MULTIPLE NON-REDUNDANT ROLES FOR PLASTIDIC 6-PHOSPHOGLUCONATE DEHYDROGENASE (6PGDH) IN MAIZE

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
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To my Mom and Dad

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The oxidative pentose phosphate pathway (OPPP) serves multiple roles in primary metabolism. Enzymes for the oxidative section of the OPPP are found both in the cytosol and plastid. Several mutant studies have suggested that cytosolic and plastidic OPPP enzymes are redundant including 6-phosphogluconate dehydrogenase (6PGDH). 6PGDH enzymes catalyze the third non-reversible step of the oxidative section of the OPPP. Maize mutations in the cytosolic 6PGDH enzymes, *pgd1* and *pgd2*, do not show obvious phenotypes beyond loss of enzyme activity. In this thesis, two knockout alleles of the maize *Pgd3* locus were identified to investigate the role of this gene in central carbon metabolism. The *pgd3* mutants disrupt plastid-localized 6PGDH activity and cause a *rough endosperm* (*rgh*) phenotype that affects both grain-fill and embryo development. Consistent with the reduced grain-fill phenotype, ^{13}C-glucose labeling experiments during seed development suggested that *pgd3* mutants disrupt carbohydrate flux for starch synthesis. PGD1, PGD2, and PGD3 are all active in both the endosperm and embryo. These data suggest that PGD3 has a non-redundant role for seed development. Moreover, homozygous *pgd3* seeds can be rescued through tissue culture experiment. The addition of asparagine in the tissue culture medium increases the rescue of mutant seeds,

suggesting that amino acid synthesis is limiting in *pgd3* mutants. The homozygous *pgd3* mutant plants show normal morphology but are slow to green and late flowering. PGD3 activity is restricted to sink tissues suggesting that the slow to green phenotype is due to disruptions in carbon metabolism during leaf expansion.

CHAPTER 1
INTRODUCTION

General Background

Oxidative Pentose Phosphate Pathway (OPPP)

The oxidative pentose phosphate pathway (OPPP) is a central process in plant metabolism. The OPPP includes a series of enzymes that converts glucose 6-phosphate into a pool of phosphorylated sugars with 3 to 7 carbons. Those sugars can be converted back into glucose 6-phosphate allowing the pathway to undergo cycles. Figure 1-1 shows a generalized schematic of the OPPP showing the reactions and intermediates through the pathway. The OPPP has two major metabolic roles: providing reducing power to the cell and providing carbon intermediates for multiple biosynthetic pathways. Both of these roles are thought to be essential to the cell and will be discussed in greater detail in section 1.2.

The OPPP provides reductant to the cell in the form of NADPH, which is synthesized in the oxidative section. The oxidative section includes three enzymes catalyzing three reactions. Initially, glucose 6-phosphate dehydrogenase (G6PDH) oxidizes glucose 6-phosphate (G6P) to phosphogluconolactone. 6-Phosphogluconolactonase then converts phosphogluconolactone to 6-phosphogluconate very quickly. After that, 6-phosphogluconate dehydrogenase (6PGDH) oxidatively decarboxylates 6-phosphogluconate to ribulose 5-phosphate. Importantly, all three of the oxidative reactions are non-reversible and loss of any of these enzyme activities is expected to disrupt the entire OPPP.

The non-oxidative section of the OPPP provides the pool of phosphorylated sugars that are needed in a variety of metabolic pathways. The five enzymes working in the non-oxidative section are: ribose 5-phosphate isomerase (RPI), ribulose 5-phosphate 3-epimease (RPE), transaldolase, transketolase, and glucose 6-phosphate isomerase. These enzymes convert the

product of 6PGDH, ribulose 5-phosphate, to other sugar phosphates. The pentose phosphate sugar pool includes: 5 carbon sugars (ribose 5-phosphate and xylulose 5-phosphate), a 7 carbon sugar (sedoheptulose 7-phosphate), a 4 carbon sugar (erythrose 4-phosphate), a 3 carbon sugar (triose 3-phosphate), and 6 carbon sugars (fructose 6-phosphate and glucose 6-phosphate). The non-oxidative enzymes catalyze reversible reactions, so it is possible to synthesize all of the phosphate sugar pool by utilizing glucose-6-phosphate and ATP (ap Rees, 1985; reviewed in Kruger and von Schaewen, 2003).

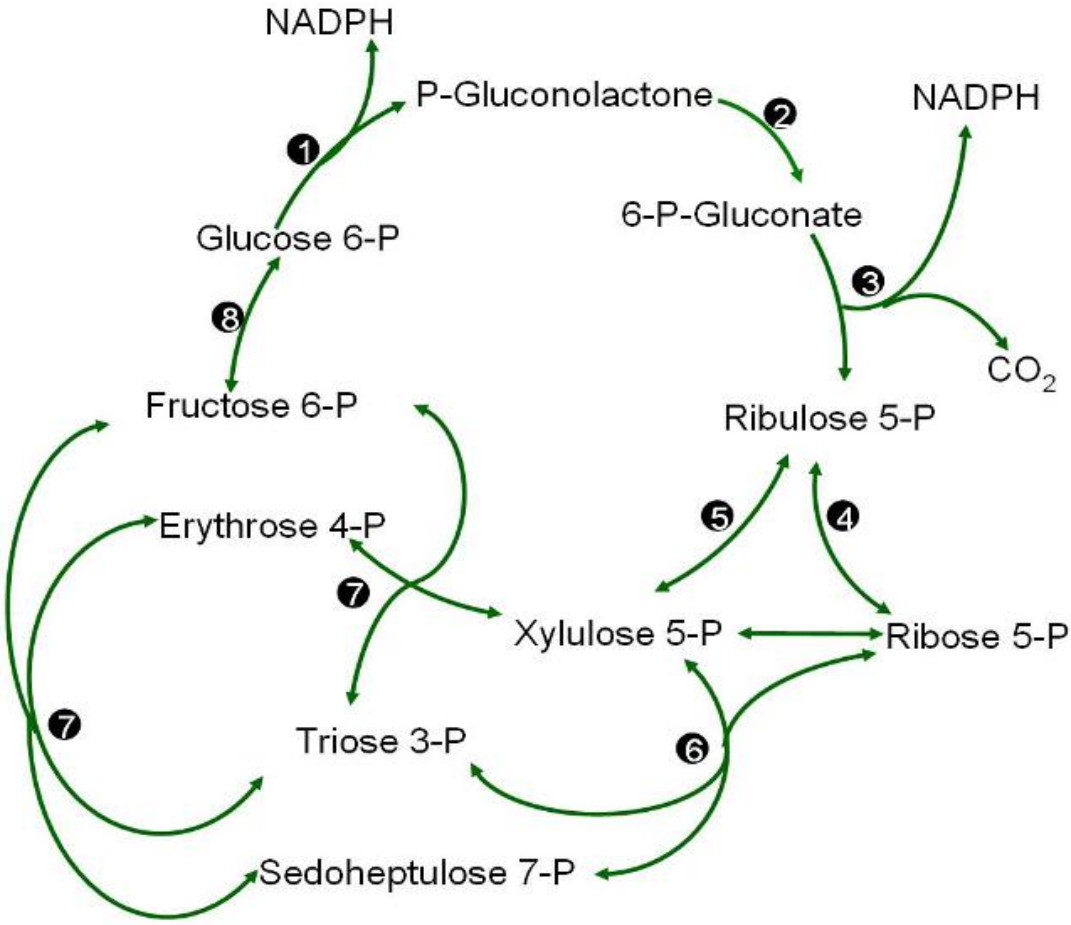

Figure 1-1. Schematic of the oxidative pentose phosphate pathway. The number in black circle denotes the enzyme that catalyzes each of the steps: 1. Glucose 6-phosphate dehydrogenase; 2. 6-Phosphogluconolactonase; 3. 6-Phosphogluconate dehydrogenase (6PGDH); 4. Ribose 5-phosphate isomerase; 5. Ribulose 5-phosphate 3-epimerase; 6. Transketolase; 7. Transaldolase; 8. Glucose 6-phosphate isomerase.

Subcellular Localization of OPPP Enzymes

OPPP enzymes in animal and yeast cells are located exclusively in the cytosol. In higher plants, the complete set of OPPP enzymes are found in the plastids (Nishimura and Beevers, 1979; Journet and Douce, 1985; Hong and Copeland, 1990).

A subset of plant OPPP enzymes are present in the cytosol, with most plant cells containing a cytosolic oxidative branch. Genes encoding cytosolic G6PDH have been identified in Arabidopsis, potato, tobacco, and maize (Schnarrenberger et al., 1995; von Schaewen et al., 1995; The Arabiopsis Genome Initiative, 2000; Knight et al., 2001). Non-oxidative enzymes are also found in the cytosol of some plant species. The global genome analysis of Arabidopsis showed except for transaldolase and transketolase, all other non-oxidative enzymes have both cytosolic and plastidic isozymes (The Arabidopsis Genome Initiative, 2000).

Connections between Cytosolic and Plastidic OPPP Enzymes

In at least some species, OPPP enzymes are not completely duplicated in the cytosol or plastid, and the cytosol is likely to produce intermediates that need to be utilized in the plastid. A number of transporters on the plastid envelope membrane connect the cytosolic and plastidic OPPP.

In the Arabidopsis genome, six genes encode functional plastidic phosphate transporters that can be grouped into four classes: the glucose 6-phosphate/phosphate transporters (AtGPT1 and AtGPT2), the triose-phosphate/phosphate transporter (AtTPT), the phosphoenolpyruvate/phosphate transporters (AtPPT1 and AtPPT2), and the xylulose 5-phosphate (Xul 5-P)/phosphate transporter (AtXPT) (reviewed in Weber, 2004).

Generally, all transporters have broad substrate specificity. For example, GPT can accept glucose 6-phosphate (G6P), triose phosphate, 3-phosphoglyceric acid, and Xul 5-P as counter-exchange substrates for inorganic phosphate. XPT, the most recently identified transporter in the

plastidic phosphate transporter family, can transport triose phosphates, Xul 5-P, and inorganic phosphate (Eicks et al., 2002).

The reversible reactions in the non-oxidative section, gene redundancy and subcellular distribution of OPPP enzymes combined with a set of transporters on the plastid membrane, suggests that the OPPP in plants has multiple levels of redundancy (Figure 1-2).

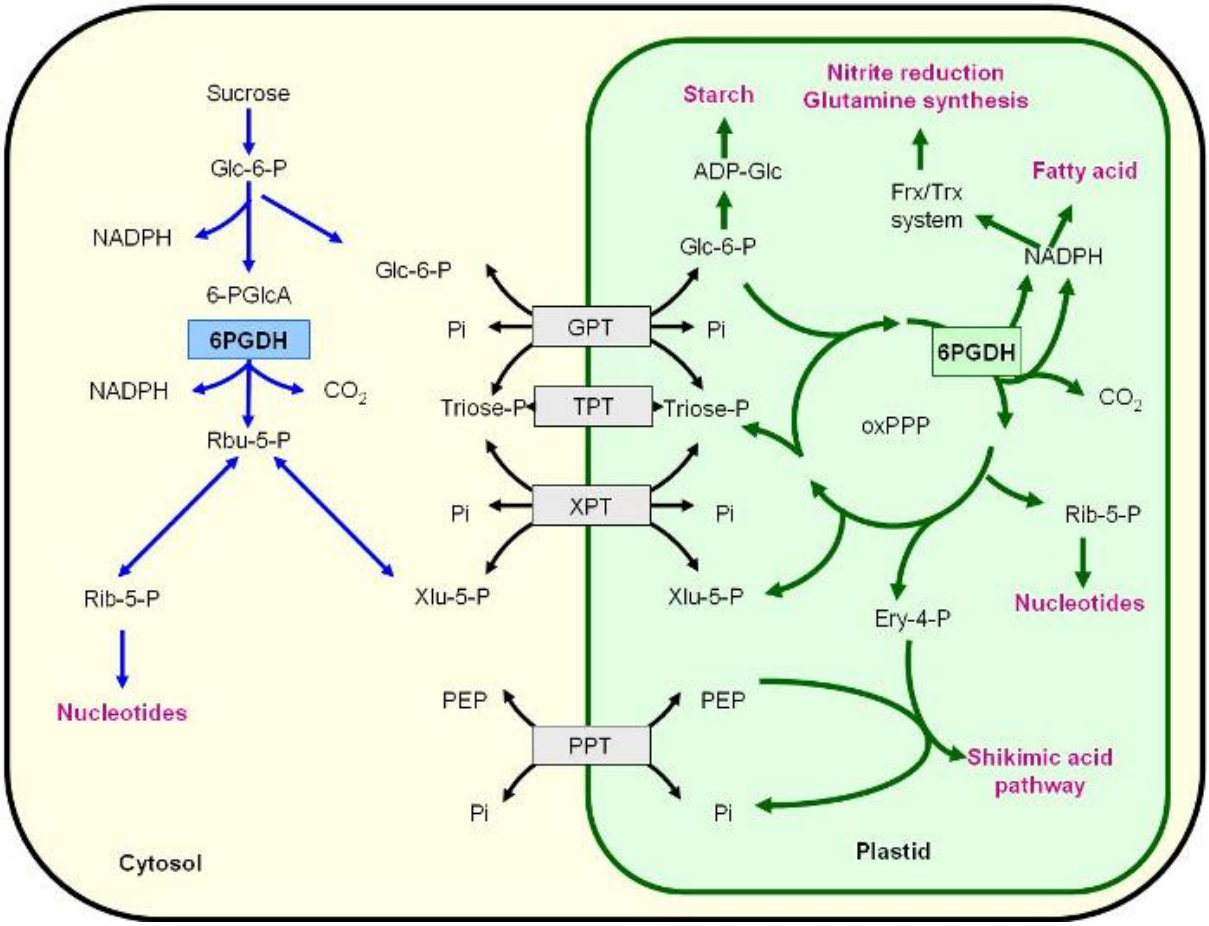

Figure 1-2. Multiple levels of redundancy of the OPPP in Arabidopsis. The OPPP provides reducing power and carbon skeletons for many biosynthetic pathways (shown in pink). Adapted from Kruger and von Schaewen, 2003, pp240, Figure 2.

