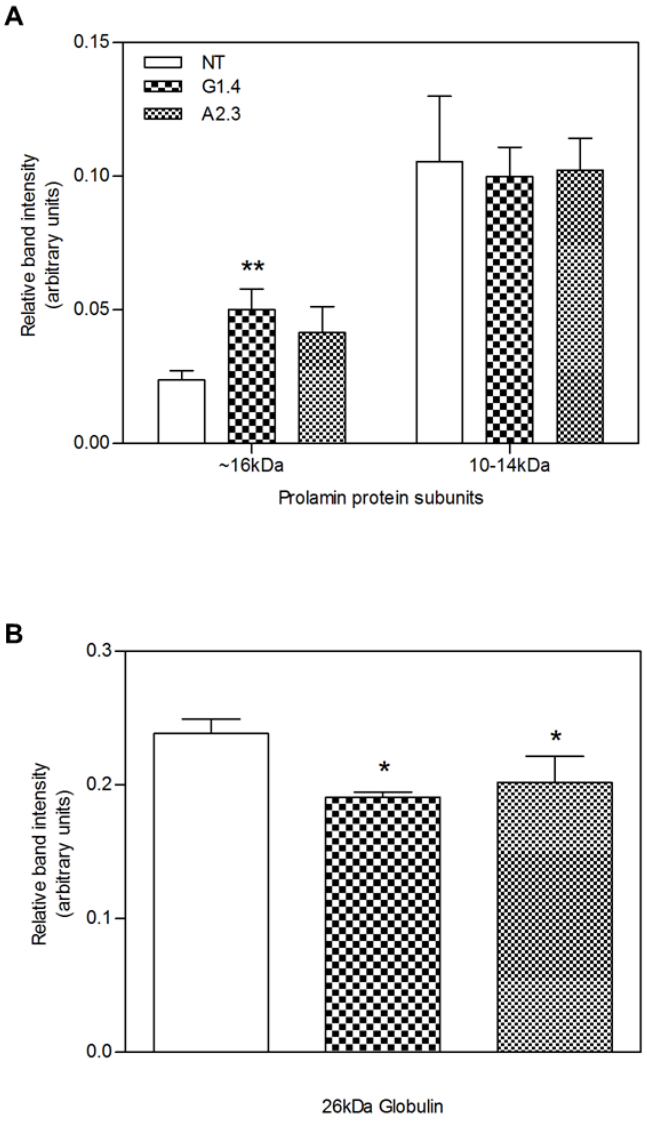


Figure 4.3 Relative quantification of prolamin polypeptides and 26kDa globulin

Densitometry to determine relative quantities of prolamin polypeptides from CBB stained gels from Pro storage protein fraction (A) and relative quantity of the 26kDa globulin protein in the Alb/Glob storage protein fraction (B). Densitometry using Image Lab software (Biorad) was used to quantify the intensity of each band relative to total protein loaded. Values are means + SD (n=3). Significant differences according to One way ANOVAs are *p<0.05, **p<0.01

Table 4.1 Protein identification by analysis of peptide sequences obtained by MALDI-TOF/TOF MS

Bands from CBB stained gels (~ 55kDa band cut from albumin/globulin gel and ~18kDa band cut from prolamin gel) were digested and the resulting peptides analysed by MALDI-TOF/TOF. Only individual ion scores >36 that indicate extensive homology have been included (Statistically significant at $p<0.05$). Putative uncharacterised protein matches have been excluded.

Fraction	Accession #	Total peptides	Unique peptides	MW (Da)	Protein ID	Seq. coverage %
Alb/Glob	P14323	7	1	56515	GluB1-A	16.2
	A1YQH5	7	1	56011	Glutelin	16.4
	Q02897	7	1	56012	GluB2	16.4
	A1YQG5	4	2	56211	Glutelin	13.0
	P07728	4	1	56212	GluA1	13.0
	P07730	3	1	56271	GluA2	8.0
	Q09151	2	1	55980	GluA3	4.0
Pro	Q10EK7	5	5	17211	OLE18 18kDa	22.1

two members of the GluB family, GluB1 and GluB2. All polypeptides had predicted masses of approximately 56 kDa.

Five unique peptides extracted and identified from the 18 kDa band prepared from the prolamin storage protein fraction aligned to one sequence in the protein database for *Oryza sativa* ssp. japonica. The only protein sequence that was recognised as a statistically significant match to those peptides was OLE18, the rice 18 kDa Oleosin protein. Other peptides identified from the sample had low ions scores, were not statistically significant and hence have not been included in these analyses.

4.3.3 Amino acid content and composition

The content of 19 free amino acids was analysed in unpolished and polished rice using LC-MS. Total amino acid content was much higher in unpolished rice samples compared with polished (Table 4.2). Putrescine, taurine and GABA were also measured however their contents were very low and did not vary between plant lines (data not shown). There were trends towards higher Asn and Cys and lower Arg, Pro, Ser, Thr, Tyr and Val in unpolished rice of line G1.4 when compared to both the NT control and line A2.3, however due to the large variation between replicate samples the differences were not significant. The only amino acid that showed any significant change in either transgenic line in unpolished rice was tryptophan (Trp) which was significantly increased in A2.3. The three amino acids with the highest contents, Glu, Asp and Asn were significantly increased in polished rice from both line G1.4 and A2.3. There were also significant increases in Glu and Asn in A2.3. Cys concentration was increased ten-fold and Trp, Tyr, Gln and Lys were also significantly higher in line G1.4 compared to the NT control. Trp concentration in A2.3 however, was significantly lower compared to the non-transgenic control.

4.3.4 Lipid fractions and fatty acid composition

Total lipid content was not significantly different between plant lines (data not shown). Therefore, fatty acid composition of the lipid fractions triacylglycerols (TAG), phospholipids (PL), free fatty acids (FFA) and total lipids (TL) were compared between the NT control and both SUT-OE lines. The fatty acids were first broadly classified as saturates, monounsaturated FA (MUFA) and polyunsaturated FA (PUFA) (Fig. 4.4). In the TAG lipid fraction, lines G1.4 and A2.3 had lower contents of saturates but higher PUFA content compared to the NT control (Fig. 4.4 A). Likewise in the PL fraction, line G1.4 had lower saturates and the proportion of fatty acids in MUFA and PUFA was not changed in either G1.4 or A2.3 (Fig. 4.4 B). In the FFA fraction, there was no difference

Table 4.2 Free amino acid content in polished rice flour analysed by LC-MS

Amino acid contents are arranged from highest to lowest and expressed as mg/100g dry weight basis. Values displayed are means (n=3). Statistically significant differences according to One way ANOVAs are, **p<0.01, ***p<0.001.

	unpolished			polished		
	NT	G1.4	A2.3	NT	G1.4	A2.3
Glutamic acid	24.9	20.7	24.1	0.4	1.1***	0.9***
Aspartic acid	19.1	18.7	21.2	0.3	0.8***	0.6*
Asparagine	7.2	19.7	7.3	0.3	0.6*	0.7***
Arginine	11.8	5.5	10.5	0.1	0.1	0.1
Proline	3.9	1.6	4.6	0.1	0.2	0.1
Alanine	3.3	3.3	3.9	0.1	0.2	0.2
Serine	4.2	2.9	4.8	0.1	0.3	0.2
Cysteine	0.3	1.7	0.4	0.1	0.5**	0.2
Glycine	1.7	1.4	2.1	0.04	0.03	0.11
Tryptophan	0.9	0.7	1.9***	0.06	0.11***	0.04***
Threonine	1.2	0.6	1.1	0.03	0.05	0.03
Tyrosine	1.6	0.9	1.6	0.02	0.06***	0.04
Glutamine	0.8	0.7	1.1	0.02	0.04*	0.03
Valine	1.1	0.6	0.9	0.02	0.04	0.03
Lysine	0.6	0.4	0.6	0.02	0.04*	0.03
Methionine	0.3	0.2	0.4	0.02	0.03	0.02
Leucine	0.7	0.4	0.7	0.02	0.03	0.02
Phenylalanine	0.9	0.5	0.7	0.02	0.03	0.02
Isoleucine	0.5	0.3	0.4	0.01	0.02	0.01
Totals	85.0	80.8	88.4	1.7	4.2*	2.8

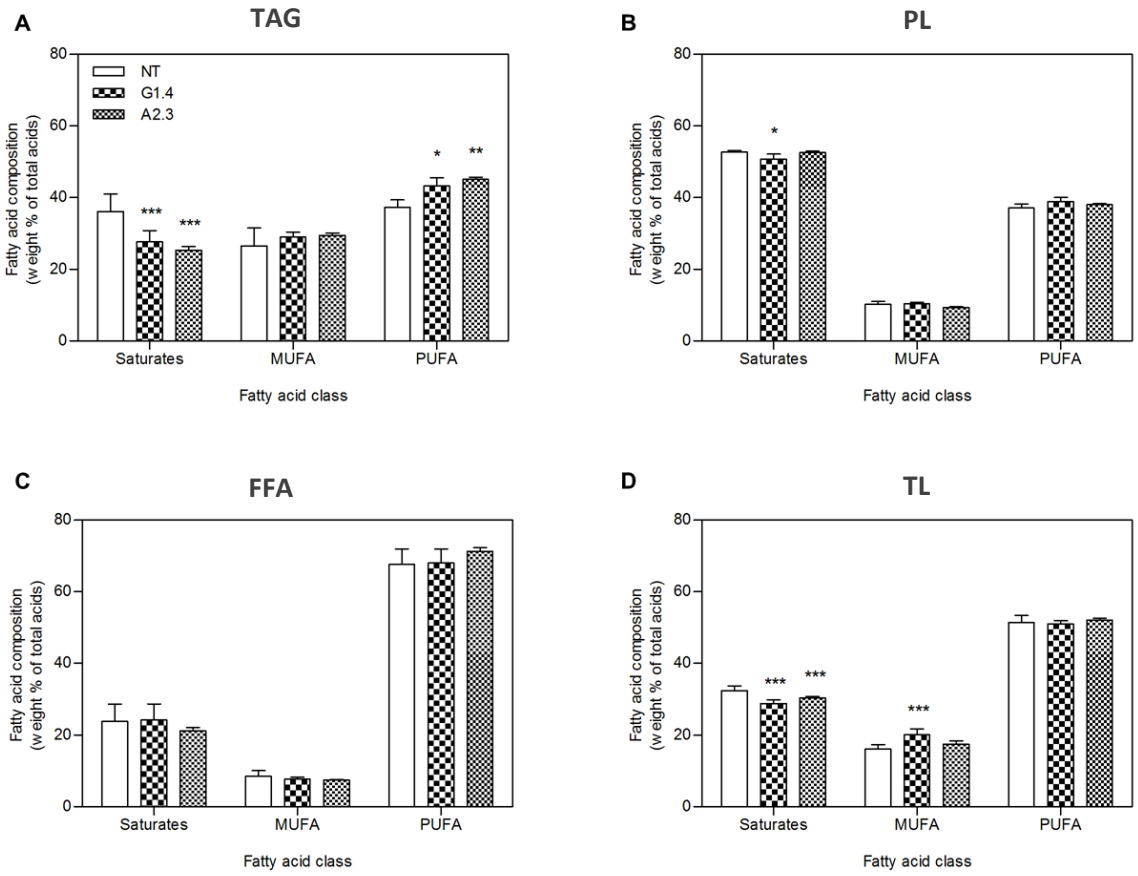


Figure 4.4 Comparison of broad classes of fatty acids distributed into each lipid fraction

Fatty acid compositions are presented for each lipid class; triacylglycerols TAG (A), phospholipids PL (B), free fatty acids FFA (C) and total lipids TL (D). Fatty acids have been divided into classes that relate to their nutritional value to humans, namely saturates, monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). Differences according to two way ANOVAs are significant as follows; *p<0.05, **p<0.01, ***p<0.001

in FA profiles amongst the three plant lines (Fig. 4.4 C). The decrease in saturated fatty acids observed in the TAG fraction was responsible for the overall decrease in saturates in the total lipids of G1.4 and A2.3 (Fig. 4.4 D). Notably, the proportion of MUFA was increased in G1.4 in total lipids, even though there was no obvious increase in each lipid fraction. Cumulatively, the slight increases in MUFA in G1.4 of each lipid fraction led to a significant increase when combined.

FA distribution was then further analysed by looking at the proportions of the separate major FA that make up the saturates, MUFA (omega-7 and 9) and PUFA (omega-3 and 6) classes (Table 4.3). The dominant FA in the saturates is palmitic (16:0), followed by much lower contents of myristic (14:0) and stearic (18:0) acids (Table 4.3). Amongst the lipid fractions, the polar lipids (PL) generally had more saturated FA content than the neutral (TAG) and insoluble (FFA) lipids. There was significantly less palmitic acid in both G1.4 and A2.3 in the TAG fraction. The comparatively lower contents of myristic and stearic acids in the three lipid fractions were not different between the three plant lines. Oleic acid (18:1n-9) was the dominant MUFA in all the plant lines, although the proportion of oleic acid in the TAG fraction was higher than in either the PL or FFA fractions. Oleic acid content was similar across all lipid fractions for all plant lines. Eicosenoic acid (20:1n-9) was only detected in the TAG fraction and did not differ between lines. Vaccenic (18:1n-7) and palmitoleic acid (16:1n-7) content was consistently low between plant lines in each lipid fraction. α-linolenic acid (ALA, 18:3n-3) content was similarly low between all three lipid fractions and between plant lines, ranging from 0.8 to 2.2% of total weight. In contrast, linoleic acid (LA, 18:2n-6) made up the highest proportion of fatty acids in TAG and FFA, and the second highest in PL and showed the greatest change in the two transgenic lines G1.4 and A2.3. Linoleic acid content was significantly increased in the TAG fraction for both lines compared to NT.

4.3.5 Correlations between storage sinks and minerals in polished rice grains

Scatterplots that show the relationship between phytate and P, Fe and Zn in polished rice between plant lines are presented in Fig. 4.5. Correlation coefficients (Pearson's r) for each plant line were computed to determine the strength and direction of the relationships between the variables. Unsurprisingly, there was a very strong positive correlation between phytate and P in all three plant lines (Fig 4.5 A). However, while phytate and Fe was positively correlated in G1.4 and A2.3, there was no correlation between phytate and Fe in the NT control (Fig. 4.5 B). There was also no

Table 4.3 Fatty acid composition of lipid classes TAG, PL and FFA

Individual fatty acid composition of the saturates, MUFA and PUFA in each lipid fraction compared between plant lines. Values are means (n=4). Statistically significant differences according to One way ANOVAs are, **p<0.01, ***p<0.001. TAG, triacylglycerols; PL, phospholipids; FFA, free FA; MUFA, monounsaturated FA; PUFA, polyunsaturated FA. Fatty acids are myristic (14:0), palmitic (16:0), stearic (18:0), oleic acid (18:1n-9), eicosenoic acid (20:1n-9), palmitoleic acid (16:1n-7), vaccenic (18:1n-7), α-linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6).

	Fatty acid composition of each plant line (weight % of total acids)								
	TAG			PL			FFA		
Fatty acid	NT	G1.4	A2.3	NT	G1.4	A2.3	NT	G1.4	A2.3
Saturates									
14:0	2.1	1.2	1.0	5.4	4.7	6.3	2.1	2.1	1.9
16:0	24.7	20.7**	20.0***	43.9	42.6	43.3	17.0	16.7	16.8
18:0	3.8	2.7	2.6	2.4	2.4	1.9	1.4	1.5	1.2
MUFA									
18:1n-9	24.8	27.5	28.0	9.4	9.5	8.5	8.0	7.1	6.8
20:1n-9	0.3	0.4	0.4	-	-	-	-	-	-
16:1n-7	0.6^	0.2	0.2	0.2	0.2	0.2	0.1	0.2	0.2
18:1n-7	0.9	0.9	0.9	0.7	0.8	0.7	0.5	0.5	0.5
PUFA									
18:3n-3	1.4	1.1	1.2	0.8	0.8	0.8	2.2	2.2	2.2
18:2n-6	35.8	42.1**	43.9**	36.0	37.8	37.2	65.3	65.7	69.1

^ mean value is skewed by one outlier. Removal of the outlier gives a mean value of 0.2 which is similar to the other values

