THE EFFECT OF MODIFIED ADP-GLUCOSE PYROPHOSPHORYLASE ACTIVITY ON STARCH METABOLISM *IN PLANTA*

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of YASUKO NAGAI find it satisfactory and recommend that it be accepted.

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Chair

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Abstract

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Increasing plant productivity and crop yield depends on modification in the capacity of source tissues and sink strength. Starch is not only a dominant reserve in plants, but also the major carbohydrate in the human diet. Although starch metabolism in source and sink organs is different, ADP-glucose pyrophosphorylase (AGPase) catalyzes the rate-limiting step of starch biosynthesis in both tissues. In order to investigate the effect of up-regulated AGPase, transgenic plants having enhanced AGPase activity were examined in Arabidopsis leaves and rice seeds.

Source leaves play a crucial role as the site of CO_2 fixation by photosynthesis as well as a transient sink for accumulating starch to support plant growth. Up-regulated transgenic *Arabidopsis* having single or double mutations in the *ApL1* gene encoding the AGPase large subunit were successfully transformed in a TL46 starch-deficient mutant which lacks a functional large subunit. All up-regulated transgenic plants had two to four-fold higher leaf AGPase activity and showed higher CO_2 assimilation rates, greater leaf starch turnover and larger plant biomass compared to wildtype, TL46 mutant and T65 control having intact wildtype *ApL1* gene. These results indicate that increased leaf AGPase activity contributed to an enhancement of leaf starch accumulation and turnover and increase plant development.

In rice, long-term starch storage accounts for 75-80% of the seed dry weight. Up-regulated transgenic rice containing *Escherichia coli glgC*-triple mutant gene which encodes a bacterial AGPase led to three to six-fold increase in AGPase activity and 7-24% enhanced seed biomass compared to wildtype Kitaake. In order to obtain additional understanding of these transformants, metabolite analysis of up-regulated transgenic lines along with wildtype was performed. Increased levels of ADP-glucose, a product of AGPase, and hexose pools such as glucose 1-phosphate and glucose 6-phosphate, were observed in all up-regulated transgenic rice. The change in hexose-phosphate pools indicates that some other factor besides AGPase limits starch biosynthesis in rice endosperm. Transport of ADP-glucose into amyloplasts and/or restricted activities of starch biosynthesis enzymes are the likely processes which may be limiting rice starch production in these transgenic plants.

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DEDICATION

This thesis is dedicated to my dear husband Kenji Takizawa,

my lovely daughter Yuri Takizawa and my family

who supported and encouraged me during this difficult moment.

CHAPTER ONE

GENERAL INTRODUCTION

Due to an increasing world population, global improvement of plant productivity and crop yield is a high priority. Plant productivity and yield are dependent on the capacity of CO_2 fixation by photosynthesis in leaves and on the capacity of sink organs to convert fixed carbon into dry matter. Various approaches have been undertaken towards enhancing yield such as increasing source capacity and/or sink strength as well as modifying assimilate partitioning.

Starch is the dominant reserve in source and sink organs in many plants. During the vegetative stage, plants accumulate sugars and starch by photosynthesis in source leaves and utilize the photosynthates for plant development and growth. However, during the reproductive stage, most of the photoassimilates synthesized in leaves are transported to sink organs (e.g. seeds, tubers and fruits) for long-term storage in the form of starch, lipids and proteins.

ADP-glucose pyrophosphorylase (AGPase) catalyzes the first committed step in the starch biosynthesis pathway of plants. Recent studies [e.g. the modified bacterial genes into potato (Stark *et al.* 1992), rice (Sakulsingharoj *et al.* 2004) and cassava (Ihemerell *et al.* 2006); the maize genes into maize (Giroux *et al.* 1996), wheat (Smidansky *et al.* 2002) and rice (Simidansky *et al.* 2003); and the *Arabidopsis* genes into *Arabidopsis* (Gibson *et al.* 2003; Obana *et al.* 2006)] have utilized an engineered AGPase to enhance plant productivity and yields. Gibson *et al*. (2003) and Obana *et al*. (2006) demonstrated that transgenic *Arabidopsis* having higher leaf AGPase activity than

wildtype plants showed a greater turnover of transitory starch, higher capacity for starch biosynthesis and elevated plant biomass (e.g. leaf area and seed weight) compared to wildtype and a starch-deficient mutant, TL46. A similar approach was undertaken to modify sink organs by the expression of a modified *Escherichia coli glgC* triple mutant (*glgC*-TM) gene, which encodes a highly active and allosterically insensitive AGPase, in rice endosperm (Sakulsingharoj *et al*. 2004). The *glgC* -TM lines exhibited higher levels of AGPase activity, higher rates of conversion of ^{14}C-sucrose into starch, and increased seed weight. These results demonstrated that AGPase is one of the limiting factors in starch biosynthesis in both source and sink organs. It also suggests that starch metabolism is an important process in influencing plant productivity.

Increasing plant productivity

In order to eliminate the hunger caused by the explosion of the world population growth, especially in India, the green revolution was initiated at the International Maize and Wheat Improvement Center (CIMMYT) in Mexico and the International Rice Research Institute (IRRI) in the Philippines in the 1960s. High yielding varieties of rice and wheat having semi-dwarf properties were created using traditional breeding techniques as well as the introduction of new agricultural management practices using nitrogen fertilizer, pesticides and irrigation. Global production of cereal crops has more than doubled in developing countries over the thirty years since the start of the green revolution (Surridge, 2002). Although the green revolution led to a dramatic increase in cereal production, it also created problems such as land salinity which led to desertification and the disappearance of genetic resources. While the global population

is still increasing, the amount of arable lands on the Earth is limited. In order to feed the increasing world population in the future, enhancing the genetic yield potential of the major crop plants for increased yields is the only feasible approach.

The genetic yield potential of crop plants depends on source-sink relationships. The photoassimilates produced by photosynthesis in source leaves are transported into sink tissues such as young leaves for growth and development or reserves such as tubers, seeds and fruits for long-term storage. Increasing photosynthetic efficiency in leaves leads to greater growth development and the export rate of photosynthates into the sink and in turn, improvement of plant productivity.

Besides source capacity, sink strength is also a vital factor in determining yield. In non-photosynthetic sink organs, transported sugars and photosynthates are converted into reserve compounds (e.g. starch, lipids and proteins). Increased sink capacity will enhance photosynthesis efficiency in source leaves (Smidansky *et al*. 2002, Smidansky *et al*. 2003). Therefore, the improvement of source-sink relationships will result in increases in plant productivity and crop yield.

The structure and regulation of plant AGPase

AGPase (EC 2.7.7.27) catalyzes a pivotal, reversible reaction [glucose-1-phosphate + ATP ↔ADP-glucose + pyrophosphate] which controls carbon flux in the starch/glycogen pathway in plants and bacteria. The bacterial AGPase is comprised of four identical subunits (α_4) of 48 kilodaltons (kDa) (Ballicora *et al*. 2003). The subunits are encoded by a single gene locus, *glgC* (Leung and Preiss, 1987, Okita *et al*. 1981). In contrast, higher plant AGPases are a heterotetramer ($\alpha_2\beta_2$) consisting of two different

subunits, a regulatory large subunit of 51-60 kDa and a catalytic small subunit of 50-54 kDa, which share about 50% sequence identity (Nakata *et al*. 1991). Sequence comparisons of the large subunit and small subunit genes from many plant species by Smith-White and Preiss (1992) indicated that the primary sequences of the small subunits are highly conserved (> 90% identity) while those of the large subunits have greater diversity (< 85% identity). Georgelis *et al*. (2007) proposed that the small subunit is less prone to mutation as it must assemble with different isoforms of the large subunit, which display much more sequence diversity. In *Arabidopsis*, four genes for large subunits (*ApL1, ApL2, ApL3* and *ApL4*) and two genes for small subunits (*ApS1* and *ApS2*) were identified (Crevillen et al. 2003, Crevillen et al. 2005). Large subunit isoforms have tissue-specific expression such as *ApL1* for leaf and *ApL3* for endosperm. Like *Arabidopsis*, rice has the same number of AGPase encoding genes in the AGP gene family (Ohdan *et al*. 2005, Lee *et al*. 2007). Based on mutant studies in *Arabidopsis* (Lin *et al.* 1988a, Lin *et al.* 1988b) and maize (Tsai and Nelson, 1966, Nelson and Pan 1995), both subunits are required for maximum catalytic activity.

Despite their structural differences, the bacterial and plant AGPases are subject to allosteric regulation by small effector molecules. The AGPases from enteric bacteria are activated by fructose-1, 6-bisphosphate and inhibited by AMP, whereas the plant AGPases are activated and inhibited by the major photoassimilates 3-phosphoglyceric acid (3-PGA) and inorganic phosphate (Pi), respectively. Many of the higher plant AGPases are also controlled by redox regulation where the pair of small subunits in heterotetrameric enzyme is linked by a disulfide bond (Jin *et al.* 2005). Light and sugars act as a trigger of the posttranslational redox modification via ferredoxin-

thioredoxin and via sugar signaling pathways involving SnRK1 (sucrose-non-fermenting-1-related protein kinase) and hexokinase (Tiessen *et al.* 2003, Hendriks *et al.* 2003, Geigenberger *et al.* 2005). It has also been found that trehalose-6-phosphate regulates redox activation of AGPase via the involvement of 14-3-3 signal transduction proteins (Huber *et al.* 2002, Kolbe *et al.* 2005, Lunn *et al.* 2006).

The regulation of starch biosynthesis in leaf tissues (Figure 1)

In leaves, AGPase is localized in the chloroplast, the site of starch synthesis. ADP-glucose, the product of AGPase, is used as a substrate by starch synthases, which add glucose units to the non-reducing end of α-1, 4-glucosidic polymer chains. Branches in the chain are introduced by starch branching enzyme, which hydrolyzes α-1, 4-glucosidic bonds and creates an α-1, 6-glucosidic bond within the same or neighboring chain. Though debranching enzyme is required for starch breakdown, the enzyme activity also plays a role in the starch synthesis pathway by trimming (redesigning) long side chains of glucosyl residues in amylopectin.

Plants synthesize triose-phosphates which are the initial product of photosynthesis. The bulk of the triose-phosphate is exported to the cytoplasm where it is metabolized to sucrose, which is exported to sink tissues to support growth and development. When the levels of sucrose become saturated in the cytosol due to sink limitations, the rate of export of triose-phosphate from the chloroplast is decreased via the triose-phosphate/Pi transporter. Starch synthesis is then activated because of the increased ratio of 3-PGA/Pi in the chloroplast which is required for activation of AGPase. High level of Pi in chloroplast stroma is essential to maintain photophosphorylation,

which is the production of ATP using absorbed light energy, and the synthesis of ribulose-1, 5-bisphosphate (RuBP), a substrate for Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco), in the Calvin cycle. Preventing the recycling of Pi results in photosynthetic feedback because of the repression of photosynthetic efficiency by decreased photophosphorylation and RuBP formation. Therefore, leaf starch serves not only as a reserve of carbon and energy required for plant maintenance during the night period, but also as a transient sink to recycle Pi.

Starch synthesis in sink tissue (Figure 2)

AGPase has two forms in developing cereal endosperm. In maize (Denyer *et al.* 1996), barley (Thorbjørnsen *et al.* 1996) and rice (Sikka *et al.* 2001), the dominant isoform is localized in the cytoplasm while a minor form is in the amyloplast, a specialized starch containing plastid in endosperm tissue. The main pathway for starch synthesis from sucrose in rice endosperm consists of UDP-glucose, glucose-1-phosphate, and ADP-glucose. Sucrose unloaded from the source tissue via phloem in the seed enters the cytosol where it can be metabolized by two different routes. The main pathway for sucrose metabolism is catalyzed by sucrose synthase which converts sucrose to UDP-glucose and fructose as the initial step. UDP-glucose is then converted to glucose-1-phosphate by UDP-glucose pyrophosphorylase. Cytoplasmic AGPase converts glucose-1-phosphate to ADP-glucose. ADP-glucose is transported from the cytoplasm into amyloplast via the ADP-glucose transporter (Brittle-1) in counter-exchange with ADP which is a by-product of the starch synthase reaction in amyloplasts (Bowsher *et al.* 2007).

In order to gain further insight on the relationship between starch metabolism and plant productivity at the physiological and metabolic levels, studies using transgenic *Arabidopsis* and rice plants expressing up-regulated AGPase were performed. Chapter two describes the physiological effects of up-regulated leaf AGPase on leaf starch metabolism, photosynthesis and biomass. Transgenic *Arabidopsis* plants were examined for the correlation between AGPase activity, leaf starch levels, CO_2 assimilation rates and overall plant growth compared to wildtype and TL46 starch-deficient mutant. In Chapter three, metabolite analysis of up-regulated cytoplasmic AGPase in developing rice seeds was performed in an effort to understand how the up-regulated AGPase contributes to increased seed weight. The results of this experiment give an increased understanding of the regulation of starch biosynthesis and sink strength in sink tissues.

References

Ballicora, M. A., A. A. Iglesias, *et al*. (2004). "ADP-Glucose pyrophosphorylase: a regulatory enzyme for plant starch synthesis." Photosynth Res 79(1): 1-24.

Bowsher, C. G., E. F. Scrase-Field, *et al*. (2007). "Characterization of ADP-glucose transport across the cereal endosperm amyloplast envelope." J Exp Bot 58(6): 1321-32.

Crevillén, P., M. A. Ballicora, *et al*. (2003). "The different large subunit isoforms of *Arabidopsis thaliana* ADP-glucose pyrophosphorylase confer distinct kinetic and regulatory properties to the heterotetrameric enzyme." J Biol Chem 278(31): 28508-15.

Crevillén, P., T. Ventriglia *et al*. (2005) "Differential pattern of expression and sugar regulation of *Arabidopsis thaliana* ADP-glucose pyrophosphorylase-encoding genes." J Biol Chem 280(9): 8143-9.

Denyer, K., F. Dunlap, *et al*. (1996). "The major form of ADP-glucose pyrophosphorylase in maize endosperm is extra-plastidial." Plant Physiol 112(2): 779-85.

Geigenberger, P., A. Kolbe, *et al*. (2005). "Redox regulation of carbon storage and partitioning in response to light and sugars." J Exp Bot 56(416): 1469-79.

Georgelis, N., E. L. Braun, *et al*. (2007). "The two AGPase subunits evolve at different rates in angiosperms, yet they are equally sensitive to activity-altering amino acid changes when expressed in bacteria." Plant Cell 19(5): 1458-72.

Gibson, K. M., S. K. Hwang, *et al*. (2003). "Metabolic engineering of starch for enhanced plant productivity and yields." J Applied Glycoscience 50: 201-206.

Giroux, M. J., J. Shaw, *et al*. (1996). "A single mutation that increases maize seed weight." Proc Natl Acad Sci U S A 93(12): 5824-9.

