The Capacity of Green Oilseeds to Utilize Photosynthesis to Drive Biosynthetic Processes[1]

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Seeds of many plant species are green during embryogenesis. To directly assess the influence of light on the physiological status of green oilseeds in planta, *Brassica napus* and soybean (*Glycine max*) seeds were rapidly dissected from plants growing in the light or dark. The activation state of malate dehydrogenase, which reflects reduced thioredoxin and NADP/NADPH ratios, was found to be as high in seeds exposed to light as in leaves and to decrease in the dark. Rubisco was highly activated (carbamylated) in both light and dark, most likely reflecting high seed CO_2 concentrations. Activities of Rubisco and phosphoribulokinase were sufficient to account for significant refixation of CO_2 produced during *B. napus* oil biosynthesis. To determine the influence of light on oil synthesis in planta, siliques on intact plants in full sunlight or detached siliques fed ^3H_2O were partly covered with aluminum foil. Seeds from light and dark sections were analyzed, and fatty acid accumulation was found to be higher in seeds exposed to light than seeds from dark sections. The spectrum of light filtering through silique walls and the pigment composition of developing *B. napus* embryos were determined. In addition to a low chlorophyll a/b ratio, the carotenoid pigments of seeds can provide additional capture of the green light that filters through siliques. Together, these results demonstrate that even the low level of light reaching seeds plays a substantial role in activating light-regulated enzymes, increasing fatty acid synthesis, and potentially powering refixation of CO_2.

Oilseeds provide a major source of calories for human consumption and are an increasingly significant source of renewable industrial materials. Interest in engineering enhanced seed oil quantity and quality has prompted efforts to better understand the biosynthesis of oil and other storage products of seeds. Several of the major oilseed crops (e.g. soybean [*Glycine max*], rapeseed, cotton, and linseed) produce seeds that are green during their development, and this fact has prompted questions regarding the contributions of seed photosynthesis to oilseed metabolism.

In leaf chloroplasts, fatty acid synthesis (FAS) is light dependent and utilizes ATP and reducing power generated by photosynthesis (Browse et al., 1986; Roughan and Ohlrogge, 1996). By contrast, plastids isolated from heterotrophic tissues, such as pea roots and cauliflower floral buds, require externally supplied ATP and/or reducing power for FAS (Möhlmann et al., 1994; Xue et al., 1997). Similarly, plastids isolated from developing chlorophylless seeds of safflower and castor bean are heterotrophic in nature and require added ATP or reducing equivalents (Browse and Slack, 1985; Smith et al., 1992). The situation is more complicated in photosynthetic oilseeds that are green during development. Although these seeds are predominantly sink tissues, they contain chloroplasts with the thylakoid structures and enzymes of typical photosynthetic machinery. However, rather than producing carbon for export as in source tissues, photosynthesis in seeds could participate in metabolism in at least three ways. First, light reactions could produce NADPH and ATP for energetically expensive FAS; second, O_2 evolution may help prevent anoxia inside seeds (Rolletschek et al., 2002); and third, the dark reactions of photosynthesis could improve the biosynthetic efficiency of seeds by refixing respiratory CO_2 and providing intermediates for metabolism.

Several previous studies on the role of light and photosynthesis in green oilseeds have not reached a consensus. Browse and Slack (1985) examined plastids from green (linseed) and nongreen (safflower) seeds and concluded that light could provide cofactors for FAS in green plastids but was unlikely to contribute to the carbon economy of seeds. Asokanthan et al. (1997) studied plastid structure in *Brassica napus* embryos as well as measured O_2 evolution and CO_2 incorporation rates. Their conclusion was that embryo chloroplasts are shade-like, photoheterotrophic, and use light to produce some NADPH and ATP for FAS. Eastmond et al. (1996) concluded that *B. napus* embryo plastids may have some capacity for photosynthesis, but because of the low-light environment inside the siliques, this makes no net contribution to carbon economy in vivo. By contrast, in a comprehensive study, King et al. (1998) evaluated both light and dark reactions of photosynthesis in *B. napus* seeds and suggested that embryos use light reactions mainly to drive CO_2 fixation by Rubisco. Based on differences in CO_2 and O_2 gas exchange by soybean fruits in light or

dark, Willms et al. (1999) concluded that light stimulates soybean fruit and seed FAS, although diffusion barriers or internal recycling of gases made quantitative conclusions about seeds inside the fruits difficult.

In addition to these studies of photosynthetic or gas-exchange capacity, there are reports that indicate that light stimulates FAS in developing green oilseeds. For example, after removing *B. napus* seeds from siliques, the incorporation of ^{14}C from different substrates into lipids increased significantly in light (Aach and Heise, 1997) and decreased when photosynthesis was inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (Fuhrmann et al., 1994). Similarly, after labeling seeds with ^{14}C-Suc, ^{14}C-acetate, or ^3H_2O, Bao et al. (1998) reported that light enhanced *B. napus* de novo FAS in plastids but not the elongation of fatty acids (FA) in the cytosol.

Most studies cited above were conducted in vitro, using excised embryos exposed to light, and, therefore, the situation of the seeds in planta within siliques could not be assessed. Conclusions from such studies may be limited because dissecting plant tissues is known to impair metabolism (e.g. Geigenberger et al., 1994). In addition, one argument against a significant role of seed photosynthesis in vivo is that the low transmittance of light through silique walls and seed coats does not provide sufficient energy to produce substantial amounts of cofactors (Eastmond et al., 1996). To address these limitations, we took an in vivo approach to study the role of light and photosynthesis in developing green seeds of *B. napus* and soybean. We harvested seeds from natural light conditions or from the dark and assessed the influence of light on the seed physiological status and on FA accumulation. In addition, we have assessed the activation state and capacity of Rubisco to refix CO_2.

