

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
Montana State University
© Copyright by Fletcher Damien Meyer (2002)

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.

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

MONTANA STATE UNIVERSITY
Bozeman, Montana

February 2002

N378
M574

APPROVAL

of a thesis submitted by

Fletcher Damien Meyer

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.

Dr. Michael J. Giroux Michael J. Giroux (Signature) 2/26/03 Date

Approved for the Department of Plant Sciences and Plant Pathology

Dr. Norman F. Weeden N F Weeden (Signature) 2/26/03 Date

Approved for the College of Graduate Studies

Dr. Bruce R. McLeod Bruce R. McLeod (Signature) 3-10-03 Date

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a master's degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library.

If I have indicated my intention to copyright this thesis by including a copyright notice page, copying is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for permission for extended quotation from or reproduction of this thesis in whole or in parts may be granted only by the copyright holder.

Signature Elizabeth Meyer

Date 2-28-03

TABLE OF CONTENTS

1. INTRODUCTION 1

SINK/SOURCE INTERACTIONS 1
ALTERING SOURCE TISSUE STRENGTH 2
ALTERING SINK TISSUE STRENGTH 3
CEREAL YIELD IS SINK LIMITED 4
THE STARCH BIOSYNTHETIC PATHWAY 5
AGP: THE RATE LIMITING ENZYME IN STARCH BIOSYNTHESIS 7
EXPERIMENTS INVOLVING AGP 8
SH2/BT2: MAIZE AGP SUBUNIT GENES 9
PRODUCING *SH2R6HS* 11
HISTORY OF GENETIC ENGINEERING 12
TRANSFORMING WHEAT WITH *SH2R6HS* 16
FAVORABLE FIELD CONDITIONS 17

2. MATERIALS AND METHODS 20

PLASMID CONSTRUCTS 20
WHEAT TRANSFORMATION 23
SELECTION OF PSH2R6HS TRANSFORMANT LINES 24
SELECTION OF PGS TRANSFORMANT LINES 25
GREENHOUSE TRIALS 26
Winter 2000/2001: pSh2r6hs F2 Trial 26
Spring 2002: pGS T2 Trial 26
Greenhouse Conditions 27
FIELD TRIALS 27
Summer 2001: pSh2r6hs F3 Trial, Bozeman, MT 27
Summer 2002: pSh2r6hs F4 Trials, Bozeman, MT 28
Summer 2002: pGS T3 Trials, Bozeman, MT 29
Summer 2002: pGS F3 Trial, Bozeman, MT 29
Summer 2002: pGS T3 Trial, Minto, Manitoba 30
Summer 2002: pSh2r6hs/pGS Biomass Experiments 30
Field Conditions 31
SOUTHERN BLOTTING 33
NORTHERN BLOTTING 33
WESTERN BLOTTING 35
AGP ACTIVITY ASSAYS 36

TABLE OF CONTENTS- continued

3. *SH2R6HS* MOLECULAR CHARACTERIZATION 38

RESULTS 38
Screening pSh2r6hs Transformants 38
Screening pGS Transformants 40
T1 RNA Expression Analysis, *Glutenin* Lines 47
Integration of *Sh2r6hs* into the Wheat Genome 48
T3 RNA Expression Analyses, *Glutenin* Lines 50
Western Blot Analyses, *Glutenin* Lines 57
AGP Activity Assays 62
DISCUSSION 68

4. GREENHOUSE AND FIELD TRIALS: *SHRUNKEN-2* PROMOTER *SH2R6HS* TRANSFORMANTS 76

RESULTS 76
Greenhouse pSh2r6hs F2 Trial, Winter 2000/2001 76
Bozeman pSh2r6hs F3 Field Trial, 2001 (Space-seeded, Irrigated) 78
Bozeman pSh2r6hs F4 Field Trial, 2002 (Space-seeded, Irrigated) 81
Bozeman pSh2r6hs F4 Field Trial, 2002 (Dense-seeded, Irrigated) 85
Bozeman pSh2r6hs F4 Field Trial, 2002 (Dense-seeded, Dryland) 88
Bozeman pSh2r6hs Biomass Study, 2002 (Dense-seeded, Irrigated) 91
DISCUSSION 93

5. GREENHOUSE AND FIELD TRIALS: *GLUTENIN* PROMOTER *SH2R6HS* TRANSFORMANTS 101

RESULTS 101
Greenhouse pGS T2 Trial, Spring 2002 101
Bozeman pGS T3 Field Trial, 2002 (Space-seeded, Irrigated) 104
Bozeman pGS T3 Field Trial, 2002 (Dense-seeded, Irrigated) 108
Bozeman pGS F3 Field Trial, 2002 (Space-seeded, Irrigated) 112
Minto, Canada pGS T3 Field Trial, 2002 (Dense-seeded, Irrigated) 115
Bozeman pGS Biomass Study, 2002 (Dense-seeded, Irrigated) 120
DISCUSSION 122

LITERATURE CITED 128

LIST OF TABLES

Table Page

1. T0 pSh2r6hs Transformant Data....39
2. T0 pGS Transformant Data....41
3. pSh2r6hs F2 Greenhouse Data....77
4. A. pSh2r6hs F3 Trial Yield Data (Space-seeded, Irrigated)....79
   B. pSh2r6hs F3 Trial Seed Data (Space-seeded, Irrigated)....81
5. A. pSh2r6hs F4 Trial Yield Data (Space-seeded, Irrigated)....83
   B. pSh2r6hs F4 Trial Seed Data (Space-seeded, Irrigated)....84
6. A. pSh2r6hs F4 Trial Yield Data (Dense-seeded, Irrigated)....85
   B. pSh2r6hs F4 Trial Seed Data (Dense-seeded, Irrigated)....87
7. A. pSh2r6hs F4 Trial Yield Data (Dense-seeded, Dryland)....89
   B. pSh2r6hs F4 Trial Seed Data (Dense-seeded, Dryland)....90
8. A. pSh2r6hs Biomass Study Yield Data....92
   B. pSh2r6hs Biomass Study Seed Data....93
9. A. pGS T2 Greenhouse Tiller Data....102
   B. pGS T2 Greenhouse Head Data....103
10. A. pGS T3 Trial Yield Data (Space-seeded, Irrigated)....105
    B. pGS T3 Trial Seed Data (Space-seeded, Irrigated)....107
11. A. pGS T3 Trial Yield Data (Dense-seeded, Irrigated)....109
    B. pGS T3 Trial Seed Data (Dense-seeded, Irrigated)....111
12. A. pGS F3 Trial Yield Data (Space-seeded, Irrigated)....113
    B. pGS F3 Trial Seed Data (Space-seeded, Irrigated)....114
13. A. Minto, Canada pGS T3 Trial (Dense-seeded, Irrigated)....116
    B. Minto, Canada pGS T3 Trial Seed Data (Dense-seeded, Irrigated)....118
    C. Minto, Canada pGS T3 Trial Group Analysis (Dense-seeded, Irrigated)....119
14. A. pGS Biomass Study Yield Data....121
    B. pGS Biomass Study Seed Data....122

LIST OF FIGURES

Figure Page

1. A. Construct pRQ101A....20
   B. Construct pSh2r6hs....21
   C. Construct pGS....22
2. Western Blot T1 Progeny Tests, *Glutenin* Lines....25
3. *Sh2r6hs/Bt2* mRNA Expression Analysis, T1 *Glutenin* Lines....44
4. SH2R6HS Expression Analysis, T1 *Glutenin* Lines....46
5. Integration of *Sh2r6hs*, T2 *Glutenin* Lines (Southern blot)....50
6. *Sh2r6hs/Bt2* mRNA Expression Analysis, T3 *Glutenin* Lines....51
7. Developmental *Sh2r6hs/Bt2/AGPL* T3 Expression Analysis....53
8. Comparison of 161-12 and GS8 *Sh2r6hs/Bt2* T3 Transcript Levels....54
9. *Sh2r6hs, Bt2, AGPL, Sut, SS, Wx*, and *Glu* Transcript Analysis....56
10. Western Blot SH2/BT2 Quantification, T3 *Glutenin* Lines (21-dpa only)....59
11. Western Blot SH2R6HS Quantification, Mature T4 *Glutenin* Lines....60
12. Developmental SH2R6HS/BT2 Analysis....61
13. Comparison of 161-12 and GS8 SH2R6HS/BT2 Protein Levels....62
14. A. AGP Activity Assays: 21-dpa Seeds, 0 mM Pi....63
    B. AGP Activity Assays: 21-dpa Seeds, 5 mM Pi....64
    C. AGP Activity Assays: 21-dpa Seeds, 10 mM Pi....65
15. A. AGP Activity Assays: 7, 14, 21, 28-dpa Seeds, 0 mM Pi....66
    B. AGP Activity Assays: 7, 14, 21, 28-dpa Seeds, 5 mM Pi....67
    C. AGP Activity Assays: 7, 14, 21, 28-dpa Seeds, 10 mM Pi....68

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.

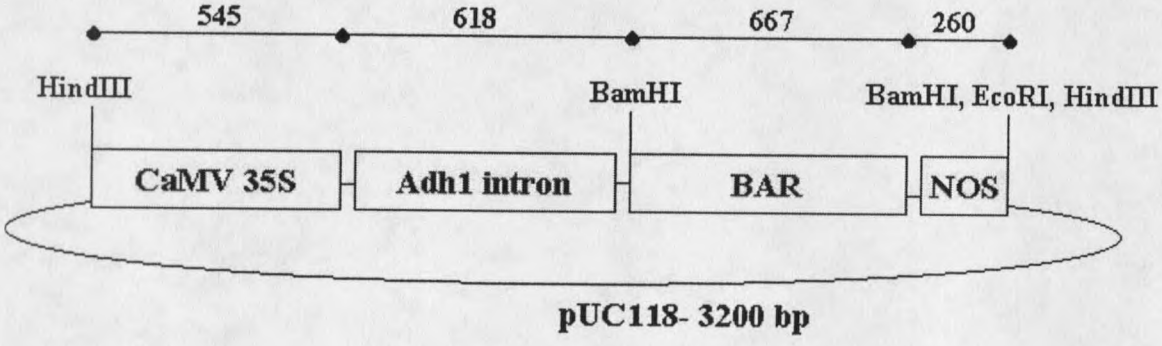


Figure 1A. Construct pRQ101A (5,290 bp). CaMV 35S, Cauliflower mosaic virus 35S promoter; Adh1 intron, alcohol dehydrogenase intron sequence; *bar*, confers resistance to the herbicides bialaphos and glufosinate; NOS, nopaline synthase terminator.

Construct pSh2r6hs (Figure 1b) has a modified 1,557 bp *Sh2* cDNA coding sequence. This modified coding sequence, *Sh2r6hs*, includes two mutations that change the allosteric properties and subunit stability of endosperm AGP. The first mutation *(rev6)* is a 6 bp insertion, resulting in an additional tyrosine and serine at amino acid position 495/496 of the AGP large subunit. This insertion makes AGP less sensitive to allosteric inhibition by inorganic phosphate (Giroux et al. 1996). The second mutation *(hs33)* is a point mutation that substitutes tyrosine for histidine at amino acid position 333, which gives rise to an AGP heterotetramer with increased stability (Greene and Hannah, 1998). The *Sh2r6hs* coding sequence was ligated into pUC19 along with the maize endosperm *Sh2* promoter (Shaw and Hannah, 1992), a *Sh1* intron cassette (Clancy et al. 1994), and the NOS terminator.

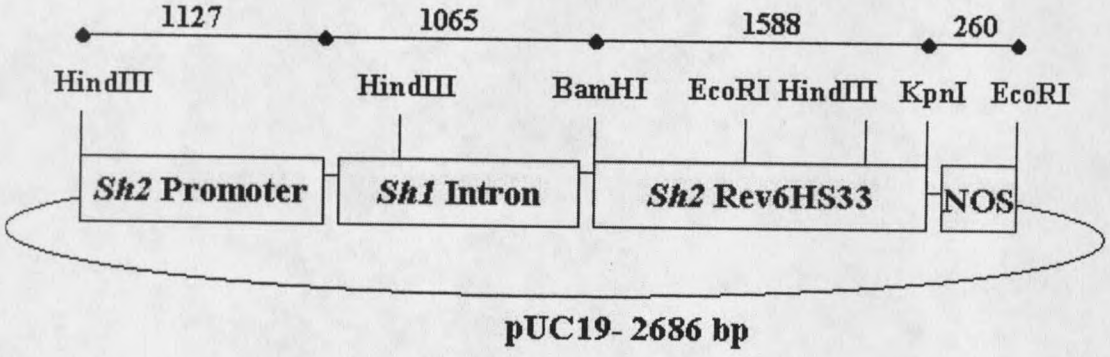


Figure 1B. Construct pSh2r6hs (6,726 bp). *Sh2* promoter, 5' terminal sequence from wild-type *Sh2*; *Sh1* intron, intron cassette; *Sh2* Rev6HS33, modified *Sh2* coding region with Rev6 and HS33 mutations; NOS, nopaline synthase terminator.

Construct pGS (Figure 1c) was generated in our laboratory by modification of construct pGB4.20 (Beecher et al. 2002), which contains untranslated *Glutenin (Glu)* gene flanking sequences from pGlu10H5 (Blechl and Anderson 1996). Primers

complementary to the 5' and 3'end of *Sh2r6hs* (SH2Snter and SH2Acter) were used to amplify a 1,598 bp sequence from pSh2r6hs:

SH2Snter: 5'-**GGGGATCC*GATATGCAGTTTGCACTTGCATTGGA-3'

SH2Acter: 5'-***ATCTAGA*GGTACCCCGCACACGCAGCCGATCTAT-3'

**Bam*HI linker sequence (*italics*); ***Xba*I linker sequence (*italics*)

The PCR consisted of a 3-min initial denaturation step at 94 °C, followed by 40 cycles of 94 °C for 45 sec, 55 °C for 30 sec, and 72 °C for 90 sec, followed by a 5 min final extension at 72 °C. This PCR product was digested with *Bam*HI and *Xba*I and inserted into the *BamHI/XbaI*-digested pGB4.20, thus replacing the original *PinB* coding sequence of pGB4.20 with *Sh2r6hs*. The resulting construct contains the *Sh2r6hs* coding sequence under the control of the *Dy10 Glu* regulatory 5' sequence and the *Dx5 Glu* 3' sequence, in a modified pET28a (Invitrogen) backbone.

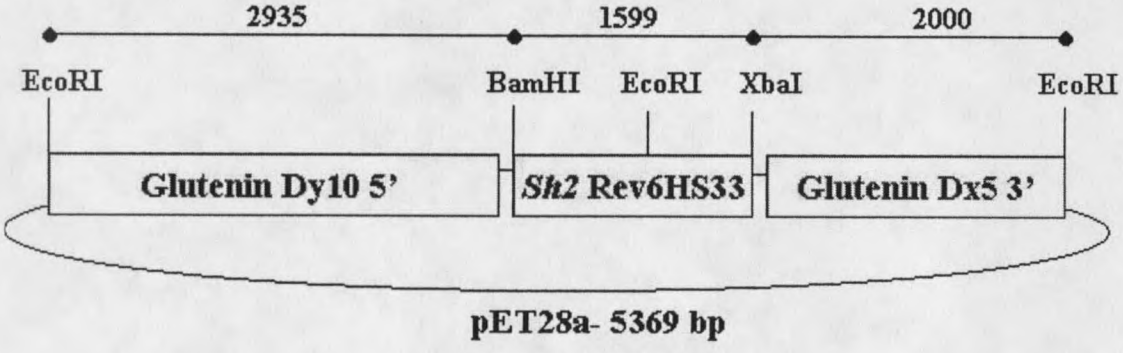


Figure 1C. Construct pGS (11,903 bp): *Glu* Dy10 5', *Glu* promoter; *Sh2* Rev6HS33, modified *Sh2* coding region with Rev6 and HS33 mutations; Dx5 3', *Glu* gene 3' untranslated region.

Wheat Transformation

Immature embryos were dissected from the hard red spring wheat variety HiLine (Lanning et al. 1992). These embryos were placed on S1 callus induction media [(4.32 g MS Basal Medium (Sigma Chemical, St. Louis, MO), 150 mg L-asparagine, 40 mg thiamine, 20 g maltose, 2 mg 2,4-D, and 2.5 g phytagel per liter, pH 5.7-5.8)] for 5-8 days in the dark at 25 °C. Calli were moved to S1 media with 0.4 M sorbitol before co-transformation with either pSh2r6hs/pRQ101A or pGS/pRQ101A constructs. The constructs were co-precipitated onto gold particles in a 1:1 molar ratio for pSh2r6hs/pRQ101A and a 4:1 molar ratio for pGS/pRQ101A. Calli were shot twice with 1,550-psi rupture disks at a distance of 6-cm, using the Bio-Rad Biolistic PDS-1000/He Particle Delivery System (Bio-Rad, Hercules, CA). After 17 hours, calli were moved onto S1 selection media containing bialaphos (5 mg/l) and maltose (20 g/l) and put in the dark for 3 weeks at 25 °C. Potential transformants were plated onto regeneration medium (same as S1 media ingredients except for an additional 1 mg/l kinetin, 0.5 mg/l IAA, no 2,4-D) until ready for rooting media. Plantlets growing in rooting media (2.16 g MS Basal Medium, 75 mg L-asparagine, 20 mg thiamine, 10 g maltose, 2.5 g phytagel, 5 mg bialaphos, and 0.01 mg NAA per liter, pH 5.7-5.8) in the light were transplanted to soil once they reached a height of approximately 3-4 cm. Plantlets reaching the two-leaf stage (approx. 2 weeks) were sprayed with 0.1% glufosinate to eliminate potential escapes.

Selection of pSh2r6hs Transformant Lines

Several T0 plants were glufosinate-resistant, indicating a successful integration of the *bar* gene (construct pRQ101A) into the wheat genome. Using an upstream PCR primer complementary to a segment of *Sh2r6hs* and a downstream primer complementary to a segment of the nopaline synthase terminator, some of these T0 plants were positively identified for *Sh2r6hs* (Smidansky et al. 2002).

T1 plants were again sprayed with 0.1% glufosinate and resistant plants were progeny tested to obtain T2 *Sh2r6hs* homozygotes. Two *Sh2r6hs* positive lines (155-4 and 161-12) and one *Sh2r6hs* negative line (161-1) were backcrossed with untransformed HiLine to improve plant vigor. These three lines were chosen for backcrossing based on expression in maturing homozygous positive T2 kernels: 161-12, 155-4, and 161-1 exhibited high, medium, and no *Sh2r6hs* expression, respectively.

Hiline ovules were fertilized with T3 pollen from these three transgenic lines. The seed produced was planted in the greenhouse and screened with glufosinate to identify successfully backcrossed F1 heterozygous *Sh2r6hs* plants. F2 seed from resistant plants was planted (see 'Greenhouse Studies') and homozygous F2 lines were found by testing F3 progeny for glufosinate resistance. F2 plants were considered homozygous positive if twelve or more consecutive progeny showed no susceptibility to 0.1% glufosinate.

Selection of pGS Transformant Lines

After being transplanted to soil-filled pots in the greenhouse, plants were sprayed with glufosinate to eliminate lines not expressing *bar*. Genomic DNA from *bar*-positive plants was probed with the *Sh2r6hs* sequence (see 'Southern Blotting') to isolate lines with cointegration of *Sh2r6hs/bar*. 25 T1 seeds from each *Sh2r6hs/bar* positive plant were grown in the field (see 'Field Studies'), and T2 seed was collected from individual plants. Homozygous *Sh2r6hs* T1 lines were identified by herbicide testing this T2 seed as described above. T1 pGS homozygotes were also examined for levels of SH2. Protein was extracted from mature T2 seed and probed with a polyclonal antibody specific to SH2 (see 'Western Blotting'). T1 plants producing only T2 seeds with high levels of SH2 (>12 progeny) were considered homozygous (Figure 2).

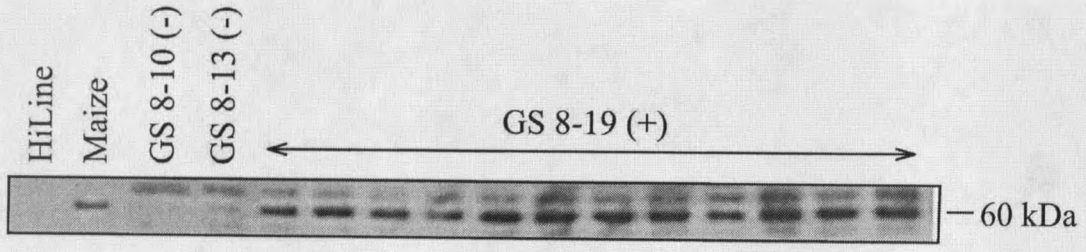


Figure 2. Western blot analysis of HiLine (untransformed control), maize, two homozygous negative *Sh2r6hs* lines (GS8-10 and GS8-13), and one homozygous positive *Sh2r6hs* line (GS8-19). Proteins were extracted from mature T2 kernels, run on an SDS-PAGE gel, and probed with a SH2-specific polyclonal antibody. Twelve or more T2 seeds were tested to distinguish heterozygous *Sh2r6hs* T1 parents from homozygous positive *Sh2r6hs* T1 parents.

Four lines (GS4, 5, 8, and 16) with strong herbicide resistance and high levels of pGS expression were backcrossed to HiLine. Successfully backcrossed heterozygous

Sh2r6hs F1 lines were identified as described above, as were homozygous F2 *Sh2r6hs* plants. Homozygous F2-derived F3 segregants were used for field trial analysis: homozygous *Sh2r6hs* positive segregants comprised the experimental group, while homozygous *Sh2r6hs* negative segregants comprised the control group.

Greenhouse Trials

Winter 2000/2001: pSh2r6hs F2 Trial

Fifty F2 segregants from each of the three crosses were grown in a random arrangement, two plants per pot, at the Montana State University Bozeman Plant Growth Center. Plants were harvested at maturity, dried, and measured for the number of heads produced, biomass, total seed weight, and average individual seed weight. Subsequent testing of F3 seed with 0.1% glufosinate identified approximately 10-15 homozygous positive/negative *Sh2r6hs* F2 parents from each collection of backcrosses.

Spring 2002: pGS T2 Trial

Nine lines of T1-derived homozygous T2 segregants were planted for yield/biomass analysis. 15 positive/15 negative plants per line were used. One positive and one negative homozygous seed (see 'Selection of *Glutenin* Transformant Lines') was planted in an 8" pot, and pots were randomly arranged with respect to different transformant lines. 270 plants were grown to maturity and quantified for the traits listed above. *Glutenin-Shrunken* (GS) lines 4, 5, 8, 9, 14, 16, 17, 28, and 54 were chosen for the trial because of their high levels of SH2R6HS expression.

Greenhouse Conditions

Temperatures of 22 °C and 14 °C were targeted for day and night, respectively, with 1000-W metal halide lamps providing illumination from 6:00 a.m. to 10:00 p.m. (16 hr photoperiod). Plants were watered as needed with 100 ppm Peters 20-20-20 General Purpose NPK plant food (The Scotts Company, Marysville, OH).

Field Trials

Summer 2001: pSh2r6hs F3 Trial, Bozeman, MT

Ten positive and ten negative homozygous F2-derived F3 segregants from three crossed pSh2r6hs transformant lines (155-4, 161-1, and 161-12) were planted on 4/26/01. These segregant lines were replicated three times in a randomized block split plot design. Each block (or main plot) consisted of one of three crosses. Main plots were divided into *Sh2r6hs* positive and *Sh2r6hs* negative subplots. A total of 20 seeds were planted at 6" intervals in 10' rows. At the two-leaf stage, plots were thinned to 10 plantlets/row. Plots were irrigated on three occasions in varying amounts (see 'Field Conditions'). On 8/23/01, rows were hand-sickled, bundled, and weighed for biomass. Heads were thrashed (Vogel, St. Louis, MO) and extracted seed was set in the greenhouse for 5-7 days to drive out excess moisture. The seed was cleaned, weighed, and analyzed for individual seed characteristics and protein content. Individual seed weight and seed diameter were obtained using a Single Kernel Characterization System 4100 (Perten, Huddinge, Sweden). Protein percentages were obtained using a Infratec 1225 Whole Grain Analyzer (Tecator, Hoganas, Sweden). Mean values for total seed weight,

biomass, shoot weight, and harvest index were compared between positive and negative groups of 155-4, 161-1, and 161-12. A one-tailed, two-sample *t* test with equal variance was used to compare positive and negative means at the 0.05 significance level. All field trial tables (Chapters 4 and 5) present trait means for positive and negative groups, as well as the ratio of positive:negative means. Asterisks accompanying ratios (*, **) indicate 0.05 and 0.01 levels of significance, respectively. However, *t* statistics were derived from the difference between replication means, and not ratio differences.

Summer 2002: pSh2r6hs F4 Trials, Bozeman, MT

F4 seed collected from the 2001 F3 trial was planted in two environments with two different seeding densities. Three experiments were conducted with the same F2-derived lines (homozygous segregants derived from crosses of 155-4, 161-1, and 161-12 with HiLine) described for the 2001 trial. The three experiments were designed as follows: Dense-seeded irrigated, space-seeded irrigated, and dense-seeded dryland. Dense-seeded rows consisted of 10 g of seed per 10' row, whereas space-seeded rows had 3 seeds planted every 6" in a 10' row. Plants were grown in a randomized block split plot design with four replications per line per experiment. All rows were planted on 4/30/02, and space-seeded sections were thinned to 20 equally-spaced plants at the two-leaf stage. Irrigated regions were watered twice (see 'Field Conditions'), and rows were harvested and analyzed as described above.

Harvest dates: Dense-seeded, dryland- 8/19/02

Dense-seeded, irrigated- 8/26/02

Space-seeded, irrigated- 9/9/02

Summer 2002: pGS T3 Trials, Bozeman, MT

The T3 seed obtained from the pGS T2 greenhouse trial was dense-seeded and space-seeded in irrigated plots on 5/2/02. Five grams of seed was planted in 5' rows for the dense-seeded experiment, and space-seeded rows were identical to those previously described. Three homozygous positive and three homozygous negative *Sh2r6hs* lines from GS4, 5, 8, 14, 28, and 54 were replicated in both irrigated and dryland environments. Two other lines, GS11 and GS12, were included in the space-seeded experiment only. Space-seeded rows were thinned to 20 plants per row at the two-leaf stage. All rows were harvested and analyzed as described above.

Harvest dates: Dense-seeded, irrigated- 8/26/02

Space-seeded, irrigated- 9/9/02

Summer 2002: pGS F3 Trial, Bozeman, MT

F2-derived *Sh2r6hs* F3 homozygotes from GS4, 5, 8, and 16 (see 'Selection of *Glutenin* Lines') were space-planted on 5/3/02 and grown under irrigated conditions. Approximately 60 F2 plants from each cross were grown in the greenhouse the previous spring; these plants were progeny tested to determine which were homozygous for *Sh2r6hs*. The total number of F2 plants, positive *Sh2r6hs* F2 homozygotes, and negative *Sh2r6hs* F2 homozygotes for each cross are as follows:

GS4: 60, 12+/14-

GS5: 60, 9+/20-

GS8: 55, 13+/16-

GS16: 58, 8+/26-

Two replications (each rep consisting of all homozygotes, eg 12 positive/14 negative *Sh2r6hs* GS4 homozygotes) were grown in a completely randomized design. Three seeds were planted every 6" in 5-foot rows, and rows were thinned to 10 equally spaced plants per row at the two-leaf stage. The F3 experiment was sickled on 9/9/02 and analyzed as described above.

Summer 2002: pGS T3 Trials, Minto, Manitoba

9-row plots were grown for seven *Glu*-promoter lines (GS4, GS5, GS6, GS8, GS11, GS12, GS54), two *Sh2*-promoter lines (152-7, 161-12), a line expressing *bar* only (161-1), and one untransformed control (HiLine). All *Sh2r6hs*-positive and negative GS groups consist of plants derived from three homozygous T2 plants. Each line was replicated six times (six positive *Sh2r6hs* plots, six negative *Sh2r6hs* plots) in a randomized complete block. Plots (5.5 m x 1.5 m) consisted of nine rows- each row was planted with 10 grams of seed. The soil type was clay loam, the seeding date was 5/14/02, and the harvest date was 9/13/02. 60 kg/Ha of N was applied, Fall 2001, and 40 kg/Ha P was applied during planting. The trial was irrigated with two inches of water on 7/12/02. Lodging and yield data was collected in Minto. Samples from each plot were mailed back to Bozeman for protein and single kernel analysis.

Summer 2002: pSh2r6hs/pGS Biomass Experiments

Vegetative plant weights and immature seed weights were measured four times before maturity for positive/negative *Sh2r6hs* lines. The goal was to determine if

increased AGP activity would have an effect on non-reproductive organs, and if so, at what developmental stage. F4 seed was pooled from ten positive 161-12 lines and ten negative 161-12 lines, respectively. Similarly, two pools were created from five positive and five negative GS8 lines. These composite pools were made from T3 seed grown in the T2 greenhouse trial. Each experiment (161-12 and GS8) consisted of twenty alternating rows with positive and negative-pooled seed. Ten-foot rows were planted with 10 grams of seed on 5/2/02. At the two-leaf stage, five 1-ft sections within each row were sectioned and thinned to 20 plants. One of the five 1-ft sections was cut from each row on the following dates: 2 weeks before anthesis (6/26/02), anthesis (7/8/02), 2 weeks after anthesis (7/22/02), 4 weeks after anthesis (8/5/02), and at maturity (8/19/02). Plants were dried and measured for vegetative weight, number of heads, and immature seed weight (if applicable).

Field Conditions

Trials were conducted in 2001 and 2002 at the Arthur H. Post Field Research Farm, 8 miles west of Bozeman. The elevation is 1,439 m and the soil is an Amsterdam silt loam.

