Analysis and manipulation of the starch biosynthesis pathway in hexaploid spring wheat (*Triticum aestivum* L.)

Shalini Mukherjee

Thesis submitted to the Faculty of Graduate Studies of
the University of Manitoba
in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSPHY

Department of Plant Science
University of Manitoba
Winnipeg

ABSTRACT

Mukherjee, Shalini. Ph.D. The University of Manitoba, May, 2014. Analysis and manipulation of the starch biosynthesis pathway in hexaploid spring wheat (*Triticum aestivum* L.). Ph.D. supervisor: Dr. Belay T. Ayele

Starch is an important component of a wheat grain, comprising 50-70% of its dry weight. Its biosynthesis involves a complex pathway mediated by several enzymes, each of which is encoded by genes that have more than one family member. To better understand starch synthesis in wheat grains, this study characterized the sucrose-starch metabolic pathway using physiological, molecular, biochemical and metabolic approaches. These analyses led to the identification of genes that appear to have predominant expression during grain development in wheat including, *TaSUT1, TaSuSy2, AGPL1, SSI, SSIIIa* and *SBEIIa*, suggesting that these genes play a regulatory role in starch accumulation. This was further confirmed by comparative analyses of starch synthesis between cultivars with contrasting thousand kernel weights, which revealed a closer association of the expression of the same set of genes with starch accumulation in developing wheat grains. The effect on starch yield of one of the candidate genes identified, AGPase, was examined through a transgenic approach, which involved expression of a gene encoding modified version of maize AGPase large subunit, designated as *Sh2r6hs*, in wheat under the control of maize's constitutive Ubiquitin1 promoter. This manipulation of the wheat AGPase activity produced wheat lines with increased AGPase activity, grain weight and grain starch level, suggesting that the wheat grain size can be enhanced through increasing the capacity of starch synthesis both in the source and sink tissues. The study also identified and characterized a partial fragment of wheat rbcS promoter, and indicated that the promoter fragment can potentially be used as a tool for targeting the expression of genes of interest in photosynthetic source tissues.

ACKNOWLEDGEMENTS

I appreciate the support of everyone in the Plant Science Department towards the completion of this thesis. First of all, I want to thank my supervisor Dr. Belay T. Ayele for giving me the opportunity to work with him and extending his expert advice, encouragement and support throughout this project. I also thank my co-supervisor Dr. Claudio Stasolla for his valuable guidance and feedback, and Drs. Anita Brûlé-Babel and Georg Hausner for being on my advisory committee and providing important suggestions. I thank Dr. Robert Gulden for his time and advice on using the Statistical Analysis System. I extend my sincerest thanks to Drs. Fouad Daayf and Genyi Li for allowing me to use their laboratory facilities. I sincerely thank Dr. Mark Jordan of AAFC, Winnipeg and his technicians for allowing me to learn the technique of cereal transformation.

I would like to acknowledge the help of capable technicians at the Crop Technology Centre and the Department of Plant Science. My sincerest thanks to Ms. Zhen Yao and Ms. Chelsey McDougall for sharing their technical expertise. I thank Mr. Douglas Durnin, Mr. Lorne Adams and Mr. Ralph Kowatsch for their help. I thank all the past and present members of the Ayele group at Crop Technology Center for their help, support and discussions. A big thank-you to my dear friends at the University for the good times at the graduate lounge!

I am grateful to the University of Manitoba for providing the International Graduate Student Scholarship during the length of my study. I am very appreciative of Husky Energy Inc. and Natural Science and Engineering Research Council of Canada (NSERC) for their financial support.

I thank my husband Shankar for being by my side through the thick and thin with a smile on his face. And finally, none of this would have been possible without my parents Somen Mukherjee who kept his trust in me till the very end, and Minoti Mukherjee who made me who I am. Thank-you dear Sohini for all your attention and being the best sister.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

FOREWORD

This thesis is written in manuscript style following the guidelines set by the University of Manitoba. A general introduction about the research project and literature review precedes the four manuscripts that comprise the main part of the thesis. Each manuscript has an abstract, introduction, materials and methods, results and discussion. The manuscripts are formatted according to the Plant Physiology journal, and they are followed by general discussion and conclusions, literature cited and appendices section.

1. INTRODUCTION

Since its origin in the Fertile Crescent several thousand years ago to its being the world's third most produced crop in 2011 (FAOSTAT), common wheat has made a long journey globally and has rightfully attained its attribute as the "cosmopolite of edible plants" (Carver, 2009). From appetizers to desserts and from barns to bio-ethanol, wheat starch has found its application in a wide variety of industries. Other than protein, the starch quality also contributes towards this versatility. One of the potential solutions to reduce the competition for starch between food, feed and fuel is maximizing its yield. An understanding of the biosynthetic machinery of starch and identifying the associated molecular components provides important insights into its further improvement both in yield and quality. Wheat is one of the most researched crops, and both its starch and protein has been well studied at the physiological and biochemical level. However, the molecular basis of starch metabolism in wheat is still poorly understood.

Sucrose transport and metabolism precede starch synthesis and are mediated by several gene families including sucrose transporters, sucrose synthases and invertases. Sucrose, being the most transportable form of photoassimilate, is translocated from the source (leaves) to the sink (grains) by the sucrose transporter proteins (Aoki *et al*., 2002; Deol *et al*., 2013). Sucrose synthases and invertases break sucrose into simple hexoses that can enter into the starch biosynthesis pathway. The formation of starch employs gene families belonging mainly to three groups: AGPases, starch synthases and starch branching enzymes. Once synthesized, starch is further modified by debranching enzymes and several other proteins (Emes *et al*., 2003; Hannah, 2007). Although previous studies have identified several genes involved in sucrose and starch metabolism, the molecular mechanisms underlying the coordination of sucrose and starch

metabolism remain poorly understood. This thesis investigated the sucrose to starch metabolic pathways in developing grains by employing physiological, molecular and enzyme activity studies as well as measurement of grain sucrose and starch content. Moreover, the thesis examined the possibility of manipulating starch yield in wheat grains through constitutive expression of the mutated AGPase large subunit from maize in both source and sink tissues using a transgenic approach. The main objectives of the thesis are:

1. Analysis of sucrose to starch metabolism pathway in developing grains of common wheat
2. Investigation of the role of natural variation in starch biosynthesis: a comparison between spring wheat cultivars with differing grain starch content
3. Investigating the effect of constitutive expression of mutated maize AGPase (*Sh2r6hs*) on starch accumulation in wheat grains

The thesis also reports the isolation and characterization of rubisco small subunit promoter from common wheat (Appendix 12). The outcomes of this thesis project contribute to increase our knowledge in sucrose-starch metabolism in wheat grains, and the findings also have the potential to further our knowledge of dry matter partitioning. The knowledge gained can be applied to generate tools to improve the wheat starch yield and quality for different end-use applications.

2. LITERATURE REVIEW

2.1 The origin and domestication of wheat cultivation

Wheat is one of the oldest cultivated crops. Originating in the "fertile crescent" near Mesopotamia (the modern Syria and Turkey), this Triticeae crop has established its importance all the way from being a wild grass to being the world's third most cultivated cereal (SOFA, 2011). Agriculture began at least 10,000 years ago (pre-pottery Neolithic age) (Lev-Yadun *et al*., 2000) as humans transitioned from the practices of foraging to farming with the domestication of wild progenitors of wheat around 7500 BC (Nesbitt, 2001). The earliest forms of wheat to be cultivated were *einkorn* with a diploid genome and *emmer* with a tetraploid genome. Domestication of a crop species involves the loss of characters which would otherwise be an asset to thrive in the wild. Accordingly, domestication of the wild wheat involved loss of seed shattering to prevent loss of grains, selection of "naked" over 'hulled' grains for ease of threshing, and development of a tough rachis over a brittle one to prevent grain loss during harvest. Larger grain size evolved for better germination capacity, higher starch to bran ratio for enhanced food value, and other changes in the morphological and physiological traits, all of which contributed to the improvement of wheat yield and quality (Nesbitt, 2001; Zohary, 1989; Nevo, 2011; Peleg *et al*., 2011). Hexaploid wheat or the modern day bread wheat (*Triticum aestivum)* is said to have entered cultivation more or less simultaneously with the *emmer* and *einkorn* wheat (Feldman, 1976) and probably has multiple origins (Brown, 1999). The current geographical region of cultivated wheat stretches from Argentina at 45^{o}S to parts of Russia and Finland at 67^{o}N (Feldman, 1976; Gustafson *et al*., 2009). The major wheat producing regions of the world include China, India, the Prairie Belt of USA and southern Canada, the Mediterranean

basin and Australia, and their combined harvest in 2012 accounted for about 28% of the world's total cereal production (FAO: World Food Situation, accessed 2012).

2.2 History of wheat cultivation in western Canada

Wheat cultivation, although originated in Eurasia amongst pastoral nomads, spread across the whole world in no time owing to the unique characteristics of the grain. Cultivation of wheat in western Canada can be traced back to 1812 when Scottish settlers in Selkirk tried growing winter wheat that they had brought from Scotland. Due to their lack of knowledge in agriculture, the crop could not be made to flourish and after repeated damage by floods and pests, the stock was exhausted (Buller, 1919). However, one of the first varieties of wheat that survived the harsh weather of Canada, Red Fife, was publicized much for its productivity. Grains of different varieties were later imported from parts of USA, Ukraine, Russia, Australia and Japan, and were established in the Prairie belt of Canada (Buller, 1919). In the early twentieth century, the use of mechanization in wheat production such as the uses of gas tractors, gang plows, and threshing machines, resulted in surpluses of wheat in Canada. Subsequently, the development of Marquis as a globally premium valued variety became one of the biggest Canadian innovations in wheat agriculture (The Canadian Encyclopedia, accessed 2013). By 1920, Marquis accounted for 90% of the area seeded for growing hard red spring wheat in the Canadian praires (McInnis, 2004). However, in the period following World War I, there were droughts, insect pests such as grasshoppers, and crop diseases that affected wheat production. Finally in 1935, the establishment of the Canadian Wheat Board brought some stability over the situation. It was a marketing board for wheat in western Canada and controlled the grain prices in order to benefit farmers affected by the great depression (The Canadian Encyclopedia, accessed 2014). At

present Canada is the world's sixth biggest producer of wheat. From 2002 to 2012 only, wheat production in Canada has increased from 16 million metric tons (MMT) to 27 MMT (FAOSTAT, accessed 2013).

2.3 Evolution of the hexaploid wheat

Hexaploid wheat (*Triticum aestivum*) has a large genome of 16000 Mb (Gupta *et al*., 2008) with a large amount of repetitive DNA and three distinguishable sub-genomes, each with seven chromosomes (n=21). The first set, also known as the A genome, has close resemblance with both the wild (*T. uratu)* as well as cultivated *(T. monococcum)* einkorn (Shewry, 2009). The second sub-set is the B genome. Although its origin is not clearly known, there are indications that it is obtained from the S genome of the *Sitopsis* section of the genus *Aegilops* with *A. speltoides* being the closely related progenitor (Sarkar and Stebbins, 1956; Gustafson *et al*., 2009). At this stage of evolution, the tetraploid wheat originated and two prominent forms, which may or may not have arisen through a single independent cross between the wild progenitors, existed (Brown, 1999; Shewry, 2009; Feldman, 1976). One form was *T. turgidum* (AABB) and the other one *T. timopheevii* (AAGG), both of which show significant difference in the coding as well as non-coding regions of the DNA of the B/G genome donor, also identified as the maternal donor (Gustafson *et al*., 2009). The third set, also known as the D genome, comes from *T. tauschii*, which appears to have been hybridized with the cultivated emmer *T. turgidum* after its domestication (Feldman, 1976; Gustafson *et al*., 2009). The chromosomes of *T. aestivum* (AABBDD) behave however like a diploid owing to the suppression of pairing of the homoeologous chromosomes due the presence of the Pairing homoeologous 1 (*Ph1*) gene on

chromosome 5B (Sears, 1969; Sears, 1976; Gupta *et al*., 2008), and this has helped in retaining the integrity and dominance of bread wheat globally.

2.4 Wheat as a source food, feed and fuel

Wheat is primarily used as human food, accounting for about a third of the world's dietary consumption. On account of the visco-elastic properties of the gluten proteins, wheat flour is uniquely suited for the production of bread. Apart from this, the western diet includes cakes, pastries, cookies and crackers, all of which are made from wheat flour. It also remains the main constituent of different food products such as soups, sausages, thickening agents, condiments and sauces. The flour of wheat has some unique properties which promoted its wide use in the food industry including protein content, water absorption capacity, dough quality, viscosity of the starch, and strength and extensibility of the resulting dough (Wrigley *et al*., 2009). These same qualities of the flour also make it an important candidate for a variety of industrial applications. Apart from these uses, the whole grain is often used directly as animal feed. The end use of wheat flour in other non-food and non-feed industries such as the pharmaceutical and paper industries largely depends on its content of gluten, a wheat protein that has a unique property of binding with water and becoming firm. Wheat is also used in the production of various health foods and dietary supplements (Wheat- a plant that changed the world, accessed 2014). The most recent use of wheat in a completely new avenue is in the field of bio-ethanol production. Wheat is very rich in carbohydrates, mainly starch, which can be distilled through various industrial processes to produce biofuels. This end-use mainly requires wheat varieties with high starch yield and low protein content. In addition, the protein in Dried Distillers Grains with Solubles

(DDGS), which is the co-product of bioethanol, is valuable as an animal feed (Graybosch *et al*., 2009).

2.5 Wheat as a biofuel feedstock

According to the Global Renewable Fuels Alliance (GRFA) and the Renewable Fuels Association (RFA), approximately 83 billion litres of bioethanol were produced globally in 2012 (Renewable Fuels Association, accessed 2013). The USA and Brazil are the top producers of bioethanol, mainly from corn and sugarcane, respectively. Until August 2013, total biofuel sales were approximately 1.37 billion gallons and 2.54 billion litres in the USA and Brazil, respectively. Canada has gradually emerged as the world's fifth largest producer of bioethanol (CTBI - Fuel & Energy, accessed 2013) owing to the 21 bioethanol (15 currently operational) plants established in the country (Canadian Renewable Fuels Association, accessed 2013). Corn is the main feedstock for bioethanol production in eastern Canada, especially in Ontario, where it is a major crop. In western Canada, however, wheat is the major substrate for bioethanol production (Alberta- Agriculture and Rural Development, accessed 2013). At the national level, it is predicted that wheat would account for 22% of the bioethanol feedstock for the year 2013-2014 (The Crop Site, accessed 2014). TerraGrain Fuel, one of the largest bioethanol plants in North America located in Saskatchewan, produces an average of 150 million litres of bioethanol from 15 million bushels of wheat annually (TerraGrain Fuels, accessed 2013). Some of the factors that contribute to wheat's potential candidacy for use as a bioethanol feedstock include its high amylopectin to amylose ratio, tolerance to mycotoxic contaminants and the amount of total protein and starch in the grain (Graybosch *et al*., 2009). The wheat grain can be processed to bioethanol by both wet and dry milling methods, the latter being more economical. This quality

makes wheat starch a lucrative choice for bioethanol feedstock. Apart from Canada, wheat has been in use as a major feedstock for bioethanol production in Europe since 2005. For example, it is the most economically viable feedstock in the UK (Smith *et al*., 2006) and Germany (Renewables made in Germany, accessed 2013).

Wheat production in Canada ranges between 22 to 24 million tonnes (MT) per year, the majority coming from Saskatchewan (Alberta- Agriculture and Rural Development, accessed 2013). There is an increasing demand for different wheat varieties from different end-users. For example, high-yielding and low-protein wheat varieties such as Winter wheat and Canadian Prairie Spring (CPS) wheat are preferred for the bioethanol industry (Alberta- Agriculture and Rural Development, accessed 2013). The CPS class of wheat was established in 1985 as a lower protein alternative to the Canada Western Red Spring (CWRS) class. It has been used in blends for flat breads, crackers and noodles, and very recently as a viable feedstock for bioethanol production (Govt. of Saskatchewan- Agriculture, accessed 2013). Among the wheat cultivars in the Canadian Western Soft White Spring (CWSWS) class, AC Andrew, which consists of about 2% to 3% lower grain protein than CWRS is considered as the variety of choice for bioethanol production in Western Canada (Austin, 2009).

2.6 The wheat grain – morphology and anatomy

Wheat is a typical member of the *Poaceae* family. Its grain has a fruit wall (pericarp) fused with a seed coat (testa), resulting in a characteristic feature of a caryopsis. A wheat grain is typically 5-9 mm in length and quite often weighs between 30-50 mg (NZFMA, accessed 2013). Each grain of wheat can be broadly divided into three parts: the bran (~ 14.5% of the grain weight), the endosperm (~ 83% of the grain weight), and the germ (~ 2.5% of the grain weight)

(Roastech, accessed 2013). A diagrammatic representation of the grain has been reproduced in Figure 2.1 from www.grainchain.com. The grain of wheat has the embryo on one end and a bundle of hairs known as the beard (or brush) on the other end. The bran layers of the wheat grain are comprised of four separate layers: the pericarp, testa, nucellar layers and the aleurone cells. The pericarp is comprised of the epidermis covered by the cuticle, hypodermis, parenchyma and the inner epidermis or the tube cells. The embryo sac wall consists of the outer and inner integument beneath which lies the nucellar epidermis and the nucellus. This is followed by the aleurone layer. Each cell of the aleurone layer contains a high concentration of niacin bodies and is enclosed within a fibrous cell wall. The aleurone cell wall is composed mainly of arabinoxylans and beta glucans in various ratios and is thicker than endospermic cell walls. The starchy endosperm is the main part of the wheat grain. The endosperm is the major site of starch biosynthesis in a wheat grain, although a certain amount of starch is also synthesised in the pericarp during the initial stages of grain development (Tetlow, 2011). The wheat germ consists of the embryo and the scutellum. The scutellum is adjacent to the endosperm and is used by the developing embryo to obtain food from the starchy endosperm during its germination. The ventral side of the grain has a deep crease also known as the ventral groove (WheatBP, accessed 2013), and the vascular bundle extends into the grain from the base to the apex (Frazier and Appalanaidu, 1965).

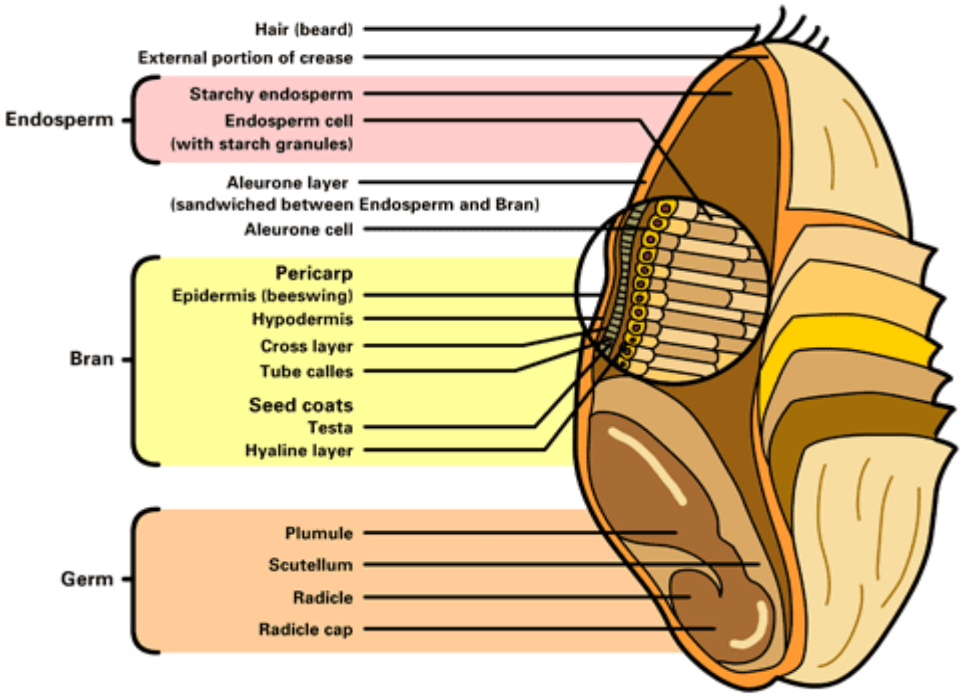


Figure 2.1: A diagrammatic representation of a wheat grain with its different parts (Image taken from: http://www.grainchain.com)

2.7 The starch biosynthesis pathway

Following carbon fixation in higher plants, the allocation of triose phosphate is a strictly regulated process. A portion of the triose phosphate is channeled to support the synthesis of transitory starch in the chloroplast, whereas another portion is exported to the cytoplasm as 3-Phosphoglyceric Acid (3PGA) for sucrose synthesis. Starch can be found in different plant tissues including stems, leaves, pollen, flowers, embryos, fruits, seed endosperm, tubers, bulbs and roots. It can be found either in transitory form or as a storage reserve (Ball and Morell, 2003; Emes *et al*., 2003). The partitioning of carbon fixed in the form of sugar to the sink tissues is largely influenced by the size and metabolic activities present in the sink, as well as its proximity and vascular connections to the source (Simmons and Crookston, 1979). Being the most transportable form of sugar, sucrose is translocated from the source to the sink tissues such as

developing grains (Fig. 2.2). The sugar molecules once in the sink tissues are then converted by sucrose synthases and invertases to hexose phosphates (Fig. 2.2), which in grains are used as substrates for the synthesis of starch via ADP-glucose (Taiz and Zeiger, 2010).

Starch is an insoluble carbon polymer made up of amylose, a glucan polymer composed of linearly linked α-1,4-linked glucose molecules, and amylopectin, another glucan polymer composed of α-1,4-linked glucose molecules branched by α-1,6-glycosidic bonds. The enzyme ADP-glucose pyrophosphorylase (AGPase) catalyzes the first committed step of starch synthesis (Fig. 2.2), converting glucose-1-phosphate and ATP to ADP-glucose and PPi. ADP-glucose is subsequently used by starch synthases and starch branching enzymes (Fig. 2.2) to elongate and branch the glucan chains of the starch granule (Kolbe *et al*., 2005). Amylose has a predominantly linear structure formed by starch synthases. This involves the transfer of α-D-glucose from ADP-glucose to the glucan moiety, elongating the chain with α-1,4 glycosidic linkage (Vrinten and Nakamura, 2000) with infrequently dispersed branching points formed by α-1,6 linkages. When the branching points are more frequent in a polymer of 10,000 to 100,000 glucosyl residue, it forms amylopectin (Keeling and Myers, 2010). Unlike amylose, amylopectin is essential for starch granule formation as evidenced by various altered granule morphology on account of disruption of the amylopectin synthesis (Keeling and Myers, 2010).

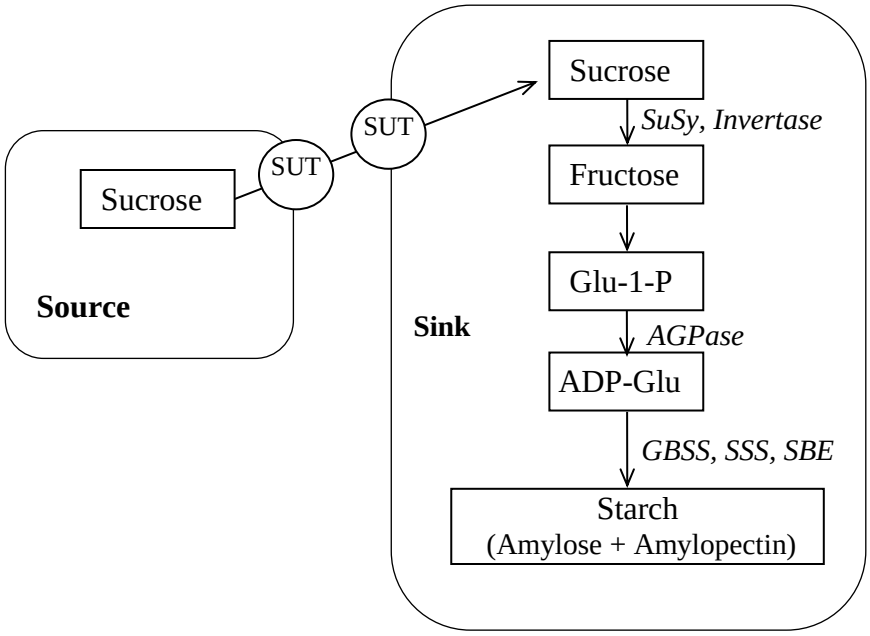


Figure 2.2: A simplified schematic representation of sucrose transport and hydrolysis, and the subsequent starch biosynthesis pathway (the major metabolites and enzymes/proteins involved are indicated). SUT= sucrose transporters; SuSy= sucrose synthase; AGPase= ADP-glucose pyrophosphorylase; GBSS= granule bound starch synthase; SSS= soluble starch synthase; SBE= starch branching enzyme; Glu-1-P, Glucose-1-phosphate; ADP-Glu, ADP Glucose. (Prepared on the basis of the literature review from Kolbe et al., 2005; Taiz and Zeiger, 2010 and Keeling and Myers, 2010).

2.7.1 The synthesis, transport and hydrolysis of sucrose

The Calvin cycle fixes carbon dioxide (CO_2) to produce triose phosphate which is the precursor for transitory starch synthesis and sucrose synthesis. During dark, when photosynthesis is not operational, the transitory starch is converted to sucrose. Sucrose is either stored in the vacuole or transported to sink tissues for use as a source of energy or for the synthesis of storage compounds such as starch (Tretheway and Smith 2000). In many plants, it is the only detectable sugar molecule in the phloem sap (Aoki *et al*., 2012), and being a non-reducing sugar, it can be easily translocated. However, not all plants translocate photosynthates in the form of sucrose;

there are some plant families such as Cucurbitaceae, which translocate raffinose, stachyose and verbascose along with sucrose. Other species belonging to Apiaceae and Rosaceae transport sugar in the form of mannitol and sorbitol, respectively (Zimmermann, 1975; Aoki *et al*., 2012). Recent studies demonstrated that some herbaceous species belonging to the Ranunculaceae and Paraveraceae families translocate hexoses (glucose and fructose) (Van Bel and Hess, 2008).

The triose phosphate and dihydroxyacetone phosphate, produced during the Calvin cycle are exported from the chloroplasts, and along with another triose phosphate, glyceraldehyde-3-phosphate, forms hexose phosphates, a major portion of which enter into the sucrose synthesis pathway. Sucrose phosphate synthase catalyzes the synthesis of sucrose-6-phosphate from UDP-glucose and fructose-6-phosphate. Sucrose phosphatase then hydrolyzes and releases free sucrose by an irreversible reaction. Sucrose synthase (SuSy) is another enzyme which, by a reversible reaction, catalyzes the formation of sucrose from UDP-glucose and fructose. However, this enzyme is mainly involved in the cleavage of sucrose, and this is discussed more in details in section 2.7.3.

Sucrose is translocated to the sink tissues such as developing grains via sieve elements of the phloem. Sieve elements exist in association with companion cells by numerous intercellular connections also known as plasmodesmata. The companion cells provide energy and proteins to the sieve elements as they have a high metabolism and form the sieve element-companion cell (SE-CC) complex (Kühn and Grof, 2010). The process of sucrose delivery into the SE-CC complex of the phloem can be either symplastic or apoplastic. The symplastic pathway involves the plasmodesmatal connections between cells whereas the apoplastic pathway involves sucrose transporter proteins which co-transport sucrose along with H^+-ions (Kühn *et al*., 1996; Kuhn *et al*., 1999). Upon arrival at the sink organs, the sucrose molecules are transported to the

endosperm. The transport of sucrose to the grains is a critical determinant for crop yield and quality (Halford *et al*., 2011). In developing grains, some of the sucrose is hydrolyzed by cell wall and vacuolar invertases and forms glucose and fructose. Invertases are also present in the apoplast, cytosol, plastids and mitochondria (Halford *et al*., 2011). They are involved in supplying hexoses for cellular respiration, osmoregulation, and stress related responses (Roitsch and González, 2004). The SuSy which is present in the cell wall as well as in the cytoplasm catalyzes the reversible conversion of sucrose and UDP to UDP-glucose and fructose, which are used as substrates for the synthesis of starch via ADP-glucose (Nelson and Cox, 2008).

2.7.2 Sucrose transporter proteins in cereals

Sucrose transporters are integral membrane proteins present on the plasma membrane of phloem cells, and are involved in the active transport of sucrose across the membrane coupled with proton transport (Aoki *et al*., 2002). They belong to the major facilitator family and are also structurally related to the hexose transporters of bacteria, fungi, plants and mammals (Reinders and Ward, 2001; Abramson *et al*., 2003; Sauer, 2007; Kühn and Grof, 2010). Sucrose transporters have been studied in rice (Aoki *et al*., 2003), maize (Aoki *et al*., 1999) , barley (Weschke *et al*., 2000) and wheat (Aoki *et al*., 2002; Aoki *et al*., 2006; Deol *et al*., 2013). They have been shown to play active roles in phloem loading, unloading, and in the retrieval of sucrose leaked from phloem (Aoki *et al*., 1999; Aoki *et al*., 2002; Rosche *et al*., 2002; Scofield *et al*., 2004).

In rice, five SUTs have been reported; each having different function (Hirose *et al*., 1997; Aoki *et al*., 2003). The protein encoded by *OsSUT1* is involved in sucrose transport to the developing grains during grain filling and to the growing embryo in germinating seeds. It is also involved in retrieving sucrose leaked out of the phloem during the long distance transport

(Matsukura *et al*., 2000; Furbank *et al*., 2001; Scofield *et al*., 2007). Recent studies have shown that high temperature during grain filling can downregulate the expression of *OsSUT1*. This results in earlier ripening due to earlier termination of assimilate supply to the grains and grain filling, and ultimately reduced yield (Phan *et al*., 2013). It has been shown that *OsSUT2* is expressed equally in different tissues, suggesting its housekeeping role, whereas *OsSUT3*, *OsSUT4* and *OsSUT5* are highly expressed in the sink leaves and in the developing grains immediately after flowering, implying their role in transporting sucrose from source leaves to growing caryopsis or developing leaves at different stages (Aoki *et al*., 2003).

The vascular bundles in barley grain are located at the bottom of the crease, and supply the photoassimilate into the grain. After the phloem unloads at the crease of the grain, the unloaded sugars move inward towards the maternal tissue symplastically. This inward movement to the maternal tissue takes place from the sieve tubes to the nucellar projection of the transfer cells (Weschke *et al*., 2000). In barley, two SUTs have been identified (Weschke *et al*., 2000); the *HvSUT1* is mainly localized in the endospermal transfer layer and the cells of the nucellar projections that serve as sites of sucrose exchange between the maternal and the filial tissues. The *HvSUT2* like *OsSUT2* has been found to be expressed at similar levels across tissues, and is thought to have a housekeeping function (Weschke *et al*., 2000).

2.7.2.1 Sucrose transporters in wheat

Two wheat SUTs have been identified to date. Three homeologues of *TaSUT1* (*TaSUT1A, TaSUT1B, TaSUT1D*) are located on chromosome 4 of hexaploid wheat (Aoki *et al*., 2002). All three are reported to be capable of transporting sucrose in yeast mutant strains and are localized in the aleurone layer, scutellar epidermis, and companion cells of germinating wheat seeds (Aoki *et al*., 2006). This suggests 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 source tissue, immunolocalization based study showed the presence of SUT1 protein on the plasma membrane of the sieve elements in the leaf tissue, implying its role in phloem loading (Scofield *et al*., 2004). This is supported by its expression pattern, which is at maximum from 4 days before heading (in flag leaves) to about 12 days after anthesis (DAA), after which it decreased (Aoki *et al*., 2002). The *TaSUT2* has been found to have a housekeeping role similar to that of *HvSUT2* and *OsSUT2* as it is expressed almost equally across different tissues (Deol *et al*., 2013).

2.7.3 Sucrose hydrolyzing enzymes

2.7.3.1 The role of sucrose synthase in cereal crops

Sucrose synthase (SuSy) catalyzes the conversion of sucrose and UDP to UDP-glucose and fructose in a reversible reaction. The activity of this enzyme has been studied in various plant species and has been shown to play a major role in energy metabolism, controlling the mobilization of sucrose into different pathways that are important for the metabolic, structural, and storage functions of a plant cell (Riffkin, 1995). The fructose produced by SuSy mediated hydrolysis of sucrose serve as substrate for starch biosynthesis. Although catalyzing a reversible reaction, the physiological conditions and high concentration of sucrose in the storage and vascular tissues favour SuSy to catalyze the cleavage of sucrose over its synthesis (Halford *et al*., 2011).

Genes encoding SuSy have been isolated from major cereal crops like wheat, maize, rice and barley. For example, rice contains six SuSy genes, of which *OsSuSy3* and *OsSuSy4* are important for starch formation in the grains. While *OsSuSy1* and *OsSuSy2* are expressed in the elongating cells of roots, leaf and internodes; *OsSuSy5* and *OsSuSy6* are expressed at low levels in almost all the tissues (Hirose, *et al*. 2008). In barley, two isoforms of SuSy gene have been

identified *HvSuSy1* and *HvSuSy2*. Both *HvSuSy1* and *HvSuSy2* are localized in vascular bundles, nucellar projection, and at the base of the endosperm region demonstrating their role in hexose supply to the developing endosperm (Guerin and Carbonero, 1997). The *HvSuSy1* is also expressed in the vascular bundles of young leaves and roots, and in the root cap cells. Maize has at least three *SuSy* genes. Two of them, designated as *Shrunken1 (Sh1) and Sucrose Synthase 1 (Sus1*), encode biochemically similar isozymes, SS1 and SS2, respectively (Chourey, 1981). A third gene, designated as *Sus3* (Carlson *et al*., 2002), is suggested to have a role in cytoplasmic sucrose metabolism (Duncan *et al*., 2006). Mutational studies onf *Zmsh1* showed 2% to 6% reduction in SuSy activity, resulting in a 60% loss of kernel starch (Chourey, 1981; Werr *et al*., 1985; Chourey *et al*., 1998).

Wheat has at least two sucrose synthase genes, *TaSuSy1* (reported as *SS1*) and *TaSuSy2* (reported as *SS2*) (Marana *et al*., 1990). *TaSuSy2* has been shown to be associated with grain weight, and is mapped to chromosomes 2A, 2B and 2D (Dale and Housley, 1986; Jiang *et al*., 2011). The activity of SuSy in wheat is associated with endosperm development and is reported to be at a maximum level during rapid grain filling (Chevalier and Lingle, 1983).

2.7.3.2 The role of invertase in cereal crops

Invertase (INV) catalyzes a conversion of sucrose to glucose and fructose, and this enzyme plays a number of roles in plant growth and development. It has a significant role in the early stages of grain development in cereals. Genes encoding INV have been isolated from maize (Kim *et al*., 2000; Chourey *et al*., 2005), barley (Obenland *et al*., 1993; Weschke *et al*., 2003), wheat (Krishnan *et al*., 1985) and rice (Hirose, 2002; Cho *et al*., 2005). Invertases have been classified into different types based on pH optima (neutral, alkaline and acid INVs) and their location (cell wall and vacuolar INVs) (Sturm, 1999). Acid INVs are mostly found in the cell wall, apoplast

and vacuole, whereas the neutral/alkaline are localized in the cytosol and plastids (Roitsch and González, 2004; Halford *et al*., 2011). Yet, a different form has been reported as an apoplastic INV (Kim *et al*., 2000). These apoplastic INVs are capable of hydrolyzing sucrose into hexoses in the apoplast, and are quite often found in plants with apoplastic mycorrhizal associations (Schaarschmidt and Hause, 2008).

The cell wall form of INV is most studied in grains, because of its role in the supply of hexoses during the early stages of grain growth. This was demonstrated by using a maize mutant *miniature-1* (*mn-1*), which is deficient in cell wall INV5 (CWINV5). Less INV activity in the basal part of the endosperm during kernel development causes withdrawal of the maternal cells in the palentochalazal area, resulting in inhibition of assimilate transport into the developing endosperm and pedicel (Miller and Chourey, 1992). All the hexoses supplied by invertase are not necessarily involved in starch formation; rather some are used for mitosis and cell growth (Roitsch and González, 2004). Rice contains 19 INV genes, of which nine are cell wall bound (Hirose, 2002). Among them, *OsCIN1, OsCIN2*, and *OsCIN7* are suggested to play important roles in the partitioning of sucrose to the embryo and endosperm. The expression of *OsCIN3* is shown to be flower-specific, indicating its specific role in pollen development (Cho *et al*., 2005). In maize, the activity of INV in developing grains is attributed to four genes, designated as *Incw1*, *Incw2, Incw3* and *Incw4* (Kim *et al*., 2000; Chourey *et al*., 2005). While the expression of *Incw3* is found to be tissue specific and developmentally regulated, *Incw4* is expressed constitutively in all vegetative and reproductive tissues (Kim *et al*., 2000). In the case of barley, the hexose content of the filial tissues in developing grains reaches a peak during internal cell divisions and elongation. This is suggested to be related to the expression of *HvCWINV1* and

HvCWINV2 that are localized in the endospermal transfer cell layers flanking the nucellar projections (Weschke *et al*., 2003; Sreenivasulu *et al*., 2004).

In wheat, INV activity is mainly studied in response to drought (Yang *et al*., 2004a; Yang *et al*., 2004b). To date, only two wheat INV genes have been reported, cell wall (*TaCWINV1*) and vacuolar (*TaVINV5*) forms (Krishnan *et al*., 1985). In wheat anthers, INV acts as a sucrolytic enzyme supplying energy for pollen viability, and any disruption to the activity of this enzyme has been shown to restrict the availability of assimilate during anther development (Dorion *et al*., 1996). The activity of INV is not found to change appreciably during endosperm development in wheat (Riffkin, 1995).

2.7.4 ADP-glucose pyrophosphorylase

The enzyme ADP-glucose pyrophosphorylase (AGPase) catalyzes the first committed step of starch synthesis, converting glucose-1-phosphate to ADP-glucose and inorganic pyrophosphate (PPi). AGPase normally occurs in more than one form within a plant; the form of AGPase found in sink tissue (such as endosperm of seeds) is different from the one in the leaves (Lohot *et al*., 2010). While the plant and unicellular chlorophytic AGPases are heterotetramers composed of two small (SSU) and two large (LSU) subunits, each of which is encoded by distinct genes (Emes *et al*., 2003), the bacterial AGPase is a homotetramer encoded by a single gene (Preiss, 1984).

2.7.4.1 The role and regulation of AGPase in plants

Earlier studies with spinach leaves have indicated the existence of two subunits of AGPase, one with molecular mass of 51 kD and the other one exhibiting 54 kD (Morell *et al*., 1987). The subunit with the 54 kD molecular mass has been reported to show a homology to the AGPases of maize and potato. The potato AGPase subunit also exhibits a size similar to the spinach AGPase

subunit (Sowokinos and Preiss, 1982). The SSUs of AGPase is more conserved across species as compared to the LSUs, possibly because it is less tissue specific and would have to bind to the large subunits that have evolved and diverged based on their tissue specificity (Smith-White and Preiss, 1992; Georgelis *et al*., 2007). *Shrunken-2* (SH2) and *Brittle-2* (BT2) from maize show 24.4% and 29% identity with the AGPase of *E.coli*, respectively (Georgelis *et al*., 2007). A close examination of the amino acid sequences of the small and large subunits of maize revealed sequence identity of 43.2%, and gene duplication could have produced the two subunits in the course of evolution (Georgelis *et al*., 2007). The large subunits of AGPase have been considered to be responsible mainly for regulatory properties, whereas the smaller subunits are of catalytic importance. Studies using *E.coli* strains were able to produce LSU homotetramers from potato with very limited enzymatic activity (Iglesias *et al*., 1993). On the other hand, SSU homotetramers from potato, were found to be catalytically active but had decreased sensitivity towards allosteric regulation.

In maize, the AGPase LSUs are encoded by *Shrunken-2* (*Sh2*), whereas the SSUs are encoded by *Brittle2* (*Bt2*) (Hannah and Nelson 1975; Giroux and Hannah 1994; Wang *et al*. 2007). The *E. coli* cells expressing only *Sh2* subunits had slightly higher AGPase activity than cells exclusively expressing *Bt2* subunits (Burger *et al*., 2003). Other studies clearly showed that both subunits are important for catalysis as well as regulation. Although the potato and maize subunits of AGPase have been separated by speciation and evolution long time ago, they are still sufficiently similar and are able to form active mosaic enzymes. It has been shown with the use of the mosaic AGPase made from potato and maize that the two subunits participate equally in controlling the allosteric properties of the mosaic enzyme (Cross, 2004). More recently, overexpression of *Sh2* and *Bt2* separately or together showed that the AGPase activity increased

when either gene was overexpressed separately, but further increase in activity was observed when both are overexpressed together (Li *et al*., 2010).

Rice has two genes encoding the SSUs of AGPase; *OsAPS1*, which is expressed during the later stage of grain development, and *OsAPS2* that is expressed constitutively throughout seed development. The LSUs of AGPase in rice is encoded by four genes; *OsAPL1 and OsAPL2* are expressed during the later stages of grain development, while *OsAPL3* is expressed predominantly during the middle stages of grain development. The fourth gene, *OsAPL4*, exhibits constitutive expression (Akihiro, 2005). Barley has three different genes encoding AGPase; two genes (*HvX* and *HvY*) are reported to encode two different isoforms of the large subunit of the enzyme in the leaf and the endosperm (Villand *et al*., 1992; Kilian *et al*., 1994; Eimert *et al*., 1997), and the small subunit in these tissues is encoded by a single gene (*Hvx*) (Villand *et al*., 1992; Thorbjornsen *et al*., 1996; Doan, 1999; Rösti *et al*., 2006). Unlike dicot plants, cereals possess, extra-plastidial AGPase, which is involved in the synthesis of cytosolic ADP-glucose, and this AGPase accounts for 85% to 96% of the endospermic AGPase activity (Denyer *et al*., 1996; Sikka *et al*., 2001; Burton *et al*., 2002).

In photosynthetic tissues, it has been established that AGPase is restricted within the plastids, and is subjected to allosteric regulation with activation by 3-phosphoglycerate (3-PGA) and inhibited by inorganic phosphate (Pi) (Ghosh and Preiss, 1966). Allosteric regulation of AGPases in the sink tissue has also been widely studied. For example, ADP-glucose synthesis in potato tubers is regulated by 3-PGA and inhibited by Pi (Sowokinos and Preiss, 1982). Apart from allosteric regulation, this enzyme is also subjected to transcriptional regulation. Several studies highlight the transcriptional regulation of AGPase by sucrose (Müller-Röber *et al*., 1990; Li *et al*., 2002), phytohormones such as ABA (Akihiro, 2005) and nitrate (Scheible *et al*., 1997).

Post-translational modification of AGPase has also been studied in potato (Tiessen, 2002) and *Arabidopsis* (Hendriks *et al*., 2003). AGPase activity is under strict redox control in leaves, where reduction during the light phase causes a thioredoxin-mediated reaction of a regulatory disulfide bond present between the two small subunits of the enzyme (Hendriks *et al*., 2003). Such a reduction of AGPase leads to increased activity and at the same time enhanced activation by 3-PGA, as compared to that obtained from the oxidised form present during the dark phase. The regulation of AGPase by redox and allosteric mechanisms reflects that the production of ADP-glucose, which serves as a substrate for starch synthesis, is tightly coordinated between the circadian cycles and carbon status of the source tissue (Gibon *et al*., 2004). Due to such sensitivity to inhibitors and activators, AGPase is widely acknowledged to mediate the rate limiting step in starch synthesis (Emes *et al*., 2003).

2.7.4.2 AGPase genes in wheat encoding the different subunits

Previous studies that involved southern hybridization and restriction analysis of wheat AGPase genes suggested the presence of two different families of AGPase the leaf and endospermic *AGPase* (Olive *et al*., 1989). They revealed 55% identity between the wheat leaf and endosperm *AGPase*, and 96% identity between members within each sub-family of endosperm *AGPase* (Olive *et al*., 1989). This study, however, was based on the cDNA sequence of a partial fragment of the AGPase gene, which later facilitated the identification and cloning of the full length cytosolic LSU of *AGPase* from wheat endosperm (Ainsworth *et al*., 1995) and leaf (Smith-White and Preiss, 1992) tissues.

A full length cDNA for the SSU isolated from a wheat endosperm library (Ainsworth *et al*., 1993) showed similarity with the barley cytosolic SSU (Thorbjornsen *et al*., 1996). Hence, it was considered as the most likely cytosolic SSU of wheat AGPase (Burton *et al*., 2002). This

study provided evidence for the presence of two distinct genes encoding SSU of wheat AGPase that are formed by alternative splicing. However, it could not identify the gene encoding for the plastidial subunit. Later, the *TaAGPS1b* was identified (Kang *et al*., 2010) and owing to the abundance of its transcripts in the leaf tissue, this gene was suggested to encode for the plastidial isoform of the enzyme.

2.7.4.3 Biotechnological approaches for manipulation of AGPase

Biotechnological approaches have been used in several studies to modify the activity of AGPase, and thereby maximize starch yield in several economically important crops. Genetic manipulation of the Russett-Burbank variety of potato with the bacterial gene *glgC* gene (encodes the bacterial AGPase) led to 30% increase in starch content but did not have any effect when the Prairie variety was used (Stark *et al*., 1992). Expression of an altered form of maize *Sh2* (*Sh2r6hs*), which is a heat stable form and not allosterically inhibited by Pi, in wheat resulted in increased photosynthesis, carbon metabolism and biomass, and enhanced sink strength in controlled environment (Smidansky *et al*., 2002, 2007). However, under field conditions no apparent *Sh2r6hs* mediated enhanced carbon partitioning was observed (Meyer *et al*., 2007). Genetic manipulation of rice with *Sh2r6hs* also showed an overall increase in biomass and seed weight, although no significant change in starch deposition was observed (Smidansky *et al*., 2003). Furthermore, studies with transgenic rice expressing the *E.coli* triple mutant *glgC* gene showed that the intracellular location where *AGPase* is expressed has a significant influence on its ability to affect starch synthesis. Rice plants with elevated cytoplasmic AGPase activity showed up to 11% increase in seed weight, while modification of the amyloplastic AGPase did not result in any significant changes in seed weight when compared to the wild type (Sakulsingharoj *et al*., 2004). In maize, enhancing the expression of both *Sh2* and *Bt2* genes in

elite lines increased starch content in the kernels to 74%, as compared to the 65% of starch found in the grains obtained from the control plants (Li *et al*., 2010). Given its roles in regulating starch yield, *AGPase* has been a gene of choice to manipulate starch biosynthesis and increase starch yield in crops.

2.7.5 Starch synthases

Apart from AGPase, there are a number of other enzymes, including starch synthase that catalyze the biosynthesis of starch. Starch synthases play major roles in elongating the glucan chain and developing the complex starch structure. There are two groups of starch synthase; the granule-bound starch synthases (GBSS), and the soluble starch synthases (SS).

2.7.5.1 Granule-bound starch synthases in crops

The GBSS is primarily involved in the formation of long chain amylose chains. This enzyme is found within the granule matrix. Encoded by the *Waxy* locus in many cereals, mutations in this locus have resulted in the production of varieties completely devoid of amylose. It is also believed to have a role in increasing the glucan chains in longer than average amylopectins that are found in species such as peas (Denver *et al*., 1995), potatoes (Edwards *et al*., 1999) and rice (Hanashiro *et al*., 2008). GBSS can also have an effect on the granule morphology of starch in potato tubers (Fulton *et al*., 2002). Studies of waxy mutants in crops are related to a variety of maize identified first in China and later in Burma (Collins, 1909). The endosperm of the waxy maize mutant, which arose from the wild starchy maize by a mutation that modified the starch molecule, is reported to contain only one kind of carbohydrate (Weatherwax, 1922). Studies on the natural glutinous variety of rice showed the presence of highly branched chain of amylopectin and the lack of ADP-glucose transglucosyl transferase (GBSS) activity in the starch granules and showed only the activity of the soluble form of starch synthase (Murata *et al*.,

1965). In addition, substantial efforts have been placed in developing waxy or glutinous rice mainly because of its commercial value as being the 'sticky rice'.

The activity of GBSS was studied in wild type and waxy mutants of rice in which the waxy mutant showed lowest GBSS activity and lowest amylose content as compared to the wild type. However, the waxy mutant carrying the Wx^a allele showed a higher amylose content throughout grain filling, likely due to a superior GBSS activity (Jeng *et al*., 2007). Furthermore, the amylose-free mutants (*amf*) of potato (Hovenkamp-Hermelink *et al*., 1987) and wheat (Nakamura *et al*., 1995) are characterized by the absence of the waxy protein, and this was coincidental with the lack of amylose in the storage starch. This waxy protein was later identified to be encoded by *GBSSI*. In contrast to its function in storage organs, GBSSI does not contribute significantly to the synthesis of amylose in green non-storage tissues. For example, amylose content of the leaf and stem tissues of waxy rice (25-35%) was found to be similar to that found in the non-waxy rice, suggesting the existence of additional isoforms of GBSS (Igaue, 1964).

It has been reported that the transcription of *GBSSI* is regulated developmentally and also by a circadian clock in Snapdragons (Mérida *et al*., 1999). Similar diurnal regulation of *GBSSI* was also observed in the photosynthetic tissues of sweet potato (Wang *et al*., 2001). Sucrose also stimulates *GBSSI* transcription of sweet potato, although it has no effect on the rhythmic pattern of the *GBSSI* gene expression (Wang *et al*., 2001).

2.7.5.2 Granule-bound starch synthases in wheat

The genetic loci of the waxy gene (*wx*) in bread wheat are located on chromosomes 7A, 4A and 7D. It has been suggested that a reciprocal translocation that occurred during the course of evolution has resulted in exchange of genetic material between chromosomes 7B and 4A, resulting in the current loci of the gene (Yamamori *et al*., 1994). The presence of several lines of

wheat from Japan, Korea, Turkey and China that are partially waxy has been documented (Graybosch, 1998). A complete waxy wheat was produced by Nakamura *et al*. (1995), and this work eventually led to the establishment of different properties and functions in GBSS isoforms (Nakamura *et al*. 1995; Nakamura *et al*. 1998; Vrinten and Nakamura 2000). The findings of this study have been used as a foundation to modify starch in wheat. For example, a double mutant of *GBSSI* and *SSIIa*, designated as 'sweet wheat', was produced by Shimbata *et al*., (2011). The double mutant exhibited alterations in granule structure, amylose content and other grain morphological characteristics in immature as well as mature seeds. In addition, the whole grain of the double mutant showed higher levels of maltose, protein, lipid, and total dietary fiber as compared to the wild-type lines. These compositional changes are suggested to have health benefits (Shimbata *et al*., 2011; Vrinten *et al*., 2012). It has been reported that GBSSI has a lower affinity for ADP-glucose as compared to the soluble starch synthases (Morell *et al*., 2001). This has led to a suggestion that a reduction in the concentration of ADP-glucose in a given tissue may result in decreased amylose content (Clarke *et al*., 1999). To date, two genes encoding GBSS have been identified in wheat. *GBSSI* is found to encode the enzyme responsible for amylose synthesis in storage organs; whereas the other isoform of GBSS is responsible for the synthesis of transient starch in the non-storage tissues such as the leaf, pericarp and culm, and is encoded by *GBSSII* (Vrinten and Nakamura, 2000).

2.7.5.3 Soluble starch synthase in plants

The soluble starch synthases (SSs) are exclusively involved in the synthesis of amylopectin, and their distribution in the granule matrix and stroma is dependent on species, developmental stage, and tissue (Ball and Morell, 2003; Tetlow, 2006). This enzyme is also regulated by environmental factors. Temperature regulation of the starch synthases has been studied, and the

results of these studies have shown that heat reduces the activity of SS (Rijven, 1986; Hurkman *et al*., 2003; Prakash *et al*., 2004; Keeling and Myers, 2010a). Starch synthase in plants is encoded by several genes including *SSI, SSII, SSIII* and *SSIV*. The individual isoforms from each of these are believed to have a specific role in the synthesis of amylopectin (Martin and Smith, 1995; James, 2003; Tetlow, 2006; Jeon *et al*., 2010) and exhibit differential expression pattern during seed development (Martin and Smith, 1995).

2.7.5.4 Isoforms of starch synthase

The SSI isoform is required primarily for the synthesis of short outer chains of amylopectin (less than 10 glucans) and it accounts for 70% of the total SS activity (Commuri and Keeling, 2001; Delvallé *et al*., 2005; Fujita *et al*., 2006). SSI is the only class of SS with no known isoform to date (Fujita *et al*., 2006). The expression of *SSI* is found to be consistently high throughout seed development in rice (Ohdan *et al*., 2005). In barley seeds, the expression of *SSI* showed a rapid increase at the beginning of starch accumulation, and remained high during the seed filling phase before declining as the endosperm matures (Radchuk *et al*., 2009). Mutation in *SSI* does not have an effect on grain size and starch crystallinity (Fujita *et al*., 2006) while a double mutant of *SSI* and *SSIII* produced sterile plants (Fujita *et al*., 2011).

The SSII isoform of most monocotyledon species has two isoforms, each encoded by *SSIIa* and *SSIIb*. The activity of SSIIa is localized in the endosperm of cereal seeds, whereas that of SSIIb is restricted in the green tissues (Imparl-Radosevich *et al*. 1999; Morell *et al*. 2003; Tetlow 2006). It has been reported that SSII contributes mostly towards the synthesis of intermediate glucan chains and plays a major role in determining both starch content and composition in grains (Morell *et al*., 2003). Consistently, mutations in the *rug5* locus encoding SSII in pea showed accumulation of amylopectin with fewer chains of intermediate length that

could alter the amylopectin structure (Craig *et al*., 1998). The abundance of *SSIIa* transcripts in rice has been shown to increase rapidly during grain filling (Ohdan *et al*., 2005). A comparative study between the *japonica* and *indica* varieties of rice revealed reduced functional activity of SSIIa in the *japonica* variety, and this results in varietal difference in grain structure (Umemoto *et al*., 2002). Maize has two forms of SSII, encoded by genes designated as *zSSIIa* and *zSSIIb*. The *zSSIIa* gene is predominantly expressed in the endosperm, whereas *zSSIIb* is primarily expressed in the leaves (Harn *et al*., 1998). Studies with maize *sugary2* (*su2*) mutants (*su2* codes for SSIIa) revealed increased abundance of short glucan chains and a reduction in intermediate chain length (Zhang *et al*., 2004). In barley, a mutant of *SSIIa* (designated as *sex6*) exhibits a 20% reduction in starch content as compared to the wild type, suggesting that *SSIIa* plays a role in the elongation of amylopectin chains (Morell *et al*., 2003). In agreement with this, the expression of *SSIIa* showed rapid increase at the onset of starch accumulation and remained high during grain filling (Radchuk *et al*., 2009).

The SSIII isoform plays an active role in synthesizing the long chains of glucans that stretch between amylopectin structures (James, 2003), and it is the second most abundant of all soluble starch synthases next to SSI (Fujita *et al*., 2007). Two isoforms of SSIII have been reported to date, one for its activity in the endosperm and the other one in photosynthetic tissues (Tetlow, 2006). In rice, the two isoforms are known to exist and they are encoded by *SSIIIa* and *SSIIIb*, which are expressed mainly in the endosperm, and leaf, respectively (Hirose and Terao, 2004; Duan and Sun, 2005). Studies of mutants have also revealed a role for *SSIII* in the synthesis of long chains of glucans (Fujita *et al*., 2007). Double recessive mutants of *SSI* and *SSIII* are found to be sterile, hence it is more likely that SSI and SSIII together play an important role in starch biosynthesis, contributing approximately 60% of the normal starch produced

(Fujita *et al*., 2011). The expression of *SSIIIa* has been shown to increase from early stages of grain development, reaching a maximum level at 7 DAA and this level was maintained through grain filling in both rice and barley (Ohdan *et al*., 2005; Radchuk *et al*., 2009). A deficiency in the activity of SSIII in rice also resulted in increased transcript abundance of *GBSSI* and *SSI*, and this affects the structure of amylopectin, amylose content and the physico-chemical property of the starch granules (Fujita *et al*., 2007).

The SSIV isoform is the least known amongst the isoforms of soluble starch synthases. The exact function of SSIV could not be determined but is most likely related to granule initiation (Tetlow, 2006; Szydlowski *et al*., 2009). Two genes encoding SSIV have been found in rice, *SSIV-1* and *SSIV-2* (Hirose and Terao, 2004). Expression analysis of these genes showed the *SSIV-1* is mainly expressed in leaves being responsible for transitory starch synthesis, while *SSIV-2* is mainly expressed in developing endosperm (Dian *et al*., 2005). In barley seeds, it has been shown that the expression of *SSIV* increases gradually from 2-10 DAA and remain constant thereafter (Radchuk *et al*., 2009).

2.7.5.5 Soluble starch synthase in wheat

It has been reported previously that the wheat SSI is localized in both starch granules as well as in the soluble fractions of wheat endosperm (Denyer *et al*., 1995). The corresponding gene, *SSI*, has been found to be expressed in the leaves, stems, roots and developing endosperms. Two-thirds of the activity of soluble starch enzymes in developing wheat endosperm is attributed to SSI (Peng *et al*., 2001). The transcripts of *SSI* are present in the endosperm as well as within starch granules at the early stage of endosperm development (Li *et al*., 1999).

The gene encoding SSII was initially identified as starch granule protein, *SGP1*, and it has been reported that SGP1 has three isoforms (Yamamori *et al*., 2000) that are encoded by the

loci *Sgp-A1, Sgp-B1* and *Sgp-D1* on short arms of chromosomes 7A, 7B and 7D (Yamamori and Endo, 1996). Further study has identified the three homeologous genes, designated as *wSSII-A, wSSII-B* and *wSSII-D* (Li *et al*., 1999; Gao and Chibbar, 2000). A wheat line lacking all three SSII homeologous proteins was later developed, and this line showed an increase in apparent amylose content and notable variations in the granule structure with altered distribution of amylopectin chains (Yamamori *et al*., 2000). These results suggest that *SSII* plays a major role in the biosynthesis and distribution of amylopectin. The loss of *SSIIa* function has been shown to increase apparent amylose starch, giving rise to resistant starch, a name so earned because it is difficult to be broken down completely to simple sugars during digestion (Yamamori *et al*., 2006). Interestingly, the loss of *SSIIa* caused the losses of *SBEIIa, SBEIIb* and *SSI* in the granule-bound phase of endosperm development (Kosar-Hashemi *et al*., 2007). Due to its significance in starch synthesis and grain-yield-related traits in wheat, efforts are being made to develop markers from *SSIIa* genes (Huang and Brûlé-Babel, 2012). The wheat gene encoding SSIII has been isolated, and its expression was detected in leaves, pre-anthesis florets and developing endosperms from very early to mid-developmental phases (Li *et al*., 2000). The *SSIV* cDNA of wheat shows similarity with rice *SSIVb* (Leterrier *et al*., 2008). It is expressed in the wheat leaves, but doesnot exhibit any circadian regulation.

2.7.6 Starch branching enzymes

Starch branching enzymes (SBE) catalyze the formation of α-1,6 linkage by breaking the already existing α-1,4 linkage, and this usually occurs after 20-30 glucan moieties, enabling the formation of amylopectin. SBEs have been classified into two different isoforms - SBEI (SBE B) and SBEII (SBE A). SBEIs are known to produce longer chains as compared to SBEIIs, which are more involved in producing branched amylopectin (Rydberg *et al*., 2001; Tetlow, 2006; Jeon

et al., 2010). The SBEI and SBEII isoforms have different substrate preferences; SBEI has a higher affinity for amylose as compared to SBEII, thus SBEI makes use of longer glucan chains unlike SBEII, which prefers the shorter ones (Martin and Smith, 1995; Tetlow, 2006; Tetlow, 2011).

2.7.6.1 Starch branching enzymes in crops

The type I starch branching enzyme (SBEI) has been identified in several crops such as pea (Burton *et al*., 1995), cassava (Salehuzzaman *et al*., 1992), rice (Kawasaki *et al*., 1993), maize (Baba *et al*., 1991) and wheat (Morell *et al*., 1997). In maize kernels, the activity of SBEI is reported to be abundant by 10 DAA and the activity decreases through 28 DAA (Baba *et al*., 1991). Similarly, the expression of *SBEI* of barley, which is localized only in the endosperm tissues, was detected from 7 DAA. Its expression showed a rapid increase through 12 DAA before exhibiting a gradual decline thereafter (Sun *et al*., 1998). It is expressed only in the endosperm tissues (Sun *et al*., 1998; Radchuk *et al*., 2009). Loss of *SBEI* function in rice results in loss of amylopectin's fine structure and physico-chemical properties of the rice endosperm starch (Satoh *et al*., 2003).

