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BIOCHEMICAL AND FUNCTIONAL CHARACTERIZATION
OF ARABIDOPSIS PLASTIDIC PYRUVATE KINASES

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Major Professor's Signature

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BIOCHEMICAL AND FUNCTIONAL CHARACTERIZATION OF ARABIDOPSIS PLASTIDIC PYRUVATE KINASES

By

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ABSTRACT

BIOCHEMICAL AND FUNCTIONAL CHARACTERIZATION OF ARABIDOPSIS PLASTIDIC PYRUVATE KINASES

By

Carl Andre

In plants, flux through the Embden-Meyerhoff pathway (glycolysis) is vital for supplying precursors for many biosynthetic pathways, including fatty acid, amino acid, and isoprenoid biosynthesis. Additionally, the production of ATP by glycolysis is expected to be important in non-photosynthetic tissues. Pyruvate kinase (PK) catalyzes the final reaction of glycolysis and in plants many of the upstream glycolytic enzymes are regulated directly or indirectly by its activity. The regulation of individual PK isoforms is thus tailored such that glycolytic flux is appropriate for the tissue in which they reside. The specific roles of individual PKs are further defined by compartmentation between the cytosol and plastid. The Arabidopsis genome has fourteen putative PK-encoding genes. Three of these encode proteins which are located in the plastid and constitute 470 kDa plastidic PK (PK_p) complexes composed of four α- and either four β_1- or four β_2-subunits. Interaction of the α-subunit with either β-subunit confers unique regulatory properties to the enzymes which suggest specific roles in plant metabolism. Notably, the $\alpha\beta_1$ enzyme has higher specific activity and is less sensitive to allosteric regulation by metabolites. Developing Arabidopsis embryos synthesize triacylglycerol (TAG) to fuel seed germination and establishment. A mutant disrupted in the β_1-encoding gene (named *pkp1*) has impaired seed-PK_p activity and a 60% reduction in TAG accumulation with a reciprocal increase in carbohydrate content. Rescue of this phenotype can be achieved by ectopic overexpression of either β-subunit encoding gene, although β_2 is not capable of

full restoration. Thus, a specific role for the $\alpha\beta_1$ enzyme is in catabolizing imported sugar to provide the precursors for high rates of fatty acid synthesis in embryos. As expected, *pkp1* seeds do not germinate and establish as efficiently as wild type. However, this defect is not completely attributable to a lack of TAG and it seems that PK_p activity is also necessary for proper metabolism in germinating seeds. It was found that *pkp1* seedlings and mature plants have much in common with mutants deficient in isoprenoid and tocopherol biosynthesis, but there was no obvious defect in fatty acid metabolism. Apparently, the function of PK_p in leaves is also to provide precursors for whichever biosynthetic processes are in highest demand.

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Firstly, I am gratefully indebted to my thesis advisor, Dr. Christoph Benning. He worked tirelessly to insure that I had a place in his lab to develop my scientific skills, despite a seeming lack of interest from funding agencies in supporting this project. He allowed for creativity and exploration while still establishing clear goals and demanding work to be performed at the best of my abilities. My thesis advisory committee, Dr. Jianping Hu, Dr. John Ohlrogge, and Dr. Andreas Weber also deserve my thanks. They made themselves accessible for discussion at their own inconvenience and contributed thoughtful insight about my research. I am thankful to the Michigan State University U.S. Department of Energy Plant Research Laboratory and to the Department of Plant Biology for accepting me as a graduate student and for fostering some of the best plant science in the world. I only hope that I can help uphold the reputation for which these departments are known. I would also like to acknowledge the Department of Biochemistry and Molecular Biology for hosting me as one of their own and for allowing me to use their numerous facilities. Included are the labs of Dr. Jack Preiss and Joe Leykam where I invaded and used equipment for extended periods of (sometimes frustrating) time. I have also benefited from the community here at MSU where numerous discussions with graduate students and postdocs, especially the members of the Benning lab, past and present that always made work a pleasant place, helped guide me in the right direction. I can leave MSU knowing that I worked and socialized with some of the best scientists and people there are. Thanks.

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Chapter 1

Seed storage compound accumulation and related metabolisms in plants

1.1. Introduction

The reproductive success, and in part evolutionary fitness, of most plants is largely dependent on sexual reproduction involving fertilization of an ovule and subsequent embryo development. The challenges of reproduction vary, and are greatly influenced by the environment in which the plants take root. Regardless of such factors, the packaging of nutrient reserves in seeds has evolved as a major strategy to insure the establishment and survival of the next generation. Germinating seeds must metabolize their own storage reserves until the seedlings establish photosynthetic rates capable of sustaining growth. Carbohydrates, oils in the form of triacylglycerol (TAG), and proteins constitute the reserves used for this establishment. In addition to their indispensability for plant survival, these compounds have broad economic importance as agricultural commodities. For this reason, we need to investigate and understand the metabolism which leads to the accumulation of these compounds with the eventual goal of engineering plants to be more productive. Seed oil, in particular, is of utmost interest given the current Zeitgeist. The model plant Arabidopsis serves as an excellent organism in which to study seed oil biosynthesis, as it is very closely related to canola (*Brassica napus*) which is a major oil seed crop in the Northern hemisphere. In this chapter, I will review the metabolisms involved in storage compound accumulation, with a focus on oil, and I will introduce some of the current hypotheses pertaining to regulation of biosynthesis and turnover. I will establish that the glycolytic enzyme pyruvate kinase is pivotal in these processes and that it represents a step that needs to be researched further to fully understand the metabolism of developing oil seed embryos.

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1.2. Embryo development

Plant embryogenesis is initiated by a fertilization event in which one male gametophyte unites with a single female gametophyte to form a zygote. An additional fertilization occurs between another male gametophyte and a specialized diploid cell within the embryo sac resulting in the formation of the triploid endosperm. The function of the endosperm is to nourish the embryo during development and germination and in some cases (*e.g.* cereals) it is the site of storage product accumulation (Berger 2003, Penfield et al. 2004). Once fertilized, the development of plant embryos generally occurs in three distinct phases: 1) cellularization and establishment of a body plan, 2) deposition of storage reserves and maturation, and 3) acquisition of desiccation tolerance (Goldberg et al. 1994). Immediately following the double fertilization event, the seed rapidly grows mostly due to syncytial development of the endosperm (Berger 1999). During this time (~ 5 days in Arabidopsis) the zygote goes through many rounds of asymmetrical cell division and establishes tissues that will eventually become the hypocotyl and cotyledons (Laux and Jurgens 1997). Upon entering the maturation phase, cell division slows in lieu of expansion as cotyledonary cells begin to fill with storage compounds. This phase of development is depicted in Chapter 3, Figure 3.3A, and is associated with distinct changes in gene expression as discussed below. During this time, the water content of the seed steadily declines (Baud et al. 2002). The acquisition of desiccation tolerance and seed dormancy is characterized by nearly complete loss of water content from the seed and is associated with expression of presumably cryo-protective protein-encoding *LEA* genes (Wise and Tunnacliffe 2004) and programmed cell death of maternal tissues which will eventually become the seed coat.

1.3. Signals that control embryo maturation and storage compound accumulation

Embryo maturation, as described above, is the phase during which storage compounds are biosynthesized and deposited in developing seeds. The regulation of this process is at best loosely understood. There are, however, known transcriptional and metabolic cues which exert some control over this process. It is difficult to tease apart these two means of regulation as metabolism is directly influenced by the genes which are active in a given tissue, the expression of which may in turn be controlled by cellular metabolites.

1.3.1. Genetic regulators

Arabidopsis has served as the model for the study of the transcriptional control of seed maturation. The changes in metabolic gene expression during the shift from embryo morphogenesis to storage compound accumulation have been documented (Girke et al. 2000, Ruuska et al. 2002, White et al. 2000). Additionally, some genetic factors which control such changes in metabolic gene expression have been elucidated. The most upstream factor identified to date is *PKL* (PICKLE). The *PKL* gene encodes a chromatin-remodeling factor that specifically represses embryo associated transcription and metabolism in non-embryo tissues (Ogas et al. 1999, Rider Jr et al. 2003, Rider Jr. et al. 2004). Indeed, *pkl* mutants ectopically accumulate seed storage compounds in roots (Ogas et al. 1997). Some of the specific targets of *PKL*-mediated repression encode the transcription factors LEC1, LEC2, and FUS3. These three *LEAFY COTYLEDON*-class transcription factors are regulators of embryo identity and are so named because of the leaf-like appearance of cotyledons of the respective mutants. Overexpression of these genes results in spurious embryo formation in vegetative tissues (Lotan et al. 1998, Stone

et al. 2001). These genes also control storage product accumulation as evidenced by reduced amounts of storage protein and oil in mutant seeds (Meinke et al. 1994). The LEC-class transcription factors are considered master regulators and are hypothesized to target a subset of transcription factors controlling embryo maturation. One potential target of LEC1 is the *WRI1* gene. Overexpression of *WRI1* in the *lec1* background rescues the desiccation intolerant phenotype and the promoter of *WRI1* contains a potential DNA binding site of LEC1 (Cernac and Benning, unpublished). The *WRI1* gene encodes an AP2/EREB domain transcription factor that regulates sugar metabolism (*i.e.* glycolysis) in tissues where *WRI1* is expressed, namely, seeds and roots (Cernac and Benning 2004, Ruuska et al. 2002). The *wri1* mutant was originally identified in a screen for mutants specifically reduced in seed oil accumulation (Focks and Benning 1998). Overexpression of *WRI1* in seedlings was subsequently found to cause an embryo identity characterized by the synthesis and accumulation of TAG and seed storage proteins (Cernac and Benning 2004). While this is an incomplete review of all the genes involved in regulating embryo maturation, it is clear that there is a strong relationship between transcriptional regulation of embryo identity and seed storage compound biosynthesis.

1.3.2. Small molecule regulators

The list of small molecules which potentially regulate seed maturation, and thus storage compound accumulation, is growing. The phytohormone abscisic acid (ABA) is one well documented regulator of these processes. There are two peaks of ABA accumulation during seed maturation of most species (Karssen et al. 1983). The first peak potentially

regulates the transition from embryo cell division to cell enlargement in Arabidopsis as ABA is known to induce the expression of cell cycle inhibitory factors (Wang et al. 1998). During this time ABA also induces the expression of seed storage protein genes (Crouch and Sussex 1981). An Arabidopsis mutant defective in the sensing of ABA, *abi3-3*, fails to accumulate seed storage proteins (Koornneef et al. 1989). However, as mentioned above, it is difficult to tease apart transcriptional and metabolite-based regulation and it has been shown that ABI3 interacts in the signaling mediated by the LEC-class transcription factors described earlier (Parcy et al. 1997).

Non-hormonal metabolite signals are gaining recognition as potential regulators of seed maturation. Among these, the most attention has fallen upon sugars. Sugars, especially glucose, have been identified as regulators of photosynthetic gene expression and as components of signal transduction networks in Arabidopsis vegetative tissue (Jang et al. 1997, Jang and Sheen 1994). Developing seeds import large amounts of sugar from maternal tissue, and it is expected that sugar signaling networks would exist here as well. In legume seeds, the transition from cell division to storage product accumulation is correlated with a shift from a high hexose to sucrose ratio to a high sucrose to hexose ratio (Borisjuk et al. 2003, Weber et al. 1996). It has been proposed that the concentrations of the sugars themselves provide signals that regulate this developmental transition (Weber et al. 1997). A similar switch in the ratio of hexose to sucrose is observed in Arabidopsis; however, it comes after the onset of high rates of oil biosynthesis and may be a result of changed metabolism rather than triggering those changes (Baud et al. 2002, Hill et al. 2003). The *apetala2* (*ap2*) mutant of Arabidopsis produces seeds with higher mass and yield (Ohto et al. 2005). The increased cell size and

number in *ap2* embryos is correlated with an increased hexose to sucrose ratio throughout embryo development. The modulation of sugar metabolism appears to have an effect on embryo development in this mutant, although again, it is unclear whether the altered sugar ratio causes the phenotype or is the result of it. Transgenic tobacco plants with seed-specific overexpression of Invertase were used to examine importance of the switch from hexose to sucrose in oil seed crops. The transgenic lines maintained a very high hexose to sucrose ratio throughout embryogenesis but developed normally, suggesting that the switch from hexose to sucrose accumulation is not a signal (Tomlinson et al. 2004). Another sugar, trehalose-6-phosphate (T6P), is involved in embryo maturation. As opposed to sucrose and hexoses, T6P exists at very low concentrations in Arabidopsis embryos and cannot serve as an important energy source, which buttresses the notion of it as a signaling molecule. The importance of this molecule in regulating embryo development is evidenced by an Arabidopsis mutant deficient in T6P-synthase which is embryo lethal (Eastmond et al. 2002). Furthermore, an analysis of the aborted embryos revealed a dearth of oil accompanied by an increase in starch accumulation (Gomez et al. 2006). Components involved in the T6P signaling network remain to be identified. The amino acid asparagine may be another metabolite regulator of embryo maturation. An analysis of soybean seeds from high- and low-seed protein lines revealed a tight correlation between the amount of aspargine present in the embryo and the amount of seed storage protein in the dry seed (Hernandez-Sebastia et al. 2005). It is unknown whether this regulation is due to effects on transcription of storage protein genes or due to substrate availability.

1.4. Considerations for seed oil biosynthesis

The composition of seed storage compounds varies between plants, but a focus on Arabidopsis and other oil seed crops is pertinent in the context of the research presented in later chapters. Approximately 30% of the dry weight of an Arabidopsis seed is in the form of TAG, while another 30% is composed of seed storage proteins (Focks and Benning 1998). Starch transiently accumulates in developing embryos, but is not a major product in mature Arabidopsis seeds. The timing of these processes has been studied at the metabolic and transcriptional levels using Arabidopsis (Baud et al. 2002, Ruuska et al. 2002). At the onset of embryo cell expansion, which is 5 days after flowering (DAF), starch is synthesized in the plastids and accumulates through 9 DAF at which point it is degraded. Coincident with starch degradation are the synthesis of TAG and seed storage proteins, both of which continue to accumulate linearly until about 17 DAF, when the seed begins to desiccate and become metabolically quiescent. Figure 1.1 depicts a simplified scheme for the biosynthesis of seed storage compounds in oil seeds. Sucrose and some amino acids are provided by the maternal source organs through the endosperm liquid and are then metabolized by the embryo (Schwender and Ohlrogge 2002). The precursors of each storage compound are derived from connected biochemical pathways, of which fine regulation is necessary to balance the final reserve composition of the seed. Such carbon partitioning will be discussed in more detail in the following section.

1.4.1. Competition between biochemical pathways

The biosynthetic pathways in Figure 1.1 are simplified to emphasize the potential for competition for resources between the biosyntheses of starch, oil, and protein. Seed

storage protein biosynthesis is the most independent pathway. Stable isotope labeling of canola embryos revealed that 30% of the amino acids used in the synthesis of storage proteins are imported and 70% arise from *de novo* biosynthesis from sugars (Schwender and Ohlrogge 2002). Labeled amino acids were not catabolized and incorporated into fatty acids. Moreover, there appears to be independent sugar metabolism for protein and fatty acid synthesis, which is supported by numerous Arabidopsis mutants in which decreases in seed oil or protein does not result in compensatory increases in the other major storage compound. The *wri1* mutant, for instance, is reduced 80% in oil with no effect on the protein content (Focks and Benning 1998). Conversely, abscisic acid biosynthetic and signaling mutants exhibit reductions in seed storage protein content with no affect on oil (Finkelstein and Somerville 1990).

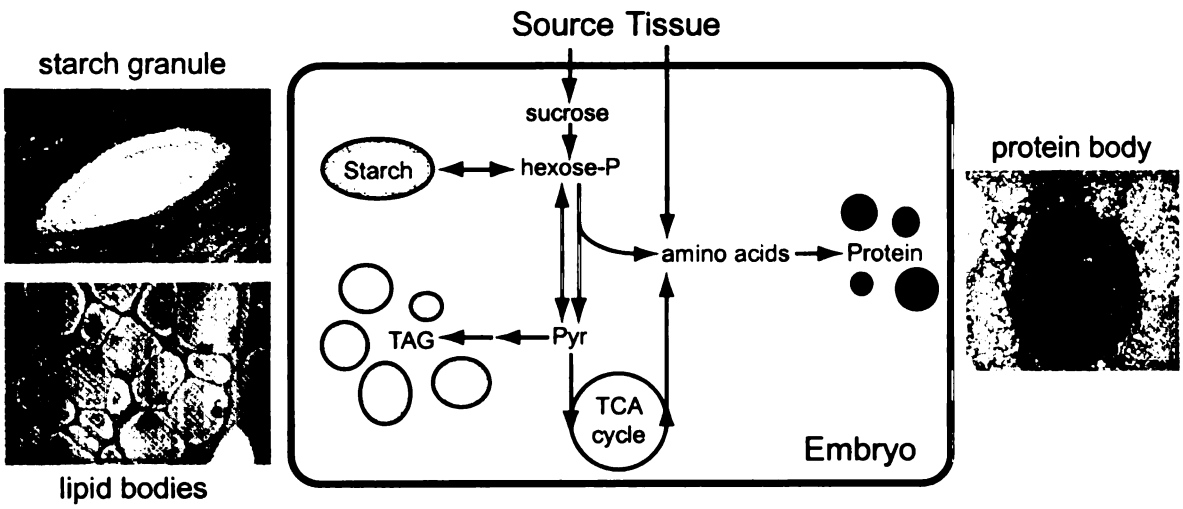

Figure 1.1. Competition for resources between seed storage compounds

Sucrose and amino acids are imported from source tissue via the phloem and are incorporated into the seed metabolic network. Images are TEM micrographs of representative storage organelles from Arabidopsis developing embryos. Pyr, pyruvate; TAG, triacylglycerol; TCA, tricarboxylic acid.

In oil seeds, the true competition occurs between starch and oil biosynthesis. The transient accumulation of starch in Arabidopsis seeds is hypothesized to serve several possible functions: 1) to maintain the sink strength of the embryonic tissue during fatty acid biosynthesis (Da Silva et al. 1997), 2) to supply carbon precursors for fatty acid synthesis upon its degradation (Norton and Harris 1975), and 3) to supply sugar (and thus osmolarity) needed for the acquisition of desiccation tolerance (Leprince et al. 1990). Two studies have directly addressed the question of the function of transitory starch in Arabidopsis seeds. In one a mutant of plastidic phosphoglucomutase (*pgm1*) which is compromised in starch biosynthesis was analyzed with respect to seed storage compounds (Periappuram et al. 2000). A 40% reduction in seed oil content was found in *pgm1*. The authors, however, could not conclude whether the reduction in starch compromised the sink strength or the carbon supply for fatty acid synthesis. Another study utilized embryo specific reduction of ADP-glucose pyrophosphorylase to impair transient starch accumulation in canola embryos (Vigeolas et al. 2004). While starch accumulation was severely compromised in the transgenic lines, oil biosynthesis was compromised only during the early stages of development. The authors concluded that starch was not a source of sugar for the production of fatty acid precursors during the main stages of oil biosynthesis. These two studies present no clear conclusions about the role of transitory starch accumulation. There is additional correlative evidence, though, that links starch accumulation to fatty acid synthesis in Arabidopsis embryos. Three mutants defective in oil accumulation, *wri1*, *lec1*, and *shrunken seed 1* (*sse1*, encoding a homolog of the peroxisome biogenesis factor Pex16p), all display compensatory increases in starch accumulation (Focks and Benning 1998, Lin et al. 1999, Lin et al.

2004, Meinke et al. 1994). In the case of *wri1* and *sse1* which both have observed decreases in the rate of fatty acid synthesis, sugars (hexose and sucrose) also accumulated to higher than wild-type levels in the developing seeds. These findings can be explained by two hypotheses: 1) starch biosynthesis functions to lessen the osmotic potential of the embryo by consuming excess sugar, thus maintaining the embryo status as a sink tissue, or 2) excess sugar inhibits the degradation of starch which would normally be needed to maintain a sugar supply to feed fatty acid synthesis. As the starchless mutants described above are desiccation tolerant, it is unlikely that starch serves to facilitate sugar accumulation for this purpose. Yet, no definitive conclusions can be drawn about the role of transitory starch accumulation in oil seeds.

1.4.2. Supply of energy

Fatty acid biosynthesis is dependent on a steady supply of adenosine triphosphate (ATP). Starch and amino acid synthesis along with the assembly of storage proteins (4 ATP or GTP per peptide bond) occurring in the embryo no doubt compete for the same ATP (Regierer et al. 2002). The committed step of fatty acid synthesis catalyzed by acetyl-CoA carboxylase (ACCase) uses ATP in a two step reaction converting acetyl-CoA into malonyl-CoA. It is the first step that is ATP depedent, in which a carboxyl group from bicarbonate is transferred to a biotin prosthetic group on the enzyme (Ohlrogge and Browse 1995). The synthesis of one 18 carbon fatty acid thus requires 9 ATP for the ACCase reaction alone. ACCase is considered the rate limiting reaction of fatty acid synthesis and its activity is light dependent by means of redox-regulation, pH, and Mg^{2+} concentration (Hunter and Ohlrogge 1998, Sasaki et al. 1997). Indeed, fatty acid

synthesis in canola embryos is stimulated by light (Bao et al. 1998, Goffman et al. 2005, Ruuska et al. 2004). Such light-dependent activation may be one means of coordinating ACCase activity with the production of ATP by photosynthesis in green tissues. However, not all oil seeds are green, and even then only as much as 30% of ambient light may reach the embryo (Eastmond et al. 1996, King et al. 1998). Other means of producing ATP include the lower half of glycolysis and mitochondrial respiration. The contribution of glycolysis to ATP production has not been directly tested to date, however, mitochondrial respiration has been studied in oil seeds. Stable isotope labeling studies revealed that only as much as 22% of the needed ATP can be generated through oxidative phosphorylation in canola embryos (Schwender et al. 2006). Increasing the respiration rate through indirect activation of the mitochondrial pyruvate dehydrogenase complex in Arabidopsis resulted in higher oil yield, suggesting that increased ATP production in mitochondria may stimulate fatty acid synthesis (Marillia et al. 2003). Export of ATP from the mitochondria occurs through the adenine nucleotide carrier and subsequent uptake by plastids would be through a known ATP/ADP transporter (Resier et al. 2004), both in counter exchange with ADP.

Oxygen tension may have a role in regulating mitochondrial respiration in seeds. In Arabidopsis siliques, the ambient oxygen concentration is low, and further reduction brought on by growth in sub-ambient [O_2] limited seed growth and oil accumulation (Porterfield et al. 1999). Additional studies in canola revealed that oxygen concentration in seeds is also low, and that increasing [O_2] in low light conditions results in elevated ATP and UDP concentrations, accompanied by faster lipid synthesis (Vigeolas et al. 2003). Although, when light is not limiting increasing atmospheric [O_2] has no

stimulatory effect, suggesting that in green seeds photosynthesis alone is capable of supplying the needed ATP (Goffman et al. 2005). Glycolysis is known to be induced in hypoxic tissues such as roots and seeds (Plaxton and Podesta 2006); however, its contribution to ATP pools has not been investigated.

In any case, ATP must be imported into (if not generated in) the plastid for use in the relevant biosynthetic processes. The Arabidopsis genome encodes two plastidic ADP/ATP transporters, and a double mutant lacking both displays 40% reduced lipid and and about 30% reduced protein accumulation in seeds, presumably due to a lack of ATP in plastids (Reiser et al. 2004). Interestingly, mRNA levels of genes encoding subunits of plastidic pyruvate kinase (examined in this thesis) were higher in this mutant. The authors speculate this was a mechanism to compensate for reduced ATP import by instead producing it in the plastid, although, pyruvate kinase enzyme activity was not measured.

The importance of ATP supply is further highlighted by an observed shift from ATP to pyrophosphate (PPi) consuming metabolism during the phase of maximum fatty acid biosynthesis in Arabidopsis seeds. This shift was observed at the level of transcription and enzyme activity (Baud and Graham 2006, Ruuska et al. 2002). In particular, ATP-dependent phosphofructokinase and invertase (which leads to downstream ATP consumption) activities are replaced by PPi-dependent phosphofructokinase and sucrose synthase (for the cleavage of sucrose). The current hypothesis is that in response to a limited oxygen supply, and thus ATP, this shift is induced in an effort to conserve the adenylate pool for use in fatty acid synthesis. A similar pattern is seen in potato tubers where ATP is required for starch synthesis (Appeldoorn et al. 1997).

1.4.3. Supply of reducing equivalents

Reducing equivalents in the form of NADH and NADPH are necessary for the synthesis, elongation, and desaturation of fatty acids. The reductases involved in fatty acid synthesis specifically use NADPH (3-ketoacyl-ACP reductase) and NADH (enoyl-ACP reductase) (Caughey and Kekwick 1982, Shimakata and Stumpf 1982, Slabas et al. 1986). Synthesis of a saturated 18 carbon fatty acid requires 8 NADH and 8 NADPH. Subsequent desaturation of fatty acids indirectly requires the input of NAD(P)H (Shanklin and Cahoon 1998). The very long chain fatty acids found in wax, suberin, spingolipids and seed oil, are elongated in the cytosol via a similar mechanism as fatty acid synthesis in the plastid, and thus also require 2 NAD(P)H for each two carbon addition (Barrett and Harwood 1998). The NADH required for fatty acid biosynthesis could be supplied from the plastidic pyruvate dehydrogenase complex, which generates NADH and fatty acid precursors (acetyl-CoA) in a 1:1 ratio. In green seeds exposed to light photosynthesis could produce the required NADPH, as is the case with ATP. As described above, light stimulates fatty acid synthesis in embryos and this could likely be a result of not only ATP, but also NADPH production (Bao et al. 1998, Goffman et al. 2005, Ruuska et al. 2004). Indeed, canola embryos contain chloroplasts similar to shade grown leaves and are capable of photosynthesis (Asokanthan et al. 1997, Eastmond et al. 1996, King et al. 1998). Calculations based on reported O_2 evolution rates from canola embryos indicate that photosynthesis could provide all of the NADPH required for fatty acid synthesis (Ruuska et al. 2004). Indeed, carbon use efficiency for storage oil synthesis is greatly reduced in dark-cultured canola embryos compared to those grown in even low light

conditions (50 μmol m^{-2} s^{-1}, Goffman et al. 2005). This effect could be due to the role of photosynthesis in producing reductant and ATP.

The oxidative pentose phosphate pathway (OPPP) generates 2 NADPH for each glucose molecule metabolized, and this could help fulfill the requirements for the reductant necessary for high rates of fatty acid synthesis (Eastmond and Rawsthorne 2000). The first enzyme of the OPPP, glucose-6-phosphate dehydrogenase (G6PDH), is feedback inhibited by NADPH and would presumably be inactivated in reducing conditions (Scheibe and Anderson 1981, Wakao and Benning 2005). Additionally, *in vivo* labeling revealed that a maximum of 25% to 45% of the reductant required for oil biosynthesis is provided by the OPPP (Schwender et al. 2003). Furthermore, an Arabidopsis double mutant which is defective in both cytosolic G6PDH isoforms (resulting in a 50% decrease in total enzyme activity) has no reduction in oil content (see Appendix A). Plastidic NADP-dependent malic enzyme (NADP-ME) also produces NADPH and a role for this enzyme in canola oil metabolism has been proposed (Kang and Rawsthorne 1994, Singal et al. 1995). However, metabolic flux analysis of cultured canola embryos revealed that malate is not a major contributor to fatty acid synthesis (Schwender and Ohlrogge 2002). NADP-ME mutants have been isolated from Arabidopsis, and loss of seed specific isoforms has no effect on oil accumulation (Wheeler et al. 2005). Taken together, it seems that the OPPP and NADP-ME play little or no role in green seeds, but in non-photosynthetic seeds there is evidence to the contrary. Labeling studies with sunflower (*Helianthus annuus*) found that malate is the preferred substrate of fatty acid synthesis when fed to isolated leucoplasts (Pleite et al. 2005). Moreover, the consumption of malate was reduced when co-fed with glucose-6-

phosphate (G6P), and vice versa. Labeled G6P was not incorporated into fatty acids, but instead was entered into the OPPP. The balancing of flux between the OPPP and NADP-ME suggests that these pathways both contribute to NADPH pools in isolated leucoplasts. However, more recent steady-state isotopic-labeling experiments using cultured embryos indicated very little flux of malate into oil (Alonso et al. 2007). NADP-ME has also been implicated in the supply of reductant in castor (*Ricinus communis*) seeds. As with sunflower, malate was the preferred substrate for fatty acid synthesis in isolated leucoplasts (Smith et al. 1992) and a correlation was observed between leucoplast NADP-ME activity and the onset of fatty acid synthesis (Shearer and Dennis 2005). Clearly, green and non-green oil seeds differ in how they supply ATP and reducing equivalents for fatty acid synthesis, but the unifying theme is that in either there are multiple overlapping and interacting pathways for the production of both.

1.5. The biosynthetic pathway from sucrose to oil

The biosynthetic pathways leading to the accumulation of TAG in oil seeds have been extensively studied in Arabidopsis and other oil seed crops. Sucrose imported from maternal tissues is the main carbon source for these metabolisms. There is no symplasmic connection between maternal and filial tissues and so sucrose (or hexose) is released to the seed apoplasm and is then imported into cotyledonary cells of the embryo by membrane localized transporters (Rosche et al. 2002). Once inside the embryo hexose is converted eventually to fatty acids and incorporated into TAG which is deposited into oil bodies (see Figure 1.1). Oil bodies originate from the endoplasmic reticulum (ER) and are delineated by a phospholipid monolayer heavily embedded with proteins (Galili et al.

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1998). Figure 1.2 is based on metabolic flux analysis and transcript profiling of green oil seeds (Ruuska et al. 2002, Schwender et al. 2004a, Schwender et al. 2004b) and depicts the major pathway of carbon metabolism from the loading of sucrose to the budding of oil bodies from the ER. Emphasis is given to the supply of carbon precursors for fatty acid synthesis, but all metabolisms will be covered in forthcoming sections.

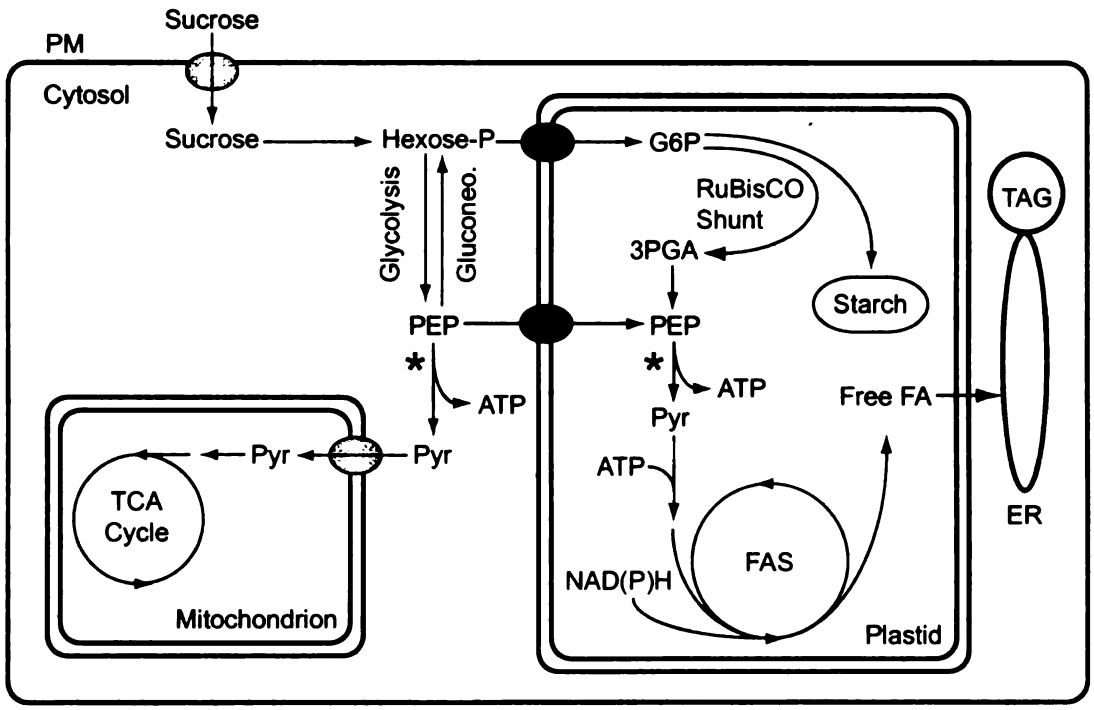

Figure 1.2. Simplified scheme of carbon metabolism in developing seeds

Emphasis is on oil production. Asterisks demark the pyruvate kinase reaction. Single arrows can indicate multiple reactions. 3PGA, 3-phosphoglycerate, ATP, adenosine triphosphate; ER, endoplasmic reticulum; FA, fatty acid; FAS, fatty acid synthase; G6P, glucose 6-phosphate; Gluconeo., gluconeogenesis; PEP, phosphoenolpyruvate; PM, plasma membrane; Pyr, pyruvate; TAG, triacylglycerols; TCA, tricarboxylic acid.

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1.5.1. Synthesis of fatty acids and triacylglycerol

The site of almost all *de novo* fatty acid synthesis in plants is the plastid (Ohlrogge et al. 1979). Fatty acids are synthesized by cycles of elongation in two carbon increments, with malonyl-CoA acting as the carbon donor. The synthesis of malonyl-CoA in plastids is performed by a multimeric bacterial-type ACCase and as mentioned above is subject to regulation by light through various mechanisms. Attempts to boost seed oil yield by increasing the activity of ACCase have had limited success to date and indicate a more complex regulation of fatty acid synthesis (Thelen and Ohlrogge 2002). To be used as a substrate by the fatty acid synthase complex (FAS), malonate must first be transfered to acyl carrier protein (ACP). Malonyl-CoA:ACP transacylase performs this transfer and the activity of this enzyme may also be subject to regulation by light through redox mechanisms (Lemaire et al. 2004). All subsequent steps in the synthesis of fatty acids in the plastid involve ACP and a bacterial type II (multimeric) FAS. A carbon-carbon bond and the release of one CO_2 from malonate are the products of condensation of malonyl-ACP with acyl-ACP (or acetyl-CoA). Three separate condensing enzymes known as 3-ketoacyl-ACP synthases (KAS) can perform this reaction depending on the initial acyl chain length. The first round of condensation to form a four-carbon product, which uses acetyl-CoA as a primer, is carried out by KASIII (Jaworski et al. 1989). Elongation from 4 carbons up to 16 is done by KASI and the final addition to make an 18-carbon fatty acid is done by KASII. Each condensation yields a 3-ketoacyl-ACP product which must be reduced (using NADPH), dehydrated, and then reduced again (using NADH) to yield a saturated product ready for the next round of condensation. These steps are catalyzed by 3-ketoacyl-ACP reductase, 3-hydroxyacyl-ACP dehydratase, and enoyl-ACP

reductase, which together with KAS make up the multimeric FAS complex. As a final step, a portion of the 18-carbon ACP can be desaturated by a plastid localized Δ^9 desaturase to form oleoyl-ACP. Additional desaturation steps are carried out in both the ER and the plastid by membrane bound desaturases. These modifications occur once fatty acids are esterified to a glycerol backbone and are the targets of metabolic engineering as the production of very long-chain polyunsaturated fatty acids in plants is becoming an attractive alternative to natural sources (Truksa et al. 2006).

Fatty acid synthesis is completed by cleavage of a 16- or 18-carbon chain from ACP by an acyl-ACP thioesterase (FAT). Two forms of FAT exist, FATA and FATB, which preferentially cleave unsaturated and saturated acids, respectively. Overexpression of a lauric acid-specific FATB encoding cDNA in Arabidopsis or canola resulted in a dramatic increase in the lauric acid content of seed oil, thus underscoring the importance of FAT enzyme activity in regulating seed oil composition (Voelker et al. 1992, Voelker et al. 1996). Once cleaved from ACP fatty acids are exported from the plastid in an as of yet undetermined manner and are subsequently esterified to CoA in the cytosol. The cytolsol is the site of fatty acid elongation which yields the 20 to 24-carbon very long-chain fatty acids (VLCFAs) present in many seed oils. A homodimeric, eukaryotic-type ACCase and a membrane bound acyl elongation complex participate in elongation in the cytosol and are regulated differently than their plastidic counterparts (Bao et al. 1998). Although, ACCase is still a bottleneck and reducing the activity of the cytosolic enzyme resulted in reduced VLCFA content in Arabidopsis seed oil (Baud et al. 2003). Many other modifications to fatty acids occur, such as hydroxylation, methylation, etc.., and these unusual fatty acids are typically specific to seed oil (Voelker and Kinney 2001).