RESULTS

Pigment Composition of *B. napus* Developing Seeds Reflects Shade Adaptation

The utilization of light by green oilseeds is determined by two major factors. First, the transmittance of light through the silique or pod wall is fairly low, on average between 20% and 30% (Eastmond et al., 1996; King et al., 1998). Second, the transmitted light available for a seed is enriched with green wavelengths and thus may not be effectively absorbed by chlorophylls (Chl). This is illustrated in Figure 1, where the light transmission spectra of a *B. napus* leaf and silique wall are presented. Light that has passed through Chl-containing tissue has peak transmission around 550 nm in the green region.

We analyzed the pigment composition of developing *B. napus* seeds to evaluate their capacity to effectively use light that penetrates through the silique wall. Plants acclimated to deep-shade conditions adapt to the low-light environment by increasing the
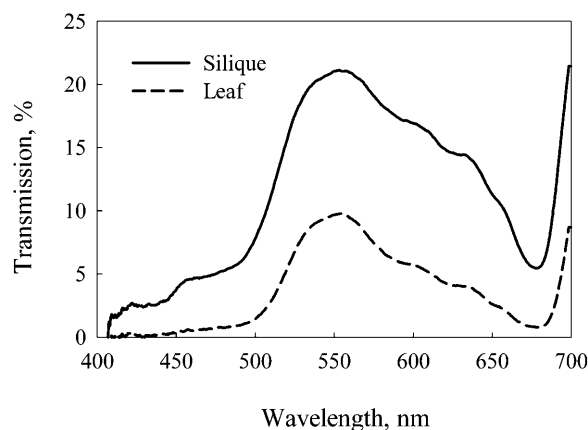
Figure 1. Transmission spectra of a *B. napus* leaf and about a 3-week-old green silique. Each spectrum is an average of two measurements over different areas.

ratio of Chl *b* to Chl *a*. In addition, carotenoids (especially violaxanthin and lutein) are significant for low-light harvesting by absorbing light energy mainly in the blue-green spectral range and transferring the excitation energy to Chl *a* (Siefermann-Harms, 1985; Frank and Cogdell, 1996). Midstage developing *B. napus* seeds had abundant Chl, on average 3 nmol (about 2.6 μg) seed^{-1}, and, as reported previously (Eastmond et al., 1996; King et al., 1998), the seeds had a lower Chl *a/b* ratio than leaves, indicating their adaptation to deep shade (Table I). HPLC analysis indicated that *B. napus* developing seeds also contained all major carotenoid pigments (violaxanthin, antheraxanthin, zeaxanthin, lutein, neoxanthin, and β-carotene), and the ratio between carotenoids and Chl was similar in both seeds and leaves (Table I). The xanthophyll conversion state (Z + A/VAZ; %) in dark-adapted *B. napus* leaves was low (9%) and higher in leaves harvested from a sunny greenhouse (range 13%–44%; average 27%). In seeds, the conversion state was around 20% regardless of the light conditions (Table I). In summary, both the carotenoid and Chl *a/b* analysis indicated that developing *B. napus* seed chloroplasts are well equipped to utilize weak, green light that has filtered through the silique wall.

Light Causes Activation of NADP-MDH in Developing Seeds

To provide an in planta measure of how light influences the physiological status of developing *B. napus* seeds, we measured the activation state of chloroplast NADPH-dependent malate dehydrogenase (MDH), an enzyme that is inactive in darkness or at low light and activates upon illumination. This light activation is mediated by the ferredoxin-thioredoxin (Fd/Tx) system, which signals the onset of light to several chloroplast enzymes (Scheibe et al., 1986). However, this reductive activation of MDH is

Table I. *Biochemical characteristics of B. napus leaves and seeds and developing soybean seeds*

The values are an average ± SD of three to six determinations. Developing seeds were analyzed at the stage of maximum lipid accumulation (see "Materials and Methods"). A, Antheraxanthin; Z, zeaxanthin; V, violaxanthin. n.d., Not determined.

	B. napus Leaves	*B. napus* Seeds	Soybean Seeds
Chl *a/b* ratio	2.4 ± 0.2	1.9 ± 0.2	n.d.
Carotenoids/Chl (mmol mol^{-1})	313 ± 29	371 ± 17	n.d.
Carotenoids: A + Z/VAZ in dark (%)	9.7 ± 2.7	20 ± 1.5	n.d.
Carotenoids: A + Z/VAZ in light (%)	27 ± 16	24 ± 1	n.d.
Rubisco/Chl (mmol mol^{-1})	5.9 ± 1.2	2.0 ± 0.3	2.4 ± 0.6
Rubisco (μg seed^{-1})	–	3.2 ± 1.3	35 ± 4
PRK activity (μmol min^{-1} mg^{-1} Chl)	49 ± 3	23 ± 1	n.d.

inhibited by $NADP^+$, making the enzyme also sensitive to the $NADP^+$/NADPH ratio (Scheibe and Jacquot, 1983), and, therefore, the activation state of MDH also reflects the stromal NADP pool reduction state (Foyer, 1993). NADPH-MDH activity was measured from *B. napus* seeds and leaves that were rapidly harvested and quick frozen from light, or after 1- to 2-h dark adaptation. Developing soybean seeds collected from similar conditions were also analyzed. As shown in Figure 2, the activation state for MDH was low (<20%) in dark and increased to an average of 50% in material harvested from light. Thus, MDH is activated similarly by light in both seeds and leaves implicating photosynthetic electron transport in seed NADPH synthesis and thioredoxin-mediated enzyme activation.

Seeds Contain Highly Active Rubisco

Expressed sequence tag sequencing of Arabidopsis and soybean developing seeds indicates that transcripts encoding Rubisco's small subunit and photosystem components are relatively abundant seed mRNA species (White et al., 2000; http://www.tigr.org/tdb/tgi/gmgi). Furthermore, Rubisco and photosystem gene expression are correlated temporally with expression of transcripts for enzymes of FAS (Ruuska et al., 2002). King et al. (1998) previously measured in vitro activities of phospho*enol*pyruvate (PEP) carboxylase (PEPC) and Rubisco in developing *B. napus* seeds, but these experiments did not reveal whether Rubisco in the seeds is in an active form. Many enzymes of the Calvin cycle are regulated by the Fd/Tx system, but the activation of Rubisco is more complex, involving Rubisco activase. Although the main influencing factor in leaves is light, activation is also determined by available CO_2 because the activation requires carbamylation of the active site (Lorimer

et al., 1976). Full activation in leaves often requires light intensities close to 1,000 μmol quanta m^{-2} s^{-1} (von Caemmerer and Edmondson, 1986). Considering that only 20% to 30% of the ambient light reaches the seeds, this could restrict the activation state of seed Rubisco.