The type II SBE (SBEII) has also been reported from several crops such as pea (Bhattacharyya *et al*., 1990), cassava (Baguma *et al*., 2003), sorghum (Mutisya *et al*., 2003), maize (Gao *et al*., 1997), rice (Mizuno *et al*., 1993), barley (Sun *et al*., 1998) and wheat (Nair *et al*., 1997). In monocots, the SBEII has been further categorized into SBEIIa and SBEIIb (Jobling *et al*., 1999). In pea, the activity of SBEII is reported to increase during early seed developmental phases after which it remained constant and was accompanied by increases of SBEI (Smith, 1988). In potato, the expression of *SBEII* is detected in both leaf and tuber tissues, whereas *SBEI* is mainly found in the tuber (Jobling *et al*., 1999). Exclusive elimination of *SBEII* in potato led to

increased accumulation of amylose (Jobling *et al*., 1999), whereas the down regulation of both *SBEI* and *SBEII* results in less than 1% of the total activity of SBE, increased accumulation of amylose and altered starch granule morphology (Schwall *et al*., 2000). In maize suppression of SBEI (Blauth *et al*., 2002) or SBEIIa (Blauth *et al*., 2001; Blauth *et al*., 2002) did not affect the endosperm amylose content These studies have led to a suggestion that SBEI causes minimal effects on starch synthesis, and its function can be carried out by other branching enzymes. However, the loss of function of SBEII cannot be compensated by the other existing isoforms (Flipse *et al*., 1996; Blauth *et al*., 2002; Ball and Morell, 2003; Tetlow, 2006).

The activity of SBE (both SBEI and SBEII together) in rice seeds was found to increase by 6 DAA and reaches at maximum level by 13 DAA before showing a decline (Nakamura and Yuki, 1992). The transcripts of *SBEII* of barley were detected from 7 DAA and showed a rapid increased through 12 DAA and then declined gradually (Sun *et al*., 1998). In maize, *SBEIIa* was expressed in the leaves, whereas *SBEIIb* was found to be the predominant form in the endosperm (Gao *et al*., 1997). In barley, *SBEIIa* has been found to be ubiquitous while *SBEIIb* is mainly expressed in the endosperm tissues (Sun *et al*., 1998; Radchuk *et al*., 2009). In general from the expression and enzyme activity studies, it can be noted that SBEII plays a role during the earlier phases of grain development, while SBEI is important during the latter part of grain development in maize, barley, rice and wheat (Mizuno *et al*., 1993; Gao *et al*., 1997; Morell *et al*., 1997; Mutisya *et al*., 2003).

2.7.6.2 Starch branching enzymes in wheat

In wheat, three isoforms of *SBE* have been identified; *SBEI*, *SBEIIa* and *SBEIIb*. *SBEI*s are known to produce longer chains as compared to SBEIIs, which are mainly involved in producing branched amylopectin (Tetlow, 2006; Jeon *et al*., 2010; Rydberg *et al*., 2001). The genes for

SBEIIa and SBEIIb are located on the long arm of chromosome 2 of wheat (Regina *et al*., 2005). Expression analysis of *SBEI* and *SBEII* has shown that *SBEII* transcripts are detected at 5 DAA and the abundance increased to a maximum by 10 DAA. The abundance of *SBEI* transcripts showed an increase at 10 DAA, reaching a maximum level by 15 DAA. The transcript of both genes became low at 20 DAA (McCue *et al*., 2002). However, it has been shown that the expression of *SBEIIa* was four-times more than that of *SBEIIb* at 15 DAA (Morell *et al*., 1997; Regina *et al*., 2005). Mutant lines with repressed expression of both *SBEIIa* and *SBEIIb* produced over 70% amylose unlike the mutant lines suppressing *SBEIIb* alone (Regina *et al*., 2006).

The concept of multi-enzyme complex formation during starch synthesis is supported by co-immunoprecipitation of SBEIIb and SBEI in a phosphorylation dependent manner, suggesting that these enzymes may form protein complexes within the wheat amyloplast *in vivo* (Tetlow *et al*., 2004b). This process requires phosphorylation of serine residues on the target protein. The co-existence of SSIII, SSIIa, SBEIIa and SBEIIb in the same complex has also been studied in maize along with wheat (Hennen-Bierwagen *et al*., 2008; Hennen-Bierwagen *et al*., 2009). The regulation of complex formation by these enzymes may be affected by pyruvate orthophosphate dikinase, which indirectly regulates the abundance of Pi and scarcity of ATP, and thereby inhibits AGPase activity (Méchin *et al*., 2007; Hennen-Bierwagen *et al*., 2009).

2.8 Transgenic approaches to modify wheat starch yield

The ever increasing world population is exerting a pressure for the need to increase crop productivity. The average wheat yield of approximately three metric tons per hectare may soon fall short of meeting the global demand for this crop (USDA- World Agricultural Production,

accessed 2013). This concern has prompted the need to improve the efficiency of wheat breeding methods. The application of biotechnological approaches has a paramount significance with this respect. Although the first fertile transgenic wheat was developed as early as 1992 (Vasil *et al*., 1992), there has not been any commercial cultivation to date (GMO Compass, accessed 2013). Like in the cases of other major crops, there have been debates over the genetic manipulation of wheat through biotechnological approaches. Although most of the genes that are incorporated into transgenic crop plants come from other plants (or animals), it is very difficult to rule out unknown consequences associated with the targeted modification. The proponents of genetically modified organisms (GMOs) believe that, with sufficient research, these organisms would be safe to commercialize.

2.8.1 Quantitative and qualitative modifications of starch in crops

Modifications of the starch biosynthetic pathway have been investigated in several crops such as cassava (Ihemere *et al*., 2006), potato (Stark *et al*., 1992), maize (Greene and Hannah, 1998a; Li *et al*., 2010; Hannah *et al*., 2012), rice (Smidansky *et al*., 2003) and wheat (Smidansky *et al*., 2002). The quantitative modifications primarily focused on overexpression or alterations of the AGPase enzyme, which has been considered to catalyze a rate limiting step in starch biosynthesis. Expression of a modified version of *E. coli* AGPase encoded by a mutated *glgC* resulted in up to 70% increase in AGPase activity (Ihemere *et al*., 2006). Furthermore, expression of the *glgC* triple mutant gene, encoding a highly active and allosterically insensitive AGPase, in rice resulted in 13-fold higher AGPase activity and 11% increase in yield as compared to the control (Sakulsingharoj *et al*., 2004). Other studies have also employed AGPase derived from higher plants. For example, expression of mutated *Sh2* of maize (*Sh2r6hs*), which is less sensitive to regulation by inhibitors and exhibits more heat stability, was shown to cause

increased AGPase activity and yield (64%) in maize (Greene and Hannah, 1998a; Hannah *et al*., 2012), and increased yield in wheat (38%) and rice (28%) (Smidansky *et al*., 2002, 2003). The yield increase due to the expression of *Sh2r6hs* in maize, wheat and rice is attributed primarily to an increase in seed number, while the yield increase exhibited by the expression of the bacterial isoform is mainly due to increases in seed size (Smidansky *et al*., 2002; Smidansky *et al*., 2003; Hannah *et al*., 2012).

In addition to the quantity, modifying the quality of starch in crops has gained importance due to the end use application of starch in various industries. This, for example, has led to the generation of high amylose wheat, consisting of 70% amylose, through downregulation of *SBEIIa* and *SBEIIb* (Regina *et al*., 2006). Such a starch with high amylose content has been shown to have a positive effect on the bowel health in mammals. The waxy mutation, which on the other hand eliminates amylose (Nakamura *et al*., 1995), is preferred for the 'sticky rice' properties, and production of commercial products such as maltodextrins and adhesives.

2.8.2 Method for DNA delivery in wheat

Several methods have been used for DNA delivery into wheat cells including particle bombardment (Vasil *et al*., 1992), electroporation (He *et al*., 1994), microinjection (Simmonds *et al*., 1992), laser micropuncture (Badr *et al*., 2005), *Agrobacterium* mediated delivery (Cheng *et al*., 1997). However, the particle bombardment (or biolistics) and *Agrobacterium* mediated gene delivery have gained prominence due to their reproducibility and efficiency. Like any other moncot crops, earlier methods of DNA delivery into wheat embryos employed the particle bombardment method (Vasil *et al.*, 1992). This method utilizes gold or tungsten particles (microcarriers) that are coated with the plasmid DNA. The particles are then forced into the cells of immature wheat embryos with the help of a pressure pulse of Helium gas. The other procedure

that has gained popularity in wheat is gene transfer via *Agrobacterium*, where the bacterial strain is co-cultivated along with immature wheat embryos so that the tumour inducing (Ti) plasmid of the *Agrobacterium* serves as a source for the transfer of DNA into the host plant. The *Agrobacterium* mediated gene transformation forms a host-pathogen recognition system with the plant, and this enables DNA delivery. The entire process of host-pathogen interaction encompasses a series of virulence (*Vir*) gene expression in the bacteria that is involved in the type IV secretion system (T4SS). The T4SS are multiprotein complexes that the bacteria use to transfer DNA as well as proteins to cells of other organisms (Zupan *et al*., 2000; Llosa *et al.*, 2002; Wallden *et al.,* 2010). These could be of two types. The first kind is involved in conjugative transfer of plasmid DNA between bacteria while the second one is involved in pathogenesis, and transfers protein and/or DNA into eukaryotic host cells (Juhas *et al.,* 2008; Aguilar *et al*., 2011). The complex series of molecular machinery in the plant signals this entire process towards the successful completion of the infection. The expression of the *vir* genes in the bacteria is triggered on recognizing the plant-derived compounds. Since wheat is a monocot species, natural phenolic compounds and other elicitors, which can facilitate the activation of T4SS in the *Agrobacterium*, are absent. Therefore, there is a need to apply such compounds (e.g. acetosyringone) exogenously in order to achieve successful infection and gene transfer. This enables the bacteria to transfer the protein coated transfer DNA (T-DNA) into the wheat cell and integration of a discrete segment of the T-DNA delimited by the left and the right border within the plant DNA (Pitzschke and Hirt, 2010). Typically transformation experiments include the expression of two gene cassettes: one involving the gene of interest, and the other involving the selection gene (which would allow selection of transformed cells).

2.8.3 Factors affecting transformation

Both the biolistic and the *Agrobacterium*-mediated gene transformation methods involve the following steps: DNA delivery followed by callus formation, selection and regeneration of plantlets. Apart from the methods of DNA delivery, the efficiency of transformation is often affected by several factors including the type of cultivar, explants, selection marker and the culture conditions. Furthermore, in the case of *Agrobacterium*-mediated DNA delivery, the choice of the bacterial strain is an important factor in determining the efficiency of transformation.

2.8.3.1 Choice of cultivar

Wheat is a recalcitrant species for transformation. Thus, cultivar choice plays an important role for the success of a transformation event. Both callus induction as well as plant regeneration are to be influenced by the genotype (Varshney and Altpeter, 2001). Amongst the European winter wheat cultivars; Certo, Tarso and Alidos exhibit higher regeneration rates as well as desirable agronomic traits. The American cultivars Florida and Cadenza are popular winter wheat cultivars used for transformation, mainly because of their good regeneration capacity (Wu *et al*., 2003). Of the spring wheat cultivars, Bobwhite has been reported to have a higher regeneration frequency (Przetakiewicz *et al*., 2003; Wu *et al*., 2003). The cultivar Fielder also exhibits good transformation efficiency when transformed with either biolistics or via *Agrobacterium* strains (Nehra *et al*., 1994; Weir *et al*., 2001). Oriental cultivars such as Akadaruma, Norin 12, Huhikari and Chinese Spring have also been used for transformation via particle bombardment (Takumi and Shimada, 1997), however, only the first two cultivars showed better transformation efficiencies. Unfortunately, most of the cultivars that show very good callus induction and regeneration are not high yielding (e.g., Bobwhite and Fielder). This triggers the need of

incorporating several generations of backcrossing with an adapted line in order to obtain a line with desired traits as well as high yield.

2.8.3.2 Choice of explants

The choice of explants for wheat transformation varies from protoplasts (He *et al*., 1994), mature embryos, and callus regenerated from immature embryos (Vasil *et al*., 1992). Regeneration and transformation efficiencies of calli derived from mature embryos and those initiated from immature embryos are found to be comparable (Patnaik and Khurana, 2003). There are instances where basal segments of young seedlings, immature seeds (*in planta*) and florets clipped from pre-anthesis spikes are used as explants for wheat transformation. For example, basal segments from 5-day old seedlings of hexaploid and tetraploid wheat cultivars have been used as explants through culturing on callus induction medium (Chugh and Khurana, 2003). The regenerated embryogenic calli were transformed further by particle delivery method, resulting in a transformation efficiency of 8.6%, 7.5% and 4.9% in *T. aestivum* cvs. CPAN1676 and PBW343, and *T. dicoccum* cv. DDK1001, respectively (Chugh and Khurana, 2003). Experiments that tested *in planta* inoculation of immature seeds produced an average transformation efficiency of 5% (Risacher *et al*., 2009). Dipping of clipped florets from pre-anthesis spikes at the single spore uninucleate stage resulted in transformation efficiency of 0.9% to 10% (Agarwal *et al*., 2009). Although there are several methods of transformation and types of explants, the most common choices are biolistic and *Agrobacterium*-mediated transformation using immature embryos, which yields a higher percentage of regeneration than the others.

2.8.3.3 Choice of selection marker

The selection marker used in plant transformation procedures is often related to the efficiency of transformation and intended acreage of the resulting transgenic crop to be grown. In Europe for

example, antibiotic markers are not used for any transformation events in order to avoid contamination of feral species. In wheat, genetic transformation has been carried out using herbicides like Basta (Patnaik and Khurana, 2003; Pellegrineschi *et al*., 2004) as selection markers, yielding transformation efficiency of up to 8.6% (Patnaik and Khurana, 2003). The other very commonly used herbicide selection marker is the *bar* gene (Jones *et al*., 2005), as it is part of commercial binary vectors that are manufactured in the European countries. A relatively less commonly used selection marker gene is phosphomannose isomerase (Wright *et al*., 2001; Wenck *et al*., 2003). Weeks *et al* (2000) have also reported the use of the *cah* gene encoding cyanamide in transforming wheat. Amongst the antiobiotic markers, *nptII* and *hptII* genes are used to confer resistance to kanamycin and hygromycin, respectively. The *nptII* gene also confers resistance against geneticin, paramomycin and G418 (similar in structure to gentamicin B1). Compared to kanamycin, which produces transformation efficiency of 1% (Bhalla *et al*., 2006), selection with hygromycin yields a relatively higher efficiency of up to 8.6% (Chugh and Khurana, 2003; Permingeat *et al*., 2003). There are also reports indicating the use of glyphosate as a selection marker for generating large-scale Roundup-ready transgenic wheat (Hu *et al*., 2003).

2.8.3.4 Choice of strain of Agrobacterium

When the transformation process is mediated via *Agrobacterium* infection, the choice of bacterial strain to be used for initiating the infection is crucial. Both the chromosomal as well as the plasmid DNA of the bacteria are involved in encoding all the proteins and virulence factors that cause the attachment and DNA transfer into the plant (Fuqua, 2008; Gelvin, 2010; Pitzschke and Hirt, 2010). Some of the bacterial strains that have been used successfully in the transformation of dicot species and wheat include LBA4404 (Khanna and Daggard, 2003) and

C58 (Cheng *et al*., 1997; Cheng *et al*., 2003; Hu *et al*., 2003). These bacterial strains have been used with a wide range of Ti and binary vectors. Other, more virulent strains, also known as hypervirulent strains, that have been used for cereal transformations include AGL0 and AGL1. These strains are engineered to contain the hypervirulent Ti plasmid carrying additional *vir* genes (Weir *et al*., 2001; Wu *et al*., 2003). However, there is not sufficient information to precisely underline which *vir* genes are required for optimal transformation of cereal crops such as wheat (Jones *et al*., 2005).

Globally, wheat is one of the most important crops. Its grain starch finds wide application in various industries. This has prompting breeders, geneticists and molecular biologists to improve the quality of the crop. This study focuses on the analysis and manipulation of the wheat starch biosynthesis pathway using three different studies. It analyzes the sucrose-to-starch metabolism at molecular, biochemical and metabolite levels in developing grains of wheat. It also investigates the role of natural variation in starch biosynthesis by comparing three cultivars with different genetic backgrounds. Finally the study uses transgenic approaches to manipulate AGPase activity in spring wheat and examine its implications for future application.

3. Analysis of sucrose to starch metabolism pathway in developing grains of common wheat (*Triticum aestivum* L.)

3.1 Abstract

Starch is the major storage reserve in wheat, comprising over 50% of grain dry weight. During the reproductive growth of wheat, most of the sucrose synthesized in the source tissues is transported into the developing grains, where it is used as a substrate for the synthesis of starch. To better understand the coordination of sucrose-starch metabolic pathways, this study investigated the dynamics of sucrose transport and catabolism and starch synthesis in wheat grains at both molecular and/or metabolite levels. Our data show that one of the sucrose transporter genes (*TaSUT1*) is highly expressed during the early stages of grain development suggesting its involvement in the transporter activity. A gene encoding sucrose synthase (*TaSuSy2*) is highly expressed during the rapid phase of starch accumulation; reflecting that sucrose hydrolysis in developing wheat grains is catalyzed mainly by the TaSuSy2 isoform. ADP-glucose pyrophosphorylase (AGPase) has been shown previously to be a regulatory starch biosynthetic enzyme in plants. Thus, the high expression of *AGPL1* and *AGPS1a*, encoding the large and small subunits of AGPase, respectively, found in the grains during the same period of development, suggest that these two genes contribute the majority of AGPase activity required for converting the hexose sugars to starch. Furthermore, the upregulation of *TaSuSy2*, *AGPL1* and *AGPS1a* was coordinated with increased expression of specific members of genes encoding granule bound (*GBSSI*) and soluble starch synthases (*SSI, SSIIa, SSIIIa*).

3.2 Introduction

Starch is the major storage reserve in the grains of cereal crops and determines their yield. In wheat (*Triticum aestivum* L.), it constitutes about 50% to70% of the grain dry weight. Allocation of the triose phosphate in the photosynthetic tissue at the end of the dark phase is a critically regulated process; a portion of it is allocated for transient starch synthesis, while the remaining portion is used for making sucrose. In the absence of photosynthesis in dark, the transitory starch is converted into sucrose and exported into the cytoplasm which, being chemically inert and on account of its high solubility, is then translocated to the sink organs including the developing grains via the phloem. In the endosperm, the sucrose is converted into storage starch by different enzymes involved in the starch biosynthesis pathway.

Sucrose transporters (SUTs) are integral membrane proteins that actively transport sucrose across the membrane (Aoki *et al*., 2002) and retrieve sucrose leaked from the phloem during translocation (Rosche *et al*. 2002; Scofield *et al*. 2004) . They are mostly localized on the plasma membrane or tonoplast of phloem cells. The SUTs have been isolated from cereal crops including rice (Aoki *et al*., 2003), maize (Aoki *et al*., 1999), barley (Weschke *et al*., 2000) and wheat (Aoki *et al*., 2002; Deol *et al*., 2013). In rice, *OsSUT1* is mainly involved in transporting sucrose to the developing grains, and in remobilising sucrose from starch reserves deposited in the leaf sheath and in the endosperm of germinating seeds (Matsukura *et al*., 2000; Furbank *et al*., 2001). While *OsSUT2* is thought to have a housekeeping role, *OsSUT3*, *OsSUT4* and *OsSUT5* are predominantly expressed in the sink leaves and young caryopsis, suggesting their involvement in supplying sucrose to growing tissues (Aoki *et al*., 2003). In barley, the transcripts of *SUT1* have been detected in the endospermal transfer layer and cells of the nucellar projection of developing grains. These are the regions involved in sucrose unloading; reflecting the

functional association of SUT1 with grain filling (Weschke *et al*., 2000; Aoki *et al*., 2002). Hexaploid wheat has three homeologous genes of *SUT1* (*TaSUT1A, TaSUT1B, TaSUT1D*) that are located on chromosome 4A, 4B and 4D (Aoki *et al*., 2002). The transcripts of all three localize in the aleurone layer, scutellar epidermis, and companion cells of germinating wheat seeds (Aoki *et al*., 2006). All the homeologues of this gene are also expressed at high levels during grain filling with the expression decreasing by 8 days after heading (DAH) and reaching to a maximum level by 16 DAH, and showing a decline thereafter (Aoki *et al*., 2002).

Sucrose synthase (SuSy) and invertase (INV) are crucial enzymes involved in sucrose catabolism, which occurs before the onset of starch synthesis. SuSy converts sucrose and UDP to UDP-glucose and fructose in a reversible reaction, while INV catalyzes an irreversible conversion of sucrose to glucose and fructose. These hexoses then enter into the starch biosynthesis pathway. SuSy has been studied in various plants and is associated with energy metabolism. Amongst the six genes encoding SuSy in rice, *SuSy3* and *SuSy4* are predominantly expressed in the developing grains during filling and have been associated with carbon allocation in grains (Hirose *et al*., 2008). Two genes encoding SuSy, *HvSuSy1* and *HvSuSy2*, have been identified from barley (Guerin and Carbonero, 1997). The *HvSuSy1* was found to be localized in vascular bundles of young leaves and roots as well as in the grain endosperm. The *HvSuSy2*, was abundantly expressed in the endosperm (Guerin and Carbonero, 1997). This underlines the role of SuSy in supplying hexose sugars to the developing endosperm during grain filling. Two *SuSy* genes have been identified from wheat, *TaSuSy1* and *TaSuSy2*. The SuSy enzyme was seen to have a higher activity in grains with higher weight (Dale and Housley, 1986). Through association mapping it has also been shown *TaSuSy2* is linked with thousand grain weight (Dale

and Housley, 1986; Jiang *et al*., 2011). In wheat, the expression of the SuSy genes during grain filling needs to be studied further.

Invertase plays an important role during the early stages of grain development in cereals. They have been classified into different types based on pH as neutral, alkaline and acid invertases (Sturm, 1999), and on their location as cell wall, vacuolar (Sturm, 1999) and apoplastic invertases (Kim *et al*., 2000). Genes encoding INV have been studied in maize (Kim *et al*., 2000; Chourey *et al*., 2005), barley (Obenland *et al*., 1993; Weschke *et al*., 2003), wheat (Krishnan *et al*., 1985) and rice (Hirose, 2002; Cho *et al*., 2005). Most of the hexoses contributed by INV are utilized for the overall energy metabolism during cell growth while the remaining are directed towards starch biosynthesis (Roitsch and González, 2004). Although invertase activity in wheat is studied in relation to drought (Saeedipour, 2011), the role of this enzyme with respect to starch formation is poorly understood.

A normal starch granule is made up of one-quarter amylose (glucan polymer composed of glucose molecules linearly linked by α-1,4 glycosidic bond) and three-quarters amylopectin (glucan polymer composed of α-1,4-linked glucose molecules branched by α-1,6-glycosidic bonds). ADP-glucose pyrophosphorylase (AGPase) catalyzes the first committed step of starch synthesis, converting glucose1-phosphate to ADP-glucose and PPi (inorganic pyrophosphate). This enzyme naturally occurs in more than one form in plants; the one present in the endosperm of seeds is different from the one found in the leaves (Lohot *et al*., 2010). The plant AGPases are heterotetramers composed of two small (AGPS) and two large (AGPL) subunits (Boehlein *et al*., 2008), each of which is encoded by distinct genes such as *Brittle2* (*Bt2*) and *Shrunken-2* (*Sh2*), respectively, in maize (Wang *et al*., 2007). In rice, *AGPL1* and *AGPS1* genes have been suggested to encode the plastidial AGPase, and their expression appeared to be prominent at the

early stage of endosperm development. The expressions of *AGPL2* and *AGPS2b* increase at the grain filling stage and possibly encode for the cytosolic AGPase (Ohdan *et al*., 2005). Both the small and large subunits are important for catalysis as well as regulation (Cross, 2004). It is well established that the location of AGPase in photosynthetic tissues is restricted within the plastids, and it is subjected to allosteric regulation by 3-PGA (activator) and inorganic phosphate (inhibitor) (Ghosh and Preiss, 1966). Due to its sensitivity to these regulators, AGPase is widely considered to catalyze the rate limiting step in starch synthesis.

ADP-glucose, which is produced by the AGPase-catalyzed-reaction, is used by starch synthases (SS) and starch branching enzymes (SBE) to elongate the glucan chains of the starch granule (Kolbe *et al*., 2005). Starch synthases play major roles along with SBEs in elongating the glucan chain, thereby developing a complex starch structure. There are two types of starch synthases; granule-bound starch synthases (GBSS) and soluble starch synthases (SSS) (Tetlow, 2006). Granule bound starch synthase has two isoforms GBSSI and GBSSII. In rice the expression of *GBSSI* is at its highest level starting from 5 DAA through the end of endosperm development, whereas that of *GBSSII* remain at low level (Ohdan *et al*., 2005). In barley, the expression of *GBSS1b* is found in pericarp as well as embryo tissues (Sreenivasulu *et al*., 2006) and its transcripts were highly abundant until 4 DAA in the pericarp. The expression of *GBSS1a*, however, occurs mainly during grain filling and is restricted to the endosperm (Radchuk *et al.*, 2009). Two genes encoding GBSS have been isolated from wheat, *GBSSI* and *GBSSII*. *GBSSI* of wheat encodes the isoforms responsible for amylose synthesis in the storage organs. *GBSSII* encodes the isoform responsible for the synthesis of transient starch in the non-storage organs including the leaf, pericarp and culm tissues (Vrinten and Nakamura, 2000).

The soluble starch synthase is encoded by *SSI, SSII, SSIII* and *SSIV*. Each has a specific role in the entire process of amylopectin synthesis (Martin and Smith, 1995; James, 2003; Tetlow, 2006; Jiang *et al*., 2011). *SSI* is mainly responsible for the manufacture of short chains of glucans and is not yet known to have an isoform. It contributes to about 70% of the total soluble starch synthase activity (Fujita *et al*., 2006). SSII is involved in the synthesis of intermediate glucan chains and plays a major role in both composition and total content of starch in grains (Morell *et al*., 2003). Wheat has two genes of *SSII, SSIIa* and *SSIIb*. While *SSIIa* is mostly expressed in the cereal endosperm, *SSIIb* is restricted to the green tissues (Morell *et al*., 2003; Tetlow, 2006). SSIII plays an active role in synthesizing the long chains of glucans that stretch between amylopectin structures (James, 2003) and has two isoforms; SSIIIa is the isoform active in the endosperm while SSIIIb is found in the photosynthetic tissue (Tetlow, 2006). SSIV is the least known amongst all the soluble starch synthases involved in the initiation of starch granule formation. Two genes encoding SSIV have been found in rice *SSIVa* and *SSIVb* (Hirose and Terao, 2004). Studies in *Arabidopsis* have shown that mutations in *SSIV* have resulted in the formation of a single starch granule per plastid (Szydlowski *et al*., 2009) .

Starch branching enzymes (SBE) catalyze the formation of α-1,6 linkage between amylose molecules formed by α-1,4 glycosidic bond, enabling the formation of amylopectin. There are two types of SBEs, SBEI that produces longer chains as compared to SBEII, which is involved in the formation of more branched amylopectin (Rydberg *et al*., 2001; Tetlow, 2006; Jiang *et al*., 2011). In barley, *SBEI* has been found to be expressed only in the endosperm tissues (Radchuk *et al.* 2009). Both barley *SBEIIa* and *SBEIIb* show similar level of expression in the endosperm at 12 DAA, although the expression of *SBEIIa* was reported not to be endosperm specific (Sun *et al*., 1998). The maize *SBEIIa* is also expressed in the leaves while *SBEIIb* is

predominant in the endosperm (Gao *et al*., 1997). In contrast, *SBEIIa* of wheat was found to have four-fold more expression in the endosperm than that of *SBEIIb* at about 15 DAA (Regina *et al*., 2005, 2006).

Sucrose and starch metabolism are influenced by a number of endogenous factors including plant hormones. Several studies have shown that the plant hormone ABA plays a crucial role in grain development and grain filling. For example, a study by King (1979) showed that the endogenous level of ABA increases during wheat grain development, but decreases towards grain maturation. Exogenously applied ABA, however, acts as a negative regulator of starch biosynthesis in developing grains by affecting the transport of sucrose (Ahmadi and Baker, 1999). The SuSy enzyme is regulated by endogenous and exogenous ABA level in the grains. Low endogenous ABA was identified as a limiting factor to SuSy expression and applications of a controlled amount of exogenous ABA stimulated this expression. ABA in conjunction with sucrose, resulted in an even higher expression of SuSy (Tang *et al*., 2009). In the presence of sucrose, ABA was also found to increase the expression of *AGPL3* and starch level in rice (Akihiro *et al.*, 2005). Hence the role of ABA in grain filling is complicated, with low doses enhancing the activity of genes and enzymes, while high doses hindering starch biosynthesis altogether.

The focus of this study was to investigate the molecular bases of sucrose transport and its hydrolysis and downstream involvement in the starch biosynthesis in the developing grains of wheat. Furthermore, the study examined the potential roles of ABA in regulating these processes during grain development. An overview of the sucrose-starch metabolic pathway with the genes studied along with the enzymes they encode is presented in Figure 3.1.

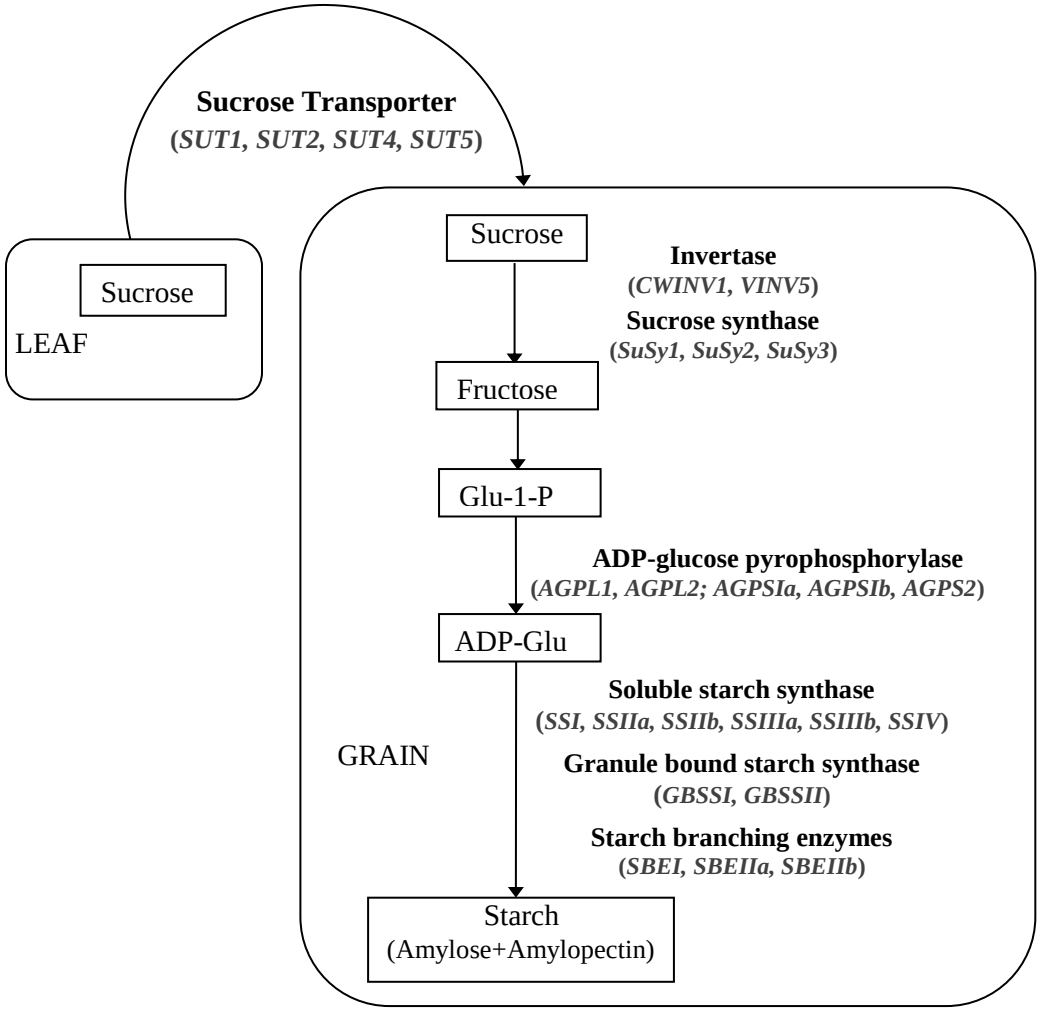


Figure 3.1: A simplified representation of the sucrose-to-starch metabolic pathway in plants showing the genes involved and the enzymes they encode. The rounded rectangles represent leaf and grain tissues. The metabolites are in rectangular boxes. The enzymes are in black bold fonts and the respective genes encoding them are in grey bold italics. Most of the genes are full length and some are partial fragments (see Table 3.1).

3.3 Materials and methods

3.3.1 Plant material

The spring wheat cv. Whitebird was grown in the greenhouse at 16-22^{o}C /14-18^{o}C (day/night) in a 16/8 h photoperiod under an average light intensity of 313.3 ± 9.2 µEm^{-2}sec^{-1}. One seed per pot (one gallon) was planted in a soil containing Sunshine Mix 4, also known as LA4 (Sungro Horticulture, Bellevue, WA, USA) and Cornell foliage mix [150 g osmocote, 120 g

superphosphate, 100 g calcium carbonate, 15 g chelated iron (13.2%), 2 g fritted trace elements, 0.7 g chelated zinc (14%) per 20 gallons of Sunshine LA4 mix]. Plants were fertilized from two-week old stage till flowering once every two weeks with N-P-K (20:20:20). One tablespoon (~20 g) of the fertilizer was added to a gallon of water, which was used for watering the plants. Spikes were tagged at the first extrusion of a yellow anther (day of anthesis) and developing grains were harvested from primary and secondary tillers at 4, 8, 16, 25, 30, 35 and 70 (mature) days after anthesis (DAA) in liquid nitrogen and stored at -80°C freezer until further use. Spikes were harvested in the morning (approximately 2 to 4 h after the lights in the green house had been turned on) and spikes from one plant in each individual pot were considered as a biological replicate. Three independent biological replicates (spikes from three different plants) were collected for each developmental stage. One individual spike was used for RNA extraction and upto two spikes from the same plant were used for enzyme assay (if two spikes were used, they were harvested at the same day and time). Grains harvested from the middle region of a single spike, which is considered as a biological replicate, were used for both sucrose and starch assays.

3.3.2 Wheat gene sequences and specific primers

In order to identify the genes encoding the membrane proteins and enzymes involved in the sucrose-to-starch metabolism pathway, sequence similarity searches were performed by BLASTN using publicly available databases (e.g., GenBank and http://compbio.dfci.harvard.edu/tgi/plant.html). Full length sequences and expressed sequence tags (ESTs) from wheat with a high percentage of identity with genes from other monocots were selected. Starch biosynthesis genes considered in this study are listed in Table 3.1. Gene specific primers for each gene were designed from their respective nucleotide sequences using Primer Express 3 and DNAMAN softwares (Appendix 1; Deol *et al.*, (2013) for *TaSUT2*; Sakthivel,

(2011) for *TaSUT1*, *TaSuSy1* and *TaSuSy2*), and their specificity were verified by BLAST searching the GenBank and PCR analysis.

3.3.3 RNA isolation and cDNA synthesis

Total RNA was prepared from 80-120 mg of developing wheat grains harvested at different stages (4, 8, 16, 25 and 30 DAA) as described previously (Li and Trick, 2005). Following extraction, 10 µg of the RNA was treated with DNase (Ambion, Austin, TX, USA) at 37^{o}C for 30 min. The RNA concentration was then quantified on NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA) and the quality verified by agarose gel (0.8%) electrophoresis. The DNase digested RNA (1µg) was used to synthesize first strand cDNA using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD, USA) following the manufacturer's instructions.

3.3.4 Real time RT-PCR assay

For real time PCR analysis of the expression of the target and reference genes, the cDNA samples were diluted to a final concentration of 2.5 ng/µL (for all starch biosynthesis genes except for *SSIV*) and 20 ng/µL (for *SSIV* and sucrose metabolism genes). The real time qPCR reaction consisted of 5 µL of the cDNA, 0.6 µL of each primer (10 µM; final concentration of 0.3 µM), 10 µL Maxima® SYBR Green/ROX qPCR Master Mix (2X) (Fermentas), and nuclease free water to a final reaction volume of 20 µL. The thermocycling conditions were as follows: initial denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 15s, annealing at 60°C for 30s, and extension at 72°C for 30s. Assays were performed on a Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA, USA). *β-actin* was used as a reference gene for normalization, and the relative transcript abundance of the target genes was determined by 2^{-}

$^{\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Grain developmental stage with the lowest normalized value was taken as a calibrator to determine the relative expression level of each gene.

Table 3.1: List of genes involved in sucrose-starch metabolism of wheat

Enzyme	Gene	EST or Accession #	Reference	Highest BLAST Hits	Description	E - value	Max Identity
AGPase Small subunit	*AGPS1a*	X66080.1	Ainsworth *et al*. (1993) Plant Mol. Biol. 23 (1) 23-33	FN179370.1	*H. vulgare plastid mRNA for AGPS1a gene*	0.0	97%
	AGPS1b	FJ643609.1	Unpublished	FN179370.1	*H. vulgare mRNA for AGPS1a*	0.0	98%
	AGPS2	AY727927.1	Roti and Denver (2007) J. Mol. Evol 65 (3) 316-327	FN179371.1	*H. vulgare* plastid partial mRNA for AGPS2 gene	0.0	94%
Large subunit	*AGPL1*	Z21969.1	Ainsworth *et al*. (1995) Planta 197 (1) 1-10	FN179372.1	*T. aestivum* AGPase large subunit mRNA	0.0	99%
	AGPL2	DQ406820.1	Unpublished	FN179373.1	*H. vulgare* plastidial AGPL2	0.0	97%
Starch synthase	*SSI*	AF091803	Li *et al*. (1999) Theor. Appl. Genet. 98, 1209-1216	FN179374.1	*H. vulgare* partial mRNA for SSI gene	0.0	95%
	SSIIa	AF155217	Li *et al*. (1999) Plant Physiol. 120 (4) 1147-1156	AY133249.1	*H. vulgare* starch synthase II	0.0	94%
	SSIIb	EU333947.3	Unpublished	AF395537.1	*O. sativa* soluble starch synthase II-2 mRNA	0.0	88%
	SSIIIa	AF258608.1	Li *et al*. (2000) Plant Physiol. 123 (2) 613-624	AF258609.1	*Aegilops tauschii* starch synthase III	0.0	100%
	SSIIIb	EU333946.2	Unpublished	EU620721.1	*Sorghum bicolor* starch synthase IIIb precursor	0.0	84%
	SSIV	AY044844.1	Unpublished	AY224560.1	*O. sativa* starch cynthase-like protein mRNA	0.0	84%
Granule-bound starch synthase	*GBSSI*	AF163319.1	Vrinten and Nakamura (2000) Plant Physiol. 122 (1) 255-264	FN179380.1	*H. vulgare* GBSS1a	0.0	92%
	GBSSII	AF109395.1	Unpublished	AF486521.1	*H. vulgare* GBSSIb precursor	0.0	95%
Starch-branching enzyme	*SBE1*	AF076679.1	Rahman *et al*. (1999) Theor. Appl. Genet. 98, 156-163	AY304541.1	*H. vulgare* SbeI	0.0	92%
	SBEIIa	Y11282.1	Plant Sci, in press, Nair *et al*.	FN179383.1	*H. vulgare* SbeIIa	0.0	99%
	SBEIIb	AY740401.1	Regina *et al*. (2005) Planta 222 (5) 899-909	AF064561.1	*H. vulgare* starch branching enzyme IIb (Sbe IIb)	0.0	95%
Sucrose Transporter	*SUT1*	AF408845	Aoki,N. *et al* (2002) Plant Mol. Biol. 50 (3), 453-462	AF408843.1	*Triticum aestivum* sucrose transporter SUT1B mRNA	0.0	100%
	SUT2	Pending	Deol *et al*. (2013) BMC Plant Biol. 13:181-196	AJ272308.1	*H. vulgare* SUT2 mRNA	0.0	92.6%
	SUT4	HX007930.1	EST	XM_003570440.1	PREDICTED: *Brachypodium distachyon* sucrose transport protein SUT4-like	3e-35	89%
	SUT5	TC368620	EST	BT086192.1	Zea mays full length cDNA clone	0.0	83%
Sucrose Synthase	*SuSy1*	AJ001117.1	Unpublished	X65871.1	*H.vulgare* mRNA for sucrose synthase type I	0.0	94%
	SuSy2	AJ000153.1	Unpublished	X69931.1	*H.vulgare* mRNA for sucrose synthase	0.0	95%
	SuSy3	TC383379	EST	FN421447.1	*Hordeum vulgare* subsp. vulgare ss3 gene for sucrose synthase	4e-151	98%
Invertase	*TaCWINV1*	AF030420.1	Minhas,J.S. and Saini,H.S. Plant Physiol. 118, 1535 (1998)	AY578160.1	*Oryza sativa* (japonica cultivar-group) cell-wall invertase 3	0.0	83%
	TaVINV5	AF069309.1	Minhas,J.S. and Saini,H.S.	AY575717.1	*Triticum monococcum vacuolar invertase1 (vin1) mRNA*	0.0	95%

3.3.5.1 Enzyme extraction

Frozen developing grains (15-55; depending on the stage) were ground to a fine powder with liquid nitrogen in a pre-cooled mortar and pestle. The ground tissue was divided into two parts and then weighed. One part was used for AGPase, GBSS and SSS assays and the other portion for the SuSy assay. The extractions of AGPase, SSS and GBSS were performed according to Nakamura *et al*. (1989). Ground tissues were homogenised with 5-10 mL of ice-cold extraction buffer [100 mM HEPES-NaOH (pH 7.5), 8 mM $MgCl_2$, 2 mM EDTA, 1 mM dithiothreitol (DTT), 12.5% (v/v) glycerol, and 5% (w/v) soluble polyvinylpyrrolidone PVP-10]. The homogenate of each sample was filtered through two layers of moistened Miracloth (EMD Millipore, Billerica, MA, US) and centrifuged at 12,000*g* for 10 min at 4°C. The supernatant was kept aside for the assay while the yellow layer of storage protein formed on the pellet of starch granules was scraped off and discarded. The starch granules were then washed by centrifugation with 2-4 mL of extraction buffer twice to remove any trace of SSS activity. The supernatants from these two washes were combined with the supernatant that was kept aside previously, and used for the AGPase and SSS assays. The pellet of the starch granules was then re-suspended with 0.5-2 mL of extraction buffer and used for the GBSS activity assay.

Extraction of sucrose synthase (SuSy) was performed according to Ranwala and Miller (1998) with minor modifications. Briefly, ground grains were homogenized with 100 mM HEPES-NaOH (pH 7.5) containing 10 mM isoascorbate, 3 mM $MgCl_2$, 5 mM DTT, 2 mM EDTA, 5% (v/v) glycerol, 3% (w/v) polyvinylpyrrolidone (PVP-10), and 0.01% Triton X-100. The homogenate was then centrifuged at 15,000g for 30 min. The supernatant was desalted on a Sephadex G-25 column. After column equilibration, the protein was eluted using a reaction

buffer that contained 50 mM HEPES-NaOH (pH 7.5), 10 mM $MgCl_2$, 2 mM EDTA, and 3 mM DTT.

3.3.5.2 Enzyme assay

All enzyme assays were optimized for pH and substrate concentration and were within the linear phase with respect to incubation time and protein concentration. The incubation time was optimized by testing the crude enzyme extract with its substrates for 5, 10, 15 and 20 min and the linear phase was chosen for the assays. The protein concentration was optimized by testing different concentrations of the extract and examining whether their absorbances were within the linear range of the spectrophotometer readings. While performing the assay, absorbance recorded at a reaction time of zero was considered as background value.

The **AGPase** (EC 2.7.7.27) assay was carried out according to Nakamura *et al*. (1989). The reaction mixture consisting of 50 mM Hepes-NaOH (pH 7.5), 1.2 mM ADPG, 5 mM PPi, 6 mM $MgCl_2$, 3 mM DTT and 50 µL of enzyme extract in a total reaction volume of 450 µL was incubated at 30°C for 2 min prior to the addition of ADP-glucose as a substrate to initiate the reaction. After incubation of the mixtures containing ADP-glucose at 30°C for 15 min, the reaction was stopped by boiling for 1 min and then centrifuged at 10,000*g* for 10 min. The supernatant was separated and mixed with 100 µL of 6 mM $NADP^+$, 300 µL of 50 mM HEPES-NaOH (pH 7.5), 0.08 unit (U) phosphoglucomutase, and 0.07 U glucose-6-phosphate dehydrogenase (G6PDH). Following incubation of this mixture for 10 min at 30°C, the activity of AGPase was determined by measuring the increase in absorbance at 340 nm with spectrophotometer (Biochrom, Holliston, MA, USA).

SSS and **GBSS** (EC 2.4.1.21) assays were performed as described in Nakamura *et al*. (1989). The SSS was assayed with a reaction mixture that contained 50 mM HEPES-NaOH (pH

7.5), 1.6 mM ADPG, 1.4 mg amylopectin, 15 mM DTT, and SSS extract in a total volume of 450 µL. The assay for the insoluble SS (GBSS) was prepared as described above, but without priming with amylopectin. The aliquot of the crude insoluble SS added to the reaction mixture was taken while the suspension was being mixed vigorously. The mixtures were incubated at 30°C for 20 min and the reaction terminated by boiling for 1 min. Settlement of the granules during the assay was prevented by slow and continuous shaking of the samples. Following incubation, the reaction mixtures were centrifuged at 10,000*g* for 10 min. The supernatant was separated and mixed with 200 µL of solution consisting of 50 mM HEPES-NaOH (pH 7.5), 4 mM phosphoenolpyruvate, 200 mM KCl, 10 mM $MgCl_2$, 1.2 U pyruvate kinase, and then incubated further for 20 min at 30°C. The ADP produced by the starch synthase reaction was converted to ATP and the resulting solution was incubated in boiling water for 1 min. The supernatant was mixed with 400 µL of a solution of 50 mM HEPES-NaOH (pH 7.5), 10 mM glucose, 20 mM $MgCl_2$, and 2 mM $NADP^+$. The enzymatic activity was measured spectrophotometrically as increase in absorbance at 340 nm, which is the result of reduction of $NADP^+$ to NADPH by the addition of 1.4 U hexokinase and 0.35 U G6PDH.

SuSy (EC 2.4.1.13) was assayed in the cleavage direction according to Ranwala and Miller (1998). The reaction mixture contained 50 mM MES-NaOH (pH 6.3), 100 mM sucrose, 3.75 mM UDP, and 50 µL of enzyme extract in a total volume of 450 µL. The buffer and enzyme were pre-incubated in a water bath at 30°C for 2 min prior to the addition of UDP to initiate the reaction. Standard reaction time was 15 min. After incubation, the reaction was terminated by placing the mixture in boiling water for 1 min. The UDP-glucose produced by the reaction was determined by incubating a 200 µL aliquot of the mixture in 0.5 M glycine/NaOH (pH 8.7) containing 2.5 mM NAD^+ and 0.03 U of UDP-glucose dehydrogenase (EMD Millipore)

in a total volume of 1.0 mL. The conversion of NAD^+ to NADH was measured spectrophotometrically as increase in absorbance at 340 nm. One unit of enzyme activity was defined as the amount of enzyme catalyzing the cleavage of 1 µmol of sucrose per min at 30°C under the assay conditions.

Protein content was determined according to Bradford (1976), using BioRad Protein Assay (BioRad, Hercules, CA, USA), and bovine serum albumin as a standard.

3.3.6 Analysis of starch and sucrose levels in developing grains

Starch was determined from developing grains at 8, 16, 25, 30, 35 DAA and mature grains while that of sucrose was determined from grains at 4, 8, 16, 25, 30 and 35 DAA. Freeze dried grain samples (23-27 mg) were finely ground and mixed with 1 ml of 70% ethanol, and then 5 ml of 100% ethanol was added onto the mixture. After centrifugation of the suspension at 3000*g* for 15 min at room temperature, the ethanol extract was separated. The pellet was re-suspended with 1.5 ml of 95% ethanol, centrifuged as described above, and the ethanol extract was pooled. The pellet was used for determination of starch content and starch composition using the Amylose/Amylopectin Assay Kit (Megazyme International Ireland Ltd., Wicklow, Ireland) following the manufacturer's instructions. The ethanol extract was used for determining sucrose content and basal tissue glucose content. Ethanol was completely evaporated by incubation in boiling water, and then the remaining extract was dissolved in 1:1 (v/v) distilled water and sodium acetate buffer (pH 4.5). After adding chloroform and mixing it vigorously, the mixture was centrifuged at 3000*g* at room temperature for 15 min. The upper aqueous phase was separated and then mixed with sodium acetate buffer to a final volume of 10 mL, and an aliquot (1mL) of this was digested with 0.1 µL invertase enzyme (1 mg/mL) from *Candida utilis* (MP Biomedicals,, Santa Ana, CA, USA) at 55°C for 15 min. Both the digested (sucrose) and

undigested (glucose) samples were incubated with GOPOD (glucose oxidase peroxidase) (Megazyme International) for 20 min at 40°C and analyzed spectrophotometrically at 510 nm.

3.3.7 ABA treatment

Developing spikes at 8 and 16 DAA were treated with exogenous ABA as follows. The spikes were harvested with the peduncle and immediately placed in water. Three replicates (individual spike from one plant as a replicate) were used for each stage. After removing the flag leaf, the entire length of peduncle was first sterilized with 70% ethanol for 2 min and then with 1.1% sodium hypochlorite with Tween-20 for 15 mins. The peduncle was then washed with sterile water to remove all traces of ethanol and sodium hypochlorite. Finally, the peduncle was cut with a sterile blade 10 to 12 cm from the bottom end of the spike and immediately placed in a liquid MS medium (pH 5.8) containing 25 mM ABA, which was then incubated at 16-22^{o}C/14-18^{o}C (day/night) in a 16/8 h photoperiod with 2800-3000 lux of light intensity for 24 h. The spikes were harvested in liquid nitrogen and stored at -80^{o}C until further use. Total RNA was isolated from ABA treated grains, and used to synthesize cDNA to investigate the effect of ABA on the expression of all target genes. For each developmental stage, the untreated control sample was used to determine the relative expression of each gene.

3.4 Results

3.4.1 Grain development- increase in fresh and dry weight

Grain development was studied from 4 DAA to maturity (Fig. 3.2), and its growth in fresh weight can be characterized by three phases: tissue proliferation phase (0 to 8 DAA), rapid grain filling phase (8 to 25 DAA), and grain maturation phase (after 25 DAA) (Fig. 3.2). These three phases of grain development were also evident when its growth is expressed on dry weight basis.

The rates of fresh and dry weight accumulation were determined by first calculating the difference in average weight between the two time points and then dividing the result by the number of days in between the two time points [For example, accumulation rate per grain per day between 8 and 16 DAA = (Mean weight at 16 DAA (mg) – Mean weight at 8 DAA (mg))/ (16-8) mg per grain per day]. The rate of fresh weight accumulation per grain per day was highest between 8 and 16 DAA, whereas that of dry weight accumulation reached maximum between 25 and 30 DAA (Table 3.2). In general, after 25 DAA, grain fresh weight was determined by dry matter accumulation (Table 3.2). As the grain developed from 8 to 35 DAA, moisture content (MC) declined steadily from 74.1% to 50.8%, after which it continued to decline substantially to the level of 11.5% by full maturity. The mature stage was selected at a stage when the spikes had visibly become golden in color, about 70 DAA (Fig. 3.2).

3.4.2 Expression pattern of genes involved in sucrose transport

Expression patterns of sucrose transporter (SUT) genes including *TaSUT1*, *TaSUT2*, *TaSUT4* and *TaSUT5* were determined in the developing grains (4 to 30 DAA, Fig. 3.3 A) using real time qPCR. The transcripts of all the genes were detected at all stages of grain development (Fig. 3.3 B). However, the expression of *TaSUT1* was substantially higher during the grain filling period (8 to 25 DAA) than the expression of the other SUT genes studied (Fig. 3.3 B).

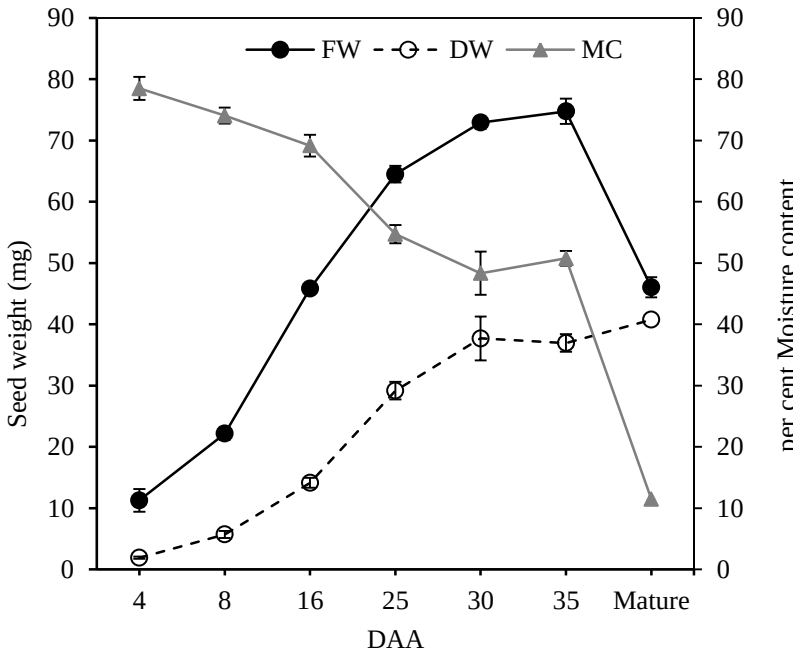


Figure 3.2: Fresh weight (FW) and dry weight (DW) and per cent moisture content (MC) of the developing grains of cv. Whitebird at 4, 8, 16, 25, 30, 35 DAA and mature stage (70 DAA). Data are means ±SE (n=3)

Table 3.2: Fresh and dry weight accumulation rates of developing grains (mg grain^{-1} day^{-1})

Accumulation rate (mg grain^{-1} day^{-1})							
DAA	0-4	4-8	8-16	16-25	25-30	30-35	35-Mature
FW[a]	2.32	3.22	2.96	2.08	1.68	0.37	-28.71
DW[b]	0.48	0.95	1.05	1.67	1.71	-0.15	3.8
Dry weight accumulation as a percent of fresh weight accumulation							
DW % FW[c]	20.69%	29.50%	35.6%	80.5%*	101.3%**	n.d.***	n.d.***

Starch accumulation rate (mg grain^{-1} day^{-1})					
DAA	0-8	8-16	16-25	25-30	30-35
Starch	0.12	0.60	1.33	0.81	-0.29

[a]FW=fresh weight, [b]DW=dry weight, [c]DW % FW= Dry weight accumulation as a percent of fresh weight accumulation

*Dry weight accumulation is more than 75% of fresh weight accumulation. **Dry weight accumulation exceeds that of fresh weight. ***No further dry weight accumulation is observed

With respect to temporal expression patterns, the mRNA level of *TaSUT1* showed a rapid increase from 4 to 16 DAA (6-fold), when it peaked. It showed a rapid decline from 16 to 30 DAA (5-fold). The expression of *TaSUT2* decreased from 4 to 8 DAA (4-fold) followed by an increase (7.5-fold) when it peaked at 16 DAA and then showed a decline (3.9-fold) from 16 to 25 DAA. A two-fold increase in its expression was observed from 25 to 30 DAA. The transcript level of *TaSUT4* showed a very slight increase from 4 to 8 DAA (1.5-fold). However, from 8 to 16 DAA its transcript level showed a decrease (2.5-fold) and remained at a comparable level through to the end of grain development (30 DAA). Expression of *TaSUT5* was low throughout the developmental stages considered.

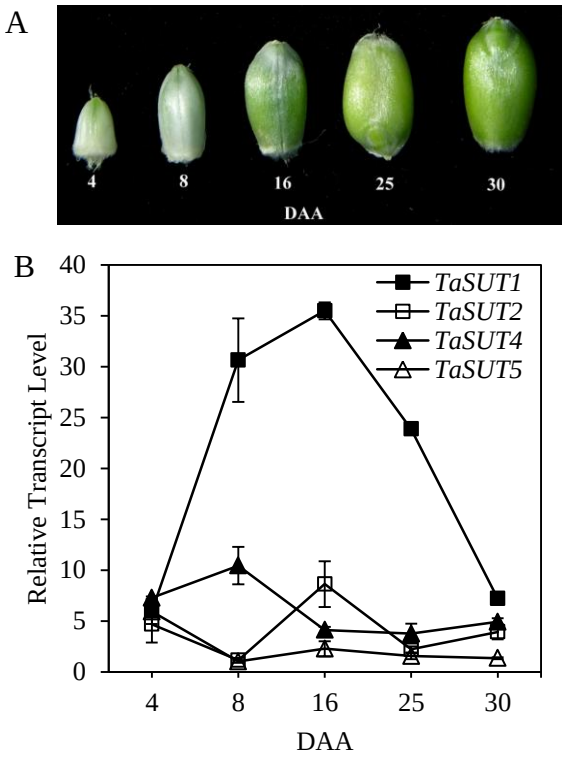


Figure 3.3: (A) Developing grains of wheat cv. Whitebird at the five stages studied for gene expression. (B) Expression of sucrose transporter genes during grain development in wheat cv. Whitebird grown under greenhouse conditions described in the materials and methods. Transcript levels were expressed relative to that of *TaSUT5* in the 8 DAA sample, which was set at a value of 1. Data are means ± SE, n=2-3.

3.4.3 Expression pattern of genes involved in sucrose hydrolysis

The expression patterns of genes encoding wheat sucrose synthase (SuSy) (*TaSuSy1*, *TaSuSy2 and TaSuSy3*), and cell wall (*TaCWINV1)* and vacuolar (*TaVINV5*) invertases were examined in the developing grains. The transcripts of all *SuSy* genes were detected at all the stages studied (Fig. 3.4 A), however, *TaSuSy1* (95- to 163-fold) and *TaSuSy2* (61- to 316-fold) showed much higher expressions than *TaSuSy3* from 4 to 16 DAA. With respect to time, the expression of *TaSuSy1* was maintained at a similar level throughout the grain developmental stages studied (Fig. 3.4 A). The transcript level of *TaSuSy2* showed a rapid increase in its transcript level from 4 to 8 DAA (4.5-fold) and declined afterwards to a very low level by 25 DAA. With respect to the *INV* genes analyzed, the transcripts of *TaVINV5,* but not that of *TaCWINV1,* were detected in the developing grains (Fig. 3.4 B).

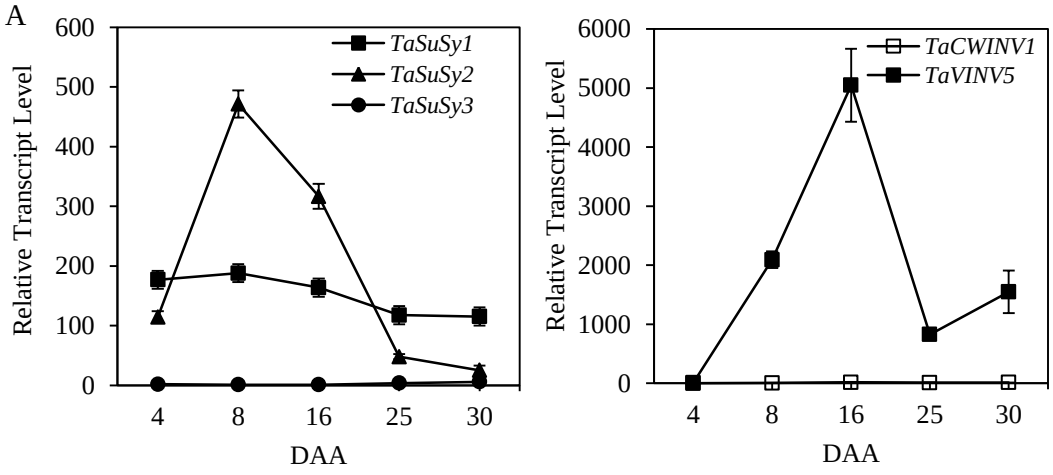


Figure 3.4: Expression of sucrose synthase (A) and invertase (B) genes during grain development in wheat cv. Whitebird grown under greenhouse conditions described in the materials and methods. Transcript levels of *TaSuSy*s and *TaINV*s were expressed relative to that of *TaSuSy3* in 16 DAA sample and *TaCWINV1* in 4 DAA samples, respectively, which were set at a value of 1. Data are means ± SE, n=2-3.

Although it was expressed at low level during the early stage of grain development (4 DAA), *TaVINV5* was expressed at substantially high level (2000- to 5000-fold as compared to 4 DAA)

during the rapid grain filling phase (8 to 16 DAA). By 25 DAA, the expression of *TaVINV5* decreased by 5-fold (relative to that observed at 16 DAA) and remained at similar level through 30 DAA (Fig. 3.4 B).

