Modification of a starch biosynthetic enzyme : potential for increased seed yield in wheat (Triticum aestivum)
by Fletcher Damien Meyer

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Plant Sciences
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Abstract:
During photosynthesis in cereal crops, sugars are transported from source tissues (mainly leaves) to sink tissues (mainly seeds) for storage. The seed endosperm is the most important storage tissue since it consists largely of starch. The rate of endosperm starch biosynthesis is determined largely by the activity of starch biosynthetic enzymes within developing endosperm cells. ADP-glucose pyrophosphorylase (AGP) controls the first committed step in starch synthesis, and is allosterically inhibited by phosphate. AGP is a heterotetramer consisting of two large and two small subunits. An altered large AGP subunit (Sh2r6hs) sequence that confers decreased AGP phosphate inhibition and increased heat stability has been identified. We have transformed wheat with Sh2r6hs to assess whether wheat yield is limited by low AGP activity. We have developed two populations of transgenic wheat, which express SH2R6HS. The populations were created using either the native maize large subunit (Sh2) promoter or the wheat glutenin promoter. The Sh2 promoter confers much weaker levels of Sh2r6hs mRNA and protein expression than does the glutenin promoter. Northern blot analyses detect Sh2r6hs transcription less than one week after anthesis in both populations. The Sh2r6hs AGP heterotetramer in Glutenin (Glu) promoter transformants exhibits ten times as much enzyme activity as untransformed AGP in the presence of phosphate. Greenhouse studies indicate that Sh2r6hs lines created using the Sh2 promoter have increased yield and an increased number of seeds per plant. Field test results indicate that this yield enhancement is also seen under various field conditions in high-expressing Sh2r6hs genotypes (Glu and Sh2 promoter populations). Increased seed yields are credited to the higher number of similar-sized seeds established on Sh2r6hs-positive plants. Our results indicate that low levels of important starch biosynthetic enzyme normally limit wheat yield.

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

During photosynthesis in cereal crops, sugars are transported from source tissues (mainly leaves) to sink tissues (mainly seeds) for storage. The seed endosperm is the most important storage tissue since it consists largely of starch. The rate of endosperm starch biosynthesis is determined largely by the activity of starch biosynthetic enzymes within developing endosperm cells. ADP-glucose pyrophosphorylase (AGP) controls the first committed step in starch synthesis, and is allosterically inhibited by phosphate. AGP is a heterotetramer consisting of two large and two small subunits. An altered large AGP subunit (*Sh2r6hs*) sequence that confers decreased AGP phosphate inhibition and increased heat stability has been identified. We have transformed wheat with *Sh2r6hs* to assess whether wheat yield is limited by low AGP activity. We have developed two populations of transgenic wheat, which express SH2R6HS. The populations were created using either the native maize large subunit (*Sh2*) promoter or the wheat *glutenin* promoter. The *Sh2* promoter confers much weaker levels of *Sh2r6hs* mRNA and protein expression than does the *glutenin* promoter. Northern blot analyses detect *Sh2r6hs* transcription less than one week after anthesis in both populations. The *Sh2r6hs* AGP heterotetramer in *Glutenin (Glu)* promoter transformants exhibits ten times as much enzyme activity as untransformed AGP in the presence of phosphate. Greenhouse studies indicate that *Sh2r6hs* lines created using the *Sh2* promoter have increased yield and an increased number of seeds per plant. Field test results indicate that this yield enhancement is also seen under various field conditions in high-expressing *Sh2r6hs* genotypes (*Glu* and *Sh2* promoter populations). Increased seed yields are credited to the higher number of similar-sized seeds established on *Sh2r6hs*-positive plants. Our results indicate that low levels of important starch biosynthetic enzyme normally limit wheat yield.

CHAPTER 1

INTRODUCTION

In the last 50 years, cereal crop yields have increased from improved agricultural practices and the use of superior cultivars developed through breeding efforts. Application of fertilizers and better water management led to higher wheat yields during the so-called "green revolution"; this yield effect was also seen when breeders increasingly selected varieties with dwarfed stems, giving a higher percentage of seed weight per plant. An assessment of wheat cultivars released from 1908 to the present found that harvest index (ratio of seed weight: vegetative plant weight) increased with the year of release from about 0.35 to 0.5 (Sinclair 1998). The upper harvest index potential for wheat may be as high as 0.6 (Austin et al. 1980), but obtaining this level with conventional breeding practices is difficult today, given wheat's long history of intense selection. Novel ways of increasing harvest index are needed to supply the world's growing demand for carbohydrates.

