Expression analysis of genes involved in sucrose transport and metabolism during grain development in wheat

BY

GEETHALAKSHMI SAKTHIVEL

A Thesis
Submitted to the Faculty of Graduate Studies of
The University of Manitoba
in partial fulfillment of the requirements of the degree of

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GENERAL ABSTRACT

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To gain an understanding of the transcriptional regulation of sucrose transport and metabolism in wheat and to examine their relationships with dry matter accumulation in seeds, this study has characterized the expression patterns of two sucrose transporters (*TaSUT1* and *TaSUT2*) and two sucrose synthase (*TaSuSy1* and *TaSuSy2*) genes in five spring wheat cultivars at different seed developmental stages. Cultivar 'AC Andrew' with higher *TaSUT1* and *TaSuSy2* transcript abundance particularly during the early grain filling stage, exhibited higher dry grain weight than other cultivars. The result, overall, suggests the significance of coordinated expression between *TaSUT1* and *TaSuSy2* for grain growth. This study also demonstrated the seed specificity of high molecular weight glutenin promoter subunit Dy10, which can be used as an important tool to drive the expression of any sucrose and starch related genes specifically in wheat grains to further our understanding of carbon partitioning and/or increase wheat starch yield.

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1.0 INTRODUCTION

Concerns about climate change due to global warming, depletion of fossil fuels and high fuel prices has led to a search for a cost effective and sustainable alternative to fossil fuels such as biofuels. Bioethanol is one form of liquid biofuels obtained by fermenting plant sugars and can be used as a transportation fuel. Various biological materials can be used for bioethanol production. Cereal grains with high starch content such as those from corn and wheat have become potential resources for producing bioethanol. For example, U.S.A. which ranks first in bioethanol production with a capacity of 40 billion litres (L), produces most of its bioethanol from corn (Licht, 2010) whereas Brazil, the second largest producer with a production capacity of approximately 25 billion L in 2009, produces it mainly from sugarcane. Wheat is the major feedstock for bioethanol in western Canada and Europe (Carver, 2009).

Canada is the sixth largest wheat producing country in the world with an average production of 24 million metric tonnes (MT) of wheat grains, according to Canadian Wheat Board (CWB) 2009 report. About 67% (16 million MT) of wheat is exported to foreign countries and about 29% (7 million MT) remained as year ending carry over stock in 2009. As of 2010, Canada has 15 bioethanol processing plants in operation, producing 1.82 billion L of bioethanol, and 20% of the production comes from wheat, as reported by the Canadian Renewable Fuels Association (CRFA). The mandatory regulation of 5% bioethanol use in transport fuels from 2010 onwards has increased the demand for wheat grains to be used as a feedstock for ethanol production (CRFA, 2011). Increasing wheat cropping area for the purpose of bioethanol will have a negative effect on the production of wheat grains for food and feed. Therefore, it is necessary to increase starch content per grain which will increase

bioethanol production and minimize the land use competition for food and fuel. One approach to improve starch yield in wheat is genetic manipulation of the starch biosynthetic pathway, and this requires a detailed understanding of starch formation in wheat.

Starch formation in wheat grain depends on rate of assimilate supply during grain development and its subsequent conversion to hexoses for starch biosynthesis (Emes et al., 2003). Photosynthesizing source cells produce triose phosphates as the end product of photosynthesis, which is either used for starch synthesis in the chloroplast or transported to the cytoplasm for sucrose synthesis (Flugge, 1999). Sucrose is the transportable form of photoassimilate in most crops including wheat. The transport of sucrose occurs through the phloem. The process of sucrose entry into the phloem (phloem loading) and its delivery into the sink cells (phloem unloading) involves membrane transporter proteins called sucrose transporters (SUTs). Transport of sucrose inside phloem cells occurs through plasmodesmata by mass flow of water from adjacent xylem cells and the process is called symplastic transport (Slewinski and Braun, 2010). In wheat grains, developing endosperm is separated from maternal cells by a thin layer of cells called transfer cells. Sucrose entering grain cells are released into the cell wall space (apoplasm), and are taken up by sucrose transporters present on the plasma membrane of transfer cells into the endosperm and this process is called apoplastic transport (Wang et al., 1995).

Genes encoding sucrose transporter proteins have been isolated in many dicot and monocot species. The *SUT1* genes of rice, maize and barley have been reported to play an important role in transporting sucrose to the developing endosperm (Kuhn, 2003). In wheat, only one *SUT* has been identified thus far, and it has three homeologous genes in the A, B and D genomes of the hexaploid wheat, *TaSUT1A*, *TaSUT1B*, and *TaSUT1D* respectively. It

has been reported that the expression of *TaSUT1* increases between 8-20 days after anthesis (DAA), suggesting its important role in transporting sucrose to developing grains (Aoki et al., 2002). Its expression in the source tissues such as leaf blades and internodes, also suggest its role in phloem loading (Aoki et al., 2004).

Sucrose transport is accompanied by sucrose hydrolysis into hexose sugars by sucrose synthases (SuSy) and invertases (INV), and the resulting hexoses serve as the major substrate for starch synthesis (Emes et al., 2003). Sucrose synthase activity predominates throughout grain development whereas INV activity decreased after anthesis which suggests the temporal specific activity of these two enzymes (Riffkin et al., 1995). Genes encoding SuSys and INVs have been isolated from a number of crops including rice, maize, barley and wheat (Chourey et al., 1998). Sucrose synthases of maize, ZmSH1 and ZmSuSy1 (Carlson et al., 2002), OsSuSy3 in rice (Wang et al., 1999), HVSuSy2 in barley (Guerin and Carbonero, 1997) are highly involved in hydrolyzing sucrose to glucose and fructose mainly for starch synthesis. The expressions of genes encoding these enzymes were found to be high in developing endosperms between 4 and 20 DAA.

To date two sucrose synthase genes, *TaSuSy1* and *TaSuSy2*, have been reported in wheat. The expression of *TaSuSy2* is specific to developing grains and is associated with thousand grain weight, whereas that of *TaSuSy1* was observed in leaves and roots under anaerobic conditions (Marana et al., 1990). Wheat grain dry weight is composed of carbohydrates (65% to 75% starch; Dai et al., 2009) and proteins (7% to 22%; Carver, 2009). It has been suggested by Dale and Housley (1986) that SuSy activity contributes to dry matter accumulation, and variation in kernel weight among cultivars. Therefore, difference in SuSy activity affects starch content of wheat grains (Jiang et al., 2011). Cell-wall invertase of

wheat, *TaCWINV1* is involved in sucrose hydrolysis during early stages of seed development, as the enzyme activity was found to be high until 4 DAA, and decreased afterwards (Koonjul et al., 2005). Water stress during meiosis reduces invertase activity and results in sterile pollen, indicating its importance during seed development (Dorion et al., 1996). Most of the studies in wheat have been focused on enzymes involved in the starch biosynthesis pathway, with only little information available on the role of sucrose transport and its metabolism during grain development.

The first part of this study investigates the expression pattern of two wheat sucrose transporter genes (*TaSUT1* and *TaSUT2*) and two sucrose synthase genes (*TaSuSy1* and *TaSuSy2*) at five stages of seed development (4, 8, 16, 25 and 30 DAA) in five spring wheat cultivars that exhibited marked variation in seed size and weight, Chinese Spring (CS), Yecora Rojo (YR), CDC Teal (CDC), White Bird (WB), and AC Andrew (ACA). The relationship between the expression of the genes studied and dry matter accumulation has been examined.

Transport of sucrose during grain development is the most important step for starch biosynthesis. The downstream process of sucrose metabolism and starch synthesis depends on sucrose supply. Various studies on sucrose transporter proteins indicate that SUTs plays a vital role in sucrose transport during seed development. Sucrose uptake capacity of sucrose transporter proteins involves two components, high affinity/low capacity and low affinity/high capacity (Ayre, 2011). High affinity transporters saturate at lower sucrose concentration resulting in a higher transport rate than low affinity transporters (Lin et al., 1984). It has been suggested by Weschke et al. (2000) that sucrose uptake capacity of wheat grains can be increased by expressing high affinity transporter system. Over-expression of a

barley *SUT1* gene, *HvSUT1* in wheat grains enhanced sucrose uptake capacity of seeds by 30% to 40% (Weichert et al., 2010). Moreover, over-expression of high affinity potato sucrose transporter gene (*StSUT1*) in pea has been shown to increase sucrose uptake by pea seeds (Rosche et al., 2002). Therefore, we proposed that expression of the high affinity sucrose transporter (*StSUT1*) specifically in wheat seeds will increase their sucrose uptake capacity and thereby starch accumulation.

The second part of this study analyzed the tissue specificity of the high molecular weight glutenin promoter subunit Dy10 and used this promoter for preparing construct**s** to express *StSUT1* in wheat grains. Moreover, another construct that involved the use of maize ubiquitin 1 promoter was prepared to express *StSUT1* constitutively in wheat, which can be used as a tool to study carbon allocation between source and sink tissues.

2.0 LITERATURE REVIEW

2.1 Bioethanol

2.1.1 World bioethanol production

Increased amount of greenhouse gases (GHGs) in the atmosphere has led to drastic climate change known as global warming. Combustion of coal, natural gas and petrol in industries and transport vehicles lead to emission of carbon dioxide (CO_2), one of the GHGs. It has been indicated in the Natural Resources Canada (NRC) 2004 report that about 27% of GHG emissions come from the transportation sector and it is expected to reach 40% in 2020 due to increased use of private transport vehicles. This concern, along with the depletion of fossil fuel resources and ever increasing demand for fuels, creates the necessity to find a suitable alternative such as biofuels. Bioethanol is generated by fermenting plant based sugars, to produce ethanol which can be used as liquid fuel for transportation. Blending of bioethanol with fossil fuels plays an important role in reducing GHG emission. The U.S.A, Brazil, members of the European Union, Canada and China are among the leading countries that blend bioethanol with gasoline for use in transportation vehicles to reduce GHG emissions. In Europe and Canada, wheat is used as the major feedstock for bioethanol production (Smith et al., 2006).

2.1.2 Bioethanol production in Canada

In 2007, annual bioethanol production in Canada was 798 million L which increased to 1.8 billion L in 2010 (CRFA, 2010). The provinces of Ontario and Quebec, mainly use corn as the feedstock, and contribute 73% of the total bioethanol produced in Canada (1.3 billion L/year). Western provinces, mainly Saskatchewan and Manitoba, produce 20% of the total

bioethanol produced in Canada using wheat as the main feedstock (CRFA, 2010). Husky Energy Inc., an integrated energy and energy related company based in Calgary is the largest producer of wheat based bioethanol in western Canada. It requires 7 million metric tonnes (MT) of wheat grains annually to produce 260 million L of ethanol (Husky Energy Inc., 2009). Wheat such as the Canadian Western Soft White Spring (CWSWS), which is characterized by high starch/low protein, is used for bioethanol production. In order to cut GHG emissions, the Canadian government has set a mandatory use of 5% ethanol in gasoline from 2010 onwards. This will require 2.2 billion L of annual bioethanol production, which is 40% more than the current level of production (Ackom, 2010).

2.1.3 Bioethanol production from wheat

Wheat can be used for a wide range of products such as food, feed and bioproducts. Some form of wheat flour is found in almost every meal and is used for making a variety of food all over the world including flat bread, pan bread, noodles, pasta, cookies, cakes, pastries, breakfast cereals and thickening agents (Carver, 2009). The by-products of the flour milling industry are used as livestock and poultry feed, as it has a unique functional protein property and is cost effective. Wheat straw is used for livestock bedding. The green forage may be grazed by livestock or used as hay or silage. In many areas, wheat is grown for dual purpose by being grazed in the fall and early spring and then harvested as a grain crop (Carver, 2009). Starch and gluten from wheat provide raw materials for the manufacturing of paper, paste, oil, adhesives, and feed for aquaculture and pets. A recent industrial application of wheat is its utilization for bioethanol production. The amount of starch in wheat grain is the most important criterion for bioethanol yield (Kindred et al., 2008; Zhao et al., 2009). Conversion

of grain starch into ethanol involves milling, cooking, fermenting, distilling and dehydration (Husky Energy Inc., 2009). Bioethanol production through dry milling method involves breaking wheat grains into fine particles before cooking (Dale and Tyners, 2006). Cooking involves steaming and subsequent liquefaction with thermostable α-amylase and amylo glucosidase to breakdown the starch. Steaming enables disintegration of the crystalline granular structure and makes it more susceptible to enzymatic degradation, a process often called gelatinization. Immediately after liquefaction, the slurry is subjected to saccharification and fermentation (Das Neves et al., 2006). Saccharification involves conversion of oligomers into glucose units by α-amylase, and fermentation involves conversion of glucose to ethanol by yeast (*Sachromyces cerevisiae*; Das Neves et al., 2006). The fermented product is purified by distillation, condensed and then passed through a dehydration system to remove all water molecules and produce pure ethanol. The resulting ethanol is treated or "denatured" by adding a small amount of gasoline to turn it into fuel grade ethanol (Cardona and Sanchez, 2007).

2.2 Wheat

Wheat (*Triticum aestivum* L) is one of the most important food crops grown worldwide as it serves major portion of carbohydrates to human diet. Wheat grain consists of 65% to 75% starch and 7% to 22% storage protein, making it superior than other cereal crops (Dai et al., 2009; Carver, 2009). Wheat accounts for 30% of the world's total cereal production. For example, in 2008, global wheat production was 685 million MT and it was ranked as the third most cultivated cereal crop, next to rice and maize. According to the Food and Agriculture organization (FAO) 2008 report, wheat stands as the second cereal crop with the

most market value, preceded only by rice. Demand for wheat is increasing every year due to rapid population growth; therefore it is essential to increase wheat productivity. Yield improvement in wheat can be achieved by employing biotechnological approaches.

2.2.1. Wheat origin and distribution

The origin of cultivated wheat dates back to 9500 B.C. Archaeological remains of wheat grains were found at various sites in the fertile crescent of Tigers and Euphrates rivers, now known as Turkey and Syria (Lev-Yadun et al., 2000). Earlier in 19th century, landraces existed as heterogenous gene pools. The wild relatives (diploids and tetraploids) of cultivated wheat species are derived later from landraces by continuous selection. In those days, non-shattering, free threshing, non-brittle rachis and hull-less spikes were the main characteristics used for selection towards higher yield (Carver, 2009). Modern selection and breeding techniques for increased yield and enhanced quality traits resulted in the development of genetically uniform cultivars, which displaced heterogeneous land-races and thereby narrowed the genetic base. However, a large number of traits including high protein content, high yield, genes conferring resistance to different types of biotic and abiotic stresses, are still available in wheat germplasm (Brown et al., 1990) and are being used as a resource in wheat breeding programs for improving wheat yield. Wheat is mainly cultivated between 25°-60° N and 25°-45° S latitude, including southern Russia, the central plains of U.S.A., southern Canada, northern China, India, Argentina and Australia (Sleper and Poehlman, 2006).

2.2.2 Wheat genome

Cultivated wheats are derived from a basic chromosome number of n=1x=7 and are classified into diploids, 2n=2x=14, AA (einkorn), tetraploids, 2n=4x=28, AABB (emmer, durum wheat), hexaploid, 2n=6x=42, AABBDD (bread wheat). The wild diploid wheat *Triticum urartu* is the donor of A genome in all polyploid wheat spieces (Dvorak et al., 1998a). The tetraploid durum wheat, *Triticum turgidum,* is formed by hybridization between the wild progenitors of A and B genomes. The wild relative *Aegilops speltoides* is proposed to be one of the putative B genome donors*;* however the origin of the B genome still remains unclear (Belyayev et al., 2000). The hexaploid wheat *Triticum aestivum* is derived by hybridization between the (AABB) tetraploid as a female donor and the diploid *Aegilops tauschii* (DD) as a male donor (Dvorak et al., 1998b). Bread wheat and durum wheat have a wide range of applications in the food and feed industries.

2.2.3 Wheat production in Canada

Canada is one of the major wheat producing countries in the world, and is well known for its high quality wheat in the world market. Wheat production in Canada exceeds the domestic consumption. According to the CWB's 2009 report, Canada exports 80% (18 MT ± 3.23) of its production, which accounts for 5% of world's wheat production and 20% of the international wheat trade (Bonjean and Angus, 2001). In North America, wheat is grown in two seasons, spring and winter. Winter wheat is planted in the fall, and harvested in the spring or summer. It accounts for 70% to 80% of the wheat production in the U.S.A. but considerably less (6%) in Canada due to the harsher winters, according to United States Department of Agriculture (USDA) 2004 report. Spring wheat, is planted in the spring and

harvested in late summer or early fall and accounts for 75% of Canadian wheat production, and the remaining 19% accounts for durum spring wheat (USDA, 2004).

Wheat is classified as eastern Canadian and western Canadian wheat classes. The Canadian Grain Commission follows strict standards in grading wheat grains. Canada Western Red Spring (CWRS) is the largest exported class of wheat with 15 million MT productions per year (Carver, 2009). From the total wheat production, 7 to 8 million MT is used domestically for different purposes: 43% for milling and baking, 45% for animal feed, 11% as seed material, and 2% for industrial uses, as reported by Agriculture and Agri-Food Canada (AAFC; 2010). In recent years, the biofuels industry in the Prairie Provinces has been using wheat as the primary feedstock for bioethanol production (Olar et al., 2004).

2.3 Wheat starch

2.3.1 Starch and sucrose synthesis in source tissues

Plants capture sunlight and converts it to chemical form of energy, namely adenosine- tri-phosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH), through a process called the light reaction. The next stage of photosynthesis, the Calvin cycle, involves the use of ATP and NADPH to fix and reduce the atmospheric CO_2 to form three carbon compound, glyceraldehyde-3-phosphate. The assimilated carbon in the form of triose phosphate, is allocated either to the chloroplast for transitory starch synthesis, or to the cytoplasm for sucrose synthesis (Schulz et al., 1993). Triose phosphate translocator, a chloroplast membrane protein, acts as antiporter and facilitates the transfer of triose phosphate from the chloroplast to the cytosol in a counter-exchange for inorganic phosphate (Flugge, 1999). Most of the triose phosphates transported from the chloroplast are converted

to sucrose in the cytosol of mesophyll cells (Raven et al., 2004). Fructose-1, 6-bisphosphatase and sucrose phosphate synthase are the key enzymes for sucrose synthesis in the cytosol. Fructose-1,6-bisphosphatase catalyzes the conversion of fructose-1,6-bis phosphate produced during the Calvin cycle, into fructose-6 phosphate, whereas sucrose phosphate synthase catalyzes the conversion of UDP-glucose and fructose-6 phosphate to sucrose (Huber and Huber, 1996). Sucrose carries the major part of energy fixed by photosynthesis and distributes it to various sink tissues. Starch formation in wheat is a function of several physiological processes, involving photosynthesis, transport of photoassimilates to the developing grain and its efficient utilization to form starch in the grain. Therefore, a detailed knowledge of these processes is essential for understanding starch synthesis and accumulation in wheat grains.

2.3.2 Starch synthesis in wheat grains

Wheat grain is formed by double fertilization, which involves the fusion of two male nuclei; one fuses with the egg to form the diploid zygote that develops into an embryo and the other fuses with the two polar nuclei to form triploid nuclei that develops into the endosperm (Emes et al., 2003). During the reproductive phase, the majority of sucrose synthesized in leaves is ascribed to starch formation in the endosperm of wheat (Dale and Housley, 1986). To this effect, sucrose is transported through phloem cells to the developing grains. Sucrose entering the developing endosperm is converted by sucrose synthase into UDP- glucose and fructose. These resulting hexoses are transported to the amyloplast (starch storing plastid) through hexose/H^+ antiporter protein and converted to ADP glucose by ADP glucose pyrophosphorylase (Kammerer et al., 1998). ADP glucose serves as substrate for starch

synthesis by starch synthases, and starch branching and de-branching enzymes, that are located in plastids (Smith et al., 1997; Smith, 1999).

2.3.3 Strategies to increase wheat starch

The biosynthesis of starch in wheat is the major determinant of yield, not only for food, but for several industrial applications such as bioethanol production. Starch accumulation in grain is influenced by a number of factors including, rate of sucrose supply during grain filling, ATP supply, and activity of sucrose hydrolyzing enzymes, AGPase, starch synthases and starch branching and de-branching enzymes. Plant biologists tend to increase starch yield by manipulating the starch biosynthetic pathway (Smith, 2008). AGPase is the key enzyme for conversion of hexose phosphate to ADP-glucose, which is the first step in starch synthesis (Ballicora et al., 2004). Attempts to increase the activity of AGPase in maize, rice and wheat plants resulted in a yield increase only under controlled conditions, but the same type of result was not observed under field conditions (Giroux et al., 1996; Smidansky et al., 2003; Meyer et al., 2007). In another study, over-expression of the single isoform of glycogen synthase, which catalyses the conversion of ADP-glucose to starch in *E. coli*, resulted in decreased starch content (Smith, 2008). Starch synthases have multiple isoforms with complementary function, thus any loss or change in the activity of one isoform results in altered starch structure (Shewmaker et al., 1994). Adenosine-tri-phosphate supplies phosphate for the conversion of glucose-1-phosphate to ADP-glucose, the substrate for starch synthesis and is reported to be one of the major factors influencing starch biosynthesis (Regierer et al., 2002). For example, increasing the supply of ATP, through manipulation of adenylate transporter activity in potato, resulted in 16% to 30% more starch per gram fresh

weight (Tjaden et al., 1998; Geigenberger et al., 2001). However, in cereal grains, there is no such definitive evidence for the role of ATP in controlling starch synthesis (Smith, 2008). It is important to understand the fate of transported assimilates in starch storing sink tissues. A comparative analysis of sink strength of tubers and source strength of leaves in potato indicated that altering the sink capacity to metabolize sucrose resulted in an increase in the number of tubers but created competition for assimilate supply between tubers (Muller-Rober et al., 1992; Sonnewald et al., 1997). This resulted in relatively little or no impact on tuber weight (Smith, 2008). Therefore, it is essential to study the factors that controls the rate of assimilate transfer from the synthesizing tissues to the storage organs.

2.4 Source-sink relationship

Higher plants developed specific tissues for production, utilization and storage of photosynthates. According to its function, plant organs are basically classified into two types: source and sink tissues. Source tissues such as mature leaves synthesize photo- assimilates, and export them to sink organs. Sink tissues such as young leaves, roots, flowers and developing grains import photosynthates for growth and maintenance. Appropriate partitioning of photoassimilates from source to sink is necessary for sustainable growth and development. The distribution of photoassimilates takes place through a specialized network of cells collectively called phloem (Ishimaru et al., 2001; Emes et al., 2003). Assimilate transport is a complex process involving several cell membranes connecting source and sink organs. As sucrose is the main transportable sugar, understanding sucrose transport will have a significant contribution to our knowledge on the source-sink relationship, which is an important determinant of crop yield.

2.4.1 Sucrose transport during seed development

Sucrose transport in higher plants takes place through phloem which consists of sieve elements (SE), companion cells (CC) and phloem parenchyma cells. During its maturation, the SE undergoes selective autophagy and loses essential organelles including the nucleus (Lemoine, 2000). The cells are arranged adjacent to each other forming sieve tubes, and the plasmodesmata at the poles widen to form sieve pores to facilitate movement of solutes through it (Lemoine, 2000). Sieve elements are interconnected with companion cells through plasmodesmata and form SE-CC complex. Companion cells contain all the cellular components necessary for further divisions and differentiation, and also supply proteins, and energy to SEs (Williams et al., 2000). The higher sucrose concentration at the source leaves and lower sucrose levels at the sink tissues create a concentration gradient, as a result water moves from adjacent xylem cells into phloem and assists sucrose movement by mass flow (Williams et al., 2000). Phloem transport involves loading and unloading mechanism. Phloem loading takes place at source leaves during which the sucrose formed in mesophyll cells is delivered to the SE-CC complex, whereas unloading takes place at sink tissues. Two ways of phloem loading are reported in plants: symplastic and apoplastic (Lemoine, 2000). In the case of symplastic loading, sucrose moves to the SE-CC complex through plasmodesmata. Apoplastic loading involves the release of sucrose to the apoplast (cell space) and its transport across the membrane by sucrose transporter (SUT) proteins located on plasma membranes of SE-CC complex. The mode of sucrose transport in plants depends on the number of plasmodesmatal connections between mesophyll and phloem cells, which varies between species (Turgeon, 1996). For example, cucurbits have multiple plasmodesmatal connections between SE-CC complex and mesophyll cells, and this promotes symplastic

sucrose transport (Riesmeier et al., 1994; Truernit, 2001). In contrast, solanaceous plants such as potato and tomato have less plasmodesmatal connections between mesophyll and phloem cells, thus sucrose transport involves SUTs (Riesmeier et al., 1994).

2.4.2 Sucrose metabolism during seed development

Carbohydrate enters grain in the form of sucrose. In wheat or barley, sucrose is unloaded at the crease phloem to the endosperm cavity through plasmodesmata. Subsequent transfer into the endosperm occurs through transfer cells with the help of membrane proteins called sucrose transporters (Weschke et al., 2000). The fate of sucrose in the cells of developing grain has been investigated in a number of cereal crops including wheat, barley, maize and rice (Marana et al., 1990; Sanchez de la Hoz et al., 1992; Carlson et al., 2002; Hirose et al., 2008). According to Huber and Akazawa (1986) there are two distinct pathways for sucrose degradation. The one mediated by sucrose synthase (SuSy) and the other one by invertase (INV). Sucrose synthase catalyzes the bi-directional conversion of sucrose and UDP into UDP-glucose and fructose and vice-versa, whereas INV catalyzes uni-directional hydrolysis of sucrose into glucose and fructose. The sucrose degradation pathway varies with species and tissues. For example, SuSy exhibited high activity during the early to mid stages of barley grain development, 5-21 DAA (Weschke et al., 2000). In maize, INV showed high activity during early kernel development (Shannon, 1972). In general, INV mediated sucrose degradation is predominant during pre-anthesis and 1-4 DAA anthesis, whereas sucrose synthase activity predominates after 4 DAA (Sung et al., 1994).

2.5 Genes involved in sucrose transport and metabolism

Phloem loading and unloading of sucrose, and its subsequent conversion to hexose phosphates are important processes for starch synthesis in developing grains (Emes et al., 2003). Sucrose transporter proteins play an important role in transporting sucrose from the source to the sink tissues. Genes encoding SUTs have been isolated and characterized in many dicot and monocot species including wheat (Kuhn, 1999; Aoki et al., 2002). Similarly, genes encoding sucrose hydrolyzing enzymes SuSy and INV have been identified in a number of plant species including rice, maize, barley and wheat (Cho et al., 2005; Hirose et al., 2008). Functional analysis of these genes is of paramount significance in understanding the roles of assimilate transport and sucrose metabolism during seed development.

2.5.1 Sucrose transporters

2.5.1.1 Classification

A gene encoding SUT was first isolated and characterized in spinach, designated as *SoSUT1* (Reismeier at al., 1992). Heterologous screening of cDNA or genomic libraries using spinach and potato *SUT*s as probes led to the identification of several other *SUT* genes from various dicot and monocot species (Kuhn et al., 1999). The number and type of SUTs vary with species. Phylogenetic analysis of all plant SUTs available in public databases, classified them into four types (Sauer, 2007): type I contains monocot SUTs, type II contains dicot SUTs, types III and IV contain both monocot and dicot SUTs. Genes encoding type I SUTs have been isolated and functionally characterized from a number of crop species including barley (*HvSUT1* and *HvSUT2*; Weschke et al., 2000), rice (*OsSUT1*; Hirose et al., 1997), sugarcane (*ShSUT1*; Reinders et al., 2006) and maize (*ZmSUT1*; Carpaneto et al., 2005). All type I

SUTs are involved in phloem loading and unloading of sucrose (Kuhn, 2003). Genes encoding type II SUTs have been isolated from dicot crop plants including spinach (*SoSUT1*; Riesmeier et al., 1992), potato (*StSUT1*; Boorer et al., 1996), tobacco (*NtSUT1*; Burkle et al., 1998) and Arabidopsis (*AtSUT1*; Sauer and Stolz, 1994, (*AtSUT5*; Baud et al., 2004). These type II SUTs are reported to be involved in phloem unloading of sucrose for seed development and have high affinity for sucrose, with Km values ranging from 1.5 to 11 mM (Kuhn, 2003). Cellular localization studies indicated the presence of type I and II SUTs in CCs of developing seeds, indicating their role in phloem unloading (Aoki et al., 2002; Scofield et al., 2007). In some plants, duplication of genes encoding type I and II proteins has been reported. The homeologous copies of SUT1 in wheat (*TaSUT1A, TaSUT1B,* and *TaSUT1D*) are examples of gene duplication (Aoki et al., 2002). Genes encoding type III SUTs have been identified from a number of crop species including rice (*OsSUT2*; Aoki et al., 2003), Arabidopsis (*AtSUT3*; Meyer et al., 2004) and tomato (*LeSUT2*; Hackel et al., 2006). These SUTs have longer (approximately 15% to 20% more) amino acid sequences than the other two types, and are reported to act as sucrose sensors. Type III SUTs are mainly localized in the SEs of sink tissues, indicating their role in phloem unloading (Hackel et al., 2006). Genes encoding type IV SUTs have been cloned from several plant species including Arabidopsis (*AtSUT4*), potato (*StSUT4*) and tomato (*LeSUT4*; Weise et al., 2000). Type IV SUTs are localized in the minor veins of SEs and tonoplast membrane of sink tissues, suggesting their role of sucrose transport in vacuoles (Endler et al., 2006).

2.5.1.2 Structure and function

All SUTs encode hydrophobic proteins which contain 12 transmembrane spanning segments connected by a central loop (Riesmeier et al., 1992; Sauer and Stolz, 1994). The transmembrane region is highly conserved among various SUTs, with variations present in the amino (N) and carboxyl (C) termini. Immunolocalization studies with site specific antibodies confirmed the presence of N and C termini on the cytoplasmic side of the membrane (Stolz et al., 1999). Sequence alignment of all available plant sucrose transporters with first and second halves of other sucrose symporter proteins showed similarity in conserved region (Marger and Saier, 1993). Comparision of 2-dimentional models of all types of SUTs showed differences in the N and C terminal domains. Variations are also observed in the central cytoplasmic loop (VI and VII helices) or in the loop facing extracellular space of the plasma membrane (Sauer, 2007). Type III SUTs have additional amino acid sequences in the N terminus and cytoplasmic loop region, but have a C terminus shorter than that in type I and type II transporters. Type IV transporters that are involved in vacuolar transport of sucrose have a short C terminal end. The linker sequence between VII and VIII helices of type IV transporters have a shorter amino acid sequence than that in any other types of SUTs. Histidine residues have been reported to be involved in sucrose binding (Lu and Bush, 1998) and are conserved across all functional SUTs identified so far.