Hendriks, J. H., A. Kolbe, *et al*. (2003). "ADP-glucose pyrophosphorylase is activated by posttranslational redox-modification in response to light and to sugars in leaves of *Arabidopsis* and other plant species." Plant Physiol 133(2): 838-49.

Huber, S. C., C. MacKintosh, *et al*. (2002). "Metabolic enzymes as targets for 14-3-3 proteins." Plant Mol Biol 50(6): 1053-63.

Ihemere, U., D. Arias-Garzon, *et al*. (2006). "Genetic modification of cassava for enhanced starch production." Plant Biotechnol J 4(4): 453-65.

Jin, X., M. A. Ballicora, *et al*. (2005). "Crystal structure of potato tuber ADP-glucose pyrophosphorylase." Embo J 24(4): 694-704.

Kolbe, A., A. Tiessen, *et al*. (2005). "Trehalose 6-phosphate regulates starch synthesis via posttranslational redox activation of ADP-glucose pyrophosphorylase." Proc Natl Acad Sci U S A 102(31): 11118-23.

Leung, P. S. and J. Preiss (1987). "Cloning of the ADP-glucose pyrophosphorylase (*glgC*) and glycogen synthase (*glgA*) structural genes from *Salmonella typhimurium* LT2." J Bacteriol 169(9): 4349-54.

Lin, T. P., T. Caspar, *et al*. (1988a). "Isolation and characterization of a starchless mutant of *Arabidopsis thaliana* (L.) Heynh lacking ADP-glucose pyrophosphorylase activity." Plant Physiol 86: 1131-1135.

Lin, T. P., T. Caspar, *et al*. (1988b). "A starch deficient mutant of *Arabidopsis thaliana* with low ADP-glucose pyrophosphorylase activity lacks one of the two subunits of the enzyme." Plant Physiol 88: 1175-1181.

Lunn, J. E., R. Feil, *et al*. (2006). "Sugar-induced increases in trehalose 6-phosphate are correlated with redox activation of ADP-glucose pyrophosphorylase and higher rates of starch synthesis in *Arabidopsis thaliana*." Biochem J 397(1): 139-48.

Nakata, P. A., T. W. Greene, *et al*. (1991). "Comparison of the primary sequences of two potato tuber ADP-glucose pyrophosphorylase subunits." Plant Mol Biol 17(5): 1089-93.

Nelson, O. and D. Pan (1995). "Starch synthesis in maize endosperms." Annu Rev Plant Physiol Plant Mol Biol 46: 475-496.

Obana, Y., D. Omoto, *et al*. (2005). "Enhanced turnover of transitory starch by expression of up-regulated ADP-glucose pyrophosphorylases in *Arabidopsis thaliana*." Plant Sci 170: 1-11.

Ohdan, T., P. B. Jr Francisco, *et al*. (2005). "Expression profiling of genes involved in starch synthesis in sink and source organs of rice." J Exp Bot 56(422): 3229-44.

Okita, T. W., R. L. Rodriguez, *et al*. (1981). "Biosynthesis of bacterial glycogen. Cloning of the glycogen biosynthetic enzyme structural genes of *Escherichia coli*." J Biol Chem 256(13): 6944-52.

Sakulsingharoj, C., S.-B. Choi, *et al*. (2004). "Engineering starch biosynthesis for enhanced rice yields: the role of the cytoplasmic ADP-glucose pyrophosphorylase." Plant Sci 167: 1323-1333.

Sikka, V. K., S. B. Choi, *et al*. (2001). "Subcellular compartmentation and allosteric regulation of the rice endosperm ADP-glucose pyrophosphorylase." Plant Sci 161: 461-468.

Smidansky, E. D., M. Clancy, *et al*. (2002). "Enhanced ADP-glucose pyrophosphorylase

activity in wheat endosperm increases seed yield." Proc Natl Acad Sci U S A 99(3): 1724-9.

Smidansky, E. D., J. M. Martin, *et al*. (2003). "Seed yield and plant biomass increases in rice are conferred by deregulation of endosperm ADP-glucose pyrophosphorylase." Planta 216(4): 656-64.

Smith-White, B. J. and J. Preiss (1992). "Comparison of proteins of ADP-glucose pyrophosphorylase from diverse sources." J Mol Evol 34(5): 449-64.

Stark, D. M., K. P. Timmerman, *et al*. (1992). "Regulation of the amount of starch in plant tissues by ADP-glucose pyrophosphorylase." Science 258: 287–292.

Surridge, C. (2002). "The rice squad." Nature 416: 576-578.

Thorbjørnsen, T., P. Villand, *et al*. (1996). "A single gene encodes two different transcripts for the ADP-glucose pyrophosphorylase small subunit from barley (*Hordeum vulgare*)." Biochem J 313 (Pt 1): 149-54.

Tiessen, A., K. Prescha, *et al*. (2003). "Evidence that SNF1-related kinase and hexokinase are involved in separate sugar-signalling pathways modulating post-translational redox activation of ADP-glucose pyrophosphorylase in potato tubers." Plant J 35(4): 490-500.

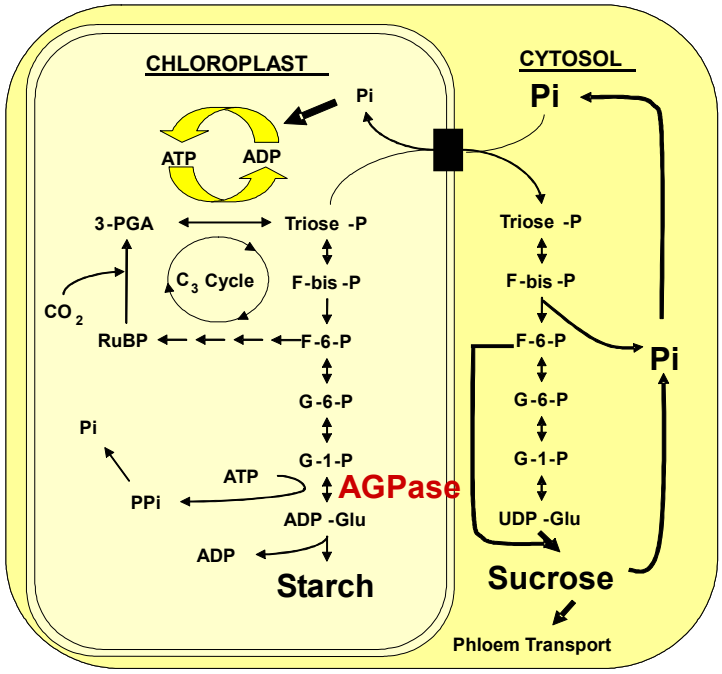
Tsai, C. Y. and O. E. Nelson (1966). "Starch-deficient maize mutant lacking adenosine dephosphate glucose pyrophosphorylase activity." Science 151(708): 341-3.

Figure Legends

Figure 1. Carbon flow in leaf tissue and the biochemical basis of feedback. At the beginning of light period, photoassimilates are transported into cytosol and utilized for sucrose synthesis. After saturation of sucrose synthesis by sink limitation, starch synthesis in chloroplasts becomes active because of the high ratio of triose-phosphate/Pi in the stroma. Released Pi by plastidial AGPase is recycled for RuBP formation and ATP production to avoid photosynthetic feedback, thereby maintaining continuous starch biosynthesis. Black box: Triose-phosphate/Pi transporter

Figure 2. Carbon metabolic pathway from sucrose to starch in cereal endosperm. In the major pathway of starch synthesis, ADP-glucose is synthesized in the cytoplasm by cytosolic AGPase and transported into amyloplasts via Brittle-1 transporter. 1. Cell wall invertase, 2. Cytoplasmic invertase, 3. Sucrose synthase, 4. Hexokinase, 5. Phosphohexose isomerase, 6. UDP-glucose pyrophosphorylase, 7. Cytoplasmic phosphoglucomutase, 8. Cytoplasmic AGPase, 9. Plastidial AGPase, 10. Plastidial phosphoglucomutase, Black box on amyloplast membrane: ADP-glucose transporter (Brittle-1)

Figure 1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ADP
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CO2
RuBP
F-6-P
F-6-P
Pi
G-6-P
G-6-P
Pi
G-1-P
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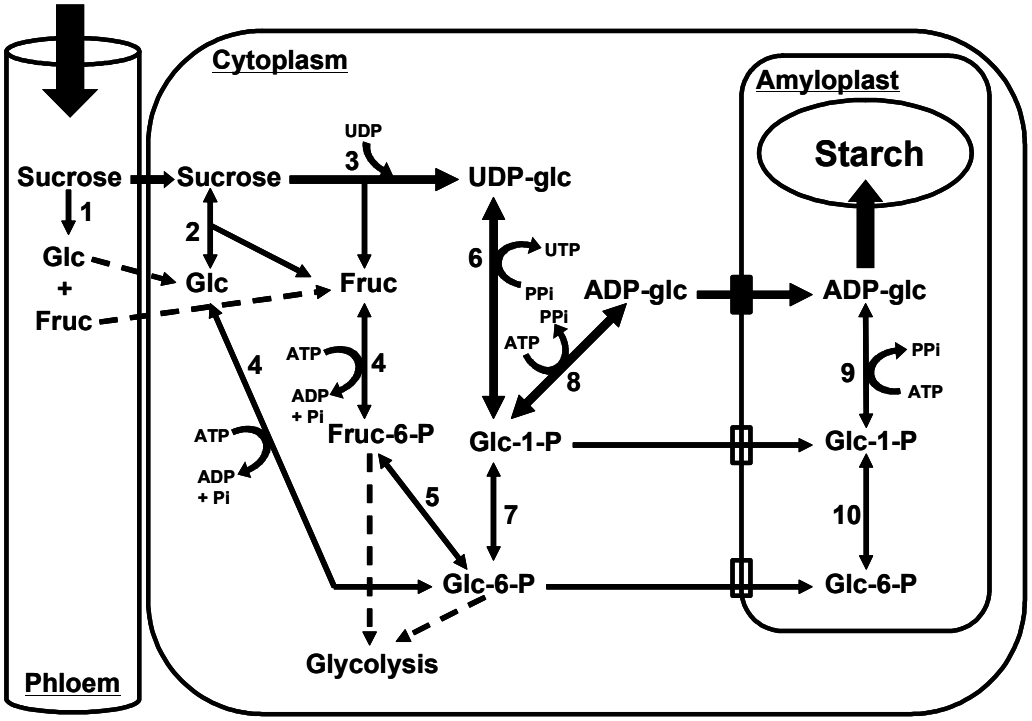
ATP
AGPase
PPi
ADP -Glu
UDP -Glu
ADP
Starch
Sucrose
Phloem Transport

Figure 2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Amyloplast
Starch
Sucrose
Sucrose
UDP
3
UDP-glc
1
2
Glc
+
Fruc
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Fruc
6
UTP
PPi
ADP-glc
ADP-glc
PPi
ATP
8
9
PPi
ATP
4
ATP
4
ADP
+ Pi
ATP
ADP
+ Pi
Fruc-6-P
Glc-1-P
Glc-1-P
5
7
10
Glc-6-P
Glc-6-P
Glycolysis
Phloem

CHAPTER TWO

Genetic modification of leaf ADP-glucose pyrophosphorylase in *Arabidopsis* for enhanced leaf starch turnover and plant productivity

Abstract

Increasing photosynthetic capacity in source leaves and enhancement of sink strength in storage organs are major considerations for improvement of plant productivity. Results from previous studies from the Edwards' and Okita's laboratories showed a correlation between photosynthetic capacity, plant growth and leaf starch levels in *Arabidopsis* mutants restricted in starch biosynthesis (Sun *et al*. 1999a, Sun *et al*. 2002). Leaf starch may act as a transient sink to accommodate excess photosynthate and thereby diminish the potential reduction in photosynthetic capacity due to feedback effects. In order to test this hypothesis, I analyzed transgenic *Arabidopsis* expressing an up-regulated AGPase, a key enzyme in starch biosynthesis in source leaves, along with wildtype and the parent TL46 starch-deficient mutant in terms of enzyme activity, leaf starch metabolism and plant biomass.

Backcrossed (TL46-background) transgenic lines having up-regulated forms of AGPase large subunit *ApL1* genes successfully showed higher AGPase activity, greater leaf starch turnover and increased biomass than wildtype and the parent TL46 mutant. Among these transgenic lines, there were two leaf types with different chlorophyll levels observed. To eliminate the potential effect of leaf phenotype, all transgenic lines were crossed to wildtype. The experiments using wildtype-background up-regulated transgenic *Arabidopsis* provides evidence that manipulation of starch biosynthesis via

expression of up-regulated AGPase enhances CO_2 assimilation by increasing sink capacity for starch biosynthesis in up-regulated transgenic lines. The combination of increased photosynthetic capacity and transitory starch turnover contributed to increased plant biomass. Thus, manipulation of AGPase activity has high potential for increasing plant productivity.

Introduction

Efforts at increasing plant productivity and crop yields center on the manipulation of source-sink relationships. Improvement of source-sink relationships is not a simple process because it involves the interaction of multiple plant organs and tissues and complicated metabolic pathways. Plant tissues can be briefly divided into two types; source (e.g. photosynthetic tissues such as leaves and stems) and sink (e.g. developing seeds and young leaves). Organs such as seeds are a storage sink which accumulates long-term reserves for future carbon and energy resource. Leaves, which serving as a source, are also a transient sink which store and utilize reserve compounds on a diurnal basis. The balance of influx and efflux of carbon reserves in both source and sink tissues is a key concept for improvement of plant productivity.

Plant leaves, especially mature green leaves, play an important role as a transient sink. Transitory starch accumulates gradually after the light regime starts and maximum starch level is attained at the end of day (Fondy and Geiger, 1982). It is totally dependent on the capacity of photosynthesis in leaves and the conditions of the surrounding environment such as temperature. In experimental model systems such as spinach and *Arabidopsis*, most of the starch accumulated during the day is degraded during the following night (Gerhardt *et al*. 1987, Lin *et al.* 1988, Zeeman *et al.* 1998). Exported carbon assimilates from mature leaves are utilized for maintenance of plant growth and development of young leaves and roots. Thus, plant leaves work not only as a source tissue but also as a transient sink organ during the day.

Starch is a major carbohydrate reserve in both source and sink tissues in many plants. It consists of two forms of polysaccharides. One is amylose which is mainly a

linear glucose polymer, and the other is amylopectin which is a highly branched complex form. The percentage of amylose and amylopectin in starch varies depending on the plant species examined. Numerous amylose and amylopectin chains are packed into a single starch granule, thereby minimizing osmotic pressure in plastids of source and sink organs.

