Chloroplast-localized 6-phosphogluconate dehydrogenase is critical for maize endosperm starch accumulation

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Abstract

Plants have duplicate versions of the oxidative pentose phosphate pathway (oxPPP) enzymes with a subset localized to the chloroplast. The chloroplast oxPPP provides NADPH and pentose sugars for multiple metabolic pathways. This study identified two loss-of-function alleles of the Zea mays (maize) chloroplast-localized oxPPP enzyme 6-phosphogluconate dehydrogenase (6PGDH). These mutations caused a rough endosperm seed phenotype with reduced embryo oil and endosperm starch. Genetic translocation experiments showed that pgd3 has separate, essential roles in both endosperm and embryo development. Endosperm metabolite profiling experiments indicated that pgd3 shifts redox-related metabolites and increases reducing sugars similar to starch-biosynthetis mutants. Heavy isotope-labelling experiments indicate that carbon flux into starch is altered in pgd3 mutants. Labelling experiments with a loss of cytosolic 6PGDH did not affect flux into starch. These results support the known role for plastid-localized oxPPP in oil synthesis and argue that amyloplast-localized oxPPP reactions are integral to endosperm starch accumulation in maize kernels.

Key words: Defective kernel, endosperm, maize, pentose phosphate pathway, PGD3, starch.

Introduction

The pentose phosphate pathway (PPP) is central to plant metabolism (Kruger and von Schaewen, 2003). The oxidative section of the PPP (oxPPP) converts glucose-6-P into ribulose-5-P. Glucose-6-P dehydrogenase (G6PDH, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGDH, EC 1.1.1.44), catalyse the irreversible oxidation of glucose-6-P, generating two molecules of NADPH and one molecule of CO2. The product of the first oxidative reaction, 6-phosphoglucono-δ-lactone, can hydrolyse to 6-phosphogluconate spontaneously or by the catalysis of 6-phosphogluconolactonase (6PGL) (Miclet et al., 2001). The non-oxidative part of the PPP (noxPPP) delivers carbon intermediates for the biosynthesis of amino acids, nucleic acids, lignin, and polyphenols.

The oxPPP is an important source of reducing power in multiple cellular compartments. Non-photosynthetic cells generate NADPH and NADH through the oxPPP, glycolysis, malic enzyme, and the tricarboxylic acid cycle whereas photosynthetic cells generate reducing equivalents primarily from the light reactions. Although multiple transporters move metabolic intermediates between membrane-bound subcellular compartments (Linka and Weber, 2010), direct transport mechanisms for NADH and NADPH are not known. Therefore, subcellular compartments produce reductants independently and plant oxPPP operates at least in the plastid and cytosol based on subcellular localization.
of enzymes (Kruger and von Schaewen, 2003). In pea and Arabidopsis, G6PDH, 6PGL, and 6PGDH were localized to peroxisomes (Corpas et al., 1998; Reumann et al., 2007; Xiong et al., 2009; Meyer et al., 2011), but not all of these enzymes have peroxisomal targeting signals. By contrast, noxPPP enzymes are always found in the plastid, while localization to other cellular compartments is dependent on plant species and tissue (Debnam and Emes, 1999; Kopriva et al., 2000).

G6PDH is considered the rate-limiting and flux-controlling enzyme of the oxPPP due to feedback inhibition by NADPH in spinach chloroplasts (Lendzian and Basmajian, 1975). G6PDH is associated with nitrogen assimilation, oxidative stress, and defence responses (Bowsher et al., 1992; Debnam et al., 2004; Scharte et al., 2009). Tobacco antisense lines of a plastidic G6PDH isoform and Arabidopsis G6PDH mutants disrupting one of two plastidic or two cytosolic isozymes show no obvious phenotype (Debnam et al., 2004; Wakao et al., 2008). By contrast, loss of chloroplast-localized 6PGL in Arabidopsis are seed lethal (Xiong et al., 2009).