Sucrose transporter is coupled with proton transport in a 1:1 ratio (Boorer et al., 1996). The H^+ ions are released in the extracellular space by proton pumps during ATP hydrolysis and this activates the plasma membrane localized sucrose transporter proteins. A proton molecule binds to the external surface of SUT protein, which is followed by a binding of a sucrose molecule. This creates a conformational change in the SUT protein; as a result

sucrose is released into the cytoplasm against a concentration gradient using a proton motive force. The ligand binding site opens again in the external surface allowing the next proton and sucrose to pass through the membrane (Boorer et al., 1996).

2.5.1.3 Cereal sucrose transporters

The deposition of starch in cereal grains depends partly on the supply of photoassimilates from source tissues in the form of sucrose. Sucrose transporter proteins play a vital role with this regard. Genes encoding SUTs with distinct functions have been isolated from a number of cereal crops including rice, barley, maize and wheat. Five SUTs have been reported in rice, and each has a distinct function. For example *OsSUT1,* encodes a SUT protein that is involved not only in transporting sucrose to developing grains, but also in remobilizing sucrose from starch reserves in leaf sheaths and germinating seeds (Matsukura et al., 2000; Furbank et al., 2001; Hirose et al., 2002). Antisense suppression of *OsSUT1*, for example, retarded the germination of rice seeds (Scofield et al., 2002). Unlike that of *OsSUT1*, the expression patterns of *OsSUT2* have been shown to be uniform in various tissues, suggesting its housekeeping role (Aoki et al., 2003). The remaining rice *SUT* genes *OsSUT3*, *OsSUT4*, and *OsSUT5* are highly expressed in mature leaves, demonstrating their role in sucrose loading (Aoki et al., 2003). The role of cereal SUTs during grain development is also well examined in other cereal crops. Two *SUT* genes, *HvSUT1* and *HvSUT2*, have been isolated and characterized in barley. An in-situ hybridization study has indicated the localization of *HvSUT1* mainly in the endospermal transfer layer and gene expression studies showed high transcript abundance of *HvSUT1* at 5-7 DAA, coinciding with high sucrose levels in developing grains (Weschke et al., 2000). This result suggests the role of *HvSUT1* in

transporting sucrose to developing endosperm. Similar to that of *OsSUT2*, the other *SUT* gene of barley (*HvSUT2*) has been shown to be expressed uniformly across all the tissues, indicating its housekeeping role (Weschke et al., 2000). A single sucrose transporter protein can also carry out the loading and unloading functions. For example, *SUT1* of maize (*ZmSUT1*) plays two distinct roles in loading sucrose from source cells such as leaf blades and germinating seeds into phloem and unloading sucrose into sink cells such as pedicels (Aoki et al., 1999).

2.5.1.4 Wheat sucrose transporters

Thus far, only one *SUT* has been isolated from wheat (*TaSUT1*). This gene is located on chromosome 4 of hexaploid wheat, and has three homeologous genes, *TaSUT1A*, *TaSUT1B*, *TaSUT1D* (Aoki et al., 2002). The nucleotide sequences of these three homeologous genes showed more than 96% homology to each other with a few base polymorphisms in the 3' untranslated region. Their amino acid sequences showed 98% similarity, and also have high similarity with *SUT*s isolated from other cereal crops such as rice, barley and maize. Expression analysis of *TaSUT1* indicated high expression of this gene in source tissues including the flag leaf sheath, flag leaf blade, and stem at 4 days before heading (Aoki et al., 2002). However, its expression in the stem decreased by 12 days after heading. The transcripts of *TaSUT1* were observed in CCs, and immunolocalization study showed the presence of SUT1 protein on the plasma membrane of SEs in leaves (Aoki et al., 2004). This is clear evidence for active phloem loading of sucrose in wheat leaves and the involvement of SUT1 in this process. Cellular localization studies indicated high accumulation of *TaSUT1* transcripts on the plasma membrane of the scutellum at 3 DAA (Aoki et al., 2006). This is in

agreement with the role of *TaSUT1* in the uptake of sucrose from the endosperm cavity into the transfer cells of the embryo (Aoki et al., 2002). In germinating wheat seeds, starch in the endosperm is hydrolyzed to glucose and maltose (Bewley and Black, 1994) and the resulting glucose is actively loaded by hexose transporters into the scutellum, where it is used for sucrose synthesis by sucrose phosphate synthase (Aoki et al., 2006). Sucrose is then loaded into the phloem cells of newly emerging shoots and roots by SUTs. Although expression and localization studies suggest that SUT1 is involved in phloem loading and unloading of sucrose in wheat, functional studies such as gene silencing/over-expression of *TaSUT1* may be useful for determining its definitive physiological role.

2.5.2 Sucrose synthase

2.5.2.1 Structure and classification

Sucrose synthase (SuSy) catalyzes bi-directional conversion of sucrose and UDP into UDP-glucose and fructose. The direction of this reaction depends on the cytosolic pH. Sucrose synthase cleaves sucrose at acidic pH of 6 to 6.5, whereas sucrose synthesis occurs at alkaline pH of 8 to 8.8 (Hardin et al., 2004). In Arabidopsis, SuSy is encoded by a multigene family. Six putative genes have been reported and all shared two common domains: sucrose synthase and glycosyl-transferase domains that are involved in the catalytic property of the enzyme (Baud et al., 2004). These conserved domains are also found in *SuSy* genes cloned from all other species. Based on amino acid sequence similarity, Komatsu et al (2002) classified plant SuSy into four groups: dicot SuSy1, dicot SuSy2, monocot SuSy1, monocot SuSy2. The exon/intron structure within each group shows high similarity across all SuSy genes identified thus far. Functional characterization of almost all of the identified members

of the dicot SuSy2 group has shown that SuSy proteins in this group are involved in sucrose degradation and provide hexoses for starch synthesis in the sink cells (Hirose et al., 2008).

2.5.2.2 Function

Sucrose synthase plays a major role in energy metabolism. It controls the mobilization and utilization of sucrose in various sink tissues, thereby connecting the metabolic, structural and storage functions of the plant cell (Hesse and Willmitzer, 1996; Sturm and Tang, 1999). The activity of SuSy enzyme has been studied in various plant species including pea (Dejardin et al., 1997), sunflower (Pfeiffer et al., 1995), cotton (Ruan et al., 2003), potato (Zrenner et al., 1995), Arabidopsis (Baud et al., 2004), rice (Hirose et al., 2008), maize (Carlson et al., 2002), barley (Guerin and Carbonero, 1997) and wheat (Marana et al., 1988). Higher plants contain multiple genes for SuSy and each member of the family has a distinct function. The SuSy enzymes associated with vascular tissues such as those in red beet and maize hydrolyze sucrose for energy generation during germination (Hanggi and Fleming, 2001). Some SuSy enzymes of cotton are involved in cell wall biosynthesis along with cellulose synthase (Haigler et al., 2001). Few *SuSy* genes in cereal crops (rice, wheat, maize and barley) are reported to be involved in determining sink strength by supplying assimilates for starch synthesis (Zrenner et al., 1995).

2.5.2.3 Cereal sucrose synthases

Sucrose synthases are important in providing hexoses to the developing endosperm in cereal crops. This functionality of *SuSy* genes has been reported in rice (Hirose et al., 2008), maize (Carlson et al., 2002), barley (Guerin and Carbonero, 1997) and wheat (Kunjool et al., 2005).

Members of *SuSy* gene family in rice consists of six genes, (*OsSuSy1, OsSuSy2, OsSuSy3, OsSuSy4, OsSuSy5 and OsSuSy6*; Hirose et al., 2008). Spatio-temporal expression pattern of these genes indicated that *OsSuSy1* and *OsSuSy2* are expressed mainly in elongating cells of roots, leaf blades and uppermost internodes with very low expression detected in mature tissues. The expressions of *OsSuSy3* and *OsSuSy4* were reported to be specific in the panicles. As compared to the other members, *OsSuSy5* and *OsSuSy6* are expressed in all tissues at low level (Hirose et al., 2008).

Three different *SuSy* genes have been identified in maize (*ZmSuSy1, ZmSuSy2* designated as *ZmSH1, ZmSuSy3*). These genes were shown to have distinct but complementary functions (Carlson et al., 2002). A mutation in the *sh1* gene, which encodes type II sucrose synthase, resulted in 2% to 6% decrease in sucrose synthase activity that led to a 60% reduction in kernel starch and produced shrunken seeds (Werr et al., 1985; Chourey et al., 1998). It indicates the importance of type II SuSy during seed development in maize (Chourey et al., 1998). Further analysis of the double mutant *sh1susy1-1* that lacks functional SuSy of both types, indicated the presence of a third SuSy gene (*ZmSuSy3*) which has a house keeping role (Carlson et al., 2002). Genes encoding SuSy proteins have also been identified and characterized in barley (*HvSuSy1* and *HvSuSy2*; Guerin and Carbonero, 1997). These two barley genes are located on chromosomes 7HS and 2HS respectively (Sanchez de la Hoz et al., 1992). In contrast, the maize SS2 and SH1 loci correspond to *ZmSuSy1* and *ZmSuSy2*, respectively and are linked and associated with chromosome 9, possibly because of the intra chromosomal duplication that occurred prior to branching out to wheat, barley and maize (McCarty et al., 1986). Immunohistochemical analyses in barley tissues showed that localization of SuSy1 and SuSy2 proteins in the vascular region and root cap cells,

whereas that of *SuSy1* protein specifically in the nucellar projection, chalazal, vascular bundles and at the base of the endosperm region, indicating its key role in supplying hexose to the developing endosperm (Guerin and Carbonero, 1997). Therefore, in cereals, at least one isoform of SuSy is involved in supplying substrate for starch biosynthesis in developing grains.

2.5.2.4 Wheat sucrose synthase

In wheat, two types of linked non allelic sucrose synthase genes (*TaSuSy1* and *TaSuSy2*) have been reported (Marana et al., 1988). Recently, *TaSuSy2* gene was mapped on chromosomes 2A, 2B and 2D, and contains two haplotypes (Hap-L and Hap-H) for *TaSuSy2*-2B locus. These haplotypes were associated with thousand grain weight (Jiang et al., 2011). This result indicates that, a single *SuSy* gene may have different haplotypes that affect different agronomic traits. The type II sucrose synthase genes of maize and barley (*ZmSH1* and *HvSuSy2*, respectively) have been shown to play specific roles in generating precursors for starch synthesis in the endosperm (Guerin and Carbonero, 1997; Chourey et al., 1998). Similarly, RNA blot hybridization with specific probes showed that *SuSy2* mRNA was more abundant during the early stages of grain development, and the *SuSy1* mRNA level increased rapidly in the later stages (Marana et al., 1988). As it is still unclear if the expression of these genes is tightly associated with grain size and yield, characterizing and comparing their expression pattern during grain filling across cultivars with varying grain yield (thousand kernel weight) will provide an insight into their role during grain development.

2.5.3 Invertase

2.5.3.1 Structure and classification

Unlike SuSy, INV catalyzes uni-directional conversion of sucrose into glucose and fructose. Invertase exists in several isoforms with varying biochemical properties and subcellular locations (Tymowska-Lalanne and Kreis, 1998). Each of these isoforms has a specific function of regulating the allocation of sucrose into different utilization pathways. Sugars are not only needed as nutrients but acts as signalling molecule for regulating the expressions of various genes involved in embryogenesis, germination, seedling development, root and leaf morphogenesis, flowering, seed development, and various biotic and abiotic stress responses (Sheen et al, 1999). It indicates the indirect role of INVs in controlling cell differentiation and plant development. Irrespective of their origin (either from monocot or dicot), plant INVs can be classified based on their subcellular location and pH (Roitsch and Gonzalez, 2004).

Based on pH, INVs of higher plants are classified into two isoforms: alkaline/neutral and acid invertases. Based on their sub cellular location, the acid INVs are further divided into two groups, 1) soluble invertases located in vacuoles (VINV) and 2) insoluble invertases present in apoplastic cell walls (CWINV). The alkaline INVs are located in the cytoplasm (Hirose et al., 2002). Unlike the acidic INVs, alkaline INVs are not glycosylated and substrate specific. They are highly conserved in the C terminal region, and have low homology with acid INVs. It is believed that modern alkaline INVs might have originated from prokaryotes by endosymbiosis (Vargas et al., 2003). As its name indicates, acid INV cleaves sucrose at a pH of 4.5-5.0, whereas the alkaline INV requires a pH of 7.0-7.8 for its activity. In general, most of the plants *INV*s contain conserved seven exons/six introns

structures except for a *CWINV* in carrot and Arabidopsis, which contain only two exons that code for the conserved tripeptide motif, DPN (Lorenz et al., 1995; Haouazine-Takvorian et al., 1997). All other acidic INVs are N-glycosylated and share conserved pentapeptide sequence NDPNG (β-fructosidase motif) close to the N terminus of mature proteins which are responsible for cleaving disaccharide from fructose residue. It also can hydrolyze other β-Fru containing oligosaccharides such as raffinose and stachyose. Vacuolar INVs are thought to be derived from acidic INVs. Cell wall and vacuolar INVs can be distinguished by a single amino acid difference in the cysteine catalytic domain, with CWINV having proline (WEC**P**D), and VINV a valine residue (WEC**V**D) in the catalytic site (Goetz and Roitsch, 1999; Maddison et al., 1999; Sturm, 1999). It has been proposed by Tymowska-Lalanne and Kreis (1998) that CWINVs are synthesized as pre-proproteins with a long leader sequence that is cleaved off during transport and protein maturation. The leader sequence has a signal peptide that may have a role in locating secreted protein to cell wall (Blobel, 1980). In contrast to CWINVs, VINV proteins contain short hydrophobic C terminal extension that might be involved in vacuolar targeting (Unger et al., 1994).

2.5.3.2 Function

The physiological functions of INV isoforms vary with their cellular location and tissue type (Roitsch and Gonzalez. 2004). Cell wall bound INVs play a key role in apoplastic loading of hexose sugars into the sink cells, which is used in glycolysis for the generation of ATP (Godt and Roitsch, 1997; Tang et al., 1999). Vacuolar invertase studies in wheat and maize highlight their importance during drought stress. Water stress during grain development, represses the expression of *INV* and causes yield loss. For example, moisture stress during

meiosis in wheat decreases *VINV* activity and causes male sterility, indicating its role in seed set (Dorion et al., 1996). Similarly in maize, water stressed ovaries showed reduced VINV activity and restricted utilization of sucrose that led to kernel abortion (Zinselmeier et al., 1999). The activity of INV is tissue specific. For example, Arabidopsis INV (*Atβfruct2)* is expressed only in the anthers (Tymowska-Lalanne and Kreis, 1998) and the maize INV (*CWINV3*) is expressed only in reproductive tissues but not in vegetative tissues (Kim et al., 2000).

2.5.3.3 Cereal invertases

Many genes encoding CWINV and VINV have been identified from various monocot species including rice, maize, barley and wheat (Kim et al., 2000)**.** Rice contains 19 *INV* genes, in which 11 are grouped into acid *INVs* and eight are categorized into neutral/alkaline *INVs* (*OsNIN1*-8). The acid INVs of rice are further classified into CWINVs and VINVs. Nine of the 11 acid INVs are classified as CWINVs (OsCWINV1-9), and the remaining two as VINVs (*OsVINV1* and *OsVINV2*; Ji et al., 2005). Expression of rice *INV* genes is reported to be organ specific and stage dependent. Specific rice *CWINV* genes, *OsCWINV1*, *OsCWINV4*, and *OsCWINV7* are expressed in leaves, roots, flowers and immature seeds, whereas *OsCWINV3* in flowers and *OsCWINV6* is roots and flowers (Cho et al., 2005). Analysis of the expression of *CWINV* genes during seed development showed that the expressions of *OsCWINV4* and *OsCWINV7* are associated with the seed coat. Localization experiments indicated the presence of *OsCWINV1* transcripts in the maternal tissues of developing seeds (Hirose et al., 2002). The *OsCWINV1* gene exhibited high expression in the ovary at a very early stage of seed development (1-2 DAA), which decreased afterwards. The other *CWINV*

gene, *OsCWINV7*, was found to be expressed highly during the mid-stage of seed development (9-15 DAA) and thought to be involved in starch accumulation (Cho et al., 2005).

In maize, four *CWINV*s (*ZmCWINV1, ZmCWINV2, ZmCWINV3* and *ZmCWINV4*) and two *VINV*s (*ZmVINV1* and *ZmVINV2*) have been reported (Xu et al., 1996; Carlson and Chourey, 1999; Kim et al., 2000). The expression of *ZmCWINV3* is specific to differentiating and vascular cells of root tips including phloem parenchyma, sieve tubes and companion cells; whereas that of *ZmCWINV4* in dividing cells (Kim et al., 2000). Strong evidence for the role of maize *ZmCWINV2* in seed development has been demonstrated by the, *miniature1* mutant (*mn1*; loss of function mutation at *Mn1* locus that encodes *ZmCWINV2*) that caused over 70% weight loss in the mutant seeds as compared to that of wild type seeds (Miller and Chourey, 1992; Cheng et al., 1996). In barley, two cell wall bound *INV*s, *HvCW1NV1* and *HvCWINV2*, have been identified. The transcripts of these two genes were detected in the maternal and filial tissues, and the expression of *HvCWINV2* was shown to be pericarp specific (Weschke et al., 2003). These studies indicate that CWINV play an important role during the early stages of seed development.

2.5.3.4 Wheat invertases

Wheat INVs play a key role in cleaving sucrose to glucose and fructose, which are used as a substrate for starch synthesis and deposition in the anther (Koonjul et al., 2005). During reproductive development in wheat, pollen grains accumulate starch, which serves as energy reserve for pollen germination and pollen tube growth. It was found that water stress during meiosis in pollen cells resulted in male sterility, which accounted for a 40-50% reduction in

grain formation (Koonjul et al., 2005). Unlike other enzymes involved in starch synthesis, a decrease in INV activity due to water deficit caused the formation of developmental lesions that could not be reverted by re-watering (Dorion et al., 1996). Invertase is also suggested to be the dominant sucrolytic enzyme in wheat anthers and its activity is crucial for pollen viability (Clement et al., 1994). Three putative *INV* cDNAs: two cell-wall type (*CWINV1* and *CWINV3*) and one vacuolar form (*VINV5*) have been isolated from a wheat anther cDNA library and were reported to play essential roles during anther development (Koonjul et al., 2005). Like any other monocot type vacuolar and cell wall isoforms, wheat INVs also contains WEC**V**DFY and WEC**I**DFY protein domains conserved among vacuolar and cell wall types respectively (Sturm, 1999). The *CWINV1* gene was expressed at low levels during early anther development but increased during the mid-developmental stage and decreased at anther maturity (Koonjul et al., 2005), whereas *CWINV3* showed low expression during initial and mid developmental stages, but increased at late stage through pollen maturity. The vacuolar *INV* (*VINV5*) showed high expression in anther and pistils at pollen maturity and in the glume during meiosis. These data indicate that wheat INV isoforms are differentially activated during reproductive development and are tissue specific. However, little is known about the expression pattern of invertase genes during grain filling. Such information is crucial in providing an insight into the role of these genes in sucrose metabolism, and its relation to starch accumulation in wheat grains.

2.5.4 Role of sucrose transport proteins and sucrose cleaving enzymes in starch formation

Seed development is one of the major plant developmental processes that are tightly regulated by the activities of SUT proteins and sucrose hydrolysing enzymes. In addition, sucrose acts as a signalling molecule for various regulatory mechanisms. Sucrose hydrolysis by INV and SuSy play a vital role in the formation of starch as it makes the required precursors available. Even though, both INV and SuSy have the same function, the activity of INV is essential for cells undergoing cell division and expansion; particularly anthers whereas SuSy activity is necessary in storage sink tissues (Riffkin et al., 1995). Therefore, studying these two enzymes at the molecular level during seed development will provide valuable information to better understand the relationship between sucrose metabolism and starch formation.

2.6 Biotechnology as a tool to improve wheat productivity

In order to meet the ever increasing demand of crops for food, feed and bioproducts, there is a pressing need to increase crop yield. Identification of novel phenotypes with improved characteristics in a large population may be difficult and time consuming in conventional breeding method (Carver, 2009). The genetic redundancy of crop plants due to gene duplication and polyploid nature including hexaploid wheat reduces mutational effects (Carver, 2009). The modern molecular approaches are capable of introducing new traits, by enhancing or suppressing endogenous proteins that control various metabolic processes in crop plants (Bhalla et al., 2006). One approach of crop improvement involves manipulation of metabolic pathways, which requires complete knowledge of the corresponding

physiological processes in crop plants. In this regard, mutational studies are known to produce plants with superior traits that can serve as genetic resource in breeding programs (Xu et al., 2009). For example, RNAi based genetic suppression of the starch branching enzyme SBEIIa, which catalyzes the synthesis of amylopectin, resulted in 70% increase in amylose content (Sestili et al., 2010). High amylose starch is reported to have nutritional importance as it provides protection against colon cancer and type II diabetes (Behall and Howe, 1995). In general, the availability of complete or partial genomic data of crop plants such as rice, wheat, barley and maize, and generation of molecular markers have the potential to facilitate the development of new varieties with higher productivity.

2.6.1 Methods of gene delivery in wheat

Transgenic expression of a target gene requires three components: a promoter sequence to control expression of the targeted gene, the gene of interest, and a transcription terminator sequence to end transcription (Carver, 2009). Direct gene delivery by particle bombardment and Agrobacterium mediated gene transformation are the two most widely used methods for transformation in wheat (Shrawat and Loerz, 2006). Each of these methods has its own advantages and drawbacks. Particle bombardment involves delivery of gold coated DNA particles at a high velocity and pressure into the explants (Jones et al., 2005). It enables complex transgene integration (more than one copy) and needs several steps to confirm transgene integration. The gene of interest can be co-bombarded with another plasmid containing a plant selectable marker gene. Agrobacterium mediated transformation requires the use of binary vectors that contain a selectable marker and a target gene cassette in one plasmid between the right and left border of transfer (T) DNA region (Jones, 2005). The T-

DNA region in binary vectors enables transfer of single copy gene. Various binary vectors that consist of either tissue specific or constitutive promoters are commercially available (Himmelbach et al., 2007). Agrobacterium is host specific and naturally infects dicot plants, thus Agrobacterium mediated transformation of monocot crops such as wheat requires careful optimization with respect to bacterial strains, plasmids, genotype, selection systems and culture conditions (Jones, 2005).

In wheat, scutellar cells of immature embryos from 10-15 DAA have the ability to produce undifferentiated callus that can be regenerated to new plants (Gopalakrishna et al., 2003; Hu et al., 2003). Only a few cultivars tolerate wound responses to biolistic or agrobacterial gene transfer and have high regeneration capacity to develop shoots and roots from callus. Two spring wheat cultivars, Fielder and Bobwhite, and a winter wheat cultivar Cadenza are widely used for genetic transformation and have high regeneration frequency (Wu et al., 2003; Bhalla et al., 2006). In the case of particle bombardment, factors such as target distance, pressure applied for bombardment and DNA concentration affect the efficiency of transformation, whereas in Agrobacterium mediated transformation, specific strains control the efficiency of infection. AGL-0, AGL-1, LBA4404 are hypervirulent strains widely employed for transgene integration (Hensel et al., 2008). Various antibiotic resistance genes such as hygromycin tolerance (hptII) and herbicide (bialaphos and gluphosinate) tolerance (bar gene) are used as selectable marker in wheat transformation (Chugh and Khurana, 2003; Meyer et al., 2004a). Wheat transformation requires standardized tissue culture techniques that can successfully be used to generate transgenic plants. Various disease resistant genes such as rice chitinase for resistance against fungal pathogens (Chen et al., 1998); barley β-1, 3 glucanase gene for resistance against fusarium

and powdery mildew (Mackintosh et al., 2007) have been introduced into wheat. Some abiotic stress tolerance genes such as *DREB-1A* gene, encoding transcription factor involved in regulating the transcription of various genes for drought, salinity and cold tolerance (Pellegrineschi et al., 2004) and the *ATNHX1* gene encoding Na^+/H^+ antiporter protein which sequester sodium ions in vacuole for salinity tolerance (Xue et al., 2004) have also been introduced in wheat. Over-expression of hybrid high-molecular weight glutenin subunit genes Dx5:Dx10 resulted in enhanced dough quality (Blechl and Anderson, 1996). Transgenic expression of the maize gene (*SH2*) which encodes the large subunit of ADP-glucose pyrophosphorylase, that catalyzes the conversion of glucose-1-phosphate to ADP-glucose for starch formation, resulted in a 30% increase in total biomass and a 38% increase in seed weight/plant (Smidansky et al., 2002). However, commercialization of these crops needs further evaluation for environmental safety and health issues.

2.6.2 Use of tissue specific promoters

Promoters are DNA sequences located upstream (5' end) of the coding sequence of a gene. Promoter sequences can direct either constitutive, or temporal and spatial expression of a transgenes. The 35s cauliflower mosaic virus (CaMV) sequence is a widely used constitutive promoter in both monocots and dicots (Pauli et al., 2004). Since its identification, the maize ubiquitin 1 (*ubi1*) promoter has gained special interest in monocots (Christensen and Quail, 1995). However, constitutive expression of a transgene in all tissues poses certain problems including unwanted expression of the gene in certain tissues that causes accumulation of transgenic proteins which are not needed for plant growth. Such an effect may lead to abnormal plant growth and development. Hence, the use of stage and tissue specific

promoters is crucial for transgenic based approach crop improvement. Seed storage proteins and their promoter sequences such as glutelin in rice (Leisey et al 1989), zein in maize (Schernthaner et al., 1988), hordein in barley (Marris et al. 1988) have been reported to have seed specific activity.

In wheat, high molecular weight glutenin subunit (HMW-GS) accounts for 5-10% of total seed protein and controls dough quality. Genes encoding HMW-GS were located on the Glu-1 loci of 1A, 1B and 1D chromosomes of hexaploid wheat and found to be inherited as linked pairs. Each pairs encodes x and y type glutenin that are homologous in structure but differ in their repeat length and N-terminal domains. This difference is responsible for different bread making quality across cultivars. Blechl and Anderson (1996) reported the hybrid subunits of x and y type glutenins, Dx5:Dy10 which encodes a protein with 15 additional amino acids and 5 cysteine residues in the N terminal region. This additional region contains regulatory sequences for seed specific expression of glutenin in wheat. Transgenic expression of the Dx5:Dy10 hybrid promoter sequence fused with the GUS reporter gene in wheat, showed an increase in the total HMW-GS protein compared to that in the native subunits (Blechl and Anderson, 1996). The expression of GUS was observed in the endosperm of developing seeds and found to be stable in the subsequent four generations.

2.7 Manipulation of sucrose transporter to improve starch yield

The dry matter of plants is composed of carbon, therefore an increase in photosynthetic rate, and supply of photoassimilates, especially during grain filling, may enhance crop yield (Gifford et al., 1984). As sucrose is the major transportable form of plant sugars and is a principal source of carbon for starch synthesis in storage organs such as wheat grains,

increasing sucrose transporter activities especially that in developing seeds has a significant potential in increasing sucrose supply to the grains, and thereby yield (Weschke et al., 2000). For example, seed specific over-expression of potato sucrose transporter (*StSUT1*) in pea cotyledons resulted in high sucrose influx (185% to 240%) as compared to the control in the storage parenchyma cells of developing pea seeds (Rosche et al., 2002). Plasma membrane vesicles isolated from transgenic potato plants expressing spinach sucrose transporter (*SoSUT1*) showed higher rates of sucrose uptake (4-fold more) as compared to that in wild type plants (Leggewie et al., 2003). A comparative study on the expression of maize AGPase large subunit (*Sh2*) gene with the seed specific HMW-GS promoter and maize *Sh2* gene promoter in wheat seeds showed 20-fold higher transgene expression in HMW-GS lines than those with maize promoter line (Smidansky et al., 2007). Thus, the HMW-GS promoter can be used to target over-expression of high affinity sucrose transporter genes in wheat grains, and thereby increase starch yield.

3.0. EXPRESSION ANALYSIS OF GENES INVOLVED IN SUCROSE TRANSPORT AND CATABOLISM DURING GRAIN DEVELOPMENT IN WHEAT

3.1 Abstract

Sucrose is the principal source of carbon for plant growth and development including starch formation in cereal grains. Sucrose transport into developing grains and its conversion to glucose and fructose are the basic steps prior to starch biosynthesis. Sucrose transporter proteins and sucrose synthases play very important roles in these processes during wheat grain development, and analyzing their expression patterns provides an insight into their relationship. To this end, we characterized the expression patterns of sucrose transporter (*TaSUT1* and *TaSUT2*) and sucrose synthase (*TaSuSy1* and *TaSuSy2*) genes using real time PCR during grain filling. Our gene expression analysis showed coordinated expression of *TaSUT1* and *TaSuSy2* genes in AC Andrew, a cultivar with larger seed size at all stages of development examined in this study. It suggests the role of these genes in contributing the majority of sucrose transporter and hydrolysis activity required for dry matter accumulation in developing wheat grains, respectively.

3.2 Introduction

Cereal grains are the widely used starch based bioethanol produced in North America and Europe. About 20% of the Canadian bioethanol industry depends on wheat starch (CRFA, 2010). Wheat grains with high starch/low protein content are mainly used for biofuel production. Mandatory use of 5% ethanol in transport fuels, from 2010 onwards, increased the demand for bioethanol and wheat grains (CRFA, 2011). Increasing cropping area to meet the demand of wheat as a raw material for bioethanol production will affect the production of wheat grain for food and feed. This calls for the need to increase wheat starch yield per seed or per unit area, which can be achieved by manipulation of the starch biosynthetic pathway. To this end, it is necessary to have a detailed understanding of the mechanisms involved in starch formation in grains.

