Light Enables a Very High Efficiency of Carbon Storage in Developing Embryos of Rapeseed[1]

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The conversion of photosynthate to seed storage reserves is crucial to plant fitness and agricultural production, yet quantitative information about the efficiency of this process is lacking. To measure metabolic efficiency in developing seeds, rapeseed (*Brassica napus*) embryos were cultured in media in which all carbon sources were [U-^{14}C]-labeled and their conversion into CO_2, oil, protein, and other biomass was determined. The conversion efficiency of the supplied carbon into seed storage reserves was very high. When provided with 0, 50, or 150 μmol m^{-2} s^{-1} light, the proportion of carbon taken up by embryos that was recovered in biomass was 60% to 64%, 77% to 86%, and 85% to 95%, respectively. Light not only improved the efficiency of carbon storage, but also increased the growth rate, the proportion of ^{14}C recovered in oil relative to protein, and the fixation of external $^{14}CO_2$ into biomass. Embryos grown at 50 μmol m^{-2} s^{-1} in the presence of 5 μM 1,1-dimethyl-3-(3,4-dichlorophenyl) urea (an inhibitor of photosystem II) were reduced in total biomass and oil synthesis by 3.2-fold and 2.8-fold, respectively, to the levels observed in the dark. To explore if the reduced growth and carbon conversion efficiency in dark were related to oxygen supplied by photosystem II, embryos and siliques were cultured with increased oxygen. The carbon conversion efficiency of embryos remained unchanged when oxygen levels were increased 3-fold. Increasing the O_2 levels surrounding siliques from 21% to 60% did not increase oil synthesis rates either at 1,000 μmol m^{-2} s^{-1} or in the dark. We conclude that light increases the growth, efficiency of carbon storage, and oil synthesis in developing rapeseed embryos primarily by providing reductant and/or ATP.

In seed crops, yield is primarily a function of the production of assimilates by the leaves and other green parts of the plant and the utilization of these assimilates to synthesize reserve materials in the seeds. In addition to its importance to agricultural productivity, efficient storage of assimilates by seeds is essential to provide metabolic precursors and chemical energy to power the young seedling until it can capture its own energy from the sun. Within a species, seedling growth is positively correlated with seed size (Howe and Richter, 1982; Stanton, 1984; Vaughton and Ramsey, 1998; Sousa et al., 2003) and seedlings grown from large seeds have higher rates of establishment than those from small seeds (Black, 1958; Grime and Jeffery, 1965; Armstrong and Westoby, 1993; Burke and Grime, 1996). Thus, the amount of reserves stored in the seed will in large part determine the success of the young seedling.

Seeds are not simply passive receptacles for the assimilates and minerals provided by the mother plant. They synthesize complex molecules from simple raw materials in relatively precise amounts and proportions (Egli, 1998). Green seeds are photosynthetically active and are able to fix carbon (Watson and Duffus, 1991; Eastmond et al., 1996). As a consequence, the light reactions of photosynthesis potentially can influence the seed's carbon economy, how carbon is stored, and also may play an important role in defining plant fitness and productivity.

In oilseeds, the conversion of carbon supplies into oil may lead to a substantial loss of carbon as CO_2. The primary substrate for oil synthesis in the seeds is the Suc imported from the photosynthetic tissues that is metabolized to generate acetyl-CoA units, the basic building blocks of fatty acids. Acetyl-CoA is formed from pyruvate by an oxidative decarboxylation catalyzed by the pyruvate dehydrogenase complex. In this reaction, for each acetyl-CoA unit produced, one carbon dioxide is also generated, and as a result, 33% of the carbon supplied as to fatty acid synthesis is potentially released as CO_2. This will reduce carbon storage efficiency if mechanisms of CO_2 conservation or recapture in the seeds are lacking. Furthermore, fatty acid synthesis, which primarily occurs in the plastids, requires stoichiometric amounts of ATP, NADPH, and NADH for each sequential addition of an acetyl unit to the growing chain of the fatty acid. In heterotrophic tissues, the plastids must import these cofactors or generate them through carbohydrate oxidation or metabolite shuttles (Rawsthorne, 2002). Several studies with developing rapeseed (*Brassica napus*) embryos showed that lipid accumulation is stimulated by light (Fuhrmann et al., 1994; Aach and Heise, 1998; Bao et al., 1998; Ruuska et al., 2004),

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suggesting that fatty acid synthesis may be dependent on the supply of reducing power and ATP provided by the light reactions of photosynthesis. Moreover, Ruuska et al. (2004) calculated that under ambient light conditions, photosynthetic electron transport can meet the demand for NADPH and ATP for rapeseed fatty acid synthesis. Schwender et al. (2004) recently demonstrated that in rapeseed embryos under low light conditions, Rubisco participates in an alternative pathway for carbohydrate to oil conversion that yields 20% more acetyl-CoA and produces 40% less CO_2. Together, these reports suggest that different levels of light may improve the efficiency of carbon storage in green oilseeds like rapeseed.

Despite decades of study of the factors that determine seed yield in crops (Egli, 1998) and the recent improved understanding of pathways involved in carbon supply to fatty acid synthesis in oil-storing seeds (Rawsthorne, 2002; Schwender et al., 2004), we could find no previous study that directly addresses the efficiency by which carbon supplies provided by the mother plant are converted into storage reserves. Such information may be useful for several reasons, including the identification of futile cycles or other potentially inefficient processes that could limit seed yield. Indeed, several studies have indicated that much of the ATP synthesized in heterotrophic plant cells may be consumed by futile cycling (Dieuaide-Noubhani et al., 1995; Rontein et al., 2002; Alonso et al., unpublished data), and we have found that sugars taken up by developing rapeseed embryos undergo potentially wasteful cycling between hexose and triose (Schwender et al., 2003).

To provide a direct measurement of the efficiency of seed carbon metabolism, we developed methods that provide accurate assessment of the conversion of carbon supplies into storage materials and CO_2 by developing rapeseed embryos. Contributions of photosynthesis to the carbon economy were determined at different light levels and by inhibiting PSII using 1,1-dimethyl-3-(3,4-dichlorophenyl) urea (DCMU). Measurements of oil synthesis rates by seeds within detached siliques under different light and oxygen levels were conducted as well. The implications for seed metabolism, physiology, and carbon economy are discussed.

RESULTS

Carbon mass balancing is widely used for estimating the efficiency of microorganism performance in processes involving the conversion of substrates into products (Novák and Loubiere, 2000; Converti and Perego, 2002; Sáez et al., 2002). This approach requires that the carbon content of all carbon-containing substrates and products, namely, medium components, biomass, and CO_2 are accurately assessed. To provide a similar assessment of seed carbon use efficiency we took advantage of methods that allow culture of developing embryos in defined media (Smith, 2000). When embryos of rapeseed are dissected from siliques and cultured in medium containing sugars and amino acids (at levels found in endosperm liquid), the embryos develop at rates, and produce storage products, that closely mimic those formed in planta (Schwender and Ohlrogge, 2002). By supplying uniformly ^{14}C-labeled sugars and amino acids in the medium, and by measuring the ^{14}C consumption, ^{14}C incorporation into biomass, and $^{14}CO_2$ production, we could accurately determine carbon balances in developing embryos. In this study, we define the percent carbon conversion efficiency (CCE) as % CCE = ^{14}C biomass $\times$ 100/(^{14}C biomass + $^{14}CO_2$) (see "Materials and Methods"). We also express carbon use efficiency as the ratio of the moles of carbon incorporated into seed oil to the molar amount of CO_2 developed (oil to CO_2 ratio; ^{14}C in oil/$^{14}CO_2$). Considering that the dominant biosynthetic pathway in rapeseed embryos is fatty acid synthesis and that plastid synthesis of acetyl-CoA from pyruvate is the major source of CO_2 produced, this ratio is a useful measure of the carbon economy of oil synthesis.

Light Increases Embryo Growth and Enables Very High Efficiency of Carbon Metabolism

Table I presents data on ^{14}C-carbon uptake, biomass accumulation, CO_2 production, oil content, CCE, and the oil to CO_2 ratio after growth of embryos at two light levels and in the dark. Total biomass increase (dry weight) was similar when embryos were grown under 50 or 150 μmol m^{-2} s^{-1} light. However, in the dark, biomass accumulation was less than one-third that in

Table I. *Effect of light irradiance on carbon balance and CCE in rapeseed embryos*

Values correspond to the mean and SD of three to four independent replicates. Embryos were cultured under sealed conditions in the presence of U-^{14}C carbon sources for 14 d at 150 and 50 μmol m^{-2} s^{-1} and 21 d at 0 μmol m^{-2} s^{-1}.

