Functional Analyses of Cytosolic Glucose-6-Phosphate Dehydrogenases and Their Contribution to Seed Oil Accumulation in Arabidopsis[1][OA]

Setsuko Wakao[2], Carl Andre, and Christoph Benning*

Department of Biochemistry and Molecular Biology (S.W., C.B.), Department of Plant Biology (C.A.), and United States Department of Energy-Plant Research Laboratory (C.A.), Michigan State University, East Lansing, Michigan 48824

Glucose-6-phosphate dehydrogenase (G6PDH) has been implicated in the supply of reduced nicotine amide cofactors for biochemical reactions and in modulating the redox state of cells. In plants, identification of its role is complicated due to the presence of several isoforms in the cytosol and plastids. Here we focus on G6PDHs in the cytosol of Arabidopsis (*Arabidopsis thaliana*) using single and double mutants disrupted in the two cytosolic G6PDHs. Only a single G6PDH isoform remained in the double mutant and was present in chloroplasts, consistent with a loss of cytosolic G6PDH activity. The activities of the cytosolic isoforms G6PD5 and G6PD6 were reciprocally increased in single mutants with no increase of their respective transcript levels. We hypothesized that G6PDH plays a role in supplying NADPH for oil accumulation in developing seeds in which photosynthesis may be light limited. G6PDH activity in seeds derived from G6PD6 and a plastid G6PDH isoform and showed a similar temporal activity pattern as oil accumulation. Seeds of the double mutant but not of the single mutants had higher oil content and increased weight compared to those of the wild type, with no alteration in the carbon to nitrogen ratio or fatty acid composition. A decrease in total G6PDH activity was observed only in the double mutant. These results suggest that loss of cytosolic G6PDH activity affects the metabolism of developing seeds by increasing carbon substrates for synthesis of storage compounds rather than by decreasing the NADPH supply specifically for fatty acid synthesis.

Glc-6-P dehydrogenase (G6PDH) is the first of the two NADPH-generating enzymes of the oxidative pentose phosphate pathway (OPPP), G6PDH and 6-phosphogluconate dehydrogenase. All eukaryotic G6PDHs studied are feedback inhibited by NADPH, hence they are presumed to act as cellular redox sensors. This together with the fact that this enzyme catalyzes the committing step makes G6PDH the regulatory enzyme of the OPPP. Its role in oxidative stress responses has been suggested for various organisms. In humans, certain alleles of the G6PDH gene are associated with hypersensitivity of erythrocytes to oxidative stress such as exposure to drugs, infection, and ingestion of fava beans (*Vicia faba*; for review, see Vulliamy et al., 1992; Martini and Ursini, 1996). In yeast (*Saccharomyces cerevisiae*) and *Escherichia coli*, genes coding for G6PDH are up-regulated by oxidative stress (Kletzien et al., 1994). In mammalian cells, cytosolic G6PDH is also regulated through alternative splicing (Tao et al., 2002) in response to hormonal and nutritional cues such as sugar and lipids (Salati et al., 2004). Increase in G6PDH gene expression has been associated with increasing glutathione levels and resistance to oxidative stress (Salvemini et al., 1999) or the protein modulating the activity of a DNA repair protein (Ayene et al., 2002).

Plants are unique in that the OPPP resides in the cytosol and the plastid (Schnarrenberger et al., 1973; Debnam and Emes, 1999). In addition to the feedback inhibition by NADPH, G6PDH of cyanobacteria (Cossar et al., 1984) and the plastidic isoforms of plants and algae (Lendzian, 1980; Scheibe and Anderson, 1981; Fickenscher and Scheibe, 1986; Graeve et al., 1994; Wenderoth et al., 1997; Wendt et al., 2000) are subject to regulation by the thioredoxin/ferredoxin system. This inactivation of G6PDHs under reduced conditions is presumed to prevent unnecessary oxidation of carbon when NADPH generation by photosynthesis is sufficient. In plants G6PDH has been most frequently described for its involvement in nitrogen assimilation. The induction of its activity or transcript has been described in various systems including pea (*Pisum sativum*) roots (Bowsher et al., 1992), *Chlamydomonas* (Huppe et al., 1994), barley (*Hordeum vulgare*) roots (Wright et al., 1997), maize (*Zea mays*) roots (Redinbaugh and Campbell, 1998), tobacco (*Nicotiana tabacum*) roots and leaves (Debnam

et al., 2004), and Arabidopsis (*Arabidopsis thaliana*; Wang et al., 2003). G6PDH activity is involved in the supply of the reducing equivalents necessary for nitrogen assimilation in root cells (Bowsher et al., 1992; Wright et al., 1997; Jin et al. 1998; Esposito et al., 2001, 2003) and in algae (Huppe and Turpin, 1996). G6PDH has been shown to respond to various oxidative stresses at the level of mRNA abundance or enzyme activity. Inhibitors of photosynthetic electron transport, such as methyl viologen, activate a plastidic isoform that is associated with the loss of phosphorylation of the protein (Hauschild and von Schaewen, 2003). Other stresses such as high salt (Nemoto and Sasakuma, 2000), fungal elicitor-induced pathogenesis response (Batz et al., 1998), and viral infection (Sindelar and Sindelarova, 2002) have been accompanied by increased activity or mRNA levels of G6PDH. Transgenic tobacco plants with decreased levels of a plastidic isoform by antisense expression showed an unexpected increase in resistance to methyl viologen (Debnam et al., 2004). An increase of cytosolic G6PDH activity in response to sugar was found to occur at the mRNA level, which was associated with the presence of sugar response DNA cis-elements in the promoter region (Hauschild and von Schaewen, 2003).

Aside from nitrogen assimilation, G6PDH has been speculated to be an important source of NADPH in nonphotosynthetic tissues (Emes and Neuhaus, 1997) and those that produce fatty acids (FAs) at high rates, such as pollen (Niewiadomski et al., 2005) and oil seeds (Eastmond and Rawsthorne, 1998). Green oil seeds such as those of Arabidopsis and canola (*Brassica napus*) contain plastids similar to those of shade-adapted leaves (Asokanthan et al., 1997) and light transmission through the silique walls has been shown to be approximately 20% to 30% (Eastmond et al., 1996; King et al., 1998). On the other hand, these seeds synthesize large amounts of FA accumulating approximately 30% to 40% dry weight as lipids by maturation (Eastmond et al., 1996). It has been long debated what the source of NADPH is for oil accumulation in green seeds. Several recent studies have reported on the importance of photosynthesis for oil accumulation in green seeds (Ruuska et al., 2004; Schwender et al., 2004; Goffman et al., 2005).

The unique attributes of the plant OPPP, such as enzyme isoforms in different compartments, complicate the interpretation of its role in whole cell physiology. Few studies address such questions. Maize mutants with the cytosolic 6-phosphogluconate dehydrogenases disrupted were affected in the capacity of nitrogen assimilation (Averill et al., 1998), indicating the presence of redox communication between the cytosol and the plastid. The discovery of the pentose-phosphate transporter (Eicks et al., 2002) demonstrated the physical connection between the nonoxidative branches of the pentose phosphate pathway (PPP) in the plastid and cytosol, but how the two pathways coordinate in supplying NADPH to the cell still remains unknown. Additionally, the close connection between biochemical pathways in central metabolism

makes interpretation of an effect observed in plants with altered biochemical activity difficult. It is not always clear whether the effect is due to decreased supply of NADPH or precursors generated from the PPP because of the cyclic nature of PPP and OPPP. Overlap of intermediates with other metabolic pathways such as glycolysis, tricarboxylic acid cycle, and amino acid and nucleotide biosynthesis also makes it difficult to discern whether the effect is primarily due to the OPPP. For example, transgenic tobacco plants decreased in plastidic transketolase activity had severe effects that extended to phenylpropanoid metabolism and photosynthesis (Henkes et al., 2001), and demonstrated the complex network of metabolic flux and regulation.