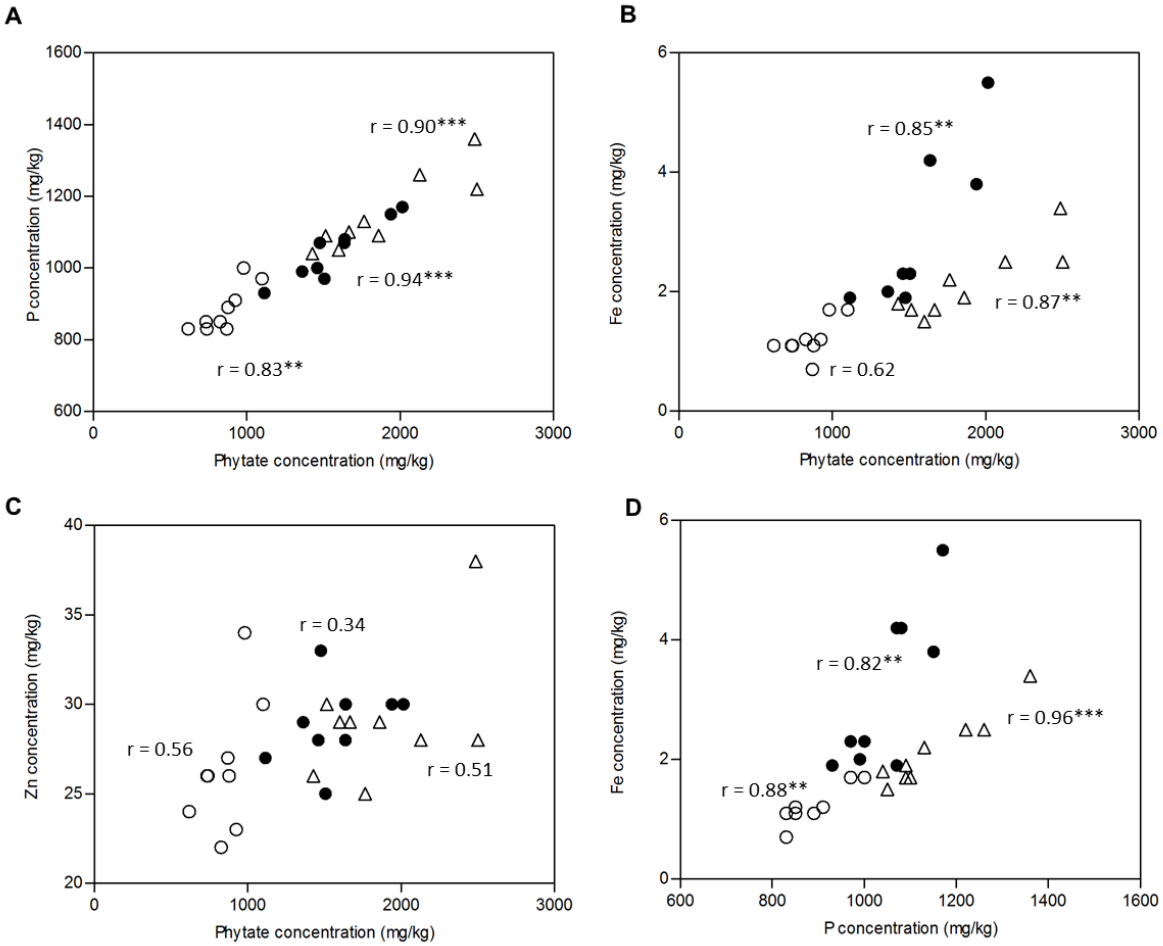


Figure 4.5 Correlations between phytate, P, Fe and Zn concentrations in polished rice

Scatterplots were generated to examine the relationships between phytate and P, Fe and Zn concentrations in polished rice grain. Plant lines are represented by symbols as follows; NT (white circles), G1.4 (black circles) and A2.3 (white triangles) for n=9. Statistically significant correlations according to Pearson's r correlation coefficient are *p<0.05, **p<0.01, ***p<0.001

correlation between phytate and Zn in any of the plant lines (Fig. 4.5 C). A statistically significant strong positive correlation between P and Fe was found in NT ($r=88$, $p<0.01$), G1.4 ($r=82$, $P<0.01$) and A2.3 ($r=96$, $p<0.001$). (Fig. 4.5 D).

The relationship between crude protein content and S, Zn and Fe concentration was also examined for each plant line. The strong positive correlation between protein content and S concentration in rice endosperm of NT and A2.3 was not observed in line G1.4 (Fig. 4.6 A). Moreover, protein and Zn was positively correlated in NT and A2.3, but not in G1.4 (Fig. 4.6 B). There was no correlation between protein and Fe in any of the plant lines investigated (Fig. 4.6 C). However, there were significant positive correlations between S and Zn concentration in NT ($r=83$, $p<0.01$), G1.4 ($r=77$, $P<0.05$) and A2.3 ($r=94$, $p<0.001$) (Fig. 4.6 D).

4.3.6 Phytate and mineral bioavailability

The phytate concentration of both transgenic lines, G1.4 and A2.3 was significantly higher than the control NT line (Table 4.4). Similarly, the P concentrations determined by ICP-OES were also higher in the transgenic lines, but P concentrations were lower than corresponding phytate concentrations in both transgenic lines. As determined previously (Chapter 3), there was no change in Zn and Fe concentrations between the plant lines. In addition, Ca concentration was the same in NT, G1.4 and A2.3 plant lines.

Higher phytate concentrations in polished rice of G1.4 and A2.3 compared to NT are reflected in the corresponding phytate:Zn and phyate:Ca ratios for these two lines. While the phyate:Zn ratios were higher in G1.4 and A2.3, the ratios for all three lines were below the critical level (<18). The phytate:Ca ratios on the other hand were much higher than the critical level (<0.24) in all three lines. The phytate x Ca/Zn molar ratios for all three lines were below the critical value (<200), even though the transgenic lines G1.4 and A2.3 were higher than NT. In contrast, phytate:Fe ratios for NT, G1.4 and A2.3 were 93.3, 73.2 and 128.9 respectively, which are much higher than the desirable limit of <1. It is noteworthy that the phytate:Fe molar ratio in line G1.4 was not significantly different from the NT line despite the higher phytate concentrations and lack of measurable change in Fe concentrations between these two lines.

4.3.7 Growth and yield comparisons

To analyse the multi-generational effects of *HvSUT1* overexpression on rice plants, seedling growth parameters were monitored from germination to 30 days DAT. All plant lines displayed approximately 100% germination and healthy seedlings were able to be transferred to soil 7 days after germination. Shoot and root dry weight was

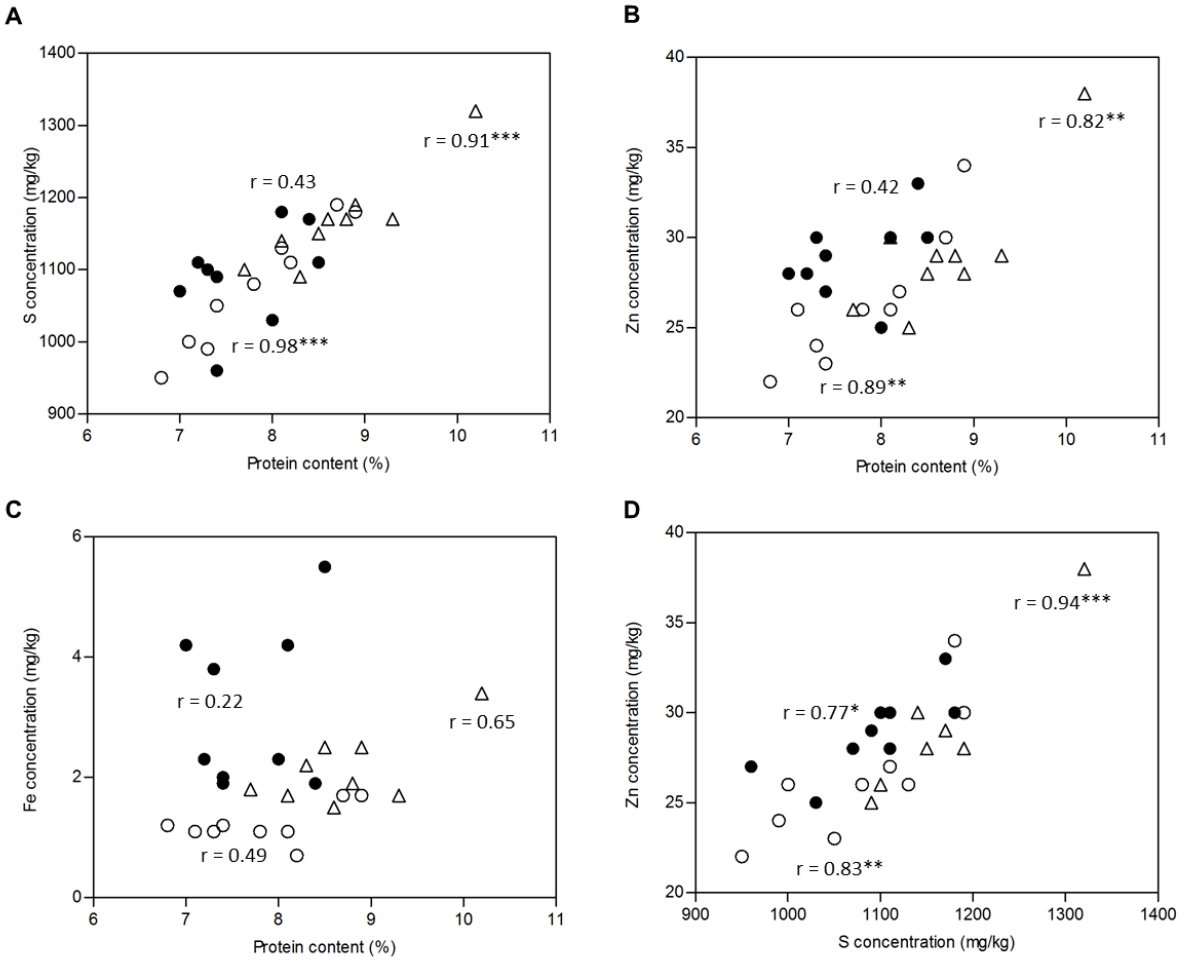


Figure 4.6 Correlations between protein content and S, Fe and Zn concentrations in polished rice

Scatterplots were generated to examine the relationships between protein content and S, Zn and Fe concentrations in polished rice grain. Plant lines are represented by symbols as follows; NT (white circles), G1.4 (black circles) and A2.3 (white triangles) for n=9. Statistically significant correlations according to Pearson's r correlation coefficient are *p<0.05, **p<0.01, ***p<0.001

Table 4.**4** **Nutrient content and bioavailability estimated by phytate:mineral ratios**

Phytate and mineral contents in polished rice and phytate:mineral molar ratios as indirect estimates of micronutrient bioavailability. Values are means ± SD (n=9). Statistically significant differences according to One way ANOVAs are, * p<0.05, **p<0.01, ***p<0.001. Desirable limits of phytate:mineral ratios obtained from Fordyce *et al*. (1987), Ma *et al*.(2007) and Gibson *et al*. (2010).

	Nutrient content (mg/100g dry weight)		
	NT	G1.4	A2.3
Phytate	85.3 ± 14.43	157.3 ± 27.82***	188.4 ± 40.2***
P	88.4 ± 6.39	104.8 ± 8.14**	114.9 ± 10.80***
Zn	2.6 ± 0.38	2.9 ± 0.22	2.9 ± 0.35
Fe	0.1 ± 0.01	0.2 ± 0.13	0.1 ± 0.06
Ca	6.2 ± 0.79	6.2 ± 0.68	6.3 ± 0.47
	Phytate:mineral molar ratio		
	NT	G1.4	A2.3
Phytate:Zn (<18)	3.2 ± 0.48	5.4 ± 0.87***	6.4 ± 1.21***
Phytate:Fe (<1.0)	93.3 ± 10.81	73.2 ± 31.53	128.9 ± 28.13*
Phytate:Ca (<0.24)	0.9 ± 0.21	1.5 ± 0.24***	1.9 ± 0.45***
Phytate x Ca:Zn (<200)	0.5 ± 0.08	0.8 ± 0.18***	1.0 ± 0.15***

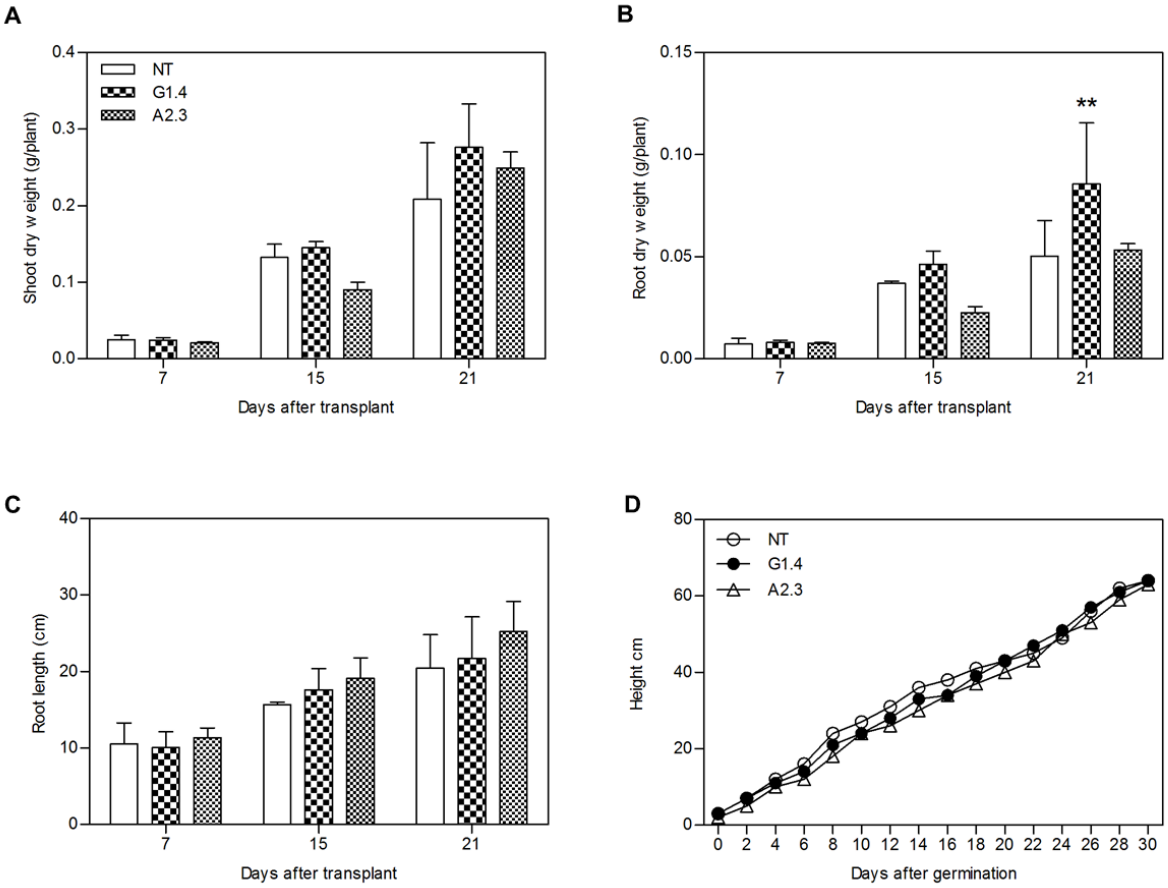


Figure 4.7 Growth parameters of transgenic seedlings and non-transgenic controls

Destructive analysis of seedling dry weights of shoots (A) and roots (B) and root length (C) was carried out at 7, 14 and 21 days after transplant (DAT). Seedling height (D) was also recorded from 0 to 30 DAT. Bars represent means + SD (n=4) and height values are means (n=16). Significant differences according to two way ANOVAs are, **p<0.01

indistinguishable at 7 DAT between all three lines (Fig. 4.7). Shoot dry weight of seedlings measured at 7, 15 and 21 DAT was not significantly different between non-transgenic NT and transgenic G1.4 and A2.3 lines. Line G1.4, however, showed a trend toward higher shoot dry weight at 15 DAT, while line A2.3 was lower than NT (Fig 4.7 A). Root dry weight was not different between the three lines at 7 or 15 DAT, however, at 21 DAT, root dry weight was significantly higher in G1.4 (Fig. 4.7 B). There was no significant difference in root length at 7, 15 or 21 DAT. Seedling height was similar between all three lines from transplant to soil until 30 DAT (Fig. 4.7 C).

Under greenhouse conditions, plants from the different lines showed no significant differences in the number of days to reach anthesis or maturity (Table 4.5). Although there was a trend for decreased plant height at the end of the vegetative stage and higher final straw weight in A2.3, the differences were not significant when compared to the non-transgenic NT or G1.4 lines. There were more panicles per plant in the transgenic lines A2.3 and G1.4 than the non-transgenic NT line, however the panicle length was decreased in A2.3. There was also highly significant decreases in the number of filled grains and the percentage grain fill of panicles from line A2.3. As a consequence, grain yield per plant was significantly lower in this line. There was no change in the number or percentage of filled grains between NT and G1.4. Compared with the non-transgenic line, grain yield per plant was higher in line G1.4, however the increase was not statistically significant. In contrast, 100 grain weight was significantly decreased in line G1.4 and A2.3.

4.4 Discussion

Changes in the protein profile, amino acid and fatty acid composition of *HvSUT1* expressing rice would have potential impacts on the nutritional quality of the rice grains. These nutritional changes could have implications for human health if such a rice variety were to be developed for human consumption. Sucrose is the primary assimilate transported to rice grains and a substrate for the synthesis of a range of grain storage products. Hence, it is crucial to examine a range of effects on rice engineered to have changes in sucrose transport. Rice grains overexpressing *HvSUT1* in the present study were shown to exhibit a range of changes in grain storage products, most likely related to changes in sucrose uptake. Changes in storage product content and composition did not appear to affect the growth of the next generation of developing rice seedlings. However, mature plants expressing *HvSUT1* showed a range of unexpected changes in yield parameters that were different between the constitutive

Table 4.5. Comparison of growth and yield of plants transformed with HvSUT1 and non-transgenic controls

Growth and yield components of T_2 non-transgenic NT and transgenic G1.4 and A2.3 plants grown in a greenhouse were measured during development and maturity. Values are means ± SD (n=16). Statistically significant differences according to One way ANOVAs are, * p<0.05, **p<0.01, ***p<0.001

Variable	Plant line (T_2)		
	NT	G1.4	A2.3
Days to anthesis	55.4 ± 2.48	54.4 ± 2.55	54.4 ± 3.18
Days to maturity	60.0 ± 2.83	60.6 ± 2.90	61.9 ± 3.72
Plant height	85.1 ± 4.61	82.6 ± 4.53	80.4 ± 5.33
Straw weight (g)	9.8 ± 1.50	9.9 ± 1.23	10.7 ± 1.26
Panicle number	9.6 ± 1.75	11.7 ± 1.35**	11.3 ± 3.03*
Panicle length (cm)	16.6 ± 0.93	16.9 ± 0.96	15.3 ± 1.2**
No. filled grains	56.5 ± 9.88	53.4 ± 5.99	38.5 ± 9.99***
Grain fill (%)	87.8 ± 7.47	83.2 ± 5.61	72.9 ± 8.41***
100 grain weight (g)	2.19 ± 0.10	2.06 ± 0.09***	2.12 ± 0.08*
Yield per plant (g)	11.7 ± 2.43	12.8 ± 2.17	9.2 ± 2.45**

and endosperm driven lines, which implies that the changes may be attributable to the temporal and spatial differences in sucrose transport.