Available soil nitrogen, 2001: 64 LB/AC
Applied N-P-K, 2001: 150-35-35 LB/AC (tilled into soil before planting)

Available soil nitrogen, 2002: 210 LB/AC
Applied N-P-K, 2002: 25-10-10 LB/AC (tilled into soil before planting)

Average Moisture (1958-2002), April-July: 8.28"
2001 Moisture, April-July: 7.71"
2002 Moisture, April-July: 8.64"

Irrigation, 2001:	5/23, 1.5"	2002:	6/28, 3.25"
	6/29, 3.0"		7/19, 3.25"
	7/10, 2.0"		

2001 July Temperatures

	High	Low	Average
1-Jul	88	56	82
2-Jul	84	54	80
3-Jul	88	50	85
4-Jul	87	55	78
5-Jul	87	55	70
6-Jul	88	55	80
7-Jul	83	53	77
8-Jul	87	61	83
9-Jul	85	61	77
10-Jul	80	57	75
11-Jul	87	50	81
12-Jul	88	51	86
13-Jul	88	48	74
14-Jul	80	47	71
15-Jul	76	46	56
16-Jul	84	43	71
17-Jul	85	49	72
18-Jul	76	44	69
19-Jul	79	44	77
20-Jul	86	51	83
21-Jul	86	51	83
22-Jul	74	49	73
23-Jul	80	47	76
24-Jul	83	45	79
25-Jul	82	46	79
26-Jul	83	52	80
27-Jul	87	48	83
28-Jul	94	44	87
29-Jul	89	44	68
30-Jul	73	47	67
31-Jul	72	51	68
Average	83.5	50.1	76.2

2002 July Temperatures

	High	Low	Average
1-Jul	83	45	76
2-Jul	80	41	77
3-Jul	86	48	69
4-Jul	80	50	72
5-Jul	82	52	79
6-Jul	85	52	74
7-Jul	85	46	67
8-Jul	81	52	79
9-Jul	81	44	76
10-Jul	87	45	84
11-Jul	97	49	72
12-Jul	98	50	96
13-Jul	99	57	79
14-Jul	95	57	69
15-Jul	91	59	84
16-Jul	89	55	73
17-Jul	84	53	87
18-Jul	90	55	81
19-Jul	83	58	69
20-Jul	80	55	63
21-Jul	82	53	63
22-Jul	79	55	74
23-Jul	87	52	75
24-Jul	90	48	87
25-Jul	90	54	66
26-Jul	77	49	73
27-Jul	77	51	65
28-Jul	80	48	76
29-Jul	86	50	82
30-Jul	90	50	84
31-Jul	82	52	83
Average	85.7	51.1	76.1

Southern Blotting

Approximately 200 mg of leaf tissue from 3-4 week old plants was collected and stored at –80 °C until DNA isolation. DNA was precipitated as described previously (Riede and Anderson, 1996) and resuspended in 50 µl TE (10mM Tris/1 mM EDTA, pH 7.4). 10 µl of the extracted DNA (approx. 40 µg) was digested (2 h, 37 °C) with 1.5 µl *Bam*HI or *Eco*RI enzyme (Promega), 2 µl of appropriate enzyme buffer, and 6.5 µl H_20. The entire digestion reaction (20 µl) was separated on a 0.8% agarose gel for 4 hours at 40 V and transferred to a nylon membrane (Osmonics Inc.). A ^{32}P-labeled *Sh2r6hs* coding sequence was generated using a random primer method (Gibco BRL). The *Sh2r6hs* probe was hybridized at 65 °C to the membrane in a solution of 0.5 M sodium phosphate (NaH_2PO_4, pH 7.3), 0.25 g bovine serum albumin (BSA), and 1.75 g SDS. Hybridized membranes were washed twice in 2X sodium chloride (NaCl)/sodium phosphate/EDTA (SSPE) (3.6 M NaCl / 0.2 NaH_2PO_4 / 0.02 EDTA, pH 7.3) and twice in 0.2X SSPE at 65 °C (15 min per wash). Blots were dried and exposed to Kodak Biomax MS film at –80 °C with an intensifying screen.

Northern Blotting

RNA was extracted from 7, 14, 21, and 28 days postanthesis (dpa) seeds. Seeds were frozen in liquid nitrogen and ground to a powder using a mortar and pestle. All samples were kept frozen at –80 °C until the following steps were performed on ice. 200 mg of the frozen seed powder was combined with 0.5 ml of extraction buffer (50 mM Tris / 200 mM NaCl / 1% sarcosyl / 20 mM EDTA / 5 mM DTT) in a 2 ml tube and

vortexed briefly. 0.5 ml phenol/chloroform/isomyl alcohol (49:49:2 v/v) was added, samples were vortexed again, and the mixture was spun at 14,000 RPM for 5 min (4 °C). Most of the resulting supernatant (0.5 ml) was added to 1 ml Trizol Reagent (Invitrogen) in a new 2 ml tube. This mixture was vortexed, 0.5 ml chloroform was added, and samples were vortexed again before centrifuging for 5 min at 14,000 RPM (4 °C). The upper aqueous layer (0.75 ml) was transferred to a clean 2 ml tube containing 0.5 ml chloroform, vortexed, and centrifuged as described above. 0.6 ml of supernatant was put into a new tube with 1.2 ml 100% ethanol and 60 µl 3M sodium acetate. The tube was vortexed and RNA was allowed to precipitate for 1 hr, -80 °C. Afterwards, tubes were spun at 14,000 RPM for 5 min, the supernatant was discarded, pellets were washed in 1 ml 70% ethanol, and RNA was suspended in 50 µl Tris-EDTA (TE) (10 mM Tris-Cl / 1 mM EDTA, pH 8.0).

2 µl of the RNA extract was diluted in 200 µl TE and assayed for A260 concentration. The volume required for 1 µg of RNA (according to A260 readings) was pipetted onto a 1% agarose gel. The intensity and intactness of ethidium bromide-stained 18S/28S ribosomal bands confirmed sample concentrations and lack of RNA degradation. 3 µg of RNA (ranging from 2-8 µl of total extract) was heated at 65 °C for 8 min and separated on a formaldehyde-denaturing gel (1.5% formaldehyde, 1.6% agarose, 4 mM MOPS, 1 mM sodium acetate, 0.1 mM EDTA) for 1 h, 80 V. RNA was transferred with 10X SSPE to a nylon membrane (Osmonics, Minnetonka, MN), then probed with either the ^{32}P-labeled *Sh2r6hs* coding sequence or the 1.7 kb ^{32}P-labeled *Bt2*

cDNA sequence (Bae et al. 1990). Hybridized blots were washed and exposed to film as described above.

Western Blotting

For mature kernels, 15 µl SDS Sample Buffer (SDS-SB) (90 mM Tris, pH 6.8 / 19% vol/vol glycerol / 2% SDS / 5% B-mercaptoethanol / 0.1% bromophenol blue / 0.1% xylene cyanol) was added per 1 mg ground kernel powder. Immature kernels were collected 7, 14, 21, and 28 days after flowering, frozen in liquid nitrogen, and ground to a fine powder with mortar/pestle. 1 mg of frozen ground tissue was mixed with 40 µl SDS-SB. Samples were vortexed every 5 min during a 15 min (70 °C) incubation and were spun at 13,000 RPM for 5 min, RT. 4 µl of supernatant was loaded for SH2 westerns, whereas an additional 1:19 sample dilution was needed for BT2 westerns. After the second 1:19 dilution, 4 µl of supernatant was loaded for BT2 westerns. Samples were run for 2 h, 50 mA on a 10-20% Tris-HCl gradient SDS-PAGE gel (Bio-Rad).

Total protein was transferred from SDS-PAGE gels to a nitrocellulose membrane (Osmonics Inc.) overnight at 10 V. Membranes were blocked with 5% nonfat milk and hybridized with SH2/BT2 polyclonal antibodies (as described in Giroux et al. 1994) in Tris-buffered saline/Tween 20 (TBSt: Tris, NaCl, HCl, 0.1% Tween 20, pH 7.6). The SH2 and BT2 antibody were diluted 1:10,000 and 1:3,000, respectively (TBSt dilution). Membranes were washed three times for 5 min in TBSt. A secondary antibody (Goat Anti-Rabbit Ig HRP, Southern Biotechnology Associates, Birmingham, AL) was applied, and membranes were washed several more times in TBSt. The western blots were

developed with an ECL-Plus Western Blotting Detection System (Lumigen Inc., Buckinghamshire, England). The resulting luminescent images were captured on Kodak Biomax MS Film.

AGP Activity Assays

7, 14, 21, and 28 dpa kernels were ground in liquid nitrogen and 30 mg frozen ground powder was added to 0.6 ml extraction buffer (80 mM Hepes, 0.5 mM EDTA, 0.1 mM DTT, 2 mM $MgCl_2$, BSA at 1.25 mg/ml) in 2 ml tubes. Samples were vortexed and centrifuged for 2 min at 13,000 RPM. 5 µl of this extract was set aside for later use. 20 µl aliquots of extraction buffer, including AGP substrates (glucose-1-phosphate and ATP, 2 mM each), 3-phosphoglycerate (4 mM), ^{14}C-labeled glucose-1-phosphate (5.7 µM, 0.08 µCi per 25 µl reaction), and varying amounts of potassium phosphate (0, 5, and 10 mM) were combined with 5 µl of protein extract in 0.5 ml tubes. The 25 µl reactions were mixed, centrifuged briefly, and kept on ice. Samples were put into a 37 °C water bath for 10 min. Enzymatic activity was killed by boiling (92.5 °C) all tubes for 5 min. After a 20 min cooling period, 6 µl (6 units) alkaline phosphatase (Promega) was added to each tube. Tubes were mixed again, centrifuged, and incubated 1 hr at 37 °C. The entire reaction was pipetted onto a 2.3 cm DEAE cellulose paper disk (Whatman, Maidstone, England). Disks were washed five times with water: twice for 1 min, three times for 10 min. The disks were arranged on paper towels and dried in a hybridization oven for 30 min at 65 °C. Whatman disks were transferred to scintillation tubes containing 10 ml Sigma-Fluor LSC Cocktail (Sigma, St. Louis, MO). Counts per minute (CPM) were

measured in a scintillation counter (Tricarb 1905 AB/LA) according to the protocol manual (Packard Instrument Co., Meriden, CT).

CPM was measured for two replications of each transgenic line at three different phosphate concentrations (0, 5, 10 mM). CPM was converted to nMoles ADP-glucose/ug total protein. This was done accordingly:

First, total protein content per 5 µl of protein extract (representative of the quantity loaded onto a Whatman disk for activity analysis) was calculated using the procedure described by Bradford (1976). A 1-10 µg BSA calibration curve was established by taking OD^{595} spectrophotometer readings on a series of samples stained with Pierce (Rockford, Illinois) reagent. OD^{595} readings from 5 µl of transgenic or HiLine protein extract were compared to the BSA calibration curve to obtain total protein content/5 µl sample.

Secondly, the quantity of ADP-glucose produced in each reaction was calculated as: nMol ADP-glucose = 0.006 * CPM

The activity of a given sample, or nMol ADP-glucose produced/µg protein, was calculated by dividing total nMol ADP-glucose by total protein/5 µl sample. Means and standard deviations were figured for seven GS lines (21 dpa), BAR (21 dpa), and for HiLine, 161-12, and GS8 at 7, 14, 21, and 28 dpa. Figures present data as percent activity of 14-dpa HiLine without phosphate. Percent activity was calculated as the ratio of sample activity (7, 14, 21, or 28 dpa and 0, 5, or 10 mM phosphate) to HiLine activity (21 days post anthesis, 0 mM phosphate).

CHAPTER 3

SH2R6HS MOLECULAR CHARACTERIZATION

Results

Screening pSh2r6hs Transformants

A total of 1,298 calli derived from HiLine embryos were cotransformed with a 1:1 molar ratio of pSh2r6hs and pRQ101A (Smidansky et al. 2002). 28 fertile wheat lines were resistant to 0.1% glufosinate (indicating the presence and functionality of the *bar* gene from construct pRQ101A). Among these, 17 were PCR-positive for *Sh2r6hs* (data not shown). Composite T1 seed samples from eight *Sh2r6hs*/*bar*-positive lines were used for northern blot and western blot anlayses. Several *Sh2r6hs* PCR-positive lines had no detectable *Sh2r6hs* transcript production or protein detected by a SH2R6HS antibody (Table 1). The following *Sh2r6hs* integration, transcription, and translation frequencies were observed for 28 T0 herbicide-resistant plants:

61% (17/28) of *bar*-positive T0 lines (tissue-culture survivors) contain *Sh2r6hs* (PCR-positive).

63% (5/8) of PCR-positive lines produce *Sh2r6hs* mRNA.

50% (4/8) of PCR-positive lines produce SH2R6HS.

Table 1. Presence of *Sh2r6hs* (PCR), expression of *Sh2r6hs* (northern and western), and T1 *bar* segregation data in nine *bar*-positive T0 lines cotransformed with pSh2r6hs.

Line[a]	PCR[b]	Northern[c]	Western[d]	Resistant/Sensitive Progeny of T0 Plants[e]	$\chi^{2(f)}$
161-1	-	-	-	9/8	4.4
152-2	+	-	-	17/3	1.07*
152-9	+	-	-	7/5	1.77*
161-5	+	-	-	10/10	6.67
152-10	+	+	-	16/5	0.016*
152-7	+	+	+	21/3	2.0*
155-4	+	+	+	17/2	2.12*
161-8	+	+	+	17/4	0.31*
161-12	+	+	+	7/4	0.76*

[a] Line number is comprised of bombardment number followed by transgenic plant number from that bombardment.
[b] "+" indicates a PCR product in a reaction containing leaf DNA from immature T0 plants and primers complementary to the flanking regions of *Sh2r6hs*.
[c] "+" indicates presence of *Sh2r6hs* mRNA transcript in 20-dpa T1 seed for each line.
[d] "+" indicates detectable levels of SH2R6HS protein for each line.
[e] Resistant plants are those expressing *bar* from either one (heterozygous) or two (homozygous positive) chromosomes. Susceptible plants do not express *bar* (homozygous negative).
[f] χ^2 (chi-square) values test fit of counts of resistant/sensitive progeny of T0 plants to 3:1 ratio.
* χ^2 (chi-square) values consistent with integration of *bar* at a single locus.

Particle bombardment generally inserts constructs into a single locus (Becker et al. 1994), thereby generating hemizygous T0 transformants. T1 progeny from a hemizygous parent segregate in the same manner as a heterozygous parent. A hemizygous *Sh2r6hs* parent will produce 25% homozygous *Sh2r6hs*-positive progeny, 50% heterozygous *Sh2r6hs* progeny, and 25% homozygous *Sh2r6hs*-negative progeny. The same segregation ratios (1:2:1) are expected when *bar* is inserted into a single chromosome. Because homozygous-positive and heterozygous *bar* plants are

glufosinate-resistant, progeny will be 75% resistant and 25% susceptible (a 3:1 phenotypic ratio).

T1 plants (two-leaf stage) derived from nine transgenic lines were sprayed with 0.1% glufosinate. Ratios of resistant:susceptible progeny are shown in Table 1. χ^2 analysis of glufosinate-resistant versus sensitive progeny of T0 plants was performed, testing a fit to a 3:1 ratio. A 3:1 ratio of resistant:susceptible progeny indicates that the *bar* gene resides at one locus and is segregating in a Mendelian fashion. Seven of nine transgenic lines produced segregation ratios consistent with a single-locus transgene integration. *Sh2r6hs* segregation perfectly correlated with *bar* segregation in subsequent generations, indicating tight linkage between the two transgenes.

Two lines with the highest levels of *Sh2r6hs* transcript and SH2R6HS protein, 152-7 and 161-12, were chosen for further expression analysis. 161-1 has a functional copy of *bar* but lacks *Sh2r6hs*.

Screening pGS Transformants

1,965 calli (derived from immature HiLine embryos) were bombarded twice using a 4:1 molar ratio of pGS:pRQ101A. Seventy transformants surviving the tissue-culturing process (bialaphos selection media, regeneration media, and rooting media) were transplanted to soil-filled pots in the greenhouse. Leaf tissue DNA from 37 bialaphos - resistant T0 plants were digested with *Eco*RI, separated on an agarose gel, Southern-blotted onto nylon, and probed with a ^{32}P-labeled *Sh2r6hs* coding sequence (data not shown). DNA from 24 of 37 (65%) plants hybridized with the *Sh2r6hs* probe, indicating host uptake of *Sh2r6hs*. Out of 24 plants, 5 had strong hybridization, 10 had medium

hybridization, and 9 had weak hybridization with the *Sh2r6hs* probe (Table 2, 'Southern'), indicating copy number differences.

Table 2. Presence of *Sh2r6hs* (Southern), expression of *Sh2r6hs* (northern and western), and T1 *bar* segregation data in 70 *bar*-positive T0 lines co-transformed with pGS.

Line[a]	Southern[b]	Northern[c]	Western[d]	Resistant/Sensitive Progeny of T0 Plants[e]	$\chi^{2(f)}$
1	Neg	n/a[g]	Neg	8/13	15.25
2	Low	Low	Neg	0/12*	
3	Neg	n/a	Neg	13/8	1.92**
4	Low	Med	High	12/8	2.4**
5	Med	High	High	21/3	2.0**
6	High	Low	Low	0/12*	
7	Neg	n/a	Neg	0/23*	
8	Med	High	High	20/7	0.012**
9	High	High	High	19/3	1.52**
10	Neg	n/a	Neg	13/4	0.02**
11	Low	Low	Low	0/12*	
12	Low	Low	Low	0/12*	
13	Neg	n/a	Neg	15/5	0**
14	Med	High	High	9/3	0**
15	Neg	n/a	Neg	15/8	1.17**
16	Med	Med	High	20/8	0.19**
17	Med	High	High	22/2	3.56
18	Med	Low	Neg	0/12*	
19	n/a	n/a	Neg	17/7	0.22**
20	n/a	n/a	Neg	15/7	0.55**
21	n/a	n/a	Neg	12/11	6.39
22	n/a	n/a	Neg	22/0	7.33
23	n/a	n/a	Med	15/7	0.55**
24	n/a	n/a	Med	14/4	0.074**
25	n/a	n/a	Neg	20/4	0.89**
26	n/a	n/a	Neg	16/9	1.61**
27	High	Med	Med	0/12*	
28	Med	Med	High	0/12*	
29	n/a	n/a	Neg	15/9	2.0**
30	n/a	n/a	Low	19/5	0.22**
31	n/a	n/a	Neg	16/8	0.89**
32	Low	n/a	Low	11/7	1.85**
33	Neg	n/a	Neg	0/18*	
34	Neg	n/a	Neg	0/19*	

35	Med	Neg	Neg	0/12	
37	Low	n/a	Low	3/12	24.2
38	High	High	Med	0/12*	
39	n/a	n/a	Neg	9/7	3.0**
40	Neg	n/a	Neg	3/9	16.0
41	n/a	n/a	Neg	5/2	0.048**
42	n/a	n/a	High	7/8	6.42
43	n/a	n/a	High	12/4	0**
44	Low	n/a	Low	12/8	2.4**
45	Low	n/a	Med	14/1	2.69**
46	n/a	n/a	Low	15/2	1.59**
47	Neg	n/a	Neg	0/7*	
48	Low	n/a	Low	16/7	0.36**
49	Med	Low	Neg	0/12*	
50	n/a	n/a	Neg	10/14	14.2
51	Neg	n/a	Neg	0/24*	
52	n/a	n/a	Neg	11/4	0.022**
53	n/a	n/a	High	12/12	8.0
54	Med	High	High	8/4	0.44**
55	Neg	n/a	Neg	16/8	0.89**
56	n/a	n/a	Neg	0/24*	
57	High	Low	Low	6/6	4.0
58	n/a	n/a	High	11/13	10.89
59	n/a	n/a	High	17/7	0.22**
60	n/a	n/a	Neg	14/5	0.017**
61	n/a	n/a	Neg	0/24*	
62	n/a	n/a	Med	18/6	0**
63	n/a	n/a	Med	0/22*	
64	n/a	n/a	Neg	24/0	8.0
65	n/a	n/a	Neg	20/3	1.75**
66	Neg	n/a	Neg	12/12	8.0
67	n/a	n/a	High	0/24*	
68	n/a	n/a	Neg	18/6	0**
69	n/a	n/a	Neg	7/17	26.89
70	n/a	n/a	High	22/2	3.56**

[a] Line number indicates the number designated to embryos following bombardment. All lines were cotransformed with a *bar* construct and the pGS construct (*Sh2r6hs* with the *Glu* promoter).

[b] Genomic DNA was probed with ^{32}P-labeled *Sh2r6hs* (Southern blotting). "High, Med, Low" indicate relative intensities of *Sh2r6hs* hybridization. "Neg" indicates no integration of *Sh2r6hs* into the wheat genome.

Table 2, cont.
[c] "High, Med, Low" indicate relative levels of *Sh2r6hs* mRNA transcript accumulation among pGS lines (northern blotting). Low, medium, and high levels correspond to 5, 10, and 20 times the level of *Sh2r6hs* transcript detected in an untransformed HiLine control, respectively. Transcript level for each line was extrapolated from a T1 northern-blot analysis. RNAs from 20-dpa T1 seeds were probed with ^{32}P-labeled *Sh2r6hs* (Figure 3). GS lines identified as *Sh2r6hs* Southern-negative did not produce *Sh2r6hs* mRNA transcript, and are designated "Neg."
[d] "Low, Med, High" indicate levels of protein detected by a SH2R6HS antibody for each line. Low, medium, and high levels correspond to 2, 4, and 6 times the level of protein detected in an untransformed HiLine control, respectively (data not shown). "Neg" denotes GS lines without production of SH2R6HS.
[e] Resistant plants are those expressing *bar* from either one (heterozygous) or two (homozygous positive) chromosomes. Susceptible plants do not express *bar* (homozygous negative).
[f] χ^2 (chi-square) values test fit of counts of resistant/sensitive progeny of T0 plants to 3:1 ratio.
[g] 'n/a' indicates lack of data for a particular GS line. Not all lines were Southern or northern blot tested.
* Indicates silencing of *bar* in T1 seedlings.
** χ^2 (chi-sqare) values consistent with integration of *bar* at a single locus, fits 3:1 ratio.

We measured the quantity of *Sh2r6hs* mRNA for 19 of 24 *Sh2r6hs* Southern-positive plants. Total RNA extracted from 20-dpa (days post anthesis) T1 kernels was probed with *Sh2r6hs* cDNA (Figure 3). 7 transformants were high *Sh2r6hs* mRNA expressers, 4 were medium expressers, 7 were low expressers, and one transformant lacked *Sh2r6hs* mRNA completely (Table 2, 'Northern').

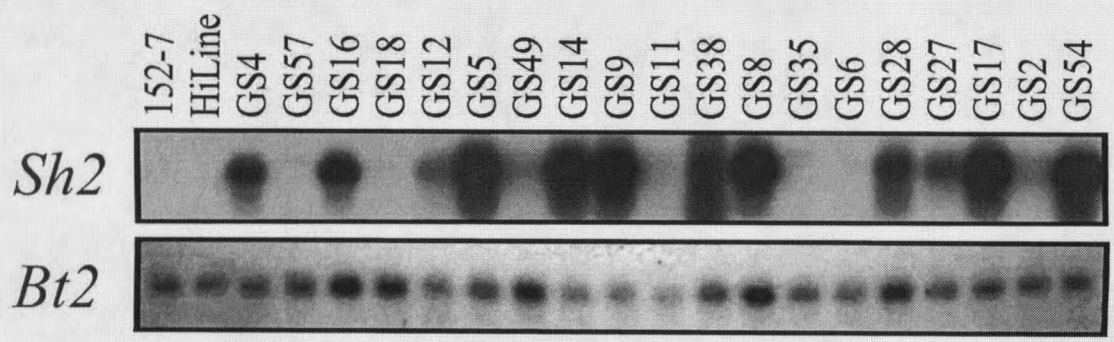


Figure 3. Analyses of mRNA in one pSh2r6hs transformant line (152-7), one untransformed control line (HiLine), and 19 pGS transformant lines. T1 seeds were collected 20 days after flowering (DAF) and frozen in liquid nitrogen. Total RNA extracted from each line was separated on a formaldehyde gel and probed with ^{32}P-labeled *Sh2r6hs* (*Sh2*) or *Brittle-2* (*Bt2*) sequences. GS6, 11, 18, 35, and 57 appear to lack *Sh2r6hs* transcript, however, a longer northern blot exposure time and subsequent western blot analysis found that *Sh2r6hs* was indeed expressed for these five lines.

The level of T1-SH2R6HS expression in 18 *Sh2r6hs* mRNA-positive GS lines (Figure 3) are as follows: 9 high expressers, 2 medium expressers, 4 low expressers, and 3 non-expressers (Figure 4). Table 2 summarizes SH2R6HS expression data for all *bar*-positive T1 transformants. High, medium, and low levels of SH2R6HS antibody hybridization were detected for 16, 7, and 10 *Glu* lines, respectively. Overall, 47% (33/70) of transformants produced SH2R6HS, 53% (37/70) did not. Not all 70 transgenic lines were Southern and northern-blotted, making it impossible to calculate how many SH2R6HS-negative lines originally transcribed *Sh2r6hs,* or even contained the *Sh2r6hs* DNA insert. Southern and northern-blotted lines integrated, transcribed, and translated *Sh2r6hs* at the following frequencies:

65% (24/37) of TO *bar*-positive lines (tissue-culture survivors) contain *Sh2r6hs.*

95% (18/19) of Southern-positive T1 lines produce *Sh2r6hs* mRNA.

79% (15/19) of Southern-positive T1 lines produce SH2R6HS.

Several T1 seed, harvested from 70 *bar*-positive T0 plants, were planted in the greenhouse. T1 plantlets were sprayed with glufosinate at the two-leaf stage to determine segregation patterns for both *bar* and *Sh2r6hs*. 51% (36/70) of transformant lines fit a 3:1 resistant:susuceptible segregation ratio, according to χ^2 analysis (Table 2). Surprisingly, 26% (18/70) of T1 lines completely lacked *bar*-conferred glufosinate resistance. Only three lines (GS17, 22, 64) segregated in a 15:1 manner, indicating a two-locus insertion of *bar*. The remainder of lines had segregation ratios higher than 3:1, likely reflecting the misclassification of transgenic seedlings as nontransgenic.

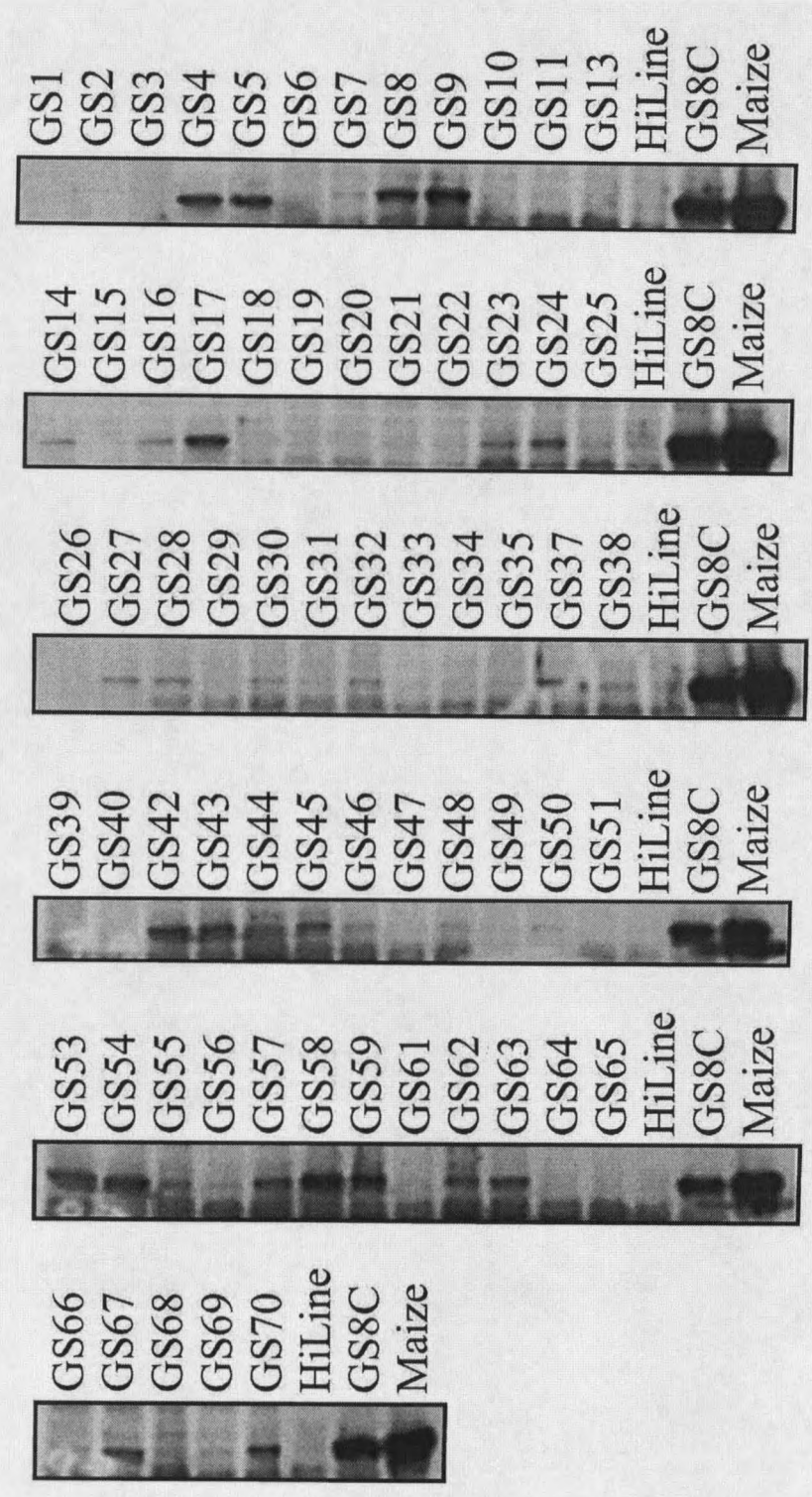


Figure 4. Western blot analysis showing SH2R6HS expression in T1 pGS transformants.

Figure 4, cont. Total protein was extracted from five mature T1 seeds per *Glu*-promoter *Sh2r6hs* (GS) line. Proteins were separated using SDS-PAGE and probed with a SH2R6HS monoclonal antibody. GS denotes pGS transformant lines, HiLine is an untransformed control, GS8C denotes a homozygous *Sh2r6hs* positive T4 seed sample derived from GS8, and Maize indicates protein extracted from a single maize kernel. Note the slight hybridization between HiLine's large AGP subunit and the monoclonal antibody. The size range shown is approximately 60 kDa.

The segregation of *Sh2r6hs* was also monitored in *bar*-positive lines. The presence of *Sh2r6hs* highly correlated with *bar* segregation- *Sh2r6hs* segregated 3:1 positive:negative along with 3:1 glufosinate-resistant:susceptible plantlets (data not shown). These data confirm that *bar* and *Sh2r6hs* cosegregate and are expressed from a single, well-defined locus in these wheat lines.

Sh2r6hs copy number (determined from Southern blotting) did not always correlate to *Sh2r6hs* mRNA/protein expression in each line. Some lines with low *Sh2r6hs* copy numbers highly expressed SH2R6HS (GS4), and vice versa (GS6). Further northern blots (Figure 6) and western blots (Figures 10, 11) confirmed stability and expression of *Sh2r6hs* in T3 and T4 generations.