Previously we have conducted a genome-wide characterization of Arabidopsis G6PDH isoforms. In this study, by taking advantage of T-DNA insertion lines, we attempted to determine the unique and redundant roles of the cytosolic G6PDHs, particularly in seed metabolism.

RESULTS

Cytosolic Localization of G6PD5 and G6PD6

To test that *G6PD5* and *G6PD6* indeed encode cytosolic isoforms the subcellular localization of the proteins was examined by transient expression of the respective cDNAs fused to a GFP gene. For both constructs containing *G6PD5* and *G6PD6*, green fluorescence was observed dispersed in the cytosol and surrounding what is presumably the nucleus (Fig. 1, A and B). The same patterns were observed in multiple experiments. A similar pattern was observed in cells expressing GFP alone, which localizes to the cytosol and to the nucleus (Fig. 1C). This result together with the lack of a potential targeting sequence in the proteins suggests that G6PD5 and G6PD6 are both likely to be cytosolic proteins.

There are three major active isoforms in vivo, G6PD5, G6PD6, and an unidentified isoform that is ubiquitous (Wakao and Benning, 2005). To rule out the possibility that the third isoform is localized in the cytosol, we examined G6PDH activity in isolated chloroplasts from Arabidopsis leaves. As shown in Figure 1D, isolated chloroplasts contain a single band on a zymogram with similar mobility as the unidentified band detected in protein extract from buds. This result strongly suggests that the unidentified ubiquitous isoform is localized in the plastid and that G6PD5 and G6PD6 are the only cytosolic isoforms with major activity in Arabidopsis.

Generation of the Double Mutant of *G6PD5* and *G6PD6*

To specifically examine the in vivo roles of the cytosolic G6PDHs, T-DNA insertion lines (SALK Institute) for the two genes were obtained. Their insertion sites were identified as previously described (Wakao
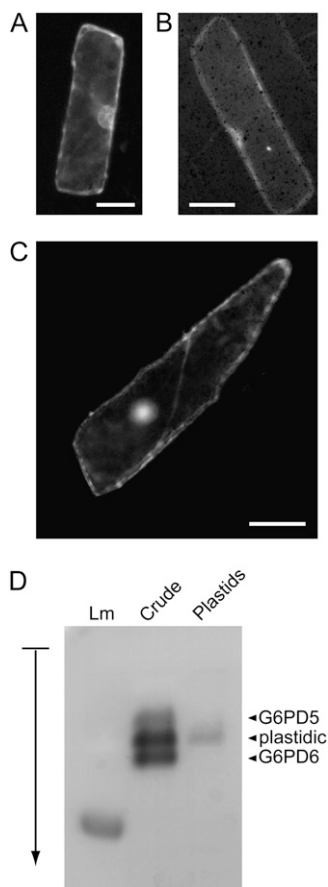
Figure 1. Cytosolic localization of G6PD5 and G6PD6. Onion cells were bombarded with either pCAMBIA1302 inserted with the coding sequence of *G6PD5* (A) or *G6PD6* (B) or the vector alone (C). D, Zymogram with isolated chloroplasts shows enrichment in the G6PDH band that is neither G6PD5 nor G6PD6. The arrow indicates origin and direction of electrophoresis. Arrowheads indicate the position of G6PDH isoforms previously identified (Wakao and Benning, 2005), and the plastidic isoform. Lm, Standard G6PDH from *Leuconostoc mesenteroides*.

and Benning, 2005). The single mutants did not have any obvious morphological phenotypes (Fig. 2A). To address whether this was because of the redundant functions of the two G6PDH isoforms, crosses between the two lines were performed to generate plants homozygous for both T-DNA insertions. All possible genotypes were detected by PCR in the F$_2$ population, some of which are shown in Figure 2B. From here on, the mutant lines will be referred to as *g6pd5* (*g6pd5/g6pd5 G6PD6/G6PD6*), *g6pd6* (*G6PD5/G6PD5 g6pd6/g6pd6*), and double mutant (*g6pd5/g6pd5 g6pd6/g6pd6*).

G6PDH activity was examined in plants of different genotypes using zymograms from bud tissue in which all three isoforms were easily detected (Fig. 2C). We chose zymograms to detect the activity of G6PDH rather than immunoblot analysis because the cytosolic isoforms have similar amino acid sequence (92%; data

not shown) and have different sensitivity to redox effectors in vitro (Wakao and Benning, 2005). Moreover, we have been successful in reproducibly separating and detecting relative activity levels of G6PDH isoforms from Arabidopsis tissues using this method (Wakao and Benning, 2005). The zymogram pattern of the plants homozygous for one T-DNA insertion and heterozygous for the other looked similar to the respective single mutants, which were distinguishable by the loss of one band and the increase of another (Fig. 2C, compare lanes 2 and 5, 3 and 6). This result suggests there is no gene dosage effect on cytosolic G6PDH activity. In the double mutant, the increase of both bands was lost, indicating that the intensified bands observed in *g6pd5* and *g6pd6* were indeed G6PD6 and G6PD5 and not an alternative G6PDH. Residual G6PD5 activity was detected in bud tissue of the double mutant and not the single mutant (Fig. 2C, lanes 3 and 4), similarly to that in wild-type leaf tissues in which G6PD5 activity is the predominant isoform (Wakao and Benning, 2005). The T-DNA insertion site in *g6pd5* is 3 bp downstream of the stop codon (Wakao and Benning, 2005) and thus a transcript containing the entire coding sequence and protein could be made. The activity of G6PD5 in the *g6pd5* and double mutant may become more necessary in *g6pd5* leaves where G6PD5 is presumed to be the major cytosolic activity, and when both cytosolic isoforms are lost in the double mutant. Surprisingly the double mutant was also indistinguishable in growth from the wild-type plant (Fig. 2A), despite the loss of most of the cytosolic G6PDH activity as observed in zymograms. Therefore we conclude that a nearly complete loss of cytosolic G6PDH activity does not result in severe morphological phenotypes of the plant under laboratory growth conditions. Attempts to complement the single and double mutants with a cDNA or G6PDH fused to GFP at the N terminus were unsuccessful (data not shown). Only when genomic fragments containing *G6PD5* or *G6PD6* were introduced did we observe the recovery of the lost bands on zymograms (Fig. 2D). This result together with the zymogram pattern of the double mutant proves the previously defined zymogram bands (Wakao and Benning, 2005) were indeed due to G6PD5 and G6PD6.