4.4.1 Differential changes in storage protein fractions in rice endosperm

Profiles of total rice storage proteins were similar between plant lines when examined on CBB stained gels. However, rice storage protein fractions extracted sequentially from polished rice flour revealed a number of differences in protein profiles between NT and transgenic rice lines. The albumins and globulins were extracted together with a saline solution and there was a more noticeably prominent band at 55 kDa in G1.4. Densitometry confirmed that there was significantly more protein at this molecular weight in G1.4 compared with NT or A2.3, and within this size range peptide mass fingerprinting using MALDI-TOF/TOF detected a number of polypeptides. Surprisingly however, they were identified as members of the glutelin family of proteins, including components of the GluA and GluB subfamilies (Kawakatsu *et al*., 2008). Specifically, uncleaved immature glutelin precursor protein normally resolves at 55 kDa (Yamagata, 1982). This was puzzling as glutelin proteins are not normally extractable in saline solutions. However, this phenomenon has been observed before by Wakasa *et al*. (2009). Their results led them to determine that the glutelin precursor is assembled as a trimer made up of GluA and/or GluB subunits and that this saline soluble complex requires further processing *in planta* to form a more complex mature glutelin protein. Mature acidic and basic subunits of the mature glutelin are held together by the interaction of strong disulphide bonds and require alkaline solutions for solubilisation, and the subunits can only be resolved using a reducing agent such as βME (Wakasa *et al*., 2009). Hence, proteins extracted sequentially from rice flour with glutelin buffer containing βME showed the expected bands corresponding to glutelin acidic and basic subunits, and densitometry revealed that there was a decrease in abundance of GluB acidic subunits with no change in basic GluA subunits in G1.4. Taken together, the greater abundance of saline soluble proglutelin and the changes in the proportion of GluB acidic subunits to basic GluA subunits in G1.4 suggests that there is an accumulation of the glutelin precursor, specifically immature GluB, in grains from this line. The changes in glutelin protein observed in G1.4 were not evident in the constitutive *HvSUT1* overexpressing line A2.3. These results suggest that *HvSUT1* expression specifically in rice endosperm affects glutelin protein accumulation in rice, possibly due to disruption of normal processing of the precursor glutelin into its mature subunits and/or the formation of PSVs.

PSVs contain both glutelins and globulins that are synthesized and packaged together (Takahashi *et al*., 2005). The abundant 26 kDa globulin storage protein was noticeable in the Alb/Glob protein fraction extracted from rice flour and appeared similar in all plant lines when analysed by CBB staining. However, densitometry revealed that the 26kDa globulin was significantly decreased in G1.4 compared with NT and also slightly decreased in A2.3. Since globulins are thought to provide the framework in PSVs (Kawakatsu *et al*., 2010), it is possible that with less globulin, the processing and inclusion of mature glutelin into PSVs was hindered, resulting in the build-up of immature pro-glutelin and decline in mature basic subunits in G1.4. It has been shown that mis-targeting of globulin RNA dramatically alters the packaging of both globulin and glutelin proteins in the PSV, with small globulin particles irregularly distributed in the cell cytoplasm (Washida *et al*., 2012). Compared with G1.4, the slight decrease in globulin protein in A2.3 was not associated with a decrease in glutelin, hence it is likely processing and accumulation of mature glutelin into PSV was not affected in the constitutively driven *HvSUT1* line.

Weichert *et al*. (2010) observed increased prolamin accumulation in wheat overexpressing *HvSUT1*. However, they did not separate out the different prolamin polypeptides for analysis. In this study, prolamin was extracted from rice and examined by separating polypeptides on CBB stained gels. The abundance of the 16 kDa prolamin subunit was increased in G1.4 and A2.3 but only significantly in G1.4, while the 10-14 kDa polypeptides were the same between plant lines. Hence, in rice and wheat overexpressing *HvSUT1*, prolamin was increased. However, in rice this is attributed to an increase in the sulphur rich 16 kDa prolamin polypeptide (Mitsukawa *et al*., 1999). Kawakatsu *et al*. (2010) hypothesized that the content of sulphur containing amino acids highly regulates the proportions of storage proteins in rice because reduced prolamin results in increased globulin and glutelin and conversely rice with less PSV protein (globulin and glutelins) accumulate more prolamin. In transgenic line G1.4, an increase in sulphur rich prolamin is associated with a decrease in sulphur rich globulin (Krishnan *et al*., 1992) thus the redistribution of sulphur in the grain appears to be in agreement with findings by Kawakatsu *et al*. (2010).

There is a large body of evidence gathered from studying storage protein mutants (Nagamine *et al*., 2011), or transgenic plants (Kawakatsu *et al*., 2010), that storage protein composition in cereal grains is in equilibrium such that increases in storage proteins of one type is compensated by decreases in other types. Furthermore, the regulation of protein synthesis may be limited by redistribution of the available amino acids as to maintain the total seed nitrogen.

4.4.2 Stimulatory effects of sucrose on amino acid metabolism or transport

Weichert *et al*. (2010) saw strongly up-regulated expression of genes essential in amino acid synthesis at later grain filling in wheat grains transformed with *HvSUT1*. However, while they measured lower levels of Asp and Glu in later stages of grain filling, *HvSUT1* overexpressing rice had the same levels of these AA in mature unpolished rice and accumulated more Glu, Asp, Asn and Gln in mature rice endosperm. These four amino acids are central to the regulation of N metabolism in plants and are involved in multiple processes including carbon/nitrogen partitioning and synthesis of other AA (Tabuchi *et al*., 2007). Furthermore, Glu and Asn are the dominant free AA found in rice phloem sap (Fukumorita and Chino, 1981; Hayashi and Chino, 1990) and are important nitrogen sources remobilised from leaves to grains at maturity (Osaki *et al*., 1992). Hence, the concurrent increases in these four AA in line G1.4 suggests that the increased sink of rice grains overexpressing *HvSUT1* in endosperm appears to promote the accumulation of amino acids and/or differentially stimulate pathways leading to the synthesis of amino acids derived from this metabolic network (Yamakawa *et al*., 2010).

Trp and Tyr, both aromatic amino acids, are products of the Shikimate pathway and are in turn, important precursors of secondary plant metabolites such as antioxidants and plant hormones (Tzin and Galili, 2010). Significant increases of both these AA in line G1.4 could be explained in a number of ways. Increased carbon flux could either have stimulated Trp and Tyr synthesis and/or inhibited the downstream processing of these AA into their respective metabolites. In addition, evidence exists that there are numerous feedback loops in the biosynthetic pathways that these aromatic AAs are involved in inhibiting or stimulating (for detailed review of these, see Tzin and Galili, 2010). What is clear is that these effects were only observed in rice endosperm by endosperm specific expression of *HvSUT1*. In contrast, line A2.3 had much higher Trp in unpolished rice but lower Trp content in polished rice compared with NT controls, which implies that Trp preferentially accumulated in the aleurone and/or the embryo and was removed after polishing. Therefore constitutive expression of *HvSUT1* resulted in a very different pattern of aromatic AA accumulation. Trp is an important precursor for Indole Acetic Acid (IAA), a plant hormone critical for plant growth and development including normal growth of rice embryos (Sazuka *et al*., 2009). The effects, if any, of the changed distribution of Trp in rice grain on seedling growth of A2.3 will be discussed in section 4.4.6.

Increased free Cys in G1.4, a sulphur rich AA, suggests that there was increased stimulation of Cys synthesis which indirectly implies changes in sulphur reduction and processing in the grain. Weichert *et al*. (2010) suggested that increased sucrose uptake capacity increases sulphate assimilation in wheat. There was no obvious overall decrease in sulphur rich proteins in G1.4, therefore excess Cys was probably not due to a breakdown in storage protein synthesis and could reflect an over-stimulated production of sulphur-rich AA in the grain due to excess carbon or increased Cys uptake into transgenic grains.

Lysine is an essential amino acid that makes up less than 1% of total grain nitrogen in most cereals (Shewry, 2007), and hence is often a target for studies striving to improve the nutritional content of cereal grains. Line G1.4 had significantly higher free Lys content in polished rice grains. Kawakatsu *et al*. (2010) suggested that the glutelin/prolamin protein ratio could be a determinant of higher lysine in seeds, however this is in relation to total amino acid content. Higher content of free Asp in G1.4, a precursor for Lys, along with higher Lys indicates the distinct activation of this particular branch of AA biosynthesis.

Like others have reported (Schaeffer and Sharpe 1997; Shewry, 2007), the free amino acid (AA) content measured in mature polished rice grain was generally very low as most AAs in grain had been incorporated into storage proteins. Together with storage protein content, the free AA in mature grain can give an indirect indication of the nitrogen status of the whole plant at maturity as AAs are translocated from the stem and leaf reserves of nitrogen into grains at senescence (Osaki *et al*., 1992). The increased free amino acid pool in rice grains and the changes in storage proteins most likely indicate that increased sucrose flux due to *HvSUT1* overexpression resulted in modifications to normal nitrogen metabolism. A large free amino acid pool in cereal grains could also be related to decreased protein content or could indicate a disruption in storage protein synthesis (Schaeffer and Sharpe 1997). For example, Ashida *et al*. (2006) knocked down globulin protein expression in rice which resulted in an increase in total free amino acid content in the grain. Likewise, maize mutants impaired in zein (analogous to rice prolamin) synthesis, had higher concentrations of AA (Wang and Larkins, 2001). However, in this study there was no clear association between increased free AA content and decreased storage protein content in *HvSUT1* overexpressing rice. There was no evidence that there was less storage protein content in either G1.4 or A2.3 or decreased total crude protein (see Chapter 3) in polished rice of transgenic rice grains. However, changes in protein profiles and content of some amino acids suggest that different branches of the amino acid biosynthetic pathways were differentially affected. Therefore, in agreement with Weichert *et al*. (2010),

increased carbon supply results in changes in metabolic processes in the grains, although the extent and consequences of those changes varies between wheat and rice.

4.4.3 Lipid and fatty acid distribution and oil bodies in *HvSUT1* overexpressing rice

The contribution of FA to the total nutritional content of rice is low however interactions between rice lipids and starch can have implications for rice quality and storage and human health (Yoshida *et al*., 2011; Liu *et al*., 2013). In Arabidopsis seeds that accumulate more fatty acids than rice, Baud *et al*. (2005) measured a decrease in fatty acids and a change in fatty acid profile of an AtSUC5 mutant that had decreased sucrose transport in the endosperm. Oats also accumulate lipids in the endosperm, which is unusual for a cereal, and can switch carbon partitioning from starch to lipid synthesis during the course of grain development (Banas *et al*., 2007). These examples provide some points for comparison and insights into the partitioning of sucrose into oils and lipids that could potentially be affected by changing the sucrose uptake capacity in rice.

Lipid extracts are classified according to their fractionation by thin layer chromatography. The three most abundant lipids are TAG, PL and FFA (Yoshida *et al*., 2011). Proportions of the major lipid classes were divided into broad components for comparison of fatty acid composition of polished rice between the rice lines. An increase in the FFA content of rice is often used to assess improper storage of harvested rice (Yasumatsu and Moritaka, 1964). The consistency of the FFA (insoluble lipid) proportions across all plant lines in the present study indicate that there was no differential change in the deterioration of rice grain lipids between samples. Free fatty acids are thought to associate with the surface of starch granules while bound lipids are located inside starch granules where they are complexed with amylose (Zhou *et al*., 2003).

Of the bound lipids, there was a decrease in saturated FA concurrent with an increase in PUFA observed in both transgenic lines in the TAG (neutral lipid) fraction. Upon further inspection, the changes were due specifically to a 15 to 20% decrease in palmitic acid (16:0) and a significant increase of 17 to 22% of the proportion of linoleic acid, also known as omega 6 (18:2n-6), in each sample. The proportional decrease in saturated 16:0 and increase in the PUFA 18:2n-6 suggests that particular processes in fatty acid elongation and desaturation has been stimulated. Broadly speaking, fatty acid chains are lengthened by the addition of two carbon groups aided by the activity of the enzymes acetyl-CoA carboxylase and fatty acid synthetase (Rawsthorne, 2002).

Specifically, KASII, a member of the β-ketoacyl-carrier protein (ACP) synthases, controls the conversion of 16:0 to 18:0 in fatty acid biosynthesis (Pidkowich *et al*., 2006). In addition, PUFAs are synthesized from saturated fatty acids by the activity of desaturases. In soybean, the seed specific *FAD2-1* gene that encodes an ω-6 desaturase plays a major role in PUFA conversion in seed development (Heppard *et al*., 1996). It is possible that similar processes are altered due to increased sucrose supply in HvSUT1 overexpressing rice grains, leading to the changes observed in lipid composition.

In the present study, PL in the endosperm of all the plants analysed was composed mostly of palmitic (~43%), followed by linoleic (~36-37%) and a small proportion of oleic acid (18:1n-9) (8-9%) which is consistent with rice FA reported in Liu *et al*. (2013). There were no significant changes in the proportion of individual fatty acids from the PL fraction between plant lines although the total saturated fatty acids in the PL fraction of G1.4 was slightly decreased. Hence the modest changes in fatty acids composition in the transgenic rice lines was mostly due to changes in the TAG fraction of lipids.

Lipids are present as components of oil bodies in seeds. In oil bodies extracted from seeds from a wide range of plant species, it was found that neutral lipids (TAG) make up approximately 94 to 98% of the oil body structure, along with much lower proportions of proteins, PL and FFA (Tzen *et al*., 1993). One of the non-lipid components of the oil body is the Oleosin (OLE) protein, that Tzen *et al*. (1992) suggests is crucial for stabilising the shell of the oil body. They propose that seed oil bodies are filled with TAG and held together by a PL shell associated with embedded OLE proteins in a 80:20 ratio. *HvSUT1* overexpression in wheat was found to alter the expression of a group of genes including storage proteins and oleosin (Weichert *et al*., 2010). The promoter regions of the oleosin genes share conserved sequences that are extremely similar to those found in storage protein promoter sequences which suggest similar regulation, perhaps by the same or similar transcription factors that may be expressed at specific time points during rice grain development (Wu *et al*., 1998). In the present study, an 18 kDa OLE protein was identified from the prolamin storage protein fraction extracted from polished rice grains, and which was considerably more abundant in both transgenic lines, but particularly in G1.4. This was an unexpected finding as oil bodies are generally found in the aleurone and embryo of rice grains, with very little oil distributed in the endosperm (Wu *et al*., 1998b). However, since the change in protein abundance was clearly visible in the polished rice samples in the CBB stained gel, the increase in OLE abundance was therefore significant. One reason why changes in TAG and OLE are observed together could be because they are assembled together in the seed during seed development (Wu *et al*., 1998b). Accumulation of TAG

and OLE occurs together in the seed during seed maturation (Tzen *et al*. 1993) and TAG:OLE ratios have been found to affect the size of oil bodies in many plant species (Wu *et al*., 2010). Tzen *et al*. (1993) also speculate that oil body size is related to the nutritional status of the plant and reliant on the available assimilates for conversion into storage products. Furthermore, evidence from oats suggests that regulation of sucrose partitioning determines final oil composition of grains (Banas *et al*., 2007). Thus increased sucrose partitioning in rice could potentially stimulate genes or modify downstream processes in FA biosynthesis. Consequently, significant changes in the proportions of fatty acids in the TAG lipid fraction and the increase in OLE, particularly in rice grains overexpressing *HvSUT1*, suggests that oil body distribution and synthesis has been affected by sucrose.

4.4.4 Speciation of Fe and Zn in *HvSUT1* overexpressing rice

Phytate is thought to be localized to the aleurone and embryo of cereal grains and therefore removed with polishing (Prom-u-thai *et al*., 2008). However, although high resolution sub-cellular imaging of the distribution of Fe in cereals has shown that Fe is strongly associated with phytate in the aleurone, phytate also is present as uncharacterised complexes associated with starch grains in endosperm cells (Moore *et al*., 2012). In addition, Iwai *et al*. (2012) found *OsIPK1* mRNA and OsIPK1 protein, which catalyses the final step of phytic acid synthesis, in endosperm cells. These studies provide convincing evidence that support starchy endosperm cell involvement in phytate synthesis and storage. Furthermore, like other grain storage products, synthesis of InsP6 involves glucose-6-phosphate (Glc-6-P), an intermediate derived from sucrose (Suzuki *et al*., 2007). In the present study, rice grains were over-polished so that a large proportion of the outer layers were removed (~20% of grain weight) to thoroughly remove contaminating aleurone and subaleurone tissues from endosperm. Thus, the increased phytate content in the endosperm of *HvSUT1* overexpressing rice seeds could be due to a stimulation of phytate synthesis in response to increased sugar supply.