The total amount and carbamylation status of Rubisco were determined in developing *B. napus* and soybean seeds using the [^{14}C]CPBP-binding method (Collatz, 1978). At midstage development, the average Rubisco contents in *B. napus* and soybean seeds were 3.2 and 35 μg, respectively (Table I). The ratio of Rubisco to total Chl was low in seeds, as is typical for low-light-adapted plants. Transfer from dark to sunlight caused the expected increase in Rubisco carbamylation status in leaves, from about 40% to almost 80% (Fig. 3). By contrast, in developing *B. napus* and soybean seeds, carbamylation was equally high in both light and dark (approximately 80%), and even removing soybean seeds from pods and exposing them to full sunlight did not further increase carbamylation (data not shown). The activation states were similar when Rubisco activation was measured using a rapid spectrophotometric assay for Rubisco initial and total activity (Kane et al., 1998). This confirmed that the high carbamylation levels in seeds were not artifacts of the CPBP assay method, where it is crucial that the assay mixture for determination of the initially carbamylated Rubisco sites is essentially CO_2 free.

An explanation for high Rubisco carbamylation levels in developing seeds in the absence of high light is most likely the high concentration of dissolved CO_2 in cells, resulting from high CO_2 release during FAS. King et al. (1998) measured 1% to 2% gaseous CO_2 inside developing *B. napus* siliques (which would result in dissolved CO_2 of 350–700 μM). Recent direct measurements of total CO_2 in seeds indicate levels over 10 mM accumulate in *B. napus* and soybean seeds
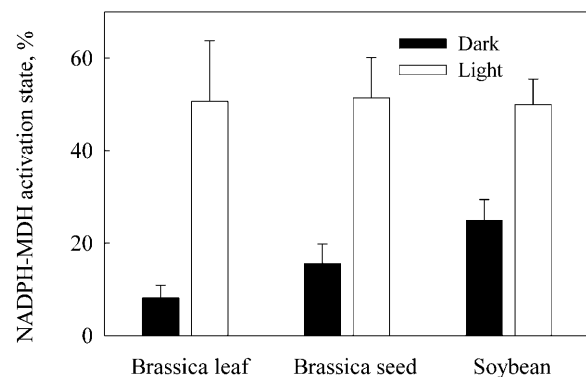
Figure 2. The activation state of chloroplast NADP-dependent MDH in *B. napus* leaves and developing seeds and in soybean seeds measured from dark-adapted (2 h) material and samples harvested from sunlight. In all samples, the activation state is significantly higher in light, demonstrating the presence of photosynthetic electron transport in light. The values are an average ± SD of three to four independent determinations.
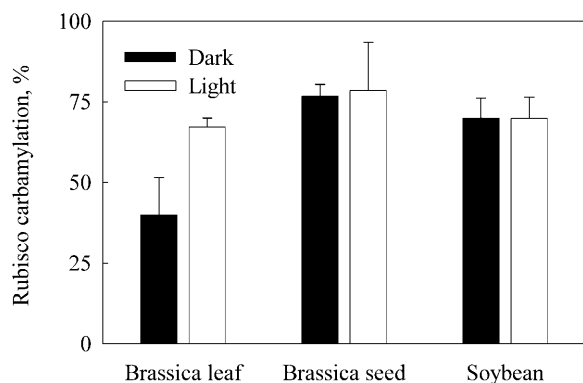
Figure 3. Carbamylation state of Rubisco in *B. napus* leaves and seeds harvested from dark or light. For comparison, carbamylation ratios were also measured from soybeans. The values are an average ± SD of three to four independent determinations.

(Goffman et al., 2004). The K_{act} (CO_2) for spontaneous carbamylation of Rubisco is approximately 30 μM (Lorimer et al., 1976) and, therefore, in seeds, spontaneous carbamylation would be sufficient to keep Rubisco highly active in light or dark. Thus, Rubisco should function in seeds even under low-light conditions, providing that a ribulose 1,5-bisphosphate (RuBP) regeneration pathway is active.

Developing Seeds Also Contain Phosphoribulokinase

In the presence of saturating CO_2 and with a high carbamylation state, Rubisco activity in developing *B. napus* and soybean seeds would be limited by the availability of RuBP. To assess the capacity for RuBP production, the activity of phosphoribulokinase (PRK) was measured after rapid extraction from *B. napus* leaf and seeds harvested from light. In addition, the presence of PRK protein was confirmed from soybean seeds with immunoblotting.

PRK activity in *B. napus* seeds was on average 36 nmol min^{-1} $seed^{-1}$. Similar to the Rubisco to Chl ratio, PRK activity expressed on a Chl basis was about 50% lower in developing *B. napus* seeds than in leaves (Table I). Because PRK is activated by the same Fd/Tx system as NADP-MDH (Fig. 2), PRK is likely to be highly active in seeds under light conditions. Considering that the average Rubisco initial site concentration in *B. napus* seeds was 0.04 nmol $seed^{-1}$ (approximately 3 μg of 80% carbamylated Rubisco), with in vivo K_{cat} of 3 s^{-1} $site^{-1}$ (von Caemmerer et al., 1994), this equals a potential CO_2-fixing capacity of Rubisco of about 7.2 nmol min^{-1} $seed^{-1}$. Accordingly, the measured PRK activity (36 nmol min^{-1} $seed^{-1}$) is about 5-fold higher than Rubisco activity, a ratio comparable to that found from tobacco leaves (Paul et al., 1995). Although we could not reliably measure the activity of PRK from developing soybean seeds due to a high background consumption of NADH in the spectrophotometric assay, immunoblot analysis confirmed the existence of PRK in developing soybean seeds (Fig. 4).

