Maternal starch turnover affects seed composition

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Biochemical Processes and Macromolecular Structures
Altered starch turnover in the maternal plant has major effects on Arabidopsis fruit growth and seed composition

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Abstract

Mature seeds of both the high-starch sex1 mutant and the almost starchless pgm mutant of Arabidopsis have 30-40% less lipid than seeds of wild-type plants. We show that this is a maternal effect, and is not attributable to the defects in starch metabolism in the embryo itself. Low lipid contents and consequent slow post-germinative growth are seen only in mutant embryos that develop on maternal plants with mutant phenotypes. Mutant embryos that develop on plants with wild-type starch metabolism have wild-type lipid contents and post-germinative growth. The maternal effect on seed lipid content is attributable to carbohydrate starvation in the mutant fruit at night. Fruits on sex1 plants grow more slowly than those on wild-type plants, particularly at night, and have low sugars and elevated expression of starvation genes at night. Transcript levels of the transcription factor WRI1, implicated in lipid synthesis, are reduced at night in sex1 but not wild-type seeds, and so are transcript levels of key enzymes of glycolysis and fatty acid synthesis. sex1 embryos develop more slowly than wild-type embryos. We conclude that the reduced capacity of mutant plants to convert starch to sugars in leaves at night results in low night-time carbohydrate availability in the developing fruit. This in turn reduces the rate of development and expression of genes encoding enzymes of storage product accumulation in the embryo. Thus the supply of carbohydrate from the maternal plant to the developing fruit at night can have an important influence on oilseed composition and on post-germinative growth.
INTRODUCTION

Developing embryos of *Arabidopsis thaliana* and oilseed rape (*Brassica napus*), initially accumulate starch, but then starch levels decline as the rates of storage lipid and protein synthesis increase (Hills, 2004; Andriotis et al., 2010c). Starch is undetectable at maturity whereas lipids account for up to 45% of the seed weight (O’Neill et al., 2003; Andriotis et al., 2010a).

Starch turnover during oilseed embryo development may be important in determining the final lipid content of the seed (Norton and Harris, 1975; da Silva et al., 1997). Although starch accumulation in embryos of both Arabidopsis and oilseed rape is small compared with the final lipid content of the seed, at the point of maximum starch content this reserve constitutes a significant fraction of embryo weight and represents a major fate for carbon entering the embryo (Kang and Rawsthorne, 1994; Eastmond and Rawsthorne, 2000; Andriotis et al., 2010c). Mutations that affect starch metabolism throughout the Arabidopsis plant, including the embryo, can bring about substantial reductions in seed lipid content at maturity. Mature seeds of the almost starchless mutant *pgm1* (lacking plastidial phosphoglucomutase) contain up to 40% less lipid than wild-type seeds (Periappuram et al., 2000). Mature seeds of the starch-degradation mutant *sex1* (lacking glucan, water dikinase GWD1; Yu et al., 2001) have ten times more starch than wild-type seeds, and about 30% less lipid (Andriotis et al., 2010c).

Although these observations suggest a direct relationship between starch metabolism and lipid accumulation in the embryo, other evidence is not consistent with this view. For both Arabidopsis and oilseed rape seeds, there are examples of specific disruptions of embryo starch metabolism that have no effect on the final lipid content of the seed. In Arabidopsis, loss of sucrose synthases SUS2 and SUS3 reduced peak levels of starch during embryo development by more than half and accelerated the accumulation of fatty acids, but the final lipid content of the seed was normal (Barratt et al., 2009; Angeles-Núñez and Tiessen, 2010). In oilseed rape, an embryo-specific reduction of 50% in activity of the starch biosynthetic enzyme ADPglucose pyrophosphorylase (AGPase) reduced starch levels at 25-30 days after
anthesis (the point of maximum starch content) by 50%. The onset of lipid accumulation was delayed, but the final lipid content of the seed was only marginally lower than that of wild-type plants (Vigeolas et al., 2004).