Relationships between Fe, Zn, phytate and protein in polished rice endosperm were examined in order to compare the speciation of these micronutrients in rice grains transformed with *HvSUT1* with non-transgenic rice. Rice nutrient quality is determined by a number of seed components including S containing storage proteins (Hagan *et al.,* 2002) and phytate, which is the main storage form of P in cereals (Hunt, 2003). S and P have been found to correlate with Zn and Fe in wheat, respectively

(Zhao *et al*., 2009). There was a very strong positive correlation between P and phytate concentrations in the plant lines in this study. It is known that a range of cations, including Fe and Zn, are strongly associated with phytate, however it is not known whether phytate has a preference for binding particular cations (Iwai *et al*., 2012). The present study revealed strong positive correlations between phytate and Fe, and showed that there was no correlation between phytate and Zn in the endosperm of grains from the transgenic lines. These results suggest that phytate binds preferentially to Fe, rather than Zn, in the endosperm of *HvSUT1* overexpressing rice grains. However, while there was a strong correlation between P and Fe for all three lines, there appeared to be no significant correlation between phytate and Fe in the non-transgenic control. These results were surprising, and given the unlikely conclusion that phytate was not associated with Fe in the controls, a closer examination of the data was undertaken. The scatterplot of phytate and Fe in polished grains (Fig. 4.5 B) shows one particular NT line had a low value for Fe (0.7 mg/kg) which had a significant effect on the correlation coefficient. Other mineral concentrations for this line were consistent with the other NT replicates hence this Fe value was either a true value or an unforeseen experimental error had occurred. Regardless, a repeat of the analysis and/or analysing larger number of replicates would help in resolving this issue.

S is an important component of seed storage proteins from a nutritional aspect and S incorporation into protein in rice seeds has been demonstrated by radioactive labelling of developing rice panicles (Luthe, 1983). Relationships between protein, S and the micronutrients Zn and Fe were explored here to determine the relationships between these grain components. There was no correlation between S and Fe concentration in rice endosperm, which agrees with findings by Hansen *et al*. (2012) who mapped the differential distribution of these two elements in rice grain. There was very strong correlation between protein content and S concentration in the NT and A2.3 lines, however there was no correlation in G1.4. Protein content and Zn concentration were also strongly positively correlated in NT and A2.3, however there was no correlation in G1.4. There was, however, a strong positive correlation between S and Zn concentration in all three lines. These findings suggest that in line G1.4, a proportion of the S in the endosperm was not associated with proteins and that the Zn was preferentially associating with these non-protein forms of S. There is a complex range of Zn and S-ligand interactions in grain tissues (Perrson *et al*., 2009). It has been postulated that Zn can be associating with Cys in glutathione (GSH) and metallothioneins (MT) or complexed with nicotianimine (NA) (Haydon and Cobbett, 2007; Tauris *et al*., 2009; Nishiyama *et al*., 2012), though this has primarily been

discussed in relation to phloem and long distance transport. In the endosperm, Zn is thought to be bound to phytate and NA (Tauris *et al*., 2009), although the present study does not support the association of Zn with phytate in rice endosperm. Significantly higher Cys content in line G1.4 could indicate that Zn could be binding to S-rich free Cys, GSH or MT in this line. In NT controls and A2.3 on the other hand, Zn appeared to be associated primarily with protein. There was also no evidence that Fe and protein was associated in the rice endosperm.

In agreement with Persson *et al*. (2009) evidence from the present study suggests that Fe and Zn are differentially associated with phytate and protein respectively in rice grain. However, it is hypothesized that increased sucrose flux into rice endosperm by endosperm specific *HvSUT1* expression, changed the proportion of S-rich ligands in the form of Cys containing metabolites, thereby changing the speciation of Zn. Line A2.3 behaved similarly to NT controls as Cys content was not changed by constitutive expression of *HvSUT1* and Zn was correlated with proteins in both these lines. Iwai *et al*. (2012) found that Zn and phytate were not distributed together in rice grains when examined by μ-XRF, which is in agreement with data presented in this study. Colocation of S and Zn together in the endosperm and P and Fe in the aleurone of rice could also explain the strong association between these elements (Hansen *et al*., 2012).

4.4.5 Bioavailability is unchanged in rice transformed with *HvSUT1*

Apart from the critical roles of phytate in seed viability (Raboy *et al*., 2001), phytate is a strong chelator of mineral ions and is therefore considered an anti-nutrient affecting the absorption of micronutrients in human diets (Lonnerdal *et al*., 2000; Lott *et al*., 2000). Conversely, some evidence suggests phytate also has important antioxidant and anti-cancer properties (Graf *et al*., 1987). Therefore, a balance in the phytate and micronutrients in cereals is required to ensure bioavailability of micronutrients while still retaining adequate phytate favourable for human and plant health. In this context, phytate concentration was measured and calculations of the relative molar ratios of phytate and important micronutrients was undertaken to determine the potential nutritional impact of rice transformed in this study.

The significantly higher phytate (InsP6) in the endosperm of the transgenic lines mirrored the significantly higher P in these two lines. Total phytate contents for the rice reported in this study are consistent with phytate contents of natural rice varieties reported in Ma *et al*. (2007). However, the higher phytate content compared to P in G1.4 and A2.3 implies that phytate content had been overestimated. In other

methods for estimating phytate, overestimation can arise from the measurement of lower inositol phosphates (InsP1 to InsP5). However, the method used in this study is able to distinguish InsP6, and is therefore not likely to contribute to phytate overestimation. There is some evidence that increased fat content of samples can lead to interference with the assay (Talamond *et al*., 2000). The polished rice flour samples in this study were not defatted prior to extraction, and although the lipid contents were low, it is possible that they could have contributed to overestimation of phytate, particularly in the transgenic rice samples. Despite this, P content was higher in these two lines and P correlates strongly with phytate so it can be concluded that although the values may be a slight overestimation, phytate was higher in the transgenic lines compared with NT.

To determine whether increased phytate in the transgenic lines had consequences for the bioavailability of Fe and Zn, a number of calculations of phytate:mineral molar ratios were used for comparison. The molar ratios of phytate:minerals in rice calculated in this study are similar to those reported in Ma *et al*. (2007). Although phytate:Zn ratios of lines G1.4 and A2.3 were significantly higher than NT, their values were still below the desirable threshold of 18 proposed by Fordyce *et al*. (1987) and Gibson *et al*. (2010). Hence, increased phytate in the transgenic rice is estimated to have little effect on Zn bioavailability. In some cases, it has been found that phytate x Ca:Zn is a better estimate of Zn bioavailability rather than phytate:Zn (Fordyce *et al*., 1987). Hence the contribution of Ca in reducing Zn absorption was also explored. Again, the significantly higher values calculated for G1.4 and A2.3 did not affect the Zn bioavailability as all values were <200. In contrast, all plant lines exceeded the limits for phytate:Fe and phytate:Ca molar ratios which implies that absorption of Fe and Ca are inhibited at usual levels of phytate and minerals found in rice grain. Therefore, the modest increase in phytate in transgenic rice expressing *HvSUT1* did not affect the already low bioavailability of these minerals. The low bioavailability of Fe and Ca, and high bioavailability of Zn in rice predicted from the present study is corroborated by values calculated from a range of raw and cooked rice products from Malaysia (Norhaizan and Nor Faizadatul, 2009) and China (Ma *et al*., 2007).

4.4.6 Nutrient changes in grains changes yield parameters in transgenic rice plants

In order to determine if increased sucrose capacity and changes in nutrient composition and distribution in transgenic rice grains had any effects on the growth or

development of plants overexpressing *HvSUT1*, growth and yield parameters were measured between the lines. Plant development, as defined by the number of days for plants to reach anthesis and maturity, was not changed in the transgenic lines compared to the non-transgenic line. Plant height and straw weight was highly variable, and therefore the changes in these parameters were not significantly different between plant lines. However, yield parameters did show some interesting changes. In both rice lines overexpressing *HvSUT1* in endosperm and constitutively, there was a significant increase in the number of panicles per plant, which was also observed in a larger scale study on field grown wheat plants overexpressing *HvSUT1* (Saalbac *et al*., 2014). Increased nutrition to the whole plant promoting the growth of more tillers could explain this phenomenon in A2.3. Although *HvSUT1* expression should be limited to the endosperm in G1.4, it is possible that there could be leaky expression of the *Glb-1* promoter in vegetative tissues as observed by Wu *et al*., (1998) and Qu and Takaiwa (2004). Carbohydrate supply and its effects on plant architecture, including tiller number were explored in Lafarge *et al*. (2010). They found that rice plant phenotypes were highly responsive to manipulations in sugar supply and that biomass in early vegetative stages was preferentially allocated to leaf elongation and tiller emergence. Thus, it is possible that increased sucrose flux could have promoted the growth of a greater number of tillers in the transformed rice in this study.

In A2.3, the increase in panicle number is associated with a decrease in panicle length, grain filling and number of filled grains as well as grain weight. Hence, the perceived advantage in the greater number of panicles is not realised due to a decrease in the other yield parameters. Unexpectedly, constitutive expression of *HvSUT1* had negative effects on yield. However, possible negative effects from plant transformation on the constitutive driven line also cannot be ruled out. Further analysis of more generations of plant lines is necessary to determine if these findings are consistent. In G1.4, there was no decrease in panicle length, grain filling and filled grains. In contrast to Saalbac *et al*. (2014) who recorded significant increases in thousand grain weight of wheat expressing endosperm specific *HvSUT1,* transgenic rice in this study had substantially smaller grains. Unlike A2.3 this did not translate to lower plant yields in G1.4 due to the increased number of panicles. Unlike in wheat, grain size in rice is limited by the husk (Yoshida, 1976), therefore it appears that increased sucrose cannot be utilized in grain expansion, and thus changes in sucrose partitioning could explain the wide range of changes observed in storage product composition in G1.4.

Overall, germination, seedling height and root length, did not vary significantly between plant lines. The contribution of grain nutrient stores on seedling growth would be expected to show some difference 7 days after germination, before seedlings

were transferred to soil. Seedling height, however, was the same across all lines. Although not all changes were significant, the data for seedling shoot and root dry weight suggests that the grains from G1.4 plants had a growth advantage particularly between 14 and 21 DAT. Line A2.3 on the other hand showed a slight delay in dry weight accumulation, with seedlings at 15 DAT having lower shoot and root dry weights, although the changes were not significant and the dry weights were similar to NT by 21 DAT. Any effect of the transgene on G1.4 seedlings can be attributed to the changes in the nutrient content in the grain whereas in A2.3, constitutive expression of *HvSUT1* could have a more direct effect on seedling growth. Constitutive knockdown of *OsSUT1* was shown to reduce rice seed germination and growth (Scofield *et al*., 2002) while *SUT1* expression in the rice embryo is positively regulated by sugars (Matsukura *et al*., 2000; Chen *et al*., 2010). Therefore enhanced germination and seedling growth due to increased sucrose supply to the embryo would be expected for line A2.3. However, there did not appear to be any definitive changes in seedling growth between lines that could be explained by these observations. Indeed, G1.4 showed more potential stimulation of seedling growth, and hence it is more likely that enhanced nutrient content of the grain was a greater factor in enhancing seedling vigour. However, the modest growth advantage in G1.4 does not emphasize the positive effects of increased mineral nutrients and seedling vigour or more long term crop productivity (Graham *et al*., 1999; Welch and Graham 2002). These effects may not be evident in a pot trial in a greenhouse where environmental conditions are controlled and nutrients are not limiting, hence the need for more trials of this material grown under field conditions, especially in mineral depleted soils. Other traits such as grain growth could also be measured to compare plant lines, and together with evidence from sucrose uptake data (Chapter 3), may provide a clearer picture of the effects of enhanced sucrose supply, not just on plant growth but also grain development.

4.5 Conclusions

A number of striking changes in the storage product content and composition of rice grains transformed with the *HvSUT1* transgene suggests that increasing sucrose flux in rice can modify a range of metabolic processes leading to modifications in storage protein, free amino acid, lipid and fatty acid contents in rice grains. Furthermore, yield impacts of *HvSUT1* expression in plants was explored, however the results were inconclusive. Further investigation is required on larger samples of more independent transgenic lines to explore the complex relationships between seed storage products and seedling growth.

In terms of human nutrition, a number of changes in transgenic rice overexpressing *HvSUT1* impacted positively on the nutritional quality of the rice grains. For example, the higher levels of 16kDa prolamin and increased free Cys content in the G1.4 line, both contribute to higher sulphur in the seed. It is thought that Cys peptides can enhance the absorption of non-haem iron in humans (Lucca *et al*., 2001). In addition, grains from this plant line were also higher in other essential amino acids, namely tyrosine, glutamine and lysine. The increased distribution of polyunsaturated fatty acids in TAG fraction of polished rice in both transgenic lines also has implications for human health. The health benefits of the essential Omega 3 and Omega 6 fatty acids have been widely publicised. Scientifically, a number of studies have attributed positive impacts of Omega 6 on lowering cholesterol and cardiovascular disease (see Simopolous *et al*., 2002). The phytate content in the endosperm was increased in the transgenic rice, although it does not appear to impact on the inherent bioavailability of micronutrients.

The evidence presented here suggests that manipulating sucrose transport in rice can be one strategy that can be utilised to change grain composition potentially with positive outcomes for human nutrition.

General Discussion

This aim of this research was to investigate the effect of increasing sucrose transport to grain as a biofortification strategy for altering the nutritional content of polished rice. To study sucrose effects on nutritional components of rice grains, the main objectives of this research were to:

- Generate and fully characterise independent transgenic rice lines overexpressing the barley *HvSUT1* transgene,
- Investigate the effects of potentially increased sucrose transport in transgenic rice on the plant phenotype, and the concentration, content and distribution of nutrients in the rice grain.

5.1 Summary and main findings

To address the first objective, transgenic rice plants overexpressing the barley sucrose transporter *HvSUT1* driven by two different promoters; the constitutive Act-1_{pro} and endosperm specific Glb-1_{pro}, were generated by *Agrobacterium* transformation (Chapter 2). Until now, cereal transformation with an exogenous *SUT* gene had previously only been performed in wheat (Weichert *et al*., 2010; Saalbach *et al.,* 2014). Tissue specificity of the promoters was verified by GUS staining. A combination of Southern blot and PCR analysis was used to show that a number of transgenic rice lines were generated containing one or more copies of *HvSUT1.* Stable transformation and expression of transgene transcript was demonstrated by RT-PCR. A novel polyclonal antibody raised in rabbits against a synthetic peptide from the putative central loop of HvSUT1 was used to analyse the accumulation of protein in developing rice grains. The endosperm specific rice lines consistently had stronger HvSUT1 protein expression than the constitutive lines and unexpectedly, transgene copy number did not correlate with increased protein expression. The present study is the first to demonstrate specific grain targeted expression of the barley HvSUT1 protein in rice. Unfortunately, the choice of plant lines for further analysis was restricted as there were a limited number of lines that contained only 1 transgene copy. In addition, the restricted physical containment space for growing plants did not allow for a large number of plants to be grown. Therefore, based on the available data, the most suitable lines of independently generated plants were chosen for analyses. Ideally, analysis of more independently transformed plant lines is necessary to support the findings in this study.

To investigate the effects of incorporating the sucrose transporter on mineral nutrients in rice, transgenic rice grains were analysed by sucrose uptake assays and

quantification of rice grain carbohydrates and minerals (Chapter 3). Accumulation of mineral nutrients were also analysed during grain filling and distribution of minerals in the mature grain was mapped by LA-ICP-MS. Expression of *HvSUT1* in rice endosperm increased sucrose transport activity specifically at 7 DAA and prolonged active sucrose uptake at later stages of grain filling. This did not appear, however, to affect mineral accumulation in the whole grain during grain filling. Grains from plants constitutively expressing *HvSUT1* were similar to controls with respect to sucrose uptake behaviour at late grain filling. Analysis of minerals in polished rice revealed that there was greater retention of minerals in the endosperm of *HvSUT1* overexpressing rice. In particular, there was increased retention of Fe and Zn in the starchy endosperm. This was supported by distribution maps of elements in the rice grain which showed markedly different distribution of minerals in the transgenic lines. Interestingly, K retention was lower in both transgenic lines which led to the general conclusion that K transport processes are closely linked to sucrose transport in rice grains. This tentative conclusion finds support from studies in dicots and plant vascular tissues (Zhang *et al.*, 1997; Bauer *et al.*, 2000; Deeken *et al.*, 2002; Zhang *et al.*, 2002), but has not yet been reported in cereal grains. As the results reported in this study are derived from only two plant lines, one endosperm specific and one constitutive *HvSUT1* overexpressing line, further experiments are necessary to provide more data to support these findings.

Sucrose partitioning into non-starch storage products in the rice grain and the effects of changed nutrient composition on mineral bioavailability was examined in Chapter 4. In addition, possible effects of these changes on seedling growth and plant yield parameters were also examined. Significant changes in storage protein profile and amino acid contents were observed in one representative endosperm specific (G1.4) and one constitutive (A2.3) plant line, but more dramatic changes were observed in G1.4. Fatty acid composition was also noticeably changed in the transgenic lines, particularly in the TAG lipid fraction. In general, phytate and P were correlated with Fe, and protein and S were correlated with Zn, although the results possibly suggest that the speciation of Fe and Zn may be altered in the transgenic lines. The changes in the transgenic grains however did not affect values that estimate mineral bioavailability. There appeared to be no effect on seedling growth from over expression of the *HvSUT1* transgene, but mature plants of line A2.3 had a yield disadvantage due to changes in panicle architecture. G1.4 on the other hand had decreased one hundred grain weight but this did not impact on the overall yield in this plant line.