The second dehydrogenase, 6PGDH, is not thought to have regulatory properties similar to G6PDH and is not considered a rate-limiting step. All higher plants so far examined have distinct cytosolic and plastidic 6PGDH isoforms (Debnam and Emes, 1999; Krepinsky et al., 1995; Kopriva et al., 1999; Alfenito, 1993). The maize genome encodes three copies of 6PGDH. Double mutants of the maize cytosolic isozymes, PGD1 and PGD2, have no obvious phenotype (Bailey-Serres et al., 1992). The third 6PGDH isoyme, PGD3, is predicted to be plastid localized, based on the available data in the literature and protein targeting prediction algorithms (Averill et al., 1998; Settles et al., 2007). However, multiple sources of inference are needed to hypothesize that PGD3 is plastid localized, with no one experiment being conclusive. The current study group previously identified a transposon insertion mutant disrupting enzyme activity of PGD3 (Settles et al., 2007). The pgd3-umul knock-out allele co-segregates with a rough endosperm (rgh) kernel phenotype generating a plausible hypothesis that PGD3 has a role in seed development. In addition, a small fraction of the rgh kernels, which was pgd3 loss-of-function due to genetic linkage, could be rescued by tissue culture allowing the conclusion that loss of PGD3 is not completely lethal to maize plants.

This paper proves that loss of PGD3 causes severely reduced grain-fll phenotypes. A combination of isozyme activity, protein subcellular localization, and translocation genetic data indicate that plastidic 6PGDH activity is associated with sink tissues in maize and has distinct functions in the endosperm versus the embryo. Histology, metabolite profiling, and stable isotope-labelling experiments suggest an active involvement of plastidic 6PGDH in starch biosynthesis. Combined, these results suggest the oxPPP has a more important role for starch synthesis in the cereal endosperm than recent models indicate (Hannah and James, 2008; Comparot-Moss and Denyer, 2009).

Materials and methods

Chemicals and plant materials

Chemicals were from Sigma-Aldrich (St Louis, MO, USA) or VWR (Radnor, PA, USA). [U-14C]Glucose was from Isotec (Miamisburg, OH, USA). UniformMu (McCarty et al., 2005) alleles of pgd3-umul and pgd3-um2 were maintained as heterozygotes. Homozygous pgd1;pgd2 double mutants were from Julia Bailey-Serres (University of California, Riverside). The TB-4Sa, B-A translocation stock was maintained as a transposon-marked hyperloid (Birchler and Alfenito, 1993) and crossed onto pgd3+/-, bi2/lr2, and dec11/+ plants. Mature non-concordant kernels were selected visually. Maize plants were either grown in field conditions in Citra, FL or in greenhouse conditions in Gainesville, FL.

Screen for pgd3-umul2

Genomic DNA of 72 rgh isolates was pooled into a 6 × 12 grid. The pgd3-umul2 insertion was amplified and sequenced using Mutator (Mu) and Pgd1 specific primers as described (Settles et al., 2007). Genetic complementation was tested by crossing genotyped heterozygous plants.

Morphology

Mature kernels and sagittal hand sections, cut with a utility knife, were imaged on a flatbed scanner. Normal and pgd3 kernels from segregating ears were harvested for microscopic analysis at 16 days after pollination (DAP). Toluidine blue-stained 500 nm sections were prepared as described (Kang et al., 2009).

Biochemical analyses

6PGDH activity was assayed on a total protein basis by native PAGE as described (Settles et al., 2007). Tissues were harvested from seedling roots and shoots 7 days after planting, tassels and immature ears just before flowering, and adult leaves after flowering. Protein was extracted from 1.05 g tissue using a 1:1 (ml:g) ratio of extraction buffer to tissue freshweight for kernels and a 2:1 (ml:g) ratio for all other tissues. Plastids were purified and subfractionated as described (Cline et al., 1993; Mori et al., 1999).

For metabolomics, three ears per genotype were harvested at 20 DAP, and metabolite analysis was performed on 3–7 replicates per ear. Kernels were frozen in liquid nitrogen and dissected, and lyophilized tissues were ground using a MiniBeadBeater-96 (BioSpec Products, OK). Analysis of metabolites using GC-MS was completed on 100 mg endosperm as described (Witt et al., 2012). Ribitol was the quantitative internal standard. Normalized relative ratios of each metabolite were calculated based on sample dryweight and percentage of solubles (normalized relative ratio = response ratio × 100/sample dryweight%/solubles). Remaining endosperm tissue after extraction was analysed for starch and protein as described (Spielbauer et al., 2009). Principal component analysis was performed with XLSTAT.