An alternative explanation for the impact on seed oil content of mutations causing high-starch or starchless phenotypes is that seed lipid accumulation is affected by altered starch metabolism in leaves or other parts of the maternal plant, rather than in the embryo itself. pgml and sexl mutants have drastic reductions in the supply of carbohydrate from starch for growth and metabolism at night: pgml mutants have no leaf starch and sexl mutants are almost unable to degrade leaf starch (Caspar et al., 1985; 1991; Yu et al., 2001; Streb et al., 2009). Both mutants also exhibit strong reductions in growth specifically at night (Wiese et al., 2007; Smith and Stitt, 2007; Yazdanbakhsh et al., 2010; Pantin et al., 2011). These observations raise the interesting possibility that seed composition of pgml and sexl mutants may be altered because of a reduced supply of carbohydrate from the maternal plant to the reproductive structures at night.

The aim of this work was to establish whether altered starch metabolism in the maternal plant can result in reduced lipid content in the seeds. First, we investigated whether seed lipid content is affected by alterations in starch metabolism specifically in the embryo. Genetic approaches were used to generate phenotypically wild-type plants bearing embryos defective in PGM1 or GWD1. Despite having strongly disrupted starch metabolism, these embryos had wild-type lipid contents at maturity. Second, we examined the effects of the sexl mutation on growth of reproductive structures. Relative to wild-type plants, elongation of young sexl siliques was reduced specifically at night, accompanied by very low levels of sucrose and elevated expression of starvation reporter genes in the inflorescence. In maturing sexl siliques, expression of starvation reporter genes was also elevated specifically at night, and there was reduced expression of the transcription factor WRINKLED1 (WRI1) and of genes that encode key enzymes of glycolysis and fatty acid synthesis. The rate of development of sexl embryos was slower than that of wild-type plants. These results show that seed oil content is strongly dependent on the supply of carbohydrate from the maternal plant to the reproductive structures during the night.
RESULTS

Embryo-specific Down-regulation of PGM1 Expression Does Not Affect the Lipid Content of Mature Seeds

To achieve an embryo-specific reduction in starch content, antisense RNA for PGM1 (At5g51820) was expressed on the B. napus oleosin embryo-specific BN-III promoter (Poleosin; Keddie et al., 1994). PGM1 transcript levels are high early during embryogenesis (early torpedo stage) and peak at walking-stick stage [data from www.genevestigator.com (Zimmermann et al., 2004); Supplemental Fig. S1A). In order to determine the earliest time point during embryogenesis at which the oleosin promoter is active we expressed a translational fusion between Poleosin and uidA [encoding β-glucuronidase (GUS): Poleosin::uidA] in Arabidopsis plants. GUS activity was reproducibly detected from early torpedo stage onwards, and it increased throughout the maturation phase of development (Supplemental Fig. S1, B and C). Transcripts for the Arabidopsis homologs of oleosin BN-III (At3g01570, At3g27660, At4g25140, and At5g40420; Shimada et al., 2008) are readily detectable in torpedo-stage embryos and abundance increases thereafter (Supplemental Fig. 1D). Thus, the Poleosin promoter is active during the same period as PGM1 is expressed.

Several independent homozygous lines expressing PGM1 antisense constructs displayed wild-type rates of growth, phenology and vegetative and reproductive morphology (not shown). Starch accumulation in leaves was like that of wild-type plants (Supplemental Fig. 2A). Native PAGE followed by specific in-gel activity staining revealed three bands of PGM activity in extracts of wild-type leaves. The fastest migrating band was missing from extracts of pgm1 mutants, thus this band corresponds to the plastidial isoform of PGM (Supplemental Fig. 2B). All three bands were present in extracts of leaves of Poleosin::asPGM1 transgenic plants, at similar intensity to wild-type plants. We examined phosphoglucose isomerase (PGI) as a control: two bands of activity were detected from all plants (Supplemental Fig. 2B). Thus the specificity of the Poleosin promoter is retained in the transgenic plants.
and interpretation of any seed phenotypes is unlikely to be complicated by alterations in PGM activity and primary carbohydrate metabolism in other parts of the plant.

To assess the effect of down-regulation of \textit{PGM1} on plastidial PGM activity, embryos were isolated at 10-12 days after flowering (DAF), the point at which starch content is maximum and embryo PGM activity peaks (Baud and Graham, 2006; Andriotis et al., 2010c). The activity of the plastidial isoform was much lower in embryos of two independent \textit{Poleosin::asPGM1} lines than in wild-type embryos. No effect of the transgene was observed on the activity of the PGI isoforms (Fig. 1A).