Starch formation in wheat grains depends on the transfer of photoassimilates from the source to the developing grains and its subsequent conversion to starch (Emes et al., 2003). Sucrose is the transportable form of carbohydrate in plants, and its transport occurs through a specialized tissue called phloem (Wind et al., 2010). The movement of sucrose inside phloem cells occurs by mass flow involving plasmodesmata, whereas phloem loading and unloading mechanisms involve membrane localized sucrose transporters (Slewinski and Braun, 2010). Genes encoding SUT proteins have been identified from a number of plant species. In wheat, only one gene encoding a SUT (*TaSUT1*) is known so far (Aoki et al., 2002). This gene has three homologs residing in the three wheat genomes, namely *TaSUT1A*, *TaSUT1B* and *TaSUT1D*, and was found to be involved in phloem loading in source tissues and unloading in developing grains (Aoki et al., 2002; Aoki et al., 2004; Aoki et al., 2006). Previous study has shown that *TaSUT1* transcripts are present at high levels in source tissues before heading,

and its transcript level decreased by 12 days after heading (DAH). In contrast, high accumulation of the transcripts of *TaSUT1* was evident in developing grains. Its expression was detected at 8 DAH, increased from 16 to 20 DAH before showing a decline afterwards through 32 DAH (Aoki et al., 2002). This developmental expression pattern of *TaSUT1* indicates its involvement in sucrose transport; thereby starch synthesis during grain development.

The role of sucrose synthase in starch synthesis has been studied extensively in a number of cereal crops including rice (Hirose et al., 2008), maize (Carlson et al., 2002) and barley (Sicilia et al., 2011). For example, rice has six *SuSy* genes, and tissue specific expression studies of these rice *SuSy* genes suggested that each of these genes has distinct functions. Immunolocalization and western blot analysis indicated the presence of *OsSuSy1* in vegetative tissues including developing leaves, internodes and elongating roots, whereas *OsSuSy2* was distributed equally in all the tissues and suggested to have a housekeeping role (Wang et al., 1999). Real time PCR analysis showed that the transcript levels of *OsSuSy5* and *OsSuSy6* were reduced in germinating shoots under submerged conditions whereas *OsSuSy2* transcript level increased in germinating seedlings under hypoxic condition (Hirose et al., 2008), indicating the role of *OsSuSy2* in sucrose metabolism under submerged conditions. Of the six members of *SuSy* gene family, *OsSuSy3* and *OsSuSy4* were predominantly expressed in developing grains, with the maximum transcript level observed at 10 DAA, and their expression pattern was associated with dry weight gain (Hirose et al., 2008). This is in agreement with Wang et al. (1999) who suggested the role of *OsSuSy3* in hydrolyzing sucrose to provide precursors for starch synthesis. In maize, three *SuSy* genes (*ZmSuSy1*, *ZmSH1* and *ZmSuSy3*) have been reported to encode isoforms of SuSy (Carlson et al., 2002).

The two genes, *ZmSuSy1* and *ZmSH1*, are highly expressed in developing endosperm, and contribute 90% of SuSy activity during grain development (Chourey et al., 1998). The *ZmSuSy3* gene was found to be expressed equally in all the tissues (Carlson et al., 2002), suggesting its housekeeping role. Two *SuSy* genes have been reported in wheat (Marana et al., 1990). The *TaSuSy2* gene is located on chromosomes 2A, 2B and 2D, and two of the *TaSuSy*2-2B haplotypes, Hap-H and Hap-L, were reported to be associated with thousand grain weight (Jiang et al., 2011). The profile of SuSy activity in the developing wheat grains of two cultivars varying in dry weight indicated a strong correlation between sucrose synthase activity and rate of dry matter accumulation (Dale and Housley, 1986) suggesting the role of SuSy in determining grain dry weight in wheat. However, the expressions of *SuSy* genes and their relation to dry matter accumulation and starch biosynthesis have to be studied in detail.

Previous studies have indicated a strong relationship between sucrose transport and metabolism. For example, *TaSUT1* has been shown to have a similar expression pattern with that of SuSy activity (Aoki et al., 2002) and overexpression of barley *SUT1* in wheat led to increased activity of SuSy (Weichert et al., 2010). Furthermore, expression of *SUT1* was dramatically reduced in the *sh1susy1-1* double mutant of maize (Chourey et al., 1998). These two physiological processes play important roles in the formation of starch that accounts for 65% to 75% of total dry weight of a wheat grain. The variation in kernel weight among cultivars is attributed to differences in dry matter accumulation during grain development (Adhikary et al., 2009; Dai et al., 2009). Though sucrose transport and metabolism during cereal grain development have been studied to a certain extent, their relationship during grain filling/dry matter accumulation and how this relationship is affected by natural variation has

not been investigated in detail. Therefore, in order to understand the coordination of sucrose supply and its subsequent metabolism with dry weight accumulation, we have characterised the expression patterns of genes encoding sucrose transporters and sucrose synthases in the developing grains of five spring wheat cultivars, and the resulting gene expression patterns are discussed with respect to seed size/dry weight.

3.3 Materials and methods

3.3.1 Plant material and growth conditions

Five spring wheat cultivars, Chinese spring (CS), Yecora Rojo (YR), CDC-Teal (CDC), White Bird (WB), and AC Andrew (ACA) were used in this study, mainly because of their difference in seed size. Seeds were germinated on moisten Whatman filter paper placed in petriplates and kept in darkness for 3 days at room temperature. The germinated seeds were planted (1 plant/pot) in 1-gallon pot filled with LA4 mix (containing Canadian sphagnum peat moss, coarse sand, starter nutrient with gypsum and dolomitic limestone; Sungrow) and grown at 16-22°C/14-18°C (day/night) in a 16/8 h photoperiod in a greenhouse at the Crop Technology Centre, University of Manitoba. Fifteen to twenty plants were grown from each cultivar; watered every other day and fertilized with 20:20:20 (N: P: K) mixture once a week. For grain fresh and dry weight measurements, and gene expression analysis, developing spikes from primary and secondary tillers were tagged at the onset of anthesis (the first protrusion of yellow anthers). Two to three spikes were harvested at 4, 8, 16, 25 and 30 days after anthesis (DAA) from three different plants (one plant per replication; 3 replications) and immediately frozen in liquid nitrogen and stored at -80°C until further use.

3.3.2 Fresh weight and dry weight analysis

Grain fresh weight data of each cultivar were obtained by weighing 20 grains individually. Developing grains were taken from the middle region of 3 spikes (6 to 7 grains per spike) harvested from three different plants at 4, 8, 16, 25 and 30 DAA. Dry weight measurements were performed after drying the samples at 105°C for 38 h. Rates of fresh and dry matter accumulation were calculated by dividing the differences in fresh or dry weights with the number of days between two successive stages.

3.3.3 RNA extraction

Grains from the middle region of each spike (90-110 FW/mg; one spike per replication; 3 replications) were ground to a fine powder in liquid nitrogen, and homogenized with 400 µl of extraction buffer I (100 mM Tris, pH 8.0, 150mM LiCl, 50mM EDTA, 1.5% sodium dodecyl sulphate, and 1.5% 2-mercaptoethanol). After vigorous vortexing, 250 µl of phenol: chloroform: isoamyl alcohol (25:24:1, pH 3) was added to the mixture and centrifuged at 13,000*g* for 15 min at 4°C. The resulting supernatant was precipitated by using 250 µl of extraction buffer II (4.2 M guanidinium sulphate (w/v), 25 mM sodium citrate, 0.5% lauryl sarcosine, and 1M sodium acetate, pH 4); and extracted with 200 µl of phenol: chloroform: isoamyl alcohol (25:24:1, pH 3) followed by centrifugation at 13,000*g* for 15 min at 4°C for more purification. The resulting supernatant was then precipitated sequentially using 300 µl of isopropanol and 250 µl of 1.2 M sodium chloride to remove polysaccharides. The precipitate was then washed with 70% ethanol, air dried and finally dissolved in 50 µl of RNase free water. The purity of the RNA sample was determined spectrophotometrically (260 to 280 ratio), whereas the integrity of RNA was examined by agarose gel

electrophoresis. To eliminate genomic DNA contamination, 30 µg total RNA was digested with 1µl DNaseI (DNA-free kit, Ambion) in 10X DNaseI buffer in a total reaction volume of 100 µl. The reaction mixture was incubated at 37°C for 30 min and inactivated by DNaseI inactivation reagent provided by the manufacturer. The RNA concentration was then measured with a micro-volume spectrophotometer (ND-1000, Thermo Scientific) and the samples were stored at -80°C until use for cDNA synthesis.

3.3.4 cDNA synthesis

The synthesis of cDNA from the total RNA samples was performed with RevertAid H Minus First Strand cDNA synthesis kit (Fermentas) with a minor modification of the manufacturer's protocol. Briefly, RNA samples (1 µg) were mixed with oligo $(dT)_{18}$ primers (100 pmol) in a total volume of 12 µl, and incubated at 65°C for 5 min. After a brief incubation on ice, 4 µl of 5X reaction buffer, 0.5 µl of RNase inhibitor (20 U/µl), 1 µl of dNTP mix (10 mM), 0.5 µl of RevertAid H Minus M-MuLV Reverse transcriptase (200 U/µl) and 2 µl of water were added to the mixture, with a total volume of 20 µl, and subjected to 42°C for 1 h. Termination of the reaction was performed by incubation at 70°C for 5min. The cDNAs were quantified by spectrophotometer (ND-1000, Thermo Scientific), diluted to 100 ng/µl concentration, and then stored at -20°C until further use.

3.3.5 Primer design

Forward and reverse primers sequences that yield an amplicon size of 60-150 bp for the target and reference genes were designed with primer 3 software (Rozen and Skaletsky, 2000;

Table 3.1) using the following parameters: primer length of 18-22 base pairs with annealing temperature of 60°C ± 2°C, and 35%- 55% Guanine-Cytosine (GC) content.

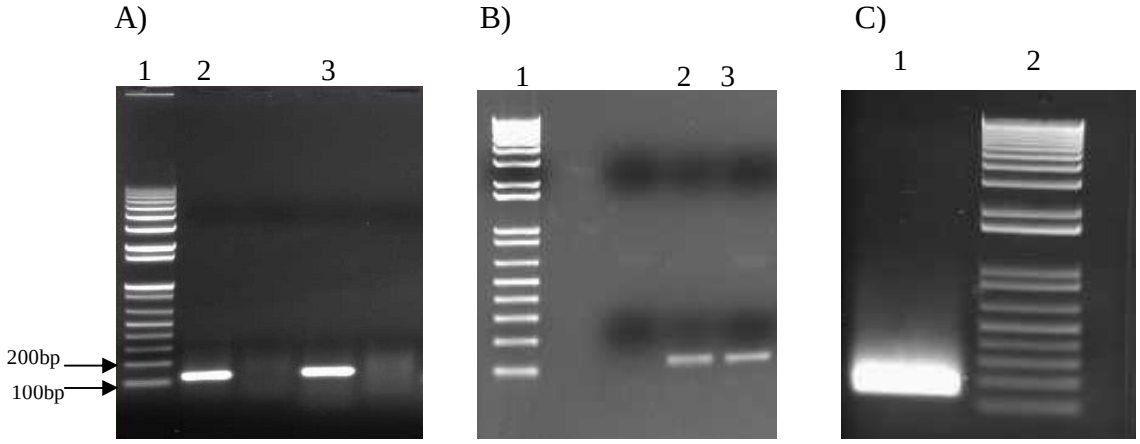

Figure 3.1. Confirmation of the specificity of primer sets of sucrose transporter and sucrose synthase genes by PCR. A) Expected band sizes for amplicons of *TaSUT1* (lane 2) and *TaSUT2* (lane 3). B) Expected band sizes for amplicons of *TaSuSy1* (lane 2) and *TaSuSy2* (lane 3). C) Expected band sizes for amplicons of *Taβ-actin* (lane 1). Lane 1 in A, B and lane 2 in C shows 1 Kb+ DNA marker.

Primers were designed based on publicly available sequences of *TaSUT1* (Aoki et al., 2002; Appendix 7.1), *TaSUT2* (unpublished), *TaSuSy1* and *TaSuSy2* (Marana et al., 1988; Appendix 7.2, 7.3) and *Taβ-Actin* (Himi and Noda, 2004; Appendix 7.4). Specificity of primers was confirmed by blasting primer and amplicon sequences against all sequences available in National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). The blasted primer and amplicon sequences showed exact match with corresponding gene sequences. Further confirmation of primer specificity was performed by PCR amplification, which produced a single band of expected size (Figure 3.1)

Table 3.1. Primer sequences used in the measurement of relative transcript levels by qPCR

Gene Name	Primers	Sequence	Amplicon length (bp)
	FP[a]	TGGATTCTGGCTCCTTGAC	
TaSUT1	RP[b]	GCCATCCAAGAACAGAAGATT	150
	FP	TACGGAGTCCTGCTCTGTCA	
TaSUT2	RP	CTCGTCGCTTCCGAAAGTA	150
	FP	TGAAGTGTCGGCTGCGTTAT	
TaSuSy1	RP	TATGGGCAGGCGTTTATTCC	163
	FP	CCGAGCCACTGGAACAAGAT	
TaSuSy2	RP	GGGCGTAGAGCATTTCAAGGTA	175
	FP	GCTGTTCCAGCCATCTCATGT	
Taβ-actin	RP	CGATCAGCAATTCCAGGAAAC	156

[a]Forward primer; [b]Reverse primer

3.3.6 Real-time PCR

Real-time PCR assays were performed on Mx3005p real time PCR machine (Stratagene) using Maxima SYBR green/ROX qPCR mix (Fermentas). For each 20 µl reaction, 2 µl cDNA (200 ng) were mixed with 0.3 µl of forward primer (0.15 µM/ µl) and 0.3 µl of reverse primer (0.15µM/ µl), 10 µl of 2X Maxima SYBR Green Master Mix (containing Hot Start Taq DNA polymerase, SYBR Green dye, ROX passive reference dye and dUTP) and 7.4 µl of RNase free water. Samples were subjected to thermal cycling conditions at 95°C for 10 min of initial denaturation to activate Maxima Hot Start Taq DNA polymerase, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 sec. PCR amplification of each sample was performed in duplicates in 96-well optical reaction plate (Bio-Rad), and the average of the duplicates was used to determine transcript level for each sample. A pooled cDNA sample was used to correct plate to plate variation in amplification in which a Ct value of pooled sample from one run was used to normalize the Ct values of samples in other runs by using the following equation as described in Ayele et al. (2006):

*Normalized Ct value of sample = (Ct value of pooled sample in standard run/Ct value of pooled sample in sample run)*Ct value of sample*.

The relative transcript level of the target genes in the plant samples under investigation was calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) where ΔCt is the difference between Ct values of target sample and actin internal control. The highest ΔCt (least expressed) of all samples was used as the calibrator to calculate ΔΔCt (ΔΔCt = individual sample ΔCt - samples with highest ΔCt).

3.3.7 Statistical analysis

Analysis of variance was conducted for each variable using PROC GLM procedure of SAS program (Ver. 9.2., SAS institute Inc, 2008). The sources of variation were cultivars, stages, and cultivars x stages interaction. All sources of variation were treated as fixed effects. Least-square (LS) means were calculated for each cultivar, stage and cultivar x stage interaction, and least significant difference (LSD) was calculated to compare significant difference in LS means of all cultivar x stage combinations. Difference in LS mean value greater than the LSD indicates a significant difference at $p \leq 0.05$.

3.4 Results

3.4.1. Changes in fresh and dry weights during grain development

Wheat grain development was studied from 4 to 30 DAA in five spring wheat cultivars (Figure 3.2A). Grain fresh weight exhibited an increase from 4 to 25 DAA (5-fold in CS, 5-

fold in YR, 6-fold in CDC, 5-fold in WB and 5-fold in ACA; Figure 3.2B), and the patterns were similar across all the cultivars studied. Similarly, grain dry weight increased from 4 through 25 DAA in all of the five cultivars (14-fold in CS and WB; 13-fold in CDC and ACA; 11-fold in YR; Figure 3.2C). The fresh and dry weights were maximum at 25 DAA in all cultivars. ACA showed greater grain fresh and dry weights at all stages of grain development as compared to CS, YR, CDC and WB. The significant difference in fresh and dry weight accumulations between cultivar * stage interaction at $p \leq 0.05$ are shown in Appendix 7.11 and 7.12, respectively.

3.4.2. Rates of fresh and dry weight accumulation during grain development

The rate of fresh weight accumulation (mg/day) was calculated across the five cultivars (Table 3.2). Fresh weight accumulations per day were high in the developing grains of the five cultivars between 0 and 4 DAA, and 8 and 16 DAA, with the highest rate of fresh weight accumulation occurred between 8 to 16 DAA. Between 4 to 8 DAA, CS and ACA showed higher rate of fresh weight accumulation than that of YR, CDC and WB (Table 3.2), whereas YR and ACA, which are characterized by their bigger and heavier grains, had higher rate of fresh weight accumulation between 16 and 25 DAA than that of CS, CDC and WB (Figure 3.2A). As compared to that observed in the other cultivars, ACA showed higher rates of fresh weight accumulations from 4 through 25 DAA, and CDC exhibited the lowest rate of fresh weight accumulation per day at all stages, except between 8 and 16 DAA, when it had the highest rate of accumulation (Table 3.2).

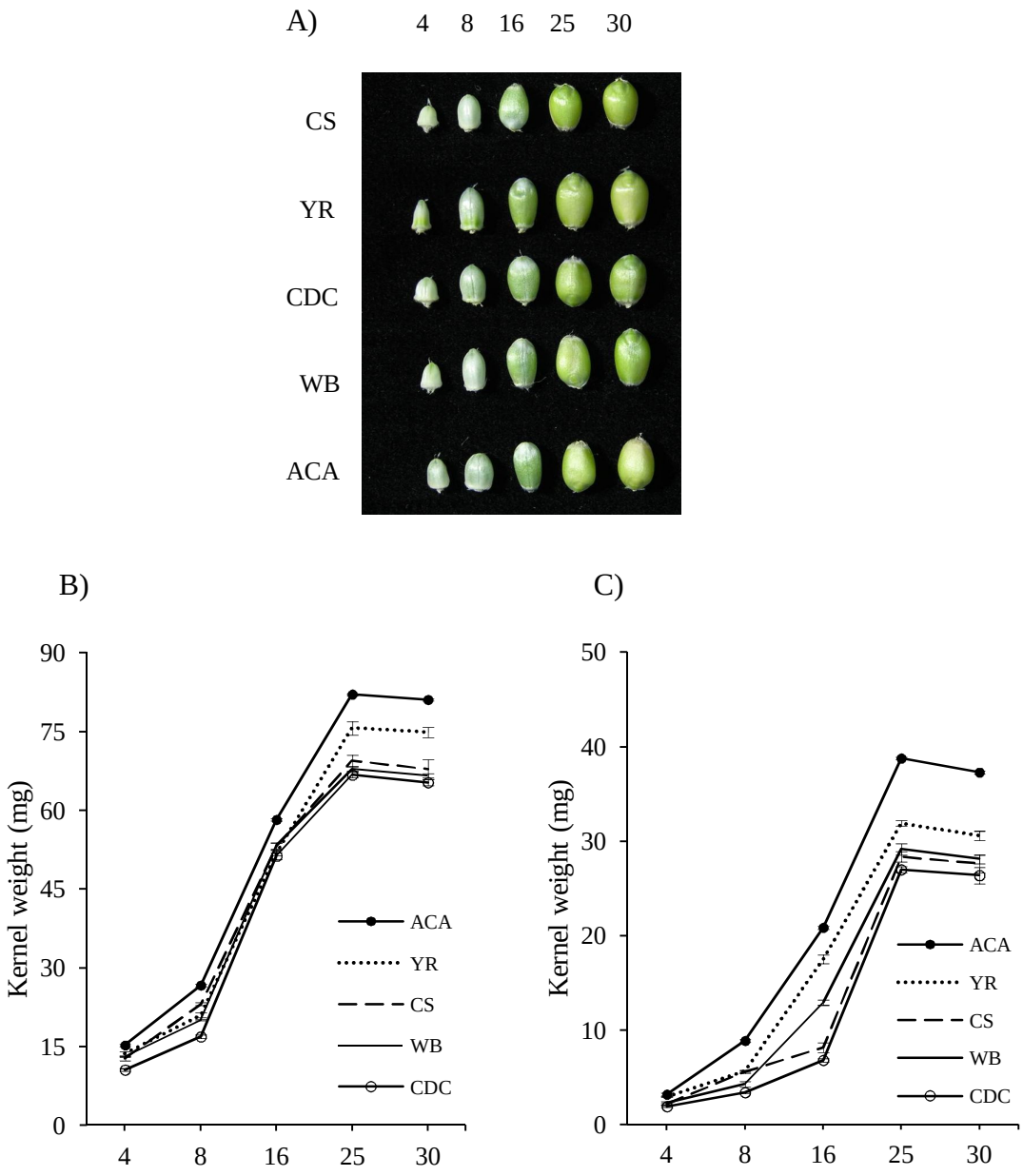

Figure 3.2. Developing grains of five spring wheat cultivars Chinese Spring (CS), Yecora Rojo (YR), CDC Teal (CDC), White Bird (WB) and AC Andrew (ACA) from 4 to 30 days after anthesis (DAA; A). Fresh (B) and dry (C) weights of the developing grains. Data are means ± SE, n=20, except for WB at 4 and 8 DAA where n=19.

Table 3.2. Rate of fresh weight accumulation (mg/day) in the developing grains of five spring wheat cultivars

	Cultivar				
DAA	CS	YR	CDC	WB	ACA
0-4	3.1	3.4	2.6	3.3	3.8
4-8	2.6	1.8	1.6	1.7	2.8
8-16	3.7	3.8	4.2	4.0	4.0
16-25	1.9	2.6	1.7	1.7	2.6
25-30	-0.2	-0.1	-0.3	-0.2	-0.2

Data are difference in mean values at specific time interval (rate of accumulation); where n= 20. Except for WB at 4 DAA and WB at 8 DAA, where n=19.

The rates of dry weight accumulation were also compared across the five cultivars. It was higher during 16-25 DAA in all the cultivars than that observed at 4-8 DAA. ACA showed higher rate of dry matter accumulation between 4 and 16 DAA than the other four cultivars. The rate of dry weight accumulation in YR was also higher than CS, WB and CDC during 8 to 16 DAA (Table 3.3). Dry weight accumulation in WB showed an increase from 8 to 25 DAA; however, it was lower than that observed in ACA at this stage (Table 3.3). Both CS and CDC exhibited lower rates of dry weight accumulation from 4 to 16 DAA, though showed rapid increases between 16 and 25 DAA.

3.4.3 Expression pattern of wheat sucrose transporter genes during grain filling

The expression patterns of *TaSUT1* and *TaSUT2* were determined in the five spring wheat cultivars at five different stages of development (Figure 3.3) by using real time qPCR. Both *TaSUT1* and *TaSUT2* genes were expressed at all stages in all the five cultivars, and the expression of *TaSUT1* was higher than that of *TaSUT2* in each cultivar at all developmental stages examined in this study (Figure 3.3).

Table 3.3. Rate of dry weight accumulation (mg/day) in the developing grains of five spring wheat cultivars

	Cultivar				
DAA	CS	YR	CDC	WB	ACA
0-4	0.5	0.7	0.4	0.6	0.8
4-8	0.9	0.7	0.4	0.5	1.4
8-16	0.3	1.5	0.4	1.0	1.5
16-25	2.2	1.5	2.3	1.8	2.0
25-30	-0.17	-0.1	0.2	-0.1	-0.3

Data are difference in mean value at specific time interval (rate of accumulation) n= 20. Except for WB at 4 DAA and WB at 8 DAA, where n=19.

However, a temporal variation in the expression pattern of *TaSUT1* was evident across cultivars. Its expression in CS and CDC showed an increase from 4 through 25 DAA (9-fold in CS and 6-fold in CDC), when it reached a maximum. From 25 to 30 DAA, the transcript level of *TaSUT1* decreased to minimum (4-fold in CS and 2-fold in CDC), compared to that detected at 25 DAA (Figure 3.3A; Figure 3.3C). In YR and WB, its expression peaked earlier, at 16 DAA (2-fold in YR and 15 fold in WB as compared to that observed at 4 DAA), and then decreased to a low level by 25 DAA (4-fold in YR and 9.5-fold in WB) and maintained at a similar level through 30 DAA. The expression of *TaSUT1* gene was significantly higher ($p<0.05$) in ACA at all developmental stages than that observed in the other four cultivars (Figure 3.3; Appendix 7.13). A very high transcript abundance was detected at 4 DAA (8- to 70-fold than that observed in the other four cultivars) and the level increased further (over 2-fold) by 8 DAA, when it reached the maximum (Figure 3.3E). Though a gradual (between 8 and 25 DAA) and rapid decrease (from 25 to 30 DAA) was observed, the transcript abundance of *TaSUT1* found at 30 DAA was still significantly higher ($p<0.05$) in ACA than that of other four cultivars (Figure 3.3E). The expression of the other *SUT* gene *TaSUT2*, was very low as compared to that of *TaSUT1* however, it still exhibited

temporal variation among cultivars (Figure 3.3). Similar to that of *TaSUT1*, the expression of *TaSUT2* was maximum at 8 DAA in ACA and it decreased (16-fold) thereafter, reaching a minimum by 30 DAA. Its expression in CS was maximum at 4 DAA and decreased gradually afterwards (1.6- to 3-fold), whereas in YR its expression was maintained at similar level from 4 to 16 DAA, after which it decreased (5-fold). In WB, the expression of *TaSUT2* increased from 4 through 16 DAA (6-fold) and then decreased (6-fold) by 25 DAA. The significant difference in *TaSUT2* expression between cultivar * stage interaction at $p \leq 0.05$ is shown in Appendix 7.14.

3.4.4 Expression patterns of wheat sucrose synthase genes during grain filling

Expression patterns of two sucrose synthase genes, *TaSuSy1* and *TaSuSy2*, were studied in the developing grains of all the five cultivars (Figure 3.4). Between the two genes, *TaSuSy2* exhibited higher expression than that of *TaSuSy1* during the early stage of grain filling (at 8 DAA) in CS, YR and ACA cultivars. In WB, both genes showed a similar expression level. Chinese Spring and ACA exhibited comparable expression of *TaSuSy2* at 8 DAA, but higher than that observed in the other three cultivars. The expression of *TaSuSy2* showed a 3.3-fold decrease in CS but only 2-fold in ACA by 16 DAA. The temporal expression pattern of *TaSuSy2* was similar across the four cultivars, except for CDC. Its expression increased in CS (35-fold), YR (2-fold), WB (8-fold) and ACA (16-fold) from 4 to 8 DAA, when it reached maximum, resulting in its expression level at 8 DAA in the following order, CS > ACA > YR > WB.

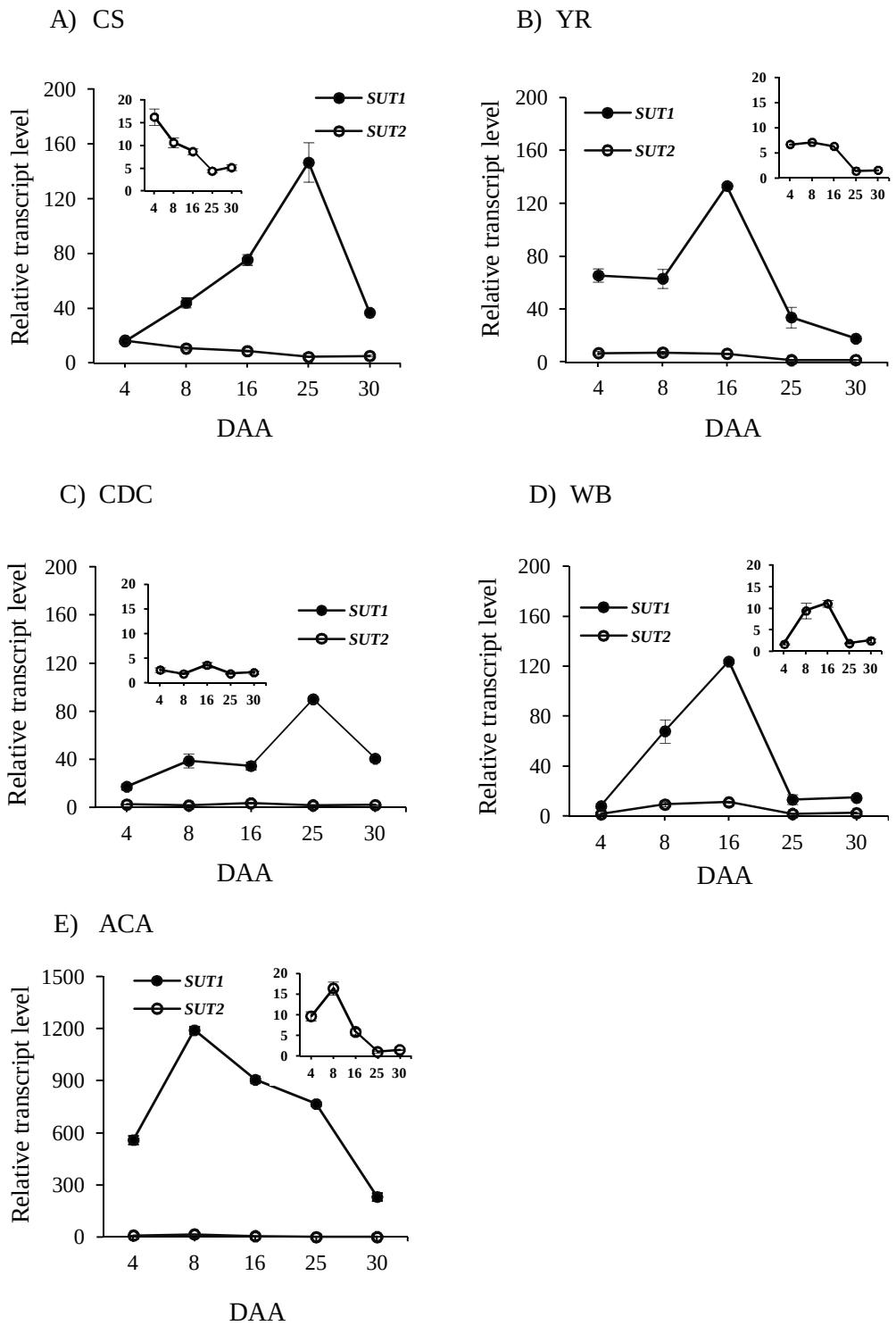

Figure 3.3. Expression pattern of *SUT1* and *SUT2* genes during grain development in spring wheat cultivars, Chinese Spring (CS, A), Yecora Rojo (YR, B), CDC Teal (CDC, C), White Bird (WB, D) and AC Andrew (ACA, E). The inset graphs show *SUT2* gene expression with expanded scale. Data are means ± SE, n=3 except for *SUT1* of CS at 25 DAA, YR at 16 DAA and WB at 16 DAA; *SUT2* of CDC at 8 DAA where n=2. Transcript levels were compared across genes, developmental stages and cultivars using the transcript abundance of *SUT2* for ACA at 25 DAA, which was set to 1 as a calibrator.