NADPH and NADP+ were determined with a modified cycling assay (Kolbe et al., 2005). For this, 400 µg DAPI-b-DAP kernels were ground in a mortar for 2 min with 1.5 ml boiling 0.1 M NaOH or HCl for NADPH or NADP+, respectively. Extracts were transferred to ice, neutralized with 1.05 mM MTT, 12.5 mM glucose-6-P). For the cycling reaction, 10 µl of G6PDH (Sigma G5885, 35 U ml−1 and 0.1 M Tricine) was added, incubated for 30 min at 37 °C, and absorbance was measured at 570 nm.
13C labelling experiments

W22, pgd3-umu1/+ and homozygous pgd1;pgd2 were grown in field conditions. Homozygous pgd3-umu1 plants were recovered via embryo culture as described (Settles et al., 2007). Ears were self-pollinated, harvested at 8 DAP and ear block tissue cultures were initiated in a modified MS medium as described (Ettenhuber et al., 2005), except that 2,4-dichlorophenoxyacetic acid was omitted from all media. Kernel blocks were transferred onto fresh culture media containing a 1:30 mixture of [U-13C6]glucose and naturally labelled glucose from 11–18 DAP. Three biological replicates were performed for each genotype with one replicate deriving from one plant for W22 and homozygous pgd3 or pgd1;pgd2. The 25% frequency of mutant kernels on pgd3-umu1/+ blocks required 3–5 plant pools per replicate. Starch was purified from endosperm tissue, and the isotopologue distribution from starch-derived glucose moieties was determined by isotope ratio mass spectrometry and 1H- and 13C-NMR spectroscopy as described (Spielbauer et al., 2006). 1H and 13C NMR spectra were recorded at 500.13 MHz and 125.76 MHz, respectively, using a Bruker DRX500 spectrometer at 25 °C. The experimental setup, signal assignments, coupling constants, isotope shifts, and the notation of isotopologues of the biolabelled α- and β-glucose were as described (Eisenreich et al., 2004). The analysis of 13C abundance and isotopologue composition was as described (Spielbauer et al., 2006). Percentage 13C-fractions (f) were calculated from the excess enrichment (e) of each isotopologue, the total excess amount (t) of 13C incorporated into starch during the labelling period, and the number (n) of 13C atoms of a certain isotopologue by applying \( f = \frac{n \times e}{t \times 6} \).

Results

The pgd3-umu1 allele was identified within UniformMu Mu transposon flanking sequence tags and perfectly co-segregates with an rgh phenotype at a map resolution <0.31 cM (Settles et al., 2007). A PCR screen of 72 independent rgh isolates also from UniformMu found an additional pgd3 allele with an insertion in the 3' end of the Pgd3 ORF (Fig. 1A). This pgd3-umu2 allele co-segregated with an rgh phenotype at <1 cM map resolution (0 recombinants/100 meiotic products) and failed to complement pgd3-umu1 in reciprocal crosses between heterozygotes (Fig. 1B). To determine the effect of pgd3-umu2 on enzyme activity, 16-DAP seeds were assayed for 6PGDH activity (Fig. 1C). The native PAGE assay detects two mobility classes of 6PGDH (Settles et al., 2007). Single mutants of pgd1 or pgd2 reduced the activity of the fast migrating band and pgd1;pgd2 double mutants eliminated this band. Both pgd3 alleles eliminated the slower migrating, PGD3 band. The enzyme, seed, and molecular lesions of pgd3-umu1 and pgd3-umu2 indicated that the alleles were equivalent null mutations. Thus, two independent alleles of pgd3 conferred the same seed phenotype.

Similar to pgd3-umu1 (Settles et al., 2007), pgd3-umu2 mutants had severely reduced grain-fill, and sagittal hand-sections of mature kernels showed developmental defects in endosperm and embryo (Supplementary Fig. S1, available at JXB online). The bulk seed composition of mature kernels from segregating ears revealed that pgd3 alleles reduced fat and starch content while increasing fibre (Supplementary Table S1). Dissected pgd3 seeds showed reduced dryweight of the embryo, endosperm, and maternal pericarp by 30-, 8-, and 2-fold, respectively (Supplementary Table S2). The reduction in fat and embryo weight is consistent with the role of oxPPP in lipid biosynthesis (Schwender et al., 2003; Hutchings et al., 2005; Alonso et al., 2010). The pericarp is predominantly cell-wall material, and the apparent increase of fibre in pgd3 mutants is likely to derive from the increased proportion of maternal tissue. The reduction of starch content was still significant when measured directly from endosperm at 20 DAP and maturity, suggesting a starch synthesis defect in pgd3 (Supplementary Table S3).