Mechanism of 6-Phosphogluconate Dehydrogenase (6PGDH) Enzymes

One method to investigate the biological roles of the plastidic and cytosolic OPPP is to identify OPPP mutants. 6PGDH is and ideal enzyme to target for mutant studies, because of its non-reversible mechanism. Complete loss of the enzyme activity is lethal since a high

concentration of 6PG is toxic to eukaryotic cells, including *Drosophila melanogaster* (Gvozdev et al., 1976; Hughes and Lucchesi, 1977; He et al., 2007), *Saccharomyces cerevisae* (Lobo and Maitra, 1982) and *Trypanosoma brucei* (Hanau et al., 2004). However, plants have multicopies of 6PGDH enzymes, and these isozymes are localized both in the cytosol and plastid. A loss of 6PGDH activity has not yet been reported in higher plants.

6PGDH(EC 1.1.1.44) converts 6-phosphogluconate (6-PG) to ribulose 5-phosphate and CO_2 by a three-step acid-base mechanism: dehydrogenation, decarboxylation and keto–enol tautomerization (Cervellati et al., 2008). Two residues in 6PGDH assist all those three steps, one acting as an acid and the other as a base (Figure 1-3). In *Trypanosoma brucei*, the catalytic residues are Glu192 and Lys185. When the enzyme binds to the substrate, the lysine residue is unprotonated, and it receives a proton from the 3-hydroxyl of 6-PG to give a 3-keto intermediate. Then this same residue lysine donates the proton to help decarboxylation and form 1,2-enediol of ribulose 5-phosphate, which is converted to ribulose 5-phosphate (Montin et al., 2007). In this oxidative decarboxylation reaction, NADP works as the oxidant to accept a proton from aqueous environment to give NADPH, one of the major reductants in the cell. The release of CO_2 in the decarboxylation step makes the reaction being non-reversible.

In yeast as well as many other species, the 6PGDH monomer contains two domains, N-terminal domain and C-terminal domain. The N-terminal α/β "co-enzyme binding" domain of 6PGDH is a $NADP^+$ binding domain**.** The C-terminal domain is almost fully helical, contributing to the dimerization (He et al., 2007). It has been shown that 6PGDH isozymes can form heterodimers and homodimers in maize (Bailey-Serres and Nguyen, 1992).

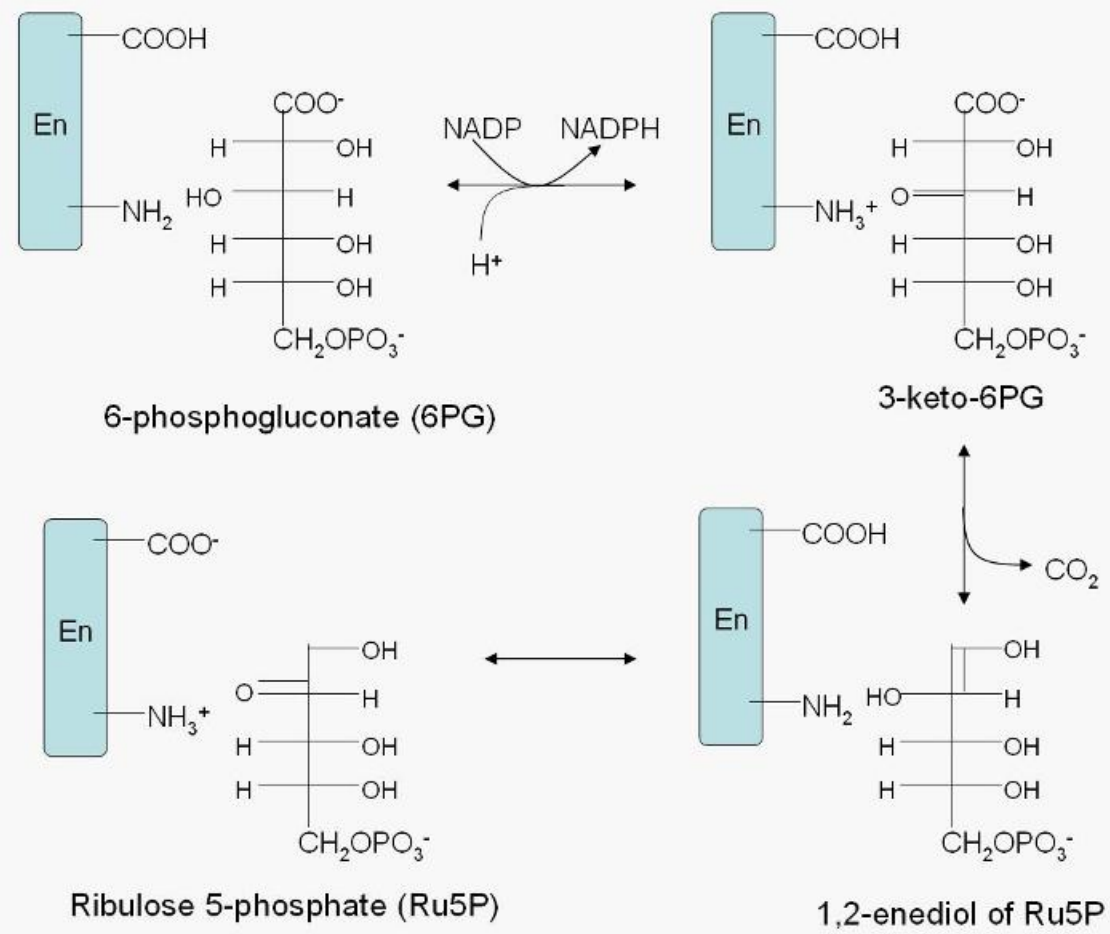

Figure 1-3. 6-Phosphogluconate dehydrogenase (6PGDH) convertes 6-phosphogluconate to ribulose 5-phosphate a three-step acid-base mechanism: dehydrogenation, decarboxylation and keto–enol tautomerization, producing NADPH and CO_2.

The Importance of the OPPP and 6PGDH to Cellular Metabolism

The oxidative pentose phosphate pathway (OPPP) is a major source of reducing power and metabolic intermediates in central carbon metabolism. NADPH produced in the non-reversible oxidative section of this pathway is the major source of reductant in nonphotosynthetic cells. The reversible non-oxidative section of this pathway provides substrates for glycolysis and several biosynthetic pathways, such as biosynthesis of nucleic acids, lignin, polyphenols, amino acids.

Substrates for Nucleotide Synthesis

In plants, *de novo* synthesis of nucleotides requires 5- phosphoribosyl-1-pyrophosphate (PRPP). PRPP is synthesized from ribose 5-phosphate by PRPP synthase. The OPPP provides ribose 5-phosphate through the action of ribose 5-phosphate isomerase (RPI), which converts ribulose 5-phosphate to ribose 5-phosphate.

The Arabidopsis radial swelling 10 (*rsw10*) mutant suggests that a primary requirement for nucleotides is in cellulose synthesis. The *rsw10* mutant is mutated in a gene predicted to encode a cytosolic ribose 5-phosphate isomerase (RPI). The root elongation in *rsw10* mutants is greatly reduced, suggesting a defect in cell wall biosynthesis. Since the orientation of microfibrils assembled by cellulose influences the balance between longitudinal and radial growth, the level of cellulose in *rsw10* mutant was analyzed and it was lower than in wild type (Howles et al., 2006). The mutation in RPI and the defect of cellulose synthesis in *rsw10* can be connected by UDP-glucose, theoretically. UDP-glucose is the substrate for the growing cellulose chain (Carpita and Delmer, 1981). Uridine nucleotides, the substrate of UDP-glucose, are products from the activity of RPI in the OPPP (reviewed in Boldt and Zrenner, 2003). It has been shown that with exogenous uridine and UDP–glucose, the phenotype of *rsw10* mutant can be suppressed. In contrast, *rsw1* mutant, which is defective in the enzyme believed to use UDP–glucose as substrate, cannot be rescued by exogenous uridine or UDP–glucose. Thus, the cellulose defect in *rsw10* mutants is caused by the defect of nucleotides synthesis (Howles et al., 2006).

In Arabidopsis, three nuclear genes are predicted to encode RPIs (reviewed in Kruger and von Schaewen, 2003). Generally, there is a duplication of the pyrimidine synthesis pathway between the cytosol and plastid, and transporters on the plastid membrane will enhance the redundancy. Since the *rsw10* mutation only changed a single amino acid residue in one of two

cytosolic RPI enzymes, those RPI isoforms should have a very different expression pattern, which has been confirmed on the transcript level. A further support is that both the cytosolic RPIs can complement the *rsw10* phenotype when expressed behind a constitutive promoter (Howles et al., 2006).

Substrates for Aromatic Amino Acid Synthesis

Another important cabon skeleton provided by OPPP is erythrose 4-phosphate, which is a downstream product in the non-oxidative sugar pool. Both erythrose 4-phosphate and phosphoenolpyruvate (PEP) are condensed and reduced to give shikimic acid. After that, shikimic acid is condensed with another PEP to give chorismic acid, the precursor of aromatic amino acids: phenylalanine, tryptophan, and tyrosine (reviewed in Herrmann and Weaver, 1999).

The Arabidopsis *cue1* mutant is the indirect evidence suggesting that the substrate defect of the shikimate pathway will cause aromatic amino acid defect. This mutant is mutated in AtPPT1, which transports another precursor of shikimate pathway phosphoenolpyruvate (PEP) into plastids. The *cue1* mutant was originally isolated because of its defect in the light-induced expression of the chlorophyll a/b binding protein. The mutant is unable to produce anthocyanins and several other products that are derived from the shikimate pathway. Moreover, the reticulate leaf phenotype of *cue1* can be rescued by feeding of aromatic amino acids (Streatfield et al., 1999).

Additionally, the phenotype of *cue1* can be complemented by constitutive overexpression of a heterologous PPT from cauliflower. Also, the defect in plastidic PEP import could be bypassed by overexpression of plastid-targeted pyruvate orthosphophate dikinase. This is because that the overexpression of pyruvate orthosphophate dikinase allows pyruvate imported into the plastids and converted to PEP in the stroma (Voll et al., 2003).

The reason that the biosynthesis of aromatic amino acids is not severely affected in the whole *cue1* mutant plant is because of the gene redundancy. As there are two PPTs in Arabidopsis, AtPPT2 is suggested to be a more housekeeping functional in providing chloroplasts with PEP as a precursor for the shikimate pathway, while AtPPT1 is involved in provision of signals for correct mesophyll development (Knappe et al., 2003). Therefore, the import of PEP is reduced but not eliminated in *cue1* mutant plant (Voll et al., 2003).

NADPH for Fatty Acid Synthesis

NADPH, which can be produced by the 6PGDH reaction, is the major power resource in nonphotosynthetic tissue for maintaining the redox potential necessary to protect against oxidative stress, especially for fatty acid biosynthesis and nitrogen assimilation.

In almost all plants, *de novo* fatty acid synthesis occurs in plastids. The first committed step of fatty acid synthesis is the formation of malonyl-CoA from acetyl-CoA and bicarbonate which is catalysed by acetyl-CoA carboxylase (Harwood, 1988). Since acetyl-CoA cannot cross the plastid membrane, it must be generated within the plastid using precursors which are synthesized inside the plastid or actively imported from the cytosol. The imported precursors include glucose 6-phosphate (G6P), dihydroxyacetone phosphate, phosphoenolpyruvate (PEP), pyruvate, malate, and acetate, and their relative rates of utilization depend on the plant species, the tissue studied, and also the developmental stage (reviewed in Rawsthorne, 2002).

The production of fatty acids requires the provision of reducing power in the form of NADPH and NADH (Slabas and Fawcett, 1992). Those reducing equivalents are used for the reduction of 3-ketoacyl-ACP to acyl-ACP, a reaction catalyzed by two subunits of the fatty acid synthase complex 3-ketoacyl reductase and enoyl-ACP reductase. Photosynthesis can provide reductants directly. In nonphotosynthetic tissues, those reductants can be generated during the synthesis of acetyl-CoA from glucose 6-phosphate (G6P), malate or pyruvate (Smith et al., 1992;

Kang and Rawsthorne, 1996), or via the OPPP. *B. napus* plastids have a full complement of glycolytic enzymes as well as OPPP oxidative reaction enzymes, so they have both photosynthetic and heterotrophic properties. But it has been confirmed that in *B. napus*, OPPP does provide a source of NADPH for fatty acid synthesis, contributing an estimated about 35% of the total required (Schwender et al., 2003). Thus, the 6PGDH enzyme is likely to be involved in providing reducing power for fatty acid synthesis in *B. napus*.