Transient starch is accumulated in the chloroplasts of source leaves when the amount of photosynthates by CO_2 fixation exceeds the demand for sucrose in sink tissues. Since the export of carbon assimilates (e.g. triose-phosphate) is regulated by a triose-phosphate/ Pi transporter on the membrane of chloroplasts, increased organic-phosphate and decreased Pi levels in the cytoplasm caused by sink limitation inhibits Pi recycling and entry to the chloroplasts. Therefore, starch synthesis is promoted because of the increased ratio of 3-phosphoglycerate over Pi in chloroplasts. Under this condition, the activated AGPase enzyme catalyzes the first committed step in starch biosynthesis. This enzyme converts glucose-1-phosphate and ATP to ADP-glucose and pyrophosphate. Pyrophosphorylase breaks down immediately into two molecules of Pi by a potent plastidial inorganic pyrophosphatase, thereby driving the reaction to ADP-glucose formation. Released Pi is utilized for ATP production and RuBP regeneration which is required for optimal rates of photosynthesis. Source leaves as transitory sinks are important for maintaining Pi recycling in chloroplasts as well as for synthesizing photosynthates via the Calvin cycle.

There is direct evidence that AGPase activity affects starch production in leaves. *Arabidopsis* mutants having only functional large subunits or small subunits contain significantly lower amounts of starch compared to non-mutated wildtype (Lin *et al*.

1988a, Lin *et al*. 1988b). By contrast, Obana *et al*. (2006) demonstrated that up-regulated forms of AGPase enzyme led to increased leaf starch turnover in *Arabidopsis*. It suggested that AGPase is a limiting factor for the conversion of photoassimilates into starch in leaves.

Sun *et al*. (1999a and 2002) demonstrated that there was a correlation between AGPase activity, leaf starch levels, photosynthetic capacity and plant growth using *Arabidopsis* Columbia wildtype and starch-deficient mutants. This relationship suggested that leaf starch serves as a transient sink to accommodate excess photosynthate and thereby diminishes potential reduction in photosynthetic capacity due to feedback effects. To further test this hypothesis, I studied *Arabidopsis* plants expressing up-regulated AGPases and showed that these plants have higher starch turnover, photosynthetic capacity and growth than wildtype plants.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana L. Heynh cv. Columbia wildtype, TL46 starch-deficient mutant, and two series of up-regulated transgenic plants (T65, T66-3, T66-9, T67 and T68) having either starch-deficient TL46 background or wild type background were used in this study. Wildtype and TL46 mutant *Arabidopsis* seeds were obtained from The *Arabidopsis* Biological Resource Center, Ohio State University, USA. TL46 contains a missense mutation in the ApL1 gene, which encodes the dominant AGPase large subunit in leaf tissues (Crevillen *et al*. 2005, Ohdan *et al*. 2005, Lee *et al*. 2007). The mutation results in a G118E substitution and a loss of subunit stability (Wang *et al*. 1997). TL46

has only about 5% of AGPase activity and accumulates 40% of starch levels compared to wildtype (Lin *et al*. 1988b). *Arabidopsis ApL1* versions of the potato large subunit Upreg-1 (E38K) and Upreg-2 (G101N) genes (Greene *et al.* 1998) as well as a wildtype gene were introduced into the starch-deficient TL46 mutant which contains a defective large subunit gene (Gibson *et al.* 2003, Obana *et al.* 2006). Four up-regulated transgenic *Arabidopsis* lines along with one T65 control line having an intact wildtype AGPase were selected at the F_3 generation for further analysis (Figure 3). These backcrossed lines are called "TL46-background" transgenic plants. After crosses between wildtype Columbia and all TL46-background lines, the generated *Arabidopsis* lines are called "wildtype-background" transgenic lines. During the selection for homozygous plants, F_1 and F_2 candidates were grown under Okita lab's growth cabinet or the semi-controlled environmental greenhouse of the Institute of Biological Chemistry. The growth conditions of the greenhouse were 16-h photoperiod with natural light and/or supplemental high pressure sodium light, and 26/22^{o}C day/night temperatures. Fertilizer comprised of a solution containing Peter's 20-20-20, iron chelate, trace elements, magnesium sulfate and micronutrients was applied three times per week.

Sterilized seeds of F_3 plants were germinated on 1/2 Murashige-Skoog medium (1/2 MS mineral salts, 3% (w/v) sucrose, 3 mg/L Thiamine Hydrochloride, 5 mg/L Nicotinic acid, 0.5 mg/L Pyridoxine, 0.7% agarose, pH 5.8) for 7 days and then transplanted to potting soil in one gallon pots (one plant per pot). These plants were grown in controlled environmental growth chambers with a 12 h photoperiod and an incident photosynthetic quantum flux density (PPFD) of 450 μmol m^{-2} s^{-1}. Day and night temperatures were 23^{o}C and 18^{o}C, respectively, with 70% humidity in the growth

chambers. Peter's fertilizer 20-20-20 was applied to the plants twice a week.

Iodine staining for early selection

During the initial selection for up-regulated transgenic lines, all candidate *Arabidopsis* were grown in the Okita lab's growth cabinet at 100 PPFD with 18 h/6 h photoperiod and 23^{o}C/19^{o}C day/night temperatures. Rosette leaves at the same age were harvested at the end of light regime. The leaf samples were treated with 99.5% of ethanol for removal of carotenoids and chlorophylls, and then stained with iodine solution (0.06% I_2, 0.1% KI and 4 mM HCl).

AGPase enzyme assay

Leaf samples were collected at the end of the light period about 30-40 days after germination and stored at -20^{o}C freezer until used. The frozen tissues consisting of 0.35 cm^2 leaf discs harvested from young leaves (about 5 cm in length) for TL46-background and 0.1g leaf powders derived from whole plant leaves for wildtype-background lines, were homogenized in the presence of liquid nitrogen. The powdered tissue was incubated on ice for 30 min with extraction buffer (50 mM Hepes-NaOH pH 7.5, 5 mM $MgCl_2$, 5 mM DTT, 1 mM EDTA pH 8.0, 10% (v/v) Glycerol, 1 mM PMSF, 0.5 mM Leupeptin, 0.5 mM Pepstatin and 0.5 mM Benzamidine). The homogenate was centrifuged at 13,000 x g for 10 min at 4^{o}C and the supernatant collected and examined for enzyme activity. Protein concentrations were measured by a spectrophotometer at 595 nm using the Advanced Protein Assay reagent (5x concentrated, Cytoskelton Inc., USA). AGPase activity was determined in the reverse (pyrophosphorylysis) direction in the presence of 5 mM 3-PGA with/without 5 mM Pi using ^{32}PPi and ADP-glucose as

substrates as described previously (Hwang *et al*. 2007). One unit of activity is defined as the amount of enzyme that produced 1μmol of [^{32}P]ATP per minute.

Chlorophyll content

0.35 cm^2 frozen leaf discs collected from young leaves were harvested at the end of the light regime from plants about 35-40 days old. The leaf tissue was homogenized in 80% (v/v) acetone, centrifuged at 13,000 x g for 10 min at 4^{o}C and the resulting supernatant collected. After diluting with 80% (v/v) acetone, chlorophyll content was assessed by measuring the absorbance at 646.8 nm and 663.2 nm (HP 8452A Diode Array Spectrophotometer with HP 845x UV-visible system software, HP Chemstation or Smart SpecTM Plus spectrophotometer, Bio-Rad) and then the amount calculated using the equation described in Lichtenthaler and Wellburn (1983).

Starch determination

Whole plants were collected at the end of light and dark periods, respectively, weighed and ground to a fine powder under liquid nitrogen. 0.1 g of leaf powder was used for the measurement of leaf starch content. After removal of chlorophyll by repeated extraction with 80% (v/v) acetone, the starch-containing pellets were then washed with 50% (v/v) acetone, followed by dH_2O. The pellets were dried under vacuum. Dried pellets were boiled for 40 min at 90-95^{o}C in 1 ml acetate buffer (0.1 M sodium acetate and 0.1 N acetic acid). Starch was hydrolyzed and converted into glucose by overnight incubation at 40^{o}C using 3 units of α-amylase and 0.25 units of amyloglucosidase suspended in 200 ul of the acetate buffer. The concentration of glucose was determined with the Amplex-Red glucose assay kit (Molecular Probes, catalogue No. A22189). For leaf starch measurements, 4 to 5 individual plants per line

were used. Starch turnover was calculated from the average of starch levels at the end of the day and night periods.

Leaf gas exchange assay

Around 30-40 days after germination, the rate of CO_2 assimilation was measured with the FastEst gas system (Tartu, Estonia) using one of the youngest fully-expanded leaves (around 5 cm) as described in detail in Laisk and Edwards (1997). The measurement conditions of TL46- or wildtype-background *Arabidopsis* were slightly different. The light density in the leaf sample cuvette was 1400 PPFD for lines studied in the TL46-background *Arabidopsis* experiment, whereas it was 800 PPFD for lines studied in the wildtype-background experiment. The temperature during the measurements was 23-24^{o}C and humidity was 25%. Measurements were made at two levels of CO_2 (380 ppm and 800 ppm) and at 2% and 21% O_2 to investigate the effect of varying the sink capacity for starch synthesis. The gas exchange measurements were performed under steady-state conditions after illumination for 1 hour at maximum light density. After leaf gas exchange analysis, the leaf area was measured with a leaf area meter (Li-cor**,** LI-3000**,** Lambda Instrument. Corporation, USA). The percent stimulation of CO_2 assimilation by reduction from 21 to 2% O_2 was calculated from the rates of CO_2 assimilation at 21% ($A_{O21\%}$) versus 2% O_2 ($A_{O2\%}$) using the equation $[(A_{O2\%}/A_{O21\%}-1) \times 100]$.

Biomass measurements

Total leaf area was measured using a leaf area meter for TL46-background *Arabidopsis* lines or by imaging the leaves and then analyzing the image using image-J software (NIH, USA) for wildtype-background lines. Leaf dry weights were obtained

after drying the plants at 60°C for two weeks.

Statistical analysis

Analysis of variance (ANOVA) was done with the MINITAB software (Minitab Inc.). Tukey's multiple comparison tests were used to compare values between wildtype, TL46 mutant and up-regulated transgenic lines. All analyses were performed at $p \leq 0.05$ significance level.

Results

TL46-Background Transgenic Lines

Greater starch pool in TL46-background transgenic lines than wildtype and the parent TL46 mutant Under low light (100 PPFD) conditions, all TL46-background transgenic leaves including T65 were darkly stained by iodine solution (Figure 4). By contrast, wildtype and the parent TL46 mutant showed weaker staining and showed a light-brownish to tan color.

Increased AGPase activity by introduction of up-regulated AGPase ApL1 genes in the TL46 mutant In order to investigate changes due to introduction of the modified AGPases, the enzyme activities of five TL46-background transgenic lines along with wildtype and the parent TL46 mutant were determined using leaf samples collected at the initial bolting stage of plant development. When assayed in the presence of 3-PGA, an activator of AGPase, the enzyme activity in the transgenic lines was 1.8-3.5 fold higher than wildtype and more than 100-fold higher than the parent TL46 mutant (Table 1). Wildtype showed a significant reduction by Pi, an inhibitor of AGPase, (22% activity

remained), while transgenic lines had 28-43% remaining activity or 3-5 fold higher activity than wildtype. Contrary to my expectations, the T65 line containing a wildtype *ApL1* gene showed similar stimulated enzyme activity by 3-PGA and high residual activity in the presence of Pi similar to that seen in the up-regulated transgenic lines.

Similar CO_2 assimilation rates in wildtype, TL46 mutant, and TL46-backgound transgenic Arabidopsis CO_2 fixation depends on the intercellular concentration of CO_2 and O_2 around the Rubisco enzyme which catalyzes the first major step of the Calvin cycle. The CO_2 assimilation rates of wildtype, TL46 mutant and five TL46-background transgenic lines were determined under two levels of CO_2 (380 ppm and 800 ppm) and O_2 (2% and 21%) using 35-40 days old plants (Table 2). At ambient conditions (380 ppm CO_2 and 21% O_2), TL46 mutant showed a lower CO_2 assimilation rate (A = 8.9) than wildtype (A = 12.3). Despite the increased AGPase activity, CO_2 assimilation rates of up-regulated transgenic lines had a broad range between those of the parent TL46 mutant and wildtype (A = 8.9 to 12.3). Transgenic lines may be divided into two groups. CO_2 assimilation rates of T65, T66-9 and T68 lines were similar to that of wildtype, while T66-3 and T67 lines were similar to the parent TL46 mutant. Such a trend was also evident when CO_2 assimilation rates were measured at 800 ppm CO_2 and 2% O_2 conditions. To assess the relationship between the extent of feedback limitation on photosynthesis and the capacity for starch synthesis, the % stimulation of CO_2 assimilation rate by reducing O_2 levels from 21% to 2% between wildtype, TL46 mutant and transgenic lines were compared (Table 3). The parent TL46 mutant and T65 control had a slightly lesser enhancement compared to wildtype and up-regulated transgenic lines at the low CO_2 concentration. At the high CO_2 concentration, there is a significant

difference between TL46 which showed reversed sensitivity to O_2 and the other plants at the 95% confident level. Enhancement by low O_2 in wildtype and all transgenic lines were similar at high CO_2.

Higher leaf starch turnover in up-regulated leaf AGPase transgenic lines Leaf starch synthesis is highly regulated by AGPase in chloroplast. To determine the outcome of increased AGPase activity in TL46-background transgenic lines, leaf starch contents at the end of light and dark regimes were measured. Even though some transgenic lines retained more starch during the night, all up-regulated transgenic lines showed 12-95% higher starch turnover than wildtype and 3-5 fold greater than the parent TL46 mutant (Table 4). T65 control plants had similar turnover to wildtype. Among up-regulated transgenic lines, T66-3 and T67 lines which showed smaller CO_2 assimilation rates also had lower starch turnover than T66-9 and T68 lines which showed higher photosynthetic and starch turnover rates.

Two types of plant growth development observed in TL46-background transgenic plants In order to quantify the consequence of increased starch turnover by up-regulated AGPase, leaf area and leaf dry weight were measured using ~40 day-old wildtype, the parent TL46 mutant and TL46- background transgenic *Arabidopsis* lines (Table 5 and Figure 5). TL46 mutant has only 56% of leaf area and 38% of leaf dry weight compared to wildtype. There is also a significant difference from wildtype and transgenic lines at 95% confidence level. The transgenic lines, T65, T66-9 and T68 accumulated higher biomass than T66-3 and T67 lines consistent with results of photosynthesis and leaf starch turnover compared to wildtype (100%). Three high biomass lines, T65, T66-9 and T68, had 117-131% of leaf area and 105-126% of leaf dry

weight, while two low biomass strains, T66-3 and T67, were 87-100% and 82-90%, respectively.