Light Irradiance	Biomass[a]	CO_2 Efflux	Oil Content[b]	^{14}C Uptake[c]	CCE[d]	Oil to CO_2 Ratio[e]
μmol m^{-2} s^{-1}	*mg embryo^{-1}*	*μmol*	*%*	*%*	*%*	
0	1.9 ± 0.2	711 ± 56	44.9 ± 0.3	16.5 ± 1.8	60.4 ± 0.3	1.1 ± 0.0
50	7.3 ± 0.1	316 ± 52	55.8 ± 1.2	23.5 ± 3.5	86.1 ± 1.4	4.2 ± 0.5
150	6.4 ± 1.2	82 ± 16	58.3 ± 1.9	21.2 ± 1.1	94.7 ± 0.3	12.5 ± 0.6

[a]Final biomass, expressed as milligrams dry weight per embryo. [b]Oil content, expressed as percentage of dry weight (percent w/w). [c]^{14}C uptake, expressed as percentage of initial ^{14}C content in medium (Ci/100 Ci). [d]CCE, expressed as percent of the sum of ^{14}C biomass and $^{14}CO_2$. [e]Oil to CO_2 ratio, calculated from the ratio of ^{14}C-labeling in oil and CO_2.

light. CO_2 production was significantly ($P < 0.01$) influenced by light levels, decreasing 2-fold between dark and 50 μmol m^{-2} s^{-1} light and almost 4-fold when light irradiance increased from 50 to 150 μmol m^{-2} s^{-1}. Thus, both increased biomass and reduced CO_2 production suggest that seed metabolism is more efficient under increasing light irradiance. This was further confirmed by the distribution of ^{14}C-labeling between biomass and CO_2 (Fig. 1). The CCE or proportion of ^{14}C-labeling in biomass (sum of labeling in oil, protein, and starch + cell wall compounds) increased from 60% in the dark to 86% and 95% of the total ^{14}C-labeling (^{14}C biomass + $^{14}CO_2$) at 50 and 150 μmol m^{-2} s^{-1}, respectively. Since the two light treatments (50 and 150 μmol m^{-2} s^{-1}) showed similar ^{14}C uptake and biomass gain (Table I), the ^{14}C-label not released as CO_2 is instead incorporated into biomass when light irradiance is increased from 50 to 150 μmol m^{-2} s^{-1}.

As indicated by Figure 1, B and C, light also preferentially enhanced oil biosynthesis. The ratio of ^{14}C-label recovered in oil to that in protein increased when light level increased from 50 to 150 μmol m^{-2} s^{-1}. Oil to CO_2 ratios were also significantly ($P < 0.01$) different among treatments. Between 50 and 150 μmol m^{-2} s^{-1}, the oil to CO_2 ratio increased from 4 to 12.5, mainly due to reduced CO_2 efflux.

Shorter incubations yielded similar results to those observed in the 14- to 21-d experiments presented in Table I and Figure 1. After 3 d of culture, biomass accumulation and lipid synthesis did not differ between the two light levels (50 and 150 μmol m^{-2} s^{-1}) but were 3-fold lower in the dark. CCE was significantly ($P < 0.01$) increased from 64% in the dark to 77% and 85% at 50 and 150 μmol m^{-2} s^{-1}, respectively. The ratio of carbon stored in oil to carbon released as CO_2 also significantly ($P < 0.01$) increased with light, from 0.9 to 2.1 to 3.8 at 0, 50, and 150 μmol m^{-2} s^{-1}, respectively. These results confirm that light significantly increases embryo growth and carbon conversion efficiency while reducing loss of carbon as CO_2.

CO_2 Fixation at Different Light Levels

Figure 2 indicates that assimilation of externally supplied $^{14}CO_2$ into biomass increased 3.6-fold when light irradiance was increased from 50 to 150 μmol m^{-2} s^{-1}, suggesting that CO_2 reassimilation by the embryos contributes to the higher carbon conversion efficiency observed at increasing light levels (see above results). Oil labeling at 150 μmol m^{-2} s^{-1} was 8.4-fold higher than at 50 μmol m^{-2} s^{-1}, whereas protein and cell wall + starch labeling were only 2.8-fold and 2.2-fold higher, respectively, indicating that light irradiance not only increases the total amount of fixed CO_2 but also alters its distribution into different storage products, with an increasing proportion of fixed CO_2 being incorporated into oil at higher light irradiances.

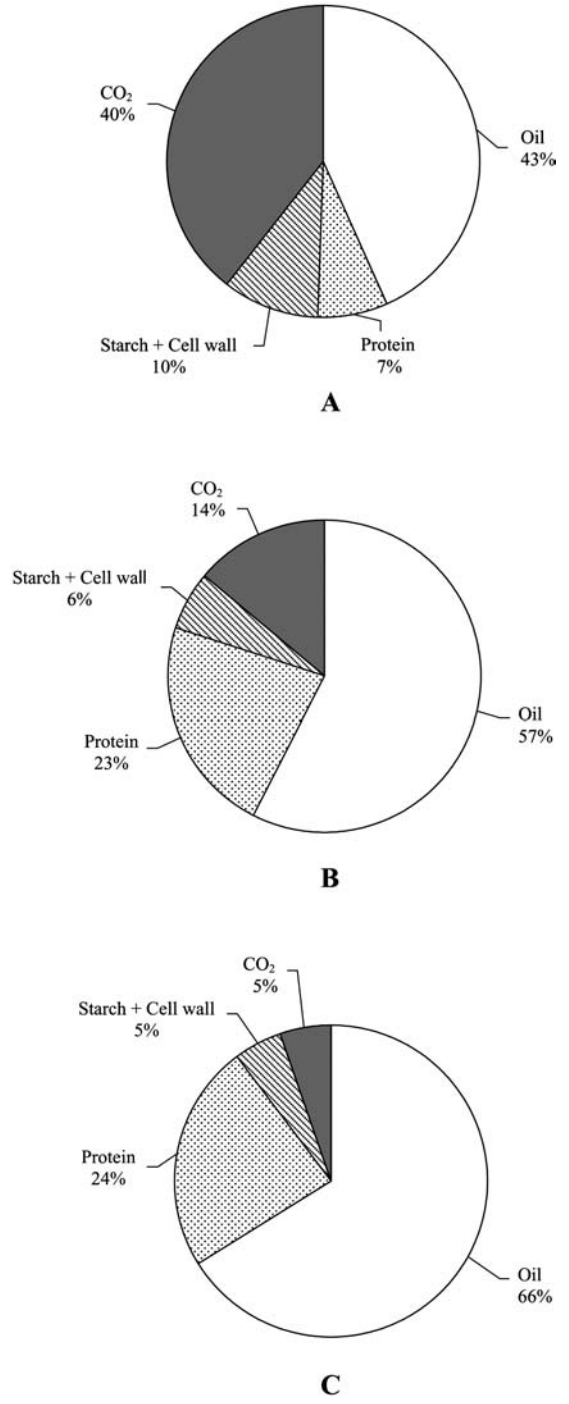

Figure 1. Distribution of ^{14}C-labeling among biomass fractions and CO_2 (100% = ^{14}C biomass + $^{14}CO_2$): (A) 0 μmol m^{-2} s^{-1}, (B) 50 μmol m^{-2} s^{-1}, and (C) 150 μmol m^{-2} s^{-1}. Embryos were cultured at 21°C for 14 d (at 150 and 50 μmol m^{-2} s^{-1}) and 21 d (at 0 μmol m^{-2} s^{-1}) in the presence of U-^{14}C carbon supplies. After culture, the $^{14}CO_2$ was collected by flushing the flasks through a trapping system, and the embryos were frozen in liquid N_2 for analysis of ^{14}C-labeling in biomass components. The percent values shown represent the percentage of ^{14}C recovered in the respective fractions. Because oil, protein, and carbohydrate contain, respectively, 77%, 53%, and 40% carbon by weight, these values differ from weight percent values such as presented in Table I.