An additional evidence of the relationship between NADPH produced by OPPP and fatty acid synthesis is found from sunflower. In sunflower embryo plastids, pyruvate utilization for fatty acid synthesis can be stimulated by the addition of glucose 6-phosphate (G6P). In contrast, glucose 6-phosphate (G6P) addition has no effect on the utilization of malate. Furthermore, while addition of pyruvate stimulated the activity of the OPPP, malate suppressed its activity (Pleite et al., 2005). This is because malate utilization can provide NADPH, NADH and acetyl-CoA via plastidic NADP-malic enzyme and pyruvate dehydrogenase complex. Under these conditions there would be no demand for additional NADPH from the OPPP.

Furthermore, an Arabidopsis *gpt* mutant shows a large reduction of the number of oil bodies in pollen with gametogenesis defects (Niewiadomski et al., 2005). The *gpt* mutant is mutated in AtGPT1, which transport glucose 6-phosphate (G6P) into plastids. Since there is a 10-fold increase in the accumulation of AtGPT1 transcripts in guard cells relative to mesophyll cells in wild type plants (Niewiadomski et al., 2005), it is suggested that the role of glucose 6-phosphate in lipid synthesis in Arabidopsis pollen is to provide reducing power via OPPP rather than the precursor of acetyl-CoA.

NADPH for Nitrate Assimilation

The NADPH provided by OPPP is also important in nitrogen assimilation and glutamine synthesis. As nitrate is reduced to nitrite in the cytosol, the uptake of nitrite into the plastids and

its subsequent reduction by nitrite reductase and glutamate synthase are potentially important control points (Bowsher et al., 2007). Several studies have shown how electrons from the plastid-localized OPPP go to nitrite in wheat and pea roots (Oji et al., 1985; Bowsher et al., 1992). Generally, OPPP-generated NADPH acts as the initial reductant to generate reduced ferredoxin via a ferredoxin- NADP oxidoreductase (FNR). Then, the reduced ferredoxin provides electrons to the nitrite reduction process.

Moreover, it has been shown that carbohydrate flux through the plastidic OPPP can be stimulated by feeding NO^{2-} or glutamine to isolated chloroplasts of green pepper fruits (Thom and Neuhaus, 1995). Finally, the treatment of nitrate induced the increase of 6PGDH activity as well as protein and transcript level in maize root plastids (Redinbaugh and Campbell, 1998).

Leaves of C4 plants such as maize have two kinds of photosynthetic cells: the bundle sheath cells (BSC) and the mesophyll cells (MC). The distribution of enzymes involved in nitrogen metabolism is different in these two cell types. Nitrate reductive reaction occurs in MC (Harel et al., 1977; Moore and Black, 1979) while the photorespiratory pathway is in BSC (Ohnishi and Kanai, 1983). In maize, although different photosynthetic ferredoxin- NADP oxidoreductases (FNRs) are localized in MC and BSC, respectively nonphotosynthetic ferredoxin- NADP oxidoreductases (FNRs) are predominantly detected in MC rather than BSC (Matsumura et al., 1999). Thus, even in photosynthetic organs, the reductant for nitrogen assimilation is supplied, at least partially, via OPPP (Favery et al., 1998).

Starch Synthesis in Maize Kernels

In plants, the glucose 6-phosphate (G6P) is either the substrate of OPPP or the precursor of starch biosynthesis. Glucose 6-phosphate is converted to glucose 1-phosphate by phosphoglucomutase. Then ADP-glucose is synthesized from glucose 1-phosphate and ATP by

the action of ADP-glucose pyrophosphorylase (AGPase). Next, ADP-glucose is transferred to the elongating starch chain by the activity of starch synthase isoforms.

The localization of ADP-glucose production is different between plant species and tissues. In most plants or tissues, this enzyme reaction is localized in the plastids. Thus, the import of G6P is required for starch synthesis. For example, Arabidopsis wild type pollen contains many starch granules in plastids. However, the *gpt* mutant pollen only contains starch-free plastids (Niewiadomski et al., 2005). In fact, other evidence suggests that not only G6P uptake but also the plastidic OPPP is involved in starch synthesis.

In cereal endosperm such as maize, the AGPase is known to be largely extraplastidic (Beckles et al., 2001). In this tissue, sucrose is the major nutrient. Thus, ADP-glucose could also be converted from glucose 1-phosphate, the product of sucrose degradation with UDP–glucose as the intermediate. As ADP-glucose is synthesized in cytosol, cereal endosperm has an additional transporter to transport ADP-glucose across the plastid envelope membrane. In maize, this transporter was identified by the *brittle1* mutant, which has a reduced starch content and accumulates ADP-glucose in the cytosol (Shannon et al., 1996). Also, the amyloplasts of the *brittle1* mutant do not synthesize starch from exogenously supplied ADP-glucose (Shannon et al., 1998). A similar phenotype has been found for barley mutants carrying mutations in the Hv.Nst1 gene (Patron et al., 2004). It is suggested that ADP-glucose is exchanged with AMP by those adenylate transporters (reviewed in Emes and Neuhaus, 1997). Thus, a model of starch synthesis in maize kernel is presented in Figure 1-4. Although many starch biosynthetic mutants, such as *brittle2* and *shrunken2* mutants, suggested that major ADP-glucose is synthesized in cytosol, ^{13}C-labeling experiment suggested that about 80% of the carbons must go to glycolysis or the OPPP before they are incorporated into starch in maize kernels (Spielbauer et al., 2006).

Since the precursor for starch synthesis is not from the plastidic OPPP, the NADPH produced by OPPP may be more important in explanation of this phenomenon.

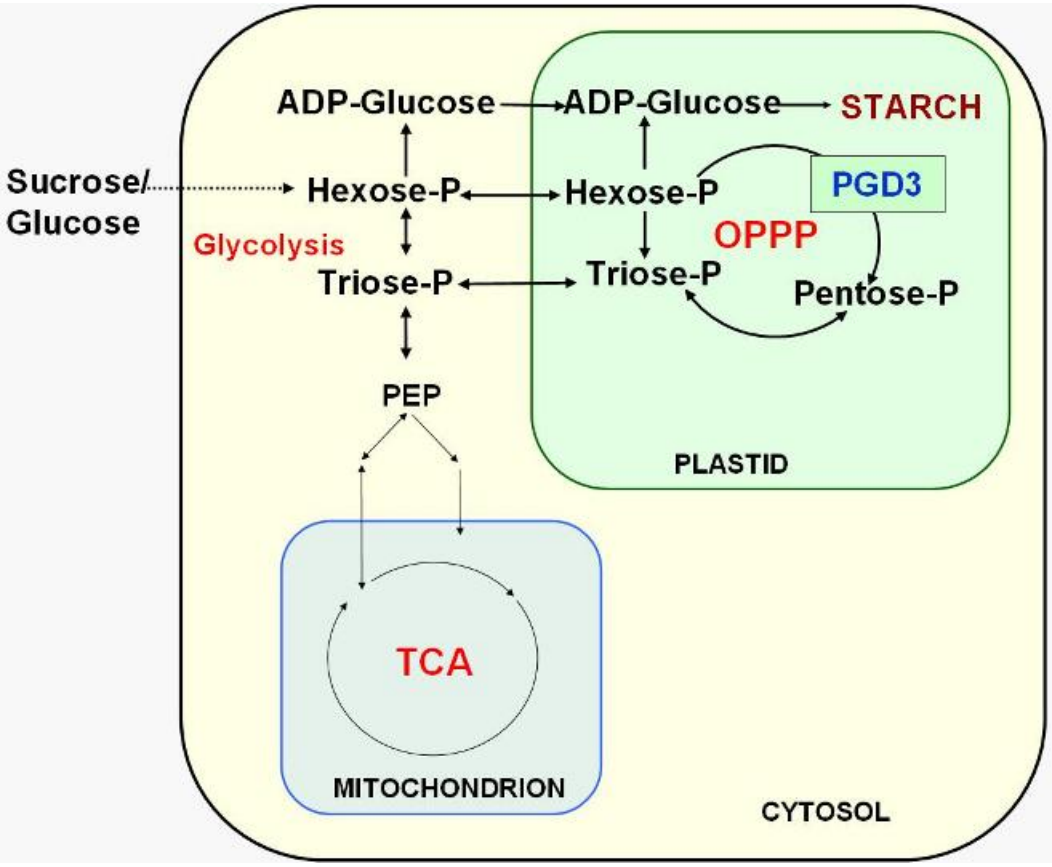

Figure 1-4. Generalized schematic of starch biosynthesis in maize kernels utilizing central carbon metabolism. The conventional flow of starch biosynthesis is: sucrose/glucose is transported into cell and converted to ADP-glucose in cytosol; then ADP-glucose is transported into plastid and incorporated into starch. Glycolysis, OPPP and TCA cycle in mitochondrion contribute carbon skeleton for starch biosynthesis, too.

Sugar Induction of Transporter Expression

Although it has been shown that the products of 6PGDH, ribulose 5-phosphate and NADPH, are very important in organisms, there was no direct phenomenon caused by 6PGDH defect in plants until 6-amino nicotinamide (6-AN), an inhibitor of 6PGDH, is applied on Arabidopsis **(**Lejay et al., 2008**)**. 6-AN is converted *in vivo* to an analogue of $NADP^+$, which is a

potent inhibitor of 6PGDH and G6PD in neural tissue (Favery et al., 1998) and restricts flux through the OPPP (Garlick et al., 2002).

Ion transporter gene expression in the roots is up-regulated by light and sugars, such as NRT1.1 and NRT2.1 (NO_3^- transporter), AMT1.3 (NH_4^+ transporter), SULTR1.1 (SO_4^{2-} transporter) (Lejay et al., 2003). However, after applying 6-AN to Arabidopsis roots, sugar induction at the transcript level of the transporter genes is reduced (Lejay et al., 2008). As the sulfur and nitrogen assimilatory pathways are well coordinated, it is not surprising that the availability of one element regulates the other pathway. Since the NADPH-dependent regulation has been found in animals for the redox regulation of fertilization in the mouse (Urner and Sakkas, 2005), the reducing power produced by the OPPP might be the key element in regulation of root ion transporters.

Questions

In a word, OPPP is a central portion of carbon metabolism in plants as it serves multiple roles, such as providing substrates and reducing power for many nutrient biosynthesis. This pathway has a great redundancy in plants, since there are multicopies of OPPP enzymes in the cytosol and plastid, and many transporters on the plastid membrane connect the carbohydrate pools of the cytosol and plastid. 6PGDH, the enzyme working in the third step of OPPP, has three copies in maize: *Pgd1*, *Pgd2*, and *Pgd3* (Bailey-Serres et al., 1992). In this thesis, I will show that mutation in *Pgd3* gives a visible phenotype, and this phenotype is caused by the some roles non-redundant of PGD3.

CHAPTER 2
RESULTS: MUTATIONS IN PGD3 CAUSES RGH SEED PHENOTYPES

Background

In maize, mutants in *Pgd1* and *Pgd2* have been previously identified (Averill et al., 1998). A mutant in *Pgd3* was identified recently via forward genetic screen from a maize mutagenic population. Most mutagenesis experiments in maize apply maize transposons, such as *Ac*/*Ds* (*Activator* and *Dissociation*) and *MuDR*/*Mu* (*Robertson's Mutator*) elements (Walbot, 2000). Mutations in *Pgd3* were identified in the UniformMu transposon-tagging population (McCarty et al., 2005). UniformMu has the *MuDR*/*Mu* elements in the W22 inbred, which create tagged mutations at a high rate.

Initial applications of transposon tagging in maize relied on correlating the inheritance of a plant phenotype with a band on a DNA hybridization blot (Walbot, 2000). Several techniques have been developed for amplifying and sequencing genomic DNA flanking to transposon insertions. A specific band can be amplified by PCR primers specific to transposons TIRs with a gene specific primer, priming from the flanking genomic DNA. Since this specific band indicates a specific insertion, it can be determined whether this transposon insertion segregates with a phenotype.

UniformMu is a high-copy transposon population, and the numerous *Mu* elements create challenges for the molecular analysis of the tagged mutations. MuTAIL PCR was developed to identify transposon insertion sites in genetic backgrounds with high-copy transposons (Settles et al., 2004). By conducting the optimized PCR with the Mu-specific primer and a series of arbitrary primers, a collection of genomic sequences flanking to the transposon insertions can be obtained. Informatic analysis showed that only a small fraction of the flanking sequences have a significant similarity to maize repetitive sequences. Also, those sequences are matched to the

TIGR *Zea mays* Gene Index (ZMGI) to get the annotations of the loci disrupted by those novel insertions. Moreover, by matching to assembled genomic islands (MAGI, http://www.plantgenomics.iasate.edu/maize/), we can design locus-specific primers as the co-dominant markers for those novel insertions. Thus, it is possible to know whether those novel insertions may be the cause of the mutant phenotype by co-segregation analysis. For example, a mutant allele *pgd3-umu1* has been shown to co-segregate with a *rough endosperm* (*rgh*) phenotype (Settles et al., 2007).