3.4.4 Expression pattern of genes involved in starch biosynthesis

AGPase genes: The transcripts of genes encoding AGPase large subunits (*AGPL1* and *AGPL2*) and small subunits (*AGPS1a, AGPS1b* and *AGPS2*) were all detected in the developing grains, but each member within each group showed differential expression patterns (Fig. 3.5 A and B). With respect to *AGPL* genes, the abundance of *AGPL1* transcripts was higher (23- to 1061-fold)

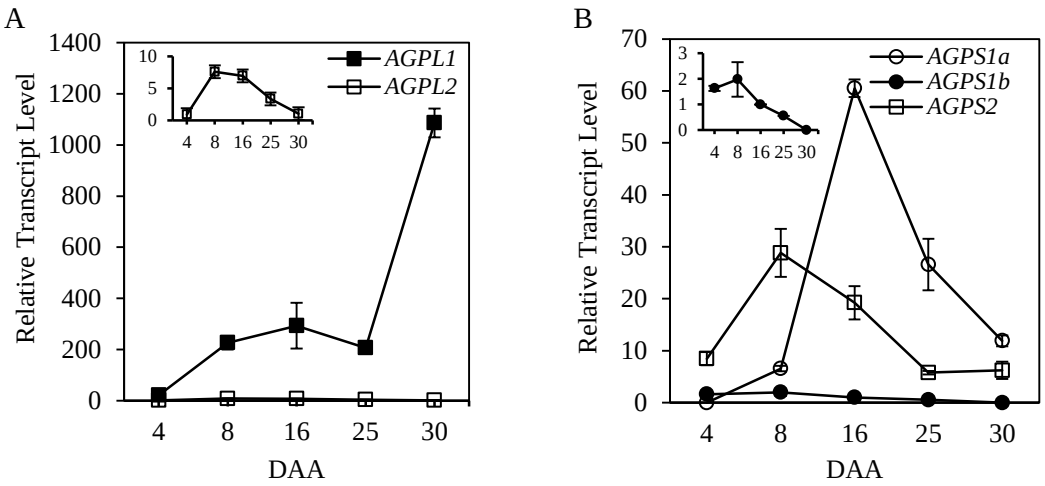


Figure 3.5: Expression of AGPase large subunit genes, *AGPL1* and *AGPL2* (A) and AGPase small subunit genes, *AGPS1a, AGPS1b,* and *AGPS2* (B) during grain development in wheat cv. Whitebird grown under greenhouse conditions described in the materials and methods. Transcript levels of *AGLs* and *AGPSs* were expressed relative to that of *AGPL2* in 30 DAA sample and *AGPS1b* in 16 DAA samples, respectively, which were set at a value of 1. Data are means ± SE, n=2-3.

The expression of the other *AGPL* gene, *AGPL2,* was very minimal throughout the grain development stages examined. Its expression showed an increase from 4 to 8 DAA (8.2-fold) and

then decreased gradually to a low level by 30 DAA (7.3-fold; Fig. 3.5 A inset). With respect to the expression of *AGPS* genes, *AGPS1a* was not detected until after 8 DAA. Its transcript abundance increased markedly from 8 to 16 DAA (9.2-fold) before showing a gradual decline from 16 to 25 DAA (2.2-fold). Following 25 DAA, the transcript level of this gene was maintained at almost similar level (Fig. 3.5 A). The expression of *AGPS1b* was very low throughout grain developmental; however, its transcript abundance at 4 and 8 DAA was higher than that observed during the later grain developmental stages examined. The expression of *AGPS2* was increased (3-fold) from 4 to 8 DAA and peaked at 8 DAA, after which it decreased gradually through 30 DAA (4.6-fold; Fig. 3.5).

Soluble and granule bound starch synthases: The transcripts of all genes identified as encoding soluble starch synthase which is involved in the formation of amylopectin during starch biosynthesis, were detected at almost all stages of grain development (Fig. 3.6). The expressions of *SSI, SSIIa* and *SSIIIa* were higher relative to the other genes in the family (*SSIIb* and *SSIIIb*). The transcript level of all these genes was higher at 8 and/or 16 DAA, except for *SSIIIb*, whose expression was higher at the later (25 and 30 DAA) than the earlier stages of grain development. Transcript abundance of *SSIV* during grain development was very low when compared to that of the other members of starch synthase gene family, as a result its transcripts could only be detected at a higher concentration of cDNA as a template (200 ng instead of the 12.5 ng used to characterize the expression of the other genes). Relative to that observed in the other grain developmental stages, its expression was higher by 30 DAA (2-fold; Fig. 3.6).

Very low transcript levels of both genes encoding GBSS (*GBSSI and GBSSII*) were detected at the early stages of grain development (4 DAA). However, the expression of *GBSSI* showed a sharp increase from 4 to 16 DAA (723-fold), after which it exhibited a 2.3-fold

decrease (Fig. 3.7). Whereas, the other gene, *GBSSII*, was expressed at a relatively low level throughout grain developmental stages, although a slight increase (4-fold) was evident during the later stages of grain development (from 25 to 30 DAA; Fig. 3.7).

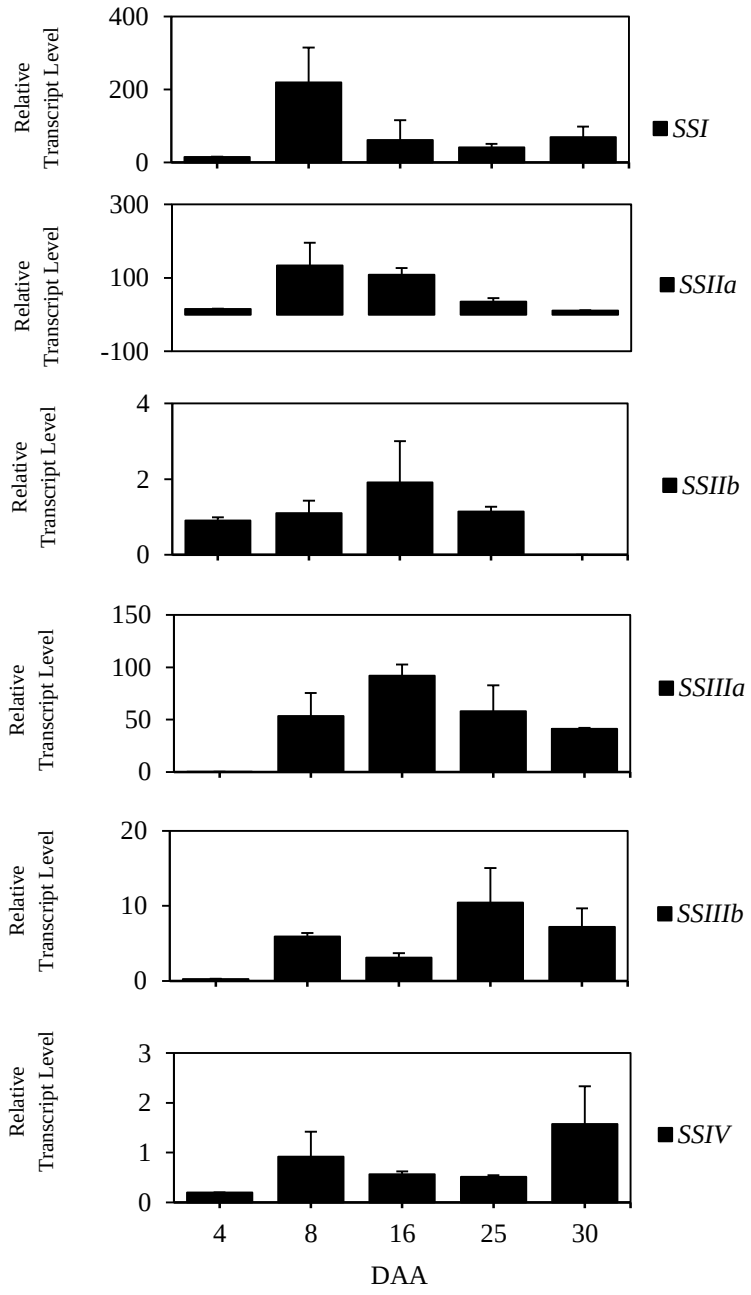


Figure 3.6: Expression of starch synthase genes (*SSI*, *SSIIa*, *SSIIb*, *SSIIIa*, *SSIIIb* and *SSIV*) during grain development in wheat cv. Whitebird grown under greenhouse conditions described in the materials and methods. Transcript levels were expressed relative to that of *SSIIb* in 8 DAA sample, which was set at a value of 1. Data are means ± SE, n=2-3.

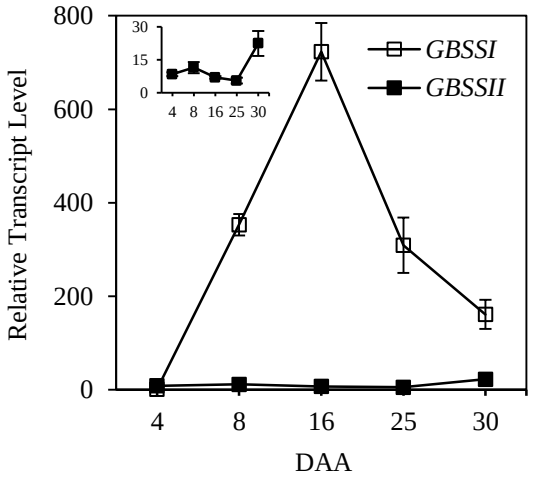


Figure 3.7: Expression of granule bound starch synthase genes (*GBSSI* and *GBSSII*) during grain development in wheat cv. Whitebird grown under greenhouse conditions described in the materials and methods. Transcript levels were expressed relative to that of *GBSSI* in 4 DAA sample, which was set at a value of 1. Data are means ± SE, n=2-3.

3.4.5 Expression pattern of genes involved in starch branching

High expression level of both *SBEI* and *SBEIIa* was found in the developing grains at 16 DAA, after which their expression declined (2.6-fold) (Fig. 3.8). However, these genes showed differential expression during the earlier and later stages of grain development. By 8 DAA, the transcript abundance of *SBEIIa* was higher (12.5-fold) than that of *SBEI*; whereas that of *SBEI* exhibited more transcript abundance (2.8-fold) than that of *SBEIIa* by 30 DAA. The transcripts of *SBEIIb* were detected only after 8 DAA but at a very low abundance.

3.4.6 Activities of enzymes involved in starch synthesis and sucrose hydrolysis

The assays for activities of SuSy, AGPase, SSS and GBSS were performed at four time points of grain development. The activity of SuSy per grain increased from 4 to 8 DAA (10-fold) and reached a maximum level at 16 DAA, after which it exhibited a decrease (2-fold) (Fig. 3.9).

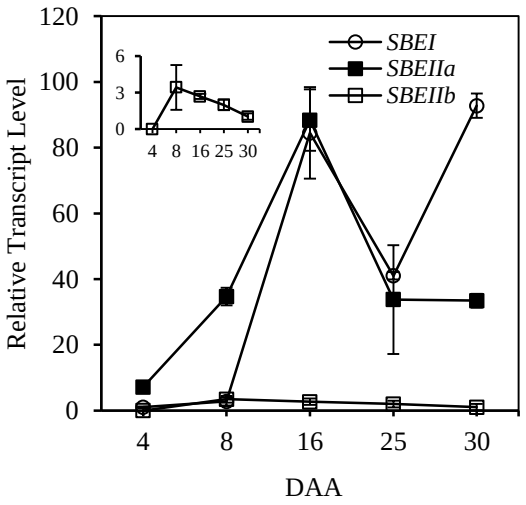


Figure 3.8: Expression of starch branching enzyme genes (*SBEI, SBEIIa* and *SBEIIb*) during grain development in wheat cv. Whitebird grown under greenhouse conditions described in the materials and methods. Transcript levels were expressed relative to that of *SBEIIb* in 30 DAA sample, which was set at a value of 1. Data are means ± SE, n=2-3.

Analyses of the activity of starch synthesizing enzymes revealed that AGPase activity per grain showed an increase from a minimal level at 4 DAA to its maximum at 16 DAA (94-fold). Its activity slightly decreased by 25 DAA (Fig. 3.9). The SSS showed maximum activity at the earlier stages of grain development (4 to 8 DAA). Its activity declined by 16 DAA and remained at low level through 25 DAA (Fig. 3.9). The GBSS showed increased activity towards the later stages of grain filling. Its activity increased from 4 to 16 DAA (648-fold) and remained at similar level through 25 DAA (Fig. 3.9). The enzyme activity observed per gFW per minute and per mg protein showed a similar trend (Appendix 3).

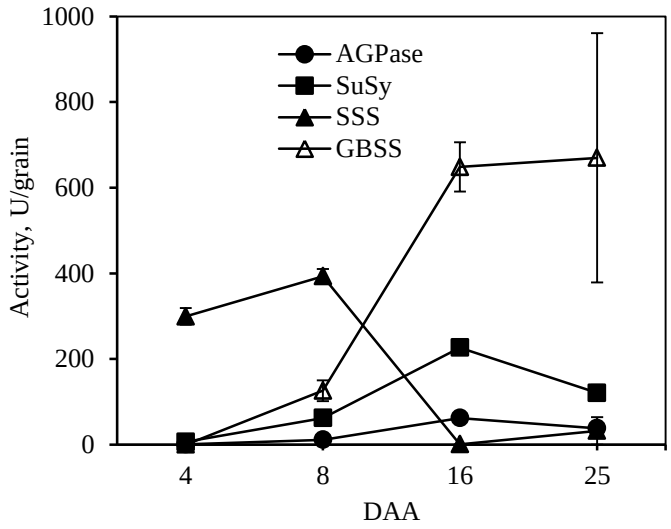


Figure 3.9 Activities of sucrose synthase (SuSy), AGPase, soluble (SSS) and granule bound starch synthases (GBSS) in developing grains of wheat cv. Whitebird grown under greenhouse conditions described in the materials and methods. Activities are expressed per grain.Data are means ± SE, n=3.

Table 3.3: Sucrose content at different stages of grain development in wheat cv. Whitebird [a]

	Days after anthesis					
	4	**8**	**16**	**25**	**30**	**35**
	mg/g fresh weight (FW)					
Sucrose	13.36±2.40	21.46±2.78	15.87±2.47	9.80±0.74	8.07±0.66	8.12±0.71
Glucose	5.14±1.19	6.75±0.95	1.81±0.61	0.50±0.12	0.33±0.15	0.38±0.10
	mg/g dry weight (DW)					
Sucrose	61.45±4.28	82.79±8.89	52.27±10.41	21.58±0.82	15.66±1.42	16.46±0.28
Glucose	24.03±5.48	25.96±2.71	5.98 ±2.20	1.09 ±0.23	0.63 ±0.27	0.80 ±0.24
	mg/grain					
Sucrose	0.11±0.02	0.472±0.046	0.726±0.118	0.631±0.040	0.587±0.041	0.610±0.069
Glucose	0.04±0.01	0.15±0.02	0.08±0.03	0.03±0.01	0.02±0.01	0.03±0.01

Data are means ± SE, n=3. [a] Cultivar was grown under greenhouse conditions as described in the materials and methods.

3.4.7 Sucrose content in developing grains

A relatively high amount of sucrose per gram fresh weight (gFW) was found at 4 DAA, and its level increased to a maximum by 8 DAA (Table 3.3). Afterwards, sucrose content exhibited a gradual decrease, and grains at 30 DAA contained approximately 40% of that detected at 8

DAA. The pattern of sucrose content per gDW followed a similar trend as that observed on a gFW basis. When expressed on grain basis, there was an increase of sucrose content per grain from 4 to 16 DAA, when it reached to a maximum (Table 3.3). It then showed a gradual decline afterwards; however, 84% of the sucrose detected at 16 DAA was still present at 30 DAA. The initial level of glucose was also concurrently quantified during grain development, and its level was found to be minimal during the main grain filling phase (Table 3.3).

Table 3.4: Starch content and composition at different stages of grain development in cv. Whitebird[a]

	Days after anthesis					
	8	**16**	**25**	**30**	**35**	**70**
	mg/g fresh weight (FW)					
Total Starch	43.84±1.83	126.79±8.00	275.86±11.92	299.27±20.50	271.64±26.79	506.90±15.80
Amylose	7.38±0.75	25.46±3.15	63.26±2.64	72.92±7.47	67.29±8.47	129.35±8.84
Amylopectin	36.46±1.18	101.32±4.86	212.60±10.25	226.35±13.37	204.34±18.45	377.55±9.32
Ratio	1:5	1:4	1:3.3	1:3.1	1:3	1:2.9
	mg/g dry weight (DW)					
Total Starch	170.2±10.10	410.5±10.50	609.2±2.60	578.1±25.70	549.7±15.10	573.0±22.40
Amylose	28.67±3.16	82.02±6.93	139.87±4.53	140.55±10.68	135.71±7.60	146.40±11.81
Amylopectin	185.83±3.24	328.50±5.40	469.30±4.80	437.50±16.50	413.90±7.90	426.60±11.70
Ratio	1:6.5	1:4	1:3.3	1:3.1	1:3	1:2.9
	mg/grain					
Total Starch	0.97±0.05	5.79±0.38	17.77±0.57	21.84±1.59	20.41±2.53	23.39±1.52
Amylose	0.16±0.02	1.16±0.15	4.08±0.19	5.33±0.58	5.06±0.76	5.98±0.57
Amylopectin	0.81±0.03	4.63±0.23	13.69±0.45	16.51±1.02	15.35±1.78	17.41±1.03
Ratio	1:5	1:4	1:3.3	1:3.1	1:3	1:2.9

Data are means ± SE, n=3. [a]Cultivar was grown under greenhouse conditions as described in the materials and methods. Ratio= ratio between amylose:amylopectin.

3.4.8 Starch accumulation in developing grains

Starch accumulation showed a steady increase with grain development from as early as 8 DAA to 25 DAA, after which it did not show any substantial change. As the grain developed from 0 to 8 DAA, starch was accumulated at a rate of 0.12 mg per grain per day (Table 3.2). During the

rapid grain filling phases (8 to 16 DAA) the rate of starch accumulation increased by 5-fold (0.60 mg) per grain per day (Table 3.2). The highest rate of starch accumulation occurred between 16 and 25 DAA, (1.33 mg per grain per day). From 25 to 30 DAA, the rate of starch accumulation declined slightly (1.6-fold) (Table 3.2). With respect to starch composition, the amylopectin to amylose ratio on gFW (5:1), gDW (6.5:1) and per grain (5:1) basis was higher at the early stage of grain development (8 DAA) (Table 3.4). The ratio showed a decrease as the grain developed. By maturity, the amylopectin to amylose ratio was found to be 2.9:1.

3.4.9 Regulation of genes involved in sucrose-starch synthesis by ABA

In order to investigate whether sucrose catabolism and starch synthesis are regulated by ABA, developing spikes (at 8 and 16 DAA) were treated with ABA. The ABA treatment significantly reduced the expression of *TaSUT1* (4-fold), which was found to be the predominant SUT gene in developing grains, while it increased the expression of *TaSUT4* at both 8 and 16 DAA (5- and 13-fold, respectively) (Table 3.5). ABA treatment also caused a significant reduction in the expression of *TaSuSy2* at 8 (2-fold) and 16 DAA (5-fold), while the expression of *TaSuSy1* showed an increase at 8 DAA (5-fold). Furthermore, the expression of genes encoding both the cell wall and vacuolar invertases were significantly increased by ABA treatment at both 8 and 16 DAA. The transcripts for *TaCWINV1* were not detected in the untreated samples but showed upregulation both at 8 and 16 DAA (973- and 395-fold, respectively). Similarly, the expression of *TaVINV5* was increased by ABA treatment at both 8 and 16 DAA (5- and 8-fold respectively). The ABA treatment caused a decrease in the transcript abundance of genes involved in starch synthesis during grain development (Table 3.6). This included a slight decrease in transcripts abundances of *SSIIa* (1.5-fold) and *SSIIIa* (1.3-fold) and a 5-fold decrease in that of *GBSSI, SBEI* and *SBEIIa* (5-fold) at 16 DAA, when compared to the untreated controls. The genes involved in

starch synthesis in the pericarp and other green tissues showed over 3-fold upregulation by ABA treatment at both 8 and 16 DAA, including *AGPL2*, *GBSSII* and *SSIIb*. The *AGPSIb*, *AGPS2* and *SSIIIb* genes, on the other hand, showed over 3-fold upregulation only at 16 DAA.

Table 3.5: Effect of ABA on the expression (fold change) of sucrose transporter and hydrolysis genes

	8 DAA		16 DAA	
	Control	ABA	Control	ABA
		Sucrose transporter genes		
SUT1	1 ± 0.20*	0.33 ± 0.02	0.70 ± 0.11 a	0.19 ± 0.01 b
SUT2	0.83 ± 0.21	0.33 ± 0.02	0.10 ± 0.01	0.18 ± 0.03
SUT4	0.26 ± 0.05 a	1.51 ± 0.29b	0.15 ± 0.01 a	2.07 ± 0.29 b
SUT5	0.03 ± 0.01	0.01 ± 0.00	0.04 ± 0.02	0.04 ± 0.01
		Sucrose hydrolysis genes		
		Sucrose synthase		
SuSy1	1 ± 0.05 a*	5.23 ± 0.73 b	0.46 ± 0.02 a	0.19 ± 0.08 b
SuSy2	2.46 ± 0.44	1.16 ± 0.22	2.87 ± 0.86	0.25 ± 0.13
		Invertase		
CWINV1	1 ± 0.16 a*	972.85 ± 38.48 b	1.78 ± 0.41 a	703.36 ± 27.85 b
VINV5	231.91 ± 9.38 a	1170.38 ± 113.85 b	117.72 ± 11.59 a	932.26 ± 109.56 b

Values are mean fold changes ± SE, n=2-3. * Expression level of the genes within each family member is determined relative to that of the first member in 8 DAA control samples, which was assigned a value of 1. Any significant difference between control and treated samples are denoted by letters.

Table 3.6: Effect of ABA on the expression of starch biosynthesis genes

	8 DAA		16 DAA	
	Control	ABA	Control	ABA
AGPase large and small subunit genes				
AGPL1	1 ± 0.37*	1.25 ± 0.81	2.77 ± 0.83	0.26 ± 0.08
AGPL2	0.46 ± 0.11 a	1.40 ± 0.06 b	0.16 ± 0.04 a	0.56 ± 0.08 b
AGPS1a	3.72 ± 1.32	0.23 ± 0.01	2.38 ± 0.39	0.13 ± 0.05
AGPS1b	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.01 a	0.71 ± 0.08 b
AGPS2	0.41 ± 0.15	0.01 ± 0.01	0.06 ± 0.01 a	0.21 ± 0.03 b
Soluble and granule bound starch synthase genes				
SSI	1 ± 0.26*	0.46 ± 0.15	0.58 ± 0.12	0.22 ± 0.06
SSIIa	0.41 ± 0.13	0.25 ± 0.03	0.28 ± 0.06	0.21 ± 0.00
SSIIb	0.00 ± 0.00 a	0.08 ± 0.01 b	0.00 ± 0.00 a	0.04 ± 0.00 b
SSIIIa	0.10 ± 0.01	0.09 ± 0.02	0.05 ± 0.00 a	0.04 ± 0.00 b
SSIIIb	0.14 ± 0.00 a	0.05 ± 0.00 b	0.02 ± 0.00 a	0.08 ± 0.01 b
GBSSI	0.67 ± 0.06 a	0.47 ± 0.05 b	1.06 ± 0.20	0.31 ± 0.02
GBSSII	0.17 ± 0.05	0.51 ± 0.09	0.03 ± 0.01 a	0.28 ± 0.04 b
Starch branching enzymes				
SBEI	1 ± 0.34*	0.15 ± 0.03	0.93 ± 0.07 a	0.14 ± 0.06 b
SBEIIa	1.40 ± 0.24	0.27 ± 0.03	0.45 ± 0.04 a	0.11 ± 0.01 b
SBEIIb	0.41 ± 0.15	0.22 ± 0.05	0.22 ± 0.05	0.04 ± 0.01

Values are mean fold changes ± SE, n=2-3. * Expression level of the genes within each family member is determined relative to that of the first member in 8 DAA control samples, which was assigned a value of 1. Any significant difference between control and treated samples are denoted by letters.

In order to gain insights into grain carbohydrate remobilization, we also analyzed the expressions of *1-FEH* and *6-FEH*, encoding fructan exohydrolases, which are involved in removing fructan units from the end of the existing fructan chains. The *1-FEH* and *6-FEH* genes showed upregulation by over 11-fold and 3-fold, respectively at both 8 and 16 DAA (Fig. 3.11).

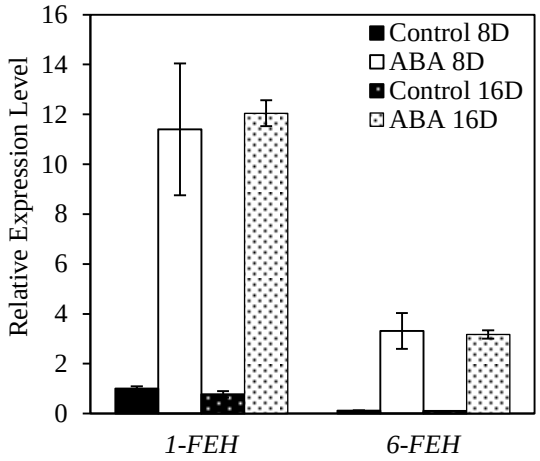


Figure 3.10: The effect of ABA on the expression of fructan exohydrolase genes *1-FEH* and *6-FEH*. Expression level of the genes within each family member is determined relative to the control samples at each developmental stage, which was assigned a value of 1. Data are mean ± SE, n=3).

3.5. Discussion

This study analyzed sucrose transport and metabolism, and starch biosynthesis in developing wheat grains at gene, enzyme and metabolite levels. The findings of the study have identified different molecular elements involved in the regulation of the physiological processes under consideration during grain development.

Genes encoding sucrose transporters in developing wheat grains showed a lower to moderate expression at the early stages of development. Of the two functionally characterized wheat sucrose transporters (Aoki *et al*., 2002; Deol *et al*., 2013), *TaSUT2* is expressed at a lower level as compared to Ta*SUT1*; however, the expression of both genes peaked at 16 DAA and gradually declined afterwards (Fig.3.3). Previously *TaSUT1* has been reported to be important in sucrose unloading into the endosperm (Aoki *et al*., 2002) and the main site of sucrose uptake was identified as the transfer cells located in the endospermic tissue (Wang and Fisher 1995). A recent report by Deol *et al*. (2013) has indicated that TaSUT2 may be involved in the regulation

of intercellular sucrose partitioning between the cytoplasm and the vacuole. This might point to possible complementary role of the two proteins in regulating the transport of sucrose from the source to the sink tissues. The gene encoding TaSUT4 was expressed at a higher level at 8 DAA. Given that the level of sucrose increased from 4 DAA to 8 DAA and remained at an elevated level through 16 DAA, it is likely that *TaSUT1* and *TaSUT4* genes have overlapping roles of sucrose transport during the early to mid-phases of grain filling. The detection of higher levels of sucrose during the early phase of grain development is consistent with a previous study that showed higher content of soluble sugars in grains during anthesis (Mi *et al*., 2002). In our study, the endospermal accumulation of starch started after 8 DAA and terminated around 30 DAA (Fig. 3.2, Table 3.4). This phase of starch synthesis would require a continuous supply of sucrose from the source into the endosperm, the meristematic division of which stops around 14 DAA to 16 DAA when the grain size is determined (Evers, 1970; Brocklehurst, 1977). Since sucrose serves as a direct precursor of starch deposited in the storage organs like grain or tubers (Smith *et al.,* 1997), the gradual decline of sucrose from 16 DAA can be attributed to increased synthesis of starch, which makes use of the hexose phosphate sugars produced via the cleavage of sucrose by TaSuSy1 and TaSuSy2. Consistent with this, the transcript abundance of *TaSuSy1* and *TaSuSy2* was at maximum by 8 DAA and 16 DAA, respectively (Fig. 3.4) and the total activity of SuSy was at maximum by 16 DAA (Fig. 3.9). Furthermore, the sharp increase in the expression of *TaSuSy2* during 4 DAA to 16 DAA corresponds well with the increase in starch accumulation rate (Table 3.4). The gene encoding cell wall invertase of wheat, *TaCWINV1*, was found at almost non-detectable levels throughout grain filling. Although cell wall invertase is reported to play a major role during the early stages of grain development (Tatsuro *et al.,* 2002; Ji *et al.,* 2007), recent reports have indicated its role during grain filling. For example, the maize

Incw2 has been shown to play a major role in grain development (Chourey *et al*., 2005), and the acid invertase activity in barley increases at about 5 days after flowering (Weschke *et al*., 2003). It is therefore likely that cell wall invertase genes other than *TaCWINV1* are important in the developing grains of wheat. The expression of the vacuolar invertase gene (*TaVINV5*) increased from 4 DAA to 16 DAA and then decreased, followed by an increase by 30DAA. The high expression of *TaVINV5* during the initial phase of endosperm development suggests a possible role of invertase in cell proliferation of the growing endosperm (Roitsch and González, 2004; Ruan *et al*., 2010) However, the high expression of the gene towards the end of grain filling could be due to the contribution of *TaVINV5* transcripts from the pericarp, where it is predominantly expressed and the corresponding enzyme is active (Chevalier and Lingle, 1983; Dale and Housley, 1986).

Based on their temporal expression patterns, the 16 starch biosynthesis genes considered in this study are categorized into three groups: those expressed during the tissue proliferation phase of grain development (0-8 DAA), the rapid phase of grain filling (8-25 DAA) and the maturation phase (25-30 DAA). The first group comprises *AGPL2, AGPS1b, AGPS2, GBSSII, SSIIb, SSIV* and *SBEIIa*; the second group includes *AGPL1, GBSS1, SSI, SSIIa, SSIIIa* and *SBE,* and the third group mainly consists of *SBEI*. However, it is important to note that there are some genes whose expression was maintained almost uniformly throughout the entire phase of grain filling with slight alterations, including *SSIIb, SSIIIb* and *SBEIIb.*

The role of AGPase in regulating starch biosynthesis is well established (Emes *et al*., 2003). In barley and wheat, there is evidence for the presence of two distinct AGPS subunits which are formed by the alternative splicing of the same gene (Thorbjornsen *et al*., 1996; Kang *et al*., 2010**).** The expression of genes encoding these sub units have been shown to be organ-

specific, one being expressed mainly in the plastids (hence leaves), and the other being expressed as a cytosolic form predominantly in the developing endosperm (Johnson *et al*., 2003; Rösti *et al*., 2006). In cereal endosperms, the ADP-glucose produced by the cytosolic isoform is imported into the plastids by an ADP-glucose transporter. In our study, both *AGPS1b* and *AGPS2* are found to be expressed during the initial phase of grain development, and this might be due to transcript contribution from the pericarp where they are known to encode the plastidial isoforms of the enzyme. These results, along with the expression of *AGPL2*, which is also known to be of plastidial origin, during the initial phases of grain development, could explain starch synthesis in the pericarp of wheat caryopsis. The activity of AGPase increased during the peak phase of starch synthesis (16-25 DAA) and this could be due to the cumulative expression of *AGPL1* and *AGPS1a* (Fig. 3.5 A and B). The expression pattern of *GBSSII*, which is responsible for amylose synthesis in the pericarp (Vrinten and Nakamura, 2000), is in concert with that of *AGPases* and *SBEIIb*, whose expression increased from 4 DAA to a maximum level at 8 DAA. This is suggestive of the key roles played by these genes during the initiation of starch biosynthesis in the pericarp at the early stage of wheat grain development. The synthesis of this transitory starch diminishes gradually as it is hydrolysed and translocated into the developing endosperm to initiate storage starch synthesis in the endosperm (Chevalier and Lingle, 1983; Radchuk *et al*., 2009). Recent studies in barley have shown that *SSI* and *SSIIb* are expressed in the pericarp, whereas all the isoforms (*SSI, SSIIa, SSIIb SSIIIa, SSIIIb* and *SSIV*) are expressed in the endosperm (Radchuk *et al*., 2009). Considering the amylose: amylopectin ratio, it can be suggested that the amylopectin synthesis starts before that of amylose and continues further after the synthesis of the former ceases. This hypothesis can be supported by the fact that expression of all the *SS* genes started at a very early phase of grain development, *SSI, SSIIa and SSIIIa* being

the predominant family members during this phase (Fig. 3.6). These genes continued to express at a moderate level through the last stage studied (30 DAA). In addition, the activity of soluble starch synthase was detected during the earlier stages of grain development and showed more activity during grain filling (Fig. 3.9). The expression of *GBSSI*, on the other hand, was moderately expressed at the beginning of grain filling but reached the maximum level 16 DAA and gradually declined to a low level thereafter (Fig. 3.7). The GBSS activity per grain was shown to increase after 8 DAA and was highest at 25 DAA. In agreement, the rate of amylopectin accumulation was higher than that of amylose except between 25-30 DAA, a phase following rapid decline in the expression of *GBSSI.* The presence of a considerable amount of starch accumulation from 30 DAA to maturity (Fig. 3.4) indicated the continuation of starch synthesis even after the rapid grain filling phase was completed. This was supported by the presence of high level of *AGPL1* and *SBEI* transcripts at 30 DAA (Fig. 3.5 A and Fig. 3.8), and GBSS activity by 25 DAA (Fig. 3.9).

Treatment of developing grains with ABA induced upregulation of the expression of one of the SuSy genes, *SuSy1*, at 8 DAA (Table 3.6). In agreement with this, the promoter region of *HvSuSy1* is shown to have several ABA-responsive elements (Seiler *et al*., 2011). In addition, ABA treatment induced the expression of *INV* genes at 8 and 16 DAA, including that of *TaCWINV1*, which was not detected in the untreated grains. These results might suggest that ABA increases the hydrolysis of sucrose possibly to increase sucrose import from the source into the sink (Yang *et al*., 2004b). This hypothesis is in agreement with the increased expression of *TaSUT2* observed at 16 DAA. It has also been shown previously that ABA promotes grain filling in wheat, although it shortens the grain filling phase (Yang *et al*., 2004b). It appears from our data that the expression of genes encoding most of the plastidial isoforms of starch biosynthesis

enzymes including *AGPL2, AGPS1b, AGPS2, SSIIb, SSIIIb* and *GBSSII* are upregulated by ABA (Table 3.6). In agreement with this, the plastidial *AGPL1*, *AGPL2* and *AGPS1* have shown upregulation by ABA in rice (Akihiro *et al*., 2005).

In contrast, ABA treatment downregulated the expressions of *SUT1* and *SuSy2*, and most of the endosperm starch biosynthesis genes including, *AGPL1, AGPS1a, SSI, GBSSI, SBEI, SBEIIa* and *SBEIIb*. This may partly contribute to the negative effect of ABA on grain growth and grain weight (Ahmadi and Baker 1999; Zhu *et al.* 2011). Therefore, although our results suggested that ABA induced an initial increase in sucrose transport into the pericarp and transient starch synthesis, the expression of the genes that were responsible for carrying out starch synthesis in the endosperm were suppressed, suggesting an overall decreased synthesis of starch in the developing grains.

Fructans are accumulated in the grains during its establishment as a sink organ. The initial accumulation acts as protection against sugar-induced feedback regulation of photosynthesis, hence maintaining a constant phloem loading (Pollock, 1986). Application of ABA to the grains upregulated the expressions of genes encoding fructan exohydrolases *1-FEH* and *6-FEH*, which remove fructan units from the end of the existing fructan chains (Ritsema and Smeekens, 2003), suggesting the possible involvement of ABA in the degradation and hence re-mobilization of the hexose released from fructans in the grains towards starch metabolism.

Our results overall highlight the significance of *SUT1, SuSy2, AGPL1, AGPS1a, SSI, SSIIa, SSIIIa, GBSSI, SBEI* and *SBEIIa* genes in controlling sucrose metabolism and its eventual use for starch biosynthesis. The model in Figure 3.11 shows the genes identified as important in regulating starch synthesis in wheat grains. Treatment with exogenous ABA downregulates the expressions of all these genes during the grain filling phase, suggesting its role in suppressing

starch synthesis. ABA upregulated the expression of *FEH* genes, and this suggests its role in the remobilization of water soluble carbohydrate in the grain towards starch metabolism. This study provides insights into the roles of genes and gene family members involved in sucrose transport, sucrose hydrolysis and starch synthesis in determining starch yield in wheat grains, however, further studies are required to determine their physiological roles.

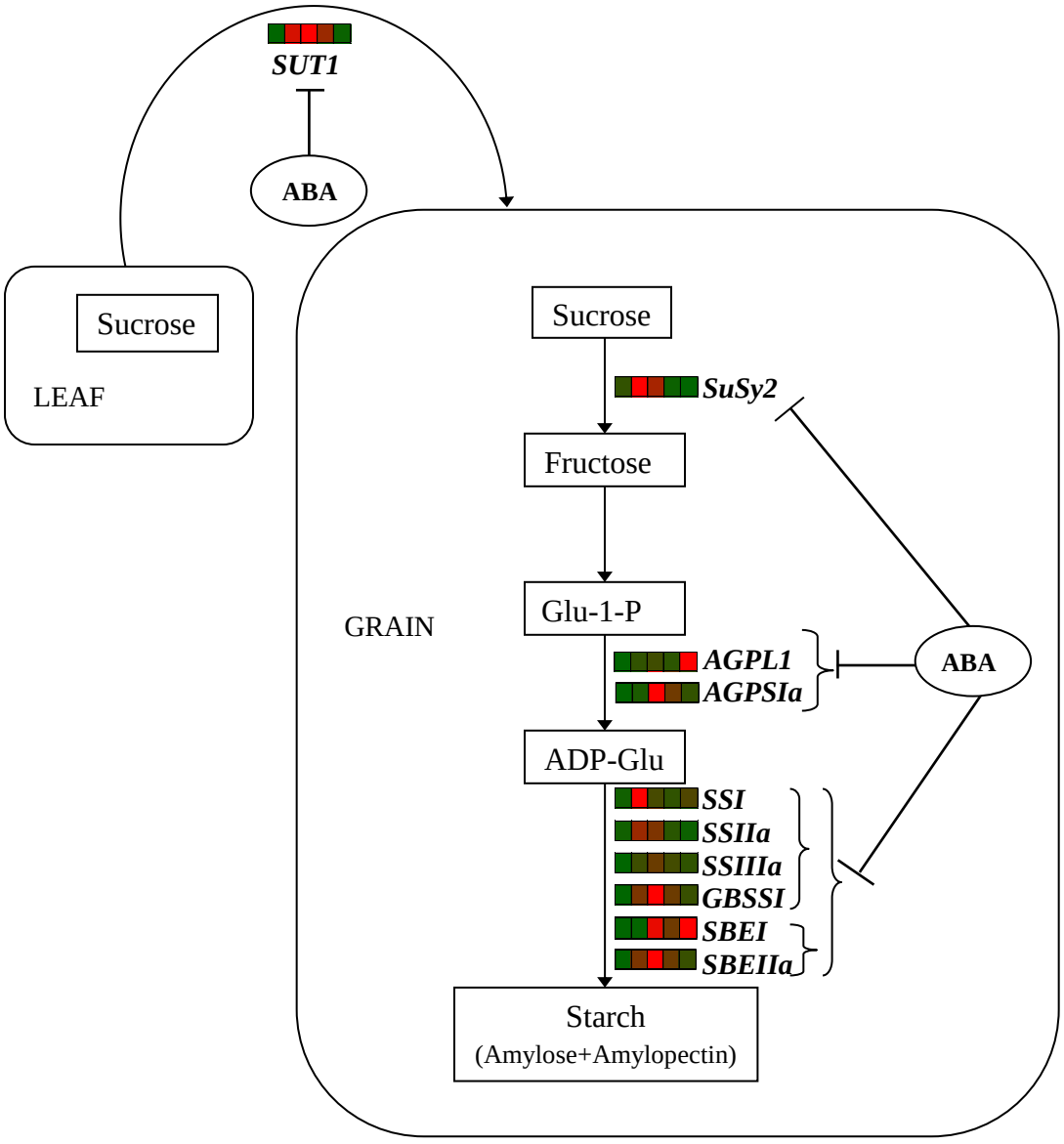


Figure 3.11: Summary of the expression patterns of sucrose-starch metabolic genes identified as predominant in developing wheat grains: *SUT1*, *SuSy2*, *AGPL1*, *AGPS1a*, *GBSSI*, *SSI*, *SSIIa*, *SSIIIa*, *SBEI* and *SBEIIa*. The colored boxes beside each gene indicate its expression levels during grain development (boxes from left to right represent 4, 8, 16, 25 and 30 DAA developmental stages). Red color represents increased expression while green color is for decreased expression. The effect of ABA on the expression of the genes identified as predominant is also shown; all genes are downregulated by ABA. The leaf as source organ and the grain as sink organ are represented by rounded rectangles in which the major metabolites and intermediates are shown.

4. Investigation of the role of natural variation in starch biosynthesis: A comparison between spring wheat cultivars with differing grain starch content

4.1 Abstract

Wheat cultivars or germplasm lines vary in their starch synthesis capacity in the grain. This study investigated whether the differences in starch accumulation are associated with the expression of starch biosynthesis genes. To this effect, comparative analyses were performed with three cultivars of spring wheat that show varying level of starch per grain, Chinese Spring (Chinese land race), AC Andrew (high yielding Canadian soft white wheat cultivar) and CDC Teal (top grade of Canadian western red spring wheat). The results of this study showed that AC Andrew has the highest thousand kernel weight and the highest starch content per grain amongst the three cultivars. This was associated with elevated expressions of the starch biosynthetic genes, *AGPL1*, *GBSSI*, *SSI*, *SSIIIa* and *SBEIIa*. Cellular localization study of the *SSI* gene showed the presense of its transcripts maily in the endosperm of developing grains. Furthermore, cv. AC Andrew exhibited higher activities of the major starch synthesis enzymes including, ADP glucose pyrophosphorylase, soluble and granule bound starch synthases. These results suggest that specific members of the starch biosynthetic genes play a regulatory role in determining starch yield in wheat.

4.2 Introduction

Starch is a primary metabolite that determines grain yield in cereal crops such as wheat. It consists of over 50% of the total dry weight of a mature wheat grain, and contributes significantly to the end use of wheat in various industries. The pathway through which starch is synthesized in plants has been well established (Tetlow, 2011). Its biosynthesis takes place in both source and sink tissues, and involves several enzymes, mainly adenosine diphosphate (ADP)-glucose pyrophosphorylase (AGPase), soluble and granule bound starch synthases (SS, GBSS), and starch branching enzymes (SBE). Most of these enzymes have multiple isoforms encoded by different gene family members. ADP-glucose pyrophosphorylase catalyzes the first committed step of starch biosynthesis, catalyzing the conversion of glucose-1-phosphate and adenosine triphosphate (ATP) to ADP-glucose and inorganic pyrophosphate (PPi). It is a heterotetramer protein composed of two small (AGPS) and two large (AGPL) subunits (Boehlein *et al*., 2008), each of which is encoded by distinct genes. In wheat, for example, the large subunits are encoded by *AGPL1* (cytosolic) and *AGPL2* (plastidial) whereas the small subunits are encoded by *AGPS1a* (cytosolic), *AGPS1b* (plastidial) and *AGPS2* (plastidial) (Emes, 2003; Tetlow, 2011). In the photosynthetic source tissues, it has generally been accepted that AGPase is restricted within the plastids, and is subjected to allosteric regulation in which it is activated by 3-Phosphoglyceric acid (3-PGA, a product of photosynthesis) and inhibited by inorganic phosphate (Pi) (Ghosh and Preiss, 1966). On account of its sensitivity to inhibitors and activators, AGPase is widely acknowledged to mediate the rate limiting step in starch biosynthesis. ADP-glucose is subsequently used by starch synthases and branching enzymes (Kolbe *et al*., 2005). Starch synthases play major roles in elongating the glucan chain, thereby developing the complex starch structure. There are two types of starch synthases; one that

encodes granule-bound starch synthases with the primary function of elongating amylose chains, and the soluble starch synthases that are exclusively involved in the synthesis of amylopectin (Tetlow, 2011). To date, two genes, encoding GBSS have been identified in wheat, *GBSSI* and *GBSSII* (Vrinten and Nakamura, 2000). The *GBSSI* encodes the isoform responsible for amylose synthesis in sink organs, whereas the *GBSSII* encodes the one responsible for transient starch synthesis in the non-sink organs such as the leaf, pericarp and culm tissues (Vrinten and Nakamura, 2000). The soluble form of starch synthase is encoded by several genes including *SSI, SSII, SSIII* and *SSIV*; each of which is believed to have a specific role in amylopectin synthesis (Martin and Smith, 1995; James, 2003; Tetlow, 2006; Hannah, 2007).

Previous studies have indicated that synthesis and accumulation of starch in grains are affected by several genetic and environmental factors. For example, water stress (Yang *et al*., 2004b) and temperature (Hurkman *et al*., 2003) are amongst the environmental factors that influence the synthesis of starch in wheat endosperm. Kernel size is one of the genetic factors that determine starch formation and deposition in grains (Dai, 2008). It has been shown that wheat cultivars with larger kernels exhibit higher activities of starch biosynthetic enzymes such as AGPase, SS and SBEs and have a higher rate of starch accumulation when compared with wheat cultivars producing smaller kernels (Jenner *et al*., 1991). The capacity of wheat grains to accumulate dry matter is established during the tissue proliferation phase, and mitotic activity of the endosperm and photoassimilate concentration have been considered the most important determinants of the grain's strength as a sink organ (Jenner *et al*., 1991).

Natural variation in the genetic makeup of different cultivars/varieties of a given crop plays a significant role in determining yield and other agronomic traits. Grain yield in wheat can be defined on the basis of number of spikes per unit area, number of grains per spike and grain

weight (Brdar *et al*. 2008). Yield is dependent on grain weight, which in turn is a function of the duration and rate of grain filling (Whan *et al*., 1996). The duration and rate of grain filling has been found to be more correlated to the genotype rather than environmental factors (Darroch and Baker, 1995). For example, in rice, dry matter accumulation in the grains of different cultivars of *indica* and *japonica* was found to be linearly related to increase in endosperm cell number as well as the activities of sucrose synthase and AGPase (Liang *et al*., 2001). More recent study showed the importance of metabolite-specific genetic diversity in rice through analysis of single nucleotide polymorphisms (SNPs) in genes that are believed to determine nutritional quality (Heuberger *et al*., 2010). It was revealed by this study that the presence of SNPs in the 5' and 3' untranslated regions of genes involved in the synthesis of phenolics and vitamin E, was attributed to the genetic diversity underlying the difference in the level of the respective metabolites. In maize, six genes have been reported to play important roles in starch synthesis, including *ae1* (encoding starch branching enzyme IIb), *bt2* (encoding AGPase small subunit), *sh1* (encoding sucrose synthase), *sh2* (encoding AGPase large subunit), *su1* (encoding isoamylase) and *wx1* (encoding granule bound starch synthase I) (Whitt *et al*., 2002). The *bt2*, *sh1* and *sh2* genes work upstream and are involved in the formation of glucose. These genes exhibit low genetic diversity among different cultivars, suggesting the significance of artificial selection for these genes in improving yield and amylopectin qualities of grains (Whitt *et al*., 2002; Wilson *et al*., 2004). Furthermore, investigation of the basis of genetic diversity for 48 metabolites, including eight sugars, in three millet (*Panicum miliaceum*) cultivars, has led to the identification of a candidate cultivar for future breeding programs (Kim *et al*., 2013).

Wheat breeding programs are focused mainly on improving traits of agronomic importance such as grain yield, protein content, and disease and drought tolerance. Given the

increased industrial application of wheat starch, it is very important to identify molecular components that play significant roles in starch formation and accumulation in wheat grains. As genetic variation forms an integral part of factors controlling agronomic traits, it is likely that comparison of starch accumulation among different germplasm lines or cultivars provides insights into the regulation of starch yield in wheat. Using gene expression, enzyme activity, and starch quantification approaches, Chapter 3 of this thesis reported on the identification of starch biosynthesis genes that appear to play regulatory roles in wheat grains. However, little information is available that investigates whether such regulatory starch biosynthetic genes exhibit differential expression across wheat cultivars differing in grain size and starch yield.

The objectives of this study were to investigate starch biosynthesis in three spring wheat cultivars, Chinese Spring, AC Andrew and CDC Teal at molecular, biochemical and metabolite levels. Chinese Spring is a land variety from China (Lee, 1995) and well characterized genetically (Sears, 1969). Although unsuitable agronomically it is widely used for genetic studies notably in the wheat genome sequencing (Brenchley *et al*., 2012). AC Andrew is a high yielding Canada Western Soft White Spring (CWSWS) cultivar with an average yield of 121% of AC Barrie (4393.91 kg/ha considering AC Barrie yield to be 3631 kg/ha) (Seed Manitoba, 2010). It was developed by Agriculture and Agri-Food Canada (AAFC)-Lethbridge Research Centre (Government of Alberta). CDC Teal is marketed in the top grades of Canadian Western Red Spring Wheat (CWRSW) and is developed by the University of Saskatchewan (Hughes and Hucl, 1993). It has a grain yield of 98% of AC Barrie (3558.38 kg/ha considering AC Barrie yield to be 3631 kg/ha) (Seed Manitoba, 2010).

4.3 Materials and methods

4.3.1 Plant material and measurement of yield parameters

Spring wheat cultivars Chinese Spring, CDC Teal and AC Andrew were grown on a bench in the greenhouse at 16-22^{o}C/14-18^{o}C (day/night) in a 16/8 h photoperiod. One seed per pot (one gallon) was planted and grown in Sunshine Mix 4 **(**LA4; Sungro Horticulture, Bellevue, WA, USA) and Cornell foliage mix [150 g osmocote, 120 g superphosphate, 100 g calcium carbonate, 15 g chelated iron (13.2%), 2 g fritted trace elements, 0.7 g chelated zinc (14%) per 20 gallons] as described in Chapter 3. Plants were fertilized once every two week with one tablespoon of N-P-K (20:20:20) per gallon from their two-week old stage until the onset of flowering. Spikes in each cultivar were tagged at the first extrusion of a yellow anther (day of anthesis) and developing grains were harvested from primary and secondary tillers at 4, 8, 16, 25 and 30 days after anthesis (DAA), in liquid nitrogen and then stored at -80°C freezer until further use. Mature grains were collected at 70 DAA, when the spikes were completely dried. Three independent biological replicates (spikes from one plant per pot were considered one replicate) were collected for each developmental stage. The number of spikes used for RNA isolation, enzyme assays and starch level determination were same as described under materials and methods section of Chapter 3.

Number of spikes (primary and secondary) per plant, number of grains per spike, total grain weight per spike, thousand kernel weight (TKW), and total grain yield per plant were recorded for three different biological replicates of each cultivar. For each biological replicate, weights of 100 kernels were taken in a technical replicate of three. The mean value was multiplied by ten to get the TKW.

4.3.2 Wheat gene sequences and specific primers

The wheat genes and the respective nucleotide sequences of the primers used for expression analysis are shown in Table 3.1 and Appendix 1.

4.3.3 RNA isolation and real time RT-PCR assay

Total RNA was prepared from developing wheat grains harvested at different stages (4, 8, 16, 25 and 30 DAA) and then treated with DNase (Ambion, Austin, TX, USA) as described in Chapter 3. After determining their quality and integrity, the RNA samples were used to synthesize first strand cDNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD, USA) following the manufacturer's instructions.

For real time qPCR analysis of the expression of the target and reference genes, the cDNA samples were diluted (final concentration of 2.5 ng/µL) prior to their use as a template for qPCR. The real time qPCR reaction contained 5 µL of the diluted cDNA, 0.6 µL of each primer (10 µM; final concentration of 0.3 µM), 10 µL Maxima® SYBR Green/ROX qPCR Master Mix (2X) (Fermentas), and nuclease free water to a final reaction volume of 20 µL. Assays were performed on a Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA, USA) using thermocycling conditions described in Chapter 3. The wheat *β-actin* was used as a reference gene, and the relative transcript abundance of the target genes was determined by $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Changes in the expression of each gene were determined relative to that in Chinese Spring 4 DAA samples, which were set to a value of 1.

4.3.5 RNA in situ localization

Wheat grains were harvested at 4 DAA and after removing the upper one-fifth portion of the spike with a razor blade. Samples were fixed in 4% paraformaldehyde (w/v) in 1X Potassium Buffer Saline (PBS) under slight vacuum at room temperature. The grains were embedded in

paraffin Paraplast (FisherScientific, Hampton, New Hampshire, USA). A pGEMTeasy vector was constructed to harbour the cDNA sequence of 578 bp from the *TaSSI* and riboprobes. Digoxigenin (DIG)-labeled riboprobes were synthesized by in vitro transcription from the vector using T7 (antisense) and SP6 (sense) polymerases (Promega, Fitchburg, WI, USA) using DIG-RNA labeling kit following the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). Both sense and antisense probes were subjected to alkaline hydrolysis at 65^{o}C to generate fragments of approximately 150 bp lengths and stored at -80^{o}C until further use. Sections of the embedded tissue (10 μm thickness) were prepared on a microtome (RM2145, Leica, Wtzlar, Germany) and transferred to Superfrost Plus slides (Fisher). Tissue dehydration, pre- and post-hybridization treatments, washes, and antibody treatments were performed as described previously (Belmonte *et al.*, 2007). Following overnight staining in Western Blue (Promega), the sections were observed under a microscope under 5X magnification (DC500, Leica, Wetzlar, Germany).

4.3.5 Enzyme extraction and assay

Frozen developing grains (15-30; depending on the stage) of Chinese Spring and AC Andrew cultivars were ground to a fine powder in liquid nitrogen using a pre-cooled mortar and pestle. Ground tissues were used for extraction and preparation of AGPase, granule bound and soluble starch synthase assays. The extractions and assays for AGPase, SSS and GBSS were performed according to Nakamura *et al*. (1989). Protein content was determined according to Bradford (1976) using BioRad Protein Assay (BioRad, Hercules, CA, USA) and bovine serum albumin as a standard.

4.3.6 Starch analysis

Starch (amylose and amylopectin) levels of the developing and mature grains of the three cultivars were determined using the Amylose/Amylopectin Assay Kit (Megazyme International Ireland Ltd) and the protocol described in Chapter 3.

4.3.7. Statistical analysis

Statistical analyses of the gene expression and starch level data were performed with Analysis of Variance (ANOVA) using the PROC Mixed procedure of SAS software (Ver. 9.2., SAS institute Inc, 2008). In order to comply with normal distribution, the fold change values in gene expressions were converted to their corresponding log 10 values. Cultivar, stage, and the cultivars x stage interaction were considered as sources of variation with fixed effects. Least significant difference (LSD) was calculated to compare statistically significant difference in least squares (LS) means of all cultivar x stage combinations. The type 3 test of Fixed Effects (tests for the significance of each of the fixed effects, cultivar and stage) was determined and those with $p \leq 0.05$ were considered significant. Grain yield and grain weights were analyzed by one way ANOVA with SigmaPlot 12 software.

4.4 Results

4.4.1 Measurement of yield parameters

Under our greenhouse conditions (see materials and methods), Chinese Spring produced more spikes per plant than the other two cultivars, however AC Andrew had a higher number of grains and grain weights per spike (Table 4.1). The cultivar AC Andrew also had about 17% higher thousand kernel weight than CDC Teal and about 48% higher than that of Chinese Spring. There was no significant difference in grain yield per plant (number of grains per spike*grain weight*number of spike per plant) amongst the three cultivars (Table 4.1).

Table 4.1: Yield parameters of Chinese Spring, CDC Teal and AC Andrew[a]

Cultivar	Country	Spike/plant	Grain/Spike	Grain wt/spike(g)	TKW(g)	Grain yield/ plant (g/plant)
Chinese Spring	China	13.3 ± 0.9	52.3 ± 3.8	1.73 ± 0.10	35.0 ± 2.8	23.3 ± 2.5
CDC Teal	Canada	10.7 ± 0.3	40.0 ± 2.0	1.53 ± 0.23	44.6 ± 1.4	18.9 ± 1.8
AC Andrew	Canada	8.3 ± 0.3	55.7 ± 1.8	2.53 ± 0.34	52.1 ± 0.5*	26.7 ± 1.9

* AC Andrew showed a significant difference ($p \leq 0.05$) compared to Chinese Spring for TKW

[a] Plants were grown under greenhouse conditions described in materials and methods.

4.4.2 Grain development- accumulation of fresh and dry weight

Grain development was studied for all the three cultivars from 4 DAA to maturity (Table 4.2, Fig. 4.1). Based on the fresh and dry matter accumulation, the grain development can be grouped into three phases, tissue proliferation phase (0-8 DAA), rapid grain filling phase (8-25 DAA), and grain maturation (25-Maturity). Accumulation rates were determined as described in Chapter 3. During the tissue proliferation phase, grain fresh matter accumulation was faster than that of dry matter. The dry weight accumulation from 0 to 8 DAA was approximately 23% of the fresh weight for Chinese Spring, CDC Teal and AC Andrew (Table 4.2; Fig. 4.2). In the rapid grain filling phase, from 8-16 DAA, the rate of dry weight accumulation was 30-36% of fresh weight accumulation in all the three cultivars. However, from 16 to 25 DAA, the rate of dry matter accumulation increased by over 60% of its fresh weight accumulation rate for Chinese Spring and over 75% for CDC Teal and AC Andrew (Table 4.2). No fresh matter accumulation was apparent after 30 DAA in Chinese Spring and after 25 DAA in both AC Andrew and CDC Teal. The rate of dry matter accumulation in CDC Teal exhibited substantial decrease (over 7-fold) following 25 DAA while only a slight decrease (1.9- to 2.1-fold) was observed in the other two cultivars between 25 to 30 DAA. After 30 DAA, the rate of dry matter accumulation decreased

considerably in Chinese Spring while AC Andrew and CDC Teal still showed some accumulation although at a much decreased rate (Table 4.2).

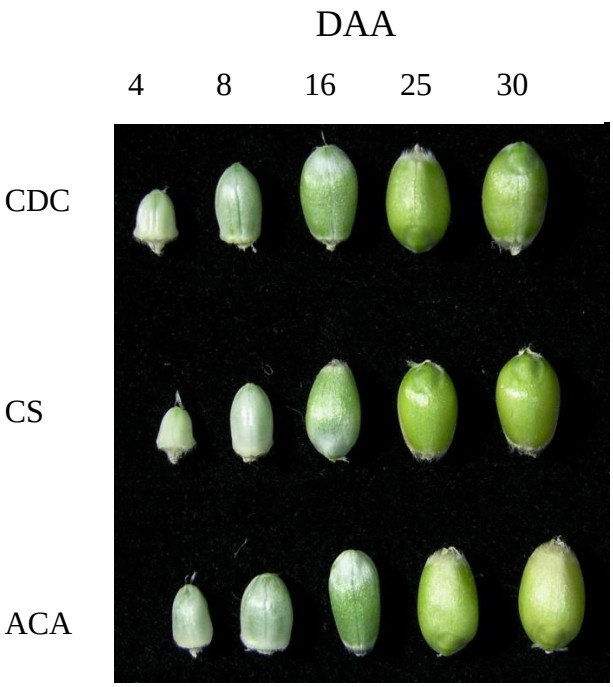


Figure 4.1: Developing grains of CDC Teal (CDC), Chinese Spring (CS) and AC Andrew (ACA) at 4, 8, 16, 25 and 30 DAA.

Table 4.2: Fresh and dry weight accumulation rates of developing grains of wheat[a]

Fresh weight accumulation rate (mg grain^{-1} day^{-1})						
	DAA					
	0-4	4-8	8-16	16-25	25-30	30-70*
Chinese Spring	2.07	3.22	2.28	2.97	1.06	-0.87
CDC Teal	2.24	2.54	4.30	2.28	-1.22	-0.60
AC Andrew	3.45	3.23	4.45	2.48	-0.42	-0.63
Dry weight accumulation rate (mg grain^{-1} day^{-1})						
	DAA					
	0-8	8-16	16-25	25-30	30-70*	
Chinese Spring	0.63	0.84	1.89	1.02	-0.02	
CDC Teal	0.54	1.30	1.75	0.24	0.22	
AC Andrew	0.80	1.51	1.94	0.91	0.28	

*70 DAA is chosen as harvest maturity. [a] cultivars were grown under greenhouse conditions as described in the materials and methods. Data are means (n=3).

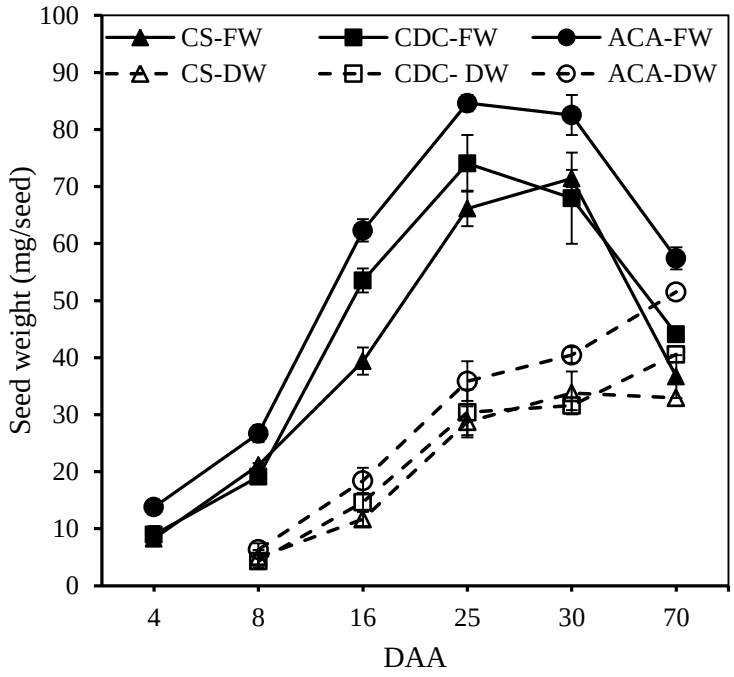


Figure 4.2: Fresh (FW) and dry weight (DW) of developing grains of Chinese Spring (CS), CDC Teal (CDC) and AC Andrew (ACA) at 4, 8, 16, 25, 30 and 70 (mature) DAA. Values are means ± SE, n= 3.