A small fraction of pgd3-umu1 embryos can be rescued with tissue culture (Settles et al., 2007). Although morphologically normal, pgd3 plants were shorter in stature and grew slower with recently emerged leaves being pale green. The seed and plant phenotypes of pgd3 mutants suggested that PGD3 activity is most critical in the developing seed. Potentially, PGD3 activity is only required in a limited number of tissues or plant developmental stages. This study surveyed 6PGDH enzyme activity with wild-type plants to determine tissues in which PGD3 was active (Fig. 2A). PGD1 and PGD2 activity was detected at similar levels in all tissues

Fig. 1. Cloning of Pgd3. (A) Schematic of the Pgd3 locus; two Mu insertions disrupt the predicted ORF of 6PGDH. (B) Complementation test of pgd3-umu1/+ with pgd3-umu2/+; self-pollinated ears and reciprocal crosses segregate for the pgd3 mutation (arrows). (C) 6PGDH native PAGE activity assays for 16-DAP kernels mutated for 6PGDH isoforms.
sampled. By contrast, PGD3 activity showed greater tissue specificity. PGD3 activity was low in fully expanded photosynthetic leaves and was relatively low in roots when compared to PGD1 and PGD2. PGD3 showed a gradient of activity in partially emerged leaves, with the proximal region of the leaf having the greatest activity and the photosynthetic tip having the lowest (Fig. 2B). High levels of PGD3 activity were observed in other sink tissues, such as pre-emergent leaf, shoot apex, tassel, and immature ear, with the most intense activity in 16-DAP embryos (Fig. 2A). PGD3 activity may also show a gradual increase during seed development (Fig. 2C), and the loss of PGD1 and PGD2 did not alter PGD3 activity in developing seeds, indicating independent regulation of 6PGDH isoforms (Fig. 2D).

6PGDH has been co-purified with peroxisomes in pea and Arabidopsis (Corpas et al., 1998; Reumann et al., 2007; Reumann et al., 2009). In maize, the majority of PGD3 enzyme activity is found in the soluble fraction of total protein extracts with approximately one-third of the activity associated with crude membrane fractions (Averill et al., 1998). The current study found PGD1, PGD2, and PGD3 to all be associated with purified chloroplasts and etioplasts, although PGD3 was enriched relative to PGD1 and PGD2 in the plastid stroma fraction (Supplementary Fig. S2C). Protein sequence alignment of higher plant 6PGDH enzymes showed two major clades (Supplementary Fig. S2A). Based on purified spinach enzymes, one clade was predicted to be cytosolic and many enzymes of this clade contain PTS1-type targeting signals (Neuberger et al., 2003). The other clade was predicted to be plastid localized (Krepinsky et al., 2001), but subcellular localization prediction algorithms such as TargetP (Emanuelsson et al., 2000), PSORT (Horton et al., 2007), MultiLoc2 (Blum et al., 2009), and Predotar (Small et al., 2004) gave low confidence or conflicting predictions. Only the spinach chloroplast isoform had the characteristic, long N-terminal extension for a chloroplast transit peptide in a multiple sequence alignment (Supplementary Fig. S2B). Predicted chloroplast-localized enzymes from Populus and Arabidopsis lack a transit peptide, while cereal proteins, including PGD3, had short N-terminal extensions that are atypical for transit peptides. To resolve these data, PGD3 subcellular localization was determined by expressing a C-terminal GFP fusion in Arabidopsis protoplasts (Supplementary Fig. S2D). PGD3-GFP signal was found in a subcellular compartment that overlapped completely with chlorophyll autofluorescence, indicating that PGD3 is a plastid-localized enzyme.