Leaf type differentiation of TL46-background transgenic Arabidopsis Two leaf phenotypes were observed among TL46-background transgenic lines (Figure 6). T65, T66-9 and T68 showed a wildtype leaf phenotype where the leaves were thick and dark-green, whereas T66-3 and T67 had TL46 type leaves which were thinner and paler in color. The latter was due to the lower amounts of chlorophyll which was about 80% of wildtype (Table 6). Chlorophyll levels for T65, T66-9 and T68 were significantly higher than T66-3 and T67 at 95% confident level. TL46 and two transgenic lines had significantly less chlorophyll than wildtype.

Normalized data with chlorophyll content for TL46-background Arabidopsis

Results obtained using TL46-backgrond *Arabidopsis* might be affected by the difference of chlorophyll levels. Therefore, I normalized the data of gas exchange, leaf starch content, and biomass with the chlorophyll content. After normalization, all transgenic lines except T66-3 showed 10-20% higher CO_2 assimilation rate than wildtype, while the parent TL46 mutant had 8% lower rate of photosynthesis compared to wildtype (Table 7). With respect starch, the parent TL46 mutant has significantly less starch at both the end of day and end of night resulting only about 70% of leaf starch turnover compared to wildtype (Table 8). All up-regulated transgenic lines have 1.5-2.0 fold higher starch turnover, whereas T65 control plants showed similar turnover to wildtype. In addition, all transgenic lines including T65 had 17-58% greater leaf area and 10-43% higher leaf dry weight than wildtype (Table 9). In both measures of biomass, T66-9 and T68 lines were significantly greater than wildtype at 95% significant level. The parent TL46

mutant has only 72% of leaf area and 49% of leaf dry weight compared to wildtype.

Elimination of leaf type and chlorophyll differences In order to eliminate the differences in leaf type morphology, I crossed all transgenic lines to wildtype plants and selected homogeneous transgenic lines having the wildtype leaf type morphology. Newly generated wildtype-background transgenic F_3 lines had chlorophyll levels similar to that of wildtype (Table 10). The TL46 mutant with 76% of wildtype chlorophyll was significantly different compared to wildtype and transgenic lines at 95% confident level.

Wildtype-Background Transgenic Lines

Up-regulated AGPase activities in wildtype-background Arabidopsis AGPase activities of wildtype-background transgenic lines (T65, T66-3, T66-9, T67 and T68) along with wildtype and TL46 mutant, were determined using 30-35 day-old plants. AGPase activity of transgenic lines in the presence of 5 mM 3-PGA showed about 1.9-2.9 fold higher activities than that of wildtype (Table 11). TL46 has only 10% activity of wildtype. In contrast, AGPase activity of transgenic lines in the presence of 5 mM 3-PGA and 5 mM Pi showed a much higher activity (about 2.7-6.6 fold) than that of wildtype and up to 110 fold higher than TL46 mutant. Although AGPase activity of the T65 control lines was higher than wildtype and TL46 mutant under both effector conditions, T65 had a similar reduction in activity by Pi. About 40% of the enzyme activity was retained in the presence of Pi for enzyme both T65 and wildtype, while the up-regulated transgenic lines, T66-3, T66-9, T67 and T68, showed similar levels of activity in the presence or absence of Pi.

Increased AGPase activity leads to enhanced photosynthesis, leaf starch turnover and biomass without interrupting chlorophyll discrimination Gas exchange, leaf starch contents, leaf area and leaf dry weight of wildtype, TL46 mutant and wildtype-background transgenic lines were assayed just at the beginning of the bolting stage (30-35 days old). TL46 starch-deficient mutant had the lowest CO_2 assimilation rate (84%, Table 12), the lowest leaf starch turnover (72%, Table 13) and the smallest biomass (74% in leaf area and 59% in leaf dry weight, Table 14 and Figure 7). There was significant difference between TL46 mutant and other lines including wildtype in leaf starch and plant biomass. By contrast, the up-regulated transgenic plants having higher AGPase activities showed 5-11% enhanced CO_2 fixation, 16-46% increased starch turnover and 29-61% greater biomass compared to wildtype. T66-9 plants in rate of CO2 fixation, T67 and T68 lines in leaf area, and T68 plants in leaf dry weight were significantly greater than wildtype and the TL46 mutant at 95% significant level, respectively. There was no significant difference between wildtype and T65 control line in all the parameters measured.

Discussion

The effects on enzyme activity, leaf starch metabolism, photosynthesis and biomass by introduction of up-regulated AGPase were investigated in this study. Transgenic plants expressing up-regulated large subunit *ApL1* gene showed higher AGPase activity compared to wildtype and TL46 mutant forms under 3-PGA activation and/or Pi-inhibitory conditions. A positive control transgenic line expressing wildtype *ApL1* gene demonstrated similar inhibitor sensitivity to wildtype under Pi-inhibitory

conditions even if increased AGPase activity was observed as high as up-regulated transgenic plants in the presence of 3-PGA.

The response of photosynthesis to introduction of up-regulated AGPase was a relatively small effect compared to enzyme activity. CO_2 fixation is tightly regulated by multiple aspects including the levels of intercellular CO_2 and O_2, the activity of Rubisco enzyme and the amount of reducing power (e. g. NADPH and ferredoxin) in the chloroplast, and the capacity for synthesizing and utilizing sucrose. Almost all up-regulated transgenic lines except for TL46-background T66-3 line revealed up to 120% higher CO_2 assimilation rate compared to wildtype.

At the same time, enhancement of leaf starch turnover was observed in all up-regulated lines in both TL46- and wildtype-background *Arabidopsis*. Since AGPase is known to catalyze the rate-limiting step of starch biosynthesis, the increase leaf starch turnover among all up-regulated transgenic lines was expected.

During accumulation of transitory starch, pyrophosphate is generated from the synthesis of ADP-glucose via AGPase. Thus, increased starch synthesis in the up-regulated transgenic lines is expected to result in faster rate of pyrophosphate release which is then broken down to generate two Pi in the chloroplast stroma. The released Pi can then be utilized for photophosphorylation and RuBP formation. Triose-phosphate utilization is necessary for continuous plastidial Pi recycling and function of the Calvin cycle. In studies using the wildtype-background transgenic lines, high starch turnover lines such as T66-9 had an increased rate of CO_2 assimilation because of higher starch synthesis and higher rate of Pi recycling. By contrast, TL46 mutant having restricted AGPase activity and starch turnover have reduced capacity to utilize triose-phosphate,

lower rate of Pi recycling in the chloroplast stroma, lower rate of RuBP and ATP synthesis and decreased rate of photosynthesis. The latter is documented by the reversed sensitivity of photosynthesis of low O_2, a property symptomatic of feedback limitation (Sun *et al.* 1999b)(Table 3).

In addition to the regulation of photosynthesis, transitory starch plays an important role for plant development. Most of the leaf starch accumulated during the day is degraded the following night and utilized for maintenance of plant growth during the night. Thus, transgenic lines with up-regulated AGPase having higher leaf starch turnover exhibited greater leaf biomass than wildtype, TL46 mutant and T65 positive control *Arabidopsis*.

Unexpectedly, the TL46-background transgenic plants showed two leaf types, wildtype- versus TL46-leaf types. This difference was difficult to detect during the early selection under low light conditions, however it was very obvious when plants were grown under high light conditions. Photosynthesis, leaf starch turnover and biomass of TL46-background T66-3 and T67 lines seem to be substantially influenced by having a low chlorophyll level with TL46-leaf type.

The next possible limiting factor in TL46-leaf type plants is Rubisco in Calvin cycle which is the enzyme responsible for CO_2 fixation. Under hydroponic conditions with 300 PPFD, TL46 mutant has slightly less Rubisco activity and less protein than wildtype with ambient CO_2 and high nitrogen fertilizer treatment (Sun *et al*. 2002). Since the difference of leaf thickness were observed between wildtype and TL46 leaves in this experiment, smaller total leaf mass of TL46 mutant would also affect the contents of Rubisco and chlorophyll. Sun *et al*. (2002) and Obana *et al*. (2006) did not report on

different leaf types, so this phenotype difference may be induced by high light, greater than 450 PPFD. Initial studies with the two leaf types showed all up-regulated transgenic plants but T66-3 had higher CO_2 assimilation rates than wildtype and TL46 mutant when normalized on a chlorophyll basis (Table 7). However, the results showed it is important to work with the same leaf type in order to study the consequence of increasing capacity for AGPase and starch biosynthesis on photosynthesis and growth.

The results of this experiment support to the hypothesis that increased synthesis and turnover of starch by introduction of up-regulated AGPase enzyme contributed to enhance photosynthesis and increase plant biomass. This research assessed only the effect on vegetative tissue. As the next assignment, the influence on reproductive organs such as seeds should be analyzed in order to obtain more depth-insight in the role of transitory starch on plant growth and reproductive development.

References

Crevillén, P., M. A. Ballicora, *et al*. (2003). "The different large subunit isoforms of *Arabidopsis thaliana* ADP-glucose pyrophosphorylase confer distinct kinetic and regulatory properties to the heterotetrameric enzyme." J Biol Chem 278(31): 28508-15.

Crevillén, P., T. Ventriglia *et al*. (2005). "Differential pattern of expression and sugar regulation of *Arabidopsis thaliana* ADP-glucose pyrophosphorylase-encoding genes." J Biol Chem 280(9): 8143-9.

Fondy, B. R., and D. R. Geiger (1982) **"**Diurnal pattern of translocation and carbohydrate metabolism in source leaves of *Beta vulgaris* L." Plant Physiol 70:671-676

Gerhardt, R., M. Stitt, *et al.* (1987). "Subcellular metabolite levels in spinach leaves. " Plant Physiol. 83: 399-407.

Gibson, K. M., S. K. Hwang, et al. (2003). "Metabolic engineering of starch for enhanced plant productivity and yields." J Applied Glycoscience 50: 201-206.

Greene, T. W., I. H. Kavakli, *et al*. (1998). "Generation of up-regulated allosteric variants of potato ADP-glucose pyrophosphorylase by reversion genetics." Proc Natl Acad Sci U S A 95(17): 10322-7.

Hwang, S. K., S. Hamada, *et al*. (2007) "Catalytic implications of the higher plant ADP-glucose pyrophosphorylase large subunit." Hytochemistry 68(4):464-77

Laisk, A. and G. E. Edwards (1997). "CO_2 and temperature-dependent induction in C4 photosynthesis: An approach to the hierarchy of rate-limiting processes." Aust J Plant Physiol 24: 505-516.

Lee, S. K., S. K. Hwang, *et al.* (2007). "Identification of the ADP-glucose

pyrophosphorylase isoforms essential for starch synthesis in the leaf and seed endosperm of rice (Oryza sativa L.).” Plant Mol Biol. 65(4): 531-46.

Lichtenthaler, H. K. and A. R. Wellburn (1983). "Determination of total carotenoids and chlorophylls a and b of leaf extracts in different solvents." Biochem Soc Trans 11: 591–592.

Lin, T. P., T. Caspar, *et al*. (1988a). "Isolation and characterization of a starchless mutant of *Arabidopsis thaliana* (L.) Heynh lacking ADP-glucose pyrophosphorylase activity." Plant Physiol 86: 1131-1135.

Lin, T. P., T. Caspar, *et al*. (1988b). "A starch deficient mutant of *Arabidopsis thaliana* with low ADP-glucose pyrophosphorylase activity lacks one of the two subunits of the enzyme." Plant Physiol 88: 1175-1181.

Obana, Y., D. Omoto, *et al*. (2006). "Enhanced turnover of transitory starch by expression of up-regulated ADP-glucose pyrophosphorylases in *Arabidopsis thaliana*." Plant Science 170: 1-11.

Ohdan, T., P. B. Jr Francisco, *et al*. (2005). “Expression profiling of genes involved in starch synthesis in sink and source organs of rice.” J Exp Bot 56(422): 3229-44.

Sun, J., K. M. Gibson, *et al*. (2002). "Interactions of nitrate and CO_2 enrichment on growth, carbohydrates, and rubisco in Arabidopsis starch mutants. Significance of starch and hexose." Plant Physiol 130(3): 1573-83.

Sun, J., T. W. Okita, *et al*. (1999a). "Modification of carbon partitioning, photosynthetic capacity, and O_2 sensitivity in Arabidopsis plants with low ADP-glucose pyrophosphorylase activity." Plant Physiol 119(1): 267-76.

Sun, J., G. E. Edwards, et al. (1999b). “Feedback inhibition of photosynthesis in rice measured by O_2 dependent transients”. Photosynthesis Res 59(2-3): 187-200.

Wang, S. M., B. Chu, *et al*. (1997). "*adg2-1* represents a missense mutation in the ADPG pyrophosphorylase large subunit gene of *Arabidopsis thaliana*." Plant J 11(5): 1121-6.

Zeeman, S. C., F. Northrop, *et al.* (1998) “A starch-accumulating mutant of Arabidopsis thaliana deficient in a chloroplastic starch-hydrolysing enzyme.” Plant J. 15(3): 357-65.

Figure Legends

Figure 3. Structure of the *ApL1* gene, which encodes the large subunit of AGPase, in the *Arabidopsis* transgenic lines. The *ApL1* gene was engineered by site-directed mutagenesis and transformed into the *Arabidopsis* starch-deficient TL46 mutant. T65 line was TL46 transformed with wildtype *ApL1* gene. T66 and T67 lines had single mutations replacing Ala99 with Lys, and Gly162 with Asn, respectively, in *ApL1*. T68 expresses an *ApL1* gene containing a double mutation A99K and G162N. Pnos: promoter of nopaline synthase gene, Km^R: kanamycin resistance gene, Tnos: terminator of nopaline synthase gene, P35S: 35S mRNA promoter of cauliflower mosaic virus, Hyg^R: hygromycin resistance gene, BR and BL: right and left border, respectively.

Figure 4. Iodine staining of wildtype, TL46 mutant and backcrossed transgenic lines. Rosetta leaves of wildtype, TL46 mutant and up-regulated leaf AGPase (TL46-background) transgenic lines grown under low light conditions were stained by iodine solution after removal of color pigments. All transgenic lines showed darker iodine staining compared to wildtype and TL46.

Figure 5. Images of wildtype, TL46 mutant and backcrossed transgenic lines. Images of about 35 day-old plants were taken for wildtype, TL46 mutant and up-regulated leaf AGPase (TL46-background) transgenic lines.

Figure 6. Leaf morphology of wildtype and TL46 mutant *Arabidopsis*. Images of fully expanded mature leaves were taken for wildtype and TL46 mutant.

Figure 7. Images of wildtype, TL46 mutant and wildtype-background transgenic lines. Images of about 30 day-old plants were taken for wildtype, TL46 mutant and up-regulated leaf AGPase transgenic lines.