Unexpectedly, an apparent decrease in the activity of the plastidic isoform was observed in the single and double mutants (Fig. 2C; also in Wakao and Benning, 2005). Gene expression analysis of the plastidic isoforms in whole seedlings of the single and double mutants did not indicate any plastidic isoform transcript to be reduced (data not shown), suggesting a posttranscriptional regulation for the plastidic isoform as well. Intriguingly, complementation of the single mutants also results in the recovery of the plastidic G6PDH activity (Fig. 2D). This does not occur when the double mutant is complemented with the genomic region containing the gene for a single isoform, excluding the possibility that the genomic sequence of the cytosolic G6PDH genes also contains that of the
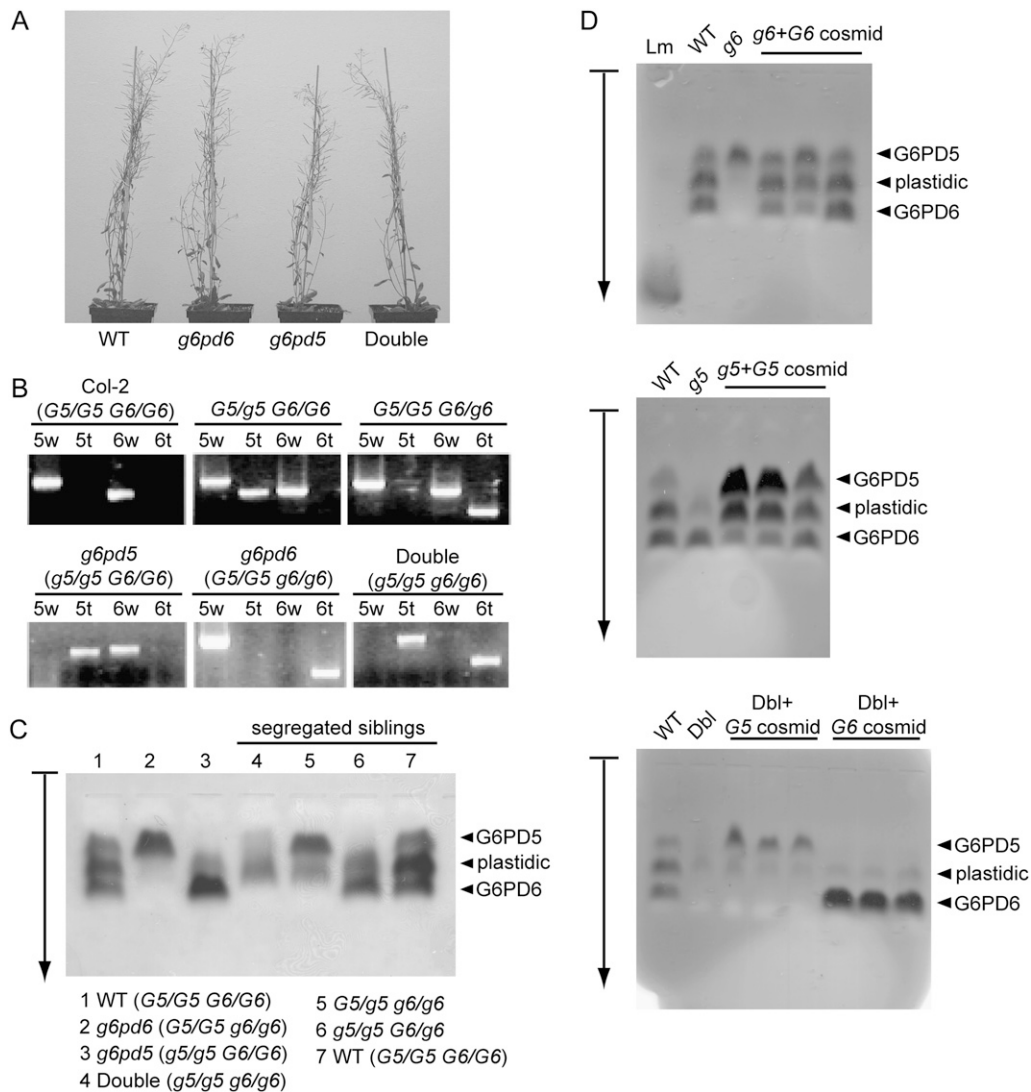
Figure 2. PCR genotypes and zymogram phenotypes of single and double mutants for G6PD5 and G6PD6. A, Morphological phenotypes of the single and double mutants. B, PCR genotyping. 5w, 6w, wild-type allele-specific PCR for *G6PD5* and *G6PD6*, respectively; 5t, 6t, T-DNA insertion-specific PCR for *G6PD5* and *G6PD6*, respectively. C, G6PDH zymogram patterns in bud tissues of different G6PDH genotypes. The arrow indicates origin and direction of electrophoresis. *G5*, *G6PD5*; *g5*, *g6pd5*; *G6*, *G6PD6*; *g6*, *g6pd6*; Dbl, double mutant. D, G6PDH zymograms of single and double mutants rescued by cosmid complementation. The arrow indicates origin and direction of electrophoresis. Bud tissues were used for visualization of the three bands. Lm, Standard G6PDH from *L. mesenteroides*.

plastidic isoform. These results suggest a possible coregulation of G6PDH activity in the two subcellular compartments at the posttranscriptional level.

Gene Expression and Activity of the Cytosolic Isoforms and the Effect of Suc

To address whether the reciprocal increase in activity of the cytosolic G6PDH occurs in other tissues and at the mRNA level, activity and gene expression were examined in seedlings by zymogram and quantitative reverse transcription (RT)-PCR, respectively. G6PD5 activity increased in the *g6pd6* mutant as was observed in bud tissues (compare Figs. 3A and 2C). In contrast, the increase in G6PD6 activity in the *g6pd5* mutant was not as pronounced as that in bud tissues (Figs. 3A and 2C). This may be explained by the low activity of G6PD5 in wild-type seedlings compared to buds (wild type in Figs. 2C and 3A), hence no compensation for its loss by G6PD6 is necessary in the *g6pd5* mutant. When primers were designed against regions upstream of the T-DNA insertion, no large increase of transcript levels of *G6PD5* and *G6PD6*, in *g6pd6* and *g6pd5* mutants was observed that accompanied any changes in their activity (Fig. 3B, top section). In fact, the transcripts of both cytosolic G6PDH genes were wild-
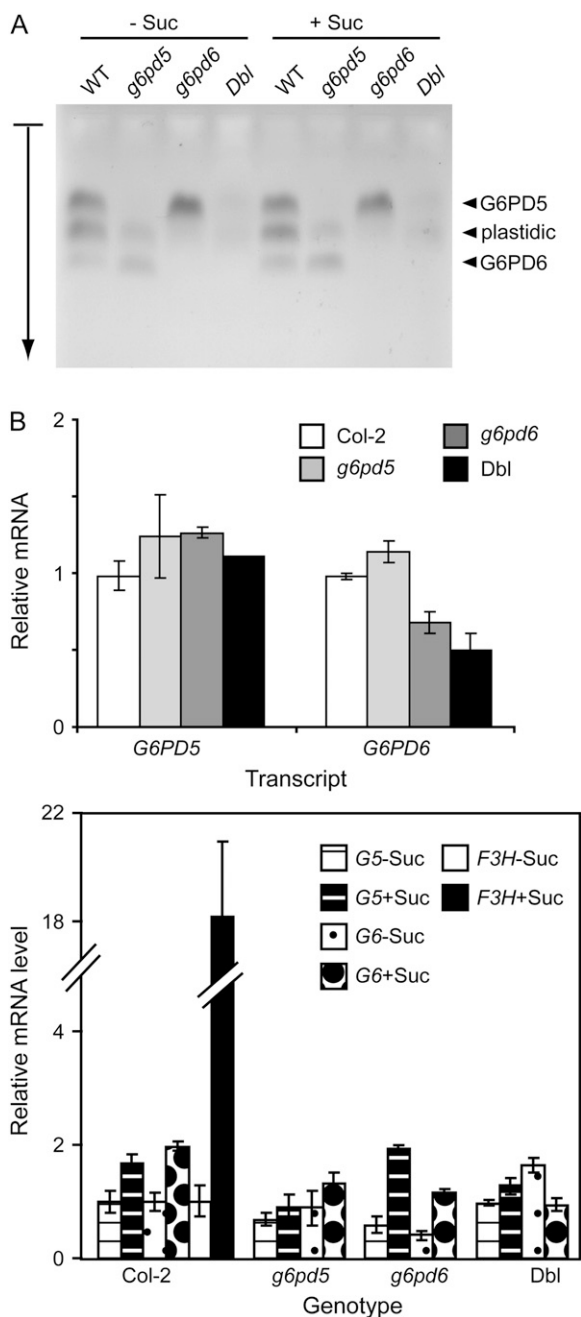
Figure 3. Activity and gene expression of cytosolic isoforms in single and double mutants and the effect of Suc. A, Zymogram of seedlings grown on presence and absence of Suc. The arrow indicates origin and direction of electrophoresis. g5, g6pd5; g6, g6pd6; Dbl, double. B, Levels of mRNA of *G6PD5* and *G6PD6* analyzed by quantitative RT-PCR in the different genotypes. Top section shows mRNA levels only in −Suc samples. Bars indicate SD from technical triplicates. Experiment was repeated three times with similar results. Graph shows one representative data set. The relative level of mRNA was normalized to wild-type-Suc values defined as 1.