Sink/Source Interactions

The harvest index of wheat is greatly affected by the production and allocation of photosynthetic sugars. A plant's components are broadly categorized as either sink tissue or source tissue. Source tissues generate photosynthetic sugars that are shipped to sink tissues. Sink tissues use these delivered assimilates to maintain cell function, to fuel growth, or as storage for later plant development. Whether a given organ is composed of

sink or source tissue depends upon its stage of development (Turgeon 1989). Young leaf tissue is a utilization sink for resources, requiring constant import of sugars for immediate use. This type of sink differs from storage sinks, such as seed endosperm, which sequester sugars for later use (Ho 1988). As leaf tissue matures, it is able to photosynthesize on its own and gradually becomes an exporter, or source, of photosynthate.

The strength of sink and source tissues vary relative to one another. Source tissue strength is determined by the rate of assimilate production and the rate of assimilate deportation. Sink tissue strength is the competitive ability of a sink organ to attract these assimilates (Marcelis 1996), and is dependent upon two factors. The first factor is the amount of assimilates the sink tissue is capable of taking up, or "reservoir" size. The second, perhaps more important factor that determines sink strength is the activity of the sink tissue, or rate at which assimilates are immediately used or stored within that specific organ.

Altering Source Tissue Strength

Many studies have altered source tissue activity indirectly by manipulating sink tissues, mainly maturing kernels. Increasing assimilate allocation to kernels (increasing sink strength) should indirectly increase overall photosynthesis (Richards 2000). The exchange between photosynthesizing leaves (particularly the flag leaf) and kernel endosperm is the source/sink system most closely examined. The endosperm is primarily a storage facility for starch molecules needed during embryonic germination, and

constitutes the largest sink tissue of a developing kernel (Duvick 1992). It is not surprising that starch production in the endosperm and photosynthetic activity are so tightly linked. King et al. (1967) recorded a 50% drop in flag leaf photosynthesis 15 hours after cutting off maturing heads. A similar reduction in photosynthesis was observed when tubers were excised from developing potato plants (Basu 1999). Accumulation of leaf carbohydrates (sucrose and hexose) leads to feedback inhibition of photosynthesis in the leaf. This feedback inhibition is relieved when new sink tissues are generated, or alternative sinks utilize photosynthate. After heads were cut in two winter wheat varieties, photosynthetic sugars were rerouted into stem and leaf growth, as indicated by an increase in non-grain plant weight (Martinez-Carrasco and Thorne, 1979).

Altering Sink Tissue Strength

Changing sink tissue strength via source organ manipulation has also been well documented. Providing the optimal amount of sunlight, water, and ambient CO2 allow maximum photosynthetic efficiency in source tissues and a steady supply of assimilates to newly formed kernels. Under greenhouse conditions, head number and yield per plant strongly correlated with the number of high-radiation days (Thorne and Wood, 1986). Similarly, space-seeded plants grown in the field had more available sunlight for photosynthesis, resulting in higher yields per plant (Fischer and Laing, 1976). Destroying source organs has the opposite effect on fertilization rates and seed yield. By removing leaves at anthesis, source tissue could not supply the sugars needed for seed

initiation, and a significant reduction in seed number per plant was recorded (Prioul and Shwebel-Dugue, 1992).

There is little doubt that sink and source tissue strength are tightly intertwined; increasing sink strength may alter source strength, as demonstrated by Sun et al. (1999). CO2 uptake, photosynthesis, and total leaf area was measured in normal and mutant *Arabidopsis* plants. The mutant plants lacked an enzyme critical in the biosynthesis of starch, ADP-glucose pyrophosphorylase. Without this starch-producing enzyme, sink tissue strength in growing seeds was greatly diminished. Sun concluded that several source tissue characteristics were affected by this reduction in sink strength. Mutant lines had much lower CO2 uptake, photosynthetic rates and leaf area compared to normal *Arabidopsis* plants.