Seeds Inside Siliques Exposed to Light Produce More FA Than Seeds in the Dark

Seed slices have higher rates of FAS when incubated in light compared to dark (e.g. Aach and Heise, 1997; Bao et al., 1998). In addition, when intact embryos are cultured as described by Schwender et al. (2003), rates of fresh-weight accumulation and ^{14}C-Suc incorporation into lipids are approximately 2-fold higher in the light than in the dark (data not shown). To extend these studies to seeds growing within siliques, we performed two types of experiments. The first experiment involved intact *B. napus* plants growing outdoors on a sunny day. In the morning, an opaque, reflective aluminum foil tube was slipped over 13 siliques such that either the distal or basal half was covered. After 10 h of sunlight, the siliques were removed from plants, the seeds dissected, and the weight, FA, and Suc content of seeds from the dark and light were determined. As shown in Figure 5A, although biological variability between siliques resulted in a wide range of values, the FA content was higher in the seeds exposed to light than to dark. On average, seeds in the light had 28 μg more FA than seeds in the dark. This difference was 23%, whether expressed as milligrams FA per seed or milligrams FA per gram fresh weight, and statistically significant (paired Student's t test; $P < 0.001$). Suc content was slightly higher in seeds exposed to the dark, as might be expected if its utilization by the seeds was slowed (Fig. 5B). Thus, any differences in FA production were not due to limitations of carbon supply to the seeds. For this experiment, we selected seeds at the early stage of oil accumulation (about 20–25 d after flowering [DAF], with an average weight of 5.5 mg), so that a large background of preformed oil was not present, and the increase in oil content over 10 h could be more easily observed. Based on data from field-grown *B. napus* (Turnham and Northcote, 1983; Murphy and Cummins, 1989), seeds at this stage would be expected to produce approximately 20 to 50 μg of FA in 1 d. The average difference between the light and dark seeds after the 10-h period was 28 μg of FA. The simplest interpretation of this comparison is that seeds in the field produce a major portion of their FA during the light portion of the diurnal cycle.
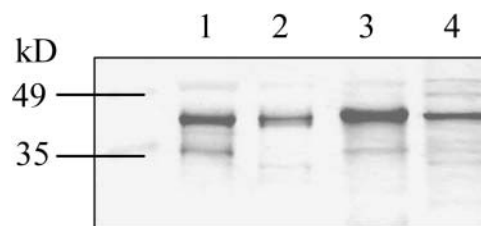
Figure 4. Immunoblot of protein extracts from leaves and developing seeds of *B. napus* and soybean. Analysis was performed using antibody against soybean PRK. Lanes 1 and 2 contained 5 μg of protein extracted from *B. napus* and soybean leaf, respectively, and lanes 3 and 4 contained 20 μg of protein extracted from developing *B. napus* and soybean seeds. PRK size is about 43 kD.
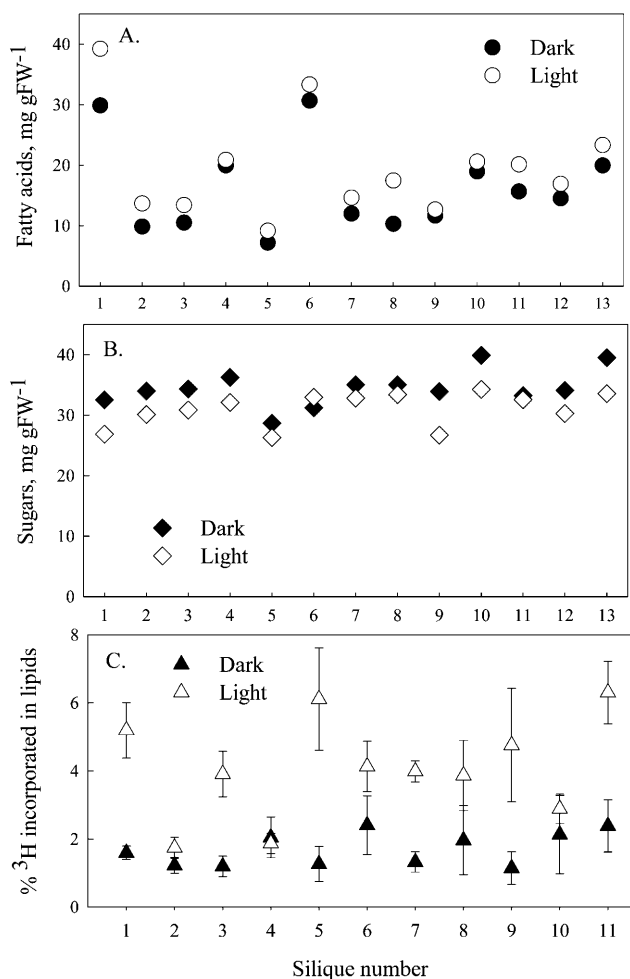
Figure 5. Influence of light on lipid synthesis in seeds inside siliques. A, Comparison of FA content of *B. napus* seeds exposed to light or dark. Either the basal or the top half of 13 siliques on plants growing outside was covered with aluminum foil so that one-half of the seeds was shaded. After a 10-h period of sunlight, the seeds of each half of the 13 siliques were analyzed. The difference between the light treatments was statistically significant (paired Student's *t* test; $P < 0.001$). For siliques 2, 3, and 8, the tip was dark, for others the base. B, Sugar contents of same seeds analyzed in A. C, Incorporation of ^{3}H from ^{3}H$_2$O into lipids of individual seeds from sections of a silique either exposed to light or shaded with foil tubes. Results are expressed as 100 × (dpm in lipid extract/dpm in aqueous extract of seeds). The mean and SD for five seeds is shown. For odd-number siliques, the tip was dark; for even-number siliques, the base was covered.