From 8 to 25 DAA, the transcript level of *TaSuSy2* exhibited a marked decrease to a very minimal level, which was maintained through 30 DAA. In CDC, the expression of *TaSuSy2* was maintained at a similar level from 4 through 16 DAA, and then increased slightly (1.8-fold) from 16 to 25 DAA, after which its expression declined (8-fold) to a minimum level. The *TaSuSy2* gene was expressed at a maximum level in CS, YR and ACA at 8 DAA, but at a lower level in CDC (7-fold) and WB (3-fold) than that observed in CS. The expression of *TaSuSy2* decreased to a minimum level by 25 and 30 DAA in all the cultivars except for CDC (Figure 3.4). Statistical analysis of *TaSuSy2* expression between cultivars at each developmental stage is shown in Appendix 7.16.

The *TaSuSy1* gene exhibited variable expression pattern across the cultivars studied. Its expression was highest at 4 DAA in YR and ACA, but showed a gradual decrease through 30 DAA, whereas in CDC, its expression was maintained almost at a similar level from 4 through 30 DAA. Unlike that observed in the other three cultivars, the temporal expression patterns of *TaSuSy1* in CS and WB cultivars were very similar to that exhibited by the *TaSuSy2*. Significant difference in *TaSuSy1* gene expression between cultivars at each stage of grain development is shown in Appendix 7.15.

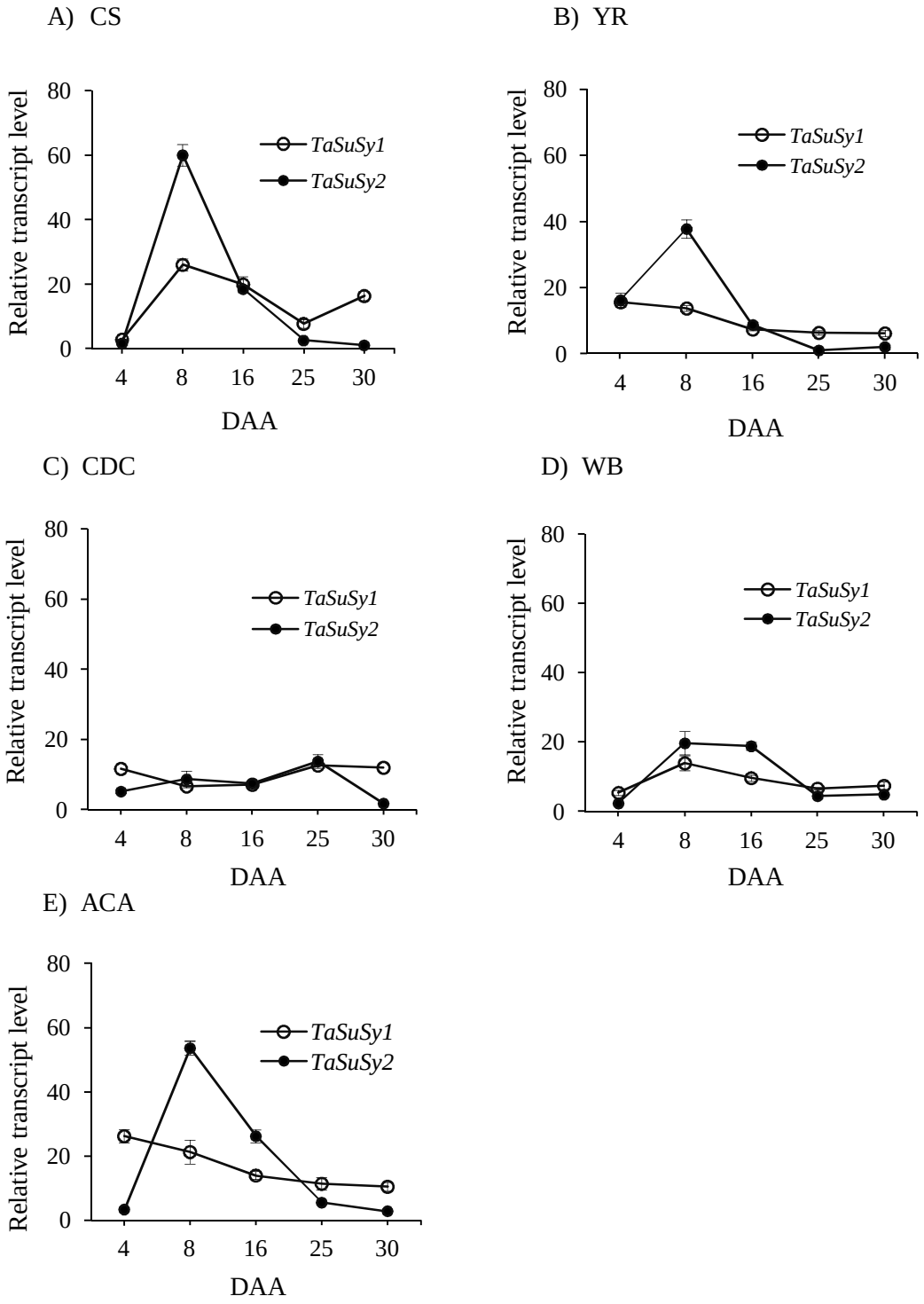

Figure 3.4. Expression patterns of *TaSuSy1* and *TaSuSy2* genes during grain development in spring wheat cultivars, Chinese Spring (CS, A), Yecora Rojo (YR, B), CDC Teal (CDC, C), White Bird (WB, D) and AC Andrew (ACA, E). Data are means ± SE, n=3 except for *TaSuSy1* of CS at 25 DAA, CDC at 4 DAA, ACA at 8 DAA and *TaSuSy2* of CS at 25 DAA, CDC at 8 DAA and WB at 8 DAA where n=2. Transcript levels were compared across genes, developmental stages and cultivars using the transcript abundance of *SuSy2* for YR at 25 DAA, which was set to 1 as a calibrator.

3.5 Discussion

3.5.1 Temporal variation in dry matter accumulation

In this study, the expression patterns of genes involved in sucrose transport and sucrose metabolism have been studied during grain development in five different spring wheat cultivars. Cereal grain development involves two stages: the first stage is grain enlargement, during which rapid cell division and maximum influx of water into the cells takes place. Thus, increase in the volume of grain cells and subsequent determination of grain size occurs at this stage. In wheat, the duration between 3 and 20 DAA is reported as the period of grain enlargement (Briarty et al., 1979). This is in agreement with our data that showed the occurrence of increase in the size of grains during 4 to 25 DAA in all cultivars (Figure 3.2). The second stage of grain development is grain filling, during which dry matter accumulation takes place (Briarty et al., 1979). This stage of grain development starts around 10 DAA and extends until maturity. Consistent with this result, the majority of the dry matter accumulation in all cultivars occurred from 8 to 25 DAA (Figure 3.2C).

The activities of proteins/enzymes in sucrose supply and metabolism have been reported to affect dry matter accumulation in developing grains (Yang et al., 2004). The profile of dry matter accumulation in the five cultivars (Figure 3.2C) coincides with the expression pattern of one of the sucrose transporter genes, *TaSUT1*. ACA showed earlier and gradual (4 to 8 DAA) followed by rapid (8 to 25 DAA) rate of increase in fresh and dry matter accumulation (Table 3.2 and 3.3). As compared to the other four cultivars, fresh and dry matter accumulation in ACA was higher at all stages of grain development, resulting in significantly higher ($P \leq 0.05$) fresh and dry weights at almost all stages of grain development than that found in the other cultivars (Appendix 7.11 and 7.12).

Our data shows that the fresh and dry matter accumulation patterns of ACA are closely associated with the expression pattern of *TaSUT1* during grain development (4-30 DAA). These results imply that *TaSUT1* contributes to the majority of sucrose transporter protein activity to supply the assimilate required to support grain growth and starch biosynthesis during wheat grain filling. Similar expression pattern of this gene was reported by Aoki et al. (2002) where its expression peaked at 16-20 DAH and decreased afterwards. Our gene expression analysis showed higher expression of *TaSUT1* than that of *TaSUT2* throughout grain development, and the expression of *TaSUT2* was very low almost at all stages examined (Figure 3.3). These expression patterns of *TaSUT1* and *TaSUT2* are in agreement with those reported in barley and led to a conclusion that barley *SUT1* plays a role in seed development, whereas *HvSUT2* has a house keeping role (Weschke et al., 2000).

Sucrose synthase is one of the enzymes involved in the hydrolysis of the incoming sucrose into hexose sugars, glucose and fructose, that are utilized as starch in developing grains (Dale and Housley. 1986). It has been shown recently that over- expression of one of the barley sucrose transporter gene (*HvSUT1*) in wheat induced the activity of sucrose synthase (Weichert et al., 2010). The expression patterns of *TaSUT1* and *TaSuSy2* in ACA (Figure 3.3 and 3.4), also suggest that coordinated increased expressions of *TaSUT1* and *TaSuSy2* are critical for increased dry matter accumulation. Variation in the activity of sucrose synthase between cultivars has been reported to lead to differences in dry matter accumulation (Dale and Housley, 1986). Consistently, difference between cultivars in the expression pattern of sucrose synthase genes during the early stage of grain development is associated with differences in seed size and dry weight (Figure 3.2C, Jiang et al., 2011), suggesting the transcriptional regulation of wheat sucrose synthase genes to produce active

enzyme. Our results indicated that coordinated expression of both the genes *TaSUT1* and *TaSuSy2* is important for grain growth and starch accumulation in the endosperm.

Yecora Rojo exhibited larger and heavier seeds next to ACA (Figure 3.2A), and this can be accounted for the shorter duration of rapid dry matter accumulation rate observed in YR (8-25 DAA, 17 days) as compared to that in ACA (4-25 DAA, 21 days, Table 3.3). Our result shows substantial amount of *TaSUT1* transcript in YR from 4 to 16 DAA, and this is accompanied by increased expression of *TaSuSy2* during the early stages of grain development (4 to 8 DAA; Figure 3.4B). This further supports our previous hypothesis that coordinated expression of genes involved in sucrose transport and metabolism are critical for grain development and filling.

Chinese Spring exhibited temporal variation in the up-regulation of *TaSUT1* and *TaSuSy2* genes, wherein the expression of *TaSuSy2* peaked at 8 DAA when the expression of *TaSUT1* was very low, and the expression of *TaSUT1* peaked at 25 DAA when *TaSuSy2* was expressed at low level. Two important roles of sucrose have been proposed during seed development (Weber et al., 1997). One foremost role is it serves as the main source of sugars for starch synthesis, and the second important role is to influence the activity of enzymes in sucrose-starch metabolic pathway. Though the high transcript level of *TaSuSy2* at 8 DAA may indicate high activity of sucrose hydrolysing enzyme, the low transcript abundance of *TaSUT1* at 4 to 8 DAA might suggest limitation in sucrose supply and its availability as a substrate for SuSy enzyme. The lower grain dry weight exhibited by CS, as compared to that of YR and ACA (Figure 3.2B and 3.2C), can therefore be explained by the lack of temporally coordinated up-regulation of the two genes.

Conversely, in CDC and WB cultivars, expression of *TaSUT1* at its maximum was evident later during grain filling (16-25 DAA), and this late up-regulation of *TaSUT1* was accompanied by low expression of both *TaSuSy1 and TaSuSy2*. As these genes are involved in hydrolyzing the incoming sucrose to hexose sugars, precursors for starch synthesis, our data indicates the critical role played by sucrose synthase in detrimental to dry matter accumulation during grain development. In general, our data suggest that temporal shifts in the expression of *TaSUT1* and *TaSuSy2* might affect sucrose supply and hydrolysis, and thereby starch synthesis, and grain size/dry weight.

Sucrose hydrolysis is also catalyzed by another enzyme, INV. Our attempt to quantify the expression of one of the *INV* genes of wheat (*TaCWINV1*) was not successful as the set of primers used for amplifying the gene produced non-specific band. This enzyme has been shown to have predominant activity during the early stage of seed development and hypothesized to play very minimal role in producing hexose sugars for starch synthesis (Sung et al., 1994). Characterization of the expression patterns of INV genes is important to gain better understanding of dry matter allocation into the reproductive organ. In summary, our data suggest that temporal shifts in the expression of TaSUT1 and TaSuSy2 might affect sucrose supply and hydrolysis, and thereby starch synthesis and grain size/dry weight.

4.0. PREPARATION OF TWO CONSTRUCTS TO EXPRESS HIGH AFFINITY POTATO SUCROSE TRANSPORTER (*StSUT1*) GENE INTO WHEAT

4.1 Abstract

Sucrose transporter proteins (SUTs) present on the plasma membrane of transfer cells in wheat grains play a vital role in active transport of sucrose into the developing endosperm. Recent attempts to increase sink strength have been focused on enhancing sucrose uptake capacity by over-expressing *SUT* genes in storage organs. It was suggested that wheat grain yield can be increased by manipulating SUT protein activity, and this requires identifying a specific promoter to drive the gene of interest. However, promoter analysis with respect to their tissue and stage specificity, and stability in expression is very limited in wheat. In this study, we examined the tissue specificity of High Molecular Weight Glutenin, Dy10 subunit (HMW-GS) in detail by analyzing the expression of a reporter gene, β-glucuronidase (GUS). Transgenic lines expressing Dy10 subunit fused with GUS showed strong GUS expression in ovary/endosperm at all stages of development but with little expression in the anthers protruding after antheis, lemma, palea, stem and leaf tissues. The expression was stable in all three generations, T_0, T_1 and T_2, indicating that this promoter can be used to express foreign genes into wheat seeds. With the ultimate aim of increasing sucrose uptake capacity of developing wheat grains, this promoter was used to prepare constructs to express high affinity potato *SUT* (*StSUT1*) into wheat seeds.

4.2 Introduction

Sucrose is the major transportable form of sugar in cereal crops and is utilized in various cellular growth, developmental and storage processes. Wheat grain comprises 65% to 75% starch synthesized by utilizing the products of sucrose hydrolysis (Dai et al., 2009). Transport of sucrose into the developing endosperm is important for starch biosynthesis. However, the sucrose uptake capacity of SUTs varies with species and even with tissue type within the same plant. It is reported to be biphasic involving high affinity/low capacity and low affinity/high capacity components (Ayre, 2011). The effect of sucrose concentration on transport rate is studied by enzyme kinetics. Sucrose transporter protein reaches maximum rate of transport (V_{max}) when the substrate binding site is saturated (completely occupied; Lin et al., 1984; Lin, 1985). High affinity SUT proteins saturate and attain maximum rate at low sucrose concentration, whereas low affinity transporters take longer time to saturate, and requires higher concentration to attain maximum rate of transport (Lin et al., 1984). Therefore, the maximum velocity of SUT (V_{max}) depends on the rate of transporter activity rather than substrate concentration (Lin, 1985). Affinity properties of SUTs are studied by using radiolabelled (^{14}C) sucrose as substrate with modified yeast cells. Modified yeast cells lack extracellular invertase activity, and thus can use sucrose as sole carbon source for their survival, sucrose affinity is determined by enzyme kinetics and represented as K_m values, which are the concentration of substrate at which the transporter protein reaches half its maximum velocity.

According to Kuhn (2003) plant SUTs are classified into three sub groups: SUT1, SUT2 and SUT4 based on their amino acid sequences. The members of these three subgroups vary in their kinetic properties, substrate affinity and tissue specificity. The SUT1

sub family members are high affinity transporters with K_m values ranging between 139 µM to 1.5 mM. They are mainly from crops including potato (StSUT1), Arabidopsis (AtSUC1), pea (PsSUT1), castor (RcSUT1) and grape (VvSUT1). The SUT2 sub group comprises both monocot and dicot SUTs. Members of the monocot SUTs of SUT2 subgroup exhibit moderate affinity for sucrose with K_m values ranging between 4-20 mM and include SUTs of rice (OsSUT1 and OsSUT5), maize (ZmSUT1), barley (HvSUT1) and wheat (TaSUT1). Dicot members of this group include SUTs from Arabidopsis (AtSUT3), potato (StSUT2), and tomato (LeSUT2). These proteins possess 30 more aminoacids in their N terminal region and have elongated central loop that is similar to yeast sugar sensors. Indeed, the AtSUT3 and LeSUT2 of Arabidopsis and tomato are reported as sugar sensors (Barker et al., 2000; Meyer et al., 2004). The SUT4 subgroup comprises low affinity SUTs from dicots including Arabidopsis (AtSUT4), tomato (LeSUT4) and potato, (StSUT4), and monocots including barley (HvSUT2) and rice (OsSUT2) with Km values between 5 mM and 6 mM.

In an attempt to modify sucrose uptake capacity in pea cotyledons, high affinity potato *SUT* (*StSUT1*) was over-expressed in pea seeds. The resulting transgenic plants showed 2-fold increase in endogenous SUT activity and similar influence on seed growth rate (Rosche et al., 2002). Constitutive over-expression of spinach SUT (*SoSUT1*) in potato also resulted in increased (4-fold) sucrose uptake in plasma membrane vesicles and also a shift in carbon partitioning between leaves and tubers (Leggewie et al., 2003). However, the over-expression had less impact on tuber morphology, growth and metabolism of potato tubers. Furthermore, over-expression of barley *SUT* (*HvSUT1*) in wheat by using endosperm specific hordein B1 promoter resulted in 30% to 40% increase in sucrose uptake by developing endospermic cells as compared to that in the control wild type (Weichert et al.,

2010). Thus, it is evident from these studies that high affinity SUT genes from other plants can be over-expressed to increase sucrose uptake capacity of cultivated plants.

The rate of starch synthesis in wheat grains depends on activity of SUT protein and rate of sucrose supply during grain development. In wheat, one *SUT* gene (*TaSUT1*) that has three homologous in the three genomes (*TaSUT1A*, *TaSUT1B* and *TaSUT1D*) has been isolated and reported to play an important role in transporting sucrose into the developing endosperm (Aoki et al., 2002). Their amino acid sequences showed more than 98% similarity among themselves, and 86% to 97% similarity to other cereal SUT1 sequences (Aoki et al., 2002).

Use of tissue specific promoters targets transgenes in specific tissues and stages of development, and minimizes the accumulation of transgenic proteins in unwanted plant tissues and stages of development. Until recently, it has been difficult to analyze promoter activity in monocots due to lack of standard protocols and hence various monocot promoter genes have been heterologously tested in dicot plants. For example, the maize zein promoter (Z4) was studied in a tobacco system. Fusion of the zein promoter sequence with *gusA* reporter gene showed GUS expression in the seeds of transgenic tobacco lines, indicating its seed specificity (Schernthaner et al., 1988). Similarly, expression of GUS reporter gene under the control of rice glutelin promoter (Gt3) in tobacco resulted in seed specific GUS activity (Leisy et al., 1989; Zhao et al., 1994). Furthermore, expression of the GUS reporter gene under the control of wheat HMW-GS promoter in tobacco showed seed specific GUS activity (Thomas and Flavell, 1990).

Expression of a target gene promoter with a reporter gene in a heterologous system provides a similar qualitative result as that observed in a native system. However,

quantitative characterization of the reporter gene expression often shows a difference between the two systems (Stoger et al., 1999). In wheat, glutenin is the major seed storage protein synthesized in the endosperm, and is an important flour processing characteristic (Halford et al., 1992). Glutenin belongs to prolamines family of triticeae, which is further divided into two major subunits, High Molecular Weight Glutenin Subunit (HMW-GS) and Low Molecular Weight Glutenin Subunit (LMW-GS; Masci et al., 1998). The gene *Glu-1* encodes HMW-GS and *Glu-3* encodes LMW-GS. The temporal and spatial expressions of both subunits are similar but differ in their expression level (Lamacchia et al., 2001). The *HMW-GS* genes have alleles that are tightly linked, each of which encodes proteins that account for 2% of total seed protein, and together they have higher expression than LMW-GS (Lamacchia et al., 2001). Studies with HMW-GS indicated that it has tightly linked genes (x and y) in *Glu-1* locus. The alleles *Glu-D1-1b* encoding the Dx5 subunit and *Glu-D1-2b* encoding the Dy10 subunit are the most studied glutenin subunits because of their dough making characteristics (Blechl and Anderson, 1996). Because of their tightly linked nature, it had been difficult to study the contributions of each gene (x and y genes) in dough making quality by conventional breeding. However, isolation of genes encoding different glutenin subunits and genetic engineering techniques have made it possible to express each subunit individually and study its end use properties.

The transformation of *LMW-GS* expressing *GUS* reporter gene in wheat indicated its seed specific expression (Stoger et al., 1999). However, the authors indicated the requirement of two or more copies for stable integration of the transgene in successive generations. In contrast, over-expression of *Glu-D1-1b* that encodes HMW-GS Dx5 fused with the GUS reporter gene showed stable GUS expression over three generations and was

seed specific (Lamacchia et al., 2001). This indicates the higher expression level of HMW-GS in wheat seeds. It was also suggested that transgene expression of Dx5 and Dy10 along with expression of endogenous glutenin subunits provided transgenic lines with high quality flour for bread making (Blechl et al., 2007). Fusion of two native glutenin subunits *Dy10* at 5' and *Dx5* at 3' sites resulted in effective transcriptional regulation of high molecular weight glutenin subunit (HMW-GS) and increased seed storage protein accumulation in wheat seeds than native HMW-GS (Blechl and Anderson, 1996). This promoter employed successful transfer of the AGPase large subunit from maize (shrunken 2) into wheat endosperm and resulted in 20 times higher expression of the transgene as compared to that driven by native maize promoter (Meyer et al., 2004a).

This study aims at characterizing the Dy10 subunit of glutenin which can be used to alter source-sink relationship. We employed Dy10 subunit of $pGSH_2$ plasmid and analyzed its activity in wheat tissues by transgenic GUS expression. Histochemical analysis of the temporal and spatial pattern of Dy10 subunit expression was performed in transgenic lines. Also, with a view to increase sucrose uptake capacity in wheat grains, two constructs were prepared, one to express high affinity potato *SUT* (*StSUT1*) in wheat by using seed specific glutenin promoter (Dy10 subunit) and another one to express *StSUT1* constitutively by using a maize ubiquitin1 promoter.

4.3 Materials and methods

4.3.1 Preparation of glutenin: GUS construct

4.3.1.1 Restriction digestion

Seed specific high molecular weight glutenin promoter subunit Dy10 from pGSH2 plasmid, kindly provided by Mike Giroux at University of Montana (Meyer et al., 2004a, Appendix 7.17) was sub cloned into pCAMBIA1391Z vector between *EcoRI* and *BamHI* restriction sites in the PUC9 multiple cloning site regions. pCAMBIA1391Z is a binary and promoterless vector with a GUS scorable reporter gene, the Nopaline synthase (*Nos*) gene for transcription termination, the Lac Z for bacterial selection and the hygromycin resistance (*hptII*) gene for plant selection, map shown in Appendix 7.18. Restriction digestion of pCAMBIA1391Z and pGSH2 plasmids with *EcoRI* and *BamHI* sites provided complementary sites for ligation. The plasmid pGSH2 contains three *EcoRI* sites, and yields two bands of almost similar size (2.9 Kb), which created a difficulty to isolate a specific band corresponding to glutenin promoter. To this end, pGSH2 was digested with three enzymes, which facilitated the separation of glutenin specific band. The digestion reaction of the pGSH2 vector contained 2 µl of 10X Fast Digest buffer, 4.5 µl of pGSH2 plasmid (230 ng/µ1), and 1 µl each of three Fast Digest enzymes (1 U/µl) *EcoRI*, *XbaI* and *BamHI*, in a total volume of 20 µl. The digestion mixture of pCAMBIA1391Z vector contained 2 µl of 10X Fast Digest buffer, 4.5 µl (200 ng) of pCAMBIA1391Z, 1 µl of each of two Fast Digest enzymes *EcoRI* and *BamHI* (1 U/µl) in a total volume of 20 µl.

4.3.1.2 Agarose gel electrophoresis and purification of digested products

The digested products were mixed with 1X DNA loading buffer [(0.126% (w/v) xylene cyanol, 0.126% (w/v) bromophenol blue, 10% (w/v) sodium dodecyl sulphate (SDS), 62.5% glycerol (v/v)], and loaded into a 1% agarose gel for separation by gel electrophoresis in 1X TAE buffer (1 L of 50X stock contained 242 g Tris, 57.1 mL glacial acetic acid, 100 mL of 0.5 M EDTA, pH 8.0) at 120 V following staining with EtBr, the band was visualized through ultraviolet light emitted from a transilluminator (Fisher Scientific).

Bands with expected size for glutenin promoter (2935bp) and pCAMBIA1391Z plasmid (11 Kb) were extracted from the gel using QIAquick Gel Extraction Kit (Qiagen). DNA fragments were sliced out and mixed with 3 volumes of guanidinium thiocyanate (buffer QG) and incubated at 50°C until the gel is dissolved. After adding one gel volume of isopropanol, the mixture was transferred into a QIAquick spin column and centrifuged at 13,000*g* for 1 min to bind the DNA to the spin column. Any residual gel was removed by adding 0.5 mL buffer QG to the QIAquick column followed by centrifugation and a wash with 0.75 mL 100% ethanol (buffer PE). The column was centrifuged further to remove any remnant ethanol, and then transferred to a clean tube for elution of the DNA with nuclease-free water. The eluted DNA was stored at -20°C until ligation.

4.3.1.3 Ligation

The ligation reaction was set up in an eppendorf tube containing 5 µl of 2X ligation buffer, 6 µl of the gel purified vector (pCAMBIA1391z, 36 ng/µl) and 3 µl of the insert (HMW glutenin, Dy10 subunit 33 ng/µl) in a 2:1 insert: vector ratio, and 1 µl of T4 DNA ligase in a total volume of 15 µl. The mixture was incubated at 4°C overnight.

4.3.1.4 Bacterial transformation

For bacterial transformation of the ligation product, frozen DH5α competent cells (50 µl) were thawed on ice and approximately 5 µl of the ligation mixture was added into it. The reaction mixture was incubated on ice for 20 min before they were given a heat shock at 37 °C for 40 sec. The cells were then subjected to incubation on ice to condition the cells for uptake of the transformed plasmids. The transformed cells were mixed with Luria-Bertani (LB) medium (1ml, pH 7.0) that contains 10 g tryptone, 5 g yeast extract and 10 g NaCl per litre and incubated on a shaker (200 rpm) at 37°C for 1 h (New Brunswick Scientific). The cells were then plated on LB agar plate containing 50 mg/l kanamycin, 0.5 mM/ml isopropyl β-D-1-thiogalactopyranoside (IPTG; Fermentas) and 0.08 mg/ml X-gal (Promega), and incubated at 37°C overnight. As the IPTG and X-gal favours blue/white selection, the positive clones with insert were expected to be white in colour.

4.3.1.5 Colony PCR

White colonies that grown on plates were selected and screened by PCR using gene specific forward and reverse primers designated as GluF and GluR (Table 4.2). The PCR reaction mixture contained 4 µl of 5X iproof buffer (1X/µl), 0.4 µl dNTP mix (0.2 mM/µl; Bio-Rad), 1 µl of GluF (0.5 µM/µl), 1 µl of GluR (0.5 µM/µl), 0.2 µl of iproof high-fidelity DNA polymerase (0.02 U/µl) and a selected colony in a total volume of 20 µl. The PCR amplification was carried out in thermal cycler (Bio-Rad) under the following conditions: initial denaturation and activation of polymerase at 98°C for 30 s, 35 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 30 s and extension at 72°C for 90 s followed by final extension at 72°C for 10 min. PCR products were electrophoresed in agarose gel (1%), and

the colony samples showing the expected band size (3 Kb) were grown overnight in LB medium containing kanamycin (0.5 mg/ml) at 37°C on a shaker (150 rpm).

4.3.1.6 Plasmid extraction

Plasmid extractions were performed with QIAprep Spin Miniprep Kit (Qiagen). The cells grown overnight were centrifuged at 3800*g* for 5 min, and pelleted cells were resuspended in 250 µL RNase A (Buffer P1) and 250 µL of buffer P2 (contains SDS and NaOH) and incubated at room temperature for 2 min. Then the buffer N3 (350 µL) containing guanidinium chloride and acetic acid was added and mixed thoroughly. The mixture was centrifuged at 16200 *g* for 10 min and the supernatant containing the isolated plasmids was transferred to a QIAprep spin column, which was centrifuged at 13,000 *g* for 1 min. After the flow through was discarded, the column was washed with 0.5 mL of buffer PB (containing guanidinium chloride and isopropanol) and then with 0.75 mL of 100% ethanol. Then the column was further centrifuged for an additional 1 min to remove any residual buffer, and transferred to a clean tube for elution of the DNA with sterile water (40 µl) by centrifugation at 13,000*g* for 1 min. The presence of the insert in the plasmid was verified by restriction digestion and sequencing.