T1 RNA Expression Analysis, *Glutenin* Lines

Nineteen pGS transformants positively identified for *Sh2r6hs* were chosen for northern analysis. Total mRNA from each line was separated on a formaldehyde gel and probed with a ^{32}P-labeled *Sh2r6h* sequence. RNA from 152-7 (containing the *Sh2* promoter) and untransformed HiLine were also probed with ^{32}P-labeled *Sh2r6h* (Figure 3). All RNA was isolated from T1 seeds collected from *Sh2r6hs*-positive T0 plants.

Because *Sh2r6hs* is segregating in T1 seeds, multiple seeds (20-dpa) from one T0 plant were pooled to represent the average amount of *Sh2r6hs* transcript per line.

No *Sh2r6hs* hybridization signal was observed for untransformed HiLine or the *Sh2*-promoter line 152-7. Although 152-7 expresses *Sh2r6hs* (Smidansky et al. 2002), the *Sh2r6hs* transcript levels from *Glu*–promoter (GS) lines were much greater, and blot exposure time was lessened to accommodate the blot's increased signal strength. This reduced exposure time made detection of 152-7 signal much more difficult. Out of the nineteen GS lines examined, 37% were weak expressers (GS2, 6, 11, 12, 18, 49, 57), 22% were medium expressers (GS4, 16, 27, 28), and 36% were high expressers (GS5, 8, 9, 14, 17, 38, 54). One line, GS35, did not transcribe *Sh2r6hs* (Figure 4). Medium-expressing GS lines (*Glu*-promoter *Sh2r6hs* lines) have at least twenty times more expression than 152-7 (*Sh2*-promoter *Sh2r6hs* line). Samples from each line were also probed with the small AGP subunit sequence from maize, *Brittle-2* (*Bt2*). The small AGP subunit is more conserved among species than the large AGP subunit. Unlike *Sh2r6hs*, which does not hybridize to the wheat AGP large subunit transcript, *Bt2* will hybridize to the wheat AGP small subunit transcript. RNA from the *Glutenin* lines described above, 152-7, and HiLine were probed with ^{32}P-labeled *Bt2*. Signal intensities did not differ among lines (Figure 3), indicating that wheat AGP small subunit transcript levels remained relatively unaltered, despite *Sh2r6hs* transcript increases in GS lines.

Integration of *Sh2r6hs* into the Wheat Genome

All *Sh2r6hs*-positive GS T0 transformants were harvested in the greenhouse, Spring 2001, and planted the following summer at the Post Agronomy Farm. Mature T2

seed harvested from each T1 plant was tested for SH2R6HS levels (See 'Selection of *Glutenin* transformant lines,' Chapter 2). Some T0 *Glutenin* transformants identified as high expressers of *Sh2r6hs* (GS4, 5, 8, 9, 14, 16, 17, 54, see Figure 4) were selected for another Southern analysis.

Construct pGS contains one *Bam*HI restriction site at the 5' end of the *Sh2r6hs* coding region (Figure 1C, Chapter 2). Young leaf tissue (2-leaf stage) from each *Glutenin* line was taken from T2 greenhouse plants for DNA extraction. As stated above, most bombardments generate transformants with many construct copies inserted at a discrete locus. However, different-sized fragments of host sequence usually lie between copies of the inserted construct. If individual transformants originated from a unique integration of pGS, a unique collection of variable-length fragments would be expected from each *Glutenin* line when digested with *Bam*HI.

Genomic DNA from each GS line was cut with *Bam*HI and probed with *Sh2r6hs* as described above. HiLine and BAR (lacking *Sh2r6hs*) DNA did not hybridize with the *Sh2r6hs* probe (Figure 5). 161-12 (a high expresser among pSh2r6hs transformants) showed three strong regions of hybridization in the 9-20 kb size range. All *Glutenin* lines (GS4, 5, 8, 9, 14, 16, 17, 54) had unique signature banding patterns when probed with *Sh2r6hs*. Hybridizations with differently-sized fragments suggests that *Sh2r6hs* integration was independent among *Glutenin* lines, and that each integration may include several copies of *Sh2r6hs*.

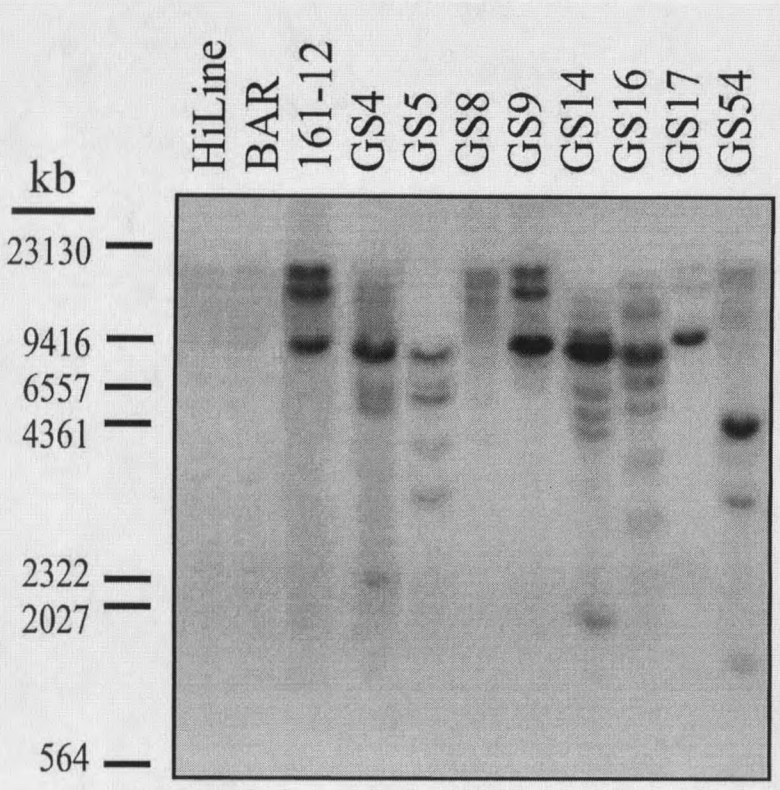


Figure 5. Southern blot analysis of two null *Sh2r6hs* lines (HiLine, BAR) and nine *Sh2r6hs*-positive lines (161-12, GS4, GS5, GS8, GS9, GS14, GS16, GS17, GS54). 20 μg of genomic DNA (extracted from T2 seedlings) was digested with *Bam*HI, run on a 0.8% agarose gel, and probed with ^{32}P-labeled *Sh2r6hs*. The probe does not hybridize to the native AGP large subunit gene. HiLine is an untransformed control, BAR is a line containing the herbicide-resistance *bar* gene but not *Sh2r6hs*, 161-12 has moderate expression of *Sh2r6hs* driven by the *Sh2* promoter, and GS lines have variable expression of *Sh2r6hs* driven by the *Glu* promoter.

T3 RNA Expression Analysis, *Glutenin* Lines

T2 seed grown in the field, Summer 2001, was progeny tested to isolate the homozygous-positive *Sh2r6hs* plants among T1 segregants. Homozygous-positive *Sh2r6hs* T2 seed was planted in the greenhouse for seven GS lines, along with HiLine and a *bar*-positive, *Sh2r6hs*-negative line (BAR). T3 seeds collected from these T2 GS plants were ground in liquid nitrogen. Precipitated RNA was quantified based on A260

spectrophotometer readings. The calculated RNA concentration of each sample was verified by running 1 ug of extract on an agarose gel. The intensity of the two ribosomal RNA bands reflected the accuracy of A260 readings (Figure 6).

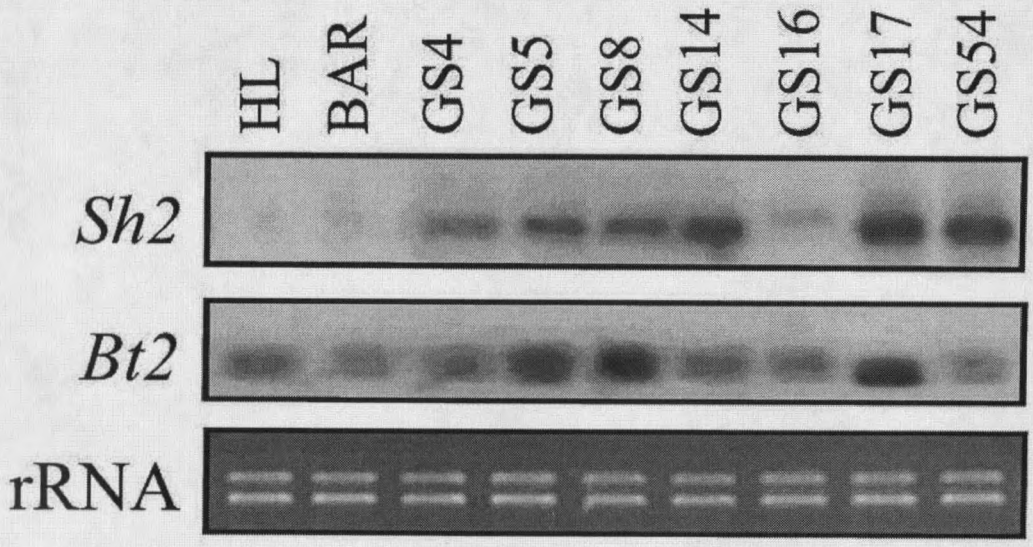


Figure 6. Northern blot analyses of 21-dpa T3 *Glutenin* seeds. RNA separated on a denaturing gel was blotted onto nylon and hybridized with *Sh2r6hs* and *Bt2* probes. Comparable ethidium bromide staining of rRNA fractionated in an agarose gel indicates similar loading from lane to lane and lack of RNA degradation. HL denotes HiLine, an untransformed control; BAR denotes a *Sh2r6hs* negative line carrying the herbicide resistance marker gene; GS denotes *Sh2r6hs* positive lines containing the *Glu* promoter.

RNA samples were heated, separated on a denaturing gel, and probed with ^{32}P-labeled *Sh2r6hs* and *Bt2* sequences as described in 'T1 Expression Analysis.' As expected, no *Sh2r6hs* transcripts were detected in HiLine or BAR (Figure 6). The seven GS lines (GS4, 5, 8, 14, 16, 17, 54) varied in accumulation of *Sh2r6hs* transcript. Expression levels within each line did not change from the T1 to the T3 generation: GS14, GS17, and GS54 maintained high expression, GS5 and GS8 maintained medium

expression, and GS4 and GS16 maintained low expression. Interestingly, accumulation of *Bt2* transcript seemed to be considerably higher for some lines, particularly GS5, GS8, and GS17.

One *Glutenin* line, GS8, was chosen for a developmental expression analysis based on three desirable qualities: (1) Decent 20-dpa expression (Figure 4), (2) *Bar* and *Sh2r6hs* cosegregate in GS8 progeny, indicating linkage of the two genes at a single locus (Table 2), and (3) GS8 plants are easily identified as glufosinate resistant/susceptible. Seeds were obtained 7, 14, 21, and 28 days after flowering from the T2-GS8 greenhouse plants mentioned above. HiLine and 161-12 were also included so that expression levels from an untransformed line and a *Sh2* promoter *Sh2r6hs* line could be compared with a *Glu* promoter *Sh2r6hs* line.

No HiLine *Sh2r6hs* transcript was detected at any developmental time point (Figure 7). 161-12 increasingly produced *Sh2r6hs* transcript from 14-dpa to 28-dpa, with peak accumulation occurring at 28-dpa. A 96-hour exposure was required to develop the signal generated from ^{32}P-labeled *Sh2r6hs* hybridization in 161-12. GS8 also began producing *Sh2r6hs* transcript at 14-dpa, and a 2-fold increase in transcript was seen from 14 to 21-dpa. GS8 differed drastically from 161-12 in the exposure time required to obtain equal signal intensities. Whereas 161-12 took 96 hours, GS8 only needed 5 hours to produce visible signal. Based on exposure times, it appears that GS8 has ~20-fold more expression than 161-12 at 14, 21, and 28-dpa.

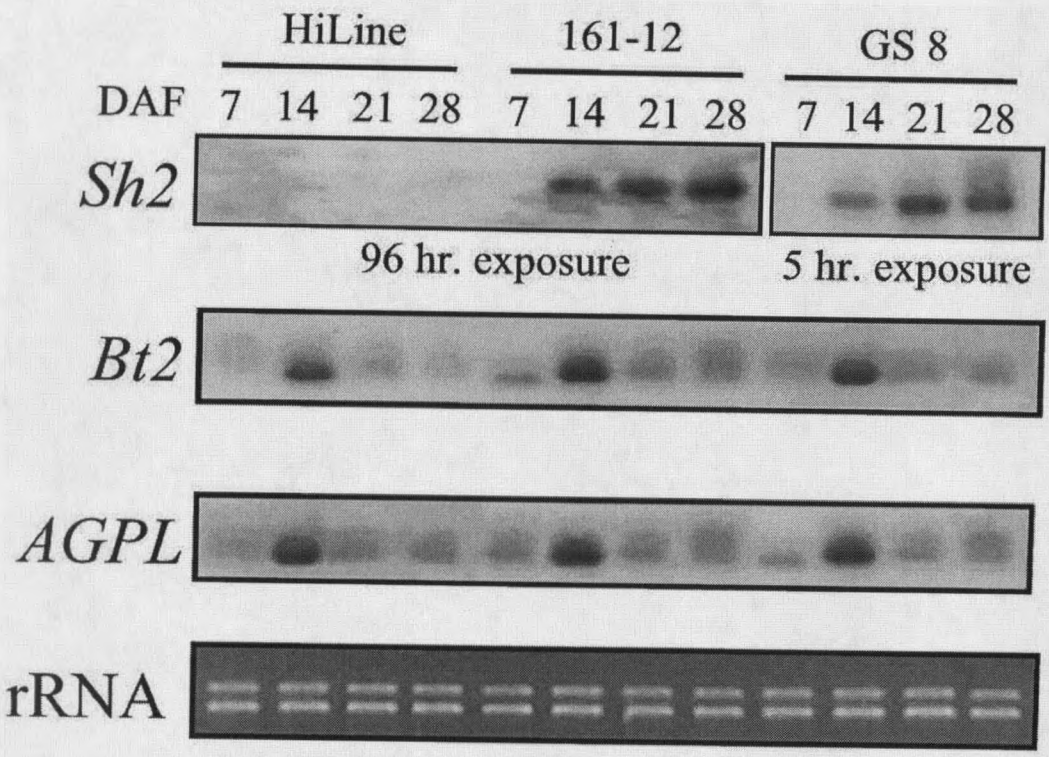


Figure 7. Northern blot assays of *Sh2r6hs*, *Bt2,* and wheat AGP large subunit gene (*AGPL*) transcripts in two transgenic (161-12 and GS8) and one untransformed control line (HiLine). Homozygous-positive seeds of F3 161-12 plants, T2 GS8 plants, and HiLine were plucked 7, 14, 21, and 28 days after flowering (DAF) and stored at –80° C to preserve RNA. The blot probed with *Sh2r6hs* was exposed for different lengths of time so that signal could be detected from the weaker 161-12 *Sh2r6hs* hybridization. RNA was separated on an agarose gel prior to northern analysis to ensure RNA intactness and equal loading of samples.

Signal arising from *Bt2* hybridization was alike in all three lines: *Bt2* transcription peaked at 14-dpa, then tapered off 21/28-dpa for HiLine, 161-12, and GS8 (Figure 7). The same trend was observed in all three lines (HiLine, 161-12, GS8) for the wheat AGP large subunit gene (*AGPL*). The peak level of AGPL expression occurred at 14-dpa, unlike the AGP large subunit transcript encoded by *Sh2r6hs*, which steadily increased from 14-dpa to 28-dpa.

In order to determine exactly how much more *Sh2r6hs* transcript was being synthesized in GS8 versus 161-12, a series of diluted GS8 RNA samples were probed with *Sh2r6hs* alongside an undiluted sample of 161-12 (Figure 8). The original 1X GS8 sample was diluted to 0.5X, 0.2X, 0.1X and 0.05X, using TE. When probed with *Sh2r6hs*, the 0.05X GS8 sample matched 161-12 in signal intensity, indicating that GS8 produces twenty times as much *Sh2r6hs* transcript as 161-12. This level of overexpression seen in GS8 matches the level derived from the exposure times necessary to develop equal signal intensities in 1X RNA samples from GS8 and 161-12 (Figure 7). Levels of *Bt2* transcript were equal in HiLine, 161-12, and GS8.

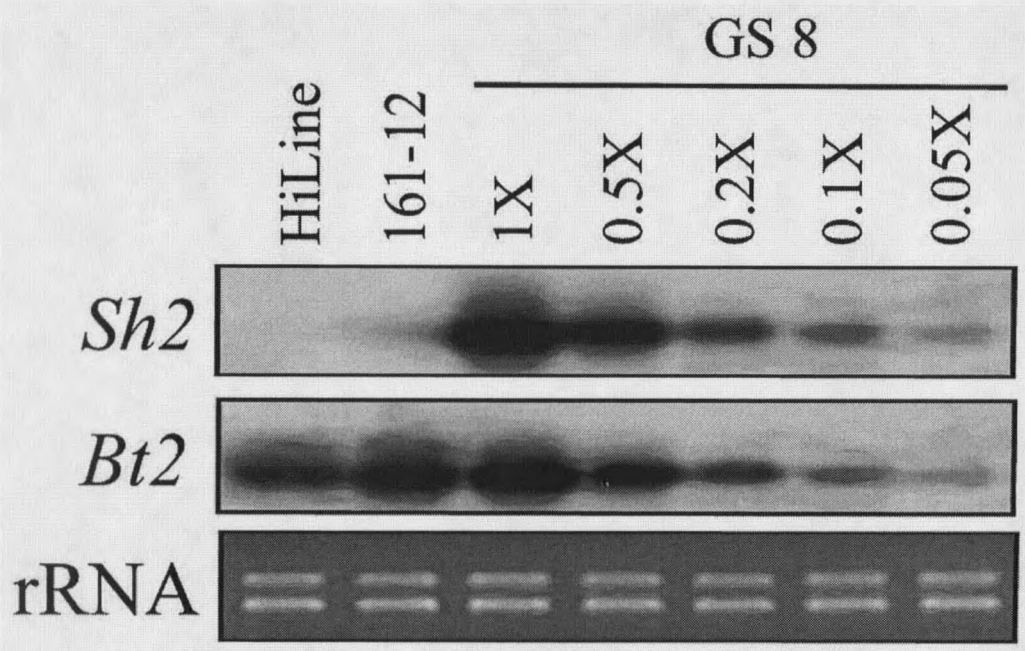


Figure 8. RNA expression comparisons of GS8 (diluted series) to HiLine and 161-12. RNA from 21-dpa seeds was probed with either *Sh2r6hs* or the maize AGP small subunit sequence (*Bt2*). HiLine is an untransformed control, while 161-12 and GS8 both express *Sh2r6hs*. 161-12 regulates transgene transcription with the *Sh2* promoter; GS8 uses the *Glu* promoter. An agarose gel stained with ethidium bromide shows equal loading of rRNA among samples.

The three northern blots above (Figures 6, 7, 8) were all probed with *Sh2r6hs* cDNA. *Sh2r6hs* does not hybridize to the wheat native AGP large subunit sequence, making it difficult to estimate the contribution of *Sh2r6hs* to total AGP large subunit transcript production in transgenic lines. To overcome this barrier, an additional ^{32}P-labeled wheat AGP large subunit sequence (*AGPL*) was hybridized to HiLine, 161-12, and GS8 RNA (Figure 9). Comparison of *Sh2r6hs* and *AGPL* mRNA levels from 161-12 (14-dpa) reveals that expression from *Sh2*-promoter *Sh2r6hs* is 90% lower than expression from *AGPL*. In contrast, *Glu*-promoter *Sh2r6hs* expression in GS8 (14-dpa) is ~4 times higher than *AGPL* expression (Figure 9). The *Glu* promoter confers much greater *Sh2r6hs* expression than the *Sh2* promoter.

Isolating PolyA RNA from repetitive RNA makes it possible to probe a greater concentration of mRNA with a given probe. Previously, 7-dpa 161-12 and GS8 total RNA was probed with *Sh2r6hs* (Figure 7). No hybridization was detected with 7-dpa total RNA samples, indicating that *Sh2r6hs* was not expressed at this stage of development. However, 7-dpa 161-12 and GS8 PolyA RNA samples showed noticeable hybridization with *Sh2r6hs* (Figure 9). *Sh2r6hs* is transcribed much earlier than we had originally hypothesized. *Sh2r6hs* expression quickly follows anthesis, and may be turned on early enough to influence the number of seeds established per head.

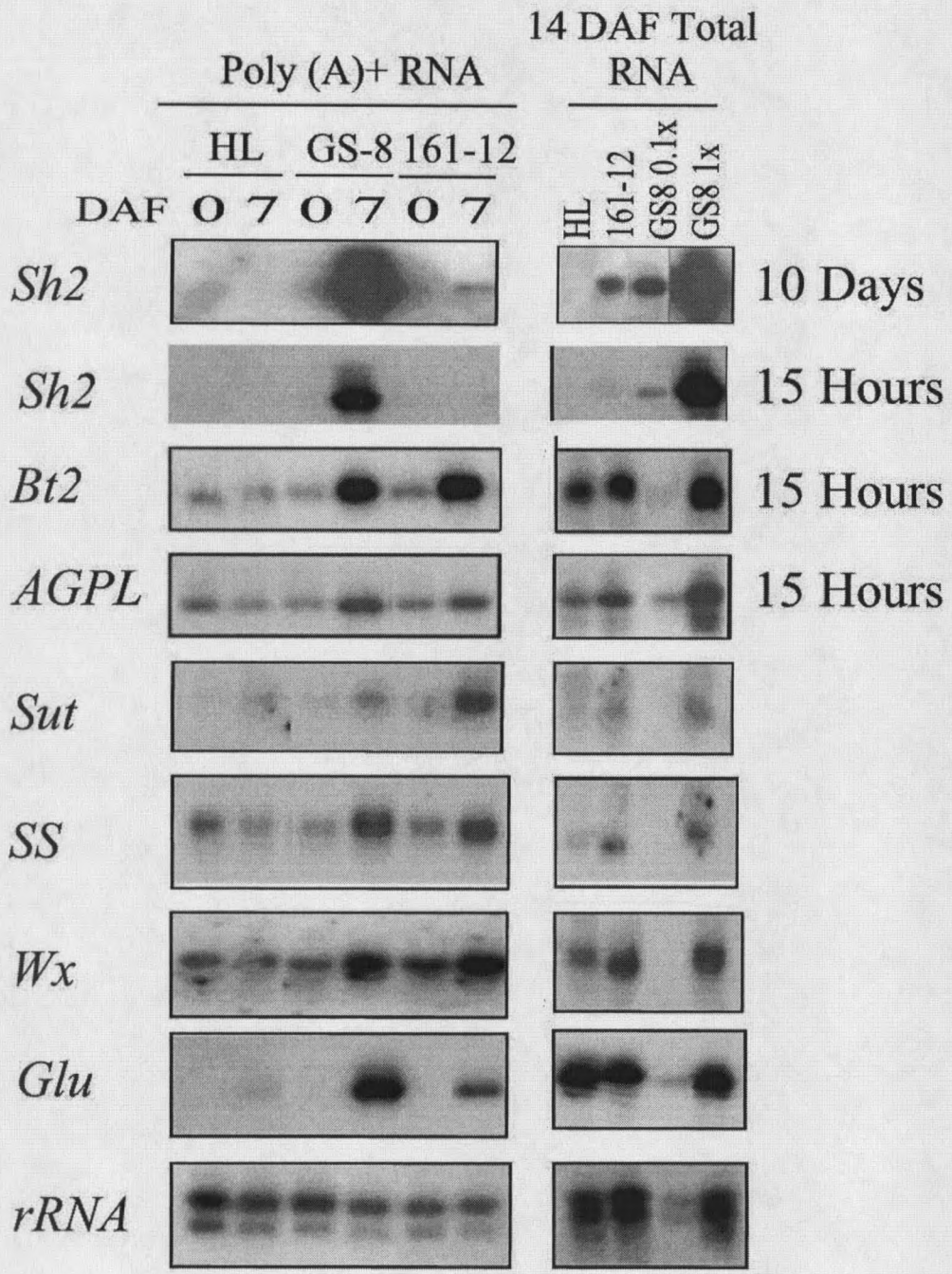


Figure 9. Quantification of mRNA from seven genes expressed in seed tissue.

Figure 9, cont. Developing seed mRNA levels from *Glu*-promoter *Sh2r6hs* line GS8, *Sh2*-promoter *Sh2r6hs* line 161-12, and untransformed HiLine (HL). 0, 7, and 14 DAF refers to days after flowering. PolyA RNA was extracted from 0 DAF and 7 DAF kernels, and total RNA was extracted from 14 DAF kernels. All samples were probed with seven cDNA sequences: *Sh2r6hs*, *Brittle-2* (*Bt2*), AGP large subunit (*AGPL*), sucrose transporter (*Sut*), soluble starch synthase (*SS*), granule-bound starch synthase, or *waxy* (*Wx*), and *Glu*. Exposure times are listed in the right-most column. The *Sh2r6hs* blot was exposed for an extended period (10 days) so that faint signals could be detected. An agarose gel stained with ethidium bromide shows equal loading of rRNA among samples (bottom panel).

Total and PolyA RNA was also probed with three other starch biosynthetic gene sequences: Sucrose transporter (*Sut*, Aoki et al. 1999), soluble starch synthase (*SS*, Gao et al. 1998), and granule-bound starch synthase (*Wx*, Shure and Federoff, 1983). The gene encoding an endosperm storage protein, *Glutenin* (Blechl and Anderson, 1996) was also hybridized to PolyA RNA samples. Transgenic lines (161-12 and GS8) have higher levels of *Sut*, *SS*, *Wx*, and *Glu* expression than untransformed HiLine 7 days after anthesis (Figure 9). The upregulation of *Sh2r6hs* expression (especially in GS8) may affect the expression patterns of other genes involved in starch synthesis. According to Figure 9, the upregulation of *Sut*, *SS*, and *Wx* in GS8 (and 161-12 to a lesser extent) occurs soon after anthesis (7-dpa or less).

Western Blot Analyses, *Glutenin* Lines

Many *Glu*-promoter *Sh2r6hs* lines produced variable quantities of SH2R6HS in mature T2 seeds, depending on whether a specific T1 parent was homozygous positive, heterozygous, or homozygous negative for *Sh2r6hs* (See 'Selection of *Glutenin* transformant lines,' Chapter 2). These progeny tests, however, did not compare SH2R6HS levels between *Sh2*-promoter *Sh2r6hs* lines (namely 161-12, a high expresser

of *Sh2r6hs*) and *Glu*-promoter *Sh2r6hs* lines. The progeny tests also did not allow for direct comparison of SH2R6HS level between GS4, 5, 8, 9, 14, 16, 17, and 54.

T2 homozygous-positive *Sh2r6hs* seed from eight *Glu*-promoter lines was planted in the greenhouse, Winter 2001-2002. Seeds were collected at 7, 14, 21, and 28-dpa, ground in liquid nitrogen, and total extractable protein was fractionated using SDS-PAGE. Blots were hybridized with a SH2R6HS polyclonal antibody that binds to the wheat AGP large subunit and the altered maize AGP large subunit encoded by *Sh2r6hs*. A BT2 polyclonal antibody was also hybridized to blots to see if wheat AGP small subunit levels were affected by fluctuation in SH2R6HS accumulation. The wheat large and small AGP subunits are approximately 60 kDa and 50 kDa, respectively (Ainsworth et al. 1995). Hybridization regions of SH2R6HS/BT2 antibodies were defined by a molecular weight standard (Gibco) loaded adjacent to protein samples. Hybridization of SH2R6HS and BT2 antibodies occurred at the expected 60 and 50 kDa regions.

Figure 10 shows the accumulated level of SH2R6HS ('SH2'), wheat AGP large subunit ('SH2') and wheat small AGP subunit ('BT2') in HiLine, BAR, and eight *Glutenin* lines. The faint signal seen for HiLine and BAR is attributable to SH2R6HS antibody binding to the native wheat AGP large subunit only. GS5, 8, 9, 17, and 54 have highest levels of SH2R6HS, while GS4, 14, and 16 are slightly lower. The small AGP subunit is not variable among *Glutenin* lines.



Figure 10. Large (SH2R6HS) and small (BT2) AGP subunit accumulation in T3 seeds. Soluble protein was extracted from 21-dpa kernels, fractionated with SDS-PAGE, and probed with SH2R6HS and BT2 polyclonal antibodies. Untransformed control (HiLine), a *Sh2r6hs*-negative line carrying the *bar* gene (BAR), and eight *Sh2r6hs*-positive lines utilizing the *Glu* promoter (GS) are shown. The SH2R6HS antibody recognizes both the *Sh2r6hs*-deriven protein and the native wheat AGP large subunit, while the maize BT2 antibody recognizes the wheat small AGP subunit.

Production of SH2R6HS in *Glutenin* lines was verified for the third time, this time using mature T4 seeds (Figure 11). GS4, 5, 8, 14, 28, and 54 strongly hybridized with a monoclonal SH2R6HS-specific antibody. GS11 and GS12 had weak hybridization in comparison. The first blot in Figure 11 used five T4 seeds from each GS line. To verify SH2R6HS expression in individual seeds, GS4 and GS8 were selected for two more western blots (Blots 2 and 3, Figure 11). Eight single homozygous-positive seeds and three homozygous-negative seeds from GS4 and GS8 were probed for SH2R6HS. Signal can be detected in each homozygous *Sh2r6hs*-positive seed.

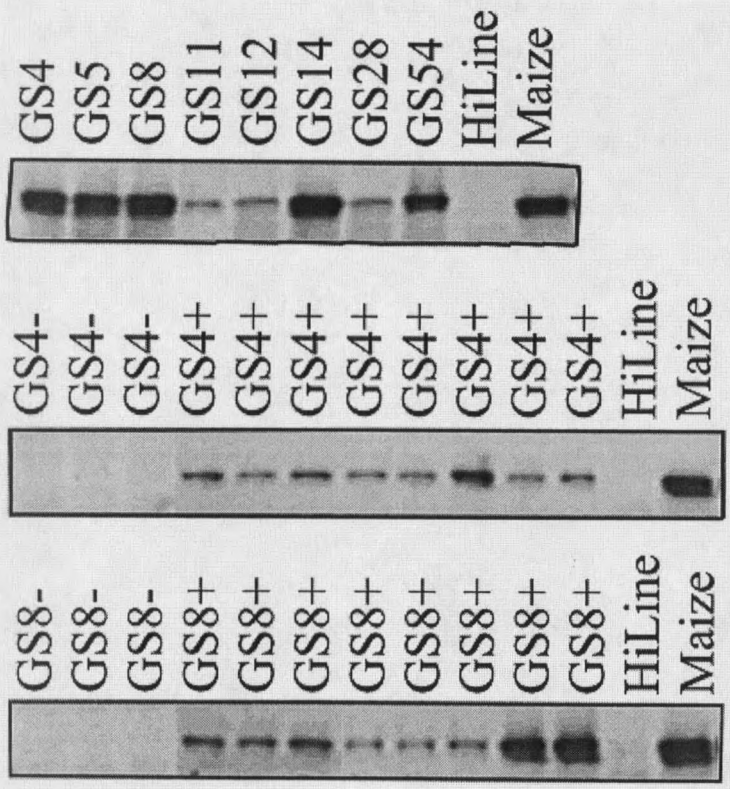


Figure 11. Immunoblot assay of SH2R6HS production in T4 *Glutenin* lines. Blot 1 verifies transgene expression in eight GS lines. Composite samples consisting of 5 seeds were probed with the SH2R6HS-specific antibody for each GS line. HiLine and Maize protein were included as controls. Blots 2 and 3 are SH2R6HS probings of individual T4 seed protein extracted from GS4 and GS8 homozygous segregants.