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The enzymes responsible for such modifications are being elucidated (*e.g.* Bao et al. 2002), and progress is being made in understanding how these unusual fatty acids are edited out of membrane lipids (Voelker and Kinney 2001).

The assembly of triacylglycerol from acyl-CoAs and membrane lipids occurs by two pathways: the Kennedy pathway, and the recently described phosphatidylcholine:diacylglycerol acyltransferase (PDAT) pathway (Dahlqvist et al. 2000). In both pathways, the initial steps are identical. Fatty acids in the ER are esterified to the *sn*-1 position of a glycerol-3 phosphate backbone followed by a second transfer to the *sn*-2 position. The resulting phosphatidic acid is then converted to diacylglycerol (DAG) by the action of a phosphatidic acid phosphatase. In the Kennedy pathway, DAG is converted to TAG by the action of DAG acyltransferase (DAGAT). In the PDAT pathway, an acyl chain from the *sn*-2 position of phosphatidylcholine (PC) is transferred to DAG to form TAG. An Arabidopsis mutant defective in a seed specific DAGAT has reduced seed oil (Katavic et al. 1995, Routaboul et al. 1999, Zou et al. 1997). Conversely, overexpression of the same gene results in enhanced seed oil content (Jako et al. 2001). While no direct role in seed oil biosynthesis has been attributed to any of the six genes encoding PDAT isoforms in Arabidopsis, one is specifically expressed in developing seeds (Stahl et al. 2004). The PDAT pathway may be a means of discriminating TAG-specific fatty acids out of membrane lipids. In any case, the genetic evidence for Arabidopsis points to the DAGAT pathway as the major source of TAG.

1.5.2. Supply of carbon precursors for fatty acid synthesis

Plastidic fatty acid synthesis and subsequent elongation in the cytosol are dependent on a

steady supply of carbon precursors. Imported photosynthate in the form of sucrose or hexose is the major source of carbon for a developing oil seed and must be broken down to serve this purpose. The initial steps involve glycolysis and the newly discovered RuBisCO shunt, and these will be discussed later. Here, I will address the direct supply of acetyl-CoA for ACCase in the plastid and cytosol. Because acetyl-CoA is membrane impermeable (Liedvogel 1986), its supply will be considered separately for each compartment.

The generation of cytosolic acetyl-CoA has only recently been extensively studied in plants. A cytosolic ATP-citrate lyase (ACL) from Arabidopsis has been identified and characterized (Fatland et al. 2002). This enzyme is the main source of cytosolic acetyl-CoA in animals and it was speculated that a similar situation might be occurring in plants. Indeed, when the activity of this enzyme is ablated by expression of antisense cDNA nearly all anabolic processes involving cytosolic acetyl-CoA pools were perturbed, including VLCFA biosynthesis (Fatland et al. 2005). It appeared that no other pathways could compensate for the loss of ACL activityin leaves suggesting that it is the sole source of cytosolic acetyl-CoA in that tissue. The substrate of this enzyme, citrate, is synthesized in the TCA cycle, and ultimately from glycolysis of imported sugars. It should be noted, however, that the synthesis of VLCFA in seeds was relatively unaffected in the ACL antisense plants.

Two enzymes exist in plastids which potentially supply acetyl-CoA to ACCase: the pyruvate dehydrogenase complex (PDC), and acetyl-CoA synthetase (ACS). Expression analysis of ACS and components of the PDC in Arabidopsis suggest that ACS makes little contribution to acetyl-CoA production in plastids of developing seeds

(Ke et al. 2000). Additionally, pyruvate is preferred over acetate as a substrate for fatty acid synthesis in isolated canola embryos (Kang and Rawsthorne 1994). When combined this evidence strongly supports the PDC as being the source of plastidic acetyl-CoA. The PDC reaction also generates NADH at a one to one ratio with acetyl-CoA, which as described earlier could support the demands of fatty acid synthesis. One consequence of PDC activity is the release of CO_2, which results in high concentrations of CO_2 in developing seeds (Goffman et al. 2004). The concentration of CO_2 is potentially not as damaging though as the fact that one third of fixed carbon in pyruvate is lost at this step. However, a refixation shunt involving RuBisCO has been discovered which partially makes up for the loss of carbon by PDC, at least in green seeds (Schwender et al. 2004a).

The amount of acetyl-CoA generated by PDC is entirely dependent on the amount of pyruvate available in the plastid. Figure 1.3 depicts pathways that can contribute to the steady state pool of plastidic pyruvate. The most direct route is the import of pyruvate from the cytosol. A plastid localized pyruvate transporter is hypothesized to exist based on the need for pyruvate translocation during C4 carbon fixation. However, no report has yet been made of the transporter's identity. This pathway would also require a supply of cytosolic PEP and the activity of cytosolic pyruvate kinase (PK_c). The activity of PK_c is high in developing embryos of castor and soybean (*Glycine max*) (Turner et al. 2005). Moreover, isolated plastids from canola embryos are capable of incorporating ^{14}C-labeled pyruvate into fatty acids (Eastmond and Rawsthorne 2000, Kang and Rawsthorne 1994). However, combined data from metabolic flux analyses of cultured canola embryos estimates that a maximum of 30% of the pyruvate used in fatty acid synthesis is generated in this manner (Schwender et al. 2004b).

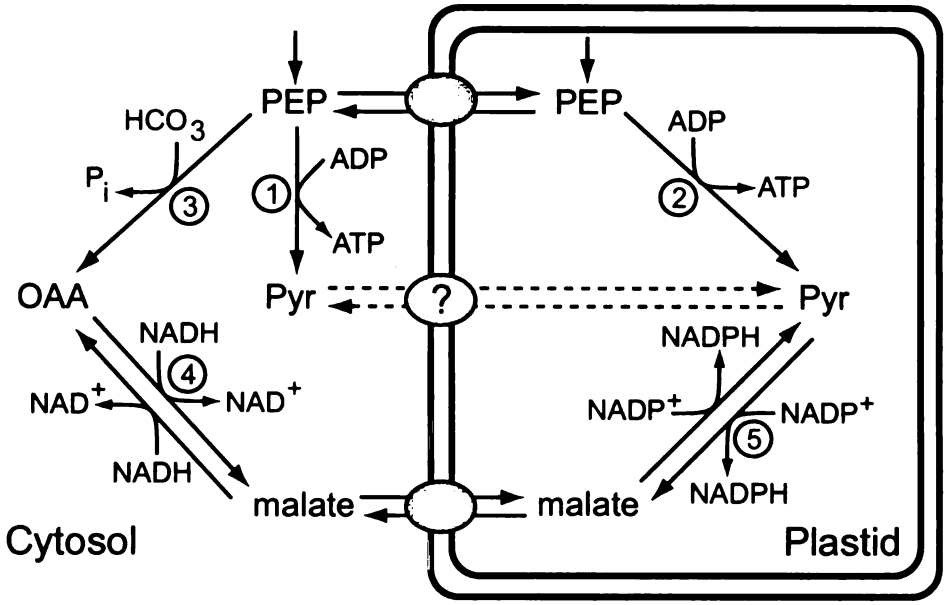

Figure 1.3. Alternative pathways of pyruvate production

Arrows represent reactions catalyzed by single enzymes. Gray ovals are plastid envelope transporters. The pyruvate transporter is marked with '?'. 1) cytosolic pyruvate kinase; 2) plastidic pyruvate kinase; 3) PEP carboxylase; 4) NAD-malate dehydrogenase; 5) NADP-malic enzyme. OAA, oxaloacetate; PEP, phosphoenolpyruvate; Pyr, pyruvate.

Alternatively, plastidic pyruvate kinase (PK_p) can generate pyruvate directly in the plastid. In this case, plastidic or cytosolic PEP can serve as the substrate. The import of PEP by plastids of canola embryos has been demonstrated (Kubis et al. 2004) and microarray analysis of developing Arabidopsis embryos suggested that most PEP is generated in the cytosol (Ruuska et al. 2002). A mutant of the seed resident PEP transporter (*chlorophyll a binding protein underexpressed, cue1*) has been identified, but unfortunately has not been analyzed with a direct focus on the role of this transporter in oil biosynthesis (Knappe et al. 2003, Li et al. 1995, Voll et al. 2003). A recent steady-state carbon flux analysis on cultured embryos of canola led to the proposal of a

RuBisCO shunt involving reactions of the reductive pentose phosphate pathway in the plastid (Schwender et al. 2004a). The proposed pathway bypasses the initial glycolytic reactions in the cytosol and generates PGA in the plastid, which could then be converted to PEP in the plastid, which could compensate for import deficiency in the *cue1* mutant. Although, the labeling studies of Schwender and others (2004a) cannot define whether PGA is converted to PEP in the plastid or cytosol.

Figure 1.3 also contains a three enzyme pathway which bypasses pyruvate kinase altogether. In the first step, cytosolic PEP is converted to oxaloacetate (OAA) by PEP carboxylase (PEPC). Then, malate dehydrogenase (MDH) converts OAA to malate, which can be imported into the plastid and metabolized by NADP-ME to generate pyruvate. Malate is transported across the plastid envelope by a malate/Pi translocator and supports the highest rate of fatty acid synthesis in isolated plastids from castor embryos (Eastmond et al. 1997, Smith et al. 1992). However, NADP-ME mutants have been isolated from Arabidopsis, and loss of seed specific isoforms has no effect on oil accumulation (Wheeler et al. 2005). Generally, PEPC is thought to serve an anaplerotic role in developing seeds, replenishing TCA intermediates consumed during storage protein biosynthesis (Chollet et al. 1996).

The mystery of the source of pyruvate in developing seeds is further complicated by the need for ATP generation in the cytosol or plastid versus the need for the transfer of reducing potential to the plastid (see section 1.4). The pathways illustrated in Figure 1.3 are capable of doing both. The combined evidence leads to the hypothesis that in green oil seeds, the pyruvate kinase pathway(s) are predominant, but that in non-green or protein-storing seeds, the pathway is flexible and may involve PK bypasses.

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1.5.3. Glycolysis in oil seeds

The PEP used for the synthesis of pyruvate is ultimately produced by some or all of the reactions of glycolysis. Stable isotope labeling has been used to demonstrate that 90% of glucose fed to developing canola embryos is converted to pyruvate by the RuBisCO bypass and the second half of glycolysis (Schwender et al. 2002, Schwender et al. 2004a). Furthermore, a link between glycolysis and seed oil metabolism is apparent in the *wri1* mutant of Arabidopsis, in which a general reduction in glycolytic activity results in an 80% reduction in seed oil (Focks and Benning 1998). Glycolysis in plants occurs in both the cytosol and the plastid and both compartments are connected through plastid membrane transporters (Plaxton 1996, Weber 2004). The glycolytic intermediates of developing canola embryos appear to be in near equilibrium between the cytosol and plastid (Schwender et al. 2003), and activities of the full glycolytic sequence have been detected in both compartments in embryos (Eastmond and Rawsthorne 2000). It is therefore likely that changes in glycolytic enzyme activities influence the transport of related intermediates across the plastid envelope. This compartmentation raises the question of a preferred route of glucose metabolism in developing oil seed embryos. Expressed sequence tag (EST) analysis of developing Arabidopsis seeds indicated that mRNAs encoding cytosolic enzymes for the entire glycolytic pathway are abundant, but that only mRNAs encoding plastidic enzymes for the second half of the pathway metabolizing trioses are abundant (White et al. 2000). The recently discovered RuBisCO shunt (Figure 1.2, (Schwender et al. 2004a) bypasses the initial reactions of glycolysis in the plastid and could explain the dearth of ESTs for these genes. Microarray data from Arabidopsis extended these findings by detecting a shift in expression from genes

encoding cytosolic glycolytic enzymes to those encoding plastidic glycolytic enzymes at the onset of storage compound accumulation (Ruuska et al. 2002).

The regulation of glycolysis in oil seeds occurs at least on the transcriptional and metabolic levels. The WRI1 transcription factor has been shown to induce glycolytic gene expression in germinating seedlings and the same trans-activation is thought to occur in developing seeds (Cernac and Benning 2004). Specifically, the activities of PPi-dependent phosphofructokinase (PFP), enolase, and pyruvate kinase are reduced in the *wri1* seeds (Focks and Benning 1998). Furthermore, genes encoding all three enzymes are coordinately expressed with *WRI1* in developing seeds (Schmid et al. 2005). These activities represent the most highly regulated or regulatory steps of plant glycolysis and are shown in Figure 1.4. The most highly regulated step in Figure 1.4 is the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate by the ATP- and PPi-dependent phosphofructokinases (PFK and PFP, respectively). In Arabidopsis PFK activity is high early during embryo morphogenesis while PFP activity is induced during the phase of storage compound accumulation (Baud and Graham 2006). The inhibition of these enzymes (directly or indirectly) by PEP constitutes the bottom-up regulation of glycolysis in plants (Plaxton 1996) and, at least for PFP, this inhibition may be intensified by the hypoxic conditions present in developing seeds (Podesta and Plaxton 2003). Furthermore, these reactions are both reversible and may have roles in controlling the partition of carbon between starch biosynthesis and the production of precursors for fatty acid synthesis. The activities of enolase and PK can have great influence over the concentration of the regulatory metabolite PEP. Little is known about the enolase present in seed tissues, and for that matter, from plants at all. An enolase encoding gene is

upregulated during hypoxic conditions in rice and may suggest a mechanism by which this enzyme is induced in seed tissues (Umeda and Uchimiya 1994). On the other hand, much is known about pyruvate kinase and given its potential importance in seed metabolism it will be discussed separately.

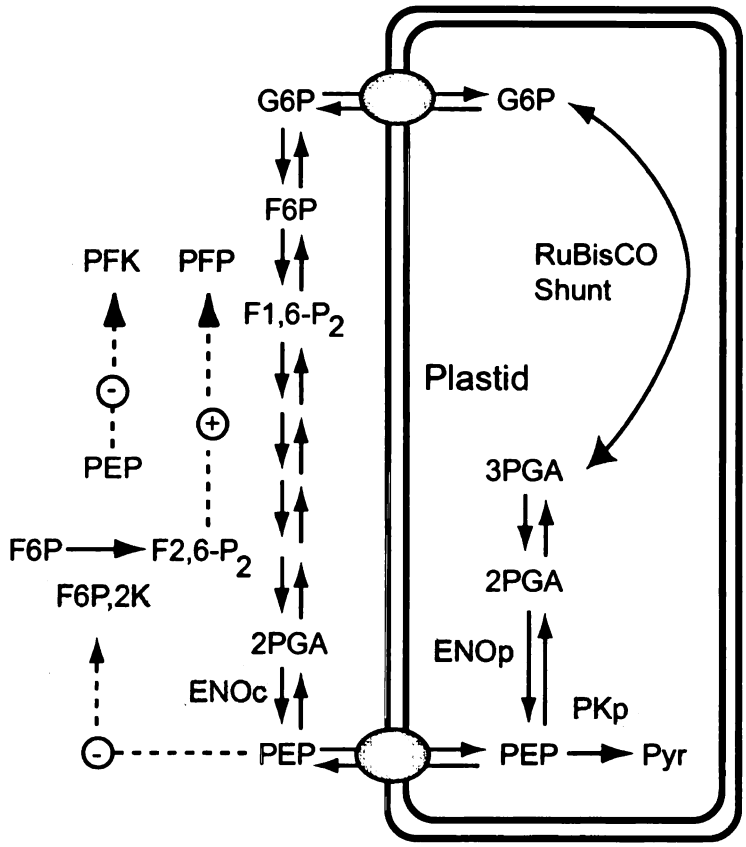

Figure 1.4. The glycolytic network in green oil seeds

Enzymes and transporters are shown as gray ovals. Individual arrows represent single reactions. Dashed lines represent metabolite based regulation. 2PGA, 2-phosphoglycerate; 3PGA, 3-phosphoglycerate; ENO, enolase; F1,6-P_2, fructose 1,6-bisphosphate; F2,6-P_2, fructose 2,6-bisphosphate; F6P, fructose 6-phosphate; F6P,2K, fructose 6-phosphate 2 kinase; G6P, glucose 6-phosphate; PEP, phosphoenolpyruvate; PFP, pyrophosphate-dependent phosphofructokinase; Pyr, pyruvate.

1.6. Pyruvate kinase

Pyruvate kinase catalyzes the conversion of pyruvate to PEP, coupled to the substrate level phosphorylation of ADP to generate ATP (Figure 1.5). In most eukaryotes studied PK is cytosolic, but in plants the enzyme also occurs in the plastid. Plant PK activities arise from the expression of multiple isozymes with different biochemical properties that depend on the tissue and plant source.

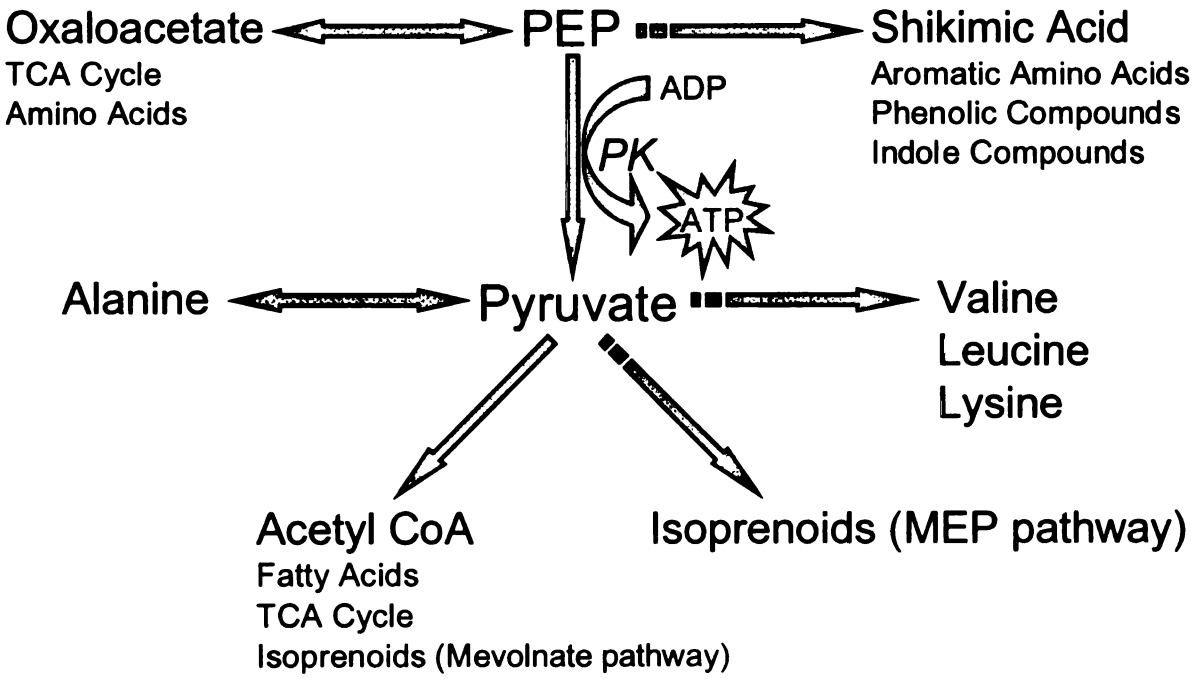

Figure 1.5. Pyruvate kinase centrality to plant metabolism

Solid arrows represent direct metabolic conversions. Metabolic pathways are represented by broken arrows. MEP, methyl-erythritol phosphate; PEP, phosphoenolpyruvate; PK, pyruvate kinase.

Arabidopsis, for instance, has 14 annotated PK genes that likely exhibit a large degree of variation with respect to regulation of gene expression and enzyme activity (Arabidopsis Genome Initiative 2000). The potential influence on the energy status, glycolytic activity, and fatty acid precursor pool in seeds alone makes PK an interesting enzyme to study. However, as shown in Figure 1.5, PK lies at a crossroad and its activity may have an impact on nearly all of plant metabolism.

1.6.1. Pyruvate kinase phylogeny

Pyruvate kinase is an ancient enzyme and occurs in all kingdoms of life. Previous phylogenetic analyses of diverse PK nucleotide (Munoz and Ponce 2003) or amino acid sequences (Hattori et al. 1995, Oria-Hernandez et al. 2006, Schramm et al. 2000) revealed common clustering patterns. Two major families of PK exist; one contains animal, fungal, some bacterial, and plant cytosolic enzymes, while the other includes archaebacterial, cyanobacterial, and plant plastidic enzymes. This segregation suggests distinct ancestral origins of the cytosolic and plastidic enzymes. Most notably, a cyanobacterial enzyme appears to be the origin of plant plastidic PK, which may be a result of the endosymbiotic event which gave rise to plastids. In mammals, two genes encode four separate PK isoenzymes by way of alternative splicing (Noguchi et al. 1986, Satoh et al. 1988). The phenomenon has not been documented in other systems but raises the possibility of much more PK enzyme diversity than can be assessed by gene sequence alone.

1.6.2. Pyruvate kinase molecular architecture

In all but one of the organisms studied to date, PK exists as a protein complex. Most non-plant PKs exist as homotetramers with individual subunits having molecular masses of 56-60 kDa (Muirhead 1990, Munoz and Ponce 2003). The subunit structure of the plant enzymes differs and is somewhat ambiguous. Purified cytosolic PK (PK_c) from germinating castor seeds is an $\alpha_2\beta_2$ heterotetramer with subunits of 56- and 57 kDa (Plaxton 1988). Leaf PK_c from castor has been found as both a homo- and heterotetramer and the enzymes from developing seeds and cotyledons are homoteteramers (Hu and Plaxton 1996, Plaxton 1989). Analyses of PK_cs from canola are consistent with this heterogeneity. In one study, PK_cs from developing and germinating canola seeds were isolated as heterotetramers with subunits the proportions of which differed between the tissues (Sangwan et al. 1992). However, the enzyme from a suspension cell culture was later shown to be a homotetramer (Smith et al. 2000).

Plastidic PKs (PK_p) are also non-uniform in their subunit organization and in one case independent analyses of the same enzyme have resulted in conflicting conclusions. The PK_p from developing castor endosperm is composed of α and β subunits. Studies of recombinant versions of these proteins led to the conclusion that PK_p-α and PK_p-β are distinct enzymes (Blakeley et al. 1995, Blakeley and Dennis 1993, Wan et al. 1995). However, others have determined that a native version of the same enzyme is a heterohexamer composed of both subunits (Negm et al. 1995, Plaxton et al. 1990, Plaxton 1991). Analysis of leucoplast PK from canola suspension cells revealed a single heterohexameric enzyme composed of equal amounts of α and β subunits (Plaxton et al. 2002). Combined, the data from canola and castor suggest PK_p is normally a

heterohexamer of $\alpha_3\beta_3$ stoichiometry. However, the relative expression and actual amount of the individual subunits varies depending on the tissue (Blakeley et al. 1995, Sangwan et al. 1992) and may indicate variable subunit stoichiometry.

1.6.3 Mechanisms of regulation

Pyruvate kinase enzyme activity is dependent on monovalent and divalent cations , with K^+ and Mg^{2+} typically filling the role (Munoz and Ponce 2003). There are some K^+-independent enzymes and phylogenic analysis correlates this feature with specific amino acid residues and suggests that K^+-dependence evolved from K^+-independent enzymes (Oria-Hernandez et al. 2006). The evolution of K^+-dependence is thought to have been driven by the fact that K^+ facilitates active conformation acquisition and subsequent binding of ADP (Oria-Hernandez et al. 2005).

Most non-plant PK's are allosterically regulated, often involving activation by adenosine monophosphate (AMP), fructose 1,6-bisphosphate and other hexose metabolism intermediates. The contact region between subunits is responsible for the allostery and mutation of a single amino acid in this region is sufficient to modify the allosteric properties of an enzyme (Ikeda et al. 1997, Valentini et al. 2000). Plant PKs are typically insensitive to allosteric regulation by FBP but are regulated more by central carbon metabolites and pH effects (Plaxton 1996). Plant cytosolic PKs typically have pH optima of approximately pH 7.0, whereas plastidic PKs are most active at pH 8.0 (Hu and Plaxton 1996, Plaxton et al. 2002, Smith et al. 2000). The most common metabolite regulators of plant PK activity are tricarboxylic acid cycle intermediates and amino acids, and they typically act as inhibitors (Plaxton and Podesta 2006). In general, non-plant

enzymes are activated by upstream metabolites, whereas plant PKs are inhibited by downstream metabolites. This generalization reflects the role of PK in the respective organisms. In non-plants, PK functions primarily in carbohydrate catabolism and the production of energy. In plants energy can be produced by photosynthesis and the role of PK is mainly to supply various metabolic pathways with carbon precursors. In summary, non-plant enzymes are activated when energy is needed and plant enzymes are inhibited when carbon precursors are abundant.

The multimeric structure of PK allows for additional levels of regulation. Catalysis only occurs when individual subunits associate and an active complex is formed. *In vitro*, this can be stimulated by the addition of polyethylene glycol, resulting in enzyme activation (Podesta and Plaxton 1993). This suggests that *in vivo*, association and dissociation of subunits may influence PK activity. Each PK subunit has intrinsic characteristics that confer regulatory properties to the complexes in which they are found. Thus, the potential for multiple subunit stoichiometries for heteromeric enzymes introduces the possibility of multiple regulatory states. The novel regulatory properties of PK hybrids formed by combining subunits of normally homomeric mammalian enzymes provides proof of this concept (Dyson and Cardenas 1973, Hubbard and Cardenas 1975). Individual subunits can be further regulated by covalent modification. Mammalian liver PK is inhibited by phosphorylation on serine residues and recently a soybean PK_c was shown to be targeted for degradation by phosphorylation (Munoz and Ponce 2003, Tang et al. 2003). The availability of a particular subunit for complex formation is also controlled by effects on gene expression, translation, compartmentation, and protein turnover and these (and other) factors likely help govern PK activity in any given tissue.

1.6.4 Analysis of pyruvate kinase mutants

Pyruvate kinase deficiency occurs or has been induced in many organisms but only one case has been reported in plants. In humans, PK deficiency is the most common glycolytic defect and is the leading cause of hereditary non spherocytic haemolytic anaemia with 180 known mutations which result in the disease (Zanella et al. 2007). Erythrocytes are entirely dependent on glycolysis for ATP production and so a severe reduction in PK activity can result in death at infancy (Zanella et al. 2007). In yeast (*Saccharomyces cerevisiae*), PK deficiency results in an inability to grow on glucose or other fermentable sugars (Sprague, Jr. 1977). Interestingly though, and counter intuitively, flux through the tricarboxylic acid cycle is stimulated in the absence of PK activity (Pearce et al. 2001). A similar glucose non-fermentable phenotype is observed in *Escherichia coli* PK mutants (Pertierra and Cooper 1977). It is clear from the above cases that PK plays a pivotal role in regulating glycolytic flux and energy production in cells lacking respiratory capacity.

Pyruvate kinase deficiency has been induced in tobacco (*Nicotiana tabacum*) inadvertently by ectopic expression of a potato (*Solanum tuberosum*) tuber PK_c fused to a chloroplast transit peptide (Gottlob-McHugh et al. 1992). Instead of increasing plastid localized PK activity, PK_c was silenced. As a result, root biomass was reduced relative to the shoot suggesting an altered source:sink relationship (Knowles et al. 1998). Further analysis revealed impaired export of photosynthate from leaves at night along with a higher rate of respiration (Grodzinski et al. 1999). Whether the reasons for higher respiration are the same as those for yeast and bacterial PK mutants is unknown.

1.6.5. Pyruvate kinase moonlighting

Ancient enzymes such as PK often have roles in addition to their primary enzymatic function. Hexokinase1 from Arabidopsis, for instance, has recently been identified as a glucose sensor and can translocate into the nucleus where it mediates expression of sugar responsive genes (Cho et al. 2006, Moore et al. 2003). Another glycolytic enzyme, enolase, acts as transcriptional regulator in response to cold stress in Arabidopsis (Lee et al. 2002). Most recently, a mammalian PK was found to translocate into the nucleus in response to interleukin-3 stimulation, where it promotes cell proliferation (Hoshino et al. 2007). Several studies using other systems find PK in other non-traditional roles. Using a rat cDNA library and a K_{ATP} channel as bait in a yeast two-hybrid experiment, PK along with glyceraldehyde-3-phosphate dehydrogenase and triose-phosphate isomerase were found to form a complex which regulates flux of K^+ (Dhar-Chowdhury et al. 2005). Another study identified PK as a lysophosphatidic acid (LPA) binding protein (Desmaret et al. 2005). PK activity was inhibited upon binding LPA, implying LPA as a signaling molecule which controls metabolism. No moonlighting functions have yet been determined for a plant PK.

1.7. Rationale and outlook

Modeling the seed metabolic network based on transcript abundance and metabolic flux analysis places PK_p at an important node that connects catabolic and anabolic processes (Figure 1.2, Figure 1.5). A hypothesis based on this model is that most of the carbon precursors used in fatty acid synthesis are produced in the plastid by PK_p, and that PK_p also provides energy to the developing embryo in the form of ATP. While native PK_ps

have been isolated and described from castor and canola, little has been done to directly test the function of these enzymes *in vivo*. Use of the model plant Arabidopsis makes investigating the connection between *in vitro* properties and *in vivo* function readily doable. Embryo tissue is not abundant in Arabidopsis but the availability of the entire genome sequence makes possible the rapid cloning of putative PK genes and heterologous production of the respective proteins. Publicly available gene expression data can help this process by hastening the identification of those genes which are actively transcribed in the tissue of interest. By this method I have identified and expressed in *E. coli* the genes which encode PK_ps involved in seed metabolism, *in lieu* of purifying the native enzyme from embryo tissue. Molecular and kinetic characterizations of the recombinant proteins indicated that they perform their annotated function and were used to make predictions about how they function *in vivo*. A shortcoming of previous studies of seed resident PK_ps is the lack of any genetic evidence to confirm any such predictions. To this end, I took advantage of the Arabidopsis T-DNA insertion mutant population. Plants that are impaired in PK_p activity were used to test the hypothesis that PK_p is crucial for the production of precursors for fatty acid synthesis in green oil seeds. Further analysis of the mutants also lead to the discovery of novel functions for PK_p. By taking such a two-prong approach, I was able to draw conclusions that unite *in vitro* and *in vivo* data for the synthesis of a more complete picture of seed oil metabolism. Finally, I will outline some strategies for the application of my findings towards metabolic engineering for the production of valuable phytochemicals.

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Chapter 2

Molecular and kinetic analysis of Arabidopsis plastidic pyruvate kinase[1]

[1] This work has been published in Andre, C., Froehlich, J.E., Moll, M.R., and Benning, C. (2007) A heteromeric plastidic pyruvate kinase complex involved in seed oil biosynthesis in Arabidopsis. Plant Cell **19**:1-17. Figure 2.3B was provided by J.E. Froehlich and M.R. Moll contributed to Figure 2.5A-E

Abstract

Plastidic pyruvate kinase (PK_p) catalyzes a highly regulated, ATP-producing reaction of glycolysis. PK_p occupies a highly branched node in the triacylglycerol biosynthetic network of developing seeds and is expected to be an important point for the regulation of carbon partitioning. A detailed biochemical characterization of this enzyme will provide a framework for future manipulations of seed metabolism. The Arabidopsis genome encodes 14 putative isoforms of pyruvate kinases. Three genes encode subunits α, β_1, and β_2 of plastidic pyruvate kinase (PK_p). Recombinant protein production and subsequent kinetic analysis was used to show that active PK_p complexes are composed of α and either β_1 or β_2 subunits. Enzyme activity is dependent the formation of a complex between α and β subunits, although the presence of either β_1 or β_2 results in unique kinetic and regulatory properties. The plastid enzyme prevalent in developing seeds likely has a subunit composition of $4\alpha4\beta_1$, is most active at pH 8 and is inhibited by glutamate.

Introduction

The biochemical reactions leading to the accumulation of seed oil are well characterized (Ohlrogge and Browse 1995). Furthermore, the use of stable isotope labeling and forward genetics has linked the supply of precursors for fatty acid biosynthesis in embryos to glycolysis (Focks and Benning 1998, Schwender and Ohlrogge 2002). A key regulatory step in plant glycolysis is pyruvate kinase, as its products inhibit the upstream glycolytic reaction catalyzed by phosphofructokinase (Plaxton 1996). Pyruvate kinase (EC 2.7.1.40) occurs as both cytosolic and plastidic isoforms and catalyzes the ADP-dependent conversion of phosphoenolpyruvate (PEP) to pyruvate while producing ATP. With respect to seed oil, plastidic pyruvate kinase (PK_p) activity and concentration have been shown to correlate with the most active stage of lipid biosynthesis in developing *Brassica napus* embryos (Sangwan et al. 1992). Microarray data of developing Arabidopsis seeds show that the transcript level of a putative PK_p encoding gene coincides with the most active period of TAG synthesis (Ruuska et al. 2002, Schmid et al. 2005). In addition, embryo PK_p from *B. napus* is activated by 6-phosphogluconate, an intermediate of the OPPP, suggesting a coordination between the production of precursors and reducing equivalents for fatty acid synthesis (Plaxton et al. 2002).

Plant PK activities arise from the expression of multiple isozymes with different biochemical properties that depend on the tissue and plant source. Arabidopsis, for instance, has 14 annotated PK genes that likely exhibit a large degree of variation with respect to regulation of gene expression and enzyme activity (Arabidopsis Genome Initiative 2000). Plastidic pyruvate kinase has been purified and characterized from castor (*Ricinus communis*) endosperm and *B. napus* suspension cell cultures (Negm et al. 1995,

Plaxton et al. 1990, Plaxton et al. 2002). Both enzymes consist of α and β subunits and exist as 3α3β heterohexamers. Both are regulated by metabolites of central carbon metabolism and have pH optima of approximately 8.0. In the case of the *B. napus,* a scheme was formulated in which the kinetic properties of PK_p were used to infer a role for the enzyme in regulating the supply of precursors for fatty acid synthesis. The characterization of orthologous PK_p(s) from the model plant Arabidopsis is pertinent as such a similarly derived model could then be tested using available genetic tools. Here, potential PK_p s from Arabidopsis were identified and heterologously produced using *E. coli*. The resulting recombinant proteins were then characterized at the molecular and biochemical levels to gain insight into this enzyme's role in seed oil biosynthesis.

Materials and Methods

Bioinformatics

Pyruvate kinase genes highly expressed in seeds were initially identified in the seed EST Database (White et al. 2000). The 14 putative Arabidopsis PK-annotated sequences from the TAIR website (www.arabidopsis.org) were used for the current work. Other annotated PK sequences were down loaded from NCBI. Predicted full-length protein sequences were aligned using ClustalW (Li 2003) available from the Biology Workbench (San Diego Supercomputer Center, University of California, San Diego; http://workbench.sdsc.edu/). A phylogenetic tree was generated in Phylip format and bootstrapped using a random number generator seed of 111 and 1000 bootstrap trials. The phylogram was visualized using the TreeView program (version 1.6.6; Page 1996). No manual adjustments were made to the initial alignment. Global gene expression data was mined from the AtGenExpress developmental database (Schmid et al. 2005).

GFP fusion localization

The cDNAs for the three PK subunits were amplified using primers specific for GFP fusion construct generation as listed in Table 2.1. Fully sequenced products were then inserted into the T-DNA vector pCAMBIA1302 (CAMBIA, Canberra, Australia), which contains a CaMV 35S promoter and a C-terminal GFP encoding sequence. The resulting constructs were electroporated into *Agrobacterium tumefaciens* as described below and were then transiently expressed in *Nicotiana benthamiana* using a published protocol (Voinnet et al. 2003). After 3-4 days, leaf samples were mounted in water on slides and were directly examined using a Zeiss LSM5 confocal microscope. Excitation light was

provided by an argon laser at 488 nm. GFP fluorescence was observed with a band-pass filter of 505 to 530 nm and chlorophyll fluorescence with a 650-nm long-pass filter. Enhanced-quality images were acquired with the LSM5 imaging system software, and post acquisition image processing was performed with the LSM5 image browser and Adobe Photoshop software. This work was performed at the Center for Advanced Microscopy (Michigan State University).