Cereal Yield is Sink-Limited

Much research has tried to resolve the question of whether cereal yield is limited by sink or source tissue strength. The hypothesis that yield is source limited has bred several experiments in which environmental conditions are controlled to give optimal photosynthetic levels. Boosting photosynthesis under elevated CO2 conditions did give higher yields in rice (Imai et al. 1985). However, this was because more vegetative biomass was produced, yet harvest index was unchanged. Another study compared wild-type and stay-green phenotypes of maize (Rajcan and Tollenaar, 1999). Stay-green phenotypes senesce later than normal maize hybrids, giving them a longer window to photosynthesize, and therefore greater source tissue strength. Similarly, the extra supply

of photosynthate was channeled into vegetative growth in stay-green maize, and no significant harvest index change was seen compared to control lines.

While some studies report yield enhancement by increasing source tissue strength (Ziska and Teramura, 1992), most do not show a significant increase in harvest index. If wheat yield is source-limited, then raising photosynthetic rates would increase seed weight but not shoot weight. It seems more likely that yield is sink limited, and that harvest index increase comes from increasing sink tissue strength (Choi et al. 1998). The task of increasing sink strength to test if yield is sink limited is much more difficult, as it cannot be done by controlling elements that source tissues depend on: water, soil conditions, atmosphere, and radiation. The sink strength of a maturing wheat kernel is determined more by biotic factors, especially the activity of starch biosynthetic enzymes. In order to understand key steps in the conversion of photosynthetic sugars to starch, an outline is given here.

The Starch Biosynthetic Pathway

Sucrose is the major carbon source for starch synthesis in storage tissues. Leaf cells, containing actively photosynthesizing chloroplasts, send sucrose through phloem conduits to newly formed seeds (Geiger, 1979). A wheat seed consists primarily of the embryo and the endosperm, the latter being a nutritive tissue that supplies starch molecules during embryo germination. Sucrose provides the carbon molecules needed for starch synthesis in the endosperm.

During the grain filling period, seed endosperm cells uptake sucrose several ways. Sucrose can be transported into the cell intact through plasmodesmata (Aoki et al. 1999), where it is cytosolically broken into UDP-glucose and fructose by sucrose synthase (Chourey 1981). Sucrose is also cleaved into glucose and fructose upon crossing the cell wall barrier by the irreversible invertase reaction (Fernie et al. 2002). Many isoforms of invertase and sucrose synthase have been identified as being expressed at discrete times in diverse tissues (Sturm and Tang, 1999), making this catabolic process very complex. UDP-glucose and free glucose are converted to glucose-1-phosphate by UDP-glucose pyrophosphorylase and an ATP-dependent phosphorylation reaction, respectively (Preiss 1982).

Glucose-1-phosphate is the substrate for the first unique step in starch synthesis, the conversion of G1P to ADP-glucose by ADP-glucose pyrophosphorylase (AGP) which utilizes G1P and ATP to yield pyrophosphate and ADP-glucose (Espada 1961). Even though there is evidence for a plastidial AGP in several organs (Denyer and Smith, 1988), cereal endosperm AGP is believed to be located in the cytosol, and not the amyloplast, for two reasons. The first reason stems from cell-fractionation studies showing 80% of AGP activity in the cytosol in developing barley endosperm (Beckles et al. 2001). Researchers have also characterized a gene that codes for an adenylate transporter, *Bt1*, which shuttles the product of AGP, ADP-glucose, into the amyloplast (Shannon et al. 1998). Granule-bound starch synthase, located in the amyloplast, cleaves ADP from ADP-glucose and ligates free glucose onto a growing starch chain (Recondo and Leloir, 1961). This straight chain of α-(1-4)-glucan linkages is called amylose. Starch

molecules are also composed of branched chains of α-(1-6) and α-(1-4)-linked glucose units, known as amylopectin (Buleon et al. 1998). Researchers have identified two soluble starch synthases (located in the stromal phase of the plastid) that coordinate with starch branching enzyme to elongate amylopectin (Edwards et al. 1999). Amylopectin and amylose constitute approximately 75% and 25% of endosperm starch, respectively.