In the second experiment, ^{3}H$_2$O was fed to siliques and the incorporation of ^{3}H into lipid was determined in light- and dark-exposed seeds. ^{3}H$_2$O is advantageous as a lipid precursor because labeled water equilibrates with endogenous pools to a uniform and easily measurable specific activity, and therefore total FAS activity can be measured without assumptions about internal pool dilutions. Eleven siliques at approximately 30 DAF were detached from plants and fed ^{3}H$_2$O via the transpiration stream. After 24 h (to allow distribution of label throughout the silique and

seeds), foil tubes were again used to cover either the distal or basal half of the silique. After an additional 24 h in the light, five seeds were harvested from each light and dark section, and ^{3}H incorporation into lipids of each seed was determined. As shown in Figure 5C, for 10 of the 11 siliques, lipid synthesis from ^{3}H$_2$O was higher in seeds that were exposed to light. The average difference between light and dark seeds from the same silique was 2.5-fold. We note that this value underestimates the difference in rates because some ^{3}H-lipid was synthesized during the initial 24-h light period.

DISCUSSION

In this study, we have examined how light influences the metabolism of green seeds. Because earlier studies were performed with isolated plastids, excised seeds, detached siliques, or theoretical calculations based on gas exchange of tissues, our objective in this study was to use methods that reflect metabolism in intact seeds on the plant.

The spectrum of light transmitted through the silique wall (Fig. 1) indicates that *B. napus* seeds will be exposed primarily to green light. Thus, not surprisingly, both the contents and compositions of Chls and carotenoids of developing seeds were similar to those characteristic of shade plants that are adapted to both low light and green-enriched light. This situation inside the silique may be analogous to the inner cells of many green leaves. In fact, although blue and red light are better captured by Chl, even in leaves, a large portion of total photosynthesis is dependent on green light (Sun et al., 1998; Evans, 1999).

In both leaves and seeds, exposure to high light led to an increase in NADPH-MDH activation from <20% to around 50% of total activity. This result provides direct evidence that light leads to biochemical responses for plastid enzymes in green seeds that are similar to those in leaves. The increase in NADP-MDH activation state also implies that the chloroplast redox state increases, and, consequently, we can assume that other enzymes that are under regulation by the Fd/Tx pathway, such as acetyl-CoA carboxylase, Fru bisphosphatase, seduheptulose bisphosphatase, PRK, and NADP-GAPDH, are also activated. The latter enzymes may be important in terms of functioning of a CO_2 assimilation pathway (see below). Also, NADPH production in plastids is consistent with O_2 evolution by PSII as measured for isolated embryos (Eastmond et al., 1996; King et al., 1998). Photosystem activity in green seeds may play a dual role of supplying cofactors and also of avoiding hypoxia. Recent studies have shown that even at ambient O_2 levels, the supply of O_2 may limit metabolism in developing *B. napus* seeds (Vigeolas et al., 2003), and seeds may experience hypoxia, as observed in developing legumes (Rolletschek et al., 2002). It has been reported that photosynthesis by *B. napus* pod walls increases the O_2 concentration in the silique locule

(Porterfield et al., 1999). However, as the developing seeds themselves are mostly impermeable to gases (King et al., 1998), O_2 evolution by the seed itself may be important to avoid anoxia.

Calculations for *B. napus* FAS and Reductant Production and CO_2 Exchange

To provide a quantitative context to the above experiments, in the following paragraphs we calculate the potential gas and reductant fluxes in developing *B. napus* seeds based on our biochemical measurements and literature values.

During the maximal oil accumulation period (3–5 weeks after flowering), a developing *B. napus* embryo synthesizes up to 75 μg FA per day (Murphy and Cummins, 1989; Bao et al., 1998; S.A. Ruuska, unpublished data). Assuming that this takes place predominantly during a 16-h light period, the FAS rate is 4.7 μg h^{-1} seed^{-1} or 16.7 nmol h^{-1} seed^{-1} for 18 carbon FAs. During plastidic FAS, each elongation of the growing chain by 2 carbons requires 1 NADH and 1 NADPH or 8 mol each per 18 carbons, or 135 nmol h^{-1} seed^{-1}. Pyruvate dehydrogenase (PDH) produces NADH and acetyl-CoA in equimolar amounts. However, the source(s) of NADPH for FAS is uncertain. Several different mechanisms could deliver cytosolic reducing equivalents to the plastid, including the oxaloacetate (OAA)/malate shuttle (Scheibe, 1987) or exchange of dihydroxyacetonphosphate with 3-phosphoglyceraldehyde (PGA) by the triose phosphate transporter (Heineke et al., 1991), combined with cytosolic reduction of 3-PGA and plastidic oxidation of glyceraldehyde-3-phosphate (GAP) by GAPDH. In these two scenarios, net fluxes of carbon and reductant move in the opposite direction to those in source leaf tissues. In addition, NADP(+)-malic activity has been described in *B. napus* embryos (Kang and Rawsthorne, 1994; Singal et al., 1995) and proposed as a source of reductant. However, analysis of in vivo labeling patterns from ^{13}C-precursors indicates that malate makes a very low contribution to *B. napus* plastid FAS (Schwender and Ohlrogge, 2002). Finally, NADPH could be produced by the plastidic oxidative pentose phosphate pathway (OPPP; Eastmond and Rawsthorne, 2000). Recent in vivo labeling results with *B. napus* embryos, however, indicate that flux of carbon through the OPPP during storage oil synthesis can account for only 25% to 45% of seed NADPH demand (Schwender et al., 2003). Furthermore, plastid Glc-6-P dehydrogenase (G6PDH), the first enzyme of the OPPP, is inactivated by reductive modification via the Fd/Tx system (Scheibe and Anderson, 1981). Therefore, seed plastid G6PDH presumably has low activity in light when the chloroplast redox state is high (Fig. 2).