Co-segregation Analysis of *pgd3-umu1*

The co-segregation between *pgd3-umu1* and a *rgh* phenotype was analyzed by PCR with co-dominant markers, and this analysis has been extended up to 323 meiotic products. All PCR results showed that, with the gene specific left and right primer, the wild type allele can be amplified from both heterozygous and wild type seeds, and cannot be amplified from the homozygous mutants. However, the mutant allele *pgd3-umu1* can only be amplified when there is a *rgh* phenotype allele by conducting PCR with a gene specific primer and a TIR primer (Figure 2-1). Thus, *pgd3-umu1* is tightly linked with the *rgh* phenotype, and the linkage is less than 0.31cM.

Identification of the *pgd3-umu2* Allele

An additional mutant allele was identified from the UniformMu population by a reverse genetic screen (Figure 2-2). DNA was extracted from the remaining independent *rgh* mutants from the UniformMu transposon-tagging population. The *Pgd3* gene-specific primer and the TIR primer were used to screen for mutants that can give a positive amplification. Those PCR products which had different size with the product of *pgd3-umu1* amplification were sequenced to confirm the insertion. The insertion site of the second allele, named as "*pgd3-umu2*", is 308 bp

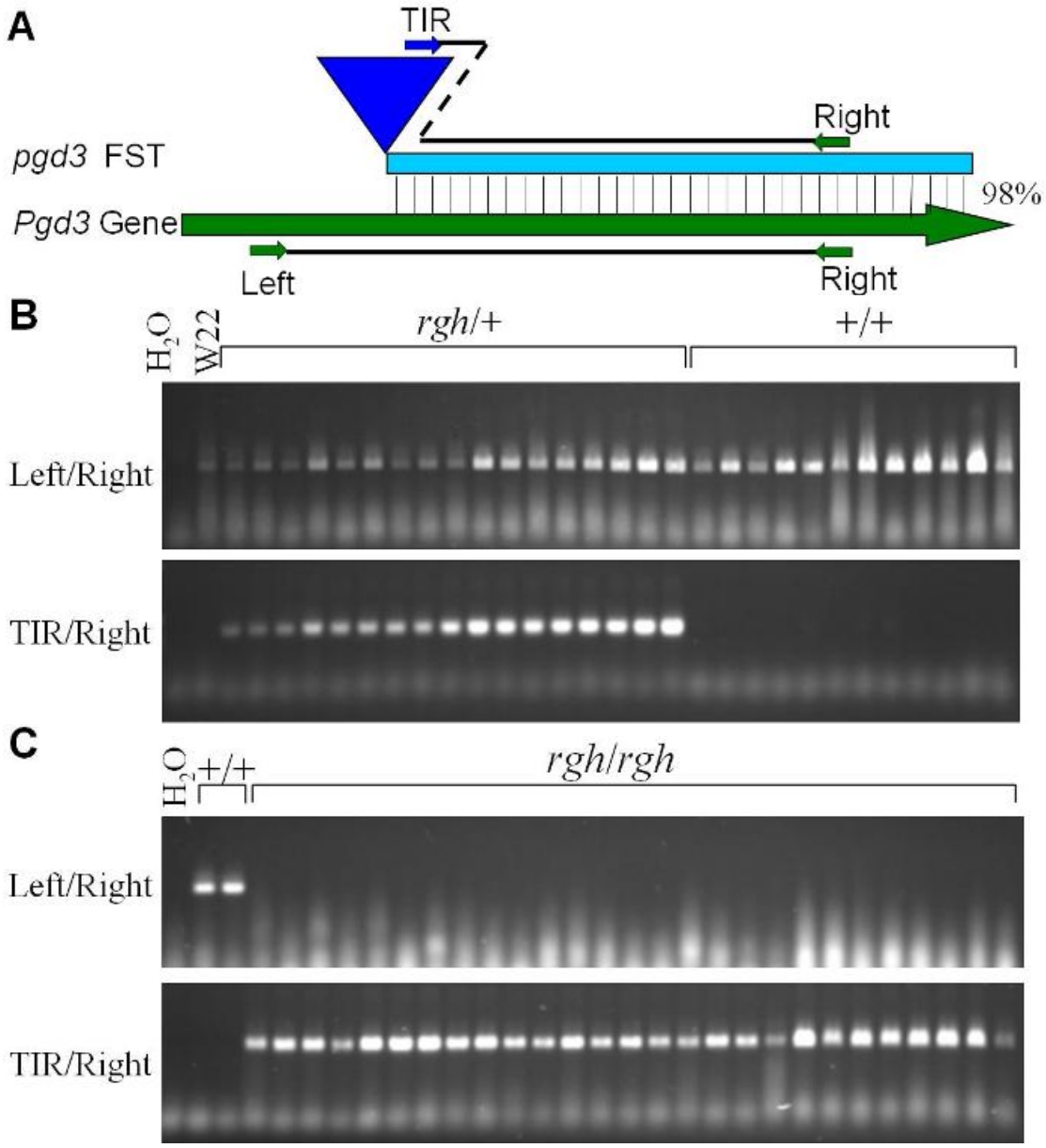

Figure 2.1. *pgd3-umu1* co-segregates with a *rough endosperm* (*rgh*) seed phenotype. A) Schematic of the *pgd3-umu1* insertion site. The identity between *pgd3* FST and *Pgd3* gene sequence is 98%. The left and right primers are specific to genomic region of the insertion site as marked by green arrows. The TIR primer is specific to transposon sequence. B-C) *pgd3-umu1* co-segregates with a *rough endosperm* (*rgh*) seed phenotype. Left/Right primer pair can amplify normal alleles. TIR/Right primer pair can amplify the insertion site. PCR was completed with genomic DNA extracted from homozygous normal, heterozygous and homozygous *rgh* mutant kernels.

3' further from the one of *pgd3-umu1* (Figure 2-2). The homozygous *pgd3-umu2* seeds give the same *rgh* phenotype as *pgd3-umu1* and the heterozygous *pgd3-umu2* plants also exhibit a *rgh*

segregating ear (Figure 2-3). As *pgd3-umu1*, co-segregation analysis showed that *pgd3-umu2* is linked with the *rgh* phenotype.

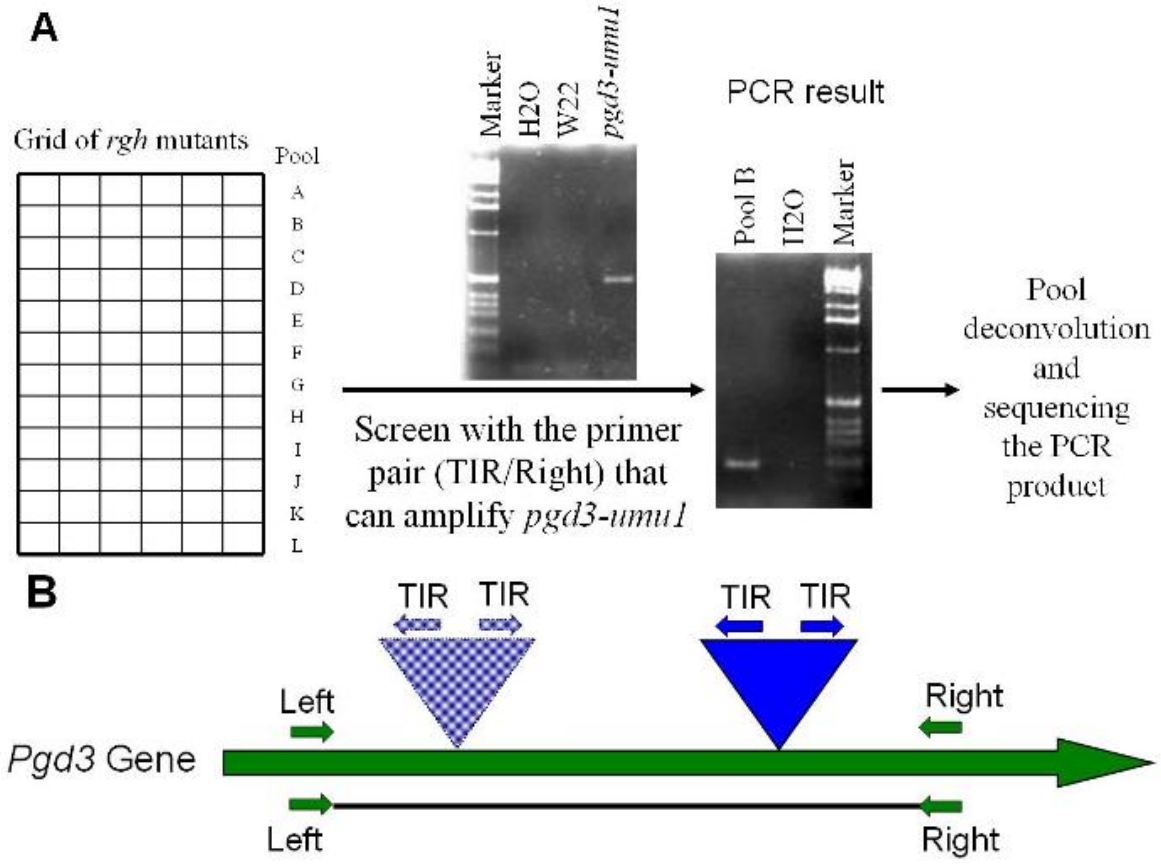

Figure 2.2. *pgd3-umu2*, the second mutant allele of *Pgd3*, was identified by reverse genetic screen. A) Schematic of the reverse genetic screen procedure. Genomic DNA was extracted from the remaining *rgh* mutants to make pools. B) Schematic of the *pgd3-umu2* insertion site, which is 308bp 3' further from the one of the *pgd3-umu1*.

Complementation Test

Reciprocal crosses between the heterozygous plants of *pgd3-umu1* and *pgd3-umu2* have been done in greenhouse and field. Before the crosses, both parents were genotyped using PCR markers. Also, they were self-pollinated to confirm the transmission of the mutation. All reciprocal crosses of two alleles failed to complement the *rgh* phenotype (Figure 2-4). Thus, mutation in *Pgd3* causes a *rgh* phenotype.

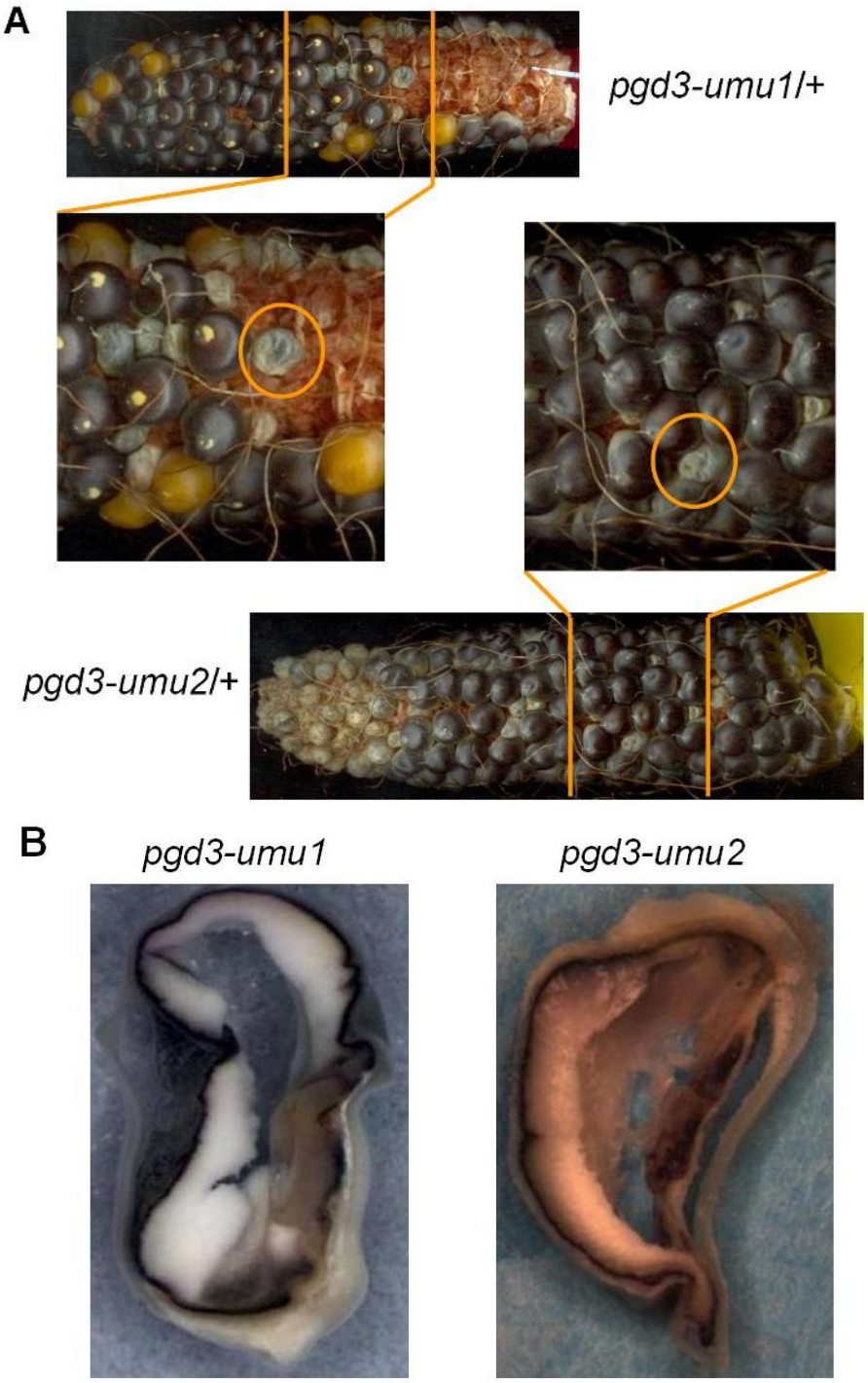

Figure 2-3. The phenotype of *pgd3-umu2* is same as the one of *pgd3-umu1*. A) Both heterozygous *pgd3* ears segregate for *rgh* mutant seeds. B) The longitudinal hand section of the homozygous *pgd3* mutant shows that they have same *rgh* phenotype.