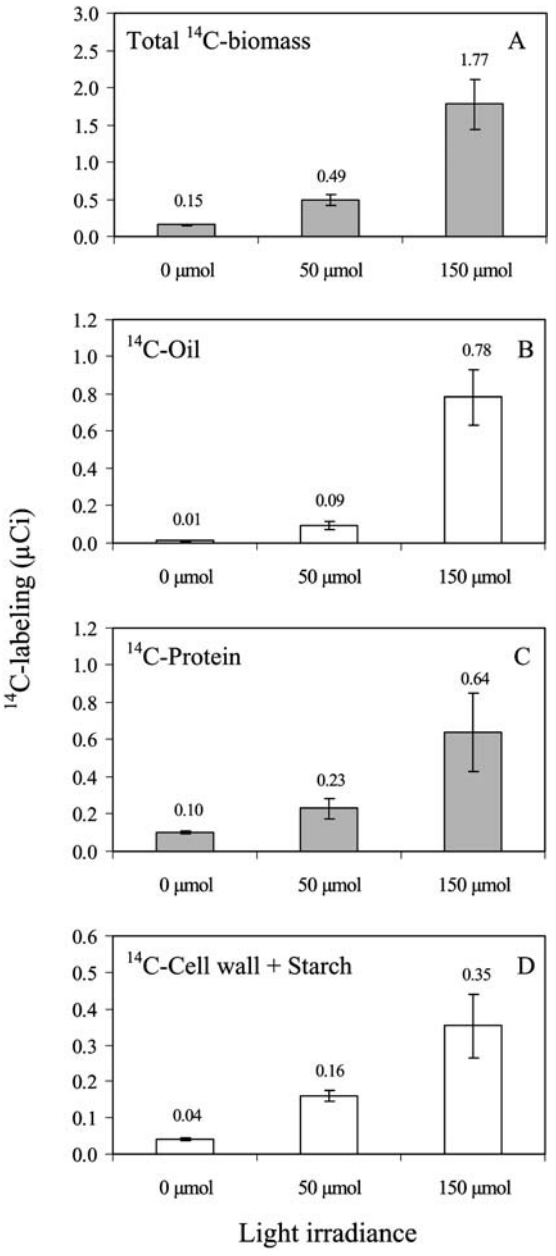

Figure 2. Effect of light irradiance on $^{14}CO_2$ incorporation into biomass. Embryos were grown under a 2% $^{14}CO_2$ atmosphere (specific activity of C = 44.9 mCi/mol) for 3 d at 150, 50, and 0 μmol m^{-2} s^{-1}. After culture, the embryos were rapidly frozen in liquid N_2, and the ^{14}C-labeling in biomass compounds was measured. A, Total biomass ^{14}C-labeling (microcuries). B, ^{14}C-labeling in oil (microcuries). C, ^{14}C-labeling in protein (microcuries). D, ^{14}C-labeling in starch + cell wall (microcuries). Columns and error bars correspond to the mean ± SD of three independent replicates. Means with the same letter are not significantly different at the $P < 0.05$ level (Tukey's studentized range test).

Inhibiting PSII Reduces the Carbon Conversion Efficiency

Under light conditions, the addition of 5 μM DCMU to the culture medium reduced total biomass accumulation and oil biosynthesis by 3.2-fold and 2.8-fold, respectively, and resulted in DCMU-treated embryos exhibiting values similar to those observed in the dark (Fig. 3). When embryos were cultured in the dark, the presence of DCMU did not significantly affect either total biomass accumulation or oil biosynthesis, indicating that effects of DCMU were specific to light

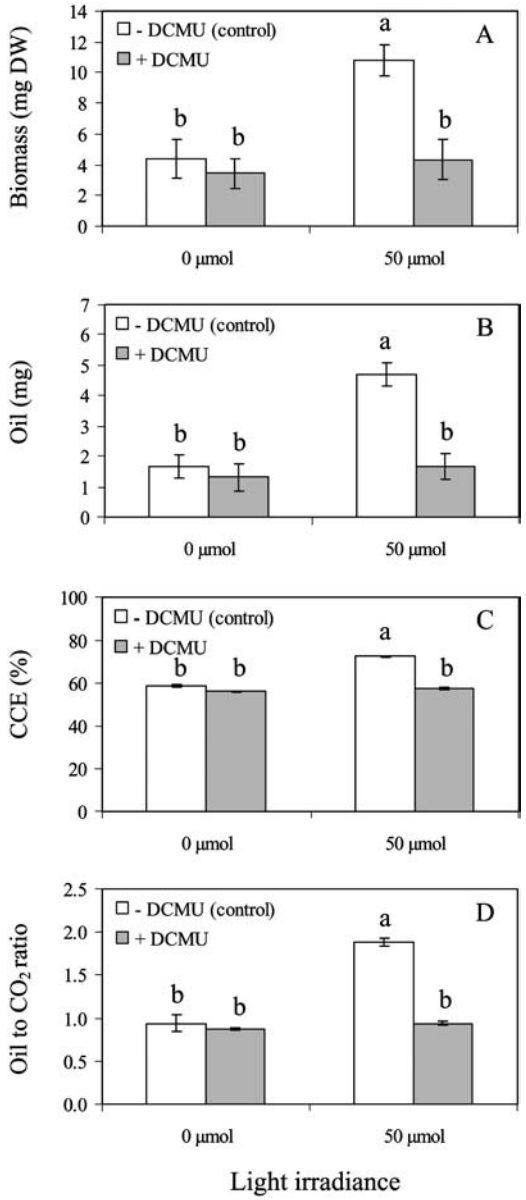

Figure 3. Effect of inhibition of PSII by DCMU on biomass production, oil synthesis, carbon conversion efficiency, and the oil to CO_2 ratio under light and dark conditions. Embryos were cultured in the presence of U-^{14}C carbon sources for 3 d at 50 μmol m^{-2} s^{-1} or in the dark with or without DCMU. After culture, the ^{14}C-labeling in the CO_2 and biomass compounds was determined. A, Total biomass production expressed in milligrams dry weight (seven embryos). B, Oil synthesis expressed in milligrams (seven embryos). C, CCE, expressed as percent of the sum of ^{14}C biomass and $^{14}CO_2$. D, Oil to CO_2 ratio, calculated from the ratio of ^{14}C-labeling between oil and CO_2. Columns and error bars correspond to the mean ± SD of three independent replicates. Means with the same letter are not significantly different at the $P < 0.05$ level (Tukey's studentized range test).

grown cultures (via PSII inhibition) without causing other major effects on embryo metabolism. The CCE and the oil to CO_2 ratio were also reduced by the DCMU treatment in the light, the control (0 μM DCMU) showing 28% and 110% higher CCE and oil to CO_2 ratio, respectively compared to the DCMU treatment. These results indicate that inhibition of PSII significantly reduces biomass and oil synthesis as well as the conversion efficiency of carbon sources into storage products.

Influence of Oxygen on Carbon Balance in Embryos and Lipid Synthesis in Seeds within Siliques

When oxygen levels in the culture flask were reduced to 1% O_2, embryos in the dark grew little or not at all and CCE was severely reduced to 35% (Fig. 4). Thus, under hypoxic conditions, the efficiency of conversion of assimilates into storage materials is strongly diminished. At superambient O_2 levels (64%), growth in the dark was increased by 30% but CCE did not increase, suggesting that higher oxygen availability in the dark does not increase the efficiency of carbon utilization in the developing embryos.

We also examined the influence of increased oxygen on lipid synthesis of seeds growing inside siliques. When the level of oxygen was increased by 3-fold, there was no significant change in the incorporation of ^3H_2O into lipids in the light or dark (Fig. 5A). Thus, under ambient light conditions similar to those of plants in the field, rapeseed embryos may produce sufficient oxygen via PSII to avoid limitation of lipid synthesis. Under dark conditions, the incorporation of ^3H into oil was also not significantly different from the initial ^3H-prelabeling, suggesting that lipid synthesis is strongly reduced in the dark. We conducted an additional experiment using [U-^{14}C]Suc as a lipid precursor. As shown in Figure 5B, increasing the oxygen levels from 21% to 60% did not significantly increase lipid synthesis in seeds inside siliques in the dark, again suggesting that low oxygen is not the major explanation for low rates of fatty acid synthesis in the dark.

DISCUSSION

To meet the biosynthetic and energy needs required for germination and seedling growth, developing seeds must maximize the conversion of photosynthate provided by the mother plant into storage reserves packaged in the mature seed. Carbon utilization efficiency, which is the result of the balance of fluxes through anabolic, catabolic, and maintenance pathways, is therefore a significant parameter of seed biology. Quantifying this parameter and determining the factors that control it are also important to increase our understanding of the basis of seed yield. For example, seed yield could be reduced by "futile cycles" that use cellular energy without contributing to biomass formation. In this context, three studies have suggested that a major fraction of the ATP produced by heterotrophic plant cells is consumed by futile cycles associated with Glc formation from hexose-phosphates (Dieuaide-Noubhani et al., 1995; Rontein et al., 2002; Alonso et al., unpublished data).