type level in *g6pd5* and the double mutant, only *G6PD6* was slightly reduced in the *g6pd6* and double mutant (Fig. 3B, top section). This indicates that the T-DNA insertions did not lead to degradation of the entire

mRNA, while transcript regions downstream of the insertion appeared reduced (data not shown). The reduced activity of the isoform in which the T-DNA insertion is in the 3′ end of the gene may be attributed to decreased protein levels from lower translational efficiency. Most importantly, this result showed that the increase in activity of the remaining cytosolic G6PDH in the single mutants did not occur at the level of mRNA.

Because cytosolic G6PDH transcript was found to increase in response to sugars in potato (*Solanum tuberosum*; Hauschild and von Schaewen, 2003), the sugar inducibility of the cytosolic G6PDH genes was tested in seedlings. In wild-type seedlings *G6PD5* and *G6PD6* showed small (1.7- and 2.0-fold, respectively) but significant induction by Suc, though not to the extent of a known sugar-inducible gene flavonoid 3′-hydroxylase (F3H; Solfanelli et al., 2006), which in this experiment increased 18-fold (Fig. 3B, bottom section). In *g6pd5* and double mutant, the sugar induction of the genes was attenuated and altered, respectively, while the induction was retained in *g6pd6* (Fig. 3B, bottom section). Although a relatively small Suc induction was detected for the *G6PD5* and *G6PD6*, this did not translate into increased activity in wild type or mutants as seen in Figure 3A.

G6PDH Activity in Developing Seeds

In light of recent findings that photosynthesis in seeds contributes to oil accumulation, the role of G6PDH was directly addressed using the single and double mutants. It was shown previously that G6PD6 and a ubiquitous plastidic isoform are active in Arabidopsis seeds 5 d after flowering (DAF; Wakao and Benning, 2005). In developing seeds the three isoforms *G6PD2*, *G6PD5*, and *G6PD6* are produced (White et al., 2000; Ruuska et al., 2004). In the AtGenExpress atlas, all isoforms are found to be expressed at various levels (Schmid et al., 2005). In particular, *G6PD2* has constitutive levels, *G6PD5* transcript increases throughout development, and *G6PD6* has the highest amount of mRNA that decreases during seed development. Zymograms and liquid assays showed that G6PDH activity decreases from 5 to 7 DAF during seed development and increases again reaching highest activity at 11 to 13 DAF (Fig. 4), correlating with the temporal pattern of FA accumulation in Arabidopsis seeds (Focks and Benning, 1998). The isoforms active were G6PD6 and the ubiquitous plastidic isoform, and no new isoform appeared during seed development and no change in the relative intensity of the two bands on the zymogram was observed (Fig. 4A).

It has been a long debate what the major source of reducing equivalents for biosynthesis of storage lipid is in green developing seeds. While several studies suggested G6PDH as a potential source of NADPH in light-limited embryos (Eastmond et al., 1996; Kang and Rawsthorne, 1996), recent reports have demonstrated the importance of photosynthesis in green
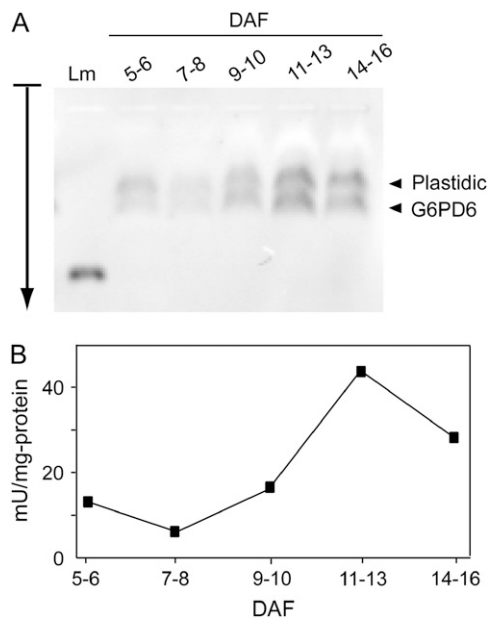
Figure 4. G6PDH activity in developing seeds. Measured by zymogram (A) and by liquid assay (B). The arrow indicates origin and direction of electrophoresis. Lm, Standard G6PDH from *L. mesenteroides*.

seeds (Ruuska et al., 2004; Schwender et al., 2004; Goffman et al., 2005). G6PDH has a seemingly inverse correlation with photosynthesis due to its feedback inhibition by NADPH and the plastidic isoforms' sensitivity to reductive inactivation through the thioredoxin/ferredoxin system, and thus we expected that a loss of photosynthesis would lead to activation of G6PDH. To gain insight into the relative contributions of photosynthesis and G6PDH in supplying NADPH for seed oil accumulation in Arabidopsis, we used the *pds1* mutant. This mutant is unable to desaturate phytoene and photobleaches due to the lack of tocopherol when homozygous for the mutation (Norris et al., 1995). In the siliques of heterozygous plants, a 1-to-3 segregation of white and green seeds was observed (Fig. 5A). A time course of FA accumulation in developing seeds showed that white *pds1* seeds contain 80% as much FA as wild-type green seeds at 15 DAF (Fig. 5B). No large change in the pattern of G6PDH activity was observed for the white seeds on zymograms (Fig. 5C). When total G6PDH activity was measured by liquid assay in these samples the white seeds were reduced by approximately 25% to 30% and approximately 50% compared to the green seeds in 10 to 13, and 14 to 15 DAF, respectively (data not shown). Apparently no compensation for the lack of photosynthesis occurred through G6PDH activity.