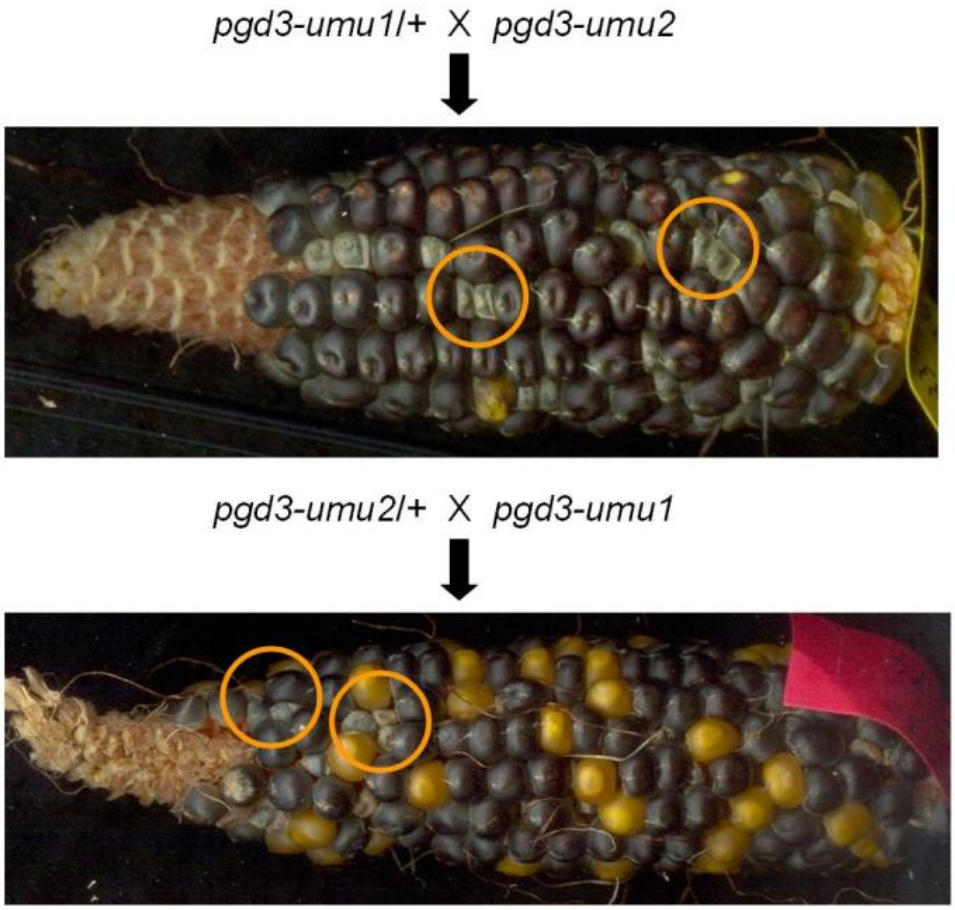

Figure 2-4. Reciprocal crosses between heterozygous *pgd3-umu1* and *pgd3-umu2* plants fail to complement resulting in F1 ears segregating for the *rgh* mutant phenotype (shown in the circle).

CHAPTER 3
RESULTS: PGD3 HAS MULTIPLE NON-REDUNDANT ROLES IN MAIZE

Background

In maize, there are three copies of 6PGDH: PGD1, PGD2 and PGD3. The cytosolic loci, *Pgdl* and *Pgd2*, are located on long arm of chromosome 6 and the long arm of chromosome 3, respectively (Stuber and Goodman, 1984). The gene products, PGD1 and PGD2, respectively, form homodimeric and heterdimeric isozymes that can be visible as separate bands by activity staining after native gel electrophoresis (Bailey-Serres et al., 1992). Cytosolic activity is present in extracts from roots and leaves, but the double-null homozygous mutant (*pgd1; pgd2*) has no detectable cytosolic isozymes in those tissues (Averill et al., 1998). However, there is about 30% of wild type activity in the *pgd1; pgd2* double mutant seedling roots. Also, the double-null homozygous mutant has no visible phenotype and is still reproductively viable, so the wild type levels of cytosolic 6PGDH are not required for development (Averill et al., 1998).

The plastidic 6PGDH is more difficult to purify because of the degradation and contamination from the cytosolic activity. Krepinsky et al. 2001 have separated the plastidic 6PGDH from the cytosolic 6PGDH from spinach leaves by anion-exchange chromatography and have sequenced the digested peptide, showing that the chloroplast 6PGDH had a blocked N-terminus (Krepinsky et al., 2001).

In maize, the predicted *Pgd3* locus has been assembled from ZMGI and MAGI sequences. The predicted PGD3 protein contains a short N-terminal extension, which is absent in PGD1 and PGD2 sequences and is predicted to be a chloroplast targeting signal. In addition, the predicted PGD3 protein is more similar to the spinach chloroplast-localized 6PGDH than to the maize PGD1 and PGD2 (Settles et al., 2007). Finally, the residual activity in homozygous

pgd1;pgd2 double mutants is 30% of wild type and confined to the plastid (Averill et al., 1998). Thus, PGD3 is predicted to be plastid-localized 6PGDH.

pgd3 Seed Phenotype and Plant Phenotype

Although *pgd1; pgd2* homozygous mutants do not have any visible phenotype, the *pgd3* mutant seeds show a *rough endosperm* (*rgh*) seed mutant phenotype (Figure 3-1). The longitudial hand sections of mature mutant kernels showed both reduced grain-fill as well as the failure of embryo development (Figure 2-3). The homozygous mutant seeds could not germinate under normal conditions, such as in soil or wet paper towels. However, a small fraction of those homozygous *pgd3* mutant seeds could be rescued by growth in culture. Those homozygous mutant plants show normal plant morphology except for a pale green leaf phenotype and a reduced growth speed (Figure 3-1).

Both *pgd3-umu1* and *pgd3-umu2* Mutants Are Enzymatic Knockouts in Seeds

After activity staining, there are two bands on the native gel loaded with the whole protein extract of wild type seeds: one slower migrating band, and one faster migrating wide band, which in fact contains several closely migrating bands (Figure 3-2). The faster migrating band indicates cytosolic 6PGDH activity, homodimers or heterodimers (Bailey-Serres et al., 1992). It is missing in the *pgd1; pgd2* double homozygous mutant seed protein extract. Also, this faster migrating band showed a reduced level in *pgd1* and *pgd2* single mutant seeds. The slower migrating band is PGD3 or the plastidic 6PGDH activity. Although both cytosolic and plastidic 6PGDH are active in wild type endosperm and embryo, the enzyme activity assay showed that the plastidic activity is lost in both *pgd3-umu1* and *pgd3-umu2* homozygous mutant seeds. Thus, both *pgd3* mutant alleles are enzymatic knockouts in maize seeds.

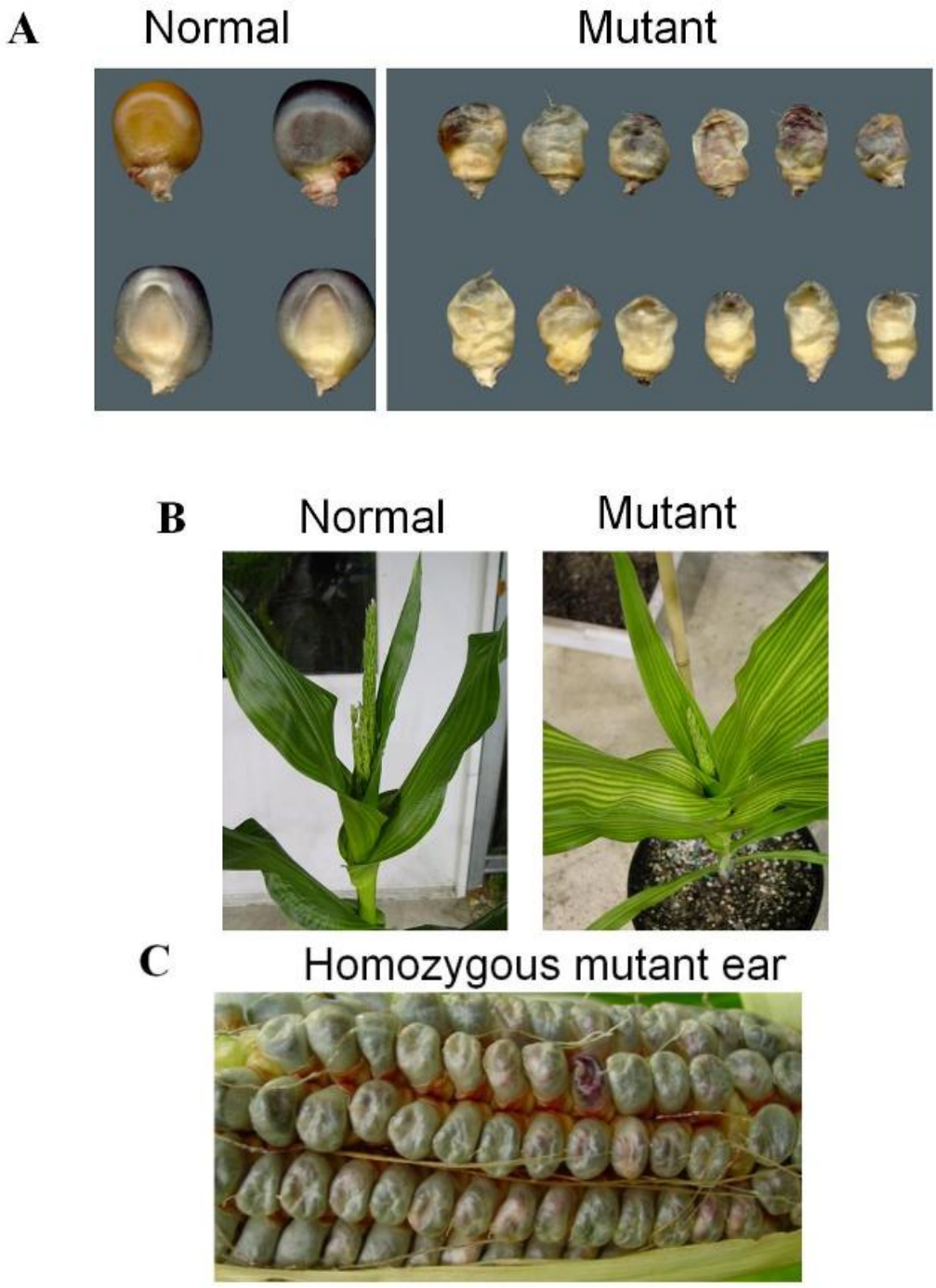

Figure 3-1. Phenotype of the *pgd3* mutants. A) Mature normal and *pgd3* kernels. The top row shows the abgerminal side of the kernels, and the bottom row shows the germinal side of the kernels. B) Normal and *pgd3* mutant plants. C) Homozygous *pgd3* mutant ear.

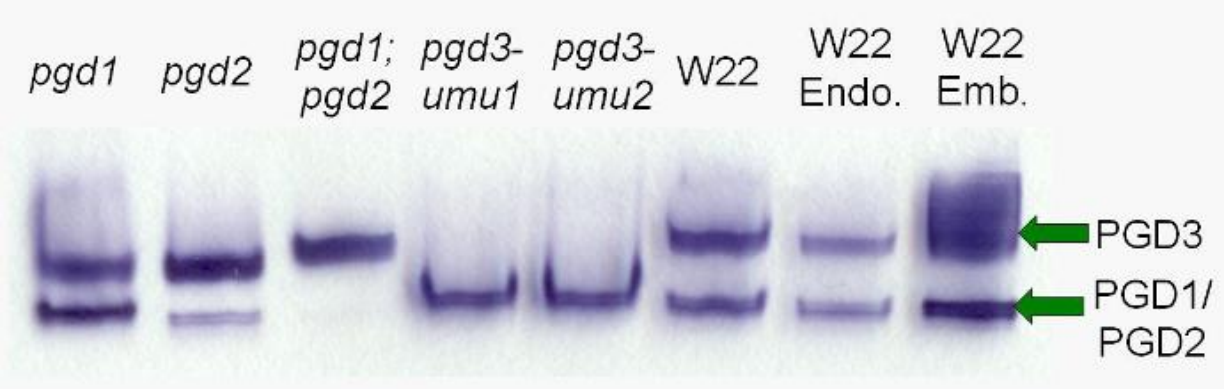

Figure 3-2. 6PGDH activity of *pgd* mutant and normal seeds. The fast migrating band on this native PAGE gel indicates PGD1/PGD2 isozyme activities. The slow migrating band on the native PAGE gel indicates PGD3 activity. Thus, both *pgd3-umu1* and *pgd3-umu2* are enzymatic nulls. The PGD1/PGD2 isozymes are active in *pgd3* mutants indicating that the cytosolic activity is not sufficient to complement the loss of plastidic 6PGDH.