Earlier work (Flinn et al., 1977; Pate et al., 1977; Peoples et al., 1985) reported carbon balances conducted

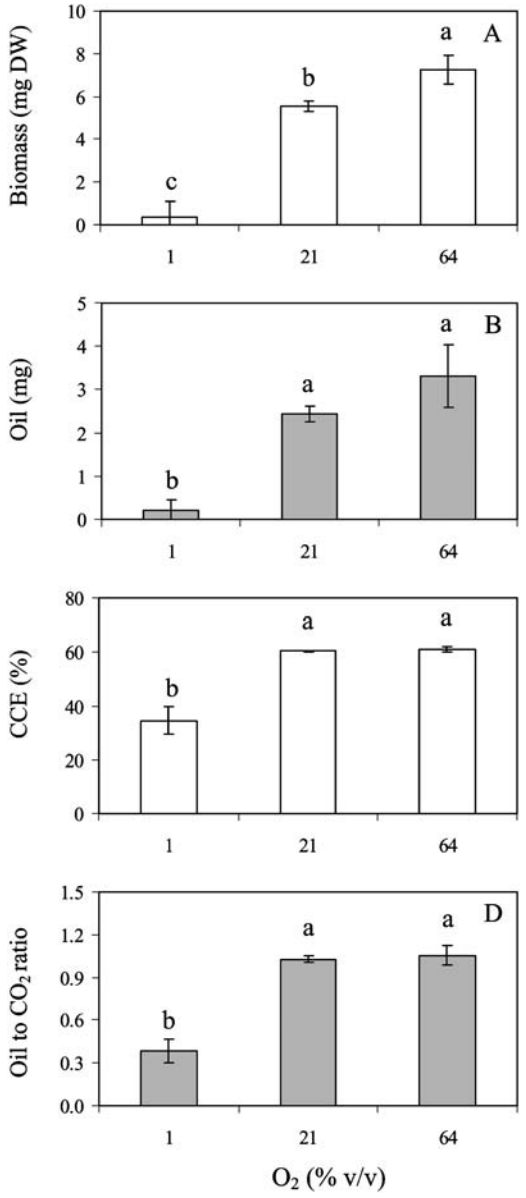

Figure 4. Effect of oxygen on biomass production, oil synthesis, CCE, and oil to CO_2 ratio under dark conditions. Embryos were cultured in the dark for 3 d under approximately 1%, 21% (ambient oxygen), and 64% (v/v) O_2 in the presence of U-^{14}C carbon sources. A, Total biomass production expressed in milligrams dry weight (seven embryos). B, Oil synthesis, expressed in milligrams (seven embryos). C, CCE expressed as percent of the sum of ^{14}C biomass and $^{14}CO_2$. D, Oil to CO_2 ratio, calculated from the ratio of ^{14}C-labeling between oil and CO_2. Columns and error bars correspond to the mean ± SD of three independent replicates. Means with the same letter are not significantly different at the $P < 0.05$ level (Tukey's studentized range test).

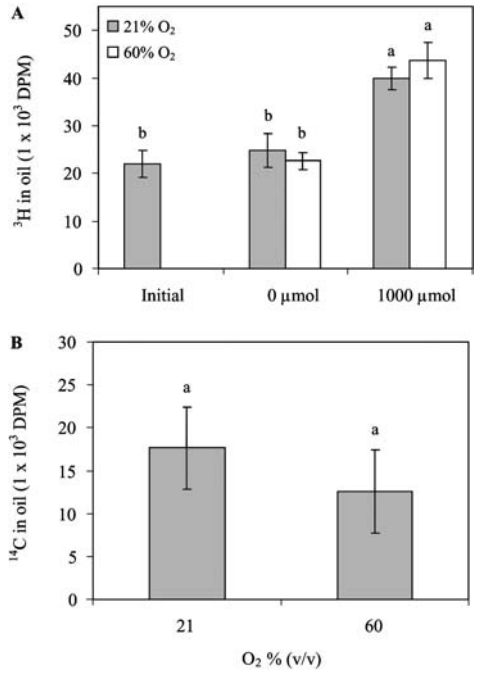

Figure 5. Incorporation of radiolabel from (A) ^3H_2O and (B) [U-^{14}C]Suc into lipids in seeds inside siliques as influenced by light and oxygen levels. A, detached siliques were prelabeled for 24 h (initial) in a buffered solution containing ^3H_2O. They were then incubated at 1,000 μmol (light treatment) for further 24 h either at ambient air or at 60% oxygen with one-half of the siliques being loosely covered with aluminum foil (dark treatment). After incubation, four seeds from the central region of each silique were removed, the lipids extracted, and the ^{3}H incorporation into fatty acids determined. Columns and error bars correspond to the mean ± SE of 12 independent replicates. Means with the same letter are not significantly different at the $P < 0.05$ level (Tukey's studentized range test). B, Detached siliques were cultured in the dark in the presence of [U-^{14}C]Suc for 4 d either at ambient air or at 60% oxygen. After incubation, the seeds from each silique were removed, the lipids extracted, and the ^{14}C incorporation into oil determined. Columns and error bars correspond to the mean ± SD of 12 independent replicates. Means with the same letter are not significantly different at the $P < 0.05$ level (Tukey's studentized range test).

in planta in developing fruits of grain legumes that store primarily carbohydrate and protein and little oil. In those studies, the carbon balance was estimated from intake and utilization of carbon and water and from CO_2 exchange measurements. Carbon intake was indirectly deduced from measurements of the amounts of water lost in fruit transpiration during growth, the concentration of solutes delivered to the fruit in xylem and phloem, and the relative ratio of xylem and phloem flows. These previous studies estimated that the conversion of photoassimilates to seed biomass (mainly storage carbohydrates and proteins) is very efficient. In pea (*Pisum sativum*) fruit, for example, from 18 to 36 d after flowering, almost 80% of the translocate from the parent plant and pod walls was incorporated into dry matter in the seeds (Flinn et al., 1977). Peoples et al. (1985) estimated that 70% of carbon entering the fruit was incorporated into seeds of cowpea (*Vigna unguiculata*).

Uncertainties about xylem and phloem flow rates and recirculation between the two in those studies (Köckenberger et al., 1997) make these earlier estimates uncertain. Furthermore, these studies and others (e.g. Furbank et al., 2004) have shown that for species such as chickpea, refixation by the fruit wall of CO_2 respired by seeds makes a significant contribution to final carbon recoveries in seeds. For plants with both green seeds and green pod walls, it is difficult to distinguish between contributions of fruit walls and seeds to final seed mass (Willms et al., 1999).

Direct measurement of C uptake and utilization by developing seeds should therefore provide more accurate data for estimating the efficiency of carbon utilization and the factors that influence it. In this study we report a direct analysis of the efficiency of carbon metabolism for developing embryos. Our results indicate that embryo carbon metabolism can be extremely efficient when light is provided, converting up to 95% of carbon input into storage material. For comparison, microorganisms growing aerobically convert approximately 50% of input carbon into biomass (Neidhardt et al., 1990).

Light Increases Biomass Accumulation, Lipid Synthesis, and the Efficiency of Carbon Storage in Rapeseed Embryos

Our results (Figs. 1–3) quantitatively demonstrate the importance of light to achieve high carbon use efficiency in embryos and extend results from several earlier studies. Light may affect carbon utilization by the developing embryos by driving the production of reductant and ATP for fatty acid synthesis. Browse and Slack (1985) concluded that light could provide cofactors for fatty acid synthesis in linseed plastids, and in canola embryos, light stimulates oil synthesis (Fuhrmann et al., 1994; Aach and Heise, 1998; Bao et al., 1998; Ruuska et al., 2004).