Figure 3

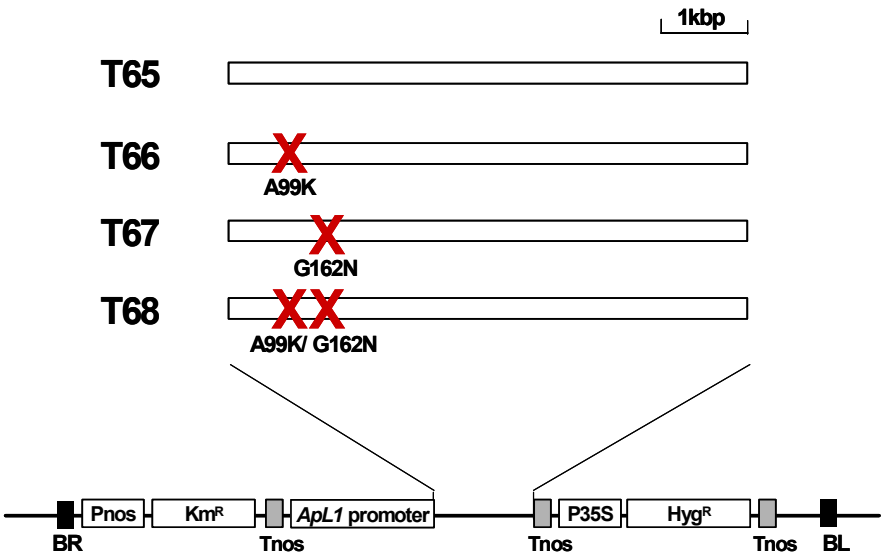
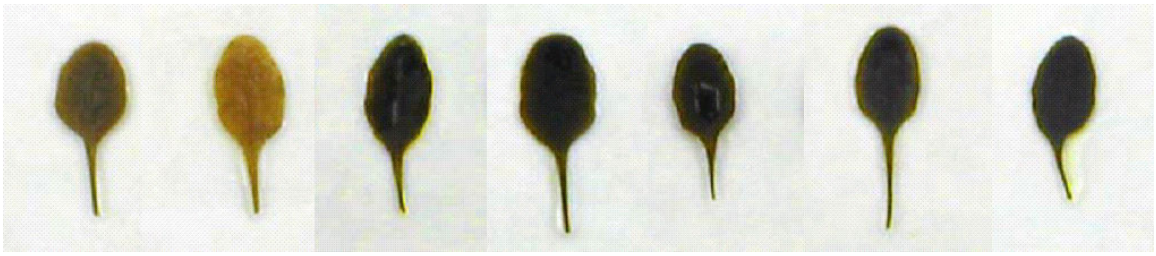

1kbp
T65
T66
A99K
T67
G162N
T68
A99K/ G162N
Pnos
KmR
ApL1 promoter
P35S
HygR
BR
Tnos
Tnos
Tnos
BL

Figure 4



WT TL46 T65 T66-3 T66-9 T67 T68

Table 1. AGPase activity of wildtype, TL46 mutant and backcrossed transgenic lines. AGPase activity under 3-PGA activation and Pi-inhibitory conditions was determined using leaf crude extracts prepared from about 40 day-old wildtype, TL46 mutant and up-regulated leaf AGPase (TL46-background) transgenic lines. Mean values ±SE were calculated from five plants per lines. * and ** denote a significant statistical difference at the 95% confidence level compared to wildtype.

Lines	3-PGA		3-PGA, Pi	
	μmol min^{-1} mg^{-1}	%	μmol min^{-1} mg^{-1}	%
WT	0.035 ± 0.009	100	0.008 ± 0.001	100
TL46	0.001 ± 0 **	2	0.001 ± 0.001	14
T65	0.080 ± 0.005 *	227	0.028 ± 0.003 *	354
T66-3	0.064 ± 0.006	183	0.025 ± 0.003 *	316
T66-9	0.090 ± 0.009 *	257	0.039 ± 0.004 *	495
T67	0.124 ± 0.003 *	354	0.035 ± 0.002 *	449
T68	0.107 ± 0.012 *	306	0.037 ± 0.005 *	468

Table 2. Photosynthesis of wildtype, TL46 mutant and backcrossed transgenic lines. CO_2 assimilation rate was measured in 35 to 40 day-old wildtype, TL46 mutant and up-regulated leaf AGPase (TL46-background) transgenic lines. Measurement was performed under two levels of CO_2 (380ppm and 800ppm) and O_2 (2% and 21%) at 23-24^oC with 1400 PPFD saturating light conditions. Mean values ±SE were calculated from three to six plants per lines. ** denotes a significant statistical difference at the 95% confidence level compared to wildtype.

Lines	380 ppm CO_2				800 ppm CO_2			
	21% O_2		2% O_2		21% O_2		2% O_2	
	µmol m^{-2} s^{-1}	%	µmol m^{-2} s^{-1}	%	µmol m^{-2} s^{-1}	%	µmol m^{-2} s^{-1}	%
WT	12.3 ± 0.6	100	15.0 ± 0.8	100	18.4 ± 0.8	100	20.2 ± 0.5	100
TL46	8.9 ± 0.5 **	72	10.1 ± 0.6 **	67	12.3 ± 0.6 **	67	10.6 ± 0.1 **	53
T65	12.5 ± 0.5	101	14.5 ± 0.7	97	18.0 ± 0.5	98	19.8 ± 0.6	98
T66-3	8.9 ± 0.4 **	72	11.1 ± 0.6 **	74	14.5 ± 0.5 **	79	15.6 ± 0.7 **	77
T66-9	12.3 ± 0.2	100	15.4 ± 0.5	103	19.8 ± 0.3	107	21.3 ± 0.5	105
T67	10.0 ± 0.7	81	12.8 ± 0.6	85	15.7 ± 0.4 **	85	17.3 ± 0.5	85
T68	11.4 ± 0.6	92	13.7 ± 0.7	91	17.5 ± 0.6	95	19.1 ± 0.5	95

Table 3. Stimulation of CO_2 assimilation by 2% O_2 in wildtype, TL46 mutant and backcrossed transgenic lines. The sensitivity of photosynthesis when O_2 was reduced from 21% to 2% was calculated from CO_2 assimilation rates of wildtype, TL46 mutant and up-regulated leaf AGPase (TL46-background) transgenic lines. The percent stimulation was calculated from the rates of CO_2 assimilation at 21% ($A_{O21\%}$) versus 2% O_2 ($A_{O2\%}$) using the equation [($A_{O2\%}/A_{O21\%}$-1) x 100]. Mean values ±SE were calculated from three to six plants per lines. ** denotes a significant statistical difference at the 95% confidence level compared to wildtype.

Lines	380 ppm CO_2	800 ppm CO_2
WT	22.0 ± 5.5	9.9 ± 2
TL46	13.9 ± 2.7	-13.3 ± 2.7 **
T65	16.7 ± 2.6	9.7 ± 2
T66-3	25.6 ± 4.3	7.8 ± 2.5
T66-9	25.7 ± 3	7.5 ± 2.9
T67	28.4 ± 3.5	10.0 ± 1.9
T68	20.5 ± 2.3	9.3 ± 1.7

Table 4. Leaf starch content of wildtype, TL46 mutant and backcrossed transgenic lines. The amount of leaf starch at end of day/night was measured in 30 day-old wildtype, TL46 mutant and up-regulated leaf AGPase (TL46-background) transgenic lines. Mean values ±SE were calculated from four to five plants per lines. * and ** denote a significant statistical difference at the 95% confidence level compared to wildtype.

Lines	End of night	End of day	Turnover	
		μmol glc. plant^{-1}		%
WT	17 ± 3	103 ± 13	86	100
TL46	5 ± 1 **	40 ± 3 **	35	41
T65	13 ± 1	94 ± 9	81	94
T66-3	25 ± 4	121 ± 11	96	112
T66-9	22 ± 2	163 ± 9 *	141	164
T67	13 ± 2	117 ± 4	104	121
T68	42 ± 4 *	210 ± 14 *	168	195

Table 5. Biomass measurements of wildtype, TL46 mutant and backcrossed transgenic lines. Leaf area and leaf dry weight were determined from about 40 day-old wildtype, TL46 mutant and up-regulated leaf AGPase (TL46-background) transgenic lines. Mean values ±SE were calculated from four to five plants per lines. ** denotes a significant statistical difference at the 95% confidence level compared to wildtype.

Lines	Leaf area		Leaf dry weight	
	cm^2 $plant^{-1}$	%	mg $plant^{-1}$	%
WT	124 ± 8	100	373 ± 19	100
TL46	70 ± 7 **	56	143 ± 14 **	38
T65	145 ± 12	117	391 ± 38	105
T66-3	124 ± 9	100	336 ± 19	90
T66-9	157 ± 14	127	470 ± 42	126
T67	108 ± 8	87	305 ± 23	82
T68	162 ± 9	131	440 ± 17	118

Figure 5

Figure 6

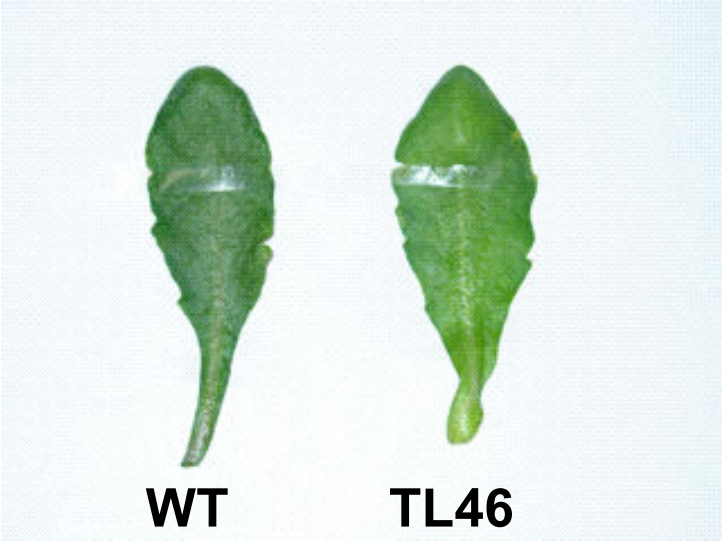


Table 6. Chlorophyll content of wildtype, TL46 mutant and backcrossed transgenic lines. The amount of chlorophyll in leaves was measured from about 40 day-old wildtype, TL46 mutant and up-regulated leaf AGPase (TL46-background) transgenic lines. Mean values ±SE were calculated from three to four plants per lines. ** denotes a significant statistical difference at the 95% confidence level compared to wildtype.

Lines	mg Chl m^{-2}	%
WT	763 ± 32	100
TL46	597 ± 37 **	78
T65	651 ± 20	85
T66-3	564 ± 28 **	74
T66-9	635 ± 42	83
T67	565 ± 45 **	74
T68	631 ± 4	83

Table 7. Normalized CO_2 assimilation rate of wildtype, TL46 mutant and backcrossed transgenic lines.

Lines	21% O_2, 380 ppm CO_2	
	μmol s^{-1} g^{-1} Chl	%
WT	16.2 ± 0.8	100
TL46	14.9 ± 0.8	92
T65	19.1 ± 0.8	118
T66-3	15.7 ± 0.8	97
T66-9	19.3 ± 0.3	120
T67	17.8 ± 1.2	110
T68	18.0 ± 0.9	111

Table 8. Normalized leaf starch content of wildtype, TL46 mutant and backcrossed transgenic lines. The statistical analysis was performed using the values of end of night/day. * and ** denote a significant statistical difference at the 95% confidence level compared to wildtype. Turnover of the leaf starch was subtracted from the average value of day to that of night.

Lines	End of night	End of day	Turnover	
	mmol glc. g^{-1} Chl			%
WT	3.5 ± 0.4	22.2 ± 0.9	18.7	100
TL46	1.3 ± 0.2 **	13.5 ± 0.7 **	12.3	66
T65	2.6 ± 0.1	22.2 ± 1.4	19.6	105
T66-3	5.6 ± 0.6 *	38.1 ± 1.0 *	32.5	173
T66-9	5.1 ± 0.3 *	34.6 ± 1.5 *	29.5	158
T67	3.3 ± 0.2	31.8 ± 1.2 *	28.5	152
T68	7.4 ± 0.3 *	44.7 ± 2.5 *	37.3	199

Table 9. Normalized biomass measurements of wildtype, TL46 mutant and backcrossed transgenic lines. Correction factors for chlorophyll were calculated from the values of chlorophyll contents (Chl) using the equation of [Chl of wildtype/ Chl of individual lines]. * and ** denote a significant statistical difference at the 95% confidence level compared to wildtype.

Lines	Correction factor for Chl	Leaf area		Leaf dry weight	
		cm^2 $plant^{-1}$	%	mg plant-1	%
WT	1.00	124 ± 8	100	373 ± 19	100
TL46	1.28	89 ± 8	72	183 ± 18 **	49
T65	1.17	170 ± 14	136	458 ± 44	123
T66-3	1.35	167 ± 12	135	455 ± 26	122
T66-9	1.20	188 ± 17 *	151	565 ± 50 *	151
T67	1.35	146 ± 11	117	412 ± 31	110
T68	1.21	196 ± 11 *	158	532 ± 20 *	143

Table 10. Chlorophyll content of wildtype, TL46 mutant and wildtype-background transgenic lines. The amount of chlorophyll in leaves was measured from about 35 day-old wildtype, TL46 mutant and up-regulated leaf AGPase transgenic lines. Mean values ±SE were calculated from three to five plants per lines. ** shows that there is a significant statistical difference at the 95% confidence level compared to wildtype.

Lines	mg Chl m^{-2}	%
WT	812 ± 44	100
TL46	619 ± 11 **	76
T65	787 ± 25	97
T66-3	790 ± 44	97
T66-9	817 ± 27	101
T67	764 ± 59	94
T68	829 ± 57	102

Table 11. AGPase activity of wildtype, TL46 mutant and wildtype-background transgenic lines. AGPase activity under 3-PGA activation and Pi-inhibitory conditions was determined using leaf crude extracts prepared from about 30 day-old wildtype, TL46 mutant and up-regulated leaf AGPase transgenic lines. Mean values ±SE were calculated from five plants per lines. * and ** denote a significant statistical difference at the 95% confidence level compared to wildtype.