AGP: The Rate-Limiting Enzyme in Starch Biosynthesis

Endosperm sink strength and yield potential could possibly be limited by the endosperm's rate of sucrose cleavage. Experiments done on sucrose synthase (*Sh1*) mutants reported a 30% decrease in starch production in maize kernels (Chourey and Nelson, 1976). Despite these findings, it seems unlikely that sucrose synthase or invertase is rate-limiting, since none of the isozymes found to date have any known allosteric effectors. Without allosteric regulation, it is doubtful that the activity of sucrose-degrading enzymes has any impact on the rate of starch synthesis. The same can be said for starch synthase and the starch branching enzymes: none of the isoforms appear to be allosterically controlled. A mutant stock of maize lines lacking starch branching enzyme had a lower percentage of amylopectin, but a higher percentage of amylose compared to wild type maize (Preiss et al. 1991). If branching enzyme was rate limiting, one would expect not only a decrease in branched starch granules, but a decrease in total starch content as well. Due to the multitude of genes responsible for these starch biosynthetic enzyme activities, it would be extremely difficult to increase endosperm sink strength by selecting specific allelic combinations. Endosperm sink

strength could be increased if one enzyme was responsible for a unique step in starch biosynthesis. To be rate-limiting, this activity of this enzyme would need to be heavily allosterically controlled.

AGP meets the allosteric criteria for this rate-limiting enzyme. In higher plants, AGP is a heterotetramer consisting of two large and two small subunits (Morell et al. 1987). Bacterial AGP is a homotetramer that plays a key role in producing glycogen in bacteria, a reaction highly analogous to starch production in higher plants. Because of this similarity, Iglesias et al. (1993) was able to express potato AGP in *E. coli*. This heterologous bacterial expression system later helped elucidate the function of each potato AGP subunit (Salamone et al. 2001). Mutant *E. coli* strains lacking the potato small subunit gene formed AGP large subunit homotetramers that were incapable of enzymatic activity. On the other hand, small subunit AGP homotetramers were catalytically active, but exhibited much less sensitivity to the allosteric effectors. This led researchers to believe that the small subunit contains the catalytic sites, while the large subunit is involved in allosteric regulation. Inorganic phosphate (Pi) allosterically inhibits AGP and 3-phosphoglycerate (3-PGA) is a positive allosteric effector of AGP (reviewed in Hannah 1997).

Experiments Involving AGP

A number of studies in bacteria, *Arabidopsis*, potato, and several cereals support the hypothesis that AGP is the strongest determinant of starch productivity. A mutant *E. coli* strain was noted to have 33% more glycogen production than normal *E. coli* (Leung

et al. 1986). The mutation in this strain affected AGP regulatory properties, specifically a reduced sensitivity of AGP to the allosteric inhibitor adenosine monophosphate (AMP). The DNA sequence for the mutant AGP, *glgC-16*, was cloned and transformed into a strain lacking the branching enzyme gene. After transformation, Leung observed a 40-fold increase in AGP expression and an increase in glycogen production in this strain. Lin et al. (1988) also recorded a direct correlation between starch production and AGP activity in plants. A mutant of *Arabidopsis thaliana* with 0.2% the leaf starch of the wild type had 0.2% the leaf AGP as the wild type.

It could be argued that the effect AGP has on basal organisms such as *E. coli* is not representative of higher plant AGP *in situ*, or that observing leaf AGP activity in *Arabidopsis* may not predict the AGP activity changes in storage tissues. This argument falls short in light of the work done on potato and maize. Potatoes transformed with the E. coli *glgC-16* mutant allele had 35% more starch content versus control potato tubers (Stark et al. 1992). Detailed molecular analyses were performed to characterize *glgC-16* for RNA and protein levels. Potatoes transformed with the mutant *E.coli* gene had 200-400% as much AGP activity as lines expressing native potato AGP (Sweetlove et al. 1996). Western immunoblotting of the transgene indicated that the level of *glgC-16* protein was strongly correlated with the amount of AGP activity.