An alternative for NADPH production within plastids of green seeds is photosynthesis, provided that enough light is available. Full sunlight in summer can reach an intensity of 2,000 μmol photons m^{-2} s^{-1}, with an average of >1,000 over 12 h. Considering that 20% to 30% of this light may penetrate the *B. napus* silique wall (Fig. 1; Eastmond et al., 1996; King et al., 1998), 200 to 600 μmol photons m^{-2} s^{-1} could reach the seed surface. Since only minor amounts of Chl are present in seed coats (King et al., 1998), most of the light will reach and be absorbed by the green embryo. The reported *B. napus* embryo gross O_2 evolution rate at 200 to 400 μmol photons m^{-2} s^{-1} is about 180 nmol h^{-1} (Eastmond et al., 1996; King et al., 1998). This can provide 360 nmol NADPH h^{-1} embryo^{-1}, which is at least 2-fold above the demand for FAS. Therefore, we conclude that seed photosynthesis contributions quantitatively match the major demand for NADPH for seed FAS and other plastid metabolism. In addition, the photosynthetic electron transport is coupled to proton translocation and photophosphorylation, and, thus, the reported O_2 evolution rate corresponds to an ATP synthesis rate of about 240 nmol ATP h^{-1}. Since FAS requires equimolar amounts of ATP and NADPH, the photosynthetic light reactions can produce almost 2 times more ATP than required for FAS.

CO_2 and the Carbon Economy of Seeds

In terms of carbon economy, FA biosynthesis is an inefficient process. During the conversion of pyruvate to acetyl-CoA by the plastid PDH, one carbon is lost as CO_2. Thus, in oilseeds such as *B. napus*, with approximately 50% oil content, it can be calculated that more than 70% of the carbon entering the embryo as sugars is processed via pyruvate to acetyl-CoA, and CO_2 release could represent up to 20% or more of the seed carbon economy. This high CO_2 release rate may cause specific problems in seeds, such as cellular acidification and repression of photosynthetic rates as observed in leaves at high CO_2 partial pressures (Sage et al., 1990; Ögren and Evans, 1993; Ruuska et al., 2000). Because of the thickness of the seed tissue and the presence of the seed coat, CO_2 formed inside seeds may not be able to easily escape to the silique locule. Moreover, low O_2 concentration further promotes cytosolic acidification (Ratcliffe, 1995; Savchenko et al., 2000).

Two enzymes of developing *B. napus* seeds have potential for refixation of this respiratory CO_2. PEPC uses dissolved bicarbonate to produce OAA from PEP. King et al. (1998) suggested that OAA could be used to replenish Krebs cycle intermediates or to make pyruvate and acetyl-CoA for FAS in developing *B. napus* seeds. However, reformation of pyruvate (via malic enzyme) would again release CO_2. In order to store the fixed carbon in a stable form rather than releasing it again during conversion to pyruvate, incorporation of the carbon into storage proteins can be considered. However, based on the amino acid composition of storage proteins (Norton, 1989), the amount of CO_2 potentially stored in Asp, Thr, Met, Ile, and Lys in seed protein is only about 4% of the CO_2 produced by FAS,

and, therefore, CO_2 cannot be accommodated quantitatively into seed protein.

The expression of Rubisco provides a second potential route to CO_2 fixation, provided sufficient reductant and cofactors are available. Synthesis of an 18-C FA from pyruvate produces 9 mol CO_2 mol^{-1} FA, which, in midstage *B. napus*, is 150 nmol CO_2 h^{-1} seed^{-1}. The CO_2 concentration in the silique cavity is 1% to 2% and much higher in seeds, and, therefore, Rubisco in seeds functions at CO_2 saturation and without photorespiration. In *B. napus* seeds, an average of 3 μg of 80% carbamylated Rubisco per seed (Table I) amounts to a catalytic site concentration of 0.04 nmol seed^{-1}. The Rubisco K_{cat} (in vivo) is about 3 s^{-1} site^{-1}, and, therefore, the maximum flux through Rubisco would be approximately 430 nmol CO_2 h^{-1}. Although these calculations assume RuBP saturation of Rubisco, which is not likely to be the case, even if only 50% of the Rubisco catalytic sites have RuBP available, the CO_2 fixation rate can still be over 200 nmol h^{-1}. These calculations indicate that Rubisco levels present in *B. napus* seeds are sufficient to accommodate CO_2 produced by FAS and therefore could represent a major carbon flux in the oil seed.

Based on the above considerations, we outline a comparative overview of photosynthesis and FAS in developing green oilseeds versus leaves, as summarized in Figure 6. This model proposes that photosynthesis in green oilseeds can be defined as follows. (1) The nonoxidative pentose phosphate pathway transforms Glc-6-P to Ru-5-P, which is activated to RuPB by PRK. (2) Rubisco produces 3-PGA, which is further transformed to acetyl-CoA. (3) Photosynthetic NADPH and ATP are used to incorporate acetyl-CoA into FAs. Obviously, this is a simplified and speculative scheme and requires more detailed flux analysis. Nevertheless, our calculations demonstrate that the overall demand and production capacity for reductant, as well as CO_2 evolution and fixation capacity, in whole *B. napus* seeds are of the same order of magnitude.