The high level of PGD3 activity in the embryo and the requirement for oxPPP in oil synthesis (Alonso et al., 2010) suggests PGD3 may only be required in the embryo during seed development. This hypothesis predicts that pgd3 mutant embryos would have a non-autonomous effect on endosperm growth and development. The current study generated kernels with non-concordant endosperm and embryo genotypes for the pgd3-umu2 allele by crossing pgd3-umu2/+ with the TB-4Sa B-A translocation stock. B-A translocations undergo non-disjunction in the second mitosis of microsporogenesis resulting in one sperm cell carrying a deletion for the translocated segment and the other being hyperploid (Roman, 1947). Double-fertilization of pgd3 female gametes produced non-concordant endosperm and embryo tissues that ‘uncovered’ either the pgd3 endosperm or embryo phenotype. If pgd3 mutant embryos caused defective endosperm development, a single uncovering class similar to what is observed for defective kernel24 (dek24) would be expected (Chang and Neuffer, 1994). However, two classes of uncovered kernels were observed. A pgd3 embryo had relatively little impact on wild-type endosperm development and growth (Fig. 3A, Supplementary Table S2). In contrast, the pgd3 endosperm disrupted wild-type embryo growth with a reduction in embryo weight (Supplementary Table S2) and development (Fig. 3A). The same TB-4Sa translocation was crossed to two mutant controls. The dek11 locus is a non-viable seed mutant with autonomous defects in the embryo and endosperm (Neuffer and Sheridan, 1980; Fouquet et al., 2011), while brittle2 (bt2) is defective for the small subunit of the rate-limiting enzyme of endosperm starch synthesis, ADP-glucose pyrophosphorylase (AGPase) (Preiss et al., 1990). Fig. 4B shows the autonomous endosperm and embryo phenotypes in non-concordant dek11 kernels, although dek11 endosperm uncovering reduced embryo weight (Supplementary Table S2). The bt2 mutant only showed a phenotype in the endosperm-uncovering kernel class (Fig. 4C) with no effect on embryo
development or weight (Supplementary Table S2). The controls showed that pgd3 non-concordant kernel development is not a general phenomenon for seed mutants on chromosome arm 4S or endosperm starch-biosynthesis mutants. The data showed that PGD3 is specifically required within the endosperm and that the endosperm function of PGD3 has an impact on embryo development.

To understand the cellular basis of pgd3 endosperm development defects, this study sectioned 16-DAP kernels and found multiple morphological differences. In the wild type, the starchy endosperm was densely filled with starch granules (Fig. 4A) while pgd3 kernels contained fewer and smaller starch granules (Fig. 4B). Moreover, 20-DAP and mature endosperm starch content was reduced in pgd3-umu1 and pgd3-umu2 (Supplementary Table S3). The basal endosperm transfer cell layer (BETL) showed aberrant morphology in pgd3 mutants. The pgd3 BETL appeared separated from the densely stained closing layer (Fig. 4C, D). At higher magnification, the nucellar pedicel parenchyma of pgd3 mutants was not as compressed as in the wild type, and pgd3 BETL cells were defective (Fig. 4E, F). Wild-type BETL cells had extensive secondary cell-wall ingrowths for efficient uptake of nutrients and were visualized with dark staining throughout the entire cell (Fig. 4G). Short ingrowths were found in pgd3 BETL cells (Fig. 4H). Similar BETL cell morphology was found in 12-DAP wild-type plants at the onset of starch biosynthesis, but the ingrowths were fully developed at 17 DAP at the peak of starch production (Kang et al., 2009). Aberrant BETL morphology similar to pgd3 was found in multiple grain-fill mutants, including defective endosperm17 (de17), mutant1 (m1l), baseless1 (bsl1), and rough endosperm3 (rgh3) (Lowe and Nelson, 1946; Brink and Cooper, 1947; Gutierrez-Marcos et al., 2006; Kang et al., 2009; Fouquet et al., 2011). BETL defects are interpreted as an indicator of reduced sink strength (Cheng et al., 1996; Kang et al., 2009).