Pea chloroplast import assays

The cDNAs encoding PK_p-α, PK_p-β_1, and PK_p-β_2 inserted into pBluescript II (Stratagene) were used in this study. These genes were transcribed/translated, and proteins were subsequently labeled with [^{35}S]-Met using the TNT-coupled wheat germ extract system according to the manufacturer's recommendations (Promega, Madison, WI, USA). The PK_p plasmids were linearized prior to translation with the T3 or T7 RNA Polymerase TNT-coupled wheat germ extract system. The plasmid containing the gene encoding the RuBisCO small subunit used for control purposes has been described (Olsen and Keegstra 1992). Pea plants (*Pisum sativum* var Little Marvel; Olds Seed Co., Madison, WI, USA) were grown under natural light in the greenhouse at 18 to 20°C. Chloroplasts were isolated from 8- to 12-d-old plants as described previously (Bruce et al., 1994). Binding or import reactions were performed according to published protocols (Tranel et al. 1995). Post-treatments of import reactions with either thermolysin or trypsin were performed as described previously (Jackson et al. 1998). All fractions were analyzed by SDS-PAGE (Laemmli 1970) and fluorography (Tranel et al. 1995).

cDNA cloning and recombinant protein production

The cDNAs corresponding to At3g22960, At5g52920, and At1g32440 were generated from total silique RNA isolated as previously described (Verwoerd et al. 1989). Reverse transcription was done with the Qiagen (Valencia, CA, USA) Omniscript RT kit and 600 ng of total RNA. Primers listed in Table 2.1 were used for PCR amplification of cDNAs to generate products with and without predicted chloroplast transit peptides (cTPs) and with or without epitope tags. All cDNAs were inserted into pBluescript II (Stratagene, LaJolla, CA, USA) and sequenced at the MSU Research Technology Support Facility. The vector pET-15b (Novagen, San Diego, CA, USA) was used for recombinant protein expression in *E. coli* strain BL21 (DE3) pLysS (Novagen). The PK_p-α encoding fragment was inserted into the *Bam*HI and Klenow-filled *Nde*I sites of pET-15b by digesting with *Kas*I, filling in with Klenow, and then by digesting with *Bam*HI and ligating. The open reading frame for PK_p-β_1 was inserted into the *Bam*HI and Klenow-filled *Nde*I sites of pET-15b by digesting with *Bgl*II, filling in with Klenow, and then by digesting with *Bam*HI and ligating. The PK_p-β_2 encoding fragment was inserted into the *Xho*I and Klenow-filled *Nde*I sites of pET-15b by first digesting with *Spe*I, filling in with Klenow, and then by digesting with *Xho*I and ligating. Proteins were expressed at 28°C by inducing at an OD_{600} of 0.6 with 0.5 mM IPTG and allowing the cultures to grow for 4 more hours. His-tagged proteins were recovered over Ni-NTA resin using standard protocols. Purified proteins were exchanged into a buffer of 50 mM $Na_xH_xPO_4$ pH 7.9, 150 mM NaCl, 5 mM $MgCl_2$, and 10% glycerol. The 6X-His tags were cleaved using a Thrombin cleavage capture kit available from Novagen. Proteins were quantified with the Bradford method using reagent from Sigma-Aldrich (St. Louis, Mo, USA).

Table 2.1 Primer sequences

Gene or SALK Line	Primer
PKp-β_2	P,PE(f) 5'-ACTAGTATTAAAATCTCCGAAGATAG-3'
	P(r) 5'-CTCGAGTCATCCACCTATCTTTATCT-3'
	PE(r) 5'-CTCGAGTCATCCCTTGTCATCGTCATCCTT ATAATCTCCACCTATCTTTATCTT-3'
	O,OE(f) 5'-GGTACCCCTCAGGTTTCTCTGCTCAT-3'
	O(r) 5'-GGTACCACTGTGAGTGATTCAAAAAA-3'
	OE(r) 5'- GGTACCTCATCCCTTGTCATCGTCATCCTT ATAATCTCCACCTATCTTTATCTT-3'
	G(f) 5'-GATATCGCTGCTTATGGTCAAATCTC-3'
	G(r) 5'-GATATCTCCACCTATCTTTATCTTAC-3'
	RT(f) 5'-GGGGATGTACCGCAGCCGATA-3'
	RT(r) 5'-GGATGCCGAGGTTCTGACAGG-3'
SALK_013574	RP 5'-TTTCACACAACAAATTCGTTCATT-3'
	LP 5'-CAGCTTCCGCGAGTTTCCAAATCA-3'
SALK_142845	Same as SALK_013574
PKp-α	P,PE(f) 5'-GGCGCCTCCTCGTCATCATCTCC-3'
	P(r)-5'-GGATCCTTACGGGACGTTCATTACCT-3'
	PE(r) 5'-GGATCCTTACAAATCCTCCTCACTAATCAA CTTTTGCTCCGGGACGTTCATTACCTG-3'
	OE(f) 5'-GGTACCAGCCAACTGTCCTGAGATTT-3'
	OE(r) 5'-GGTACCTTACAAATCCTCCTCACTAATCAA CTTTTGCTCCGGGACGTTCATTACCTG-3'
	G(f) 5'-ACATGTCTCAGTCTATTCAATTCTCC-3'
	G(r) 5'-ACTAGTCGGGACGTTCATTACCTGGA-3'
SALK_096141	LP 5'-CCAAATTCAACACTCTCACACTTCG-3'
	RP 5'-CCATCCCACCATCAACCAAAA-3'
	RT(f) 5'-GCTGCTCGTTCCCGTGGAGG-3'
	RT(r) 5'-TTGAAGCGGTACAGACTCAT`-3'
SALK_024870	LP 5'-TCTCGGACATGCTGCAATCAA-3'
	RP 5'-TTCGCATCAGTCATCTTCGTCTTC-3'
PKp-β_1	P,PE(f) 5'-AGATCTGCTCGTGTTGAGACTGA-3'
	P(r) 5'-GGATCCTTAAACCTTGCGGACTTGGA-3'
	PE(r) 5'-GGATCCTTATGCATAATCGGGAACATCATA GGGATAAACCTTGCGGACTTGGAT-3'
	O,OE(f) 5'-GGTACCCTTCACTACTCTGTCTCAGC-3'
	O(r) 5'-GGTACCCAAAAACGAGGTTCTACATA-3'
	OE(r) 5'-GGTACCTTATGCATAATCGGGAACATCATA GGGATAAACCTTGCGGACTTGGAT-3'
	G(f) 5'-CCATGGCTCAAGTGGTTGCTACCAGG-3'
	G(r) 3'-ACTAGTAACCTTGCGGACTTGGATGT-3'

Table 2.1 Continued

SALK_042938	LP 5'-TGAGATAGCATTTCAATTTGATGCG-3'
	RP 5'-GGCAAATCATTCACTTAGGATGGA-3'
	RT1(f) 5'-CTGGGATGAATGTTGCTAGG-3'
	RT2(r) 5'-GTCAACTTTGTTCTCCACTCC-3'
SALK_042681	LP 5'-ACAGCCAATCTGGCGATCTCA-3'
	RP 5'-TATTACAGGTCTATTTCTTTCGG-3'
T-DNA LB	5'-GTTCACGTAGTGGGCCATCG-3'
ACTIN-1	RT(f) 5'-AACAATCGATGGACCTGACTCG-3'
	RT(r) 5'-TGCGACAATGGAACTGGAATGG-3'

Primers used for cloning of recombinant proteins without predicted cTP with (PE) or without (P) eptiope tags, for cloning of full length proteins for overexpression in plants with (OE) or without (O) epitope tags, for GFP fusion construct making (G), for genotyping of SALK_KO lines (LP, RP) and for expression analysis (RT)

Native-PAGE analysis and gel filtration chromatography

Native-polyacrylamide gel electrophoresis (PAGE) was performed using 7.5% acrylamide Ready-Gels from Bio-Rad (Hercules, CA, USA). Freshly prepared protein was used and 5 pmols of each PK subunit were loaded per well. The gels were run at 4°C at 140 V. PK activity staining was done as previously described, except that 50 mM HEPES-KOH pH 8.0 buffer was used (Rivoal et al. 2002). Immunoblotting was done using standard protocols and monoclonal anti-c-myc, anti-FLAG, and anti-HA antibodies from Sigma-Aldrich. Antibodies were tested for specificity against epitope tagged and untagged versions of all three PK subunits. Gel filtration was accomplished using a Superdex 200 HR10/30 column with a flow rate of 0.4 mL min^{-1} and a buffer of 50 mM HEPES-KOH pH 8.0, 10% glycerol, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, and 0.04% NaN_3. A standard curve was generated with the HMW gel filtration calibration kit

from GE Healthcare (Piscataway, NJ, USA). For the samples, 300 µL of 0.5 mg mL^{-1} (per subunit) solution was injected and 0.35 mL fractions were collected.

Co-immunopreciptation analysis

Full length epitope tagged versions of PK_p-α, PK_p-β_1, and PK_p-β_2 were produced by PCR with primers listed in Table 2.1. The respective DNA fragments were then inserted into the KpnI site of a modified pCAMBIA1300 vector (CAMBIA), which contained the EcoRI/HindIII expression cassette from pBIN121 (Clontech, Palo Alto, CA, USA). Arabidopsis was stably transformed with these constructs and protein expression was monitored by immunoblotting. Arabidopsis plants were prepared for transformation as previously described (Cernac and Benning 2004). When ready, plants were transformed using the floral dip method (Clough and Bent 1998). Competent cells of *Agrobacterium tumefaciens* strain C58C1 GV3101 pMP90 (Koncz and Schell 1986) were prepared and transformed as previously described (Shen and Forde 1989).

Silique tissue was ground in 3 volumes (w/v) of extraction buffer containing 50 mM HEPES-KOH pH 8.0, 5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1mM DTT, 0.1% Triton-X100, 10% glycerol, 2 mM benzamidine, 2 mM ε-amino-n-caproic acid, 1 mM PMSF, and 1 mM PEP. Debris was removed by centrifugation at 16,000*g* for 10 min and the supernatant was used for SDS-PAGE or co-IP. For co-IP, 250 µL of supernatant was pre-cleared by incubation for 1 hour at 4°C with 30 µL of a 50% slurry of Protein-A sepharose. The supernatant was kept and mixed with 5 µg of the appropriate antibody and was then nutated at 4°C. After 1 hour, 30 µL of Protein-A sepharose slurry was added and the mixture rocked for an additional hour. After this time, the Protein-A

sepharose with bound antibody and proteins was washed 4 times in extraction buffer and then mixed with SDS-PAGE sample buffer, heated, and the supernatant loaded on gels. Excised bands were submitted to the MSU Research Technology Support Facility for tryptic digest and LC/MS/MS. The generated data was then compared against the Arabidopsis proteome using MASCOT software (Matrix Science, Boston, MA, USA).

PK enzyme assays and kinetic analysis

All chemicals were from Sigma-Aldrich. Pyruvate kinase activity was detected by coupling the production of pyruvate to the conversion of NADH to NAD^+ by lactate dehydrogenase unless otherwise noted. Reactions were kept at 25°C, were started by the addition of enzyme mix, and were linear for at least 5 minutes. Absorbance at 340 nm was monitored using a FLUOstar Optima 96-well plate reader (BMG Labtech, Offenburg, Germany). Standard PK_p reaction mixtures contained 50 mM HEPES-KOH pH 8.0, 5% PEG-8000, 50 mM KCl, 15 mM $MgCl_2$, 1 mM DTT, 2 mM PEP, 1 mM ADP, 0.2 mM NADH, and 2 U ml^{-1} desalted rabbit muscle lactate dehydrogenase. PEP phosphatase activity was corrected for by omitting ADP from the reaction. Reactions at pH 7.0 were done using 50 mM MOPS pH 7.0 instead of HEPES.

For kinetic analysis 2.5 pmol of each subunit were mixed and used per reaction. $S_{0.5}$ and V_{max} values were determined by fitting the Hill equation to plots of initial velocity versus substrate concentration using origin 7.0 (OriginLab Corporation, Northampton, MA, USA). pH optimum curves were generated using a 25 mM MES, 25 mM Bis-Tris-propane buffer over a range of pH's. For inhibitor/activator studies metabolite stocks were made equimolar with $MgCl_2$ and were pH adjusted to 8.0.

Metabolites were tested at pH 8.0 with 100 μM PEP and 150 μM ADP for $\alpha\beta_1$ and 150 μM PEP and 300 μM ADP for $\alpha\beta_2$. The metabolites tested were: glucose, fructose, 6GP, G1P, F6P, F16bP, DHAP, G3P, 2PG, acetate, OAA, citrate, iso-citrate, 2-oxoglutarate, succinate, fumerate, malate, gro3P, 2P-glycolate, glycolate, Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Leu, Lys, Met, Ser, AMP, ADP, ATP, ADP-Glc, UDP-Glc, UDP-Gal, R5P, 6PG, KPi, $NaNO_3$, and NH_4Cl, all at 10 mM; MgPPi, NADH, NADPH, NAD^+, $NADP^+$, oxalate, Ile, Phe, Pro, Thr, Trp, Tyr, Val, CoA, Mal-CoA, Ac-CoA, and shikimate, all at 0.5 mM; F26bP, Oleoyl-CoA, Oleate, and LPA all at 0.05 mM. Oxalate, glyoxylate, and OAA were found to inhibit the LDH reaction so PK activity was measured by coupling to the ATP dependent conversion of glucose to glucose-6-phosphate by hexokinase followed by the NAD^+-dependent conversion of glucose-6-phosphate to 6-phosphogluconate by glucose-6-phosphate dehydrogenase. The reaction mix was adjusted to contain no NADH or LDH, but instead to have 1 mM NAD^+, 5 mM glucose, 2 U mL^{-1} hexokinase, and 2 U mL^{-1} G6PDH. I_{50} and K_a values are the concentration of a metabolite required for 50% maximum inhibition or activation, respectively. They were calculated by fitting a modified Hill equation to plots of initial velocity versus effector concentration as previously described (Ballicora et al. 2005).

Site-directed mutagenesis of PK subunits was done using the QuikChange-XL Site-Directed Mutagenesis Kit using primers designed to the manufacturer's specifications (Qiagen). An absolutely conserved lysine residue in the PK active site was mutated to leucine for each subunit (PK_p-α, K344L; PK_p-β_1, K325L; PK_p-β_2, K314L). This mutation has previously been shown to abolish PK activity (Sakai 2005). Chemical inactivation was achieved by incubating 5 μM solutions of purified subunits with either

water or 100-fold molar excess of 2,4,6-trinitrobenzenesulfonic (TNBS) acid in the dark at room temperature for 1 hour. This treatment has been shown to inactivate PK subunits by covalent modification of lysine residues without abolishing protein interactions (Hollenberg et al. 1971) Excess TNBS was quenched with an equal volume of 100 mM Tris-Cl pH 7.5 for 20 minutes on ice and the proteins were used directly in enzyme assays.

Accession numbers

Arabidopsis Genome Initiative locus identifiers (www.arabidopsis.org) used in this study are as follows: At3g22960 (encoding PK_p-α), At5g52920 (encoding PK_p-β_1), At1g32440 (encoding PK_p-β_2), At2g36580, At3g04050, At3g25960, At3g49160, At3g52990, At3g55650, At3g55810, At4g26390, At5g08570, At5g56350, At5g63680. Genbank accession numbers for non-Arabidopsis protein sequences used are as follows: *Nt* PK_p-A, Q40545; *Os* PK_p-A, NP_001059042; *Rc* PK_p-A, Q43117; *Nt* PK_p-G, Q40546; *Se PCC6301* PK, YP_172116; *Se WH8102* PK, NP_897391; *Pfu* PK, NP_578917; *Sa* PK, YP_256251; *Ec* PK-1, AAA24392; *Hs* PK-L, BAA02515; *An* PK, Q12669; *Sc* PYK1, NP_009362; *Os* PK_c, BAD81116; *Nt* PK_c, Q42954; *St* PK_c, P22200; *Gm* PK_c, Q42806

Results

Identification of plastid localized and seed resident pyruvate kinases

The Arabidopsis genome encodes 14 putative PK isoforms (Arabidopsis Genome Initiative 2000). All but one (encoded by At3g49160) of the predicted PKs contain a fully conserved PK active site of [LIVAC]-x-[LIVM]-[LIVM]-[SAPCV]-K-[LIV]-E-[NKRST]-x-[DEQHS]-[GSTA]-[LIVM] (listed at European Bioinformatics Institute, http://www.ebi.ac.uk/) and are presumably active enzymes. Several cross-kingdom PK phylogenies have been published (*e.g.* Hattori et al. 1995, Munoz and Ponce 2003, Schramm et al. 2000), but only one putative PK_c from Arabidopsis was included in these studies. When the 14 Arabidopsis PK amino acid sequences are aligned with *bona fide* PKs from other organisms they segregate into cytosolic and plastidic clades (Figure 2.1A). This amino acid sequence similarity-based segregation is supported by the exclusive prediction of chloroplast transit peptides at the N-termini of the four predicted PK_ps using ChloroP and TargetP (Emanuelsson et al. 1999, Emanuelsson et al. 2000). The PK_ps in Figure 2.1A are further divided between α and β subunits. One Arabidopsis PK_p subunit (encoded by At3g22960) is most similar to described PK_p-αs. Two others, which show 63% amino acid identity to each other (encoded by At5g52920 ,PK_p-β_1; At1g32440, PK_p-β_2), are most similar to known PK_p-βs (Figure 2.1A).

Three of the four predicted Arabidopsis PK_p genes were identified as seed expressed by EST analysis of developing seeds (White et al. 2000). Two, PK_p-α and PK_p-β_1, represent the highest level of induction of any PK gene in seed and are coordinately expressed in all tissues while the PK_p-β_2 encoding gene has very low transcript accumulation in any tissue (Figure 2.1B, Schmid et al. 2005).

Figure 2.1. Pyruvate kinase similarity and selected gene expression in Arabidopsis

(A) Pyruvate kinase phylogeny. Amino acid sequences of Arabidopsis (gene loci in bold) and other *bona fide* pyruvate kinases were used. Bootstrap values are indicted at branches; α and β represent plastidic PK subunit families. The scale represents 10% difference. *An*, *Aspergillus niger*; *Ec*, *Eschericia coli*; *Gm*, *Glycine max*; *Hs*, *Homo sapiens*; *Nt*, *Nicotiana tabacum*; *Os*, *Oryza sativa*; *Pfu*, *Pyrococcus furiosus*; *Rc*, *Ricinus communus*; *Sa*, *Sulfolobus acidocaldarius*; *Se*, *Synechocystis sp.*; *Sc*, *Saccharomyces cerevisiae*; *St*, *Solanum tuberosum*

(B) Relative gene expression of putative Arabidopsis PK_p-α (At3g22960), PK_p-β_1 (At5g52920), and PK_p-β_2 (At1g32440) encoding genes (derived from published microarray data (Schmid *et al.*, 2005)). DAF, days after flowering. Values are the mean ± SD (n=3).

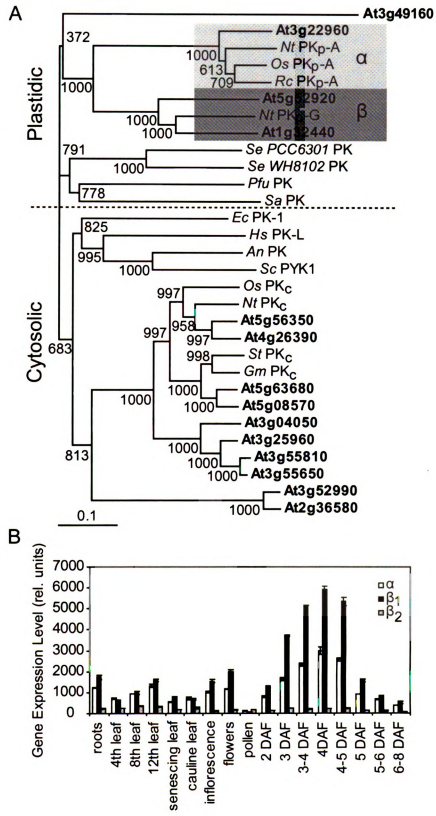


Figure 2.1. Pyruvate kinase similarity and selected gene expression in Arabidopsis

Figure 2.2. Phylogenetic analysis of plant PK protein sequences.

Amino acid sequence-based phylogeny of annotated PKs from plant and algal sources. Some sequences are the same as used in Figure 2.1. The rice (Os), corn (*Z. mays*), Chlamydomonas (*C. reinhardtii*), *Cyanidioschyzon merolae* (*C. merolae*), and *Physcomitrella patens* (*P. patens*) sequences are new and accession numbers are listed after the genus identifier.

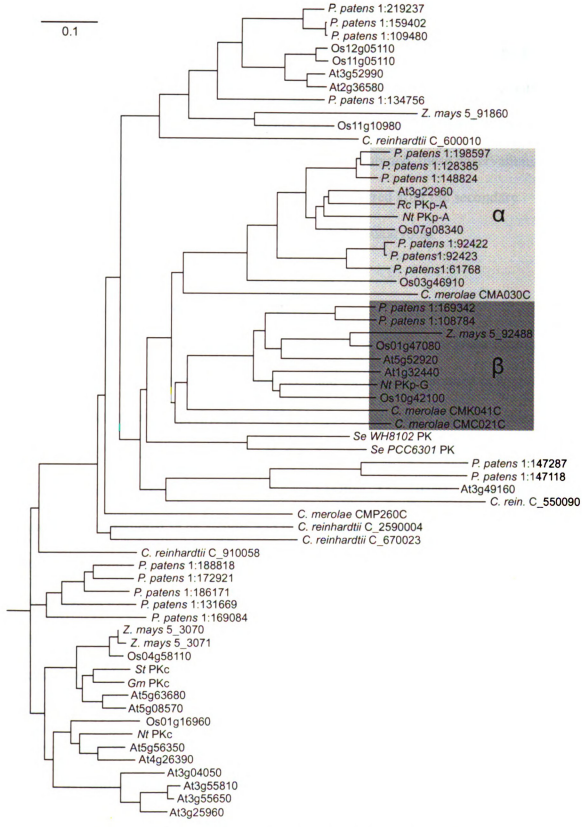


Figure 2.2. Phylogenetic analysis of plant PK protein sequences

An additional phylogenetic analysis was performed using amino acid sequences of annotated plant and algal PKs to understand the evolutionary history of the PK_p-α and PK_p-β subfamilies (Figure 2.2). Both the α- and β-subunit subfamilies are populated by proteins from algae, moss, and higher plants. Furthermore, both subfamilies originate from a branch containing cyanobacterial PKs. When combined, these observations suggest that the evolution of separate PK_p subunits occurred after the secondary endosymbiotic event which gave rise to photosynthetic eukaryotes.

Predicted PK_p subunits are plastid localized

To study the subcellular localization of the putative PK_ps, C-terminal green fluorescent protein (GFP) fusion constructs were generated and transiently expressed in *Nicotiana benthamiana* under the control of the CaMV 35S promoter. Figur2.3A shows that for all three putative PK_ps a punctate GFP signal was observed at the periphery of chloroplasts. No GFP signal was associated with structures other than chloroplasts. All three proteins in question are predicted to be soluble and stromal. The localized patterns observed in Figure 2.3A could be due to the abundance of the fusion proteins, caused by overexpression and resulting in possible overloading of the import apparatus. *In vitro* chloroplast import followed by protease protection assays were conducted to obtain independent evidence for plastid localization of the PK_p subunits. When *in vitro* produced ^{35}S-labeled PK_p subunits were incubated with isolated pea chloroplasts, the proteins were imported and processed to their presumably mature forms (Figure 2.3B). All three were resistant to treatment with thermolysin and trypsin and were found in the soluble fraction.

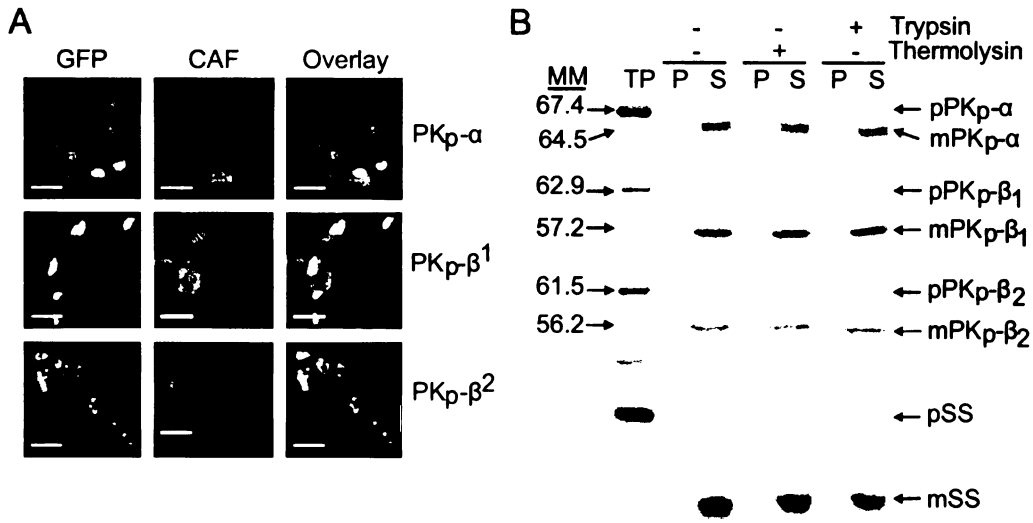


Figure 2.3. Subcellular localizations of pyruvate kinase subunits

(A) Transient expression of GFP fusion constructs in *N. benthamiana*. GFP, green fluorescent protein fluorescence; CAF, chlorophyll autofluorescence; the bar is 5 μM long.

(B) *In vitro* import of PK_p subunits into isolated pea chloroplasts. After import, chloroplasts were subjected to either no treatment (-) or to post-treatment (+) with either Thermolysin or Trypsin. Intact chloroplasts were subsequently recovered by centrifugation through 40% Percoll cushion and fractionated into a total membrane (P) and a supernatant (S) fraction. All fractions were analyzed by SDS-PAGE and fluorography. MM, molecular masses of precursor and mature proteins based on R_f analysis; TP, represents 10% of translation reaction added; p, precursor or m, mature form; pSS, precursor of the small subunit of RuBisCO included as control.

Based on the data, the three putative PK_p subunits are localized to chloroplasts and are imported and processed into mature, soluble, stromal proteins as predicted by the analysis of their amino acid sequence. The molecular masses indicated in Figure 2.3B were derived from R_f analysis and are in agreement with the transit peptide cleavage site predictions made by ChloroP. The full length precursor proteins were calculated to be 65.1 kDa, 63.5 kDa, and 62.6 kDa with predicted transit peptides of 47, 63, and 55 amino acids for PK_p-α, PK_p-β_1, and PK_p-β_2, respectively. The mobility shifts of all three mature proteins reveal no major discrepancies between the predicted and observed transit peptide cleavage sites.

Heteromeric subunit composition of recombinant PK_ps

A recombinant approach was taken to study Arabidopsis seed PK_p subunit composition due to the scarcity of embryo tissue for native protein purification. The cDNAs encoding the three putative PK_p subunits lacking the predicted transit peptides were isolated by reverse transcription and PCR and were inserted into an *E. coli* expression vector with an N-terminal 6X-His tag and a thrombin site for removal of the tag after purification. Gel electrophoresis (SDS-PAGE) and subsequent immunoblotting were used to confirm purification of single proteins and complete cleavage of the tag (Figure 2.4A). Initial tests indicated that in liquid assays none of the subunits had PK activity on their own and that only $\alpha\beta_1$ and $\alpha\beta_2$ combinations were active (Figure 2.4B). Epitope-tagged versions of the proteins were also generated that in addition to the N-terminal His-tag had short C-terminal epitope tags. PK_p-α was fused with c-myc (EQKLISEEDL), PK_p-β_1 with HA (YPYDVPDYA), and PK_p-β_2 with FLAG (DYKDDDDKG). The antibodies used for

detection of the epitopes were shown to be lacking of any cross reactivity. Native-PAGE with epitope-tagged proteins was used to explore this subunit requirement in more detail. Figure 2.5, panels A-E show five identical native-PAGE gels developed in different ways. The gel in Figure 2.5A was stained for PK activity. Only the $\alpha\beta_1$ and $\alpha\beta_2$ mixtures were active. Moreover, the activities coincided with less mobile bands as shown by the Coomassie brilliant blue (CBB) stained gel (Figure 2.5B). In the case of $\alpha\beta_1$, detection of the individual α-myc and β_1-HA fusion proteins with specific antibodies revealed a higher molecular mass complex coinciding in mobility with the active complex in Figure 2.5A, suggesting that the active complex is composed of both α and β_1 subunits (Figure 2.5C, D).

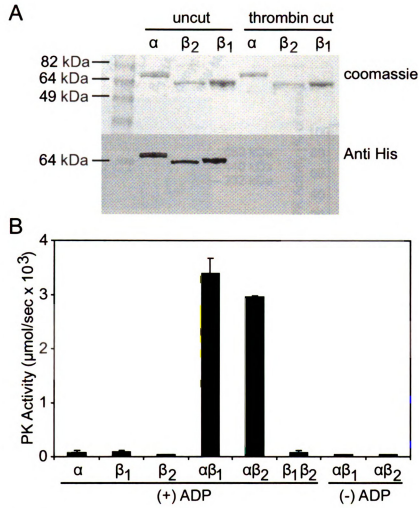


Figure 2.4. PK_p protein purification and initial activity assay

(A) SDS-PAGE and anti-His immunblot of purified PK_p subunits. Individual subunits were subjected to no treatment or treatment with thrombin to remove the His tag. 500 ng protein was loaded per well. Immunoblot shows His tag has been removed after treatment with thrombin.

(B) Pyruvate kinase activity of various subunit mixtures. Equal volumes of purified subunits were assayed alone or in combination. Approximately 0.1 ng of each subunit was used in each assay with saturating substrate concentrations. No PEP phosphatase activity was observed in control reactions without ADP.

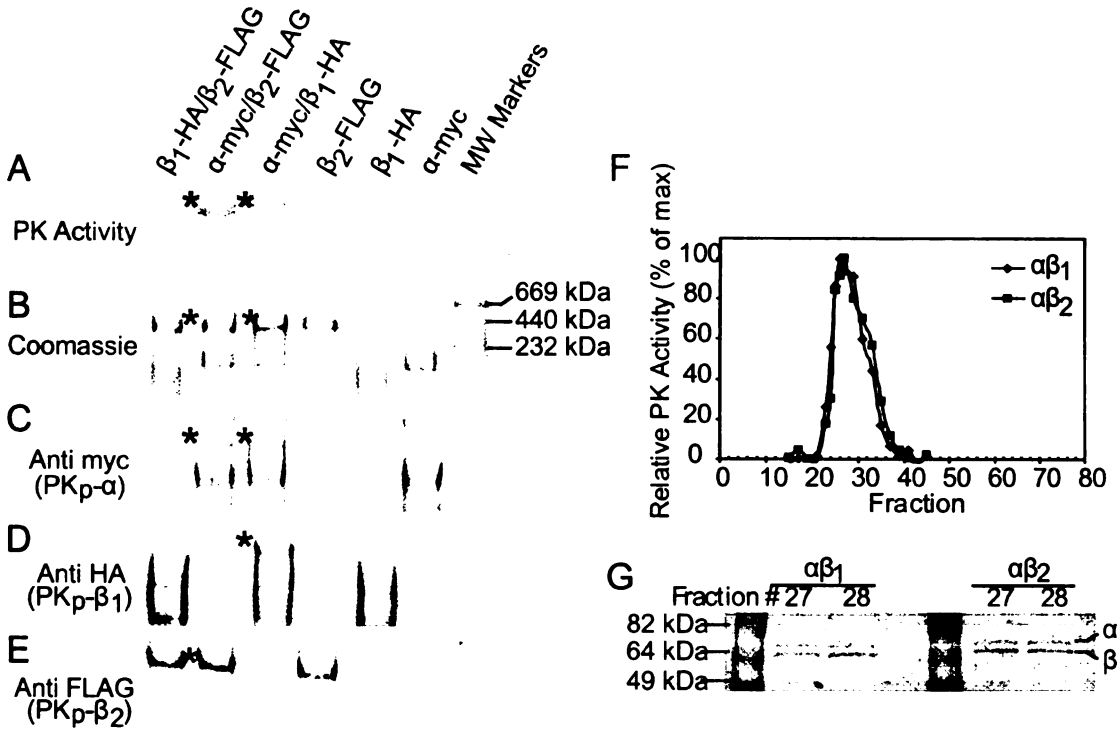


Figure 2.5. *In vitro* interaction of PK_p subunits

(A-E) Identically loaded native-PAGE gels showing *in vitro* interaction of PK_p subunits. 10 pmol (~0.6 μg) of each subunit was used per lane (A) PK activity stained gel. (B) Coomassie stained gel. (C) Immunological detection of α-myc with anti c-myc (D) Immunological detection of β_1-HA with anti HA (E) Immunological detection of β_2-FLAG with anti FLAG. * is used to denote bands corresponding to the active PK complexes.

(F) PK activity elution profile after FPLC over Superdex-200

(G) SDS-PAGE gel of most active fractions from (F). 75ng of protein was loaded per lane.

The result is less clear for the $\alpha\beta_2$ complex α and β_1 subunits (Figure 2.5C, D). The result is less clear for the $\alpha\beta_2$ complex because the β_2 subunit alone forms a higher molecular mass complex with the same mobility as the active enzyme (Figure 2.5A, B, E). However, the α-myc protein is present in the $\alpha\beta_2$ higher molecular complex (Figure 2.5C). Thus, it is likely that the higher molecular mass active complex in the $\alpha\beta_2$ mixture is also composed of both subunits.

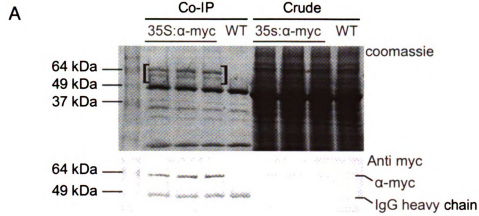
Gel-filtration chromatography was used to estimate the molecular masses of the active PK_p heteromers. Figure 2.5F shows that for both $\alpha\beta_1$ and $\alpha\beta_2$ PK activity eluted as a single peak. The molecular masses of these active complexes were calculated to be 463 $\pm$ 10 kDa for $\alpha\beta_1$ and 476 $\pm$ 10 kDa for $\alpha\beta_2$, which is consistent with octomeric complexes of 60 kDa α subunits and 57 kDa β subunits. Based on SDS-PAGE analysis, the most active FPLC fractions contained equal amounts of α and β subunits (Figure 2.5G). Thus, the active PKs appear to be heterooctomers composed of 4 α and 4 β subunits.

The *in vivo* interaction of the PK_p subunits was tested using co-immunoprecipitation (co-IP). Three constructs containing full-length, epitope-tagged cDNAs encoding the α, β_1, and β_2 subunits driven by the CaMV 35S promoter were introduced into Arabidopsis. Only the α-myc fusion protein could be directly immunoprecipitated from plant tissue. One possible explanation is that the epitope tags in the β subunits were not accessible in the native complex. The SDS-PAGE gel in Figure 2.6A show the result of co-IP experiments with silique tissue from wild type and three 35S:α-myc plants.

Figure 2.6 *In vivo* co-immunoprecipitation of PK_p subunits

(A) Coomassie Brilliant Blue stained gel and anti myc immunoblot of co-immunoprecipitated (Co-IP) proteins. Total proteins were extracted from wild-type and 35S:α-myc silique tissue and were subjected to Co-IP. About 37.5 μg of crude protein and half of the total eluate were loaded per lane. Bands unique to the 35S:α-myc co-IP lanes (in brackets) were excised and identified. IgG heavy chain is indicated on immunoblot for reference. WT, wild type.