4.3.2 Biolistic transformation

4.3.2.1 Plant material

Seeds of wheat cultivar Fielder kindly provided by Dr. Mark Jordan's research group (Cereal Research Centre- Agriculture and Agri-Food Canada) were germinated a Petri plates system, and transferred into pots containing LA4 soil mix. Plants were grown in a greenhouse at 18°C/16°C (day/night) with a photoperiod of 16/8 h. Plants were watered in alternative days and fertilized with 20:20:20 (N: P: K) mixture once a week and the developing spikes were tagged at anthesis (when protrusion of the first yellow anther observed). Spikes were then collected at 12-14 DAA and the embryo isolated from the middle region of the spike.

4.3.2.2 Embryo isolation and pre-culture

Seeds were separated from the spikes and sterilized with 70% ethanol for 2 min followed by 20% of commercial bleach for 20 min and rinsed (3 times) with sterile distilled water. Embryos of 0.5-0.8 mm in size were isolated and pre-cultured with scutellar side up for 2 days in RG5-N media. The medium consisted of MS salts (Murashige and Skoog, 1962; Table 4.1) supplemented with 2, 4- Dichlorophenoxyacetic acid (2 mg/l); thiamine (0.4 mg/l), glutamine (100 mg/l), niacinamide (1 mM/l) and phytagel 2.5 g/l (Jordan, 2000).

4.3.2.3 Preparation of DNA coated particles and particle bombardment

Particle bombardment of the DNA construct was performed according to Jordan (2000). Pre-cultured embryos were transferred to osmoticum media (RG5-O) containing 36.44 g/l sorbital and 36.44 g/l mannitol in RG5-N without niacinamide.

Table 4.1. Components of Murashige and Skoog (MS, 10X) stock solution (Murashige and Skoog, 1962)

Component	Final Concentration (mg/L)
$MgSO_4$ $7H_2O$	370
$MnSO_4$ H_2O	16.9
$CaCl_2$ $2H_2O$	435
NH_4NO_3	1650
KNO_3	1900
KH_2PO_4	170
H_3BO_3	6.2
$ZnSO_4$ $7H_2O$	8.5
$CuSO_4$ $5H_2O$	0.025
KI	0.75
$CoCl_2$ $6H_2O$	0.025
Na_2MoO_4 $2H_2O$	0.25
Fe(III)EDTA	424
Nicotinic Acid	0.5
(B6) Pyridoxine	0.5
(B1) Thiamine HCl	0.1
Myo-inositol	100

Embryos were placed in the form of circle of 1-2cm diameter at the centre of a Petri plate and kept under darkness for 4 h. Plasmid DNA (5 µl, 1 µg/µl) was mixed with 25 µl of gold particle (0.6 µM) solution to which 10 µl spermidine (0.1 M) and 25 µl $CaCl_2$ (2.5 M) were added. The mixture was vortexed and then purified by centrifugation and ethanol (70%) precipitation. The resulting gold coated DNA particles were resuspended in 95% ethanol, air

dried and then loaded to the centre of a macro carrier. The PDS1000-He particle delivery system was adjusted to 1100 psi of helium pressure and 25 mm Hg of vacuum pressure to bombard the gold coated DNA particles into the embryos in a petriplate placed on a sample holder shelf at a 7 cm target distance. After particle bombardment, the embryos were incubated overnight at room temperature under darkness.

4.3.2.4 Callus induction, selection and regeneration of transgenic plants

Bombarded embryos were transferred to callus induction media (RG5 solidified with phytagel [2.5 g/l] without niacinamide) and incubated at room temperature in darkness for two weeks. The callus formed on RG5 media was transferred to selection media (½ MS media containing hygromycin [50 mg/l]) and kept in a growth chamber at 24^{o}C under 16/8 h (light/dark) conditions for three weeks. The calli that formed green shoots were transferred to regeneration media (the same as callus induction medium but containing hygromycin [50 mg/l]) and incubated in a growth chamber for two weeks. Hygromycin resistant plantlets were transferred to pots containing LA4 mix and Cornell fertilizer mix (26% $CaCo_3$, 40% osmocote, 18:6:12 NPK, 32% superphosphate, 0.53% micro nutrients, 0.4% chelated iron and 0.2% chelated zinc) for rooting. Generated transgenic plants were grown in a growth chamber at 20°C/16°C (day/night) in a 17/7 h photoperiod.

4.3.2.5 GUS assay

Individual spikes from the transgenic plants were tagged at the first day of anthesis. Flag leaf, peduncle and spikelets were collected at different seed developmental stages (1 to 30 DAA). Thin sections of each tissue samples were treated with 1 ml of X-Gluc solution, which

contained (50 ml of 200 mM $NaPO_4$; 0.5 ml of 0.1 M potassium ferricyanide; 0.5 ml of 0.1 M potassium ferrocyanide; 2 ml of 0.5 M EDTA (pH 8), 100 µl of Triton-100 in 100ml) and incubated at 37°C for two days. The green tissues (flag leaf, peduncle, spikelets) were de-chlorophyllized in 70% ethanol to visualize the blue staining as described previously (Hirose et al., 2010).

4.3.3 Preparation of constructs to express potato sucrose transporter (*StSUT1*) gene

4.3.3.1 Construct for constitutive expression of *StSUT1*

For constitutive expression of *StSUT1*, a construct was prepared using gateway technology. Gateway cloning involves two types of vectors: an Entry vector to clone gene of interest and a destination vector with a T-DNA region for DNA delivery into plants. The attLl and aatL2 regions of the entry vector, and attR1 and attR2 of the destination vector contain unique restriction sites which ensure site specific recombination. The destination vector employed in this study is pBract214, which is a binary vector containing a maize ubiquitin 1 promoter for constitutive expression of the transgene, a hygromycin resistance gene for screening transgenic plants, and a kanamycin resistance gene for barcterial selection. Schematic representation of the entry vector pENTR™1A and destination vector pBract214 is shown in Appendices 7.19 and 7.20, and the steps involved in preparing the *ubiquitin*: *StSUT1* construct are described below:

i) Cloning of *StSUT1* into pENTR1A entry vector

The *StSUT1* gene isolated from potato cv. Russet Burbank (Appendix 7.22) and cloned into pGEM-T-Easy vector was prepared by Zhen Yao, a research technician in the lab. The

restriction enzyme recognition sites, *Sal1* and *Xhol1*, were added to the 5' and 3' ends of *StSUT1*, respectively, by PCR amplification. The PCR reaction contained 5 µ1 of 10X reaction buffer, 5 µl of 10 Mm dNTPs (2 mM), 1 µl of forward primer *StSUT1* linked with *SalI* (0.5 µM/µl), 1 µl of reverse primer *StSUT1* linked with *XhoI* (0.5 µM/µl), 1 µl plasmid (100 ng), and 0.2 µl of thermostable Taq DNA polymerase enzyme (1.25 U/µl). The sequence information for primers (with restriction sites) is shown in Table 4.2. The PCR amplification was carried out in a thermal cycler (Bio-Rad) under the following conditions: initial denaturation and polymerase activation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 60 s, followed by final extension at 72°C for 10 min. The PCR product was separated by agarose gel electrophoresis. The band corresponding to *StSUT1* fragment (1.5 Kb) was extracted from the gel using the QIAquick gel extraction kit as described previously. For ligating the *StSUT1* gene into pENTR1A gateway entry vector, both the purified insert and plasmid vector pENTR1A were digested with *SalI* and *XhoI* restriction enzymes (Fermentas). The reaction mix for plasmid digestion contained 2 µl of 10X Fast Digest buffer, 2 µl of plasmid (50 ng/µl), and 1µl of each Fast Digest enzyme *SalI* and *XhoI* (1 U/µl). The digestion reaction for PCR product of *StSUT1* consisted of 2 µl of 10X Fast Digest buffer, 6 µl of purified PCR product (150 ng/µl) and 1 µl of each Fast Digest enzymes *SalI* and *XhoI* (1 U/µl). The components were mixed gently and incubated at 37 °C for 3 h, after which the digested products were separated by agarose gel electrophoresis. The digested fragment of the PCR product (*StSUT1*) and pENTR1A plasmid that contained complementary sites for ligation were gel purified as described above. The ligation reaction contained 4 µ1 of 2X ligation buffer, 2 µl of digested pENTR1A (50 ng/µl), 3 µl of insert *StSUT1* (50 ng/µl) in a 3:1 insert: vector ratio, and 1 µl

of T4 DNA ligase (Promega) in a total volume of 10 µl. The reaction mix was incubated overnight at 4°C, and then used for bacterial transformation.

ii) Screening of transformed colonies and plasmid extraction

Colonies grown on the LB media containing plate were screened by colony PCR using gene specific *StSUT1* primers linked with *SalI* and *XhoI* sites in forward and reverse primers respectively. Positive colonies that yielded expected the band size (1.5 Kb) were cultured in LB broth, plasmids were extracted using QIAprep Spin Miniprep Kit and used for ligation reaction (Invitrogen).

iii) Ligation

The ligation reaction was set up in a total volume of 10 µl according to the manufacturer's instruction containing 1 µl of pENTR1A carrying *StSUT1* (50 ng/µl), 2µl of pBract 214 (100 ng/µl), 5 µl of Tris EDTA buffer, and 2 µl of LR clonase II enzyme kit (Invitrogen) and incubated at 25°C overnight. The ligated product was transformed to *E.coli* cells, plated on kanamycin (50 mg/l) containing media, and then incubated at 37°C overnight. Positive colonies were selected by PCR screening using backbone primer Ubi promD provided by the manufacturer as forward primer and gene specific *StSUT1R* (Table 4.2). Plasmids were extracted from positive colonies and the *StSUT1* gene sequence was verified by sequencing (Macrogen).

4.3.3.2 Construct for seed specific expression of *StSUT1*

For seed specific expression of *StSUT1*, glutenin promoter subunit Dy10 was used. The construct was prepared in pBluescript II SK^+ vector, which was modified to include a Nos terminator gene in the *SacI* site of its multiple cloning region (Appendix 7.24).

Table 4.2. Primers used for construct preparation

Construct	Primers	Sequence	Amplicon length (bp)
Glutenin: GUS	GluF[a]	GAATTCACTAGAATTGTGTTACACCCCAC	2935
	GluR[b]	GTGGACTGTCAGTGAATTGATCTCTATT	
Ubi1: StSUT1	SalIF	ATAGTCGACATGGAGAATGGTACAAA	1500
	XhoIR	TACTCGAGTTATTTAATGGAAAGCCC	
Glutenin: StSUT1	XbaIF	CATGTCTAGAATGGAGAATGGTACAAAAAGAGAAGG	1500
	BstXIR	TACGCCAAGCGATTGGTTGTAATTATTTAA	
StSUT1	F	ATGGAGAATGGTACAAAAAGAGA	1500
	R	GTAATTATTTAATGGAAAGCCCC	
Ubi promD	F	GCATATGCAGCAGCTATATGTG	

[a] Forward primer; [b]Reverse primer

i) Cloning of glutenin promoter into pBluescript II SK^+ vector

Ligation of the glutenin promoter to pBluescript II SK^+ vector was performed as follows: The plasmid pGSH2 (containing a glutenin promoter) and pBluescript II SK^+ were digested with *EcoRI* and *BamHI* restriction enzymes. The reaction mix for digesting pGSH2 vector (to isolate the glutenin promoter) contained 2 µl of 10X Fast Digest buffer, 4.5 µl of pGSH2 plasmid (230 ng/µ1), and 1 µl of each Fast Digest enzymes, *EcoRI, XbaI* and *BamHI* (1 U/µl) in a total volume of 20µl. The reaction mix for the digestion of pBluescript II SK^+ vector contained 2 µl of 10X Fast Digest buffer, 2.5 µl of pBluescript II SK^+ plasmid (445 ng/µ1), and 1 µl of each Fast Digest enzyme, *EcoRI* and *BamHI* (1 U/µl) in a total reaction volume of 20 µl. The reactions mixtures were incubated at 37°C for 30 min, and the digested

products were separated by agarose gel electrophoresis. Then bands with expected size of glutenin promoter and pBluescript II SK^+ plasmid DNA were gel purified using QIAquick Gel Extraction Kit as described previously. Purified digested products provide complementary sites at the 5' and 3' ends for ligation. The ligation mixture consisted of 1.5 µl of digested plasmid pBluescript II SK^+ (35 ng/µl) and 5 µl of insert glutenin (30 ng/µl) in a 3:1 insert:vector ratio and 1 µl of T4 DNA ligase kit (Promega) in a total reaction volume of 15 µl, and the mixture was incubated at 4°C overnight. The ligated product (5 µl) was transformed to *E.coli* cells (DH5α), and plated on ampicilin (100 µg/ml) containing media. Colonies were screened by PCR using glutenin specific primers (*Glu* forward and *Glu* reverse; Table 4.2), and plasmids extracted from the positive colonies were used for cloning *StSUT1*.

ii) Cloning *StSUT1* into pBluescript II SK^+ containing glutenin promoter

The *Xba1* and *BstX1* sites were added to *StSUT1* gene at its 5' and 3'ends, respectively, by PCR amplification using *Xba1* linked forward and *BstX1* linked reverse primers (Table 4.2). The PCR products were gel purified and digested with 1µ1 each of *Xba1* and *BstX1* restriction enzymes (1 U/µl). The pBluescript II SK^+ containing glutenin promoter was digested with the same restriction enzymes. The ligation reaction was performed using T4 ligase kit (Promega) following the manufacturer's protocol, and then the ligated products were transformed to *E.coli* (DH5α) cells. Positive colonies were selected by PCR screening using *Xba1F* and *BstX1R* linked primers. The insertion of *StSUT1* into the pBluescript II SK^+ vector was verified by sequencing as described previously.

4.4 Results

4.4.1 Promoter analysis

4.4.1.1 Generation of glutenin: GUS construct

In order to generate Glutenin: GUS construct in a pCAMBIA1391Z plasmid, the glutenin promoter was digested out from pGSH2 (Figure 4.1A), and ligated into a pCAMBIA1391Z plasmid digested with the same restriction enzymes as pGSH2. The size of glutenin promoter ligated into pCAMBIA1391Z was verified by restriction digestion, which produced DNA fragment with expected band size (Figure 4.1B). The cloned glutenin promoter showed 100% similarity with the original glutenin subunit Dy10 in pGSH2 (Appendix 7.23).

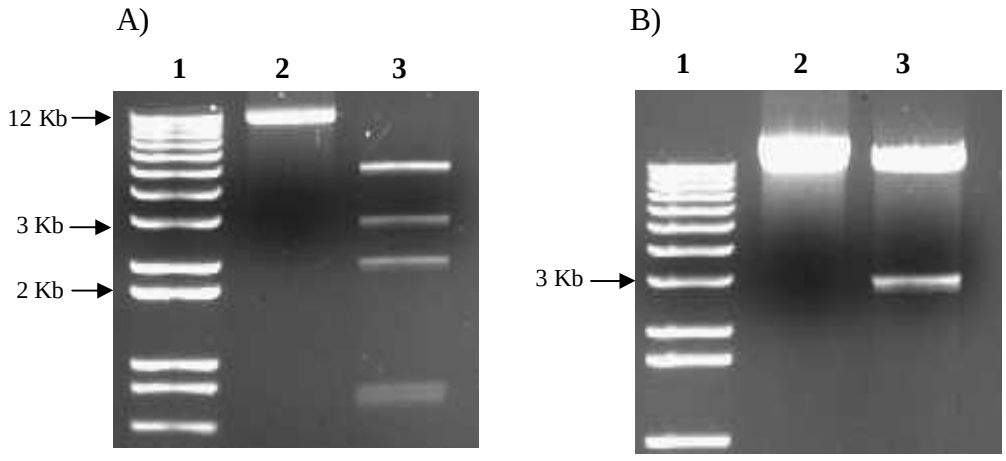

Figure 4.1. Digestion analysis of pGSH2 with *EcoRI*, *BamHI* and *XbaI* in 1% agarose gel (A). From left to right, DNA marker 1 Kb+ (lane 1), undigested pGSH2 (lane 2) and digested pGSH2 (lane 3). Digestion analysis of pCAMBIA1391z containing glutenin subunit Dy10 (B). From left to right, DNA marker 1Kb+ (lane 1), undigested pCAMBIA1391Z (lane 2), digested pCAMBIA1391z (lane 3). The bands with expected size of 3Kb that corresponding to glutenin subunit Dy10 is shown in both cases.

4.4.1.2 Biolistic transformation

Out of 400 bombarded embryos, almost all the embryos developed callus after two weeks of incubation in darkness, however, only 12 of them developed green shoots within two weeks

of incubation on a media containing hygromycin (50 mg/l, Figure 4.2 A). After two successive selections with hygromycin, two putative transgenic plants were obtained and designated as transgenic line 1 and line 2 (Figure 4.2 B). The product of GUS gene reacts with X-Gluc solution, and thereby develops blue colour, which is used as a marker for GUS expression. Blue staining analysis in both vegetative and reproductive tissues revealed strong expression of GUS in developing ovaries and seeds at one day before anthesis, first day of anthesis, 4, 8, 6 and 25 DAA (Figure 4.3). Expression of GUS was also observed in the anthers that protruded out after antheis. Its expression was relatively weak in the lemma and palea tissues. No expression of GUS was observed in the vegetative tissues (stem and flag leaf) at any of the stages examined (Figure 4.3). This confirms the seed/reproductive organ specific activity of HMW glutenin Dy10 subunit.

A) B)

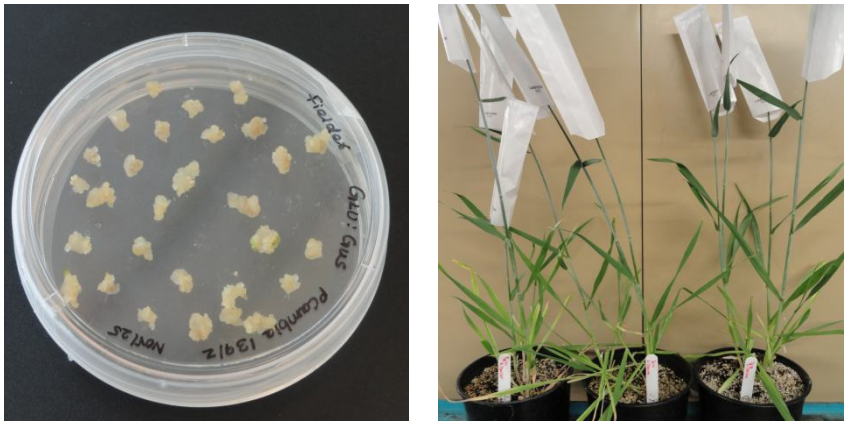

Figure 4.2. Developing callus (A) and the resulting transgenic plants expressing GUS under the control of HMW glutenin Dy10 subunit (B).

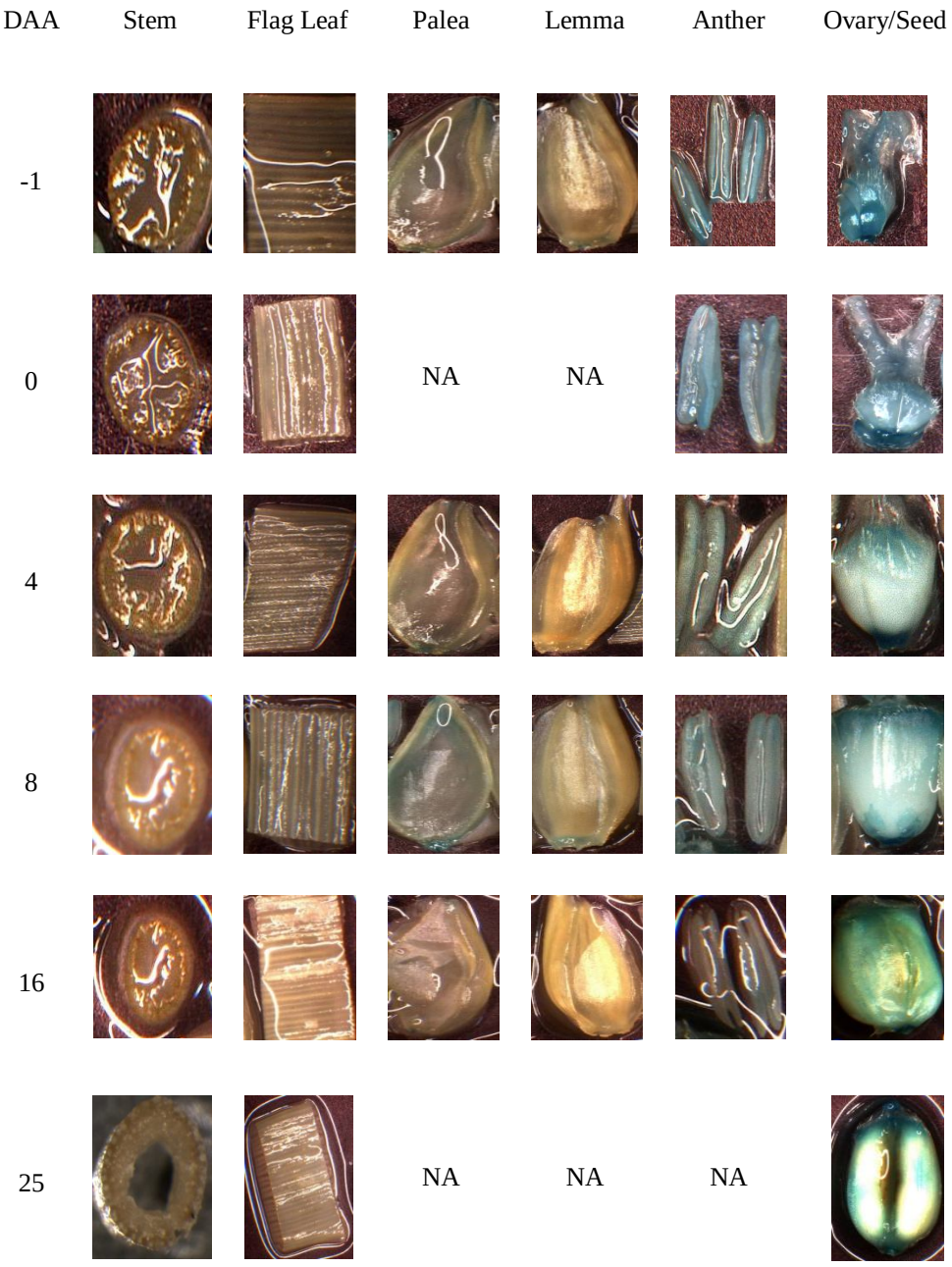

Figure 4. 3. GUS expression (blue staining) in different tissues of wheat: stem, flag leaf blade, palea, lemma, anthers protruding to seeds after antheis, and ovary tissues at different stages of seed development.

4.4.2 Generation of ubi1: StSUT1 construct

The *StSUT1* fragment cloned into the entry vector pENTR1A was verified by colony PCR, and the PCR products produced a band with expected band size of *StSUT1* (1.5Kb, Figure 4.4A). The pENTR1A containing *StSUT1* was used for ligation reaction to transfer it to the destination vector, pBract214. The presence of *StSUT1* in the destination pBract214 vector was verified by colony PCR, and the positive colonies produced a band with expected size of 1.5 Kb (Figure 4.4B). Subsequent sequencing of the *StSUT1* cloned into pBract214 showed 99% similarity with the *StSUT1* sequence available in the NCBI database (Appendix 7.26).

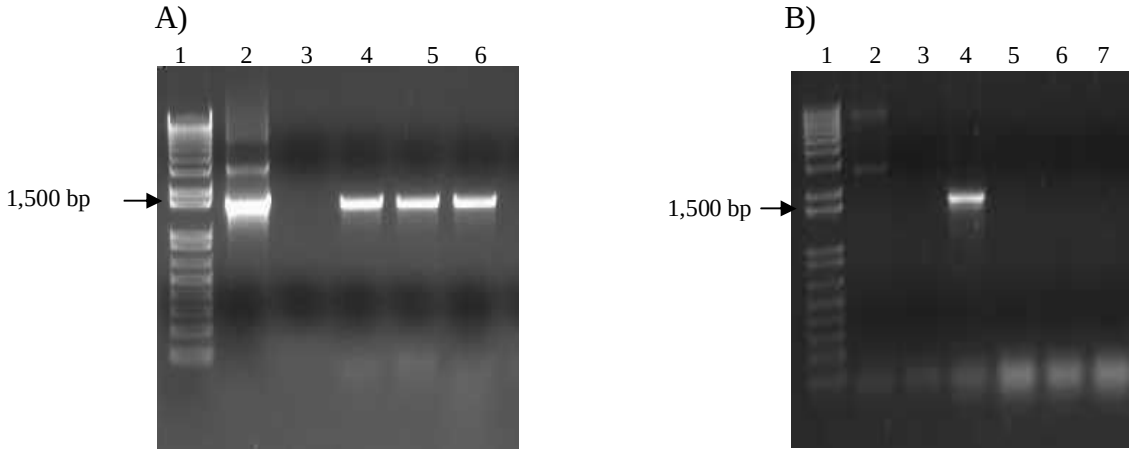

Figure 4.4. PCR analysis of transformed *E.coli* cells containing *StSUT1* in pENTR1A (A). From left to right, 1 Kb^+ DNA marker (lane 1); *StSUT1* positive control (lane 2); *StSUT1* from positive colonies (lane 4, 5 and 6). PCR analysis of transformed *E.coli* cells containing *StSUT1* in pBract 214 (B) from left to right, 1 Kb^+ DNA marker (lane 1); 1.5 Kb band corresponding to *StSUT1* from a positive colony (lane 4).

4.4.3 Generation of glutenin: StSUT1 construct

i) Cloning glutenin promoter into pBluescript II SK$^+$ vector

The presence of glutenin sequence in pBluescript II SK$^+$ was verified by PCR amplification using *GluF* and *GluR* primers (Table 4.2), which produced the expected band size of 3 Kb (Figure 4.5).

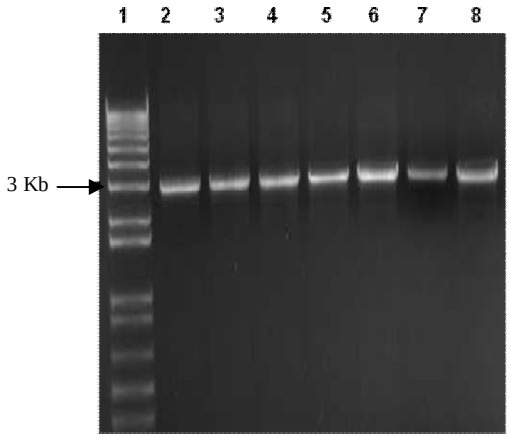

Figure 4.5. PCR analysis of transformed *E.coli* cells containing glutenin promoter Dy10 subunit in pBluescript II SK$^+$. From left to right, DNA marker 1Kb+ (lane 1) and amplified products (3 Kb) of glutenin promoter (lanes 2-8).

ii) Cloning *StSUT1* to glutenin containing pBluescript II SK$^+$

The ligation of the *StSUT1* to pBluescript II SK$^+$ was verified by transforming ligated products to *E.Coli* cells and screening the colonies by PCR using *StSUT1F* and *StSUT1R* primers (Table 4.2). The positive clones containing the gene of interest gave expected band size of 1.5 Kb, which corresponds to *StSUT1* (Figure 4.6A). Restriction digestion on pBluescript II SK$^+$ containing glutenin and *StSUT1* with *XbaI* and *BstXI* gave expected bands of size 6 Kb (vector backbone + Glutenin) and the 1.5 Kb *StSUT1* as shown in Figure 4.6B. The cloned *StSUT1* sequence showed 99% sequence homology with the *StSUT1* sequence available in the NCBI database (Appendix 7.25).

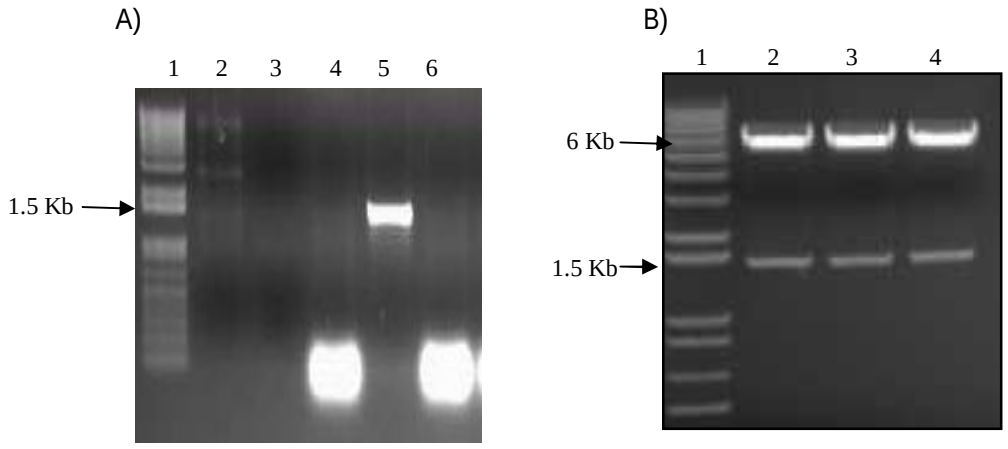

Figure 4.6. PCR analysis of transformed *E.coli* cells containing *StSUT1* cloned into pBluescript II SK^+ (A). DNA marker 1Kb+ (lane 1), PCR product corresponding to the *StSUT1* gene from a positive colony (lanes 5). Digestion analysis of pBluescript II SK^+ containing glutenin promoter and *StSUT1* with *XbaI* and *BstxI* restriction enzymes (B). DNA marker $1Kb^+$ (lane 1), digested products *StSUT1* 1.5 Kb (lane 2-4), vector backbone and glutenin promoter 6 Kb (lanes 2-4).

4.5 Discussion

4.5.1 High molecular weight glutenin promoter subunit Dy10 is seed specific

The success of genetic transformation depends on several factors including delivery of the transgene at a specific developmental stage into a particular tissue, which avoids unwanted accumulation of proteins in other tissues. Tissue specific promoters play an important role in spatial and temporal expression of a transgene. Regulatory sequences of seed storage proteins were used to express foreign proteins in grains of rice (Sindhu et al., 1997), maize (Russell and Fromm, 1997), barley (Choi et al., 2003) and wheat (Lamacchia et al., 2001). In rice, over-expression of the pea legumin gene (*LegA*) under the control of a seed specific rice glutelin promoter resulted in legumin accumulation in transgenic rice seeds (Sindhu et al., 1997). The lysine content in maize has been increased from 16% to 54% in transgenic maize, over-expressing potato gene *sb401* that encodes lysine rich protein under the control of maize

seed specific zein promoter (p19z). Analysis of transgenic lines over six generations showed stable transgene expression (Yu et al., 2005).