The accumulation of SH2R6HS and AGP small subunit was measured in HiLine, 161-12, and GS8 seeds harvested 7, 14, 21 and 28 days after anthesis (Figure 12). HiLine did not produce SH2R6HS at any developmental point, nor did 7-dpa 161-12 or 7-dpa GS8 seeds. Both 161-12 and GS8 noticeably hybridized with SH2R6HS at 14-dpa. These levels progressively increased at 21 and 28-dpa.

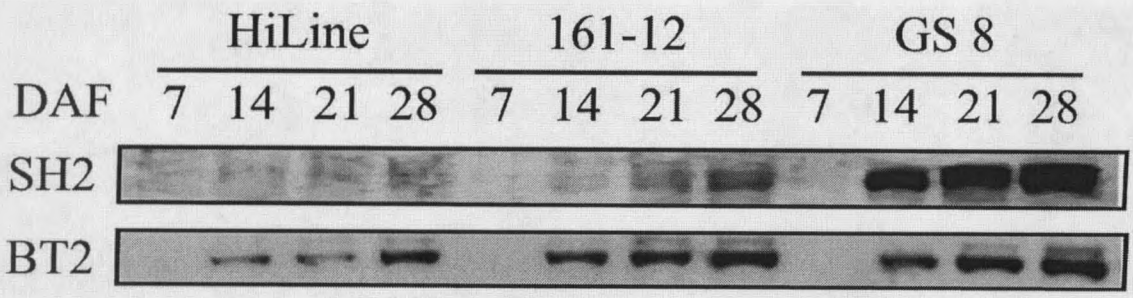


Figure 12. Western blot assays of SH2R6HS and BT2 levels in untransformed HiLine, the *Sh2* promoter *Sh2r6hs* line 161-12, and the *Glu* promoter *Sh2r6hs* line GS8. Developing seeds were collected 7, 14, 21, and 28 days after flowering (DAF). Western blotting followed the protocol described for Figure 9.

HiLine small subunit accumulation was slightly less than transformant lines at each sampling point. Wheat AGP small subunit levels, indicated by probing with a BT2-specific antibody, were equal for 161-12 and GS8. Like SH2R6HS levels, AGP small subunit levels "ramped up" in intensity from 14-dpa to 28-dpa. The observable peak occurred 28 days post anthesis. This peak in BT2 is in contrast with the AGP small subunit mRNA peak (14-dpa, See Figure 7).

A western blot with diluted GS8 protein samples was probed with SH2R6HS and BT2-specific antibodies to see the level SH2R6HS overproduction in GS8 compared to 161-12. The signal received from the 0.2X GS8 dilution matched the signal produced from 1X 161-12 seeds (Figure 13). However, the percentage of AGPL differs between 161-12 and GS8. 161-12 contains a higher percentage AGPL and a lower percentage of SH2R6HS compared to GS8. Taking this difference into account, GS8 21-dpa SH2R6HS production is likely ten times greater than 161-12.

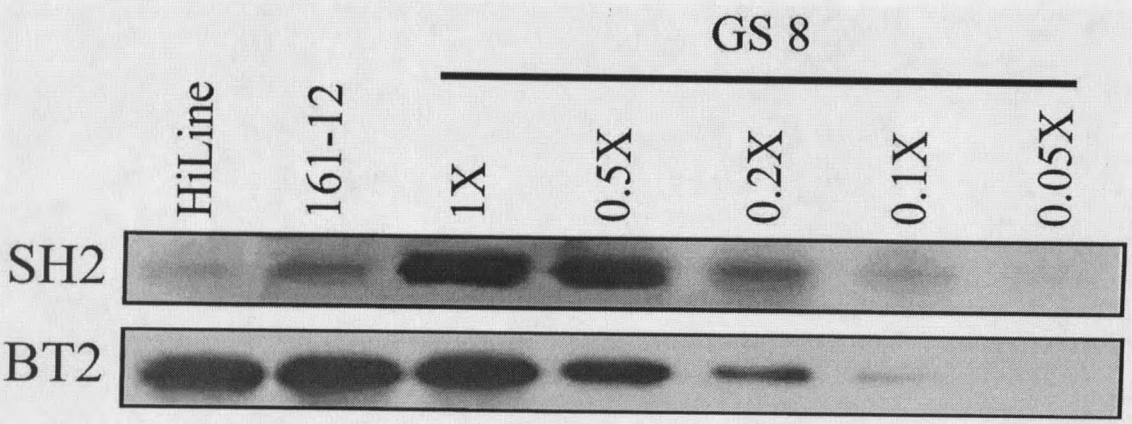


Figure 13. Immunoblot analyses of SH2R6HS and BT2 levels in HiLine, 161-12, and a diluted series of GS8 protein extracted from 21-dpa seeds. The 1X GS8 sample was mixed with the following proportions of 1X protein extract to Tris-EDTA- 1:1 (0.5X), 1:5 (0.2X), 1:10 (0.1X), and 1:20 (0.05X). Protein samples were run on a 10-20% Tris-glycine gel and hybridized with a polyclonal antibody specific to wheat AGP large subunit/SH2R6HS and BT2 binding sites.

AGP Activity Assays

The endosperm AGP activity of 21-dpa HiLine, BAR, 161-12, and eight GS lines were measured *in vitro*, using a ^{14}C-labeled glucose-1-phosphate (G1P) substrate (Figures 14a-c). 5 µl of 21-dpa seed protein extract was pipetted into a 20 µl reaction containing labeled/unlabeled G1P, ATP, 4 mM 3-phosphoglycerate (3-PGA, an AGP positive allosteric effector), and one of three concentrations (0, 5, or 10 mM) of phosphate (an AGP negative allosteric effector). AGP activity for each sample is presented as a percentage of the activity of 21-dpa HiLine seeds at 0 mM phosphate.

At 0 mM phosphate, BAR and 161-12 had approximately 90% as much activity as HiLine, and all *Glu*-promoter *Sh2r6hs* (GS) lines were slightly higher in activity (5-30%) than HiLine (Figure 14a). HiLine, BAR, and 161-12 all show inhibited AGP activity when 5 mM (Figure 14b) and 10 mM (Figure 14c) phosphate concentrations are

introduced. Interestingly, AGP activity in GS lines was not inhibited as much at 5mM and 10 mM phosphate. While HiLine, BAR, and 161-12 had about 10% the activity at 5 mM phosphate, some GS lines maintained 80-90% of their original AGP activity (Figure 14b). The same trend was seen at 10 mM phosphate: GS line activity was reduced (approximately 50%) but still much higher than HiLine or 161-12 (Figure 14c).

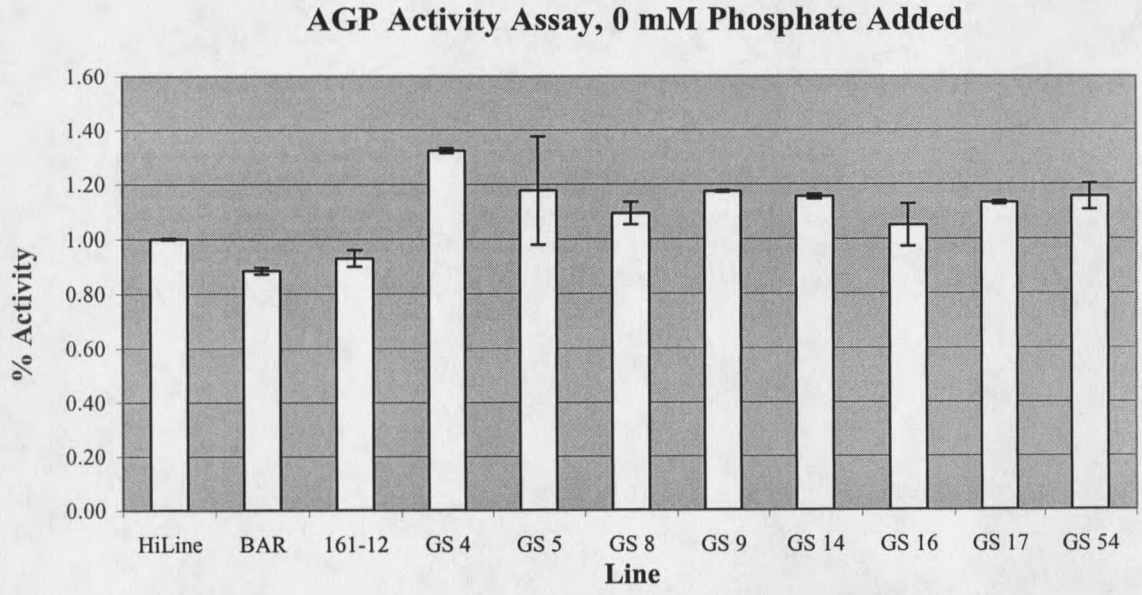


Figure 14a. AGP activity assay, 0 mM phosphate added. Total protein was extracted from 21-dpa seeds of two *Sh2r6hs*-negative lines (HiLine, BAR) and nine *Sh2r6hs*-positive lines (161-12, GS4, 5, 8, 9, 14, 16, 17, 54). 5 µl of extract was added to a 20 µl reaction mixture containing no phosphate. 100% AGP activity is defined by the activity measured in 21-dpa HiLine seeds. Error bars denote standard deviation between replications.

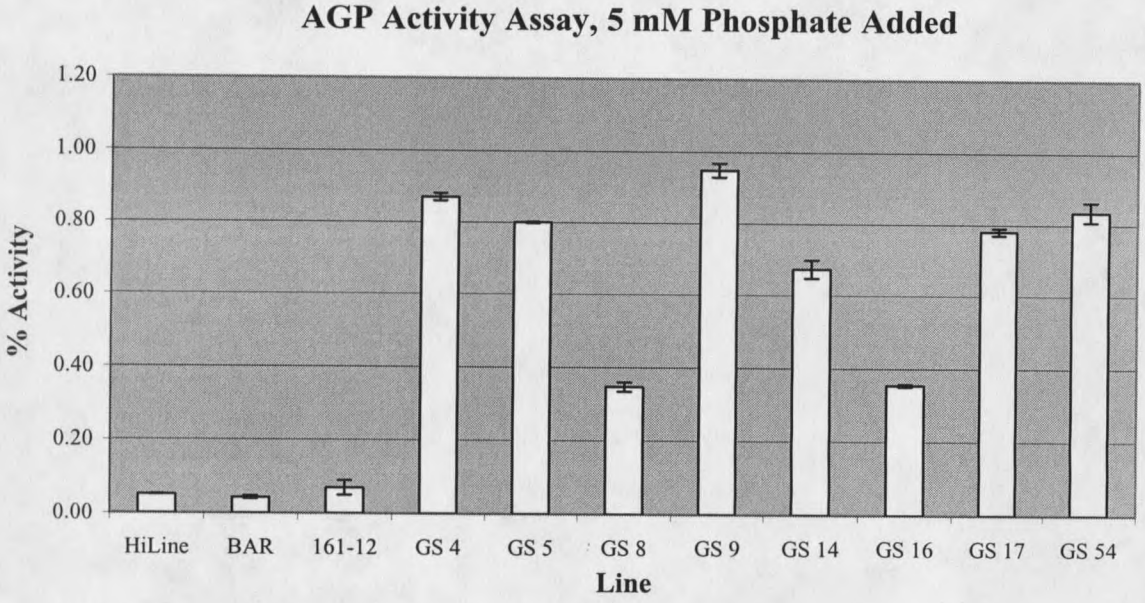


Figure 14b. AGP activity assay, 5 mM phosphate added. Total protein was extracted from 21-dpa seeds of two *Sh2r6hs*-negative lines (HiLine, BAR) and nine *Sh2r6hs*-positive lines (161-12, GS4, 5, 8, 9, 14, 16, 17, 54). *In vitro* reactions contained 5 mM phosphate, an allosteric inhibitor of AGP. 100% AGP activity is defined by the activity measured in 21-dpa HiLine seeds with 0 mM phosphate. Error bars denote standard deviation between replications.

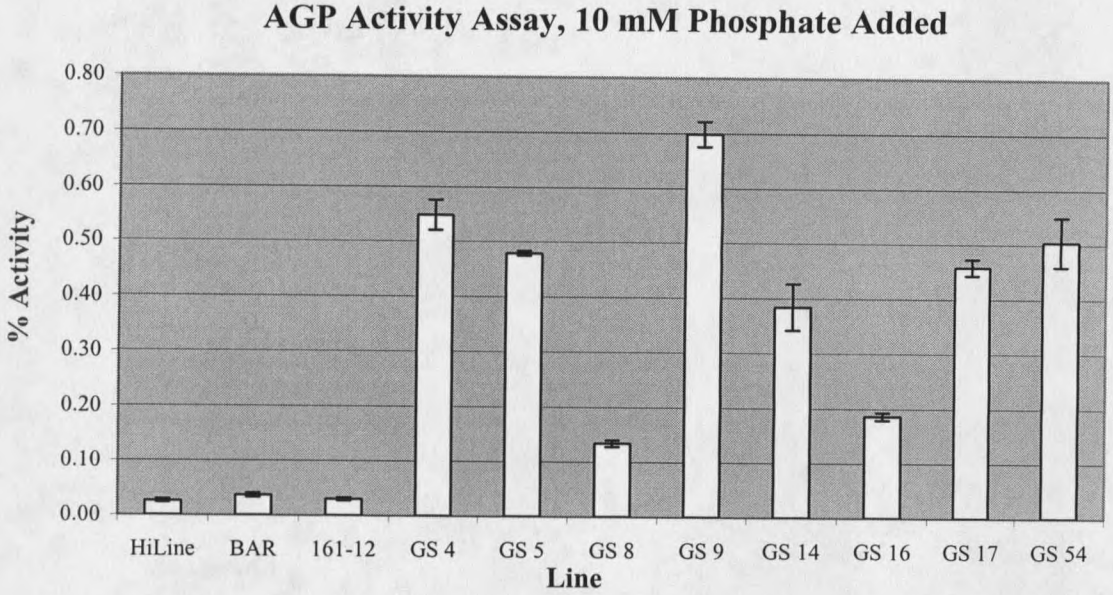


Figure 14c. AGP activity assay, 10 mM phosphate added. Total protein was extracted from 21-dpa seeds of two *Sh2r6hs*-negative lines (HiLine, BAR) and nine *Sh2r6hs*-positive lines (161-12, GS4, 5, 8, 9, 14, 16, 17, 54). *In vitro* reactions contained 10 mM phosphate, an allosteric inhibitor of AGP. 100% AGP activity is defined by the activity measured in 21-dpa HiLine seeds with 0 mM phosphate. Error bars denote standard deviation between replications.

The same set of reaction conditions were used with HiLine, 161-12, and GS8 seeds collected 7, 14, 21, and 28 days after flowering (Figure 15a-c). There was negligable activity (<5%) for all three lines at 7-dpa, no matter how much phosphate was present. At 0 mM phosphate, HiLine, 161-12, and GS8 all had sharp increases in activity at 14-dpa (Figure 15a). HiLine and 161-12 had equal activities, and GS8 was slightly higher 14 days after anthesis. Adding 5 mM phosphate dropped HiLine and 161-12 activity to less than 10% of its original activity for 14, 21, and 28-dpa samples (Figure 15b). GS8 had 35% activity at 14-dpa and peaked at 75% activity at 21-dpa in the

presence of 5 mM phosphate. 10 mM phosphate dropped GS8 activities to 13% and 33% for 14-dpa and 21-dpa samples, respectively (Figure 15c).

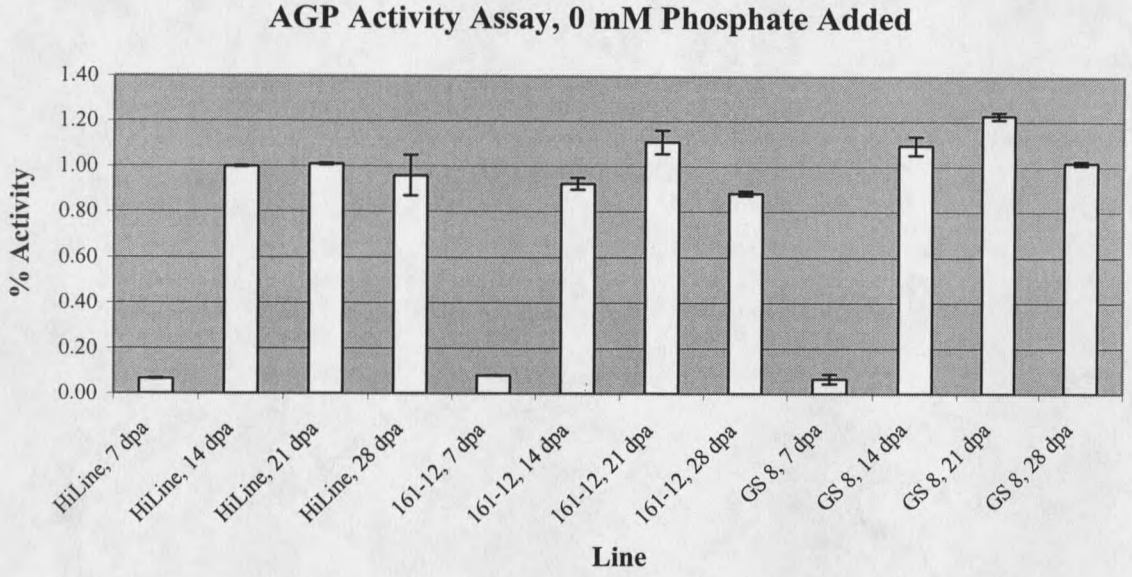


Figure 15a. AGP activity assay, 0 mM phosphate added. Total protein was extracted from 7, 14, 21, and 28-dpa seeds of two *Sh2r6hs*-positive lines (161-12, GS8) and one *Sh2r6hs*-negative line (HiLine). 5 µl of extract was added to a 20 µl reaction mixture containing no phosphate. 100% AGP activity is defined by the activity measured in 21-dpa HiLine seeds. Error bars denote standard deviation between replications.

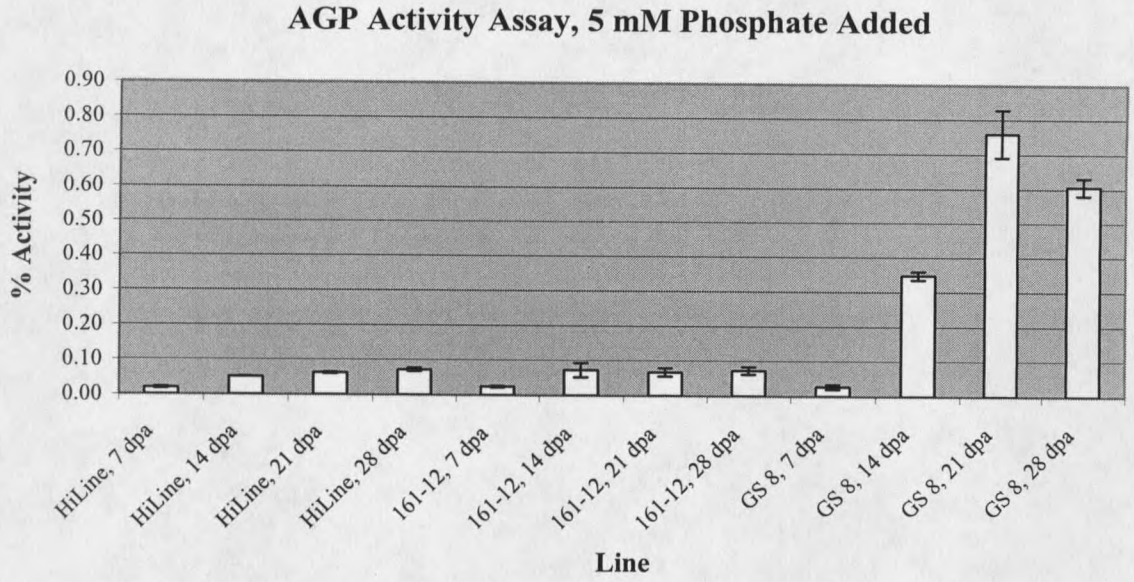


Figure 15b. AGP activity assay, 5 mM phosphate added. Total protein was extracted from 7, 14, 21, and 28-dpa seeds of two *Sh2r6hs*-positive lines (161-12, GS8) and one *Sh2r6hs*-negative line (HiLine). *In vitro* reactions contained 5 mM phosphate, an allosteric inhibitor of AGP. 100% AGP activity is defined by the activity measured in 21-dpa HiLine seeds with 0 mM phosphate. Error bars denote standard deviation between replications.

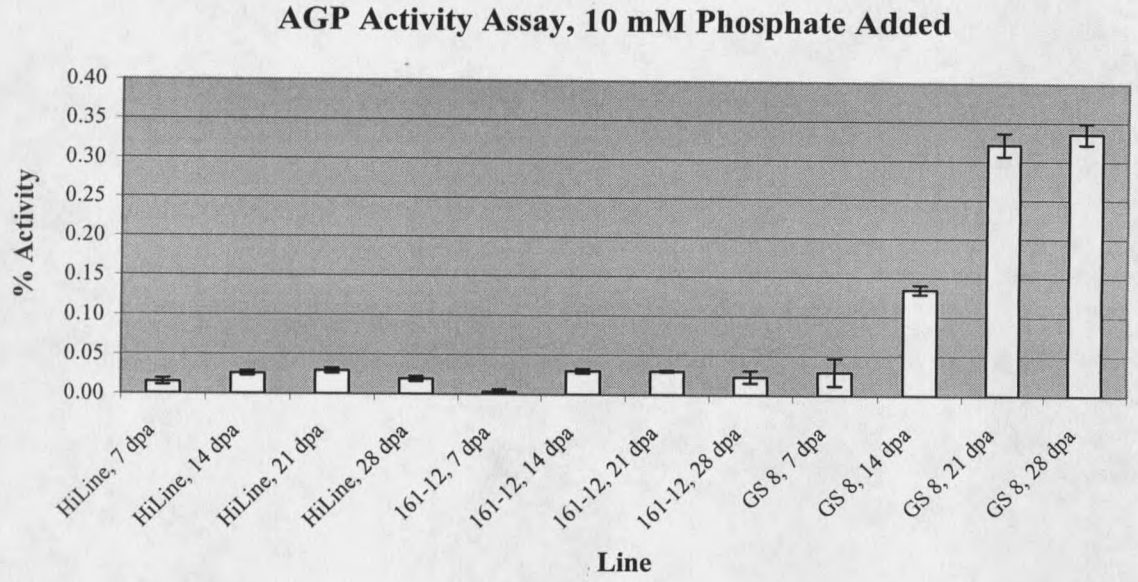


Figure 15c. AGP activity assay, 10 mM phosphate added. Total protein was extracted from 7, 14, 21, and 28-dpa seeds of two *Sh2r6hs*-positive lines (161-12, GS8) and one *Sh2r6hs*-negative line (HiLine). *In vitro* reactions contained 10 mM phosphate, an allosteric inhibitor of AGP. 100% AGP activity is defined by the activity measured in 21-dpa HiLine seeds with 0 mM phosphate. Error bars denote standard deviation between replications.

Discussion

The sink strength of developing endosperm is determined largely by the rate-limiting step in starch biosynthesis, the conversion of glucose-1-phosphate to ADP-glucose. The maize endosperm enzyme catalyzing this rate-limiting step, AGP, has been modified with two properties that could potentially raise starch biosynthetic rates in wheat. The first modification is a two-amino-acid insertion in the maize AGP large subunit coding sequence (*rev6*). This mutation results in formation of an altered AGP heterotetramer with decreased sensitivity to the AGP allosteric inhibitor, Pi (Giroux et al. 1996). The second maize AGP modification is a single amino acid substitution in the

AGP large subunit gene (*HS33*), giving rise to AGP heterotetramers with greater subunit stability (Greene and Hannah, 1998). *Rev6* and *HS33* were incorporated into a novel maize AGP large subunit gene, *Sh2r6hs*. Increased AGP subunit stability and decreased AGP allosteric inhibition resulting from *Sh2r6hs* expression could potentially increase cereal endosperm sink strength. The hard red spring wheat variety HiLine was transformed with construct pSh2r6hs (*Sh2r6hs* with the maize AGP large subunit promoter, *Sh2*) and pGS (*Sh2r6hs* with the wheat storage-protein promoter, *Glutenin*).

2.2% of all pSh2r6hs/pRQ101A-bombarded calli (28/1,298) and 3.6% of all pGS/pRQ101A-bombarded calli (70/1,965) were successfully transformed with *bar*. Plating bombarded calli onto selective media containing the optimal concentration of bialaphos is critical to identifying co-transformed plants. If the concentration of the selection agent is too low, the frequency of untransformed plants surviving tissue culture will be much higher. One experiment bombarded wheat calli with *bar* and the B-glucuronidase gene, *uidA* (Becker et al. 1994). Only 6 of 53 plantlets (11%) surviving on selective media were positively identified for GUS activity. We report much higher frequencies of *Sh2r6hs* transformation from selection media survivors. PCR analysis confirmed co-integration of pSh2r6hs and pRQ101A in 61% (17 of 28) of herbicide-resistant lines. Similarly, 65% (24 of 37) of *bar*-positive pGS transformants were identified as *Sh2r6hs* positive, indicating successful co-integration of pGS and pRQ101A.

26% (18/70) of T0 pGS plants surviving on selection media produced T1 progeny that lacked glufosinate resistance. This is due to one or more of the following: (1) Some T0 plants survived herbicide selection despite being non-transgenic (perhaps bialaphos

concentrations were too low), (2) *Bar* was not inherited in T1 progeny, or (3) *Bar* was not expressed in T1 progeny. Gene silencing has occurred in transgenic plants with both multi and single-copy insertions (Elmayan and Vaucheret, 1996). The exact cause(s) for the deficiency of T1 herbicide resistance is uncertain. Regardless, *bar* was expressed in 73% (51/70) of all lines bombarded with pGS and pRQ101A. 51% (36/70) of all pGS transformants segregated 3:1 for glufosinate-resistance and glufosinate susceptibility, respectively (Table 2). The 3:1 pattern of segregation is the result of a single-locus *bar* insertion. *Sh2r6hs* co-segregates 3:1 with *bar* in subsequent generations, indicating tight chromosomal linkage between the two transgenes. Co-segregation of two plasmid DNA sequences is a phenomenon reported in many biolistic transformation experiments (Lammachia et al. 2001).

Some *Sh2r6hs*-positive pSh2r6hs plants did not express *Sh2r6hs* mRNA or protein. Five of eight *Sh2r6hs* PCR-positive pSh2r6hs transformants produced *Sh2r6hs* mRNA; only four of these produced *Sh2r6hs* protein. Even though the sample size is small (n=8), half of the pSh2r6hs population analyzed did not produce SH2R6HS. Jackson et al. (2001) concluded that the type of promoter used, rather than the chromosomal site of transgene integration, was most critical for transgene expression. Indeed, many experiments using various promoters report higher expression frequencies than those obtained with the *Sh2* promoter. GUS activity, driven by the cauliflower mosaic virus (35S) promoter, was verified in 9 of 10 (90%) *uidA*-positive wheat plants (Demeke et al. 1999).

A construct containing a high molecular weight *Glutenin* subunit (HMW-GS) hybrid gene (composed of *Dy10* and *Dx5 Glu* subunit genes) was transformed into 26 independent lines (Blechl and Anderson, 1996). Eighteen lines (69%) co-expressed *bar* and the HMW-GS hybrid gene, leading to the accumulation of HMW-GS and the alteration of seed composition. The 5' *Dy10* and 3' *Dx5* regulatory sequences were excised and ligated onto flanking regions of *Sh2r6hs*. We expected higher levels of *Sh2r6hs* expression from the resulting *Glu*-promoter construct, pGS.

Nineteen biaphalos-resistant T0 plants transformed with pGS were analyzed for *Sh2r6hs* mRNA (Figure 3) and protein content (Figure 4). 79% (15/19) of transformants transcribed and translated *Glu*-promoter *Sh2r6hs,* a much higher expression frequency than the frequency obtained with pSh2r6hs transformants. The *Glu* promoter not only raises the percentage of *Sh2r6hs* expressers, it also raises the upper limit of *Sh2r6hs* expression in individual plants. GS8, a medium expresser compared to other GS lines, has twenty times as much *Sh2r6hs* mRNA accumulation in 21-dpa kernels as the *Sh2*-promoter line, 161-12 (Figures 7 and 8). Furthermore, *Glu*-promoter *Sh2r6hs* expression is four times higher than *AGPL* expression in GS8 (Figure 9), while *Sh2*-promoter *Sh2r6hs* expression is only 10% of *AGPL* expression in 161-12.

One argument is that 161-12 expression is lower because it contains fewer *Sh2r6hs* copies than GS8. However, Southern-blot analysis (Figure 5) suggests otherwise. 161-12 has more copies of *Sh2r6hs* than GS8 and many other pGS transformants. It is interesting to note the high *Sh2r6hs* expression from low-copy lines such as GS5 and GS8. This finding suggests that copy number has little impact on

Sh2r6hs expression. Other research coincides with this finding- expression levels are not closely proportional to transgene copy number (Stoger et al. 1998, Jackson et al. 2001). The data above support the hypothesis that the *Glu* promoter confers much greater *Sh2r6hs* expression than the *Sh2* promoter. *Glu*-promoter transformants have a greater probability of producing yield increases, due to higher *Sh2r6hs* expression.