Shrunken-2 and Brittle-2 are Maize AGP Subunit Genes

Much of the work done on cereal endosperm, particularly from maize, has helped elucidate the structure and properties of AGP, as well as the effect AGP has on the

production of starch. Tsai and Nelson (1966) identified several maize lines with a mutation at the *Shrunken-2 (Sh2)* locus. The kernels from these lines had 25 percent as much starch as normal maize, and were later found to be low in AGP activity in the endosperm tissue. Later work on mutant maize lines discovered a separate locus affecting AGP activity, named *Brittle-2 (Bt2)* (Hannah and Nelson, 1976). The contribution of *Sh2* and *Bt2* to AGP synthesis was not fully understood until the quaternary structure of AGP was known. Molecular characterization of these genes found that *Sh2* and *Bt2* encode the large and small subunit of maize endosperm AGP, respectively (Bhave et al. 1990; Bae et al. 1990). The ability of AGP to produce ADP-glucose relies on the assembly of large and small subunits. It has been shown that the stability of the SH2 or the BT2 protein is dependent on the presence of the other subunit (Giroux and Hannah, 1994).

Identification of the *Shrunken-2* and *Brittle-2* maize mutants enabled the characterization of the genes encoding both subunits of the maize seed AGP heterotetramer. These null mutants were easily identified by their starchless kernels. Researchers took interest in finding out how strongly maize yield is affected by AGP small and large subunit activity. It is conceivable that a mutation at either the *Sh2* or *Bt2* locus could somehow confer higher AGP activity, and therefore higher starch content. However, identifying these higher-yielding mutants would be much more difficult than identifying the original *Sh2/Bt2* null mutants, since mutated kernels would only be slightly larger than wild-type kernels. To avoid the difficult task of screening for these potential AGP-enhanced mutants, Giroux et al. (1996) utilized a transposable element

mutagenesis system to generate isoalleles of *Sh2*. One of these isoalleles might alter the functionality of AGP, resulting in higher starch content in developing maize kernels.

Producing *Sh2rev6hs*

The increase in starch content described in transgenic potatoes came from the expression of a bacterial homotetrameric AGP with decreased sensitivity to a major allosteric inhibitor. If transposable elements could mutate *Sh2* or *Bt2* in such a way that affected the allosteric properties of AGP, perhaps the same effect could be seen in maize kernels. Nelson isolated a mutant (*sh2-m1*) with an insertion of the transposable element, *dissociation* (*Ds*), from the *A1* gene into the *Sh2* locus. This mutant had shrunken kernels due to the disruption in large AGP subunit production. Rare progeny of *sh2-m1* were noted to have restored function of the *Sh2* gene, due to an *Ac*-mediated transposition of *Ds* from the *Sh2* locus. Of the five revertant *Sh2* isoalles identified, one allele (*Sh2rev6*) gave rise to an 18% increase in seed weight. Unlike the increase in potato starch content, which came at the expense of water, the revertant maize seeds did not change in water content. The increase in maize seed weight came from an increase in several seed components, including starch. Upon closer examination, the *Sh2rev6* isoalle was shown to have an insertion of an additional tyrosine and serine residue at amino acid positions 495 and 496. The insertion of these two amino acids appears to confer less sensitivity of AGP to the allosteric inhibitor, inorganic phosphate (Giroux et al. 1996).

There has been speculation that the low cereal yields associated with high temperatures is a result of the heat lability of AGP during grain filling. Greene and

Hannah (1998) induced mutations of the *Sh2* cDNA in a plasmid vector. This mutated plasmid DNA was then transformed into *E.coli* along with the wild-type *Bt2* sequence. Greene then measured glycogen production in both wild-type and transformed *E. coli* at elevated temperatures. The wild-type *E.coli* had a severe reduction in glycogen production at 42°C, but one transformant strain (containing a mutated *Sh2*, termed *Sh2hs33*) had near-normal levels of glycogen synthesis at this temperature. Sequencing *Sh2hs33* identified the mutation as being a single substitution of histidine for tyrosine at amino acid 333 of the *Sh2* coding region. These experiments showed that replacement of the wild type SH2 with SH2HS33 enhanced the interaction between small and large AGP subunits. *Sh2hs33* may confer less susceptibity to AGP heterotetramer degradation when environmental conditions are less than favorable during the grain filling period.

We hypothesize that combining these two mutations, *Sh2rev6* and *Sh2hs33* (*Sh2r6hs*), would give rise to an AGP with less allosteric inhibition to phosphate and greater subunit stability. The altered large subunit conferred by *Sh2r6hs* may potentially increase endosperm sink strength, and ultimately seed weight in wheat. To test this hypothesis, a method of gene transfer is necessary to express the maize-deriven *Sh2r6hs* in wheat. The advent of transformation technology now makes the expression of foreign genes in wheat possible.