Glutenins account for 80% of storage protein in wheat seeds, and their HMW subunits play important role in dough making properties (Stoger et al., 1999). The characterization of HMW-GS (Dx5) in transgenic wheat expressing GUS reporter gene indicated its seed specificity (Lamacchia et al., 2001). Histochemical analysis of the GUS assay indicated its expression in endosperm but not in embryos or other vegetative tissues such as leaves and roots. The GUS staining was prominent at 10, 12, 14 and 21 DAA. Blechl and Anderson (1996) reported the expression of hybrid glutenin subunits Dy10: Dx5 that resulted in an increase in total HMW glutenin proteins in transgenic lines as compared to the control (untransformed) bobwhite cultivar. It has also been suggested that the regulatory sequences of HMW-GS could be employed to control expression of other endospermic genes or to express a foreign gene in wheat endosperm. Expression of the modified AGPase gene from maize (*SH2*, which is a rate limiting enzyme in starch biosynthesis) driven by the glutenin subunit Dy10: Dx5 resulted in 20 times more expression of the transgene than that driven by maize shrunken gene promoter (Meyer et al., 2004a).

In this study, we examined the temporal and spatial expression of HMW-GS (Dy10) by transgenic GUS assay. The GUS expression observed in ovary and anthers before antheis indicates the promoter activity occurs in reproductive tissues even before anthesis. It supports the hypothesis by Meyer et al. (2004a), in which it was suggested that, a possibility of *SH2R6HS* expression in pre-flowering ovules might trigger source tissues to respond to sink strength manipulation, resulting in altered seed set and number of spikes per plant. The prominent GUS activity in the endosperm at 4, 8, 16 and 25 DAA indicates its temporal

expression pattern. This is consistent with the results of Lamacchia et al. (2001), where the GUS expression driven by HMW-GS (Dx5) was studied by RT-PCR and histochemical staining. The transverse and longitudinal analysis of caryopsis from GUS transgenic lines showed endosperm specific expression at 10, 12, 14 and 21 DAA. The GUS activity was not observed in stem or flag leaf tissues at any of the analyzed stages, indicating the seed specificity of HMW-GS (Dy10). The similar pattern of GUS activity in two subsequent generations demonstrates transgene stability over generations. According to Blechl and Anderson (1996), a single copy of hybrid subunits Dy10: Dx5 is sufficient to enhance glutenin protein level in wheat seeds. Our result demonstrates that expression of Dy10 subunit is sufficient to express transgene in wheat seeds. However, comparative analyses of transgenic GUS lines generated under the same condition using the Dy10 subunit and Dy10: Dx5 hybrid subunit will provide a better picture with this regard.

4.5.2 Novel approach to increase wheat starch

Starch has a wide range of applications in industries including bioethanol production. However, wheat starch for ethanol processing requires higher starch rather than seed protein. Hence recent research activities are focused on developing high starch and high yielding wheat varieties. A number of strategies have been employed to increase starch content in wheat grains, mostly involving manipulation of genes in starch biosynthetic pathway (Smith, 2008). However, starch formation in wheat grains depends on sucrose supply, which is the foremost determinant for starch synthesis. Thus, alteration of sucrose transport to developing grains can be considered as one approach to increase starch accumulation in wheat grains. Weschke et al. (2000) suggested that uptake capacity of SUT proteins can be increased by

complementation with high affinity SUT. Expression of barley *SUT* (*HvSUT1)* in wheat seeds resulted in increase in sucrose uptake into endosperm cells (Weichert et al., 2010). However, the performance of transgenic plants in semi-controlled conditions did not give similar results as that obtained under greenhouse conditions (Weichert et al., 2010). Since *HvSUT1* is a low affinity/high capacity transporter protein, over-expression of the *HvSUT1* gene may lead to an increase in the number of transporter proteins but not in the rate of sucrose transport.

Hence, we propose that expression of high affinity SUTs such as the one from potato (StSUT1 with Km of 1mM), which has high affinity for sucrose, might increase the rate of sucrose supply and thereby starch accumulation in the grains. The constructs generated in this project (Glutenin: StSUT1 and Ubiquitin1:StSUT1) will be useful to understand the carbon partitioning between source and sink tissues.

5.0 GENERAL DISCUSSIONS AND CONCLUSIONS

Wheat starch has wide range of industrial applications including bioethanol production. Increasing starch content per grain in wheat requires detailed understanding of various physiological, biochemical and molecular mechanisms underlying starch biosynthesis. Several studies have been targeting starch biosynthetic enzymes as a means to increase starch content in wheat grains. In addition to these enzymes, starch synthesis is interdependent on other upstream process including sucrose transport and metabolism. However, the molecular mechanisms underlying these processes are still unclear. The current study (Chapter 3) characterizes the expression pattern of genes involved in sucrose transport and metabolism in wheat during grain development in five cultivars that exhibited marked variation in grain dry weight. Our results suggest that genotypic variation in seed size and dry weight is attributed to temporal variation in sucrose transporter gene expression, particularly of *TaSUT1*. It is evident from ACA cultivar, which has a greater grain dry weight than the other four cultivars, that higher transcript abundance of *TaSUT1* during grain filling phase (4-25 DAA) is associated with increased *TaSUT1* activity and rate of sucrose transport. The unloading of sucrose in developing grains is accompanied by sucrose hydrolysis catalyzed with sucrose synthases (SuSy). Expression analysis of two *SuSy* genes of wheat, *TaSuSy1* and *TaSuSy2*, in spring wheat cultivars indicated that *TaSuSy2* plays important role in sucrose hydrolysis in wheat seeds and is influenced by *TaSUT1* expression. We suggest that the cultivar ACA with higher *TaSUT1* expression would transport more sucrose and the accompanying high transcript level of *TaSuSy2* during early to mid stages of grain development has a better sucrose hydrolyzing capacity that helps in the production of substrates for starch synthesis. In

agreement with this, CDC and WB that exhibited lower *TaSUT1* and *TaSuSy2* expression had lower dry weight accumulation during grain development.

It can be observed from our study that majority of sucrose transport and hydrolysis occurs in a short period of time between 8-16 DAA and dry weight accumulation occurs between 8-25 DAA. Any temporal shift in sucrose transport and hydrolysis affects dry matter accumulation. However, this study is based on only two *SUT* genes; thus identification and characterization of other *SUT* genes in wheat will provide better understanding on the role of sucrose transport during grain development. Our results indicated the importance of *TaSuSy2* in providing precursors for starch synthesis. Over-expression or silencing of *TaSuSy2* gene using constitutive or seed specific promoters will shed more light on their function and significance during grain filling. Furthermore, detailed characterization of invertase genes is necessary to gain in depth understanding of carbon allocation between source and sink tissues.

The second part of our study (Chapter 4) provides suitable promoter sequence, high molecular weight glutenin subunit Dy10 that can be used to express transgenes in wheat grains. The constructs prepared to express high affinity potato transporter system *StSUT1* in wheat, can be employed as important tools to understand sucrose transport and assimilation in wheat.

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7.0 Appendix

Appendix 7.1: mRNA sequence for *TaSUT1A* (accession No: AF408842; Aoki et al., 2002). The underlined and highlighted nucleotide sequences represent forward and reverse primers used for real time-PCR based gene expression analysis.

```
1       ATGGCGCGCG GCGGCGGCAA CGGCGAGGTG GAGCTCTCGG TGGGGGTCGG CGGAGGCGGC
61      GCCGGCGCCG GCGGGGCGGA CGCCCCCGCC GTGGACATCA GCCTCGGCAG GCTCATCCTC
121     GCCGGCATGG TCGCCGGCGG CGTGCAGTAC GGATGGGCGC TCCAGCTCTC CCTGCTCACC
181     CCCTACGTCC AGACTCTGGG ACTTTCGCAT GCTCTGACTT CATTCATGTG GCTCTGCGGC
241     CCTATTGCTG GATTAGTGGT TCAACCATGC GTTGGGCTCT ACAGTGACAA GTGCACTTCA
301     AGATGGGGAA GACGCAGACC GTTCATTCTG ACAGGATGTA TCCTCATCTG CATTGCTGTC
361     GTGGTCGTCG GCTTCTCGGC TGACATTGGA GCTGCTCTGG GTGACAGCAA GGAAGAGTGC
421     AGTCTCTATC ATGGGCCTCG TTGGCACGCT GCAATTGTGT ATGTTCTTGG ATTCTGGCTC
481     CTTGACTTCT CCAACAACAC AGTGCAAGGA CCAGCGCGTG CTCTGATGGC TGATTTATCA
541     GCCCAGCATG GACCCAGTGC AGCAAATTCA ATCTTCTGTT CTTGGATGGC ACTGGGAAAT
601     ATCCTAGGAT ACTCATCTGG TTCCACAAAT AACTGGCACA AGTGGTTTCC GTTCCTCCGG
661     ACAAGGGCTT GCTGTGAAGC CTGCGCAAAT CTGAAAGGCG CATTTCTGGT GGCAGTGCTG
721     TTCCTGGCCT TCTGTTTGGT GATAACCGTG ATCTTCGCCA AGGAGATACC GTACAAGGCG
781     ATTGCGCCCC TCCCAACAAA GGCCAATGGC CAGGTTGAAG TCGAGCCCAC CGGGCCGCTC
841     GCCGTCTTCA AAGGCTTCAA GAACTTGCCT CCTGGAATGC CGTCAGTGCT CCTCGTCACC
901     GGCCTCACCT GGCTGTCCTG GTTCCCCTTC ATCCTGTACG ACACCGACTG GATGGGTCGT
961     GAGATCTACC ACGGTGACCC CAAGGGAACC CCCGACGAGG CCAACGCGTT CCAGGCAGGT
1021    GTCAGGGCCG GGGCGTTCGG CCTGCTACTC AACTCGGTCG TCCTGGGGTT CAGCTCGTTC
1081    CTGATCGAGC CGCTGTGCAA GAGGCTAGGC CCGCGGGTGG TGTGGGTGTC AAGCAACTTC
1141    CTCGTCTGCC TCTCCATGGC CGCCATTTGC ATCATAAGCT GGTGGGCCAC TCAGGACCTG
1201    CATGGGTACA TCCAGCACGC CATCACCGCC AGCAAGGAGA TCAAGATCGT CTCCCTCGCC
1261    CTCTTCGCCT TCCTCGGAAT CCCTCTCGCC ATTCTGTACA GTGTCCCTTT CGCGGTGACG
1321    GCGCAGCTGG CGGCGAACAG AGGCGGTGGC CAAGGGCTGT GCACGGGCGT GCTGAACATC
1381    GCCATCGTGA TACCCCAGGT GATCATCGCG GTGGGGGCGG GGCCGTGGGA CGAGCTGTTC
1441    GGCAAGGGCA ACATCCCGGC GTTCGGCGTG GCGTCCGCCT TCGCGCTCAT CGGCGGCATC
1501    GTCGGCATAT TCCTGCTGCC CAAGATCTCC AGGCGCCAGT TCCGGGCCGT CAGCGGCGGC
1561    GGTCACTGA
```

Appendix 7.2: mRNA sequence for *TaSuSy1* (accession No: AJ001117; Marana et al., 1988). The underlined and highlighted nucleotide sequences represent forward and reverse primers used for real time-PCR based gene expression analysis.

```
1       ATGGCTGCCA AGCTGACCCG CCTCCACAGC CTCAGGGAGC GCCTTGGTGC CACCTTCTCC
61      TCCCATCCCA ATGAGCTCAT TGCACTCTTT TCCAGGTATG TTCACCAGGG CAAGGGCATG
121     CTTCAGCGCC ACCAGCTGCT TGCTGAGTTT GACGCCCTGT TCGAATCCGA CAAGGAGAAG
181     TATGCACCGT TTGAGGACAT CCTCCGTGCT GCGCAGGAAG CAATTGTGCT GCCCCCATGG
241     GTTGCACTTG CCATCAGGCC CAGGCCTGGT GTCTGGGACT ACATTCGGGT GAATGTTAGT
301     GAGCTCGCTG TGGAAGAACT GACTGTTTCT GAATACTTAG CATTCAAGGA GCAGCTTGTC
361     GACGAACATG CCAGCAGCAA GTTTGTGCTA GAGCTTGATT TTGAGCCTTT CAATGCCTCC
421     TTCCCGCGCC CTTCCATGTC CAACTCCATC GGAAAACGGG TGCAGTTCCT TAACCGTCAC
481     CTGTCTTCCC AGTTGTTCCA GGACAAGGAG AGCCTCTACC CACTGCTCAA CTTCCTGAAA
541     GCCCATTACT ACAAGGGCAC GACAATGATG TTGAACGACA GAATTCAGAG CCTTCGCGGT
601     CTCCAGTCAG CCCTTAGGAA GGCAGAAGAG TATCTAGTCA GCATTCCTGA AGACACTCCC
661     AGCTCTGAAT TCAACCACAG GTTCCAAGAG CTTGGCTTGG AGAAGGGTTG GGGTGACACC
721     GCAAAGCGTG TACACGACAC CATCCATTTG CTTCTGGACC TTCTTGAGGC CCCTGATCCG
781     GCCAGCTTGG AGAAGTTCCT TGGAACCATT CCAATGATGT TCAATGTTGT CATCCTGTCT
841     CCCCATGGAT ACTTTGCTCA ATCCAATGTG TTGGGATACC CTGATACCGG TGGCCAGGTT
901     GTGTACATCT TGGATCAAGT CCGTGCTTTG GAGAATGAGA TGCTTCTGAG GATTAAGCAG
961     CAAGGCCTTG ATATAACTCC CAAGATCCTC ATTGTAACCA GGTTGTTGCC TGATGCTGTT
1021    GGAACCACAT GTGGCCAGCG GCTGGAGAAA GTCATTGGAA CTGAGCACAC TGACATTCTC
1081    CGTGTTCCAT TCAGAACTGA TAATGGGATC CTTCGTAAGT GGATCTCGCG TTTTGATGTC
1141    TGGCCATACC TGGAGACATA CACCGAGGAT GTTGCAAACG AACTCATGAG GGAAATGCAG
1201    ACCAAGCCTG ATTTCATCAT TGGTAACAAC AGTGATGGTA ACCTTGTGGC CACTCTGCTT
1261    GCACATAAAT TGGGAGTTAC CCAGTGCACC ATTGCCCATG CCTTGGAGAA AACCAAGTAC
1321    CCCAACTCAG ACATATATTT GGACAAATTT GACAGCCAGT ACCACTTTTC ATGCCAATTC
1381    ACAGCTGACC TGATTGCCAT GAACCACACT GATTTCATCA TTACCAGCAC ATTCCAGGAA
1441    ATCGCTGGAA GCAAGGATAG CGTGGGCCAA TATGAGTCTC ACATTGCTTT CACCCTTCCT
1501    GATCTGTACC GGGTTGTCCA TGGGATTGAT GTGTTTGATC CTAAGTTCAA CATCGTCTNT
1561    CCTGGAGCAG ACATGACTGT CTACTTCCCA TACACCGAGA CTGACAAGAG GCTCACTGCC
1621    TTCCACTCTG AAATTGAGGA GCTCCTGTAC AGCGATGTTG AGAACGATGA ACACAAATTT
1681    GTGTTGAAGG ACAGGAACAA GCCAATCATC TTTTCAATGG CCCGTCTTGA CCGTGTGAAG
1741    AACATGACTG GCTTGGTTGA GATGTACGGC AAGAATGCTC ATCTGAAGGG ATTTGGCAAA
1801    CTTGTGATTG TTGCTGGCGA CCATGGCAAG GAGTCCAAGG ATAGGGAGGA GCAGGCTGAG
1861    TTCAAGAGGA TGTACAGTCT CATTGAGGAG TACAAGCTGA AGGGCCATAT CCGTTGGATC
1921    TCTGCTCAGA TGAACCGTGT TCGCAATGGT GAGCTGTACC GCTACATCTG TGACACCAAG
1981    GGAGCATTTG TGCAGCCTGC ATTCTATGAA GCATTTGGCC TGACTGTCAT TGAGGTCCAT
2041    GAATGTGGTT TGCCGACAAT TGCGACATGC CACGGTGGCC CTGCTGAAAT CATTGTGAAC
2101    GGGGTGTCTG GCCTGCACAT TGATCCTTAC CACAGCGACA AGGCCGCAGA TATCCTTGTC
2161    AACTTCTTTG AGAAGTGCAG CGAGGATCCA AGCTACTGGG ACAAAATGTC TGAAGGAGGC
2221    CTGAAGAGAA TTTATGAGAA GTACACCTGG AAGCTGTACT CAGAGAGGCT GATGACCCTG
2281    ACCGGCGTGT ATGGGTTCTG GAAGTACGTG AGCAACCTGG AGAGGCGCGA GACTCGCCGT
2341    TACCTGGAGA TGTTCTACGC TCTGAAGTAC CGCAGCCTGG CTGCTGCAGT TCCATTGGCA
2401    GTTGATGGCG AGAGCTCCGA CAACTAGCGC GGTGCAATGG GGCATGAAGA GGCGCATTCA
2461    GCGGGAGGGT GAAGTGTCGG CTGCGTTATG ATTTGTCTGT CTACCGTAGT TTCCATTTGG
2521    TCTGGTCGTG GGGTGTGTTA ATTTGTTGTC TCGGCACATT TGTGAGGTCC TAGGCAGTGC
2581    TCGCTGGTTC ATGCCAGCCG CATTTGTTGC GTGGAATAAA CGCCTGCCCA TATTGTTCAT
2641    CTTTCATCAG AGTTCAATGC AATGTTTCGT TGTCTG
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Appendix 7.3: mRNA for *TaSuSy2* (accession No: AJ000153; Marana et al., 1988). The underlined and highlighted nucleotide sequences represent forward and reverse primers used for real time-PCR based gene expression analysis.

```
1       GAAGAGGACA GCAATGGGGG AGACTGCTGG AGGGCGCGCC CTGAGCCGCA TCCACAGCGT
61      GAGGGAGCGC ATCGGTGATT CCCTCTCTGC GCACACCAAT GAGCTCGTCG CCGTCTTCTC
121     AAGGCTTGTT AACCAAGGAA AGGGGATGCT GCAGCCCCAT CAGATCACTG CTGAGTACAA
181     TGCCGCGATC CCTGAGGCCG AGCGCGAGAA GCTCAAGGAC ACCGCCTTTG AGGATCTCCT
241     AAGGGGCGCA CAGGAGGCAA TTGTCATCCC TCCATGGGTT GCTCTCGCCA TCCGGCCAAG
301     GCCCGGCGTC TGGGAGTATG TGAGGGTCAA TGTGAGCGAA CTTGGTGTTG AAGAATTGAA
361     CGTCCTGAAT TATTTGCAGT TCAAGGAACA ACTGGCGAAT GGAAGCATCG ATAACAACTT
421     TGTGCTTGAG CTGGACTTTG AGCCATTCAA CGCCTCCTTC CCGCGCCCAT CGCTGTCGAA
481     GTCCATTGGC AACGGTGTGC AGTTCCTGAA CAGGCACTTG TCATCGAAGC TGTTCCATGA
541     CAAGGAGAGC ATGTACCCAT TGCTCAACTT CCTTCGCGCG CACAACTACA AGGGGATGAC
601     CATGATGTTG AACGACAGGA TTCGCAGTCT CGGTACCCTC CAAGGTGCAC TCAGGAAGGC
661     AGAGACACAT CTGTCAGGCC TTCCAGCTGA CACCCCTTAC TCAGAGTTCC ACCACCGGTT
721     CCAGGAACTT GGTTTGGAGA AAGGTTGGGG CGACTGTGCT CAGCGTGCGA GCGAGACTAT
781     CCACCTTCTC TTGGACCTTC TCGAGGCCCC TGATCCATCC TCCTTGGAGA AGTTCCTCGG
841     GACAATCCCA ATGGTGTTCA ATGTCGTTAT CCTCTCTCCT CATGGTTACT TTGCTCAGGC
901     CAATGTCTTG GGGTACCCTG ATACTGGTGG ACAGGTTGTT TACATTTTGG ACCAAGTCCG
961     TGCTATGGAG AATGAGATGC TGTTGAGAAT CAAGCAGCAA GGTCTTGACA TTACACCAAA
1021    GATTCTAATA GTCACCAGGT TGCTCCCTGA TGCACATGGC ACCACCTGTG GCCAGCGCCT
1081    TGAGAAGGTC CTTGGCACCG AGCACACCCA CATCCTGCGT GTGCCATTCA AAACAGAAGA
1141    TGGTATTGTT CGCAAATGGA TCTCCCGCTT TGAAGTCTGG CCTTACCTGG AAGCTTACAC
1201    TGATGATGTG GCACACGAGA TCGCCGGAGA GCTGCAGGCC ACTCCTGACC TGATCATTGG
1261    AAACTACAGT GATGGCAACC TAGTCGCGTG TTCGTTGGCT CACAAGTTGG GAGTTACTCA
1321    TTGTATTGCG CATGCACTCG AGAAAACCAA GTATCCCAAC TCCGACCTTT ACTGGAAGAA
1381    ATTTGAGGAT CACTACCACT TCTCCTGCCA GTTCACAGCT GACCTGATTG CAATGAATCA
1441    TGCTGACTTC ATCATCACCA GTACTTTCCA AGAGATTGCC GGAAACAAGG ACACCGTAGG
1501    GCAGTACGAG TCGCACATGG CATTCACAAT GCCAAGCCTC TATCGTGTTG TCCATGGTAT
1561    TGATGTCTTC GACCCCAAGT TCAACATCGT CTCCCCTGGT GCTGACATGT CCATCTACTT
1621    CCCATACACT GAACAGCAGA AGAGGCTTAC CTCCCTCCAT ACTGAGATTG AGGAGCTACT
1681    CTTCAGTGAT GTTGAGAATG CTGAGCACAA ATTTGTGCTG AAGGACAAGA AGAAGCCGAT
1741    CATCTTCTCG ATGGCTAGGC TGGACCGTGT CAAGAATATG ACTGGCCTAG TAGAAATGTA
1801    TGGGCGGAAT CCTCGCCTAC AGGAGCTGGT AAACCTAGTG GTTGTTTGTG GTGACCATGG
1861    AAAGGTGTCC AAGGACAAGG AGGAGCAGGC AGAGTTCAAA AAGATGTTTG ATCTTATCGA
1921    ACAGTACAAC CTGATTGGTC ACATCCGCTG GATCTCTGCT CAGATGAACC GTGTCCGCAA
1981    TGGTGAGCTC TACCGCTACA TCTGCGACAT GAAGGGAGCC TTTGTGCAGC CTGCTTTCTA
2041    TGAGGCTTTC GGTCTTACCG TGATAGAGGC CATGACATGT GGCCTTCCAA CATTCGCCAC
2101    TGCATATGGT GGTCCAGCTG AGATCATTGT GCACGGTGTG TCCGGCTACC ACATCGATCC
2161    TTACCAGAAT GACAAGGCCT CCGCACTGCT TGTGGACTTC TTTGGGAAGT GCCAGGAAGA
2221    CCCGAGCCAC TGGAACAAGA TCTCGCAGGG AGGACTCCAG CGCATCGAGG AGAAGTACAC
2281    CTGGAAGCTG TACTCTGAGA GGCTGATGAC CCTTTCTGGT GTCTATCGTT TCTGGAAGTA
2341    TGTCTCCAAC CTCGACAGGC GCGAGACTCG TCGCTACCTT GAAATGCTCT ACGCCCTCAA
2401    GTACCGCAAA ATGGCTGCAA CTGTCCCATT GGCTGTTGAG GGCGAGACCT CGGGCAAATG
2461    ATTTGTCCTT ACCAGAGAAA TAAATGGCGG GCGAGCGCTC CGCTTTACCG TTCTTGATTC
2521    AGTGATGAAG CACAGATCGG AGAGTGTTAT GCCTTTGATT GTCCTTTGTT ACCGTTCTTG
2581    ATTCAGTATG AACTTTAGTT CCTGTTGAGG CCCCGGCCGT GTTGTCGCGG TAGGGGAACT
2641    GGATGGATGC TGTGTTGTTG GTACTTTCTC ATGGATACAG TATTTGAATG AATGAATAAG
2701    ATAGTCTTTG TTTTACTCCC A
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Appendix 7.4: mRNA sequence for *Taβ-actin* (accession No: AB181991; Himi and Noda, 2004). The underlined and highlighted nucleotide sequences represent forward and reverse primers used for real time-PCR based gene expression analysis.

```
1       TCAACAACTG GGATGACATG GGGAAAATAT GGCATCACAC GTTCTACAAC GAGCTCCGTG
61      TCGCACCAGA GGATCATCCT GTGTTGCTGA CTGAGGCCCC TCTCAACCCC AAGGCCAACA
121     GAGAGAAAAT GACCCAGATC ATGTTCGAAA CCTTCAGTTG CCCAGCAATG TATGTCGCAA
181     TCCAGGCCGT TCTGTCCTTG TATGCCAGCG GTCGAACAAC TGGTATTGTG CTCGACTCTG
241     GTGATGGTGT GAGCCACACT GTTCCAATCT ATGAGGGATA CACGCTTCCT CATGCTATCC
301     TTCGTTTGGA CCTTGCTGGC CGGGACCTCA CGGATAATCT AATGAAGATC CTGACAGAAA
361     GAGGGTACTC CCTCACAACA ACCGCTGAGC GGGAAATTGT CAGAGACATA AAGGAGAAGC
421     TCGCTTACGT GGCCCTTGAT TATGAGCAGG AGCTGGAAAC GGCTAGGAGC AGCTCCTCTG
481     TGGAGAAGAG CTATGAGATG CCTGATGGTC AGGTTATAAC AATTGGTTCA GAAAGGTTCA
541     GGTGCCCTGA GGTGCTGTTC CAGCCATCTC ATGTTGGTAT GGAAGTTCCT GGTATACACG
601     AAGCGACATA CAATTCCATC ATGAAGTGTG ATGTTGATAT CAGAAAGGAT CTGTATGGTA
661     ATGTTGTTCT CAGTGGAGGT TCTACCATGT TTCCTGGAAT TGCTGATCGC ATGAGCAAAG
721     AGATCACGGC CCTTGCTCCT AGCAGTATGA AGGTTAAAGT TATTGCACCA CCTGAAAGGA
781     AATACAGTGT CTGGATCGGT GGCTCTATTT TGGCCTCTCT TAGCACTTTC CAGCAGATGT
841     GGATCTCCAA GGCGGAGTAC GATGAGTCTG GTCCCGGCAT TGTCCACATG AAGTGCTTTT
901     GAAGAGTCGG TGAAGGGGAC TTACAAAGGG GACTTGCTGC CAAGAATATA TAGTACATGG
961     AGTACATGGT TAGTGTTCTG TAGAAGATGT GTACCCTCAG AGGAATAAGG GGTACAGGAA
1021    AATCAGTCTC GGTTCAGCTT TTCCTTTTGG CACAATTGCA TGGCCTTGCA TGGTGCTTGC
1081    CTGCTGTATG AAATAATGAA ATGGGACATA TGTGTATGCC TAAGCGACAT TGTGTGGTAC
1141    GCCAAAAAAA AAAAAAAAAA AAA
```

Appendix 7.5: Analysis of variance for fresh weight accumulation (mg) in five spring wheat cultivars measured at five stages of seed development at $p \leq 0.01$

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Cultivar	4	6527.79	1631.95	143.93	<.0001
Stage	4	306007.85	76501.96	6747.17	<.0001
Cultivar*Stage	16	1960.91	122.56	10.81	<.0001
Error Term	473	5363.05	11.34		
Total	497				

Appendix 7.6: Analysis of variance for dry weight accumulation (mg) in five spring wheat cultivars measured at five stages of seed development at $p \leq 0.01$

Source	DF	Type III SS	Mean Square	F Value	Pr > F
cultivar	4	4392.97	1098.24	310.89	<.0001
stage	4	72993.59	18248.40	5165.74	<.0001
cultivar*stage	16	1787.77	111.735	31.63	<.0001
Error Term	473	1670.91	3.53		
Total	497				

Appendix 7.7: Analysis of variance for *TaSUT1* relative transcript levels in five spring wheat cultivars at five stages of seed development at $p \leq 0.01$

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Cultivar	4	5426898.68	1356724.67	4312.06	<.0001
Stage	4	451430.78	112857.70	358.69	<.0001
Cultivar*Stage	16	1193045.58	74565.35	236.99	<.0001
Error Term	47	14787.85	314.63		
Total	71				

Appendix 7.8: Analysis of variance for *TaSUT2* relative transcript levels in five spring wheat cultivars at five stages of seed development at $p \leq 0.01$

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Cultivar	4	356.17	89.04	39.48	<.0001
Stage	4	567.90	141.97	62.95	<.0001
Cultivar*Stage	16	514.56	32.16	14.26	<.0001
Error Term	49	110.51	2.25		
Total	73				

Appendix 7.9: Analysis of variance for *TaSuSy1* relative transcript levels in five spring wheat cultivars at five stages of seed development at $p \leq 0.01$

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Cultivar	4	630.12	157.53	23.88	<.0001
Stage	4	442.43	110.61	16.77	<.0001
Cultivar*Stage	16	1667.87	104.24	15.80	<.0001
Error Term	47	310.01	6.60		
Total	71				

Appendix 7.10: Analysis of variance for *TaSuSy2* relative transcript levels in five spring wheat cultivars at five stages of seed development at $p \leq 0.01$

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Cultivar	4	1197.09	299.27	44.58	<.0001
Stage	4	10033.99	2508.50	373.70	<.0001
Cultivar*Stage	16	5006.74	312.92	46.62	<.0001
Error Term	47	315.49	6.71		
Total	71				

Appendix 7.11: Least Square (LS) mean values for fresh weight accumulation of the cultivar*stage interaction

Cultivar	Stage	LS Mean value (mg)
CS	4	12.75
CS	8	23.03
CS	16	52.79
CS	25	70.19
CS	30	68.97
YR	4	13.63
YR	8	21.14
YR	16	52.02
YR	25	75.34
YR	30	74.61
CDC	4	10.58
CDC	8	16.89
CDC	16	50.97
CDC	25	66.70
CDC	30	65.14
WB	4	13.10
WB	8	20.06
WB	16	52.42
WB	25	68.10
WB	30	66.84
AC	4	15.28
AC	8	26.61
AC	16	58.48
AC	25	82.03
AC	30	81.07
	LSD α0.05	2.10

Note: Samples are of equal size, n=20 except for WB at 4 days after anthesis (DAA) and WB at 8 DAA where n=19. Difference in mean value greater than Least Significant Difference value (LSD) indicates significant difference. CS- Chinese Spring, YR- Yecora Rojo, CDC- CDC Teal, WB- White Bird, ACA- AC Andrew.