The *pds1* seeds cannot be distinguished when they are mature due to the brown seed coat. The population of mature seeds from a *PDS1/pds1* heterozygous plant shows a bimodal distribution when plotted for oil content (Fig. 6A). The group with less oil consists of approximately 25% of the total number of seeds and its

distribution is maximal at 5 to 5.5 μg/seed, in contrast to the larger group with a maximum in the distribution at 8.5 to 9 μg/seed. Extrapolating from this distribution the white seeds are likely to have accumulated approximately 60% wild-type level oil in the absence of photosynthesis by the time of maturation. Interestingly the seeds with less oil contain a higher ratio of very long-chain FAs (Fig. 6B), possibly due to increased FA elongation to compensate for reduced de novo FA synthesis. These results indicate that 60% seed oil of wild type can be produced in the absence of photosynthesis with little change in G6PDH activity.

Seed Oil Content per Seed Is Increased Only in the Double Mutant

Oil content in seeds of wild type, *g6pd5*, *g6pd6*, and double mutants was measured to examine the contribution of the cytosolic G6PDHs. The data consists of values obtained from seeds from five or six individual
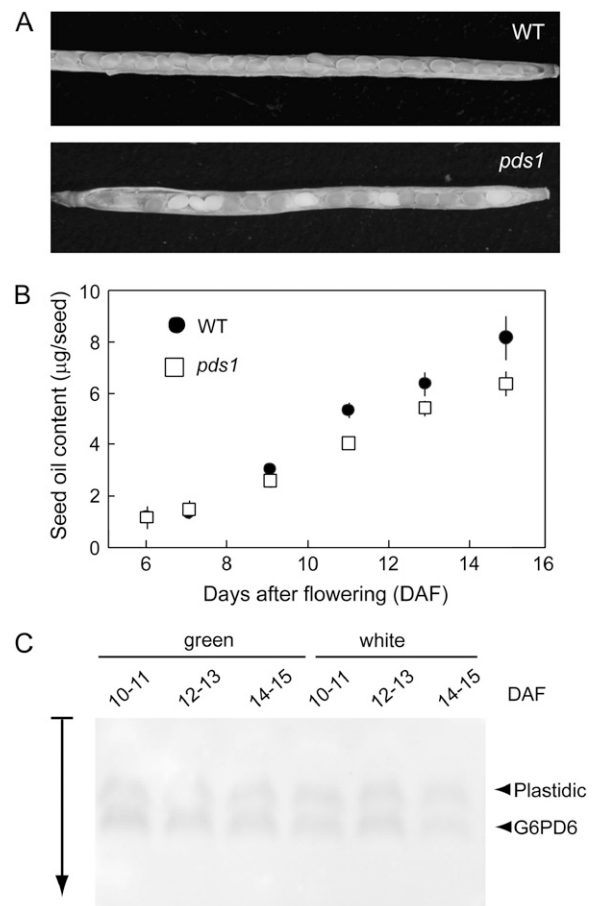

Figure 5. Developing *pds1* seeds. A, Siliques of wild-type and *PDS1/pds1* plants. B, White seeds accumulate oil at a lower rate than wild type. Ten white or green seeds were pooled and the oil content was measured. Bars indicate SD of five technical replicates. C, G6PDH activity in wild-type and white seeds measured by zymogram. Arrow indicates direction of electrophoresis and origin.

plants of each genotype that were grown at two separate times but under the exact same conditions in the same growth chamber. In a single experiment, approximately 50 seeds were analyzed per plant. The mean of the values for seed oil content from each plant and the SD are shown in Table I. The single mutants and the double mutant were compared to wild type by Student's t test. For *g6pd5*, *g6pd6*, and the double mutant the probability of assuming the null hypothesis (that they are not different from wild type) were 0.52, 0.35, and 0.056, respectively. This result suggests a difference between wild type and the double mutant with a 94% confidence, close to the typically accepted 95% (Kimble, 1978) but not for the single mutants and wild type.

To address whether the possible increase in oil in the double mutant occurred at the expense of storage protein accumulation, the content of total carbon and nitrogen in the seeds was analyzed for wild type and double mutant. No significant difference was observed between the carbon to nitrogen ratios of the two genotypes (Table I). The seeds of wild type and the double mutant were counted to 500 each, dried, and weighed. For the double mutant, seeds were on average heavier than those of wild type (Table I). A plot for the two variables, seed weight and oil content, gives a r^2 value of 0.54, suggesting some correlation. This may imply that the seeds of the double mutant contain more oil due to larger seeds rather than the effect being specific to oil accumulation. The number of seeds in siliques was counted and compared between the mutants and wild type to address whether a decrease in seed number accompanied the increase in seed mass and no difference between genotypes was observed (data not shown). Because FA elongation beyond C_{16} and C_{18} requires NADPH in the cytosol, it was possible that the accumulation of long-chain FAs would be affected in the mutant lines that lack cytosolic G6PDHs. The FA compositions of seeds of the four genotypes closely resembled each other (Table II). The loss of either of the cytosolic G6PDH isoform did not affect FA content or FA composition, however, the loss of both isoforms increased FA content without altering the composition.

Total G6PDH Activity Is Reduced Only in the Double Mutant

Only seeds of the double mutant accumulated more oil per seed than wild type. We hypothesized that this was due to increased substrate availability for glycolysis and subsequent FA synthesis as a result of the block in the cytosolic OPPP and that the redundancy of G6PD5 and G6PD6 prevented such a phenotype in the single mutants. We analyzed G6PDH activity in whole siliques to explore this hypothesis. The typical reciprocal increase in activity of the cytosolic isoforms was observed in the single mutants by zymogram (Fig. 7A). When G6PDH activity was measured in these extracts by liquid assay, a decrease in total activity was ob-

served only for the double mutant (Fig. 7B). The large decrease in total activity could be due to the loss of both cytosolic or the plastidic isoform or all three (because the plastidic activity decreases pleiotropically in the mutants; Fig. 2D). Nonetheless, these results indicate that total G6PDH activity is reduced only in the double mutant and not in the single mutants, coincident with the increased seed oil content and mass. In this experiment whole siliques were used instead of isolated seeds to simplify the harvesting procedure. Although the isoforms present are the same, the temporal pattern differs in whole siliques from that observed in isolated seeds (compare Figs. 4B and 7B), which is likely to be due to the contribution of G6PDH activity in the silique walls. We have observed that the zymogram patterns comparing whole siliques and seeds of the same stage are similar (Fig. 4A; Wakao and Benning, 2005; data not shown) and thus we presume our results resemble that in seeds.

DISCUSSION

Role of Cytosolic G6PDHs

The activity of cytosolic G6PDH shows tissue specificity in wild-type plants; G6PD6 activity is ubiquitous in all tissues except leaves, while that of G6PD5 is the dominant cytosolic activity in leaves and is also present in roots (Wakao and Benning, 2005). Assuming each isoform has its role in specific tissues it was surprising to find that the mutant lines had no obvious
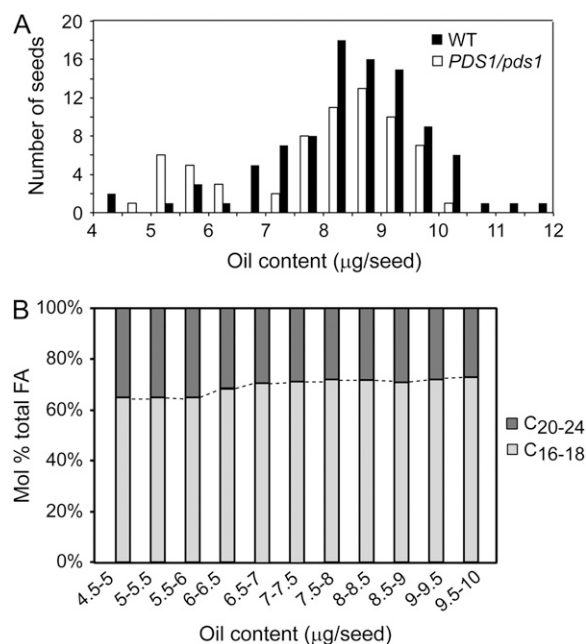

Figure 6. Oil in mature seeds from a *PDS1/pds1* plant. A, Distribution of single seed oil content from wild-type and *PDS1/pds* plants. B, Average composition (mol%) of C_{16-18} and C_{20-24} FAs in seeds with different total oil content.