(B) Protein sequences, predicted transit peptides, and proteomics coverage of PK_p-α ,PK_p-β_1 ,and PK_p-β_2. Gene Loci, encoded subunit, and percent coverage by proteomics are listed. ChloroP predicted chloroplast transit peptides are in bold. Peptide fragments identified by proteomics are highlighted in gray.



B

At3g22960, PK_p-α, 67% coverage

msqsiqfstpshtphlllhlphsqfnrplssisfrrfplttikytsirassssspspdldsssssssqvll spngtgavksdersvvatavttdtsgievdtveaelkengfrsrrtklictigpatcgfeqlealavggmnv arlnmchgtrdwhrgvirsvrrlneekgfavaimmdtegseihmgdlggeasakaedgevwtftvrafd ssrpertisvsydgfaedvrvgdellvdggmvrfeviekigpdvkclctdpgillpranlttwrdgslvrema mlptisskdwldidfgiaegvdfiavsfvksaevinhlksylaarsrggeigviakiesidsltnleeiilasdg amvargdlgaqipleqvpaaqqrivqvcralnkpvivasqllesmieyptptraevadvseavrqrsdal mlsgesamgqfpdkaltvlrtvslrierwwreekrhesvplqaigssfsdkiseeicnsaakmannlgvd avfvyttsghmaslvsrcrpdcpifaftttsvrrrlnlqwglipfrlsfsddmesnlnktfslllksrgmiksgdlvi avsdmlqsiqvmnvp

At5g52920, PK_p-β_1, 21% coverage

maqvvatrsiqgsmlspnggsvstrsekllkpasfavkvlgneakrsgrvsvrsrrvvdttvrsa rvetevipvspedvpnreeqlerllemqqfgdtsvgmwskptvrrktkivctvgpstntremiwklaeag mnvarmnmshgdhashkkvidlvkeynaqtkdntiaimldtkgpevrsgdlpqpimldpgqefttier gvstpscvsvnyddfvndveagdmllvdggmmsfmvksktkdsvkcevvdggelksrrhlnvrgksa tlpsitekdwedikfgvenkvdfyavsfvkdaqvvhelkkylqnsgadihvivkiesadsipnlhsiitasdg amvargdlgaelpieevpilqeeiinlcrsmgkavivatnmlesmivhptptraevsdiaiavregadav mlsgetahgkfplkaagvmhtvalrteatitsgemppnlgqafknhmsemfayhatmmsntlgtstvvf trtgfmaillshyrpsgtiyaftnekkiqqrlalyqgvcpiymeftddaeetfanalatllkqgmvkkgeeiaiv qsgtqpiwrsqsthniqvrkv

At1g32440, PK_p-β_2, 10% coverage

maaygqissgmtvdpqvlsssrnigvslsplrrtligagvrstsislrqcslsvrsikisedsrkpka yaengafdvgvldsssyrladsrtssndsrrktkivctigpssssremiwklaeagmnvarlnmshgdh ashqitidlvkeynslfvdkaiaimldtkgpevrsgdvpqpifleegqefnftikrgvslkdtvsvnyddfvnd vevgdillvdggmmslavksktsdlvkcvvidggelqsrrhlnvrgksatlpsitdkdwedikfgvdnqvdf yavsfvkdakvvhelknylktcsadisvivkiesadsiknlpsiisacdgamvargdlgaelpieevpllqe eiirrcrsihkpvivatnmlesminhptptraevsdiaiavregadaimlsgetahgkfplkavnvmhtval rteaslpvrtsasrttaykghmgqmfafhasimantlssplivftrtgsmavllshyrpsatifaftnqrrimqr lalyqgvmpiymefsddaedtyarslkllqdenmlkegqhvtlvqsgsqpiwreesthliqvrkikigg

Figure 2.6 *In vivo* co-immunoprecipitation of PK_p subunits

A small amount of α-myc protein was detected by an anti myc immunoblot in crude extracts from 35S:α-myc plants. After immunoprecipitation, the α-myc protein was enriched and became visible on CBB-stained gels. In addition, another protein running slightly faster than α-myc was visible on the CBB-stained gel. The CBB-stained and immuno-reactive bands running at 49 kDa and at a slightly less molecular mass were also present in the wild-type control. These were presumably products of the degradation of the anti-myc IgG during elution from the Protein-A sepharose. All three PK_p subunits are very close in size and could co-migrate during SDS-PAGE. Therefore, a gel slice including a section above and below the α-myc protein from the co-IP reaction (indicated by brackets in Figure 2.6A) was excised and the contained proteins were subjected to tryptic digest and mass spectrometry (LC/MS/MS). Analysis of the mass spectrometry data identified peptides of all three PK_p subunits with significant individual ion scores (Mowse scores >31, $p<0.05$): PK_p-α (encoded by At3g22960), PK_p-β_1 (encoded by At5g52920), and PK_p-β_2 (encoded by At1g32440), with 29, 7, and 3 non-redundant peptides representing 67%, 21%, and 10% coverage of the predicted mature proteins, respectively (Figure 2.6B).

Kinetic characterization of PK complexes

Enzyme activity analysis was performed using reconstituted PK_p complexes. The maximum PK activity for the $\alpha\beta_1$ and $\alpha\beta_2$ heteromers was reached within 1 minute of mixing the subunits (Figure 2.7A). Reciprocal titrations in saturating substrate conditions showed that plots of PK activity versus subunit equivalents follow hyperbolic curves when one subunit is held constant and the other titrated (Figure 2.7B).

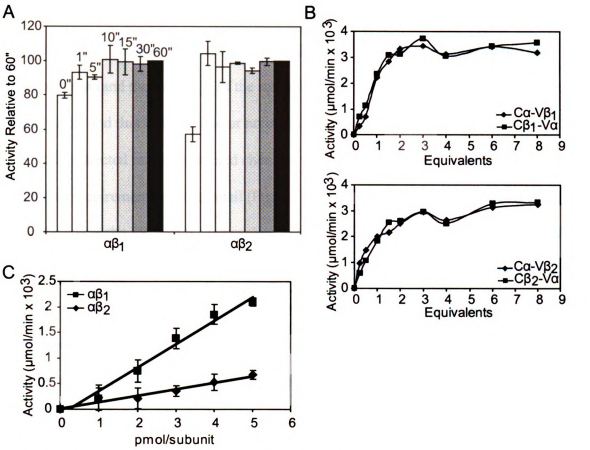


Figure 2.7 Kinetics of active PK_p complex formation

(A) Time course of PK activity after mixing subunits. Numbers above bars represent the time (minutes) incubated prior to assay. 2 pmol of each subunit was used per assay under saturating substrate conditions. Activity is expressed relative to the 60 minute sample.

(B) Subunit titration curves with PK_p subunits. For $C\alpha$-$V\beta_1$, the α subunit was held constant and the β_1 subunit was variable. For $C\beta_1$-$V\alpha$, the β_1 subunit was held constant and the α subunit was variable. The same notation applies to the β_2 titrations. 2.5 pmol of the constant subunit was used per reaction in saturating substrate conditions. Equivalents refers to the molar ratio of the variable subunit to the fixed one.

(C) Pyruvate kinase activity relative to protein concentration. Equal amounts of either α and β_1 or α and β_2 were mixed and assayed under sub-saturating substrate conditions.

Furthermore, when equal molar ratios of subunits were used, enzyme activity increased linearly with increasing protein concentration (Figure 2.7C). These results suggest that the association (and thus activity) of the subunits is dependent only on protein concentration and that there is little or no cooperativity of subunit association.

Site-directed mutagenesis and chemical inactivation were performed to explore the subunit requirement in more detail (Figure 2.8). Both treatments were directed at a lysine residue in the PK active site required for phosphoryl group transfer. In the case of the site-directed mutant proteins (Figure 2.8A,B), PK activity was only observed when both a wild-type α and a wild-type β subunit were present. No activity was seen when only one subunit had a wild-type active site. The inclusion of a mutant protein extract did not inhibit the activity of wild-type complexes, indicating the contaminating bands seen in the SDM lanes of the SDS-PAGE gel of Figure 2.8A did not interfere with PK activity. Thus, the loss of PK activity was due only to mutation of the active site of either subunit of the PK_p complex. Treatment with 2,4,6-trinitrobenzenesulfonic acid was employed to chemically inactivate the PK subunits. Inactivation was incomplete as determined by mixing TNBS-treated α and β subunits. Nonetheless, maximum PK activity was only observed with mixtures of mock-treated proteins. Activity was reduced 60-80% when either subunit was chemically inactivated. Addition of an inactivated subunit to an active mixture did not affect PK activity, meaning carryover of a component of the TNBS treatment was not responsible for reductions in PK activity. Therefore, the observed reductions in PK activity when using one TNBS-treated subunit in combination with a mock-treated one were due only to modification of lysine residues on the protein. Unmodified active sites in both subunits were required for PK catalytic activity.

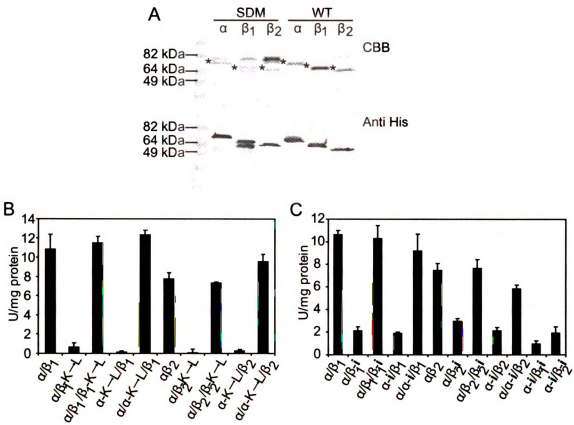


Figure 2.8. PK_p subunit inactivation

(A) SDS-PAGE and anti-His immunoblot of affinity purified site-directed mutant (SDM) and wild-type (WT) PK_p subunits. Approximately 15 pmol of each subunit was loaded per well. Conditions for protein purification were the same for all six proteins, but contaminating protein bands can be seen in the mutant protein lanes. The immunblot reveals which bands are the actual his-tagged PK_p subunits (marked with * on CBB gel).

(B) PK activity using wild-type and site-directed mutant (denoted with K→L) proteins. Assays were done at pH 8.0 with saturating substrates and 2 pmol of each subunit.

(C) PK activity using wild-type and chemically inactivated (denoted with **i**) proteins. Assays were done at pH 8.0 with saturating substrates and 2 pmol of each subunit.

Further kinetic experiments were done using equal molar ratios of $\alpha\beta_1$ or $\alpha\beta_2$ mixtures. The enzymes had a strict requirement for Mg^{2+} and K^+ and were completely inactivated after 3 min incubation at 50-55°C. The pH optima were found to be pH 7.8-8.0 (Table 2.2) and subsequent reactions were conducted at pH 8.0. The V_{max} of $\alpha\beta_1$ was approximately 2-fold higher than that of $\alpha\beta_2$ and the $S_{0.5}$(s) for ADP and PEP were 1.5 and 3-fold lower, respectively, for $\alpha\beta_1$ (Table 2.2). Both complexes displayed sigmoidal saturation kinetics for ADP and PEP, with $\alpha\beta_1$ having greater Hill coefficients (Table 2.2). Both enzymes were capable of catalyzing phosphoryl group transfer from PEP to NDPs other then ADP. Activities detected using 10 mM CDP, GDP, or UDP were 85, 57, and 67% and 13, 24, and 19% for $\alpha\beta_1$ and $\alpha\beta_2$, respectively, when compared to an ADP control. Clearly, the $\alpha\beta_2$ enzyme preferred ADP while $\alpha\beta_1$ was less discriminatory.

Table 2.2 PK_p kinetic constants

	Enzyme	
Constant	$\alpha\beta_1$	$\alpha\beta_2$
pH Optimum	8.0	7.8
V_{max} (U/mg)	13.5 ± 0.3	7.6 ± 0.4
PEP $S_{0.5}$ (μM)	75.1 ± 7.0 (2.0)	118.6 ± 11.5 (1.2)
ADP $S_{0.5}$ (μM)	113.9 ± 9.1 (1.9)	303.6 ± 27.8 (1.2)
K_{cat} (s^{-1})	9.5×10^4	5.3×10^4

Hill coefficients for PEP and ADP are given in parentheses. Values are the mean ± SD (n=4). PEP, phosphoenolpyruvate; ADP, adenosine diphosphate.

Numerous metabolites and signaling compounds (fully listed in Materials and Methods) were tested as effectors of Arabidopsis PK_p activity at subsaturating concentrations of PEP and ADP. Those compounds which had a significant effect are listed in Table 2.3. Only one activator, 6-phosphogluconate, was identified and it only activates $\alpha\beta_2$. The rest of the effectors acted as inhibitors with the most effective being glutamate and oxalate (Table 2.3). Values for the constants I_{50} and K_a were calculated (see Materials and Methods for definitions) for effectors capable of 50% relative activation or inhibition and they clearly show that $\alpha\beta_2$ is the more sensitive enzyme with respect to these compounds. Neither enzyme was sensitive to treatment with dithiothreitol (DTT) or sodium tetrathionate (NaTT) suggesting a lack of redox regulation contrary to what was previously observed for plastidic glucose 6-phosphate dehydrogenase isoforms of Arabidopsis (Wakao and Benning 2005).

Table 2.3 Metabolite effectors of PK_p activity

	Concentration Tested (mM)	Relative Activity (%)		I_{50} (mM)		K_a (mM)	
Metabolite		$\alpha\beta_1$	$\alpha\beta_2$	$\alpha\beta_1$	$\alpha\beta_2$	$\alpha\beta_1$	$\alpha\beta_2$
6PG	0.05	96	192	-	-	-	0.02
Glutamate	5	59	30	6.2	2.1	-	-
Oxalate	0.2	71	50	0.41	0.21	-	-
Iso-Citrate	10	70	75	N.D.	N.D.	-	-
AMP	1	77	96	N.D.	N.D.	-	-
ATP	1	77	87	N.D.	N.D.	-	-
Glyoxylate	5	83	79	N.D.	N.D.	-	-
OAA	2	82	88	N.D.	N.D.	-	-

Values represent the mean of at least four repeats. Activity is percent relative to a no effector control (set at 100). 6-PG, 6-phoshpogluconate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; OAA, oxaloacetate; N.D., not determined

Discussion

Arabidopsis has two heterooctomeric PK_ps

Previous kinetic analyses of plant PKs have been limited to enzymes which can be purified in the native state from dissected tissue samples (*e.g.* Hu and Plaxton 1996, Plaxton et al. 2002, Smith et al. 2000, Turner et al. 2005). The results of such studies have revealed that PK isoforms vary depending on the tissue and subcellular compartment in question. Arabidopsis tissues, and especially developing seed, represent a challenge to the traditional enzyme purification approach due to the difficulty in amassing the quantities of tissue required. However, this work is facilitated by the available resources and model characteristics of Arabidopsis and the findings are crucial if we want to achieve a complete understanding of the biology of this plant. A bioinformatics approach was taken to identify the candidate seed expressed PK_p encoding genes in the Arabidopsis genome sequence. Four potential plastid targeted, seed-resident PKs were identified based on phylogenetics and gene expression data (Figure 2.1). The prediction of plastid localization of these proteins was subsequently confirmed using GFP fusion protein localization and pea chloroplast *in vivo* import assays (Figure 2.3). Indeed, PK_p-α and PK_p-β_1 have been found in the stromal fraction of chloroplasts using a proteomics approach (Friso et al. 2004). PK_p-β_1 was also previously identified in a mitochondrial proteomics study, but plastid contamination could not be ruled out (Giege et al. 2003). Previous research on PK_p-α orthologs from *B. napus* and *R. communis* detailed the proteins' transit peptide cleavage sites (Plaxton et al. 2002, Wan et al. 1995). The N-terminal sequence of the mature *B. napus* protein aligns with a region about 40 amino acids c-terminal to the predicted Arabidopsis cleavage site, while the *R. communis*

protein has a transit peptide of 83 amino acids. These data suggest that the transit peptide of PK_p-α may be longer than the predicted 55 amino acids. However, analysis of the observed molecular masses of the precursor and mature proteins after pea chloroplast import support the predictions made by TargetP and ChloroP (Fig 3). Moreover, the Arabidopsis PK_p-α protein contains a domain (79% amino acid identity to *R. communis* PK_p-A) which is responsible for altered import characteristics of *R. communis* PK_p-A (Wan et al. 1995). Our results indicate that this sequence does not affect the ability of Arabidopsis PK_p-α to be imported into and processed in pea chloroplasts.

It was determined that the Arabidopsis PK_p enzymes are most likely ~460 kDa heterooctomers of 60 and 57 kDa subunits with 4α4β stoichiometry (Figure 2.5). The reconstitution of active PK_ps from individually purified inactive subunits in this study as well as the *in vivo* Co-IP results leave little ambiguity as to the heteromeric structure of the Arabidopsis PK_ps. Some studies of recombinant PK_P polypeptides from developing *R. communis* endosperm concluded that the PK_p-α and PK_p-β homologs are distinct enzymes (Blakeley and Dennis 1993, Blakeley et al. 1995, Wan et al. 1995), while others have determined that the same proteins are actually subunits of a single heteromeric PK_p (Negm et al. 1995, Plaxton 1991, Plaxton et al. 1990). The data presented here support the conclusion that PK_p is a complex composed of two different subunits.

Arabidopsis PK_p activity is determined by two β subunits

Kinetic analysis revealed that the reconstituted Arabidopsis enzymes behave much like previously documented PK_ps (pH optima of approximately 8.0, Mg^{2+} and K^+ requirement, and $S_{0.5}$ values for PEP and ADP in the 100-300 μM range). A pH optimum of 8.0 is

potentially important for these enzymes. Such a pH is generated in the plastid stroma in response to light. As the plastids of Arabidopsis seeds are green and presumably photosynthetic, it is possible that *in vivo* PK_p is light-activated via alkalinization of the stroma. Such regulation of PK_p could contribute to the light-induced stimulation of fatty acid synthesis in green seeds (Goffman et al. 2005). What else was discovered were distinct differences between the two isoforms in Arabidopsis. The $\alpha\beta_1$ form is a more efficient enzyme with a higher specific activity and lower $S_{0.5}$ values for both PEP (75 μM vs 120 μM) and ADP (Table 2.2). The concentration of PEP in potato tuber tissue has been estimated to be around 50-100 μM (Farre et al. 2001) and assuming the same is true for metabolically active Arabidopsis tissues, $\alpha\beta_1$ would be expected to be more active *in vivo*. In addition, $\alpha\beta_1$ is 3-5 times more efficient at utilizing alternative nucleoside diphosphates. The $\alpha\beta_2$ enzyme, on the other hand, is more responsive to the strong metabolite effectors glutamate, oxalate, and 6-PG (Table 2.3). In fact, $\alpha\beta_1$ is completely insensitive to the activating effect of 6-PG. This is interesting since what has been described as the major PK_p from *B. napus* is activated by 6-PG (Plaxton et al. 2002). Apparently, Arabidopsis and *B. napus* have diverged in this regard as the gene for the β_2 subunit present in the the 6-PG-regulated PK_p ($\alpha\beta_2$ enzyme) from Arabidopsis is hardly expressed in any tissue (Figure 2.1B). A distinct feature of the Arabidopsis PK_ps is their inhibition by glutamate (Table 2.2). Regulation by this effector has been reported for PK_cs from other plants but not PK_ps (Hu and Plaxton 1996, Smith et al. 2000). It should be noted that while the presence of either β_1 or β_2 in the Arabidopsis PK_p complex results in different regulatory properties, these subunits should not be considered purely regulatory. They both contain fully conserved PK active sites in which chemical or

genetic modification results in an inactive PK complex (Figure 2.8). These experiments, in combination with the fact that the α subunit has no activity when assayed alone (Figure 2.4B), support a model in which the both α and β_1 or β_2 subunits are required for enzyme activity and the specific interactions between the subunits result in differential kinetic properties.

The primary structures of the recombinant PK_ps may not be the same as the native enzymes and thus the kinetic data must be considered with caution. However, as the transit peptide cleavage site predictions agree well with the observed molecular masses of precursor and mature PK subunits (Figure 2.3B), the differences are likely to be minimal between the native and recombinant proteins. It should also be noted that specific post-translational modifications could result in changes to the enzyme structure and or function, and these possibilities were not explored. Soybean PK_c, for instance, can be partially degraded at the c-terminus *in vivo*, which results in altered regulatory properties (Tang et al. 2003).

Perhaps most significant is the realization that PK_p from Arabidopsis exists as differentially regulated heteromers resulting from the interaction of the α subunit with one or the other β subunit. The two PK_p-β subunits of Arabidopsis likely arose through gene duplication as indicated by the similarity of their amino acid sequence and gene structure and subsequently evolved unique regulatory features. The $\alpha\beta_1$ enzyme is likely to be dominant in most tissues based on gene expression (Figure 2.1), but it is possible that under conditions when glycolysis needs to be more regulated the $\alpha\beta_2$ enzyme is produced at higher rates. The regulatory properties of the $\alpha\beta_1$ enzyme, notably the lower $S_{0.5}$ for ADP and PEP, higher V_{max}, lower sensitivity to metabolic regulation, and ability

to utilize other NDPs more efficiently, make it a better enzyme for processing carbon at high rates, independent of the metabolic status of the tissue. The $\alpha\beta_2$ enzyme, however, is less active and more susceptible to metabolite-based regulation and may serve more specialized roles. It will be interesting to study the *in vivo* relationship of these subunits and whether or not one enzyme complex can contain a mixture of β subunits that would further fine tune PK activity for specific metabolic demands.

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Chapter 3

Analysis of carbon metabolism and storage compound accumulation in seeds of an Arabidopsis mutant deficient in a plastidic pyruvate kinase[2]

[2] This work has been published in Andre, C., Froehlich, J.E., Moll, M.R., and Benning, C. (2007) A heteromeric plastidic pyruvate kinase complex involved in seed oil biosynthesis in Arabidopsis. Plant Cell **19**:1-17. I performed all of the experiments shown here.

Abstract

Glycolysis is a ubiquitous pathway thought to be essential for the production of oil in developing seeds of Arabidopsis and oil crops. Compartmentation of primary metabolism in developing embryos poses a significant challenge towards testing this hypothesis and for the engineering of seed biomass production. It also raises the question whether there is a preferred route of carbon from imported photosynthate to seed oil in the embryo. Disruption of the gene encoding the β_1 subunit of plastidic pyruvate kinase (PK_p) causes a 75% reduction in enzyme activity and a 60% reduction in seed oil content. The seed oil phenotype is fully restored by expression of the β_1 subunit-encoding cDNA, and partially by the β_2 subunit-encoding cDNA. Additionally, carbohydrates accumulate in the mutant seeds, possibly due to reduced activity of upstream glycolytic enzymes. Therefore, the identified pyruvate kinase catalyzes a crucial step in the conversion of photosynthate into oil suggesting a preferred plastid route from its substrate phosphoenolpyruvate to fatty acids.

Introduction

An important metabolic function of a developing Arabidopsis (*Arabidopsis thaliana*) seed is the deposition of storage reserves: oil in the form of triacylglycerols (TAG), but also proteins, oligo- and polysaccharides (Baud et al. 2002). As sucrose is the major photosynthetic product transported in the phloem the embryo is required to catabolize incoming sucrose and convert it into the more efficient storage compounds mentioned above. Glycolysis is central to this process as it converts sugars into precursors for protein and fatty acid synthesis while concomitantly producing ATP by substrate level phosphorylation. In fact, stable isotope labeling has been used to demonstrate that 90% of glucose fed to developing canola (*Brassica napus*) (a close relative of Arabidopsis and oilseed crop) embryos is converted to pyruvate by the RuBisCO bypass and the lower half of glycolysis (Schwender and Ohlrogge 2002, Schwender et al. 2004a). Furthermore, a clear link between glycolysis and seed metabolism is apparent in the *wrinkled1* (*wri1*) mutant of Arabidopsis, in which a general reduction in glycolytic activity results in an 80% reduction in seed oil (Focks and Benning 1998).

Glycolysis in plants occurs in both the cytosol and the plastid and both compartments are connected through plastid membrane transporters (Plaxton 1996, Weber 2004). The intermediates of upper glycolysis of developing *B. napus* embryos appear to be in near equilibrium between the cytosol and plastid (Schwender et al. 2003), and enzymes of the full glycolytic sequence have been detected in both compartments in embryos (Eastmond and Rawsthorne 2000). It is therefore likely that changes in glycolytic enzyme activities influence the transport of related intermediates across the plastid envelope. This compartmentation raises the question of a preferred route of

glucose metabolism in developing oil seed embryos. Expressed sequence tag (EST) analysis of developing Arabidopsis seeds indicated that mRNAs encoding cytosolic enzymes for the entire glycolytic pathway are abundant, but that only mRNAs encoding plastidic enzymes for the second half of the pathway metabolizing trioses are abundant (White et al. 2000). Microarray experiments extended these findings by detecting a shift in expression from genes encoding cytosolic glycolytic enzymes to those encoding plastidic glycolytic enzymes at the onset of storage compound accumulation. While gene expression does not necessary reflect enzyme activity or metabolite flux, the microarray data led to the hypothesis that glucose is broken down in the cytosol to phosphoenolpyruvate (PEP), which is then imported into the plastid and metabolized by plastidic pyruvate kinase (PK_p) (Figure 1.2, Ruuska et al. 2002). Revisions to this scheme were introduced based on more recent experiments (*i.e.* RuBisCO bypass, see Schwender et al. 2004a) but in the model the lower half of glycolysis metabolizing PEP remains unaltered. While the import of PEP by plastids of *B. napus* embryos has been demonstrated (Kubis et al. 2004), the *chlorophyll a binding protein underexpressed* (*cue1*) mutant of Arabidopsis lacking a seed-expressed PEP transporter has no reported seed reserve phenotype (Li et al. 1995). A recent steady-state carbon flux analysis on cultured embryos of *B. napus* led to the proposal of a RuBisCO shunt involving reactions of the reductive pentose phosphate pathway in the plastid (Schwender et al. 2004a). The proposed pathway bypasses the initial glycolytic reactions in the cytosol and generates PEP in the plastid, which could compensate for the PEP import deficiency in the *cue1* mutant. Alternatively, pyruvate generated by cytosolic pyruvate kinase (PK_c) could be imported and used directly for fatty acid biosynthesis. Isolated plastids from *B. napus*

embryos are capable of incorporating ^{14}C labeled pyruvate into fatty acids (Eastmond and Rawsthorne 2000, Kang and Rawsthorne 1994), but no plastidic pyruvate transporter has been reported. A current model of primary metabolism in developing Arabidopsis seeds is shown in Figure 1.2. Direct molecular or genetic corroboration of this scheme is generally lacking and the focusing on PK_p in developing Arabidopsis seeds should be highly informative in the testing of this hypothesis.

Materials and Methods

Plant growth and transformation

All Arabidopsis plants were of the Col-2 ecotype, except for the SALK T-DNA lines which were Col-0. All seeds were first sterilized in 20% bleach, 0.05% TritonX-100 for 20 min and were then rinsed 5 times with water and plated on half-strength MS medium, pH 5.9, 0.9% agar, and 2% sucrose. When appropriate, kanamycin or hygromycin B were included in the medium at 50 $\mu g\ mL^{-1}$ and 25 $\mu g\ mL^{-1}$, respectively. Seeds were stratified at 4°C for 3 days prior to being germinated in an incubator (AR-75; Percival Scientific, Boone, IA, USA) at a photon flux density of 60–80 $\mu mol\ m^{-2}\ sec^{-1}$ and a light period of 16 h (22°C), and a dark period of 8 h (18°C). Seedlings were transferred to 3.5 inch square pots and were grown in a soil mix as described previously (Xu et al. 2002) and were grown under a 16-h photoperiod with a day temperature of 22°C and a night temperature of 20°C at a photon flux density of 100–120 $\mu mol\ m^{-2}\ sec^{-1}$. The plants were fertilized with half-strength Miracle-Gro (Scotts, Marysville, OH, USA) plant food every fifteen days. Wild-type and mutant Arabidopsis plants were prepared for transformation as previously described (Cernac and Benning 2004). When ready, plants were transformed using the floral dip method (Clough and Bent 1998). Competent cells of *Agrobacterium tumefaciens* strain C58C1 GV3101 pMP90 (Koncz and Schell 1986) were prepared and transformed as previously described (Shen and Forde 1989).

T-DNA mutant isolation and characterization

T-DNA insertion lines were obtained from the SALK T-DNA insertion population (Alonso et al. 2003). Mutants were selected on growth medium containing kanamycin

and T-DNA insertions were confirmed using PCR primers specific for gene sequences and the T-DNA left border. Primers were designed using the i-sect tool (http://signal.salk.edu/tdnaprimers.html). Insertion sites were confirmed by sequencing the PCR product of the left border primer and a gene specific primer. Expression of the target gene was analyzed with RT-PCR using primers listed in Table 2.1. Total RNA was isolated from seedlings using the Qiagen RNeasy kit. 600 ng of total RNA was used for reverse transcription using the Qiagen Omniscript RT kit. PCR was done using 5% of the RT product. *Actin1* (At2g37620) was used for control purposes. The PCR consisted of 25 cycles of 95°C for 30 sec, 60°C for 45 sec, and 72°C for 1 min, followed by a 10 min 72°C extension. Complementation of the mutant was done using full length cDNAs inserted into the *Kpn*I site of the CaMV 35s containing pCAMBIA1300 derivative mentioned above. An antisense construct was generated using the same full length cDNA for PK_p-β_1 inserted in the antisense orientation into the *Kpn*I site of the binary vector pBinAR-Hyg (Dörmann and Benning, 1998) containing the 12S seed storage protein promoter from Arabidopsis (Ohlrogge J, Benning C, Gao H, Girke T, and White J, Inventors; Plant seed specific promoters. US patent 7,081,565. 2006 July 25). Analysis of gene expression in the rescued lines was done using RNA gel blots. Total silique RNA was extracted using a previously described protocol (Verwoerd et al. 1989) followed by DNase treatment and cleanup using the Qiagen RNeasy kit. Northern analysis (5 μg total RNA) was performed as previously described (Dörmann and Benning 1998). The blots were analyzed using a phosphor imager (Molecular Dynamics, Amersham, Piscataway, NJ, USA).

Analysis of chlorophyll content

Chlorophyll was quantified in seeds as previously described (Lichtenthaler 1987). Seeds were imaged using a Leica MZ 12.5 dissecting microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Spot Insight color camera (Diagnostic Instruments, Sterling Heights, MI, USA).

Transmission electron microscopy

For electron microscopy, seeds were dissected out of staged silques and soaked in water for 1 hour. Embryos were then expelled from their seed coats by pressing the soaked seeds between two glass microscope slides and were then embedded in 2% agarose. The embryos were fixed for 2 hours at room temperature with 2.5% glutaraldehyde, 2.5% paraformaldehyde in 0.1 M cacodylate buffer and then post-fixed in 1% (w/v) osmium tetrachloride in 0.1 M cacodylate buffer. The samples were then dehydrated in a graded series of acetone, embedded in Poly BD 812 resin, and sectioned. The thin sections (~70 to 100 nm) were stained with uranyl acetate and lead citrate prior to examination in a JEOL 100CX electron microscope (JEOL, Japan).

Developing seed enzyme assays

Enzyme activities were measured from developing seed proteins extracted in a buffer of 50 mM Tris-Cl pH 7.5, 5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1mM DTT, 0.1% Triton-X100, 10% glycerol, 2 mM benzamidine, 2 mM ε-amino-n-caproic acid, and 1 mM PMSF. Pyruvate kinase activity was detected by coupling the production of pyruvate to the conversion of NADH to NAD^+ by lactate dehydrogenase. Reactions were kept at

25°C, were started by the addition of enzyme mix, and were linear for at least 5 minutes. Absorbance at 340 nm was monitored using a FLUOstar Optima 96-well plate reader (BMG Labtech, Offenburg, Germany). Standard PK_p reaction mixtures contained 50 mM HEPES-KOH pH 8.0, 5% PEG-8000, 50 mM KCl, 15 mM $MgCl_2$, 1 mM DTT, 2 mM PEP, 1 mM ADP, 0.2 mM NADH, and 2 U ml^{-1} desalted rabbit muscle lactate dehydrogenase. PEP phosphatase activity was corrected for by omitting ADP from the reaction. Reactions at pH 7.0 were done using 50 mM MOPS pH 7.0 instead of HEPES. ATP-dependent phosphofructokinase (PFK) and pyrophosphate-dependent phosphofructokinase (PFP) activities were measured as previously described (Burrell et al. 1994).

Seed metabolite analysis

Seed oil quantification by fatty acid methyl ester analysis was done as previously described (Focks and Benning 1998). Seed storage proteins were extracted from 50 mg of dry seeds by first grinding in a mortar and pestle followed by 2 extractions of the tissue with 30 volumes of hexane. The delipidated seed material was pelleted by centrifugation at 13,000 g for 10 min. The pellet was then dried in a speed-vac and extracted twice for 15 min with 0.5 volumes of 50 mM Tris-HCl pH 8, 200 mM NaCl, 5 mM EDTA, 0.1% Tween-20, 2 mM benzamidine, 2 mM ε-amino-n-caproic acid, 1 mM PMSF. Water bath sonication was used to resuspend the pellets. The supernatants from the extractions were combined and a 1:5 dilution was used to quantify protein using the Bio-Rad DC Protein Assay Kit. For SDS-PAGE, the equivalent 3 seeds worth of total protein was loaded per lane. Mature seed free amino acids were extracted from 25 mg of seed tissue 3 times with

400 μL of 70% methanol. The supernatants were combined and extracted 2 times with an equal volume of chloroform to remove the lipids. The remaining aqueous phase was dried under vacuum and then resuspended in 20 mM HCL. Extracts (20 μL) were then loaded and run on over a strong anion exchange column using a Hitachi Amino Acid Analyzer (Hitachi High Technologies America, Inc., San Jose, CA, USA) at the MSU Macromolecular Structure, Sequencing, and Synthesis Facility. Glucose, fructose, sucrose, and starch were extracted from developing seeds and quantified as previously described (Focks and Benning 1998). PEP and pyruvate were extracted from developing seeds with perchloric acid and quantified using a NADH fluorescence assay as previously described (Hausler et al. 2000). ADP and ATP were measured in the same extracts with an ATP Bioluminescence Assay Kit (Sigma) using a previously described protocol (Ruuska et al, 2000).

Results

Disrupting the PK_p-β_1 encoding gene causes a reduction of PK_p activity and seed oil content

Multiple independent SALK T-DNA Insertion lines were obtained to study the in vivo function of the Arabidopsis PK_ps (Alonso et al. 2003). Six lines were reanalyzed for exact insertion site location (PK_p-α; SALK_096141, SALK_024870, PK_p-β_1; SALK_042938, SALK_042681, PK_p-β_2; SALK_013574, SALK_142845), but only one (SALK_042938 in PK_p-β_1) carried an insertion in a translated portion of the gene (Figure 3.1A). The line SALK_096141 has an insertion in an intron of the PK_p-α encoding gene, but RNA levels were unaffected in these plants. Genotyping by PCR revealed homozygous SALK_042938 mutants completely lacking transcripts from the PK_p-β_1 encoding gene as detected by RT-PCR (Figure 3.1B, C). Transcript amounts for the PK_p-α and PK_p-β_2 encoding genes were unchanged (Figure 3.2). The mutant will be referred to as *pkp1*. Notably, of the six analyzed mutants, only *pkp1* had any visible seed phenotype. The mature seeds of *pkp1* were wrinkled when observed under a dissecting microscope and developing mutant embryos contained less chlorophyll (Figure 3.3A, B). Rescue of *pkp1* with ectopic expression of the PK_p-β_1 encoding gene restored chlorophyll content to nearly wild-type levels, however, overexpressing the PK_p-β_2 encoding gene was much less effective. Transmission electron microscopy was done to assess any ultrastructural perturbations in the *pkp1* mutant. The 5k-magnification (Figure 3.3C, upper panels) micrographs show representative cotyledonary cells from 13 DAF embryos. Wild-type cells are full of oil bodies (ob) by this time and still contain some starch (st) in the plastids. The *pkp1* mutant has much smaller oil bodies than wild type, but has much

larger starch granules. The 67k-magnification images (Figure 3.3C, lower panels) show in more detail the thylakoid structure of the mutant. While organized similarly, the *pkp1* thylakoids are less extensive than wild type.