CONCLUSION

In this study, we have shown that developing *B. napus* seeds are adapted to utilize low light in photosynthesis. We have also shown that, under normal growth conditions, light filtered through the silique wall is sufficient to activate seed enzymes that depend
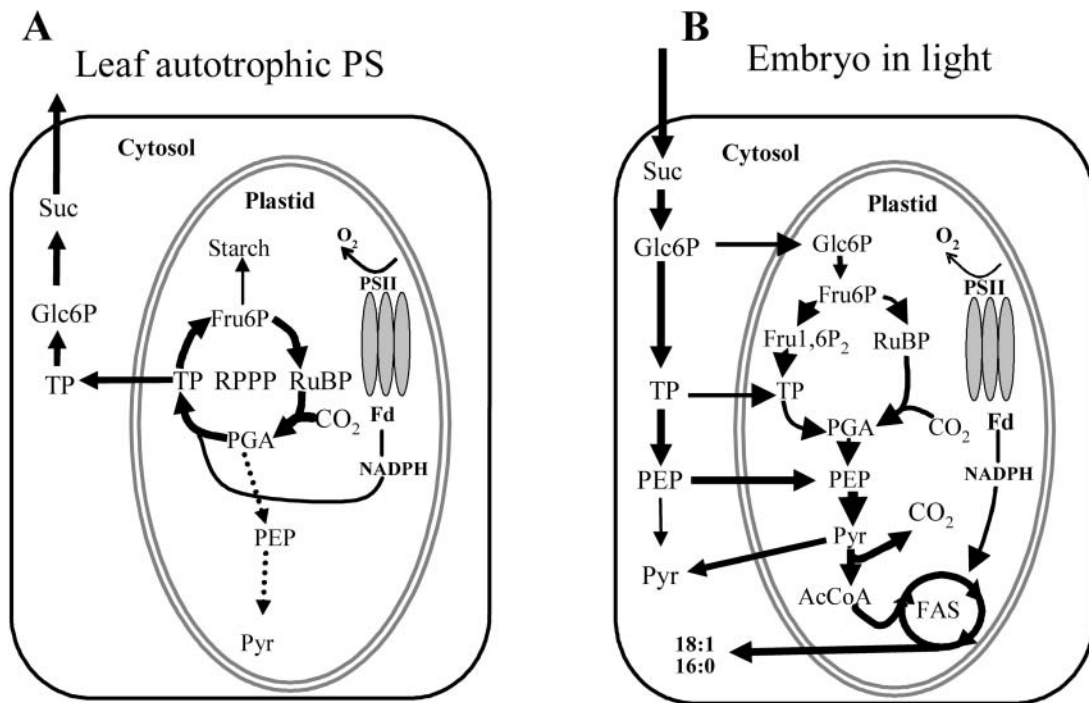
Figure 6. Simplified comparison of leaf photosynthesis with the metabolism of green oilseeds in light. Only the major reactions involved in photosynthesis or conversion of Suc to oil are shown. For cofactors, only the fate of NADPH, produced by photosynthesis, is indicated. A, In an autotrophic leaf, photosynthetic CO_2 is incorporated by Rubisco into PGA, which is reduced to TP. Altogether, five-sixths of the produced TP is used to recycle RuBP, the acceptor molecule for CO_2. B, In *B. napus* embryos, the major pathway of carbon flow during oil synthesis, based on biochemical analyses, stable isotope-labeling patterns, expressed sequence tag, and microarray analysis, is indicated by thick arrows. Embryo chloroplasts contain the full enzymatic set to catalyze glycolysis. The Rubisco reaction is added to the scheme by considering synthesis of RuBP from imported hexose and considering PGA metabolism to pyruvate rather than recycling. OPPP production of NADPH contributes approximately 38% of NADPH (Schwender et al., 2003) but is omitted from the figure for simplicity. Suc, Sucrose; Glc, glucose; Glc-6-P, glucose-6-phosphate; TP, triose phosphate; Pyr, pyruvate; AcCoA, acetyl-CoA; RPPP, reductive pentose phosphate pathway.

on photosynthetic electron transport. Moreover, seed Rubisco is highly active, and there is considerable PRK activity present to provide potential to refix CO_2 released by PDH. Our calculations also show that photosynthesis has the capacity to produce enough reductant for FAS and for a large portion of CO_2 refixation as well. This notion is reinforced by our finding that in planta seeds exposed to light produce more FAs than seeds that are shaded. Our conclusion is that light harvesting provides several advantages to the seeds, including reductant and O_2 generation, and CO_2 fixation. The net result is activation of enzymes and increased rates of oil synthesis.

MATERIALS AND METHODS

Growth and Sampling of the Plant Material

Plants were grown in an air-conditioned greenhouse under natural light supplemented with lamps to provide 16/8-h photoperiod and an approximate light intensity of 800 μmol photons m^2 s^{-1}. Developing seeds were collected at the time of maximal lipid accumulation. *Brassica napus* L. cv Reston seeds were used at the age of 3 to 5 weeks after flowering, and soybean (*Glycine max* L. cv NKS-1990) seeds at 4 to 5 weeks after flowering (Ohlrogge and Kuo, 1984). Whole seeds were used for experiments. Seeds or leaf discs were harvested either from light on a sunny day, or, after 1 to 2 h of dark adaptation, seeds were removed from the siliques and immediately frozen with liquid nitrogen for storage at −80°C before the biochemical assays.

Light Transmission and Pigment Analysis

Seed Chl and carotenoid composition were analyzed by HPLC as outlined in Tian and DellaPenna (2001). The total amount of Chl in samples used for enzyme assays was routinely analyzed in 80% acetone buffered with 25 mM HEPES, pH 7.5 (Porra et al., 1989). Leaf and silique transmission spectra were recorded with a S2000 Fiber Optic Spectrometer (Ocean Optics, Dunedin, FL), using a 50-μm fiber-optic tungsten source for illumination. A collimating lens (f/2; Ocean Optics) was placed onto the fiber-optic source to increase the sampling angle.

NADP-MDH Assays

The activation state of chloroplast NADP-dependent MDH was assayed as outlined in Scheibe and Stitt (1998). The extraction buffer (100 mM Na-acetate, pH 6, 4 mM dithiothreitol [DTT], 0.5% bovine serum albumin, 0.25% Triton X-100, 0.25% CHAPS, 2% polyvinylpolypyrrolidone [PVPP], and 0.5 mM of each benzamidin, γ-aminocaproic acid, and AEBSF) was sparged with humidified nitrogen to maintain low oxygen concentration. Seeds and leaf discs were extracted with ice-cold buffer using a glass homogenizer, the crude extract was centrifuged for 5 min at 10,000g, and the supernatant was used for duplicate assays. Initial activity was assayed immediately in 1 mL volume containing 100 mM Tris-HCl, pH 8, 1 mM EDTA, 1 mM DTT, 0.2 mM NADPH, 2 mM OAA, and 30 to 50 μL of supernatant. The decline in A_{340} nm was monitored. Reductive activation of MDH was achieved by incubating an aliquot of the supernatant in 250 mM Tris-HCl, pH 9 (sparged with nitrogen), and 125 mM DTT under nitrogen atmosphere at room temperature for 15 to 20 min.