Previous studies that investigated SUT overexpression in plants tended to focus only on carbon partitioning. However, the present study investigated a comprehensive range of effects on the nutritional composition of seed sinks that could be attributed to SUT overexpression. Overall, there were a number of changes in the storage products of rice grains transformed with *HvSUT1,* which were more dramatic in the endosperm specific line G1.4 compared with the modest changes in the constitutive line A2.3. It is unclear whether this difference between endosperm specific and constitutive expression is related to the higher levels of HvSUT1 protein measured in G1.4 compared with A2.3 or the activity of HvSUT1 in other plant tissues, such as leaf and stem, in the constitutive line. Regardless, the findings from this research suggest that *HvSUT1* overexpression driven by an endosperm specific promoter results in the re-distribution of the available minerals transported into the grain. Furthermore, given the range of changes in storage products observed in rice grains overexpressing *HvSUT1*, the results suggests that a number of metabolic pathways for grain storage product synthesis have been altered. This strategy could thus be exploited to produce more nutrient dense rice grains with altered nutritional composition.

5.2 Proposed mechanisms to explain the main outcomes of *HvSUT1* overexpression in rice endosperm

A pictorial representation of a rice grain and cross sections of the tissues involved in assimilate transport are shown in Fig. 5.1. This diagram attempts to explain the re-distribution of Fe and Zn in transgenic compared to non-transgenic rice grains. Fig 5.1 A shows the localization of endogenous SUTs in the plasma membrane of aleurone cells and their role in active sucrose transport from the apoplast into the symplast. Under normal circumstances, the contribution of SUTs to sucrose transport within the endosperm is minimal, and sucrose movement into the inner endosperm is thought to occur primarily by diffusion through plasmodesmata (Oparka and Gates, 1981; Wang *et al*., 1995).

The proposed positions of SUTs in the plasma membranes of endosperm cells in grains overexpressing *HvSUT1* are shown in Fig 5.1 B. Results suggest that sucrose uptake was modified in the transgenic grains. Hence, for the activity of exogenous SUTs to increase sucrose uptake capacity requires sucrose to be present in the endosperm apoplast. This implies that as well as the primary symplastic pathway for sucrose movement in endosperm proposed by Oparka and Gates (1981), and measured in wheat grains by Wang *et al.* (1995), there must be some sucrose that is transported in the apoplast of endosperm cells. Presumably sucrose could leak out into the apoplast

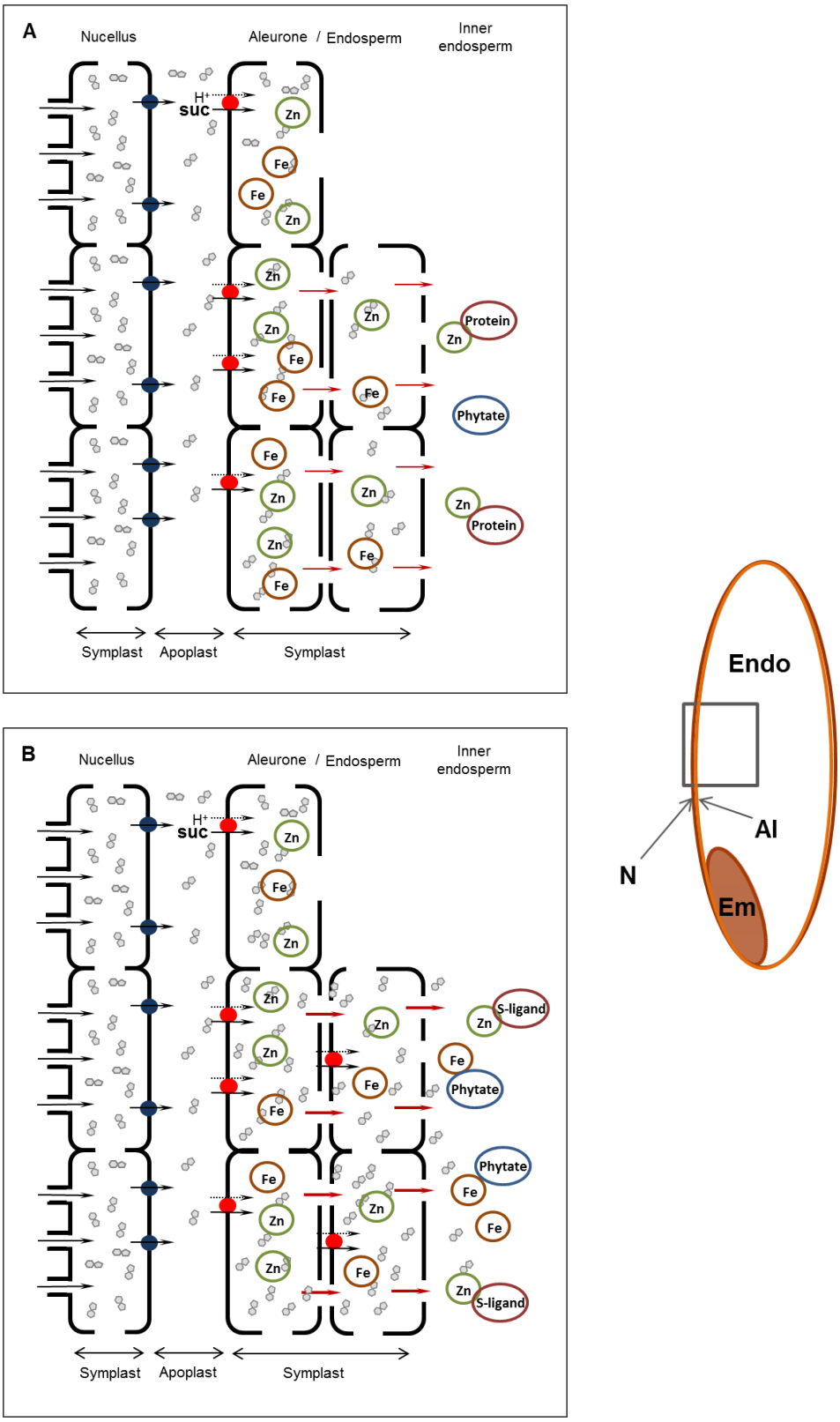


Figure 5.1 Proposed explanation for redistribution of Zn and Fe in the endosperm of HvSUT1 overexpressing rice grains

Pictorial representation of a rice grain (right) showing the locations of the embryo (Em), nucellus (N), aleurone (Al) and endosperm (Endo). The grey box is a cross section of the grain tissues depicted in the non-transgenic (A) and HvSUT1 overexpressing (B) rice grain.

at numerous points along the transport pathway, such as in the transfer of sucrose from the nucellus to the aleurone and leakage through plasmodesmata. Thus, sucrose that has leaked into the apoplastic space and usually would be transported out of the caryopsis via the pathway proposed for water (Oparka and Gates, 1981) could be recovered through active transporters. Consequently, the exogenous SUTs are proposed to have this role in sucrose retrieval, thus increasing the sucrose uptake capacity of endosperm cells. Mineral ions that are minor components of the solutes transported in phloem are therefore potentially also being transported, along with sucrose, into the inner endosperm. This model explains only the mechanical aspects of sucrose transport into the rice grain and for simplicity does not incorporate the activity of metal transporters that may be involved. As Weichert *et al.* (2010) reported in wheat, a wide range of genes with differing functions were stimulated by *HvSUT1* overexpression, therefore the effects of increased sucrose uptake capacity on the expression of metal transport genes in rice cannot be ruled out. In this study, it is further proposed that in transgenic rice grains with endosperm specific expression of *HvSUT1*, excess Fe is binding to phytate, while Zn associates with unspecified compounds containing sulphur.

Another key finding from this study was the decreased K concentration and changed K distribution in endosperm cells. Fig. 5.2 A depicts transporters in the plasma membrane and tonoplast involved in sucrose and K uptake and sequestration in the vacuole respectively. Under normal circumstances, SUTs, H^+-ATPases and a K transporter such as AKT2/3 work in concert maintaining concentrations of sucrose and ions across both sides of the plasma membrane while also regulating membrane potential. This coupling of sucrose and potassium transport has also been hypothesized to occur in sucrose loading in phloem tissue (Geiger, 2011). A number of ion transporters and channels in the tonoplast along with V-ATPases are involved in ion homeostasis by sequestering ions in the vacuole and also in regulating cell turgor. Fig. 5.2 B attempts to explain the possible effects of increased sucrose transport into endosperm cells on K distribution and transport. When *HvSUT1* was expressed in endosperm cells, more sucrose and H+ were taken up into the cytosol from the extracellular space (1). To prevent depolarization of the membrane, the activity of the PM K transporter changed from an inward rectifying to a non-rectifying channel, as demonstrated in computer models by Gajdanowicz *et al.* (2011), to transport K+ back out into the extracellular space (2). A second but related hypothesis involves vacuolar transport processes. Increasing sucrose in the cytosol could stimulate accumulation of sucrose into the vacuole (3). The activities of a number of monovalent ion transporters

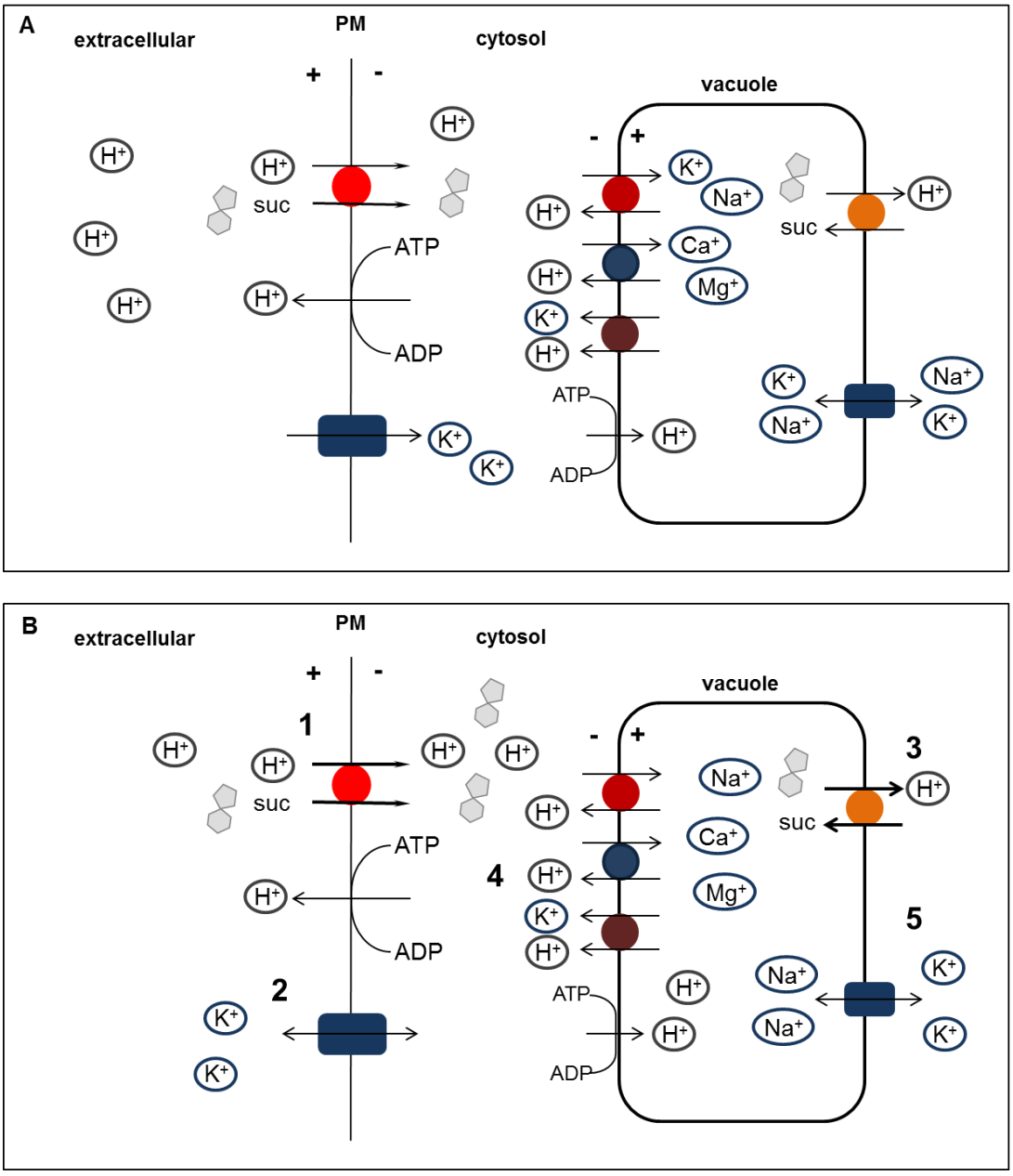


Figure 5.2 **Proposed roles of K in the membrane and vacuole of rice grains**

Location of various transporters and channels involved in sucrose and K transport across the plasma membrane (PM) and in the tonoplast of the vacuole of rice endosperm cells. Simplified pictorial representations of proposed differences in transporter activity compared between non-transgenic (A) and transgenic rice grains overexpressing HvSUT1 (B). The numbers in image B are explained in the text.

in the tonoplast along with the V-ATPase could have been modified (4). Although K is the main osmoticum, K+ ions may be limiting due to their role in maintaining the polarity of the PM, hence ions such as Na, Ca and Mg could be compensating for K in maintaining cell pressure by vacuolar sequestration through active transporters (4) or channels (5). Oparka and Gates (1981) suggested that water and assimilates enter the rice grain together, however water then leaves via the apoplast (see Chapter 1, Fig. 1.1) during grain filling. This could provide a pathway for the elimination of extracellular K, along with water, and thus provide an explanation for the decreased K concentration and retention of K in endosperm cells of HvSUT1 expressing rice grains.

Simplified starch, amino acid and lipid biosynthetic pathways in a rice endosperm cell are depicted in Figure 5.3. Only the tentative pathways and components to explain the changes that were observed in transgenic grains have been included. This diagram clearly shows that sucrose supply is integrally linked to the downstream processing pathways in synthesis of many metabolites in the grain. The content of soluble sucrose, but not hexoses, fructose or glucose, was significantly increased in HvSUT1 overexpressing rice grains. This implies that there was increased sucrose uptake, probably through active transporters. The dramatic increase in sucrose content in G1.4 was aligned with a significant decrease in soluble starch content. This could mean that either sucrose hydrolysis was disrupted through alteration of the activities of sucrose synthase (Susy) and/or that starch synthesis was disrupted through altering the activities of ADP-glucose pyrophosphorylase (AGPase), granule-bound starch synthase (GBSS), starch synthase (SS) or starch branching enzyme (SBE). It is more likely that downstream enzymatic steps in starch conversion may have been disrupted rather than sucrose hydrolysis because the synthesis of all other rice storage products was not negatively impacted, implying that supply of hexose phosphates was not limiting. Considering the more global changes in grain storage products in the transgenic rice grains, enhanced partitioning of hexose phosphates Glc-6-P and Fru-6-P into biosynthetic pathways for AAs, proteins, lipids and phytate (InsP6) could also perhaps explain the decrease in starch.

Hexose phosphates are also channelled into pathways for the synthesis of amino acids via the intermediate, 3-phosphoglycerate (3PGA) (Fig. 5.3). As well as Cys, 3PGA is also involved in biosynthesis of aromatic amino acids via phosphoenolpyruvate (PEP) and the Shikimate pathway. Cys, Tyr and Trp contents were increased with endosperm specific expression of *HvSUT1*, which suggests increased carbon flux into these metabolic pathways. The process of lipid synthesis also involves the metabolism of sugars through PEP into Acetyl CoA. Downstream, the total lipid contents were not

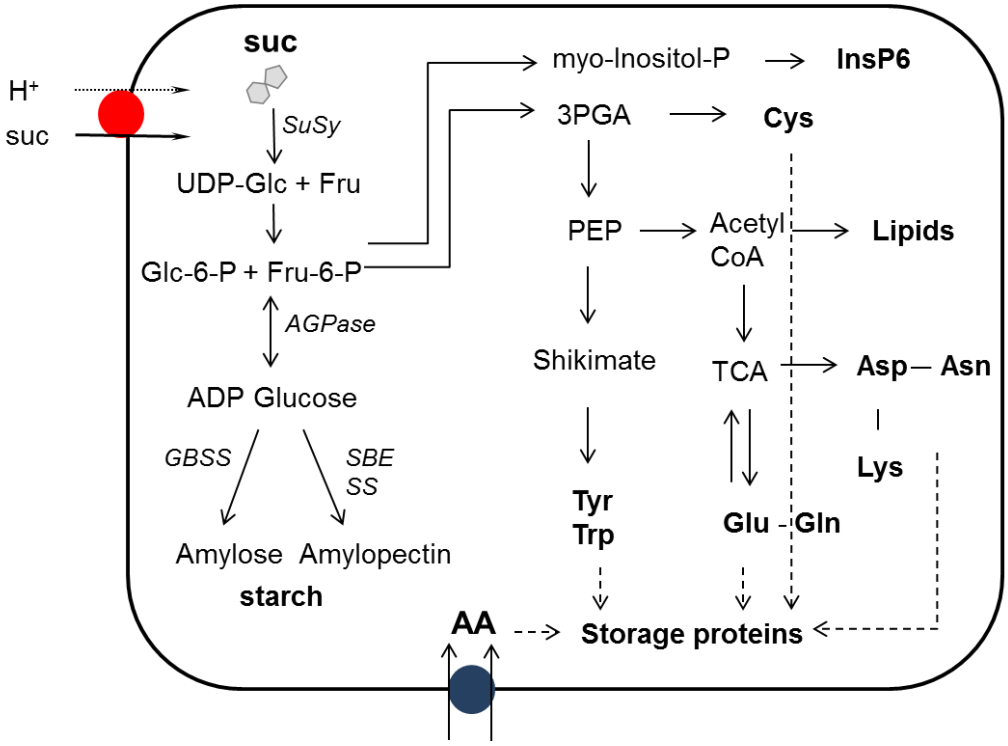


Figure 5.3 Summary of storage product biosynthetic pathways in rice endosperm cells

Simplified metabolic pathways for storage product synthesis from sucrose in endosperm cells. Only pathways relevant to changes observed in *HvSUT1* overexpressing rice grains have been included. Note that turnover of some substrates and bi-directional reactions have been omitted and there is no distinction made between reactions occurring in subcellular compartments, such as the cytosol and amyloplast. Solid arrows represent stages in the metabolic pathways and dashed arrows link amino acids to storage protein synthesis. Abbreviations are explained in the text.

increased in transgenic grains, but lipid composition was altered. This suggests that lipid content in rice grains is tightly regulated so that carbon partitioning into fatty acids may alter biosynthetic pathways to change composition but not content. Acetyl CoA is also involved in synthesis of the amino acids Glu, Gln, Asp, Asn, and Lys via the TCA cycle. The increased contents of all of these amino acids in rice grains expressing HvSUT1 specifically in endosperm suggests that there was increased carbon partitioning from Acetyl CoA into the TCA cycle for amino acid biosynthesis. Preferential partitioning of carbon into synthesis of amino acids could also possibly account for the stable lipid content in transgenic rice grains. As well as being synthesized in the grain, there is also possibly some contribution of AA transport from senescing vegetative organs for incorporation into grain storage proteins. The interconnected pathways described above and outlined in Fig. 5.3 clearly indicate that increased sucrose and hexose substrates partitioning into the various biosynthetic pathways could account for the altered storage product composition in *HvSUT1* overexpressing rice grains. Gene expression and enzyme activity can also be feedback regulated by substrates, intermediates and products of each biosynthetic pathway. A detailed discussion of these reactions is beyond the scope of this study, however future work could incorporate a transcriptome and metabolome analysis of transgenic grains to further elucidate the details of these regulatory networks.