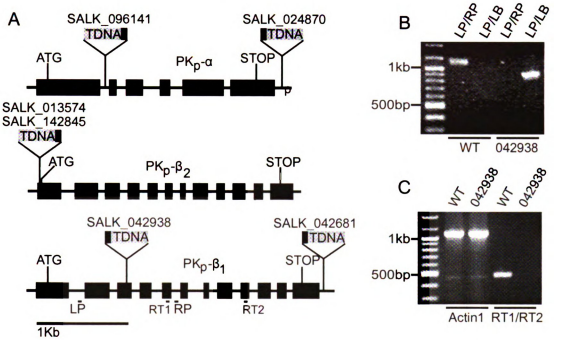

Figure 3.1. Identification of a SALK T-DNA mutant in PK_p-β_1

(A) Gene structure of the three PK subunits and locations of T-DNA insertions. Black box on T-DNA is the left border. LP and RP depict locations of primers used for genotyping in (B). RT1 and RT2 depict locations of primers used for RT-PCR in (C). ATG, start codon; STOP, stop codon.

(B) PCR based genotyping of SALK_042938. LP and RP refer to At5g52920 specific primers shown in (A) and in Table 2.1. LB refers to the T-DNA left border primer in Table 4. WT, wild type.

(C) RT-PCR to measure PK_p-β_1 encoding gene expression in SALK_042938. Actin1 (At2g37620) is the control. RT1 and RT2 refer to At5g52920 specific primers shown in (A) and in Table 2.1. WT, wild type

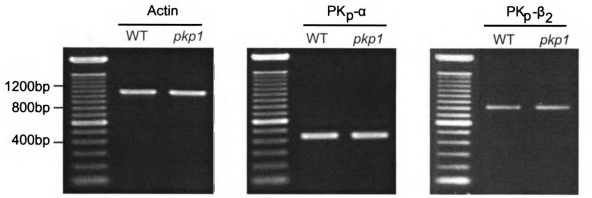

Figure 3.2. Reverse transcriptase-PCR analysis of PK_p gene expression

PK_p-α and PK_p-β_2 gene expression in 11 days after flowering seeds of the *pkp1* and WT background. Actin1 (At2g37620) is the control. WT, wild type.

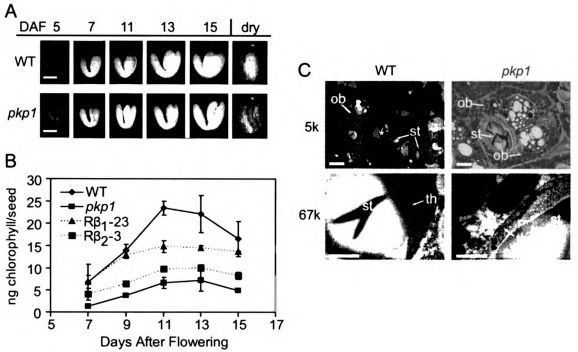

Figure 3.3. *pkp1* seed phenotypes

(A) Seed phenotypes of *pkp1* and wild type (WT). Embryos were dissected out of developing seeds at the time (DAF, days after flowering) indicated. Fully dessicated mature seeds are shown as well. The bar represents 0.2 mm.

(B) Total chlorophyll content in developing seeds of *pkp1*, wild type (WT), and lines rescued with CaMV 35S-driven expression of either PK_P-β_1 ($R\beta_1$-23) or PK_P-β_2 ($R\beta_2$-3). Forty seeds were measured per sample. Values are the mean ± SD (n=6).

(C) Electron micrographs of cells from 13 DAF wild-type (WT) and *pkp1* cotyledons. Starch granules (st) and oil bodies (ob) are marked with arrows in the upper panels. Higher magnification in the lower panels reveals thylakoid membranes (th) inside plastids. 5k and 67k denote magnification used. Asterisks (*) protein bodies. Bars in upper panels represent 2 μm. Bars in lower panels represent 0.5 μm. Left panels, wild type; right panels, *pkp1* mutant.

The smaller oil bodies, reduced thylakoid membranes, and wrinkled seeds of *pkp1* suggest a reduction in lipid biosynthesis and possibly storage compound accumulation. Therefore, oil and protein were quantified in the mature *pkp1* seeds (Table 3.1). The mutant accumulated only 40% as much oil as wild type, yet there was only a 15% reduction in protein. Test crosses and analysis of the F1 progeny indicated that the low oil phenotype of *pkp1* is a recessive trait largely dependent on the genotype of the embryo. A homozygous *pkp1* sporophyte did however result in a 15% reduction in seed oil when pollinated with wild-type pollen (Table 3.1) indicating a small maternal effect.

Table 3.1 WT and *pkp1* seed storage compound accumulation

	WT	*pkp1*	WT♂ x *pkp1*♀	*pkp1*♂ x WT♀
Total FAME (µg/seed)	6.77 ± 0.7	2.71 ± 0.2	5.68 ± 0.8	6.9 ± 0.2
Protein (µg/seed)	5.23 ± 0.6	4.39 ± 0.5	N.D.	N.D.
Seed mass (µg/seed)	19.2 ± 0.9	14.4 ± 1.1	N.D.	N.D.

Values are the mean of three repeats ± standard deviation. Seed mass determined by measuring the weight of 500 seeds three times. N.D., not determined

Enzyme activities in developing seeds were measured to determine the extent of the PK_p defect in *pkp1*. The presence of a potentially large number of different cytosolic and plastidic PK isoforms complicates the interpretation of activity assays using crude extracts. However, two factors aide in validating seed PK_p activity in crude extracts: 1. all 14 PK encoding genes are not highly expressed in any given tissue at the same time (Schmid et al. 2005), 2. cytosolic PKs typically have pH optima of approximately pH 7.0, whereas plastidic PKs have pH optima of approximately pH 8.0 (Hu and Plaxton 1996,

Plaxton et al. 2002, Smith et al. 2000, this work). Figure 3.4A shows the time course of PK specific activity using protein extracts of seed dissected from staged siliques. At pH 8.0 wild-type PK specific activity is greatest at 7 days after flowering (DAF) and steadily declines throughout seed development. This pattern agrees with the expression profiles of PK_p-α and PK_p-β_1 encoding genes as shown in Figure 2.1, but is shifted to later DAF possibly due to differences in growth conditions. The *pkp1* mutant at pH 8.0, however, has 3-fold reduced PK specific activity at 7 DAF and does not change within experimental limitations throughout the rest of the time course. Pyruvate kinase specific activity was not reduced in the mutant at pH 7.0 but was actually increased at 11 and 15 DAF. Mutant and wild-type protein extracts were also assayed in the presence of 5 mM glutamate or 0.2 mM 6-phosphogluconate (Figure 3.4B). Recall that glutamate at 5 mM inhibited recombinant $\alpha\beta_1$ by about 40%, while $\alpha\beta_2$ was inhibited by 70% (Table 2.2). Native PK specific activity in 9-11 DAF wild-type seed extract was inhibited only about 25% by 5 mM glutamate while that from *pkp1* was unaffected. As shown in Chapter 2, 6-phosphogluconate is a potent activator of $\alpha\beta_2$ (Table 2.2), yet had no effect on wild-type or *pkp1* seed PK specific activity.

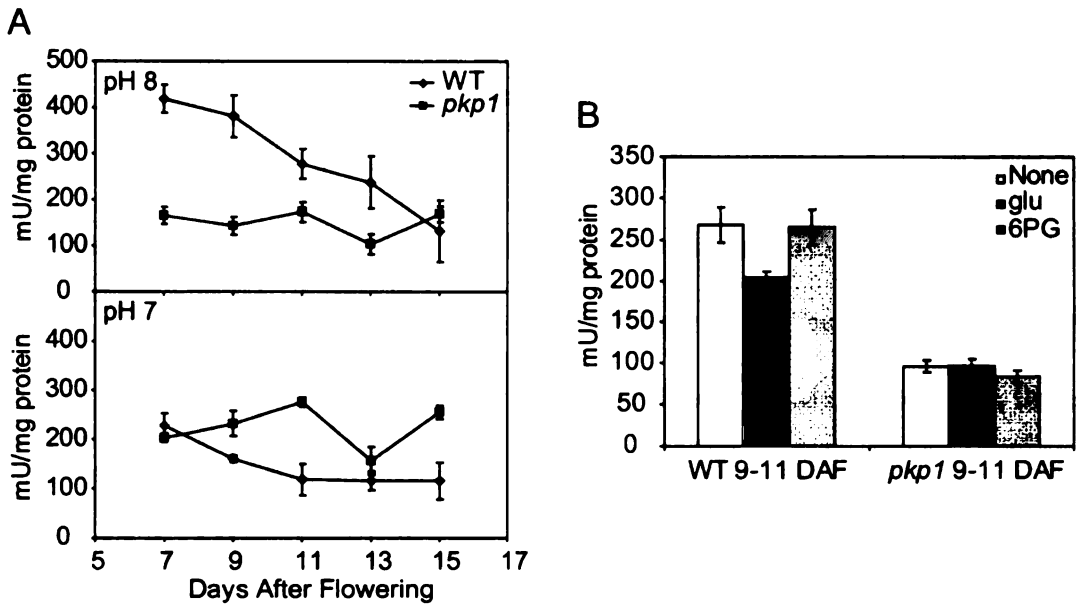

Figure 3.4. PK specific activity in *pkp1* and wild-type seeds

(A) Total PK specific activity measured at pH 8 and pH 7 in saturating substrate conditions. WT, wild type. One mU is defined a 1 nmol pyruvate formed per minute. Values are the mean ± SD (n=4).

(B) Native seed PK specific activity at 9-11 DAF in response to metabolite effectors. Activity was measured at pH 8.0 with subsaturating substrate concentrations. WT, wild type. None, no effectors; glu, 5 mM glutamate; 6-PG, 0.2 mM 6-phosphogluconate. One mU is defined a 1 nmol pyruvate formed per minute. Values are the mean ± SD (n=4).

Seed fatty acid and protein profiles in *pkp1*

Fatty acid methylester (FAME) analysis of developing seeds revealed that *pkp1* accumulates oil in the same temporal pattern as wild type, but at a much lower rate (Figure 3.5A). The fatty acid composition of the oil in mature seeds was also analyzed. The *pkp1* mutant had a decrease in stearic (18:0), oleic (18:1), and linoleic (18:2) acids along with an increase in linolenic (18:3). The proportion of very long chain (20 and 22 carbons) to long chain (16 and 18 carbons) fatty acids was also increased in *pkp1* (Figure 3.5B). Total protein extracts were made from mature wild-type and *pkp1* seeds and were run on an SDS-PAGE gel to analyze the storage protein profile. Apparently, there are no differences in the major storage protein profiles between wild type and *pkp1* (Figure 3.5C) and the 15% reduction in protein content seen in *pkp1* (Table 3.1) is not specific to any one protein. Free amino acid content of mature seeds was also compared between wild type and the mutant. Analysis of amino acid extracts by HPLC revealed increases in the proportions of glycine, arginine, and glutamine in *pkp1* (Figure 3.5D). On the other hand, aspartate, aspargine, glutamate, and valine were in greater proportion in the wild-type seeds. Statistically, however, the differences were very small between wild type and *pkp1* for any of the listed amino acids.

Restoration of seed oil content in *pkp1* expressing β-subunit-encoding cDNAs

Dealing with a single T-DNA insertion line, which could potentially harbor secondary mutations that cannot be detected by genotyping, required additional precautions to unambiguously link the target genotype with the observed phenotype, in this case the reduction in oil content.

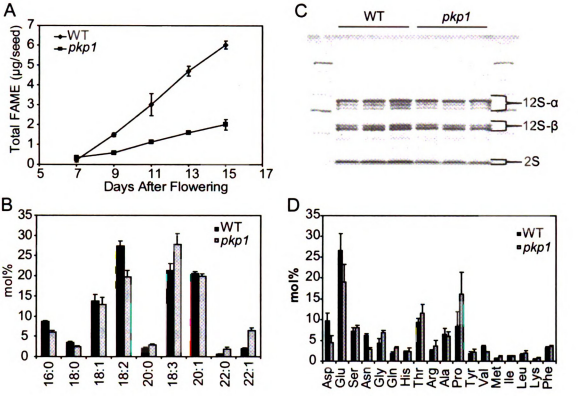

Figure 3.5. Oil and protein phenotype of *pkp1* seeds

(A) Fatty acid accumulation in developing seeds. DAF, days after flowering; FAME, fatty acid methyl ester; WT, wild type. Values are the mean ± SD (n=6).

(B) Fatty acid profile of desiccated mature seeds. Values obtained from FAME analysis of dry seeds. WT, wild type. Values are the mean ± SD (n=6).

(C) CBB stained SDS-PAGE gel of total protein extracts from wild-type (WT) and *pkp1* seeds. Major storage protein bands are denoted in the margin.

(D) Free amino acid profiles of wild-type (WT) and *pkp1* mature seeds. Values are the mean ± SD (n=3).

To address this issue we constructed transgenic lines in the *pkp1* background expressing cDNAs that encode either PK_p-β_1 or PK_p-β_1 subunits and included these transgenic lines in the analysis. Expression of these cDNAs was expected to restore the oil content if this phenotype was due to the disruption in the PK_p-β_1 encoding gene in the *pkp1* line thereby confirming the link between genotype and phenotype.

A T-DNA construct containing a CaMV 35S driven cDNA encoding PK_p-β_1 was used to rescue the lipid phenotype of *pkp1*. Antibiotic resistance was used to select transformants and rescued lines were identified by scoring for visual rescue of the wrinkled seed phenotype. Of 43 independent transformants, 8 appeared to be rescued based on seed morphology. Homozygous T3 seeds from individual rescued lines were subjected to FAME analysis (Figure 3.6A). Overexpression of the PK_p-β_1 encoding cDNA rescues the lipid phenotype of the mutant, but does not result in an increase in oil amount in excess of wild-type seed oil content in any of the lines. A PK_p-β_2 encoding cDNA was similarly overexpressed in *pkp1* and transformants were selected. Twenty independent transformants were identified and 6 of these had rescued seed morphology. FAME analysis was performed on homozygous T3 seeds from these lines and showed rescue of the low-oil phenotype of *pkp1* (Figure 3.6B). However, the restoration of oil content was not as complete as was achieved by overexpressing the PK_p-β_1 encoding cDNA. Overexpression of the cDNA encoding PK_p-β_1 was able to restore the fatty acid profile of *pkp1* to wild type, while the PK_p-β_2 encoding cDNA overexpressors had the same fatty acid profile as the *pkp1* mutant, despite being almost completely rescued in terms of oil accumulation (Figure 3.6C).

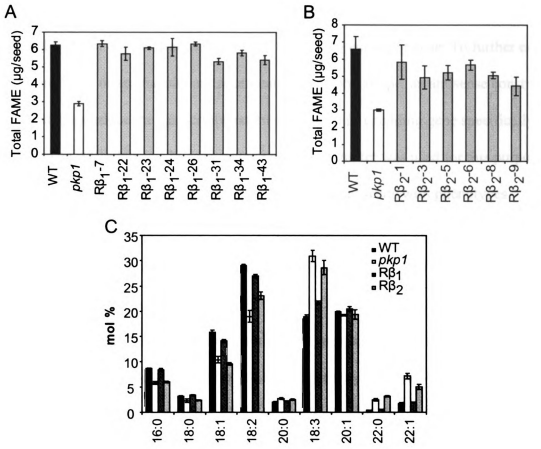

Figure 3.6. Rescue of the *pkp1* seed oil phenotype

(A) Oil amounts in mature seeds of *pkp1* overexpressing the PK_p-β_1 encoding cDNA. The 8 individual rescued lines are denoted with Rβ_1 and a number. WT, wild type. Values are the mean ± SD (n=3).

(B) Oil amounts in mature seeds of *pkp1* overexpressing the PK_p-β_2 encoding cDNA. The 6 individual rescued lines are denoted with Rβ_2 and a number. WT, wild type. Values are the mean ± SD (n=3).

(D) Average fatty acid profiles of the 8 Rβ_1 and 6 Rβ_2 lines compared to wild type (WT) and *pkp1*. Values are the mean ± SD (n=6).

The complementation data strongly suggest that the seed oil phenotype of *pkp1* is caused by the T-DNA insertion located in the PK_p-β_1 encoding gene. To further exclude the possibility of second site mutations causing the phenotype, an antisense construct was generated to reduce the transcript amount of the PK_p-β_1 encoding gene specifically in seeds. The Arabidopsis 12S seed storage protein promoter was used for this purpose. Of twenty wild-type transformants identified, three had seeds with phenotypes reminiscent of the *pkp1* mutant. None of the empty vector control lines displayed altered seed morphology (wrinkledness). Analysis of FAMEs was performed on the wrinkled seeds and the three lines contained 3.92 ± 0.6, 2.73 ± 0.3, and 2.22 ± 0.2 μg of oil per seed. The recapitulation of the *pkp1* seed phenotype using antisense repression independently corroborates that the low oil phenotype can be caused by reduction or abolishment of expression of the *PKP1* gene encoding PK_p-β_1.

Rescued lines have subunit-specific restoration of PK activity

PK gene expression and enzyme activity in the rescued lines were explored. Figure 3.7A shows RNA gel blots of 9-11 DAF silique tissue probed with PK_p gene specific probes. The top panel shows no accumulation of PK_P-β_1 transcript in *pkp1* (as demonstrated by RT-PCR in Figure 3.1C) and restoration only in the Rβ_1 lines. In the middle panel, very little PK_P-β_2 transcript is present in wild type, *pkp1,* or the Rβ_1 lines. However, the Rβ_2 lines clearly have increased amounts of the transcript. Based on gene expression, the Rβ_1 and Rβ_2 lines appear to have $\alpha\beta_1$ or $\alpha\beta_2$, respectively, as the dominant PK in silique tissue and thus provided an opportunity to further test the metabolite regulation observed for the recombinant $\alpha\beta_1$ and $\alpha\beta_2$ complexes.

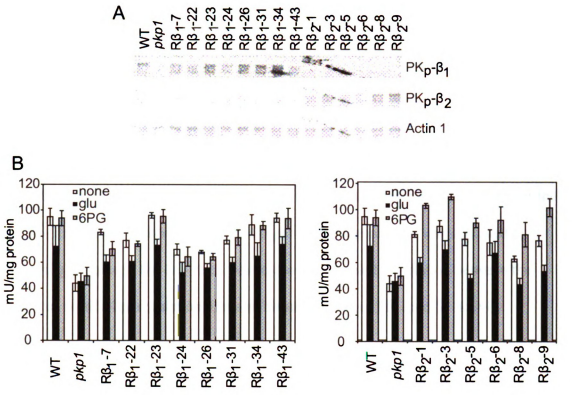

Figure 3.7. PK_p gene expression and enzyme activity in rescued *pkp1* lines

(A) Expression of PK_p encoding genes in siliques of wild type (WT), *pkp1*, and rescued lines. $R\beta_1$ is overexpression of PK_p-β_1 and $R\beta_2$ denotes overexpression of PK_p-β_2. Actin1 probe was used for loading control.

(B) Native PK specific activity of 9-11 DAF siliques in response to metabolite effectors. Left panel includes wild type (WT), *pkp1*, and *pkp1* lines rescued with overexpression of PK_p-β_1 ($R\beta_1$). Right panel includes wild type (WT), pkp1, and *pkp1* lines rescued with overexpression of PK_p-β_2 ($R\beta_2$). Activity was measured at pH 8.0 with subsaturating substrate concentrations. None, no effectors; glu, 5 mM glutamate; 6-PG, 0.2 mM 6-phosphogluconate. One mU is defined a 1 nmol pyruvate formed per minute. Values are the mean ± SD (n=4).

PK specific activity was first measured at pH 8.0 from 9-11 DAF silique material in the absence of effectors. The left and right panels of Figure 3.7B show that PK specific activity was restored in the $R\beta_1$ and $R\beta_2$ lines. The inclusion of 5 mM glutamate in the assay mixture resulted in a moderate inhibition of native PK specific activity in wild type and all of the rescued lines, as was observed for wild type in Figure 3.4B. The PK specific activity from the $R\beta_2$ lines, however, responded differently from wild type and the $R\beta_1$ lines to the presence of 0.2 mM 6-phosphogluconate. PK was activated by 6-phosphogluconate in the $R\beta_2$ lines, which provides needed correlation between the observed metabolite regulation of recombinant and native PK specific activity.

Altered substrate/product ratios and accumulation of glycolytic precursors in the *pkp1* mutant

The effects of the *pkp1* mutation on the pools of the substrates (PEP and ADP) and products (Pyr and ATP) of PK in developing seeds were analyzed. Metabolite measurements were made at 5-7 DAF and at 11-13 DAF, when PK specific activity and chlorophyll content, respectively, are most affected in the mutant seeds. Table 3.2 shows the results of these analyses. At 5-7 DAF, the amount of Pyr in *pkp1* is decreased, resulting in a 40% reduction in the Pyr/PEP ratio. At 11-13 DAF, there is no difference in the ratio of Pyr/PEP between wild type and *pkp1*. Instead, in *pkp1*, the absolute amounts of Pyr and PEP are proportionally increased. The PEP and Pyr data show that PK specific activity positively correlates with the Pyr/PEP ratio, due mostly to effects on the steady state levels of Pyr. The ATP/ADP ratio is increased almost 2 fold in *pkp1* when both PK specific activity (5-7 DAF) and chlorophyll content (11-13 DAF) are the most reduced.

Table 3.2 Metabolite levels in WT and *pkp1* developing seeds

	DAF	PEP nmol/g FW	Pyr nmol/g FW	Pyr/PEP	ADP nmol/g FW	ATP nmol/g FW	ATP/ADP
WT	5-7	17.5 ± 2.5	89.1 ± 7.9	5.1	15.8 ± 1.6	53.7 ± 3.7	3.4
	11-13	13.3 ± 1.6	36.5 ± 2.7	2.7	12.8 ± 1.2	38.8 ± 3.7	3.0
pkp1	5-7	18.6 ± 1.5	58.1 ± 6.1	3.1	13.0 ± 1.3	66.4 ± 3.4	5.1
	11-13	22.2 ± 4.1	59.1 ± 10.4	2.7	10.9 ± 2.6	57.3 ± 7.0	5.3

Values represent the mean ± SD of at least three repeats. DAF, days after flowering; PEP, phosphoenolpyruvate; Pyr, pyruvate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; FW, fresh weight

It seems that the metabolic perturbations in *pkp1* actually result in increased energy status in the developing seeds. This is somewhat surprising as PK activity and photosynthesis are expected to contribute to the ATP pool.

Pyruvate kinase is a control point for glycolysis as its activity has a direct impact on ATP and PEP, the latter of which is an inhibitor of phosphofructokinase in anoxic tissues such as seeds (Plaxton 1996). It is reasonable that a reduction in PK activity would result in an inhibition of glycolytic flux and an accumulation of carbohydrate precursors. Thus, hexose, sucrose, and starch were also measured in developing wild-type and *pkp1* seed. Figure 3.8A shows that in wild type hexoses accumulated early during development and steadily decreased, while sucrose followed the opposite trend. The *pkp1* mutant seeds followed the same trends, but contained twice as much of both compounds during peak accumulation times. Starch in the wild type showed the same transient accumulation pattern as previously documented (Focks and Benning 1998, Baud et al. 2002). However, the *pkp1* mutant continued to store starch throughout embryo development (Figure 3.8A).

A

ng/seed

ng/seed

µg/seed

Fig

pk

(A

wi

(B

ph

pg

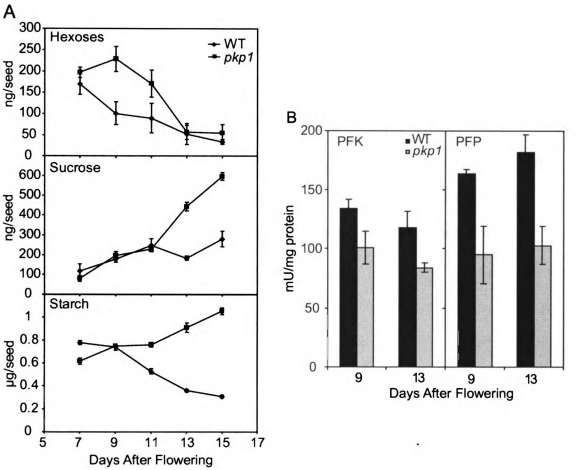

Figure 3.8. Carbohydrate accumulation and phosphofructokinase enzyme activity in *pkp1* and wild-type seeds

(A) Hexoses (glucose plus fructose), sucrose, and starch levels in developing seeds. WT, wild type. Values are the mean ± SD (n=4).

(B) ATP-dependent phosphofructokinase (PFK) and pyrophosphate-dependent phosphofructokinase (PFP) activity in developing seeds. One mU is defined a 1 nmol pyruvate formed per minute. WT, wild type.

ATP-dependent and PPi-dependent phosphofructokinase (PFK and PFP, respectively) activities were measured in developing seeds at 9 and 13 DAF (Figure 3.8B), when *pkp1* has increased hexoses only, or increased sucrose and starch, respectively. PFK activity decreases in the wild type between the two timepoints while PFP activity increases. In the *pkp1* mutant, the PFK and PFP activities change as in wild type, but are reduced by 25% and 40%, respectively. Taken together, the data in Figure 3.8 suggest that a reduction in PK_p activity impairs the catabolism of carbohydrates in developing seeds, possibly due to a reduction in upstream glycolytic activities.

Discussion

The αβ1 form of PK_p is dominant in seeds

A reverse genetic approach was taken to directly test the metabolic function of PK_p in developing Arabidopsis seeds. Mutants in the PK_p-α gene, which should result in complete inactivation of both PK_ps, could not be isolated. Only one mutant, in the PK_p-β_1 encoding gene, was identified which had no transcript accumulation and a seed phenotype (Figure 3.1, Table 3.1). Methods to separate the native Arabidopsis PK isoforms (isoelectric focusing, zymograms, native-PAGE) were unsuccessful and so PK_p activity was assayed at pH 8.0 to minimize the background from cytosolic enzymes. The PK specific activity profile at pH 8.0 in wild type very closely matches the expression of the α and β_1 subunit encoding genes (Figure 2.1B), while in *pkp1* the activity is greatly reduced and does not change (Figure 3.4A). In addition, wild-type and *pkp1* seed PK activities were insensitive to activation by 6PG (Figure 3.4B). In contrast, seed PK_p activity in *pkp1* lines that have been rescued with overexpression of the PK_p-β_2 encoding cDNA ($R\beta_2$) are activated in the presence of 6PG (Figure 3.7B) All these results agree with the *in vitro* enzyme characterization presented in Chapter 2 and corroborate a specific inactivation of the $\alpha\beta_1$ enzyme in the *pkp1* mutant. Furthermore, the *pkp1* mutant supports the initial hypothesis that the influx of photosynthate into embryo tissue and the high demand for lipid and amino acid precursors requires high PK_p activity. The regulatory properties of the $\alpha\beta_1$ enzyme mentioned above make it a prime candidate for this role. Taken together with the lack of transcript accumulation for the gene encoding PK_p-β_2 (Figure 2.1B), these data indicate that the $\alpha\beta_1$ enzyme is the major PK_p isoform present in developing Arabidopsis seeds. The increase in PK specific activity at pH 7.0 in

pkp1 suggests a compensatory mechanism in the mutant (Figure 3.4A). It is possible that more pyruvate is being generated in the cytosol by another isoform of PK. A preliminary flux map of carbon metabolism in *B. napus* embryos shows that 30% of the pyruvate used for fatty acid synthesis is cytosolic in origin (Schwender et al. 2003, Schwender et al. 2004b, Schwender and Ohlrogge 2002). If the same is true for Arabidopsis, the increase in PK specific activity at pH 7.0 only marginally compensates, as *pkp1* still has a 60% reduction in seed oil. Based on gene expression data (Figure 2.1B, Figure 3.2), a small amount of the PK_p-β_2 subunit could also be present in developing seed. The presence of this subunit could explain the incomplete loss of PK activity and seed oil in the *pkp1* mutant.

Seed metabolism is dependent on proper PK_p function

The *pkp1* mutant seeds are smaller and less green than wild-type seeds (Figure 3.3). The reduction in chlorophyll in *pkp1* developing seeds could be the result of sugar accumulation (Figure 3.8A) as sugars are known to repress chlorophyll accumulation and photosynthetic gene expression (Jang et al. 1997, Jang and Sheen 1994). It is also possible that a pleiotropic effect of the *pkp1* mutation is reduced chlorophyll biosynthesis. Dark treatment has been shown to decrease fatty acid synthesis by 23% in *B. napus* embryos (Ruuska et al. 2004). However, all light-activated processes are affected by this treatment and the reduced biosynthetic capability was linked to a lack of light induced activation of certain enzymes. In the case of *pkp1*, light still penetrates the seed and is capable of inducing enzyme activities and other processes. Thus, the primary metabolic

defect brought on by a reduction in PK_p activity is likely the major factor contributing to the low oil phenotype of *pkp1*.

In both wild-type and *pkp1* seeds, fatty acids accumulate in a linear time course from about 5 DAF until at least 15 DAF (Figure 3.5A). The rate of accumulation, though, is reduced by about 60% in the mutant, which correlates with the reduction in seed oil and the reduction in total PK specific activity at pH 8.0. The fatty acid profile of mature *pkp1* seeds (Figure 3.5B) is very similar to that of the *wri1* mutant and plants with altered biotin carboxyl carrier protein gene expression (Focks and Benning 1998, Thelen and Ohlrogge 2002). These mutations impair fatty acid synthesis either by reducing the supply of precursors or by inhibiting the ACCase reaction, respectively. It is likely that in the *pkp1* mutant a reduction in precursors for fatty acid synthesis is the cause of the altered fatty acid profile. Indeed, the steady state level of pyruvate is reduced in *pkp1* seeds compared to wild type at 5-7 DAF, which correlates with the onset of fatty acid biosynthesis (Table 3.2). It should be noted that these measurements do not distinguish the subcellular compartmentation of the metabolites in question. Interestingly, the amounts of PEP and pyruvate, but not the ratio of the two, are increased in *pkp1* at 11-13 DAF (Table 3.2). The idea of a reduction in the supply of precursors for fatty acid biosynthesis is further supported by the accumulation of carbohydrates in the mutant seed, a phenotype similar to that of *wri1* (Figure 3.8A). It is also possible that the reduction in the rate of fatty acid synthesis is brought on by a lack of ATP. Seeds of *B. napus* (and likely Arabidopsis) are a low oxygen environment (Vigeolas et al. 2003) and even with photosynthesis PK could have an important role in the production of ATP. A mutant defective in a plastidic ATP/ADP transporter with reduced ATP import capacity into

plastids has reduced oil content in its seeds (Reiser et al. 2004). Moreover, this mutant was shown to compensate for reduced ATP import by increasing the expression of the genes encoding PK_p-β_1 and PK_p-β_2. A similar increase in transcript level for any of the PK_p subunits is not seen in *pkp1* seeds (Figure 3.2). Additionally, steady state levels of ATP in the mutant seeds were actually increased relative to wild type (Table 3.2), possibly as a result of increased cytosolic PK (PK_c) activity in the mutant (Figure 3.4A). Elevated PK_c activity in *pkp1* might be an indicator of enhanced cytosolic glycolytic flux into mitochondrial respiration, which could also elevate the ATP/ADP ratio. Based on the *pkp1* mutant phenotype it is unlikely that ATP production is a major function of PK_p in developing Arabidopsis seeds. The *pkp1* mutant is rescued by ectopic overexpression of the PK_p-β_1 and PK_p-β_2 encoding cDNAs except that oil accumulation is recovered less fully in the Rβ_2 lines (Figure 3.6). A similar pattern of rescue was observed for seed chlorophyll accumulation (Figure 3.3B) and fatty acid profile (Figure 3.6C), in which the Rβ_2 lines were not rescued as completely as the Rβ_1 lines. The maximum activity observed in seed extracts was similar in all of the rescued lines (Figure 3.7B). Thus, it is likely that distinct regulatory features determined for the two PK_p complexes (detailed in Chapter 2) account for the observed physiological differences between the Rβ_1 and Rβ_2 rescued plants. No transgenics were observed that had an increase in seed oil content or in PK activity, despite higher than wild-type expression levels of the PK_p-β_1 or PK_p-β_2 encoding genes (Fig 3.7A). This might indicate that in these lines the amount of PK_p-α is limiting as it is required for activity.

Concomitant with the reduction in seed oil in *pkp1* is the accumulation of increased amounts of hexoses, sucrose, and starch (Figure 3.8A). It seems that there is a

redirection of carbon partitioning in *pkp1* in which less hexose and sucrose are catabolized via glycolysis, but are instead incorporated into starch. However, the starch accumulated in *pkp1* seeds at 15 DAF only accounts for approximately 20% of the carbon not incorporated into fatty acids (Table 3.2, Figure 3.8A). One potential mechanism for the observed accumulation of carbohydrates is that elevated PEP (Table 3.2), which is a potent inhibitor of plant phosphofructokinases (Plaxton and Podesta 2006), could slow the entry of hexose-phosphates into glycolysis, resulting in a misregulation of metabolism. The reduction of PFK and PFP activities in *pkp1* support this hypothesis (Figure 3.8B). It should be noted that the assays for PFK and PFP are set up in such a way as to maximally activate the enzymes, and thus measure activity as it relates to total protein amounts. The observed reductions in activities therefore do not reflect any potential additional regulation of the enzymes caused by altered metabolite levels. The mechanisms that down-regulate these activities in *pkp1* are unknown, but one could speculate that sugar signaling is somehow involved. It is interesting that in *pkp1* excess carbon is not redirected into storage protein synthesis. Instead, protein levels are slightly decreased (Table 3.1), which could be a result of decreased glycolytic flux and reduced substrate availability. Two plastid-localized enzymes of branched chain amino acid biosynthesis, acetolactate synthase (ALS) and dihydrodipicolinate synthase (DHPS), use pyruvate as a substrate and have K_m values of 1.6-14 mM for ALS and about 1.7 mM for DHPS (Durner and Boger 1990, Dereppe et al. 1992). The plastidic pyruvate dehydrogenase (ptPDC) complex has a K_m for pyruvate of about 300 μM (Camp et al. 1988). Thus, amino acid synthesis could be out competed by ptPDC in situations of limiting pyruvate availability. However, based on these properties alone, one would expect a decrease in

protein content greater than that of oil in *pkp1*. One possible explanation for the observed phenotype is that branched chain amino acids are only a minor component of total seed protein (Figure 3.5D) and restriction of their synthesis has little effect on protein content. Increased cytosolic PK specific activity in *pkp1* (PK at pH 7.0, Figure 3.4A) could also help maintain almost wild-type protein levels by feeding into the TCA cycle and increasing the supply of carbon skeletons available for the synthesis of other amino acids.

The results of this study are in support of the metabolic model depicted in Figure 1.2 in which PEP metabolized by PK_p in the plastid is the main source of pyruvate for fatty acid and amino acid syntheses. The compartmentation of metabolism apparently serves to isolate metabolic pathways such that specific products can be generated from distinct pools of substrates. In the case of seed oil metabolism, pyruvate generated in the plastid is used mainly for fatty acid synthesis and cannot be fully replaced by cytosolic pools. It seems that Arabidopsis seeds are programmed to make oil from plastidic pyruvate and if that pathway is perturbed, as is the case in the *pkp1* mutant, some of the carbon (20% in *pkp1*) is stored in a different form, e.g. starch. As such the *pkp1* mutant provides an example for a plant with altered carbon partitioning in developing seeds.