Table I. *Seed characteristics of wild type, single, and double mutants*

Numbers in top row represent individual plants. SD is shown in parentheses. For oil content, *n* is approximately 50, for all other values, *n* = 3. Carbon and nitrogen content is shown in % (w/w). Oil content is expressed per seed. Dry weight (DW) is expressed per 500 seeds. Ave., Average.

Plant Lines and Parameters	1	2	3	4	5	6	Ave.
Wild type							
Oil (μg)	7.24 (0.89)	6.36 (1.9)	6.82 (0.64)	6.73 (0.75)	7.81 (0.88)		6.99
DW (mg)	9.12 (0.30)	9.29 (0.38)	8.71 (0.16)	8.78 (0.30)	9.16 (0.11)		9.01
Carbon (%)	57.8 (0.52)	57.4 (0.95)	58.1 (1.0)	59.2 (0.41)	58.6 (0.48)		58.2
Nitrogen (%)	3.00 (0.04)	3.00 (0.07)	2.54 (0.1)	2.52 (0.03)	2.52 (0.03)		2.72
g6pd5							
Oil (μg)	6.93 (0.87)	6.79 (0.08)	7.14 (1.1)	7.31 (0.69)	7.8 (0.82)		7.19
DW (mg)	9.00 (0.43)	8.90 (0.24)	9.03 (0.25)	8.90 (0)	9.56 (0.42)		9.08
Carbon (%)	55.8 (0.2)	54.5 (1.5)	57.6 (1.8)	57.5 (0.4)	56.4 (1.4)		56.36
Nitrogen (%)	2.98 (0.04)	2.68 (0.07)	2.58 (0.05)	2.45 (0.03)	2.55 (0.05)		2.65
g6pd6							
Oil (μg)	7.44 (1.4)	6.34 (0.08)	8.83 (1.6)	7.14 (0.7)	7.51 (0.9)		7.45
DW (mg)	9.27 (0.26)	9.00 (0.65)	9.23 (0.48)	8.70 (0.24)	9.23 (0.38)		9.09
Carbon (%)	57.7 (1.6)	54.0 (0.7)	56.6 (0.8)	57.0 (0.5)	57.1 (1.0)		56.48
Nitrogen (%)	3.33 (0.2)	3.68 (0.1)	2.64 (0.05)	2.75 (0.04)	3.06 (0.07)		3.09
Double							
Oil (μg)	7.44 (0.63)	8.45 (1.0)	7.12 (1.0)	7.74 (0.8)	7.44 (0.69)	7.73 (0.89)	7.65
DW (mg)	10.17 (0.60)	10.83 (0.41)	9.75 (0.61)	9.51 (0.57)	9.67 (0.41)	10.2 (0.31)	10.02
Carbon (%)	58.4 (1.0)	58.0 (0.1)	58.2 (0.7)	58.0 (0.59)	57.4 (0.9)	56.8 (0.73)	57.8
Nitrogen (%)	2.58 (0.09)	2.83 (0.06)	2.71 (0.05)	2.52 (0.004)	2.58 (0.04)	2.49 (0.02)	2.62

phenotypes (Fig. 2A). This may be explained by the reciprocal increase in activity of the remaining isoform when one is lost (Fig. 2D; Wakao and Benning, 2005), demonstrating the flexibility in the tissue specificity of each isoform. When tested in mutant seedlings, no large increase of mRNA was detected despite the increased activity of the remaining isoform (Fig. 3, A and B). From this result and together with the fact that tissue-specific patterns of gene expression and enzyme activity often did not agree with each other, and that cytosolic G6PDH exhibit differential inactivation by oxidation in vitro (Wakao and Benning, 2005), we speculate that in Arabidopsis cytosolic G6PDH regulation occurs mostly at the posttranscriptional level. We observed that the mRNAs of the cytosolic G6PDHs were increased by Suc as has been shown in mammalian systems (Salati et al., 2004) and potatoes (Hauschild and von Schaewen, 2003), although not to the extent of a known sugar-induced gene F3H (Fig. 3A; Solfanelli et al., 2006). When examined by zymogram no obvious increase in the activity of any isoform was observed, despite increased transcript amount (Fig. 3B). In potato, decrease in a plastidic G6PDH activity has been associated with phosphorylation of the protein (Hauschild and von Schaewen, 2003). It is unknown whether phosphorylation or any other protein modification is involved in modulating cytosolic G6PDH activity in Arabidopsis.

The two cytosolic isoforms possibly have little or no overlapping roles in wild-type plants reflected in their tissue-specific distribution (Wakao and Benning, 2005) but exhibit redundancy in single mutants. The reciprocal increase in activity of the two cytosolic G6PDHs

suggested the presence of a common mechanism through which the loss of an isoform is sensed, perhaps through changes in the concentration of metabolites such as $NADPH/NADP^+$ or G6P/6-phosphogluconate. We have tested for enhanced sensitivity to oxidative stress in the mutants [3-(3,4-dichlorophenyl)-1,1-dimethylurea, methyl viologen, high salt, hydrogen peroxide, and low nitrate] but no obvious phenotype was observed (data not shown). It may be that the cytosolic G6PDH mutants constitutively lacking an enzyme activity have already reached a steady state in which compensating mechanisms are activated in its subcellular compartment, such as up-regulation of other $NADP^+$-utilizing enzymes (e.g. NADP-dependent malate dehydrogenase and NADP-dependent glyceraldehyde-3-P dehydrogenase) and NADH kinase, recently reported as a major source of cytosolic NADPH under oxidative stress (Chai et al., 2006). Therefore, phenotypes may not be revealed unless short-term responses to stress or steady-state perturbations are examined.

A compensatory increase in activity for the two cytosolic G6PDHs has been observed in different tissues, though in some tissues of *g6pd5* (seedlings, Fig. 3 and whole siliques, Fig. 7) the increase was less pronounced than others (buds, Fig. 2C). Because G6PD6 is the predominant isoform in all tissues examined except leaves and roots (Wakao and Benning, 2005), compensation of the loss of G6PD5 by increase in G6PD6 may not be necessary. A study on the gene expression patterns of nonessential pairs of paralogs in yeast suggests that a paralog that normally is differentially expressed serves as a backup through

Table II. *Seed FA composition of wild type, single, and double mutants*

FA composition is shown in mol%. SD is shown in parentheses (*n* is approximately 50).