To determine the metabolic consequences of pgd3 defects in the endosperm, this study measured NADP+ and NADPH levels in 21-DAP kernels (Supplementary Table S4) and profiled 74 metabolites in 20-DAP pgd3-umu1, pgd3-umu2, and corresponding normal tissues (Supplementary Table S5). For profiling, GC-MS peak area ratios were normalized to a ribitol internal quantitative standard and the percentage of methanol extractable soluble compounds (Roessner et al., 2000; Witt et al., 2012). Principal components analysis of the data separated pgd3 and normal tissue in PC1, while PC2 separated the genetic pedigrees of the alleles (Supplementary Fig. S3). The relative fold-change in metabolite levels for pgd3-normal endosperm was highly correlated between the alleles, suggesting nearly identical effects of the mutants relative to normal siblings (Fig. 5A). The product of G6PDH, gluconate-1,5-lactone, accumulated significantly in mutants consistent with loss of 6PGDH (Fig. 5B). Total levels of NADP+ and NADPH were reduced, but the ratio of NADP+:NADPH was unchanged (Supplementary Table S4). Intermediates for gluconic acid and ascorbic acid pathways were increased, while glycerol-3-phosphate, malate, glutamate, and aspartate showed shifts in relative concentration, suggesting alterations in intracellular redox shuttling mechanisms (Fig. 5B, C). Additional amino acids were changed without a clear trend (Supplementary Table S5). Several fatty acids were reduced in the mutants consistent with the requirement for NAPDH in fatty acid synthesis (Fig. 5C). Finally, pgd3 increased reducing sugars similarly to the increases found in starch-biosynthesis mutants (Creech, 1965; Fig. 5C).

Heavy isotope-labeling experiments of developing maize kernels have shown the bulk of glucose incorporated into starch derives from products of glycolysis and the PPP (Spielbauer et al., 2006). This system infers relative metabolic pathway usage based on the incorporation of different isotopologues, molecules that differ only in their isotopic composition, into starch (Ettenhuber et al., 2005). However, these experiments cannot identify the subcellular localization of the PPP enzymes that produce substrates for starch synthesis (Masakapalli et al., 2010). In the current study, the reduced starch content of mature and developing endosperm in pgd3 mutants as well as the increase in reducing sugars in 20-DAP endosperm suggested that plastidic 6PGDH activity is needed for starch synthesis. It was then predicted that metabolic flux of glucose into starch would be altered in pgd3 seeds. Steady-state labelling was performed by feeding [U-13C]glucose to cob tissue with attached developing seeds. Fig. 5D–F shows the pattern of glucose isotopologues incorporated into starch in homozygous pgd3-umu1 ears and W22 inbred controls. The most prominent effect was a strong increase in direct

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**Fig. 3.** Non-concordant seed phenotypes. Sagittal hand sections of mature kernels from self-pollinations and TB-4Sa translocation crosses of pgd3-umu2/+ (A), dek11/+ (B), and bt2/+ (C) plants. Presence of a normal embryo in the mutant endosperm class indicates that a wild-type embryo can develop in conjunction with a mutant endosperm. Bars = 2 mm.
incorporation of label into starch relative to fluxes through other pathways (11111 in Fig. 5D–F).

In vitro growth of kernels in sterile culture requires cob tissue (Felker, 1992). Potentially, isotopologue patterns in pgd3 homozygous plants are due to altered cob metabolic activities in ears lacking PGD3 (Alonso et al., 2011). To control for ear effects, the current study labelled pgd3/+ ears segregating for pgd3 mutant kernels. Mutant and normal kernels were visually separated for isotopologue analysis, resulting in the same trend, with pgd3 showing increased direct incorporation (Fig. 5E). The differences observed were not as large as in homozygous pgd3 ears, possibly due to aborted normal kernels being included in pgd3 samples. These results indicate maternal cob tissue has, if any, only minor effects on $^{13}$C distribution in the labelling system. It is concluded the altered $^{13}$C pattern of pgd3 mutants was due to metabolic reactions in the kernel. Normal kernels from heterozygous ears, W22, and pgd1;pgd2 double mutants all showed similar isotopologue patterns, indicating a specific effect on starch synthesis from loss of plastid-localized oxPPP (Fig. 5F).

Ratios of specific isotopologues can approximate relative flux through carbon metabolic pathways (Spielbauer et al., 2011).
Maize endosperm starch synthesis requires PGD3 (Ettenhuber et al., 2006). The ratio of uniformly labelled glucose \{111111\} to \{111000\} (see legend to Fig. 5F) estimates flux partitioning between direct incorporation into starch and hexose cycling, i.e. entry into glycolysis or PPP. This ratio was approximately doubled in \textit{pgd3} (Supplementary Table S6). Reforming glucose via triose-hexose exchange by glycolytic reactions should give an equal ratio of \{000111\} to \{111000\}, while exchange reactions of transaldolase in the noxPPP will only produce \{000111\}. Thus, a ratio of \{000111\} to \{111000\} above 1 indicates flux through noxPPP (Ettenhuber et al., 2005). Mutant \textit{pgd3} kernels showed a statistically significant 5–10% increase in this ratio compared to wild-type and \textit{pgd1;pgd2} seeds, suggesting increased flux through noxPPP (Supplementary Table S6). Finally, \{110000\} is an indicator of oxPPP as it is primarily formed after loss of the C1-atom in the oxPPP and glucose-6-P is regenerated via the noxPPP. Surprisingly, neither \textit{pgd1;pgd2} double mutants nor \textit{pgd3} changed the ratio of \{110000\} to \{111000\}, suggesting that pentoses produced in the \textit{pgd3} cytosol can enter the amyloplast to regenerate into glucose. In summary, isotopologue patterns of \textit{pgd3} starch
suggest that fluxes through glycolysis and PPP are reduced and that flux through noxPPP may be increased.