History of Genetic Engineering

The first report of plant transformation came in 1984, when DeBlock et al. (1984) inserted foreign genes into tobacco using *Agrobacterium tumefaciens* as a delivery

vector. *Agrobacterium*-mediated transformation appeared to be limited to dicotyledonous plants, and wheat transformation using *Agrobacterium* seemed particularly difficult due to the genomic complexity of the system (Songstad et al. 1995). It was not until 1992 that wheat was transformed with a herbicide-resistance gene (Vasil et al. 1992) using a biolistic delivery device, or gene gun. Biolistic transformation is accomplished by bombarding embryogenic cells with DNA-coated gold particles. The gold particles are high-density projectiles that allow insertion of plasmid DNA into the callus tissue. Several problems were encountered with the original biolistic wheat transformation experiments: low frequencies of transformant recovery, high frequency of escapes, instability or silencing of transgenes, and the lack of transgene transmission from parents to progeny, to name a few (Demeke et al. 1999). Since then, *Agrobacterium* has been used to generate transgenic wheat (Cheng et al. 1997), and was originally thought to have a higher transformation efficiency and less transgene rearrangement than transgenics generated using biolistics. A comparison of the two transformation systems concluded that this was not accurate- similar tranformation frequencies, trangene integration complexities, and levels of transgene expression were found for both methods in wheat, barley, maize, and rice (Stoger et al. 1998).

Studies have tried to describe the nature of biolistic transgene integration into the wheat genome. Because potential transgenic cells are literally bombarded with plasmid DNA, the chromosomal location, number of copies, and intactness of the trangenic sequence all vary among different transformation events. Researchers cannot determine if a true correlation between copy number and gene stability exists. This is not

surprising, given the data that recognizes multiple factors affecting transgene stability. These factors include the sequence homology of the transgene to endogenous genes (Muller et al. 1996), the region disrupted by the transgene insertions, and even environmental factors. Jackson et al. (2001) physically mapped the location of transgene insertions in wheat using fluorescence in situ hybridization (FISH). Several wheat lines were identified with different transgene copy numbers and chromosomal location. Jackson found that the type of promoter used, rather than the chromosomal site or copy number, determined the level of expression. It is evident that the uptake of transgenes into the wheat genome is a complex event. Because of the stresses induced by tissue culturing and the variation in transgene integration, the frequency of producing viable plants that express and trasmit transgenes is very low.

Nevertheless, some genetically modified cereals with improved agronomic traits are already being commercially produced for the agricultural community. The discovery of a soil bacterium (*Bacillus thuringiensis*, or *Bt*) that synthesizes insecticidal endotoxins led to the development of insect-resistant maize and corn. The *cry* gene, which codes for the endotoxin from *B. thuringiensis*, was highly expressed and transmitted in maize lines (Armstrong et al. 1995). In 1999, these *Bt*-resistant maize varieties were grown on over 8.9 million hectares around the world (Repellin et al. 2001). Other commercially grown transgenic maize include a herbicide resistant variety, which accounted for twenty percent of the total corn acreage grown in 1999 (ERS, USDA, 1999).

Agronomic traits are not the only targets for improvement through genetic engineering. Integration of genes from dicotyledonous species has led to the nutritive

improvement of rice. One storage protein in rice is glutelin, which has a low concentration of the essential amino acids lysine and threonine. The pea storage protein conferred by the *LegA* gene is similar to rice glutelin, but has higher levels of lysine and threonine. Expression of *LegA* with a glutelin promoter raised lysine and threonine content in transgenic rice (Sindhu et al. 1997). Rice is also deficient in B-carotene, the precursor needed to produce the essential nutrient vitamin A. The distant precursor to B-carotene is found in rice, but three subsequent enzymatic reactions in the B-carotene synthetic pathway are missing. One bacterial gene and two genes from daffodil (*Narcissus pseudonarcissus*) were found to code for these missing enzymes (Ye et al. 2000). Transgenic rice lines expressing all three of these trangenes produced grains with elevated levels of B-carotene.