T1 pGS *Sh2r6hs* expression levels were conserved in T3 seeds (Figure 6). Unlike T1-AGP small subunit transcript levels, which did not vary significantly among lines (Figure 3), T3-AGP small subunit transcript levels were up-regulated in GS5, GS8, and GS16 (Figure 6). The AGP small subunit mRNA was not necessarily the highest in GS lines with the highest *Sh2r6hs* expression levels. GS8, for example, produced considerably less *Sh2r6hs* transcript than GS4, yet had much stronger hybridization with the maize AGP small subunit sequence (*Bt2*) probe. Transgenic lines producing higher levels of non-feedback-inhibited SH2R6HS-AGP heterotetramers would certainly require higher levels of AGP small subunit gene mRNA. Without a correlation between *Sh2r6hs* expression and small subunit gene expression, this may or may not be the case for GS transformants.

The effect *Sh2r6hs* has on starch biosynthetic rates depends not only on the magnitude of *Sh2r6hs* expression, but on the timing of *Sh2r6hs* expression as well. The high rate of floret abortion observed in several wheat trials (Zhen-Wen et al. 1988) is often credited to a deficiency of photoassimilate transport to developing heads (Abbate et al. 1998). The majority of seed abortions occur from the time of flower maturation until one week after anthesis (Bindraban et al. 1998). Enhancing the sink strength of newly-

formed kernels will increase the flow of assimilates from leaves to heads, thereby decreasing the rate of seed abortion. *Sh2r6hs* must be expressed early enough to increase sink strength and the survivability of recently pollinated seeds. Northern blot analysis reveals *Sh2r6hs* expression seven days after anthesis (Figure 9). The early *Sh2r6hs* expression allows development of greater numbers of initiated seeds, thus decreasing the rate of seed abortion.

Sh2r6hs transcript accumulates from 7-dpa to 28-dpa in 161-12 and GS8 (Figure 7). The steady increase in transgene mRNA expression is unique compared to the expression patterns of native genes. The wheat AGP small subunit gene peaks at 14-dpa for 161-12, GS8, and untransformed HiLine (Figure 7). *AGPL* also reaches the highest level of mRNA accumulation at 14-dpa, tapering off at 21-dpa and 28-dpa. The similarity between 161-12 and GS8 *Sh2r6hs* expression patterns is odd, given the different promoters (*Sh2* and *Glu*) in each line. Biolistically-inserted transgenes may increase expression levels over the course of development, regardless of the type of promoter.

Transcript levels of sucrose transporter (*Sut*), soluble starch synthase (*SS*), and granule-bound starch synthase (*Wx*) were up-regulated in GS8 and 161-12 (Figure 9). These transcript levels indicate that the over-expression of *Sh2r6hs* in GS8 and 161-12 affects the transcription of many genes involved in starch biosynthesis. *Glu*, a storage protein not directly associated with starch accumulation, was also over-expressed in 161-12 and GS8 7-dpa seeds. It is not understood why *Glu* gene expression is influenced by increased *Sh2r6hs* expression. Increased *Glu* production (leading to increased protein

storage) is possibly a response to higher starch accumulation. Because of this response, seed starch and protein levels will remain proportionate to wild-type seed levels.

The quantities of SH2R6HS increase in a fashion similar to *Sh2r6hs* mRNA levels in 7, 14, 21, and 28-dpa seeds (Figure 12). Some hybridization between the SH2R6HS-specific antibody and HiLine AGPL was observed. 161-12 and GS8 AGPL also hybridizes to the antibody, but this contributes little to total antibody hybridization. GS8 SH2R6HS levels are 10-20 times greater than GS8 SH2R6HS levels at 14, 21, and 28-dpa (Figure 12). This value does not agree with Figure 13, which indicates 5 times as much SH2R6HS in GS8 as 161-12. In either case, the *Glu* promoter confers between 5 to 20 times more SH2R6HS production than the *Sh2* promoter.

AGP small subunit (AGPS), as detected by a BT2-specific antibody, increased from 14-dpa to 28-dpa (Figure 12). Interestingly, this pattern is unlike *AGPS* mRNA expression patterns (Figure 7), where mRNA levels peaked at 14-dpa for transformed (GS8, 161-12) and untransformed (HiLine) genotypes. The difference between AGPS translation patterns and *AGPS* transcript level patterns cannot be accounted for.

AGP activity was slightly higher in pGS lines than HiLine or 161-12 when no phosphate (Pi) was present (Figure 14a). The *HS33* amino acid substitution in *Sh2r6hs* gives rise to AGP heterotetramers with higher subunit stability (Greene and Hannah, 1998). This higher stability may increase total AGP activity 10-20%, as seen with several GS lines. The addition of Pi (5mM and 10mM) to reaction mixtures resulted in severe allosteric inhibition of HiLine and 161-12 AGP activity, but not to eight independent GS lines (Figures 14b, 14c). The *Rev6* insertion in *Sh2r6hs* confers reduced

sensitivity to Pi (Giroux et al. 1996). This altered AGP property allows *Glu*-promoter *Sh2r6hs* lines to retain high AGP activity in the presence of Pi. *Sh2*-promoter *Sh2r6hs* lines, on the other hand, do not express *Sh2r6hs* as highly as GS lines. This explains the diminished 161-12 AGP activity with 5 and 10mM Pi (Figures 15b, 15c).

Sh2r6hs expression was conserved in pSh2r6hs transformants across seven generations (T0 through T3 in original transgenic lines and F1 through F4 following the 155-4, 161-1, and 161-12 X HiLine cross) and across four pGS transformant generations (T0 through T1 in original transgenic lines and F1 through F3 following the GS4, GS5, GS8, and GS16 X HiLine cross). We conducted numerous yield trials on four pSh2r6hs generations (T0, F2, F3, F4) and four pGS generations (T0, T2, T3, F3) positively-expressing *Sh2r6hs*. Homozygous positive and negative *Sh2r6hs* segregants were analyzed for seed and plant qualities in the greenhouse and under several field environments. The results from these experiments are discussed in Chapters 4 and 5.

CHAPTER 4

GREENHOUSE AND FIELD TRIALS: *SH2*-PROMOTER SH2R6HS TRANSFORMANTS

Results

Greenhouse pSh2r6hs F2 Trial, Winter 2000/2001

161-12, a *Sh2*-promoter *Sh2r6hs* line with high expression, was backcrossed with HiLine to reduce tissue culture effects. 14 positive and 9 negative homozygous *Sh2r6hs* F2 plants derived from this backcross were analyzed for biomass, seed weight, harvest index, head number, seed number, and individual seed weight. Homozygous-positive *Sh2r6hs* plants produced 38% more seed weight and 31% more biomass than homozygous-negative *Sh2r6hs* plants (Table 3). The increase in biomass among *Sh2r6hs*-positives also led to an increase in the number of heads produced. Although seeds occurring on later heads account for roughly half of the yield increase, the remaining yield increase (roughly 19%) came from increased seed set on heads occurring in both positive and negative groups. Transgenic (+) and control (-) groups do not differ significantly for individual seed weight or harvest index. Overall, phenotypic data indicates that expression of *Sh2r6hs* increases seed yield in positive plants without reducing individual seed weight.

Table 3. Bozeman pSh2r6hs F2 Greenhouse Data, Winter 2000/2001. Plant and seed components are presented for F2 homozygous positive/negative *Sh2r6hs* segregants. 14 homozygous positive and 9 homozygous negative *Sh2r6hs* plants were grown under greenhouse conditions. Homozygotes were derived from 161-12, a transformant strongly expressing *Sh2r6hs* and the *bar* marker gene. Positive and negative means are given with corresponding standard deviations (in parentheses). +/- ratios are positive group means divided by negative group means.

Line	Total Plant Biomass (g)[a]	Seed Weight per Plant (g)	Harvest Index[b]
161-12 (+)	10.3 (1.8)	6.5 (1.33)	0.594 (.031)
161-12 (-)	7.9 (1.8)	4.7 (1.65)	0.553 (.091)
+/-	**1.31****	**1.38****	**1.07**

Line	Heads per Plant	Seeds per Plant	Average Individual Seed Weight (mg)[c]
161-12 (+)	6.5 (1.7)	186.5 (42.2)	35.4 (6.4)
161-12 (-)	4.7 (1.1)	137.2 (42.2)	33.8 (4.9)
+/-	**1.38****	**1.36****	**1.05**

[a] Plant biomass includes all aboveground plant structures.
[b] Harvest index is a comparison of seed weight to vegetative plant weight. It is calculated as the ratio of seed weight to biomass.
[c] Average individual seed weight was calculated by dividing total seed weight per plant by the number of seeds counted per plant.
*,** denote means significantly different at <0.05 and 0.01 level, respectively.

Two other *Sh2*-promoter lines backcrossed to HiLine were greenhouse-propagated with the 161-12 X HiLine F2 population described above. Both 155-4 (a low expresser of *Sh2r6hs,* also expresses *bar*) and 161-1 (expresses *bar* only) were backcrossed with HiLine. Fifty *Sh2r6hs*-segregating F2 plants derived from 155-4 and 161-1 backcrosses were progeny tested to identify homozygotes. 10 homozygous

positive/10 homozygous negative *Sh2r6hs* plants from 155-4 and 161-12 and 10 homozygous positive/10 homozygous negative *bar* 161-1 progeny were selected for field trials. 161-1 was chosen for field analysis so that the effects of *Sh2r6hs* expression in 155-4 and 161-12 could be compared to a line that has been tissue-cultured but does not express *Sh2r6hs*.

Bozeman pSh2r6hs F3 Field Trial, 2001: Space-seeded, Irrigated Conditions

Greenhouse-derived F3 seed from the three *pSh2r6hs* lines (155-4, 161-1, and 161-12) were space-planted at the Post Farm, Summer 2001. Twenty seeds were planted and rows were thinned to ten plants/row where possible. However, some rows did not yield ten plants, making it necessary to count the number of plants per row. Averages are presented for individual plants in Table 4a. 161-12 appears to have an overall reduction in plant germination (8.5 versus 9.1 for 155-4 and 161-1). Whether this is due to localized field effects or genetic contributions is uncertain. 161-12 seed yield is significantly higher than its control group (+12%), while 155-4 and 161-1 have lowered seed yields (-16% and -5% compared to controls, respectively). Similar trends are seen for biomass and shoot weight: 161-12 *Sh2r6hs* positives produced approximately 10% more vegetative mass than negatives. 155-4 suffered a biomass/shoot weight reduction, whereas 161-1 did not. The drop in seed yield without concurrent loss in shoot yield resulted in a significant harvest index decrease for 161-1. 155-4 and 161-12 positives do not differ in harvest indices.

161-12 *Sh2r6hs* positive plants were also compared to 155-4 and 161-1 (Table 4a) to establish relative levels of plant vigor in three transgenic lines. 161-12 plants have

higher seed weight, seed number, and shoot weight than 155-4 and 161-1. These results indicate that 155-4 and 161-1 have vigor-reducing mutations from tissue culturing and the transformation process.

Table 4a. Bozeman pSh2r6hs F3 Field Trial, 2001. Three lines were analyzed for yield and plant data: 155-4 is a weak expresser of *Sh2r6hs*, 161-1 is a null-*Sh2r6hs* line transformed with *bar*, and 161-12 is a strong expresser of *Sh2r6hs*. 10 homozygous *Sh2r6hs*-positive/10 homozygous *Sh2r6hs*-negative F2-derived F3 segregants (20 segregants total per line) were replicated three times. Plots were irrigated on two occasions.

			Yield Data per Plant				
Line[a]	**Geno[b]**	**#/Row[c]**	**Seed Weight (g)[d]**	**Seed Number[e]**	**Total Biomass (g)[f]**	**Shoot Weight (g)[g]**	**Harvest Index[h]**
155-4	+	9.1	28.9	752	90.0	61.1	0.321
	-	9.3	34.3	855	106.9	72.6	0.321
	+/-	**0.98**	**0.84****	**0.88****	**0.84****	**0.84****	**1.00**
161-1	+	9.0	30.4	745	99.9	69.5	0.305
	-	9.2	32.2	811	100.2	68.0	0.321
	+/-	**0.98**	**0.95***	**0.92***	**1.00**	**1.02**	**0.95****
161-12	+	8.8	37.9	928	118.7	80.8	0.319
	-	8.2	33.9	831	107.1	73.2	0.316
	+/-	**1.08**	**1.12***	**1.12***	**1.11***	**1.10***	**1.01**
161-12 + vs 155-4 +/-		0.95	1.20**	1.15**	1.21**	1.21**	0.99
161-12 + vs 161-1 +/-		0.96	1.21**	1.19**	1.19**	1.18**	1.02
161-12 + vs 161-12-, all 155-4, 161-1		1.00	1.14*	1.16*	1.17**	1.16**	1.01

[a] Line number is comprised of bombardment number followed by transgenic plant number from that bombardment.
[b] 'Geno' indicates genotype; + and – indicate homozygous positive and negative *Sh2r6hs* lines, respectively.

Table 4a, cont.
[c] Average number of plants per row (n=30). Forty seeds were originally space-planted (2 seeds every 6") in 10 ft rows. At the two-leaf stage, rows were thinned to 10 plants per row when applicable. Some rows did not germinate 10 plants per row, lowering the overall average.
[d] Seed weight is given on an individual plant basis. Total yield harvested per row was divided by the number of plants counted for that row.
[e] Seed number was calculated as the total seed weight divided by the average individual seed weight (shown in following table).
[f] Biomass consists of all aboveground plant weight. Rows sickled in the field were weighed prior to thrashing.
[g] Shoot weight is equal to biomass minus seed weight.
[h] Harvest index is a comparison of seed weight to vegetative plant weight. It is calculated as the ratio of seed weight to biomass.
*, ** denote means significantly different at <0.05 and 0.01 level, respectively.

Homozygous *Sh2r6hs*-positive 161-12 plants were compared to all other groups (155-4 +/-, 161-1 +/-, and 161-12 -). On average, 161-12 positives yielded 17% greater seed number, seed weight, shoot weight, and biomass than these groups (Table 4a). The data collected from 161-12 greenhouse and field tests suggests that *Sh2r6hs* boosts seed yield and overall plant vigor.

Seed qualities (protein, seed individual seed diameter/seed weight) were also recorded. 155-4 is significantly lower than its control group for protein percentage, individual seed weight/individual seed weight standard deviation, and seed diameter/seed diameter standard deviation (Table 4b). The lower averages indicate smaller seeds and less variation in seed size for positive 155-4 plants. Seeds from 161-1 positive plants have 4% more protein and 3% greater individual seed weight. 161-12 *Sh2r6hs*-positive seeds do not differ significantly from 161-12 *Sh2r6hs*-negative seed. Compared to positive/negative 155-4 seed, 161-12 seed is 4% greater for both seed weight and diameter.

Table 4b. Bozeman pSh2r6hs F3 Field Trial, 2001. Seed protein content, individual seed weight, seed diameter, and seed weight/diameter standard deviations for 155-4, 161-1, and 161-12.

Line[a]	Genotype[b]	Protein (% wt.)[c]	Individ. SW (mg)[d]	Individ. SW SD (mg)[e]	Diam. (mm)[f]	Diam. SD (mm)[g]
155-4	+	15.24	38.38	8.85	2.74	0.52
	-	15.70	40.12	9.69	2.80	0.55
	+/-	**0.97****	**0.96****	**0.91****	**0.98****	**0.94***
161-1	+	16.19	40.78	9.50	2.83	0.54
	-	15.58	39.72	9.30	2.81	0.53
	+/-	**1.04****	**1.03****	**1.02**	**1.01**	**1.01**
161-12	+	15.72	40.85	9.14	2.87	0.53
	-	15.69	40.79	9.40	2.86	0.54
	+/-	**1.00**	**1.00**	**0.97**	**1.00**	**0.97**
161-12 + vs 155-4 +/-		1.02	1.04**	0.99	1.04**	0.99
161-12 + vs 161-1 +/-		0.99	1.01*	0.97	1.02	0.98
161-12 + vs 161-12- and all 155-4, 161-1		**1.00**	**1.02****	**0.98**	**1.01**	**0.98**

[a] Line number is comprised of bombardment number followed by transgenic plant number from that bombardment.
[b] Genotypes are homozygous positive and negative *Sh2r6hs* lines.
[c] Protein percentage obtained from an Infratec 1225 Whole Grain Analyzer (Tecator, Sweden). Kernel absorbance of infrared wavelengths was taken for each sample.
[d] Individual seed weight averages. 100 kernels per sample were tested with the Single Kernel Characterization System 4100 (Perten, Sweden).
[e] Individual seed weight standard deviation averages (n=100).
[f] Seed diameter averages. 100 kernels per sample were tested with the Single Kernel Characterization System 4100 (Perten, Sweden).
[g] Seed diameter standard deviation averages (n=100).
*, ** denote means significantly different at <0.05 and 0.01 level, respectively.

Bozeman pSh2r6hs F4 Field Trial, 2002: Space-seeded, Irrigated Conditions

F4 seed collected from the previous summer's field trial was space planted in 2002. All rows consisted of 20 plants, since an excess of seed was planted (60 seeds per

row versus 20 seeds the previous summer). Because all rows were uniform for plant number, harvest data was collected and analyzed for rows as a whole, and not for individual plants.

The reduction in 155-4 *Sh2r6hs*-positive plant vigor noted in the 2001 space-planted trial is not duplicated in this trial. Curiously, 155-4 has the same seed yield, shoot mass, and total biomass as control rows (Table 5a). Seed yield is depressed again in 161-1 rows (-8%), this time enough to significantly decrease total biomass (-4%). The most unexpected finding comes from 161-12. Positive rows yielded less seed weight (mainly due to a smaller number of kernels) and less biomass than negative rows. This finding may have been due to unreliable data from the negative control group. Negative rows yielded 3% more than untransformed HiLine, an unlikely occurrence considering the yield reductions associated with biolistic transformation.

Five other lines were included: three parental lines from the previous HiLine backcrossing (P-155-4, P-161-1, P-161-12), a high-expressing *Sh2r6hs* line (152-7, not backcrossed with HiLine), and HiLine (Table 5a). Both positive and negative groups from the backcrossed 161-12 population performed as well as or better than untransformed HiLine. Backcrossing 161-12 to HiLine may reduce some tissue-culture mutations, generating a high-expressing *Sh2r6hs* line with yields equivalent to the wild-type variety. The three lines not backcrossed to HiLine (P-155-4, P-161-1, P-161-12) have much lower yields and biomasses compared to HiLine (Table 5a).

Protein is 1% higher in 161-12 positives, but seed weight standard deviation and seed diameter standard deviation is 6% and 7% lower, respectively (Table 5b). 155-4 and

161-1 follow the same trends in the 2001 trial: 155-4 produced smaller seeds with lower protein composition, while 161-1 produced larger seeds with higher protein composition.

Table 5a. Bozeman pSh2r6hs F4 Trial, Summer 2002: Space-Seeded, Irrigated Conditions. 10+/10- *Sh2r6hs* F2-derived F4 segregant lines from each transformant line (155-4, 161-1, and 161-12) were replicated four times in a randomized block design. 155-4 is a weak expresser of *Sh2r6hs*, 161-1 is a null-*Sh2r6hs* line transformed with *bar*, and 161-12 is a strong expresser of *Sh2r6hs*. The parents from each backcross (HiLine, P-155-4, P-161-1, and P-161-12) and a high-expressing *Sh2r6hs* line (152-7, not backcrossed with HiLine) were included. Seed weight, biomass, and shoot weight are presented on a 12% moisture basis. Plots were irrigated on two occasions. Each 10-ft row consisted of 20 plants spaced six inches apart.

Line[a]	Geno[b]	Seed Weight (g)[c]	Seed Number[d]	Total Biomass (g)[e]	Shoot Weight (g)[f]	Harvest Index[g]
155-4	+	454.5	11,351	999.8	548.3	0.454
	-	461.4	10,949	1014.0	555.3	0.455
	+/-	**0.98**	**1.04**	**0.98**	**0.99**	**1.00**
161-1	+	435.4	9,873	1008.4	573.0	0.433
	-	476.0	11,145	1054.4	578.4	0.453
	+/-	**0.92****	**0.88****	**0.96***	**0.99**	**0.96***
161-12	+	472.3	10,882	1025.3	553.0	0.461
	-	489.2	11,356	1061.9	572.7	0.461
	+/-	**0.97***	**0.96**	**0.97***	**0.97**	**1.00**
152-7		475.7	11,976	1105.4	629.8	0.431
P-155-4		425.2	10,577	946.0	520.8	0.450
P-161-1		418.1	9,507	968.3	550.3	0.432
P-161-12		413.7	9,762	919.2	505.5	0.451
HiLine		472.0	10,969	1037.1	565.1	0.456

[a] Line number is comprised of bombardment number followed by transgenic plant number from that bombardment.
[b] 'Geno' indicates genotype; + and – indicate homozygous positive and negative *Sh2r6hs* lines, respectively.
[c] Seed weight was measured from all plants growing in a single row. Average seed weight of 10 positive or 10 negative *Sh2r6hs* rows is given.
[d] Seed number was calculated as the total seed weight divided by the average individual seed weight (shown in following table).
[e] Biomass consists of all aboveground plant weight per row. Rows were sickled, bundled, and dried for 1-2 days prior to weighing.
[f] Shoot weight is equal to biomass per row minus seed weight per row.

Table 5a, cont.
[g] Harvest index is a comparison of seed weight to vegetative plant weight. It is calculated as the ratio of seed weight to biomass.
*, ** denote means significantly different at <0.05 and 0.01 level, respectively.

Table 5b. Bozeman pSh2r6hs F4 Field Trial, 2002: Space-Seeded, Irrigated Conditions. Seed protein content, individual seed weight, seed diameter, and seed weight/diameter standard deviations are given for 155-4, 161-1, and 161-12. Parental types (HiLine, P-155-4, P-161-1, P-161-12) and 152-7 are also included.

Line[a]	Genotype[b]	Protein (% wt.)[c]	Individ. SW (mg)[d]	Individ. SW SD (mg)[e]	Diam. (mm)[f]	Diam. SD (mm)[g]
155-4	+	15.25	40.04	9.54	2.76	0.49
	-	15.84	42.14	9.76	2.85	0.50
	+/-	**0.96****	**0.95****	**0.98**	**0.97****	**0.98**
161-1	+	15.85	44.10	9.47	2.97	0.51
	-	15.25	42.71	9.58	2.88	0.51
	+/-	**1.04****	**1.03***	**0.99**	**1.03****	**1.00**
161-12	+	15.80	43.40	9.24	2.93	0.50
	-	15.65	43.08	9.87	2.93	0.53
	+/-	**1.01***	**1.01**	**0.94***	**1.00**	**0.93***
152-7		16.35	39.72	9.30	2.75	0.49
P-155-4		15.20	40.20	10.04	2.81	0.55
P-161-1		16.50	43.98	9.58	2.97	0.46
P-161-12		15.55	42.38	10.15	2.89	0.50
HiLine		16.03	43.03	9.94	2.90	0.52

[a] Line number is comprised of bombardment number followed by transgenic plant number from that bombardment.
[b] Genotypes are homozygous positive and negative *Sh2r6hs* lines.
[c] Protein percentage obtained from an Infratec 1225 Whole Grain Analyzer (Tecator, Sweden). Kernel absorbance of infrared wavelengths was taken for each sample.
[d] Individual seed weight averages. 100 kernels per sample were tested with the Single Kernel Characterization System 4100 (Perten, Sweden).
[e] Individual seed weight standard deviation averages (n=100).
[f] Seed diameter averages. 100 kernels per sample were tested with the Single Kernel Characterization System 4100 (Perten, Sweden).
[g] Seed diameter standard deviation averages (n=100).
*, ** denote means significantly different at <0.05 and 0.01 level, respectively.

Bozeman pSh2r6hs F4 Field Trial, 2002: Dense-seeded, Irrigated Conditions

Ample seed stocks of 155-4, 161-1, and 161-12 allowed for a dense-seeded trial. Without unlimited sunlight per plant, we believe that the effect *Sh2r6hs* has on plant phenotype will be diminished. Reduced solar radiation will limit source tissues, and sink tissues will not receive abundant photoassimilates for starch synthesis. Indeed, positive 161-12 rows produced the same seed weight and shoot weight as 161-12 negative rows (Table 6a). The only significant difference ($p<0.05$) recorded is for harvest index, although positives are only 1% higher.

Table 6a. Bozeman pSh2r6hs F4 Trial, Summer 2002: Dense-Seeded, Irrigated Conditions. 10+/10- *Sh2r6hs* F2-derived F4 segregant lines from each transformant line (155-4, 161-1, and 161-12) were replicated four times in a randomized block design. 155-4 is a weak expresser of *Sh2r6hs*, 161-1 is a null-*Sh2r6hs* line transformed with *bar*, and 161-12 is a strong expresser of *Sh2r6hs*. The parents from each backcross (HiLine, P-155-4, P-161-1, and P-161-12) and a high-expressing *Sh2r6hs* line (152-7, not backcrossed with HiLine) were included. Seed weight, biomass, and shoot weight are presented on a 12% moisture basis. Plots were irrigated on two occasions.

Line[a]	Geno[b]	Seed Weight (g)[c]	Seed Number[d]	Total Biomass (g)[e]	Shoot Weight (g)[f]	Harvest Index[g]
155-4	+	545.9	15,408	1342.4	796.5	0.407
	-	610.5	16,211	1435.9	825.3	0.426
	+/-	**0.89****	**0.95***	**0.94****	**0.97***	**0.95****
161-1	+	550.2	14,947	1365.7	815.4	0.402
	-	561.7	15,814	1365.3	803.5	0.411
	+/-	**0.98**	**0.95**	**1.00**	**1.01**	**0.98***
161-12	+	578.7	15,169	1389.2	810.5	0.416
	-	577.5	15,246	1401.0	823.5	0.412
	+/-	**1.00**	**0.99**	**0.99**	**0.98**	**1.01***
152-7		590.0	16,949	1458.6	868.6	0.405
P-155-4		531.9	16,779	1329.0	797.1	0.399
P-161-1		567.3	14,621	1362.8	795.6	0.416
P-161-12		558.1	14,143	1374.5	816.4	0.406
HiLine		568.9	15,869	1366.7	797.8	0.416

Table 6a, cont.
[a] Line number is comprised of bombardment number followed by transgenic plant number from that bombardment.
[b] 'Geno' indicates genotype; + and – indicate homozygous positive and negative *Sh2r6hs* lines, respectively.
[c] Seed weight was determined for entire rows. Average seed weight of 10 positive or 10 negative *Sh2r6hs* rows is given.
[d] Seed number was calculated as the total seed weight divided by the average individual seed weight (shown in following table).
[e] Biomass consists of all aboveground plant weight. Rows were sickled, bundled, and dried for 1-2 days prior to weighing.
[f] Shoot weight is equal to biomass minus seed weight.
[g] Harvest index is a comparison of seed weight to vegetative plant weight. It is calculated as the ratio of seed weight to biomass.
*, ** denote means significantly different at <0.05 and 0.01 level, respectively.

155-4 continued to suffer yield reductions from the *Sh2r6hs* insertion. Seed weight, biomass, shoot weight, and harvest index are lower in 155-4 positives vs. negatives. 155-4 negatives yielded exceptionally well (610 grams average seed weight), while 155-4 positives set a low number of sickly seeds. 161-1, the non-expresser of *Sh2r6hs*, is equal to the 161-1 control for all harvest data.

161-12 positives did not yield more than 161-12 negatives in this trial. This may be due to the extensive lodging that occurred 3-4 weeks after anthesis. The dense-irrigated trial was almost entirely lodged from heavy wind and rain on 8/3/02. Regardless of lodging, both positive and negative 161-12 groups equal HiLine for several harvest categories (Table 6a). Backcrossing 161-12 with HiLine restored some of the plant vigor lost during the transformation process.

Protein, seed weight, and seed diameter are reduced again in 155-4 plants (-2%, -6%, and –4%, respectively, Table 6b). 161-1 has a 4% increase in protein content but

does not vary from controls in seed size or seed weight. 161-12 does not differ from controls for any individual seed characteristics.

Table 6b. Bozeman pSh2r6hs F4 Field Trial, 2002: Dense-Seeded, Irrigated Conditions. Seed protein content, individual seed weight, seed diameter, and seed weight/diameter standard deviations are given for 155-4, 161-1, and 161-12. Parental types (HiLine, P-155-4, P-161-1, P-161-12) and 152-7 are included.

Line[a]	Genotype[b]	Protein (% wt.)[c]	Individ. SW (mg)[d]	Individ. SW SD (mg)[e]	Diam. (mm)[f]	Diam. SD (mm)[g]
155-4	+	14.14	35.43	8.19	2.52	0.44
	-	14.48	37.66	8.24	2.64	0.47
	+/-	**0.98****	**0.94****	**0.99**	**0.96****	**0.93**
161-1	+	15.13	36.81	8.74	2.62	0.46
	-	14.57	35.52	8.50	2.55	0.44
	+/-	**1.04***	**1.04**	**1.03**	**1.02**	**1.04**
161-12	+	14.42	38.15	8.28	2.66	0.44
	-	14.36	37.88	8.41	2.65	0.44
	+/-	**1.00**	**1.01**	**0.99**	**1.00**	**1.00**
152-7		15.13	34.81	8.70	2.51	0.47
P-155-4		14.55	31.70	8.93	2.36	0.47
P-161-1		15.15	38.80	9.05	2.68	0.48
P-161-12		14.48	39.46	8.85	2.69	0.42
HiLine		14.60	35.85	9.07	2.56	0.52

[a] Line number is comprised of bombardment number followed by transgenic plant number from that bombardment.
[b] Genotypes are homozygous positive and negative *Sh2r6hs* lines.
[c] Protein percentage obtained from an Infratec 1225 Whole Grain Analyzer (Tecator, Sweden). Kernel absorbance of infrared wavelengths was taken for each sample.
[d] Individual seed weight averages. 100 kernels per sample were tested with the Single Kernel Characterization System 4100 (Perten, Sweden).
[e] Individual seed weight standard deviation averages (n=100).
[f] Seed diameter averages. 100 kernels per sample were tested with the Single Kernel Characterization System 4100 (Perten, Sweden).
[g] Seed diameter standard deviation averages (n=100).
*, ** denote means significantly different at <0.05 and 0.01 level, respectively.

Bozeman pSh2r6hs F4 Field Trial, 2002: Dense-seeded, Dryland Conditions

Dryland conditions provide further limitations on source tissues in developing plants. 155-4 positives yielded the lowest of any group in the experiment (296 grams, 15% less than the control, Figure 7a). Seed number, biomass, shoot weight, and harvest index are also lower in 155-4 positives. Although harvest index is unchanged for 161-1, three other harvest components (seed weight, seed number, biomass, and shoot weight) are significantly reduced. Slight increases were observed for 161-12 positive rows (+3% seed weight, +2% biomass, and +2% shoot weight) but these increases are not significant at the 0.05 level (Figure 7a). Only seed number is significantly different between 161-12 positives and the controls (+6%).