Genotype	16:0	18:0	18:1	18:2	20:0	18:3	20:1	22:0	22:1	24:1
Wild type	10.1	4.0	13.9	26.8	2.4	20.9	19.5	0.30	2.2	0.14
	(0.9)	(0.3)	(1.0)	(0.7)	(0.2)	(0.8)	(0.6)	(0.04)	(0.3)	(0.03)
g6pd5	9.8	4.0	13.3	26.9	2.5	20.9	19.9	0.32	2.2	0.16
	(0.8)	(0.5)	(1.6)	(0.7)	(0.2)	(1.1)	(0.6)	(0.04)	(0.3)	(0.06)
g6pd6	9.9	4.0	13.8	26.7	2.3	21.2	19.4	0.29	2.3	0.15
	(0.7)	(0.3)	(1.3)	(0.7)	(0.2)	(1.3)	(0.8)	(0.03)	(0.3)	(0.02)
Double	10.0	4.1	13.8	27.0	2.4	20.9	19.3	0.29	2.0	0.14
	(0.8)	(0.3)	(1.0)	(0.9)	(0.2)	(1.2)	(1.5)	(0.04)	(0.3)	(0.04)

transcriptional reprogramming when the other is disrupted (Kafri et al., 2005). In the case of Arabidopsis, cytosolic G6PDH transcript level and enzyme activity did not correlate in many cases (Wakao and Benning, 2005; this study). The modes of coordination of the two cytosolic isoforms at the transcriptional and posttranscriptional level are not exclusive, but it is likely that the former does not have a major role in regulation. Despite their similarity (92% amino acid similarity), the biochemical characteristics of G6PD5 and G6PD6 differ (Wakao and Benning, 2005). The activities of G6PD5, resistant to redox changes, and G6PD6, with high specific activity but inactivated by oxidation, may be coordinated through changes in metabolite levels. Regulation at a level beyond the abundance of mRNA, i.e. translation efficiency, protein stability, or posttranslational modification, involving functions of other proteins or changes in metabolite levels, is likely to play an important role in the regulation of their activities.

The Role of G6PDH in Seeds

In wild-type seeds, the activities of two isoforms were detected: G6PD6 and the unidentified plastidic isoform (Fig. 4A). The activity in seeds between 5 to 16 DAF was lowest at 7 to 8 DAF and increased thereafter (Fig. 4B). Before 5 DAF, seeds are nonphotosynthetic and go through rapid cell division, which is likely to require NADPH for FA synthesis to support membrane biosynthesis. A plastidic G6PDH was speculated to be important during these stages, but could not be examined due to difficulty in isolating such young seeds in a sufficient amount to perform enzyme assays. Seeds of 5 to 6 DAF are still transparent to the eye and contained relatively high G6PDH activity (Fig. 4), which may indicate the importance of G6PDH in supplying NADPH during the earlier nonphotosynthetic stages of seed development.

The seeds of the double mutant but not the single mutants accumulated more oil per seed than those of wild type, which was associated with the increase in seed mass and not specifically oil content. This was unexpected since the wild-type appearance of whole plants of the single and double mutants indicated the presence of a compensatory mechanism to supply cytosolic NADPH possibly through other enzymes

that utilize $NADP^+$. In agreement, the composition of FAs was similar among the different genotypes (Table II), indicating that the loss of cytosolic G6PDH as a NADPH source did not specifically affect FA elongation. Moreover, it was surprising that the loss of cytosolic G6PDHs resulted in an increase of seed oil content and mass rather than a decrease. Total G6PDH activity was reduced only in the double mutant and this evidence supports our hypothesis that an impairment in the OPPP leads to increased substrate availability for glycolysis. Glycolysis has been shown to be essential for seed oil biosynthesis and increasing glycolytic flux could provide more precursors for FA synthesis (Focks and Benning, 1998; Cernac and Benning, 2004; Andre et al., 2007). Whether this is a specific effect due to the loss of cytosolic or plastidic G6PDH or both remains unknown. Such alteration in carbon metabolism in the double mutant may have occurred in nonseed tissues as well but could not be detected by visual examination of plants or by our oxidative stress experiments.

Correlation of Photosynthesis, G6PDH Activity, and Seed Oil Accumulation

Despite the lack of photosynthesis, total G6PDH activity or the isoforms present during seed development did not change in the *pds1* mutant. The white seeds are presumed to have accumulated approximately 60% oil compared to wild type by maturation (Figs. 5B and 6A). The significance of photosynthesis in oil accumulation in green seeds has been an emerging focus in recent years (Schwender et al., 2003, 2004; Ruuska et al., 2004; Goffman et al., 2005) and hence it was surprising that the *pds1* mutant seeds still accumulated oil, moreover with no apparent increase or change in pattern of G6PDH activity. Apparently the wild-type level G6PDH activity or other compensating mechanisms are sufficient to support at least approximately 60% of oil accumulation. In contrast to the unaltered FA composition of the double mutant, the seeds with less oil, i.e. the fraction in which *pds1* seeds are presumably enriched, contain a higher ratio of long-chain FAs (Fig. 6B). This indicates that photosynthesis affects FA synthesis requiring plastidic NADPH and not FA elongation that utilizes NADPH in the
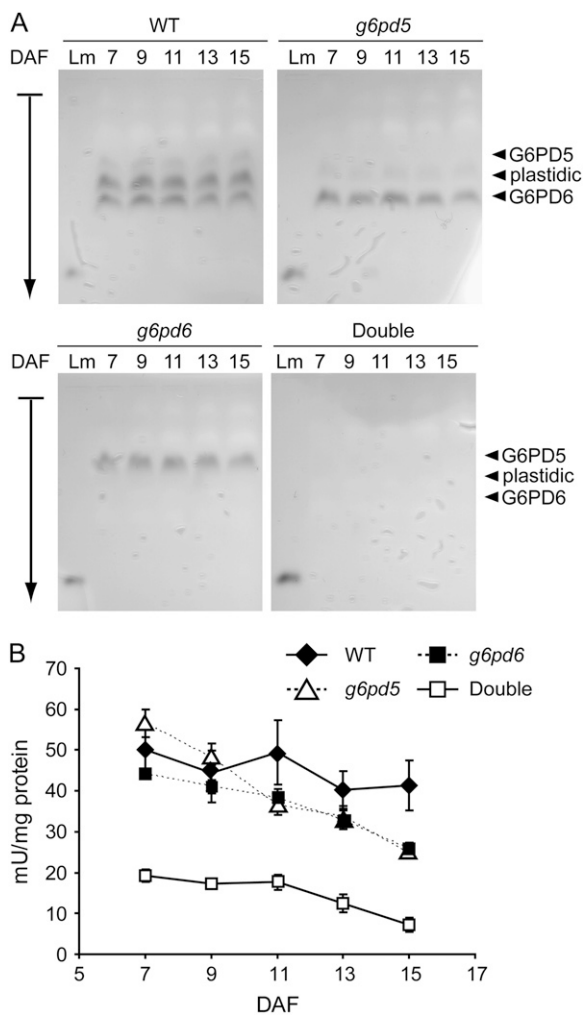
Figure 7. G6PDH activity in wild type, single, and double mutants during seed development. G6PDH activity in whole siliques during seed development was detected by zymogram (A) and liquid assay (B). Lm, Standard G6PDH from *L. mesenteroides*.

cytosol (Lardans and Tremolieres, 1992; Barrett and Harwood, 1998). It is interesting that the white seeds had accumulated approximately 80% oil of the green seeds at 15 DAF (Fig. 5B), and this difference was even larger by maturation (distribution peaks, 5–5.5 $\mu g/$ seed in white and 8.5–9 $\mu g/$seed in green seeds; Fig. 6A). This result suggests that the difference between these seeds manifests beyond the stage when they are visually distinguishable by color. One possible reason could be that the white seeds lacking photosynthetic generation of ATP may not efficiently store starch to supply carbon substrate for later FA synthesis.