Appendix 7.12: Least Square (LS) mean values for dry weight accumulation of the cultivar*stage interaction

Cultivar	Stage	LS Mean value (mg)
CS	4	2.14
CS	8	5.68
CS	16	8.43
CS	25	28.5
CS	30	27.65
YR	4	2.96
YR	8	5.64
YR	16	17.87
YR	25	31.76
YR	30	31.09
CDC	4	1.92
CDC	8	3.39
CDC	16	6.81
CDC	25	27.21
CDC	30	28.05
WB	4	2.16
WB	8	4.27
WB	16	12.5
WB	25	29.20
WB	30	28.36
AC	4	3.22
AC	8	8.84
AC	16	20.77
AC	25	38.71
AC	30	37.19
	LSD α0.05	1.17

Note: Samples are of equal size, n=20 except for WB at 4 DAA and WB at 8 days after anthesis (DAA) where n=19. Difference in mean value greater than Least Significant Difference value (LSD) indicates significant difference. CS- Chinese Spring, YR- Yecora Rojo, CDC- CDC Teal, WB- White Bird, ACA- AC Andrew.

Appendix 7.13: Least square (LS) mean values for *TaSUT1* relative transcript levels of the cultivar*stage interaction

Cultivar	Stage	LS Mean value
CS	4	15.90
CS	8	43.99
CS	16	75.38
CS	25	146.33
CS	30	36.80
YR	4	65.49
YR	8	62.94
YR	16	132.98
YR	25	33.67
YR	30	17.60
CDC	4	17.36
CDC	8	38.84
CDC	16	34.65
CDC	25	89.96
CDC	30	40.60
WB	4	8.12
WB	8	68.06
WB	16	123.86
WB	25	13.41
WB	30	14.84
AC	4	559.52
AC	8	1191.26
AC	16	905.43
AC	25	766.79
AC	30	231.53
	LSD α0.05	30.00

Note: Samples are of equal size, n=3 except for except for CS at 25 days after antheis (DAA), YR at 16 DAA, CDC at 25 DAA data, WB at 16 DAA and ACA at 8 DAA where n=2. Difference in mean value greater than Least Significant Difference value (LSD) indicates significant difference. CS- Chinese Spring, YR- Yecora Rojo, CDC- CDC Teal, WB- White Bird, ACA- AC Andrew.

Appendix 7.14: Least square (LS) mean values for *TaSUT2* gene expression of the cultivar*stage interaction

Cultivar	Stage	LS Mean value
CS	4	16.32
CS	8	10.70
CS	16	8.76
CS	25	4.47
CS	30	5.24
YR	4	6.71
YR	8	7.15
YR	16	6.33
YR	25	1.42
YR	30	1.60
CDC	4	2.68
CDC	8	1.89
CDC	16	3.67
CDC	25	1.91
CDC	30	2.16
WB	4	1.76
WB	8	9.51
WB	16	11.18
WB	25	1.95
WB	30	2.58
AC	4	9.69
AC	8	16.42
AC	16	5.88
AC	25	1.04
AC	30	1.55
	LSD α0.05	2.49

Note: samples are of equal size, n=3 except for CDC at 8 days after antheis. Difference in mean value greater than Least Significant Difference value (LSD) indicates significant difference. CS- Chinese Spring, YR- Yecora Rojo, CDC- CDC Teal, WB- White Bird, ACA- AC Andrew.

Appendix 7.15: Least square **(**LS) Mean values for *TaSuSy1* gene expression of the cultivar*stage interaction

Cultivar	Stage	LS Mean value
CS	4	2.85
CS	8	26.03
CS	16	19.97
CS	25	7.73
CS	30	16.36
YR	4	15.56
YR	8	13.71
YR	16	7.28
YR	25	6.31
YR	30	6.13
CDC	4	11.68
CDC	8	6.67
CDC	16	7.14
CDC	25	12.58
CDC	30	12.01
WB	4	5.37
WB	8	13.90
WB	16	9.62
WB	25	6.62
WB	30	7.43
AC	4	26.19
AC	8	21.29
AC	16	13.96
AC	25	11.41
AC	30	8.23
	LSD α0.05	4.34

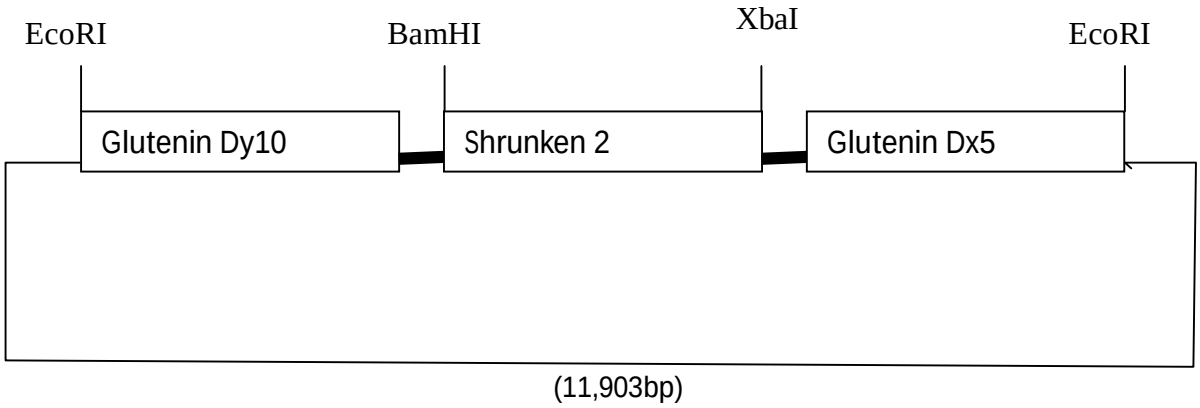
Note: Samples are of equal size, n=3 except for CS at 25 days after anthesis (DAA), CDC at 4 DAA, WB at 16 DAA, where n=2. Difference in mean value greater than Least Significant Difference value (LSD) indicates significant difference. CS- Chinese Spring, YR- Yecora Rojo, CDC- CDC Teal, WB- White Bird, ACA- AC Andrew.

Appendix 7.16: Least square **(**LS) Mean values for *TaSuSy2* gene expression of the cultivar*stage interaction

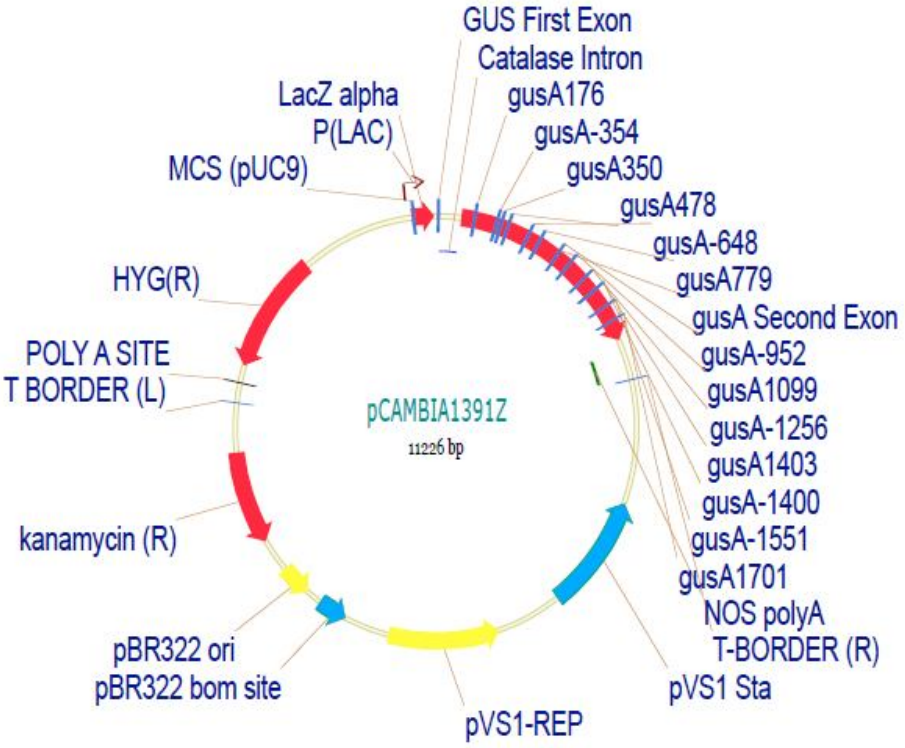
Cultivar	Stage	LS Mean value
CS	4	1.70
CS	8	60.03
CS	16	18.53
CS	25	2.57
CS	30	1.07
YR	4	16.17
YR	8	37.73
YR	16	8.73
YR	25	1.02
YR	30	2.08
CDC	4	5.21
CDC	8	8.68
CDC	16	7.51
CDC	25	11.51
CDC	30	1.75
WB	4	2.34
WB	8	19.70
WB	16	18.79
WB	25	5.78
WB	30	4.08
AC	4	3.36
AC	8	53.65
AC	16	26.18
AC	25	4.45
AC	30	2.83
	LSD α0.05	4.38

Note: Samples are of equal size, n=3 except for CDC at 8 and 25 DAA, WB at 16 DAA, and ACA at 4 and 8 DAA data where n=2. Difference in mean value greater than Least Significant Difference value (LSD) indicates significant difference. CS- Chinese Spring, YR- Yecora Rojo, CDC- CDC Teal, WB- White Bird, ACA- AC Andrew.

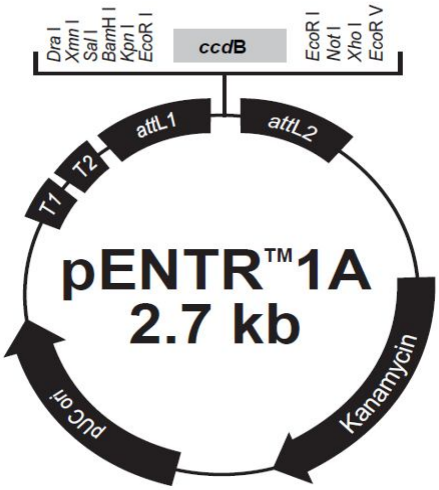
Appendix 7.17: Schematic representation of pGSH2 vector (provided by Mike Giroux's lab, University of Montana)



Appendix 7.18: Schematic representation of pCambia1391Z (Cambia)



Appendix 7.19: Schematic representation of pENTR 1A (Invitrogen)



attL1

352 GGGCCCCAAA TAATGATTTT ATTTTGACTG ATAGTGACCT GTTCGTTGCA ACAAATTGAT
CCCGGGGTTT ATTACTAAAA TAAAACTGAC TATCACTGGA CAAGCAACGT TGTTTAACTA

Dra I

412 AAG CAA TGC TTT TTT ATA ATG CCA ACT TTG TAC AAA AAA GCA GGC TTT
TTC GTT ACG AAA AAA TAT TAC GGT TGA AAC ATG TTT TTT CGT CCG AAA

Xmn I Sal I BamH I Kpn I EcoR I

460 AAA GGA ACC AAT TCA GTC GAC TGG ATC CGG TAC CGA ATT CGC--- ccdB gene ---
TTT CCT TGG TTA AGT CAG CTG ACC TAG GCC ATG GCT TAA GCG

EcoR I Not I Xho I EcoR V

915 TAG AAT TCG CGG CCG CAC TCG AGA TAT CTA GAC CCA GCT TTC TTG TAC AAA
ATC TTA AGC GCC GGC GTG AGC TCT ATA GAT CTG GGT CGA AAG AAC ATG TTT

attL2

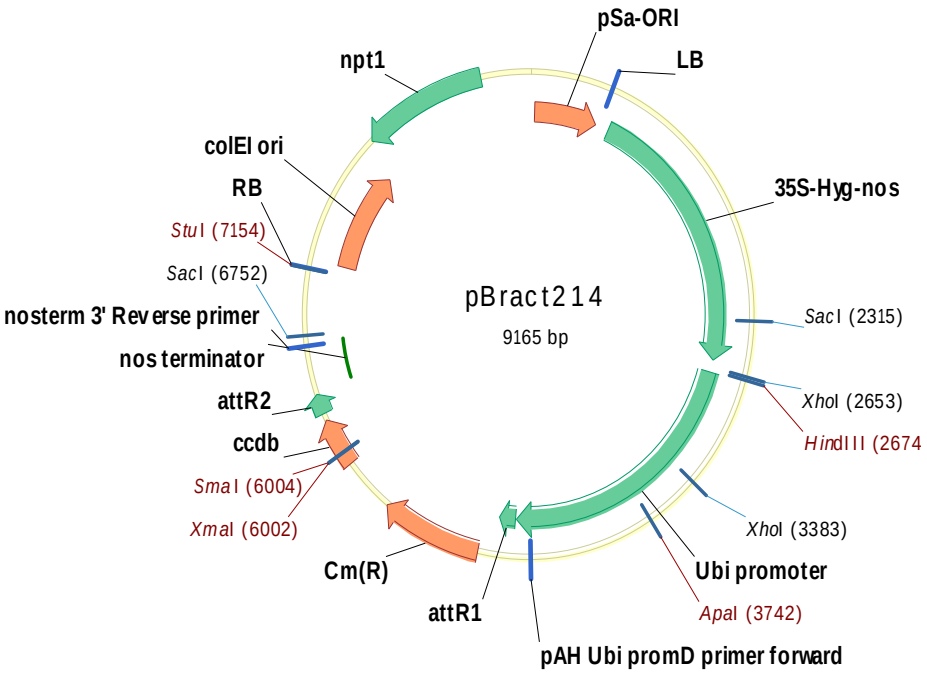
966 GTTGGCATTA TAAGAAAGCA TTGCTTATCA ATTTGTTGCA ACGAACAGGT CACTATCAGT
CAACCGTAAT ATTCTTTCGT AACGAATAGT TAAACAACGT TGCTTGTCCA GTGATAGTCA

reverse primer binding site

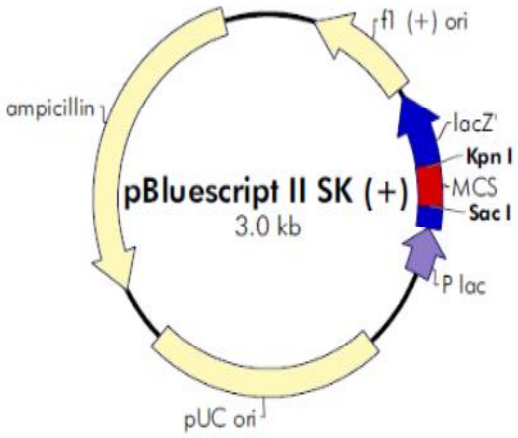
1026 CAAAATAAAA TCATTATTTG CCATCCAGCT GCAGCTCTGG CCCGTGTCTC AAAATCTCTG
GTTTTATTTT AGTAATAAAC GGTAGGTCGA CGTCGAGACC GGGCACAGAG TTTTAGAGAC

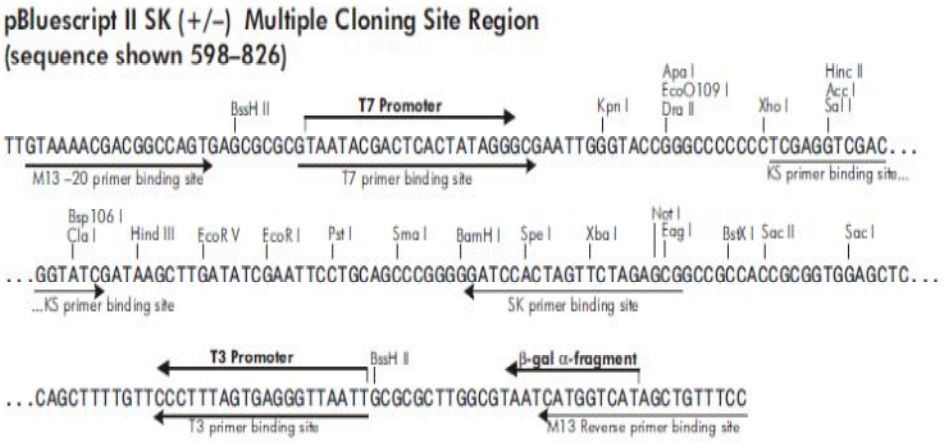
1086 ATGTTACATT
TACAATGTAA

Appendix 7.20: Schematic representation of construct pBract214 (Bract)



Appendix 7.21: Schematic representation of construct pBluescript II SK (+) vector (Stratagene)





Appendix 7.22: mRNA sequence for Potato (*Solanum tuberosum*) sucrose transporter1 gene (*StSUT1*) from cv. Russet Burbank (isolated by Zhen Yao, Dr. Ayele's lab, University of Manitoba)

ATGGAGAATGGTACAAAAAGAGAAGGTTTAGGGAAACTTACAGTTTCATCTTCTCTACAAGTTGAACAGCCTTTA
GCACCATCAAAGCTATGGAAAATTATAGTTGTAGCTTCCATAGCTGCTGGTGTTCAATTTGGTTGGGCTCTTCAG
CTCTCTTTGCTTACACCTTATGTTCAATTGCTCGGAATTCCTCATAAATTTGCCTCTTTTATTTGGCTTTGTGGA
CCGATTTCTGGTATGATTGTTCAGCCAGTTGTCGGCTACTACAGTGATAATTGCTCCTCCCGTTTCGGTCGCCGC
CGGCCATTCATTGCCGCCGGAGCTGCACTTGTTATGATTGCGGTTTTCCTCATCGGATTCGCCGCCGACCTTGGT
CACGCCTCCGGTGACACTCTCGGAAAAGGATTTAAGCCACGTGCCATTGCCGTTTTCGTCGTCGGCTTTTGGATC
CTTGATGTTGCTAACAACATGTTACAGGGCCCATGCAGAGCACTACTGGCTGATCTCTCCGGCGGAAAATCCGGC
AGGATGAGAACAGCAAATGCTTTTTTCTCATTCTTCATGGCCGTCGGAAACATTCTGGGGTACGCCGCCGGTTCA
TATTCTCACCTCTTTAAAGTATTCCCCTTCTCAAAAACCAAAGCCTGCGACATGTACTGCGCAAATCTGAAGAGT
TGTTTCTTCATCGCTATATTCCTTTTACTCAGCTTAACAACCATAGCCTTAACCTTAGTCCGGGAAAACGAGCTC
CCGGAGAAAGACGAGCAAGAAATCGACGAGAAATTAGCCGGCGCCGGAAAATCGAAAGTACCGTTTTTCGGTGAA
ATTTTTGGGGCTTTGAAAGAATTACCTCGACCGATGTGGATTCTTCTATTAGTAACCTGTTTGAACTGGATCGCG
TGGTTTCCCTTTTTCTTATACGATACAGATTGGATGGCTAAGGAGGTTTTCGGTGGACAAGTCGGTGATGCGAGG
TTGTACGATTTGGGTGTACGCGCTGGTGCAATGGGATTACTGTTGCAATCTGTGGTTCTAGGGTTTATGTCACTT
GGGGTTGAATTCTTAGGGAAGAAGATTGGTGGTGCTAAGAGGTTATGGGGAATTTTGAACTTTGTTTTGGCTATT
TGCTTGGCTATGACCATTTTGGTCACCAAAATGGCCGAGAAATCTCGCCAGCACGACCCCGCCGGCACACTTATG
GGGCCGACGCCTGGTGTTAAAATCGGTGCCTTGCTTCTCTTTGCCGCCCTTGGTATTCCTCTTGCGGCAACTTTT
AGTATTCCATTTGCTTTGGCATCTATATTTTCTAGTAATGCTGGTTCAGGACAAGGTTTGTCACTAGGAGTGCTC
AATCTTGCAATTGTTGTACCACAGATGTTGGTGTCACTAGTAGGAGGGCCATGGGATGATTTGTTTGGAGGAGGA
AACTTGCCTGGATTTGTAGTTGGAGCAGTTGCAGCTGCCGCGAGCGGTGTTTTAGCACTCACAATGTTGCCATCT
CCACCTGCTGATGCTAAGCCAGCAGTCGCCATGGGCGGTTTCCATTAAATAA

Appendix 7.23: Nucleotide sequence of glutenin promoter Glu-D1-2b (Dy 10 subunit; Meyer et al., 2004)

GAATTCACTAGAATTGTGTTACACCCCACTTGTCCAGCGAGGACCCGGCTATTTCGGAGGGCGATAGCCTCTGCC
ATGGCAGCCGTGTTGACGTAGGTTACAACGCAGTTGCTTGCCTGCCATGATTATCATGAAGCACCACGCCTGAAG
CGTCATTCTTCTCGAGAATGATGAAGGCCGCATCAACATGAAGTTTTACATAGTTCTCCGGTGGTTTCTCCCGGC
CTCCTCTGCGAGTGCCCACCTTTTTATTTTGCTCACCTGTAGTTAAGACATAGCGGTTGTTAAGTTTTACATATT
TTGTACTGCACTTAGTATAACTATAATTGTATTTTTAGTACTATTAAGTTATACTTAGAATTGGTTGCATGCTAA
ACACAATTAATAAATGGTGGATTATGTGGCACAATAAACAACTATTGGCAAATTTTCTGCATGCATGATTTCACT
TGGATTCCTTCCTAGTTTTTTTTTCCAGGAAGTCTTTCCTAGTTGATCCGCTTCCCCATTGATGCATCCTCGCCT
CAGGCAAACAATTACAATCTCTAGTGGTGATGAATGCTTTGTTGTTAATACAAGTTTCTCTTAGTTATGAGCGGA
ACAAGAAGTAATATACACAAGTTTCTGCACCAAACCTAGAGGCAAGGTGGCATTGGAGGATTGAGGATGTTTGTG
TTTTTTCTTGTGGGGATGTCTGCATTTGTTGTTGAGGTGGATTATGACAATCTATCTTTTGCCCGTTTTATTATT
TGTTCAACATTTTATTTGCTTCCATGGCTATCTATTTTTGTTGCCGATACAATGAATAAGGTTAATAAAGCATAA
TTTAGCAACCACAAAATGTGTATTAGGAGTCAGGGCCGGGCCGGCAAACTCAGGGCCCTATGCCAAACTCTAACA
ATGAGCCTACCATTAGAAGAACGTCCGGCAAAGAGATGCACTGCAACATATGACCAAAACGTCATCCATGATCGA
GTACAATTTCTTAGTGCCTTTTTCATCAAAGTTATATTTGAAACATTTTAGAAAATTTGTCTTAAATTTTTTCCC
ATGGCGTTTCGATCAAAAAAATGCCTTAAAACCTACCTATATAAAGGCAATAAGGGAAAACACTTCAGGATACCT
TCGAAGCTAAGGTGTCTTTCTTTAGATGGTATGCTAAACTGTTCTTCATGGCGCCTCAAATCATCATTCTGCAAG
TAAAGACTAAAAATAACGCTAAATATGGAGTTAAAGGTTTACAAACGACGAGCGGAAAAGGAGTCTTATATAATT
CCAGTAGCATTTATTCCATTTTGCTTCACATAGAAAATGTAGCTGAGGTGGTCGATGTTTATTTTGACTCGCGTG
AGGTCGCCGGTTCGATCTCAGAAGCAACGCTGGAATATCCCATATAGATTTATTTTTCAGGCTGAGTGATGCTCG
GGTACCTCCAGCGTATGGGCTGAAATTCGGCCCCCCTGCAAATCATGGGCCCTGTGACGTTCGCACGGGTTGCAC
ATGCCTTGGCCCGGGCCTACTAGGAGTGTACCTGGATTATGTTGGACGACGGGAGATGAAAGGGATGTATTAATT
AACAAAGATAATGAAGCTTAATTTTCTTATATGTTAATATTGACAAGAAACAAGCTGGGCCTAACTCAAAGTTAC
GGTTACATAGTCGCAACCTTTTATATCTAAATAATATATCTCTCTCAATATGCAAACATGGCCACCTTAGCATGT
AGCATGCATGGAAAATTGTCCACTTCAACATGCAACCATGCATCAAAATTTCCATTTTACTAGGCTATTTATTTG
ATAAAATTTCACAAATATACAATAATCAAACACAATAGATCATATGTGTTTTCAGTTTTGGTTCTCACATTATTA
CTCCAAATATAAATGTTTCGTATAACCAAATTTCATTCAAATATACTGCAGAACATTTCCGTGACAACAGGTGGG
GTACATCTAGTTAGTNGATGTCCTGCAAGTGATAAGGCCAAGGAGAGAAGAAGTGCACCATCTACAGAGGCCAGG
GAAAGACAATGGACATGCAGAGAGGCGGGGGCGGGGAAGAAACACATGGAGATCATAGAAGAACATAAGAGGTTA
AACATAGGAGGAGGTATAATGGCAATTAAATCCACATTACTTGAACTCATTTGGAAGTGAAAAAATCCCCTATCT
GTGTAAATCAAACTAATTGACGCGAGTTTTCTCTGAAGATTCTATGTTAATTTTAGACATGAATGACCAAAGGTT
TCAGTTAGTTGAGTTTTGTCATCGAAAGGTGTTTACATAAGTCCAAAAATTCTACCAGCTTTTGGTACGGCGCGT
CACAGAACAGATAAATGGTGTGAGTCATTGGATAGATATTATGAGTCATAGCATGGATTTGTGTTGCCTGGAAAT
CTAACTATGACAAGAAACAAAACATAAATGGGCTTTTGAAAGATGATTTATCAACTTACCTTATCCATGCAAGCT
ACCTTCCACTAGTCGACATGCTTAGAAGCTTTTAGTGACCGCAGATTTGCAAAAGCAATGGCTAACAGACACCCA
AACCCCAAGAAGCATAACCACTTCTCTTAGATAAAAATAGCAGATCGATATACAAACGGTCTACACTTCTGCAAA
CAATACCCAGAAGCCAGAATTAGGATTGAACCGATTACGTGGCTTTAGCAGACCGTCCAAAAATCTGTTTTGCAA
AGCTCCAATTGCTCCTTGCTTATCCAGCTTCTTTTGTGTTGGCAAATTGTTCTTTTCCAACCAACTTTATTCTTT
TCACACTTTCTTCTTAGGCTGAACTAACTCGCCGTGCACACAACCATTGTCCTGAACCTTCACCACGTCCCTATA
AAAGCCCAACCAATCTCCACAATTTCATCATCACCCACAACACCGAGCACCACAAAATAGAGATCAATTCACTGA
CAGTCCAC

Appendix 7.24: Alignment of glutenin Dy10 subunit cloned into pCAMBIA1391z and pGSH2 construct