4.4.3 Accumulation of total starch and its components

Starch accumulation was measured from 0-30 DAA and at harvest maturity (70 DAA) (Table 4.3). Although starch deposition is initiated prior to 8 DAA, there is increased accumulation rate closer to the rapid grain filling phase. From our data, the starch content was highest in Chinese Spring at 8 DAA followed by CDC Teal and AC Andrew. From 8-16 DAA, the starch accumulation rate (SAR) was higher in AC Andrew on both per grain and per gram dry weight basis than the other two cultivars (Table 4.3). From 16-25 DAA the SAR per grain was highest for all the cultivars as compared to the other developmental stages; however SAR per gram dry weight was lower than that observed in the preceding phase (8-16 DAA). During the same period, the SAR per grain was comparable between AC Andrew and CDC Teal while that of

Chinese Spring was the lowest. The SAR per gram dry weight was highest in CDC Teal when the starch content increased three-fold. During 25-30 DAA, SAR per grain and per gram dry weight in CDC Teal was negligible but a slight increase was evident in the other two cultivars. No starch accumulation per gram dry weight was observed in CDC Teal during this period. From 30 DAA till harvest maturity, there was no starch accumulation in Chinese Spring, whereas AC Andrew showed slight increase per grain. CDC Teal showed slight increase in SAR during this phase both per grain as well as per gram dry weight.

Table 4.3: Starch accumulation rate in developing grains of three cultivars of wheat Chinese Spring (CS), AC Andrew (ACA) and CDC Teal (CDC)[a]

	DAA				
	0-8	8-16	16-25	25-30	30-70
	Starch accumulation rate (mg•grain-1•day-1)				
CS	0.14	0.46	1.22	0.64	-0.05
CDC	0.11	0.64	1.43	0.05	0.19
ACA	0.12	0.78	1.44	0.66	0.14
	Starch accumulation rate (mg•gDW-1•day-1)				
CS	28.18	22.61	15.69	2.08	-1.23
CDC	26.42	24.54	23.66	-2.29	0.42
ACA	17.79	30.96	19.08	4.46	-0.41

*70 DAA is chosen as harvest maturity. [a] cultivars were grown under greenhouse conditions as described in the materials and methods.

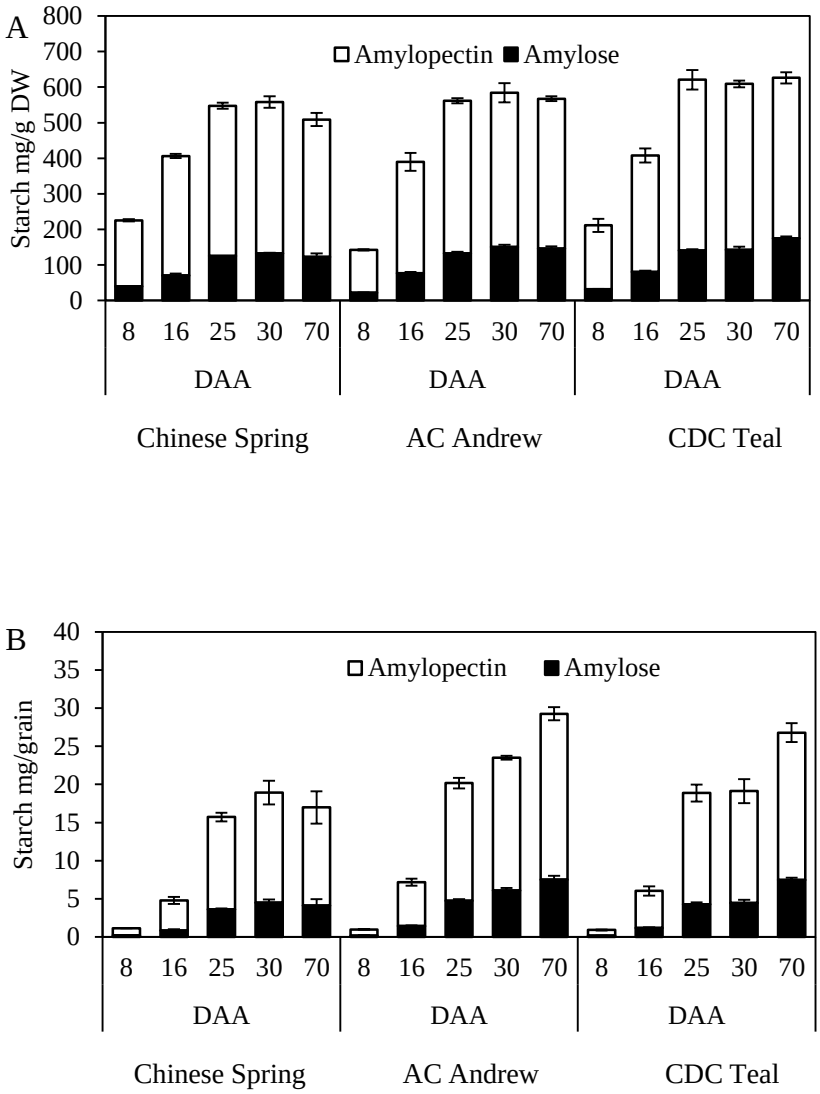


Figure 4.3: Total starch with amylose and amylopectin content (A) per gram dry weight (DW) and (B) per grain in Chinese Spring, AC Andrew, and CDC Teal at 8, 16, 25, 30 and 70 DAA (mature) grains. Plants were grown under greenhouse conditions as described in materials and methodos. Data are means ±SE, n=3.

4.4.4 Comparative analysis of the expression patterns of starch biosynthetic genes

Expression of AGPase genes

The transcripts of genes encoding the large (*AGPL1, AGPL2*) and small (*AGPS1a, AGPS1b* and *AGPS2*) subunits of AGPase were detected in the developing grains, but each member within each group showed differential expression patterns (Fig. 4.4 and 4.5). With respect to the *AGPL* genes, the transcript abundance of *AGPL1*, which encodes the cytosolic isoform of the large subunit of AGPase, was substantially higher than that of *AGPL2*, which encodes the plastidial isoform of the large subunit of AGPase, throughout the entire grain filling stages studied (Fig. 4.4). *AGPL1* showed higher expression (6-fold) at the early stage of grain development (at 4 DAA) in AC Andrew, than the other two cultivars (Fig. 4.4 A). From 4 to 8 DAA, the expression of *AGPL1* increased in all the three cultivars, Chinese Spring (4-fold), CDC Teal (8.5-fold) and in AC Andrew (2.5-fold) (Fig. 4.4 A). From 8 to 16 DAA, its expression either showed a decrease or was maintained at the level observed at 8 DAA. However, during this rapid grain filling period, cultivar AC Andrew maintained the highest expression *AGPL1* as compared to the other two cultivars (Fig. 4.4 A). The expression of *AGPL1* in the developing grains of Chinese Spring and CDC Teal showed an increase (2 to 3-fold) from 16 to 25 DAA, when it attained a level similar to that observed in AC Andrew (Fig. 4.4 A). After 25 DAA, the expression of *AGPL1* showed a decrease in all the three cultivars. The other *AGPase* gene, *AGPL2* showed higher expression in both AC Andrew and CDC Teal at 4 DAA as compared to that observed in Chinese Spring, after which its expression showed a continuous decrease in both the cultivars (Fig. 4.4 B). In Chinese Spring, the expression of *AGPL2* showed an increase from 4 DAA to 8 DAA (3-fold), however, it declined to a very low level from 8 to 16 DAA (11-fold) and remained at a similar level thereafter (Fig. 4.4 B).

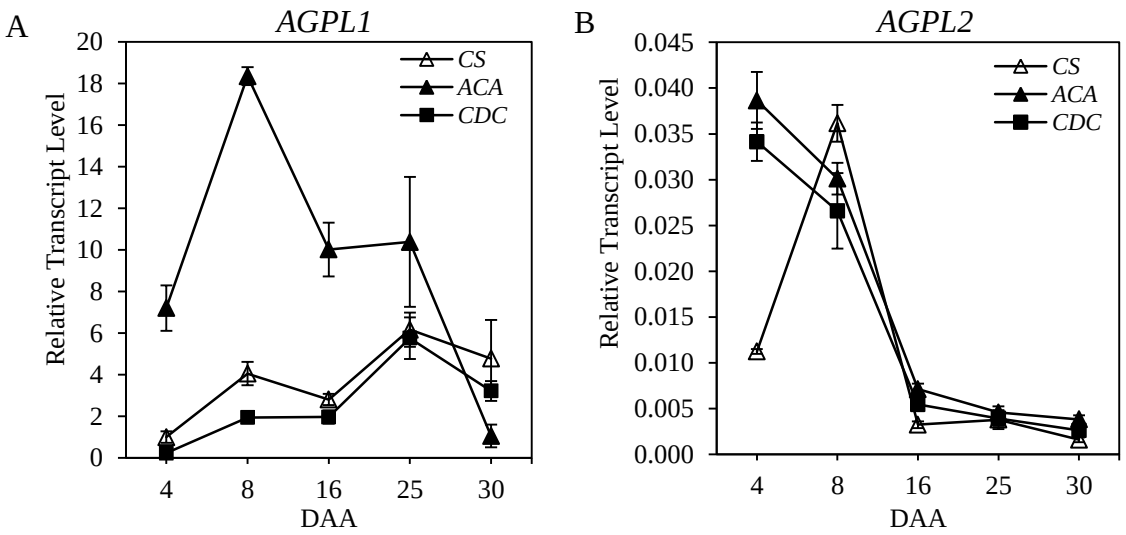


Figure 4.4: Expression of AGPase large subunit genes (A) *AGPL1* and (B) *AGPL2* in Chinese Spring (CS), CDC Teal (CDC) and AC Andrew (ACA) at 4, 8, 16, 25, 30 DAA of grain development. Transcript levels were expressed relative to that of Chinese Spring *AGPL1* in 4 DAA sample, which was set to a value of 1. Plants were grown under greenhouse conditions described in the materials and methods. Data are means ± SE where n=3.

The transcripts of *AGPS1a* that encode the cytosolic small subunit of AGPase were detected at a very low level during the early stages of grain development in all the three cultivars (Fig. 4.5 A). However, a very drastic increase in the transcript abundance of this gene was observed from 8 to 16 DAA in AC Andrew, producing a significantly higher transcript abundance (24 to 35 fold; $P \leq 0.05$) than that found in the other two cultivars. Although the expression of *AGPS1a* showed an increase (9-fold) from 16 to 30 DAA in CDC Teal, no significant difference was apparent when compared with that found in the 30 DAA grains of the other two cultivars. The expression of *AGPS1b*, encoding the plastidial isoform of AGPase small subunit, was expressed at a lower level when compared with the other two family members, *AGPS1a* and *AGPS2*. Its expression was higher during the early stages of grain development in both Chinese Spring and AC Andrew but decreased substantially afterwards (Fig. 4.5 B). In CDC Teal, the transcript abundance of

AGPS1b was maintained at a low level throughout the entire grain development. A very similar expression pattern of *AGPS2* was exhibited by the three cultivars; higher expression at early stage (by 4 DAA) and a continuous decline during the remaining periods of grain development (Fig. 4.5 C). The expression of *AGPS2* at the early stage of grain development was substantially higher in AC Andrew followed by Chinese Spring and then by CDC Teal (Fig. 4.5 C).

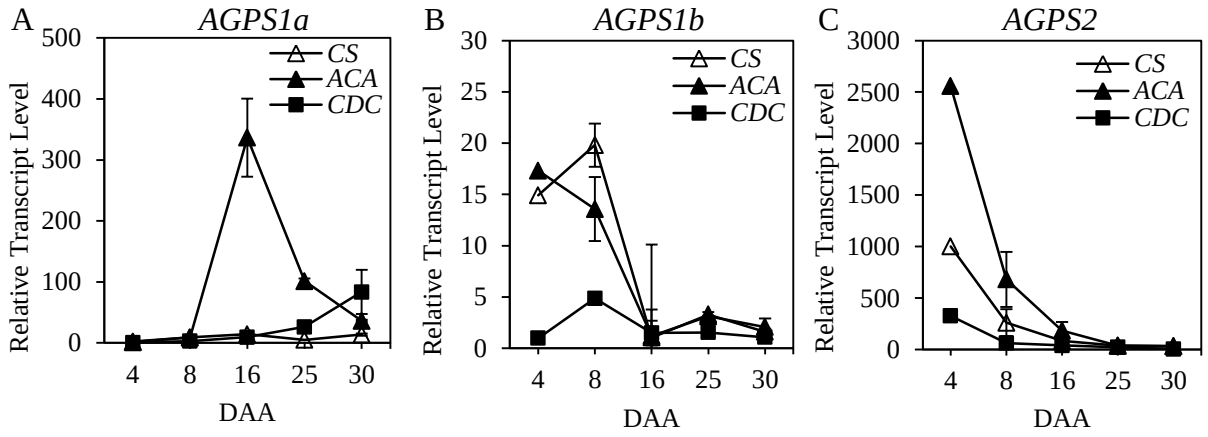


Figure 4.5: Expression of AGPase small subunit genes (A) *AGPS1a* (B) *AGPS1b* and (C) *AGPS2* in Chinese Spring (CS), CDC Teal (CDC) and AC Andrew (ACA) at 4, 8, 16, 25, 30 DAA of grain development. Transcript levels were expressed relative to that of Chinese Spring *AGPS1a* in 4 DAA sample, which was set to a value of 1. Plants were grown under greenhouse conditions described in the materials and methods. Data are means ± SE where n=3.

Expression of starch synthase genes

The expression of genes encoding both types of starch synthase enzymes- granule bound and soluble was analyzed. With respect to family members of the granule bound starch synthase gene, *GBSSI* had a higher expression during grain development in the three cultivars, when compared to that of *GBSSII* (Fig. 4.6). During the early and rapid grain filling stages of grain development (4 to 16 DAA), AC Andrew exhibited a significantly higher level of *GBSSI* transcripts ($p \leq 0.05$) than the other two cultivars, which showed similar level of its expression.

No difference in the expression of this gene was apparent afterwards, except that Chinese Spring exhibited higher transcript abundance than AC Andrew and CDC Teal at 30 DAA. *GBSSII* showed similar patterns of expression across the three cultivars, higher expression at 4 DAA and lower expression afterwards (Fig. 4.6). AC Andrew and CDC Teal appear to have higher expression of this gene as compared to that of Chinese Spring.

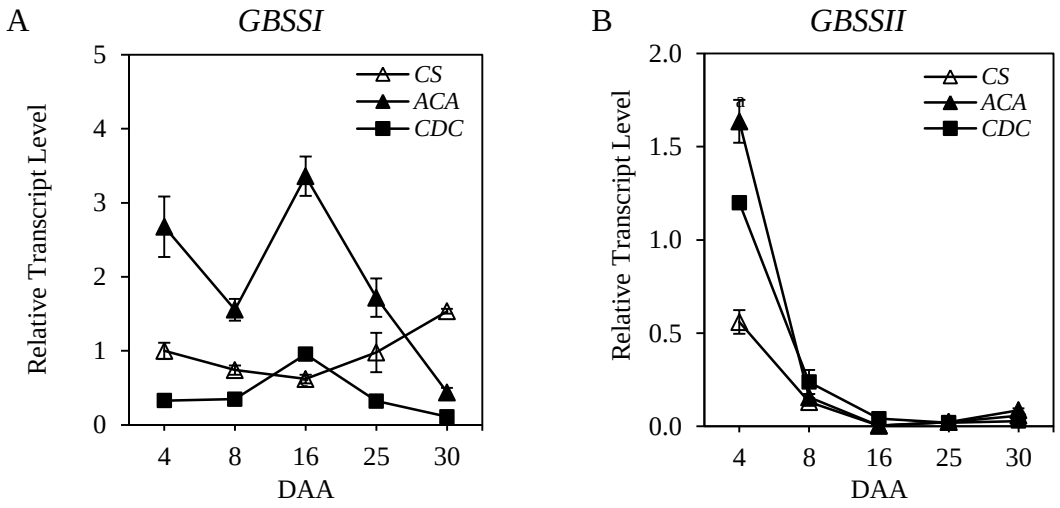


Figure 4.6: Expression of granule bound starch synthase genes (A) *GBSSI* (B) *GBSSII* in Chinese Spring (CS), CDC Teal (CDC) and AC Andrew (ACA) at 4, 8, 16, 25, 30 DAA of grain development. Transcript levels were expressed relative to that of Chinese Spring *GBSSI* in 4 DAA sample, which was set to a value of 1. Plants were grown under greenhouse conditions described in the materials and methods. Data are means ± SE where n=3.

Genes encoding soluble starch synthase include *SSI*, *SSIIa*, *SSIIIa*, *SSIIb* and *SSIIIb*. The abundance of *SSI*, *SSIIa* and *SSIIIa* transcripts were much higher than that of *SSIIb* and *SSIIIb* in the developing grains of all the three cultivars studied (Fig. 4.7). Nevertheless, the transcripts of all the genes showed higher abundance in all cultivars at 8 and/or 16 DAA, except *SSIIb*, which was expressed at a low level in CDC Teal during the same period (Fig. 4.7 C). By 16 DAA, the expression of all the five soluble starch synthase genes was either maintained at the same level or

exhibited a decrease except *SSIIIa* in ACA which reached its peak at 16 DAA. After 16 DAA, the transcript abundance of all genes exhibited a decrease except that of *SSIIIb* which showed slight increases in Chinese Spring and AC Andrew. When compared across all the cultivars, *SSI*, *SSIIb* and *SSIIIa* showed significantly higher expression ($p \leq 0.05$) at 4, 8 and 16 DAA, respectively, in AC Andrew than that found in Chinese Spring and CDC Teal. High expressions of *SSIIa*, and *SSIIIb* were also apparent in AC Andrew at 4 and 8 DAA, respectively, although similar levels of expression of these genes were found in Chinese Spring and/or CDC Teal. *SSIIb* and *SSIIIb* genes exhibited significantly higher expression in Chinese Spring at 4 DAA than that found in the corresponding grains of CDC Teal and AC Andrew.

Expression of starch branching genes

Expressions of *SBEI* showed almost similar patterns and levels with no significant difference across cultivars (Fig. 4.8 A). Increase in the expression of *SBEI* was evident through 16 (Chinese Spring) and 25 (AC Andrew and CDC Teal) DAA and before showing a decrease afterwards. *SBEIIa* was expressed at significantly higher levels ($p \leq 0.05$) at all stages of grain development in AC Andrew (2 to 23-fold) than that observed in CDC Teal and Chinese Spring. Its expression in AC Andrew was high at 4 DAA followed by a continuous decline afterwards. The transcript level of *SBEIIa* was very low in both CDC Teal and Chinese Spring throughout the entire grain developmental stages included in this study. In contrast, *SBEIIb* had lower expression in AC Andrew than in CDC Teal or Chinese Spring during grain development (Fig. 4.8 C).

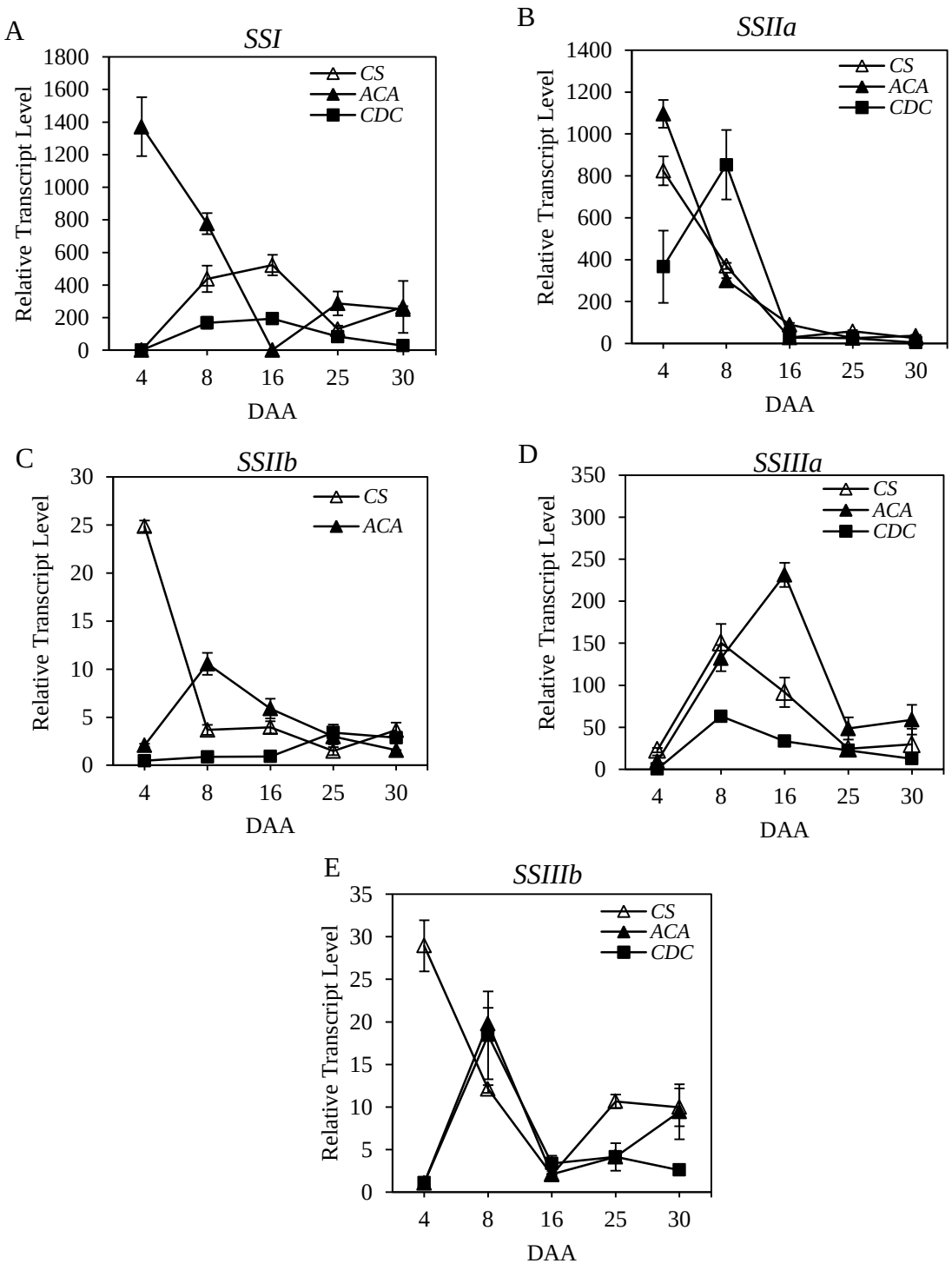


Figure 4.7: Expression of soluble starch synthase genes (A) *SSI* (B) *SSIIa* (C) *SSIIb* (D) *SSIIIa* and (E) *SSIIIb* in Chinese Spring (CS), CDC Teal (CDC) and AC Andrew (ACA) at 4, 8, 16, 25, 30 DAA of grain development. Transcript levels were expressed relative to that of Chinese Spring *SSI* in 4 DAA sample, which was set to a value of 1. Plants were grown under greenhouse conditions described in the materials and methods. Data are means ± SE where n=3.

4.4.5 Activities of starch biosynthetic enzymes

The total activities of AGPase, soluble and granule bound starch synthases were examined in the developing grains of Chinese Spring and AC Andrew as these two cultivars showed the biggest difference between their starch per grain content ($p \leq 0.05$) and also in the expression levels of genes that appear to be predominant in starch synthesis in wheat grains.

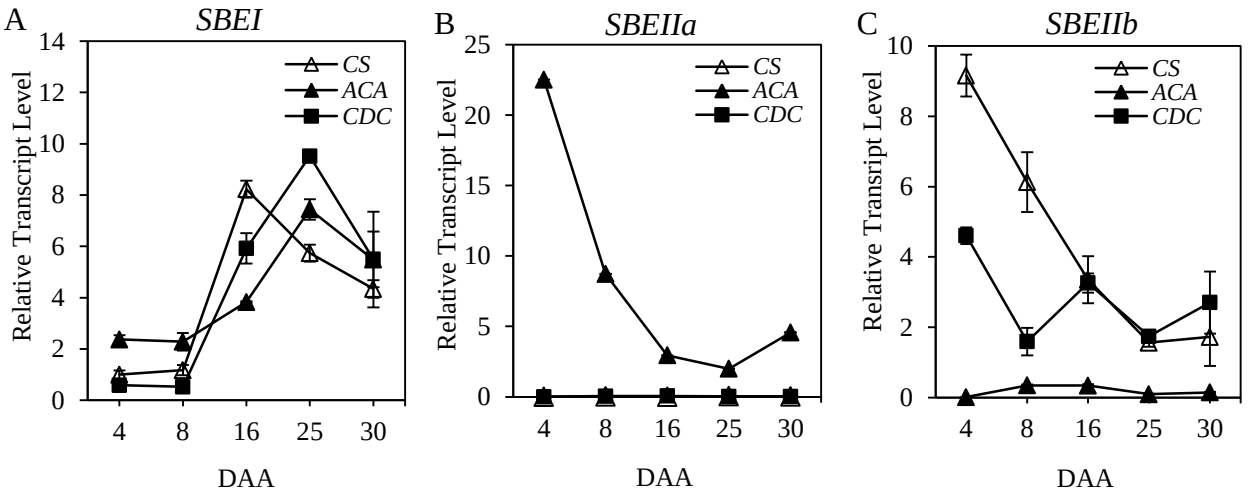


Figure 4.8: Expression of genes involved in starch branching (A) *SBEI* (B) *SBEIIa* and (C) *SBEIIb* in Chinese Spring (CS), CDC Teal (CDC) and AC Andrew (ACA) at 4, 8, 16, 25, 30 DAA of grain development. Transcript levels were expressed relative to that of Chinese Spring *SBEI* in 4 DAA sample, which was set to a value of 1. Plants were grown under greenhouse conditions described in the materials and methods. Data are means ± SE where n=3.

Figure 4.9 shows the mean enzyme activity per grain in the two cultivars. Only very low activity of AGPase was detected at 4 DAA in both cultivars, showing a continuous increase thereafter (Fig. 4.9). However, the AGPase activity in AC Andrew was 1.3 to 1.5-fold higher than that of Chinese Spring at 16 and 25 DAA. Soluble starch synthase showed similar activity in both cultivars during the earlier stages of grain development, showing an increase from 4 DAA, when substantial activity was detected, to 8 DAA, but declined to a very low level by 16 DAA. Although its activity increased from 16 to 25 DAA in both cultivars, AC Andrew contained 4.5-

fold more activity ($p \leq 0.05$) than that found in the corresponding grains of Chinese Spring. The activity of granule bound starch synthase showed similar patterns and levels of increase during the entire period of grain development considered in this study (from 4 to 25 DAA) in both cultivars (Fig. 4.9).

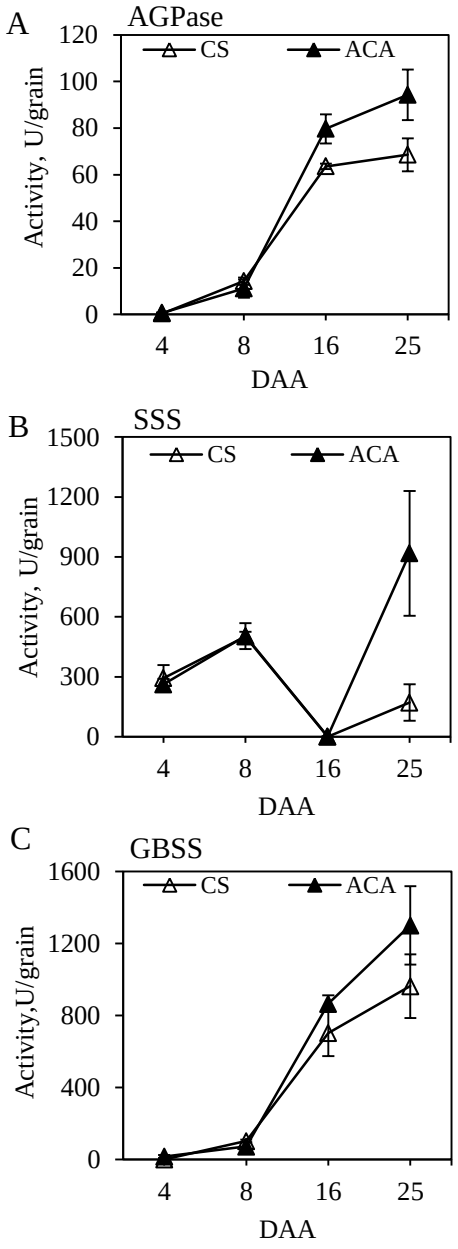


Figure 4.9: Mean activity of the enzymes (A) AGPase, (B) soluble and (C) granule bound starch synthases, per grain at 4, 8, 16, 25 DAA during grain filling in Chinese Spring (CS) and AC Andrew (ACA). Plants were grown under greenhouse conditions as described in materials and methods. Data are means ± SE, n=2-3.

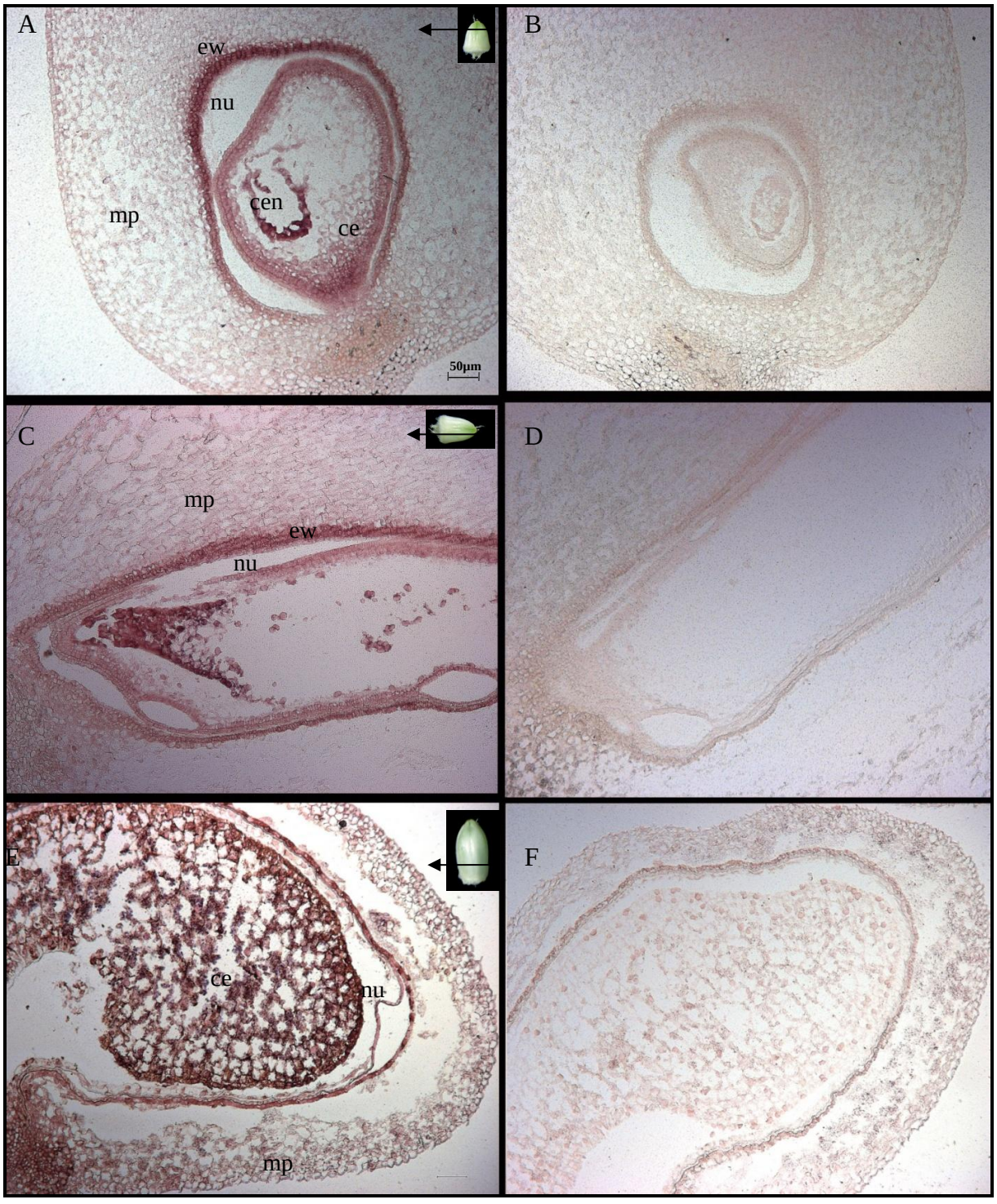


Figure 4.10: Cellular localization of *SSI* mRNAs in developing wheat grains at 4 (A, B, C, D) and 8 (E, F) DAA. The transcripts are mainly localized (stained red) in the inner walls of the maternal pericarp (mp) embryo sac wall (ew), ceonocytic endosperm nuclei (cen), and in some cells of the cellularised endosperm (ce) at 4 DAA, and mostly in the ce at 8 DAA. A, C and E show the samples treated with antisense, and B, D and F with sense riboprobes, all at 5X magnifications. Both transverse and longitudinal sections of the grain were studied at 4 DAA (see inset, A and C) and only transverse sections at 8 DAA (see inset E).

4.4.6 Cellular localization of wheat SSI

Cellular localization of the *SSI* transcripts was investigated in developing grains at 4 and 8 DAA by in situ hybridization. As shown in Figure 4.10 A and C, the transcripts of *SSI* appeared to be strongly expressed (red brown staining) in the inner walls of the maternal pericarp, embryo sac wall, ceonocytic endosperm nuclei, and in some cells of the newly formed cellularised endosperm at 4 DAA (Fig. 4.10 A, C). By 8 DAA, the expression of *SSI* was mainly localized in the cellularised endosperm (Fig. 4.10 E).

4.4 Discussion

This study investigated if natural genetic variation affects starch biosynthesis during grain development. To this end, the study involved the use of three wheat cultivars that are different in terms of grain size and starch content, namely Chinese Spring, AC Andrew and CDC Teal. Comparison of the three cultivars revealed that AC Andrew has higher thousand kernel weight (TKW) than the other two cultivars (Table 4.1), indicating that it has larger grains. Between the other two cultivars, CDC Teal exhibited higher TKW than Chinese Spring. However, when other yield parameters are considered, Chinese Spring showed the highest number of spikes followed by CDC Teal, and also highest grains per plant followed by AC Andrew. These results indicate that Chinese Spring produces more grains/plant, although the grains are smaller in size when compared to the other two cultivars.

With respect to starch content per grain, AC Andrew has the highest starch per grain followed by CDC Teal and then by Chinese Spring (Fig. 4.3 B). As starch constitutes 50-70% of total grain weight, this result is consistent with the TKW data. It has generally been accepted that the rate of grain filling in wheat is largely dependent on the sink strength, which is determined

partly by sink size. The size of the sink/grain is partly dependent on the duration of tissue proliferation and cell expansion of the endosperm (Herzog, 1986).

Comparison of the expression patterns of starch biosynthesis genes across the three cultivars provided some interesting insights. The study in Chapter 3 showed that *AGPL1* and *AGPS1a* are the predominant genes responsible for the activity of AGPase in the endosperm of developing wheat grains. The transcript abundance of *AGPL1* showed higher expression (2 to 4.5-fold) at the earlier and towards the mid-stage of grain development (4-16 DAA), in AC Andrew, than the other two cultivars (Fig. 4.4 A). These two phases correspond to the cell proliferation and expansion, and rapid grain filling of the grain. Furthermore, the developing grains of AC Andrew exhibited substantially higher expression of *AGPL2* and *AGPS2* at 4 and 8 DAA, respectively. It has been reported previously that AGPase catalyzes the rate limiting step of starch biosynthesis (Emes *et al*., 2003). AGPase consists of two sub units, the large and small sub units, and the availability of both sub units is required for efficient activity and functionality of the enzyme (Cross, 2004). Our data, thus, suggests the significance of the coordinated expression of these genes during the cell proliferation and expansion, and also in the rapid grain filling phases in determining starch accumulation and grain weight in wheat. In agreement with this, higher total activity of AGPase was observed in developing grains (16-25 DAA) of AC Andrew than that observed in Chinese Spring (Fig. 4.9 A). Given that the two genes *AGPL2* and *AGPS2* encode plastidial isoform of AGPase (Olive *et al*., 1989; Burton *et al*., 2002; Kang *et al*., 2010), the result supports that starch deposition in the endosperm is influenced by the synthesis of transient starch in the pericarp at the initial stage of wheat caryopsis development. This is because the transitory starch would eventually be hydrolysed and translocated to the endosperm

(Duffus and Rosie, 1973; Sato, 1984), where it is used as a substrate to initiate starch synthesis (Chevalier and Lingle, 1983; Radchuk *et al*., 2009).

Starch synthases are responsible for the elongation of the glucan chains to give rise to complex starch structure (Emes *et al*., 2003). Previous studies in barley have shown that soluble starch synthase genes are expressed both in the endosperm and pericarp tissues (Radchuk *et al*. 2009), suggesting the occurrence of starch synthesis in both endospermic and pericarp tissues of a developing grain. This is consistent with the expression pattern of the wheat genes encoding the plastdial isoform of AGPase shown by this study. When compared across all the cultivars, specific soluble starch synthase genes including *SSI* and *SSIIIa* exhibited significantly higher expression in the developing grains of AC Andrew at 4 and 16 DAA (cell proliferation and expansion, and rapid grain filling phases), respectively, than that found in the corresponding grains of Chinese Spring and CDC Teal. In agreement with this, a study in rice suggested that the coordinated actions of SSI and SSIIIa is a key factor responsible for the majority of starch synthesized in rice endosperm (Fujita *et al*., 2011). The transcripts of *SSI* were localized in the inner layers of the maternal pericarp at 4 DAA and predominantly in the endosperm at 8 DAA (Fig. 4.10 A, C, D). Similarly, *SSI* has been found to be expressed in the pericarp as well as in the endosperm of developing barley grains (Radchuk *et al*., 2009). Although primarily responsible for synthesizing short chain glucans, SSI is the only class of soluble starch synthase that lacks isoforms and accounts for nearly 70% of the total soluble starch synthase activity (Fujita *et al*., 2006). These results, therefore, suggest transcriptional activation of *SSI* and *SSIIIa* might play an important role in regulating starch formation and accumulation in both the pericarp and endosperm of developing wheat grains. Previous studies in higher plants, including wheat, have suggested the interaction of SSI and SSIIa proteins with other starch synthesis enzymes

such as SBEIIa/SBEIIb, and this interaction has been considered as a regulatory factor of starch formation (Tetlow *et al*. 2004; Tetlow *et al*. 2008). In support of this, the loss of *SSIIa* in wheat resulted in enhanced amylose but an altered amylopectin structure with increased shorter chains at the cost of chains of intermediate length (Yamamori *et al*., 2000). Furthermore, loss of function of the same gene in barley abolished the binding of SSI, SBEIIa and SBEIIb to the starch granules, causing reduction in the synthesis of amylopectin (Morell *et al*., 2003). Another study in barley has also suggested the association of SSI, SSII and SBEIIa of amyloplast via phosphorylation as a mechanism for the assembly of the SS and SBE proteins in order to produce starch (Alexander and Morris, 2006). Wheat *SBEIIa* is believed to play a more important role than *SBEIIb* (Regina *et al*., 2005, 2010; Tetlow *et al*., 2008). In agreement with this, *SBEIIa* was expressed at a higher level at all stages of grain development in AC Andrew (Fig. 4.8 B) than that observed in CDC Teal and Chinese Spring. Although similar activity of soluble starch synthase was observed in both AC Andrew and Chinese Spring during the cell proliferation and expansion phase, significant difference between the two cultivars was evident towards the end of the rapid grain filling phases (Fig. 4.9 B). These results suggest a possible involvement of the combination of SBEIIa with other enzymes through forming a complex in regulating starch biosynthesis. Further investigation at the protein levels and analysis of the activities of branching enzymes in these cultivars might contribute to our understanding of the molecular mechanism of starch biosynthesis during grain filling.

Apart from *SSI* and *SSIIIa* genes, our analysis revealed the presence of high expressions of other starch synthase genes including *SSIIa*, and *SSIIIb* in the developing grains of AC Andrew at 4 and 8 DAA, respectively, although this was not statistically different from the expression of these genes found in Chinese Spring and CDC Teal. This might suggest the potential

contribution of these genes to the total activity of starch synthase during the early to mid-stages of starch synthesis. Although the starch synthase gene, *SSIIb*, exhibited significantly higher expression in Chinese Spring at 4 DAA ($p \leq 0.05$), the basal expression level of the genes in developing wheat grains is very low and this might suggest its minimal role.

AC Andrew also exhibited a higher level of *GBSSI* transcripts, during the early to rapid grain filling stages of grain development (4 to 16 DAA), than the other two cultivars, which showed similar levels of expression with each other (Fig. 4.7 A). Given that *GBSSI* is the predominant gene for amylose synthesis in developing grains, the variation in *GBSSI* expression might have a contribution to the differences in grain starch content or TKW observed among the three cultivars. However, the absence of difference in GBSS activity between AC Andrew and Chinese Spring (Fig. 4.9 C) suggests the possible role of post transcriptional regulation of *GBSSI*. Such a regulation has been studied previously in rice involving alternative splicing of pre mRNA (Wang *et al*., 1995; Isshiki *et al*., 2006; Zeng *et al*., 2007). The basal expression of *GBSSII*, which has been shown to be responsible for amylose synthesis in the pericarp of wheat (Vrinten and Nakamura 2000), was found to be very low in all cultivars, suggesting its minimal role in starch synthesis during grain development in wheat.

The starch biosynthesis pathway is a complex pathway involving multiple gene families. In this study we compared the expressions of the genes involved in this pathway during the grain development across cultivars, and AC Andrew showed significantly higher expression of *AGPL1, AGPS1a, SSI, SSIIIa, GBSSI* and *SBEIIa* as compared to the other two cultivars. At the very onset of starch synthesis, AGPase activity contributes towards the production of ADP-glucose, this in turn is acted upon by starch synthases and branching enzymes in order to form starch at the end. *AGPL1, AGPS1a, SSI, SSIIIa* and *SBEIIa* show transcriptional differences

although our combined data of *GBSSI* suggests its post transcriptional regulation in the cultivars studied. The differential transcription of the starch biosynthesis genes could possibly contribute towards the difference in starch content per grain observed across the cultivars. The differences in gene expression in the case of AGPase and SSS were further correlated with differences in enzyme activity. As the cultivars have a different genetic background, determination of the specific roles of these genes with respect to grain development, requires detailed assessment of their nucleotide diversity. Previous studies have shown that the presence of reduced nucleotide diversity of genes involved in the starch pathway can be used as a target for artificial selection in maize (Whitt *et al*., 2002); however, whether or not this could have any regulatory implications remains to be studied. Although this study provides insights into some of the genetic components involved in regulating starch yield in wheat, grain filling has been reported to be of complex trait (Mashiringwani *et al*., 1994), indicating the importance of investigating the genetics of starch yield in wheat in the context of different environmental factors. As this study was limited to gene transcription, broader insights into the starch biosynthesis in wheat grains can be obtained by extending the study to protein levels and investigating other regulatory mechanisms.

5. The effect of constitutive expression of mutated maize AGPase (*Sh2r6hs*) on starch accumulation in wheat grains

5.1 Abstract

The strength of a developing grain as a sink organ contributes to the control of starch yield in cereals. It is determined by grain size and the rate of metabolic activities including that of starch synthesis taking place in the grain. It is well established that the enzyme ADP-glucose pyrophosphorylase (AGPase) catalyzes a rate limiting step in starch biosynthesis. The plant AGPase is subjected to redox and allosteric regulations, and this affects its enzymatic activity. In this study a modified form of the maize AGPase large subunit, *Shrunken-2* (*Sh2r6hs*), which is able to form a heterotetramer with decreased sensitivity to negative allosteric regulation, was expressed in wheat under the control of a constitutive promoter. The grains derived from the positive T1 generation transformants expressing the transgene showed increased AGPase activity as compared to the transformed negative lines and the wild type control plants. This increase in AGPase activity was associated with increased grain weight and starch content per grain in selected positive lines. Expression of *Sh2r6hs* also increased the expression of genes involved in sucrose metabolism (*SuSy2*) and other starch biosynthetic genes (*AGPS1a*, *SSIIa* and *SBEIIa*).

5.2 Introduction

Wheat is one of the most important cereal crops worldwide. Over 50% of the total weight of a wheat grain is composed of starch, thus starch biosynthesis and accumulation in developing grains largely affect yield. Starch is synthesised from ADP-glucose, and the synthesis of this molecule from glucose1-phosphate and ATP is catalyzed by ADP-glucose pyrophosphorylase (AGPase). This process also involves the release of inorganic pyrophosphate (PPi). ADP-glucose is subsequently used by starch synthases and branching enzymes to elongate the glucan chains of the starch granule (Kolbe *et al*., 2005). AGPase naturally occurs in more than one form within a plant. For example, the AGPase found in sink tissue (such as endosperm of seeds) is different from the one found in the source tissues such as leaves. Bacterial AGPases like the one in *E. coli* are homotetramers encoded by the glgC gene (Preiss, 1984). In contrast, the plant and unicellular chlorophytic AGPases are heterotetramers composed of two small and two large subunits, each of which is encoded by distinct genes (Emes *et al*., 2003). In maize, AGPase large subunits are encoded by the *Shrunken-2* (Sh2) gene whereas the small subunits are encoded by the *Brittle2* (Bt2) gene (Hannah and Nelson, 1975). Previous studies indicated that the large subunits of AGPase have regulatory properties; whereas the smaller subunits are of catalytic importance. Studies by Cross *et al.* (2004) clearly showed that both subunits are important for catalysis as well as regulation. By using the two isoforms of AGPases derived from potato tuber and maize endosperms, the authors have shown that both of the subunits equally participate in controlling the allosteric properties of the enzyme (Cross, 2004). A close examination of the sequence of the small and large subunits of maize revealed a 43.2% identity in their amino acid sequence and that a gene duplication could have probably given rise to the two subunits in the course of plant evolution (Georgelis *et al*., 2007), although the small subunit shows more conservation across

plant species. Sequence comparisons of the large subunit have classified AGPases into the following groups: tuber/stem, leaf, fruit/root, endosperm AGPases (Cross, 2004). In rice, two isoforms of the small subunit were detected both in the seed as well as in the leaves. The two isoforms of the large AGPase subunits, on the other hand, were detected only in the seed and a third isoform exclusively in the leaves (Koller *et al*., 2002).

In the photosynthetic tissues, AGPase is generally restricted within the plastids and is subjected to allosteric regulation where it is activated by 3-Phosphoglyceric acid (3-PGA) and inhibited by inorganic phosphate (Pi) (Ghosh and Preiss, 1966). In addition, certain isoforms of AGPases have been found to be heat labile. Cytosolic endospermic AGPases are very sensitive to heat, whereas its plastidial isoforms are more stable under heat. The activity of maize AGPase, for instance, is destroyed by 96% within 5 min when heated to 57^{o}C while the enzyme from the potato tuber can still retain its activity at 70^{o}C (Hannah *et al*., 1980). The thermal stability of this enzyme is an important property as exposure to high temperatures during the growing phase cause reduction in grain yield in important cereal crops like wheat, maize and rice (Singletary *et al*., 1994). A cysteine residue at the N- terminus of potato tuber small subunit endows heat stability to the enzyme, and the removal of this cysteine residue has been shown to reduce its heat stability (Fu *et al*., 1998). In addition, incorporation of cysteine into the maize endospermic heat labile isoform increased its stability under heat (Linebarger *et al*., 2005). In another study, a mutation that led to substitution of the histidine (His) residue by tyrosine (Tyr) in the SH2 protein at the 333 position of maize resulted in stability of the enzyme at elevated temperature, and enhanced interaction with the wild type BT2 (Greene and Hannah, 1998b). It has also been shown in the same study that all the heat-labile cereal endospermic AGPases have His residues,

whereas the heat-stable potato AGPase has Tyr at the position where His is substituted by Tyr in the SH2 of maize.

AGPase is under strict redox regulation in leaves, where reduction during the light phase causes a thioredoxin-mediated reaction of a disulfide bond present between the two small subunits of the enzyme (Hendriks *et al*., 2003). The reduction of AGPase results in its increased activity triggered by higher activation by 3-PGA, as compared to the oxidized form present during the dark cycle. Since this enzyme is subject to redox and allosteric regulation, ADP-glucose production eventually leading to starch formation is tightly coordinated between the circadian cycles and carbon status of the source tissue. Due to this sensitivity to inhibitors and activators, AGPase is widely recognized as a critical enzyme catalyzing the rate-limiting step in starch synthesis. This has led to the hypothesis that increasing AGPase activity through genetic manipulation might lead to increased starch yield. To this end, an attempt has been made to increase the yield of starch in potatoes by genetically express the bacterial AGPase that is not sensitive to the allosteric regulation by 3-PGA and Pi (Stark *et al*., 1992). However, no consistent results were obtained when different varieties are used. In variety Russett Burbank, the genetic modification of AGPase produced a 30% increase in starch content while the same genetic modification in variety Prairie did not produce any change in starch yield, although it caused a four-fold increase in AGPase activity, as compared to the control. In cassava, the introduction of a mutated *E. coli* AGPase gene (*glgC*) under the control of a class I patatin promoter (Ihemere *et al*., 2006) resulted in up to 70% increase in AGPase activity, and plants with the highest AGPase activity showed a 2.6-fold increase in total tuberous root biomass, although no significant change in starch per gram fresh weight was evident. Transformation of rice with another variant of the *E.coli glgC* resulted in 13-fold higher AGPase activity and 11%

increase in yield (Sakulsingharoj *et al*., 2004). In maize, transformation with the *E. coli glgC-16* gene led to a 13-25% increase in the kernel weight (Wang *et al*., 2007). Furthermore, a 15% increase in starch content and higher grain weight were obtained through overexpression of the wild type *Sh2* and *Bt2* (Li *et al*., 2011). The results of these studies indicate a correlation between an increase in AGPase activity and a sink starch content and grain weight in cereals.

The effect of altered wheat AGPase on starch yield has also been investigated by transformation with the modified maize Sh2 gene (*Sh2r6hs*), exhibiting decreased sensitivity to inhibition by Pi (Meyer *et al*., 2004), using a seed specific HMW glutenin promoter. This modification resulted in increased total plant biomass and yield per plant with no significant increase in starch (Smidansky *et al*., 2002; Smidansky *et al*., 2003; Meyer *et al*., 2004; Smidansky *et al*., 2007). Another study has shown that overexpression of *E. coli* pyrophosphatase in mesophyll cells using cytosolic fructose 1,6- bisphosphatase promoter and simultaneous downregulation of AGPase in leaves through antisense expression increased the strengths of both sink and source tissues (Jonik *et al*., 2012). The expression of *Ubiquitin1* gene from maize has been established in different tissues of germinating seedlings, anther, ovary, palea, glume and lemma (Blechl and Jones, 2009), and the promoter has already been proved to drive high levels of constitutive expression (Cornejo *et al*., 1993). This has led us to hypothesize that over-expression of the *Sh2r6hs* gene (from maize) in wheat under the control of the *Ubiquitin1* promoter increases the source and sink strengths and thereby wheat starch and/or grain yield. The study here outlines the construction of the transformation vector and generation of the transformed plants, and initial characterization of the heterozygous plants at the T1 generation.

5.3 Materials and methods

5.3.1 Growth of donor and transformed plants

The spring wheat cv. Fielder was grown in the growth chamber at 16-22^{o}C /14-18^{o}C (day/night) in a 16/8 h photoperiod at a light intensity of 175 µEm^{-2}sec^{-1}. Seeds were germinated and were transplanted onto pots (one seed per one gallon pot) containing Sunshine Mix 4 and Cornell foliage mix (see materials and methods, Chapter 3). The plants were fertilized once in two weeks with one tablespoon of N-P-K (20:20:20) per gallon water from two week-old stage until flowering. Spikes were tagged as described in Chapter 3 and were harvested at 11-13 days after anthesis (DAA). Developing grains were harvested from the middle region of the spikes and used for the isolation of the immature embryos.

5.3.2 Construction of transformation vector

The pGSH2 plasmid construct was obtained from Dr. Mike Giroux lab at Montana State University, Bozeman, Montana, USA. It contained a mutated version of AGPase large subunit (*Sh2r6hs*) with two alterations to the wild type maize *Sh2*. The *Sh2r6hs* is composed of *Sh2* revertant allele, *rev6*, and a second mutation that causes substitution of a tyrosine residue for a histidine residue at position 333 (Greene and Hannah, 1998b; Meyer *et al*., 2004). The mutant *rev6* has additional tyrosine and serine residues inserted between amino acids 495 and 496 that ensure a decreased allosteric sensitivity to inorganic phosphate in those lines which express this gene (Giroux *et al*., 1996). The second mutation in which a tyrosine residue is substituted for that of a histidine residue at position 333 resulted in higher catalytic activity of *Sh2rev6* even at elevated temperature. The *Sh2r6hs* derived from the pGSH2 plasmid was subcloned into pBRACT 214 vector (obtained from John Innes Centre, Norwich, UK) under the control of a

maize *Ubiquitin1* promoter (Fig. 5.1) using Gateway cloning technology (Invitrogen, Carlsbad, CA, USA). This vector was used for the subsequent wheat transformations.

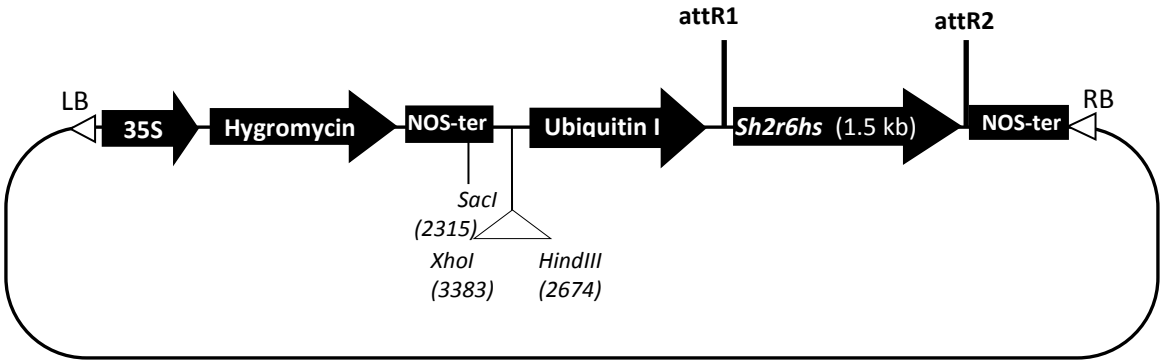


Figure 5.1: Schematic diagram of the pBRACT214 construct with *Sh2r6hs* driven by maize *Ubiquitin1* promoter. The plasmid consists of the *hpt* conferring resistance to the antibiotic hygromycin as a selection marker. Both gene cassettes contain the NOS terminator.

5.3.3 Isolation of immature embryos

The wheat grains collected from the middle regions of each spike were surface sterilized first with 70% (v/v) aqueous ethanol for 5 min and then with 10% commercial bleach for 20 min with gentle shaking in between. The grains were then rinsed with sterile water at least three times. Embryos were isolated in a sterile environment using a sharp sterile scalpel and a fine pair of sterile forceps. After isolation, the embryos were injured by an incision to facilitate infection by *Agrobacterium* and avoid precocious germination.

5.3.4 Preparation of Agrobacterium and inoculation of embryos

Agrobacterium liquid cultures were initiated by adding 500 μL glycerol inoculum of AGL1 strain containing the pBRACT vector. The culture was then allowed to grow until the optical density of 1 (at 600 nm) was reached. The cells were pelleted by centrifugation and resuspended in 4 mL inoculation medium on a shaker (at 200 rpm) for a maximum of 3 h. After adding 1%

Silwet (to a final concentration of 0.015%), the culture was poured into 100 mm X 15 mm plastic petri dishes (FisherScientific). The embryos were then placed on the inoculation medium (50 embryos per petri dish) with the scutella facing down. The embryos were co-cultivated with *Agrobacterium* at room temperature (22-23^{o}C) for 48 to 72 h in darkness.

5.3.5 Embryogenic callus formation, regeneration, selection and growth of T0 plants

After co-cultivation, all the embryos were transferred onto a fresh induction medium and then incubated at ~ 25^{o}C. After 18 days, they were transferred to the regeneration medium supplemented with zeatin (final concentration of 5 ml/L) and incubated at the same temperature and a 16/8 h (day/night) photoperiod cycle. After three weeks of growth in the regeneration media, the differentiated calli were plated on the selection medium supplemented with hygromycin (final concentration of 30 mg/L). The resulting plantlets were transferred to a fresh selection medium every three weeks or earlier if there was any overgrowth of *Agrobacterium*. After a total of six to nine weeks, the plants that survived the selection were transferred to pots containing a soil mix as described earlier (see material method section 5.3.1) to obtain T0 plants under the same conditions as the donor/control plants. A summary of the *Agrobacterium* mediated wheat transformation steps is shown in Figure 5.2.

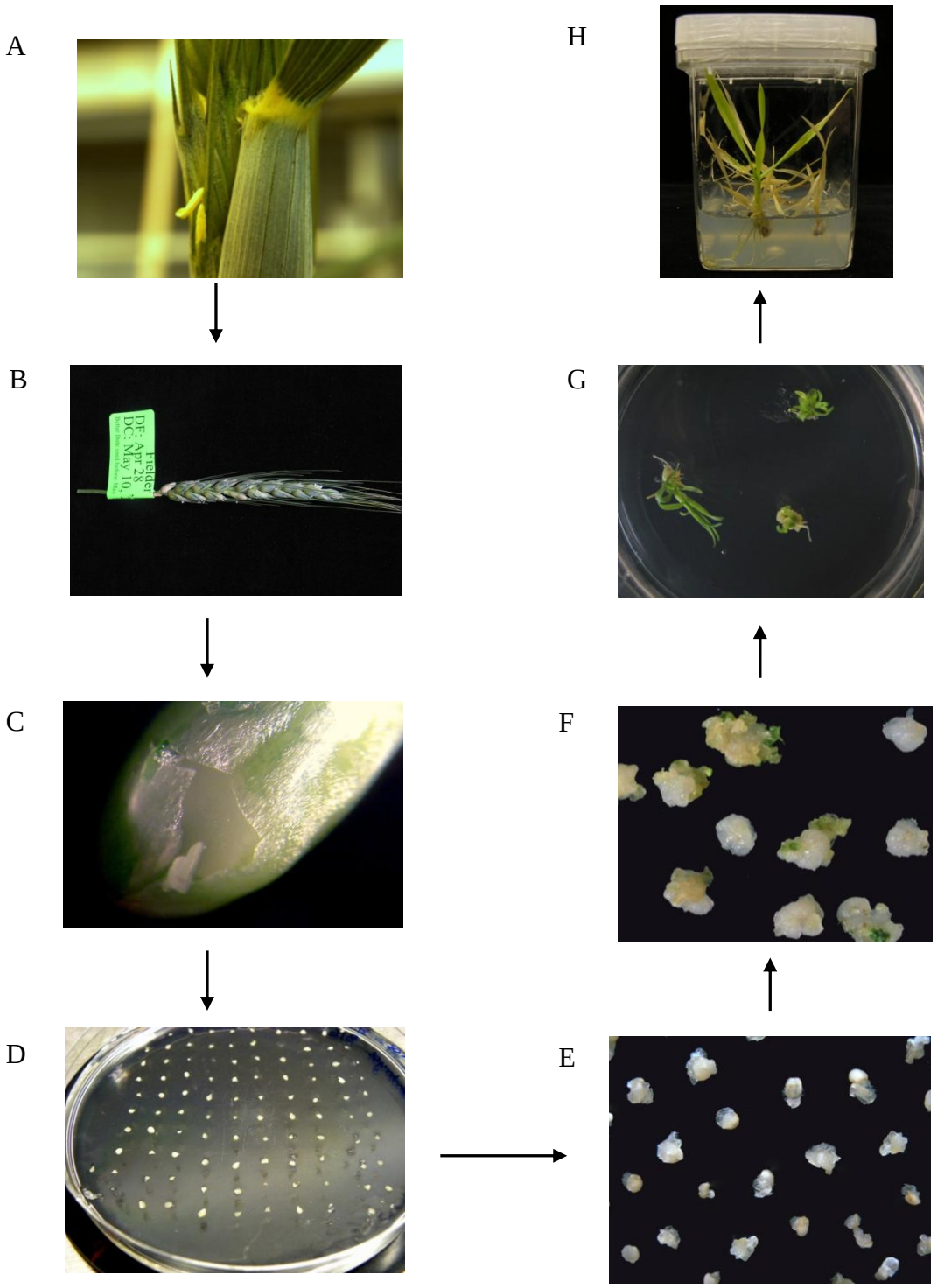


Figure 5.2: Summary of *Agrobacterium* mediated wheat transformation steps. (A) Tagging of the spikes at the first protrusion of the yellow anthers; (B) Harvesting of the spikes with developing seeds at 11 to13 DAA; (C) Isolation of the embryos; (D) Co-cultivation of the isolated embryos with *Agrobacterium*; (E) Callus formation; (F) and (G) Regeneration; and (H) Selection on a media containing hygromycin.