Lines	3-PGA		3-PGA, Pi	
	μmol min^{-1} mg^{-1}	%	μmol min^{-1} mg^{-1}	%
WT	0.055 ± 0.004	100	0.023 ± 0.003	100
TL46	0.005 ± 0.005 **	10	0.001 ± 0	6
T65	0.159 ± 0.012 *	291	0.062 ± 0.017	268
T66-3	0.128 ± 0.003 *	234	0.120 ± 0.018 *	517
T66-9	0.131 ± 0.006 *	240	0.136 ± 0.004 *	587
T67	0.104 ± 0.013 *	190	0.102 ± 0.012 *	441
T68	0.123 ± 0.013 *	223	0.154 ± 0.016 *	661

Table 12. Photosynthesis of wildtype, TL46 mutant and wildtype-background transgenic lines. CO_2 assimilation rate was measured in 30 to 35 day-old wildtype, TL46 mutant and up-regulated leaf AGPase transgenic lines. Measurements were performed under two levels of CO_2 (380ppm and 800ppm) and O_2 (2% and 21%) at 23-24^oC with 800 PPFD saturating light conditions. Mean values ±SE were calculated from seven plants per lines. ** denotes a significant statistical difference at the 95% confidence level compared to wildtype.

Lines	380 ppm CO_2				800 ppm CO_2			
	21% O_2		2% O_2		21% O_2		2% O_2	
	µmol m^{-2} s^{-1}	%	µmol m^{-2} s^{-1}	%	µmol m^{-2} s^{-1}	%	µmol m^{-2} s^{-1}	%
WT	9.9 ± 0.3	100	14.0 ± 0.2	100	17.5 ± 0.3	100	19.0 ± 0.6	100
TL46	8.3 ± 0.5	84	9.9 ± 0.6 **	71	10.9 ± 0.7 **	62	9.4 ± 0.8 **	49
T65	10.3 ± 0.5	104	13.0 ± 0.6	93	16.2 ± 0.7	93	18.3 ± 1.1	96
T66-3	11.0 ± 0.2	111	14.1 ± 0.4	101	16.9 ± 0.5	97	18.3 ± 0.3	96
T66-9	11.9 ± 0.4 *	120	15.3 ± 0.6	109	18.1 ± 0.8	103	19.4 ± 0.8	102
T67	10.4 ± 0.2	105	13.8 ± 0.3	99	16.7 ± 0.3	95	19.0 ± 0.5	100
T68	10.5 ± 0.5	106	13.8 ± 0.5	99	16.5 ± 0.8	94	17.9 ± 0.8	94

Table 13. Leaf starch content of wildtype, TL46 mutant and wildtype-background transgenic lines. The amount of leaf starch at end of day/night was measured in 30 day-old wildtype, TL46 mutant and up-regulated leaf AGPase transgenic lines. Mean values ±SE were calculated from five plants per lines. Turnover of the leaf starch was subtracted from the average value of day to that of night. * and ** denote a significant statistical difference at the 95% confidence level compared to wildtype.

	End of night	End of day	Turnover	
Lines	μmol glc. plant^{-1}			%
WT	51 ± 3	209 ± 3	158	100
TL46	13 ± 0 **	127 ± 8 **	114	72
T65	27 ± 4	180 ± 8	153	97
T66-3	102 ± 3 *	312 ± 11 *	210	133
T66-9	68 ± 8	298 ± 14 *	231	146
T67	62 ± 10	246 ± 15	184	116
T68	85 ± 4 *	289 ± 19 *	204	129

Table 14. Biomass measurements of wildtype, TL46 mutant and wildtype-background transgenic lines. Leaf area and leaf dry weight were determined from about 35 day-old wildtype, TL46 mutant and up-regulated leaf AGPase transgenic lines. Mean values ±SE were calculated from three to five plants per lines. * and ** denote a significant statistical difference at the 95% confidence level compared to wildtype.

Lines	Leaf area		Leaf dry weight	
	cm^2 $plant^{-1}$	%	mg $plant^{-1}$	%
WT	160 ± 8	100	360 ± 21	100
TL46	90 ± 4 **	57	164 ± 12 **	46
T65	148 ± 11	93	328 ± 35	91
T66-3	204 ± 16	128	454 ± 41	126
T66-9	223 ± 18	140	495 ± 49	138
T67	242 ± 14 *	151	498 ± 26	138
T68	258 ± 12 *	162	545 ± 27 *	151

Figure 7

CHAPTER THREE

Control of starch synthesis in cereals: metabolite analysis of transgenic rice expressing up-regulated cytoplasmic ADP-glucose pyrophosphorylase in developing seeds

Abstract

We have previously demonstrated that expression of a cytoplasmic-localized (but not a plastid-localized) ADP-glucose pyrophosphorylase (AGPase) mutant gene from *Escherichia coli* in rice endosperm resulted in enhanced starch synthesis and, in turn, higher seed weights (Sakulsingharoj *et al.* 2004). In this study, the levels of the major primary carbon metabolites were assessed for wildtype and four transgenic CS 8 rice lines expressing 3- to 6-fold higher AGPase activity when measured under inorganic phosphate (Pi)-inhibitory conditions. As seen earlier for mature seeds, developing seeds from the various CS 8 lines showed a 7 to 24% increase in grain weight compared to wildtype control. Consistent with the increase in AGPase activity, all four transgenic CS 8 lines showed elevated levels of ADP-glucose although the extent of increases in this metabolite were much higher than the extent of increases in starch as measured by seed weight. Surprisingly, the levels of several other key intermediates were significantly altered. Glucose 1-phosphate, a substrate of the AGPase reaction, as well as UDP-glucose and glucose 6-phosphate were also elevated in the transgenic lines, and analysis of metabolite ratios indicated reactions were near equilibrium. Moreover, glucose and fructose levels were also elevated in three transgenic lines that showed the largest differences in metabolites and seed weight over wildtype suggesting the induction of invertase. Overall, the results indicate that the AGPase catalyzed reaction is no longer

limiting in the transgenic lines, and constraints on carbon flux into starch are downstream of ADP-glucose formation, resulting in an elevation of precursors upstream of ADP-glucose formation.

Introduction

Global improvement of plant productivity and crop yield is a high priority to meet the challenge of feeding the world's growing population which is expected to reach 8 billion by the year 2020 (Choi *et al.* 1998). Crop yield is dependent on many factors including environmental conditions, nutritional inputs, and the genetic potential for plant growth and development. The latter is controlled by source-sink relationships where sugars and amino acids generated by the photochemical and dark reactions of photosynthesis and nitrogen metabolism in source leaves are transported and assimilated by heterotrophic sink organs (Edwards *et al.* 2000, Okita *et al.* 2001, Tegeder and Weber 2006). During the vegetative stage, most of the photoassimilates (e.g. sucrose and amino acids) are utilized for plant growth and development. However, during the reproductive stage, plants convert the bulk of the photosynthate into storage reserves as proteins, carbohydrates and lipids. Therefore, the capacity for synthesis of starch, proteins, and lipids in seeds of staple economic crops, like rice, maize and wheat, is an important consideration for enhancing yield.

In cereals, the principal storage reserve is starch which constitutes ~78% of the dry weight of the grain (Singh and Juliano 1977). One key regulatory step that controls the flux of carbon into starch is catalyzed by ADP-glucose pyrophosphorylase (Hannah 1997, Preiss *et al.* 1991, Sakulsingharoj *et al.* 2004, Slattery *et al.* 2000, Smith *et al.* 1997). In photosynthetic tissues, the catalytic activity of this enzyme is controlled by its allosteric regulatory responses to the activator 3-PGA and inhibitor Pi. Several of the AGPase isoforms are also finely regulated by redox control where the catalytic activity of the oxidized form is suppressed compared to the reduced form of the enzyme at low 3-PGA

levels (Tiessen *et al.* 2002). In most tissues where starch is synthesized, AGPase is localized in the chloroplast or amyloplast, the site of starch synthesis and accumulation. The exception is in starch synthesis in amyloplasts of the cereal endosperm where two forms of the enzyme are present: a major cytoplasmic form and a minor plastidal form (Denyer *et al.* 1996, Sikka *et al.* 2001, Thorbjornsen *et al.* 1996).

In previous studies from our laboratories, we introduced the *E. coli glgC* triple mutant (*glgC*-TM) gene into rice and targeted expression to either the cytoplasm or amyloplasts of developing endosperm (Sakulsingharoj *et al.* 2004). This mutant AGPase contains three amino acid replacements, R67K, P295D, and G366D which renders the enzyme essentially independent of allosteric regulation, *i.e.* does not require any activators for catalytic activity and is insensitive to the inhibitor Pi (Preiss *et al.* 1994). When assessed under Pi-inhibitory conditions to suppress the activity of the endogenous AGPase catalytic activity, the transgenic plants showed up to 13-fold higher AGPase activity. However, only transgenic plants expressing the *glgC*-TM in the cytoplasm (CS 8 lines) and not the amyloplast had a higher rate of conversion of sucrose into starch, and bore seeds having up to 11% increase grain weight compared to the wildtype control (Sakulsingharoj *et al.* 2004). These results demonstrate that the AGPase reaction is one of the limiting factors of starch biosynthesis and that the carbon flux into starch can be enhanced by increasing the net catalytic activity of this enzyme. Moreover, the results also supported the view that the major pathway leading to ADP-glucose synthesis and, in turn, starch biosynthesis, is cytoplasmic.

Results from CO_2 enrichment experiments suggested that ADP-glucose formation in the CS 8 transgenic rice plants was no longer a limitation in starch synthesis and that

one or more other steps was limiting (Sakulsingharoj *et al.* 2001). To gain further insight on this process, we analyzed the levels of the major carbon metabolites in selected transgenic CS 8 transgenic lines and wildtype. As expected, ADP-glucose levels were elevated in the transgenic lines, which accounted for the increase in grain weights of mid-developing and mature seeds. Interestingly, the precursor metabolites, glucose 1-phosphate and UDP-glucose, that lead to ADP-glucose, were also elevated to the same extent, indicating that ADP-glucose may be reaching saturating levels and that the transport or utilization of this sugar nucleotide is the major downstream limitation in starch synthesis.

Materials and Methods

Plant materials and growth conditions

Wildtype rice (*Oryza sativa L.* cv Kitaake) and four CS 8 T5 rice (CS 8-3, CS 8-13, CS 8-18 and CS 8-29) having up-regulated cytoplasm-targeted *E. coli glgC*-TM gene (Figure 3-1) were grown in a controlled environmental growth chamber with 12h-photoperiods, at 26^{o}C during the day and 22^{o}C during the night. Photosynthetic photon flux density of the growth chambers was 1000 μmol m^{-2} s^{-1} and relative humidity was 80%. Rice plants were germinated in water for 10 days after sterilizing seeds with 50% bleach, and then transplanted one plant per 2 gallon pot. Fertilizer [1 part Calcium Nitrate (15.5-0-0), 2 parts Hi Phos Special (15-30-15), ½ part Magnitrate (10-0-0 + Magnesium), ½ part Chelated Iron (Fe 330), ¼ part Soluble trace element mix (all micronutrients)] was applied twice per week. In addition, 1 mM potassium phosphate was applied once per week to prevent phosphate deficiency.

AGPase activity assay

Approximately 13 days after flowering, 10 immature rice seeds were selected and ground in the presence of liquid nitrogen, and then 1 ml of the extraction buffer [20% (w/v) sucrose, 50 mM Hepes-NaOH, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 1 mM PMSF, 1 μg/ml pepstatin, and 1 μg/ml leupeptin] was added. The homogenate was centrifuged at 12,000 x g for 15 min at 4°C and the supernatant was immediately used for assay of AGPase activity.

AGPase activity was assayed in the pyrophosphorolysis direction, which measures the formation of [^{32}P] ATP from ^{32}PPi. The reaction mixture contained 100 mM Hepes-NaOH, pH 7.5, 5 mM DTT, 7 mM $MgCl_2$, 10 mM NaF, 1 mM ADP-glucose, 0.4 mg/ml BSA, 1.5 mM NaPPi and 2000-2500 cpm/ml ^{32}PPi in the presence or absence of 10 mM potassium phosphate. The reaction was initiated by adding 5 μl crude protein extract and then incubated for 10 min at 37°C. The amount of ATP formed was measured as described in Laughlin *et al.* (1998). Protein concentrations were determinated using Advanced protein assay reagent (5x concentrated, Cytoskelton Inc., USA).

Determination of biomass and seed morphology

During the developing reproductive stage (about 13 days after anthesis), 25 immature seeds per plant were weighed and stored for metabolite analysis. At maturity, all panicles of individual plants were harvested and air-dried at room temperature. From the first 3 to 5 panicles to flower, 100 seeds were obtained per panicle, and weighed in order to calculate the average seed weight of each line. The weight of intact grains, seeds with the seed coats removed, hulls (seed coats), and the height, width and thickness

of intact grains and seeds were measured. Images of wildtype and CS 8 rice seeds without seed coat were taken using ProgRes® C12 ^{Plus}Camera and ProgRes® Capture Pro (Version 2.0) software (Germany).

LC-MS/MS metabolite analysis

Twenty-five developing seeds (about 13 days after anthesis) were collected from each plant, frozen in liquid nitrogen and then stored at -80^{o}C. Seed samples were ground to a fine powder in liquid nitrogen, and then lyophilized for 2.5 days (Virtis, Gardener, NY).

The samples were processed and analyzed by LC-MS/MS performed on an Agilent 1100 liquid chromatograph interfaced to an Applied Biosystems Sciex^{TM} 3000 triple quadrapole tandem mass spectrometer equipped with a TurboIon^{TM} Spray inlet as described by Smidansky *et al.* (2006). Quantitation was achieved by creating a calibration curve for each targeted metabolite and using linear regression analysis on each extract.

Statistical analysis

Analysis of variance (ANOVA) was done with the MINITAB software (Minitab Inc.). Dunnet's multiple comparison tests were used to compare between wildtype and CS 8 transgenic lines. All analyses were performed at $p \leq 0.05$ significance level.

Results

AGPase activity of wildtype and CS 8 rice

Four independent, homozygous transgenic CS 8 lines expressing a cytoplasmic *E. coli* AGPase TM enzyme as well as wildtype plants were grown to maturity under

controlled environmental conditions. Samples of mid-developing seeds from three plants of each line were collected and assayed for AGPase activity. When assayed under fully activating conditions in the absence of Pi, the transgenic lines showed a 1.7 to 2.8 fold increase in activity. When measured in the presence of Pi to suppress the catalytic activity of the endogenous endosperm AGPase, the various CS 8 lines showed a 3- to 6-fold increase in AGPase activity compared to wildtype plants (Figure 9). As these transgenic plants were fifth generation progeny from the initial transformation event, expression of the integrated transgene in each of these CS 8 lines was stable and not subjected to gene silencing.

Comparison of seed weight and seed morphology with wildtype and CS 8 rice

The effect of enhanced cytoplasmic AGPase activity on starch synthesis during seed maturation was evaluated by measuring the weights of developing and mature grains. From analysis during the developing stage (13 days after flowering), all of the CS 8 rice lines showed an increase in total seed weight varying from 7 to 24% as compared to wildtype (Figure 10). Likewise, mature seeds from the various transgenic lines showed a significant increase in grain weight (7 to 15%) compared to wildtype (Table 15). The increase in grain weight observed for the transgenic plant lines was accompanied by an increase in grain size (Table 16) and volume (Figure 11). Interestingly, these increases were reflected not only in the seed themselves, but also in the seed coat, where the weight of the outer seed hulls was observed to be elevated in the transgenic lines.