Differences in PGD1/PGD2 and PGD3 Activity Cannot Explain *pgd3* Mutant Phenotypes

The enzyme activity assay from wild type plant showed that almost all tissues show a great cytosolic activity in vitro. However, PGD3 activity level showed tissue differences (Figure 3-3). Protein extract from mature leaves has a relatively very low level of PGD3 activity, but almost all other non-photosynthetic tissues have a great PGD3 activity, including seeds, immature leaves, and roots. Interesting, although there is a substantial PGD3 activity in the inflorescence tissues, loss of PGD3 activity in those tissues did not affect the function and maturation of inflorescence in *pgd3* mutant plants. In fact, both the male and female gametes of the homozygous mutant are fertile, and homozygous mutant ears can be obtained (Figure 3-1). Another interesting thing is that the most obvious *pgd3* phenotype is in tissues with the lowest PGD3 activity, suggesting that the pale green leaf phenotype is caused by loss of PGD3 activity in sink tissues.

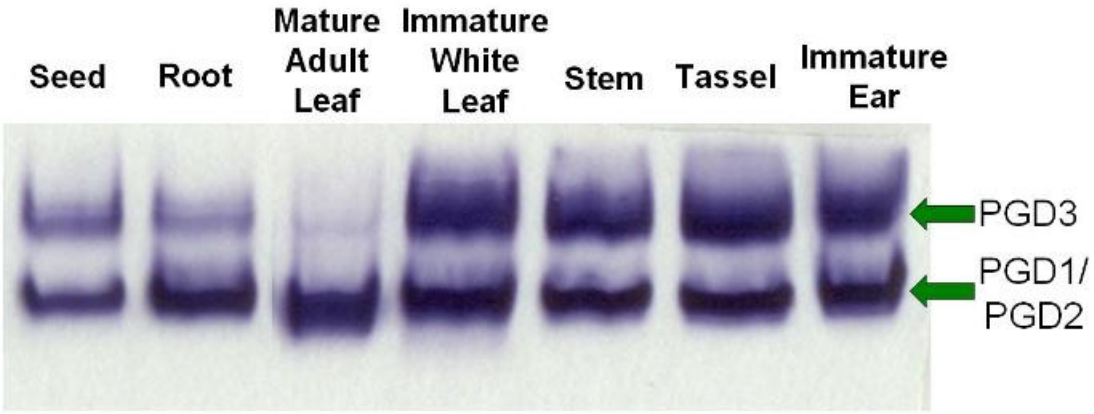

Figure 3-3. 6PGDH activity in different tissues from a wild type plant (W22). The fast migrating band on this native PAGE gel indicates PGD1/PGD2 isozyme activities. The slow migrating band on the native PAGE gel indicates PGD3 activity. It is interesting that most obvious *pgd3* phenotype in plant level (pale green leaves) is in tissues with lowest PGD3 activity (mature adult leaf).

PGD1, PGD2 and PGD3 Can be Co-purified with Plastids

PGD1 and PGD2 are cytosolic and form isoenzyme dimers (Bailey-Serres et al., 1992), and PGD3 is predicted to be plastidic-localized (Settles et al., 2007). Thus, PGD3 activity is expected to co-purify with intact plastids. Indeed, PGD3 activity is found in the intact plastid fraction of isolated chloroplasts and etioplasts from W22 seedling leaves. However, PGD1/PGD2 also co-purify with plastids, and all three enzymes also co-purify with the stroma subfraction and membrane subfraction (Figure 3-4). One possibility is that PGD1/PGD2 may stick on the plastid envelope.The protease thermolysin was used to digest proteins on the outside of the purified plastids, but this treatment did not alter the activity of any of the 6PGDH enzymes (data not shown). To test the digestion efficiency of proteases, protease K, thermolysin, and trypsin were applied on the total protein extract from leaf tissue. The results showed that even the concentration of protease is increased to 20-fold of the typical treatment, the remaining PGD1/PGD2 activity is still detectable, but PGD3 activity is completely gone (Figure 3-5). Thus

PGD1/PGD2 isozymes are highly resistant to proteases, and the PGD1/PGD2 acitivity that purifies with isolated plastids was not been conclusively shown to be cytosolic.

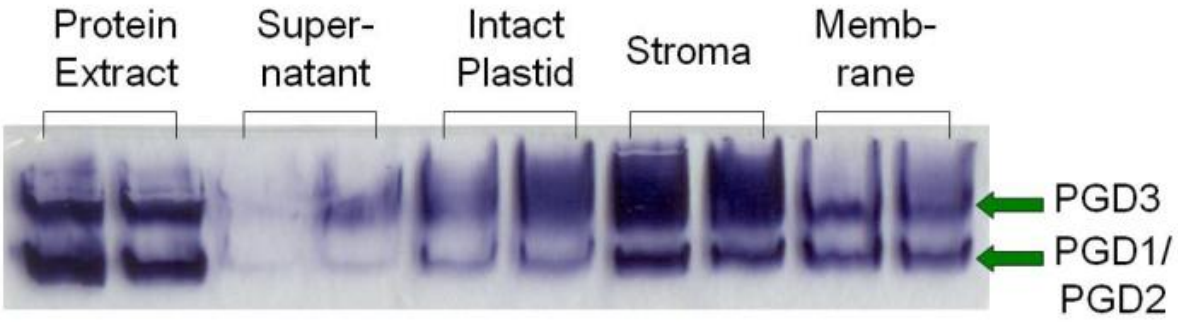

Figure 3-4. 6PGDH activity after chloroplast isolation using total protein extract from fresh leaf tissue as a control. The fast migrating band on this native PAGE gel indicates PGD1/PGD2 isozyme activities. The slow migrating band on the native PAGE gel indicates PGD3 activity. The first two lanes are control sample, total protein extract, from leaf tissue. The second two samples are supernatant after the gradient centrifuge in plastid isolation experiment. The third two samples are intact plastid layer after the gradient centrifuge. The forth two samples are stroma subfraction after breaking the intact plastid extract. The final two samples are membrane subfraction after breaking the intact plastid extract. Thus, both PGD1/PGD2 and PGD3 are active in the plastid extracts.

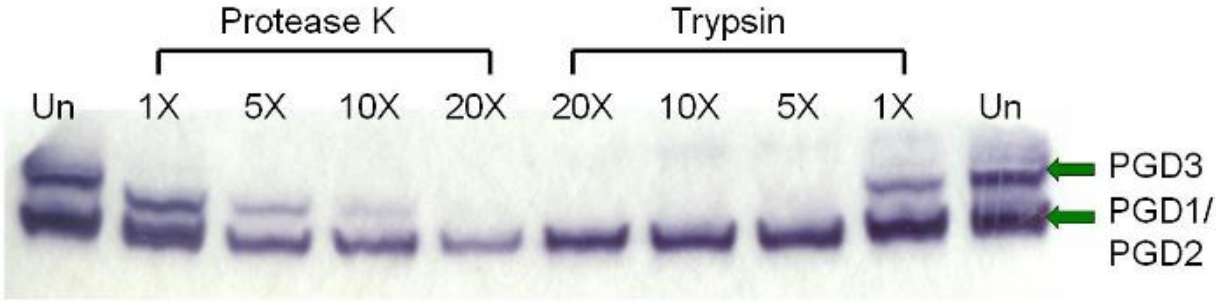

Figure 3-5. 6PGDH activity after protease treatment. The total protein extract from seedling leaf was treated with proteases in 1-fold, 5-fold, 10-fold, 20-fold concentration at 25ºC for 25min, then loaded on the native PAGE gel for enzyme activity assay. The cytosolic activity (PGD1/PGD2) is still detectable after treated with 500ug/ml protease. 1-fold: 25ug/ml.

CHAPTER 4
RESULTS: POTENTIAL NON-REDUNDANT ROLES OF PLASTIDIC 6PGDH

Background

It has been shown that the plastidic OPPP is related to starch biosynthesis in maize kernel by ^{13}C-labelling experiments (Spielbauer et al., 2006). Generally, cereal seeds utilize exogenous glucose or sucrose to synthesize starch. The exogenous hexose is converted to ADP-glucose by AGPase in cytosol, after that ADP-glucose is transported into the plastid and is incorporated into starch. Although it has been suggested that most of ADP-glucose for starch synthesis in cereal endosperm is from the cytosol, hexose in plastids can be converted to ADP-glucose as well. Also, both hexose and triose in the cytosol can be transported into the plastid, becoming a part of OPPP or starch biosynthesis. It has been shown that after providing ^{13}C-labeled glucose, only 20% of ^{13}C -labeled glucose goes into starch directly. It means that about 80% of carbons go through glycolysis or the OPPP before they are incorporated into starch in maize (Spielbauer et al., 2006). This suggests that carbohydrate fluxes are robust in maize endosperm. Also, those fluxes are very stable as the distribution of ^{13}C-labeled glucose in synthesized starch had a very similar pattern in wild type maize kernels as well as many starch synthesis defect mutants, such *brittle2* and *shrunken2* mutants (Spielbauer et al., 2006).

The plastidic OPPP also plays a very important role in plants. It has been suggested that the restriction of the plastidic OPPP in roots affects the sugar induction of nitrogen and sulfur transporter expression. (Lejay et al., 2008) Also, in non-photosynthesis tissues, the NAPDH produced by the plastidic OPPP is a major resource of reducing power for the ferredoxin regulation system, a major regulation system in many biosynthesis pathways. For example, in nitrogen assimilation and glutamine synthesis, although nitrate is reduced to nitrite in the cytosol,

nitrite needs to be reduced in plastid by ferredoxin system and nitrite reductase (Bowsher et al., 1992).

Plastidic 6PGDH is Required for Normal Starch Synthesis in Seeds

Since ^{13}C fully labeled glucose is supplied to cultured wild type kernels, if such glucose is converted into starch without going through any other metabolic flux, the carbon in the glucose digested from synthesized starch in kernels should be all ^{13}C carbons, so the percentage of the 111111 isotopolog glucose would be 100%. However, the isotopolog pattern of starch in wild type kernels showed that about 15% glucose is incorporated into starch directly (Figure 4-1). About 65% glucose is converted to triose prior to starch synthesis, indicated by the 111000 and 000111 type glucose. Also, about 10% glucose needs to be converted to pentose, then goes into starch, indicated by the 110000 and 001111 type glucose (Figure 4-1).

However, the isotopolog pattern in *pgd3* homozygous mutant kernels is very different. About 30% glucose is directly incorporated into starch, shown by the percentage of the 111111 type glucose, which is 2-fold of the percentage in wild type seeds. The percentage of triose incorporation (111000 and 000111 type glucose) is reduced, but the percentage of pentose incorporation did not have a significant change. Thus, the metabolic fluxes of normal starch synthesis are altered signigicantly by mutations in *Pgd3*.

Nitrogen Assimilation May be Limited in *pgd3* Mutants

As the plastidic OPPP is suggested to be related to nitrogen assimilation and glutamine synthesis, it is hypothesized that exogenous nutrients may help *pgd3* mutant seed germination in the embryo rescue experiments. Since the germination percentage of the *pgd3* seeds in culture rescue experiments are very low, it is important to find an assimilated nitrogen source that can be absorbed by kernels and does not impair tissue culture growth. Asparagine is common nutrient in endosperm culture medium, so this nutrient was added into the MS medium. Asparagine rescue

experiment suggests that exogenous asparagine gives a 2-fold increase in *pgd3* mutant germination (Figure 4-2). Also, this nutrient accelerated germination to some extent.

In addition, total nitrogen and carbon content in *pgd3* homozygous mutants and wild type plants were measured with a CN analyzer to investigate whether *pgd3* mutants were able to uptake nitrate. The homozygous *pgd3* mutants are very sick and hard to survive and only one comparison between mutant and wild type was possible. The mutants in this single experiment had about 8% nitrogen while normal sibling plants had about 5% nitrogen. These data gives suggestive evidence that *pgd3* mutants are not significantly impaired in nitrate transport.