5.3.6 Confirmation of gene insertion in T0 plants

Leaves were harvested from the T0 plants and used for DNA extraction. The DNA was used to verify the presence of *Sh2r6hs* by PCR using the forward primer 5'-CCATTGACAACATTGTAATCTTGAGT-3' (from the 607th bp of the gene) and the reverse primer 5'-CAGCCTCTTGGATGCCCTTAC-3' (from the 1468th bp of the gene). Since the maize *Sh2r6hs* has ~70% homology with the wheat native *AGPL1* gene, amplification of the fragments unique to *Sh2r6hs* was performed with primers specific to *Sh2r6hs*.

5.3.7 RNA isolation and cDNA synthesis

Our previous data (see Chapter 3 and Chapter 4) have shown that significant changes in the expression of starch biosynthesis genes occur during transition from the tissue proliferation phase to rapid grain filling phase (8-16 DAA). Thus, grains were collected at 8 and 16 DAA and used for RNA isolation. The synthesis of cDNA from RNA samples was performed as described earlier (see material method of Chapter 3).

5.3.8. Semi quantitative-RT-PCR and quantitative real-time RT- PCR assays

In order to verify the expression of the *Sh2r6hs* gene, cDNA samples were used directly for semi quantitative RT-PCR using Taq DNA Polymerase with forward primer 5'-GCATTCTGTGATTGGAGTCTGC-3' and reverse primer 5'-CAGCCTCTTGGATGCCCTTAC-3'. The optimized PCR conditions for the semi quantitative RT-PCR were initial denaturation at 95^{o}C for 45s, followed by 33 to 38 cycles of denaturation at 95^{o}C for 30s, annealing at 60^{o}C for 30s, and extension for 30s. The PCR products were then run on a 1% Agarose gel containing 0.3μg/mL ethidium bromide, and the corresponding band intensities quantified using Quantity One software (BioRad, Hercules, CA, USA).

The effect of expressing *Sh2r6hs* on the transcript abundance of sucrose transporter, sucrose synthase and the other starch biosynthetic genes (*SUT1, SuSy2, AGPL1, AGPL2, AGPS1a, AGPS1b, AGPS2, GBSS1, SSI, SSIIa, SSIIIa, SBEI, SBEIIa, SBEIIb*) was examined by RT-qPCR using primers listed in Appendix 1 (see Chapter 3) on CFX96 Real-Time PCR Detection System (BioRad). The relative transcript levels were determined by $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Where $\Delta\Delta Ct = [(Ct_{\text{target of control}}) - (Ct_{\beta\text{-actin of control}})] - [(Ct_{\text{target of transformed}}) - (Ct_{\beta\text{-actin of transformed}})]$. Ct (cycle threshold) refers to the number of cycles at which the fluorescent signal crosses the threshold.

5.3.8 Enzyme extraction and activity assay for AGPase

To assess the effect of expressing *Sh2r6hs* on AGPase activity, grains (10 to 15) harvested at 8 and 16 DAA from transformed and control plants were used for AGPase extraction and activity assays using the protocol described in Chapter 3. Three independent biological replicates (spikes from three different plants of the same line) were used for each line studied.

5.3.9 Starch analysis

In order to determine the effect of *Sh2r6hs* on total starch content and composition, total starch and its components were quantified from grains (10 to 15), harvested at maturity from transformed and control plants, as described before (Chapter 3). Three independent biological replicates (spikes from three different plants of the same line) were used for the analysis.

5.3.10 Yield analysis

Mature dry grains harvested from T1 plants were used for yield analysis. Number of grains per spike and grain weight data was recorded from 20 grains harvested from two negative and three

positive independent lines. Grains harvested from untransformed Fielder plants were used as controls.

5.3.11. Statistical analysis

Statistical analysis was performed with gene expression, starch content and AGPase activity data using with Analysis of Variance (ANOVA) of the PROC Mixed procedure of SAS software (Ver. 9.2., SAS institute Inc, 2008). In order to comply with normal distribution, the fold change values in gene expressions and AGPase activity were converted to their corresponding log 10 values. Cultivar, stage, and the cultivars x stage interaction were considered as sources of variation with fixed effects. Least significant difference (LSD) was calculated to compare statistically significant difference in least squares (LS) means of all cultivar x stage combinations. The type 3 test of Fixed Effects (tests for the significance of each of the fixed effects, cultivar and stage) was determined and those with $p \leq 0.05$ were considered significant. Grain yield and grain weights were analyzed by one way ANOVA with SigmaPlot 12 software.

5.4 Results

5.4.1 Screening transformant lines

Wheat cv. Fielder was transformed with *Sh2r6hs* under the control of a maize *Ubiquitin1* promoter, thus the positive and negative transformant lines were designated as US-line number-positive (P) or US-line number-negative (N), respectively. The T0 plants that survived on the hygromycin containing selection media were grown in soil and the DNA samples extracted from their leaf tissues were used to verify the integration of *Sh2r6hs* by PCR. The PCR product of the DNA derived from eight out of the nine plants selected by hygromycin produced bands with the expected size of 861 bp (Fig. 5.3). The PCR product of genomic DNA extracted from

untransformed Fielder did not produce any band (Fig. 5.3). All the lines were allowed to grow in a growth chamber with the condition described in material and method section 5.3.1, and T1 grains were collected from these plants.

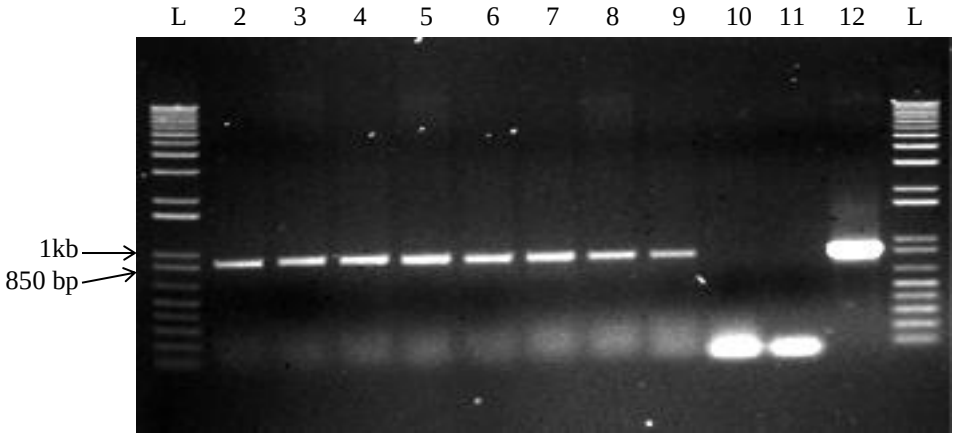


Figure 5.3: Screening of positive T0 plants with PCR. Lanes 1 and 13 designated as "L" represent 1kb plus DNA ladder. Lanes 2 to 10 represent the nine transformed lines that survived all rounds of selection on hygromycin containing medium. Lane 11 represents untransformed plant cv. Fielder used as a negative control. Lane 12 (P) represents the plasmid containing *Sh2r6hs* as a positive control.

After imbibition for a day under darkness, the embryos isolated from T1 grains (grains harvested from the positive T0 plants) were planted on hygromycin (50 mg/L) containing medium for two weeks and those that survived were transplanted into pots containing a soil mix as described before (see material and method section 5.3.1). The seedlings derived from T1 seeds were further screened with PCR using the same primers employed for screening the T0 plants. Lines designated as US4, US5, US6 and US7 were screened as positive lines. The positive lines with a PCR product of expected size (861 bp) (Fig. 5.4) were used for further analysis. Plants with no PCR product of expected size were also included in our subsequent analysis to be used as

negative controls as they had undergone the same transformation process as the positive transformants.

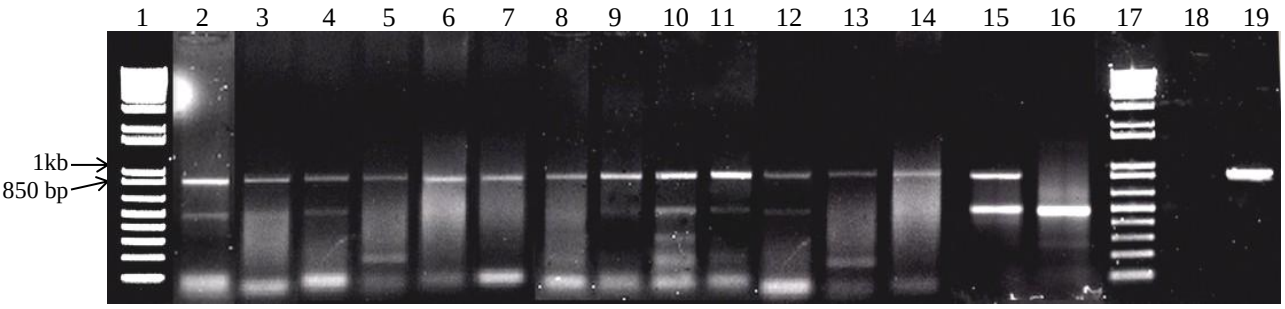


Figure 5.4: PCR screening of T1 plants. Lane 1 and lane 17 represent the 1 kb plus ladder. Lane 2 represents one positive plant from US4; lane 3-6 represent four positive plants from US5; lane 7-11 represent five positive plants from US6; lane 12-15 represent four positive plants from US7; lane 16 represents a product from gDNA of untransformed control plant; lane 19 represents a product from a plasmid containing *Sh2r6hs* used as a positive control. Lane 18 was not used.

5.4.2 Expression analysis of Sh2r6hs

Semi-quantitative RT-PCR based analysis of *Sh2r6hs* expression revealed that the leaves of T1 plants contain a very low amount of its transcripts. Analysis of the target gene in the grains of the positive transformants produced a fragment with expected size of 236 bp (Fig. 5.5 A and B). In all the positive lines analyzed, the expression of *Sh2r6hs* was found to be higher in grains collected at 16 DAA than those collected at 8 DAA (Fig. 5.5 C). For the positive line US4P, the expression of *Sh2r6hs* was analyzed only in one replicate as only one plant survived selection. However, the expression of *Sh2r6hs* in the positive lines US5P, US6P and US7P were analyzed in three biological replicates. These however are not truly replicates as this is a segregating generation, rendering different genetic make up in different individuals. Hence, biological replicates here refer to different individual plants derived from the same line. The plant from US4P showed expression of *Sh2r6hs* at both 8 and 16 DAA in all the PCR cycles tested;

however the expression observed at 8 DAA was slightly higher than that at 16 DAA. The line 5 positive plants, US5P1 and US5P3, exhibited a slightly higher expression of *Sh2r6hs* when compared to that of US5P2 at both 8 and 16 DAA; and in line 6, US6P3 showed higher expression of *Sh2r6hs* than US6P1 at both 8 and 16 DAA. Positive line US6P2 had a higher expression at 8 DAA than that observed at 16 DAA. In line 7, US7P3 exhibited a higher expression of *Sh2r6hs* at both 8 and 16 DAA while US7P1 expressed *Sh2r6hs* higher in 8 DAA than in 16 DAA. Overall, the degree of expression of *Sh2r6hs* in the positive lines was as follows US7P = US5P > US4P > US6P. As expected, none of the negative or untransformed control plants showed any expression of *Sh2r6hs*.

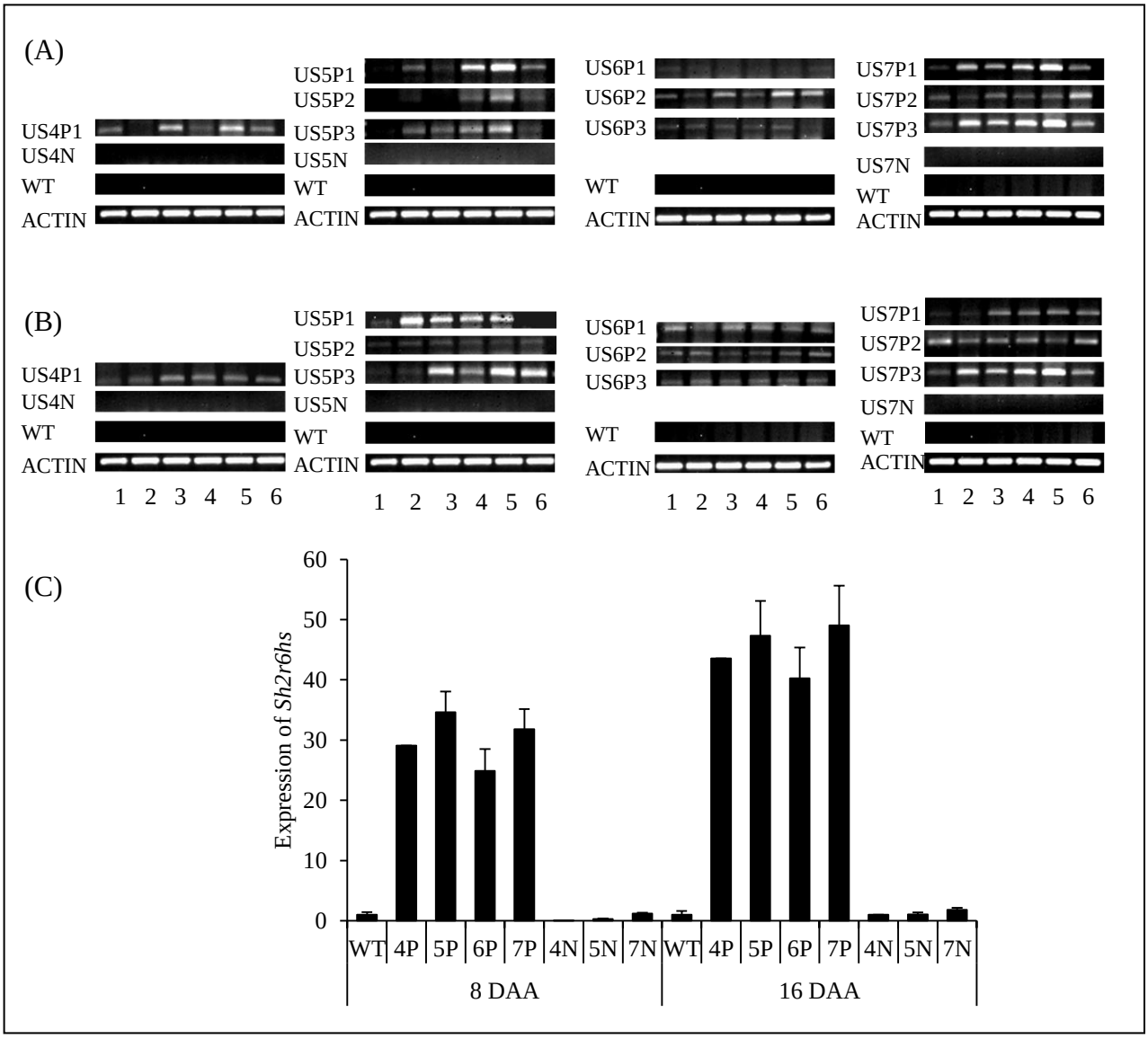


Figure 5.5: Semi-quantitative RT-PCR analysis of *Sh2r6hs* in developing grains of T1 plants (positive lines 4, 5, 6 and 7). The expression of *Sh2r6hs* was analyzed in the grains of individual positive lines at (A) 8 DAA and (B) 16 DAA. Representative images showing the expression of *Sh2r6hs* in the transformed negative lines and untransformed wild type plants, and the expression of the reference gene, β-actin, are also presented. (C) Graphical representation of the expression of *Sh2r6hs* in the grains of positive and negative lines (lines 4, 5, 6 and 7), as well as in the untransformed wild type plants after normalization with the expression of *β-actin.* The values are means ± SE (n=3 for all lines except in 4P where only one survived). The numbers at the bottom of each image (A and B) represent PCR cycle numbers: 1=33 cycle; 2=34 cycle; 3=35 cycle; 4=36 cycle; 5=37 cycle; and 6=38 cycle.

5.4.3 AGPase activity assay

The activity of endospermic AGPase was analyzed *in vitro* in the developing grains of the positive lines 5, 6 and 7 at 8 DAA and 16 DAA (Fig. 5.6). Our results showed that the activity of AGPase in the positive transformed lines was higher than that detected in the control untransformed and negative control plants. In all cases, the activity of AGPase at 16 DAA was higher than that detected at 8 DAA. Almost similar level of AGPase activity was found in the developing grains of untransformed and negative control lines at both stages of grain development.

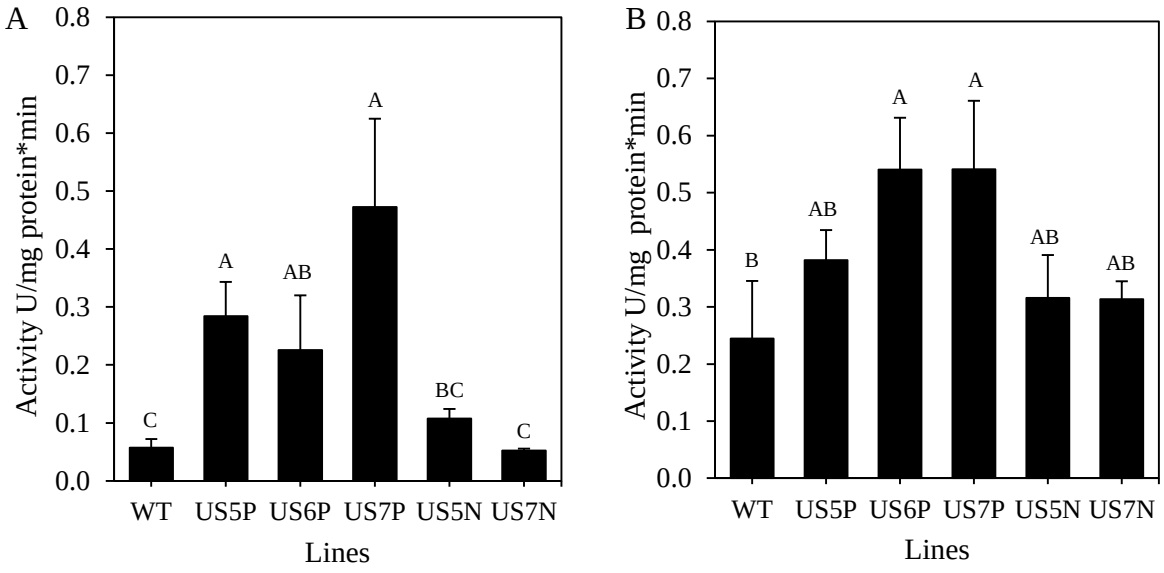


Figure 5.6: AGPase activity in developing grains of wild-type (WT), positive (US5P, US6P and US7P) and negative (US5N and US7N) lines at (A) 8 DAA and (B) 16 DAA. Data are means ± SE, n=3 (except US5P at 8 DAA where n=2)

Lines US7P showed the highest activity of AGPase at 8 DAA, while both lines US5P and US6P exhibited higher activity of AGPase than that observed in wild-type and the negative control lines (Fig. 5.6 A). Both US6P and US7P showed the highest AGPase activity at 16 DAA with line US5P showing AGPase activity similar to that observed in the controls (Fig. 5.6 B).

5.4.4 Expression of the genes involved in starch biosynthesis pathway

In order to examine if the introduction of *Sh2r6hs* under the control of ubiquitin promoter affected the expression of genes encoding enzymes/proteins acting upstream or downstream of the step catalyzed by AGPase genes in developing grains, the expression of several target genes was analyzed using real-time qPCR in untransformed control (wild-type), transformed positive lines and also in the negative control lines.

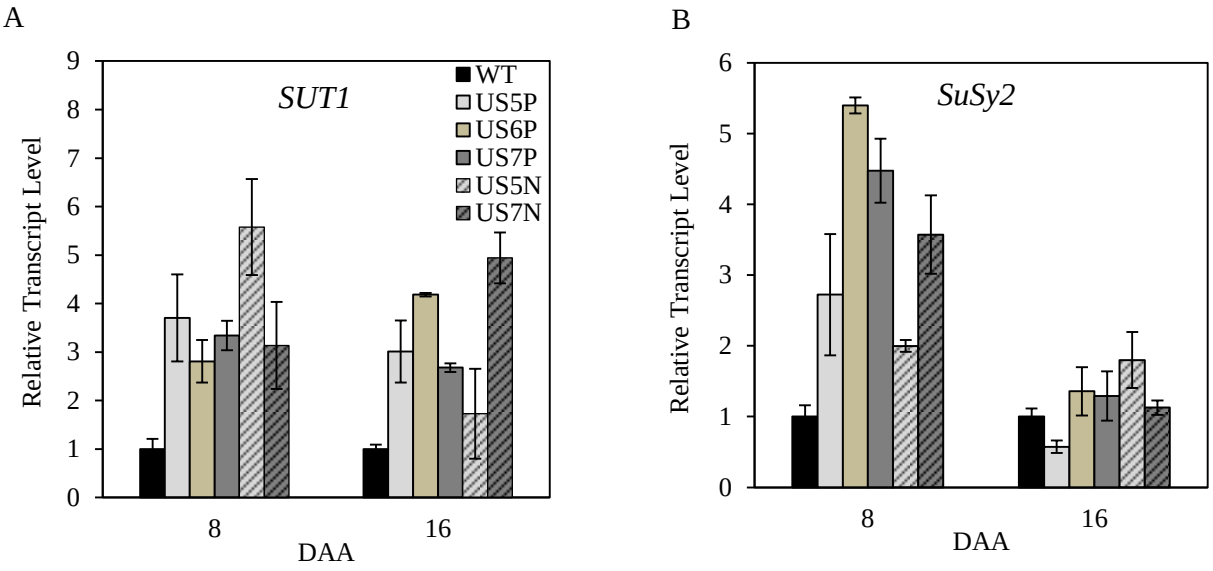


Figure 5.7: Expression profile of (A) *SUT1* and (B) *SuSy2* in the developing grains of wild type (WT), positive (US5P, US6P and US7P) and negative (US5N and US7N) lines. Data are means ± SE, n=2 to 3. Transcript levels of the genes at each stage were determined relative to that found in the wild type (WT) control, which was assigned a value of 1.

Expression profile of genes involved in sucrose transport and catabolism

There was no significant difference in the expression of *SUT1* in any of the positive lines (Fig. 5.7 A), although the plants subjected to tissue culture showed a slight elevated expression compared to the wild-type. The sucrose catabolism gene *SuSy2* had a higher expression in the line US6P at 8 DAA (Fig. 5.7 B). Plants in line US5P and US7P also showed higher expression,

however their expression was not statistically different (Appendix 8.14) from the negative controls of the same lines.

Expression profile of genes involved in starch formation

Expression of all the genes in the *AGPase* family, *SSI*, *SSIIa* and *SSIIIa* of soluble starch synthase and *GBSSI* of granule bound starch synthase were studied. At 8 DAA, the expression of *AGPL1* in the wild type, negative control and US5P lines. Plants from US7P and US6P lines showed slightly higher expression of *AGPL1* at 8 DAA (~1.8-fold higher than the wild type and ~1.2-fold higher than the negative control lines). At 16 DAA, the expression of *AGPL1* was similar across wild type, negative and positive lines (Fig. 5.8 A). *AGPL2* expression was lower than wild type in all of the transformed lines (5-fold lower in US5P, 2.5-fold lower in US7P and 2-fold lower in US6P) at 8 DAA (Fig. 5.8 B). With respect to the negative lines, the line US5N showed an expression level lower than that of the wild type (2.5-fold), while line US7N showed an expression level similar to the wild type. At 16 DAA, the US5N line showed an expression level of *AGPL2* similar to that observed in the wild type, whereas all the other positive lines still showed lower expression. Of the genes encoding small subunits of AGPase, *AGPS1a* expression was found to be higher in plants of line US5P at 16 DAA (Fig. 5.8 C) as compared to wild type. The other positive lines showed similar levels of expression with the wild type at both 8 and 16 DAA (Fig. 5.8 C). The expression of *AGPS1b* showed a similar level of expression between the wild type and all the positive lines at both 8 and 16 DAA except line US7P at 16 DAA, which showed a higher expression of this gene than the wild type (4.5-fold) and the respective negative line, US7N (2-fold). *AGPS2* exhibited a higher expression in the US7P line (2.3-fold) than that exhibited in the wild type. However, its expression in the other positive lines was comparable to that of the wild type.

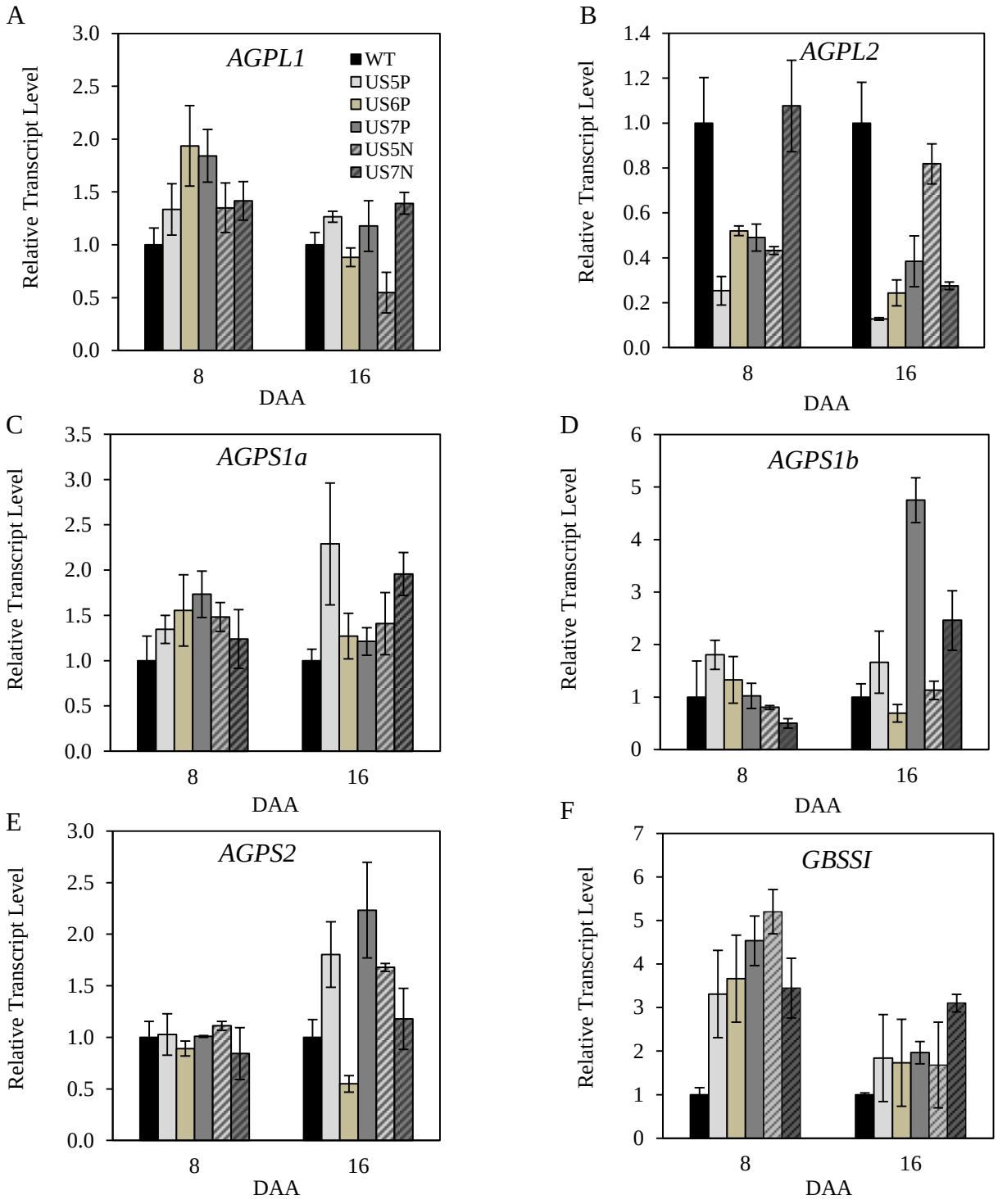


Figure 5.8: Expression of (A) *AGPL1* (B) *AGPL2* (C) *AGPS1a* (D) *AGPS1b* (E) *AGPS2* and (F) *GBSSI* at 8 and 16 DAA in wild type (WT), positive (US5P, US6P and US7P) and negative (US5N and US7N) lines. Transcript levels of the genes at each stage were determined relative to that found in the wild type (WT) control, which was assigned a value of 1. Data are means ± SE, n=2 to 3.

The expression of *GBSSI* at 8 DAA was higher (3.2- to 5.2-fold) in the other lines than that observed in the wild type (Fig. 5.8 F). By 16 DAA, *GBSSI* was expressed at lower levels than that found in 8 DAA samples. With respect to the SS gene family members, *SSI* had a comparable expression in all the lines studied at both the stages (Fig. 5.9 A). However, *SSIIa* was expressed at a higher level in lines US6P and US7P at 8 DAA than that observed in the other lines (Fig. 5.9 B). *SSIIIa* showed almost similar expression between the wild type and the other lines at both 8 and 16 DAA grain developmental stages studied.

The expression of the starch branching enzymes, *SBEI*, *SBEIIa* and *SBEIIb* was also compared. The expression of *SBEI* was similar between the wild type and the other lines at both developmental stages (Fig. 5.9 D). Compared to the wild type, the positive lines US5P (4.5-fold), US6P (6.5-fold) and US7P (4.5-fold) exhibited significantly higher expression of *SBEIIa* at 8 DAA but not at 16 DAA (Fig. 5.9 E, Appendix 8.11). The expression of this gene in US7P at 8 DAA was also significantly higher than its negative line US7N. The expression of *SBEIIb* was also higher in the US6P line at 8 DAA (2.3-fold) and in the US7P line at both 8 and 16 DAA (2.3-2.5-fold) than that observed in the wild type (Fig. 5.9 F).

5.4.5 Determination of Total starch

The starch content in all the lines was measured at the mature stage. The starch content per grain was significantly higher ($p \leq 0.05$) in Line US5P (30.19 ± 0.78 mg/grain) as compared to the rest of the lines including the wild type (23.87 ± 0.77 mg/grain). US7P had difference in its three replicates and US7P3 had a lower starch level (22.59mg/grain) compared to the other two (27.91mg and 25.95mg per grain) (Fig. 5.10). However when compared on per gram dry weight, the starch content did not seem to be significantly different across the different positive lines.

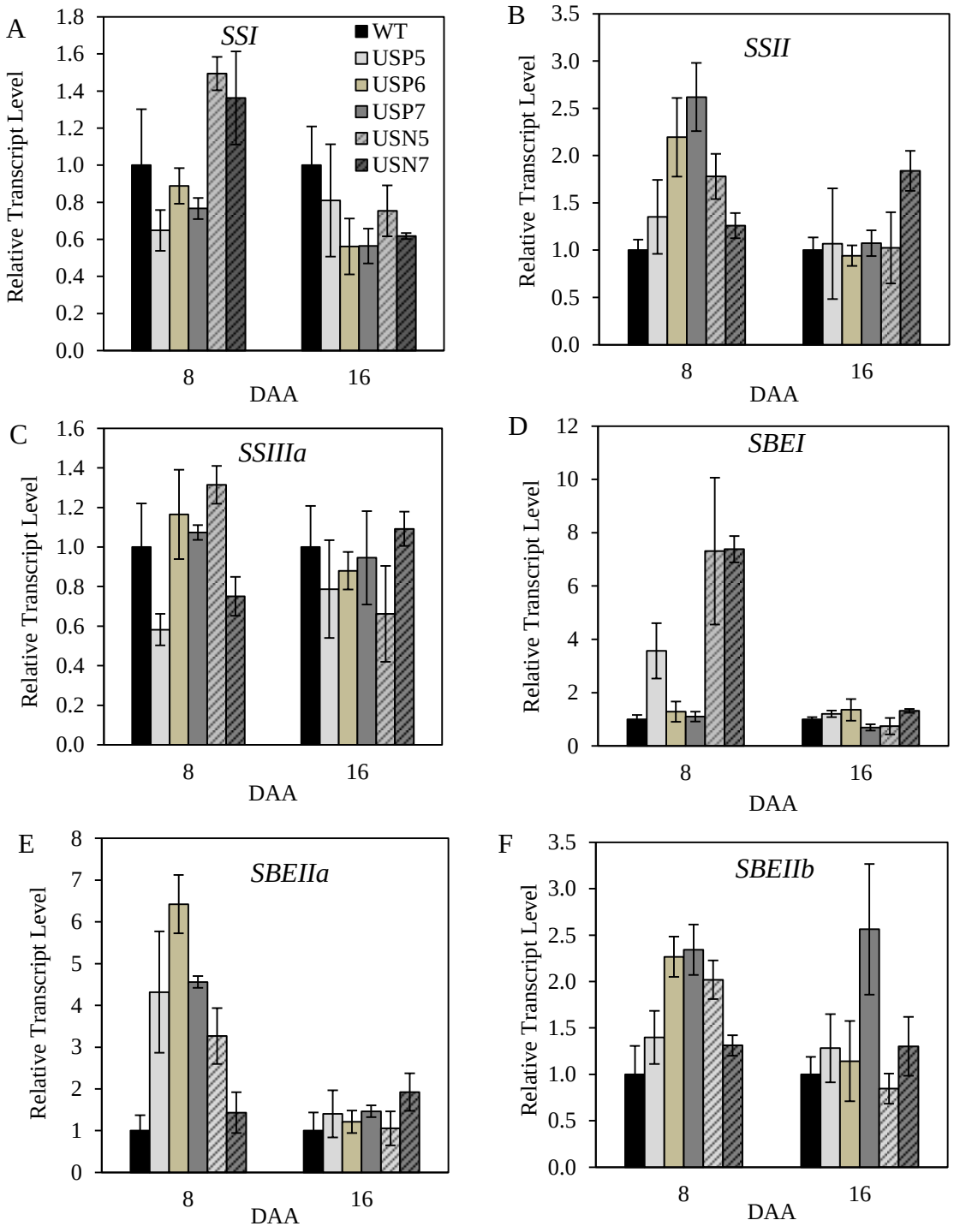


Figure 5.9: Expression of (A) *SSI* (B) *SSIIa* (C) *SSIIIa* (D) *SBEI* (E) *SBEIIa* and (F) *SBEIIb* at 8 and 16 DAA in wild type (WT), positive (US5P, US6P and US7P) and negative (US5N and US7N) lines. Transcript levels of the genes at each stage were determined relative to that found in the wild type (WT) control, which was assigned a value of 1. Data are means ± SE, n=2 to 3.

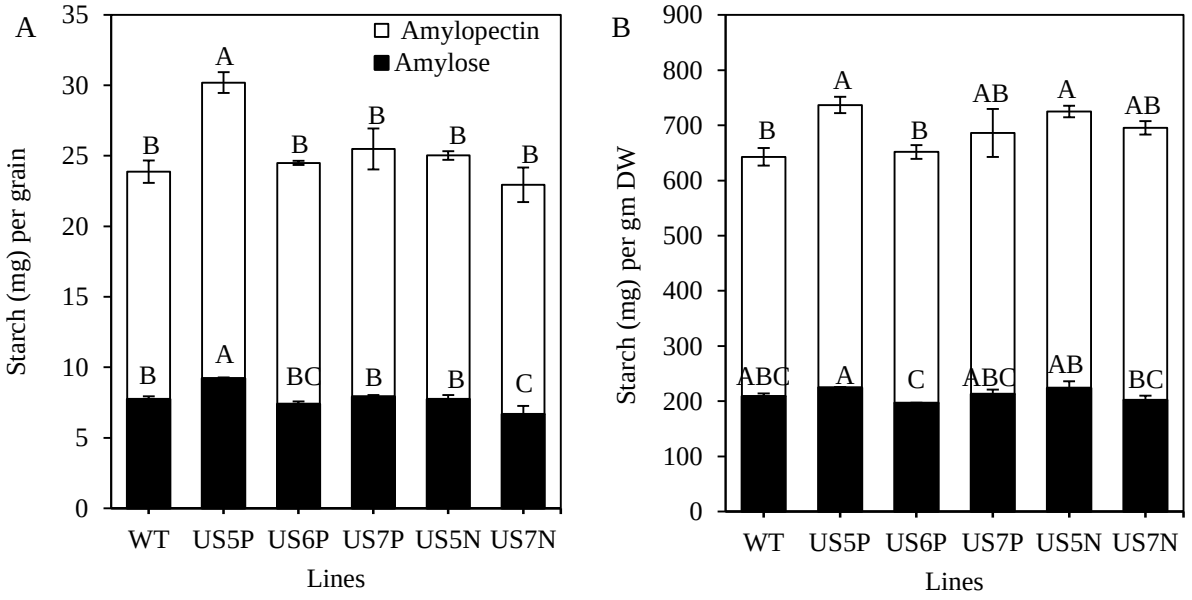


Figure 5.10 Total starch (A) mg per grain (B) mg per gram dry weight (DW) in the mature grains of wild type (WT), positive (US5P, US6P and US7P) and negative (US5N and US7N) lines. Data are means ± SE, n= 3

5.4.6 Analysis of yield parameters

The PCR analysis of genomic DNA from T1 plants along with the semi quantitative-RT-PCR analysis indicated that four lines were successfully transformed with *Sh2r6hs*. However, only three were analyzed for phenotypic characters as only one plant survived from line US4P. Summary of the phenotypic data of the grains from T1 plants is presented in Table 5.1. However, the sample sizes were very small since most of the plants had few tillers (both positive and negative lines) possibly because all these plants were initially subjected to a stress (due to exposure to the 50 mg/L of hygromycin during their early development) and other residual tissue culture effects.

Table 5.1: Parameters of mature grains harvested from the T1 generation of positive (US5P, US6P, US7P), negative lines (US5N, US7N) and the untransformed wild type plants.

Lines	Grain weight (mg)	Grain number/spike
WT	40.66 ± 0.62	27.33 ± 2.96
US5P	48.63 ± 0.06*	29.08 ± 0.08
US6P	41.62 ± 1.26	29.67 ± 2.33
US7P	44.02 ± 1.23	35.67 ± 4.10
US5N	36.86 ± 2.12	28.61 ± 1.31
US7N	42.32 ± 0.68	30.67 ± 2.03

*US5P is significantly different ($p \leq 0.05$) in grain weight from WT, US5N, US6P and US7N

Plants from US5P showed 19.5% increase in grain weight which was significantly different ($p \leq 0.05$) from WT, and 6.4% increase in number of grains per spike compared to untransformed wild type plants (Table 5.1). They also showed a significant increase in weight of 32% ($p \leq 0.05$). Plants from the US7P line showed an 8.3% increase in grain weight and 30.5% increase in number of grains per spike compared to untransformed wild type plants. However, when compared to the negative control of the same line, it showed a 4.0% increase in grain weight and 16.3% increase in number of grains per spike. Plants in line US6P showed a 2.3% increase in grain weight and 8.5% increase in the number of grains per spike as compared to wild type. Some grains in the US6P line were much shrunken and did not develop well enough to even germinate. Those were not selected for the grain weight and size measurements.

5.5 Discussion

The sink (grain) size and strength is largely dependent on the activity of the enzymes in the grains during their development (Stark *et al*., 1992; Giroux *et al*., 1996) and AGPase is one of the key enzymes responsible for this, as discussed in the preceding chapters. Although conventional breeding targets towards increase in yield, genetic manipulation of important traits

through biotechnology has gained prominence in the last two decades (Koornneef and Stam, 2001; Meyer *et al*., 2004). Several efforts have been made to manipulate the activity of AGPase, and thereby increase yield (Smidansky *et al*., 2002; Smidansky *et al*., 2003; Meyer *et al*., 2004; Sakulsingharoj *et al*., 2004; Li *et al*., 2011). For example, expression of the modified form of the maize AGPase large subunit (*Sh2r6hs*) under the control of *Sh2* promoter has been found to enhance grain AGPase activity and biomass in wheat (Smidansky *et al*., 2002; Meyer *et al*., 2004) and rice (Smidansky *et al*., 2003). This study was aimed at investigating if the expression of the mutant maize AGPase gene (*Sh2r6hs*) under the control of the maize ubiquitin1 (*Ubi1*) promoter leads to further increase in starch and/or grain yield in wheat.

The T1 plants characterised here belong to a segregating population and their zygosity has not been determined, however, the presence and expression of the transgene was confirmed by PCR of the genomic DNA and semi-quantitative RT-PCR, respectively (Fig. 5.4 and 5.5). Our analysis of the T1 positive plants showed that introduction of *Sh2r6hs* into wheat under the control of *Ubi1* promoter increased AGPase activity significantly ($p \leq 0.05$) in the positive lines at 8 DAA (Fig. 5.6). Similarly, expressing the same gene under the control of a seed specific promoter *Glu* resulted an increase in AGPase activity (5-20%) (Meyer *et al*., 2004). In previous studies, plants expressing *Sh2r6hs* driven by *Sh2* or HMW *Glu* promoter showed enhanced yield under high light intensity (625 $\mu Em^{-2}sec^{-1}$) environmental condition (Meyer *et al*., 2004) However, no difference in yield was apparent between the positive and negative lines when grown under low light intensity (260 $\mu Em^{-2}sec^{-1}$) conditions (Smidansky *et al*., 2007). In the present study, increases in grain weight, as well as number of grains per spike were observed in the positive lines US5P and US7P as compared to the wild-type or the negative lines (Table 5.1), when grown under a light intensity of 175 $\mu Em^{-2}sec^{-1}$, which is lower than the low light

conditions used in the previous study (Smidansky *et al*., 2007). Although at this stage, all of the increases were not statistically significant, grain weight from US5P was statistically different from grains of wild-type as well as the negative controls. Whether the increases in grain yield observed in the positive lines were an effect of enhanced photosynthesis in the source tissues triggered by the constitutive overexpression of *Sh2r6hs* requires further clarifications. Previous studies have reported the effect of *Sh2r6hs* expression on processes upstream of starch biosynthesis (Smidansky *et al*., 2007). For example, the expression of *Sh2r6hs* transgene specifically in the seed was associated with stimulation of photosynthesis and increased production of carbon metabolites in the flag leaves. Analysis of the expression of genes involved in the processes upstream of ADP-glucose formation, including those involved in sucrose transport and sucrose hydrolysis showed an upregulation of *SuSy2* in all the positive lines at 8 DAA (Fig. 5.7 B) as compared to the untransformed wild-type. However, no difference in *SuSy2* was evident when compared to the negative control lines. Whether this is due to the segregating population can be ascertained through further analysis of developing grains from subsequent generations. Since SuSy catalyzes the hydrolysis of sucrose into fructose and Glu-6-P, which serve as substrates for the synthesis of ADP-glucose and thereby starch, our result suggests the contribution of increased sucrose catabolism to the enhancement of starch yield observed in the positive transformed lines. This result is supported by previous studies that associated wheat *SuSy2* with increased yield in different wheat varieties (Jiang et al., 2011). However, the expression of *SUT1* in the transgenic lines did not show any significant increase, suggesting that the increase in sucrose hydrolysis is not associated with increased sucrose transport.

The native cytosolic large subunit of AGPase, which is encoded by the *AGPL1*, shows high expression at 8 DAA relative to that found 16 DAA grains in several of the positively

transformed plants (Fig. 5.8 A). All the positive lines, however, show a tendency for higher expression of the *Sh2r6hs* at 16 DAA as compared to that found in 8 DAA (Fig. 5.5), implying that enhanced transcription of *Sh2r6hs* takes place during the rapid grain filling phase. Alternatively, this could be due to the higher stability of the *Sh2r6hs* mRNA as suggested in earlier studies (Meyer *et al*., 2004). Apart from that of *AGPL1*, the expression of *Sh2r6hs* as a transgene likely influences other components of starch synthesis during the rapid grain filling phase. For example, increased expression of *AGPSes* (1.3- to 2.3-fold) was observed in lines US5P and US7P at 16 DAA (Fig. 5.8), suggesting a possible increase in the amount of AGPase small subunit protein, and thereby increased activity of AGPase. Although, the positive lines US7P (for *AGPS1b* and *AGPS2* genes) and US5P (for *AGPS1a* and *AGPS2*) show significant differences in expression of *AGPS* genes, this pattern can further be verified in subsequent generations. Also, the higher mean AGPase activity observed in the positive lines either at 8 DAA or 16 DAA (Fig. 5.6) is well in support of this. Furthermore, higher starch content per grain was observed in two of the transformed positive lines, US5P (significantly higher than the other lines) and US7P, as compared to the wild-type (Fig. 5.10 A, B). Interestingly, *AGPL2*, which encodes the plastidial large subunit of AGPase was expressed at lower levels in the transformed positive lines than that observed a wild type plants (Fig. 5.8 B). Its transcripts have been found to be abundant in the developing grains, especially at 8 DAA (see Fig. 3.5 B and Fig. 4.3 B of Chapter 3 and 4, respectively).

Furthermore, grains of the transformed positive lines US6P and US7P exhibited high expression of *SSIIa* (2.3- to 2.5-fold) at 8 DAA (Fig. 5.9 B). It has been shown in rice that the SSI accounts for at least 50% of the total starch synthase activity (Fujita *et al*., 2007). However, in wheat the SSIIa isoform contributes to the majority of starch synthase and starch branching

enzyme complex formation required for the synthesis of starch in the endosperm (Kosar-Hashemi *et al*., 2007). Consistent with this result, the *SSIIa* triple null lines of wheat exhibit reduced grain weight and starch content (Konik-Rose *et al*., 2007). It may be likely that the increased synthesis of ADP-glucose in the transformed plants leads to increased expression of *SSIIa*, and thereby enhanced starch yield per grain. It would be interesting to investigate this in the next generations. As reported by Mutisya *et al*. (2003), SBEI is largely responsible for starch branching towards the later stages of grain development. In agreement with this, *SBE1* was expressed at low level at 8 DAA and 16 DAA grains (Fig. 5.9 D). However, *SBEIIa* showed upregulation (4 to 6.5-fold) at 8 DAA in all of the positive lines compared to the wild-type (Fig. 5.9 E), and *SBEIIb* had higher transcript abundance in line US7P than that observed in the wild-type grains at both 8 and 16 DAA (Fig. 5.9 F). Overall, the higher expression of *Sh2r6hs* and the higher AGPase activity in lines US5P and US7P appear to be associated with the upregulation of *SSIIa* and *SBEIIa* at 8 DAA. Interestingly, line US5P had higher grain weight and starch content per grain when compared to the wild-type (Table 5.1; Fig. 5.10 A). The increase in starch content is due to a 30% increase in the amount of amylopectin and 19% increase in that of amylose (Fig. 5.10 A). As discussed before, the synthesis of amylopectin involves the *SS* and *SBE* genes, whereas that of amylose involves *GBSS* genes. Although the increase in the amounts of amylopectin in US5P grains coincides with the trend of increased expression of *AGPS1a*, *SSIIa* and *SBEIIa* genes, no significant change in the expression of *GBSSI* was observed at the stages considered in this study. Previous studies have shown that rice genes involved in amylopectin synthesis are regulated transcriptionally (Wang *et al*., 2013a), whereas *GBSS* is regulated at both the transcriptional (Zhu *et al*., 2003) and post-transcriptional levels (Wang *et al*., 1995; Isshiki *et al*., 2006; Zeng *et al*., 2007). Therefore, it is possible that either our study missed the stages at

which *GBSSI* is transcriptionally regulated or that it is subjected to post-transcriptional regulation in wheat. Although not statistically significant, line US7P shows a higher grain weight (Table 5.11) and increased number of grains per spike (Table 5.1). This trend is accompanied by increased expression of *SSIIa* and *SBEIIa* genes. Since the T1 plants belong to a segregating population, the gene dosage and zygosity of these plants could be attributed to the differences among biological replicates, resulting in larger standard errors. Interestingly, a role for *Sh2r6hs* in increasing grain number has also been shown in maize (Hannah *et al*., 2012).

In conclusion, this study showed that constitutive expression of *Sh2r6hs* in wheat shows a trend to increased starch content per grain and total grain weight in the transformed positive lines. These were accompanied by increased activity of AGPase in the grains although starch content on gram dry weight basis was unaffected by the expression of the transgene. This study was focused on analysis of the T1 generation of the transformed plants mainly to accommodate the limited duration allowed for completeing a doctoral study. Further investigations are required to determine the stability of the transgene inheritance and its effect on yield and yiled parameters in the subsequent generations. Furthermore, investigating copy number of the transgene and the effect of the gene dose would be useful in correlating the gene expression to the difference in some of the yield characteristics observed in this study.

7. GENERAL DISCUSSION AND CONCLUSIONS

Wheat starch has several end uses from food to pharmaceuticals, from animal feed to bioethanol and beyond. This diversified use of wheat starch has triggered continually increasing demand of this crop in various industrial sectors. The yield of starch in wheat grains is defined at molecular level by many genes involved in sucrose transport and catabolism, and starch biosynthesis pathway. Several wheat genes or their corresponding ESTs involved in these processes have been identified. The expression of these genes has been shown in previous studies to be regulated developmentally, and is also influenced by a number of environmental factors. The development of a wheat grain can be classified into three different phases: the first phase represents the tissue proliferation of the endosperm and the growing embryo; the second phase is usually characterized as the phase of rapid grain filling, and the third phase, involves the maturation and dehydration of the grain. Therefore, a detailed characterization of the determinants of starch biosynthesis during these phases of wheat grain development contributes to our understanding of the mechanisms regulating starch yield in wheat grains. This in turn will enhance the scope of genetic manipulation or breeding of wheat for improved starch yield and quality.

Using a soft white spring wheat cultivar, Whitebird, this study characterized the sucrose-to-starch metabolic pathway in detail. The study analyzed the expression of 25 genes and the activity of four major enzymes during grain development. In addition, it quantified sucrose and starch levels in developing grains. The three phases of grain development were studied, and the first tissue proliferation phase represent the period between anthesis and 8 DAA, the second rapid grain filling phase from 8 to 25 DAA, and the period beyond 25 DAA represent the grain maturation phase. During these phases, the gene expression analyses suggested that *TaSUT1*, *TaSuSy2*, *AGPL1*, *AGPS1a*, *SSI*, *SSIIa*, *SSIIIa*, *GBSSI*, *SBEI* and *SBEIIa* appear to be the

predominant genes regulating starch formation in wheat endosperm. The expressions of *TaSUT1* and *TaSuSy2* encoding for proteins involved in transporting sucrose to the developing grain and hydrolyzing it respectively, was more abundant from the tissue proliferation through the rapid grain filling phase (8-16 DAA), highlighting the importance of sucrose transport into the developing grain and its subsequent hydrolysis into hexose sugars that are used as substrates for starch synthesis, respectively. This was accompanied by increasing grain sucrose content through 8 DAA and high SuSy activity through 16 DAA. These factors contributed to the increasing grain weight accumulation during 8-25 DAA. The onset of starch synthesis in developing wheat grains (8-25 DAA) coincides with the increased transcript abundance of *AGPL1*, *AGPS1a*, *SSI, SSIIa, SSIIIa, SBEIIa* and *SBEI.* The temporal expression patterns of the *SS* and *SBE* genes imply that the synthesis of amylopectin, which forms 75% of total starch, starts early. Although the abundance of *GBSSI* transcripts show a gradual increase from 8 DAA, the activity of the corresponding enzyme was not found to increase during the same period, suggesting a possible post-transcriptional regulation, which can be a subject of future investigation. However, the GBSS activity showed an increase towards the latter stages of grain filling, and this result suggests that majority of amylose synthesis occurs during the later stages of grain development as compared to the synthesis of amylopectin. The results of this study showed *AGPL1,* encoding the large subunit of AGPase, as one of the most highly expressed gene throughout grain development. The expression of this gene started very early and continued through with major increases observed during grain filling, suggesting its involvement in synthesizing starch at every stage. The finding of this study could be further enriched by investigating the contribution of each genome of hexaploid wheat to the total expression of *AGPL1* at the different stages of grain development.

The expression profile of the genes involved in starch biosynthesis during rapid grain filling (8 and 16 DAA), were examined in response to ABA treatment to understand how this affects starch synthesis. Our observations indicated downregulation of the major genes involved in endospermic starch synthesis including *AGPL1*, *SSI, SSIIa, SSIIIa*, and *SBEIIa*. However, the genes previously known to be encoding the plastidial isoforms of the enzymes involved in starch synthesis showed upregulation by ABA treatment. In a wheat grain, these genes would be contributing towards the formation of transitory starch in the pericarp. This led us to hypothesize that ABA enhances the synthesis of transient starch but negatively affects that of endospermic starch during the rapid grain filling phase. Our study also revealed the upregulation of genes encoding fructan exohydrolase in response to ABA, suggesting ABA's role in the remobilization of water soluble carbohydrates towards endospermic starch synthesis. It would be interesting to investigate how the fructan degradation pathway is coordinated with starch synthesis in wheat grains.

This study also compared starch synthesis in three spring wheat cultivars (AC Andrew, CDC Teal and Chinese Spring) that have different genetic backgrounds and varying grain starch content. These three cultivars showed differences in thousand kernel weight (TKW) and starch per grain. AC Andrew showed elevated expression of *AGPLI* and *AGPS1a*, which encode the large and small subunits of cytosolic AGPase, respectively, during the rapid grain filling phase. This was also reflected at the level of enzyme activity which was higher than Chinese Spring by the end of the rapid grain filling. The high expression of *AGPL1* and *AGPS1a*, and increased activity of AGPase in AC Andrew was coordinated with upregulation of *SSI, SSIIIa* and *SBEIIa* genes as compared to that observed in other cultivars. SSI is known to account for nearly 70% of the total soluble starch synthase activity. Cellular localization of *SSI* transcripts in this study

revealed its presence in the inner layers of the maternal pericarp at 4 DAA and mostly in the endosperm at 8 DAA. These results suggest that *SSI* is involved in the synthesis of transitional starch in the pericarp at the early stages of grain development but later, during the onset of starch accumulation, it acts predominantly in the endosperm. It is therefore likely that the transcriptional activation of starch synthase genes play an important role in regulating starch formation and accumulation in both pericarp and endosperm tissues of developing wheat grains. AC Andrew also showed enhanced activities of AGPase and SSS at specific developmental stages. Overall, the difference in the expression of *AGPase* (*AGPL1* and *AGPS1a*) and *SS* (*SSI* and *SSIIIa*) and *SBE* (*SBEIIa*) genes appear to determine variation in grain starch yield in the three cultivars. Analysis of the activity of starch branching enzymes in these cultivars could further our understanding of the molecular mechanism of starch biosynthesis during grain filling. As these cultivars, however, have different genetic background, investigating the nucleotide diversity of the targeted genes may help to determine their specific roles.

Following the identification of genes that appear to regulate grain starch content in wheat, a transgenic approach was applied to over-express the modified form of maize AGPase large subunit *Sh2* gene (*Sh2r6hs*), in wheat under the control of a constitutive maize *Ubiquitin1* promoter. This mutant gene has previously exhibited decreased sensitivity to inhibition by inorganic phosphate. Four lines carrying the *Sh2r6hs* gene designated as US4P, US5P, US6P and US7P were analyzed, and expression of *Sh2r6hs* was shown to be in the following order, US7P = US5P > US4P > US6P. The expression of *Sh2r6hs* was found to be higher at 16 DAA in comparison to 8 DAA. The expression of this gene was also associated with increased AGPase activity in the grains obtained from the transformed lines. Further, characterization of the three lines, US5P, US6P and US7P, for their grain starch content and other yield parameters showed

that US5P had higher grain weight and grain starch content as compared to the transformed negative lines and the untransformed controls (wild-type). Characterization of the expression of the other genes involved in the sucrose-to-starch metabolism pathway showed that expressing the *Sh2r6hs* gene in wheat induced the expression of *SuSy2*, which is involved in sucrose catabolism (at 8 DAA), and *AGPS1a* (at 16 DAA), and *SSIIa* and *SBEIIa* (at 8 DAA) that catalyze endosperm starch formation. Our results also indicated that introduction of *Sh2r6hs* into wheat has a positive effect on the activity of AGPase and thereby starch yield. Since the results presented here are obtained from T1 generation plants, which belong to a population that is segregating, further characterization of the positive tranformant lines in the subsequent generation is necessary to verify the stability of the transgene and its effect on starch biosynthesis. It would also be interesting to study if the expression of the transgene alters the level of ADP-glucose and whether that triggers any feedback regulation of starch metabolism.

A part of this study was focused on isolation and functional characterization of a partial promoter sequence of the wheat RuBisCO small subunit (rbcS) gene (Appendix 12). Analysis of the sequence of the promoter fragment revealed the presence of light responsive elements such as the I-box and GT1 consensus sequences in the fragment. Moreover, the promoter consists of the GATA sequence, which is associated with light induced and tissue-specific expression, the CAAT sequence and the TATA-box, both of which are associated with the binding site for transcription factor. This partial sequence of the *rbcS* promoter was able to drive the expression of GUS gene in tobacco leaves and immature wheat embryos when incubated in light. The intensity of the expression driven by *rbcS* promoter was much less than that of *CaMV35S*. However, it was not able to direct the expression of *GUS* in tissues that are not normally exposed

to light such as the young roots of wheat. These results indicate that the *rbcS* promoter fragment isolated in this study can be used to direct the expression of target genes in source tissues.

In conclusion, this study enhances our understanding of the transcriptional regulation of the genes involved in the sucrose-to-starch pathway and their contribution in controlling starch accumulation in wheat grains. However, further analysis of the physiological roles of these genes is necessary to develop molecular tools for improving starch yield and quality of wheat, and thereby its end use applications.