Many cereals have also been modified with genes that enhance grain quality. The major constituents of wheat endosperm are starch (approx. 70%) and protein (approx. 15%). The viscoeleastic property of dough is attributable to the largest protein polymer in the endosperm, gluten (Wrigley 1996). Gluten is composed of the polymeric glutenins and the monomeric gliadins. Blechl and Anderson (1996) transformed wheat with a high molecular weight glutenin subunit (HMW-GS) hybrid gene, whose expression could be monitored independent of native HMW-GS gene expression. High levels of the hybrid gene transcript were measured, resulting in increased total glutenin content and different viscoelastic properties in dough-making experiments. A similar HMW-GS gene, *1Ax1*, was cloned and expressed in wheat. Additional copies of *1Ax1* also boosted gluten content relative to controls, and increased dough elasticity (Vasil et al. 2001).

Improving starch quality or quantity is an area less explored by transgenic research. The amylopectin content differed significantly among transgenic potato lines expressing variable levels of two soluble starch synthase genes (Edwards et al. 1999). Alternatively, a reduction in amylose content was observed in transgenic oat lines with anti-sense inhibition of the granule-bound starch synthase gene (Rasmussen et al. 1998). There are only preliminary reports describing the change in wheat amylose/amylopectin composition caused by the modification of soluble and granule-bound starch synthase isozyme activity (Lutticke et al. 1999; Barsby et al. 1999) or starch branching enzyme activity (Baga et al. 1999). However, no one has attempted to increase the *rate* of wheat starch synthesis by changing the allosteric properties and expression levels of AGP.

Transforming Wheat with *Sh2r6hs*

Using plasmid DNA containing *Sh2* with the *Sh2rev6* and *Sh2hs33* mutation (*Sh2r6hs*), we bombarded immature embryos from the variety Hi-Line. It is hopeful that *Sh2r6hs* will express an AGP large subunit in wheat with different allosteric properties, and that this large subunit will combine with the native AGP small subunit to form a functional AGP homotetramer. We will attempt to find phenotypical changes induced in the reproductive and vegetative parts of wheat carrying the *Sh2r6hs* transgene. Some of these plant characteristics include seed weight, harvest index, plant biomass, individual seed weight, and protein content.

The process of transforming wheat can have very negative effects on agronomic traits. The insertion of a forgeign gene into the wheat genome can often disrupt flanking

gene expression, and many tissue-culture-induced mutations may occur at sites essential for plant survivability. This reduction in transgenic plant vigor (compared to the parental variety) is commonly reflected in transgenic wheat trials. A trial conducted with lines carrying a wheat streak mosaic virus resistance gene noted a significant drop in yield in transgenic lines versus the parental (HiLine) variety (Sharp et al. 2002). Barro et al. (2002) found that heading dates were later and yields were lower in *glutenin* transformant lines than the untransformed parents. It appears that many vigor-associated traits are lowered because of transformation and the tissue culturing process. Increased AGP activity in *Sh2r6hs* transformants could potentially increase starch synthesis, however, it is uncertain whether or not this increase will be significant enough to overcome transformation-associated yield losses. If *Sh2r6hs* does not have a significant impact on AGP activity and starch biosynthesis, we expect lower yields in transgenics versus parental types, due to the yield penalties acquired during transformation.

Favorable Field Conditions

There are several environmental factors that will strongly determine whether or not *Sh2r6hs* will affect yield in wheat. Slafer and Rawson (1994) reported that the amount of solar radiation received (photoperiod) and temperature are the largest contributing factors to plant development. Level of nutrition, water availability, and plant density also contribute to development, but to a lesser degree. The modified AGP heterotetramer in *Sh2r6hs* transgenics has the best chance of producing higher total yield if seed set is plentiful. We hypothesize that *Sh2r6hs* will induce a slight increase in

individual kernel size. However, this small kernel weight increase will not give rise to higher overall transgenic yields unless seed set is high. Although seed set is affected by a combination of all environmental factors, solar radiation and temperature appear to have the greatest influence.

The size of developing heads, and therefore the number of potential seeds per head, is affected as early as 30 days prior to anthesis (Fischer 1985). Plants grown under short photoperiods with weak levels of solar radiation produced, on average, smaller heads with fewer flowers than plants grown in high radiation/long photoperiod environments. At anthesis, high temperatures decreased the survivability of pollen, which drastically reduced the percentage of pollinated ovules (Thorne and Wood, 1986). Reducing the number of pollinated ovules decreased seed set, and yield was reduced compared to plants pollinating at cooler temperatures. For *Sh2r6hs* field experiments, low solar radiation prior to anthesis or extremely high temperatures after anthesis (>95° F, 2002 trials) could significantly drop seed set, making detection of increased transgenic yield much more difficult.