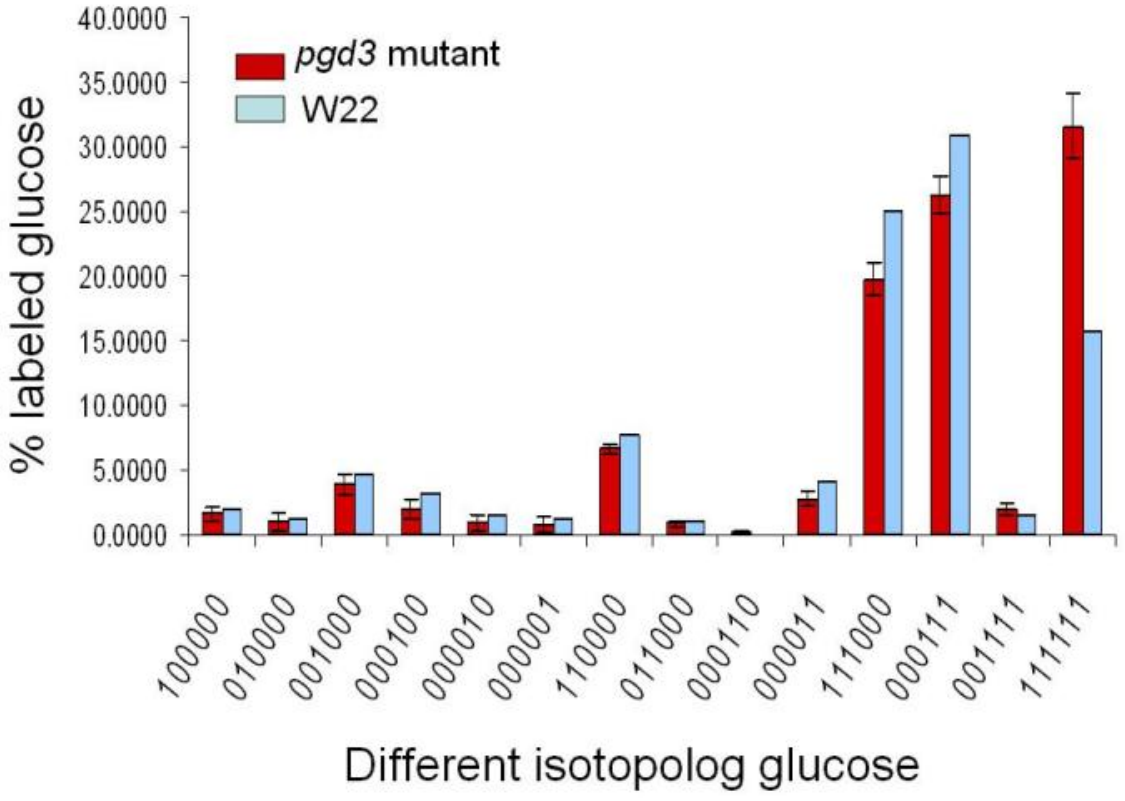

Figure 4-1. Mutation in *Pgd3* changes isotopolog patterns of glucose in starch from the maize kernels. The percentage of 111111 isotopolog type glucose in *pgd3* mutant is 2-fold of the one in wild type, indicating that the percentage of direct incorporation of glucose into starch in *pgd3* mutant is greatly increased.

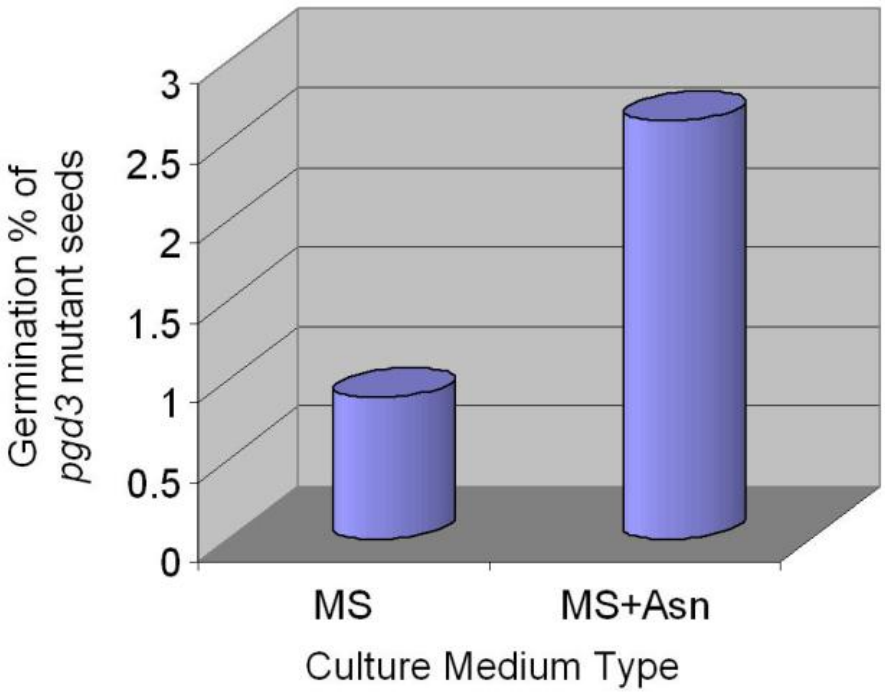

Figure 4-2. The germination percentage of the *pgd3* homozygous mutant seeds in the culture rescue experiment. The addition of the asparagine monohydrates in medium helped germination as the germination percentage in MS+Asn medium is 2-fold higher than the one in MS medium.

CHAPTER 5
DISCUSSION

The characteristics of 6PGDH enzyme have been studied extensively in *Drosophila melanogaster*, *Saccharomyces cerevisa*, *Ovis aries* and *Trypanosoma brucei*. Although the crystal structure of yeast Gnd1 protein suggested that there are three domains in 6PGDH (He et al., 2007), the third domain (C-terminal tail) can be included into the alpha domain structure. Thus, all 6PGDH enzymes are separated into two domains. The C-terminal is an all-alpha domain, and the NAD binding domain forms a Rossman fold.

The predicted PGD3 protein shows many similarities to other 6PGDH enzymes, as the BLAST search of this sequence on the NCBI webpage gives hit on NAD-binding domain and C-terminal domain. The insertion in *pgd3-umu1* and *pgd3-umu2* alleles occured at 1151 and 1459 bp 3' further of the predicted ATG site, respectively. Thus, the mutation is likely disrupted the C-terminal domain of 6PGDH, causing the loss of activity.

It is very hard to eliminate cytosolic contamination when purifying the plastidic enzyme from spinach, so the evidence to confirm purification of plastidic 6PGDH is based on sequence analysis. However, although the localization of PGD3 is predicted to be plastidic (Settles et al., 2007), the N-terminal signal in PGD3 is a very weak evidence since it is very short compared to the plastidic 6PGDH from spinach. Thus, it is not as clear that PGD3 is plastid-localized.

As maize has three copies of 6PGDH, it has been suggested that the complete loss of PGD1/PGD2 can be complemented by the PGD3 activity (Averill et al., 1998). The enzyme activity assay suggested that both PGD1/PGD2 and PGD3 were highly active in endosperm and embryo. However, the visible phenotype of *pgd3* mutants suggested that the PGD3 protein has a non-redundant role in maize kernels. As *pgd3* seeds show a *rgh* phenotype with a greatly reduced grain-fill, it is reasonable to hypothesize that mutation in *Pgd3* affects starch biosynthesis in

maize kernels. In maize, *brittle2* and *shrunken2* mutants are starch biosynthesis defect mutants that mutated in cytosolic AGPase, which converts glucose 6-phosphate to ADP-glucose in the cytosol. Thus, those two mutants should reduce the incorporation of glucose from cytosolic carbon flux. However, the ^{13}C-labelling experiments showed that the carbon fluxes pattern in those two mutants did not have a significant change (Spielbauer et al., 2006). It has shown that the metabolic fluxes in *pgd3* mutant seeds are significantly different from those in wild type seeds, as the percentage of directly incorporation is doubled. Thus, PGD3 is required for normal starch biosynthesis. Then, there are two possible models for starch biosynthesis in maize kernels. Both two models required exogenous hexose to go through OPPP cycles before starch incorporation. After OPPP cycles, hexose may be converted into ADP-glucose in the plastid to synthesize starch, requiring plastidic AGPase. Alternatively, hexose may be transported out of the plastid, converted to ADP-glucose in the cytosol, and ADP-glucose is transported back to plastid. The reason why the plastidic OPPP is required is still unknown. There are several possibilities. The ADP-glucose transporter might be affected by *Pgd3* mutation, as the sequence analysis of this transporter suggested that it is a target of ferredoxin regulation, related to reducing power provided by OPPP. Also, OPPP provides substrates for nucleotide biosynthesis, such as AMP, ATP. AMP is the exchanger for ADP-glucose transporter. ATP is a substrate for synthesizing ADP-glucose.

Although the plastidic 6PGDH is essential in maize seeds, it is interesting that both female and male gametes of homozygous *pgd3* mutant plants are fertile. Thus, the cytosolic 6PGDH might complement the mutation of *Pgd3* in some tissues. Also, 6PGDH activity assays of various plant tissues suggests that both cytosolic and plastidic activity maintain a high level in almost all non-photosynthetic tissues, including roots and developing leaves. The homozygous

pgd3 plants give a visible phenotype, suggesting that the plastidic 6PGDH has a non-redundant role at the whole plant level. However, the study of the whole plant level is limited, as the homozygous mutant plants exhibit very poor growth and very few can survive. Only 1~5% of the homozygous mutants can germinate by embryo rescue experiments on the basic MS medium.

Interestingly, exogenous asparagine helped mutant seed germination suggesting that *pgd3* mutants have a defect in amino acid biosynthesis. There are several possible explanations for this phenomenon. First, plastidic OPPP provides NADPH, which is required for nitrite reductase to convert nitrate into ammonia in plastids. Second, is has been shown that inhibition of 6PGDH activity will suppress the sugar induction of the nitrate transporter expression. A mutation in *Pgd3* may limit nitrate uptake. However, it is still not clear that whether the amino acid biosynthesis defect is the primary defect of *Pgd3* mutation, as there are many difficulties in comparing the metabolites in *pgd3* plants to wild type plants. First, all mutants are come from the culture medium and are transplanted to the soil, but the wild type plants are germinated directly from soil. Also, the mutants grow much slower than wild type plants, so it is very difficult to make the mutant and wild type plants in the same development level for sampling. A possible resolution would be to transplant both mutants and wild type plants to hydroponic growth. In such case, environment effects will be reduced. Also, it would provide the possibility to give different nutrient sources to know the key nutrient for complementing or worsening the *pgd3* phenotype.

CHAPTER 6
MATERIALS AND METHODS

Genomic DNA Extraction

Fresh leaf tissues were ground with urea extraction buffer, or frozen leaf tissues were ground with liquid nitrogen, and then urea extraction buffer was added in a g F.W. : mL ratio of 1:2.5. The urea extraction buffer was made from 168 g urea, 25 mL of 5M NaCl, 20 mL of 1M Tris–HCl pH 8, 16 mL of 0.5M EDTA pH 8, 20 mL of sarkosine and 190 mL of H_2O. The mixture was transferred to a 2 mL-microfuge tube. An equal volume of phenol:chloroform :Isoamylalcohol (25:24:1) was added to the tubes and mixed well by gently shaking the tubes for 15 min. The tubes were centrifuged at room temperature for 10 min at 1600 Xg. The upper aqueous phase was collected in a new tube. The DNA was precipitated from the solution by adding 0.1 volume of 3 M sodium acetate pH 7.0 and 0.7 volume of isopropanol. After 10 min of incubation on ice the tubes were centrifuged for 15 min at 1600 Xg. The DNA pellet was washed with 70% ethanol and air dried. The DNA was dissolved in TE (10 mM Tris–HCl pH 8, 1 mM EDTA pH 8). Each DNA sample was diluted to approximately100 ng/µL in water and 1 µL were added to a 20 µL PCR assay reaction mixture.

PCR Assay

The wild type allele was amplified with gene specific primer that is 5’ to the transposon insertion (02S-2018L1: 5’-GGTTAATGTCGACAAGAAGGTGCTG-3’) and 3’ to the transposon insertion (02S-2018R1: 5’-CCCTTCTCATACCAACCAATTCCTC-3’). The mutant allele was amplified with one gene specific primer and TIR8 primer. The TIR8 primer was composed of the TIR8.1 (5’-CGCCTCCATTTCGTCGAATCCCCTS-3’), TIR8.2 (5’-CGCCTCCATTTCGTCGAATCCSCTT-3’), TIR8.3 (5’-SGCCTCCATTTCGTCGAATCCCKT-3’) and TIR8.4 (5’-

CGCCTCCATTTCGTCGAATCACCTC-3') primers mixed in a 2:4:4:1 ratio, respectively. The PCR amplification was carried out in a volume of 20 μL containing 100 ng of template DNA, 1.0 uL of 40 mM $MgCl_2$, 1uL of 100%DMSO, 2.0 μL of 10X PCR buffer, 2.0 uL of 2 mM each dNTP, 25 pmole of primer and 0.3 uL 50 Unit Taq DNA Polymerase. Thermocycling conditions were generally 94°C for 1 min, 60°C for 1 min, 72°C for 1 min for 40 cycles. The same DNA extraction and PCR assay were used for co-segregation analysis, reverse genetic screen of *pgd3-umu2*, and genotyping plants.