The substantial changes in storage reserves in rice grains from G1.4 plants may be considered in relation to the temporal transgene expression corresponding with phases of grain development. As well as prolonged grain filling, endosperm specific overexpression of HvSUT1 stimulated increased sucrose transport specifically at 7 DAA and significantly, these grains showed the most dramatic changes in storage product composition. The critical point for grain filling appears to be the switch between grain expansion and storage product synthesis and accumulation which occurs around 7 DAA. Coincidentally, this specific time point has been identified as the time when a crucial switch occurs in grain filling and maturation. Rice grain development has been well studied and a number of specific phases have been identified (Ishimaru *et al.*, 2005). The pre-storage phase (1 to 4 DAA) is characterised by lengthening of the grain but there is no deposition of storage products. The early storage phase (4 to 8 DAA) encompasses the period when cellularization of rice endosperm is complete (Li *et al.*, 2008) followed by periods of rapid storage product synthesis and accumulation and hence is a critical phase that determines the sink strength (Liang *et al.*, 2001; Tang *et al.*, 2009). Dramatically increased sucrose uptake at 7 DAA in rice transformed with *Glb-1*$_{pro}$ driving *HvSUT1* coincides with endogenous globulin protein synthesis at 5 to 6

DAA and deposition in protein bodies at 7 DAA (Yamagata *et al.*, 1982). Rapid accumulation of starch in rice grains also occurs between 4 and 9 DAA (Teo *et al.*, 2011). Accumulation of TAGs and oleosins in rice seed also commences one week after flowering (Wu et al., 1998b). The endosperm is primarily liquid at this point and the rate of increase in grain fresh weight between 5 and 7 DAA can be attributed foremost to assimilate flux driving storage product synthesis (Iwai et al., 2012). Thus, although there was no overall measurable change in sucrose transport at most stages of grain filling in rice grains expressing *HvSUT1*, enhanced sucrose uptake at the point of storage product accumulation could explain the wide ranging changes in the grain composition of rice specifically expressing HvSUT1 in the endosperm.

5.3 Recommendations for future work

It will be necessary to conduct further examination of other independent transgenic lines generated in this study to provide support for the hypotheses proposed to explain changes in grain storage products due to *HvSUT1* overexpression. Although the findings presented here with limited transgenic lines clearly demonstrate that manipulating sucrose transport in rice has wide ranging effects on the nutritional composition of rice grains, the mechanisms that control those changes remain to be investigated.

HvSUT1 protein was shown to accumulate in rice grains driven by both the *Glb-1* and *Act-1* promoters. However, the question remains to be confirmed as to whether the *Glb-1* promoter drives *HvSUT1* expression only in the endosperm of transgenic rice grains and/or whether the *Act-1* promoter drives expression in all plant tissues. Immunolocalisation of HvSUT1 protein in rice tissues, using the anti-HvSUT1 produced in this study would provide support for the tissue specificity of both promoters. In addition, sucrose uptake assays using other tissues such as stem, leaf and roots could determine if constitutive expression of HvSUT1 protein results in functional changes in sucrose transport in plant organs other than the grain. These analyses would identify tissue expression and possible functionality of the transgene in rice tissue, enabling the effects between endosperm specific versus constitutive expression of HvSUT1 to be more clearly distinguished. In addition to the experiments looking at nutrient loading into grains, *Act-1* SUT-OE lines generated in this study could be used to investigate the as yet undetermined mechanisms of phloem loading in rice source tissues.

Elemental maps generated by LA-ICP-MS showed the differential distribution of micronutrients in transgenic rice grains. The association of these mineral elements with other storage products such as proteins or phytate were inferred from the data in

this study, but results were not definitive. The storage protein composition of grains overexpressing HvSUT1 in the endosperm was changed, however it is not known whether this affected the packaging of proteins into PSV. There were also changes in fatty acid composition that could affect oil body structure in the grain. To resolve these issues, microscopic imaging of the grain cellular structure could determine whether there had been modifications and redistribution of storage products in the endosperm. Moreover, high resolution sub cellular elemental distribution could then be linked back to the images of grain cellular structure to elucidate Fe and Zn speciation.

One of the most intriguing findings from this study was the change in K distribution in the transgenic rice grains. Further investigation of the role of K in ion homeostasis and cell turgor in the transgenic lines would first involve the isolation of protoplasts from both non transgenic and transgenic endosperm cells. The differential responses of the plasma membranes from the different plant lines could be explored *in vitro* by modifying the conditions in the protoplast extracellular media. Subsequent effects on the membrane potential and cell turgor could be measured to test the hypothesis that K transport is modified to prevent the depolarization of the plasma membrane caused by increased sucrose transport across the plasma membrane in HvSUT1 overexpressing rice grains.

Despite a higher retention of minerals in mature grain and changes in the distribution of elements in the grain endosperm, patterns of mineral accumulation during grain filling in developing rice grains were not significantly different in the transgenic plant lines. Mineral accumulation from 5 to 25 DAA was analysed only in unpolished grains, however, while mineral content was measured in both unpolished and polished mature rice grains. Significant changes in nutrients in mature rice grains were identified in the endosperm, therefore tracing mineral accumulation specifically in the endosperm of transgenic rice grains could highlight important time points for peak mineral nutrient loading. Measuring mineral contents in the endosperm of developing grains is possible by dissecting grains and collecting endosperm cells for mineral analysis. The sample volume would be very small, but robust methods have recently been developed to quantify mineral concentrations from nanolitre volumes (Palmer *et al.*, 2014).

Samples of developing endosperm tissue could also be used to analyse gene expression profiles in the transgenic lines. Further insights could be gained from metabolite profiling of endosperm tissue. In particular, measuring gene expression and metabolite levels for intermediates in carbon metabolism and amino acid and lipid synthesis at critical points in grain filling could establish the mechanisms that

potentially control nutrient deposition in the transgenic rice grains. Furthermore, there are possible links between sucrose and expression of genes related to metal transport that could be identified by gene profiling. Together, these experiments would enable a more comprehensive picture of the overall effects of increased assimilate uptake on the regulation of storage product synthesis and accumulation of minerals in rice grains.

One of the major constraints in this study was the space restrictions and inability to test the transgenic plant lines under field conditions due to the strict laws governing the growth of transgenic plants in South Australia. Ideally, testing for transgene effects on seed quality characteristics would be conducted in field conditions, in various locations with differing soil characteristics. In addition, several years of growth associated with natural seasonal variation is required to test the stability of the transgene effects. Future work could involve collaborations with international research labs where the testing of transgenic plants under field conditions could be undertaken. Additional plant lines could also be generated from further rounds of plant transformation with the plasmids that were generated in this research. This would provide more single copy homozygous plant lines for investigation. From a human nutrition and biosafety regulation viewpoint, production of marker-free transgenic lines could also be a target for future work. Furthermore, gene stacking *HvSUT1* with genes that are involved in metal transport, such as *NAS2* could be a dual approach to increasing both the concentration and retention of micronutrient in rice endosperm.

Appendix A PCR primers, components and conditions

PCR 1			Conc.
Glb1Fv2	GGAGAACTAGTGGCGCCTGGAGGGA		0.5µM
Glb1R	GAGAGGCGCGCCTGATGATCAATCAGACAATCACTAG		0.5µM
HF buffer (Finzymmes)	5x stock		1x
dNTPs	10mM stock		200µM
Phusion polymerase	2 U/µl		0.02U/µl
gDNA template	15ng/ µl		60ng
	Temperature	Time	Cycles
Initial denaturation	98°C	30sec	1
Denaturation	98°C	10sec	30
Annealing	66°C	30sec	
Extension	72°C	30sec	
Final extension	72°C	5min	1
Hold	4°C	∞	

PCR 2			Conc.
HvSUT1F	CTCGGCAGGCTCATCCTC		0.5µM
HvSUT3R	GTACAGGATGAACGGGAACC		0.5µM
Thermopol buffer (NEB)	10x stock		1x
dNTPs	10mM stock		200µM
Vent polymerase	2 U/µl		0.02U/µl
E.coli colony template	5 µl of colony resuspended in 20µl milliQ		
	Temperature	Time	Cycles
Initial denaturation	95°C	20sec	1
Denaturation	95°C	20sec	25
Annealing	55°C	20sec	
Extension	72°C	50sec	
Final extension	72°C	5min	1
Hold	4°C	∞	

PCR 3			Conc.
attB1 HvSUTF	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAGGGCGGCCTCCCTGCGA		0.5µM
attB2 HvSUTR	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTCTAGCGGAAGTCGGCCGCTC		0.5µM
HF buffer (Finzymmes)	5x stock		1x
dNTPs	10mM stock		200µM
Phusion polymerase	2 U/µl		0.02U/µl
HOSUT plasmid	5ng/µl stock		5ng
	Temperature	Time	Cycles
Initial denaturation	98°C	30sec	1
Denaturation	98°C	5sec	35
Annealing	72°C	10sec	
Extension	72°C	60sec	
Final extension	72°C	5min	1
Hold	4°C	∞	

PCR 4			Conc.
HvSUT3F	GGTTCTGGGGTTTAGCTCGT		0.5µM
NosTR	AAGACCGGCAACAGGATTC		0.5µM
Thermopol buffer (NEB)	10x stock		1x
dNTPs	10mM stock		200µM
Vent	2 U/µl		0.02U/µl
E.coli colony template	2µl of colony resuspended in 20µl milliQ		
	Temperature	Time	Cycles
Initial denaturation	95°C	30sec	1
Denaturation	95°C	20sec	30
Annealing	54°C	20sec	
Extension	72°C	1min	
Final extension	72°C	5min	1
Hold	4°C	∞	

PCR 5			Conc.
GUSF	CAGCCAAAAGCCAGACAG		0.5µM
GUSR	GGCACAGCACATCAAAGA		0.5µM
Thermopol buffer (NEB)	10x stock		1x
dNTPs	10mM stock		200µM
Vent polymerase	2 U/µl		0.02U/µl
Plasmid DNA	2ng/µl stock		2ng
	Temperature	Time	Cycles
Initial denaturation	95°C	30sec	1
Denaturation	95°C	15sec	25
Annealing	49°C	15sec	
Extension	72°C	40sec	
Final extension	72°C	5min	1
Hold	4°C	∞	

PCR 6			Conc.
HvSUT4F	ACTGACTGACCTGAGCATGG		0.5µM
NosTR	AAGACCGGCAACAGGATTC		0.5µM
Standard buffer (NEB)	10x stock		1x
dNTPs	10mM stock		200µM
Taq polymerase	2 U/µl		0.02U/µl
Plasmid DNA	2ng/µl stock		2ng
	Temperature	Time	Cycles
Initial denaturation	95°C	30sec	1
Denaturation	95°C	30sec	30
Annealing	50°C	30sec	
Extension	68°C	30sec	
Final extension	68°C	5min	1
Hold	4°C	∞	

PCR 7			Conc.
eEf1α F	TTTCACTCTTGGTGTGAAGCAGAT		0.5µM
eEf1α R	GACTTCCTTCACGATTTCATCGTAA		0.5µM
Standard buffer (NEB)	10x stock		1x
dNTPs	10mM stock		200µM
Taq polymerase	2 U/µl		0.02U/µl
cDNA	2µl of RT rxn (500ng RNA)		
	Temperature	Time	Cycles
Initial denaturation	95°C	2min	1
Denaturation	95°C	15sec	26
Annealing	51°C	30sec	
Extension	68°C	45sec	
Final extension	68°C	5min	1
Hold	4°C	∞	

PCR 8			Conc.
OsSUT F	CCTCTTTGCGTTTCTTGGAG		0.5µM
OsSUT R	CCACCTCACCCCTTCCTAA		0.5µM
Standard buffer (NEB)	10x stock		1x
dNTPs	10mM stock		200µM
Taq polymerase	2 U/µl		0.02U/µl
cDNA	2µl of RT rxn (500ng RNA)		
	Temperature	Time	Cycles
Initial denaturation	95°C	2min	1
Denaturation	95°C	15sec	26
Annealing	53°C	30sec	
Extension	68°C	45sec	
Final extension	68°C	5min	1
Hold	4°C	∞	

PCR 9			Conc.
HvSUT F	ACTGACTGACCTGAGCATGG		0.5µM
HvSUT R	GCTGCTGAAATTTCACCATCCATTC		0.5µM
Standard buffer (NEB)	10x stock		1x
dNTPs	10mM stock		200µM
Taq polymerase	2 U/µl		0.02U/µl
cDNA	2µl of RT rxn (500ng RNA)		
	Temperature	Time	Cycles
Initial denaturation	95°C	2min	1
Denaturation	95°C	15sec	26
Annealing	56°C	30sec	
Extension	68°C	45sec	
Final extension	68°C	5min	1
Hold	4°C	∞	

Sequencing primers		Tm (°C)
HvSUT1 1F	CTCGGCAGGCTCATCCTC	55
HvSUT1 2F	CCAGTGCTGCAAATTCAATC	50
HvSUT1 3F	GGTTCTGGGGTTTAGCTCGT	54
HvSUT1 4F	ACTGACTGACCTGAGCATGG	54
HvSUT1 2R	AGGCCCATGATAGAGACTGC	54
HvSUT1 3R	GTACAGGATGAACGGGAACC	54
HvSUT1 4R	ATCACCTGCGGTATCACGA	51

Appendix B Stock solutions and media used in rice transformation

Antibiotic stocks

Rifampicin stock	(20mg/ml)
Spectinomycin	(200mg/ml)
Hygromycin stock	(50mg/ml)
Kanamycin stock	(50mg/ml)
Timentin stock	(150mg/ml)

LB

Bacto-Tryptone	10g
Yeast extract	5g
Sodium Chloride	10g

Add milliQ to 1L and autoclave

LB preparations

LB broth
LB plates + 20 µg/ml rif for AGL1
25µg/ml spec for pIPKb
20µg/ml rif + 25µg/ml spec for AGL1+pIPKb

Sucrose stock solution (120g/L)

Sucrose 30g
milliQ to 250 ml
Autoclave

Sucrose (120g/L) + Glucose (40g/L) stock solution

Glucose 5g
Sucrose 15g
milliQ to 250ml
Autoclave

Sucrose (120g/L) + Sorbitol stock solution (120g/L) stock solution

Sorbitol 30g
Sucrose 30g
milliQ to 500ml
Autoclave

Phytohormone/Phytochemical stocks

2, 4-D Stock (2 mg/ ml)

2,4-D 20 mg
1N KOH a few drops to dissolve
milliQ to 10 ml
Filter sterilize and store 1ml aliquots at -20 ℃

Kinetin Stock (2 mg/ ml)

Kinetin 20 mg
1N KOH a few drops to dissolve
milliQ to 10 ml
Filter sterilize and store 1ml aliquots at -20 ℃

Napthaleneacetic acid NAA Stock (1 mg/ ml)

NAA 10 mg
1N KOH a few drops to dissolve
milliQ to 10 ml
Filter sterilize and store 1ml aliquots at -20 ˚C

Acetosyringone stock AS 20mg/mL

AS 200mg
DMSO to 10ml
Store as 1ml aliquots at -20˚C (short term storage)

YM Media

Mannitol	10g
Yeast extract	0.4g
K2HPO4 (10% w/v stock)	1ml
KH2PO4 (10% w/v stock)	4ml
NaCl (10% w/v stock)	1ml
MgSO4.7H2O(10% w/v stock)	2ml

Top up ddH_2O to 1L pH 6.8
Agar 15g/L 15g
Autoclave

YM plates

YM + rif (20 µg/ml) +spec(15µg/ml)

N6D medium

Chu's Basal Salt Mixture w/vitamins	3.99g
Casamino acid (or Casein hydrolysate)	300mg
myo-inositol	100mg
L proline	2.88g

Add 450ml ddH_2O and adjust pH 5.8 by 1N KOH
Add (0.4% final volume) 4g Gelrite
Top up ddH_2O to 749ml
Autoclave
Add

Sucrose stock solution	250mL
2,4-D (2mg/ml)	1ml

N6D preparations

N6D plates only (1L makes ~ 30 plates)
N6D plates + hyg (50 µg/ml) + tim(150µg/ml)