Once more, 161-1 positives have larger seeds than negatives, despite 161-1 reduction in seed yield (Table 7b). 161-12, on the other hand, produced seeds with smaller weights (-4%) and diameters (-3%) with less variability. It is likely that smaller seeds in 161-12 positives played a large role in reducing overall yield. Even if yield increase potential in 161-12 positives existed (ie. greater sink strength), it would be difficult to detect without complete grain filling in positive plants.

Table 7a. Bozeman pSh2r6hs F4 Trial, Summer 2002: Dense-Seeded, Dryland Conditions. 10+/10- *Sh2r6hs* F2-derived F4 segregant lines from each transformant line (155-4, 161-1, and 161-12) were replicated four times in a randomized block design. 155-4 is a weak expresser of *Sh2r6hs*, 161-1 is a null-*Sh2r6hs* line transformed with *bar*, and 161-12 is a strong expresser of *Sh2r6hs*. The parents from each backcross (HiLine, P-155-4, P-161-1, and P-161-12) and a high-expressing *Sh2r6hs* line (152-7, not backcrossed with HiLine) were included. Seed weight, biomass, and shoot weight are presented on a 12% moisture basis.

Line[a]	Geno[b]	Seed Weight (g)[c]	Seed Number[d]	Total Biomass (g)[e]	Shoot Weight (g)[f]	Harvest Index[g]
155-4	+	295.9	10,224	887.8	591.9	0.333
	-	348.8	12,170	975.0	626.2	0.358
	+/-	**0.85****	**0.84****	**0.91****	**0.95****	**0.93****
161-1	+	347.9	11,062	940.8	592.9	0.370
	-	359.6	12,586	982.4	622.8	0.366
	+/-	**0.97***	**0.88****	**0.96****	**0.95****	**1.01**
161-12	+	335.8	11,713	940.8	605.0	0.357
	-	327.5	11,008	922.5	595.0	0.355
	+/-	**1.03**	**1.06***	**1.02**	**1.02**	**1.01**
152-7		344.2	12,005	974.4	630.2	0.354
P-155-4		295.4	10,804	903.1	607.7	0.327
P-161-1		329.1	9,898	900.9	571.8	0.365
P-161-12		339.7	11,712	949.0	609.3	0.358
HiLine		349.9	11,150	928.4	578.5	0.377

[a] Line number is comprised of bombardment number followed by transgenic plant number from that bombardment.
[b] 'Geno' indicates genotype; + and – indicate homozygous positive and negative *Sh2r6hs* lines, respectively.
[c] Seed weight was determined for entire rows. Average seed weight of 10 positive or 10 negative *Sh2r6hs* rows is presented.
[d] Seed number was calculated as the total seed weight divided by the average individual seed weight (shown in following table).
[e] Seed number was calculated as the total seed weight divided by the average individual seed weight (shown in following table).
[f] Biomass consists of all aboveground plant weight. Rows were sickled, bundled, and dried for 1-2 days prior to weighing.
[g] Shoot weight is equal to biomass minus seed weight.
[h] Harvest index is a comparison of seed weight to vegetative plant weight. It is calculated as the ratio of seed weight to biomass.
*, ** denote means significantly different at <0.05 and 0.01 level, respectively.

Table 7b. Bozeman pSh2r6hs F4 Field Trial, 2002: Dense-Seeded, Dryland Conditions. Seed protein content, individual seed weight, seed diameter, and seed weight/diameter standard deviations are given for 155-4, 161-1, and 161-12. Parental types (HiLine, P-155-4, P-161-1, P-161-12) and 152-7 are included.

Line[a]	Genotype[b]	Protein (% wt.)[c]	Individ. SW (mg)[d]	Individ. SW SD (mg)[e]	Diam. (mm)[f]	Diam. SD (mm)[g]
155-4	+	16.10	28.94	9.05	2.23	0.49
	-	16.37	28.66	9.28	2.23	0.50
	+/-	**0.98****	**1.01**	**0.98**	**1.00**	**0.99**
161-1	+	16.68	31.45	9.00	2.39	0.50
	-	16.44	28.57	9.26	2.23	0.51
	+/-	**1.01****	**1.10****	**0.97**	**1.07****	**0.99**
161-12	+	16.61	28.67	8.84	2.23	0.50
	-	16.55	29.79	9.66	2.29	0.53
	+/-	**1.00**	**0.96***	**0.92****	**0.97***	**0.95**
152-7		17.13	28.67	8.74	2.25	0.55
P-155-4		15.98	27.34	8.45	2.20	0.53
P-161-1		16.70	33.25	9.91	2.37	0.57
P-161-12		16.44	28.99	9.24	2.25	0.46
HiLine		16.41	31.38	11.62	2.35	0.58

[a] Line number is comprised of bombardment number followed by transgenic plant number from that bombardment.
[b] Genotypes are homozygous positive and negative *Sh2r6hs* lines.
[c] Protein percentage obtained from an Infratec 1225 Whole Grain Analyzer (Tecator, Sweden). Kernel absorbance of infrared wavelengths was taken for each sample.
[d] Individual seed weight averages. 100 kernels per sample were tested with the Single Kernel Characterization System 4100 (Perten, Sweden).
[e] Individual seed weight standard deviation averages (n=100).
[f] Seed diameter averages. 100 kernels per sample were tested with the Single Kernel Characterization System 4100 (Perten, Sweden).
[g] Seed diameter standard deviation averages (n=100).
*, ** denote means significantly different at <0.05 and 0.01 level, respectively.

Bozeman pSh2r6hs Biomass Study, 2002: Dense-seeded, Irrigated Conditions

Ten homozygous positive and ten homozygous negative *Sh2r6hs* 161-12 lines (the same homozygotes planted in the pSh2r6hs F4 trials) were combined into positive or negative seed pools. Table 8a presents data taken from 1-foot sections of these homozygous *Sh2r6hs* 161-12 rows. Immature positive plants have 20% less biomass than negative plants at anthesis. Two weeks later (14 dpa), positive plants equal negative plants for biomass. At this developmental stage, all plants are beginning to produce heads with immature seeds. Once more, + and – groups are equal in the number of heads formed and the seed weight produced. Positives appear to surpass controls at 28 dpa, although no significant differences were detected for biomass, head number, or seed weight (Table 8a). Mature positive plants are significantly higher for biomass (16% greater than negatives), head number (15% greater), and seed weight (20% greater). The depression in positive plant biomass at anthesis suggests that the *Sh2r6hs/bar* locus may disrupt processes not associated with starch development. The positive increase in biomass, seed weight, and head number at maturity, however, may be accounted for by the activation of *Sh2r6hs* expression after anthesis.

Positive and negative seeds are the same for individual seed weight and diameter at 14 dpa, 28 dpa, and maturity (Table 8b). The data acquired at maturity resembles the data taken for the F2-pSh2r6hs-greenhouse trial (see Table 3). Both experiments measured increases in total seed weight without increases in individual seed weight. Protein percentage does not differ between positive/negative seeds at 28 dpa or maturity.

Table 8a. Bozeman pSh2r6hs Biomass Study, Summer 2002. Ten rows (10 grams densely-planted seed, 10-ft long) containing 161-12 *Sh2r6hs*-positive homozygotes were grown. At the two-leaf stage, five 1-ft sections (consisting of 20 plants) were marked within each row. These sections were harvested independently on five occasions. The same was done for 10 rows containing 161-12 *Sh2r6hs*-negative homozygotes. All samples were placed in a 40° C drying chamber for 1 week before weighing to remove excess moisture (<6% water by weight). Averages derived from the 1-ft sections are presented for several plant and seed characteristics.

Sampling Date[a]	Geno[b]	Total Biomass (g)[c]	Total Heads	Seed Weight (g)	Seed Number[d]	Shoot Weight (g)[e]	Harvest Index[f]
(-) 14 DPA	+	29.3					
	-	27.9					
	+/-	**1.05**					
Anthesis	+	56.6	50				
	-	70.9	55				
	+/-	**0.80***	**0.90**				
14 DPA	+	107.2	60	23.6	1,373	83.6	0.22
	-	110.0	61	23.3	1,369	86.7	0.21
	+/-	**0.97**	**0.98**	**1.01**	**1.00**	**0.96**	**1.05**
28 DPA	+	140.9	58	60.0	1,891	80.9	0.43
	-	130.8	54	55.7	1,765	75.1	0.43
	+/-	**1.08**	**1.08**	**1.08**	**1.07**	**1.08**	**1.00**
Maturity	+	178.2	62	77.7	1,895	100.5	0.44
	-	153.1	54	64.8	1,624	88.3	0.42
	+/-	**1.16***	**1.15***	**1.20****	**1.17***	**1.14***	**1.05**

[a] Sections were harvested on four dates before maturity and at maturity. (-) 14 DPA refers to 14 days before anthesis; 14 DPA and 28 DPA refer to 14 and 28 days post anthesis, respectively.

[b] 'Geno' indicates genotype; + and – indicate homozygous positive and negative *Sh2r6hs* lines, respectively. +/- is the ratio of the positive average to the negative average.

[c] Biomass consists of all aboveground plant weight. Sections were sickled, bagged, placed in a drying chamber for 1 week to remove excess moisture, and weighed.

[d] Seed number was calculated as the total seed weight divided by the average individual seed weight (shown in following table).

[e] Shoot weight is equal to biomass minus seed weight.

[f] Harvest index is a comparison of seed weight to vegetative plant weight. It is calculated as the ratio of seed weight to biomass.

*, ** denote means significantly different at <0.05 and 0.01 level, respectively.

Table 8b. Bozeman pSh2r6hs Biomass Study, Summer 2002. Seed protein content, individual seed weight, seed diameter, and seed weight/diameter standard deviations are given for +/- *Sh2r6hs* homozygotes on each sampling date.

Sampling Date[a]	Genotype[b]	Protein (% wt.)[c]	Individ. SW (mg)[d]	Individ. SW SD (mg)[e]	Diam. (mm)[f]	Diam. SD (mm)[g]
14 DPA	+		17.19	4.60	1.60	0.29
	-		17.02	4.48	1.61	0.30
	+/-		**1.01**	**1.03**	**0.99**	**0.99**
28 DPA	+	13.10	31.73	7.70	2.57	0.50
	-	13.05	31.56	7.36	2.59	0.52
	+/-	**1.00**	**1.01**	**1.05**	**0.99**	**0.96**
Maturity	+	14.41	41.00	9.01	2.88	0.53
	-	14.55	39.89	9.30	2.80	0.54
	+/-	**0.99**	**1.03**	**0.97**	**1.03**	**0.98**

[a] Seeds were large enough for single kernel analysis 14 days post anthesis (14 DPA). Protein analysis could only be performed on 28 DPA and fully-mature seeds.
[b] Genotypes are homozygous positive and negative *Sh2r6hs* lines.
[c] Protein percentage obtained from an Infratec 1225 Whole Grain Analyzer (Tecator, Sweden). There was not enough seed produced at 14 DPA to obtain protein composition.
[d] Individual seed weight averages. 100 kernels per sample were tested with the Single Kernel Characterization System 4100 (Perten, Sweden).
[e] Individual seed weight standard deviation averages (n=100).
[f] Seed diameter averages. 100 kernels per sample were tested with the Single Kernel Characterization System 4100 (Perten, Sweden).
[g] Seed diameter standard deviation averages (n=100).
*, ** denote means significantly different at <0.05 and 0.01 level, respectively.

Discussion

Almost three-fourths of pollinated wheat flowers do not mature into seeds (Zhen-Wen et al. 1988). High seed abortion rates stem from a lack of assimilates delivered from photosynthesizing leaves to sink tissues, namely immature seeds (Abbate et al. 1998). *Sh2r6hs* codes for a modified AGP large subunit with two properties that potentially increase seed sink strength. These properties are decreased allosteric inhibition to Pi

(Giroux et al. 1996) and greater stability of large and small AGP subunits (Greene and Hannah, 1998). Three genotypes (155-4, 161-1, and 161-12) transformed with the *Sh2*-promoter *Sh2r6hs* construct (pSh2r6hs) were tested for enhanced kernel sink strength. Molecular data confirms *Sh2r6hs* expression seven days post anthesis, before most seed abortions occur (Figure 9). The probability of yield increase in *Sh2r6hs*-positive plants is highest if less seeds are aborted, compared to *Sh2r6hs*-negative plants. F2 greenhouse data taken from 161-12, the highest *Sh2*-promoter expresser of *Sh2r6hs*, support this hypothesis (Table 3). Positive plants yielded 36% more seeds than negative plants, giving positive plants a 38% boost in overall kernel yield.

Among the abiotic factors influencing kernel yield in wheat, solar radiation and temperature are the largest contributors (Slafer and Rawson, 1994). Unfortunately, these factors are much more difficult to control in the field than in the greenhouse. Fischer and Laing (1975) found that space-seeded plants generate higher yields than dense-seeded plants. Shading from neighboring plants greatly reduces photosynthetic levels, leading to a reduced sugar supply to developing endosperm cells. Dense-seeded rows may be source-tissue limited compared to space-seeded rows (Fischer 1985). To ensure that transgenic lines were not source-limited, seeds were space-planted to optimize potential *Sh2r6hs* sink enhancement.

Sh2r6hs-positive 161-12 plants produced 12% more seeds, seed weight, and biomass than *Sh2r6hs*-negative 161-12 plants (Table 4a). Positive plants produce more seeds because *Sh2r6hs* enhances sink strength early in seed maturation (Figure 9), lessening the frequency of seed abortion. Another consequence of *Sh2r6hs* sink-tissue

enhancement is increased photosynthesis (Richards 2000). Leaf carbohydrates are not accumulated in the leaf, since stronger sink tissues demand more assimilates for starch production. Feedback inhibition is lessened without leaf photoassimilate accumulation, and photosynthetic rates are raised. *Sh2r6hs*-positive plants develop greater leaf masses when photosynthetic rates are high. This explains the higher biomass average recorded for 161-12 positive plants.

Enough seed was available after the 2001 trial to allow planting of 155-4, 161-1, and 161-12 in three different environments in 2002: space-seeded, irrigated; dense-seeded, irrigated; and dense-seeded, dryland. *Sh2r6hs*-positive 161-12 rows yielded the same as controls under dense-seeded, irrigated conditions (Table 6a) and significantly less (-3%) than controls under space-seeded, irrigated conditions (Table 5a). Surprisingly, dense-seeded, dryland rows yielded 3% more than controls, setting significantly more seeds (+6%) (Table 7a). We hypothesized that 161-12 plants provided with optimal levels of sunlight and water (space-seeded, irrigated conditions) would generate the highest yield difference between *Sh2r6hs*-positive plants and control plants, since source-tissues are not resource-limited. However, space-seeded, irrigated rows did not produce higher yield differences than dense-seeded, dryland rows or dense-seeded, irrigated rows (Table 5a, 6a, 7a). The highest 161-12 yield difference (+3%), obtained from dense-seeded, dryland rows, indicates that source tissues are not limited under these conditions.

The reason for higher seed set in dense-dryland rows and not in dense-irrigated or space-irrigated rows remains unclear. 161-12 seed yield and seed number were not

consistently higher from one space-seeded, irrigated trial (2001) to the next (2002). This inconsistency indicates that seed and plant productivity are dependent on environmental factors other than seeding density and irrigation schemes. These factors may include seasonal precipitation, the ambient temperature during anthesis, and lodging induced by extreme weather.

Following space-planting, the 2001 trial did not receive rainfall for almost three weeks (data not shown). Without soil moisture to induce germination, seedlings inconsistently emerged over an extended period. 2002 space-planted rows received 3-4 inches of snow after planting. The subsequent snowmelt fueled seedling emergence within a narrow window. As mentioned above, 2001 space-seeded 161-12 plants produced 12% more yield, yet 2002 space-seeded plants produced 3% less yield than controls. Differences in the frequency and period of germination may account for the yield differences between trials.

2001 and 2002 irrigated experiments were equally watered with 6.5 inches of water on several occasions, beginning in late May (See 'Field Conditions,' Chapter 2). However, the 2002 growing season exceeded the 2001 growing season by nearly an inch of precipitation. The extra rainfall is partially responsible for delayed maturation of 2002 space-seeded rows. Even though both trials were planted on similar dates (late April), 2002 space-seeded rows were harvested nearly two weeks later than 2001 space-seeded rows. The extended time required for 2002 plants to mature may have given 161-12 *Sh2r6hs*-negative plants a chance to "catch up" to positive plants. This seems more feasible if *Sh2r6hs* contributes less to grain-filling late in plant development.

High temperatures decrease the viability of pollen, lowering the fertilization frequency of wheat florets (Thorne and Wood, 1986). Dense-seeded, dryland *Sh2r6hs* rows flowered approximately one week before space-irrigated and dense-irrigated rows (data not shown). Lower mean temperatures during dense-dryland fertilization periods may account for increased seed set, and ultimately increased yield in dense-dryland plants (Table 7a). The 2001 space-irrigated trial may have also experienced cooler temperatures during anthesis, raising the number of pollinated flowers in positive plants (Table 4a). Extremely hot temperatures the week after anthesis (See Chapter 2, Field Conditions, 2002) could account for the 4% decrease in 161-12 *Sh2r6hs*-positive plant seed number. Dense-seeded, irrigated rows were completely lodged three weeks after anthesis. Whether or not this event impacted *Sh2r6hs*-positive plants is unknown. Lodging certainly reduces leaf absorbance of solar radiation, which may negatively affect *Sh2r6hs*-driven starch biosynthesis. Because positive and negative rows were lodged equally, starch accumulation was probably affected in all plants, regardless of *Sh2r6hs* expression.

Vegetative growth and seed yield is generally depressed in transgenic lines (Sharp et al. 2002; Barro et al. 2002), mainly because transformation and tissue-culturing induce numerous mutations in the wheat genome. All three pSh2r6hs lines were backcrossed to HiLine to eliminate harmful mutations not linked to the *Sh2r6hs/bar* locus. Backcrossing to HiLine introduces a gametic set of non-transformed chromosomes, thus reducing genomic mutations by 50%. 161-12 X HiLine, 155-4 X HiLine and 161-1 X HiLine

backcross progeny generated greater yields than their respective transgenic parents (Table 5a).

The insertion of *Sh2r6hs* and/or *bar* can consistently lower transgenic plant productivity, as seen in 155-4 and 161-1 (Tables 4a, 5a, 6a, 7a). 155-4 contains numerous copies of *Sh2r6hs* interfering with native genes at a specific locus. These disrupted genes are responsible for both seed (indicated by reduced seed yield) and plant (indicated by reduced biomass) growth processes. The potentially negative effects *Sh2r6hs* has upon plant vigor can easily be distinguished from tissue-culture mutations. Tissue-culture mutations are widespread throughout the wheat genome, and plant vigor is equally reduced in *Sh2r6hs*-positive and negative progeny, compared to untranformed HiLine. If *Sh2r6hs* interferes with genes vital for plant productivity, then T1 *Sh2r6hs*-positive segregants will suffer yield and/or biomass reductions, whereas *Sh2r6hs*-negative segregants will not.

Sh2r6hs insertion can also affect protein storage genes. 155-4 yielded 16% less seed mass (space-planted, irrigated conditions) and seeds contained 3% less protein (Table 4b). 161-1 also yielded less than controls, but seeds were 4% higher in protein. The 155-4 *Sh2r6hs* locus may disrupt native starch biosynthetic and protein storage genes, which decreases both of these components in mature seeds. 161-1 protein storage genes are not affected by transgene insertion, but a reduction in starch synthesis results in higher 161-1 protein content.

Transgene insertion variably lowers seed and vegetative yield in 155-4, 161-1, and 161-12, due to the disruption of native genes at that locus. 155-4 *Sh2r6hs* positives

are sickly because many *Sh2r6hs* copies disrupt native genes, and because low 155-4 *Sh2r6hs* expression cannot boost starch production enough to compensate for vigor depression. 161-12 also suffers vigor depression from *Sh2r6hs* insertion, based on immature plant biomass data taken from the biomass experiment (Table 8a). *Sh2r6hs*-positive plants weigh 20% less than negative plants at anthesis and 15% more than negative plants at maturity. The same trend is followed for head number and seed weight. The decrease in biomass and head number at anthesis stems from several tranformation mutations. *Sh2r6hs* expression commences after anthesis, and overcomes these vigor-associated mutations at 14-dpa (Table 8a). Biomass, head number, and seed weight continue to rise at 28-dpa, and all three characteristics are significantly increased at maturity. Similar to various greenhouse and field trials (Tables 3, 4, and 7), increased seed weight (+20%) was accompanied by an increase in seed set (+17%).

Environmental factors affect several aspects of plant development, possibly even the timing of *Sh2r6hs* expression. Although *Sh2r6hs* expression was noted at 7-dpa in greenhouse-grown plants (Figure 9), the onset of expression could be shifted with differing field conditions. Temporal and spatial fluctuations in growing conditions strongly determine if seed yield, seed set and biomass will be higher in *Sh2r6hs*-positive plants. 161-12 transformants produced more seed mass than controls in the F2 greenhouse trial (Table 3), one space-seeded, irrigated field trial (Table 4), and one dense-seeded, irrigated biomass trial (Table 8). Yield data from two other trials (See Tables 6, 7) did not confirm higher or lower productivity in 161-12, while 2002 space-seeded, irrigated positive plants yielded significantly less than negative plants (Table 5).

This collection of data suggests that increasing the sink strength of endosperm tissue via *Sh2r6hs* transformation results in higher kernel yields only when environmental conditions are favorable. The term "favorable" is used loosely, as we simply do not know how many, and to what degree, environmental conditions influence starch biosynthesis.

CHAPTER 5

GREENHOUSE AND FIELD TRIALS: *GLUTENIN*-PROMOTER SH2R6HS TRANSFORMANTS

Results

Greenhouse pGS T2 Trial, Spring 2002

Nine *Glutenin*-promoter *Sh2r6hs* (GS) lines with high expression of *Sh2r6hs* were selected for a T2 greenhouse study: GS4, 5, 8, 9, 14, 16, 17, 28, and 54. Fifteen positive/fifteen negative homozygous *Sh2r6hs* plants per line (one negative, one positive plant in a pot) were planted on 10/17/01. Tiller and head number for each plant was determined on five occassions to see what type of effect *Sh2r6hs* has on plant development (Table 9a and 9b).

GS28 is the only line with significantly more tillers and heads at most sampling points (Tables 9a, 9b). GS16 has significantly more tillers toward maturity but does not differ for head number. 161-12, a positive *Sh2r6hs* line with the *Sh2* promoter, equals the control groups for both tillers and heads on all occasions. Several GS lines are sickly: GS9, GS14, and GS17 all produced fewer tillers and heads than their control groups. Three lines (GS 4, 5, 8) have the same number of heads as control lines. GS4, appearing to have tiller/head number reductions early in development, shows equal vigor at maturity (Table 9a, 9b). This may be indicative of GS4 *Sh2r6hs* activation after anthesis.

Table 9a. Bozeman pGS T2 Greenhouse Data, Spring 2002. Number of tillers counted for homozygous *Sh2r6hs*-positive and *Sh2r6hs*-negative plants on five dates. 15+/15-*Sh2r6hs* plants were grown for each pGS line (GS4, 5, 8, 9, 14, 16, 17, 28, 54), one pSh2r6hs line (161-12), and one untransformed control (HiLine). Pots were planted on 10/17/01 (one negative seed next to one positive seed). Average date of anthesis: 12/17/01.

Line[a]	Genotype[b]	12/10[c] Tillers[d]	12/17 Tillers	1/2 Tillers	1/9 Tillers	1/16 Tillers
GS4	+	3.93	3.93	8.93	8.87	9.00
	-	4.07	4.33	7.60	7.73	7.73
	+/-	**0.97**	**0.91**	**1.18**	**1.15**	**1.16**
GS5	+	4.13	4.13	10.93	10.40	10.40
	-	4.20	4.27	12.07	11.67	11.80
	+/-	**0.98**	**0.97**	**0.91**	**0.89**	**0.88**
GS8	+	4.07	4.07	9.27	8.73	8.47
	-	3.87	3.93	10.13	9.67	9.20
	+/-	**1.05**	**1.03**	**0.91**	**0.90**	**0.92**
GS9	+	4.07	4.47	8.60	8.80	8.67
	-	4.53	4.67	9.87	9.67	9.47
	+/-	**0.90**	**0.96**	**0.87**	**0.91**	**0.92**
GS14	+	3.07	3.27	6.13	5.73	5.67
	-	4.13	4.60	8.20	8.07	8.07
	+/-	**0.74****	**0.71****	**0.75****	**0.71****	**0.70****
GS16	+	4.87	4.87	11.80	11.60	11.87
	-	4.53	4.53	9.20	9.53	9.53
	+/-	**1.07**	**1.07**	**1.28****	**1.22****	**1.24****
GS17	+	4.27	4.27	8.93	9.13	9.00
	-	4.47	4.53	9.73	9.13	9.47
	+/-	**0.96**	**0.94**	**0.92**	**1.00**	**0.95**
GS28	+	5.07	5.00	12.40	12.00	12.07
	-	3.60	3.73	7.93	7.40	7.33
	+/-	**1.41****	**1.34***	**1.56****	**1.62****	**1.65****
GS54	+	3.33	3.67	7.20	7.20	7.20
	-	3.93	4.27	8.13	8.27	8.27
	+/-	**0.85**	**0.86**	**0.89**	**0.87**	**0.87**
161-12	+	3.67	3.78	7.89	7.44	7.22
	-	3.67	3.78	7.22	7.56	7.56
	+/-	**1.00**	**1.00**	**1.09**	**0.99**	**0.96**
HiLine		3.79	4.00	7.05	6.95	7.00

[a] pGS transformants are indicated by 'GS' (*Glutenin-Shrunken*), followed by the bombarded plant number.

Table 9a, cont.
[b] Genotypes are homozygous positive and negative lines. Each genotypic group consists of T2 seed derived from three homozygous positive and three homozygous negative T1 plants. +/- is the ratio of positive average:negative average.
[c] Tillers were counted one week before anthesis (12/10/01), at anthesis (12/17/01), and 14, 21, and 28 days post anthesis (1/2/02, 1/9/02, and 1/16/02, respectively)
[d] Tillers per plant were averaged over a homozygous group (n=15 per group, per line).
*, ** denote means significantly different at <0.05 and 0.01 level, respectively.

Table 9b. Bozeman pGS T2 Greenhouse Data, Spring 2002. Number of heads counted for homozygous *Sh2r6hs*-positive and *Sh2r6hs*-negative plants on five dates.

Line[a]	Genotype[b]	12/17[c] Heads[d]	1/2 Heads	1/9 Heads	1/16 Heads	1/23 Heads
GS4	+	2.23	3.93	3.87	4.80	6.40
	-	2.93	4.00	4.20	4.60	6.27
	+/-	**0.76**	**0.98**	**0.92**	**1.04**	**1.02**
GS5	+	2.33	3.67	3.93	6.40	7.60
	-	2.00	3.93	3.93	6.47	8.13
	+/-	**1.17**	**0.93**	**1.00**	**0.99**	**0.93**
GS8	+	2.93	3.87	4.00	5.73	6.80
	-	2.53	3.93	3.93	5.80	7.27
	+/-	**1.16**	**0.98**	**1.02**	**0.99**	**0.94**
GS9	+	2.07	4.20	4.53	5.33	6.47
	-	2.67	4.33	4.87	6.60	8.33
	+/-	**0.78**	**0.97**	**0.93**	**0.81***	**0.78****
GS14	+	1.87	3.20	3.27	4.20	4.93
	-	2.73	4.13	4.47	5.67	7.13
	+/-	**0.68***	**0.77***	**0.73****	**0.74***	**0.69****
GS16	+	2.47	4.40	4.73	6.20	8.33
	-	3.13	4.20	4.27	6.07	7.53
	+/-	**0.79**	**1.05**	**1.11**	**1.02**	**1.11**
GS17	+	0.75	3.73	3.93	4.40	4.87
	-	1.00	4.20	4.40	5.00	6.27
	+/-	**0.75**	**0.89***	**0.89***	**0.88***	**0.78***
GS28	+	0.96	4.67	4.73	6.00	8.40
	-	1.33	3.27	3.47	4.20	5.00
	+/-	**0.72**	**1.43***	**1.37****	**1.43****	**1.68****
GS54	+	1.60	3.47	3.53	4.73	6.13
	-	1.87	3.73	4.27	4.73	6.00
	+/-	**0.86**	**0.93**	**0.83**	**1.00**	**1.02**
161-12	+	1.56	3.78	3.89	4.89	6.56
	-	1.56	3.78	3.78	5.22	7.00
	+/-	**1.00**	**1.00**	**1.03**	**0.94**	**0.94**
HiLine		2.53	3.63	4.11	5.00	5.95

Table 9b, cont.
[a] pGS transformants are indicated by 'GS' (*Glutenin-Shrunken*), followed by the bombarded plant number.
[b] Genotypes are homozygous positive and negative lines. Each genotypic group consists of T2 seed derived from three homozygous positive and three homozygous negative T1 plants. +/- is the ratio of positive average:negative average.
[c] Heads were counted for each plant at anthesis,14 DPA, 21 DPA, 28 DPA, and 35 DPA (12/17/01, 1/2/02, 1/9/02, 1/16/02, and 1/23/02, respectively).
Table 9b, cont.
[d] Heads per plant were averaged over a homozygous group (n=15 per group, per line).

Bozeman pGS T3 Field Trial, 2002: Space-seeded, Irrigated Conditions

Eight *Glu*-promoter *Sh2r6hs*-positive (GS) lines were planted identically to the 10-foot rows described for the pSh2r6hs F4 trial (see Chapter 4). Some rows contained less than 20 plants. Therefore, plant and seed characteristics are presented per plant, and not per row in Table 10a. Positive and negative *Sh2r6hs* segregant plants were quantified for the number of plants per row, seed weight, seed number, biomass, shoot weight, harvest index (Table 10a), protein content, individual seed weight, and individual seed diameter (Table 10b).

Seven of nine transgenic lines produced higher seed yield than their controls (Table 10a). GS11 is the only line to have significantly higher seed weight (+67%), seed number (+61%), biomass (+43%), shoot weight (+28%), and harvest index (+16%) in positive versus negative plants. This is largely accredited to the sickly negative GS11 control group- these plants yielded 13.6 grams of seed, whereas positive GS11 plants and HiLine produced about 23 grams of seed. GS14 also yielded significantly more seeds (+21%) and total seed weight (+23%) than GS14 controls.

Two lines have decreased seed yields: GS28 (-9%) and the *Sh2*-promoter *Sh2r6hs* line, 161-12 (-3%). Transgenic lines as a whole were not as vigorous as HiLine, (Table 10a) indicating seed and shoot yield reductions arising from the transformation process.