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Chapter 4

Germination, establishment, and growth of Arabidopsis plants lacking a plastidic pyruvate kinase

Abstract

Catabolism of storage reserves is essential for seed germination and establishment. An Arabidopsis mutant (*pkp1*) deficient in plastidic pyruvate kinase (PK_p) which is unable to amass storage oil to the same extent as wild type is abnormal in these processes. Germination is delayed in the mutant and seedling establishment is dependent on an exogenous sugar supply. It appears, however, as though these phenotypes are not entirely caused specifically by a lack of seed oil and may be related to reduced PK_p activity. Germinating seeds of *pkp1* are unable to metabolize storage oil and cannot utilize applied sucrose for hypocotyl elongation in the dark. Additionally, seed longevity is greatly reduced in *pkp1* indicating a potential lack of seed tocopherols. Mature *pkp1* plants are slightly chlorotic and contain less glucose and fructose. Thus, it appears as though PK_p is necessary for proper metabolic function during all aspects of plant growth.

Introduction

Pyruvate kinase (PK) is a ubiquitous enzyme located at a major branch point in carbon metabolism (see Figure 1.5). Native PKs have been purified and characterized from a variety of organisms and these studies have revealed a wide range of kinetic and regulatory properties for these enzymes (Munoz and Ponce 2003). This diversity of molecular and biochemical characteristics seem to define specific functions for individual PK isoforms within a single organism. For instance, mammals have four PKs which are differentially expressed depending on the metabolic demands of the tissue (Yamada and Noguchi 1999). Plant metabolism, however, is more complex than that of mammals and this is reflected by the fact that the Arabidopsis genome encodes 14 putative PKs which reside in both the cytosol (PK_c) and the plastid (PK_p; Arabidopsis Genome Initiative 2000). Its location in the metabolic network and sheer redundancy indicate that PK is a critical enzyme for plants, but few studies have focused on this enzyme with regard to its function *in vivo*.

Only one instance of a plant deficient in PK has been reported. An attempt to engineer transgenic tobacco (*Nicotiana tabacum*) with increased PK_p inadvertently resulted in co-suppression of an endogenous PK_c-encoding gene and consequent loss of enzyme activity in leaves (Gottlob-McHugh et al. 1992). At the time the only apparent phenotype was a shift from a high pyruvate/phosphoenolpyruvate (Pyr/PEP) ratio to a low one. More detailed analysis led to the discovery of a root growth defect, despite the PK_c deficiency being localized exclusively to leaves (Knowles et al. 1998). This phenotype was exacerbated in low light conditions. Later, it was observed that PK_c functions in regulating photoassimilate export at night by controlling carbon flow into

respiration (Grodzinski et al. 1999). This result is somewhat confusing as reduced PK_c activity actually resulted in increased respiratory CO_2 release. However, a yeast (*Saccharomyces cerevisiae*) mutant with a similar reduction in PK activity also has elevated respiratory flux (Pearce et al. 2001). An induction of a PK bypass (as shown in Figure 1.3) could explain this phenomenon.

The occurrence of plant PKs in the plastid and cytosol almost certainly reflects the unique roles for the respective enzymes (Plaxton 1996). For example, Pyr transported into mitochondria for entry into respiration is most likely derived from PK_c, especially when considering the lack of a reported plastidic Pyr transporter (Weber 2004). Plastid-localized metabolisms which use PEP and Pyr are no doubt influenced by PK_p activity. Two enzymes in the Shikimate pathway, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase, and 5-enolpyruvylshikimate 3-phosphate synthase, use PEP as a substrate (Herrmann and Weaver 1999). The final product of this pathway, chorismate, is metabolized into aromatic amino acids which themselves are the starting points for the synthesis of a variety of secondary metabolites (*e.g.* anthocyanins). The plastidic pyruvate dehydrogenase complex, which produces acetyl-CoA for fatty acid synthesis acts on pyruvate. The first enzyme of the methylerythritol-4-phosphate (MEP) pathway, 1-deoxy-D-xylulose-5-phosphate synthase, uses pyruvate and glyceraldehyde-3-phosphate to synthesize the first intermediate of plastidic isoprenoid synthesis (Lichtenthaler 1999). Plastid-derived isoprenoids include the carotenoids and phytol used in the biosynthesis of chlorophyll and tocopherol. In addition, pyruvate is a substrate in the biosynthesic pathways of valine, lysine, and isoleucine, and can be directly converted to alanine by a transaminase (AraCyc metabolic map, www.arabidopsis.org/tools/aracyc/).

Clearly, the ratio of PEP to Pyr must be balanced, in large part by PK_p, such that these biochemical pathways function properly.

In Chapter 3, I detailed the fatty acid biosynthetic defect (a plastid localized metabolism) of an Arabidopsis mutant (*pkp1*) deficient in seed PK_p activity. Additionally, *pkp1* seeds had much less chlorophyll than wild type and this could indicate substrate-restricted flux through the MEP pathway (which produces the phytol side chain of chlorophyll). Here, I performed experiments to determine the extent to which the lack of seed oil and perturbation of plastidic metabolism affects germination, establishment, and growth of *pkp1* offspring.

Materials and Methods

Plant growth conditions

Wild-type plants were of the Col-2 ecotype while *pkp1* and the respective rescued lines were in the Col-0 background. All seeds were sterilized with 20% bleach, 0.05% TritonX-100 for 15 min and were rinsed 5 times in sterile water. Medium used for germination and growth on agar plates was full strength MS, pH 5.8, 0.9% agar and included 0, 2, or 4% sucrose when appropriate. Seeds were stratified at 4°C for 3 d prior to being put into an incubator with a photon flux density of 60–80 μmol m^{-2} sec^{-1} and a light period of 16 h (22°C), and a dark period of 8 h (18°C). After 10 d, seedlings were either transferred to soil or to fresh agar plates. Soil grown plants were put into a 16-h photoperiod with a day temperature of 22°C and a night temperature of 20°C at a photon flux density of 100–120 μmol m^{-2} sec^{-1}. Plant growth measurements were on plants transferred to soil at 10 d after sowing. The aerial portion of six individuals was used for each time point.

Root and hypocotyl elongation assays

For these assays, seeds were sown in a straight line and the agar plates were arranged vertically. Root lengths were measured to the nearest mm every 24 h. The same set of agar plates was used throughout the experiment. Hypocotyl elongation assays were performed using 7 d after sowing seedlings as previously described (Penfield et al. 2004). Once exposed to the light hypocotyls were immediately measured and those plates were not used again.

Seed germination assays

All seeds used were produced from mother plants grown in identical conditions and were of the same age. Germination assays were routinely performed using the plant growth procedures mentioned above. When appropriate, 0, 2, or 4% sucrose was added to the medium prior to being autoclaved. Germination was scored as radicle emergence from the seed coat and was determined every 24 h using the same set of horizontally grown agar plates. Accelerated aging treatment was done as previously described (Sattler et al. 2004) except that 42°C was used instead of 40°C.

Pyruvate kinase enzyme activity measurements

Seedling and leaf crude protein extracts were prepared by grinding tissue in approximately 10 volumes (μL/mg) of buffer containing 50 mM Tris-Cl pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1mM DTT, 0.1% Triton-X100, 10% glycerol, and a protease inhibitor mix (Complete mini, Roche). Pyruvate kinase activity was coupled to the conversion of NADH to NAD^+ by lactate dehydrogenase. Reactions were kept at 25°C, were started by the addition of enzyme mix, and were linear for at least 5 minutes. Absorbance at 340 nm was measured using a FLUOstar Optima 96-well plate reader (BMG Labtech, Offenburg, Germany). The PK_p reaction mixtures contained 50 mM HEPES-KOH pH 8.0, 5% PEG-8000, 50 mM KCl, 15 mM $MgCl_2$, 1 mM DTT, 2 mM PEP, 1 mM ADP, 0.2 mM NADH, and 2 U ml^{-1} desalted rabbit muscle lactate dehydrogenase. PEP phosphatase activity was corrected for by omitting ADP from the reaction. Reactions at pH 7.0 were done using 50 mM MOPS pH 7.0 instead of HEPES. Protein was quantified using Bradford reagent (Sigma).

Lipid and carbohydrate analysis

Total leaf lipids were extracted from pre-weighed tissue by vigorously shaking for 5 min in 500 µL of methanol/chloroform/formate (2:1:0.1, v/v). Then 250 µL of 1 M KCl, 0.2 M H_3PO_4 was added and the tubes were vortexed. The phases were separated by centrifugation at 16,000 g for 5 min. The organic phase was loaded quantitatively onto a treated (soaked in 0.15 M $(NH_4)_2SO_4$ and dried, then heated to 120°C for 2.5 hrs) silica-60 TLC plate (Baker). The solvent system used was acetone/toluene/water (91:30:7, v/v) and staining was done with iodine and α-naphthol. Lipid composition was determined by fatty acid methyl ester (FAME) analysis as described previously (Focks and Benning 1998). Ten seedlings or two whole leaves were used for each sample. Glucose, fructose, sucrose, and starch were extracted and quantified as previously described (Focks and Benning 1998). Five 25 d old plants were homogenized together and about 50 mg (fresh weight) of leaf tissue was used for each extraction. Soluble sugars were resuspended in 200 µL of water and 15 µL was used for each measurement. Insoluble carbohydrate pellets were resuspended in 300 µL of 0.2N KOH and the remaining volumes were adjusted proportionally. Starch assays were done with 15 µL of the final preparation.

Leaf pigment quantification

Chlorophyll was extracted from leaves and seedlings using 100 volumes (µL/mg) of 80% acetone and was quantified as previously described (Lichtenthaler 1987). Anthocyanins were extracted and quantified using a published protocol (Martin et al. 2002).

Results

Seed germination and seedling establishment are aberrant in *pkp1*

Chapter 3 describes the seed-specific phenotypes of wild type and the *pkp1* mutant and includes some data for *pkp1* rescued by ectopic overexpression of PK_p-β_1 ($R\beta_1$-23) and *pkp1* rescued by ectopic overexpression of PK_p- β_2 ($R\beta_2$-3). During routine growth of these plants it was observed that *pkp1* seedlings do not establish (defined as development of true leaves and root elongation) when sown directly on soil. In a single repetition, 100% of wild-type seedlings and 0% of *pkp1* seedlings established after 12 days when sown on soil. This defect was rescued 100% in $R\beta_1$-23 but only 80% in $R\beta_2$-3. A common phenotype of low oil mutants such as *pkp1* is an inability to establish in the absence of an exogenous sugar source (Cernac et al. 2006, Lu and Hills 2002). Indeed, *pkp1* will not establish unless provided with 2% sucrose in the medium (Figure 4.1A) and even then growth is slow in the mutant. Root elongation assays were performed to quantify this defect. As shown in Figure 4.1B, *pkp1* roots do not grow at the same rate as wild type or either of the rescued lines. The results also indicate that the initiation of root growth may be delayed in *pkp1* as at 3 days after sowing (DAS) its roots have not elongated past 1 mm. One possible explanation is that germination itself is inhibited in these seedlings.

Germination assays were conducted in the light in the presence of 2% sucrose to explore the possibility that *pkp1* seeds do not germinate as fast as wild type. Images of seedlings taken at 3 and 6 DAS qualitatively demonstrate an abnormality in *pkp1* seedlings (Figure 4.2A). By 3 DAS, wild type seeds have germinated (defined by radicle emergence from the seed coat) and the roots are already elongating.

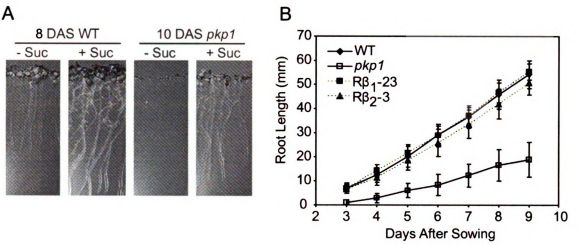

Figure 4.1. Sucrose dependent establishment and root elongation in *pkp1*

(A) Germination and seedling establishment in the presence (+) or absence (-) of 2% sucrose (Suc). Ten days after sowing (DAS) *pkp1* seedlings are at the same developmental stage as 8 DAS wild-type seedlings.

(B) Root elongation in the presence of 2% sucrose. Without sucrose *pkp1* roots do not elongate (shown in A). Values are the mean ± SD of 25 measurements.

In *pkp1*, however, only a portion of the seeds display radicle emergence at 3 DAS and not until 6 DAS have all of the seeds germinated. Figure 4.2B shows the germination rates of wild type, *pkp1*, and the rescued lines determined from independent experiments using seeds of the same age. After 1 day almost 100% of wild type and $R\beta_1$-23 and about 75% of $R\beta_2$-3 seeds have germinated while in *pkp1* less than 20% of the radicles have emerged. Not until 5 DAS does *pkp1* reach its maximum germination percent, which is about 90%. Pyruvate kinase (PK) activity was measured at pH 8.0 to ascertain if reductions in enzyme activity are correlated with the delayed germination in *pkp1*.

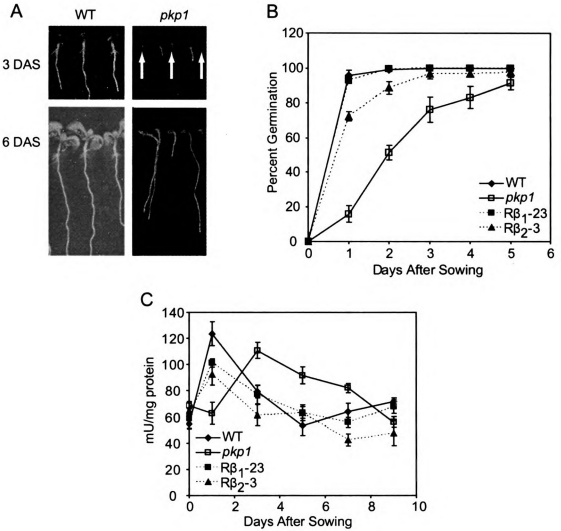

Figure 4.2. Delayed germination and induction of PK_p activity in *pkp1*

(A) Seed germination is delayed in *pkp1* in the light on MS medium with 2% sucrose. DAS, days after sowing. Un-germinated seeds are marked with white arrows.

(B) Seed germination rates on medium with 2% sucrose. Values are the mean ± SD (n=6).

(C) Pyruvate kinase activity measured at pH 8.0 using protein extracts from germinating seeds and seedlings grown in the light. Values are the mean ± SD (n=3). One mU is 1 nmol pyruvate formed per min.

Imbibed and stratified seeds served as the 0 DAS time point and at this time all of the lines have about the same PK specific activity (Figure 4.2C). Twenty-four h later, there is a doubling of PK specific activity in wild type and a similar, although less intense, induction in the rescued lines. By 5 DAS, activity has returned to the starting point where it remains for the rest of the time course. The pattern of PK induction is skewed in *pkp1* and does not peak until 3 DAS, after which it steadily declines. There is indeed a correlation between delayed induction of PK specific activity and germination in *pkp1*.

Developing seeds of *pkp1* accumulate carbohydrates in the form of sucrose and starch late during embryogenesis (see Figure 3.8). High sugar concentration in the medium has been shown to delay seed germination (Dekkers et al. 2004, Zhou et al. 1998). Therefore, it is reasonable to postulate that the sugar accumulated in *pkp1* seeds is partly responsible for the observed germination defect and that the mutant will be more sensitive to exogenous sugar in the medium. To test this hypothesis, germination assays were performed on agar plates containing 0, 2, or 4% sucrose. A very subtle delay in germination is observed for wild type with increasing sugar concentration (Figure 4.3A). In all treatments though, germination of wild-type seeds reached a maximum by 2 DAS. As predicted, *pkp1* is more sensitive to sugar in the medium. In the absence of sucrose, the maximum number of *pkp1* seeds has germinated by 3 DAS (Figure 4.3B). With 2%, this time is extended to 5 DAS. On 4% sucrose only 50% of the *pkp1* seeds have germinated at 5 DAS and not until 10 DAS is the maximum achieved.

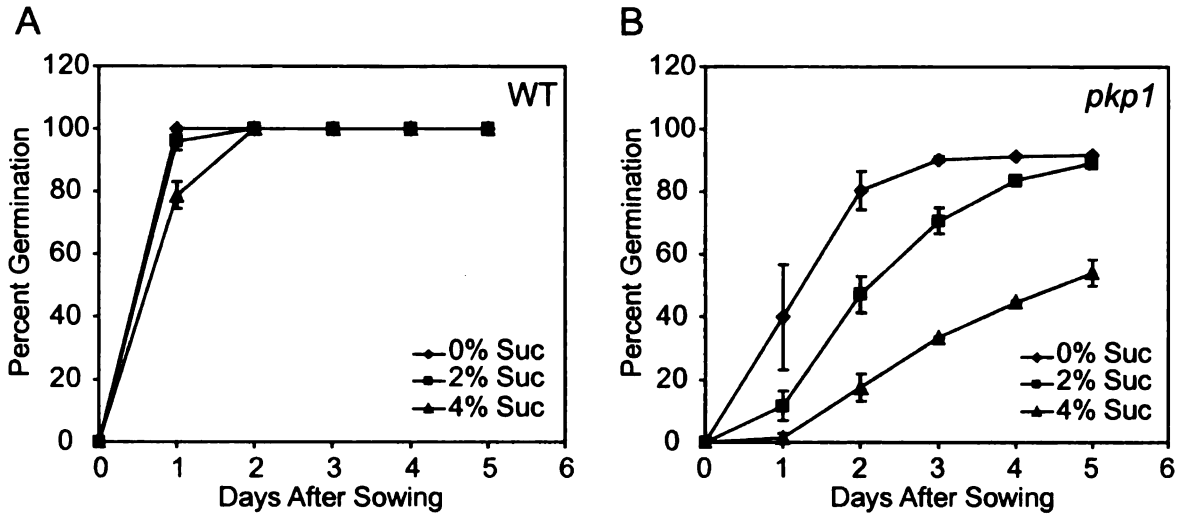

Figure 4.3. Inhibition of *pkp1* germination by exogenous sucrose

(A) Wild type germination rates in the presence of 0, 2, or 4% sucrose.

(B) Germination of *pkp1* in the presence of increasing concentrations of sucrose.

Storage lipid metabolism is defective in *pkp1*

Hypocotyl elongation assays in the dark are a standard means of examining storage oil metabolism in germinating seeds. When grown in the dark in the presence or absence of sucrose wild type hypocotyls elongate (Figure 4.4A, 4.4B). *pkp1* hypocotyls do not elongate in medium without an exogenous carbon source, which is typical of oil deficient mutants such as *wrinkled1* (*wri1*; Cernac et al. 2006). However, even when provided with 2% sucrose *pkp1* seedlings do not elongate their hypocotyls; a phenotype which is rescued in lines overexpressing either β-subunit encoding gene (Figure 4.4A, 4.4B). Dark-grown seedlings must generate ATP mainly by glycolysis and respiration, whether fueled by endogenous storage reserves or by uptake of an exogenous carbon source.

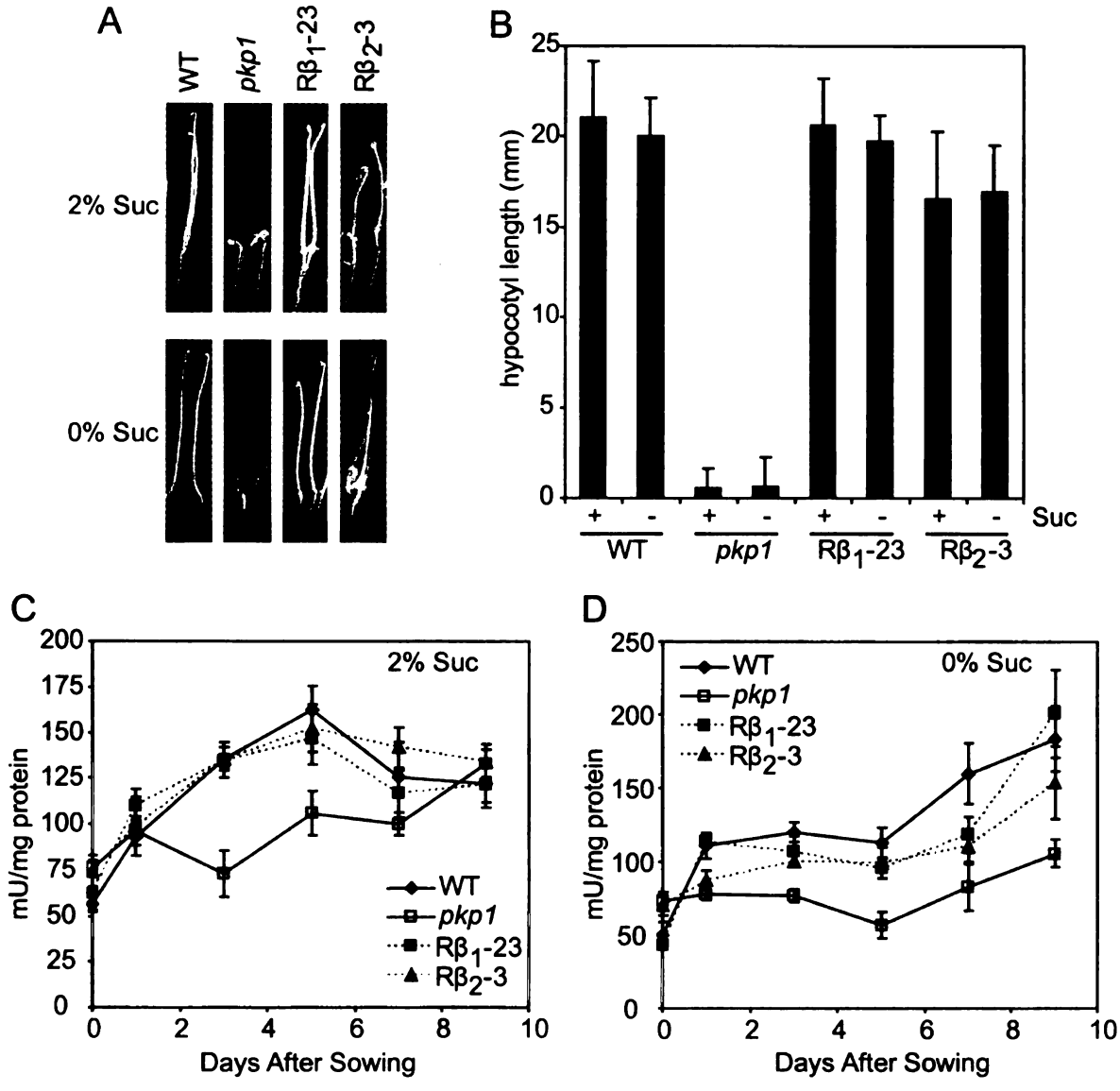

Figure 4.4. Hypocotyl elongation and PK_p activity in dark-grown seedlings

(A) Dark-grown seedlings at 7 days after sowing on MS medium with 0 or 2% sucrose.

(B) Quantification of hypocotyl lengths from seedlings in (A). Values are the mean ± SD of 25 measurments. (+), 2% sucrose; (-), 0% sucrose.

(C-D) Pyruvate kinase activity measured at pH 8.0 from dark-grown hypocotyls in the presence (C) or absence (D) of 2% sucrose. Values are the mean ± SD (n=3). One mU is 1 nmol pyruvate formed per min.

In either case, PK is important for the substrate-level phosphorylative generation of ATP and for the production of respiratory precursors. Thus, a reduction in PK activity could help explain the hypocotyl elongation phenotype of *pkp1*. Indeed, PK specific activity is relatively low in *pkp1* etiolated seedlings when grown with or without exogenous sucrose (Figure 4.4C, 4.4D).

Seedling establishment is largely fueled by seed storage oil breakdown. Figure 4.5A illustrates this process in seedlings grown on agar plates containing 2% sucrose. Very long chain fatty acids (VLCFAs), which in Arabidopsis are specific to seed triacylglycerol (TAG), were used as markers for storage oil content. In wild type and the rescued lines storage oil begins to be metabolized at 2 DAS and is essentially depleted by 6 DAS. Interestingly, storage lipids are not used by *pkp1* seedlings (Figure 4.5A).

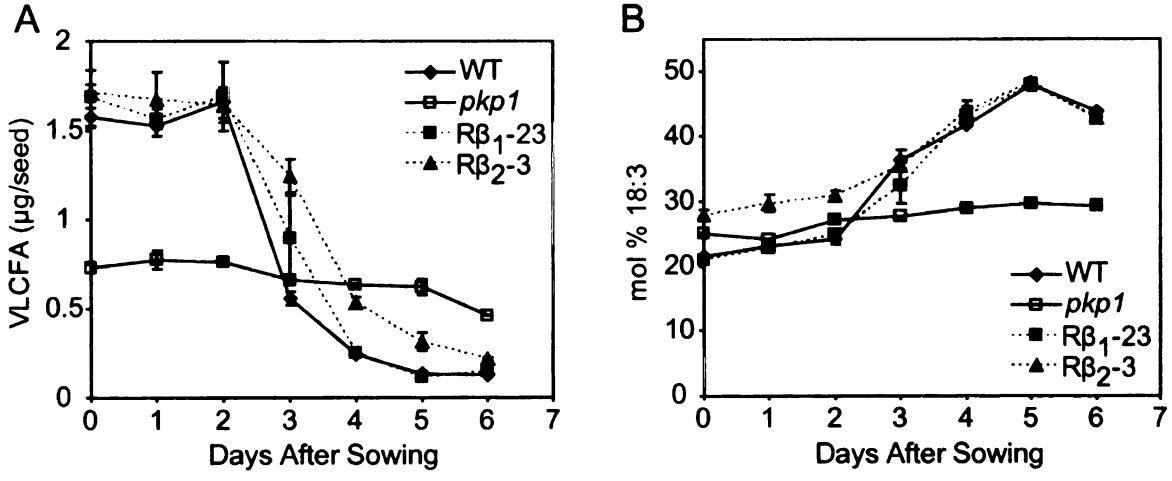

Figure 4.5. Fatty acid composition in germinating seeds and seedlings

(A) Seed oil-specific very long chain fatty acid (VLCFA; 20:0, 20:1, 21:0, 21:1) content in seedlings grown on 2% sucrose. Values are the mean ± SD (n=4).

(B) mol% of linolenic acid (18:3) in seedlings grown on 2% sucrose. Values are the mean ± SD (n=4).

An additional marker for seedling establishment is an increase in the proportion of linolenic acid (18:3) in membrane lipids, which is a major component of thylakoid membranes. By 5 DAS, the proportion of 18:3 to other fatty acids has increased by about 30% in wild type and the rescued lines (Figure 4.5B). In *pkp1* the increase is limited to about 5%. It is clear that lipid metabolism is abnormal in *pkp1* seedlings.

The lipid metabolism phenotype of *pkp1* seedlings is reminiscent of Arabidopsis mutants deficient in seed tocopherol, *vitamin e1* and *vitamin e2* (*vte1*, *vte2*; Sattler et al. 2004). Seed vitamin E deficiency leads to irreversible lipid peroxidation and thus, reduced seed longevity. Treatment with high temperature and relative humidity simulates natural aging and was used here to determine the extent to which *pkp1* resembles the *vte* mutants. Figure 4.6 shows the results of this experiment.

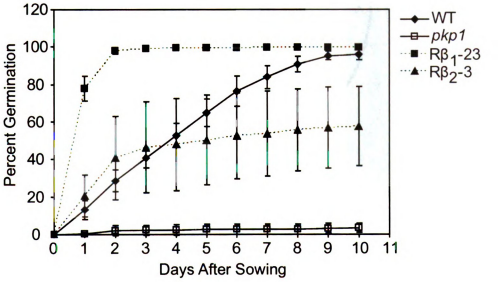

Figure 4.6. Seed germination following accelerated aging treatment
Seeds were the same as were used in previous germination assays and were produced from plants grown in identical conditions. Values are the mean ± SD (n=8).

Wild type germination was delayed relative to untreated seeds (compare to Figure 4.2B), but still reached nearly 100%. Germination of *pkp1* was almost completely arrested. Surprisingly, $R\beta_1$-23 seeds were unaffected by the treatment while $R\beta_2$-3 showed a phenotype intermediate between wild type and *pkp1*.

Altered growth and leaf metabolism in *pkp1*

The *pkp1* seedlings that successfully establish continue to experience difficulties in growth after being transferred to soil. Biomass production is limited and around the time of flowering initiation (25 DAS) *pkp1* aerial parts weigh approximately half compared to wild type (Figure 4.7A).

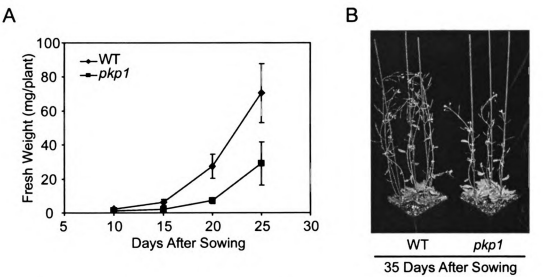

Figure 4.7. Plant growth and morphology of *pkp1*

(A) Plant growth curve of wild type and *pkp1*. Measurements were initiated at 10 days after sowing, when seedlings were transferred from MS medium with 2% sucrose to soil. Each value is the mean ± SD (n=6).

(B) Whole plant morphology of wild type and *pkp1*.

Taking into account the delay in germination, *pkp1* follows the same developmental time course as wild type. At 35 DAS, both genotypes are well into flowering and *pkp1* is nearly equal to wild type in size (Figure 4.7B). Another noticeable morphology of *pkp1* is slight chlorosis. When grown on soil, total chlorophyll content is reduced by 30%, accompanied by a moderate increase in anthocyanins (Table 4.1). This phenotype is exaggerated in plants propagated on agar plates in lower light conditions (photon flux densities of 100–120 and 60-80 μmol m^{-2} sec^{-1} for soil and agar growth, respectively).

Table 4.1 Leaf pigments in 25 day old plants grown on soil or on agar plates

	Soil-grown plants		Agar plate-grown plants	
	Chlorophyll (μg/mg FW)	Anthocyanins (A_{530}-A_{657}/g FW)	Chlorophyll (μg/mg FW)	Anthocyanins (A_{530}-A_{657}/g FW)
WT	1.06 ± 0.05	0.69 ± 0.01	1.18 ± 0.01	0.22 ± 0.03
pkp1	0.76 ± 0.09	0.80 ± 0.01	0.42 ± 0.02	0.54 ± 0.02

Values represent the mean ± SD of at least three repeats. FW, fresh weight

Pyruvate kinase enzyme assays were performed to see if the morphological differences of *pkp1* are correlated with a reduction in activity. When measured at pH 7.0, which is more specific for cytosolic enzymes, enzyme activity is the same between wild type and *pkp1* (Figure 4.8A). Plastidic PK's typically have a pH optimum of 8.0, and so any differences directly related to the *pkp1* mutation are expected to be seen at this pH. The data in Figure 4.8B establishes that PK_p activity in *pkp1* leaves is ablated and only reaches about 60% of wild-type levels.

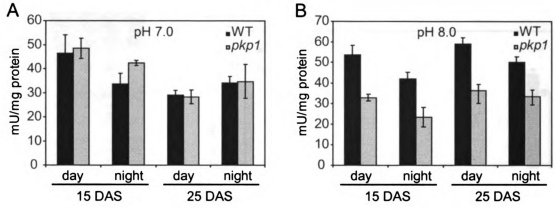

Figure 4.8. Pyruvate kinase activity in rosette leaves during the day and night

(A) Pyruvate kinase activity measured at pH 7.0 For each time point, 5 whole plants were homogenized and used to prepare protein extracts. Day = 8 hours after lights on. Night = 8 hours after lights off. Values are the mean ± SD (n=4). DAS, days after sowing.

(B) Pyruvate kinase activity measured at pH 8.0 using the same protein extracts described in (A).

Lipid metabolism is aberrant in *pkp1* seeds and seedlings and so it is logical to presume that there is also an effect in leaves of mature plants. However, limited examination of leaf lipids revealed very little qualitative, if any, difference between wild type and *pkp1*. Figure 4.9A is a thin layer-chromatogram of total leaf lipids extracted from 25 DAS plants. It is clear that there is no defect in the membrane lipid profile of *pkp1*. Analysis of the fatty acid profile revealed a very subtle, but significant decrease in 18:3 content which is accompanied by an increase in the proportion of linoleic acid (18:2; Figure 4.9B). This result is not totally unexpected as the same trend is seen in seedlings, although to a much greater extent (Figure 4.5B).

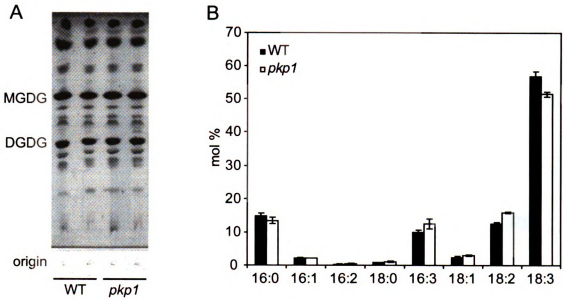

Figure 4.9. Lipid profile and composition of *pkp1* leaves

(A) Thin layer-chromatogram of total lipids extracted quantitatively from 25 days after sowing wild type and *pkp1* leaves. Plate was stained with iodine vapor and α-naphthol to maximize the number of lipids detected. DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol.

(B) Fatty acid composition of total leaf lipids from 25 days after sowing plants. Values are the mean ± SD (n=3).

Reduced biomass production and chlorophyll content of *pkp1* could reflect or cause altered carbohydrate metabolism. Therefore, soluble sugars and starch were extracted from leaves during the day and night and were quantified. Hexose content (glucose and fructose only) was reduced by more than half in *pkp1* during both the day and night. On the other hand, sucrose and starch accumulation were unaffected.

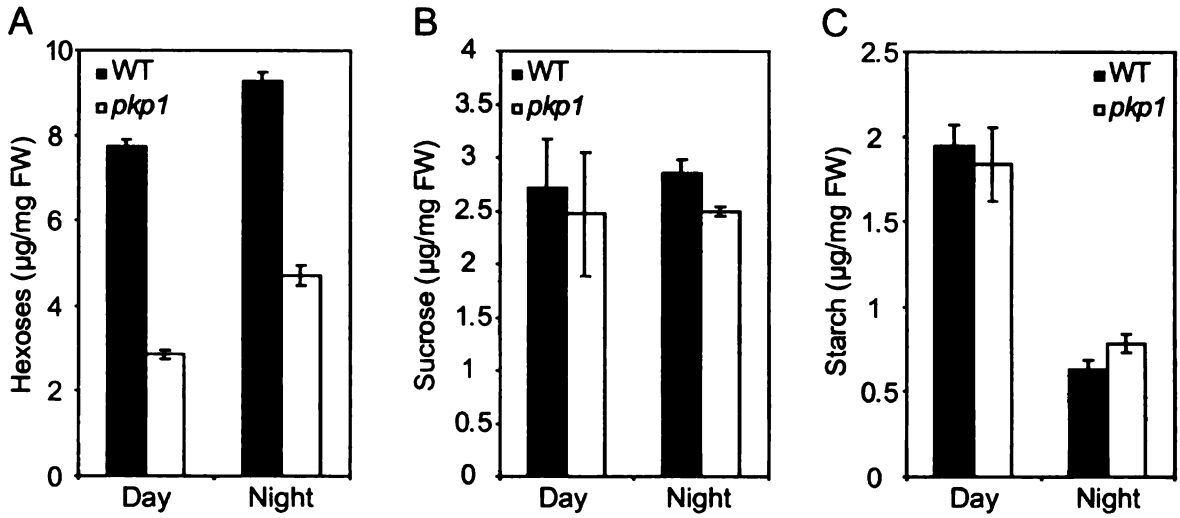

Figure 4.10. Carbohydrate content of 25 day old leaves

(A) Hexose (glucose and fructose) content of rosette leaves. 5 whole plants were homogenized and used for extraction. Day night cycle used was 16 hrs light and 8 hrs dark. Day = 8 hours after lights on. Night = 8 hours after lights off. Values are the mean ± SD (n=4). FW, fresh weight.

(B) Sucrose content of the samples described in (A). Values are the mean ± SD (n=4).

(C) Starch accumulation in the same samples as detailed in (A). Values are the mean ± SD (n=4).