The oil to CO_2 ratio is one useful indicator of how efficiently oilseeds use carbon to synthesize oil. As a result of loss of CO_2 in the pyruvate dehydrogenase reaction, conversion of carbohydrate to oil via glycolysis will yield a maximum ratio of two. The values we determined for dark-grown embryos were near one and indicate that the embryos produce CO_2 not only via pyruvate dehydrogenase but also from other respiratory pathways involving carbohydrate oxidation. At 50 μmol m^{-2} s^{-1}, the ratio was higher than two, which can be explained by the involvement of Rubisco and the nonoxidative steps of the pentose phosphate pathway in a metabolic route (the Rubisco bypass) that we recently described (Schwender et al., 2004). The cofactor requirements (ATP and NADPH) of this route are approximately 15% of those required for CO_2 fixation by the Calvin cycle and therefore this route is feasible even with the attenuated light reaching embryos (Schwender et al., 2004). The capacity of the embryos to use light energy even at low irradiances is in agreement with the fact that developing rapeseed

seed pigments are adapted to utilize low light in photosynthesis (Ruuska et al., 2004). Plants under field conditions on sunny days receive an average of approximately 1,000 μmol m^{-2} s^{-1} for up to 12 h. Since the silique wall transmits approximately 20% of incident light, seeds in planta often experience 200 μmol m^{-2} s^{-1} or more light. At 150 μmol m^{-2} s^{-1}, we observed CCE values approaching 100%, indicating that embryos receiving moderate light are extremely efficient in converting carbon sources into biomass compounds. The very high CCE values observed at 150 μmol m^{-2} s^{-1} light are higher than predicted by the Rubisco bypass (Schwender et al., 2004). In addition, at 150 μmol m^{-2} s^{-1}, we observed substantial fixation of external CO_2 into oil (Fig. 3), a labeling pattern that is characteristic of the Calvin cycle but not the Rubisco bypass. Together, these observations indicate that cofactor production by the photosystems is sufficient at 150 μmol m^{-2} s^{-1} to power the Calvin cycle fixation of additional CO_2, thereby increasing the CCE close to 100%.

Increasing light from 50 to 150 μmol m^{-2} s^{-1} also significantly ($P < 0.05$) increased the oil to protein ratio. We have observed similar results for Arabidopsis (*Arabidopsis thaliana*) plants grown under different light levels (Y. Li and J.B. Ohlrogge, unpublished data). These data suggest that in the plastid, greater cofactor production by light can preferentially stimulate fatty acid synthesis or, alternatively, greater phosphoglycerate synthesis via Rubisco may increase substrates availability for plastid fatty acid synthesis. However, light is clearly not essential for high oil production in some seeds such as castor (*Ricinus communis*) or sunflower (*Helianthus annuus*) that do not have green seeds. Our initial studies have indicated that isolated sunflower embryos have carbon conversion efficiencies of approximately 60% (data not shown). Other methods of recycling respiratory carbon involving nonseed structures may allow such plants to achieve overall high conversions of photosynthate to seed oil.

Superambient Oxygen Does Not Increase Carbon Storage Efficiency or Lipid Synthesis in Rapeseed

Several recent studies have proposed that seed metabolism may be limited by low oxygen concentrations (Geigenberger, 2003; Vigeolas et al., 2003). In our embryo culture experiments, we found that the rate and composition of biomass accumulation at ambient oxygen are very close to those observed in planta. Vigeolas et al. (2003) reported that developing rapeseed seeds growing in planta at ambient oxygen levels contain approximately 0.8% O_2 (v/v). We attempted to mimic these conditions by growing embryos at low oxygen concentrations. However, the embryos almost stopped growing, accumulating 15-times less biomass than at atmospheric oxygen (Fig. 4A). Thus, an external oxygen level of 1% added to embryo cultures apparently does not reflect in planta conditions, perhaps because the embryos consumed oxygen, resulting in strong hypoxia. Because the silique cavity contains 17% O_2 (Vigeolas et al., 2003), culture of rapeseed embryos under 17% to 21% O_2 likely better represents in planta conditions.

Increasing the oxygen levels from 21% to 64% in the dark did not increase CCE, which remained unchanged at 64%. In addition, for seeds growing inside siliques (Fig. 5), an increase in the oxygen levels from 21% to 60% did not elevate the rate of oil synthesis in the light (1,000 μmol m^{-2} s^{-1}) nor in the dark despite the fact that such an increase in external oxygen levels raises the O_2 levels in the siliques and thus inside the seeds (Vigeolas et al., 2003). Vigeolas et al. (2003) also reported that the lipid synthesis rate in planta increases about 2 times when oxygen levels are increased from atmospheric to 60%. However, the plants in that investigation were cultivated at 300 μmol m^{-2} s^{-1}, whereas in our study the siliques were exposed to 3-fold higher levels of light (1,000 μmol m^{-2} s^{-1}). Seeds and embryos are capable of evolving net oxygen via photosynthesis (Eastmond et al., 1996; King et al., 1998), and under higher levels, light may be sufficient to drive photosynthetic electron transport to provide cofactors and/or oxygen so that oil synthesis is not limited by oxygen availability. The lack of effect of elevated oxygen on embryos inside dark siliques may be because overall metabolism is slower under such conditions and oxygen demand can be met by diffusion from the environment. Whether or not oxygen in fact limits growth and biosynthesis in developing seeds under field light conditions, its utilization for respiration will reduce rather than improve carbon conversion efficiency. Thus, the production of cofactors via photosynthesis is an effective strategy for green seeds to improve carbon storage.

Why is Rapeseed Embryo Metabolism Slow in the Dark?

In this and previous studies, we have observed that rapeseed growth and metabolism are substantially

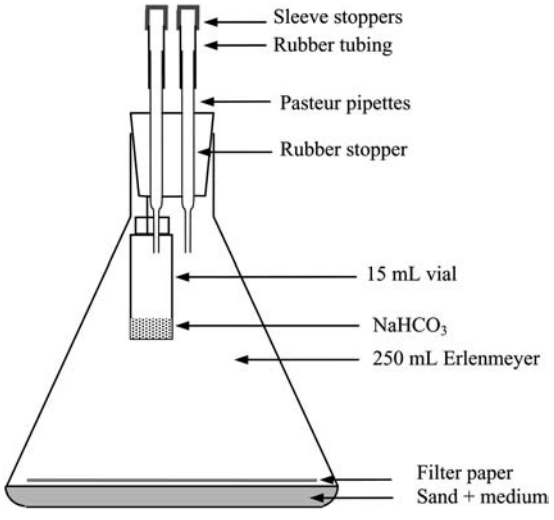

Figure 6. Diagram of the culture system used to perform 3-d carbon balances of rapeseed embryos under 2% CO_2.

reduced in the dark (Bao et al., 1998; Ruuska et al., 2004; Schwender et al., 2004). Both in culture and in planta embryos have abundant supplies of carbohydrate that could in principle provide cofactors and precursors needed to maintain high rates of metabolism. Thus, it is not immediately clear why metabolism slows at least 2- to 3-fold in the dark. We propose that lowering the rate of storage product accumulation (particularly oil) during the night may be a strategy to reduce carbon lost as CO_2. In rapeseed seeds, an oil content of 45% by weight is equivalent to 61% of seed carbon and therefore over 20% of the imported carbon would be lost at night, whereas in the light, this loss is greatly reduced (Fig. 1).

Based on data of King et al. (1998), the CO_2 produced during the night is unlikely to be recovered by the plant. Even though the sclerenchyma layer of the silique wall may act as a gaseous diffusion barrier causing a build-up of respired CO_2 within the silique locus, King et al. (1998) found that light-dependent CO_2 fixation significantly lowered cavity CO_2 only at 26 DPA, a stage when silique wall chlorophyll is high and seed respiration is low (Eastmond et al., 1996). In addition, at 26 DPA, the CO_2 refixation capacity of the silique wall is 2-fold lower than that of the seed and further decreases to 2.5 times at 30 DPA (King et al., 1998). In developing pea fruits, Flinn et al. (1977) found that more than 40% of $^{14}CO_2$ injected into the fruit gas cavity in the dark was not fixed by pod or seeds, and it was released as $^{14}CO_2$ to the outside atmosphere within 1 h. Together, these studies suggest that in oil plants, the conversion of carbon supplies to reserve materials may cause a large loss of carbon during the night or at very low light irradiances. Thus, green oilseeds like rapeseed may maximize oil synthesis during light periods and strongly reduce oil synthesis under dark conditions to avoid large losses of carbon. This diurnal carbon conservation strategy may ultimately lead to more carbon storage in seeds and thereby, a higher number of successfully established seedlings in the next generation.