Individual seed quality testing detected significant differences in some GS lines. GS4 produced smaller seeds with less protein (Table 10b). The 6% GS4 seed weight increase (Table 10a) coupled with a 4% seed size decrease contributed to 10% more seeds in GS4. GS11 produced larger seeds than controls, but this increase was probably due to more starch accumulation, and not protein accumulation. The 5% protein reduction in GS11 positives (Table 10b) supports this hypothesis.

Table 10a. Bozeman pGS T3 Trial, Summer 2002: Space-seeded, Irrigated Conditions. 3 *Sh2r6hs* homozygous negative and 3 *Sh2r6hs* homozygous positive T1-derived T3 segregant lines from eight pGS transformant lines (GS4, 5, 8, 11, 12, 14, 28, 54) were replicated twice along with several pSh2r6hs lines and HiLine. 10-ft rows consisted of 19 or 20 plants spaced six inches apart (see "#/Row" column for averages). Plant and seed weights include 12% moisture.

			Yield Data per Plant				
Line[a]	Geno[b]	#/Row[c]	Seed Weight (g)[d]	Seed Number[e]	Total Biomass (g)[f]	Shoot Weight (g)[g]	Harvest Index[h]
GS4	+	20.0	23.5	590	49.6	26.1	0.474
	-	19.3	22.2	537	49.3	27.1	0.448
	+/-	**1.03**	**1.06**	**1.10***	**1.01**	**0.96**	**1.06**
GS5	+	20.0	24.0	577	52.3	28.3	0.459
	-	19.8	23.8	567	50.9	27.0	0.470
	+/-	**1.01**	**1.01**	**1.02**	**1.03**	**1.05**	**0.98**
GS8	+	19.8	23.1	554	49.7	26.6	0.465
	-	19.7	22.4	521	46.8	24.4	0.480
	+/-	**1.01**	**1.03**	**1.06**	**1.06**	**1.09**	**0.97**
GS11	+	20.0	22.7	531	49.0	26.3	0.465
	-	20.0	13.6	330	34.3	20.6	0.400
	+/-	**1.00**	**1.67****	**1.61****	**1.43****	**1.28***	**1.16****

Table 10a, cont.

			Yield Data per Plant				
Line[a]	Geno[b]	#/Row[c]	Seed Weight (g)[d]	Seed Number[e]	Total Biomass (g)[f]	Shoot Weight (g)[g]	Harvest Index[h]
GS12	+	20.0	20.1	491	45.6	25.5	0.441
	-	20.0	16.4	415	40.5	24.2	0.402
	+/-	**1.00**	**1.23**	**1.18***	**1.13**	**1.05**	**1.10***
GS14	+	19.5	24.4	585	50.6	26.2	0.483
	-	19.5	19.9	481	45.8	26.0	0.435
	+/-	**1.00**	**1.23***	**1.21***	**1.10**	**1.01**	**1.11**
GS28	+	19.5	19.5	498	47.3	27.7	0.413
	-	20.0	21.4	516	46.3	24.8	0.467
	+/-	**0.98**	**0.91**	**0.97**	**1.02**	**1.12**	**0.88**
GS54	+	19.8	22.1	515	48.1	25.9	0.461
	-	19.0	21.6	524	47.7	26.1	0.453
	+/-	**1.04**	**1.03**	**0.98**	**1.01**	**0.99**	**1.02**
161-12	+	20.0	24.6	561	51.8	27.2	0.476
	-	20.0	25.3	559	51.4	26.1	0.492
	+/-	**1.00**	**0.97**	**1.00**	**1.01**	**1.04**	**0.97**
152-7		20.0	22.4	592	51.5	29.1	0.438
P-155-4		20.0	21.9	543	45.4	23.5	0.482
P-161-1		19.0	18.2	427	41.2	23.0	0.441
P-161-12		19.5	19.6	459	40.4	20.8	0.486
HiLine		20.0	24.1	557	50.5	26.5	0.476

[a] GS lines all contain *Sh2r6hs* under control of the *Glu* promoter. 161-12 carries the *Sh2* promoter and has been backcrossed to HiLine. 152-7, P-155-4, P-161-1, and P-161-12 also contain the *Sh2* promoter, but have not been backcrossed to HiLine.

[b] Genotypes are homozygous positive and negative lines. Each genotypic group consists of T3 seed derived from three homozygous positive and three homozygous negative T1 plants. +/- is the ratio of positive average:negative average.

[c] Average number of plants per row (n=3). Sixty seeds were originally space-planted (3 seeds every 6") in 10 ft rows. At the two-leaf stage, rows were thinned to 20 plants per row when applicable. Some rows did not germinate 20 plants per row, lowering the overall average.

[d] Seed weight was calculated for individual plants growing in a single row. Total yield harvested per row was divided by the number of plants counted for that row.

[e] Seed number was calculated as the plant seed weight divided by the average individual seed weight (shown in following table).

[f] Biomass consists of all aboveground plant weight per row. Rows were sickled, bundled, and dried for 1-2 days prior to weighing. Total biomass was divided by the number of plants per row to obtain biomass/plant.

Table 10a, cont.
[g] Shoot weight is equal to biomass per row minus seed weight per row. This figure was divided by the number of plants per row to obtain shoot weight/plant.
[h] Harvest index is a comparison of seed weight to vegetative plant weight. It is calculated as the ratio of seed weight to biomass.
*, ** denote means significantly different at <0.05 and 0.01 level, respectively.

Table 10b. Bozeman pGS T3 Trial, Summer 2002: Space-seeded, Irrigated Conditions. Seed protein content, individual seed weight, seed diameter, and seed weight/diameter standard deviations for eight GS lines, five *Sh2*-promoter lines, and HiLine.

Line	Genotype	Protein (% wt.)[a]	Individ. SW (mg)[b]	Individ. SW SD (mg)[c]	Diam. (mm)[d]	Diam. SD (mm)[e]
GS4	+	15.20	39.80	10.01	2.79	0.52
	-	16.62	41.31	9.61	2.84	0.49
	+/-	**0.91***	**0.96**	**1.04**	**0.98**	**1.04**
GS5	+	16.20	41.60	9.79	2.84	0.54
	-	16.32	41.97	9.40	2.89	0.48
	+/-	**0.99**	**0.99**	**1.04**	**0.98**	**1.14***
GS8	+	16.30	41.68	10.80	2.85	0.53
	-	16.52	42.96	9.79	2.91	0.50
	+/-	**0.99**	**0.97**	**1.10***	**0.98**	**1.06**
GS11	+	15.70	42.74	9.04	2.86	0.45
	-	16.50	41.19	9.35	2.82	0.46
	+/-	**0.95***	**1.04***	**0.97**	**1.02**	**0.97**
GS12	+	16.58	40.94	9.70	2.86	0.52
	-	17.10	39.56	8.71	2.83	0.48
	+/-	**0.97**	**1.03**	**1.11***	**1.01**	**1.08**
GS14	+	16.15	41.69	10.40	2.95	0.49
	-	16.75	41.40	10.99	2.85	0.59
	+/-	**0.96**	**1.01**	**0.95**	**1.03**	**0.83***
GS28	+	16.05	39.13	10.43	2.76	0.53
	-	15.95	41.48	10.12	2.86	0.58
	+/-	**1.01**	**0.94**	**1.03**	**0.96**	**0.91**
GS54	+	16.28	42.90	10.21	2.85	0.50
	-	16.45	41.26	9.75	2.84	0.52
	+/-	**0.99**	**1.04**	**1.05**	**1.00**	**0.97**
161-12	+	15.90	43.88	8.71	2.91	0.46
	-	15.95	45.23	9.22	3.00	0.48
	+/-	**1.00**	**0.97**	**0.94**	**0.97**	**0.95**
152-7		16.20	37.84	10.19	2.68	0.56

Table 10b, cont.

Line	Genotype	Protein (% wt.)[a]	Individ. SW (mg)[b]	Individ. SW SD (mg)[c]	Diam. (mm)[d]	Diam. SD (mm)[e]
P-155-4		15.40	40.32	8.96	2.80	0.46
P-161-1		16.70	42.60	10.94	2.96	0.59
P-161-12		15.80	42.72	11.73	2.97	0.63
HiLine		15.75	43.25	9.55	2.91	0.53

[a] Protein percentage obtained from an Infratec 1225 Whole Grain Analyzer (Tecator, Sweden). Kernel absorbance of infrared wavelengths was taken for each sample.
[b] Individual seed weight averages. 100 kernels per sample were tested with the Single Kernel Characterization System 4100 (Perten, Sweden).
[c] Individual seed weight standard deviation averages (n=100).
[d] Seed diameter averages. 100 kernels per sample were tested with the Single Kernel Characterization System 4100 (Perten, Sweden).
[e] Seed diameter standard deviation averages (n=100).
*, ** denote means significantly different at <0.05 and 0.01 level, respectively.

Bozeman pGS T3 Field Trial, 2002: Dense-seeded, Irrigated Conditions

The same space-seeded T3 GS lines (except for GS11 and GS12) were dense-seeded in five foot rows. A storm completely lodged the majority of rows 3-4 weeks after anthesis. The data was not skewed to either positive or negative *Sh2r6hs* genotypes, as both groups were equally lodged.

GS4 positives are slightly up for seed weight (+4%) and harvest index (+7%), and significantly up for seed number (+11%)(Table 11a). Compared with other GS lines, GS4 has much lower levels of plant and seed production. Most lines yielded seed weights in the upper 200-gram range, but GS4 positives and controls average approximately 230 grams. GS8 has decent vigor compared with HiLine controls, and positive groups are 17% greater for seed weight than GS8 negatives. GS8 also produced significantly more seeds, biomass and shoot weight.

Although some lines exhibit yield differences compared to controls (see GS28 and GS54, Table 11a), these differences are often not significant because of the lack of experimental replication. For example, GS28 and 161-12 are up for seed yield (22% and 11%, respectively) and GS54 is down for seed yield (-15%), but no significant difference is detected for any line. 161-12 has 17% more seeds than its negative counterpart. If all 161-12 positive seeds filled as much as 161-12 negative seeds, total positive seed yield might have been significantly higher.

GS4 has decreased protein content and decreased individual seed weight (Table 11b). GS5 positives are much more variable for individual seed weight and seed diameter, as indicated by significant standard deviation differences. GS8 is significantly lower for protein content, individual seed weight, and seed diameter (Table 11b).

Table 11a. Bozeman pGS T3 Trial, Summer 2002: Dense-seeded, Irrigated Conditions. 3 *Sh2r6hs* homozygous negative and 3 *Sh2r6hs* homozygous positive T1-derived T3 segregant lines from eight pGS transformant lines (GS4, 5, 8, 11, 12, 14, 28, 54) were replicated twice along with several pSh2r6hs lines and HiLine. 5-ft rows were planted with 5 grams of seed. Plots were irrigated on two occasions. Seed weight, biomass, and shoot weight are presented on a 12% moisture basis.

Line[a]	Geno[b]	Seed Weight (g)[c]	Seed Number[d]	Total Biomass (g)[e]	Shoot Weight (g)[f]	Harvest Index[g]
GS4	+	236.7	6,947	557.5	320.8	0.425
	-	227.3	6,231	570.0	342.7	0.395
	+/-	**1.04**	**1.11***	**0.98**	**0.94**	**1.07**
GS5	+	300.1	8,385	701.7	401.6	0.427
	-	315.4	8,725	755.8	440.4	0.417
	+/-	**0.95**	**0.96**	**0.93**	**0.91***	**1.02**
GS8	+	306.3	8,387	734.2	427.9	0.416
	-	261.7	6,910	639.6	377.8	0.409
	+/-	**1.17***	**1.21****	**1.15***	**1.13***	**1.02**
GS14	+	228.9	6,080	560.0	331.1	0.409
	-	240.6	6,228	610.0	369.4	0.394

Table 11a, cont.

Line[a]	Geno[b]	Seed Weight (g)[c]	Seed Number[d]	Total Biomass (g)[e]	Shoot Weight (g)[f]	Harvest Index[g]
	+/-	**0.95**	**0.98**	**0.92**	**0.90**	**1.04***
GS28	+	303.7	8,250	707.5	403.9	0.429
	-	248.2	7,049	607.5	359.4	0.408
	+/-	**1.22**	**1.17**	**1.16**	**1.12**	**1.05**
GS54	+	259.6	6,899	648.8	389.2	0.400
	-	305.0	8,060	695.0	390.0	0.439
	+/-	**0.85**	**0.85**	**0.93**	**1.00**	**0.91**
161-12	+	300.7	8,311	712.5	411.9	0.420
	-	270.6	7,121	652.5	382.0	0.410
	+/-	**1.11**	**1.17***	**1.09**	**1.08**	**1.02**
152-7		254.4	7,130	660.0	405.6	0.387
P-155-4		302.9	8,547	725.0	422.1	0.417
P-161-1		292.3	7,522	725.0	432.8	0.401
P-161-12		288.7	7,520	712.5	423.9	0.407
HiLine		279.0	7,327	667.5	388.6	0.416

[a] GS lines all contain *Sh2r6hs* with the *Glu* promoter. 161-12 carries the *Sh2* promoter and was previously backcrossed to HiLine. 152-7, P-155-4, P-161-1, and P-161-12 also contain the *Sh2* promoter but have not been backcrossed to HiLine.

[b] Genotypes are homozygous positive and negative lines. Each genotypic group consists of T3 seed derived from three homozygous positive and three homozygous negative T1 plants. +/- is the ratio of positive average:negative average.

[c] Seed weight was measured from all plants growing in a single row. Average seed weight of 3 positive or 3 negative *Sh2r6hs* rows is given per line.

[d] Seed number was calculated as total seed weight divided by the average individual seed weight (shown in following table).

[e] Biomass consists of all aboveground plant weight per row. Rows were sickled, bundled, and dried for 1-2 days prior to weighing.

[f] Shoot weight is equal to biomass per row minus seed weight per row.

[g] Harvest index is a comparison of seed weight to vegetative plant weight. It is calculated as the ratio of seed weight to biomass.

*, ** denote means significantly different at <0.05 and 0.01 level, respectively.

Table 11b. Bozeman pGS T3 Trial, Summer 2002: Dense-seeded, Irrigated Conditions. Seed protein content, individual seed weight, seed diameter, and seed weight/diameter standard deviations for six GS lines, five *Sh2*-promoter lines, and HiLine.

Line	Genotype	Protein (% wt.)[a]	Individ. SW (mg)[b]	Individ. SW SD (mg)[c]	Diam. (mm)[d]	Diam. SD (mm)[e]
GS4	+	14.20	34.07	8.25	2.51	0.49
	-	14.78	36.48	8.75	2.59	0.52
	+/-	**0.96****	**0.93***	**0.94**	**0.97**	**0.95**
GS5	+	14.57	35.79	9.12	2.63	0.54
	-	14.33	36.15	7.95	2.64	0.44
	+/-	**1.02**	**0.99**	**1.15****	**0.99**	**1.24***
GS8	+	14.62	36.52	8.18	2.63	0.48
	-	15.03	37.87	8.19	2.75	0.50
	+/-	**0.97***	**0.96***	**1.00**	**0.96****	**0.95**
GS14	+	14.70	37.65	8.95	2.77	0.59
	-	14.70	38.63	9.22	2.73	0.56
	+/-	**1.00**	**0.97**	**0.97**	**1.02**	**1.05**
GS28	+	14.05	36.81	8.43	2.69	0.48
	-	14.15	35.21	8.71	2.58	0.50
	+/-	**0.99**	**1.05**	**0.97**	**1.04**	**0.97**
GS54	+	14.85	37.63	8.38	2.64	0.45
	-	14.95	37.84	8.70	2.66	0.48
	+/-	**0.99**	**0.99**	**0.96**	**0.99**	**0.94**
161-12	+	14.25	36.18	8.81	2.69	0.49
	-	14.55	38.00	8.41	2.69	0.52
	+/-	**0.98**	**0.95**	**1.05**	**1.00**	**0.94**
152-7		14.80	35.68	7.81	2.58	0.48
P-155-4		14.15	35.44	8.33	2.59	0.52
P-161-1		14.95	38.86	9.57	2.71	0.54
P-161-12		14.10	38.39	8.53	2.76	0.50
HiLine		14.45	38.08	7.15	2.72	0.44

[a] Protein percentage obtained from an Infratec 1225 Whole Grain Analyzer (Tecator, Sweden). Kernel absorbance of infrared wavelengths was taken for each sample.
[b] Individual seed weight averages. 100 kernels per sample were tested with the Single Kernel Characterization System 4100 (Perten, Sweden).
[c] Individual seed weight standard deviation averages (n=100).
[d] Seed diameter averages. 100 kernels per sample were tested.
[e] Seed diameter standard deviation averages (n=100).
*, ** denote means significantly different at <0.05 and 0.01 level, respectively.

Bozeman pGS F3 Field Trial, 2002: Space-seeded, Irrigated Conditions

GS4, GS5, GS8, and GS16 were backcrossed to HiLine to eliminate some of the mutations induced from tissue culture. GS4 positives yielded poorly- seed weight, seed number, biomass, and shoot weight are all significantly lower than negatives (Table 12a). GS5 positives equal HiLine and GS5 controls, while GS8 produced 3-4% more seed weight and biomass than the GS8 control. GS16 positives channeled more resources into vegetative growth, resulting in decreased seed weight and increased shoot weight (approximately 8-9% each). The shift from endosperm starch synthesis to leaf/stem tissue synthesis also decreased the harvest index in GS16 positives.

GS5, GS8, and 161-12 *Sh2r6hs*-positive groups equal HiLine seed production. These results suggest that backcrossing can improve growth traits to some extent. GS4 and GS16-positive seed yield is much lower than HiLine and GS4/GS16 negatives. According to the F3 trial data, the pGS insert affects seed initiation and development in GS4 and GS16 differently. GS4 yielded less because fewer seeds were established (Table 12a), but GS16 has the same number of seeds as its control. GS16 seeds are much smaller (smaller for individual seed weight and diameter) than GS16 controls (Table 12b), lending to an overall reduction in yield.

Table 12a. Bozeman pGS F3 Trial, Summer 2002: Space-seeded, Irrigated Conditions. Four *Glu* promoter *Sh2r6hs* lines (GS 4, 5, 8, 16) were backcrossed to HiLine. Approximately 15 homozygous *Sh2r6hs*-positive and 15 *Sh2r6hs*-negative segregants per line (see chapter 2, 'pGS F3 Trial' for exact number of segregants used per line) were grown in 5 ft rows (10 plants per row). Plots were irrigated on two occasions.

Line[a]	Genotype[b]	Seed Weight (g)[c]	Seed Number[d]	Total Biomass (g)[e]	Shoot Weight (g)[f]	Harvest Index[g]
GS 4	+	168.8	4,238	375.8	201.3	0.457
	-	192.0	4,684	417.3	225.3	0.460
	+/-	**0.88****	**0.90***	**0.90***	**0.89***	**0.99**
GS 5	+	192.3	4,520	425.6	233.2	0.453
	-	191.7	4,577	430.5	238.8	0.445
	+/-	**1.00**	**0.99**	**0.99**	**0.98**	**1.02**
GS 8	+	193.5	4,484	424.6	231.1	0.457
	-	185.5	4,337	411.8	226.3	0.450
	+/-	**1.04**	**1.03**	**1.03**	**1.02**	**1.01**
GS 16	+	176.5	4,564	420.9	244.4	0.419
	-	193.8	4,557	419.0	225.3	0.464
	+/-	**0.91***	**1.00**	**1.00**	**1.08***	**0.90****
161-12	+	203.6	4,723	447.9	244.3	0.456
	-	212.6	4,972	455.8	243.2	0.467
	+/-	**0.96**	**0.95**	**0.98**	**1.00**	**0.98**
HiLine		193.8	4,471	427.9	234.5	0.452

[a] GS lines all contain *Sh2r6hs* with the *Glu* promoter. 161-12 carries the *Sh2* promoter and was previously backcrossed with HiLine.
[b] Genotypes are homozygous positive and negative *Sh2r6hs* lines.
[c] Seed number was calculated as row seed weight divided by the average individual seed weight (shown in following table).
[d] Seed weight was measured from all 10 plants growing in a single row.
[e] Biomass consists of all aboveground plant weight per row. Rows were sickled, bundled, and dried for 1-2 days prior to weighing.
[f] Shoot weight is equal to biomass per row minus seed weight per row.
[g] Harvest index is a comparison of seed weight to vegetative plant weight. It is calculated as the ratio of seed weight to biomass.
*, ** denote means significantly different at <0.05 and 0.01 level, respectively.

Table 12b. Bozeman pGS F3 Trial, Summer 2002: Space-seeded, Irrigated Conditions. Protein content, individual seed weight, individual seed diameter, and seed weight/diameter standard deviations for four backcrossed *Glutenin* lines, 161-12, and HiLine.

Line	Genotype	Protein (% wt.)[a]	Individ. SW (mg)[b]	Individ. SW SD (mg)[c]	Diam. (mm)[d]	Diam. SD (mm)[e]
GS 4	+	15.75	39.83	9.43	2.78	0.50
	-	16.20	40.99	9.94	2.83	0.52
	+/-	**0.97***	**0.97**	**0.95**	**0.98**	**0.96**
GS 5	+	16.33	42.54	9.74	2.87	0.51
	-	16.58	41.88	9.62	2.86	0.50
	+/-	**0.98**	**1.02**	**1.01**	**1.00**	**1.01**
GS 8	+	16.13	43.15	9.94	2.90	0.51
	-	16.33	42.77	10.03	2.91	0.49
	+/-	**0.99**	**1.01**	**0.99**	**1.00**	**1.04**
GS 16	+	16.61	38.67	9.07	2.80	0.50
	-	16.00	42.53	9.97	2.91	0.52
	+/-	**1.04***	**0.91****	**0.91****	**0.96****	**0.96**
161-12	+	16.02	43.11	9.37	2.96	0.51
	-	16.22	42.76	10.20	2.92	0.55
	+/-	**0.99**	**1.01**	**0.92***	**1.01**	**0.93**
HiLine		15.96	43.35	9.67	2.95	0.52

[a] Protein percentage obtained from an Infratec 1225 Whole Grain Analyzer (Tecator, Sweden). Kernel absorbance of infrared wavelengths was taken for each sample.
[b] Individual seed weight averages. 100 kernels per sample were tested with the Single Kernel Characterization System 4100 (Perten, Sweden).
[c] Individual seed weight standard deviation averages (n=100).
[d] Seed diameter averages. 100 kernels per sample were tested with the Single Kernel Characterization System 4100 (Perten, Sweden).
[e] Seed diameter standard deviation averages (n=100).
*, ** denote means significantly different at <0.05 and 0.01 level, respectively.

Minto, Canada pGS T3 Field Trial, 2002: Dense-seeded, Irrigated Conditions

All previous field trials relied on individual rows for data analysis. We speculated that expanding each genotype into 9-row plots would diminish some of the background variation associated with single row trials. This reduction in variation would make it much easier to detect yield differences, if any, between *Sh2r6hs* positives and controls. Both *Sh2r6hs*-positive and control (*Sh2r6hs*-negative) *Glu*-promoter groups consist of homozygous seed derived from three homozygous T2 segregants, respectively.

Two of seven *Glu*-promoter *Sh2r6hs* lines produced lower yields (GS6 and GS12, Table 13a), but neither is significantly lower at the 0.05 level. GS4 and GS11 have significantly higher yields than controls (36% and 17%, respectively). The yield increase in GS4 is mainly due to increased starch accumulation, as total seed protein is 5% less than control plots (Table 13b). GS5 and GS8 have approximately 13% more seed weight than controls, but neither is significantly different. An interesting statistic accompanying each GS line is the degree of lodging observed before harvest. Positive groups lodged much more than negative groups (Table 13a). Heavier seed masses may have made positive plots more susceptible to lodging.

Table 13c compares groups of either high-expressing *Sh2r6hs* lines (GS4, 5, 8, 54) or groups of low-expressing *Sh2r6hs* lines (GS 6, 11, 12) to the appropriate controls. High-expressing lines, on average, generated 19% more seed mass than controls, but also lodged significantly more. Low-expressing lines do not show the same trends as high-expressing lines. Yield is only 2% greater in low *Sh2r6hs* expressers, and lodging did not differ.

Table 13a. Minto, Canada pGS T3 Trial, Summer 2002: Dense-seeded 9-row plots, Irrigated Conditions. Lodging, seed yield, and protein content is presented for the following lines: Seven *Glu*-promoter *Sh2r6hs* lines (GS4, GS5, GS6, GS8, GS11, GS12, GS54), two *Sh2*-promoter *Sh2r6hs* lines (152-7, 161-12), one line expressing *bar* only (P-161-1), and one untransformed control (HiLine). Each plot was dense-seeded with 90 grams of seed. Plot Dimensions: 5.5 m x 1.5 m

Line[a]	Genotype[b]	Lodging (0-9)[c]	Yield (kg/ha)	Protein (% wt.)[d]
High Expressing GS Lines:				
GS4	+	4.00	3441.7	15.68
	-	1.60	2535.6	16.44
	+/-	**2.50***	**1.36***	**0.95***
GS5	+	3.89	3268.0	16.19
	-	1.67	2904.7	16.17
	+/-	**2.33****	**1.13**	**1.00**
GS8	+	3.11	2988.9	16.52
	-	2.63	2661.1	16.61
	+/-	**1.18**	**1.12**	**0.99**
GS54	+	3.00	2158.3	16.70
	-	2.50	2131.2	16.82
	+/-	**1.20**	**1.01**	**0.99**
Low Expressing GS Lines:				
GS6	+	2.75	2754.5	16.19
	-	1.67	3051.7	16.35
	+/-	**1.65**	**0.90**	**0.99**
GS11	+	4.13	3486.8	15.86
	-	2.20	2990.4	16.19
	+/-	**1.88***	**1.17***	**0.98**
GS12	+	3.16	3124.5	16.35
	-	4.00	3141.3	16.66
	+/-	**0.79**	**0.99**	**0.98**

Table 13a, cont.

Line[a]	Genotype[b]	Lodging (0-9)[c]	Yield (kg/ha)	Protein (% wt.)[d]
161-12	+	4.90	3284.4	16.1
	-	4.60	3393.9	16.05
	+/-	**1.07**	**0.97**	**1.00**
152-7		5.17	3077.8	16.35
P-161-1		4.33	3063.5	16.4
HiLine		3.50	3287.7	16.1

[a] GS lines all contain *Sh2r6hs* under control of the *Glu* promoter. 161-12 carries the *Sh2* promoter and has been backcrossed to HiLine. 152-7 and P-161-1 also contain the *Sh2* promoter but have not been backcrossed to HiLine.
[b] Genotypes are *Sh2r6hs* positive or negative homozygotes.
[c] Lodging scores at maturity, based on a 0-9 scale, where 0 indicates no lodging and 9 is completely lodged.
[d] Protein percentage obtained from an Infratec 1225 Whole Grain Analyzer (Tecator, Sweden). Kernel absorbance of infrared wavelengths was taken for each sample.
*, ** denote means significantly different at <0.05 and 0.01 level, respectively.

Table 13b. Minto, Canada pGS T3 Trial, Summer 2002: Dense-seeded 9-row plots, Irrigated Conditions. Individual seed weight, seed diameter, and seed weight/diameter standard deviations for seven GS lines, three *Sh2*-promoter lines, and HiLine.

Line	Genotype	Individual SW (mg)[a]	Individual SW SD (mg)[b]	Diameter (mm)[c]	Diameter SD (mm)[d]
High Expressing GS Lines					
GS4	+	36.23	9.51	2.66	0.48
	-	36.74	10.42	2.70	0.58
	+/-	**0.99**	**0.91***	**0.99**	**0.83****
GS5	+	37.29	9.73	2.78	0.55
	-	37.34	9.55	2.76	0.52
	+/-	**1.00**	**1.02**	**1.01**	**1.07***
GS8	+	36.96	9.97	2.76	0.55
	-	38.13	9.08	2.80	0.53
	+/-	**0.97**	**1.10***	**0.99**	**1.04**
GS54	+	37.19	10.59	2.75	0.56
	-	37.36	9.70	2.80	0.55
	+/-	**1.00**	**1.09***	**0.98**	**1.03**
Low Expressing GS Lines					
GS6	+	37.54	9.18	2.77	0.56
	-	37.83	9.53	2.71	0.51
	+/-	**0.99**	**0.96**	**1.02**	**1.10***
GS11	+	36.98	9.90	2.73	0.52
	-	39.16	10.08	2.88	0.58
	+/-	**0.94**	**0.98**	**0.95**	**0.91****
GS12	+	36.75	10.04	2.75	0.52
	-	36.57	9.58	2.78	0.53
	+/-	**1.00**	**1.05***	**0.99**	**0.98**
161-12	+	37.55	10.32	2.66	0.55
	-	37.25	10.45	2.48	0.54
	+/-	**1.01**	**0.99**	**1.07***	**1.02**
152-7		36.1	9.95	2.7	0.52
P-161-1		37.9	10.21	2.81	0.54
HiLine		38.28	9.93	2.82	0.56

Table 13b, cont.
[a] Individual seed weight averages. 100 kernels per sample were tested with the Single Kernel Characterization System 4100 (Perten, Sweden).
[b] Individual seed weight standard deviation averages (n=100).
[c] Seed diameter averages. 100 kernels per sample were tested with the Single Kernel Characterization System 4100 (Perten, Sweden).
[d] Seed diameter standard deviation averages (n=100).
*, ** denote means significantly different at <0.05 and 0.01 level, respectively.

Table 13c. Minto, Canada pGS T3 Trial: Means for lodging, yield, protein, and individual seed characteristics in three individually-pooled groups. Four high-expressing GS lines (GS4, 5, 8, 54) were pooled to compare positive and negative homozygous *Sh2r6hs* means. Low-expressing lines (GS6, 11, 12) were also pooled to compare homozygous means. The third group consists of positive/negative *Sh2r6hs* homozygotes from all GS lines.

	Genotype	Lodging (0-9)	Yield (kg/ha)	Protein (% wt.)
High-Expressing	+	3.54	3057.0	16.27
Lines Only	-	2.16	2567.3	16.54
(GS4, 5, 8, 54)	+/-	**1.64****	**1.19****	**0.98***
Low-Expressing	+	3.36	3121.7	16.11
Lines Only	-	2.93	3073.1	16.47
(GS6, 11, 12)	+/-	**1.15**	**1.02**	**0.98****
All Positive	+	3.46	3085.4	16.2
vs. All	-	2.45	2757.0	16.52
Negative Lines	+/-	**1.41****	**1.12****	**0.98****

Table 13c, cont.