Rubisco Assays

The total amount and carbamylation status of Rubisco were measured by the [^{14}C]CPBP-binding method (Mate et al., 1993) after rapid extraction in ice-cold CO_2-free buffer containing 50 mM HEPES-NaOH, pH 7.8, 1 mM EDTA, 5 mM MgCl$_2$, 10 mM DTT, 1 mM each of benzamidin, γ-aminocaproic acid, and AEBSF, 0.1% Triton X-100, and 1% PVPP. Both radiolabeled and unlabeled CPBP (an unresolved mixture of 2'-carboxy D-arabinitol-1,5 bisphosphate and 2'-carboxy D-ribitol-1,5 bisphosphate) was synthesized as described in Collatz

(1978). For comparison, the activation status of Rubisco in *B. napus* seeds was also measured by comparing initial and total activities using an NADPH-linked spectrophotometric assay (Kane et al., 1998). Activation of Rubisco was achieved by incubating an aliquot of the supernatant in the assay mixture for 10 min prior to adding RuBP (Sigma-Aldrich, St. Louis).

PRK Assays

The activity of PRK was measured as outlined in Wara-Aswapati et al. (1980). Samples were extracted in a buffer containing 100 mM HEPES-NaOH, pH 8, 10 mM DTT, 10 mM MgCl$_2$, 2 mM EDTA, 0.25% Triton X-100, 0.25% bovine serum albumin, 1% PVPP, 1 mM each of AEBSF, benzamidin, and γ-amino-caproic acid, and centrifuged for 3 min at 10,000g. Supernatant was used for PRK activity assays immediately. The assay mixture (volume of 1 mL) consisted of 100 mM HEPES-NaOH, pH 7.8, 10 mM MgCl$_2$, 20 mM KCl, 10 mM DTT, 1 mM ATP, 1.5 mM PEP, 0.2 mM NADH, 3 mM ribose-5-phosphate, 3 units ribose-5-phosphate isomerase, 3 units lactate dehydrogenase, and 2 units pyruvate kinase. The mixture minus NADH was incubated for 5 min before the assays to allow for the synthesis of Ru-5-P by the isomerase, and the duplicate assays were started by adding 10 to 25 μL of supernatant to a final volume of 1 mL. For each sample, a control reaction without added Rib-5-P was carried out to correct for background rates of NADH consumption, originating from an ADP impurity in ATP and other endogenous ATPase activities.

Immunoblots

Soluble proteins were extracted from leaf and seed material with a buffer containing 50 mM HEPES-NaOH, pH 7.8, 100 mM NaCl, 0.05% SDS, and 1 mM each of AEBSF, benzamidin, and γ-aminocaproic acid. Extracts were centrifuged for 5 min at 10,000g, and the protein content of the supernatant was measured. Soluble proteins were separated by SDS-PAGE for immunoblot analyses as described in Thelen et al. (2001). Primary antibody against soybean PRK was kindly provided by Dr. Steven Crafts-Brandner (U.S. Department of Agriculture-Agricultural Research Service, Western Cotton Research Lab, Phoenix).

Estimate of Oil Synthesis by Developing Seeds in Planta

Two experiments were conducted to test the influence of light on seeds within siliques. In the first, *B. napus* L. cv Reston plants were grown outdoors in 25-cm pots. For measurement of oil synthesis in planta, siliques from four different plants were selected on a warm (+25°C), sunny day and at a developmental stage of 20 to 30 DAF. While still attached to the plant, either the base or the tip half of 13 siliques was covered with a 7-mm i.d. plastic tube wrapped with aluminum foil to shade half of the seeds. After 10 h, light- and dark-exposed seeds were harvested and fresh weight was determined. To assure similar developmental stage, seeds were selected for further analysis that had an average fresh weight per seed between 5 and 6 mg and that differed not more than 0.5 mg in fresh weight per seed between light and dark sections of the same silique.

FA and sugar content of the seeds was determined by extracting 10 to 15 seeds from each half-silique for 10 min with 2 mL of hot isopropanol (90°C). After cooling to room temperature, 4 mL hexane with 2 mg triheptadecanoine as internal standard were added and heated again for 10 min (90°C). After cooling, 0.5 volume of water was added. An aliquot of the upper hexane phase was analyzed by gas chromatography/flame ionization detector after direct transesterification of FAs to FA methyl esters (Browse et al., 1986). An aliquot of the water/isopropanol phase was used for enzymatic determination of Suc, Glc, and Fru (Stitt et al., 1989; Schwender and Ohlrogge, 2002).

In the second experiment, ^3H_2O was used as a radiolabeled precursor for FAS (Bao et al., 1998). In preliminary experiments, we determined that the water in seeds at different positions within the silique became similarly labeled with ^3H_2O after 20- to 24-h feeding. Eleven siliques at approximately 30 DAF were then removed from three greenhouse-grown plants, and the pedicle was immediately placed into a solution of 5 mCi/mL ^3H_2O, 50 mM Suc, and 3 mg mL^{-1} Murashige and Skoog salts. After 24 h in the light to allow movement of water into the silique (and its seeds) via transpiration, the base or tip half of each silique was covered with aluminum foil-covered tubes and the siliques were further incubated for 24 h with 600 to 800 μE light. During this 48 h, approximately 1 mL of solution was taken up by each silique. Five seeds from each light section and five seeds from each dark section were then

removed, placed into individual screw-cap tubes, and heated with 0.1 mL isopropanol for 10 min at +85°C. After cooling, seeds were homogenized and 0.15 mL hexane were added and allowed to extract overnight at room temperature. After adding 0.8 mL of hexane and 0.2 mL of 0.1 M acetic acid, 0.5 mL of the upper-phase lipid extract were transferred to a scintillation vial, evaporated at 80° C for 15 min, and counted to determine ^{3}H incorporation into FAs. Total ^{3}H incorporation into each seed was also determined by counting an aliquot of the aqueous phase of the lipid extract. Results are expressed as $100 \times$ (dpm in lipid extract/dpm in aqueous extract of seeds).

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