Total Protein Extraction

Seeds for enzyme activity were harvest 16 days after pollination (DAP). Roots and immature white leaves were harvested from seedling 1 week after germination. Tassel and immature ear were harvested from mature plant before flowering. Mature adult leaves were harvested from mature plant after flowering. Leaves for all fresh tissues were harvested into liquid nitrogen and stored at -80 ºC. The extraction procedures were carried out at 4ºC. Frozen tissues were ground with liquid nitrogen and then added extraction buffer (100 mM Tris-HCl pH 7.5, 30 mM 1,4-Dithiothreitol (DTT), 15% (v/v) glycerol) in the mg/ul ratio of 1:1 for seeds and mg/ul ratio of 1:2 for other tissues. Then the mixtures were centrifuged for 20 min at 1600 Xg. The upper aqueous phase was collected in a new tube, and stored at -80ºC. For protease treatment, fresh extract was distributed at 50 uL per centrifuge tube, and added protease to make the final protease concentration to be 25 uL/mL for 1-fold digestion. The stock of protease K and trypsin was made in 20 mM HEPES pH 8 up to 2 mg/mL. Thermolysin was made at 2 mg/mL in import buffer (50 mM HEPES/KOH pH 8, 0.33 M sorbitol) with addition of 10 mM $CaCl_2$. After incubation on ice for 45 min or at 25ºC for 25 min, protease K was terminated with an equal volume of 4 mM PMSF (phenyl methyl sulfonyl fluoride). PMSF stock solution is made freshly up to 100 mM in ethanol, and used soon after preparation. Trypsin was terminated with equal

volume of 4 mM PMSF and 0.2 mg/mL soybean trypsin inhibitor. Thermolysin was terminated with 100 mM EDTA.

Plastid Isolation

Seeds were planted in growth chamber either in dark to obtain etioplastids or in light to obtain chloroplasts. The temperature of the growth chamber is 30ºC at day and 20ºC at night, so seeds germinated very fast. All procedures were carried out at 4ºC. Percoll gradients were prepared freshly before the isolation experiment. To make percoll gradients, 1 mg glutathione was mixed with 17.5 2X GR-buffer by vortexing, mixed with 17.5 mL Percoll by inversion, spin at 48000 Xg for 40 min, and then stored in 4ºC. Seedling leaves were harvested 6 days after planting, starting at the basal meristem, and then cut into 0.5 to 1 cm sections. Approximately 30 g leaf tissues were ground in 200 mL GR-buffer (50 mM HEPES/KOH pH 7.5, 0.33 M sorbitol, 2 mM EDTA, 5 mM Na-ascorbate, 0.1% BSA) with a Polytro w/PTA35/2M probe set at medium power. After filtering slurry through one layer of Miracloth, the collected samples were spin at 1800 Xg for 3 min, and supernatant was removed. The pellet was resuspended by swirling in 5 mL GR-buffer, and loaded on the performed Percoll gradients. After gradient centrifugation at 1800 Xg for 15 min, intact plastids were in bottom band, and other bands were removed carefully with a wide bore pipet. The intact plastid band were diluted about 1:4 with import buffer (50 mM HEPES/KOH pH 8, 0.33 M sorbitol), invert gently for mixing, and then centrifuged at 1500 Xg for 6 min. The pellet was resuspend at 1 mg chlorophyll/mL in import buffer, and stored at -80ºC. For breaking intact plastids, the pellet was resuspended at 1 mg chlorophyll/mL in HKM buffer (10 mM HEPES/KOH pH 8, 10 mM $MgCl_2$), incubated on ice for 5 min, and added an equal volume of 2X import buffer. Then the lysates were centrifuged at 42000 Xg for 30 min. The supernatant is the membrane fraction, and the stroma pellet was resuspended in import buffer at same volume of membrane fraction. For protease thermolysin

treatment, intact chloroplasts were mixed with import buffer at 100 ug chlorophyll/ 0.5 mL import buffer, and then incubated with 25 uL thermolysin (2 mg/mL in import buffer, 10 mM $CaCl_2$) at 4ºC for 45 min or at 25ºC for 25 min. The digestion was terminated by adding 100 uL 50 mM EDTA-import buffer. The plastids were repurified on 35% Percoll-5 mM EDTA-import buffer, and then resuspended in 1 mL HKM buffer for subfraction.

6PGDH Activity Assay

The protein samples were loaded onto a native polyacrylamide gel and electrophoresed at 20~25 mA and 4ºC for 2.5 hr. 6PGDH activity was revealed by incubating gels at room temperature for 30 min to 1 hr, in the dark, in 6PGDH activity stain (0.1 mg/mL $NADP^+$, 0.1 mg/mL nitro blue tetrazolium, 0.1 mg/mL phenazine methosulfate, 0.5 mg/mL 6-phosphogluconate, 100 mM Tris-HCl pH 7.5). Activity stain solutions were made just before using. Gels were stored in water overnight prior to drying.

Mutant Seeds Rescue

The medium for embryo rescue was made with MS and 3% sucrose and sterilized for 30 min, then stored at 4ºC. The addition of 0.2% asparagine monohydrates was made before sterilization. Both mutant and normal seeds were harvested 21 days after pollination from heterozygous ears. The freshly harvested seeds were sterilized with 70% ethanol for 2 min, followed by 20% (v/v) bleach for 15 min. Next, the seeds were washed by sterilized water for several times. The pericarp was carefully cut at the endosperm and embryo axis to get the immature embryo with some endosperm tissues, which was then incubated on the sterilized medium in a growth chamber (30ºC at day and 20ºC at night).

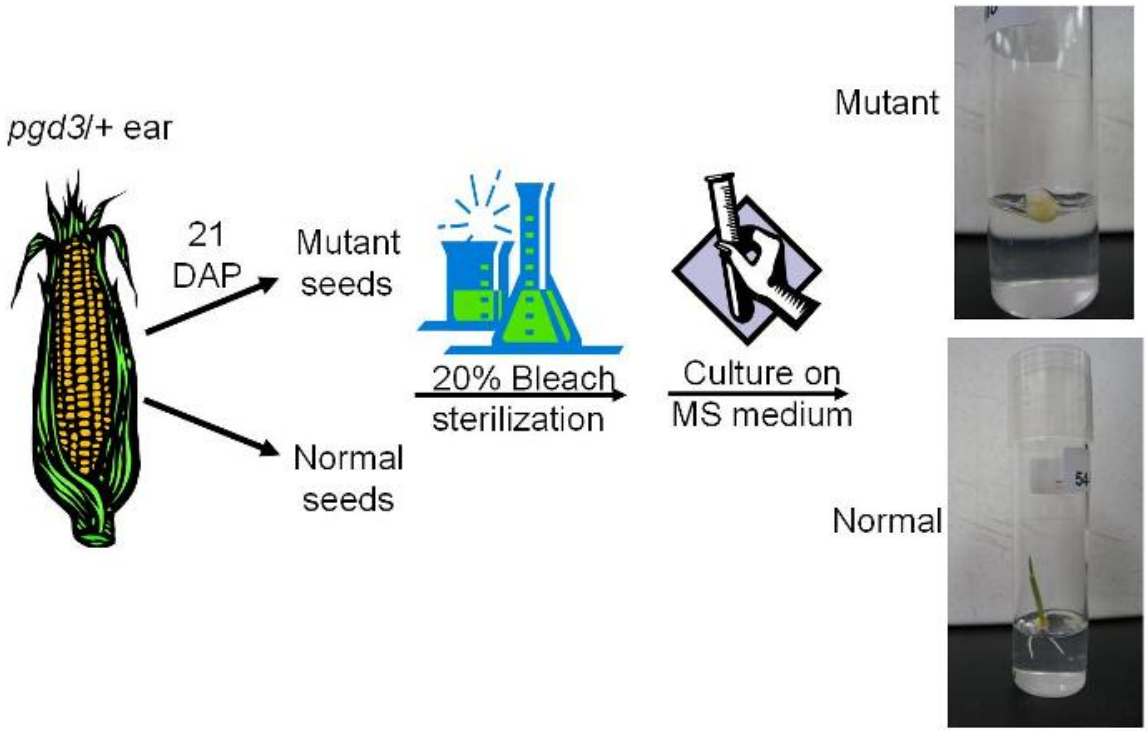

Figure 6-1. Schematic of the mutant seeds rescue experiments. Both mutant and normal seeds were selected from heterozygous ear 21 days after pollination. After sterilization and then removing pericarp, those seeds were cultured on MS medium.

^{13}C-labelling Experiments

Heterozygous ears were harvested 8–10 day after pollination and kernels were cultured as previously described in Spielbauer et al., 2006. For labeling, kernel blocks were transferred onto fresh culture media containing 77.4 g/L glucose and 2.6 g/L [U-$^{13}C_{12}$] glucose. Kernels were harvested after 7 days, frozen in liquid nitrogen and stored at -80ºC and transported to Germany for starch analysis. The measurement of the percentage of isotopolog glucose in starch by NMR spectroscopy was described in Spielbauer et al., 2006. Different isotopolog glucose was marked as 6 digit number in order like Figure 6-2.

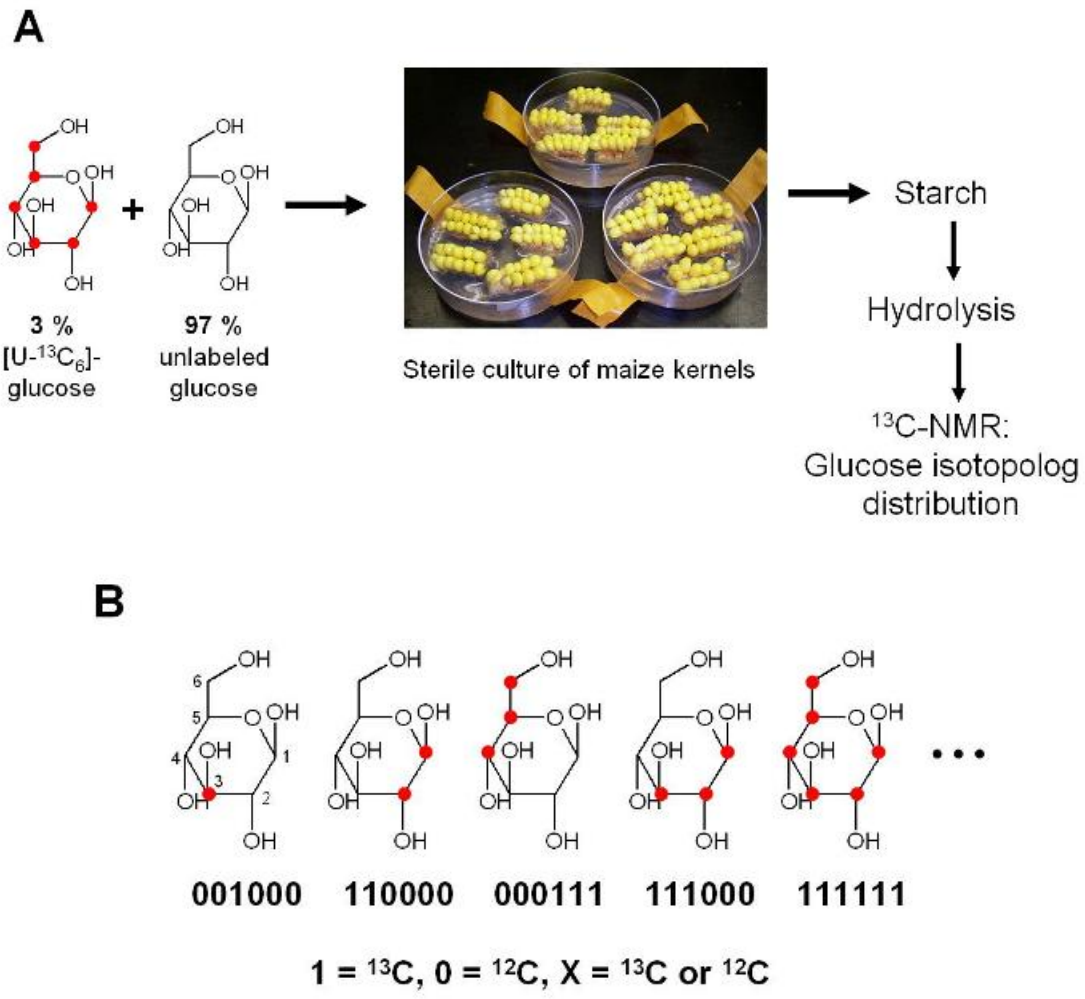

Figure 6-2. Schematic of the ^{13}C-labeling experiments. A) Procedure of the ^{13}C-labeling experiment. B) Different isotopolog glucose in starch is marked as 6 digit number in order: 0 stands for ^{12}C and 1 stands for ^{13}C.

Total Nitrogen Measurement

Homozygous mutant plants were obtained from the embryo rescue experiments. Wild type seeds were planted at the same time when mutants were transplanted into the greenhouse. The top and second leaves were harvested from those plants. Starting from 2 cm further from the tip, a 10 cm long rectangle of leaf tissue was cut and dried in 65ºC for 3 days. Then the dried leaf tissues were ground to powder. The weights of the fresh and dry tissue were recorded. About 5

mg dry powder was rolled in tin cups for the carbon and nitrogen analysis in CN analyzer. Apple standard sample in range from 3 mg to 6 mg was used for calibration in this measurement.

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BIOGRAPHICAL SKETCH

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