MATERIALS AND METHODS

Radiochemicals

[U-$^{14}C_6$]Glc (317 mCi/mmol, 11.7 GBq/mmol), [U-$^{14}C_{12}$]Suc (660 mCi/mmol, 24.4 GBq/mmol), [U-$^{14}C_5$]Gln (242 mCi/mmol, 8.95 GBq/mmol), and [U-$^{14}C_3$]Ala (162 mCi/mmol, 5.99 GBq/mmol) were from Amersham Biosciences (Piscataway, NJ); $NaH^{14}CO_3$ (7.2 mCi/mol, 265.74 GBq/ mmol) was from Sigma-Aldrich (St. Louis).

Plant Material

Rapeseed (*Brassica napus*) *L.* cv Reston was grown in 30-cm pots in a greenhouse maintained at 20°C/15°C day/night temperature and with supplemental lighting to provide irradiance of approximately 600 μmol m^{-2} s^{-1} and a 16-:8-h day/night photoperiod. At 2 weeks, seedlings were thinned to two per pot. Flowers from the main stem were tagged at anthesis and silique development recorded as DPA. Tagged siliques were harvested at 20 DPA and taken to a laminar flow bench for dissection.

Medium Composition and Embryo Culture Conditions

Siliques were surface sterilized with 5% sodium hypochlorite for 10 min and then rinsed three times with sterile water. The developing embryos were dissected under aseptic conditions and transferred into the following culture medium. Carbon and nitrogen medium sources were: Suc (80 mM), Glc (40 mM), Gln (35 mM), and Ala (10 mM). The mineral and vitamin additions were (μg mL^{-1}): $MgSO_4.7H_2O$, 370; KCl, 350; $CaCl_2.2H_2O$, 880; KH_2PO_4, 170; Na_2EDTA, 14.9; $FeSO_4.7H_2O$, 11.1; H_3BO_3, 12.4; $MnSO_4.H_2O$, 33.6; $ZnSO_4.7H_2O$, 21; KI, 1.66; $Na_2MoO4.2H_2O$, 0.5; $CuSO_4.5H_2O$, 0.05; $CoCl_2.6H_2O$, 0.05; nicotinic acid, 5; pyridoxin hydrochloride, 0.5; thiamine hydrochloride, 0.5; and folic acid, 0.5, respectively. Polyethylene glycol 4,000 was added at 20% to adjust osmotic potential (Schwender and Ohlrogge, 2002), and 50 mM MES was included as a buffer and the medium adjusted to pH 5.8 with 1 N KOH. The embryos were cultured either in a 250-mL screw-cap Erlenmeyer flask sealed with a septum closure or in a regular Erlenmeyer closed with a two-hole rubber stopper connected to two Pasteur pipettes with attached rubber tubing and sleeve stoppers (Fig. 6). The sealing was tested to ensure that the flasks were gas-tight. The septum closure and the sleeve stoppers were included in the culture system to allow CO_2 determinations.

For shorter-term experiments, embryos (20 DPA) were acclimated by preculture for 3 to 7 d at 21°C under continuous fluorescent light (50 μmol m^{-2} s^{-1}) in petri dishes containing a sterile glass prefilter and 7 mL of culture medium and were then cultured for 3 d in the presence of radiolabel as described below. Since embryos in planta develop under a high (up to 2.5%) CO_2 atmosphere (Johnson-Flanagan and Spencer, 1994; King et al., 1998, Goffman et al., 2004), we increased the initial content of CO_2 to 2%. The gas headspace was adjusted to contain 2% CO_2 by including a 15-mL glass vial containing 18.6 mg sodium bicarbonate in the culture system. By injecting 2 mL of 1 N HCl through the Pasteur pipette directly into the vial, the bicarbonate is converted to CO_2. We tested this system by quantifying the CO_2 levels inside the culture system with an Infra Red Gas Analyzer (IRGA) as described by Goffman et al. (2004), obtaining a high reproducible level of headspace CO_2 (%CO_2 = 1.92, SD = 0.01). Additionally, to provide a uniform support for the embryos, the flask's bottom was leveled by adding 20 mL of washed and ignited sand (mesh 30–40) and a 6.5-cm diameter filter paper (low ash, <0.01%; fine porosity) was placed on the top of the sand. Addition of 8 mL of medium to the flasks produced a thin liquid layer around the embryos (approximately 0.5 mm).

Effect of Light Irradiance on Carbon Balance

Flasks were incubated in a growth chamber at 21°C either under darkness or continuous fluorescent illumination of 50 or 150 μmol m^{-2} s^{-1}. Seven embryos (20 DPA) were cultured in each flask for 14 d. For dark treatment, 12 embryos were cultured for 21 d to compensate for the slow growth in the dark and to provide sufficient biomass for analysis. Uniformly ^{14}C-labeled carbon supplies (Suc, Glc, Gln, and Ala) were added to the growth medium to provide approximately 10 μCi (370 kBq) total radioactivity per flask. The radioactivity of each compound was adjusted such that each carbon atom added to the growth medium was equally represented at a specific activity of C = 0.89 mCi/mol C. For shorter-term experiments, precultured embryos were transferred to 250-mL Erlenmeyer flasks (7 embryos per flask) and cultured for 3 d in a growth chamber at 21°C either under darkness or light of 50 or 150 μmol m^{-2} s^{-1} in the presence of radiolabeled carbon supplies as described above.

$^{14}CO_2$ Fixation at Different Light Levels

Precultured embryos (7 embryos per flask) were cultured for 3 d under a 2% ^{14}C-labeled CO_2 atmosphere at three light levels (0, 50, and 150 μmol m^{-2} s^{-1}). The $^{14}CO_2$ was added to headspace by pipetting 100 μL of a $NaH^{14}CO_3$ solution in 1 N KOH (100 μCi mL^{-1}, 3.7 MBq mL^{-1}) into the internal vial containing unlabel sodium bicarbonate before injecting 1 N HCl. The resulting specific activity of the CO_2 was 44.9 mCi/mol.

Effect of Inhibiting PSII on Carbon Balance

Precultured embryos (7 embryos per flask) were cultured for 3 d under an approximately 2% CO_2 in the presence of radiolabeled carbon sources as described above. The experiment was conducted using a factorial design with light level and DCMU concentration as factors, each with two levels: 0 and 50 μmol m^{-2} s^{-1}, and 0 and 5 μM DCMU, respectively.

Effect of Oxygen on Embryo Carbon Balance

Precultured embryos were incubated in the dark for 3 d under three different O_2 levels (approximately 1%, 21%, and 64% O_2, each with 2% CO_2) in the headspace in the presence of radiolabeled supplies as described above with three replicates per treatment. The gas mixtures were created as follows: approximately 1% O_2 was produced by flushing the flasks with humidified N_2 at approximately 78 mL min^{-1} for 9 min 20 s and approximately 64% O_2 by flushing the flasks with humidified O_2 at approximately 78 mL min^{-1} for 2 min 4 s. The gases were humidified by bubbling them through water traps placed in series between the gas cylinders and the flasks. Afterward, 6.5 mL of the headspace gas was withdrawn from each flask using a syringe, and the same volume of pure CO_2 was injected into the flasks to create a 2% CO_2 internal atmosphere in all samples. The resulting gas composition of each flask was checked in duplicate analysis at the beginning of the incubation by injecting 100 μL of the headspace gas into the IRGA.

Determination of ^{14}C-Labeling in Medium, CO_2 Efflux, and $^{14}CO_2$

Immediately after culture, the flasks were placed in an ice bath and 1 mL 0.2 N HCl was injected through the septum or sleeve stopper into the medium to stop metabolism and to release inorganic carbon to the flask headspace. Duplicate 200-μL gas samples were withdrawn from the flask headspace using a 1-mL syringe and injected into an IRGA to determine total CO_2 efflux. The flasks were then flushed for 2 h with nitrogen at 45 mL min^{-1} and the exhaust gas bubbled through a 250-mL gas washing bottle containing 140 mL of 1 N KOH. The recovery efficiency of the CO_2 trap was tested with known amounts of $NaH^{14}CO3$, being 99.6% (SD% = 3.3). After trapping the CO_2, the embryos were removed and rinsed three times each with 10 mL water to remove surface radioisotope. The embryos were then frozen with liquid nitrogen and lyophilized. The rinse water used for washing the embryos was added to the flasks and the medium was filtered in vacuo through a Buechner funnel with a 55-mm ashless filter paper (fine porosity). The filter and the flask were rinsed several times with water to recover all medium, and the final volume was adjusted to 100 mL. ^{14}C-label in medium was determined in duplicate analysis by liquid scintillation counting with quenching and background correction. An aliquot (2.5 mL) of the trapping solution containing the $^{14}CO_2$ was pipetted into a 20-mL scintillation vial containing 5 mL of Hionic Fluor scintillation cocktail (Packard BioScience) and counted on a liquid scintillation counter. Analysis was performed in duplicate and all counts were corrected for background and quenching.