2N6-AS medium

Chu's Basal Salt Mixture w/vitamins	3.99g
Casamino acid (or Casein hydrolysate)	300mg
myo-inositol	100mg

Add some ddH_2O and adjust pH 5.2 by 1N KOH
Add ddH_2O to 498mL and divide into two equal volumes

	Bottle 1	*Bottle 2*
Add	*2g Gelrite*	-
Autoclave both and add to each:		
Sucrose + Glucose stock	250ml	250ml
2,4-D(2mg/ml)	0.5ml	0.5ml
Acetosyringone (20mg/mL)	0.5ml	0.5ml

2N6-AS preparations

Bottle 1 - 2N6-AS plates only (with 0.4% Gelrite)
Bottle 2 - 2N6-AS broth

RE III Medium

MS Basal media	4.33g
Casamino acid (or Casein hydrolysate)	2g
MS vitamins 1000x	1mL

Add some ddH_2O and pH 5.8 by 1N KOH
Add ddH_2O to 498mL
Gelrite (0.4% final conc.) 4g
Autoclave
Add

Sucrose + Sorbitol stock	500mL
NAA (1mg/ml stock)	50µl
Kinetin (2mg/ml stock)	1.25ml

REIII preparations

in plant tissue culture plates
REIII media + hyg(50 µg/ml) +tim(150µg/ml)

HF medium

MS Basal media	4.33g
MS vitamins 1000 x	1mL

Add some ddH_2O and pH 5.8 by 1N KOH
Add ddH_2O to 750ml and 4g Gelrite (0.4% final conc.)
Autoclave
Add
Sucrose stock
250mL

HF preparations

in clear polypropylene tubes with ventilation in lid

HF media + hyg (50 µg/ml) + tim (150 µg/ml)

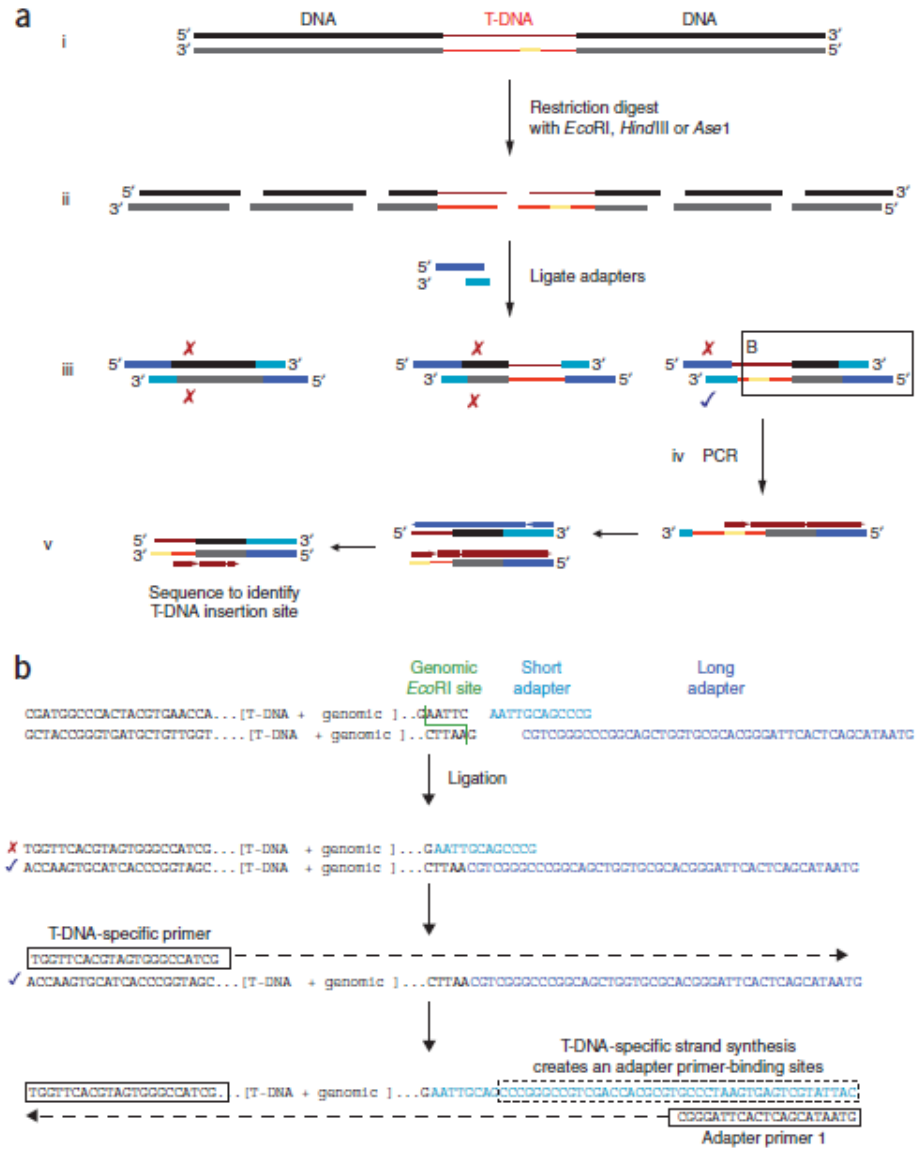
Other chemicals/reagents

- 1N KOH (5.611g in 100ml ddH_2O)
- 1M sterilized $MgSO_4$-$7H_2O$ (24.6g in 100ml ddH_2O; filter)
- 70% ethanol
- 100% ethanol
- 33-50% commercial bleach (or 2.5% hypochlorite)
- Sterile ddH_2O
- Tween 20

Plant material

- Rice seed “Nipponbare” WT

Appendix C Adapter Ligation PCR to determine transgene integration site



Protocol for mapping the location site of inserted T-DNA strands in plant genomic DNA by adapter ligation-mediated PCR (AL-PCR) developed by O'Malley et al. (2007). In summary (a) gDNA from transgenic lines is digested and ligated to long and short adapter sequences that create overhanging, asymmetric flanking sequences either side of the cut gDNA. Only amplification of the complementary long adapter sequence (by PCR with a T-DNA specific primer) will create a binding site for the Adapter primer (b). These T-DNA-gDNA-Adapter sequences will then be amplified selectively and can be prepared for sequencing.

Table C.1 Summary of flanking sequence results derived from AL-PCR of rice gDNA. Colour codes of sequences are as follows: Orange, Adapter sequence; Blue, gene in vector; Green, rice chromosome; Pink, vector sequences; Red, restriction site; Black, unknown sequences.

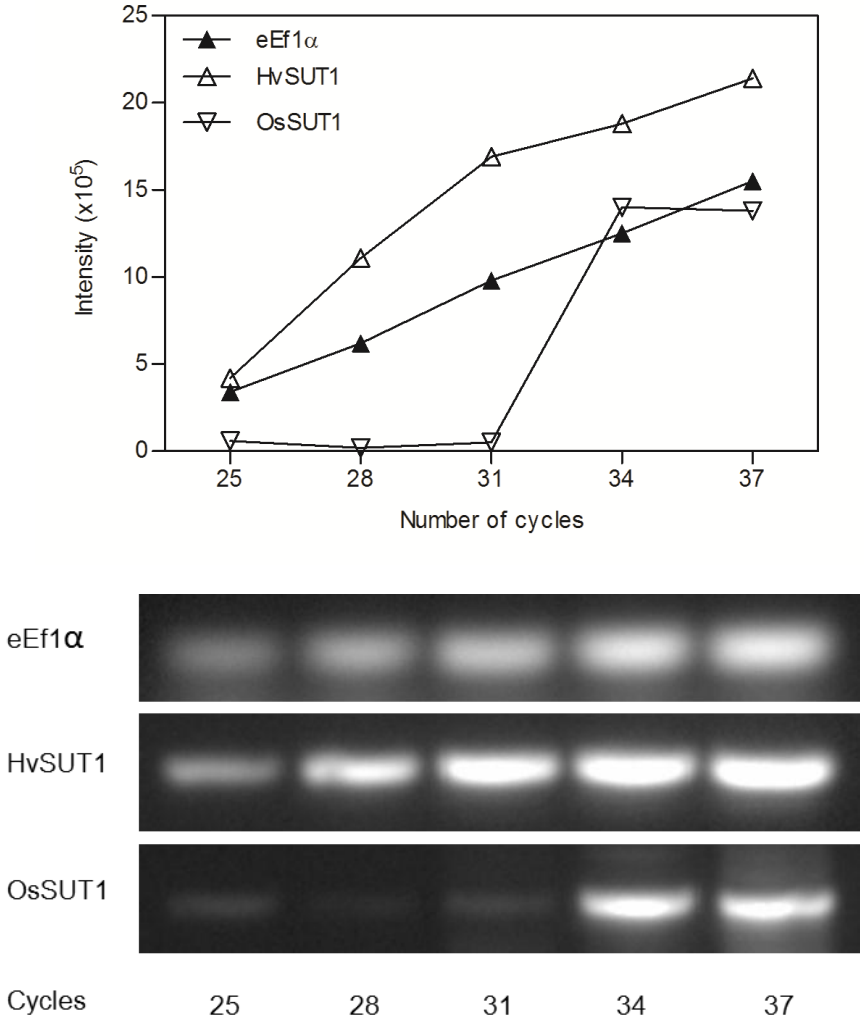
Plant line	T-DNA Border	Sequence	Flanking DNA ID/Position
G1.1	Left	GGACCGGAACACGGATCAAACACTGATAGTTTAAACCGAAGGCGGGAAACGACAATCTGATCGGGTACCGGGCCCAAGATCTGGCCCTTAAGGCCTATTTAAATACTAGTGGCGCCTGGAGGGAGGAGAGGGGAGAGATGGTGAGAGAGGAGGAAGAAGAGGAGGGGTGACAATGATATGTGGGGCCATGTGGGCCCCACCATTTTTTAATTCATTCTTTTGTTGAAACTGACATGTGGGTCCCATGAGATTTATTATTTTTCGGATCGAATTGCCACGTAAGCGCTACGTCAATGCTACGTCAGATGAAGACCGAGTCAAATTAGCCACGTAAGCGCCACGTCAGCCAAAACCACCATCCAAACCGCCGAGGGACCTCATCTGCACTGGTTTTGATAGTTGAGGGACCCGTTGTATCTGGTTTTTCGATTGAAGGACGAAAATCAAATTTGTTGACAAGTTAAGGGACCTTAAATGAACTTATTCCATTTCAAAATATTCTGTGAGCCATATATACCGTGGGCTTCCAATCCTCCTCAAATTAAAGGGCCTTTTTAAAATAGATAATTGCCTTCTTTCAGTCACCCATAAAAGTACAAAACTACTACCAACAAGCAACATGCGCAGTTACACACATTTTCTGCACATTTCCACCACGTCACAAAGAGCTAAGAGTTATCCCTAGGACAATCTCATTAGTGTAGATACATCCATTAATCTTTTATCAGAGGCAAACGTAAAGCCGCTCTTTATGACAAAAATAGGTGACACAAAAGTGTTATCTGCCACATACATAACTTCAGAAATTACCCAACACCAAGAGAAAATAAAAAAAAAATCTTTTTGCAAGCTCCAAATCTTGAAACCTTTTTCACTCTTTGCAGCATTGTACTCTTGCTCTTTTCCAACCGATCCATGTCACCCTCAAGCTGCACCCGG	*O. sativa* Chr 5
G3.1	Left	ATTACTCTTTCTTTTTCTCCATATTGACCATCATACTCATTGCTGATCATTTTATAATAACGATCTGGTGTCTTGCTGTTCCATGAGAAATATGCCTATCGATCGATTTCTATTTGGTTTCTTAATTATGTCGCTCGATGGAGCCGCTTGATTGCTACAGTGTATTTGCTAAATTAACCTAACTAGCAGGAGCATGGAGGAGACGAAAAAAATAAGCAGTGTATGCGCTTGGTTTCACGGATTGGGGTCGATTTATATGAAGGTTTAACAATAATTTGAGAGAAATCAAGCTAGCCACCACATGCAAAAACTCCTACAATGCACTTATATAAAGATAGGCAGATCATAATTAAATGCAGTTTGAAGATGGACTTACTTAGGATGAAGATCCGGGCTCTGGTGATCCATGATTATACTCAATGTACAGTTTTTTTTTTTTTGAGGGATACTCAATGTACAGTTAAAACGGTATTTGGCTAACAAAATTAGTTTAAGCGAGCACGGGATTGGGGGCGATTTATATGCATCTTCTTCACTGGACAATTAACGTGAGGGAATCGGAATCGTTACCGTGTTCTGAACAAATTACTGTATGCGTAGTACTCCCTCCATCCTTAAAAAGGCCCAACCGAACCTAAAGGAGCAATCCCTATTTTCATATCTAATTGTGATTTTACCTCTGCTATTTAGGTTTTTTTGGCCCCTGCTTTTTTTTTTGATCGAATGAATCGATTACACCTGTTTTTGACGGAAGCAAGTGGACCCGGAATTTGTTAAGGGAAAAAAAAATAGGGATTTTGCAAATCTTGTGTTACCCTTCTATTCCGGCAAGGTCACTCCGTGCTTACTGGAATAGCATTATACTCGTATGTCTTGCTCTTGGAGCCATGGTGCTCAACGCCCTCAACGGCAGCGCTGGGCTCCGGCTAGAATAGTACTAGCTC	*O. sativa* Chr 6
G4.1	Right	GGGAATGAAGTACATTTTGATCGGGTAATGACTGGGCTGTTAGTGTACCTGTGGGCAGGGTTTTCAATTTCAAAATTAGTATTTTTGCGTTGGGGGGTGCGAAATTTTGAAAATTTCGAATTGCAGCCCGGGCCGTCGACCA	*O. sativa* Chr 6
G5.1	Right	GTGTTTGAGCCTGCGCGACGGACGCACTGACGGTGTCGTCCATCACAGTTTGCCAGTGATACACATGGGGATCAGCAATCGCGCATATGAAATCACGCCATGTAGTGTATTGACCGATTCCTTGCGGTCCGAATGGGCCGAACCCGCTCGTCTGGCTAAGATCGGCCGCAGCGATCGCATCCATGGCCTCCGCGACCGGCTGCAGAACAGCGGGCAGTTCGGTTTCAGGCAGGTCTTGCAACGTGACACCCTGTGCACGGCGGGAGATGCAATAGGTCAGGCTCTCGCTGAATTGCAGCCCGGGCCGTCGACCA	Hpt^R
A1.1	Left	CTCTTTCTTTTTCTCCATATTGACCATCATACTCATTGCTGATCCATGTACATTTCCCGGACATGAAGCGTCCCGGCATCGGCCGTTTTCTCTACCGCCTGGCACGCCGCGCCGCAGGCAAGGCAGAAGCCAGATGGTTGTTCAAGACGATCTACGAACGCAGTGGCAGCGCCGGAGAGTTCAAGAAG	

		TTCTGTTTCACCGTGCGCAAGCTGATCGGGTCAAATGACCTGCCGGA GTACGATTTGAAGGAGGAGGCGGGGCAGGCTGGCCCGATCCTAGTC ATGCGCTACCGCAACCTGATCGAGGGCGAAGCATCCGCCGGTTCCT AATGTACGGAGCAGATGCTAGGGCAAATTGCCCTAGCAGGGGAAAAA GGTCGAAAAGGTCTCTTTCCTGTGGATAGCACGTACATTGGGAACCC AAAGCCGTACATTGGGAACCGGAACCCGTACATTGGGAACCCAAAGC CGTACATTGGGAACCGGTCACACATGTAAGTGACTGATATAAAAGAG AAAAAAGGCGATTTTTCCGCCTAAAACTCTTTAAAACTTATTAAAACTC TTAAAACCCGCCTGGCCTGTGCATAACTGTCTGGCCAGCGCACAGCC GAAGAGCTGCAAAAAGCGCCTACCCTTCGGTCGCTGCGCTCCCTAC GCCCCGCCGCTTCGCGTCGGCCTATCGCGGCCGACAGCCTTCTGCG ACAGACCGACGAGATCTGCTCCCTGATCACCCGCCTGCCAAGGCCG CCCAAATCCGCAGGCCAGCGCTTTTCCGCGCCCTATCTGACCGTGAT GGACTGGGGACACGACGAGCGCGATTATGCAGCTGGTCATTCCGAC AGAGCGCCGCGGCTCGATGACGCCGCGTGGGCCGGCATGATGAAC AACATACGGGCCATATCCGAGCTTGCCCGCGACAAATATGGTGTTCG TGCCACCATTCATCCGCATGCCGGTGGCTA	pVS1 ori, recombinase (Agrobacterium str. C58)
A1.1	Right	**GTTTG**AATTGCAGTGCCGTTAGGGACATACACGGGCAGCACGTTCTG ACAAATCTGCATATATATATATATATATAGATATATATAGATATATATAT CTTCCTCCTGAATATTCTTTATAGTCTACAAATTCTTAATTAATCACCAA GCAGATCATGAGCATGTGATTTCTTCTCTTAAAAGTGCCTTACAGATC AATCGTTACATGTTATCATTTCTTCCGCCGGGCTAATCATTATATTATA TATTGTCAGAATCGAATAACAAAAATAAACTGTGATCTTAATCCAAAAT CAAGCTGCAGCCCGGGCC	*O. sativa* Chr 1
A4.1	Left	GGACATGAAGCCATTTACAATTGAATGCATATCTGTATTTGAGTCGGG TTTTAAGTTCGTTTGCTTTTGTAAATACAGAGGGATTTGTATAAGAAAT ATCTTTAAAAAAACCCATATGCTAATTTGACATAATTTTTGAGAAAAAT ATATATTCAGGCGAATTGCAGCCCGGGCCGTCAACCA	Act1
A6.1*	Left	AACGACAATCTGATCGGGTACCGGGCCCAAGATCTGGCCCTTAAGGC CTTACTAGTGGATCCCCCGGGACTAGTGGCTCGTGGTCATTCATATG CTTGAGAAGAGAGTCGGGATAGTCCAAAATAAAACAAAGGTAAGATTA CCTGGTCAAAAGTGAAAACATCAGTTAAAAGGTGGTATAAAGTAAAAT ATCGGTAATAAAAGGTGGCCCAAAGTGAAATTTACTCTTTTCTACTATT ATAAAAATTGAGGATGTTTTTGTCGGTACTTTGATACGTCATTTTTGTA TGAATTGGTTTTTAAGTTTATTCGCTTTTGGAAATGCATATCTGTATTT GAGTCGGGTTTTAAGTTCGTTTGCTTTTGTAAATACAGAGGGATTTGT ATAAGAAATATCTTTAAAAAAACCCATATGCTAATTTGACATAATTTTTG AGAAAAATATATATTCAGGCGAATTGCAGCCCGGGCCGTCGACCA	**vector**
* AL-PCR yielded two bands, however only the high molecular weight band gave readable sequence			

Reliable sequence data of the T-DNA junction obtained by gel purification of bands obtained from AL-PCR (O'Malley et al., 2007) of genomic DNA from T_1 transgenic rice lines, and Sanger sequencing (Australian Genome Research Facility, Adelaide) followed by BLAST searches in NCBI rice genomic database gave results which are summarised in Table C.1.