	Genotype	Indiv. SW (mg)	Indiv. SW SD (mg)	Diam. (mm)	Diam. SD (mm)
High-Expressing	+	36.91	9.95	2.74	0.54
Lines Only	-	37.39	9.69	2.76	0.54
(GS4, 5, 8, 54)	+/-	**0.99**	**1.03**	**0.99**	**0.99**
Low-Expressing	+	37.09	9.71	2.75	0.53
Lines Only	-	37.86	9.73	2.79	0.54
(GS6, 11, 12)	+/-	**0.98**	**1.00**	**0.98**	**0.99**
All Positive	+	36.99	9.84	2.74	0.53
vs. All	-	37.59	9.71	2.77	0.54
Negative Lines	+/-	**0.98**	**1.01**	**0.99**	**0.99**

Bozeman pGS Biomass Study, 2002: Dense-seeded, Irrigated Conditions

Positive and negative *Sh2r6hs* GS8 rows were grown alongside one another and 1-foot sections were harvested on five occasions. Positive sections have fewer heads and slightly less biomass than negatives at anthesis (Table 14a). At 14 dpa, positives are nearly the same as negatives for biomass, head number, seed weight, shoot weight, and harvest index. Two weeks later (28 dpa), biomass (+13%) and seed weight (+14%) are both significantly increased in positive plants. Mature positive plants maintain higher biomass, head number, and seed weight, although not at the 0.05 level of significance. No significant individual seed quality differences were detected on any sampling date (Table 14b).

Table 14a. Bozeman pGS Biomass Study, Summer 2002. Ten rows (10 grams densely-planted seed, 10-ft long) containing GS8 *Sh2r6hs*-positive homozygotes were grown. At the two-leaf stage, five 1-ft sections (consisting of 20 plants) were marked within each row. These sections were harvested independently on five occasions. The same procedure was followed for 10 rows containing GS8 *Sh2r6hs*-negative homozygotes. All samples were placed in a 40° C drying chamber for 1 week before weighing to remove excess moisture (<6% water by weight). Averages derived from the 1-ft sections are presented for several plant and seed characteristics.

Sampling Date[a]	Geno[b]	Total Biomass (g)[c]	Total Heads[d]	Seed Weight (g)	Seed Number	Shoot Weight (g)[e]	Harvest Index[f]
(-) 14 DPA	+	27.4					
	-	25.9					
	+/-	**1.06**					
Anthesis	+	54.4	51				
	-	56.2	54				
	+/-	**0.97**	**0.94**				
14 DPA	+	98.9	58	19.5	1,133	79.5	0.20
	-	100.7	61	19.8	1,131	80.9	0.20
	+/-	**0.98**	**0.96**	**0.98**	**1.00**	**0.98**	**1.00**
28 DPA	+	142.5	62	61.1	1,946	81.4	0.43
	-	126.5	57	53.6	1,761	72.9	0.42
	+/-	**1.13***	**1.08**	**1.14***	**1.11***	**1.12**	**1.02**
Maturity	+	164.8	62	70.8	1,831	94.0	0.43
	-	153.1	57	66.8	1,689	87.6	0.44
	+/-	**1.08**	**1.09**	**1.06**	**1.08**	**1.07**	**0.98**

[a] Sections were harvested on four dates before maturity and at maturity. (-) 14 DPA refers to 14 days before anthesis; 14 DPA and 28 DPA refer to 14 and 28 days post anthesis, respectively.

[b] 'Geno' indicates genotype; + and – indicate homozygous positive and negative *Sh2r6hs* lines, respectively. +/- is the ratio of the positive average to the negative average.

[c] Biomass consists of all aboveground plant weight. Sections were sickled, bagged, placed in a drying chamber for 1 week to remove excess moisture, and weighed.

[d] Seed number was calculated as the total seed weight divided by the average individual seed weight (shown in following table).

[e] Shoot weight is equal to biomass minus seed weight.

[f] Harvest index is a comparison of seed weight to vegetative plant weight. It is calculated as the ratio of seed weight to biomass.

*, ** denote means significantly different at <0.05 and 0.01 level, respectively.

Table 14b. Bozeman pGS Biomass Study, Summer 2002. Seed protein content, individual seed weight, seed diameter, and seed weight/diameter standard deviations for +/- *Sh2r6hs* homozygotes on three sampling dates (14 DPA, 28 DPA, maturity).

Sampling Date[a]	Genotype[b]	Protein (% wt.)[c]	Individ. SW (mg)[d]	Individ. SW SD (mg)[e]	Diam. (mm)[f]	Diam. SD (mm)[g]
14 DPA	+		17.21	4.75	1.60	0.30
	-		17.50	5.00	1.59	0.31
	+/-		**0.98**	**0.95**	**1.01**	**0.95**
28 DPA	+	13.92	31.39	7.92	2.59	0.53
	-	13.68	30.43	7.74	2.53	0.53
	+/-	**1.02**	**1.03**	**1.02**	**1.02**	**1.00**
Maturity	+	14.83	38.67	8.72	2.88	0.55
	-	14.75	39.55	8.70	2.82	0.52
	+/-	**1.01**	**0.98**	**1.00**	**1.02**	**1.06**

[a] Seeds were large enough for single kernel analysis 14 days post anthesis (14 DPA). Protein analysis could only be performed on 28 DPA and fully-mature seeds.
[b] Genotypes are homozygous positive and negative *Sh2r6hs* lines.
[c] Protein percentage obtained from an Infratec 1225 Whole Grain Analyzer (Tecator, Sweden). There was not enough seed produced at 14 DPA to obtain protein composition.
[d] Individual seed weight averages. 100 kernels per sample were tested with the Single Kernel Characterization System 4100 (Perten, Sweden).
[e] Individual seed weight standard deviation averages (n=100).
[f] Seed diameter averages. 100 kernels per sample were tested with the Single Kernel Characterization System 4100 (Perten, Sweden).
[g] Seed diameter standard deviation averages (n=100).
*, ** denote means significantly different at <0.05 and 0.01 level, respectively.

Discussion

Nine homozygous pGS *Sh2r6hs* T2 segregants were planted in the greenhouse to determine: (1) How homozygous positive and negative *Sh2r6hs* plants compare to untransformed HiLine in terms of overall vigor, and (2) How *Sh2r6hs*-positive plants compare to *Sh2r6hs*-negative plants in each pGS line. Tiller and head number was recorded on five dates before maturity (Tables 9a, 9b). Data collection over several

weeks allowed us to analyze the impact of *Sh2r6hs* expression at different developmental stages. Three lines (GS9, GS14, GS17) all generated significantly less heads than HiLine and respective control groups. Only one line (GS28) has a significantly higher head number and tiller number, mainly because GS28 negative plants were much more sickly than HiLine. GS16 is exceptional because tiller number, but not head number, is significantly increased among positive plants. This growth pattern contradicts the effect we expected from *Sh2r6hs* expression. If *Sh2r6hs* does not interfere with native genes, sink-tissue enhancement should result in higher seed yield and unaltered biomass. However, GS16 contains a *Sh2r6hs* insertion that interferes with genes involved in head development. The excess resources not invested in head development are alternatively put towards GS16 biomass, resulting in increased shoot and leaf weight.

Seven of nine space-seeded, T3 GS lines produced higher seed yield than their controls (Table 10a). GS11 positive plants have significantly higher seed yield and biomass than negative plants. GS11 positives produce yields comparable to untransformed HiLine, but negative plants suffer a 60% yield reduction. *Sh2r6hs*-negative GS11 yield reduction is due to several tissue culture mutations acquired during segregation. All growth characteristics (including individual seed weight and seed number) except for protein percentage are lowered in GS11 negatives (Table 10b).

GS4 positives have slightly greater yields than negatives (+6%) and significantly more seeds than negatives (+10%). Again, yield increase is the result of greater seed initiation after anthesis. Almost all yield increases noted in trials thus far have been accompanied by seed number increases as well. Similar to GS11 positives, GS4 positives

have 9% less protein. Protein content is not universally reduced in lines exhibiting increased yield. For example, the 161-12 space-seeded trial revealed significant yield increase without a decrease in protein content (Table 4b). It is uncertain why this phenomenon occurs in some trials and not in others.

Inconsistencies exist between many of the yield trials. As seen in greenhouse studies (Table 9a), GS28 outperformed controls for many yield characteristics. This is not the case for T3-GS28 positives grown in space-seeded, irrigated rows (Table 10a). GS28 seed weight is down 9% in the T3 field trial. This drop in seed weight is attributable to either a lack of replication or failure of *Sh2r6hs*-induced sink enhancement. None of the pGS field trials were replicated as highly as pSh2r6hs trials, since seed quantities and field space were limited. Lack of replication results in much more positive and negative yield variability. In addition, *Sh2r6hs* may only increase yield when specific environmental conditions are met. These conditions, as discussed in Chapter 4, include (but are not limited to) solar radiation, mean temperature, natural precipitation quantities, and germination efficiency. GS28 greenhouse and field trial yields are inconsistent because environmental conditions vary from trial to trial, and because the number of rows per positive and negative group (n=6) is not high enough to detect yield increase conferred by *Sh2r6hs*.

Despite small positive and negative GS sample sizes, GS4 yields were consistently up in both space-seeded and dense-seeded trials (Table 11a). GS4 seed weight is up 4% and seed number is up 11%, contributing to a 7% boost in harvest index. GS4, although not significantly up for seed yield, is a model *Sh2r6hs* transformant line.

Sh2r6hs does not affect vegetative growth in GS4, but endosperm sink enhancement raises harvest index. GS8 experienced a 17% seed weight increase with 21% more seeds, contrasted with a 3% seed weight increase among space-planted positive plants. The significant seed weight increase in dense-seeded GS8 plants is in unison with a 15% increase in biomass and no harvest index increase. This yield increase could be disregarded as a chance event if a significant GS8 yield *decrease* was recorded. However, GS8 was not significantly yield depressed in the space-seeded trial. It seems more likely that GS8 space-seeded positive plants were not exposed to environmental conditions most favorable to *Sh2r6hs*-induced yield enhancement.

Dense-seeded GS4 and GS8 plants show conflicting growth patterns. *Sh2r6hs* transformation affects different aspects of plant growth and development. GS4 exclusively channels surplus photoassimilates into seed development, but not into leaf or shoot development. GS8 channels photoassimilates into seeds, leaves, and stems, resulting in increased biomass. Even though GS4 and GS8 differ in this regard, both produce seeds with lower protein content (Table 11b). In the past, conventional breeders have intensively selected wheat varieties with high levels of seed protein. Protein reduction must be taken into account when transforming current varieties to enhance yield. Transformant lines such as GS4 and GS8 are less than ideal for wheat producers, as lowered grain protein content also lowers market value.

Many T3-GS lines (GS14, GS28, GS54) performed poorly compared to HiLine in space-seeded and dense-seeded environments. Backcrossed 161-12, on the other hand, displays higher seed yield than HiLine under both space-seeded (Table 10a) and dense-

seeded conditions (Table 11a). GS4, GS5, GS8, and GS16 were backcrossed to HiLine to reduce tissue-culture mutations. A single backcross did not sufficiently raise yield, based on F3 field trial data (Table 12a). GS4 and GS16 positive plant yields are significantly lower than HiLine. GS5 and GS8 positive and negative yields are equal to HiLine, but neither line is yield-depressed in either T3 trial, making it hard to determine if backcrossing truly does improve transgenic plant vigor. Two additional backcrosses would reduce tranformation-induced mutations even more, supplementing nearly 88% of the transformant genetic makeup with that of HiLine. Backcrossing sickly GS lines such as GS14, GS28, or GS54 might result in more pronounced vigor improvements, given the large number of mutations residing within their genomes. Regardless of backcrossing, GS16 continues to invest more resources into vegetative growth than into seed growth. The 9% decrease in seed weight and 8% increase in shoot weight resemble the 24% tiller increase from the T2 greenhouse experiment (Table 9a).

The Minto T3 Trial is unique, in that it is the first experiment deriving yield data from 9-row pGS and 161-12 plots (Tables 13a, b, c). Five of eight transgenic lines yielded higher than controls; two of these are significant at the 0.05 level (Table 13a). Grouping high *Sh2r6hs*-expressing lines together reveals that yield remains significantly higher, whereas the group of low-expressing lines is not yield-enhanced (Table 13c). High *Sh2r6hs*-expressing GS lines lodged more extensively (approximately 30 degrees) than low-expressing lines. Yield increase in high-expressing lines shifted weight ratios to the upper portion of plants, making plots more susceptible to lodging. Low-expressing

lines did not significantly lodge, probably because yields were not significantly increased.

GS8 seed and biomass data, measured over several weeks, closely resembles the post-anthesis yield increase observed in 161-12 positive plants (Tables 8a, 14a). Positive and negative groups have equal biomass and head number at anthesis and 14 days post anthesis. 28 days after anthesis, once *Sh2r6hs* expression confers sink strength enhancement and grain-filling has occurred, positive plants accumulate greater seed weight. Once more, positive yield increase is associated with increased levels of seed initiation. Individual *Sh2r6hs*-positive seeds do not contain greater quantities of starch, indicated by individual seed size testing (Table 14b). *Sh2r6hs* increases seed set at flowering, but overall yield increase is not detected until seeds undergo a substantial amount of grain-filling after 14-dpa.

Glu-promoter *Sh2r6hs* lines inconsistently produce higher seed yields. Although yield increase is observed in various trials, yield inconsistencies are most likely the result of variable field conditions and inadequate replication. The Minto Trial suggests that plot-sized replications, rather than individual row replications, make *Sh2r6hs* yield-enhancement much easier to detect. Future efforts will focus on planting large plots of each genotype. Higher *Sh2r6hs* expression among GS lines raises the potential for yield enhancement, compared to *Sh2*-promoter lines. This yield potential will be fulfilled only if F3 backcrossed lines are propagated to allow multiple dense-seeded replications. Higher-yielding genotypes can be generated through further backcrossing of GS5 and GS8, as well as other non-backcrossed lines (such as GS11).

LITERATURE CITED

Abbate PE, Andrade FH, Lazaro L, Bariffi JH, Berardocco HG, Inza VH, Marturano F. 1998. Grain yield increase in recent Argentine wheat cultivars. Crop Science. Vol. 38, P. 1203-1209.

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. Vol. 197, P. 1-10.

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

Armstrong CL, Parker GB, Pershing JC, Brown SM, Sander PR, Duncan DR, Stone DA, DeBoer DL, hart AR, Morrish FM, Pajeau ME, Peersen WL, Reich BJ, Rodriquez R, Santino CG, Sato SJ, Schuler W, Sims SR, Stehling S, Tarochione LJ, Fromm ME. 1995. Field evaluation of European corn borer control in progeny of 173 trangenic corn events expressing an insecticidal protein from Bacillus thuringiensis. Crop Science. Vol. 35, P. 550-557.

Austin RB. 1980. Physiological limitations to cereal yields and ways of reducing them by breeding. In: Hud RG, Biscoe PV, Dennis C, eds. Opportunities for increasing crop yields. London: Pitman, 3-19.

Bae JM, Giroux M, Hannah LC. 1990. Cloning and characterization of the Bt2 gene in maize. Maydica. Vol. 35, P. 317-322.

Baga M, Repellin A, Demeke T, Caswell K, Leung N, Abdel-Aal ES, Huel P, Chibbar RN. 1999. Wheat starch modification through biotechnology. Starch. Vol. 51, 111-116.

Barro F, Barcelo P, Lazzeri PA, Shewry PR, Martin A, Ballesteros J. 2002. Field evaluation and agronomic performance of transgenic wheat. Theor. Appl. Genet. Vol. 105, P. 980-984.

Barsby TL, Bervas E, Coates S, Freeman JP, Weir AF. 1999. Significant alteration of wheat starch by transgenics. In: Abstracts Book, Genetic Tailoring of Novel Starch Polymers. Carry-le-Rouet, France.

Basu PS, Sharma A, Garg ID, Sukumara NP. 1999. Tuber sink modifies photosynthetic response in potato under water stress. Environmental and Experimental Botany. Vol. 42, P. 25-39.

Becker D, Brettschneider R, Lorz H. 1994. Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. The Plant Journal. Vol. 5 (2), P. 299-307.

Beckles DM, Smith AM, Rees T. 2001. A cytosolic ADP-glucose pryrophosphorylase is a feature of graminaceous endosperms, but not of other starch-storing organs. Vol. 25, P. 818-827.

Beecher B, Bettge A, Smidansky E, Giroux MJ. 2002. Expression of wild-type pinB sequence in transgenic wheat complements a hard phenotype. Theor. Appl. Genet. Vol. 105, P. 870-877.

Bhave MB, Lawrence S, Barton C, Hannah CL. 1990. Identification and molecular characterization of Shrunken-2 cDNA clones of maize. The Plant Cell. Vol. 2, P. 581-588.

Bindraban PS, Sayre KD, Soli-Moya E. 1998. Identifying factors that determine kernel number in wheat. Field Crops Research. Vol. 58, P. 223-234.

Blechl AE, Anderson OD. 1996. Expression of a novel high-molecular-weight glutenin subunit gene in transgenic wheat. Nature Biotechnology. Vol. 14, P. 875-879.

Bradford, M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Reproduction Research Laboratories, Dept. of Biochemistry, University of Georgia, Athens, Ga., Jan. 29, 1976.

Buleon A, Colonna P, Planchot V, Ball S. 1998. Starch granules: structure and biosynthesis. International Journal of Biological Macromolecules. Vol. 23, P. 85-112.

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 Physiology. Vol. 115, P. 971-980.

Choi SB, Zhang Y, Ito H, Stephens K, Winder T, Edwards GE, Okita TW. 1998. Applications of Science to Increase Yield. Oxford University Press.

Chourey PS, Nelson OE. 1976. The enzymatic deficiency conditioned by the Shrunken-1 mutations in maize. Biochemical Genetics. Vol. 14, P. 1041-1055.

Chourey PS. 1981. Genetic control of sucrose synthase in maize endosperm. Mol. Gen. Genet. Vol. 184, P. 372-376

Clancy M, Vasil V, Hannah LC, Vasil IK. 1994. Maize Shrunken-1 intron and exon regions increase gene expression in maize protoplasts. Plant Science. Vol. 98, P. 151-161.

Demeke T, Hucl P, Baga M, Caswell K, Leung N, Chibbar RN. 1999. Transgene inheritance and silencing in hexaploid spring wheat. Theoretical and Applied Genetics. Vol. 99, P. 947-953.

Denyer K, Smith AM. 1988. The capacity of plastids from developing pea cotyledons to synthesize acetyl CoA. Planta. Vol. 173, P. 172-182.

Duvick DN. 1992. Genetic contributions to advances in yield of U.S. maize. Maydica. Vol. 37, P. 69-79.

Edwards A, Fulton DC, Hylton CM, Jobling SA, Gidley M, Rossner U, Martin C, Smith A. 1999. A combined reduction in activity of starch synthases II and III of potato has novel effects on the starch of tubers. The Plant Journal. Vol. 17, P. 251-261.

Elmayan T, Vaucheret H. 1996. Expression of single copies of a strongly expressed 35S transgene can be silenced post-transcriptionally. Plant Journal. Vol. 9, P. 787-797.

Espada J. 1962. Enzymic synthesis of adenosine diphosphate glucose from glucose 1-phosphate and adenosine triphosphate. Journal of Biological Chemistry. Vol. 237, P. 3577-3581.

Fernie AR, Willmitzer L, Trethewey RN. 2002. Sucrose to starch: a transition in molecular plant physiology. Trends in Plant Science. Vol. 7, P. 35-41.

Fischer RA, Laing DR. 1976. Yield potential in a dwarf spring wheat and response to crop thinning. Journal of Agricultural Science. Vol. 87, P. 113-122.

Fischer RA. 1985. Number of kernels in wheat crops and the influence of solar radiation and temperature. J. agric. Sci., Cambridge. Vol 105, P. 447-461.

Gao M, Wanat J, Stinard PS, James MG and Myers AM. 1998. Characterization of *dull1*, a maize gene coding for a novel starch synthase. The Plant Cell. Vol. 10, P. 399-412.

Geiger DR. 1979. Control of partitioning and export of carbon in leaves of higher plants. Botanical Gazette. Vol. 140, P. 241-248.

Giroux MJ, Hannah LC. 1994. ADP-glucose pyrophosphorylase in shrunken2 and brittle2 mutants of maize endosperm. Mol. Gen. Genetics. Vol. 243, P. 400-408.

Giroux MJ, Shaw J, Barry G, Cobb GB, Greene T, Okita T, Hannah CL. 1996. A single gene mutation that increases maize seed weight. Proceedings of the National Academy of Science. Vol. 93, P. 5824-5829.

Greene TW, Hannah CL. 1998. Enhanced stability of maize endosperm ADP-glucose pyrophosphorylase is gained through mutants that alter subunit interactions. Proceedings of the National Academy of Science. Vol. 95, p. 13342-13347.

Hannah CL, Nelson OE. 1976. Characterization of ADP-glucose pyrophosphorylase from Shrunken-2 and Brittle-2 mutants of maize. Biochemical Genetics. Vol. 14, P. 547-560.

Hannah LC. 1997. In Celluar and Molecular Biology of Seed Development, eds. Larkins BA & Vasil IK., pp. 375-405.

Ho LC. 1988. Metabolism and compartmentation of imported sugars in sink organs in relation to sink strength. Ann. Rev. Plant Physiol. Plant Mol. Biol. Vol. 39, P. 355-378.

Iglesias AA, Barry, GF, Meyer C, Bloksberg L, Nakata PA, Greene T, Laughlin MJ, Okita TW, Kishore GM, Preiss J. 1993. The Journal of Biological Chemistry. Vol. 268, P. 1081-1086.

Imai K, Coleman D, Yanagisawa T. 1985. Increase of atmospheric partial pressure of carbon dioxide and growth and yield of rice (Oryza sativa L.). Japan Journal of Crop Science. Vol. 54, P. 413-418.

Jackson SA, Zhang P, Chen WP, Phillips RL, Friebe B, Muthukrishnan S, Gill BS. 2001. High-resolution structural analysis of biolistic transgene integration into the genome of wheat. Theoretical and Applied Genetics. Vol. 103, P. 56-62.

King RW, Wardlaw IF, Evans LT. 1967. Effect of Assimilate Utilization on Photosynthetic Rate in Wheat. Planta. Vol. 77, P. 261-276.

Lammacchia C, Shewry PR, Fonzo ND, 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. Journal of Experimental Botany. Vol. 52, P. 243-250.

Lanning SP, Talbert LE, McNeal FH, Alexander WL, McGuire CF, Bowman H, Carlson G, Jackson G, Eckhoff J, Kushnak G. 1992. Release of the cultivar 'HiLine.' Crop Science. Vol. 32, P. 283-284.

Leung P, Lee Y, Greenberg E, Esch K, Boylan S, Preiss J. 1986. Cloning and expression of the Escherichia coli glgC gene from a mutant containing an ADPglucose pyrophosphorylase with altered allosteric properties. Journal of Bacteriology. Vol. 167, P. 82-88.

Lin TP, Caspar T, Somerville CR, Preiss J. 1988. Isolation and characterization of a starchless mutant of Arbidopsis thaliana lacking ADP-glucose pyrophosphorylase activity. Plant Physiology. Vol. 86, P. 1131-1135.

Lutticke S, Genschel U, Block M, Kroger C, Walter L, Abel G, Lorz H. 1999. Approaches to metabolic engineering of starch biosynthesis in wheat In: Abstracts book, Genetic Tailoring of Novel Starch Polymers. Carry-le-Rouet, France, Sept. 1999.

Marcelis, LFM. 1996. Sink strength as a determinant of dry matter partitioning in the whole plant. Journal of Experimental Botany. Vol. 47, P. 1281-1291.

Martinez-Carrasco R, Thorne G. 1979. Effects of crop thinning and reduced grain numbers per ear on grain size in two winter wheat varieties given different amounts of nitrogen. Ann. Appl. Biol. Vol. 92, P. 383-393.

Morell MK, Bloom M, Knowles V, Preiss J. 1987. Subunit structure of spinach leaf ADPglucose pyrophosphorylase. Plant Physiology. Vol. 85, P. 182-187.

Muller E, Lorz H, Lutticke S. 1996. Variability of trangene expression in clonal cell lines of wheat. Plant Science. Vol. 114, P. 71-82.

Preiss J, Ball K, Hutney J, Smith-White B, Li L, Okita TW. 1991. Regulatory mechanisms involved in the biosynthesis of starch. Pure and Applied Chemistry. Vol. 63, P. 535-544.

Preiss J. 1982. Regulation of the biosynthesis and degradation of starch. Annual Review of Plant Physiology. Vol. 33, P. 431-454

Prioul JL, Schwebel-Dugue N. 1992. Source-sink manipulations and carbohydrate metabolism in maize. Crop Science. Vol. 32, P. 751-756.
Rajcan I, Tollenaar M. 1999. Source:sink ratio and leaf senescence in maize: I.Dry matter accumulation and partitioning during grain filling. Field Crops Research. Vol. 60, P. 245-253.

Rasmussen RD, Al-Saady N, Torbert KA, Smith LS, Phillips RL, Rines HW, Somers DA. 1998. Antisense inhibition of the Waxy genes in haxaploid oat. Annual Meeting Abstracts, American Society of Agronomy, Baltimore, MD, October 1998 (pg 154).

Recondo E, Leloir LF. 1961. Adenosine diphosphate glucose and starch synthesis. Biochem. Biophys. Res. Commun. Vol. 6, P. 85-88.

Repellin A, Baga M, Jauhar PP, Chibbar RN. 2001. Genetic enrichment of cereal crops via alien gene transfer: New challenges. Plant Cell, Tissue and Organ Culture. Vol. 64, P. 159-183.

Richards RA. 2000. Selectable traits to increase crop photosynthesis and yield of grain crops. Journal of Experimental Botany. Vol. 51, P. 447-458.

Riede CR, Anderson JA. 1996. Linkage of RFLP markers to an aluminum tolerance gene in wheat. Crop Science. Vol. 36, P. 905-909.

Salamone PR, Kavakli HI, Slattery CJ, Okita TW. 2002. Directed molecular evolution of ADP-glucose pyrophosphorylase. Proceedings of the National Academy of Science. Vol. 99, P. 1070-1075.

Shannon JC, Pien F, Cao H, Leu K. 1998. Brittle-1, an adenylate translocator, facilitates transfer of extraplastidial synthesized ADP-glucose into amyloplasts of maize endosperms. Plant Physiology. Vol. 117, P. 1235-1252.

Sharp GL, Martin JM, Lanning SP, Blake NK, Brey CW, Sivamani E, Qu R, Talbert LE. 2002. Field Evaluation of transgenic and classical sources of wheat sreak mosaic virus resistance. Crop Science. Vol. 42, P. 105-110.

Shaw JR, Hannah LC. 1992. Genomic nucleotide sequence of a wild-type Shrunken-2 allele of Zea mays. Plant Physiology. Vol. 98, P. 1214-1216.

Shure MSW and Federoff N. 1983. Molecular identification and isolation of the *waxy* locus in maize. Cell. Vol. 35, P. 225-233.

Sinclair TR. 1998. Historical changes in harvest index and crop nitrogen accumulation. Crop Science. Vol. 38, P. 638-643.

Sindhu AS, Zheng ZW, Murai N. 1997. The pea seed storage protein legumin was synthesized, processed, and accumulated stably in transgenic rice. Plant Science. Vol. 130, P. 189-196.

Sivamani E, Brey CW, Dyer WE, Talbert LE, Qu R. 2000. Resistance to wheat streak mosaic virus mediated by WSMV-RNA replicase gene in transgenic wheat. Mol. Breeding. Vol. 6, P. 469-477.

Slafer GA, Rawson HM. 1994. Sensitivity of wheat phasic development to major environmental factors: a re-examination of some assumptions made by physiologists and modellers. Vol. 21, P. 393-426.

Smidansky ED, Clancy M, Meyer F, Lanning SP, Blake NK, Talbert LE, Giroux MJ. 2002. Enhanced ADP-glucose pyrophosphorylase activity in wheat endosperm increases yield. Proc. Natl. Acad. Sci. USA. Vol. 99, P. 1724-1729.

Songstad DD, Somers DA, Griesback RJ. 1995. Advances in alternative DNA delivery techniques. Plant Cell Tiss. Org. Cult. Vol. 40, P. 1-15.

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. Vol. 258, P. 287-292.

Stoger E, Williams S, Keen D, Christou P. 1998. Molecular characteristics of transgenic wheat and the effect on transgene expression. Transgenic Research. Vol. 7, P. 463-471.

Sturm A, Tang G. 1999. The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning. Trends in Plant Science. Vol. 4, P. 401-407.

Sun J, Okita TW, Edwards GE. 1999. Modification of carbon partitioning, photosynthetic capacity, and O2 sensitivity in Arabidopsis plants with low ADP-glucose pyrophosphorylase activity. Plant Physiology. Vol. 119, P. 267-276.

Sweetlove LJ, Burrell MM, Rees T. 1996. Characterization of transgenic potato (Solanum tuberosum) tubers with increased ADPglucose pyrophosphorylase. Vol. 320, P. 487-492.

Thorne GN, Wood DW. 1986. Effects of radiation and temperature on tiller survival, grain number and grain yield in winter wheat. Annals of Botany. Vol. 59, P. 413-426.

Tsai C, Nelson O. 1966. Starch deficient maize mutant lacking adenosine diphosphate glucose pyrophosphorylase activity. Science. Vol. 238, P. 341-343.

Turgeon R. 1989. The sink-source transition in leaves. Annu. Rev. Plant Physiol. Plant Mol. Biol. Vol. 40, P. 119-138.

Vasil IK, Bean S, Zhao J, McCluskey P, Lookhart G, Zhao HP, Altpeter F, Vasil V. 2001. Evaluation of baking properties and gluten protein composition of field grown transgenic wheat lines expressing high molecular weight glutenin gene 1Ax1. Journal of Plant Physiology. Vol. 158, P. 521-528.

Vasil V, Castillo AM, Fromm ME, Vail IK. 1992. Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Bio/Technology. Vol. 10, P. 667-674.

Wrigley CW. 1996. Giant proteins with flour power. Nature. Vol. 381, P. 738-739.

Ye X, Al-Babili S, Kloti A, Zhang J, Lucca P, Beyer P, Potrykus I. 2000. Engineering the provitamin A (B carotene) biosynthetic pathway into (carotenoid-free) rice endosper. Science. Vol. 287, P. 303-305.

Zhen-Wen Y, Van Sanford DA, Egli DB. 1988. The effect of population density on floret initiation, development and abortion in winter wheat. Annals of Botany. Vol. 62, P. 295-302.

Ziska LH, Teramura AH. 1992. CO2 enhancement of growth and photosynthesis in rice (Oryza sativa). Plant Physiology. Vol. 99, P. 473-481.\

37 206MT TH 3037
5/03 30568-61 NLB

MONTANA STATE UNIVERSITY - BOZEMAN
3 1762 10382363 7