Many pollinated wheat florets do not develop into mature seeds. A study by Zhen-Wen et al. (1988) found that 72% of the initiated florets did not produce kernels. This is partly due to a deficiency of assimilate supply to newly formed seeds (Abbate et al. 1998). Low endosperm sink strength could lead to a deficiency in assimilate import after anthesis- this deficiency could raise the rate of seed abortion. *Sh2r6hs* may increase endosperm sink strength, but it is questionable if *Sh2r6hs*-induced sink strength enhancement will occur early enough in development to have an impact. If the modified

AGP does not affect sink strength until one or two weeks after anthesis, it is doubtful that the percentage of young seeds aborted will change.

Supplying optimal levels of water and sunlight during the grain-filling phase can only have positive effects on plants producing *Sh2r6hs*-AGP. Although we believe that sink strength is limiting to yield, source strength must be maintained in order to see a transgenic yield effect. Fischer and Laing (1975) boosted source strength by thinning dense-seeded plots, thereby increasing the amount of light/water available to individual plants. Plants in the thinned regions produced higher yields than plants in dense-planted regions. To examine how plant density affects *Sh2r6hs* transformants, we have conducted both space-seeded and dense-seeded field trials. Space-seeded plants will not be as source limited as dense-seeded plants, and have a higher probability of generating greater transgenic yield.

CHAPTER 2

MATERIALS AND METHODS

Plasmid Constructs

Construct pRQ101A (Figure 1a) was used for selection of transformed wheat lines. It contains the selectable marker gene, *bar*, which gives resistance to the herbicides bialaphos (Meiji Seika Kaisha Ltd, Japan) and glufosinate (AgrEva USA Company, Wilmington, Del.). The *bar* gene in pRQ101A is under control of the cauliflower mosaic virus 35S promoter (CaMV 35S) and the nopaline synthase (NOS) terminator (Sivamani et al. 2000). Construct pRQ101A was co-transformed with either pSh2r6hs or pGS.

Figures 1A-C. Constructs pRQ101A, pSh2r6hs, and pGS. Box lengths are proportional to base pair lengths for promoter, intron, coding, and terminator regions. Relevant restriction enzyme sites are given for each construct. Circular line segments represent plasmid vectors but are not drawn to scale.

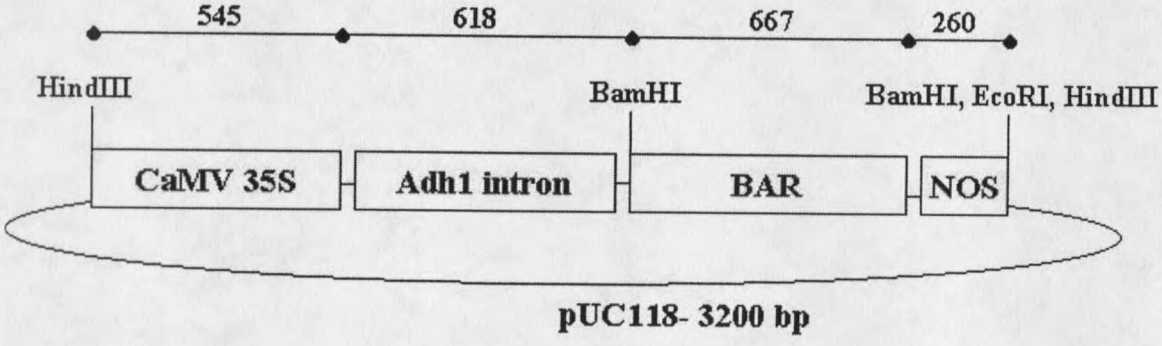

Figure 1A. Construct pRQ101A (5,290 bp). CaMV 35S, Cauliflower mosaic virus 35S promoter; Adh1 intron, alcohol dehydrogenase intron sequence; *bar*, confers resistance to the herbicides bialaphos and glufosinate; NOS, nopaline synthase terminator.