8. LITERATURE CITED

1. **Abramson J, Smirnova I, Kasho V, Verner G, Kaback HR, Iwata S** (2003) Structure and mechanism of the lactose permease of *Escherichia coli*. Science **301**: 610–615

2. **Agarwal S, Loar S, Steber C, Zale J** (2009) Floral transformation of wheat. *In* HD Jones, PR Shewry, eds, Transgenic wheat barley oats. Humana Press, pp 105–113

3. **Aguilar J, Cameron TA, Zupan J, Zambryski P** (2011) Membrane and core periplasmic Agrobacterium tumefaciens virulence Type IV Secretion System components localize to multiple sites around the bacterial perimeter during lateral attachment to plant cells. mBio **2** (6) e00218-11

4. **Ahmadi A, Baker DA** (1999) Effects of abscisic acid (ABA) on grain filling processes in wheat. Plant Growth Regul **28**: 187–197

5. **Ainsworth C, Hosein F, Tarvis M, Weir F, Burrell M, Devos KM, Gale MD** (1995) Adenosine diphosphate glucose pyrophosphorylase genes in wheat: differential expression and gene mapping. Planta **197**: 1–10

6. **Ainsworth C, Tarvis M, Clark J** (1993) Isolation and analysis of a cDNA clone encoding the small subunit of ADP-glucose pyrophosphorylase from wheat. Plant Mol Biol **23**: 23–33

7. **Akihiro T** (2005) Gene expression of ADP-glucose pyrophosphorylase and starch contents in rice cultured cells are cooperatively regulated by sucrose and ABA. Plant Cell Physiol **46**: 937–946

8. **Alexander RD, Morris PC** (2006) A proteomic analysis of 14-3-3 binding proteins from developing barley grains. Proteomics **6**: 1886–1896

9. **Altpeter F, Varshney A, Abderhalden O, Douchkov D, Sautter C, Kumlehn J, Dudler R, Schweizer P** (2005) Stable expression of a defense-related gene in wheat epidermis under transcriptional control of a novel promoter confers pathogen resistance. Plant Mol Biol **57**: 271–283

10. **Aoki, Hirose T, Scofield GN, Whitfeld PR, Furbank RT** (2003) The sucrose transporter gene family in rice. Plant Cell Physiol **44**: 223–232

11. **Aoki N, Hirose T, Furbank RT** (2012) Sucrose transport in higher plants: from source to sink. *In* JJ Eaton-Rye, BC Tripathy, TD Sharkey, eds, Photosynthesis. Springer Netherlands, pp 703–729

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

13. **Aoki N, Scofield GN, Wang X-D, Offler CE, Patrick JW, Furbank RT** (2006) Pathway of sugar transport in germinating wheat seeds. Plant Physiol **141**: 1255 –1263

14. **Aoki, Whitfeld P, Hoeren F, Scofield G, Newell K, Patrick J, Offler C, Clarke B, Rahman S, Furbank RT** (2002) Three sucrose transporter genes are expressed in the developing grain of hexaploid wheat. Plant Mol Biol **50**: 453–462

15. **Arias-Garzon DI, Sayre RT** (1993) Tissue specific inhibition of transient gene expression in cassava (Manihot esculenta Crantz). Plant Sci **93**: 121–130

16. **Austin** (2009) Ethanol Producer Magazine EthanolProducer.com. http://www.ethanolproducer.com/articles/5586/embracing-the-alternatives. Accessed: 2013-07-10 21:20:33

17. **Baba T, Kimura K, Mizuno K, Etoh H, Ishida Y, Shida O, Arai Y** (1991) Sequence conservation of the catalytic regions of amylolytic enzymes in maize branching enzyme-I. Biochem Biophys Res Commun **181**: 87–94

18. **Badr YA, Kereim MA, Yehia MA, Fouad OO, Bahieldin A** (2005) Production of fertile transgenic wheat plants by laser micropuncture. Photochem Photobiol Sci **4**: 803–807

19. **Baguma Y, Sun C, Ahlandsberg S, Mutisya J, Palmqvist S, Rubaihayo PR, Magambo MJ, Egwang TG, Larsson H, Jansson C** (2003) Expression patterns of the gene encoding starch branching enzyme II in the storage roots of cassava (*Manihot esculenta* Crantz). Plant Sci **164**: 833–839

20. **Bakhsh A, Qayyum R, Shamim Z, Husnain T** (2011) A mini review: RuBisCo small subunit as a strong, green tissue-specific promoter. Arch Biol Sci **63**: 299–307

21. **Ball SG, Morell MK** (2003) From bacterial glycogen to starch: Understanding the biogenesis of the plant starch granule. Annu Rev Plant Biol **54**: 207–233

22. **Basu C, Kausch AP, Luo H, Chandlee JM** (2003) Promoter analysis in transient assays using a GUS reporter gene construct in creeping bentgrass (*Agrostis palustris*). J Plant Physiol **160**: 1233–1239

23. **Becker D, Brettschneider R, Lörz H** (1994) Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. Plant J Cell Mol Biol **5**: 299–307

24. **Van Bel AJE van, Hess PH** (2008) Hexoses as phloem transport sugars: the end of a dogma? J Exp Bot **59**: 261–272

25. **Bhalla PL, Ottenhof HH, Singh MB** (2006) Wheat transformation – an update of recent progress. Euphytica **149**: 353–366

26. **Bhattacharyya MK, Smith AM, Ellis THN, Hedley C, Martin C** (1990) The wrinkled-seed character of pea described by Mendel is caused by a transposon-like insertion in a gene encoding starch-branching enzyme. Cell **60**: 115–122

27. **Blauth SL, Kim K-N, Klucinec J, Shannon JC, Thompson D, Guiltinan M** (2002) Identification of mutator insertional mutants of starch-branching enzyme 1 (sbe1) in *Zea mays* L. Plant Mol Biol **48**: 287–297

28. **Blauth SL, Yao Y, Klucinec JD, Shannon JC, Thompson DB, Guilitinan MJ** (2001) Identification of mutator insertional mutants of starch-branching enzyme 2a in corn. Plant Physiol **125**: 1396–1405

29. **Blechl AE, Jones HD** (2009) Transgenic applications in wheat improvement. *In* BFCE Regentsessor, ed, Wheat Science and Trade. Wiley-Blackwell, pp 397–435

30. **Boehlein SK, Shaw JR, Stewart JD, Hannah LC** (2008) Heat stability and allosteric properties of the maize endosperm ADP-glucose pyrophosphorylase are intimately intertwined. Plant Physiol **146**: 289 –299

31. **Bourdon V, Wickham A, Lonsdale D, Harwood W** (2004) Additional introns inserted within the luciferase reporter gene stabilise transgene expression in wheat. Plant Sci **167**: 1143–1149

32. **Brenchley R, Spannagl M, Pfeifer M, Barker GLA, D'Amore R, Allen AM, McKenzie N, Kramer M, Kerhornou A, Bolser D, et al** (2012) Analysis of the bread wheat genome using whole-genome shotgun sequencing. Nature **491**: 705–710

33. **Brocklehurst PA** (1977) Factors controlling grain weight in wheat. Nature **266** (5600): 348–49

34. **Brown TA** (1999) How ancient DNA may help in understanding the origin and spread of agriculture. Philos Trans R Soc Lond B Biol Sci **354**: 89–98

35. **Buchanan BB, Jones RL** (2007) Biochemistry and molecular biology of plants. I.K. International Publishing House Pvt. Ltd.

36. **Burger BT, Cross JM, Shaw JR, Caren JR, Greene TW, Okita TW, Hannah LC** (2003) Relative turnover numbers of maize endosperm and potato tuber ADP-glucose pyrophosphorylases in the absence and presence of 3-phosphoglyceric acid. Planta **217**: 449–456

37. **Burton RA, Bewley JD, Smith AM, Bhattacharyya MK, Tatge H, Ring S, Bull V, Hamilton WDO, Martin C** (1995) Starch branching enzymes belonging to distinct enzyme families are differentially expressed during pea embryo development. Plant J **7**: 3–15

38. **Burton RA, Johnson PE, Beckles DM, Fincher GB, Jenner HL, Naldrett MJ, Denyer K** (2002) Characterization of the genes encoding the cytosolic and plastidial forms of ADP-Glucose pyrophosphorylase in wheat endosperm. Plant Physiol **130**: 1464–1475

39. **Carlson SJ, Chourey PS, Helentjaris T, Datta R** (2002) Gene expression studies on developing kernels of maize sucrose synthase (SuSy) mutants show evidence for a third SuSy gene. Plant Mol Biol **49**: 15–29

40. **Carver BF** (2009) Wheat: Science and Trade. Wiley-Blackwell, pp xvii-xx

41. **Cheng M, Fry JE, Pang S, Zhou H, Hironaka CM, Duncan DR, Conner TW, Wan Y** (1997) Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiol **115**: 971–980

42. **Cheng M, Hu T, Layton J, Liu C-N, Fry JE** (2003) Desiccation of plant tissues post-*Agrobacterium* infection enhances T-DNA delivery and increases stable transformation efficiency in wheat. Vitro Cell Dev Biol - Plant **39**: 595–604

43. **Chevalier P, Lingle SE** (1983) Sugar metabolism in developing kernels of wheat and barley. Crop Sci **23**: 272-277

44. **Cho J-I, Lee S-K, Ko S, Kim H-K, Jun S-H, Lee Y-H, Bhoo SH, Lee K-W, An G, Hahn T-R, Jeon J-S** (2005) Molecular cloning and expression analysis of the cell-wall invertase gene family in rice (*Oryza sativa* L.). Plant Cell Rep **24**: 225–236

45. **Chourey, Jain M, Li Q-B, Carlson SJ** (2005) Genetic control of cell wall invertases in developing endosperm of maize. Planta **223**: 159–167

46. **Chourey PS** (1981) Genetic control of sucrose synthetase in maize endosperm. Mol Gen Genet **184**: 372–376

47. **Chourey, Taliercio EW, Carlson SJ, Ruan YL** (1998) Genetic evidence that the two isozymes of sucrose synthase present in developing maize endosperm are critical, one for cell wall integrity and the other for starch biosynthesis. Mol Gen Genet MGG **259**: 88–96

48. **Christensen AH, Sharrock RA, Quail PH** (1992) Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol Biol **18**: 675–689

49. **Chugh A, Khurana P** (2003) Regeneration via somatic embryogenesis from leaf basal segments and genetic transformation of bread and emmer wheat by particle bombardment. Plant Cell Tissue Organ Cult **74**: 151–161

50. **Clarke BR, Denyer K, Jenner CF, Smith AM** (1999) The relationship between the rate of starch synthesis, the adenosine 5′-diphosphoglucose concentration and the amylose content of starch in developing pea embryos. Planta **209**: 324–329

51. **Collins GN** (1909) A new type of Indian corn from China. http://archive.org/details/newtypeofindianc00coll. Accessed 2013-10-08 00:47:51.

52. **Commuri PD, Keeling PL** (2001) Chain-length specificities of maize starch synthase I enzyme: studies of glucan affinity and catalytic properties. Plant J **25**: 475–486

53. **Cornejo M-J, Luth D, Blankenship KM, Anderson OD, Blechl AE** (1993) Activity of a maize ubiquitin promoter in transgenic rice. Plant Mol Biol **23**: 567–581

54. **Craig J, Lloyd JR, Tomlinson K, Barber L, Edwards A, Wang TL, Martin C, Hedley CL, Smith AM** (1998) Mutations in the gene encoding starch synthase II profoundly alter amylopectin structure in pea embryos. Plant Cell **10**: 413

55. **Cross JM** (2004) Both subunits of ADP-Glucose pyrophosphorylase are regulatory. Plant Physiol **135**: 137–144

56. **CTBI - Fuel & Energy** CTBI - Fuel & Energy. http://www.ctbi.ca/index.php?option=com_content&view=article&id=17&Itemid=121. Accessed: 2013-07-10 21:11:23.

57. **Dai Z** (2008) Comparison of starch accumulation and enzyme activity in grains of wheat cultivars differing in kernel type. Plant Growth Regul **57**: 153–162

58. **Dale EM, Housley TL** (1986) Sucrose synthase activity in developing wheat endosperms differing in maximum weight. Plant Physiol **82**: 7–10

59. **Dale PJ, Clarke B, Fontes EMG** (2002) Potential for the environmental impact of transgenic crops. Nat Biotechnol **20**: 567–574

60. **Darroch BA, Baker RJ** (1995) Two measures of grain filling in spring wheat. Crop Sci **35**: 164

61. **Delvallé D, Dumez S, Wattebled F, Roldán I, Planchot V, Berbezy P, Colonna P, Vyas D, Chatterjee M, Ball S, et al** (2005) Soluble starch synthase I: a major determinant for the synthesis of amylopectin in *Arabidopsis thaliana* leaves. Plant J **43**: 398–412

62. **Denver K, Barber LM, Burton R, Hedley CL, Hylton CM, Johnson S, Jones DA, Marshall J, Smith AM, Tatge H, et al** (1995) The isolation and characterization of novel low-amylose mutants of *Pisum sativum* L. Plant Cell Environ **18**: 1019–1026

63. **Denyer K, Clarke B, Hylton C, Tatge H, Smith AM** (1996) The elongation of amylose and amylopectin chains in isolated starch granules. Plant J **10**: 1135–1143

64. **Denyer K, Hylton CM, Jenner CF, Smith AM** (1995) Identification of multiple isoforms of soluble and granule-bound starch synthase in developing wheat endosperm. Planta **196**: 256–265

65. **Deol KK, Mukherjee S, Gao F, Brûlé-Babel A, Stasolla C, Ayele BT** (2013) Identification and characterization of the three homeologues of a new sucrose transporter in hexaploid wheat (*Triticum aestivum* L.). BMC Plant Biol **13**: 181

66. **Dian W, Jiang H, Wu P** (2005) Evolution and expression analysis of starch synthase III and IV in rice. J Exp Bot **56**: 623–632

67. **Doan** (1999) The allosterically unregulated isoform of ADP-glucose pyrophosphorylase from barley endosperm is the most likely source of ADP-glucose incorporated into endosperm starch. Plant Physiol **121**: 965–975

68. **Donald RG, Cashmore AR** (1990) Mutation of either G box or I box sequences profoundly affects expression from the *Arabidopsis* rbcS-1A promoter. EMBO J **9**: 1717–1726

69. **Dorion S, Lalonde S, Saini HS** (1996) Induction of male sterility in wheat by meiotic-stage water deficit is preceded by a decline in invertase activity and changes in carbohydrate metabolism in anthers. Plant Physiol **111**: 137–145

70. **Duan M, Sun SSM** (2005) Profiling the expression of genes controlling rice grain quality. Plant Mol Biol **59**: 165–178

71. **Duffus C, Rosie R** (1973) Starch hydrolysing enzymes in the developing barley grain. Planta **109**: 153–160

72. **Duncan KA, Hardin SC, Huber SC** (2006) The three maize sucrose synthase isoforms differ in distribution, localization, and phosphorylation. Plant Cell Physiol **47**: 959–971

73. **Edwards A, Borthakur A, Bornemann S, Venail J, Denyer K, Waite D, Fulton D, Smith A, Martin C** (1999) Specificity of starch synthase isoforms from potato. Eur J Biochem **266**: 724–736

74. **Eimert K, Luo C, Déjardin A, Villand P, Thorbjørnsen T, Kleczkowski LA** (1997) Molecular cloning and expression of the large subunit of ADP-glucose pyrophosphorylase from barley (*Hordeum vulgare*) leaves. Gene **189**: 79–82

75. **Ellis RJ** (1981) Chloroplast proteins: synthesis, transport, and assembly. Annu Rev Plant Physiol **32**: 111–137

76. **Emes MJ, Bowsher CG, Hedley C, Burrell MM, Scrase-Field ESF, Tetlow IJ** (2003) Starch synthesis and carbon partitioning in developing endosperm. J Exp Bot **54**: 569 –575

77. **Evers AD** (1970) Development of the endosperm of wheat. Ann Bot **34**: 547–555

78. **Flipse E, Suurs L, Keetels CJAM, Kossmann J, Jacobsen E, Visser RGF** (1996) Introduction of sense and antisense cDNA for branching enzyme in the amylose-free potato mutant leads to physico-chemical changes in the starch. Planta **198**: 340–347

79. **Fluhr R, Kuhlemeier C, Nagy F, Chua NH** (1986) Organ-specific and light-induced expression of plant genes. Science **232**: 1106–1112

80. **FAOSTAT**. http://faostat.fao.org/site/339/default.aspx. Accessed: 2013-06-22 13:31:50

81. **Frame B, Marcy M, Rosemarie S and Kan W** (2011) Genetic Transformation Using Maize Immature Zygotic Embryos. *In* Plant Embryo Culture, eds Trevor A, Thorpe and Edward C. Yeung, Methods in Molecular Biology. Humana Press.327–41

82. **Frazier JC, Appalanaidu B** (1965) The wheat grain during development with reference to nature, location, and role of its translocatory tissues. Am J Bot **52**: 193

83. **Fu Y, Ballicora MA, Leykam JF, Preiss J** (1998) Mechanism of reductive activation of potato tuber ADP-glucose pyrophosphorylase. J Biol Chem **273**: 25045–25052

84. **Fujita N, Satoh R, Hayashi A, Kodama M, Itoh R, Aihara S, Nakamura Y** (2011) Starch biosynthesis in rice endosperm requires the presence of either starch synthase I or IIIa. J Exp Bot **62**: 4819–4831

85. **Fujita N, Yoshida M, Asakura N, Ohdan T, Miyao A, Hirochika H, Nakamura Y** (2006) Function and characterization of starch synthase I using mutants in rice. Plant Physiol **140**: 1070 –1084

86. **Fujita N, Yoshida M, Kondo T, Saito K, Utsumi Y, Tokunaga T, Nishi A, Satoh H, Park J-H, Jane J-L, et al** (2007) Characterization of SSIIIa-deficient mutants of rice: the function of SSIIIa and pleiotropic effects by SSIIIa deficiency in the rice endosperm. Plant Physiol **144**: 2009 –2023

87. **Fulton DC, Edwards A, Pilling E, Robinson HL, Fahy B, Seale R, Kato L, Donald AM, Geigenberger P, Martin C, et al** (2002) Role of granule-bound starch synthase in determination of amylopectin structure and starch granule morphology in potato. J Biol Chem **277**: 10834–10841

88. **Fuqua C** (2008) *Agrobacterium*-host attachment and biofilm formation. *In* T Tzfira, V Citovsky, eds, Agrobacterium: From Biology to Biotechnology Springer New York, pp 243–277

89. **Furbank, Scofield GN, Hirose T, Wang X-D, Patrick JW, Offler CE** (2001) Cellular localisation and function of a sucrose transporter OsSUT1 in developing rice grains. Aust J Plant Physiol **28**: 1187–1196

90. **Gao M, Chibbar RN** (2000) Isolation, characterization, and expression analysis of starch synthase IIa cDNA from wheat (*Triticum aestivum* L.). Genome **43**: 768–775

91. **Gao M, Fisher DK, Kim KN, Shannon JC, Guiltinan MJ** (1997) Independent genetic control of maize starch-branching enzymes IIa and IIb. Isolation and characterization of a Sbe2a cDNA. Plant Physiol **114**: 69

92. **Gelvin SB** (2010) Plant proteins involved in *Agrobacterium*-mediated genetic transformation. Annu Rev Phytopathol **48**: 45–68

93. **Georgelis N, Braun EL, Shaw JR, Hannah LC** (2007) The two AGPase subunits evolve at different rates in angiosperms, yet they are equally sensitive to activity-altering amino acid changes when expressed in bacteria. Plant Cell **19**: 1458 –1472

94. **Ghosh HP, Preiss J** (1966) Adenosine diphosphate glucose pyrophosphorylase. J Biol Chem **241**: 4491 –4504

95. **Gibon Y, Bläsing OE, Palacios-Rojas N, Pankovic D, Hendriks JH., Fisahn J, Höhne M, Günther M, Stitt M** (2004) Adjustment of diurnal starch turnover to short days: depletion of

sugar during the night leads to a temporary inhibition of carbohydrate utilization, accumulation of sugars and post-translational activation of ADP-glucose pyrophosphorylase in the following light period. Plant J **39**: 847–862

96. **Gilmartin PM, Chua NH** (1990) Spacing between GT-1 binding sites within a light-responsive element is critical for transcriptional activity. Plant Cell **2**: 447–455

97. **Giroux MJ, Hannah LC** (1994) ADP-glucose pyrophosphorylase in shrunken-2 and brittle-2 mutants of maize. Mol Gen Genet **243**: 400–408

98. **Giroux MJ, Shaw J, Barry G, Cobb BG, Greene T, Okita T, Hannah LC** (1996) A single mutation that increases maize seed weight. Proc Natl Acad Sci **93**: 5824–5829

99. **Giuliano G, Pichersky E, Malik VS, Timko MP, Scolnik PA, Cashmore AR** (1988) An evolutionarily conserved protein binding sequence upstream of a plant light-regulated gene. Proc Natl Acad Sci USA **85**: 7089–7093

100. **Government of Alberta AA and RD** Crop Information Portal. http://www.agric.gov.ab.ca/app95/loadCropVariety?action=display&id=133. Accessed: 2013-01-01 21:02:42

101. **Graybosch RA** (1998) Waxy wheats: Origin, properties, and prospects. Trends in Food Science & Technology **9**: 135-142.

102. **Graybosch RA, Liu RH, Madl RL, Shi Y-C, Wang D, Wu X** (2009) New Uses for wheat and modified wheat products. *In* BFCE Regentsessor, ed, Wheat Science and Trade. Wiley-Blackwell, pp 521–550

103. **Greene TW, Hannah LC** (1998a) Maize Endosperm ADP–Glucose Pyrophosphorylase SHRUNKEN2 and BRITTLE2 Subunit Interactions. Plant Cell Online **10**: 1295–1306

104. **Greene TW, Hannah LC** (1998b) Enhanced stability of maize endosperm ADP-glucose pyrophosphorylase is gained through mutants that alter subunit interactions. Proc Natl Acad Sci USA **95**: 13342–13347

105. **Guerin J, Carbonero P** (1997) The spatial distribution of sucrose synthase isozymes in barley. Plant Physiol **114**: 55–62

106. **Gupta PK, Mir RR, Mohan A, Kumar J** (2008) Wheat genomics: present status and future prospects. Int J Plant Genomics **2008**: 1–36

107. **Gustafson P, Raskina O, Ma X, Nevo E** (2009) Wheat evolution, domestication, and improvement. *In* BFCE Regentsessor, ed, Wheat Science and Trade. Wiley-Blackwell, pp 3–30

108. **Halford N g., Curtis T y., Muttucumaru N, Postles J, Mottram D s.** (2011) Sugars in crop plants. Ann Appl Biol **158**: 1–25

109. **Hanashiro I, Itoh K, Kuratomi Y, Yamazaki M, Igarashi T, Matsugasako J, Takeda Y** (2008) Granule-bound starch synthase I is responsible for biosynthesis of extra-long unit chains of amylopectin in rice. Plant Cell Physiol **49**: 925–933

110. **Hannah L** (2007) Starch formation in the cereal endosperm. Endosperm 179–193

111. **Hannah LC, Futch B, Bing J, Shaw JR, Boehlein S, Stewart JD, Beiriger R, Georgelis N, Greene T** (2012) A shrunken-2 Transgene increases maize yield by acting in maternal tissues to increase the frequency of seed development. Plant Cell **24**: 2352–2363

112. **Hannah LC, Nelson OE** (1975) Characterization of adenosine diphosphate glucose pyrophosphorylases from developing maize seeds. Plant Physiol **55**: 297–302

113. **Hannah LC, Tuschall DM, Mans RJ** (1980) Multiple forms of maize endosperm adp-glucose pyrophosphorylase and their control by Shrunken-2 and Brittle-2. Genetics **95**: 961–970

114. **Harn C, Knight M, Ramakrishnan A, Guan H, Keeling PL, Wasserman BP** (1998) Isolation and characterization of the zSSIIa and zSSIIb starch synthase cDNA clones from maize endosperm. Plant Mol Biol **37**: 639–649

115. **He DG, Mouradov A, Yang YM, Mouradova E, Scott KJ** (1994) Transformation of wheat (*Triticum aestivum* L.) through electroporation of protoplasts. Plant Cell Rep **14**: 192–196

116. **Hendriks JH., Kolbe A, Gibon Y, Stitt M, Geigenberger P** (2003) ADP-glucose pyrophosphorylase is activated by posttranslational redox-modification in response to light and to sugars in leaves of Arabidopsis and other plant species. Plant Physiol **133**: 838

117. **Hennen-Bierwagen TA, Lin Q, Grimaud F, Planchot V, Keeling PL, James MG, Myers AM** (2009) Proteins from multiple metabolic pathways associate with starch biosynthetic enzymes in high molecular weight complexes: a model for regulation of carbon allocation in maize amyloplasts. Plant Physiol **149**: 1541–1559

118. **Hennen-Bierwagen TA, Liu F, Marsh RS, Kim S, Gan Q, Tetlow IJ, Emes MJ, James MG, Myers AM** (2008) Starch biosynthetic enzymes from developing maize endosperm associate in multisubunit complexes. Plant Physiol **146**: 1892–1908

119. **Herzog H** (1986) Source and sink during the reproductive period of wheat: development and its regulation with special reference to cytokinins. P. Parey Scientific Publishers

120. **Heuberger AL, Lewis MR, Chen M-H, Brick MA, Leach JE, Ryan EP** (2010) Metabolomic and functional genomic analyses reveal varietal differences in bioactive compounds of cooked rice. PLoS One **5**: e12915

121. **Hirose T** (2002) Cell wall invertase in developing rice caryopsis: molecular cloning of OsCIN1 and analysis of its expression in relation to its role in grain filling. Plant Cell Physiol **43**: 452 – 459

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

123. **Hirose T, Scofield GN, Terao T** (2008) An expression analysis profile for the entire sucrose synthase gene family in rice. Plant Sci **174**: 534–543

124. **Hirose, Terao T** (2004) A comprehensive expression analysis of the starch synthase gene family in rice (*Oryza sativa* L.). Planta **220**: 9–16

125. **Hong B, Barg R, Ho TH** (1992) Developmental and organ-specific expression of an ABA- and stress-induced protein in barley. Plant Mol Biol **18**: 663–674

126. **Hovenkamp-Hermelink JHM, Jacobsen E, Ponstein AS, Visser RGF, Vos-Scheperkeuter GH, Bijmolt EW, Vries JN, Witholt B, Feenstra WJ** (1987) Isolation of an amylose-free starch mutant of the potato (*Solanum tuberosum* L.). Theor Appl Genet **75**: 217–221

127. **Hu T, Metz S, Chay C, Zhou HP, Biest N, Chen G, Cheng M, Feng X, Radionenko M, Lu F, et al** (2003) *Agrobacterium*-mediated large-scale transformation of wheat (*Triticum aestivum* L.) using glyphosate selection. Plant Cell Rep **21**: 1010–1019

128. **Huang X-Q, Brûlé-Babel A** (2012) Sequence diversity, haplotype analysis, association mapping and functional marker development in the waxy and starch synthase IIa; genes for grain-yield-related traits in hexaploid wheat (*Triticum aestivum* L.). Mol Breed **30**: 627–645

129. **Hughes GR, Hucl P** (1993) CDC Teal hard red spring wheat. Can J Plant Sci **73**: 193–197

130. **Hurkman WJ, McCue KF, Altenbach SB, Korn A, Tanaka CK, Kothari KM, Johnson EL, Bechtel DB, Wilson JD, Anderson OD, et al** (2003) Effect of temperature on expression of genes encoding enzymes for starch biosynthesis in developing wheat endosperm. Plant Sci **164**: 873–881

131. **Igaue I** (1964) Studies on Q-enzyme of rice plant. Memoirs of the faculty of agriculture Niigata University. Nippon Nōgeikagaku Kaishi **36**: 1–54

132. **Iglesias AA, Barry GF, Meyer C, Bloksberg L, Nakata PA, Greene T, Laughlin MJ, Okita TW, Kishore GM, Preiss J** (1993) Expression of the potato tuber ADP-glucose pyrophosphorylase in Escherichia coli. J Biol Chem **268**: 1081–1086

133. **Ihemere U, Arias-Garzon D, Lawrence S, Sayre R** (2006) Genetic modification of cassava for enhanced starch production. Plant Biotechnol J **4**: 453–465

134. **Imparl-Radosevich JM, Nichols DJ, Li P, McKean AL, Keeling PL, Guan H** (1999) Analysis of purified maize starch synthases IIa and IIb: SS isoforms can be distinguished based on their kinetic properties. Arch Biochem Biophys **362**: 131–138

135. **Isshiki M, Tsumoto A, Shimamoto K** (2006) The serine/arginine-rich protein family in rice plays important roles in constitutive and alternative splicing of pre-mRNA. Plant Cell **18**: 146–158

136. **James MG** (2003) Starch synthesis in the cereal endosperm. Curr Opin Plant Biol **6**: 215–222

137. **Jeng TL, Wang CS, Tseng TH, Sung JM** (2007) Expression of granule-bound starch synthase in developing rice grain. J Sci Food Agric **87**: 2456–2463

138. **Jenner C, Ugalde T, Aspinall D** (1991) The physiology of starch and protein deposition in the endosperm of wheat. Funct Plant Biol **18**: 211–226

139. **Jeon JS, Ryoo N, Hahn TR, Walia H, Nakamura Y** (2010) Starch biosynthesis in cereal endosperm. Plant Physiol Biochem **48**: 383–392

140. **Ji X, Van den Ende W, Schroeven L, Clerens S, Geuten K, Cheng S, Bennett J** (2007) The rice genome encodes two vacuolar invertases with fructan exohydrolase activity but lacks the related fructan biosynthesis genes of the Pooideae. New Phytol **173**: 50–62

141. **Jiang Q, Hou J, Hao C, Wang L, Ge H, Dong Y, Zhang X** (2011) The wheat (*T. aestivum*) sucrose synthase 2 gene (TaSus2) active in endosperm development is associated with yield traits. Funct Integr Genomics **11**: 49–61

142. **Jobling SA., Schwall GP., Westcott RJ., Sidebottom CM., Debet M, Gidley MJ., Jeffcoat R, Safford R** (1999) A minor form of starch branching enzyme in potato (*Solanum tuberosum* L.) tubers has a major effect on starch structure: cloning and characterisation of multiple forms of SBE A. Plant J **18**: 163–171

143. **Johnson PE, Patron NJ, Bottrill AR, Dinges JR, Fahy BF, Parker ML, Waite DN, Denyer K** (2003) A low-starch barley mutant, risø 16, lacking the cytosolic small subunit of adp-glucose pyrophosphorylase, reveals the importance of the cytosolic isoform and the identity of the plastidial small subunit. Plant Physiol **131**: 684 –696

144. **Jones HD, Doherty A, Wu H** (2005) Review of methodologies and a protocol for the *Agrobacterium*-mediated transformation of wheat. Plant Methods **1**: 5

145. **Jonik C, Sonnewald U, Hajirezaei M-R, Flügge U-I, Ludewig F** (2012) Simultaneous boosting of source and sink capacities doubles tuber starch yield of potato plants. Plant Biotechnol J **10**: 1088–1098

146. **Juhas M, Crook DW, Hood DW** (2008) Type IV secretion systems: tools of bacterial horizontal gene transfer and virulence. Cell Microbiol 10: 2377–2386

147. **Kang GZ, Zheng BB, Shen BQ, Peng HF, Guo TC** (2010) A novel Ta. AGP. S. 1b transcript in Chinese common wheat (*Triticum aestivum* L.). C R Biol **333**:716-724.

148. **Kawasaki T, Mizuno K, Baba T, Shimada H** (1993) Molecular analysis of the gene encoding a rice starch branching enzyme. Mol Gen Genet **237**: 10–16

149. **Keeling PL, Myers AM** (2010a) Biochemistry and genetics of starch synthesis. Annu Rev Food Sci Technol **1**: 271–303

150. **Keeling PL, Myers AM** (2010b) Biochemistry and Genetics of Starch Synthesis. *In* MP Doyle, TR Klaenhammer, eds, Annual Review of Food Science and Technology, Vol 1, Palo Alto, pp 271–303

151. **Khanna HK, Daggard GE** (2003) *Agrobacterium tumefaciens*-mediated transformation of wheat using a superbinary vector and a polyamine-supplemented regeneration medium. Plant Cell Rep **21**: 429–436

152. **Kilian A, Kleinhofs A, Villand P, Thorbjørnsen T, Olsen O-A, Kleczkowski LA** (1994) Mapping of the ADP-glucose pyrophosphorylase genes in barley. Theor Appl Genet **87**: 869–871

153. **Kim JK, Park S-Y, Yeo Y, Cho HS, Kim YB, Bae H, Park CH, Lee J-H, Park SU** (2013) Metabolic profiling of millet (*Paniciim miliaceum*) using gas chromatography - time-of-flight mass spectrometry (GC-TOFMS) for quality assessment. Plant Omics **6**: 73

154. **Kim J-Y, Mahé A, Guy S, Brangeon J, Roche O, Chourey PS, Prioul J-L** (2000) Characterization of two members of the maize gene family, Incw3 and Incw4, encoding cell-wall invertases. Gene **245**: 89–102

155. **Kolbe, Tiessen A, Schluepmann H, Paul M, Ulrich S, Geigenberger P** (2005) Trehalose 6-phosphate regulates starch synthesis via posttranslational redox activation of ADP-glucose pyrophosphorylase. Proc Natl Acad Sci U S A **102**: 11118 –11123

156. **Koller A, Washburn MP, Lange BM, Andon NL, Deciu C, Haynes PA, Hays L, Schieltz D, Ulaszek R, Wei J, et al** (2002) Proteomic survey of metabolic pathways in rice. Proc Natl Acad Sci U S A **99**: 11969–11974

157. **Konik-Rose C, Thistleton J, Chanvrier H, Tan I, Halley P, Gidley M, Kosar-Hashemi B, Wang H, Larroque O, Ikea J, et al** (2007) Effects of starch synthase IIa gene dosage on grain, protein and starch in endosperm of wheat. Theor Appl Genet Theor Angew Genet **115**: 1053–1065

158. **Koornneef M, Stam P** (2001) Changing paradigms in plant breeding. Plant Physiol **125**: 156–159

159. **Kosar-Hashemi B, Li Z, Larroque O, Regina A, Yamamori M, Morell MK, Rahman S** (2007) Multiple effects of the starch synthase II mutation in developing wheat endosperm. Funct Plant Biol **34**: 431–438

160. **Krishnan HB, Blanchette JT, Okita TW** (1985) Wheat invertases : characterization of cell wall-bound and soluble forms. Plant Physiol **78**: 241

161. **Kühn C, Quick WP, Schulz A, Riesmeier JW, Sonnewald U, Frommer WB** (1996) Companion cell-specific inhibition of the potato sucrose transporter SUT1. Plant Cell Environ **19**: 1115–1123

162. **Kühn, Grof CPL** (2010) Sucrose transporters of higher plants. Curr Opin Plant Biol **13**: 288–298

163. **Kuhn C, Barker L, Burkle L, Frommer W-B** (1999) Update on sucrose transport in higher plants. J Exp Bot **50**: 935–953

164. **Lam E, Chua NH** (1989) ASF-2: a factor that binds to the cauliflower mosaic virus 35S promoter and a conserved GATA motif in Cab promoters. Plant Cell Online **1**: 1147–1156

165. **Lamacchia C, Shewry PR, Di Fonzo N, Forsyth JL, Harris N, Lazzeri PA, Napier JA, Halford NG, Barcelo P** (2001) Endosperm-specific activity of a storage protein gene promoter in transgenic wheat seed. J Exp Bot **52**: 243–250

166. **Lee SJ** (1995) Characterization of loci containing microsatellite sequences among Canadian wheat cultivars. Genome **38**: 1037–1040

167. **Leterrier M, Holappa LD, Broglie KE, Beckles DM** (2008) Cloning, characterisation and comparative analysis of a starch synthase IV gene in wheat: functional and evolutionary implications. BMC Plant Biol **8**: 98

168. **Li N, Zhang S, Zhao Y, Li B, Zhang J** (2011) Over-expression of AGPase genes enhances seed weight and starch content in transgenic maize. Planta **233**: 241–250

169. **Li X, Xing J, Gianfagna TJ, Janes HW** (2002) Sucrose regulation of ADP-glucose pyrophosphorylase subunit genes transcript levels in leaves and fruits. Plant Sci **162**: 239–244

170. **Li Z, Chu X, Mouille G, Yan L, Kosar-Hashemi B, Hey S, Napier J, Shewry P, Clarke B, Appels R, et al** (1999) The localization and expression of the class II starch synthases of wheat. Plant Physiol **120**: 1147

171. **Li Z, Mouille G, Kosar-Hashemi B, Rahman S, Clarke B, Gale KR, Appels R, Morell MK** (2000) The structure and expression of the wheat Starch Synthase III gene. motifs in the expressed gene define the lineage of the Starch Synthase III gene family. Plant Physiol **123**: 613–624

172. **Li Z, Trick HN** (2005) Rapid method for high-quality RNA isolation from seed endosperm containing high levels of starch. BioTechniques **38**: 872

173. **Liang J, Zhang J, Cao X** (2001) Grain sink strength may be related to the poor grain filling of indica-japonica rice (*Oryza sativa*) hybrids. Physiol Plant **112**: 470–477

174. **Linebarger CRL, Boehlein SK, Sewell AK, Shaw J, Hannah LC** (2005) Heat stability of maize endosperm ADP-glucose pyrophosphorylase is enhanced by insertion of a cysteine in the N terminus of the small subunit. Plant Physiol **139**: 1625–1634

175. **Llosa M, Gomis-Rüth FX, Coll M, Cruz F de la** (2002) Bacterial conjugation: a two-step mechanism for DNA transport. Mol Microbiol 45: 1–8

176. **Lohot VD, Natu P, Pandey R, Ghildiyal MC** (2010) ADP-glucose pyrophosphorylase activity in relation to starch accumulation and grain growth in wheat cultivars. Curr Sci **98**: 426–430

177. **Marana C, Garcia-Olmedo F, Carbonero P** (1990) Differential expression of two types of sucrose synthase-encoding genes in wheat in response to anaerobiosis, cold shock and light. Gene **88**: 167–172

178. **Marraccini P, Courjault C, Caillet V, Lepage B, Rogers WJ, Tessereau S, Deshayes A** (2003) Rubisco small subunit of *Coffea arabica*: cDNA sequence, gene cloning and promoter analysis in transgenic tobacco plants. Plant Physiol Biochem **41**: 17–25

179. **Martin C, Smith AM** (1995) Starch biosynthesis. Plant Cell **7**: 971

180. **Mashiringwani NA, Mashingaidze K, Kangai J, Olsen K** (1994) Genetic basis of grain filling rate in wheat (*Triticum aestivum* L. emend. Thell.). Euphytica **76**: 33–44

181. **Matsukura C, Saitoh T, Hirose T, Ohsugi R, Perata P, Yamaguchi J** (2000) Sugar uptake and transport in rice embryo. Expression of companion cell-specific sucrose transporter (OsSUT1) Induced by Sugar and Light. Plant Physiol **124**: 85 –94

182. **McCue K, Hurkman W, Tanka C, Anderson O** (2002) Starch-branching enzymes Sbe1 and Sbe2 from wheat (*Triticum aestivum* cv. Cheyenne): Molecular characterization, development expression, and homoeologue assignment by differential PCR. Plant Mol Biol Report **20**: 191–192

183. **McInnis A** (2004) The wheat that won the west: - The impact of Marquis wheat development. A report prepared for Winning the Prairie Gamble: The Saskatchewan Story. http://www.wdm.ca/skteacherguide/WDMResearch/MarquisWheatPaper.pdf. Accessed 2014-06-06 17:31:13

184. **Méchin V, Thévenot C, Guilloux ML, Prioul J-L, Damerval C** (2007) Developmental analysis of maize endosperm proteome suggests a pivotal role for pyruvate orthophosphate dikinase. Plant Physiol **143**: 1203–1219

185. **Meng L, Bregitzer P, Zhang S, Lemaux PG** (2003) Methylation of the exon/intron region in the Ubi1 promoter complex correlates with transgene silencing in barley. Plant Mol Biol **53**: 327–340

186. **Mérida A, Rodríguez-Galán JM, Vincent C, Romero JM** (1999) Expression of the granule-bound starch synthase I (Waxy) gene from snapdragon is developmentally and circadian clock regulated. Plant Physiol **120**: 401–410

187. **Meyer FD, Talbert LE, Martin JM, Lanning SP, Greene TW, Giroux MJ** (2007) Field evaluation of transgenic wheat expressing a modified ADP-glucose pyrophosphorylase large subunit. Crop Sci **47**: 336

188. **Meyer, Smidansky ED, Beecher B, Greene TW, Giroux MJ** (2004) The maize Sh2r6hs ADP-glucose pyrophosphorylase (AGP) large subunit confers enhanced AGP properties in transgenic wheat (*Triticum aestivum*). Plant Sci **167**: 899–911

189. **Mi G, Tang L, Zhang F, Zhang J** (2002) Carbohydrate storage and utilization during grain filling as regulated by nitrogen application in two wheat cultivars. J Plant Nutr **25**: 213–229

190. **Miller M, Chourey P** (1992) The maize invertase-deficient miniature-1 seed mutation is associated with aberrant pedicel and endosperm development. Plant Cell **4**: 297–305

191. **Mizuno K, Kawasaki T, Shimada H, Satoh H, Kobayashi E, Okumura S, Arai Y, Baba T** (1993) Alteration of the structural properties of starch components by the lack of an isoform of starch branching enzyme in rice seeds. J Biol Chem **268**: 19084–19091

192. **Moore PJ, Moore AJ, Collins GB** (1994) Genotypic and developmental regulation of transient expression of a reporter gene in soybean zygotic cotyledons. Plant Cell Rep **13**: 556–560

193. **Morell MK, Blennow A, Kosar-Hashemi B, Samuel MS** (1997) Differential expression and properties of starch branching enzyme isoforms in developing wheat endosperm. Plant Physiol **113**: 201–208

194. **Morell MK, Bloom M, Knowles V, Preiss J** (1987) Subunit structure of spinach leaf ADPglucose pyrophosphorylase. Plant Physiol **85**: 182

195. **Morell MK, Kosar-Hashemi B, Cmiel M, Samuel MS, Chandler P, Rahman S, Buleon A, Batey IL, Li Z** (2003) Barley sex6 mutants lack starch synthase IIa activity and contain a starch with novel properties. Plant J **34**: 173–185

196. **Morell MK, Li Z, Rehman S** (2001) Starch biosynthesis in the small grained cereals-wheat and barley In Barsby TL, Donald AM, Frazier PJ eds Starch: Advances in structure and function. Royal Society of Chemistry, Cambridge pp 129-132

197. **Müller-Röber BT, Koßmann J, Hannah LC, Willmitzer L, Sonnewald U** (1990) One of two different ADP-glucose pyrophosphorylase genes from potato responds strongly to elevated levels of sucrose. Mol Gen Genet **224**: 136–146

198. **Murata T, Sugiyama T, Akazawa T** (1965) Enzymic mechanism of starch synthesis in glutinous rice grains. Biochem Biophys Res Commun **18**: 371–376

199. **Mutisya J, Sathish P, Sun C, Andersson L, Ahlandsberg S, Baguma Y, Palmqvist S, Odhiambo B, Aman P, Jansson C** (2003) Starch branching enzymes in sorghum (*Sorghum bicolor*) and barley (*Hordeum vulgare*): comparative analyses of enzyme structure and gene expression. J Plant Physiol **160**: 921–930

200. **Nair RB, Båga M, Scoles GJ, Kartha KK, Chibbar RN** (1997) Isolation, characterization and expression analysis of a starch branching enzyme II cDNA from wheat. Plant Sci **122**: 153–163

201. **Nakamura T, Vrinten P, Hayakawa K, Ikeda J** (1998) Characterization of a granule-bound starch synthase isoform found in the pericarp of wheat. Plant Physiol **118**: 451–459

202. **Nakamura T, Yamamori M, Hirano H, Hidaka S, Nagamine T** (1995) Production of waxy (amylose-free) wheats. Mol Gen Genet **248**: 253–259

203. **Nakamura Y, Yuki K** (1992) Changes in enzyme activities associated with carbohydrate metabolism during the development of rice endosperm. Plant Sci **82**: 15–20

204. **Nehra NS, Chibbar RN, Leung N, Caswell K, Mallard C, Steinhauer L, Baga M, Kartha KK** (1994) Self-fertile transgenic wheat plants regenerated from isolated scutellar tissues following microprojectile bombardment with two distinct gene constructs. Plant J **5**: 285–297

205. **Nelson D, Cox M** (2008) Lehninger Principles of Biochemistry. W. H. Freeman

206. **Nevo E** (2011) Triticum. *In* C Kole, ed, Wild Crop Relatives: Genomic and Breeding Resources Springer Berlin Heidelberg, pp 407–456

207. **Obenland DM, Simmen U, Boller T, Wiemken A** (1993) Purification and characterization of three soluble invertases from barley (*Hordeum vulgare* L.) leaves. Plant Physiol **101**: 1331–1339

208. **Ohdan T, Francisco PB Jr, Sawada T, Hirose T, Terao T, Satoh H, Nakamura Y** (2005) Expression profiling of genes involved in starch synthesis in sink and source organs of rice. J Exp Bot **56**: 3229–3244

209. **Olive, Ellis RJ, Schuch WW** (1989) Isolation and nucleotide sequences of cDNA clones encoding ADP-glucose pyrophosphorylase polypeptides from wheat leaf and endosperm. Plant Mol Biol **12**: 525–538

210. **Patnaik D, Khurana P** (2003) Genetic transformation of Indian bread (*T. aestivum*) and pasta (*T. durum*) wheat by particle bombardment of mature embryo-derived calli. BMC Plant Biol **3**: 5

211. **Peleg Z, Fahima T, Korol AB, Abbo S, Saranga Y** (2011) Genetic analysis of wheat domestication and evolution under domestication. J Exp Bot **62**: 5051–5061

212. **Pellegrineschi A, Reynolds M, Pacheco M, Brito RM, Almeraya R, Yamaguchi-Shinozaki K, Hoisington D** (2004) Stress-induced expression in wheat of the Arabidopsis thaliana DREB1A gene delays water stress symptoms under greenhouse conditions. Genome **47**: 493–500

213. **Peng M, Hucl P, Chibbar RN** (2001) Isolation, characterization and expression analysis of starch synthase I from wheat (*Triticum aestivum* L.). Plant Sci **161**: 1055–1062

214. **Permingeat HR, Alvarez ML, Cervigni GDL, Ravizzini RA, Vallejos RH** (2003) Stable wheat transformation obtained without selectable markers. Plant Mol Biol **52**: 415–419

215. **Phan TTT, Ishibashi Y, Miyazaki M, Tran HT, Okamura K, Tanaka S, Nakamura J, Yuasa T, Iwaya-Inoue M** (2013) High Temperature-Induced Repression of the Rice Sucrose Transporter (OsSUT1) and Starch Synthesis-Related Genes in Sink and Source Organs at Milky Ripening Stage Causes Chalky Grains. J Agron Crop Sci **199**: 178–188

216. **Pitzschke A, Hirt H** (2010) New insights into an old story: *Agrobacterium*-induced tumour formation in plants by plant transformation. EMBO J **29**: 1021–1032

217. **Pollock CJ** (1986) Tansley review no. 5 fructans and the metabolism of sucrose in vascular plants. New Phytol **104**: 1–24

218. **Prakash P, Sharma-Natu P, Ghildiyal MC** (2004) Effect of different temperature on starch synthase activity in excised grains of wheat cultivars. Indian J Exp Biol **42**: 227–230

219. **Preiss J** (1984) Bacterial Glycogen Synthesis and its Regulation. Annu Rev Microbiol **38**: 419–458

220. **Przetakiewicz A, Orczyk W, Nadolska-Orczyk A** (2003) The effect of auxin on plant regeneration of wheat, barley and triticale. Plant Cell Tissue Organ Cult **73**: 245–256

221. **Radchuk VV, Borisjuk L, Sreenivasulu N, Merx K, Mock H-P, Rolletschek H, Wobus U, Weschke W** (2009) Spatiotemporal profiling of starch biosynthesis and degradation in the developing barley grain. Plant Physiol **150**: 190 –204

222. **Regina A, Bird A, Topping D, Bowden S, Freeman J, Barsby T, Kosar-Hashemi B, Li Z, Rahman S, Morell M** (2006) High-amylose wheat generated by RNA interference improves indices of large-bowel health in rats. Proc Natl Acad Sci U S A **103**: 3546–3551

223. **Regina A, Kosar-Hashemi B, Li Z, Pedler A, Mukai Y, Yamamoto M, Gale K, Sharp PJ, Morell MK, Rahman S** (2005) Starch branching enzyme IIb in wheat is expressed at low levels in the endosperm compared to other cereals and encoded at a non-syntenic locus. Planta **222**: 899–909

224. **Regina A, Kosar-Hashemi B, Ling S, Li Z, Rahman S, Morell M** (2010) Control of starch branching in barley defined through differential RNAi suppression of starch branching enzyme IIa and IIb. J Exp Bot **61**: 1469–1482

225. **Reinders A, Ward JM** (2001) Functional characterization of the alpha-glucoside transporter Sut1p from *Schizosaccharomyces pomb*e, the first fungal homologue of plant sucrose transporters. Mol Microbiol **39**: 445–454

226. **Riffkin HL** (1995) Sucrose metabolism during endosperm development in wheat (*Triticum aestivum*). Physiol Plant **93**: 123–131

227. **Rijven AHGC** (1986) Heat inactivation of starch synthase in wheat endosperm tissue. Plant Physiol **81**: 448–453

228. **Risacher T, Craze M, Bowden S, Paul* W, Barsby T** (2009) Highly efficient *Agrobacterium*-mediated transformation of wheat via in planta inoculation. *In* HD Jones, PR Shewry, eds, Transgenic Wheat Barley Oats. Humana Press, pp 115–124

229. **Ritsema T, Smeekens S** (2003) Fructans: beneficial for plants and humans. Curr Opin Plant Biol **6**: 223–230

230. **Rodermel S** (1999) Subunit control of Rubisco biosynthesis – a relic of an endosymbiotic past? Photosynth Res **59**: 105–123

231. **Roitsch T, González M-C** (2004) Function and regulation of plant invertases: sweet sensations. Trends Plant Sci **9**: 606–613

232. **Rooke L, Byrne D, Salgueiro S** (2000) Marker gene expression driven by the maize ubiquitin promoter in transgenic wheat. Ann Appl Biol **136**: 167–172

233. **Rosche, Blackmore D, Tegeder M, Richardson T, Schroeder H, Higgins TJV, Frommer WB, Offler CE, Patrick JW** (2002) Seed-specific overexpression of a potato sucrose transporter increases sucrose uptake and growth rates of developing pea cotyledons. Plant J Cell Mol Biol **30**: 165–175

234. **Rösti S, Rudi H, Rudi K, Opsahl-Sorteberg H-G, Fahy B, Denyer K** (2006) The gene encoding the cytosolic small subunit of ADP-glucose pyrophosphorylase in barley endosperm also encodes the major plastidial small subunit in the leaves. J Exp Bot **57**: 3619 –3626

235. **Ruan Y-L, Jin Y, Yang Y-J, Li G-J, Boyer JS** (2010) Sugar input, metabolism, and signaling mediated by invertase: roles in development, yield potential, and response to drought and heat. Mol Plant **3:** 942-955

236. **Rydberg U, Andersson L, Andersson R, Åman P, Larsson H** (2001) Comparison of starch branching enzyme I and II from potato. Eur J Biochem **268**: 6140–6145

237. **Saeedipour S** (2011) Activities of sucrose-metabolizing enzymes in grains of two wheat (*Triticum aestivum* L.) cultivars subjected to water stress dur. J Plant Breed Crop Sci **3**: 106–113

238. **Sakthivel G** (2011) Expression analysis of genes involved in sucrose transport and metabolism during grain filling development in wheat. MSc thesis. University of Manitoba, Winnipeg, Manitoba, Canada

239. **Sakulsingharoj C, Choi SB, Hwang SK, Edwards GE, Bork J, Meyer CR, Preiss J, Okita TW** (2004) Engineering starch biosynthesis for increasing rice seed weight: the role of the cytoplasmic ADP-glucose pyrophosphorylase. Plant Sci **167**: 1323–1333

240. **Salehuzzaman SN, Jacobsen E, Visser RG** (1992) Cloning, partial sequencing and expression of a cDNA coding for branching enzyme in cassava. Plant Mol Biol **20**: 809–819

241. **Sarkar P, Stebbins GL** (1956) Morphological evidence concerning the origin of the B genome in wheat. Am J Bot **43**: 297

242. **Sasanuma T** (2001) Characterization of the rbcS multigene family in wheat: subfamily classification, determination of chromosomal location and evolutionary analysis. Mol Genet Genomics **265**: 161–171

243. **Sato K** (1984) Starch granules in tissues of rice plants and their changes in relation to plant growth. JARQ **18**: 79-86

244. **Satoh H, Nishi A, Yamashita K, Takemoto Y, Tanaka Y, Hosaka Y, Sakurai A, Fujita N, Nakamura Y** (2003) Starch-branching enzyme I-deficient mutation specifically affects the structure and properties of starch in rice endosperm. Plant Physiol **133**: 1111–1121

245. **Sauer N** (2007) Molecular physiology of higher plant sucrose transporters. FEBS Lett **581**: 2309–2317

246. **Schaarschmidt S, Hause B** (2008) Apoplastic invertases. Plant Signal Behav **3**: 317–319

247. **Scheible WR, Gonzalez-Fontes A, Lauerer M, Muller-Rober B, Caboche M, Stitt M** (1997) Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. Plant Cell **9**: 783

248. **Schnyder H, Gillenberg C, Hinz J** (1993) Fructan contents and dry matter deposition in different tissues of the wheat grain during development. Plant Cell Environ **16**: 179–187

249. **Schwall GP, Safford R, Westcott RJ, Jeffcoat R, Tayal A, Shi YC, Gidley MJ, Jobling SA** (2000) Production of very-high-amylose potato starch by inhibition of SBE A and B. Nat Biotechnol **18**: 551–554

250. **Scofield GN, Aoki N, Hirose T, Takano M, Jenkins CLD, Furbank RT** (2007) The role of the sucrose transporter, OsSUT1, in germination and early seedling growth and development of rice plants. J Exp Bot **58**: 483–495

251. **Scofield, Wang X-D, Patrick JW, Offler CE, Furbank RT** (2004) Expression and localisation analysis of the wheat sucrose transporter TaSUT1 in vegetative tissues. Planta **219**: 176–184

252. **Sears ER** (1969) Wheat cytogenetics. Annu Rev Genet **3**: 451–468

253. **Sears ER** (1976) Genetic control of chromosome pairing in wheat. Annu Rev Genet **10**: 31–51

254. **Seed Manitoba** (2010) http://issuu.com/fbcpublishing/docs/smb091210. Accessed : 2014-06-20 11:20:05

255. **Seiler C, Harshavardhan VT, Rajesh K, Reddy PS, Strickert M, Rolletschek H, Scholz U, Wobus U, Sreenivasulu N** (2011) ABA biosynthesis and degradation contributing to ABA

homeostasis during barley seed development under control and terminal drought-stress conditions. J Exp Bot **62**: 2615-2632

256. **Shewry PR** (2009) Wheat. J Exp Bot **60**: 1537–1553

257. **Shimbata T, Inokuma T, Sunohara A, Vrinten P, Saito M, Takiya T, Nakamura T** (2011) High levels of sugars and fructan in mature seed of sweet wheat lacking GBSSI and SSIIa enzymes. J Agric Food Chem **59**: 4794–4800

258. **Shirsat A, Wilford N, Croy R, Boulter D** (1989) Sequences responsible for the tissue specific promoter activity of a pea legumin gene in tobacco. Mol Gen Genet **215**: 326–331

259. **Sikka VK, Choi S-B, Kavakli IH, Sakulsingharoj C, Gupta S, Ito H, Okita TW** (2001) Subcellular compartmentation and allosteric regulation of the rice endosperm ADPglucose pyrophosphorylase. Plant Sci **161**: 461–468

260. **Simmonds J, Stewart P, Simmonds D** (1992) Regeneration of *Triticum aestivum* apical explants after microinjection of germ line progenitor cells with DNA. Physiol Plant **85**: 197–206

261. **Simmons SR, Crookston RK** (1979) Rate and duration of growth of kernels formed at specific florets in spikelets of spring wheat. Crop Sci **19**: 690

262. **Singletary, R B, P.l K** (1994) Heat stress during grain filling in maize: effects on carbohydrate storage and metabolism. Aust J Plant Physiol **21**:829-841

263. **Smart MG, O'Brien TP** (1983) The development of the wheat embryo in relation to the neighbouring tissues. Protoplasma **114**: 1–13

264. **Smidansky, Clancy M, Meyer FD, Lanning SP, Blake NK, Talbert LE, Giroux MJ** (2002) Enhanced ADP-glucose pyrophosphorylase activity in wheat endosperm increases seed yield. Proc Natl Acad Sci U S A **99**: 1724

265. **Smidansky, Martin JM, Hannah CL, Fischer AM, Giroux MJ** (2003) Seed yield and plant biomass increases in rice are conferred by deregulation of endosperm ADP-glucose pyrophosphorylase. Planta **216**: 656–664

266. **Smidansky, Meyer F, Blakeslee B, Weglarz T, Greene T, Giroux M** (2007) Expression of a modified ADP-glucose pyrophosphorylase large subunit in wheat seeds stimulates photosynthesis and carbon metabolism. Planta **225**: 965–976

267. **Smith** (2006) HGCA - Wheat as a feedstock for alcohol production. http://www.hgca.com/cms_publications.output/2/2/Publications/Final%20project%20reports/Wheat%20as%20a%20feedstock%20for%20alcohol%20production.mspx?fn=show&pubcon=3588. Accessed: 2013-07-10 20:28:42

268. **Smith AM** (1988) Major differences in isoforms of starch-branching enzyme between developing embryos of round- and wrinkled-seeded peas (*Pisum sativum* L.). Planta **175**: 270–279

269. **Smith-White BJ, Preiss J** (1992) Comparison of proteins of ADP-glucose pyrophosphorylase from diverse sources. J Mol Evol **34**: 449–464

270. **Song P, Heinen JL, Burns TH, Allen RD** (2000) Expression of two tissue-specific promoters in transgenic cotton plants. J Cotton Sci **4**: 217–223

271. **Sowokinos JR, Preiss J** (1982) Pyrophosphorylases in Solanum tuberosum III. Purification, physical, and catalytic properties of ADP-glucose pyrophosphorylase in potatoes. Plant Physiol **69**: 1459–1466

272. **Sreenivasulu N, Altschmied L, Radchuk V, Gubatz S, Wobus U, Weschke W** (2004) Transcript profiles and deduced changes of metabolic pathways in maternal and filial tissues of developing barley grains. Plant J Cell Mol Biol **37**: 539–553

273. **Sreenivasulu N, Radchuk V, Strickert M, Miersch O, Weschke W, Wobus U** (2006) Gene expression patterns reveal tissue-specific signaling networks controlling programmed cell death and ABA- regulated maturation in developing barley seeds. Plant J **47**: 310–327

274. **Stalker DM, Mcbride KE, Malyj LD** (1988) Herbicide resistance in transgenic plants expressing a bacterial detoxification gene. Science **242**: 419–423

275. **Stark DM, Timmerman KP, Barry GF, Preiss J, Kishore GM** (1992) Regulation of the amount of starch in plant tissues by ADP glucose pyrophosphorylase. Science **258**: 287–292

276. **Sturm A** (1999) Invertases. Primary structures, functions, and roles in plant development and sucrose partitioning. Plant Physiol **121**: 1–8

277. **Sun C, Sathish P, Ahlandsberg S, Jansson C** (1998) The two genes encoding starch-branching enzymes IIa and IIb are differentially expressed in barley. Plant Physiol **118**: 37–49

278. **Suzuki Y, Nakabayashi K, Yoshizawa R, Mae T, Makino A** (2009) Differences in expression of the RBCS multigene family and rubisco protein content in various rice plant tissues at different growth stages. Plant Cell Physiol **50**: 1851–1855

279. **Szydlowski N, Ragel P, Raynaud S, Lucas MM, Roldán I, Montero M, Muñoz FJ, Ovecka M, Bahaji A, Planchot V, et al** (2009) Starch granule initiation in *Arabidopsis* requires the presence of either class IV or class III starch synthases. Plant Cell **21**: 2443 –2457

280. **Taiz L, Zeiger E** (2010) Photosynthesis: The Carbon Reactions. *In* Plant physiology. Ed 5, Sinauer Associates, Sunderland, Massachusetts, USA, pp 199-242

281. **Takeda S, Matsuoka M** (2008) Genetic approaches to crop improvement: responding to environmental and population changes. Nat Rev Genet **9**: 444–457

282. **Takumi S, Shimada T** (1997) Variation in transformation frequencies among six common wheat cultivars through particle bombardment of scutellar tissues. Genes Genet Syst **72**: 63–69

283. **Tang T, Xie H, Wang Y, Lü B, Liang J** (2009) The effect of sucrose and abscisic acid interaction on sucrose synthase and its relationship to grain filling of rice (*Oryza sativa* L.). J Exp Bot **60**: 2641–2652

284. **Terzaghi WB, Cashmore AR** (1995) Light-regulated transcription. Annu Rev Plant Physiol Plant Mol Biol **46**: 445–474

285. **Tetlow** (2011) Starch biosynthesis in developing seeds. Seed Sci Res 2**1**: 5-32

286. **Tetlow IJ, Beisel KG, Cameron S, Makhmoudova A, Liu F, Bresolin NS, Wait R, Morell MK, Emes MJ** (2008) Analysis of protein complexes in wheat amyloplasts reveals functional interactions among starch biosynthetic enzymes. Plant Physiol **146**: 1878–1891

287. **Tetlow IJ, Morell MK, Emes MJ** (2004a) Recent developments in understanding the regulation of starch metabolism in higher plants. J Exp Bot **55**: 2131–2145

288. **Tetlow IJ, Wait R, Lu Z, Akkasaeng R, Bowsher CG, Esposito S, Kosar-Hashemi B, Morell MK, Emes MJ** (2004b) Protein phosphorylation in amyloplasts regulates starch branching enzyme activity and protein–protein interactions. Plant Cell **16**: 694 –708

289. **Thorbjornsen T, Villand P, Kleczkowski LA, Olsen OA** (1996) A single gene encodes two different transcripts for the ADP-glucose pyrophosphorylase small subunit from barley (*Hordeum vulgare*). Biochem J **313**: 149

290. **Tiessen A** (2002) Starch synthesis in potato tubers is regulated by post-translational redox modification of ADP-glucose pyrophosphorylase: A novel regulatory mechanism linking starch synthesis to the sucrose supply. Plant Cell **14**: 2191–2213

291. **Umemoto T, Yano M, Satoh H, Shomura A, Nakamura Y** (2002) Mapping of a gene responsible for the difference in amylopectin structure between japonica-type and indica-type rice varieties. Theor Appl Genet **104**: 1–8

292. **Varshney A, Altpeter F** (2001) Stable transformation and tissue culture response in current European winter wheats (*Triticum aestivum* L.). Mol Breed **8**: 295–309

293. **Vasil V, Castillo AM, Fromm ME, Vasil IK** (1992) Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Nat Biotechnol **10**: 667–674

294. **Villand P, Aalen R, Olsen OA, Lüthi E, Lönneborg A, Kleczkowski LA** (1992) PCR amplification and sequences of cDNA clones for the small and large subunits of ADP-glucose pyrophosphorylase from barley tissues. Plant Mol Biol **19**: 381–389