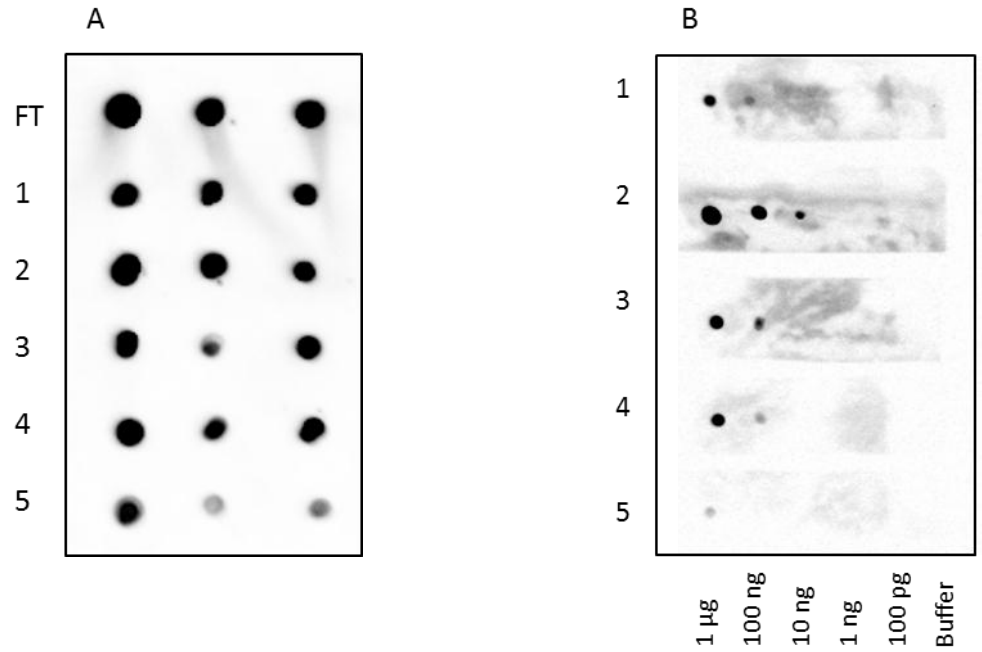
Analysis of many plant lines either did not yield specific bands on a gel or readable sequences to be analysed. Only a few samples gave any readable sequence relating to the possible location of the T-DNA in the rice genome. Other samples resulted in sequences that implied either that unintended sequences from the plasmid were integrated into the genome or gave unreliable sequence data that could not be interpreted. Multiple insertions in the plant line would yield unreadable sequence using this method as the sequencing primer is the Left Border primer that primes to all ligated Left Border adapters in the sample, and hence the sequences overlapped.

Appendix D Pilot test to determine number of cycles for exponential amplification of target genes for semi quantitative RT-PCR



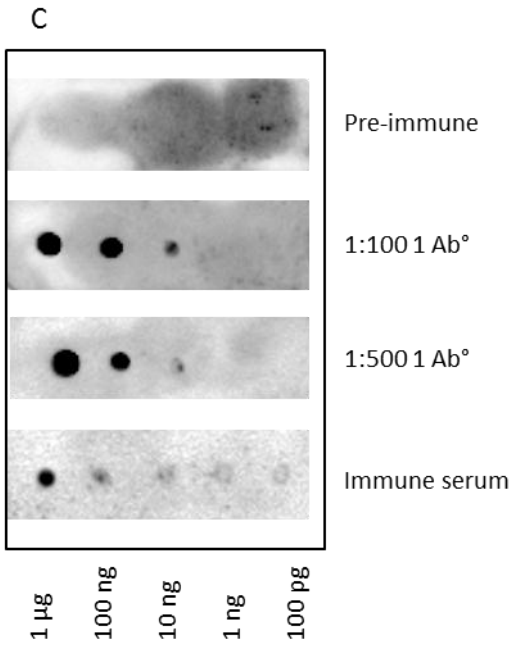
A pilot test was conducted to determine the number of cycles required for exponential amplification of the target RNA for semi quantitative densitometric analysis. Housekeeping gene *eEf1α* has been used as a standard to calculate relative ratios of the transgenic *HvSUT1* and endogenous *OsSUT1*. The graph plots the intensities of bands from PCR cycles 25 to 37 of the same cDNA on 1% agarose gels. The bands were quantified using Image Lab software (Biorad). Based on these results, 26 RT-PCR cycles were used for *eEF1α* and *HvSUT1* and 33 for *OsSUT1* (*OsSUT1* consistently gave relatively inconsistent amplification even between technical replicates).

Appendix E Titration of HvSUT1 antibody and tests of specificity and dilution of Ab° to use in immunoblotting



A: Dot blot of the various fractions eluted from the affinity column (elutions 1 to 5) plus the flow through (FT), in triplicate, showed the presence of IgGs in all fractions.

B: Dilution series of peptide on nitrocellulose (100pg to 1µg) was detected with each elution (1 to 5) from affinity purification (1:1000 in blotto) to select elution with the highest activity (elution 2).



C: Specificity and optimum dilution for use in immunoblotting was determined for elution 2 against dilution series of peptide on nitrocellulose. Pre immune serum was used as a negative control and the unpurified immune serum was also used to show the increased activity of affinity purified antibody and the specificity of the antibody for the peptide. Final dilution for use in immunoblotting was 1:500 to decrease background.

Appendix F Copy number analyses of transgenic rice plants transformed using *Agrobacterium* to overexpress barley *HvSUT1*

Transgenic line designation	*T1 Germination*			*Seg. ratio*	*Leaf assay*	*Southern Blot*	*A/L PCR*	*Possible no. of loci*
	Total	*Hyg^R*	*Hyg^S*		*Hyg ^{R}:Hyg ^{S}*			
G1.1	26	13	13	1:1		1	2	
G1.2	27	11	16	1:1			3	
G1.3	30	18	12	1:1		3 - 4	4	
G1.4	29	25	4	3:1	3:1	1-2	1	**1**
G1.5	25	13	12	1:1			4	
G1.6	29	13	16	1:1				
G1.7	30	12	18	1:1				
G2.1	13	10	3	3:1$^+$			4	
G2.2	29	29	0	1:0	1:0	3 - 4		
G2.3	29	29	0	1:0$^+$		3		
G3.1	32	24	8	3:1	3:1	2	2	**1 - 2**
G3.2	15	9	6	1:1				
G4.1	14	11	3	3:1			>5	
G4.2	28	26	2	1:0	1:0	4 - 5		
G5.1	-^						1	
G5.2	-^							
G6.1	30	23	7	3:1		2	1	
G6.2	29	22	7	1:1	1:1	2		
G6.3	29	24$^+$	5	3:1$^+$		2		
G6.4	15	10$^+$	5	3:1$^+$				
A1.1	-				3:1$^{\&}$	3	1	
A2.1	29	25*	4	3:1*		1	>2	
A2.2	28	13	15	1:1				
A2.3	29	21	8	3:1	3:1	1		**1**
A3.1	30	23	7	3:1	3:1	1		
A3.2	-						1	
A3.3	-							
A4.1	-				1:1	2 - 3	>3	
A4.2	32	25	7	3:1				
A5.1	29	19	10	3:1	3:1	1		**1**
A6.1	30	25	5	3:1		>4	>2	

nd not determined

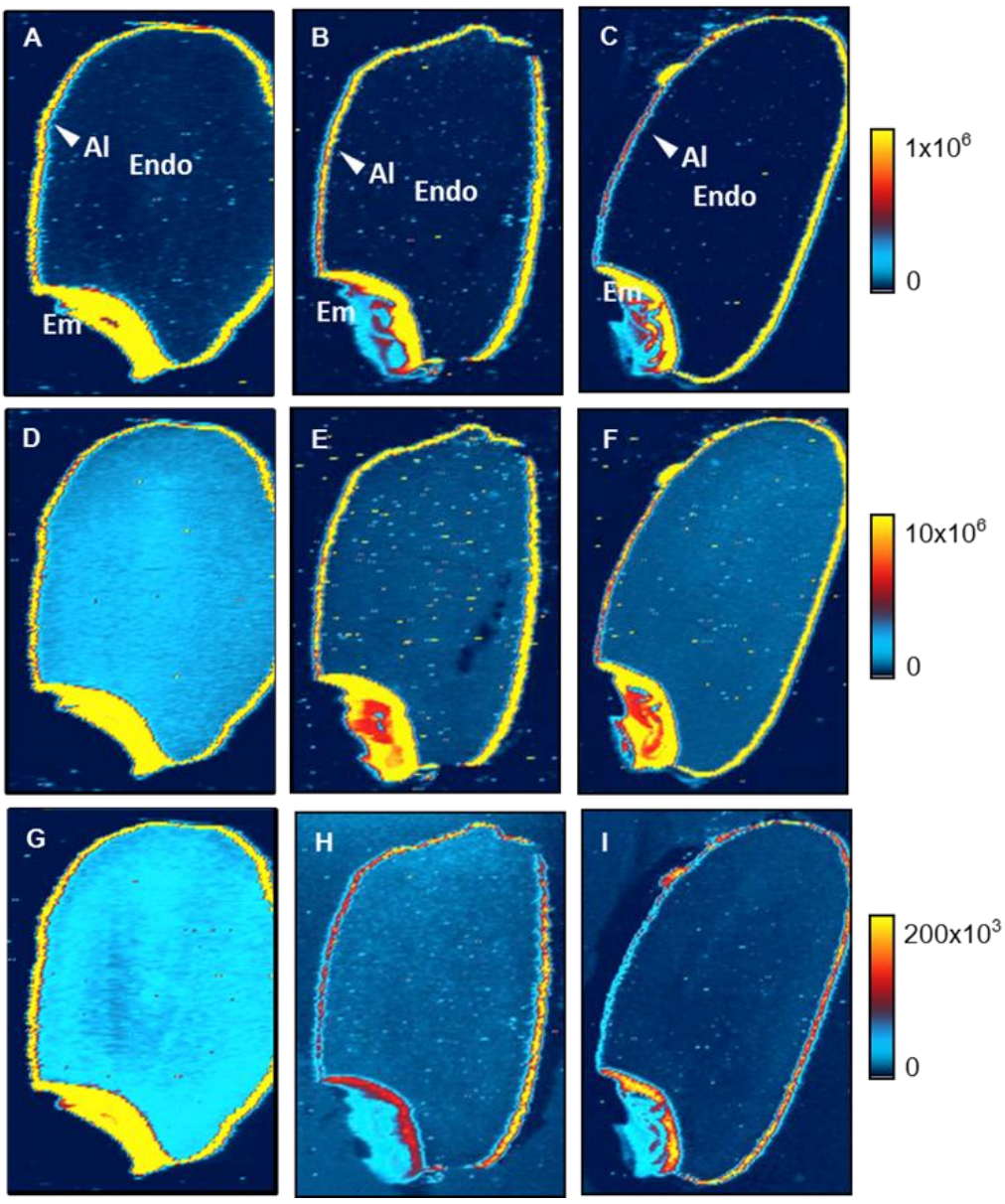
*some seedlings variegated

† some seedlings albino

$^{\&}$ mature plants gave unusual grain phenotype and not enough seed for analysis

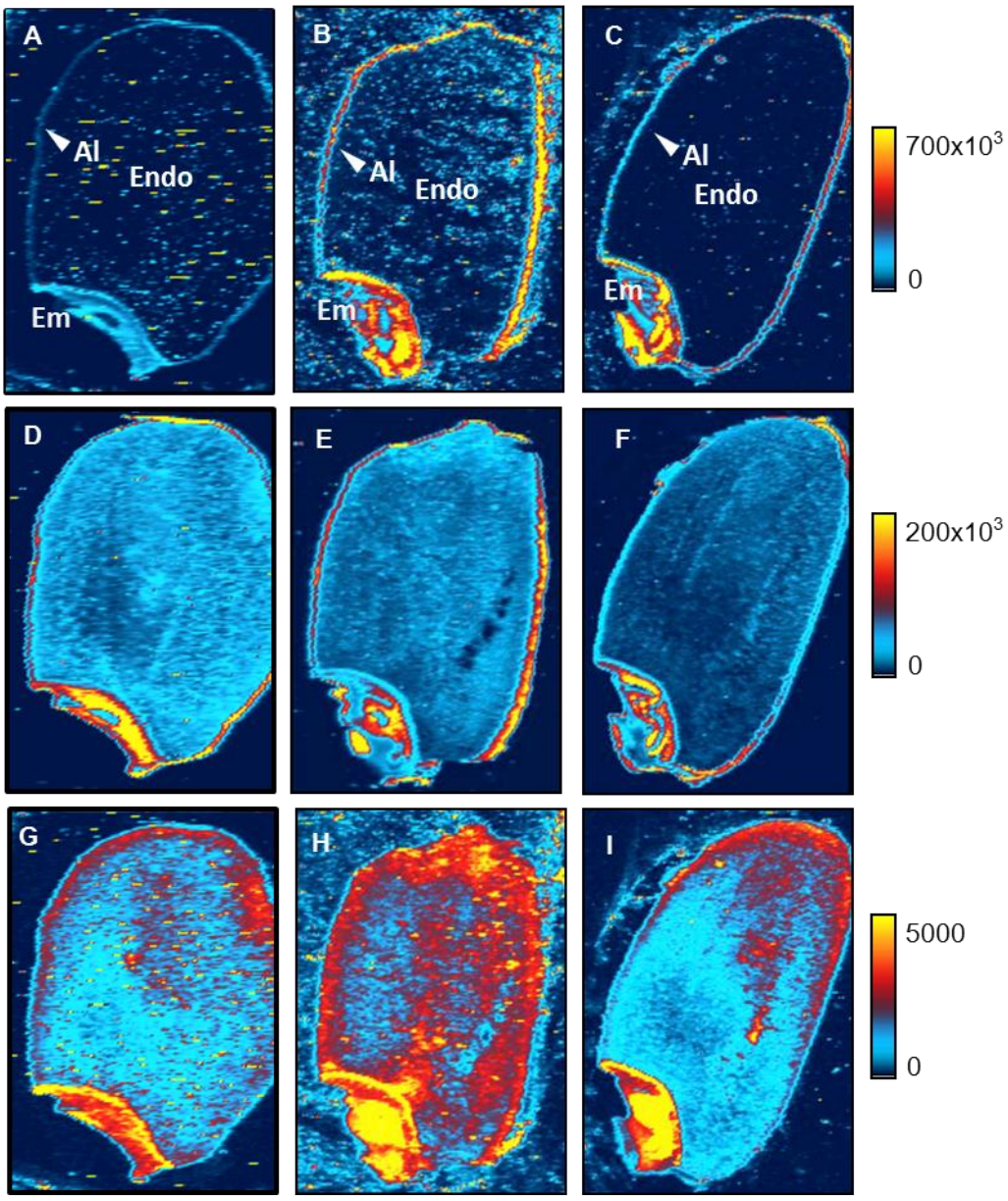
^ plants from these lines did not produce viable seed

Appendix G LA-ICP-MS elemental maps of minerals in longitudinal sections of rice grain



A LA-ICP-MS elemental maps of macronutrients in longitudinal cross sections of rice grain

Distribution of Mg in NT (A), G1.4 (B) and A2.3 (C), K in NT (D), G1.4 (E) and A2.3 (F), and P in NT (G), G1.4 (H) and A2.3 (I) derived from longitudinal sections of rice grain. Normalized counts per second for each element are scaled from dark blue (lowest) to highest (yellow). Grains are oriented with the ventral and dorsal side of the grain on the left and right respectively. Al, aleurone; Endo, endosperm; Em, embryo.



B LA-ICP-MS elemental maps of micronutrients in longitudinal cross sections of rice grain

Distribution of Fe in NT (A), G1.4 (B) and A2.3 (C), Mn in NT (D), G1.4 (E) and A2.3 (F), and Zn in NT (G), G1.4 (H) and A2.3 (I) derived from longitudinal sections of rice grain. Normalized counts per second for each element are scaled from dark blue (lowest) to highest (yellow). Grains are oriented with the ventral and dorsal side of the grain on the left and right respectively. Al, aleurone; Endo, endosperm; Em, embryo.

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