Carbon metabolism in upregulated cytoplasmic AGPase rice

The major primary carbon metabolites were analyzed in developing seeds of

wildtype and the four independent CS 8 transgenic rice lines (Table 17). Each metabolite value was an average of the quantities measured from samples of developing seeds derived from three plants of each CS 8 transgenic line. As expected ADP-glucose, the product of AGPase activity, showed a significant increase in all of the transgenic lines, CS 8-3, CS 8-13, CS 8-18 and CS 8-29, compared to wildtype. This increase in ADP-glucose levels, however, was not proportional to the increase in levels of AGPase activity measured in the various transgenic lines. Although CS 8-18 had nearly twice the levels of AGPase activity than CS 8-29, it had lower levels of ADP-glucose, and as noted earlier, lower seed weight than other transgenic lines. In general, higher ADP-glucose levels corresponded to higher seed weight. The lowest (29%) and highest (46%) increases in ADP-glucose levels were seen for CS 8-18 and CS 8-29, respectively.

Surprisingly, there were significant increases in nearly all of the other metabolites measured. With the exception of GDP-glucose and sucrose, the levels of the major primary carbon metabolites, glucose 6-phosphate, glucose 1-phosphate and UDP-glucose, were significantly elevated compared to the wildtype control. As seen earlier for ADP-glucose, CS 8-18 showed the smallest increase in the levels of these metabolites while the other three transgenic lines showing significantly larger increases.

Interestingly, three of the transgenic lines showed significant increases in fructose and glucose levels. The increases in hexoses suggest a more active invertase activity in these transgenic plants as reflected by the apparent equilibrium values (Glc)(Fru)/(Suc) for CS 8-3, CS 8-13 and CS 8-29 (Table 17). The fourth transgenic line, CS 8-18, appeared to have somewhat increased glucose levels (though not significant at $p = 0.05$) while fructose levels were essentially unchanged from that of the wildtype.

Discussion

Crop yields are governed by source-sink relationships. As starch comprises ~78% of dry weight of rice seed, the rate and capacity for carbon flux into this storage product has a large impact on sink strength in rice (Singh and Juliano 1977). A key regulatory step in starch biosynthesis is catalyzed by AGPase whose activity is governed by the relative amounts of the activator 3-PGA to inhibitor Pi. As Pi is a potent inhibitor of the enzyme even in the presence of equimolar amounts of the activator 3-PGA, it is likely that this reaction is one of the rate-limiting steps of starch biosynthesis. Indeed, the expression of a highly active, allosteric-insensitive bacterial AGPase results in a higher flux of carbon into starch in potato tubers (Stark *et al.* 1992, Sweetlove *et al.* 1996) and in developing rice seeds (Sakulsingharoj *et al.* 2004). A similar situation was observed in maize where expression of a naturally occurring Pi-insensitive AGPase coded by Shrunken 2 rev6 resulted in increase starch content and, in turn, kernel weight (Giroux *et al.* 1996).

Our studies showed that the cytoplasmic-localized, but not plastid-localized, AGPase catalyzed reaction is a limiting step in starch biosynthesis in developing rice seed. Further efforts to enhance starch synthesis in developing rice seeds by growing the transgenic CS 8 plants under elevated CO_2, however, did not further increase grain weight (Sakulsingharoj *et al.* 2004). Since elevated CO_2 enhances photosynthesis and increases sucrose levels to the developing seed (Chen and Sung 1994, Rowland-Bamford *et al.* 1996), the lack of CO_2 stimulated starch synthesis suggested that the AGPase catalyzed step was no longer limiting in rice and that one or more other processes became more important (Okita *et al.* 2001).

To further characterize these rice transgenic lines, we conducted a more in-depth study of their biochemical properties and traits. All four transgenic CS 8 lines exhibited higher AGPase activity levels than wildtype and showed higher seed weights during mid-development and at final maturation.

Although all of the CS 8 transgenic lines produced seeds of higher weight, there was no obvious correlation between the increase in AGPase activity (3 to 6 fold higher than wildtype) and extent of increases in seed weight. This suggests a 3 fold increase in AGPase activity is sufficient to overcome limitations in this step in starch synthesis. The lack of correlation between AGPase activity and starch biosynthesis was clearly apparent for CS 8-18 line which had the highest AGPase activity but the lowest increase in grain weight of the four CS 8 lines studied. This lack of correlation may infer an inhibition of starch synthesis when AGPase activity becomes excessively high. Also, sucrose levels in developing seeds of CS 8-18 were the lowest of the four transgenic lines, possibly suggesting that this line may not transport sucrose as efficiently as the others. Further studies with CS 8-18 will be required to clarify why its enhancement of seed weight is lower than that of other lines.

Interestingly, in addition to increases in grain weight, the weight of the outer seed hull of the four CS 8 transgenic rice lines was also elevated compared to the wildtype. Seed hull weights were found to increase from 6 to 24% with CS 8-18 showing the smallest increase and CS 8-29 showing the highest increase. It has been suggested that the rigid hull of rice limits the grain size (Yoshida, 1981). These increases in seed coat indicate that the growth of the outer hull is not rigidly set. Instead, the growth of the seed hull is elastic and can accommodate the increases in seed volume (Figure 11)

mediated by increase starch synthesis.

All of the transgenic CS 8 lines had significantly higher levels of ADP-glucose than wildtype plants which resulted in these CS 8 transgenic lines producing seeds of higher weight. The extent of increase of ADP-glucose levels, however, did not translate to the same increases in starch as reflected by grain weight. For example, ADP-glucose levels were elevated from 29-46% among the four transgenic lines, whereas increases in seed weight were much lower and ranged from 7-15%. These results, together with the absence of any further increase in grain weight in transgenic rice plants grown under elevated CO_2, indicate that ADP-glucose biosynthesis is no longer a limiting step in starch biosynthesis and that one or more other reactions now constrains carbon flux into starch. Analysis of the other major carbon metabolites in these transgenic lines provides important insights in this process.

The principal pathway leading to starch synthesis in cereal endosperm is the breakdown of sucrose into UDP-glucose and fructose followed by the metabolism of UDP-glucose into glucose 1-phosphate. The resulting glucose 1-phosphate together with ATP is then converted into ADP-glucose (Figure 2). The cytoplasmic produced ADP-glucose is then transported into the amyloplast where it serves as a substrate for various starch synthase activities. Analysis of the major metabolites formed from sucrose metabolism showed that they were also elevated compared to the wildtype. The largest increases were seen for glucose 1-phosphate (32-61%) followed by glucose 6-phosphate (18-50%), and, in turn, followed by UDP-glucose (15-39%). In all instances, the smallest increases in these metabolites were displayed by CS 8-18 which also showed the smallest increases in seed weight compared to the wildtype.

Despite the range of increases, these metabolites are apparently in near equilibrium as indicated when the relative ratios of these metabolites are calculated. This is best illustrated by the ratio of glucose 6-phosphate/glucose 1-phosphate which is 17.9 in the wildtype and 15.9 to 16.7 in the various CS 8 transgenic lines. The wildtype equilibrium constant (K_{eq}) value is very close to the K_{eq} of 19-20 for this reaction. The ratios of ADP-glucose/glucose 1-phosphate, ADP-glucose/UDP-glucose and glucose 1-phosphate/UDP-glucose were nearly constant among the four CS 8 transgenic and wildtype lines. These constant metabolite ratios indicate that the metabolites, although elevated in the CS 8 transgenic lines, are in equilibrium at a higher steady state level.

What accounts for the increases in the upstream metabolite precursors that lead to ADP-glucose synthesis? The simplest explanation is that the higher net catalytic activity of the *E. coli* AGPase mutant enzyme increases the synthesis and, hence, the levels of ADP-glucose in the cytoplasm. However, since the AGPase reaction has a K_{eq} of about 1.0, the reverse reaction also occurs resulting in the elevation of glucose 1-phosphate and, in turn, glucose 6-phosphate via the reversible phosphoglucomutase. The elevated glucose 1-phosphate levels would also increase UDP-glucose levels, as the UDP-glucose pyrophosphorylase reaction is also reversible. The increase in ADP-glucose as well as the upstream precursor metabolites, along with the extent of these increases which are much higher than the increases in grain weight, indicate that downstream processes in ADP-glucose utilization are now limiting. These downstream steps include the transport of ADP-glucose from the cytoplasm to the amyloplast where it is utilized by starch synthase activities. Of these two processes, the transport of ADP-glucose may be the more important limiting factor given that there are multiple forms of

starch synthase.

Our results differ substantially from those reported by Smidansky *et al.* (2002, 2003) who showed that transgenic rice and wheat lines expressing a maize AGPase large subunit Sh2r6hs gene displayed higher yields stemming mainly from a higher number of seeds per panicle and enhanced number of reproductive organs, and not from increase seed weight. The larger number of seeds per rice panicle or wheat head suggests a reduction in seed abortion by expression of the variant AGPase large subunit gene. Moreover, unlike the condition observed in this study, ADP-glucose, UDP-glucose, glucose 1-phosphate and glucose 6-phosphate levels remained essentially unchanged between the wheat transgenic and a segregating non-transgenic sibling (Smidansky *et al.* 2007).

Overall, our results show that the sink strength of developing rice seeds can be increased by enhancing the net catalytic activity of the cytoplasmic-localized AGPase activity. Analysis of the major metabolites in the transgenic rice lines showed that the catalytic reactions leading to ADP-glucose are no longer limiting and that further improvements in increasing carbon flux into starch are situated downstream of ADP-glucose formation and occur at the transport of this sugar nucleotide into the amyloplast or its utilization by starch synthases.

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References

Chen, C. L., and J. M. Sung (1994). "Carbohydrate metabolism enzymes in CO_2-enriched developing rice grains varying in grain size." Physiol Plant 90: 79-85.

Choi, S.B., Y. Zhang, *et al*. (1998). "Increasing rice productivity by manipulation of starch biosynthesis during seed development." In *Feeding a World Population of More Than Eight Billion People: A Challenge to Science*. Edited by Waterford, J. , Armstrong, D. , Fowden, L. and Riley, R. pp. 137-149. Oxford University Press, New York.

Denyer, K., F. Dunlap, *et al*. (1996). "The major form of ADP-glucose pyrophosphorylase in maize endosperm is extra-plastidial." Plant Physiol 112: 779-785.

Edwards, G., O. Kiirats, *et al*. (2000). "Requirements for CO_2 concentrating mechanism in C_4 plants relative to limitations on carbon assimilation in rice." In *Redesigning rice photosynthesis to increase yield: Proceedings of the Workshop to Reduce World Hunger: Redesigning Rice Photosynthesis*. Edited by Sheedy, J.E. , Mitchell, P.L. and Hardy, B. pp. 99-112. Elsevier Science B.V, The Netherlands.

Giroux, M.J., J. Shaw, *et al*. (1996). "A single gene mutation that increases maize seed weight." Proc Natl Acad Sci USA 93: 5824-5829.

Hannah, L.C. (1997). "Starch synthesis in the maize seed." In *Cellular and Molecular Biology of Plant Seed Development*. Edited by Larkins, B.A. and Vasil, I.K. pp. 375-405. Kluwer Academic Publishers, Netherlands.

Okita, T.W., J. Sun, *et al*. (2001). "Increasing rice productivity and yield by manipulation of starch synthesis." Novartis Found Symp 236: 135-46.

Preiss, J., K. Ball, *et al*. (1991). "Starch biosynthesis and its regulation." Biochem Soc Trans 19: 539-547.

Preiss, J., D. Stark, *et al*. (1994). "Prospects for the production of cereals with improved starch properties." In *Proceedings of the Improvement of Cereal Quality by Genetic Engineering*. Edited by Henry, R. and Ronalds, J.A. pp. 115-127. Plenum Press.

Rowland-Bamford, A.J., J. T. Baker, *et al*. (1996). "Interactions of CO_2 enrichment and temperature on carbohydrate accumulation and partitioning in rice." Environ Exp Bot 36: 111-124.

Sakulsingharoj, C., S.-B. Hwang, *et al*. (2004). "Engineering starch biosynthesis for enhanced rice yields: the role of the cytoplasmic ADP-glucose pyrophosphorylase." Plant Sci 167: 1323-1333.

Sakulsingharoj, C., S.-B.Choi, *et al*. (2001). "Manipulation of ADP-glucose pyrophosphorylase in starch biosynthesis during rice seed development." In *"Rice Genetics IV. Proceedings of the Fourth International Rice Genetics Symposium*. Edited International Rice Research Institute, P.O. Box 933, Manila, Philippines.

Sikka, V.K., S.-B. Choi, *et al*. (2001). "Subcellular compartmentation and allosteric regulation of the rice endosperm ADPglucose pyrophosphorylase." Plant Sci 161: 461-468.

Singh, R. and B.O. Juliano (1977). "Free Sugars in Relation to Starch Accumulation in Developing Rice Grain." Plant Physiol 59: 417-421.

Slattery, C.J., I. H. Kavakli, *et al*. (2000). "Engineering starch for increased quantity and quality." Trends Plant Sci 5: 291-8.

Smidansky, E., F. Meyer, *et al*. (2007). "Expression of a modified ADP-glucose pyrophosphorylase large subunit in wheat seeds stimulates photosynthesis and carbon metabolism." Planta 225: 965-976.

Smidansky, E.D., M. Clancy, *et al*. (2002). "Enhanced ADP-glucose pyrophosphorylase activity in wheat endosperm increases seed yield." Proc Natl Acad Sci U S A 99: 1724-1729.

Smidansky, E.D., J. M. Martin, *et al*. (2003). "Seed yield and plant biomass increases in rice are conferred by deregulation of endosperm ADP-glucose pyrophosphorylase." Planta 216: 656-64.

Smith, A.M., K. Denyer (1997). "The synthesis of the starch granule." Annu Rev Plant Physiol 48: 65-87.

Stark, D.M., K. P. Timmerman, *et al*. (1992). "Regulation of the amount of starch in plant tissues by ADP glucose pyrophosphorylase." Science 258: 287-292.

Sweetlove, L.J., M. M. Burrell, *et al*. (1996). "Starch metabolism in tubers of transgenic potato *(Solanum tuberosum)* with increased ADPglucose pyrophosphorylase." Biochem J 320: 493-498.

Tegeder, M. and A. P. M. Weber (2006). "Metabolite transporters in the control of plant primary metabolism." In *Annu. Plant Rev. 22, 85-120.* vol. 22 Edited by Plaxton, W. and McManus, M. pp. 85-120. Blackwell Publishing, Oxford.