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glu_gus         GAATTCACTAGAATTGTGTTACACCCCACTTGTCCAGCGAGGACCCGGCTATTTCGGAGG 60
pGSH2           GAATTCACTAGAATTGTGTTACACCCCACTTGTCCAGCGAGGACCCGGCTATTTCGGAGG 60
                ************************************************************

glu_gus         GCGATAGCCTCTGCCATGGCAGCCGTGTTGACGTAGGTTACAACGCAGTTGCTTGCCTGC 120
pGSH2           GCGATAGCCTCTGCCATGGCAGCCGTGTTGACGTAGGTTACAACGCAGTTGCTTGCCTGC 120
                ************************************************************

glu_gus         CATGATTATCATGAAGCACCACGCCTGAAGCGTCATTCTTCTCGAGAATGATGAAGGCCG 180
pGSH2           CATGATTATCATGAAGCACCACGCCTGAAGCGTCATTCTTCTCGAGAATGATGAAGGCCG 180
                ************************************************************

glu_gus         CATCAACATGAAGTTTTACATAGTTCTCCGGTGGTTTCTCCCGGCCTCCTCTGCGAGTGC 240
pGSH2           CATCAACATGAAGTTTTACATAGTTCTCCGGTGGTTTCTCCCGGCCTCCTCTGCGAGTGC 240
                ************************************************************

glu_gus         CCACCTTTTTATTTTGCTCACCTGTAGTTAAGACATAGCGGTTGTTAAGTTTTACATATT 300
pGSH2           CCACCTTTTTATTTTGCTCACCTGTAGTTAAGACATAGCGGTTGTTAAGTTTTACATATT 300
                ************************************************************

glu_gus         TTGTACTGCACTTAGTATAACTATAATTGTATTTTTAGTACTATTAAGTTATACTTAGAA 360
pGSH2           TTGTACTGCACTTAGTATAACTATAATTGTATTTTTAGTACTATTAAGTTATACTTAGAA 360
                ************************************************************

glu_gus         TTGGTTGCATGCTAAACACAATTAATAAATGGTGGATTATGTGGCACAATAAACAACTAT 420
pGSH2           TTGGTTGCATGCTAAACACAATTAATAAATGGTGGATTATGTGGCACAATAAACAACTAT 420
                ************************************************************

glu_gus         TGGCAAATTTTCTGCATGCATGATTTCACTTGGATTCCTTCCTAGTTTTTTTTTCCAGGA 480
pGSH2           TGGCAAATTTTCTGCATGCATGATTTCACTTGGATTCCTTCCTAGTTTTTTTTTCCAGGA 480
                ************************************************************

glu_gus         AGTCTTTCCTAGTTGATCCGCTTCCCCATTGATGCATCCTCGCCTCAGGCAAACAATTAC 540
pGSH2           AGTCTTTCCTAGTTGATCCGCTTCCCCATTGATGCATCCTCGCCTCAGGCAAACAATTAC 540
                ************************************************************

glu_gus         AATCTCTAGTGGTGATGAATGCTTTGTTGTTAATACAAGTTTCTCTTAGTTATGAGCGGA 600
pGSH2           AATCTCTAGTGGTGATGAATGCTTTGTTGTTAATACAAGTTTCTCTTAGTTATGAGCGGA 600
                ************************************************************

glu_gus         ACAAGAAGTAATATACACAAGTTTCTGCACCAAACCTAGAGGCAAGGTGGCATTGGAGGA 660
pGSH2           ACAAGAAGTAATATACACAAGTTTCTGCACCAAACCTAGAGGCAAGGTGGCATTGGAGGA 660
                ************************************************************

glu_gus         TTGAGGATGTTTGTGTTTTTTCTTGTGGGGATGTCTGCATTTGTTGTTGAGGTGGATTAT 720
pGSH2           TTGAGGATGTTTGTGTTTTTTCTTGTGGGGATGTCTGCATTTGTTGTTGAGGTGGATTAT 720
                ************************************************************

glu_gus         GACAATCTATCTTTTGCCCGTTTTATTATTTGTTCAACATTTTATTTGCTTCCATGGCTA 780
pGSH2           GACAATCTATCTTTTGCCCGTTTTATTATTTGTTCAACATTTTATTTGCTTCCATGGCTA 780
                ************************************************************

glu_gus         TCTATTTTTGTTGCCGATACAATGAATAAGGTTAATAAAGCATAATTTAGCAACCACAAA 840
pGSH2           TCTATTTTTGTTGCCGATACAATGAATAAGGTTAATAAAGCATAATTTAGCAACCACAAA 840
                ************************************************************

glu_gus         ATGTGTATTAGGAGTCAGGGCCGGGCCGGCAAACTCAGGGCCCTATGCCAAACTCTAACA 900
pGSH2           ATGTGTATTAGGAGTCAGGGCCGGGCCGGCAAACTCAGGGCCCTATGCCAAACTCTAACA 900
                ************************************************************


glu_gus         ATGAGCCTACCATTAGAAGAACGTCCGGCAAAGAGATGCACTGCAACATATGACCAAAAC 960
pGSH2           ATGAGCCTACCATTAGAAGAACGTCCGGCAAAGAGATGCACTGCAACATATGACCAAAAC 960
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glu_gus         GTCATCCATGATCGAGTACAATTTCTTAGTGCCTTTTTCATCAAAGTTATATTTGAAACA 1020
pGSH2           GTCATCCATGATCGAGTACAATTTCTTAGTGCCTTTTTCATCAAAGTTATATTTGAAACA 1020
                ************************************************************

glu_gus         TTTTAGAAAATTTGTCTTAAATTTTTTCCCATGGCGTTTCGATCAAAAAAATGCCTTAAA 1080
pGSH2           TTTTAGAAAATTTGTCTTAAATTTTTTCCCATGGCGTTTCGATCAAAAAAATGCCTTAAA 1080
                ************************************************************

glu_gus         ACCTACCTATATAAAGGCAATAAGGGAAAACACTTCAGGATACCTTCGAAGCTAAGGTGT 1140
pGSH2           ACCTACCTATATAAAGGCAATAAGGGAAAACACTTCAGGATACCTTCGAAGCTAAGGTGT 1140
                ************************************************************

glu_gus         CTTTCTTTAGATGGTATGCTAAACTGTTCTTCATGGCGCCTCAAATCATCATTCTGCAAG 1200
pGSH2           CTTTCTTTAGATGGTATGCTAAACTGTTCTTCATGGCGCCTCAAATCATCATTCTGCAAG 1200
                ************************************************************

glu_gus         TAAAGACTAAAAATAACGCTAAATATGGAGTTAAAGGTTTACAAACGACGAGCGGAAAAG 1260
pGSH2           TAAAGACTAAAAATAACGCTAAATATGGAGTTAAAGGTTTACAAACGACGAGCGGAAAAG 1260
                ************************************************************

glu_gus         GAGTCTTATATAATTCCAGTAGCATTTATTCCATTTTGCTTCACATAGAAAATGTAGCTG 1320
pGSH2           GAGTCTTATATAATTCCAGTAGCATTTATTCCATTTTGCTTCACATAGAAAATGTAGCTG 1320
                ************************************************************

glu_gus         AGGTGGTCGATGTTTATTTTGACTCGCGTGAGGTCGCCGGTTCGATCTCAGAAGCAACGC 1380
pGSH2           AGGTGGTCGATGTTTATTTTGACTCGCGTGAGGTCGCCGGTTCGATCTCAGAAGCAACGC 1380
                ************************************************************

glu_gus         TGGAATATCCCATATAGATTTATTTTTCAGGCTGAGTGATGCTCGGGTACCTCCAGCGTA 1440
pGSH2           TGGAATATCCCATATAGATTTATTTTTCAGGCTGAGTGATGCTCGGGTACCTCCAGCGTA 1440
                ************************************************************

glu_gus         TGGGCTGAAATTCGGCCCCCCTGCAAATCATGGGCCCTGTGACGTTCGCACGGGTTGCAC 1500
pGSH2           TGGGCTGAAATTCGGCCCCCCTGCAAATCATGGGCCCTGTGACGTTCGCACGGGTTGCAC 1500
                ************************************************************

glu_gus         ATGCCTTGGCCCGGGCCTACTAGGAGTGTACCTGGATTATGTTGGACGACGGGAGATGAA 1560
pGSH2           ATGCCTTGGCCCGGGCCTACTAGGAGTGTACCTGGATTATGTTGGACGACGGGAGATGAA 1560
                ************************************************************

glu_gus         AGGGATGTATTAATTAACAAAGATAATGAAGCTTAATTTTCTTATATGTTAATATTGACA 1620
pGSH2           AGGGATGTATTAATTAACAAAGATAATGAAGCTTAATTTTCTTATATGTTAATATTGACA 1620
                ************************************************************

glu_gus         AGAAACAAGCTGGGCCTAACTCAAAGTTACGGTTACATAGTCGCAACCTTTTATATCTAA 1680
pGSH2           AGAAACAAGCTGGGCCTAACTCAAAGTTACGGTTACATAGTCGCAACCTTTTATATCTAA 1680
                ************************************************************

glu_gus         ATAATATATCTCTCTCAATATGCAAACATGGCCACCTTAGCATGTAGCATGCATGGAAAA 1740
pGSH2           ATAATATATCTCTCTCAATATGCAAACATGGCCACCTTAGCATGTAGCATGCATGGAAAA 1740
                ************************************************************

glu_gus         TTGTCCACTTCAACATGCAACCATGCATCAAAATTTCCATTTTACTAGGCTATTTATTTG 1800
pGSH2           TTGTCCACTTCAACATGCAACCATGCATCAAAATTTCCATTTTACTAGGCTATTTATTTG 1800
                ************************************************************

glu_gus         ATAAAATTTCACAAATATACAATAATCAAACACAATAGATCATATGTGTTTTCAGTTTTG 1860
pGSH2           ATAAAATTTCACAAATATACAATAATCAAACACAATAGATCATATGTGTTTTCAGTTTTG 1860
                ************************************************************

glu_gus         GTTCTCACATTATTACTCCAAATATAAATGTTTCGTATAACCAAATTTCATTCAAATATA 1920
pGSH2           GTTCTCACATTATTACTCCAAATATAAATGTTTCGTATAACCAAATTTCATTCAAATATA 1920
                ************************************************************


glu_gus         CTGCAGAACATTTCCGTGACAACAGGTGGGGTACATCTAGTTAGTGGATGTCCTGCAAGT 1980
pGSH2           CTGCAGAACATTTCCGTGACAACAGGTGGGGTACATCTAGTTAGTNGATGTCCTGCAAGT 1980
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glu_gus         GATAAGGCCAAGGAGAGAAGAAGTGCACCATCTACAGAGGCCAGGGAAAGACAATGGACA 2040
pGSH2           GATAAGGCCAAGGAGAGAAGAAGTGCACCATCTACAGAGGCCAGGGAAAGACAATGGACA 2040
                ************************************************************

glu_gus         TGCAGAGAGGCGGGGGCGGGGAAGAAACACATGGAGATCATAGAAGAACATAAGAGGTTA 2100
pGSH2           TGCAGAGAGGCGGGGGCGGGGAAGAAACACATGGAGATCATAGAAGAACATAAGAGGTTA 2100
                ************************************************************

glu_gus         AACATAGGAGGAGGATATAATGGACAATTAAATCCACATTACTTGAACTCATTTGGGAAG 2157
pGSH2           AACATAGGAGGAGGATATAATGGACAATTAAATCCACATTACTTGAACTCATTTGGGAAG 2157
                ************************************************************

glu_gus         TGGAAAAAATCCCCTATTCTGGTGTAAATCAAACTAATTGACGCGAGTTTTCTCTGAAGA 2214
pGSH2           TGGAAAAAATCCCCTATTCTGGTGTAAATCAAACTAATTGACGCGAGTTTTCTCTGAAGA 2214
                ************************************************************

glu_gus         TTCTATGTTAATTTTAGACATGAATGACCAAAGGTTTCAGTTAGTTGAGTTTTGTCATCG 2274
pGSH2           TTCTATGTTAATTTTAGACATGAATGACCAAAGGTTTCAGTTAGTTGAGTTTTGTCATCG 2274
                ************************************************************

glu_gus         AAAGGTGTTTACATAAGTCCAAAAATTCTACCAGCTTTTGGTACGGCGCGTCACAGAACA 2334
pGSH2           AAAGGTGTTTACATAAGTCCAAAAATTCTACCAGCTTTTGGTACGGCGCGTCACAGAACA 2334
                ************************************************************

glu_gus         GATAAATGGTGTGAGTCATTGGATAGATATTATGAGTCATAGCATGGATTTGTGTTGCCT 2394
pGSH2           GATAAATGGTGTGAGTCATTGGATAGATATTATGAGTCATAGCATGGATTTGTGTTGCCT 2394
                ************************************************************

glu_gus         GGAAATCTAACTATGACAAGAAACAAAACATAAATGGGCTTTTGAAAGATGATTTATCAA 2454
pGSH2           GGAAATCTAACTATGACAAGAAACAAAACATAAATGGGCTTTTGAAAGATGATTTATCAA 2454
                ************************************************************

glu_gus         CTTACCTTATCCATGCAAGCTACCTTCCACTAGTCGACATGCTTAGAAGCTTTTAGTGAC 2514
pGSH2           CTTACCTTATCCATGCAAGCTACCTTCCACTAGTCGACATGCTTAGAAGCTTTTAGTGAC 2514
                ************************************************************

glu_gus         CGCAGATTTGCAAAAGCAATGGCTAACAGACACCCAAACCCCAAGAAGCATAACCACTTC 2574
pGSH2           CGCAGATTTGCAAAAGCAATGGCTAACAGACACCCAAACCCCAAGAAGCATAACCACTTC 2574
                ************************************************************

glu_gus         TCTTAGATAAAAATAGCAGATCGATATACAAACGGTCTACACTTCTGCAAACAATACCCA 2634
pGSH2           TCTTAGATAAAAATAGCAGATCGATATACAAACGGTCTACACTTCTGCAAACAATACCCA 2634
                ************************************************************

glu_gus         GAAGCCAGAATTAGGATTGAACCGATTACGTGGCTTTAGCAGACCGTCCAAAAATCTGTT 2694
pGSH2           GAAGCCAGAATTAGGATTGAACCGATTACGTGGCTTTAGCAGACCGTCCAAAAATCTGTT 2694
                ************************************************************

glu_gus         TTGCAAAGCTCCAATTGCTCCTTGCTTATCCAGCTTCTTTTGTGTTGGCAAATTGTCCTT 2754
pGSH2           TTGCAAAGCTCCAATTGCTCCTTGCTTATCCAGCTTCTTTTGTGTTGGCAAATTGTTCTT 2754
                ************************************************************

glu_gus         TTCCAACCAACTTTATTCTTTTCACACTTTCTTCTTAGGCTGAACTAACCTCGCCGTGCA 2813
pGSH2           TTCCAACCAACTTTATTCTTTTCACACTTTCTTCTTAGGCTGAACTAACCTCGCCGTGCA 2813
                ************************************************************

glu_gus         CACAACCATTGTCCTGAACCTTCACCACGTCCCTATAAAAGCCCAACCAATCTCCACAAT 2873
pGSH2           CACAACCATTGTCCTGAACCTTCACCACGTCCCTATAAAAGCCCAACCAATCTCCACAAT 2873
                ************************************************************

glu_gus         TTCATCATCACCCACAACACCGAGCACCACAAAATAGAGATCAATTCACT---------- 2933
pGSH2           TTCATCATCACCCACAACACCGAGCACCACAAAATAGAGATCAATTCACT---------- 2933
                **********************************************
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Appendix 7.25: Alignment of *StSUT1* sequence cloned into pBluescript II SK^{+} vector

```
X69165          AAAAATGGAGAATGGTACAAAAAGAGAAGGTTTAGGGAAACTTACAGTTTCATCTTCTCT 60
C2              ----ATGGAGAATGGTACAAAAAGAGAAGGTTTAGGGAAACTTACAGTTTCATCTTCTCT 56
                    ************************************************************

X69165          ACAAGTTGAACAGCCTTTAGCACCATCAAAGCTATGGAAAATTATAGTTGTAGCTTCCAT 120
C2              ACAAGTTGAACAGCCTTTAGCACCATCAAAGCTATGGAAAATTATAGTTGTAGCTTCCAT 116
                ************************************************************

X69165          AGCTGCTGGTGTTCAATTTGGTTGGGCTCTTCAGCTCTCTTTGCTTACACCTTATGTTCA 180
C2              AGCTGCTGGTGTTCAATTTGGTTGGGCTCTTCAGCTCTCTTTGCTTACACCTTATGTTCA 176
                ************************************************************

X69165          ATTGCTCGGAATTCCTCATAAATTTGCCTCTTTTATTTGGCTTTGTGGACCGATTTCTGG 240
C2              ATTGCTCGGAATTCCTCATAAATTTGCCTCTTTTATTTGGCTTTGTGGACCGATTTCTGG 236
                ************************************************************

X69165          TATGATTGTTCAGCCAGTTGTCGGCTACTACAGTGATAATTGCTCCTCCCGTTTCGGTCG 300
C2              TATGATTGTTCAGCCAGTTGTCGGCTACTACAGTGATAATTGCTCCTCCCGTTTCGGTCG 296
                ************************************************************

X69165          CCGCCGGCCATTCATTGCCGCCGGAGCTGCACTTGTTATGATTGCGGTTTTCCTCATCGG 360
C2              CCGCCGGCCATTCATTGCCGCCGGAGCTGCACTTGTTATGATTGCGGTTTTCCTCATCGG 356
                ************************************************************

X69165          ATTCGCCGCCGACCTTGGTCACGCCTCCGGTGACACTCTCGGAAAAGGATTTAAGCCACG 420
C2              ATTCGCCGCCGACCTTGGTCACGCCTCCGGTGACACTCTCGGAAAAGGATTTAAGCCACG 416
                ************************************************************

X69165          TGCCATTGCCGTTTTCGTCGTCGGCTTTTGGATCCTTGATGTTGCTAACAACATGTTACA 480
C2              TGCCATTGCCGTTTTCGTCGTCGGCTTTTGGATCCTTGATGTTGCTAACAACATGTTACA 476
                ************************************************************

X69165          GGGCCCATGCAGAGCACTACTGGCTGATCTCTCCGGCGGAAAATCCGGCAGGATGAGAAC 540
C2              GGGCCCATGCAGAGCACTACTGGCTGATCTCTCCGGCGGAAAATCCGGCAGGATGAGAAC 536
                ************************************************************

X69165          AGCAAATGCTTTTTTCTCATTCTTCATGGCCGTCGGAAACATTCTGGGGTACGCCGCCGG 600
C2              AGCAAATGCTTTTTTCTCATTCTTCATGGCCGTCGGAAACATTCTGGGGTACGCCGCCGG 596
                ************************************************************

X69165          TTCATATTCTCACCTCTTTAAAGTATTCCCCTTCTCAAAAACCAAAGCCTGCGACATGTA 660
C2              TTCATATTCTCACCTCTTTAAAGTATTCCCCTTCTCAAAAACCAAAGCCTGCGACATGTA 656
                ************************************************************

X69165          CTGCGCAAATCTGAAGAGTTGTTTCTTCATCGCTATATTCCTTTTACTCAGCTTAACAAC 720
C2              CTGCGCAAATCTGAAGAGTTGTTTCTTCATCGCTATATTCCTTTTACTCAGCTTAACAAC 716
                ************************************************************

X69165          CATAGCCCTAACCTTAGTCCGGGAAAACGAGCTCCCGGAGAAAGACGAGCAAGAAATCGA 780
C2              CATAGCCCTAACCTTAGTCCGGGAAAACGAGCTCCCGGAGAAAGACGAGCAAGAAATCGA 776
                ************************************************************

X69165          CGAGAAATTAGCCGGCGCCGGAAAATCGAAAGTACCGTTTTTCGGTGAAATTTTTGGGGC 840
C2              CGAGAAATTAGCCGGCGCCGGAAAATCGAAAGTACCGTTTTTCGGTGAAATTTTTGGGGC 836
                ************************************************************

X69165          TTTGAAAGAATTACCTCGACCGATGTGGATTCTTCTATTAGTAACCTGTTTGAACTGGAT 900
C2              TTTGAAAGAATTACCTCGACCGATGTGGATTCTTCTATTAGTAACCTGTTTGAACTGGAT 896
                ************************************************************

X69165          CGCGTGGTTTCCCTTTTT-CTTATACGATACAGATTGGATGGCTAAGGAGGTTTTCGGTG 959
C2              CGCGTGGTTTCCCTTTTT-CTTATACGATACAGATTGGATGGCTAAGGAGGTTTTCGGTG 955
                ***************** ******************************************
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X69165          GACAAGTCGGTGATGCGAGGTTGTACGATTTGGGTGTACGCGCTGGTGCAATGGGATTAC 1019
C2              GACAAGTCGGTGATGCGAGGTTGTACGATTTGGGTGTACGCGCTGGTGCAATGGGATTAC 1015
                ************************************************************

X69165          TGTTGCAATCTGTGGTTCTAGGGTTTATGTCACTTGGGGTTGAATTCTTAGGGAAGAAGA 1079
C2              TGTTGCAATCTGTGGTTCTAGGGTTTATGTCACTTGGGGTTGAATTCTTAGGGAAGAAGA 1075
                ************************************************************

X69165          TTGGTGGTGCTAAGAGGTTATGGGGAATTTTGAACTTTGTTTTGGCTATTTGCTTGGCTA 1139
C2              TTGGTGGTGCTAAGAGGTTACGGGGAATTTTGAACTTTGTTTTGGCTATTTGCTTGGCTA 1135
                ******************* ************************************

X69165          TGACCATTTTGGTCACCAAAATGGCCGAGAAATCTCGCCAGCACGACCCCGCCGGCACAC 1199
C2              TGACCATTTTGGTCACCAAAATGGCCGAGAAATCTCGCCGGCACGACCCCGCCGGCACAC 1195
                ************************************.*********************

X69165          TTATGGGGCCGACGCCTGGTGTTAAAATCGGTGCCTTGCTTCTCTTTGCCGCCCTTGGTA 1259
C2              TTATGGGGCCGACGCCTGGTGTTAAAATCGGTGCCTTGCTTCTCTTTGCCGCCCTTGGTA 1255
                ************************************************************

X69165          TTCCTCTTGCGGCAACTTTTAGTATTCCATTTGCTTTGGCATCTATATTTTCTAGTAAT- 1319
C2              TTCCTCTTGCGGCAACTTTTAGTATTCCATTTGCTTTGGCATCTATATTTTCTAGTAATC 1315
                ************************************************************

X69165          GCTGGTTCAGGACAAGGTTTGTCACTAGGAGTGCTCAATCTTGCAATTGTTGTACCACAG 1379
C2              G-TGGTTCAGGACAAGGTTTGTCACTAGGAGTGCTCAATCTTGCAATTGTTGTACCACAG 1375
                * ************************************************************

X69165          ATGTTGGTGTCACTAGTAGGAGGGCCATGGGATGATTTGTTTGGAGGAGGAAACTTGCCT 1439
C2              ATGTTGGTGTCACTAGTAGGAGGGCCATGGGATGATTTGTTTGGAGGAGGAAACTTGCCT 1435
                ************************************************************

X69165          GGATTTGTAGTTGGAGCAGTTGCAGCTGCCGCGAGCGCTGTTTTAGCACTCACAATGTTG 1499
C2              GGATTTGTAGTTGGAGCAGTTGCAGCTGCCGCGAGCGGTGTTTTAGCACTCACAATGTTG 1495
                ********************************** ***********************

X69165          CCATCTCCACCTGCTGATGCTAAGCCAGCAGTCGCCATGGGCGGTTTCCATTAAATAATTA 1558
C2              CCATCTCCACCTGCTGATGCTAAGCCAGCAGTCGCCATGGG-GCTTTCCATTAAATAATTA 1554
                ****************************************** ******************
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Appendix 7.26: Alignment of *StSUT1* sequence cloned into pBract 214 with the original *StSUT1*.The underlined sequences indicates ubi1 promoter sequences in pBract214

```
ubi_StSUT1      GCTATTTATTTGCTTGGTACTGTTTCTTTTGTCGATGCTCACCCTGTTGTTTGGTGTTAC 60
StSUT1

ubi_StSUT1      TTCGCCCATCACAAGTTTGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACAT 120
StSUT1          ------------------------------------------------------------AT 2
                                                                              **

ubi_StSUT1      GGAGAATGGTACAAAAAGAGAAGGTTTAGGGAAACTTACAGTTTCATCTTCTCTACAAGT 180
StSUT1          GGAGAATGGTACAAAAAGAGAAGGTTTAGGGAAACTTACAGTTTCATCTTCTCTACAAGT 62
                ************************************************************

ubi_StSUT1      TGAACAGCCTTTAGCACCATCAAAGCTATGGAAAATTATAGTTGTAGCTTCCATAGCTGC 240
StSUT1          TGAACAGCCTTTAGCACCATCAAAGCTATGGAAAATTATAGTTGTAGCTTCCATAGCTGC 122
                ************************************************************

ubi_StSUT1      TGGTGTTCAATTTGGTTGGACTCTTCAGCTCTCTTTGCTTACACCTTATGTTCAATTGCT 300
StSUT1          TGGTGTTCAATTTGGTTGGGCTCTTCAGCTCTCTTTGCTTACACCTTATGTTCAATTGCT 182
                ******************.*****************************************

ubi_StSUT1      CGGAATTCCTCATAAATTTGCCTCTTTTATTTGGCTTTGTGGACCGATTTCTGGTATGAT 360
StSUT1          CGGAATTCCTCATAAATTTGCCTCTTTTATTTGGCTTTGTGGACCGATTTCTGGTATGAT 242
                ************************************************************

ubi_StSUT1      TGTTCAGCCAGTTGTCGGTTACTACAGTGATAATTGCTCCTCCCGTTTCGGTCGCCGCCG 420
StSUT1          TGTTCAGCCAGTTGTCGGTTACTACAGTGATAATTGCTCCTCCCGTTTCGGTCGCCGCCG 302
                ************************************************************

ubi_StSUT1      GCCATTCATTACCGCCGGAGCTGCACTTGTTATGATTGCGGTTTTCCTCATCGGATTCGC 480
StSUT1          GCCATTCATTGCCGCCGGAGCTGCACTTGTTATGATTGCGGTTTTCCTCATCGGATTCGC 362
                **********.*************************************************

ubi_StSUT1      CGCCGACCTTGGTCATGCCTCCGGTGACACTCTCGGAAAAGGATTTAAGCCACGTGCCAT 540
StSUT1          CGCCGACCTTGGTCATGCCTCCGGTGACACTCTCGGAAAAGGATTTAAGCCACGTGCCAT 422
                ************************************************************

ubi_StSUT1      TGCCGTTTTCGTCGTCGGCTTTTGGATCCTTGATGTTGCTAACAACATGTTACAGGGCCC 600
StSUT1          TGCCGTTTTCGTCGTCGGCTTTTGGATCCTTGATGTTGCTAACAACATGTTACAGGGCCC 482
                ************************************************************

ubi_StSUT1      ATGCAGAGCACTACTGGCTGATCTCTCCGGCGGAAAATCCGGCAGGATGAGAACAGCAAA 660
StSUT1          ATGCAGAGCACTACTGGCTGATCTCTCCGGCGGAAAATCCGGCAGGATGAGAACAGCAAA 542
                ************************************************************

ubi_StSUT1      TGCTTTTTTCTCATTCTTCATGGCCGTCGGAAACATTCTGGGGTACGCCGCCGGTTCATA 720
StSUT1          TGCTTTTTTCTCATTCTTCATGGCCGTCGGAAACATTCTGGGGTACGCCGCCGGTTCATA 602
                ************************************************************

ubi_StSUT1      TTCTCACCTCTTTAAAGTATTCCCCTTCTCAAAAACCAAAGCCTGCGACATGTACTGCGC 780
StSUT1          TTCTCACCTCTTTAAAGTATTCCCCTTCTCAAAAACCAAAGCCTGCGACATGTACTGCGC 662
                ************************************************************

ubi_StSUT1      AAATCTGAAGAGTTGTTTCTTCATCGCTATATTCCTTTTACTCAGCTTAACAACCATAGC 840
StSUT1          AAATCTGAAGAGTTGTTTCTTCATCGCTATATTCCTTTTACTCAGCTTAACAACCATAGC 722
                ************************************************************

ubi_StSUT1      CCTAACCTTAGTCCGGGAAAACGAGCTCCCGGAGAAAGACGAGCAAGAAATCGACGAGAA 900
StSUT1          CCTAACCTTAGTCCGGGAAAACGAGCTCCCGGAGAAAGACGAGCAAGAAATCGACGAGAA 782
                ************************************************************

ubi_StSUT1      ATTAGCCGGCGCCGGAAAATCGAAAGTACCGTTTTTCGGTGAAATTTTTGGGGCTTTGAA 960
StSUT1          ATTAGCCGGCGCCGGAAAATCGAAAGTACCGTTTTTCGGTGAAATTTTTGGGGCTTTGAA 842
                ************************************************************


ubi_StSUT1      AGAATTAACTCGACCGATGTGGATTCTTCTATTAGTAACCTGTTTGAACTGGATCGCGTG 1014
StSUT1          AGAATTACCTCGACCGATGTGGATTCTTCTATTAGTAACCTGTTTGAACTGGATCGCGTG 902
                ************************************************************
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ubi_StSUT1      GTTTCCCTTTTTCTTATACGATACAGATTGGATGGCTAAGGAGGTTTTCGGTGGACAAGT 1073
StSUT1          GTTTCCCTTTTTCTTATACGATACAGATTGGATGGCTAAGGAGGTTTTCGGTGGACAAGT 962
                ************************************************************

ubi_StSUT1      CGGTGATGCGAGGTTGTACGATTTGGGTGTACGCGCTGGTGCAATGGGATTACTGTTGCA 1133
StSUT1          CGGTGATGCGAGGTTGTACGATTTGGGTGTACGCGCTGGTGCAATGGGATTACTGTTGCA 1022
                ************************************************************

ubi_StSUT1      ATCTGTGGTTCTAGGGTTTATGTCACTTGGGGTTGAATTCTTAGGGAAGAAGATTGGTGG 1193
StSUT1          ATCTGTGGTTCTAGGGTTTATGTCACTTGGGGTTGAATTCTTAGGGAAGAAGATTGGTGG 1082
                ************************************************************

ubi_StSUT1      TGCTAAGAGGTTACGGGGAATTTTGAACTTTGTTTTGGCTATTTGCTTGGCTATGACCAT 1253
StSUT1          TGCTAAGAGGTTACGGGGAATTTTGAACTTTGTTTTGGCTATTTGCTTGGCTATGACCAT 1142
                ************************************************************

ubi_StSUT1      TTTGGTCACCAAAATGGCCGAGAAATCTCGCCGGCACGACCCCGCCGGCACACTTATGGG 1313
StSUT1          TTTGGTCACCAAAATGGCCGAGAAATCTCGCCGGCACGACCCCGCCGGCACACTTATGGG 1202
                ************************************************************

ubi_StSUT1      GCCGACGCCTGGTGTTAAAATCGGTGCCTTGCTTCTCTTTGCCGCCCTTGGTATTCCTCT 1373
StSUT1          GCCGACGCCTGGTGTTAAAATCGGTGCCTTGCTTCTCTTTGCCGCCCTTGGTATTCCTCT 1262
                ************************************************************

ubi_StSUT1      TGCGGCAACTTTTAGTATTCCATTTGCTTTGGCATCTATATTTTCTAGTAAT-GCTGGTT 1433
StSUT1          TGCGGCAACTTTTAGTATTCCATTTGCTTTGGCATCTATATTTTCTAGTAATCG-TGGTT 1322
                ************************************************** * *****

ubi_StSUT1      CAGGACAAGGTTTGTCACTAGGAGTGCTCAATCTTGCAATTGTTGTACCACAGATGTTGG 1493
StSUT1          CAGGACAAGGTTTGTCACTAGGAGTGCTCAATCTTGCAATTGTTGTACCACAGATGTTGG 1382
                ************************************************************

ubi_StSUT1      TGTCACTAGTAGGAGGGCCATGGGATGATTTGTTTGGAGGAGGAAACTTGCCTGGATTTG 1553
StSUT1          TGTCACTAGTAGGAGGGCCATGGGATGATTTGTTTGGAGGAGGAAACTTGCCTGGATTTG 1442
                ************************************************************

ubi_StSUT1      TAGTTGGAGCAGTTGCAGCTGCCGCGAGCGGTGTTTTAGCACTCACAATGTTGCCATCTC 1613
StSUT1          TAGTTGGAGCAGTTGCAGCTGCCGCGAGCGCTGTTTTAGCACTCACAATGTTGCCATCTC 1502
                ***************************** ****************************

ubi_StSUT1      CACCTGCTGATGCTAAGCCAGCAGTCGCCATGGGCGGTTTCCATTAAATA..........  1631
StSUT1          CACCTGCTGATGCTAAGCCAGCAGTCGCCATGGG-GCTTTCCATTAAATAA..........  1552
                ********************************* * **************
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