**Discussion**

This study shows that loss of PGD3 enzyme activity caused a severe defective kernel and indicates a non-redundant role for Pgd3 with the other 6PGDH loci, Pgd1 and Pgd2. Prior work found PGD3 was enriched in crude membrane fractions, and it was assumed PGD3 is plastid localized based on sequence similarity (Averill et al., 1998; Kruger and von Schaewen, 2003). However, all three 6PGDH isoforms co-purified with plastids, and the PGD3 chloroplast targeting peptide was non-canonical (Supplementary Fig. S2). Thus, this study provides conclusive evidence that PGD3 is plastid localized with a GFP fusion.

The data contrast with prior conclusions that PGD1 and PGD2 enzymes act jointly with PGD3 to satisfy cellular demand for NADPH in all compartments via a proposed shuttling mechanism for reducing equivalents (Averill et al., 1998). Double mutants of pgd1:pgd2 have subtle growth phenotypes and a reduced rate of nitrite uptake in roots (Averill et al., 1998). In light of the severe pgd3 phenotype, the data argue that cytosolic 6PGDH is not essential for maize metabolism. Metabolite analysis of pgd3 endosperm suggests that reductant shuttles are shifted, yet these do not compensate for loss of PGD3 (Fig. 5B, C). These data are consistent with the general model that the cytosol and plastid produce reductant independently.

A simple interpretation of the pgd3 kernel phenotype is an essential role for oil accumulation that impacts kernel development. Almost all maize oil is stored in the embryo (Doehlert and Lambert, 1991), and oxPPP provides the majority of the NADPH needed for oil synthesis (Alonso et al., 2010). However, PGD3 is not required continuously in embryos, because a small percentage of pgd3 embryos can be rescued in culture and develop normally (Settles et al., 2007). Moreover, B-A translocation genetics indicated distinct and critical roles for PGD3 in both the endosperm and embryo (Figs. 2 and 3). PGD3 is specifically required in the endosperm and this endosperm function promotes embryo development.

The essential role for plastid oxPPP in endosperm is not easy to explain. The major storage component of the maize endosperm is starch, and demand for reductant is thought to be low (Alonso et al., 2011). The extensive changes in the pgd3 metabolite profiles suggest a critical role throughout the metabolic network, while the cellular defects, endosperm starch reduction, and heavy isotope-labelling experiments indicate a requirement for PGD3 in starch synthesis. Although pgd3 is more pleiotropic than conventional sweet corn mutants, starch reductions are comparable on a per kernel basis. Mutant pgd3 accumulates 15% of starch of the wild type, while bt1, sh2, and bt2 accumulate 12–21% starch of wild-type kernels (Spielbauer et al., 2009).

The current model of cereal endosperm starch biosynthesis is known to require ATP and pyrophosphate but not NADPH or NADH (Fig. 6, Comparot-Moss and Denyer, 2009). Sucrose enters the kernel and is hydrolysed by sucrose synthase to yield UDP-glucose and fructose. These hexoses are converted into glucose-1-P and then into ADP-glucose by AGPase, the rate-limiting step of starch biosynthesis. The major AGPase in the cereal endosperm is cytosolic, and ADP-glucose is transported into the plastid by the adenylate translocator for incorporation into starch. Heavy isotope-labelling experiments indicate that much of the glucose incorporated into endosperm starch first passes through the initial reactions of glycolysis and the PPP (Spielbauer et al., 2006). The current study found a significant shift towards direct incorporation of glucose into starch in the pgd3 mutant (Fig. 5D–F). This shift was not due to cob tissue effects, indicating that plastidic oxPPP in the endosperm influences starch synthesis.