295. **Vrinten PL, Nakamura T** (2000) Wheat granule-bound starch synthase I and II are encoded by separate genes that are expressed in different tissues. Plant Physiol **122**: 255

296. **Vrinten PL, Shimbata T, Yanase M, Sunohara A, Saito M, Inokuma T, Takiya T, Takaha T, Nakamura T** (2012) Properties of a novel type of starch found in the double mutant "sweet wheat" Carbohydr Polym **89**: 1250–1260

297. **Wang N, Fisher DB** (1995) Sucrose release into the endosperm cavity of wheat grains apparently occurs by facilitated diffusion across the nucellar cell membranes. Plant Physiol **109**: 579–585

298. **Wang S-J, Yeh K-W, Tsai C-Y** (2001) Regulation of starch granule-bound starch synthase I gene expression by circadian clock and sucrose in the source tissue of sweet potato. Plant Sci **161**: 635–644

299. **Wang, Xu H, Zhu Y, Liu Q-Q, Cai X-L** (2013a) OsbZIP58, a basic leucine zipper transcription factor, regulates starch biosynthesis in rice endosperm. J Exp Bot **64**: 3453–3466

300. **Wang Y, Fu B, Pan L, Chen L, Fu X, Li K** (2013b) Overexpression of *Arabidopsis* Dof1, GS1 and GS2 enhanced nitrogen assimilation in transgenic tobacco grown under low-nitrogen conditions. Plant Mol Biol Report **31**: 886–900

301. **Wang Z, Chen X, Wang J, Liu T, Liu Y, Zhao L, Wang G** (2007a) Increasing maize seed weight by enhancing the cytoplasmic ADP-glucose pyrophosphorylase activity in transgenic maize plants. Plant Cell Tissue Organ Cult **88**: 83–92

302. **Wang Z, Chen X, Wang J, Liu T, Liu Y, Zhao L, Wang G** (2007b) Increasing maize seed weight by enhancing the cytoplasmic ADP-glucose pyrophosphorylase activity in transgenic maize plants. Plant Cell Tissue Organ Cult **88**: 83–92

303. **Wang ZY, Zheng FQ, Shen GZ, Gao JP, Snustad DP, Li MG, Zhang JL, Hong MM** (1995) The amylose content in rice endosperm is related to the post-transcriptional regulation of the waxy gene. Plant J **7**: 613–622

304. **Weatherwax P** (1922) A Rare Carbohydrate in Waxy Maize. Genetics **7**: 568–572

305. **Weir B, Gu X, Wang M, Upadhyaya N, Elliott AR, Brettell RIS** (2001) *Agrobacterium tumefaciens*-mediated transformation of wheat using suspension cells as a model system and green fluorescent protein as a visual marker. Funct Plant Biol **28**: 807–818

306. **Wenck A, Pugieux C, Turner M, Dunn M, Stacy C, Tiozzo A, Dunder E, Grinsven E van, Khan R, Sigareva M, et al** (2003) Reef-coral proteins as visual, non-destructive reporters for plant transformation. Plant Cell Rep **22**: 244–251

307. **Werr W, Frommer WB, Maas C, Starlinger P** (1985) Structure of the sucrose synthase gene on chromosome 9 of *Zea mays* L. EMBO J **4**: 1373

308. **Weschke W, Panitz R, Gubatz S, Wang Q, Radchuk R, Weber H, Wobus U** (2003) The role of invertases and hexose transporters in controlling sugar ratios in maternal and filial tissues of barley caryopses during early development. Plant J **33**: 395–411

309. **Weschke W, Panitz R, Sauer N, Wang Q, Neubohn B, Weber H, Wobus U** (2000) Sucrose transport into barley seeds: molecular characterization of two transporters and implications for seed development and starch accumulation. Plant J **21**: 455–467

310. **Whan B, Carlton G, Anderson W** (1996) Potential for increasing rate of grain growth in spring wheat. I. Identification of genetic improvements. Aust J Agric Res **47**: 17–31

311. **Wheat- a plant that changed the world** (2014). http://www.allaboutwheat.info/default.html. Accessed 2014-05-05 17:31:13

312. **Wheat evolution**: Integrating archaeological and biological evidence. http://kew.academia.edu/MarkNesbitt/Papers/1529731/Wheat_evolution_integrating_archaeological_and_biological_evidence. Accessed 2012-08-25 13:59:01

313. **Whitt SR, Wilson LM, Tenaillon MI, Gaut BS, Buckler ES** (2002) Genetic diversity and selection in the maize starch pathway. Proc Natl Acad Sci U S A **99**: 12959–12962

314. **Wilson LM, Whitt SR, Ibáñez AM, Rocheford TR, Goodman MM, Buckler ES** (2004) Dissection of maize kernel composition and starch production by candidate gene association. Plant Cell **16**: 2719–2733

315. **World Food Situation** (2012) FAO Cereal Supply and Demand Brief. http://www.fao.org/worldfoodsituation/wfs-home/csdb/en/. Accessed 2012-08-26 00:11:16

316. **Wright M, Dawson J, Dunder E, Suttie J, Reed J, Kramer C, Chang Y, Novitzky R, Wang H, Artim-Moore L** (2001) Efficient biolistic transformation of maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) using the phosphomannose isomerase gene, pmi, as the selectable marker. Plant Cell Rep **20**: 429–436

317. **Wrigley C, Asenstorfer R, Batey I, Cornish G, Day L, Mares D, Mrva K** (2009) The Biochemical and molecular basis of wheat quality. *In* BFCE Regentsessor, ed, Wheat Science and Trade. Wiley-Blackwell, pp 495–520

318. **Wu H, Sparks C, Amoah B, Jones HD** (2003) Factors influencing successful *Agrobacterium*-mediated genetic transformation of wheat. Plant Cell Rep **21**: 659–668

319. **Lev-Yadun S, Gopher A, Abbo S** (2000) The cradle of agriculture. Science **288**: 1602–1603

320. **Yamamori M, Endo T** (1996) Variation of starch granule proteins and chromosome mapping of their coding genes in common wheat. Theor Appl Genet **93**: 275–281

321. **Yamamori M, Fujita S, Hayakawa K, Matsuki J, Yasui T** (2000) Genetic elimination of a starch granule protein, SGP-1, of wheat generates an altered starch with apparent high amylose. Theor Appl Genet **101**: 21–29

322. **Yamamori M, Kato M, Yui M, Kawasaki M** (2006) Resistant starch and starch pasting properties of a starch synthase IIa-deficient wheat with apparent high amylose. Aust J Agric Res **57**: 531–535

323. **Yamamori M, Nakamura T, Endo TR, Nagamine T** (1994) Waxy protein deficiency and chromosomal location of coding genes in common wheat. Theor Appl Genet **89**: 179–184

324. **Yang J, Zhang J, Wang Z, Xu G, Zhu Q** (2004b) Activities of key enzymes in sucrose-to-starch conversion in wheat grains subjected to water deficit during grain filling. Plant Physiol **135**: 1621–1629

325. **Yang J, Zhang J, Wang Z, Zhu Q, Liu L** (2004a) Activities of fructan- and sucrose-metabolizing enzymes in wheat stems subjected to water stress during grain filling. Planta **220**: 331–343

326. **Zeng D, Yan M, Wang Y, Liu X, Qian Q, Li J** (2007) Du1, encoding a novel Prp1 protein, regulates starch biosynthesis through affecting the splicing of Wxb pre-mRNAs in rice (*Oryza sativa* L.). Plant Mol Biol **65**: 501–509

327. **Zhang W, McElroy D, Wu R** (1991) Analysis of rice Act1 5' region activity in transgenic rice plants. Plant Cell **3**: 1155–1165

328. **Zhang X, Colleoni C, Ratushna V, Sirghie-Colleoni M, James MG, Myers AM** (2004) Molecular characterization demonstrates that the *Zea mays* gene sugary2 codes for the starch synthase isoform SSIIa. Plant Mol Biol **54**: 865–879

329. **Zhu G, Ye N, Yang J, Peng X, Zhang J** (2011) Regulation of expression of starch synthesis genes by ethylene and ABA in relation to the development of rice inferior and superior spikelets. J Exp Bot **62**: 3907-3916

330. **Zhu Y, Cai X-L, Wang Z-Y, Hong M-M** (2003) An interaction between a MYC protein and an EREBP protein is involved in transcriptional regulation of the rice Wx gene. J Biol Chem **278**: 47803–47811

331. **Zimmermann Z** (1975) List of sugars and sugar alcohols in sieve-tube exudates. In MH Zimmermann, JA Milburn, eds, Encyclopedia of Plant Physiology, NS Vol 1. Transport in Plants 1: Phloem Transport. Springer, New York, pp 480–503

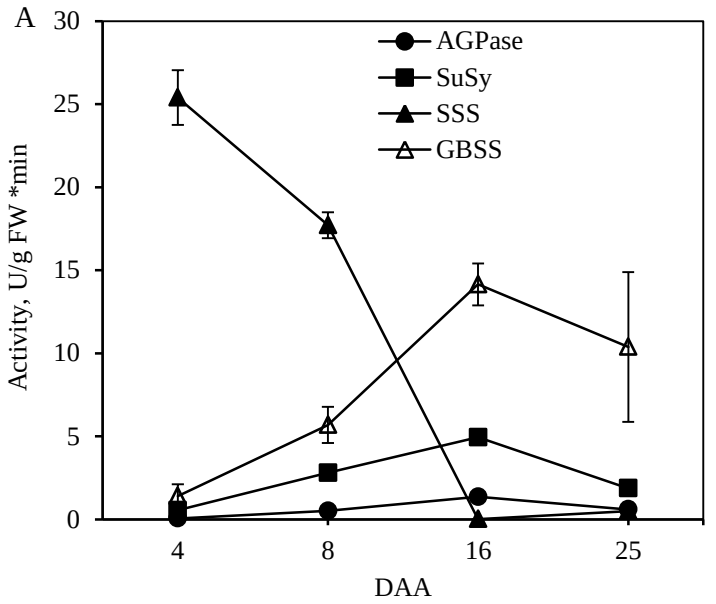
332. **Zohary D** (1989) Pulse domestication and cereal domestication: How different are they? Econ Bot **43**: 31–34

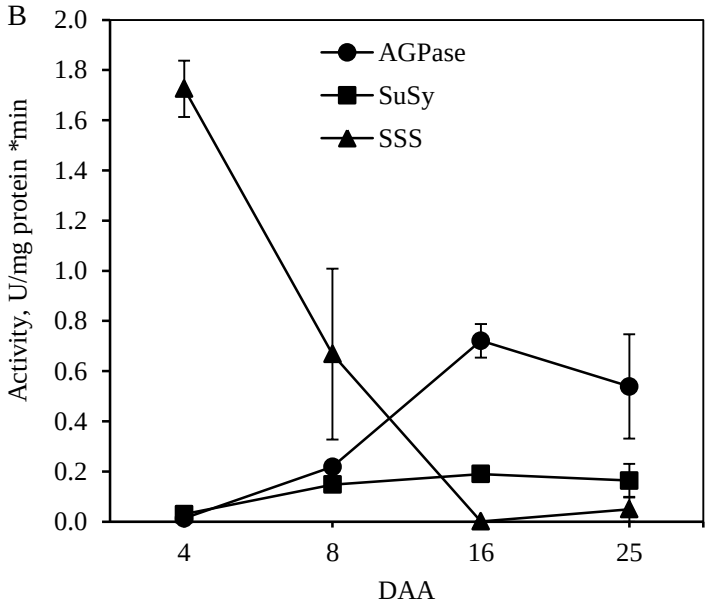
333. **Zupan J, Muth TR, Draper O, Zambryski P** (2000) The transfer of DNA from Agrobacterium tumefaciens into plants: a feast of fundamental insights. Plant J 23: 11–28

Appendix 1: List of primers and their respective sequences that are used for amplifying the target genes involved in sucrose-to-starch metabolism and fructan hydrolysis and the reference gene β-actin

Gene	Direction	Primer sequence	Amplicon length (bp)	R^2 value	PCR Efficiency%
AGPS1a	Forward	5'-GCCTTTGGCATCTAAAACCTT-3'	102	0.976	98.74
	Reverse	5'-**G**GATTGAGATCTGCATGCTTC-3'			
AGPS1b	Forward	5'-CACCTCCTGCGACTCCTT-3'	146	0.989	99.93
	Reverse	5'-GACAGGTCTGGGAGCTCTTG-3'			
AGPS2	Forward	5'-CCTCAACAGCAATGTGTCCAA-3'	137	0.998	95.79
	Reverse	5'-GCGCAGCGAGAACTTCGA-3'			
AGPL1	Forward	5'-CAACTCGGCCTCTCTTAATCGT-3'	88	0.990	95.07
	Reverse	5'-GGCCAATACCTCAACAGATCCA-3'			
AGPL2	Forward	5'-GGTAGAGCACAGTGTCGTTGGA-3'	80	0.999	103.43
	Reverse	5'-CAGCACCGAGCATTACCGTAT-3'			
SSI	Forward	5'-CGCTAACCGTGGACAAGATGT-3'	102	0.970	90.41
	Reverse	5'-TCGTCATGCCTCGCTTCA-3'			
SSIIa	Forward	5'-CAGATGATGCGGGCTCCTT-3'	147	0.996	105.14
	Reverse	5'-GCAAAGCACCGGCAACA-3'			
SSIIb	Forward	5'-AGCTGCGACCAGATCCAAAG-3'	94	0.996	129.93
	Reverse	5'-TCCAACCTGTTCAGCGACAA-3'			
SSIIIa	Forward	5'-AACTGATGTCCCTATTGTCGGAAT-3'	80	0.999	102.10
	Reverse	5'-GGTGAATTGCGTGCTTGATG-3'			
SSIIIb	Forward	5'-TGCAGGAGAAGCTTGGATTACA-3'	98	0.979	124.52
	Reverse	5'-TGCATGTCTGATAAGGTGAATTCC-3'			
SSIV	Forward	5'-CCAAGCATGTAACAGCGAAA-3'	148	0.996	96.20
	Reverse	5'-CCATAGTTCCGAAGGCATGT-3'			
GBSSI	Forward	5'-ACTACCAGTCCAATGGCATCTACAG-3'	106	0.993	94.71
	Reverse	5'-AGGTTGAGCTGCGCGAAGT-3'			
GBSSII	Forward	5'-ACCGTCGATCCAGCAGATGT-3'	91	0.990	104.24
	Reverse	5'-GAACCATTGCGTGGAACGA-3'			
SBEI	Forward	5'-CGCCAGTGGAGCCTCTCA-3'	82	0.949	101.42
	Reverse	5'-CGTCGAGCGCATTCATTG-3'			
SBEIIa	Forward	5'-GAATGGTTTCGATGGCACTGA-3'	80	0.994	94.00
	Reverse	5'-ATAGACGAGAATCCCACATCCAA-3'			
SBEIIb	Forward	5'-GATCCCTGACGGCGGTAGT-3'	82	0.994	101.18
	Reverse	5'-TGGAACCTTCAGATCATCAGAATC-3''			
SUT1	Forward	5'-TGGATTCTGGCTCCTTGAC-3'	150	0.946	103.86
	Reverse	5'-GCCATCCAAGAACAGAAGATT-3'			
SUT2	Forward	5'-TACGGAGTCCTGCTCTGTCA-3'	150	0.984	116.87
	Reverse	5'- CTCGTCGCTTCCGAAAGTA-3'			
SUT4	Forward	5'-TGGCTACAACAATACTAAGTTGGATC-'3	112	0.993	70.39
	Reverse	5'- ATAAGTGATCGAGAGTGGCAAT-'3			
SUT5	Forward	5'- TGCAGCTGTCCCTCCTCTC-'3	102	0.953	71.37
	Reverse	5'- ACGATGGGTTGCACCACAAAT-'3			
SuSy1	Forward	5'-TGAAGTGTCGGCTGCGTTAT-3'	163	0.987	101.26
	Reverse	5'-TATGGGCAGGCGTTTATTCC-3'			
SuSy2	Forward	5'- CCGAGCCACTGGAACAAGAT -3'	175	0.963	105.55
	Reverse	5'- GGGCGTAGAGCATTTCAAGGTA -3'			
SuSy3	Forward	5'- CGGTCAAGCTAATGTGCTGG -3'	81	0.964	91.66
	Reverse	5'- TCCTCCAAAGCACGAACTTG -3'			
CWINV1	Forward	5'- AGACTTTTACCCCGTGGCGGT -3'	146	0.915	96.35
	Reverse	5'- TGTAGTACTCGTAGCGCGTTACG -3'			
VINV5	Forward	5'- ATCGCTGCCGTCAACGAGGC -3'	86	0.999	96.12
	Reverse	5'- CCGAAAGGACCGAGAGCGCC -3'			
1-FEH	Forward	5'- ACAAGGCGCCAAGCATGCCC -3'	127	0.997	100.92
	Reverse	5'- CAGCCACAGCCGACGGTCATC -3'			
6-FEH	Forward	5'- GGTGAATGCCGGTGGCGTGA-3'	162	0.994	96.12
	Reverse	5'- CCTGGCACGGACGCACCATT-3'			
Actin	Forward	5'-GCTGTTCCAGCCATCTCATGT-3'	156	0.981	106.85
	Reverse	5'-CGATCAGCAATTCCAGGAAAC-3'			

Appendix 2: Activities of sucrose synthase (SuSy), AGPase, soluble (SSS) and granule bound starch synthases (GBSS) in developing grains of wheat cv. Whitebird grown under greenhouse conditions. Activities are on the basis of per gram fresh weight (A) and per mg protein (B). Data are means ± SE, n=3.





Appendix 3: Analysis of Variance (ANOVA) for expression data of *AGPL1*, *AGPL2*, *AGPS1a*, *AGPS1b*, *AGPS2*, *SSI*, *SSIIa*, *SSIIb*, *SSIIIa*, *SSIIIb*, *SBEI*, *SBEIIa* and *SBEIIb* genes in Chinese Spring (CS), CDC Teal (CDC) and AC Andrew (ACA) using the PROC Mixed procedure of SAS software. The relative transcript levels were transformed to their log10 values in order to comply with normal distribution.

Appendix 3.1 a: Type 3 tests of fixed effects for relative transcript levels of ***AGPL1*** in wheat cultivars at five grain developmental stages at $p \leq 0.05$

Effect	Num DF	Den DF	F Value	Pr > F
Cult	2	6	7.59	0.0227
Stage	4	24	8.88	0.0002
Cult*stage	8	24	3.02	0.0171

Appendix 3.1 b: Letter groups for wheat cultivars based on the least significant difference for the means of the relative transcript level of ***AGPL1*** at $p \leq 0.05$

Effect= Cult Method= LSD (P < .05) Set= 1

Obs	Cult	stage	Estimate	Standard Error	Letter Group
1	ACA		0.6525	0.1014	A
2	CS		0.3595	0.1014	AB
3	CDC		0.09413	0.1014	B

Appendix 3.1 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***AGPL1*** at $p \leq 0.05$

Effect= stage Method= LSD (P < .05) Set= 2

Obs	Cult	stage	Estimate	Standard Error	Letter Group
4		8	0.6615	0.05267	A
5		25	0.6594	0.1182	AB
6		16	0.4858	0.03831	B
7		30	0.1065	0.1822	C
8		4	-0.06962	0.1426	C

Appendix 3.1 d: Letter groups for wheat cultivars and grain developmental stages interaction based on the least significant difference for the means of the relative transcript level of ***AGPL1*** at $p \leq 0.05$

Effect= Cult*stage Method= LSD (P < .05) Set= 3

Obs	Cult	stage	Estimate	Standard Error	Letter Group
9	ACA	8	1.2826	0.09123	A
10	ACA	16	0.9055	0.06635	B
11	ACA	25	0.8670	0.2047	ABC
12	CDC	25	0.6582	0.2047	BCD
13	CS	30	0.6147	0.3156	ABCDE
14	CS	8	0.5103	0.09123	CD
15	CS	25	0.4531	0.2047	CDE
16	ACA	4	0.4315	0.2470	BCDE
17	CS	16	0.3589	0.06635	DE
18	CDC	16	0.1931	0.06635	E
19	CDC	8	0.1917	0.09123	E
20	CDC	30	-0.07125	0.3156	DEF
21	CS	4	-0.1394	0.2470	EF
22	ACA	30	-0.2241	0.3156	EF
23	CDC	4	-0.5010	0.2470	F

Appendix 3.2 a: Type 3 tests of fixed effects for relative transcript levels of ***AGPL2*** in wheat cultivars at five grain developmental stages at $p \leq 0.05$

Effect	Num DF	Den DF	F Value	Pr > F
Cult	2	6	6.69	0.0296
Stage	4	24	102.59	<.0001
Cult*stage	8	24	6.39	0.0002

Appendix 3.2 b: Letter groups for wheat cultivars based on the least significant difference for the means of the relative transcript level of ***AGPL2*** at $p \leq 0.05$

Effect= Cult Method= LSD (P < .05) Set= 1

Obs	Cult	stage	Estimate	Standard Error	Letter Group
1	ACA		-1.7092	0.02163	A
2	CDC		-1.7474	0.02163	AB
3	CS		-1.8194	0.02163	B

Appendix 3.2 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***AGPL2*** at $p \leq 0.05$

Effect= stage Method= LSD (P < .05) Set= 2

Obs	Cult	stage	Estimate	Standard Error	Letter Group
4		8	-1.4730	0.02605	A
5		4	-1.4875	0.02605	A
6		16	-1.8662	0.02605	B
7		30	-1.9666	0.02605	C
8		25	-2.0000	0.02605	C

Appendix 3.2 d: Letter groups for wheat cultivars and grain developmental stages interaction based on the least significant difference for the means of the relative transcript level of ***AGPL2*** at $p \leq 0.05$

Effect= Cult*stage Method= LSD (P < .05) Set= 3

Obs	Cult	stage	Estimate	Standard Error	Letter Group
9	ACA	4	-1.3656	0.04512	A
10	CDC	4	-1.3979	0.04512	A
11	CS	8	-1.3979	0.04512	A
12	ACA	8	-1.4812	0.04512	AB
13	CDC	8	-1.5399	0.04512	B
14	ACA	16	-1.6990	0.04512	C
15	CS	4	-1.6990	0.04512	C
16	CDC	30	-1.8997	0.04512	D
17	CDC	16	-1.8997	0.04512	D
18	CDC	25	-2.0000	0.04512	D
19	ACA	25	-2.0000	0.04512	D
20	ACA	30	-2.0000	0.04512	D
21	CS	25	-2.0000	0.04512	D
22	CS	30	-2.0000	0.04512	D
23	CS	16	-2.0000	0.04512	D

Appendix 3.3 a: Type 3 tests of fixed effects for relative transcript levels of ***AGPS1a*** in wheat cultivars at five grain developmental stages at $p \leq 0.05$

Effect	Num DF	Den DF	F Value	Pr > F
Cult	2	6	15.78	0.0041
stage	4	24	42.34	<.0001
Cult*stage	8	24	3.93	0.0043

Appendix 3.3 b: Letter groups for wheat cultivars based on the least significant difference for the means of the relative transcript level of ***AGPS1a*** at $p \leq 0.05$

Effect= Cult Method= LSD (P < .05) Set= 1

Obs	Cult	stage	Estimate	Standard Error	Letter Group
1	ACA		1.3578	0.07922	A
2	CS		0.8414	0.07922	B
3	CDC		0.7880	0.07922	B

Appendix 3.3 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***AGPS1a*** at $p \leq 0.05$

Effect= stage Method= LSD (P < .05) Set= 2

Obs	Cult	stage	Estimate	Standard Error	Letter Group
4		30	1.5126	0.1023	A
5		16	1.4337	0.1023	A
6		25	1.3687	0.1023	A
7		8	0.7143	0.1023	B
8		4	-0.05046	0.1023	C

Appendix 3.3 d: Letter groups for wheat cultivars and grain developmental stages interaction based on the least significant difference for the means of the relative transcript level of ***AGPS1a*** at $p \leq 0.05$

Effect= Cult*stage Method= LSD (P < .05) Set= 3

Obs	Cult	stage	Estimate	Standard Error	Letter Group
9	ACA	16	2.3253	0.1771	A
10	ACA	25	1.7859	0.1771	B
11	CDC	30	1.6619	0.1771	B
12	ACA	30	1.5163	0.1771	BC
13	CS	30	1.3595	0.1771	BCD
14	CDC	25	1.3587	0.1771	BCD
15	CS	16	1.0464	0.1771	CD
16	CS	25	0.9615	0.1771	D
17	CDC	16	0.9293	0.1771	D
18	ACA	8	0.8885	0.1771	D
19	CS	8	0.8880	0.1771	D
20	CDC	8	0.3666	0.1771	E
21	ACA	4	0.2732	0.1771	E
22	CS	4	-0.04827	0.1771	EF
23	CDC	4	-0.3763	0.1771	F

Appendix 3.4 a: Type 3 tests of fixed effects for relative transcript levels of ***AGPS1b*** in wheat cultivars at five grain developmental stages at $p \leq 0.05$

Effect	Num DF	Den DF	F Value	Pr > F
Cult	2	6	35.71	0.0005
Stage	4	24	71.88	<.0001
Cult*stage	8	24	14.00	<.0001

Appendix 3.4 b: Letter groups for wheat cultivars based on the least significant difference for the means of the relative transcript level of ***AGPS1b*** at $p \leq 0.05$

Effect= Cult Method= LSD (P < .05) Set= 1

Obs	Cult	stage	Estimate	Standard Error	Letter Group
1	CS		0.6317	0.04445	A
2	ACA		0.5920	0.04445	A
3	CDC		0.1530	0.04445	B

Appendix 3.4 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***AGPS1b*** at $p \leq 0.05$

Effect= stage Method= LSD (P < .05) Set= 2

Obs	Cult	stage	Estimate	Standard Error	Letter Group
4		8	1.0518	0.05164	A
5		4	0.7292	0.05164	B
6		25	0.3431	0.05164	C
7		30	0.1321	0.05164	D
8		16	0.03830	0.05164	D

Appendix 3.4 d: Letter groups for wheat cultivars and grain developmental stages interaction based on the least significant difference for the means of the relative transcript level of ***AGPS1b*** at $p \leq 0.05$

Effect= Cult*stage Method= LSD (P < .05) Set= 3

Obs	Cult	stage	Estimate	Standard Error	Letter Group
9	CS	8	1.4716	0.08945	A
10	ACA	4	1.1783	0.08945	B
11	CS	4	1.1217	0.08945	B
12	ACA	8	1.0728	0.08945	B
13	CDC	8	0.6111	0.08945	C
14	CS	25	0.4704	0.08945	CD
15	ACA	25	0.4127	0.08945	CD
16	ACA	30	0.2596	0.08945	DE
17	CS	30	0.1487	0.08945	EFG
18	CDC	25	0.1462	0.08945	EF
19	CDC	16	0.1321	0.08945	EFG
20	ACA	16	0.03658	0.08945	EFG
21	CDC	30	-0.01181	0.08945	FG
22	CS	16	-0.05376	0.08945	FG
23	CDC	4	-0.1124	0.08945	G

Appendix 3.5 a: Type 3 tests of fixed effects for relative transcript levels of ***AGPS2*** in wheat cultivars at five grain developmental stages at $p \leq 0.05$

Effect	Num DF	Den DF	F Value	Pr > F
Cult	2	6	39.57	0.0003
Stage	4	24	89.84	<.0001
Cult*stage	8	24	1.94	0.0994

Appendix 3.5 b: Letter groups for wheat cultivars based on the least significant difference for the means of the relative transcript level of ***AGPS2*** at $p \leq 0.05$

Effect= Cult Method= LSD (P < .05) Set= 1

Obs	Cult	stage	Estimate	Standard Error	Letter Group
1	ACA		2.2561	0.05770	A
2	CS		1.9993	0.05770	B
3	CDC		1.5398	0.05770	C

Appendix 3.5 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***AGPS2*** at $p \leq 0.05$

Effect= stage Method= LSD (P < .05) Set= 2

Obs	Cult	stage	Estimate	Standard Error	Letter Group
4		4	2.9266	0.07448	A
5		8	2.2816	0.07448	B
6		16	1.8840	0.07448	C
7		25	1.4213	0.07448	D
8		30	1.1451	0.07448	E

Appendix 3.5 d: Letter groups for wheat cultivars and grain developmental stages interaction based on the least significant difference for the means of the relative transcript level of ***AGPS2*** at $p \leq 0.05$

Effect= Cult*stage Method= LSD (P < .05) Set= 3

Obs	Cult	stage	Estimate	Standard Error	Letter Group
9	ACA	4	3.3610	0.1290	A
10	CS	4	2.9492	0.1290	B
11	ACA	8	2.7845	0.1290	BC
12	CDC	4	2.4695	0.1290	CD
13	CS	8	2.3600	0.1290	D
14	ACA	16	2.2235	0.1290	DE
15	CS	16	1.8758	0.1290	EF
16	CDC	8	1.7002	0.1290	FG
17	CDC	16	1.5529	0.1290	FGH
18	CS	25	1.5480	0.1290	FGH
19	ACA	30	1.4716	0.1290	GH
20	ACA	25	1.4401	0.1290	GH
21	CDC	25	1.2758	0.1290	H
22	CS	30	1.2633	0.1290	H
23	CDC	30	0.7004	0.1290	I

Appendix 3.6 a: Type 3 tests of fixed effects for relative transcript levels of ***GBSSI*** in wheat cultivars at five grain developmental stages at $p \leq 0.05$

Effect	Num DF	Den DF	F Value	Pr > F
Cult	2	6	49.07	0.0002
Stage	4	24	13.07	<.0001
Cult*stage	8	24	9.84	<.0001

Appendix 3.6 b: Letter groups for wheat cultivars based on the least significant difference for the means of the relative transcript level of ***GBSSI*** at $p \leq 0.05$

Effect= Cult Method= LSD (P < .05) Set= 1

Obs	Cult	stage	Estimate	Standard Error	Letter Group
1	ACA		0.1588	0.05079	A
2	CS		-0.1390	0.05079	B
3	CDC		-0.5498	0.05079	C

Appendix 3.6 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***GBSSI*** at $p \leq 0.05$

Effect= stage Method= LSD (P < .05) Set= 2

Obs	Cult	stage	Estimate	Standard Error	Letter Group
4		16	0.05886	0.02193	A
5		4	-0.06349	0.03269	B
6		25	-0.1470	0.05523	BC
7		8	-0.1819	0.04320	C
8		30	-0.5497	0.1226	D

Appendix 3.6 d: Letter groups for wheat cultivars and grain developmental stages interaction based on the least significant difference for the means of the relative transcript level of ***GBSSI*** at $p \leq 0.05$

Effect= Cult*stage Method= LSD (P < .05) Set= 3

Obs	Cult	stage	Estimate	Standard Error	Letter Group
9	ACA	16	0.4858	0.03798	A
10	ACA	4	0.3786	0.05662	AB
11	ACA	25	0.1847	0.09566	BC
12	ACA	8	0.1498	0.07482	C
13	CS	4	-0.04318	0.05662	CD
14	CDC	16	-0.05885	0.03798	D
15	CS	25	-0.08180	0.09566	CDE
16	CS	30	-0.1481	0.2124	CDEF
17	CS	8	-0.1713	0.07482	DE
18	CS	16	-0.2504	0.03798	E
19	ACA	30	-0.4047	0.2124	DEF
20	CDC	8	-0.5241	0.07482	F
21	CDC	4	-0.5258	0.05662	F
22	CDC	25	-0.5437	0.09566	F
23	CDC	30	-1.0962	0.2124	G

Appendix 3.7 a: Type 3 tests of fixed effects for relative transcript levels of ***GBSSII*** in wheat cultivars at five grain developmental stages at $p \leq 0.05$

Effect	Num DF	Den DF	F Value	Pr > F
Cult	2	6	4.84	0.0561
Stage	4	24	129.29	<.0001
Cult*stage	8	24	6.69	0.0001

Appendix 3.7 b: Letter groups for wheat cultivars based on the least significant difference for the means of the relative transcript level of ***GBSSII*** at $p \leq 0.05$

Effect= Cult Method= LSD (P < .05) Set= 1

Obs	Cult	stage	Estimate	Standard Error	Letter Group
1	CDC		-1.1020	0.05725	A
2	ACA		-1.2884	0.05725	AB
3	CS		-1.3419	0.05725	B

Appendix 3.7 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***GBSSII*** at $p \leq 0.05$

Effect= stage Method= LSD (P < .05) Set= 2

Obs	Cult	stage	Estimate	Standard Error	Letter Group
4		4	-0.02695	0.07355	A
5		8	-0.8342	0.07355	B
6		30	-1.4737	0.07355	C
7		25	-1.7061	0.07355	D
8		16	-2.1795	0.07355	E

Appendix 3.7 d: Letter groups for wheat cultivars and grain developmental stages interaction based on the least significant difference for the means of the relative transcript level of ***GBSSII*** at $p \leq 0.05$

Effect= Cult*stage Method= LSD (P < .05) Set= 3

Obs	Cult	stage	Estimate	Standard Error	Letter Group
9	ACA	4	0.1743	0.1274	A
10	CDC	4	0.04182	0.1274	AB
11	CS	4	-0.2969	0.1274	B
12	CDC	8	-0.7146	0.1274	C
13	ACA	8	-0.8514	0.1274	C
14	CS	8	-0.9367	0.1274	CD
15	CS	30	-1.3057	0.1274	DE
16	CDC	16	-1.4415	0.1274	EF
17	ACA	30	-1.4784	0.1274	EF
18	CDC	30	-1.6370	0.1274	EF
19	CS	25	-1.6717	0.1274	EF
20	ACA	25	-1.6878	0.1274	F
21	CDC	25	-1.7588	0.1274	F
22	CS	16	-2.4983	0.1274	G
23	ACA	16	-2.5986	0.1274	G

Appendix 3.8 a: Type 3 tests of fixed effects for relative transcript levels of ***SSI*** in wheat cultivars at five grain developmental stages at $p \leq 0.05$

Effect	Num DF	Den DF	F Value	Pr > F
Cult	2	6	4.05	0.0770
Stage	4	24	33.18	<.0001
Cult*stage	8	24	52.19	<.0001

Appendix 3.8 b: Letter groups for wheat cultivars based on the least significant difference for the means of the relative transcript level of ***SSI*** at $p \leq 0.05$

Effect=Cult Method=LSD (P<.05) Set=1

Obs	Cult	stage	Estimate	Standard Error	Letter Group
1	ACA		2.1762	0.1322	A
2	CS		1.7986	0.1322	AB
3	CDC		1.6623	0.1322	B

Appendix 3.8 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***SSI*** at $p \leq 0.05$

Effect= stage Method= LSD (P < .05) Set= 2

Obs	Cult	stage	Estimate	Standard Error	Letter Group
4		8	2.5261	0.05411	A
5		30	2.0542	0.08076	B
6		25	1.8747	0.2301	BC
7		16	1.7094	0.04988	C
8		4	1.2307	0.2843	C

Appendix 3.8 d: Letter groups for wheat cultivars and grain developmental stages interaction based on the least significant difference for the means of the relative transcript level of ***SSI*** at $p \leq 0.05$

Effect= Cult*stage Method= LSD (P < .05) Set= 3

Obs	Cult	stage	Estimate	Standard Error	Letter Group
9	ACA	4	3.0870	0.4924	ABC
10	ACA	8	2.8457	0.09372	A
11	CS	16	2.6685	0.08640	AB
12	CS	8	2.5798	0.09372	AB
13	CS	30	2.3895	0.1399	BC
14	ACA	25	2.3769	0.3986	ABCD
15	ACA	30	2.3567	0.1399	BC
16	CDC	16	2.2452	0.08640	CD
17	CDC	8	2.1527	0.09372	CD
18	CDC	25	1.8438	0.3986	BCDE
19	CDC	30	1.4165	0.1399	E
20	CS	25	1.4035	0.3986	DE
21	CDC	4	0.6533	0.4924	EF
22	ACA	16	0.2146	0.08640	F
23	CS	4	-0.04827	0.4924	F

Appendix 3.9 a: Type 3 tests of fixed effects for relative transcript levels of ***SSIIa*** in wheat cultivars at five grain developmental stages at $p \leq 0.05$

Effect	Num DF	Den DF	F Value	Pr > F
Cult	2	6	3.82	0.0850
Stage	4	24	17.73	<.0001
Cult*stage	8	24	1.86	0.1150

Appendix 3.9 b: Letter groups for wheat cultivars based on the least significant difference for the means of the relative transcript level of ***SSIIa*** at $p \leq 0.05$

Effect= Cult Method= LSD (P < .05) Set= 1

Obs	Cult	stage	Estimate	Standard Error	Letter Group
1	ACA		2.0236	0.1323	A
2	CS		1.9084	0.1323	AB
3	CDC		1.5291	0.1323	B

Appendix 3.9 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***SSIIa*** at $p \leq 0.05$

Effect= stage Method= LSD (P < .05) Set= 2

Obs	Cult	stage	Estimate	Standard Error	Letter Group
4		8	2.6095	0.1709	A
5		4	2.5508	0.1709	A
6		16	1.5097	0.1709	B
7		25	1.4235	0.1709	BC
8		30	1.0082	0.1709	C

Appendix 3.9 d: Letter groups for wheat cultivars and grain developmental stages interaction based on the least significant difference for the means of the relative transcript level of ***SSIIa*** at $p \leq 0.05$

Effect= Cult*stage Method= LSD (P < .05) Set= 3

Obs	Cult	stage	Estimate	Standard Error	Letter Group
9	ACA	4	2.9966	0.2959	A
10	CS	4	2.8712	0.2959	A
11	CDC	8	2.8653	0.2959	A
12	CS	8	2.5267	0.2959	AB
13	ACA	8	2.4364	0.2959	AB
14	ACA	16	1.9086	0.2959	BC
15	CDC	4	1.7847	0.2959	BC
16	CS	25	1.7211	0.2959	BC
17	ACA	30	1.5215	0.2959	C
18	CS	16	1.3472	0.2959	C
19	CDC	25	1.2946	0.2959	C
20	CDC	16	1.2733	0.2959	CD
21	ACA	25	1.2549	0.2959	CD
22	CS	30	1.0758	0.2959	CD
23	CDC	30	0.4273	0.2959	D

Appendix 3.10 a: Type 3 tests of fixed effects for relative transcript levels of ***SSIIb*** in wheat cultivars at five grain developmental stages at $p \leq 0.05$

Effect	Num DF	Den DF	F Value	Pr > F
Cult	2	6	50.10	0.0002
Stage	4	24	41.03	<.0001
Cult*stage	8	24	62.20	<.0001

Appendix 3.10 b: Letter groups for wheat cultivars based on the least significant difference for the means of the relative transcript level of ***SSIIb*** at $p \leq 0.05$

Effect= Cult Method= LSD (P < .05) Set= 1

Obs	Cult	stage	Estimate	Standard Error	Letter Group
1	CS		6.8400	0.3559	A
2	ACA		4.1973	0.3559	B
3	CDC		1.8040	0.3559	C

Appendix 3.10 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***SSIIb*** at $p \leq 0.05$

Effect= stage Method= LSD (P < .05) Set=2

Obs	Cult	stage	Estimate	Standard Error	Letter Group
4		4	8.7278	0.4673	A
5		8	4.5811	0.4475	B
6		16	3.2611	0.4012	C
7		30	2.4467	0.2673	C
8		25	2.3856	0.4186	C

Appendix 3.10 d: Letter groups for wheat cultivars and grain developmental stages interaction based on the least significant difference for the means of the relative transcript level of ***SSIIb*** at $p \leq 0.05$

Effect= Cult*stage Method= LSD (P < .05) Set= 3

Obs	Cult	stage	Estimate	Standard Error	Letter Group
9	CS	4	22.5967	0.8093	A
10	ACA	8	9.5967	0.7750	B
11	ACA	16	5.3600	0.6950	C
12	CS	16	3.5900	0.6950	CD
13	CS	8	3.3533	0.7750	CDE
14	CS	30	3.3167	0.4629	D
15	CDC	25	3.0967	0.7250	DEF
16	ACA	25	2.7167	0.7250	DEFG
17	CDC	30	2.6000	0.4629	DEF
18	ACA	4	1.8900	0.8093	DEFG
19	CDC	4	1.6967	0.8093	DEFG
20	ACA	30	1.4233	0.4629	FG
21	CS	25	1.3433	0.7250	EFG
22	CDC	16	0.8333	0.6950	G
23	CDC	8	0.7933	0.7750	G

Appendix 3.11 a: Type 3 tests of fixed effects for relative transcript levels of ***SSIIIa*** in wheat cultivars at five grain developmental stages at $p \leq 0.05$

Effect	Num DF	Den DF	F Value	Pr > F
Cult	2	6	32.86	0.0006
Stage	4	24	38.95	<.0001
Cult*stage	8	24	10.70	<.0001

Appendix 3.11 b: Letter groups for wheat cultivars based on the least significant difference for the means of the relative transcript level of ***SSIIIa*** at $p \leq 0.05$

Effect= Cult Method= LSD (P < .05) Set= 1

Obs	Cult	stage	Estimate	Standard Error	Letter Group
1	ACA		87.4273	5.5081	A
2	CS		58.7680	5.5081	B
3	CDC		24.3627	5.5081	C

Appendix 3.11 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***SSIIIa*** at $p \leq 0.05$

Effect= stage Method=LSD (P < .05) Set= 2

Obs	Cult	stage	Estimate	Standard Error	Letter Group
4		16	108.06	7.1109	A
5		8	100.68	7.1109	A
6		30	35.9522	7.1109	B
7		25	28.4378	7.1109	BC
8		4	11.1333	7.1109	C

Appendix 3.11 d: Letter groups for wheat cultivars and grain developmental stages interaction based on the least significant difference for the means of the relative transcript level of ***SSIIIa*** at $p \leq 0.05$

Effect= Cult*stage Method= LSD (P < .05) Set= 3

Obs	Cult	stage	Estimate	Standard Error	Letter Group
9	ACA	16	210.36	12.3165	A
10	CS	8	136.94	12.3165	B
11	ACA	8	120.23	12.3165	B
12	CS	16	83.3400	12.3165	C
13	ACA	30	53.6800	12.3165	CD
14	CDC	8	44.8667	12.3165	D
15	ACA	25	44.1333	12.3165	DE
16	CS	30	32.0733	12.3165	DEF
17	CDC	16	30.4867	12.3165	DEF
18	CDC	30	22.1033	12.3165	DEF
19	CS	4	20.8467	12.3165	DEF
20	CS	25	20.6400	12.3165	DEF
21	CDC	25	20.5400	12.3165	DEF
22	ACA	4	8.7367	12.3165	EF
23	CDC	4	3.8167	12.3165	F

Appendix 3.12 a: Type 3 tests of fixed effects for relative transcript levels of ***SSIIIb*** in wheat cultivars at five grain developmental stages at $p \leq 0.05$

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Cult	2	6	1.14	0.3793
Stage	4	24	83.50	<.0001
Cult*stage	8	24	3.36	0.0101

Appendix 3.12 b: Letter groups for wheat cultivars based on the least significant difference for the means of the relative transcript level of ***SSIIIb*** at $p \leq 0.05$

Effect= Cult Method= LSD (P < .05) Set= 1

Obs	Cult	stage	Estimate	Standard Error	Letter Group
1	CS		0.7879	0.1086	A
2	CDC		0.6028	0.1086	A
3	ACA		0.5736	0.1086	A

Appendix 3.12 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***SSIIIb*** at $p \leq 0.05$

Effect= stage Method=LSD (P < .05) Set= 2

Obs	Cult	stage	Estimate	Standard Error	Letter Group
4		8	1.1513	0.05741	A
5		30	0.7167	0.09105	B
6		25	0.6104	0.1139	B
7		4	0.4625	0.2076	BC
8		16	0.3329	0.04117	C

Appendix 3.12 d: Letter groups for wheat cultivars and grain developmental stages interaction based on the least significant difference for the means of the relative transcript level of ***SSIIIb*** at $p \leq 0.05$

Effect= Cult*stage Method= LSD (P < .05) Set= 3

Obs	Cult	stage	Estimate	Standard Error	Letter Group
9	ACA	8	1.2498	0.09944	A
10	CDC	8	1.1630	0.09944	AB
11	CS	8	1.0411	0.09944	AB
12	CS	4	0.9343	0.3596	ABCDE
13	CS	30	0.9256	0.1577	ABC
14	ACA	30	0.8633	0.1577	BC
15	CS	25	0.7708	0.1973	BCD
16	CDC	25	0.5744	0.1973	CDE
17	ACA	25	0.4858	0.1973	CDE
18	CDC	4	0.4658	0.3596	BCDE
19	CDC	16	0.4498	0.07131	DE
20	CDC	30	0.3612	0.1577	DE
21	ACA	16	0.2816	0.07131	E
22	CS	16	0.2674	0.07131	E
23	ACA	4	-0.01258	0.3596	DE

Appendix 3.13 a: Letter groups for wheat cultivars based on the least significant difference for the means of the relative transcript level of ***SBEI*** at $p \leq 0.05$

Effect= Cult Method= LSD (P < .05) Set= 1

Obs	Cult	stage	Estimate	Standard Error	Letter Group
1	ACA		1.9578	0.1617	A
2	CDC		1.4881	0.1617	AB
3	CS		0.9941	0.1617	B

Appendix 3.13 b: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***SBEI*** at $p \leq 0.05$

Effect= stage Method=LSD (P < .05) Set= 2

Obs	Cult	stage	Estimate	Standard Error	Letter Group
4		16	1.6934	0.2087	A
5		30	1.5392	0.2087	A
6		4	1.5085	0.2087	A
7		8	1.4262	0.2087	A
8		25	1.2326	0.2087	A

Appendix 3.13 c: Letter groups for wheat cultivars and grain developmental stages interaction based on the least significant difference for the means of the relative transcript level of ***SBEI*** at $p \leq 0.05$

Effect= Cult*stage Method= LSD (P < .05) Set= 3

Obs	Cult	stage	Estimate	Standard Error	Letter Group
9	ACA	8	2.3723	0.3615	A
10	ACA	16	2.1745	0.3615	AB
11	ACA	30	1.8095	0.3615	AB
12	ACA	25	1.7208	0.3615	ABC
13	ACA	4	1.7120	0.3615	ABC
14	CDC	25	1.6679	0.3615	ABC
15	CDC	16	1.6584	0.3615	ABC
16	CDC	4	1.6100	0.3615	ABC
17	CS	30	1.5377	0.3615	ABC
18	CDC	30	1.2703	0.3615	BCD
19	CS	16	1.2474	0.3615	BCD
20	CDC	8	1.2338	0.3615	BCD
21	CS	4	1.2035	0.3615	BCD
22	CS	8	0.6725	0.3615	CD
23	CS	25	0.3092	0.3615	D

Appendix 3.14 a: Type 3 tests of fixed effects for relative transcript levels of ***SBEIIa*** in wheat cultivars at five grain developmental stages at $p \leq 0.05$

Effect	Num DF	Den DF	F Value	Pr > F
Cult	2	6	582.37	<.0001
Stage	4	21	10.94	<.0001
Cult*stage	7	21	33.12	<.0001

Appendix 3.14 b: Letter groups for wheat cultivars based on the least significant difference for the means of the relative transcript level of ***SBEIIa*** at $p \leq 0.05$

Effect= Cult Method= LSD (P < .05) Set= 1

Obs	Cult	stage	Estimate	Standard Error	Letter Group
1	ACA		0.6747	0.05351	A
2	CS		-1.7811	0.05451	B
3	CDC		.	.	

Appendix 3.14 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***SBEIIa*** at $p \leq 0.05$

Effect= stage Method=LSD (P < .05) Set= 2

Obs	Cult	stage	Estimate	Standard Error	Letter Group
4		8	-0.8432	0.09666	A
5		30	-0.8694	0.09617	A
6		16	-0.9551	0.04567	A
7		25	-0.9614	0.05890	A
8		4	.	.	

Appendix 3.14 d: Letter groups for wheat cultivars and grain developmental stages interaction based on the least significant difference for the means of the relative transcript level of ***SBEIIa*** at $p \leq 0.05$

Effect= Cult*stage Method= LSD (P < .05) Set= 3

Obs	Cult	stage	Estimate	Standard Error	Letter Group
9	ACA	4	1.2921	0.006600	A
10	ACA	8	0.8777	0.1674	B
11	ACA	30	0.5985	0.1666	BC
12	ACA	16	0.3829	0.07324	C
13	ACA	25	0.2220	0.1020	C
14	CDC	16	-1.2482	0.07324	D
15	CS	25	-1.5076	0.1020	DE
16	CDC	25	-1.5986	0.1020	E
17	CS	30	-1.5986	0.1666	DE
18	CDC	8	-1.6080	0.1674	DEF
19	CDC	30	-1.6080	0.1666	DEF
20	CS	8	-1.7993	0.1674	EFG
21	CS	16	-2.0000	0.08970	FG
22	CS	4	-2.0000	0.006600	G

Appendix 3.15 a: Letter groups for wheat cultivars based on the least significant difference for the means of the relative transcript level of ***SBEIIb*** at $p \leq 0.05$

Effect= Cult Method= LSD (P < .05) Set= 1

Obs	Cult	stage	Estimate	Standard Error	Letter Group
1	ACA		0.6747	0.05351	A
2	CS		-1.7811	0.05451	B
3	CDC		.	.	

Appendix 3.15 b: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***SBEIIb*** at $p \leq 0.05$

Effect= stage Method=LSD (P < .05) Set= 2

Obs	Cult	stage	Estimate	Standard Error	Letter Group
4		8	-0.8432	0.09666	A
5		30	-0.8694	0.09617	A
6		16	-0.9551	0.04567	A
7		25	-0.9614	0.05890	A
8		4	.	.	

Appendix 3.15 c: Letter groups for wheat cultivars and grain developmental stages interaction based on the least significant difference for the means of the relative transcript level of ***SBEIIb*** at $p \leq 0.05$

Effect= Cult*stage Method= LSD (P < .05) Set= 3

Obs	Cult	stage	Estimate	Standard Error	Letter Group
9	ACA	4	1.2921	0.006600	A
10	ACA	8	0.8777	0.1674	B
11	ACA	30	0.5985	0.1666	BC
12	ACA	16	0.3829	0.07324	C
13	ACA	25	0.2220	0.1020	C
14	CDC	16	-1.2482	0.07324	D
15	CS	25	-1.5076	0.1020	DE
16	CDC	25	-1.5986	0.1020	E
17	CS	30	-1.5986	0.1666	DE
18	CDC	8	-1.6080	0.1674	DEF
19	CDC	30	-1.6080	0.1666	DEF
20	CS	8	-1.7993	0.1674	EFG
21	CS	16	-2.0000	0.08970	FG
22	CS	4	-2.0000	0.006600	G

Appendix 4: Analysis of Variance (ANOVA) for total starch content per grain of wheat cultivars Chinese Spring (CS), CDC Teal (CDC) and AC Andrew (ACA) using the PROC Mixed procedure of SAS software.

Appendix 4.1 a: Type 3 tests of fixed effects for the total starch content per grain in wheat cultivars at five grain developmental stages at $p \leq 0.05$

Effect	Num DF	Den DF	F Value	Pr > F
Cult	2	6	8.76	0.0166
Stage	4	24	280.12	<.0001
Cult*stage	8	24	6.24	0.0002

Appendix 4.1 b: Letter groups for wheat cultivars based on the least significant difference for the means of the total starch content per grain at $p \leq 0.05$

Effect= cult Method=LSD (P < .05) Set= 1

Obs	cult	stage	Estimate	Standard Error	Letter Group
1	ACA		16.2140	0.7984	A
2	CDC		14.2527	0.7984	AB
3	CS		11.5087	0.7984	B

Appendix 4.1 c: Letter groups for grain developmental stages based on the least significant difference for the means of the total starch content per grain at $p \leq 0.05$

Effect= stage Method= LSD (P < .05) Set= 2

Obs	cult	stage	Estimate	Standard Error	LetterGroup
4		70	24.1956	0.7046	A
5		30	20.5189	0.7046	B
6		25	18.2578	0.7046	C
7		16	5.9889	0.7046	D
8		8	0.9978	0.7046	E

Appendix 4.1 d: Letter groups for wheat cultivars and grain developmental stages interaction based on the least significant difference for the means of the total starch content per grain at $p \leq 0.05$

Effect= cult*stage Method= LSD (P < .05) Set= 3

Obs	cult	stage	Estimate	Standard Error	LetterGroup
9	ACA	70	29.2667	1.2205	A
10	CDC	70	26.3367	1.2205	AB
11	ACA	30	23.4933	1.2205	B
12	ACA	25	20.1667	1.2205	C
13	CDC	30	19.1233	1.2205	CD
14	CS	30	18.9400	1.2205	C
15	CDC	25	18.8800	1.2205	CD
16	CS	70	16.9833	1.2205	CD
17	CS	25	15.7267	1.2205	D
18	ACA	16	7.1833	1.2205	E
19	CDC	16	6.0167	1.2205	E
20	CS	16	4.7667	1.2205	E
21	CS	8	1.1267	1.2205	F
22	ACA	8	0.9600	1.2205	F
23	CDC	8	0.9067	1.2205	F

Appendix 5: Analysis of Variance (ANOVA) for AGPase, soluble starch synthase (SSS) and granule bound starch synthase (GBSS) activities per grain of wheat cultivars Chinese Spring (CS) and AC Andrew (ACA) using the PROC Mixed procedure of SAS software.

Appendix 5.1 a: Type 3 tests of fixed effects for AGPase activity per grain in wheat cultivars at five grain developmental stages at $p \leq 0.05$

Effect	Num DF	Den DF	F Value	Pr > F
Cult	1	4	6.74	0.0603
Stage	3	12	118.77	<.0001
Cult*stage	3	12	3.26	0.0594

Appendix 5.1 b: Letter groups for wheat cultivars based on the least significant difference for the means of the AGPase activity per grain at $p \leq 0.05$

Effect= cult Method= LSD (P < .05) Set= 1

Obs	cult	stage	Estimate	Standard Error	LetterGroup
1	ACA		46.4217	2.6506	A
2	CS		36.6900	2.6506	A

Appendix 5.1 c: Letter groups grain developmental stages based on the least significant difference for the means of AGPase activity per grain at $p \leq 0.05$

Effect= stage Method= LSD (P < .05) Set= 2

Obs	cult	stage	Estimate	Standard Error	LetterGroup
3		25	81.3850	3.7485	A
4		16	71.6133	3.7485	A
5		8	12.7117	3.7485	B
6		4	0.5133	3.7485	C

Appendix 5.1 d: Letter groups for wheat cultivars and grain developmental stages interaction based on the least significant difference for the means of AGPase activity per grain at $p \leq 0.05$

Effect= cult*stage Method= LSD (P < .05) Set=3

Obs	cult	stage	Estimate	Standard Error	LetterGroup
7	ACA	25	94.2333	5.3011	A
8	ACA	16	79.6533	5.3011	AB
9	CS	25	68.5367	5.3011	B
10	CS	16	63.5733	5.3011	B
11	CS	8	14.2867	5.3011	C
12	ACA	8	11.1367	5.3011	C
13	ACA	4	0.6633	5.3011	C
14	CS	4	0.3633	5.3011	C

Appendix 5.2 a: Type 3 tests of fixed effects for SSS activity per grain in wheat cultivars at five grain developmental stages at $p \leq 0.05$

Effect	Num DF	Den DF	F Value	Pr > F
Cult	1	4	5.65	0.0762
Stage	3	8	12.77	0.0020
Cult*stage	3	8	7.33	0.0110

Appendix 5.2 b: Letter groups for wheat cultivars based on the least significant difference for the means of the SSS activity per grain at $p \leq 0.05$

Effect= cult Method=LSD (P < .05) Set= 1

Obs	cult	stage	Estimate	Standard Error	LetterGroup
1	ACA		420.76	49.9163	A
2	CS		243.74	55.2237	A

Appendix 5.2 c: Letter groups for grain developmental stages based on the least significant difference for the means of SSS activity per grain at $p \leq 0.05$

Effect= stage Method= LSD (P < .05) Set= 2

Obs	cult	stage	Estimate	Standard Error	LetterGroup
3		25	543.52	70.9127	A
4		8	504.34	70.9308	AB
5		4	279.10	70.9308	B
6		16	2.0364	70.9308	C

Appendix 5.2 d: Letter groups for wheat cultivars and grain developmental stages interaction based on the least significant difference for the means of SSS activity per grain at $p \leq 0.05$

Effect= cult*stage Method= LSD (P < .05) Set= 3

Obs	cult	stage	Estimate	Standard Error	LetterGroup
7	ACA	25	916.00	109.83	A
8	CS	8	504.83	109.88	B
9	ACA	8	503.86	89.7340	B
10	CS	4	295.98	109.88	BC
11	ACA	4	262.22	89.7340	BC
12	CS	25	171.04	89.7340	C
13	CS	16	3.1195	109.88	C
14	ACA	16	0.9533	89.7340	C

Appendix 5.3 a: Type 3 tests of fixed effects for GBSS activity per grain in wheat cultivars at five grain developmental stages at $p \leq 0.05$

Effect	Num DF	Den DF	F Value	Pr > F
Cult	1	4	2.42	0.1948
Stage	3	12	48.56	<.0001
Cult*stage	3	12	1.13	0.3770

Appendix 5.3 b: Letter groups for wheat cultivars based on the least significant difference for the means of the GBSS activity per grain at $p \leq 0.05$

Effect= cult Method=LSD (P < .05) Set= 1

Obs	cult	stage	Estimate	Standard Error	LetterGroup
1	ACA		563.43	55.2955	A
2	CS		441.78	55.2955	A

Appendix 5.3 c: Letter groups for grain developmental stages based on the least significant difference for the means of GBSS activity per grain at $p \leq 0.05$

Effect= stage Method= LSD (P < .05) Set= 2

Obs	cult	stage	Estimate	Standard Error	LetterGroup
3		25	1131.17	78.1996	A
4		16	783.57	78.1996	B
5		8	87.4183	78.1996	C
6		4	8.2750	78.1996	C

Appendix 5.3 d: Letter groups for wheat cultivars and grain developmental stages interaction based on the least significant difference for the means of GBSS activity per grain at $p \leq 0.05$

Effect= cult*stage Method= LSD (P < .05) Set= 3

Obs	cult	stage	Estimate	Standard Error	LetterGroup
7	ACA	25	1300.05	110.59	A
8	CS	25	962.29	110.59	AB
9	ACA	16	865.11	110.59	B
10	CS	16	702.02	110.59	B
11	CS	8	102.81	110.59	C
12	ACA	8	72.0233	110.59	C
13	ACA	4	16.5500	110.59	C
14	CS	4	-426E-16	110.59	C

Appendix 6: One way ANOVA for thousand kernel weight (TKW) of Chinese Spring (CS), CDC Teal (CDC) and AC Andrew (ACA) using SigmaPlot 12 software.

Normality Test (Shapiro-Wilk) Passed (P = 0.929)

Equal Variance Test: Failed (P < 0.050)

Kruskal-Wallis One Way Analysis of Variance on Ranks

Group	N	Missing	Median	25%	75%
CS	3	0	34.542	30.406	40.170
CDC	3	0	44.524	42.290	46.996
ACA	3	0	52.536	51.047	52.752

H = 7.200 with 2 degrees of freedom. P (est.) = 0.027 P (exact)= 0.004

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.004)

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0.05
ACA vs CS	18.000	3.795	Yes
ACA vs CDC	9.000	1.897	No
CDC vs CS	9.000	1.897	No

Appendix 7: One way ANOVA for grain yield per plant among Chinese Spring (CS), CDC Teal (CDC) and AC Andrew (ACA) when grown under green house conditions using SigmaPlot 12 software

Normality Test (Shapiro-Wilk) Passed (P = 0.549)

Equal Variance Test: Passed (P = 0.506)

Group Name	N	Missing	Mean	Std Dev	SEM
CS	3	0	701.333	142.497	82.271
CDC	3	0	427.333	52.624	30.383
ACA	3	0	465.000	57.715	33.322

Source of Variation	DF	SS	MS	F	P
Between Groups	2	132348.222	66174.111	7.518	0.023
Residual	6	52811.333	8801.889		
Total	8	185159.556			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.023).

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
CS vs. CDC	274.000	3	5.059	0.027	Yes
CS vs. ACA	236.333	3	4.363	0.049	Yes
ACA vs. CDC	37.667	3	0.695	0.878	No

Appendix 8: Analysis of Variance (ANOVA) for expression data of *AGPL1*, *AGPL2*, *AGPS1a*, *AGPS1b*, *AGPS2*, *SSI*, *SSIIa*, *SSIIIa*, *SBEI*, *SBEIIa* and *SBEIIb* genes in wild type (WT) transgenic positive (US5P, US6P, US7P) and negative control (US5N, US7N) lines using the PROC Mixed procedure of SAS software. The relative transcript levels were transformed to their log10 values in order to comply with normal distribution.