Discussion

PK_p activity is required to fuel seed germination

Seed germination in Arabidopsis is largely driven by the metabolism of storage reserves other than lipids, while seed oil is more important for fueling subsequent seedling establishment (Cernac et al. 2006). Breakdown of carbohydrates to produce energy proceeds through glycolysis, with PK generating half of the ATP and providing precursors for respiration. In germinating wild-type seeds, a rapid increase of PK_p activity is observed coincident with radicle emergence (0-1 DAS, Figure 4.2C). In *pkp1* there is a delay in this induction, which could explain or result from the impaired germination rate (Figure 4.2A, 4.2B). The eventual increase in PK_p activity in *pkp1* can be explained by two possibilities: 1) enhanced expression of the PK_p-β_2-encoding gene in response to unfavorable energy status, or 2) induction of a cytosolic enzyme with higher than normal pH optimum. The first hypothesis is supported by elevated expression of the PK_p-β_2-encoding gene in dark-grown seedlings of a plastidic ATP/ADP transporter mutant, supposedly to compensate for reduced ATP import into plastids (Reiser et al. 2004). Induction of a PK_c is also not unreasonable. In seeds of *pkp1* PK_c activity is increased relative to wild type (Figure 3.4).

Addition of an exogenous carbon source does not rescue the germination defect of *pkp1*. In fact, increasing the amount of sucrose actually inhibits germination (Figure 4.3). It was not tested whether this is a result of osmotic potential in the medium. The oil deficient *wri1* and *triacylglycerol1* (*tag1*) mutants have a similar phenotype but in those cases it was likened to heightened sensitivity to osmolarity (Cernac et al. 2006, Lu and Hills 2002). However, *pkp1* differs from these mutants in that there is 50% reduction in

PK_p activity at the time when nearly 100% of wild-type seeds have germinated (Figure 4.2C). Pyruvate kinase activity in germinating *wri1* seeds is no different from wild type (Cernac et al. 2006). Therefore it is possible that in *pkp1* an accumulation of carbohydrates in the seedling brought on by a reduction in glycolytic flux is responsible for increased sensitivity to sugar in the medium. The incomplete rescue of root elongation by sugar application bolsters the idea that *pkp1* is less capable of metabolizing sucrose (Figure 4.1).

Storage compound utilization is dependent on PK_p either in seeds or seedlings

Seedling establishment and hypocotyl elongation in the dark are driven by the catabolism of seed storage oil. Arabidopsis mutants defective in β-oxidation (Footitt et al. 2002, Germain et al. 2001) fail to establish unless provided with an exogenous carbon source such as sucrose. Similar phenotypes are observed in glyoxylate cycle and gluconeogenesis mutants which are unable to convert storage reserves into carbohydrates (Cornah et al. 2004, Eastmond et al. 2000, Penfield et al. 2004, Rylott et al. 2003). The *pkp1* mutant has 60% less seed oil than wild type and does not elongate its hypocotyls in the absence of sucrose (Figure 4.4A, 4.4B). In contrast to the β-oxidation, glyoxylate cycle, and gluconeogenesis mutants, exogenous sucrose does not rescue this phenotype. Without sucrose PK_p activity is only marginally reduced in *pkp1* and hypocotyl elongation is likely inhibited due to a lack of storage reserves (Figure 4.4D). However, on 2% sucrose PK_p activity is significantly reduced and this may contribute to the apparent inability to utilize the supplied sugar (Figure 4.4C). It could also be that *pkp1* cannot

synthesize the membrane lipids necessary for expansive growth, which supported by the fact that the mol% of 18:3 does not increase in *pkp1* germinating seeds (Figure 4.5B).

The *pkp1* mutant does not efficiently metabolize its seed oil reserves (Figure 4.5A) and this could also cause faulty seedling establishment and hypocotyl elongation. Storage lipid metabolism is not completely restored in the $R\beta_1$-23 and $R\beta_2$-3 rescued lines and this is correlated with incomplete rescue of PK_p activity (Figure 4.2C). Since PK_p is not directly involved in any metabolisms associated with storage lipid breakdown another explanation for this phenotype was sought. Accelerated aging treatment followed by germination assays revealed that *pkp1* viability is severely compromised over time (Figure 4.6). This result together with the storage lipid breakdown defect is consistent with a reduction in seed tocopherol (Sattler et al. 2004). Tocopherol is synthesized in the plastid and uses phytyldiphosphate (phytyl-PP) as a precursor (Collakova and DellaPenna 2001). Phytyl-PP is synthesized exclusively in the plastid as a downsteam product of the methylerythritol-4-phosphate (MEP) pathway and as a salvage product released during chlorophyll degradation (Ischebeck et al. 2006). Developing seeds of *pkp1* expectedly have lower flux through PK_p and therefore less Pyr for entry into the MEP pathway. Additionally, seed chlorophyll content is drastically reduced in developing seeds possibly to do a lack of phytol (see Table 3.2). Moreover, the *cloroplastos alterado* (*cla1*) mutant of Arabidopsis, deficient in 1-deoxy-D-xylulose-5-phosphate synthase which catalyzes the first step of the MEP pathway (using Pyr as one substrate) has retarded germination and reduced tocopherol content at least in leaves (Estevez et al. 2001). The combined data are in agreement with reduced flux through the MEP pathway leading to lower tocopherol content in *pkp1* seeds. Most interestingly, $R\beta_1$-23 appears to be completely

resistant to the accelerated aging treatment employed, suggesting an increase in seed tocopherols (Figure 4.6). However, seed PK_p activity and chlorophyll content are not higher than wild type in this line (see Chapter 3). An increase in tocopherol could potentially be explained by altered timing of PK_p activity in these seeds. Typically, PK_p activity peaks just prior the maximum rate of oil biosynthesis. Tocopherol is synthesized later, once all of the oil has been deposited and seeds begin to degrade and recycle chlorophyll (Valentin et al. 2006). Expression of the PK_p-β_1-encoding gene in $R\beta_1$-23 is driven by the constitutive CaMV 35S promoter and it is possible that spurious PK_p activity late during development leads to elevated tocopherol biosynthesis.

PK_p activity influences chlorophyll biosynthesis and sugar accumulation in leaves

The effects of the *pkp1* mutation are evident in many aspects of whole plant morphology and physiology. The establishment and growth phenotypes of *pkp1* could not be rescued by the application of aromatic amino acids, alanine, branched chain amino acids, or with combinations of those (data not shown). Therefore, impaired amino acid biosynthesis brought on by altered PEP and pyruvate metabolism seems not to be responsible for the *pkp1* phenotypes. Leaves of *pkp1* contain 30 to 60% less chlorophyll than wild type, depending of if they were grown on soil or on agar plates (Table 4.1). As with seeds, reduced PK_p activity (Figure 4.8B) in leaves and resultant impairment of isoprenoid biosynthesis could explain this. Again, the *cla1* mutant supports this hypothesis in that it too has reduced chlorophyll content in leaves (Mandel et al. 1996). Increased anthocyanins in *pkp1* (Table 4.1) may also be related to PK_p activity as PEP is a substrate for the Shikimic acid pathway which gives rise to precursors for anthocyanin biosynthesis.

Unlike seeds, hexose content is reduced in *pkp1* leaves (Figure 4.10A) and likely has no role in repression of chlorophyll biosynthesis. Instead, reduced photosynthesis due to a lack of chlorophyll might be the reason for reduced glucose and fructose. Photosynthetic limitations could also explain the dwindled biomass of *pkp1* (Figure 4.7A). Sucrose and starch appear to be unaffected in *pkp1* suggesting that any problems with carbohydrate metabolism are localized in or around glycolysis (Figure 4.10B, 4.10C). Altered sugar levels would be expected to result in additional pleiotropic effects as 30 to 50% of Arabidopsis genes are at least partially transcriptionally regulated by sugar concentration (Blasing et al. 2005). It is interesting that lipid content and composition is roughly the same in *pkp1* as in wild type. It remains to be determined if *pkp1* has a lower rate of fatty acid synthesis, as is the case in developing seeds. In conclusion, the *pkp1* mutant has little in common with previously described tobacco plants lacking PK_c, thus detailing the unique roles of plastid- and cytosolic-localized PKs. Much work remains to be done to fully understand the role of PK_p in plant growth and development.

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Chapter 5

Conclusions and perspectives

Major Conclusions

This multifaceted study of Arabidopsis PK_p has confirmed its central location in the seed metabolic network (see Figure 1.2). Of two PK_p complexes, the one that is most active and least sensitive to feedback inhibition has been naturally selected for in seeds. Based on analysis of the *pkp1* mutant, one can conclude that at least 60% of the fatty acids in Arabidopsis seed oil are ultimately derived from a single PK_p (specifically $\alpha\beta_1$). Carbohydrates accumulated in place of 20% of the oil in *pkp1* seeds. Therefore, PK_p represents a regulatory step that controls carbon partitioning in developing embryos. Connectivity between the cytosol and plastid provided by metabolite transporters was insufficient for increased PK_c activity to fully compensate for the loss of PK_p. This result implies that pyruvate is not a major metabolite that is transported across the plastid envelope in seed tissue. Remaining questions and directions for future research will be discussed in the forthcoming sections.

Some additional questions

The now *bona fide* PK_ps described here account for only a fraction of the annotated PK-encoding genes in Arabidopsis. Phylogenetic analysis suggested the presence of one other plastid targeted PK (At3g49160; Figure 2.1A), but pilot experiments with recombinant protein were unable to detect any catalytic activity. The question remains whether the enzymes used in this study are the only PK_ps in Arabidopsis. If so, the presence of 10 genes encoding PK_cs implies either great redundancy or the ability to very precisely regulate cytosolic glycolysis. In any case, the lack of redundancy for PK_p is surprising compared to the cytosolic enzymes.

The *pkp1* mutant still accumulates up to 40% wild-type seed oil and the source of carbon precursors for this residual TAG is unknown. Basal expression of PK_p-β_2 is one explanation. The contribution of the $\alpha\beta_2$ complex to seed oil biosynthesis could be tested by crossing *pkp1* with a null mutant of PK_p-β_2. It could be that metabolic redundancy is provided by PK_c, as evidenced by induction of its activity in *pkp1* seeds (Figure 3.4A). It is also possible that the PK bypasses discussed in Chapter 1 (Figure 1.3) compensate for the reduction in PK_p activity. Preliminary experiments detected no changes in the activities of PEP carboxylase, NAD-malate dehydrogenase, or NADP-malic enzyme in *pkp1*, but more accurate measurements are needed to confirm this. There is also the question of to what extent PK_p-β_2 can function in place of PK_p-β_1. The kinetic parameters of the $\alpha\beta_2$ complex (Table 2.2) and the inability of PK_p-β_2 overexpression to fully restore oil content in rescued *pkp1* lines (Figure 3.6B) suggests that PK_p-β_2 is not able to functionally replace PK_p-β_1. However, other aspects of plant growth and development seem to be restored in the 35S:PK_p-β_2-rescued *pkp1* lines. It seems that only in situations of very high rates of fatty acid synthesis is it advantageous to have $\alpha\beta_1$ as the dominant PK_p.

In this work, enzyme assays at pH 8.0 were used to enrich detection of PK_p activity. This method was useful in sensing total activity, but was not able to pinpoint the loss of a specific PK_p isoform in *pkp1*. Procedures used to separate and detect individual PKs were unsuccessful and so loss of a specific isoform had to be assumed. Perfection of a zymogram technique as was used for glucose-6-phosphate dehydrogenases would be useful here (see Appendix A). Analysis of the reconstituted enzymes also raised some questions that deserve attention. For instance, it was not tested whether a single complex

can contain both β_1- and β_2-subunits. A mixed-composition enzyme might have regulatory properties distinct from the $\alpha\beta_1$ and $\alpha\beta_2$ enzymes, as is the case with hybrids of mammalian PKs (Hubbard and Cardenas 1975). The association of the individual PK subunits is likely a dynamic process and the factors that control the strength of protein-protein interaction were not analyzed in much detail. It was observed that K^+ and PEP were required for co-immunoprecipitation of native PK_p complexes, suggesting that these are important for subunit association. However, the effects of pH and metabolite effectors, for example, were not examined. It could be that regulation of enzyme activity by certain metabolites is mediated by abrogation of subunit interaction.

Several phenotypes of the *pkp1* mutant require additional examination. The effects of reduced fatty acid synthesis are no doubt extended beyond TAG accumulation. Other lipids such as those making up the hydrophobic layer on the seed coat might also be affected. Preliminary staining of mature seeds suggested increased permeability in *pkp1*, thus warranting investigation of lipids other than TAG. The reduction in chlorophyll content in *pkp1* is also intriguing. Reduced chlorophyll in seeds is correlated with less extensive thylakoid membranes (Figure 3.3). It would be interesting to know if the situation in leaf chloroplasts is the same. Furthermore, the cause of reduced chlorophyll is still unknown. Is it a chlorophyll biosynthetic defect or is it the result of a lack of thylakoid membranes brought on by impaired fatty acid synthesis? Either is possible. Chlorophyll biosynthesis is likely not inhibited by sugar concentration in *pkp1* leaves, since hexoses are actually decreased relative to wild type (Figure 4.10). Several lines of evidence also hint at a lack of seed tocopherols in *pkp1*, but measurements have not yet been made. Both chlorophyll and tocopherol biosynthetic defects could arise from

reduced phytol synthesis, and so it would also be informative to measure flux through the methylerythritol-4-phosphate (MEP) pathway in plastids.

Future Directions

In addition to answering the immediate questions listed above, future research on PK_p should be focused on systems biology and metabolic engineering. The biochemical properties ($S_{0.5}$, V_{max}, I_{50}, K_a) obtained for the PK_p complexes could be incorporated into a kinetic model of seed metabolism. The details of many other enzymes in the network would be needed and this dissertation provides a framework for such characterizations. Once made, a kinetic model could be used to predict the effects of specific metabolic perturbations or enhancements. For example, the effects of having $\alpha\beta_1$ versus $\alpha\beta_2$ as the dominant seed PK_p could be predicted and then tested using *pkp1* and the respective rescued lines. A similar approach has been taken to direct the engineering of glycine betaine metabolism in tobacco (McNeil et al. 2001). Simple metabolic profiling of *pkp1* seeds and seedlings would also be informative. It is expected that the defect in central carbon metabolism results in pleiotropic effects that would be readily elucidated with this method. Developing Arabidopsis seeds have temporally distinct metabolic fingerprints (Fait et al. 2006) and it would be interesting to see if PK_p controls the balance of metabolites beyond what was presented in previous chapters. Metabolic flux analysis using stable isotopomers would also be informative for *pkp1*. This method could help determine any rerouting of metabolic fluxes that are not evident based on enzyme activity alone. For example, alternative routes of pyruvate production may be more active in *pkp1*, despite there being no changes in extractable enzyme activities. In general, a systems

biology approach to investigating metabolism in *pkp1* would be the most efficient for answering the obvious questions while at the same time discovering unexpected perturbations.

Increasing oil yield in crop plants is the main driving force for this research. As PK_p is a regulatory enzyme in this process, the next logical step is to increase PK_p activity in developing seeds. This was not possible by overexpression of either β-subunit-encoding cDNA, probably because the amount of α was limiting. Therefore, simultaneous overexpression of both α- and β-genes should be pursued. This can be achieved using stacked expression constructs with seed-specific promoters. Increasing PK_p activity in non-seed tissues would also be a worthwhile endeavor. Ectopic oil production is emerging as a means to greatly increase the yield of a single plant, as seeds have evolved to be at or near their maximum potential. Use of tissue specific or inducible promoters is one means of achieving this. Another way is to take advantage of endogenous regulatory networks. The WRINKLED1 (WRI1) transcription factor induces expression of at least PK_p-α and PK_p-β_1, possibly by binding directly to their promoters. Thus, overexpression of WRI1 in the desired tissues could be used to increase PK_p activity, with the advantage that WRI1 also regulates downstream components of fatty acid and TAG biosynthesis (Ruuska et al. 2002). A similar approach was taken to stimulate nitrogen assimilation in maize (*Zea mays*). Ectopic production of the *Zm*Dof1 transcription factor (normally involved in light response) was used to activate anaplerotic carbon metabolism and resulted in a 30% improvement in nitrogen assimilation (Yanagisawa et al. 2004). Increased PK_p activity could also be used for the production of useful secondary metabolites. However, the range of metabolic influence of PK_p is not

fully known and there is (weak) precedent only for the engineering of plastid localized isoprenoid metabolism.

Another approach to modulating PK_p activity is to engineer the enzyme itself. Targeting of specific residues for increased activity or altered regulation would be greatly facilitated by a crystal structure for the PK_p in question. The crystal structure of a mammalian PK has been determined (Muirhead et al. 1986) and it was used to direct mutagenesis for the conversion of a non-allosteric PK into an allosteric enzyme (Ikeda et al. 1997). Comparison of (preferably) crystal structures or the primary sequences of $\alpha\beta_1$ and $\alpha\beta_2$ to each other and to additional plant PKs could indicate which amino acids are good targets for modification. A non-targeted approach such as directed evolution using error-prone PCR and DNA shuffling could also yield similarly modified enzymes. Error prone PCR introduces random mutations and resultant protein libraries must then be screened for the desired qualities. In DNA shuffling, PCR is used to randomly combine domains from different genes to generate a library of chimeras which must then be screened. These procedures have been very successful for improving the biodegradation pathways of microorganisms (Parales and Ditty 2005). Pyruvate kinases have distinct domains for substrate and effector binding (Munoz and Ponce 2003) and combination of these from diverse enzymes could prove fruitful. A non-mutagenic approach to altering PK_p activity would be to mix whole subunits from various organisms. In this work it was determined that the presence of either β-subunit influences the biochemical properties of the respective PK_p complexes. It is therefore possible that hybrid enzymes with novel kinetics and regulation could be constructed by taking advantage of natural variation. This method has been successful for the assembly of a hybrid ADP-glucose

pyrophosphorylase out of Arabidopsis and potato subunits (Ventriglia et al. 2007). With so many options for the engineering of PK_p enzymes, one should carefully weigh the benefits and pitfalls of each. Targeted mutagenesis involves the least amount of work, but would require inferences to be drawn from crystal structures which have not yet been determined for any plant PK. Directed evolution has a high potential for success, but the outcomes are less predictable and are entirely dependent on the screening method used to analyze the mutant proteins (which should number in the thousands). Combining subunits from various organisms takes advantage of existing enzyme diversity, but one must obtain the purified subunits to perform any experiments. In conclusion, I feel that future research on Arabidopsis PK_p should be from multiple directions and should involve collaboration with experts in various fields. Eventually, what is learned needs to be applied to a real crop plant so that humanity can benefit from their investment in science.

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Appendix A

Analysis of glucose-6-phosphate dehydrogenase activity in Arabidopsis T-DNA insertion mutants[3]

[3] This work was done in collaboration with Dr. Setsuko Wakao and is being prepared for publication in: Wakao, S., Andre, C., and Benning C. (2007) Functional analyses of cytosolic G6PDHs and their contribution to seed oil accumulation in Arabidopsis. Plant Physiol. I contributed Figures A.1D, A.2C, and all of A.3.

Introduction

Glucose-6-phosphate dehydrogenase (G6PDH) is one of the two NADPH generating enzymes of the oxidative pentose phosphate pathway (OPPP). All eukaryotic G6PDHs studied are feed-back inhibited by NADPH, and together with the fact that this enzyme catalyzes a committed step makes G6PDH the regulatory enzyme of the OPPP.
In addition to the feed-back inhibition by NADPH, the plastidic isoforms of G6PDH in plants and algae are subject to regulation by the thioredoxin/ferredoxin system (Graeve et al. 1994, Lendzian 1980, Scheibe and Anderson 1981, Wenderoth et al. 1997, Wendt et al. 2000). Hence, they are presumed to act as cellular redox sensors and are inactivated to prevent unnecessary oxidation of carbon when photosynthesis is sufficient for NADPH generation.

In plants G6PDH has been frequently described in connection to its involvement in nitrogen assimilation. The induction of its activity or transcript has been described in various systems including pea roots (Bowsher et al. 1992), barley roots (Wright et al. 1997), maize roots (Redinbaugh and Campbell 1998), tobacco roots and leaves (Debnam et al. 2004) and Arabidopsis (Wang et al. 2003). G6PDH activity is necessary to supply the reducing equivalents required for nitrogen assimilation in root cells that lack photosynthesis (Bowsher et al. 1992, Esposito et al. 2001, Esposito et al. 2003, Jin et al. 1998, Wright et al. 1997). Aside from nitrogen assimilation, G6PDH has been hypothesized to be an important source of NADPH in other non-photosynthetic tissues (Emes and Neuhaus 1997) and in those that synthesize large quantities of fatty acids (for incorporation into triacylglycerol), such as pollen (Niewiadomski et al. 2005) and oil seeds (Eastmond and Rawsthorne 1998). Green oil seeds such as those of canola

(*Brassica napus*) contain plastids similar to those of shade-adapted leaves (Asokanthan et al. 1997) and are capable of photosynthetic NADPH production, however, only 20-30% of ambient light penetrates the silique walls and reaches the embryo (Eastmond et al. 1996, King et al. 1998). Reported O_2 evolution rates from canola embryos were used to calculate that all of the NADPH required for fatty acid synthesis could be provided by photosynthesis (Ruuska et al. 2004). However, in controlled experiments only a 25% decrease in fatty acid synthesis is observed in the dark. Thus, 75% of the required NADPH could be generated by other reactions such as those of the OPPP. *In vivo* stable isotope labeling revealed that a maximum 25% to 45% of the reductant required for oil biosynthesis could come from the OPPP (Schwender et al. 2003). A recent report of G6PDHs from Arabidopsis reported the isolation of T-DNA insertion mutants for both cytosolic isoforms (Wakao and Benning 2005). Here, I examined the effects of these mutations on total G6PDH activity in relation to seed oil biosynthesis.

Materials and Methods

Plant growth conditions and transformation

All seeds were surface sterilized by incubating in 20% bleach, 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 MS medium (pH 5.8) (Murashige and Skoog 1962) with 1% sucrose, 0.9% agar and were transferred to soil after 9 days. Wild-type and mutant Arabidopsis plants were prepared for transformation as previously described (Cernac and Benning 2004).

Transient expression of G6PDH::GFP for subcellular localization analysis

The coding region of G6PD5 and G6PD6 were amplified with the following primers; for G6PD5, (+) 5'-GGACTAGTATGGGTTCTGGTCAATGGCA, (-) 5' GGACTAGTCAATGTAGGAGGGATCCAAA, and for G6PD6, (+) 5'-GGACTAGTATGGGATCTGGTCAATGGCA, (-) 5'-GGACTAGTTAGTGTAGGAGGGATCCAG. The cDNAs were cloned into the *Spe*I site of pCAMBIA1302 (Genbank accession no. AF234298). Onion epidermal peels were bombarded following the methods previously described (Varagona et al. 1992) using 1100 psi (pounds per square inch) rupture discs at ~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, Wetzlar, Germany).

Construction of complementation vectors for G6PD5 and G6PD6

The T-DNA insertion lines were transformed with BAC clones containing the regions of G6PD5 and G6PD6 that were isolated from a genomic library in a cosmid vector (pBIC20) (Meyer et al. 1994). The 3'-UTR of the respective genes was used 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

Protein extraction from various tissues and electrophoresis on cellulose acetate plates (zymogram) were performed as described previously (Wakao and Benning 2005). Roughly 8 volumes (v/w) of extaction buffer were used per sample for homogenization. Liquid assay of G6PDH was performed as described previously (Wakao and Benning 2005). For identification of the middle band, intact plastids were isolated from 5 week old wild-type Arabidopsis 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).

Results and Discussion

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 green fluorescence protein (GFP) gene. For both constructs containing G6PD5 and G6PD6, the green fluorescence was observed dispersed in the cytosol and surrounding what is presumably the nucleus (Figure A.1A, A1.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 (Figure A.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 G6PDH isoforms *in vivo*, G6PD5, G6PD6 and an unidentified isoform that is ubiquitous (Wakao and Benning 2005). To help 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 A.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 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.

To specifically examine the *in vivo* roles of the cytosolic G6PDHs T-DNA insertion lines for the two genes were obtained from the SALK institute. Their insertion sites were identified using PCR as previously described (Figure A2.B and Wakao and Benning 2005).

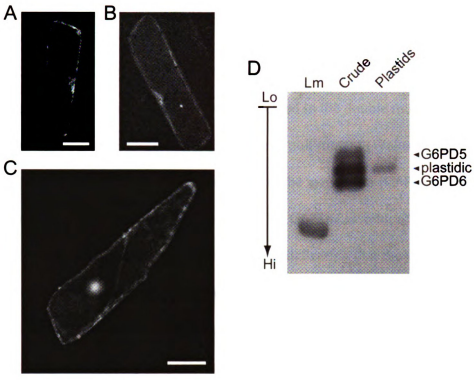

Figure A.1. Cytosolic localization of G6PD5 and G6PD6

(A-C) Onion cells were bombarded with either with pCAMBIA1302 inserted with (A) G6PD5 or (B) G6PD6 coding sequence or (C) the vector alone.

(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. Lm, standard G6PDH from *Leuconostoc mesenteroides*.

The single mutants did not have any obvious morphological phenotypes (Figure A.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. G6PDH activity was examined in plants of different

genotypes using zymograms of bud protein extracts (Figure A.2C). In each single mutant, *g6pd5* and *g6pd6*, a band is lost from the zymogram. In the double mutant, both G6PD5 and G6PD6 were lost. Interestingly, activity from the plastidic isoform is reduced in all of the mutants. Residual activity of G6PD5 was observed in the double mutant, consistent with previous observations indicating *g6pd5* is not a null mutant (Wakao and Benning 2005). In the *g6pd6* mutant, the uppermost band on the zymogram (G6PD5) is intensified relative to wild type. It is possible that an alternative G6PDH is induced in this mutant. However, in the double mutant this induction is lost indicating that the upper band in the *g6pd6* zymogram is indeed G6PD5. Surprisingly the double mutant was also indistinguishable from the WT plant, 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 normal conditions. Attempts to complement the single and double mutants with a cDNA or G6PDH fused to GFP at the N-terminal have been 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 (Figure A.2C). This result together with the zymogram pattern of the double mutant proves the previously defined zymogram bands (Wakao and Benning 2005) were indeed coded by *G6PD5* and *G6PD6*.

Seed triacylglycerol (TAG) was quantified to determine the effects, if any, of loss of one or both cytosolic G6PDHs. The single mutants had no changes in seed oil content. The double mutant had a small, albeit significant, increase in TAG (data not shown) which was associated with an increase in seed mass and not specifically oil accumulation.

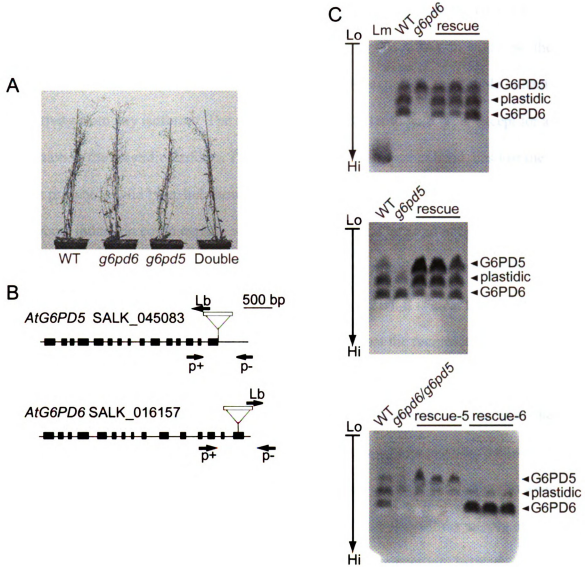

Figure A.2. Single and double mutants for G6PD5 and G6PD6

(A) Morphological phenotypes of the single and double mutants.

(B) Gene structure of G6PD5, G6PD6 and T-DNA insertion sites. Primers used for genotyping PCR were designed as shown.

(C) G6PDH zymograms of single and double mutants and lines rescued by Cosmid complementation. The arrow indicates origin and direction of electrophoresis. Lm, standard G6PDH from *Leuconostoc mesenteroides*.

To better understand the increase in seed mass for the double mutant the G6PDH isoforms present in developing seeds were examined (Figure A.3A). In wild type, the dominant isoforms are G6PD6 and the plastidic one. As expected, the double mutant has no activity from any isoforms. The *g6pd5* mutant is similar to wild type, except for a reduction in the plastidic isoform. The *g6pd6* mutant, on the other hand, has lost the bands present in wild type, but shows a reciprocal increase in G6PD5 activity. This reciprocal induction could explain why only the double mutant and not *g6pd6* has a seed mass phenotype. Liquid enzyme assays were performed with the same extracts to quantify any changes in total G6PDH activity (Figure A.3B). It is clear that G6PDH activity is only compromised in the double mutant and that the reciprocal induction of G6PD5 makes up for the loss of G6PD6 in the *g6pd6* mutant.

The unique attributes of plant OPPP, such as dual localization, complicate the interpretation of G6PDHs role in whole cells. The discovery of a plastidic pentose-phosphate transporter (Eicks et al. 2002) demonstrated a physical connection between plastidic and cytosolic pentose phosphate pathway but how the cellular supply of NADPH is coordinated remains unknown. Additionally, connectivity between biochemical pathways makes interpretation difficult. Overlap of intermediates with other metabolic pathways such as glycolysis, the TCA cycle, and amino acid and nucleotide biosynthesis makes it difficult to discern whether the effect is primarily due to reduced OPPP flux, NADPH supply, or something else. We speculate that in the double mutant but not in the single mutants (because of the G6PDH activity compensation) there is altered carbon metabolism and one of the effects is larger seeds.

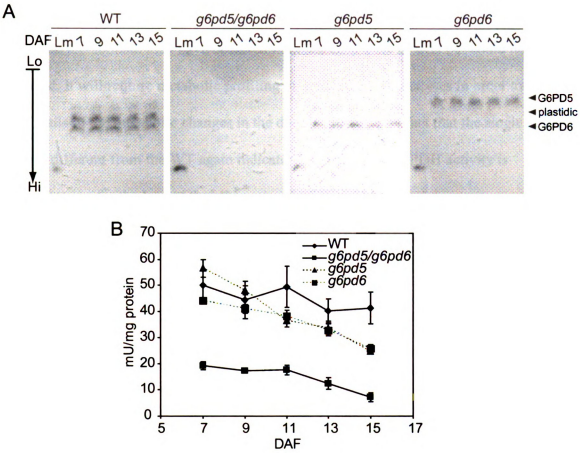

Figure A.3. G6PDH isoforms and activity in developing siliques

(A) Zymograms of protein extracts from staged siliques of the various geneotypes. An induction of the normally inactive G6PD5 is evident for the *g6pd6* mutant. The arrow indicates origin and direction of electrophoresis. Days after flowering (DAF) is indicated for each sample. Lm, standard G6PDH from *Leuconostoc mesenteroides*.

(B) Liquid enzyme assays show that only in the double mutant is G6PDH activity reduced. DAF, days after flowering.

It is possible that glycolytic flux is increased in the double mutant, since glucose-6-phosphate is not consumed by OPPP, further fueling fatty acid synthesis. Such alteration in carbon metabolism may have occurred in non-seed tissues as well but could not be detected. It will require metabolic profiling or metabolic flux analyses in order to study the details of the metabolic changes in the double mutant. The fact that the single mutants are not different from the WT again indicates the remaining G6PDH activity is compensating for the loss of the other.

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Appendix B

Analysis of glycolytic enzyme activities in seedlings of the *wrinkled1* mutant of Arabidopsis[4]

[4] This work was done in collaboration with Dr. Alex Cernac and portions have been published in: Cernac, A., Andre, C., Hoffman-Benning, S., and Benning, C. (2006) WRI1 is required for seed germination and seedling establishment. Plant Physiol. 141:745-757. I contributed Figure B.2.

Introduction

Seed germination in Arabidopsis initiates with the matrix-driven absorption of water, followed by cell expansion, splitting of the seed coat, and subsequent emergence of the radicle. The mobilization and metabolism of seed triacylglycerol (TAG) is an intricate but well-characterized process and has been reviewed recently (Penfield et al. 2005). Plants with reduced TAG mobilization have been identified, including those affected in lipid trafficking and β-oxidation (Footitt et al. 2002, Germain et al. 2001, Hayashi et al. 1998, Lawand et al. 2002, Zolman et al. 2001). The role of the glyoxylate cycle involved in the conversion of lipids into carbohydrates has also been examined through a series of mutants (Cornah et al. 2004, Eastmond et al. 2000, Hayashi et al. 2005), as has gluconeogenesis itself (Penfield et al. 2004, Rylott et al. 2003). Common phenotypes, many of which can be rescued by sugar supplementation or appropriate photosynthetic conditions, include a reduced germination rate, arrest of development after germination, inability to elongate the hypocotyl in the dark, and failure to elongate the root after opening and greening of the cotyledons. Based on the analysis of these mutants a picture has emerged suggesting that in Arabidopsis the energy for germination is derived from stored reserves other than lipids, and that seed oil becomes vital for continued growth and seedling establishment after the radicle has emerged.

The *wrinkled1* (*wri1*) mutant of Arabidopsis was originally isolated based on its low seed oil content and developing *wri1* seeds showed reduced activity of key glycolytic enzymes such as hexokinase (HXK) and pyruvate kinase (PK, Focks and Benning 1998). *WRI1* was subsequently shown to encode an APETALA2/ethylene-responsive element-binding transcription factor (Cernac and Benning 2004).

Microarray analysis of *wri1-1* and wild-type developing seeds indicated a global down-regulation of transcripts encoding enzymes involved in carbohydrate metabolism in the mutant (Ruuska et al. 2002) consistent with a role for WRI1 in the regulation of sugar metabolism. Further support for the function of WRI1 was provided when *WRI1* was recently identified in an activation-tagging screen targeting genes for which a strong expression resulted in increased transcription from a known sugar-inducible promoter (Masaki et al. 2005). The ectopic expression of the *WRI1* cDNA caused a sugar-inducible accumulation of seed oil in the transgenic seedlings, which is correlated with increased transcript amount of HXK and PK encoding genes. The general appearance of the transgenic seedlings suggested a resumption of embryonic development following germination. The overall conclusion was that the *WRI1* gene product is involved in controlling the phase of embryo maturation in which TAG accumulates, possibly by regulating the expression of specific HXK and PK encoding genes (Cernac and Benning 2004). Seed germination and establishment are also impaired in *wri1* (Cernac et al. 2006). Establishment of *wri1* seedlings is dependent on a supply of sucrose and root ultrastructure is abnormal. As the *WRI1* gene is expressed in seedling roots, we wanted to determine if misregulation of HXK and PK are responsible for the observed morphological differences between wild type and *wri1*.

Materials and Methods

Growth of seedlings for root elongation and enzyme assays

Root elongation rate assays were carried out on half-strength Murashige and Skoog medium. Seeds were sown in a straight line, 15 to 20 for wild type and rescued lines and 30 to 50 for wri1-1. Seedling root length was measured every 24 h starting on the 4th d post incubation through the 12th d. For RNA and protein extraction seeds were sown as they were for root elongation except 15 x 150 mm plates were used. The seeds were stratified as above and the plates incubated vertically for 11 d. Entire *wri1-1* seedlings were harvested for the 0 mM Suc treatment. Roots and shoots were separated by cutting along the line of plants at the base of the hypocotyl. Harvested material was wrapped in aluminum packets, weighed, and frozen in liquid nitrogen.

Protein extraction and enzyme assays

Approximately 100 mg of tissue was used for each protein extraction. Tissue was ground frozen using mortar and pestle and transferred to 1.5 mL tubes. Protein for enzyme assays was extracted as described previously except that bovine serum albumin was omitted from the extraction buffer (Focks and Benning 1998). Protein extracts were used immediately for enzyme assays in a double-beam spectrophotometer (Uvicon 930, Kontron Instruments) equipped with a cell changer (model 900, Kontron). All reagents were from Sigma-Aldrich. Pyruvate kinase was assayed in 1 mL total volume in a reaction mix consisting of 50 mM MOPS pH 7.0, 5% PEG-8000, 50 mM KCl, 15 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM phosphoenolpyruvate, 1 mM ADP, 0.2 mM NADH, and 2 units of desalted lactate dehydrogenase. Pyruvate kinase was also assayed at pH 8.0

by using 50 mM HEPES-KOH pH 8.0 instead of MOPS. The same trends were observed using both conditions. Correction for phosphoenolpyruvate phosphatase activity was carried out by the omission of ADP from the reaction mix. Glucokinase and Fructokinase were assayed as previously described, but in 1 mL total volume (Wiese et al. 1999). All reactions were done at 30°C, were initiated by the addition of protein extracts, and were linear for at least 5 min. Total protein in each extract was determined using the *DC* protein assay kit from Bio-Rad.