Separation of ^{14}C-Labeled Biomass Compounds

To extract the oil, labeled embryos (approximately 20 mg dry weight) were homogenized with a glass microgrinder at 4°C in 1 mL hexanes:isopropanol (2:1, v/v). The microgrinder tube was centrifuged for 5 min at 5,000*g* and the supernatant was pipetted into a glass test tube. This extraction was repeated two more times. The pooled lipid fractions were dried under nitrogen at 60°C and redissolved in 4 mL isooctane for scintillation counting. We examined this fraction by thin-layer chromatography (TLC) on silica gel plates developed for 30 min with chloroform/methanol/water (65:25:4 v/v), confirming that it is composed of lipids (>97% of the total counts). After lipid extraction, the pellets were extracted three times each with 1 mL 80% ethanol to recover low-M_r compounds (mostly sugars, amino and organic acids). TLC analysis on cellulose plates of the ethanolic extracts developed with *n*-butanol/acetic acid/water (4:1:1 v/v) for 2 h (three times) using U-^{14}C standards (Suc, Glc, Gln, and Ala) confirmed that this fraction mainly contains the carbon sources included in the culture medium (data not shown). The remaining pellet was then dried at room temperature under a stream of nitrogen and resuspended in 1 mL of 0.01 M sodium phosphate saline buffer, pH 7.4, containing 1 mM EDTA, 10 mM 2-mercaptoethanol, 0.02% (w/v) sodium azide,l and 0.0125% (w/v) SDS. The homogenate was transferred to a glass tube and 3 mL buffer solution along with four glass beads (4-mm diameter) were added to the samples. The proteins were extracted by mixing the samples with a Vortex mixer for 15 min. The tubes were then centrifuged at 3,200*g* for 15 min and the supernatants transferred into another glass tube. The proteins were further extracted by repeating this procedure after adding an additional 4 mL of buffer. The collected protein extracts were pooled and an aliquot sampled for scintillation counting. The pellet was then dried under nitrogen at 60°C and 0.5 mL of 67% (v/v) aqueous sulfuric acid was added to the samples to hydrolyze starch and cell wall components. The pellet was mixed with a Vortex mixer and incubated for approximately 5 min at 60°C until samples turned slightly red. The reaction was stopped by adding 4.5 mL of 1 N KOH and an aliquot of the suspensions was counted. ^{14}C-label was determined in duplicate analysis in each biomass fraction (oil, protein, and starch and cell wall components) by liquid scintillation counting with quenching and background correction.

Determination of Oil Content

Total oil content of embryos was determined by gas chromatography with flame ionization detection of fatty acid methyl esters. One milliliter of the above lipid extract together with 1 mL of a 750 μg mL^{-1} triheptadecanoin solution in isooctane (internal standard) were dried under nitrogen at 60°C, and then transmethylated for 45 min at 60°C with 1 mL of a 0.5-M solution of sodium methylate in methanol. Isooctane (1.5 mL) and 0.5 mL of 5% (w/v) $NaHSO_4$ in water were added with mixing and tubes centrifuged for 10 min at 3,200*g*. One microliter of the isooctane phase was analyzed on a DB-23 capillary column (30-m × 0.25-mm i.d. × 0.25-μm thickness; Agilent J&W, Palo Alto, CA). The carrier gas was helium at a pressure of 120 kPa. The oven temperature was programmed as follows: the initial temperature (150°C) was kept for 3 min, increased linearly to 210°C at a 5°C/min rate, and then increased to 250°C at 20°C/min, being the final temperature hold for 3 min. The injector and detector temperatures were 270°C and 280°C, respectively. The samples were injected at a split rate of 15:1.

Calculation of Carbon Balance, Carbon Recovery, and Carbon Conversion Efficiency

The carbon balance of rapeseed embryo cultures was determined by providing all carbon sources uniformly ^{14}C-labeled at the same specific activity and determining ^{14}C incorporation into biomass end products, the ^{14}C radioactivity remaining in the growth medium and released as $^{14}CO_2$. Carbon uptake was estimated as the difference between initial ^{14}C-labeling (10 μCi, 370 kBq) minus final ^{14}C-labeling present in the medium after culture. In preliminary experiments, NMR and TLC analysis of the medium after culture of embryos (data not shown) indicated that embryos do not secrete products (e.g. malate) at levels that would alter the carbon balance. The recovery of carbon after culture (carbon recovery [percent] = [^{14}C biomass + $^{14}CO_2$] × 100/^{14}C uptake) was 99.5% (SD = 5.3%), which indicates that all the major carbon components in the system are properly accounted for. Therefore, the sum of measurements of ^{14}C incorporation into embryo biomass plus $^{14}CO_2$ represents the total uptake of labeled carbon supplies. ^{14}C-labeled biomass was calculated as the sum of each biomass fraction (^{14}C biomass = ^{14}C oil + ^{14}C protein + ^{14}C starch and cell wall). The 80% ethanolic fraction (<15% of total ^{14}C in 3-d experiments) was not included in this calculation because it largely represents substrate uptake but not carbon conversion to storage products. CCE was calculated as: percent CCE = ^{14}C biomass × 100/[^{14}C biomass + $^{14}CO_2$].

Effect of Oxygen on Oil Synthesis in Seeds within Siliques

Lipid Labeling Using ^3H_2O as Precursor

Because it equilibrates rapidly with cellular water and exchanges ^{3}H with hydrogen atom of NAD(P)H that participate in reduction steps of fatty acid synthesis, ^3H_2O is an effective tracer for lipid synthesis (Bao et al., 1998; Ruuska et al., 2004). To assess the influence of oxygen on seeds within siliques, ^3H_2O was fed to the seeds via the transpiration stream as follows. Eighteen siliques at 30 DPA were removed from greenhouse grown plants and the pedicle was immediately placed into a solution of 5 mCi/mL ^3H_2O, 50 mM Suc, 50 mM HEPES, pH 5.7, and 3 mg/mL Murashige and Skoog basal salts (Murashige and Skoog, 1962). After 24 h in the light to allow movement of water into the silique (and its seeds) via transpiration, seeds from six siliques were analyzed for ^{3}H prelabeling into oil as described below. The remaining 12 siliques were further incubated for 24 h in two 1-L jars continuously flushed (1 L min^{-1}) with either ambient air or with 60% oxygen. Increasing ambient oxygen surrounding siliques has been shown to rapidly increase silique locule oxygen content (Porterfield et al., 1999). Light for each jar was provided from two 500-W projector bulbs shining through $CuSO_4$ solution in Blake bottles to

absorb infrared light. Light impinging on each silique was approximately 1,000 μmol m^{-2} s^{-1}. Temperature in the jars did not increase during the experiment. One-half of the siliques in each jar were loosely covered with aluminum foil with both ends open and allowing air space surrounding the silique. During the entire 48-h experiment, approximately 1 mL of solution was taken up (transpired) by each silique. Four seeds from the central region of each silique were then removed, placed into individual screw cap tubes, and heated with 0.1 mL isopropanol for 10 min at 80°C. After cooling, seeds were homogenized and 0.15 mL hexane added and allowed to extract overnight at room temperature. After adding 0.8 mL hexane and 0.2 mL 0.1 M acetic acid, 0.35 mL of the upper phase lipid extract was transferred to a scintillation vial, evaporated at 70°C, and counted to determine ^{3}H incorporation into fatty acids.