Appendix 8.1 a: Type 3 tests of fixed effects for relative transcript levels of ***AGPL1*** in wheat cultivars at two grain developmental stages at $p \leq 0.05$

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Line	5	13	4.09	0.0188
stage	1	13	11.81	0.0044
Line*stage	5	13	4.35	0.0151

Appendix 8.1 b: Letter groups for lines based on the least significant difference for the means of the relative transcript level of ***AGPL1*** at $p \leq 0.05$

Effect= Line Method= LSD (P < .05) Set=1

Obs	Line	stage	Estimate	Standard Error	LetterGroup
1	US6P		0.4456	0.06303	A
2	US7P		0.3996	0.07047	A
3	US7N		0.2970	0.06303	AB
4	US5N		0.2961	0.06303	AB
5	US5P		0.08214	0.09454	BC
6	WT		0.01843	0.08914	C

Appendix 8.1 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***AGPL1*** at $p \leq 0.05$

Effect=stage Method=LSD (P < .05) Set=2

Obs	Line	stage	Estimate	Standard Error	LetterGroup
7		8	0.4385	0.03788	A
8		16	0.07444	0.04814	B

Appendix 8.1 d: Letter groups for the lines (positive, negative and wild-type) and developmental stage interaction based on the least significant difference for the means of the relative transcript level of ***AGPL1*** at $p \leq 0.05$

Effect=Line*stage Method=LSD (P < .05) Set=3

Obs	Line	stage	Estimate	Standard Error	LetterGroup
9	US6P	8	0.7401	0.08914	A
10	US7P	8	0.6572	0.08914	A
11	US7N	8	0.5000	0.08914	AB
12	US5P	8	0.4239	0.1092	ABC
13	US5N	8	0.3103	0.08914	BC
14	US5N	16	0.2818	0.08914	BCD
15	US6P	16	0.1511	0.08914	CDE
16	US7P	16	0.1419	0.1092	CDE
17	US7N	16	0.09405	0.08914	CDE
18	WT	16	0.03743	0.1544	CDE
19	WT	8	-0.00057	0.08914	DE
20	US5P	16	-0.2596	0.1544	E

Appendix 8.2 a: Type 3 tests of fixed effects for relative transcript levels of ***AGPL2*** in wheat cultivars at two grain developmental stages at $p \leq 0.05$

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Line	5	12	2.32	0.1078
stage	1	10	1.22	0.2944
Line*stage	5	10	6.77	0.0053

Appendix 8.2 b: Letter groups for lines based on the least significant difference for the means of the relative transcript level of ***AGPL2*** at $p \leq 0.05$

Effect= Line Method= LSD (P < .05) Set=1

Obs	Line	stage	Estimate	Standard Error	LetterGroup
1	WT		0.04234	0.1073	A
2	US5N		-0.1436	0.1002	AB
3	US7N		-0.1896	0.1002	AB
4	US7P		-0.2907	0.1002	B
5	US6P		-0.3723	0.1002	B
6	US5P		-0.3774	0.1073	B

Appendix 8.2 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***AGPL2*** at $p \leq 0.05$

Effect=stage Method=LSD (P < .05) Set=2

Obs	Line	stage	Estimate	Standard Error	LetterGroup
7		16	-0.1911	0.05187	A
8		8	-0.2526	0.04863	A

Appendix 8.2 d: Letter groups for the lines (positive, negative and wild-type) and developmental stage interaction based on the least significant difference for the means of the relative transcript level of ***AGPL2*** at $p \leq 0.05$

Effect=Line*stage Method=LSD (P < .05) Set=3

Obs	Line	stage	Estimate	Standard Error	Letter Group
9	WT	16	0.08519	0.1416	A
10	US5N	16	0.06677	0.1191	A
11	US7N	8	0.02701	0.1191	A
12	WT	8	-0.00051	0.1191	AB
13	US5P	16	-0.1438	0.1416	ABC
14	US6P	8	-0.2738	0.1191	ABCD
15	US7P	16	-0.2777	0.1191	ABCD
16	US7P	8	-0.3036	0.1191	ABCD
17	US5N	8	-0.3539	0.1191	BCD
18	US7N	16	-0.4062	0.1191	CD
19	US6P	16	-0.4709	0.1191	CD
20	US5P	8	-0.6109	0.1191	D

Appendix 8.3 a: Type 3 tests of fixed effects for relative transcript levels of ***AGPS1a*** in wheat cultivars at two grain developmental stages at $p \leq 0.05$

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Line	5	12	1.33	0.3156
stage	1	9	2.38	0.1576
Line*stage	5	9	0.91	0.5145

Appendix 8.3 b: Letter groups for lines based on the least significant difference for the means of the relative transcript level of ***AGPS1a*** at $p \leq 0.05$

Effect= Line Method= LSD (P < .05) Set=1

Obs	Line	stage	Estimate	Standard Error	Letter Group
1	US5P		0.2763	0.08060	A
2	US7P		0.2734	0.07308	A
3	US7N		0.2104	0.07308	AB
4	US5N		0.1793	0.07308	AB
5	US6P		0.1737	0.08060	AB
6	WT		0.02725	0.08060	B

Appendix 8.3 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***AGPS1a*** at $p \leq 0.05$

Effect=stage Method=LSD (P < .05) Set=2

Obs	Line	stage	Estimate	Standard Error	Letter Group
7		16	0.2330	0.04421	A
8		8	0.1471	0.03961	A

Appendix 8.3 d: Letter groups for the lines (positive, negative and wild-type) and developmental stage interaction based on the least significant difference for the means of the relative transcript level of ***AGPS1a*** at $p \leq 0.05$

Effect=Line*stage Method=LSD (P < .05) Set=3

Obs	Line	stage	Estimate	Standard Error	Letter Group
9	US5P	16	0.4040	0.1185	A
10	US7N	16	0.3296	0.09703	A
11	US7P	16	0.2900	0.09703	AB
12	US7P	8	0.2568	0.09703	AB
13	US6P	8	0.1942	0.09703	AB
14	US5N	8	0.1925	0.09703	AB
15	US5N	16	0.1662	0.09703	AB
16	US6P	16	0.1533	0.1185	AB
17	US5P	8	0.1486	0.09703	AB
18	US7N	8	0.09118	0.09703	AB
19	WT	16	0.05491	0.1185	AB
20	WT	8	-0.00042	0.09703	B

Appendix 8.4 a: Type 3 tests of fixed effects for relative transcript levels of ***AGPS1b*** in wheat cultivars at two grain developmental stages at $p \leq 0.05$

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Line	5	12	1.24	0.3508
stage	1	6	3.14	0.1266
Line*stage	5	6	2.21	0.1807

Appendix 8.4 b: Letter groups for lines based on the least significant difference for the means of the relative transcript level of ***AGPS1b*** at $p \leq 0.05$

Effect= Line Method= LSD (P < .05) Set=1

Obs	Line	stage	Estimate	Standard Error	Letter Group
1	US7P		0.4292	0.1452	A
2	US5P		0.2393	0.1357	AB
3	US6P		0.2005	0.1236	AB
4	US7N		0.1274	0.1357	AB
5	US5N		0.06246	0.1357	AB
6	WT		-0.01961	0.1357	B

Appendix 8.4 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***AGPS1b*** at $p \leq 0.05$

Effect=stage Method=LSD (P < .05) Set=2

Obs	Line	stage	Estimate	Standard Error	Letter Group
7		16	0.2588	0.07573	A
8		8	0.08766	0.07096	A

Appendix 8.4 d: Letter groups for the lines (positive, negative and wild-type) and developmental stage interaction based on the least significant difference for the means of the relative transcript level of ***AGPS1b*** at $p \leq 0.05$

Effect=Line*stage Method=LSD (P < .05) Set=3

Obs	Line	stage	Estimate	Standard Error	Letter Group
9	US7P	16	0.7394	0.1967	A
10	US7N	16	0.4320	0.1612	AB
11	US5P	8	0.3651	0.1612	ABC
12	US6P	16	0.2013	0.1612	ABC
13	US6P	8	0.1997	0.1612	ABC
14	US7P	8	0.1191	0.1967	ABC
15	US5P	16	0.1136	0.1965	ABC
16	US5N	16	0.1057	0.1965	ABC
17	US5N	8	0.01918	0.1612	BC
18	WT	8	-0.00005	0.1612	BC
19	WT	16	-0.03917	0.1965	BC
20	US7N	8	-0.1771	0.1965	C

Appendix 8.5 a: Type 3 tests of fixed effects for relative transcript levels of ***AGPS2*** in wheat cultivars at two grain developmental stages at $p \leq 0.05$

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Line	5	12	1.52	0.2568
stage	1	10	2.99	0.1145
Line*stage	5	10	0.58	0.7178

Appendix 8.5 b: Letter groups for lines based on the least significant difference for the means of the relative transcript level of ***AGPS2*** at $p \leq 0.05$

Effect= Line Method= LSD (P < .05) Set=1

Obs	Line	stage	Estimate	Standard Error	Letter Group
1	US7P		0.2728	0.09872	A
2	US6P		0.1762	0.09872	AB
3	US7N		0.1344	0.09872	AB
4	US5P		0.1227	0.1082	AB
5	US5N		-0.03786	0.09872	B
6	WT		-0.04641	0.1082	B

Appendix 8.5 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***AGPS2*** at $p \leq 0.05$

Effect=stage Method=LSD (P < .05) Set=2

Obs	Line	stage	Estimate	Standard Error	Letter Group
7		16	0.1634	0.05608	A
8		8	0.04385	0.05207	A

Appendix 8.5 d: Letter groups for the lines (positive, negative and wild-type) and developmental stage interaction based on the least significant difference for the means of the relative transcript level of ***AGPS2*** at $p \leq 0.05$

Effect=Line*stage Method=LSD (P < .05) Set=3

Obs	Line	stage	Estimate	Standard Error	Letter Group
9	US7P	16	0.3548	0.1275	A
10	US6P	16	0.2765	0.1275	AB
11	US5P	16	0.2403	0.1552	AB
12	US7N	16	0.2383	0.1275	AB
13	US7P	8	0.1907	0.1275	AB
14	US6P	8	0.07595	0.1275	AB
15	US7N	8	0.03055	0.1275	AB
16	US5P	8	0.005051	0.1275	AB
17	WT	8	0.001254	0.1275	AB
18	US5N	16	-0.03533	0.1275	AB
19	US5N	8	-0.04040	0.1275	AB
20	WT	16	-0.09408	0.1552	B

Appendix 8.6 a: Type 3 tests of fixed effects for relative transcript levels of ***GBSSI*** in wheat cultivars at two grain developmental stages at $p \leq 0.05$

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Line	5	12	8.70	0.0011
stage	1	10	46.15	<.0001
Line*stage	5	10	4.01	0.0295

Appendix 8.6 b: Letter groups for lines based on the least significant difference for the means of the relative transcript level of ***GBSSI*** at $p \leq 0.05$

Effect= Line Method= LSD (P < .05) Set=1

Obs	Line	stage	Estimate	Standard Error	Letter Group
1	US7N		0.5140	0.06430	A
2	US5N		0.4848	0.06430	A
3	US7P		0.4777	0.06430	A
4	US6P		0.4185	0.06430	AB
5	US5P		0.2562	0.07033	B
6	WT		-0.01075	0.07033	C

Appendix 8.6 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***GBSSI*** at $p \leq 0.05$

Effect=stage Method=LSD (P < .05) Set=2

Obs	Line	stage	Estimate	Standard Error	Letter Group
7		8	0.5072	0.03369	A
8		16	0.2062	0.03627	B

Appendix 8.6 d: Letter groups for the lines (positive, negative and wild-type) and developmental stage interaction based on the least significant difference for the means of the relative transcript level of ***GBSSI*** at $p \leq 0.05$

Effect=Line*stage Method=LSD (P < .05) Set=3

Obs	Line	stage	Estimate	Standard Error	Letter Group
9	US5N	8	0.7244	0.08252	A
10	US7P	8	0.6612	0.08252	A
11	US6P	8	0.6093	0.08252	A
12	US7N	8	0.5297	0.08252	AB
13	US5P	8	0.5193	0.08252	AB
14	US7N	16	0.4983	0.08252	ABC
15	US7P	16	0.2942	0.08252	BCD
16	US5N	16	0.2451	0.08252	CDE
17	US6P	16	0.2276	0.08252	DE
18	WT	8	-0.00044	0.08252	E
19	US5P	16	-0.00698	0.1003	E
20	WT	16	-0.02105	0.1003	E

Appendix 8.7 a: Type 3 tests of fixed effects for relative transcript levels of ***SSI*** in wheat cultivars at two grain developmental stages at $p \leq 0.05$

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Line	5	12	2.56	0.0846
stage	1	9	1.38	0.2708
Line*stage	5	9	5.82	0.0114

Appendix 8.7 b: Letter groups for lines based on the least significant difference for the means of the relative transcript level of ***SSI*** at $p \leq 0.05$

Effect= Line Method= LSD (P < .05) Set=1

Obs	Line	stage	Estimate	Standard Error	Letter Group
1	US5P		0.1099	0.07612	A
2	US5N		0.08086	0.05590	A
3	US7N		0.01733	0.05590	AB
4	WT		0.01328	0.06158	AB
5	US6P		-0.1058	0.05590	B
6	US7P		-0.1267	0.05590	B

Appendix 8.7 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***SSI*** at $p \leq 0.05$

Effect=stage Method=LSD (P < .05) Set=2

Obs	Line	stage	Estimate	Standard Error	Letter Group
7		8	0.02386	0.03015	A
8		16	-0.02760	0.03577	A

Appendix 8.7 d: Letter groups for the lines (positive, negative and wild-type) and developmental stage interaction based on the least significant difference for the means of the relative transcript level of ***SSI*** at $p \leq 0.05$

Effect=Line*stage Method=LSD (P < .05) Set=3

Obs	Line	stage	Estimate	Standard Error	Letter Group
9	US5P	16	0.3740	0.1270	A
10	US5N	8	0.2171	0.07385	AB
11	US7N	8	0.1657	0.07385	ABC
12	WT	16	0.02601	0.09013	ABCD
13	WT	8	0.000561	0.07385	BCD
14	US6P	8	-0.01334	0.07385	BCD
15	US5N	16	-0.05539	0.07385	CD
16	US7P	8	-0.07260	0.07385	D
17	US7N	16	-0.1310	0.07385	D
18	US5P	8	-0.1542	0.07385	D
19	US7P	16	-0.1809	0.07385	D
20	US6P	16	-0.1983	0.07385	D

Appendix 8.8 a: Type 3 tests of fixed effects for relative transcript levels of ***SSIIa*** in wheat cultivars at two grain developmental stages at $p \leq 0.05$

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Line	5	12	1.47	0.2702
stage	1	9	18.22	0.0021
Line*stage	5	9	7.10	0.0060

Appendix 8.8 b: Letter groups for lines based on the least significant difference for the means of the relative transcript level of ***SSIIa*** at $p \leq 0.05$

Effect= Line Method= LSD (P < .05) Set=1

Obs	Line	stage	Estimate	Standard Error	Letter Group
1	US7P		0.2511	0.08912	A
2	US7N		0.2054	0.08582	A
3	US6P		0.1738	0.08582	A
4	US5N		0.1314	0.08582	A
5	US5P		0.008387	0.08912	A
6	WT		-0.01396	0.08912	A

Appendix 8.8 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***SSIIa*** at $p \leq 0.05$

Effect=stage Method=LSD (P < .05) Set=2

Obs	Line	stage	Estimate	Standard Error	Letter Group
7		8	0.1969	0.03815	A
8		16	0.05512	0.04059	B

Appendix 8.8 d: Letter groups for the lines (positive, negative and wild-type) and developmental stage interaction based on the least significant difference for the means of the relative transcript level of ***SSIIa*** at $p \leq 0.05$

Effect=Line*stage Method=LSD (P < .05) Set=3

Obs	Line	stage	Estimate	Standard Error	Letter Group
9	US7P	8	0.4151	0.09345	A
10	US6P	8	0.3295	0.09345	AB
11	US7N	16	0.3096	0.09345	ACD
12	US5N	8	0.2488	0.09345	ABCE
13	US7N	8	0.1012	0.09345	BEF
14	US5P	8	0.08838	0.09345	BCDEF
15	US7P	16	0.08709	0.1051	BCDEF
16	US6P	16	0.01812	0.09345	CDEF
17	US5N	16	0.01402	0.09345	DF
18	WT	8	-0.00142	0.09345	EF
19	WT	16	-0.02650	0.1051	EF
20	US5P	16	-0.07161	0.1051	F

Appendix 8.9 a: Type 3 tests of fixed effects for relative transcript levels of ***SSIIIa*** in wheat cultivars at two grain developmental stages at $p \leq 0.05$

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Line	5	12	0.06	0.9967
stage	1	10	0.52	0.4866
Line*stage	5	10	3.01	0.0652

Appendix 8.9 b: Letter groups for lines based on the least significant difference for the means of the relative transcript level of ***SSIIIa*** at $p \leq 0.05$

Effect= Line Method= LSD (P < .05) Set=1

Obs	Line	stage	Estimate	Standard Error	Letter Group
1	WT		1.1298	0.2022	A
2	US5P		1.1080	0.2022	A
3	US7P		1.0800	0.1839	A
4	US6P		1.0667	0.1839	A
5	US5N		1.0483	0.1839	A
6	US7N		0.9933	0.1839	A

Appendix 8.9 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***SSIIIa*** at $p \leq 0.05$

Effect=stage Method=LSD (P < .05) Set=2

Obs	Line	stage	Estimate	Standard Error	Letter Group
7		16	1.1193	0.1062	A
8		8	1.0228	0.09849	A

Appendix 8.9 d: Letter groups for the lines (positive, negative and wild-type) and developmental stage interaction based on the least significant difference for the means of the relative transcript level of ***SSIIIa*** at $p \leq 0.05$

Effect=Line*stage Method=LSD (P < .05) Set=3

Obs	Line	stage	Estimate	Standard Error	Letter Group
9	US5P	16	1.6093	0.2942	A
10	US5N	8	1.3700	0.2413	AB
11	WT	16	1.2163	0.2942	ABC
12	US6P	8	1.2133	0.2413	ABC
13	US7N	16	1.2033	0.2413	ABC
14	US7P	8	1.1200	0.2413	ABC
15	WT	8	1.0433	0.2413	ABC
16	US7P	16	1.0400	0.2413	ABC
17	US6P	16	0.9200	0.2413	ABC
18	US7N	8	0.7833	0.2413	ABC
19	US5N	16	0.7267	0.2413	BC
20	US5P	8	0.6067	0.2413	C

Appendix 8.10 a: Type 3 tests of fixed effects for relative transcript levels of ***SBEI*** in wheat cultivars at two grain developmental stages at $p \leq 0.05$

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Line	5	12	3.64	0.0309
stage	1	10	35.44	0.0001
Line*stage	5	10	5.30	0.0123

Appendix 8.10 b: Letter groups for lines based on the least significant difference for the means of the relative transcript level of ***SBEI*** at $p \leq 0.05$

Effect= Line Method= LSD (P < .05) Set=1

Obs	Line	stage	Estimate	Standard Error	Letter Group
1	US7N		0.5429	0.1023	A
2	US5P		0.4292	0.1143	AB
3	US5N		0.3631	0.1023	ABC
4	US6P		0.1620	0.1023	BC
5	WT		0.07888	0.1143	BC
6	US7P		0.05532	0.1023	C

Appendix 8.10 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***SBEI*** at $p \leq 0.05$

Effect=stage Method=LSD (P < .05) Set=2

Obs	Line	stage	Estimate	Standard Error	Letter Group
7		8	0.5306	0.05904	A
8		16	0.01323	0.06377	B

Appendix 8.10 d: Letter groups for the lines (positive, negative and wild-type) and developmental stage interaction based on the least significant difference for the means of the relative transcript level of ***SBEI*** at $p \leq 0.05$

Effect=Line*stage Method=LSD (P < .05) Set=3

Obs	Line	stage	Estimate	Standard Error	Letter Group
9	US7N	8	0.9988	0.1446	A
10	US5N	8	0.9688	0.1446	A
11	US5P	8	0.6428	0.1446	AB
12	US7P	8	0.3132	0.1446	BC
13	US6P	8	0.2617	0.1446	BC
14	US5P	16	0.2157	0.1771	BCD
15	WT	16	0.1595	0.1771	BCD
16	US7N	16	0.08710	0.1446	CD
17	US6P	16	0.06222	0.1446	CD
18	WT	8	-0.00172	0.1446	CD
19	US7P	16	-0.2025	0.1446	D
20	US5N	16	-0.2426	0.1446	D

Appendix 8.11 a: Type 3 tests of fixed effects for relative transcript levels of ***SBEIIa*** in wheat cultivars at two grain developmental stages at $p \leq 0.05$

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Line	5	12	5.26	0.0087
stage	1	8	34.16	0.0004
Line*stage	5	8	3.83	0.0455

Appendix 8.11 b: Letter groups for lines based on the least significant difference for the means of the relative transcript level of ***SBEIIa*** at $p \leq 0.05$

Effect= Line Method= LSD (P < .05) Set=1

Obs	Line	stage	Estimate	Standard Error	Letter Group
1	US6P		0.5086	0.08204	A
2	US7P		0.4602	0.09172	AB
3	US5P		0.3232	0.1005	AB
4	US5N		0.2832	0.08204	AB
5	US7N		0.2402	0.08204	B
6	WT		-0.06875	0.09172	C

Appendix 8.11 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***SBEIIa*** at $p \leq 0.05$

Effect=stage Method=LSD (P < .05) Set=2

Obs	Line	stage	Estimate	Standard Error	Letter Group
7		8	0.5026	0.04930	A
8		16	0.07965	0.05296	B

Appendix 8.11 d: Letter groups for the lines (positive, negative and wild-type) and developmental stage interaction based on the least significant difference for the means of the relative transcript level of ***SBEIIa*** at $p \leq 0.05$

Effect=Line*stage Method=LSD (P < .05) Set=3

Obs	Line	stage	Estimate	Standard Error	Letter Group
9	US6P	8	0.8696	0.1160	A
10	US7P	8	0.7248	0.1160	A
11	US5P	8	0.6715	0.1421	AB
12	US5N	8	0.5638	0.1160	ABC
13	US7N	16	0.2950	0.1160	BCD
14	US7P	16	0.1957	0.1421	CDE
15	US7N	8	0.1854	0.1160	DE
16	US6P	16	0.1476	0.1160	DE
17	US5N	16	0.002456	0.1160	DE
18	WT	8	0.000200	0.1160	DE
19	US5P	16	-0.02506	0.1421	DE
20	WT	16	-0.1377	0.1421	E

Appendix 8.12 a: Type 3 tests of fixed effects for relative transcript levels of ***SBEIIb*** in transformed lines at two grain developmental stages at $p \leq 0.05$

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Line	5	12	3.03	0.0535
stage	1	9	8.02	0.0197
Line*stage	5	9	1.50	0.2796

Appendix 8.12 b: Letter groups for lines based on the least significant difference for the means of the relative transcript level of ***SBEIIb*** at $p \leq 0.05$

Effect= Line Method= LSD (P < .05) Set=1

Obs	Line	stage	Estimate	Standard Error	Letter Group
1	US7P		0.3710	0.06916	A
2	US6P		0.2635	0.06916	AB
3	US5N		0.2043	0.06916	ABC
4	US7N		0.1903	0.06916	ABC
5	US5P		0.04705	0.09781	BC
6	WT		0.01467	0.07732	C

Appendix 8.12 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***SBEIIb*** at $p \leq 0.05$

Effect=stage Method=LSD (P < .05) Set=2

Obs	Line	stage	Estimate	Standard Error	Letter Group
7		8	0.2697	0.03993	A
8		16	0.09391	0.04753	B

Appendix 8.12 d: Letter groups for the lines (positive, negative and wild-type) and developmental stage interaction based on the least significant difference for the means of the relative transcript level of ***SBEIIb*** at $p \leq 0.05$

Effect=Line*stage Method=LSD (P < .05) Set=3

Obs	Line	stage	Estimate	Standard Error	Letter Group
9	US5N	8	0.4187	0.09781	A
10	US7P	8	0.4087	0.09781	A
11	US6P	8	0.4083	0.09781	A
12	US7P	16	0.3334	0.09781	AB
13	US7N	8	0.2078	0.09781	ABC
14	US5P	8	0.1750	0.09781	ABC
15	US7N	16	0.1727	0.09781	ABC
16	US6P	16	0.1186	0.09781	ABC
17	WT	16	0.02971	0.1198	BC
18	WT	8	-0.00037	0.09781	C
19	US5N	16	-0.01002	0.09781	C
20	US5P	16	-0.08092	0.1694	BC

Appendix 8.13 a: Type 3 tests of fixed effects for relative transcript levels of ***SUT1*** in transformed lines at two grain developmental stages at $p \leq 0.05$

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Line	5	12	10.17	0.0005
stage	1	8	0.01	0.9295
Line*stage	5	8	4.24	0.0352

Appendix 8.13 b: Letter groups for lines based on the least significant difference for the means of the relative transcript level of ***SUT1***at $p \leq 0.05$

Effect= Line Method= LSD (P < .05) Set=1

Obs	Line	stage	Estimate	Standard Error	Letter Group
1	US5P		0.7252	0.07970	A
2	US7N		0.5880	0.07128	AB
3	US6P		0.5448	0.07128	AB
4	US7P		0.4889	0.07970	AB
5	US5N		0.4647	0.07970	B
6	WT		-0.01298	0.07970	C

Appendix 8.13 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***SUT1*** at $p \leq 0.05$

Effect=stage Method= LSD (P < .05) Set=2

Obs	Line	stage	Estimate	Standard Error	Letter Group
7		16	0.4693	0.04752	A
8		8	0.4636	0.04116	A

Appendix 8.13 d: Letter groups for the lines (positive, negative and wild-type) and developmental stage interaction based on the least significant difference for the means of the relative transcript level of ***SUT1*** at $p \leq 0.05$

Effect=Line*stage Method= LSD(P<.05) Set=3

Obs	Line	stage	Estimate	Standard Error	Letter Group
9	US5P	16	0.8867	0.1235	A
10	US5N	8	0.7489	0.1008	AB
11	US7N	16	0.7010	0.1008	AB
12	US6P	16	0.6338	0.1008	AB
13	US5P	8	0.5637	0.1008	AB
14	US7P	8	0.5371	0.1008	ABC
15	US7N	8	0.4750	0.1008	BC
16	US6P	8	0.4557	0.1008	BC
17	US7P	16	0.4407	0.1235	BC
18	US5N	16	0.1804	0.1235	CD
19	WT	8	0.000815	0.1008	D
20	WT	16	-0.02677	0.1235	D

Appendix 8.14 a: Type 3 tests of fixed effects for relative transcript levels of ***SuSy2*** in transformed lines at two grain developmental stages at $p \leq 0.05$

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Line	5	11	4.52	0.0174
stage	1	7	35.33	0.0006
Line*stage	5	7	3.89	0.0523

Appendix 8.14 b: Letter groups for lines based on the least significant difference for the means of the relative transcript level of ***SuSy2*** at $p \leq 0.05$

Effect=Line Method= LSD (P<.05) Set=1

Obs	Line	stage	Estimate	Standard Error	Letter Group
1	US6P		0.4456	0.06303	A
2	US7P		0.3996	0.07047	A
3	US7N		0.2970	0.06303	AB
4	US5N		0.2961	0.06303	AB
5	US5P		0.08214	0.09454	BC
6	WT		0.01843	0.08914	C

Appendix 8.14 c: Letter groups for grain developmental stages based on the least significant difference for the means of the relative transcript level of ***SuSy2*** at $p \leq 0.05$

Effect=stage Method= LSD (P<.05) Set=2

Obs	Line	stage	Estimate	Standard Error	Letter Group
7		8	0.4385	0.03788	A
8		16	0.07444	0.04814	B

Appendix 8.14 d: Letter groups for the lines (positive, negative and wild-type) and developmental stage interaction based on the least significant difference for the means of the relative transcript level of ***SuSy2*** at $p \leq 0.05$

Effect=Line*stage Method= LSD (P<.05) Set=3

Obs	Line	stage	Estimate	Standard Error	Letter Group
9	US6P	8	0.7401	0.08914	A
10	US7P	8	0.6572	0.08914	A
11	US7N	8	0.5000	0.08914	AB
12	US5P	8	0.4239	0.1092	ABC
13	US5N	8	0.3103	0.08914	BC
14	US5N	16	0.2818	0.08914	BCD
15	US6P	16	0.1511	0.08914	CDE
16	US7P	16	0.1419	0.1092	CDE
17	US7N	16	0.09405	0.08914	CDE
18	WT	16	0.03743	0.1544	CDE
19	WT	8	-0.00057	0.08914	DE
20	US5P	16	-0.2596	0.1544	E

Appendix 9: Analysis of Variance (ANOVA) for the total starch content per grain of wild type (WT), transgenic positive (US5P, US6P, US7P) and negative control (US5N, US7N) using the PROC Mixed procedure of SAS software

Appendix 9.1 a: Type 3 tests of fixed effects for the total starch content per grain in transformed lines at mature grain stage at $p \leq 0.05$

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Line	5	12	5.52	0.0073

Appendix 9.1 b: Letter groups for lines based on the least significant difference for the means of total starch content per grain at $p \leq 0.05$

Effect=Line Method= LSD (P<.05) Set=1

Obs	Line	Estimate	Standard Error	Letter Group
1	US5P	30.1900	1.0797	A
2	US7P	25.4833	1.0797	B
3	US5N	25.2433	1.0797	B
4	US6P	24.4900	1.0797	B
5	WT	23.8700	1.0797	B
6	US7N	22.9467	1.0797	B

Appendix 10: Analysis of Variance (ANOVA) for AGPase activity per mg protein in wild type (WT), transgenic positive (US5P, US6P, US7P) and negative control (US5N, US7N) using the PROC Mixed procedure using SAS software

Appendix 10.1 a: Type 3 tests of fixed effects for the total AGPase activity per mg protein in transformed lines at 8 DAA at $p \leq 0.05$

Type 3 Tests of Fixed Effects

Effect	NumDF	DenDF	F Value	Pr > F
Line	5	11	7.51	0.0027

Appendix 10.1 b: Letter groups for the transformed lines based on the least significant difference for the total AGPase activity per mg protein in transformed lines at 8 DAA at $p \leq 0.05$

Effect=Line Method=LSD (P < .05) Set=1

Obs	Line	Estimate	Standard Error	Letter Group
1	US5P	-0.3708	0.1505	A
2	US7P	-0.3737	0.1505	A
3	US6P	-0.7454	0.1505	AB
4	US5N	-1.0240	0.1843	BC
5	WT	-1.2701	0.1505	C
6	US7N	-1.2830	0.1505	C

Appendix 10.2 a: Type 3 tests of fixed effects for the total AGPase activity per mg protein in transformed lines at 16 DAA at $p \leq 0.05$

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Line	5	12	2.21	0.1209

Appendix 10.2 b: Letter groups for the transformed lines based on the least significant difference for the total AGPase activity per mg protein in transformed lines at 16 DAA at $p \leq 0.05$

Obs	Line	stage	Estimate	Standard Error	Letter Group
1	US6P		-0.2818	0.1019	A
2	US7P		-0.2935	0.1019	A
3	US5P		-0.4238	0.1019	AB
4	US7N		-0.5081	0.1019	AB
5	US5N		-0.5258	0.1019	AB
6	WT		-0.6782	0.1019	B

Appendix 11: One way ANOVA for average grain weight of wild type (WT) transgenic positive (US5P, US6P, US7P) and negative control (US5N, US7N) lines using SigmaPlot 12 software

Normality Test (Shapiro-Wilk) Passed (P = 0.954)

Equal Variance Test: Passed (P = 0.401)

Group Name	N	Missing	Mean	Std Dev	SEM
WT	3	0	40.663	1.077	0.622
US5P	3	0	48.630	1.103	0.637
US6P	3	0	41.623	2.177	1.257
US7P	3	0	44.020	2.137	1.234
US5N	3	0	36.860	3.679	2.124
US7N	3	0	42.323	1.179	0.681

Source of Variation	DF	SS	MS	F	P
Between Groups	5	227.223	45.445	10.247	<0.001
Residual	12	53.219	4.435		
Total	17	280.442			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 0.995

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	q	P	P<0.050
US5P vs. US5N	11.7706	9.680	<0.001	Yes
US5P vs. WT	7.9676	6.552	0.006	Yes
US5P vs. US6P	7.0076	5.763	0.015	Yes
US5P vs. US7N	6.3076	5.187	0.030	Yes
US5P vs. US7P	4.6106	3.792	0.151	No
US7P vs. US5N	7.1606	5.889	0.013	Yes
US7P vs. WT	3.3576	2.761	0.419	No
US7P vs. US6P	2.3976	1.971	0.730	Do Not Test
US7P vs. US7N	1.6976	1.395	0.914	Do Not Test
US7N vs. US5N	5.4636	4.493	0.068	No
US7N vs. WT	1.6606	1.365	0.920	Do Not Test
US7N vs. US6P	0.7006	0.576	0.998	Do Not Test
US6P vs. US5N	4.7636	3.918	0.131	Do Not Test
US6P vs. WT	0.9606	0.790	0.992	Do Not Test
WT vs. US5N	3.8036	3.128	0.300	Do Not Test

A result of "Do Not Test" occurs for a comparison when no significant difference is found between two means that enclose that comparison.

Appendix 12: Isolation and characterization of rubisco small subunit promoter from common wheat (*Triticum aestivum*)

1. Abstract

In order to isolate a promoter specific to source tissues from wheat, a partial sequence upstream of the coding sequence of the wheat *rbcS* gene was targeted. The functionality of this putative promoter sequence was examined by fusing it with a GUS reporter gene. The *TarbcS:GUS* construct was bombarded into wheat immature embryos and roots, and tobacco leaf discs. Transient expression of the reporter gene was observed in both immature wheat embryos and tobacco leaf tissues but not in the wheat root tissue. Analysis of the promoter nucleotide sequence revealed the presence of light responsive elements such as the I-box and GT1 consensus sequences as well as the GATA sequence, that are associated with light induced and tissue-specific expression. These results indicate that the isolated *TarbcS* sequence can be used to localize the expression of target genes in source tissues.

2. Introduction

Promoters, regions upstream of the coding region in a DNA sequence, contain specific sequences that are recognized by proteins involved in the initiation of transcription (e.g., transcription factors) (Buchanan and Jones, 2007). Several promoters from different organisms (e.g., from virus or plants) have been used in plant molecular biology in order to express the gene of interest constitutively or in a tissue- or stage- specific manner. It is very critical to make the right choice of promoter for successful gene transfer and expression in plants (irrespective of their end use) in order to avoid unwanted consequences (Dale *et al*., 2002).

Constitutive promoters are often used to drive high levels of gene expression in nearly all tissues at all stages of development and in a wide range of environmental conditions (Blechl and Jones, 2009). The most frequently used constitutive promoters include the maize ubiquitin (*Ubi1*) (Christensen *et al*., 1992; Rooke *et al*., 2000) and the rice actin (*Act1*) promoters (Zhang *et al*., 1991) from plants and the *35S* promoter from *Cauliflower mosaic virus* (Becker *et al*., 1994). The *Ubi1* and *Act1* promoters are often used with their first introns, as this inclusion of increases the levels of gene expression (Meng *et al*., 2003; Bourdon *et al*., 2004). The other kind of promoters used in transgene expression are tissue/stage specific promoters, which are used to limit the expression of transgenes in selected tissues or developmental stages, and reduce unintended effects of the transgenes in non-target tissues/stages (Blechl and Jones, 2009). Some examples of such promoters include the high molecular weight glutenin subunit (HMW-GS), which is a seed-specific promoter (Lamacchia *et al*., 2001), and the promoter of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (Ellis, 1981; Wang *et al*., 2013b), which is specifically activated in the leaf tissue. There are also other promoters which can be induced by certain conditions such as (glutathione-S- transferase) *GstA1* which is induced

by pathogens and has been used to express defense-related wheat genes in inner leaf tissues (Altpeter *et al*., 2005) or *HvA1*, which induced by ABA related stress (Hong *et al*., 1992).

RuBisCO, the most abundant protein on earth, catalyzes the first step of carbon fixation in plants. It also functions as an oxygenase in photorespiration. In higher plants, it is composed of eight large and small subunits arranged in 4 dimers. The large subunits (rbcL) are encoded in the chloroplast, whereas the small subunits (rbcS) are encoded in the nucleus (Ellis, 1981) . In plants, the rbcS multigene family has been well characterized and has regulatory elements that confer light inducible and tissue specific expressions in transgenic plants (Gilmartin and Chua, 1990). The promoter of the gene encoding RuBisCO small subunit (*RbcS*) has been proven to be an efficient tool in expressing target genes in photosynthetic tissues, such as expressing a nitrilase gene (*bxn*) from a soil bacterium *Klebsiella ozaenae* in the leaves of tobacco to provide resistance against the herbicide bromoxynil (Stalker *et al*., 1988). It has also been used to express a truncated delta-endotoxin gene, *cryIA(b)* of *Bacillus thuringiensis*, in the leaves of rice to provide resistance against injury by insects, and a β-glucuronidase gene to examine gene expression in the leaves of cotton (Song *et al*., 2000). Furthermore, the promoter of *RbcS* from *Coffea arabica* has been shown to function as a "leaf-specific and light-regulated promoter" in tobacco plants (Marraccini *et al*., 2003).

Several light responsive elements (LREs) have long been known to be responsible for light-inducible increases in gene expressions (Fluhr *et al*., 1986). In most plants, *rbcS* is encoded by a multigene family consisting of 2 to 22 members (Rodermel, 1999; Sasanuma, 2001; Suzuki *et al*., 2009). The promoters from some of these members are known to contain *cis*-acting elements called the I-box and G-box, which are important for tissue specific expression (Giuliano *et al*., 1988; Donald and Cashmore, 1990). The I-box and G-box *cis*-elements could

confer gene expression level comparable to that produced by the 35S CaMV promoter (Song *et al*., 2000). Another consensus sequence, designated as GT-1, has been found in several light regulated genes, including the *rbcS* of pea and other dicot species (Terzaghi and Cashmore, 1995), and is thought to have a role in stabilizing the TATA box complex.

To date, there has not yet been any report of the isolation and characterization of the *rbcS* promoter from wheat. This study was aimed at identifying a promoter region of the *TarbcS* gene and characterizing its functionality in driving the expression of target genes in photosynthetic tissues in different plant species.

3. Materials and methods

3.1 Plant material

Spring wheat cv. Fielder was grown in the greenhouse at 16-22^{o}C/14-18^{o}C (day/night) in a 16/8 h photoperiod. Ten seeds (1 seed/pot) were planted in Sunshine Mix 4 and Cornell foliage mix. Following two weeks after planting, plants were fertilized biweekly with 20-20-20 (N-P-K). Spikes were tagged on the day of anthesis and harvested at 14 days after anthesis (DAA). Embryos were isolated and used for transformation. For root related experiments, seeds of cultivar Fielder were germinated with water on a filter paper, and 5 to 7 days after planting in Sunshine Mix 4, the roots were harvested and used for the assay.

Tobacco (*Nicotiana tabacam* cv. *Xanthi*) plants were grown in the greenhouse at 16-22^{o}C /14-18^{o}C (day/night) in a 16/8 h photoperiod at an average light intensity of 296.7 ± 10.2 µEm^{-2}sec^{-1}. Seeds were planted in one gallon pots containing Sunshine Mix 4 and Cornell foliage mix. Plants were fertilized from seedling stage with 20-20-20 (1 tablespoon per gallon water) every week. Leaves were harvested at 3 to 4 weeks after planting and used for transient transformation.

3.2 Isolation of RbcS promoter

The wheat *RbcS* gene sequence available in GenBank (accession no. AB042069) was used as the template to design primers for isolating a promoter fragment from the genomic DNA (upstream of the start codon) of hexaploid spring wheat (*Triticum aestivum*) namely Chinese Spring. The forward and reverse primers primers used were 5'-CCAAGCTTTCAATAGTTGCCTTGCGAG -3' and 5'- TTGGATCCATCGGAGGAGAGGAGGA -3', respectively. To facilitate the cloning of the promoter fragment into the vector (pBI121) restriction endonuclease sites, HindIII and BamHI, were incorporated in the forward and the reverse primer sequences, respectively. The PCR product separated on a 1% agarose gel was gel-purified, cloned into pGEMTeasy (Promega, Fitchburg, WI, USA), and then sequenced.

3.3 Construction of the transformation vector

The binary vector pBI121 carrying the *CaMV35S* promoter and β-glucuronidase (GUS) reporter gene was used for preparing the transformation vector. The *CaMV35S* promoter was first removed before the 576 bp fragment of the *RbcS* promoter was inserted using directional cloning with HindIII and BamHI restriction sites (Fig. 1). Once constructed, the vector was used to transform the immature embryos and roots of wheat and the young leaves of tobacco plants. Transformations were also performed with pBI121 carrying the *CaMV35S* promoter as a positive control and also with the promoter-less pCAMBIA1391Z as a negative control. Transformation of young roots was done with pBRACT214 (see Chapter 5) carrying the GUS reporter gene driven by the *Ubiquitin1* promoter.

3.4 Microprojectile bombardment

Transformation was achieved through bombardment with gold particles using the particle delivery system PDS-1000/He (BioRad, Hercules, CA, USA). Gold particles (1.0 μm) were

coated with 5 μL plasmid DNA (at the concentration of 1μg/μL) and bombarded into the leaf tissues of tobacco, and embryos and roots of wheat. To this end, the DNA coated gold particles were placed in the center of a macrocarrier, and the particle delivery system adjusted to 950 psi of helium pressure and 27mm Hg of vacuum pressure. The immature wheat embryos and roots, and the tobacco leaf discs, prepared from the tobacco leaf using a cork borer, were placed at the center of a petri dish containing MS media. Following the bombardment, the leaf, the embryos as well as the roots were incubated for 48 h in darkness at room temperature prior to histochemical staining.

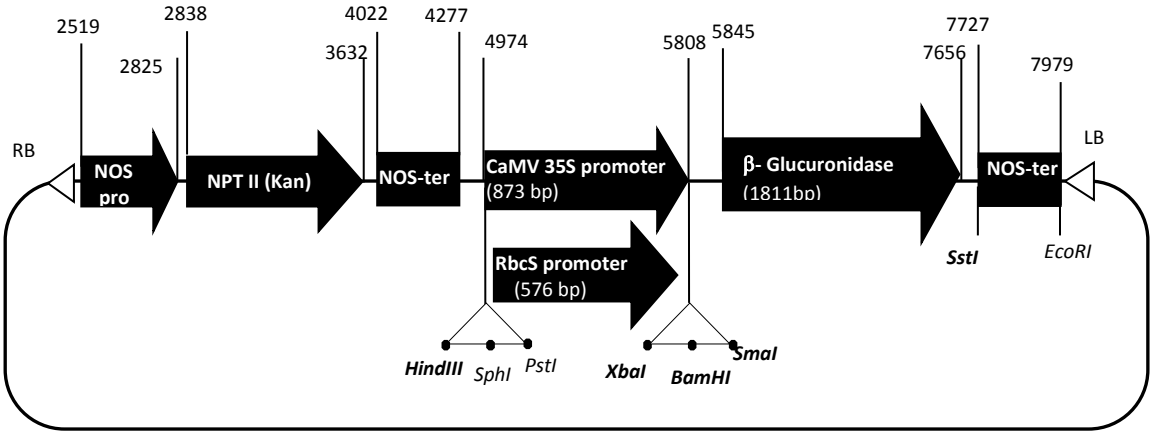


Figure 1: Schematic diagram of the pBI121 vector. The original pBI121 vector was modified by substitution of the *CaMV35S* promoter with *RbcS* promoter.

3.5 Histochemical GUS Assay

After 48 h incubation, the individual immature embryos, leaf discs and roots were removed from the media, and incubated in staining buffer (50 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.5 mM ferrocyanide, 0.5 mM ferricyanide, 0.05% Triton X-100, X-glucuronide 1mg/mL) for 24 h at 37^{o}C. The resulting blue stainings were observed under a light microscope.

4. Results

4.1 Isolation and analysis of the wheat rbcS promoter

Amplification of the genomic DNA with primers described above produced a DNA fragment of expected size (Fig. 2). Sequencing of the fragment revealed a size of 576 bp (Fig. 2). Analysis of the promoter fragment with PlantPAN (http://plantpan.mbc.nctu.edu.tw/seq_analysis.php) showed the presence of elements that are characteristic features of an *rbcS* promoter (Fig. 3). These characteristic elements include the I-Box core elements (GATAAG) at -156 to -151 bp (Fig. 3) and the GT-1 consensus sequence (GRWAAW; R= A/G, W=A/T) at -309 to -314. The I-Box core element, has been identified in the upstream region of several light regulated genes, especially in the *rbcS* of various species including Arabidopsis and tomato (Giuliano *et al*., 1988; Bakhsh *et al*., 2011). The GT-1 consensus sequence, which is present in several light induced genes of several plants (Terzaghi and Cashmore, 1995), is proposed to function in stabilizing the TATA box complex (-429 to -422 bp) (Fig. 2). Our analysis also revealed the presence of repetitive GATA sequence (at the positions -360bp, -156bp, -14 bp) (Fig. 3), which have been shown to be associated with light induced and tissue-specific expression, was present (Lam and Chua, 1989). Furthermore, the CAAT box (-581bp, -518bp, -232bp, -55bp) (Fig. 3) identified as an important sequence for promoter activity of several genes (Shirsat *et al*., 1989) was present.

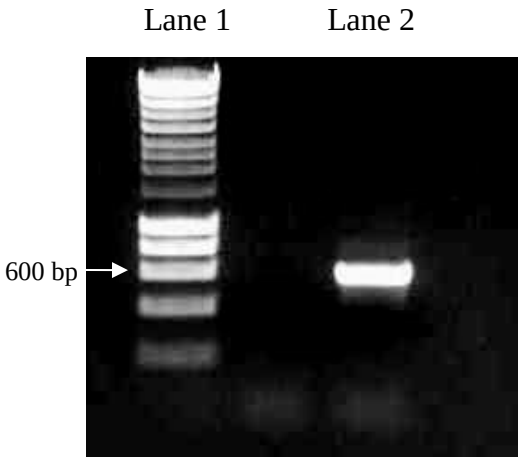


Figure 2: PCR product of the partial DNA fragment of *TarbcS* promoter amplified from genomic DNA sample prepared from leaf tissues of Chinese Spring using primers designed from upstream of the start codon of the gene *TarbcS*. Lane 1 shows the DNA ladder and Lane 2 shows the PCR product (576 bp)

Table 1: Comparative analysis of GUS reporter gene expression driven by *TarbcS* and *CaMV35S* in tobacco leaves.

Promoter driving GUS expression	**Total number of leaf discs bombarded**	**Total number of leaf discs showing GUS expression**	**Average TEUs*/GUS expressing leaf discs**
TarbcS	116	23	21.2
CaMV35S	74	57	69.6

*TEUs= average number of blue spots/GUS expressing leaves

Figure 3: Nucleotide sequence of the promoter fragment *TarbcS* (GenBank accession number AB042069.1). The start of the coding region of *TarbcS* is indicated by an asterisk. The TATA-box, CAAT-box, I-box, GT1-consensus sequences are boxed in green. The GATA sequences are shown in red fonts. Primers used for PCR are shown with arrows. Sequences in italics represent the first intron.

4.2 Transient expression of TarbcS promoter

Transient expression of the GUS reporter gene driven by the *TarbcS* promoter was examined in immature wheat embryos and young roots, and tobacco young leaves. The immature wheat embryos incubated in darkness showed almost no blue staining while those incubated in light showed a stronger staining (Fig. 4 A and C). The observed staining (both in number and instensity), however, was much less than those transformed with a vector carrying *GUS* under the control of *CaMV35S* promoter (Fig. 4 D). In tobacco leaves, the degree of GUS expression

was determined as Transient Expression Units or TEU (Moore *et al*., 1994), the number of blue spots per GUS expressing leaves. The tobacco leaves also showed higher *GUS* expression and more blue spots when transformed with the vector carrying *CaMV35S*:*GUS* (69.6 TEUs) as compared to those transformed with *TarbcS:GUS* (21.1 TEUs) (Fig. 5, Table 1). The negative controls transformed with a vector carrying the *GUS* with no promoter produced no blue coloration in both immature wheat embryo and tobacco leaf samples (Fig. 4 B). Unlike the immature embryo tissues, the wheat roots transformed with *TarbcS:GUS* did not show any blue staining (Fig. 6). Transformation of wheat roots with *Ubi1*:*GUS* produced blue staining, although a similar experiment with *CaMV35S*:*GUS* was unable to show the same result (Fig. 6). Roots transformed with a vector carrying the *GUS* with no promoter also produced no blue staining (Fig. 6). In all experiments, none of the untransformed tissue samples subjected to X-glucuronidase staining along with the transformed tissues exhibited blue coloration (Fig. 6).

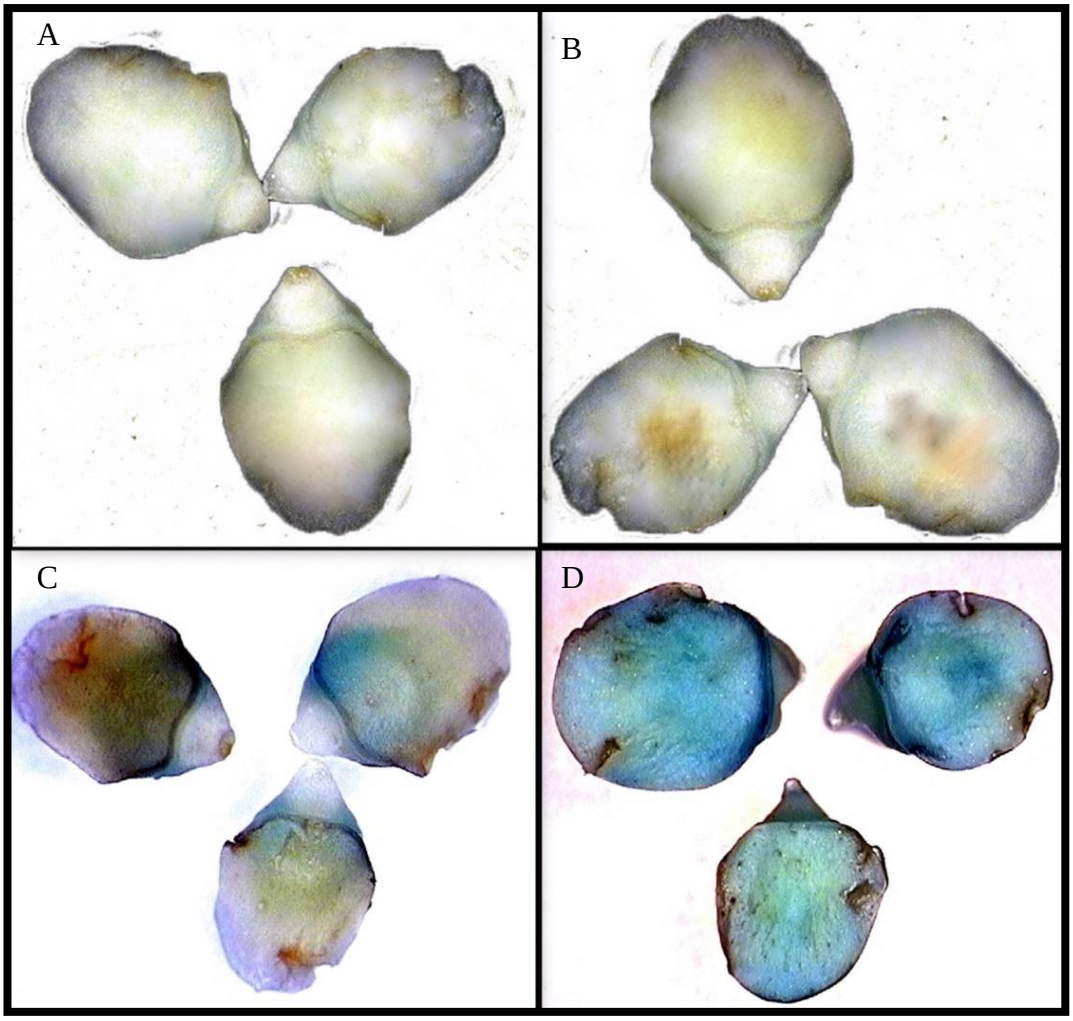


Figure 4: Wheat embryos showing transient expression of GUS. (A) Embryos incubated under darkness before and after transformation with *TarbcS*:*GUS* construct; (B) Embryos transformed with a vector carrying the *GUS* but with no promoter; (C) Embryos incubated in light before and after transformation with *TarbcS*:*GUS* construct; (D) Embryos transformed with a *CaMV 35S*:*GUS* construct.

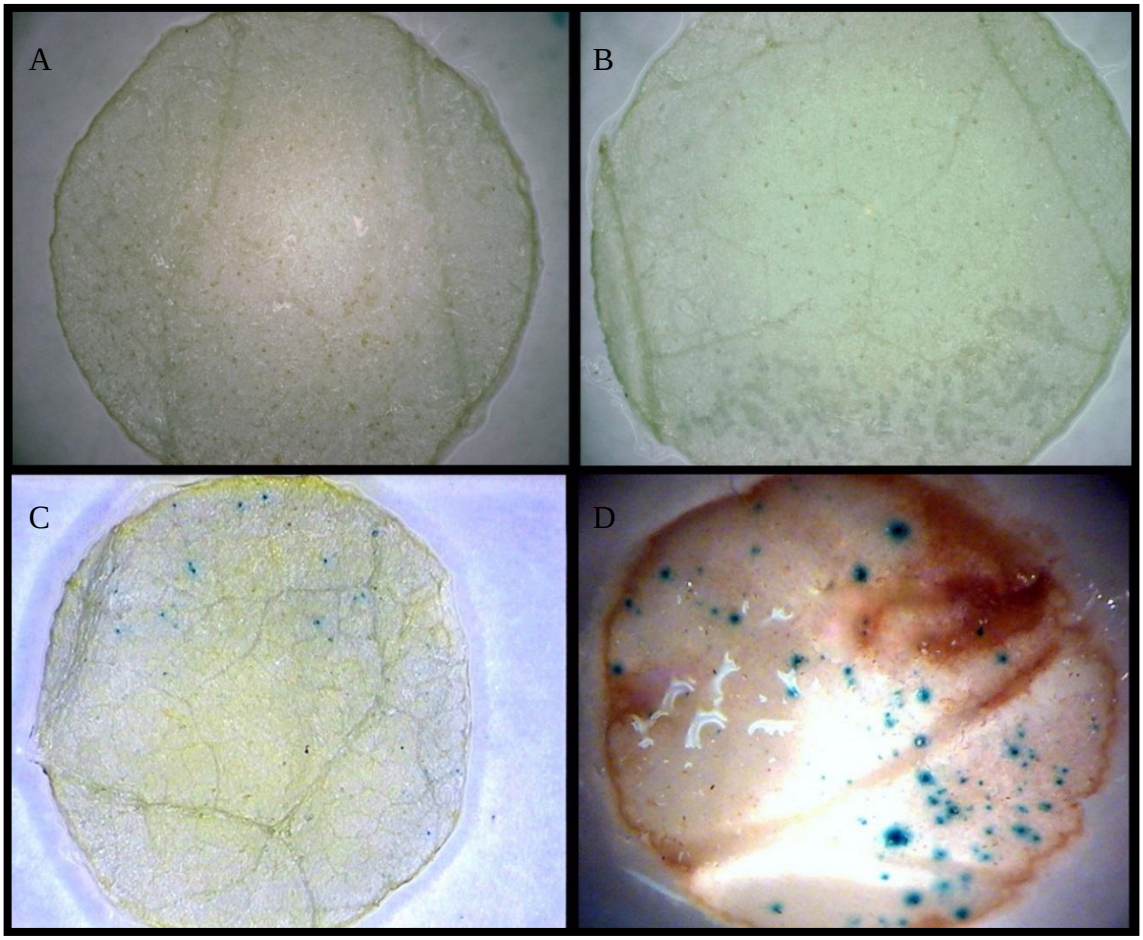


Figure 5: Young leaf discs of tobacco with transient expression of GUS. (A) Untransformed leaves subjected to X-glucuronidase staining; (B) Leaves transformed with a vector carrying the GUS gene but with no promoter; (C) Leaves transformed with *TarbcS*:*GUS* construct; (D) Leaves transformed with a *CaMV35S*:*GUS* construct.

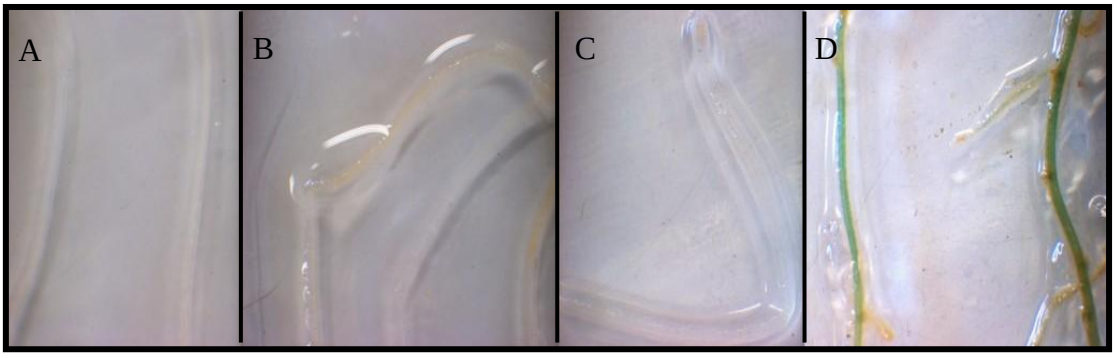


Figure 6: Young roots of wheat with transient expression of GUS. (A) Untransformed roots subjected to X-glucuronidase staining; (B) Roots transformed with a vector carrying the GUS gene but with no promoter; (C) Roots transformed with *TarbcS:GUS* construct; (D) Roots transformed with *Ubi1*:*GUS* construct .

5. Discussion

The wheat embryos show blue staining when the GUS reporter gene is driven by the *TarbcS* promoter, indicating the activity of the promoter in the immature embryos. The activity of the leaf specific promoter observed in the immature embryos might be due to the fact that immature embryos are not fully differentiated. Although differentiation in developing embryos is initiated at 8 DAA, the embryo differentiates prominently into plumule radical and even to shoot apical bud by 21 DAA (Smart and O'Brien, 1983). Undifferentiated young embryos have demonstrated totipotent nature (Frame, 2011) and hence are ideal candidates to examine the activity of promoters by transient assay. In this experiment, the embryos showed the expression of *TarbcS* more prominently when incubated under light than in darkness.

In the leaf tissues, the blue staining was higher for *CaMV35S* than for *TarbcS.* There was virtually no effect of light on the number of blue spots observed on the leaf discs transformed with GUS driven by either of the promoters. The average number of blue spots per GUS expressing leaves was expressed as Transient Expression Unit (TEUs) (Moore *et al*., 1994; Basu *et al*., 2003), which showed over 3-fold more TEUs for *CaMV35S* than for *TarbcS* (Table 1). Although it has lower efficiency than *CaMV35S*, the *TarbcS* promoter is functional in expressing GUS in leaf tissues as revealed by the GUS staining (Fig. 4, Fig. 5, and Fig. 6). Transient expression of GUS is influenced by several factors including, but not limited to, the period of incubation for GUS staining, degradation of the protein produced by *uidA* gene, and the stage of cell division in the explants (Arias-Garzon and Sayre, 1993). Optimization of these factors might improve the efficiency of *TarbcS* in driving the expression of *GUS*. Alternatively, including other regions such as the first intron of *TarbcS* with the promoter might improve its efficiency of

directing the expression of a target gene as observed for some promoters such as the *Ubi1* and *Act1* (Meng *et al*., 2003; Bourdon *et al*., 2004).

The *TarbcS*:*GUS* construct was also used to examine the functionality of the promoter in roots – a tissue which normally grows with no light exposure. No *GUS* expression was observed in roots bombarded with *TarbcS*:*GUS* or *CaMV35S*:*GUS* constructs. The reason why the *CaMV35S* promoter failed to drive any expression of the GUS gene in the wheat roots is unknown. A study that investigated a variety of promoter-reporter gene constructs through transient transformation so as to identify root specific promoters in cassava (*Manihot esculenta)* also indicated the ability of *CaMV35S* promoter in directing GUS expression in other plant tissues but not in roots (Arias-Garzon and Sayre, 1993).

To confirm if leaf specific promoter, *TarbcS*, cannot direct gene expression in roots, we compared it with the maize ubiquitin promoter (*Ubi1*). The ubiquitin promoter was able to drive the expression of GUS in the main root, as shown by the blue coloration (Fig. 6 D). A study that compared the expression of *GUS* in turfgrass under the control of four different promoters showed that the level of *GUS* expression from *CaMV35S* and a dicot (potato) Ubiquitin promoter was much lower in magnitude in comparison to that driven by rice *Act1* or maize *Ubi1* promoters (Basu *et al*., 2003). It is therefore likely that the *Ubi1* promoter resulted in a better expression owing to its monocot origin.

This study is the first to analyze the activity of *rbcS* promoter from spring wheat. The results obtained suggest that the *TarbcS* promoter is active in leaf tissues and immature undifferentiated embryos incubated when in light. However, further study is required to better understand the role of the regulatory elements directing gene expression for its potential use as a tool in crop biotechnology.