There is no simple explanation for the requirement of plastid-localized 6PGDH in maize endosperm starch biosynthesis. The products of PGD3 are ribulose-5-P and NADPH. Ribulose-5-P can be generated through the reversible reactions of the noxPPP, while NADPH could be generated from NADP-malic enzyme. Malate concentrations are increased up to 2-fold in the pgd3 mutants, while there is an overall 20% reduction of NADP+ and NADPH. These data suggest NADPH may be limiting in pgd3. A role for NADPH in starch biosynthesis is consistent with a metabolic flux model for the maize endosperm in which NADPH is produced well above demand for known endosperm metabolic processes (Alonso et al., 2011).

Redox status in the plastid is potentially important for cereal endosperm starch synthesis. Plastid-produced NADPH is required for the ferredoxin/thioredoxin system identified in wheat amyloplasts (Balmer et al., 2006). Ferredoxin/thioredoxin targets relevant to starch metabolism include α,4-glucan phosphorylase, starch branching enzyme IIa, and the BT1 transporter (Kirchberger et al., 2007). In contrast to pgd3, bt1 does not change the proportion of glucose that is directly incorporated into starch (Spielbauer et al., 2006), suggesting that BT1 transporter activity is not the primary target of reducing equivalents produced through oxPPP. Posttranslational redox-regulation for the plastid small subunit of AGPase has been established in potato tuber as well as Arabidopsis and pea leaves (Tiessen et al., 2002; Geigenberger et al., 2005; Michalska et al., 2009). There is some debate about the importance of plastid-localized AGPase in cereal endosperm. The majority of AGPase activity has been detected in the cytosol (Denyer et al., 1996). However, residual AGPase activity is found in isolated amyloplasts and in sh2 and bt2 null mutant kernels (Dickinson and Preiss, 1969; Denyer et al., 1996), suggesting that plastid-localized isoforms of APGase are present in maize endosperm. Peptides identical to both SH2 and BT2 subunits co-purify in a complex with plastid-localized starch synthase III supporting a role for plastidic AGPase (Hennen-Bierwagen et al., 2009). In maize endosperm, the genetic identity of the amyloplast-localized small subunit of AGPase has not been resolved. Two candidates are Agpsemzm and Bt2b. Agpsemzm is expressed in the embryo and endosperm (Giroux and Hannah, 1994; Rosti and Denyer, 2007; Cosse et al., 2008). BT2b is a theoretical alternative product of the Bt2 gene, which is predicted to be targeted to the plastid (Rosti and Denyer, 2007;
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Cossegal et al., 2008). In contrast to the cytosolic endosperm small subunit of AGPase (BT2) (Hendriks et al., 2003), both plastid-targeted proteins contain the redox-relevant cysteine residue and could be regulatory targets for the reductant produced by PGD3.

Despite the increasing knowledge about redox regulation of starch-biosynthetic enzymes (Geigenberger, 2011), the importance of plastidic oxPPP has been underestimated in the cereal endosperm and has not been integrated in current models (Hannah and James, 2008; Comparot-Moss and Denyer, 2009). The pgd3 mutant provides significant evidence for the requirement of oxPPP in the cereal endosperm amyloplast to enable starch synthesis.

**Supplementary material**

Supplementary data are available at JXB online.

**Supplementary Table S1.** Bulk seed composition of selected maize genotypes.

**Supplementary Table S2.** Dryweights of tissues dissected from mature maize kernels.

**Supplementary Table S3.** Endosperm composition of the pgd3 mutant and normal kernels from segregating ears.

**Supplementary Table S4.** NADP+ and NADPH levels in 21 DAP kernels.

**Supplementary Table S5.** Metabolite ratios in 20 DAP pgd3-umu1 and pgd3-umu2 mutant and normal endosperm.

**Supplementary Table S6.** Ratios of selected isotopologues from [U-13C6]glucose kernel labelling experiments.

**Supplementary Fig. S1.** Equivalent phenotypes for pgd3-umu1 and pgd3-umu2 alleles.

**Supplementary Fig. S2.** PGD3 is a chloroplast-localized 6PGDH.

**Supplementary Fig. S3.** Principal component analysis of normalized peak area ratios from endosperm metabolic profiles.

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