Lipid Labeling in the Dark Using [U-^{14}C]Suc as Precursor

We performed an additional experiment using [U-^{14}C]Suc as lipid precursor with no prelabeling. Siliques at 23 DPA were taken from greenhouse plants and surface sterilized as above. The siliques' pedicels were placed into 15-mL vials containing 150 mM [U-^{14}C]Suc (10 μCi, 370 kBq), 70 mM Gln, and 4.3 mg mL^{-1} Murashige and Skoog basal salts, pH 5.8. Three vials with two siliques per vial were placed inside 1-L screw-cap Erlenmeyers covered with aluminum foil. Two flasks per oxygen treatment were sealed after transferring the vials inside the flasks. A 60% O_2 (v/v) atmosphere was created by flushing the flasks with humidified O_2 at approximately 78 mL min^{-1} for 10 min 16 s. The gas composition was checked by injecting 100 μL of the flask gas headspace into the IRGA. After 4 d of labeling at 21°C, the siliques were then removed from the vials and immediately submerged into liquid nitrogen and lyophilized for 48 h. The seeds from each silique were removed, placed in 1.5-mL polypropylene vials containing a 6 mm-stainless steel ball, and homogenized in 0.5 mL 2:1 (v/v) hexanes:isopropanol. After centrifugation, lipids were extracted two more times each with 1 mL of solvent mixture. Pooled extracts were directly dried inside scintillation vials under N_2 at 60°C. Five milliliters of scintillation cocktail were added to each vial and ^{14}C-labeling determined by liquid scintillation counting.

Statistics

One-way ANOVA and Tukey's studentized range test were performed using the general linear model procedure of SAS statistical software (SAS Institute, NC). Unless otherwise indicated, all experiments were done with three replications per treatment.

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LITERATURE CITED

Aach H, Heise KP (1998) On the compartmentation of triacylglycerol synthesis in developing seeds of *Brassica napus*. Bot Acta **111:** 123–129

Armstrong DP, Westoby M (1993) Seedlings from large seeds tolerate defoliation better: a test using phylogenetically independent contrasts. Ecology **78:** 1092–1116

Bao XM, Pollard M, Ohlrogge J (1998) The biosynthesis of erucic acid in developing embryos of *Brassica rapa*. Plant Physiol **118:** 183–190

Black JN (1958) Competition between plants of different initial seed sizes in swards of subterranean clover (*Trifolium subterraneum* L.) with particular reference to leaf area and light microclimate. Aust J Agric Res **9:** 299–318

Browse J, Slack CR (1985) Fatty-acid synthesis in plastids from maturing safflower and linseed cotyledons. Planta **166:** 74–80

Burke MJW, Grime JP (1996) An experimental study of plant community invasibility. Ecology **77:** 776–790

Converti A, Perego P (2002) Use of carbon and energy balances in the study of the anaerobic metabolism of *Enterobacter aerogenes* at variable starting glucose concentrations. Appl Microbiol Biotechnol **59:** 303–309

Dieuaide-Noubhani M, Raffard G, Canioni P, Pradet A, Raymond P (1995) Quantification of compartmented metabolic fluxes in maize root tips using isotope distribution from ^{13}C- or ^{14}C-labeled glucose. J Biol Chem **270:** 13147–13159

Eastmond P, Kolacna L, Rawsthorne S (1996) Photosynthesis by developing embryos of oilseed rape (*Brassica napus* L.). J Exp Bot **47:** 1763–1769

Egli DB (1998) Seed growth and development. *In* DB Egli, ed, Seed Biology and the Yield of Grain Crops, Ed 1. Cab International, New York, pp 15–37

Flinn AM, Atkins CA, Pate JS (1977) Significance of photosynthetic and respiratory exchanges in the carbon economy of the developing pea fruit. Plant Physiol **60:** 412–418

Fuhrmann J, Johnen T, Heise K-P (1994) Compartmentation of fatty acid metabolism in zygotic rape embryos. J Plant Physiol **143:** 565–569

Furbank RT, White R, Palta JA, Turner NC (2004) Internal recycling of respiratory CO_2 in pods of chickpea (*Cicer arietinum* L.): the role of pod wall, seed coat, and embryo. J Exp Bot **55:** 1687–1696

Geigenberger P (2003) Response of plant metabolism to too little oxygen. Curr Opin Plant Biol **6:** 247–256

Goffman FD, Ruckle M, Ohlrogge J, Shachar-Hill Y (2004) Carbon dioxide concentrations are very high in developing oilseeds. Plant Physiol Biochem **42:** 703–708

Grime JP, Jeffery DW (1965) Seedling establishment in vertical gradients of sunlight. J Ecol **53:** 621–642

Howe HF, Richter WM (1982) Effects of seed size on seedling size in *Virola surinamensis*: a within and between tree analysis. Oecologia **53:** 347–351

Johnson-Flanagan AM, Spencer MS (1994) Ethylene production during development of mustard (*Brassica juncea*) and canola (*Brassica napus*) seed. Plant Physiol **106:** 601–606

King SP, Badger MR, Furbank RT (1998) CO_2 refixation characteristics of developing canola seeds and silique wall. Aust J Plant Physiol **25:** 377–386

Köckenberger W, Pope JM, Xia Y, Jeffrey KR, Komor E, Callaghan PT (1997) A non-invasive measurement of phloem and xylem water flow in castor bean seedlings by nuclear magnetic resonance microimaging. Planta **201:** 53–63

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant **15:** 473–497

Neidhardt FC, Ingraham J, Schaechter M (1990) Physiology of the Bacterial Cell: A Molecular Approach. Sinauer Associates, Sunderland, MA, pp 353

Novák L, Loubiere P (2000) The metabolic network of Lactococcus lactis: distribution of 14C-labeled substrates between catabolic and anabolic pathways. J Bacteriol **182:** 1136–1143

Pate JS, Sharkey PJ, Atkins CA (1977) Nutrition of a developing legume fruit. Plant Physiol **59:** 506–510

Peoples MB, Pate JS, Atkins CA, Murray DR (1985) Economy of water, carbon, and nitrogen in the developing cowpea fruit. Plant Physiol **77:** 142–147

Porterfield DM, Kuang AX, Smith PJS, Crispi ML, Musgrave ME (1999) Oxygen-depleted zones inside reproductive structures of Brassicaceae: implications for oxygen control of seed development. Can J Bot **77:** 1439–1446

Rawsthorne S (2002) Carbon flux and fatty acid synthesis in plants. Prog Lipid Res **41:** 182–196

Rontein D, Dieuaide-Noubhani M, Dufourc EJ, Raymond P, Rolin D (2002) The metabolic architecture of plant cells. Stability of central metabolism and flexibility of anabolic pathways during the growth cycle of tomato cells. J Biol Chem **277:** 43948–43960

Ruuska SA, Schwender J, Ohlrogge JB (2004) The capacity of green oilseeds to utilize photosynthesis to drive biosynthetic processes. Plant Physiol **136:** 2700–2709

Sáez JC, Schell DJ, Tholudur A, Farmer J, Hamilton J, Colucci JA, McMillan JD (2002) Carbon mass balance evaluation of cellulase production on soluble and insoluble substrates. Biotechnol Prog **18:** 1400–1407

SAS Institute (1985) SAS User's Guide: Statistics, Ed 5. SAS Institute, Cary, NC, pp 956

Schwender J, Goffman F, Ohlrogge JB, Shachar-Hill Y (2004) A novel role for Rubisco in the carbon economy of developing seeds. Nature **432:** 779–782

Schwender J, Ohlrogge JB (2002) Probing *in vivo* metabolism by stable isotope labeling of storage lipids and proteins in developing *Brassica napus* embryos. Plant Physiol **130:** 347–361

Schwender J, Ohlrogge JB, Shachar-Hill Y (2003) A flux model of glycolysis and the oxidative pentosephosphate pathway in developing *Brassica napus* embryos. J Biol Chem **278:** 29442–29453

Smith RH (2000) Plant Tissue Culture: Techniques and Experiments, Ed 2. Academic Press, San Diego, CA, pp 231

Sousa WP, Kennedy PG, Mitchell B (2003) Propagule size and predispersal damage by insects affect establishment and early growth of mangrove seedlings. Oecologia **135:** 564–575

Stanton ML (1984) Seed variation in wild radish: effect of seed size on components of seedling and adult fitness. Ecology **65:** 1105–1112

Vaughton G, Ramsey M (1998) Sources and consequences of seed mass variation in *Banksia marginata* (Proteaceae). J Ecol **86:** 563–573

Vigeolas H, van Dongen JT, Waldeck P, Hühn D, Geigenberger P (2003) Lipid storage metabolism is limited by the prevailing low oxygen concentrations within developing seeds of oilseed rape. Plant Physiol **133:** 2048–2060

Watson PA, Duffus CM (1991) Light-dependent CO_2 retrieval in immature barley caryopsis. J Exp Bot **42:** 1013–1020

Willms JR, Salon C, Layzell DB (1999) Evidence for light-stimulated fatty acid synthesis in soybean fruit. Plant Physiol **120:** 1117–1127