Results and Discussion

The poor establishment of *wri1-1* in medium lacking sugar is qualitatively shown in Figure B.1A and was quantified by measurements of root length from day 4 through day 12 post incubation (Figure B.1B). Without any sugar supplement, the *wri1-1* seedlings failed to establish and even after 12 d they did not significantly elongate their root or form true leaves. This phenotype was similar to that observed for *abi8*, *ntt2*, and *bou* mutants. The *abi8* mutant is defective in ABA signaling and allelic to *eld1* (Brocard-Gifford et al. 2004, Cheng et al. 2000), *bou* is deficient in a mitochondrial acyl carnitine transporter (Lawand et al. 2002), and *ntt2* is deficient in a plastidic ATP/ADP transporter (Reiser et al. 2004). While Suc did not enhance the germination frequency of *wri1-1* it did allow the seedlings to establish (Figure B.1A). As previously observed (Focks and Benning 1998), *wri1-1* plants grew similar to wild type on soil after they were raised on medium containing sugar. Expression of the *WRI1* cDNA in the transgenic lines was able to rescue the *wri1-1* root elongation defect almost to the level of wild type (Figure B.1B).

An impairment of sugar metabolism, i.e. glycolysis, in nonphotosynthetic tissues should have a detrimental effect on seedling establishment as the root is completely dependent upon the import and metabolism of sugar initially from the cotyledons breaking down lipids and converting them to sugars, and later from cotyledons and true leaves conducting photosynthesis. Thus, to better understand the physiological effects described above, we examined the activity of hexokinase (HXK) and pyruvate kinase (PK) in roots and shoots of 11-d-old developing seedlings of the different lines under investigation.

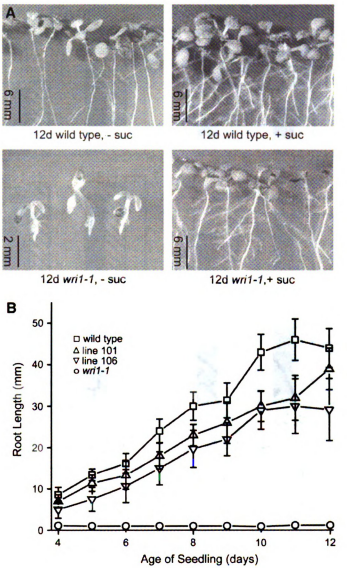

Figure B.1. Impaired seedling establishment in the *wri1-1* mutant

(A) Appearance of 12-d-old seedlings of wild type and *wri1-1* grown in the presence (+) or absence (–) of 50 mM Suc.

(B) Root growth on medium lacking Suc. The error bars represent SD of the mean of at least 15 root length measurements.

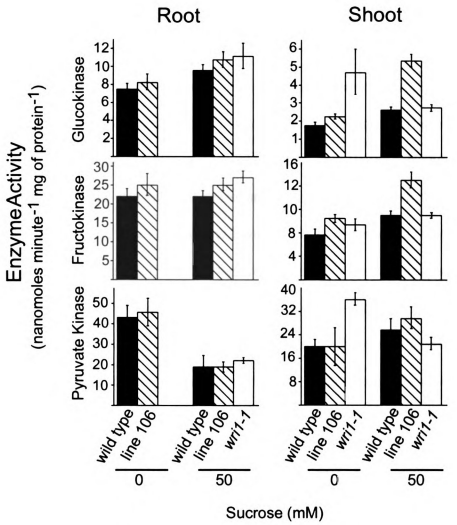

Figure B.2. Glycolytic enzyme activities in green seedlings *wri1-1*, wild type, and *WRI1* overexpressing seedlings in the presence or absence of sucrose. Values are the mean ± SD, n=5.

The seedlings shown in Figure B1.A are representative of the tissue that was used for enzyme activity measurements. The data obtained from liquid enzyme assays is depicted in Figure B2. In wild type, PK activity in roots grown on Suc was reduced. On the contrary, with Suc present the gluco- and fructokinase activities representing HXK were slightly increased. There were no gross differences between the three genotypes.

Based on these results we concluded that glycolysis is active in 11-d-old seedlings that have progressed through the establishment phase during which sugar supplementation could alleviate the deficiency in the *wri1-1* mutant. Therefore, WRI1 does not to seem to be directly involved in regulation of glycolysis once seedlings start growing during the process of establishment. However, what is presumably limiting during this phase of development in the mutant is the sparse amount of seed storage oil, which is reduced at least 80% in *wri1-1* seeds (Focks and Benning 1998). Offering Suc that can provide energy and building blocks at this stage completely restores seedling establishment (Figure B.1A). It seems that the role of WRI1 on seedling establishment is indirect, by way of its involvement in the up-regulation of carbohydrate metabolism in the developing seed thereby ensuring the presence of storage oil needed for seedling establishment.

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Appendix C

Leaf and seed lipid analysis of Arabidopsis mutants disrupted in Lipin genes

Introduction

The biochemical reactions involved in lipid homeostasis have been described, but the regulators of these processes are more elusive. Lipodystrophy is a human metabolic disorder which is characterized by the loss of body fat and concomitant deposition of fat in the liver (Reitman 2005). Mice carrying mutations in the fatty liver dystrophy (*fld*) gene have symptoms of lipodystrophy and serve as a model for the human disease. Positional cloning was used to identify the gene responsible for mouse *fld* (Peterfy et al. 2001). The gene, called *Lipin-1* (*Lpin1*) has homologues in all eukaryotic kingdoms. In mice *Lpin1* controls triacylglycerol (TAG) accumulation in adipose tissue. *Lpin1* knock outs have no TAG and do not develop mature functioning adipocytes, while overexpressors of *Lpin1* contain more TAG and are prone to obesity (Peterfy et al. 2005, Phan et al. 2004). A yeast lipin mutant is also compromised in TAG accumulation (Han et al. 2006). Combined, these phenotypes are consistent with a role for lipin in TAG biosynthesis.

The yeast and, more recently, the mammalian lipin genes have been shown to encode members of the Mg^{2+}-dependent phosphatidate (PA) phosphatase (PAP1) family (Donkor et al. 2007, Han et al. 2006). The generation of diacylglycerol from PA is a step in the synthesis of TAG in both the Kennedy and the phosphocholine:diacylglycerol acyltransferase pathways (outlined in Chapter 1). While lipin is required for TAG accumulation in yeast and mice, its contribution to TAG biosynthesis in plants has not been investigated. In addition, PA is recognized as a secondary messenger in plant (and other eukaryotic) signal transduction networks (Wang 2004), and an emerging role for PA is as a membrane lipid biosynthetic intermediate which is involved in the trafficking

of lipids from the endoplasmic reticulum to the plastid (Awai et al. 2006, Xu et al. 2005). The manipulation of PA levels through modulation of lipin gene expression provides an opportunity to explore the dynamics of such lipid signaling and trafficking. Moreover, the same mutants would provide a chance to investigate whether or not lipin plays a similar role in seeds as in adipose tissue, with respect to TAG synthesis. The relative ease of obtaining T-DNA insertion mutants for nearly any gene in the Arabidopsis genome provided enough impetus to pursue these questions.

Materials and Methods

Gene identification

Arabidopsis lipin homologues (At3g09560 and At5g42870; *LPN1* and *LPL1*, respectively) were identified using the deduced amino acid sequence of the *Mus musculus* *Lpin1* gene (accession NM_172950). Homologues were identified using the basic local alignment search tool (BLAST) available at the TAIR website (www.arabidopsis.org). Global gene expression data was mined from the AtGenExpress developmental database (Schmid et al. 2005).

T-DNA mutant isolation

Arabidopsis lipin mutants were selected from the SALK T-DNA insertion population (Alonso et al. 2003). Selection of mutants was on half-strength MS medium containing 25 $\mu g\ mL^{-1}$ Kanamycin. T-DNA insertion sites were determined by sequencing PCR products made using primers designed with the help of the i-sect tool available on the Salk Institute website (http://signal.salk.edu/tdnaprimers.html). Primers for *LPN1* were 5'-TgTTATTgTTCTCTAATTTTg-3' and 5'-gTTTTggTCAgCTCTgACTgC-3' and primers used for *LPL1* were 5'-gAATTCgCgCATAgTTgTgTC-3' and 5'-AACAAgCCCCgTATCTCCTgT-3'. The left border primer used was LBa1 as suggested by the SALK Institute website (http://signal.salk.edu/). PCR conditions used standard buffer conditions and 40 cycles of 95°C for 30 sec, 52°C for 40 sec, and 72°C for 90 sec, followed by a 10 min 72°C extension. All subsequent genotyping was done using the same PCR protocol.

Lipid analysis

Leaf samples (50-150 mg) were collected from individual plants and were weighed and frozen at -80°C. Lipids were extracted from leaves by vigorously shaking for 5 min in 500 µL of methanol/chloroform/formate (2:1:0.1, v/v). Then 250 µL of 1 M KCl, 0.2 M H_3PO_4 was added and the tubes were vortexed. The phases were separated by centrifugation at 16,000 g for 5 min. The organic phase was removed and loaded quantitatively onto a pre-treated (soaked in 0.15 M $(NH_4)_2SO_4$ and dried, then heated to 120°C for 2.5 hrs) silica-60 TLC plates (Baker). Lipids were developed in a solvent system of acetone/toluene/water (91:30:7, v/v) and were stained with iodine and α-naphthol. Fatty acid methyl ester analysis of leaf and seed lipids was done as previously described (Focks and Benning 1998). One small leaf or 10 seeds were used for each replicate.

Results and Discussion

Lipin homologue identification and analysis of gene expression

Arabidopsis lipin homologues were identified based on amino acid sequence similarity to *Mus musculus* Lpin1. Two genes were identified which encode Lpin1 homologues, At3g09560 and At5g42870. The At3g09560 gene encodes a protein most similar to Lpin1 (23% identical, 38% similar) and was named *lipin1* (*LPN1*). The other gene, At5g42870, encodes a protein less similar to Lpin1 (22% identical, 36% similar) and thus was named *lipin1-like* (*LPL1*). Lipin proteins contain a *DXDXT* motif characteristic of Mg^{2+}-dependent phosphatases. In the Lpin1 homologues from mammals, chicken, fish, *Caenorhabditis elegans, Drosophila, Ciona*, and *S. cerevisiae*, the motif is exactly DIDGT (Donkor et al. 2007). The Arabidopsis proteins vary only slightly and both contain a signature sequence of DVDGT. It is therefore likely that the Arabidopsis proteins are also Mg^{2+}-dependent phosphatases.

Publicly available Arabidopsis gene expression data reveals the tissue specific expression patterns for both *LPN1* and *LPL1* (Figure C.1). In general, *LPN1* is expressed at the same or higher level as *LPL1* in any given tissue. Both genes are induced during embryo development and have maximal expression just prior to the onset of TAG biosynthesis and accumulation. The gene expression data suggests that both *LPN1* and *LPL1* have roles in embryo development.

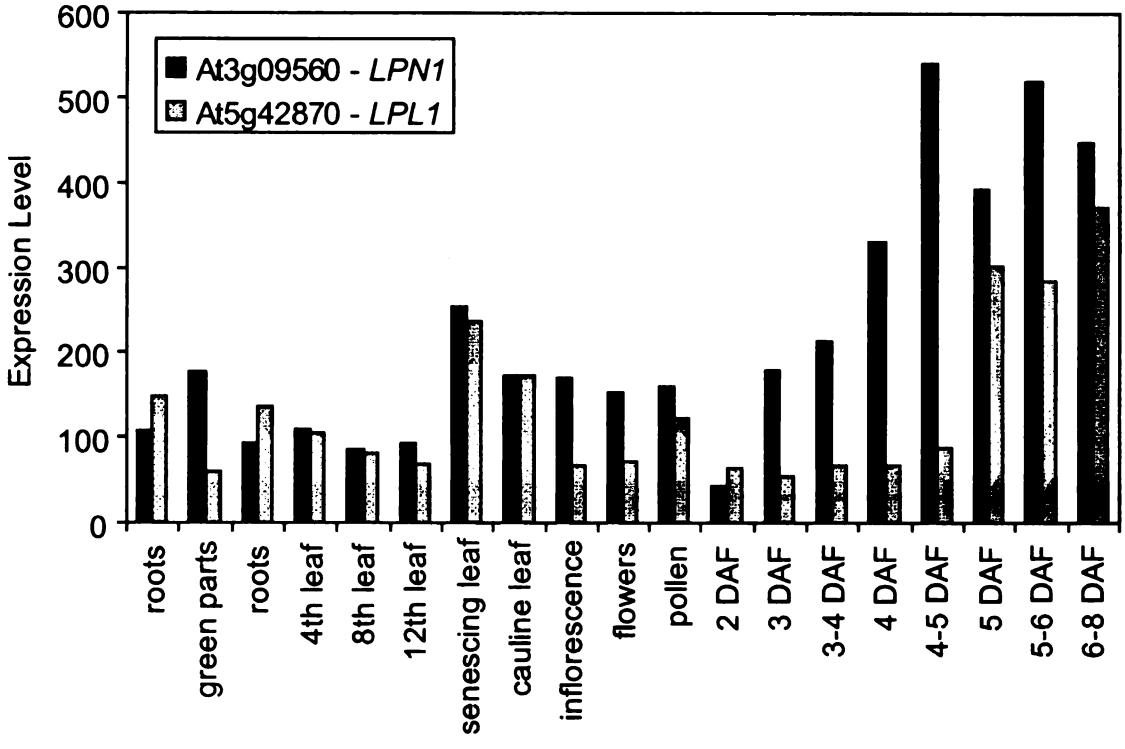

Figure C.1. Relative gene expression of putative Arabidopsis lipin homologues

Figure is derived from published microarray data (Schmid *et al.*, 2005). DAF, days after flowering.

T-DNA mutant isolation and lipid analysis

The Arabidopsis T-DNA mutant population was utilized to study the *in vitro* function of the Arabidopsis lipins (Alonso et al. 2003). Three lines were obtained which had inserts in the desired genes (Figure C.2). Genotyping of putative mutants was done with PCR as described in materials and methods and exact insertion sites were determined. As it turns out, SALK_146637 and SALK_042850, both located in *LPN1*, had identical insertion sites. For all three lines, the T-DNA was inserted into a translated portion of an exon which almost certainly causes reduced transcript abundance in homozygous lines. However, it remains to be tested whether or not transcription is actually affected by these

insertions. There were no obvious morphological or developmental differences between the single mutants and wild type. A double mutant was generated by crossing *lpn1* and *lpl1* and it too was wild-type in appearance.

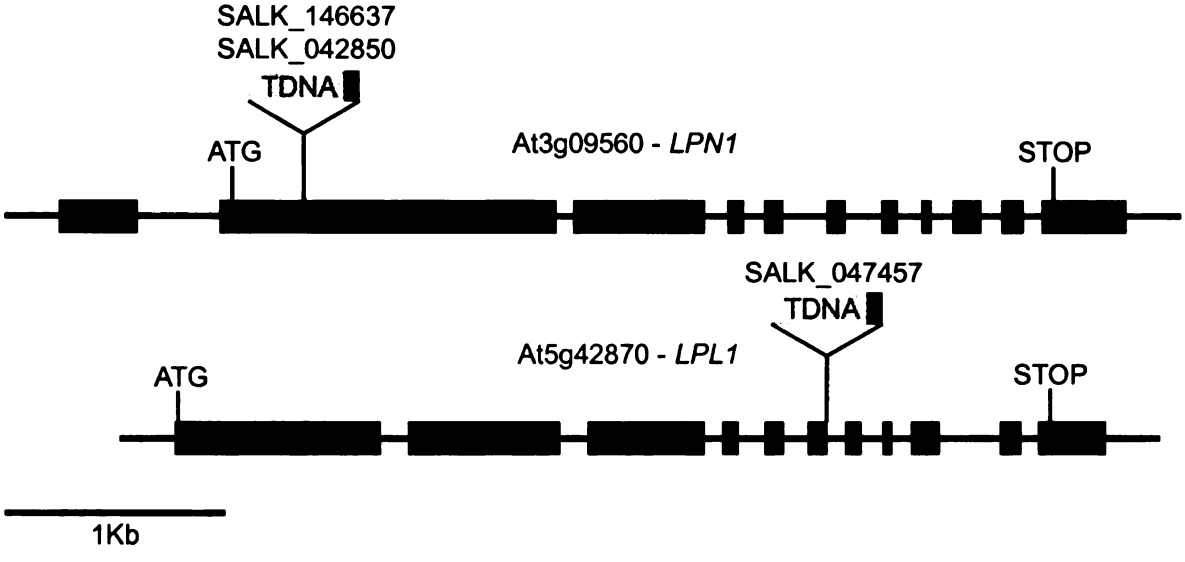

Figure C.2. Gene structure of the two Arabidopsis *Lpin1* homologues

Locations of the T-DNA insertions are indicated. Black box on T-DNA is the left border. ATG, start codon; STOP, stop codon.

Lipid analysis was carried out for the individual lipin mutants as well as for the double mutant, despite not knowing the degree of mRNA reduction in any of the lines. First, total leaf lipids were extracted quantitatively and analyzed by thin layer chromatography (TLC). Figure C.3A shows the results of this experiment. Two staining methods were used on the same plate to maximize the number of lipids detected. Membrane galacto- and phospholipids dominate the stained TLC plate. It is clear that there are no gross differences in the lipid composition between wild type and either the single or double mutants.

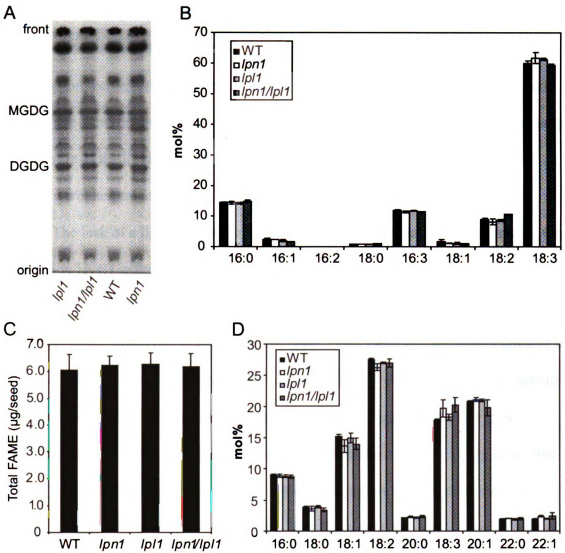

Figure C.3. Lipid analysis of Arabidopsis lipin single and double mutants

(A) Thin layer chromatogram of total leaf lipids stained with iodine and a sugar-sensitive stain. DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol.

(B) Fatty acid composition of total leaf lipids. Single leaves were subjected to fatty acid methyl ester analysis using gas chromatography. WT, wild type.

(C) Seed oil content of wild type (WT) and lipin single and double mutants as determined by fatty acid methyl ester (FAME) analysis.

(D) Fatty acid profile from seeds used for oil content determinations in (C).

The fatty acid profiles of total lipids from leaves were determined as well (Figure C.3B). Again, the mutants and wild type were remarkably similar. Seed oil content and composition were examined in mature seeds of the lipin mutants. As shown in Figure C.3C, there were no differences observed between any of the lines. And again, the fatty acid profile of the lipids in questions was determined, and again, the wild type and mutant were the same (Figure C.3D).

The lack of a lipid phenotype for these mutant lines is not totally surprising. Gene expression levels in the mutants were not analyzed and it is possible that the T-DNA insertions have no affect on mRNA or protein abundance. A simple RT-PCR or RNA blot experiment would begin to address this issue. Also, there was no confirmation of the encoded proteins' putative catalytic function. Nonetheless, even if the genes in question do encode active Mg^{2+}-dependent phosphatidate phosphatases, and even if gene expression is completely knocked out, the mutants might not have an easily detectable phenotype. Phosphatidate occurs at very low concentrations in plant cells and is not readily measured by the methods used here. Any changes in the steady state amount of PA would have to be detected by other means. Furthermore, the fatty acid profile data in Figures C.3B and C.3D is of total lipids and does not take into account any changes at the level of individual lipid species (*i.e.* PG, MGDG, DGDG). Beyond these obvious methodological shortcomings, the flexibility of plant metabolism could also account for the apparent lack of aberration in the lipin mutants. For instance, certainly LPN1 and LPL1 are not the only PA phosphatases in Arabidopsis. Future studies of lipin in Arabidopsis should be focused on mutants with documented reductions in gene expression in combination with other lipid metabolism mutants, such as TGD1-3.

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Appendix D

In vitro substrate specificity of the *Rhodobacter sphaeroides* betaine lipid biosynthetic enzyme BtaA[5]

[5] This work was done in collaboration with Dr. Wayne Riekhof and has been published in: Riekhof, W.R.., Andre, C., and Benning, C. (2005) Two enzymes, BtaA and BtaB, are sufficient for betaine lipid biosynthesis in bacteria. Arch. Biochem. Biophys. 441:96-105. I contributed Figure D.2.

`Introduction

The sessile lifestyle of many plants, bacteria, and fungi has led to the development of complex means of coping with specific mineral deficiencies. For instance, the replacement of abundant cellular phospholipids with alternative, non-phosphorous lipids is one means of dealing with environmental phosphate limitation. This phenomenon has been the most extensively documented for the α-proteobacteria *Rhodobacter sphaeroides* and *Sinorhizobium meliloti*, which synthesize the betaine lipid diacylglyceryl-*N,N,N*-trimethylhomoserine (DGTS) and ornithine containing lipids to replace depleted membrane phospholipids (Benning et al. 1995, Geiger et al. 1999).

DGTS was first discovered in a unicellular alga (Brown and Elovson 1974), but has since been found in lower plants and fungi (Kunzler and Eichenberger 1997, Rozentsvet et al. 2000). The structure and zwitterionic nature (Figure D.1A) of DGTS led to the hypothesis that it replaces phosphatydylcholine in the organisms in which it occurs. The use of radioisotope labeling in *Chlamydomonas reinhardtii* and in *R. sphaeroides* led to the identification of methionine as the biosynthetic precursor for the homoserine moiety and the adjoining methyl groups (Hofmann and Eichenberger 1996, Sato 1988). Subsequently, a genetic study using *R. sphaeroides* resulted in the identification of a two gene operon responsible for DGTS biosynthesis during phosphate starvation (Klug and Benning 2001). The two genes, designated *btaA* and *btaB*, respectively encode a proposed AdoMet/diacylglycerol 3-amino-3-carboxypropyl transferase producing the intermediate DGHS, and a putative methyltransferase adding three methyl units to the amino group of DGHS to form DGTS.

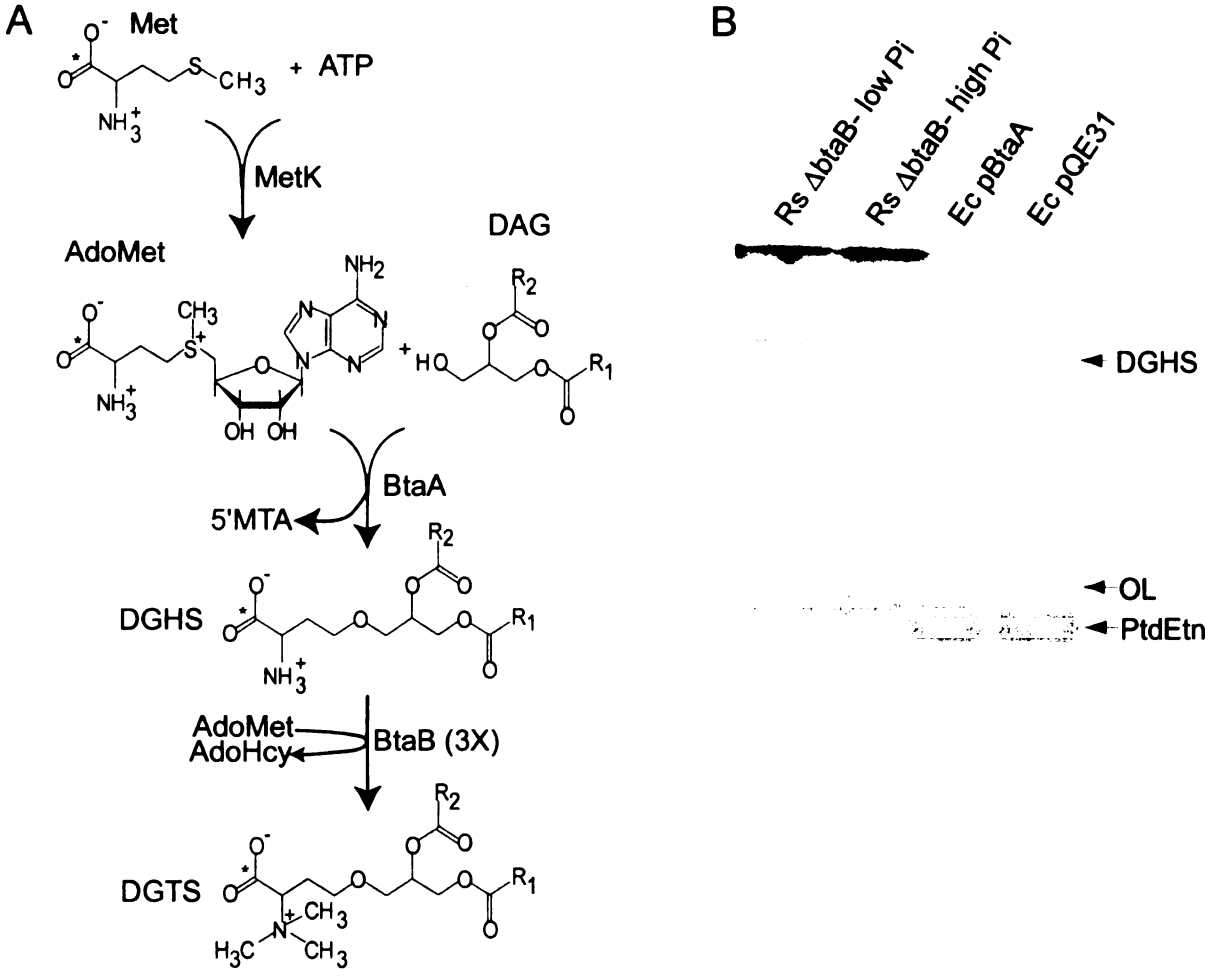

Figure D.1. DGTS biosynthetic pathway and involvement of *Rs*BtaAB

(A) BtaA is proposed to catalyze the transfer of the 3-amino-3-carboxypropyl group of AdoMet to the 3-hydroxyl of DAG to form DGHS. DGHS is then *N*-methylated by BtaB to form DGTS. AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; DAG, diacylglycerol; DGHS, diacylglycerylhomoserine; DGTS, diacylglyceryl-(*N*,*N*,*N*)-trimethylhomoserine; MetK, AdoMet synthetase; 5'MTA, 5'-methylthioadenosine.

(B) *E. coli* expressing *btaA* accumulate DGHS. The *btaA* expression strain (third lane) shows a band co-migrating with authentic DGHS from a phosphate stressed *R. sphaeroides btaB* KO strain (first lane). Phosphate-replete *R. sphaeroides btaB* KO and empty pQE-31 served as negative controls (second and fourth lanes, respectively).

This proposed DGTS biosynthetic scheme involving these two enzymes is depicted in Figure D.1A. While these genes have been shown to be necessary for DGTS biosynthesis *in vivo*, a biochemical analysis of their protein products has not been done. This work focuses on the BtaA enzyme, due to its unusual reaction, and describes an *in vitro* experiment which helped confirm its identity as an AdoMet/diacylglycerol 3-amino-3-carboxypropyl transferase.

Materials and Methods

In vitro activity assays of RsBtaA

Escherichia coli TOP10 F′ harboring pBtaA (constructed as described in Riekhof et al. 2005) was grown in 250 ml LB-ampicillin at 37 °C to an OD of 0.7, and induced with 0.25 mM IPTG followed by an additional 4 h of growth at 28 °C. Cells were harvested by centrifugation and the cell pellet was suspended in 10 ml of cold buffer (50 mM Hepes, 1 mM DTT, 1 mM EDTA, pH 7.3). The resuspended cells were sonicated 3–4 times, 30 s each with a microprobe tip, and the lysate was centrifuged at 2000 g for 10 min to remove unbroken cells and cellular debris. Aliquots (1 ml) of the cell-free extract were then frozen in liquid N_2 and stored at −80 °C prior to use. Activity under these storage conditions did not decrease appreciably for at least 1 month.

Assays were conducted in 100 µl final volume by combining 48.75 µl of cell-free extract with 48.75 µl of 100 mM Hepes, Tris–Cl, or MES, 1 mM DTT, 1 mM EDTA, at varying initial pH to give a final pH in the range of 5.5–8.6 when mixed with the cell-free extract (initial, pH 7.3). Reactions were initiated by addition of 25,000 dpm 1-[^{14}C]AdoMet (American Radiolabeled Chemicals, 2.5 µl, final concentration of AdoMet of 2.1 µM, specific activity 7.14 MBq/nmol), or 75,000 dpm [^{14}C]DAG (dioleoyl-rac-glycerol, [oleoyl 1-^{14}C]; American Radiolabeled Chemicals, final concentration 6.3 µM, specific activity 7.14 MBq/nmol). For the reaction initiated with labeled DAG, it was essential to dry [^{14}C]DAG, which was delivered suspended in toluene/ethanol (1:1, v/v) at room temperature under a stream of nitrogen. A sonicating water bath was used to disperse the DAG at the desired concentration in a buffer of 50 mM Hepes, pH 7.8, 1 mM EDTA, 1 mM EGTA, 0.1% Triton X-100. Reactions were incubated at 28 C, and

terminated by addition of 400 µl of chloroform/methanol (1:1, v/v) and 100 µl 0.9% (w/v) NaCl to separate aqueous and organic phases. The organic phase was transferred to a new tube, dried under a stream of nitrogen, and dissolved in 50 µl chloroform/methanol (1:1, v/v). This lipid extract was then spotted onto silica-60 TLC plates (Baker) and developed with the solvent chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v), followed by quantification of the signal for DGHS on a Molecular Dynamics phosphorimager screen (Amersham, Piscataway, NJ, USA) with the ImageQuant software package. Alternatively, TLC plates were stained with ninhydrin reagent to visualize primary amine-containing lipids.

Results and Discussion

The *btaA* and *btaB* genes have previously been shown to be necessary for DGTS accumulation in phosphate stressed *R. sphaeroides* (Klug and Benning 2001). Expression of *btaA* in *E. coli* resulted in the accumulation of a primary amine-containing lipid band which co-migrated with authentic DGHS produced from phosphate stressed *R. sphaeroides btaB* KO (Figure D.1B). We expected that *E. coli* expressing *Rs*BtaA could use endogenous diacylglycerol (DAG) and *S*-adenosylmethionine (AdoMet) to synthesize DGHS. Heterologous expression of *Rs*BtaA confirmed the catalytic role of the encoded protein, but did not confirm DAG or AdoMet as the substrates used for DGHS biosynthesis.

In vitro assays of *Rs*BtaA enzyme activity were conducted using 1-[^{14}C]AdoMet or [^{14}C]DAG to follow product formation. While we were able to utilize the engineered His_6-tag to purify small amounts of apparently soluble *Rs*BtaA protein using various detergents in the lysis buffer, we were unable to demonstrate activity in a reconstituted liposome system. To circumvent this problem, we developed a system to minimize the steps between expression and enzyme assay to demonstrate the proposed reaction catalyzed by *Rs*BtaA. As we wanted to test the incorporation of label from 1-[^{14}C]AdoMet as well as [^{14}C]DAG, two sets of controlled reactions were set up keeping all conditions the same except for the compound carrying the label. Both sets of reactions were supplied with the two substrates, AdoMet at 2.1 μM and DAG at 6.3 μM. In the two sets of reactions, the specific activity for one or the other substrate was kept the same. In both sets a 3-fold higher amount of DAG was used because unlike AdoMet, DAG is offered as a micelle suspension and a number of other reactions consume this precursor

as well (see below). This most suitable 1–3 ratio of substrates was empirically determined. Given that the specific activity of both substrates and their total concentrations in the two sets of reactions were identical, we expected that the rates for both sets of reactions were identical if both compounds are direct substrates of the enzyme. The result is shown in Figure D.2A. Label from AdoMet was efficiently incorporated into DGHS by transfer of the 3-amino-3-carboxylpropyl moiety to a lipid acceptor giving rise to a single labeled compound in the lipid fraction of the reaction extract. This compound was not present in the vector control consistent with it being DGHS. When labeled DAG was used, label was observed in a number of polar lipids as DAG is a general precursor for polar lipid biosynthesis. These labeled compounds were also present in the vector control. One compound co-chromatographing with DGHS present in the AdoMet reaction (Figure D.2A, box) was also present, but unlike other lipids, it was absent from the vector control thereby suggesting that it was DGHS. Thus, labeled DAG appeared to serve as a precursor for DGHS biosynthesis under the employed conditions. To determine the relative rates of incorporation, the phosphor imager outputs were quantified (Figure D.2B). The rates were linear for both labeled substrates (r^2 = 0.9964 for the AdoMet reaction and r^2 = 0.9743 for the DAG reaction) over the incubation time suggesting that the enzyme was present at non-saturating amounts in the reaction mixture. The rates were very similar (29.6 relative units/min for the AdoMet reaction and 27.7 relative units/min for the DAG reaction). Given that the specific activity for the labeled compounds and the total substrate concentrations in both reactions were identical, one can cautiously conclude that both compounds were incorporated into DGHS with similar efficiency as expected for the direct substrates of the reaction.

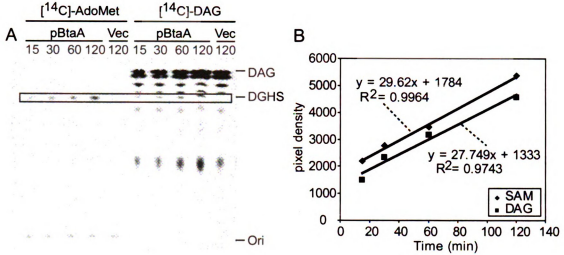

Figure D.2. BtaA-catalyzed DGHS biosynthesis from radiolabeled substrates

(A) *E. coli* cells expressing *Rs*BtaA were assayed for the ability to incorporate label from 1-[^{14}C]AdoMet or [^{14}C]DAG into DGHS. Cells either contained the pBtaA plasmid or an empty pQE31 vector control (Vec). Time courses are shown with incubation times (min) indicated. Reaction products were separated by TLC and detected using a phosphorimager. The product bands containing DGHS (missing in the vector controls) are shown inside the box. DAG, diacylglycerol; DGHS, diacylglycerylhomoserine; Ori, origin.

(B) Plot of DGHS synthesized from 1-[^{14}C]AdoMet or [^{14}C]DAG over time. DGHS amount (expressed as pixel density) was determined from the phosphorimage in (A). The rates of DGHS synthesis from radiolabeled DAG or SAM are linear and have roughly the same value.

The *Rs*BtaA protein in a crude membrane preparation was shown to transfer the 3-amino-3-carboxypropyl moiety from AdoMet to DAG by demonstrating that under identical conditions incorporation of label into DGHS from either labeled AdoMet or DAG proceeded at the same rate (Figure D.2). This result is in agreement with the role of *Rs*BtaA that had been tentatively assigned based on mutagenesis and heterologous expression, and rules out other contingencies for the activity of *Rs*BtaA. Previous work in a cell-free system on the cognate BtaA-type transferase activity in *C. reinhardtii* using radiolabeled AdoMet to follow the reaction had given conflicting results as to the identity of the hydrophobic substrate, indicating that DAG might not be the direct substrate because the addition of unlabeled DAG strongly inhibited the reaction (Moore et al. 2001). However, the point was raised that excess DAG might simply disrupt the membrane environment in which the enzyme(s) are working, and the decrease in activity might not be a result of enzyme inhibition, per se. The results presented here for recombinant *Rs*BtaA seemingly provide a solution to this question.

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