Thorbjornsen, T., P. Villand, *et al.* (1996). "Distinct isoforms of ADPglucose pyrophosphorylase occur inside and outside the amyloplasts in barley endosperm." Plant J 10: 243-250.

Tiessen, A., H. J. Hendriks, *et al*. (2002). "Starch synthesis in potato tubers is regulated

by post-translational redox modification of ADP-glucose pyrophosphorylase: a novel regulatory mechanism linking starch synthesis to the sucrose supply." Plant Cell 14: 2191-213.

Figure Legends

Figure 8. Structure of rice transgenic lines in the region of *glgC*-TM gene in transgenic CS 8 rice. Tnos: terminator of nopaline synthase gene, *gusA*: β-glucuronidase gene, P35S: 35S mRNA promoter of cauliflower mosaic virus, MCS: multiple cloning site, Hyg^R: hygromycin resistance gene, T35S: 35S mRNA terminator of cauliflower mosaic virus, Gt1P: glutelin Gt1 promoter, T3'rAGP: terminator of rice AGPase gene, BR and BL: right and left border.

Figure 9. AGPase activity of wildtype and CS 8 rice under no Pi and Pi-inhibitory conditions. AGPase activity was measured in developing seeds about 13 days after flowering from wildtype control and transgenic CS 8 lines. Mean values ±SE were calculated from three plants per line. An asterisk shows that there is a significant statistical difference at the 95% confidence level compared to wildtype.

Figure 10. Comparison of immature seed weight of wildtype and CS 8 rice. Immature seed weight was measured using developing seed about 13 days after flowering from wildtype control and transgenic CS 8 lines. Mean values ±SE were calculated from three plants per line. An asterisk indicates that there is a significant statistical difference at the 95% confidence level compared to wildtype.

Figure 11. Seed morphology of wildtype and CS 8 rice. The image of matured seeds was taken from wildtype control and transgenic CS 8 lines. The depicted seeds

for each line were selected based on their weight which was identical to the mean weight for each of the rice lines. The white bar is 1mm.

Figure 8

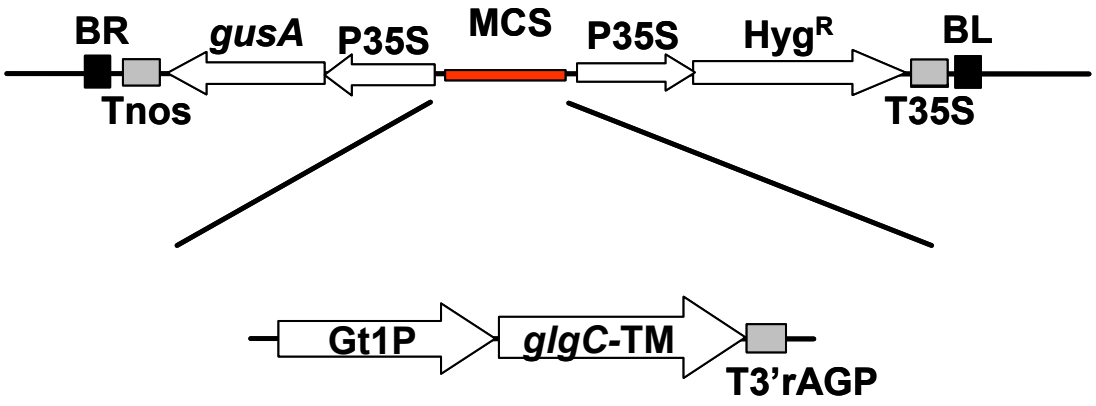
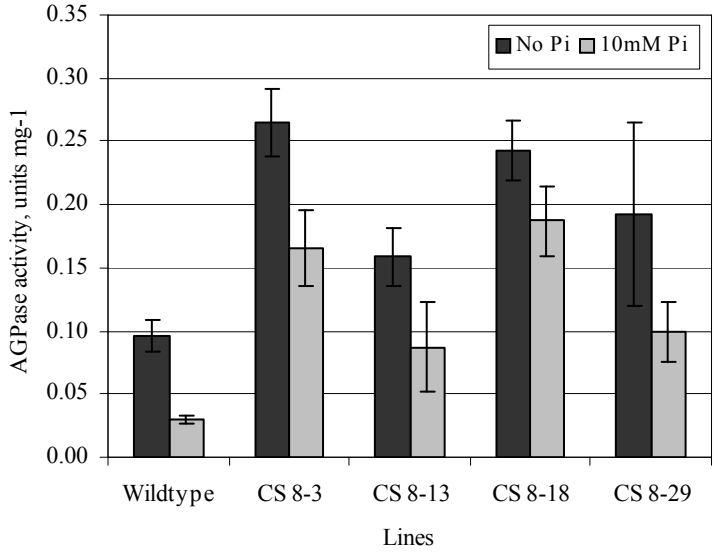
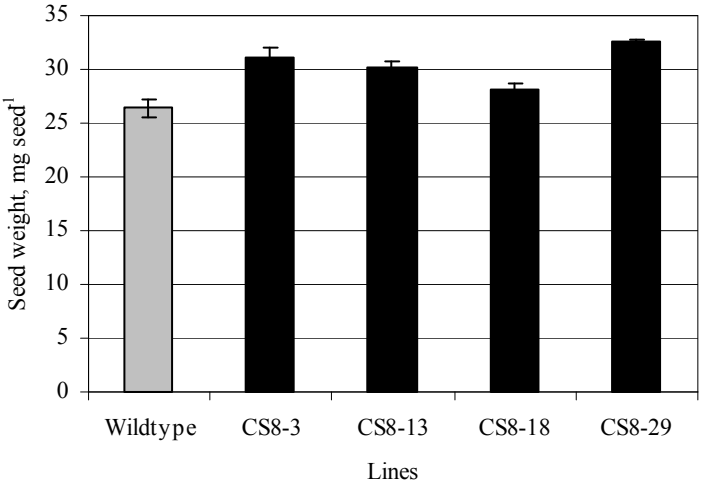


Figure 9



No Pi	100	275*	165	252	199
10mM Pi	100	550*	289	621*	329
Number of samples	3	3	3	3	3

Figure 10



Standard error	0.80	0.96	0.48	0.48	0.13
% compared to wildtype	100	118*	115*	107	124*
Number of samples	3	3	3	3	3

Table 15 The seed weights of wildtype and CS 8 rice. Matured seed weights of intact grain (seed and hull), seed, and seed hull were measured from wildtype control and transgenic CS 8 lines. Mean values ±SE were calculated from six or seven plants per lines. Parentheses indicate percentage of seed weight compared to wildtype. An asterisk indicates that there is a significant statistical difference at the 95% confidence level compared to wildtype.

		Intact grain		Seed		Hull	
Line	N	mg seed^{-1}	%	mg seed^{-1}	%	mg seed^{-1}	%
Wildtype	7	26.7 ± 0.6	(100)	22.4 ± 0.5	(100)	4.3 ± 0.1	(100)
CS 8-3	7	29.8 ± 0.2*	(111)	24.9 ± 0.2*	(111)	4.9 ± 0.1*	(114)
CS 8-13	6	30.1 ± 0.2*	(113)	25.3 ± 0.2*	(113)	4.8 ± 0.1*	(111)
CS 8-18	7	28.5 ± 0.3*	(107)	23.9 ± 0.3*	(107)	4.6 ± 0.1	(106)
CS 8-29	7	30.6 ± 0.2*	(115)	25.3 ± 0.2*	(113)	5.4 ± 0.1*	(124)

Table 16. Grain size of wildtype and CS 8 rice. Height, width and thickness of matured intact grain (seed and hull) and seed were measured from wildtype control and transgenic CS 8 lines. Mean values ±SE were calculated from six or seven plants per line. Parentheses indicate percentage of seed weight compared to wildtype. An asterisk indicates that there is a significant statistical difference at the 95% confidence level compared to wildtype.

		Intact grain, mm			Seed, mm		
Line	N	Height	Width	Thickness	Height	Width	Thickness
Wildtype	7	7.09 ± 0.04	3.58 ± 0.04	2.50 ± 0.02	4.83 ± 0.05	3.03 ± 0.03	2.25 ± 0.03
CS 8-3	7	7.78 ± 0.08 *	3.80 ± 0.04 *	2.59 ± 0.02 *	5.30 ± 0.04 *	3.18 ± 0.03 *	2.28 ± 0.02
CS 8-13	6	7.83 ± 0.07 *	3.81 ± 0.03 *	2.58 ± 0.01 *	5.30 ± 0.03 *	3.24 ± 0.02 *	2.28 ± 0.01
CS 8-18	7	7.41 ± 0.05 *	3.75 ± 0.02 *	2.60 ± 0.02 *	5.04 ± 0.03 *	3.15 ± 0.02 *	2.32 ± 0.02 *
CS 8-29	7	8.02 ± 0.06 *	3.87 ± 0.01 *	2.63 ± 0.02 *	5.42 ± 0.04 *	3.24 ± 0.01 *	2.31 ± 0.01

Figure 11

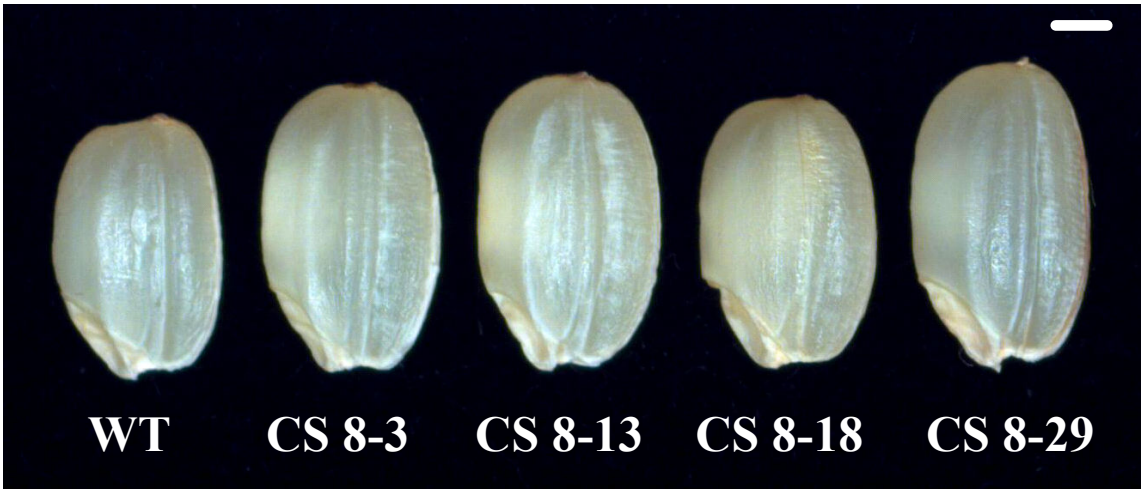


Table 17. Metabolite levels in 13-day-old developing seeds from wildtype and CS 8 rice. Metabolite analysis was measured from wildtype control and transgenic CS 8 lines. Mean values or ratio ±SE were calculated from three plants per line. Parentheses indicate percentage of metabolite content compared to wildtype. An asterisk indicates that there is a significant statistical difference at the 95% confidence level compared to wildtype.

	Wildtype	%	CS 8-3	%	CS 8-13	%	CS 8-18	%	CS 8-29	%
Metabolites	nmol seed^{-1}									
G6P	41.3 ± 0.5	(100)	56.6 ± 2.7*	(137)	54.5 ± 1.9*	(132)	48.5 ± 2.2	(118)	61.9 ± 1.0*	(150)
G1P	2.31 ± 0.05	(100)	3.45 ± 0.18*	(149)	3.30 ± 0.17*	(143)	3.06 ± 0.17*	(132)	3.71 ± 0.1*	(161)
ADPglc	0.72 ± 0.01	(100)	1.02 ± 0.04*	(142)	1.02 ± 0.06*	(142)	0.93 ± 0.06*	(129)	1.05 ± 0.05*	(146)
GDPglc	0.028 ± 0.001	(100)	0.030 ± 0.004	(107)	0.029 ± 0.003	(104)	0.035 ± 0.002	(125)	0.026 ± 0.003	(93)
UDPglc	3.82 ± 0.01	(100)	5.21 ± 0.38*	(136)	4.69 ± 0.18	(123)	4.40 ± 0.21	(115)	5.31 ± 0.12*	(139)
Fructose	1649 ± 51	(100)	1948 ± 182	(118)	1786 ± 52	(108)	1621 ± 65	(98)	1959 ± 62	(119)
Glucose	1558 ± 22	(100)	2089 ± 188*	(134)	1771 ± 19	(114)	1650 ± 6	(106)	2023 ± 62*	(130)
Sucrose	7409 ± 234	(100)	7618 ± 591	(103)	7323 ± 268	(99)	7169 ± 320	(97)	8189 ± 141	(111)
G6P+G1P	43.6 ± 0.5	(100)	60.1 ± 2.8*	(138)	57.8 ± 1.9*	(133)	51.6 ± 2.3*	(118)	65.6 ± 0.9*	(151)
ADPglc+UDPglc	4.54 ± 0.02	(100)	6.23 ± 0.42*	(137)	5.71 ± 0.24*	(126)	5.34 ± 0.27	(118)	6.36 ± 0.15*	(140)
Total sugars	10617 ± 188	(100)	11654 ± 961	(110)	10881 ± 321	(103)	10440 ± 264	(98)	12171 ± 47	(115)
	Ratio of metabolites									
(Glc)(Fruc)/Suc	348 ± 22	(100)	534 ± 57	(153)	432 ± 5	(124)	376 ± 32	(108)	486 ± 39	(140)
(Fruc)(UDPglc)/Suc	0.86 ± 0.05	(100)	1.33 ± 0.11	(155)	1.14 ± 0.04	(133)	1.01 ± 0.14	(117)	1.27 ± 0.09	(148)
G6P/Glc	0.027 ± 0.000	(100)	0.027 ± 0.002	(100)	0.031 ± 0.001	(115)	0.029 ± 0.001	(107)	0.031 ± 0.001	(115)
G1P/UDPglc	0.60 ± 0.01	(100)	0.66 ± 0.02	(110)	0.71 ± 0.04	(118)	0.69 ± 0.02	(115)	0.70 ± 0.02	(117)
G6P/G1P	17.9 ± 0.6	(100)	16.4 ± 0.2	(92)	16.6 ± 1.0	(93)	15.9 ± 0.4	(89)	16.7 ± 0.7	(93)
ADPglc/G1P	0.31 ± 0.01	(100)	0.30 ± 0.01	(97)	0.31 ± 0.02	(100)	0.30 ± 0.01	(97)	0.28 ± 0.02	(90)
ADPglc/UDPglc	0.19 ± 0.00	(100)	0.20 ± 0.01	(105)	0.22 ± 0.00*	(116)	0.21 ± 0.00*	(111)	0.20 ± 0.01	(105)