Intriguingly, the loss of photosynthesis is not accompanied by increase of G6PDH activity, suggesting compensation, if any, occurs through other mechanisms. To test whether loss of the plastidic G6PDH would affect seed oil accumulation similarly as loss of photosynthesis we have attempted to generate RNA interference lines specific to certain plastidic G6PDH

isoforms, but with no success. Because of the high sequence similarity of the isoforms, RNA interference was targeted to a short stretch of sequence in the 3' untranslated region and this may have resulted in inefficient silencing (data not shown). Because of the pleiotropic decrease in the plastidic G6PDH activity in the single and double mutants, we have indirectly observed that decrease in plastidic G6PDH activity does not lead to reduced seed oil. It remains unknown whether the plastidic G6PDH activity in wild-type seeds, without the metabolic alteration due to the loss of cytosolic G6PDH, is critical in supplying NADPH in the plastid for FA synthesis.

Our results indicated the presence of a highly dynamic metabolic network that compensates for the loss of one or both of the cytosolic G6PDHs. Overall, cytosolic G6PDHs are largely dispensable for plant growth under laboratory growth conditions. However, the increased seed oil content and mass in the double mutant suggests there is indeed a metabolic change, possibly an increase in glycolytic flux, caused by the disruption in cytosolic G6PDHs.

MATERIALS AND METHODS

Plant Growth Conditions and Transformation

All seeds were surface sterilized by incubation in 20% bleach and 0.05% Triton X. The tubes containing the seeds were inverted for 15 min and washed three times with water. The seeds were suspended in 0.1% agar and plated onto 1× Murashige and Skoog medium (pH 5.8; Murashige and Skoog, 1962) with 1% Suc, 0.9% agar unless stated otherwise. The duration of stratification was usually 1 to 2 d at 4°C. Plants were grown in incubators with photon flux density of 60 to 80 μmol m^{-2} s^{-1}, 16 to 8 h light-dark photoperiod, 22°C and 18°C, light and dark temperatures. All transformations were performed by *Agrobacterium*-mediated floral dipping (Clough and Bent, 1998).

Transient Expression of *G6PDH::GFP* for Subcellular Localization Analysis

The coding regions of *G6PD5* and *G6PD6* were amplified with the following primers: for *G6PD5*, (+) 5'-GGACTAGTATGGGTTCTGGT-CAATGGCA, (−) 5'-GGACTAGTCAATGTAGGAGGGATCCAAA, and for *G6PD6*, (+) 5'-GGACTAGTATGGGGATCTGGTCAATGGCA, (−) 5'-GGACT-AGTTAGTGTAGGAGGGATCCAG. The cDNAs were cloned into the *Spe*I site of pCAMBIA1302 (GenBank accession no. AF234298). The orientation was confirmed by restriction analysis and sequencing. Onion (*Allium cepa*) epidermal peels were bombarded with the above constructs following the methods previously described (Varagona et al., 1992) using 1,100 pounds per square inch rupture discs at approximately 4 cm distance using a biolistic gene delivery system (DuPont). For each construct, three peels were bombarded and incubated overnight at 22°C in the dark. The peels were observed with a Leica DMR A2 microscope in the fluorescence mode with the L5 filter cube (Leica Microsystems).

Sugar Response Treatments and Gene Expression Analysis

For sugar response experiment seedlings were germinated and grown for 2 weeks without Suc on Murashige and Skoog 1.5% agar plate vertically then transferred to liquid media with 90 or 0 mM Suc for 24 h. RNA was extracted and cDNA synthesized as described previously (Wakao and Benning, 2005) from 1 μg of RNA. Quantitative PCR was performed using iQ SYBR Green Supermix (Bio-Rad) on an MJ Research DNA Engine Opticon Continuous Fluorescence Detector (Bio-Rad). Each sample was run in technical triplicates and the threshold cycle of the target genes from no RT templates was 8 to 10 cycles higher compared to that from cDNA templates (data not shown).

UBQ10 gene was used as an internal reference (Czechowski et al., 2005) after confirming its levels stay constant under presence and absence of Suc. The primers used are as follows, *G6PD5* (+) 5′-CGTACAAGCAGCGTTACCAA, (−) 5′-TCCTGTGAAGCAGTGGAGTG; *G6PD6* (+) 5′-GAGCATATCCGG-GATGAGAA, (−) 5′-TGTGGCAAATGTTGGAGTGT; *UBQ10* (+) 5′-ACC-CTAACGGGAAAGACGAT, (−) 5′-AAGAGTTCTGCCATCCTCCA; F3H (+) 5′-CGGTTCCTTTGGTGGTACAT, (−) 5′-AACTCCGGATCAAACTACGC. F3H was used as a positive control for a gene induced under the conditions applied (Solfanelli et al., 2006). All primers were confirmed to be approximately 90% to 100% efficient in amplification, and $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) was used for analyses.

Construction of Complementation Vectors for *G6PD5* and *G6PD6*

The T-DNA insertion lines were transformed with cosmid clones containing the regions of *G6PD5* and *G6PD6* that were isolated from a genomic cosmid (pBIC20) library (Meyer et al., 1994) using the 3′ untranslated region of the respective genes as a probe. The T-DNA insertion lines for both *G6PD5* and *G6PD6* have lost their kanamycin resistance, and thus the transformants were selected by kanamycin resistance introduced by the cosmid vector.

G6PDH Activity Assay with Tissue and Isolated Plastids

For tissues other than seeds, protein extraction and zymogram detection were carried out as previously described (Wakao and Benning, 2005). For seed protein the following modifications were applied. Approximately 6- to 8-fold (volume/weight seeds) of extraction buffer was used for protein extraction and adjusted to approximately 1 to 2 mg protein/mL. Cellulose acetate plates were overlaid with 0.2 mM 5′-cyano-2,3-ditolyl tetrazolium chloride (excitation wavelength 450 nm, emission wavelength 630 nm; Polysciences) instead of nitroblue tetrazolium chloride for increased sensitivity. Activity was detected by scanning with a fluorescence scanner (Molecular Imager FX, Bio-Rad) with excitation wavelength of 532 nm and detection with a 640 nm filter, and the image was processed with PDQuest (Bio-Rad). No fluorescence was observed when G6P was not added. Liquid assay of G6PDH was performed as described previously (Wakao and Benning, 2005). All zymograms were repeated at least twice with reproducible results and those with highest clarity are shown in the figures. Intact plastids were isolated from 5-week-old wild-type Arabidopsis (*Arabidopsis thaliana*) plants grown on soil. Five to 10 g of tissue was used per isolation. Intact plastids were isolated from homogenized tissue using a discontinuous Percoll gradient as previously described (Xu et al., 2002).

Seed Oil Content Measurement with Gas Chromatography

Plants of different genotypes were grown in the same growth chamber and the seeds were harvested after having dried in the siliques attached to the plant. Single mature seeds from a single plant of which the genotype was confirmed by PCR were ground using a 6 mm glass bead (Fischer Scientific) in a glass tube using a paint shaker for 3 min. Lipid extraction and sample preparation for quantification by gas chromatography was carried out as previously described (Focks and Benning, 1998).

Elemental (Carbon and Nitrogen) Analysis in Seeds

Seeds were sent to the Duke Environmental Stable Isotope Laboratory for elemental analysis. Between 2 to 5 mg of seeds were sent in three replicates for each plant, and for all the plants that seed oil content was analyzed by gas chromatography.

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