The starch contents of \textit{pgm1} embryos at 10-12 DAF were close to the detection limit of the assay. Embryos from \textit{Poleosin::asPGM1} plants had 15% to 33% of the starch content of wild-type embryos (Table I) and stained with iodine only in a zone above the radicle tip (Fig. 1B; Supplemental Fig. S3 for color image). These data show that starch accumulation is strongly reduced in developing embryos of the transgenic lines.

Embryo-specific reduction of PGM1 activity resulted in reduced rates of lipid accumulation during embryo development. At 10-12 DAF, the total fatty acid content of developing seeds from \textit{Poleosin::asPGM1} transgenic plants was up to 50% lower than that of wild-type plants. However, the rate of fatty acid accumulation increased as development progressed (Fig. 1C) so that the total lipid content of mature seed from \textit{Poleosin::asPGM1} transgenic plants was only marginally lower than that of co-segregating wild-type plants (Table I). The largest difference (3%) was in the transgenic line with the lowest starch content.

**Embryo-Specific Loss of GWD1 Does Not Affect the Lipid Content of Mature Seeds**

To observe the effect of an embryo-specific reduction in starch turnover, we studied individual embryos on maternal plants heterozygous for a null allele of the \textit{GWD1} (\textit{SEX1}) gene. Heterozygous plants were generated by crossing wild-type (Col-0) with \textit{sex1-3} mutant (Yu et al., 2001) plants. The \textit{sex1-3} allele carries point
mutations at positions 5874 (from C to T) and 5877 (from A to C) and a deletion of nucleotides 5878 to 5910 relative to the ATG initiation codon of AtGWD1 (At1g10760; Supplemental Fig. S4). Whereas sex1 mutant plants had very high levels of starch in leaves, SEX1/sex1-3 plants had starch contents and a diurnal turnover of starch comparable with that of wild-type plants (Fig. 2, A and B). The testas of mature seeds of SEX1/sex1-3 plants did not stain for starch whereas those of sex1 mutant plants stained strongly (Fig. 2C; Supplemental Fig. S5 for color image).

Embryos from mature seeds on selfed SEX1/sex1-3 plants were either used for PCR-based genotyping, or stained with iodine to discover their starch contents. One quarter of embryos was homozygous for the sex1-3 mutation (ratio of wild-type : SEX1/sex1-3 : sex1-3 embryos of 1.02 : 1.8 : 1.17; 82 embryos tested). Consistent with this, most mature embryos contained no starch but about one quarter contained starch in a zone above the radicle tip (74 embryos out of 263 tested; not statistically significantly different from a 3:1 ratio; \( \chi^2 \)-test=0.64, \( P_{1d.f.}=0.05 \); Fig. 2C; Supplemental Fig. S5 for color image). This phenotype is identical to that of mature embryos of sex1-3 plants (Andriotis et al., 2010c). These data suggest strongly that starch turnover is reduced specifically in embryos homozygous for the sex1-3 mutation.

To determine the effect of genotype on lipid content, radicles from individual embryos from mature seeds on selfed SEX1/sex1-3 plants were genotyped, then the remainder of each embryo was used for fatty acid methyl ester (FAME) analysis. There was no statistically significant difference in FAME content between sex1-3 embryos and their wild-type siblings from SEX1/sex1-3 maternal plants, and between these embryos and those from wild-type plants (Fig. 2D). The FAME content of embryos from sex1-3 plants was about 40% lower than that of embryos from wild-type plants (Fig. 2D; Andriotis et al., 2010c).

**Mutant Embryos from Mutant Parents Show Reduced Seedling Growth, but Mutant Embryos from Phenotypically Wild-Type Parents Do Not**

As a further check for the influence of parental phenotype on composition of sex1 seed, we examined seedling growth. Early post-germinative growth in Arabidopsis
depends on mobilization of the oil reserves of the seed (Graham, 2008; Theodoulou and Eastmond, 2012). Seedlings of mutants with specific defects in the synthesis of seed storage lipids or in their post-germinative mobilization show reduced growth and reduced frequency of establishment (Hayashi et al., 1998; Routaboul et al., 1999; Zou et al., 1999; Eastmond et al., 2000; Cernac et al., 2006; Eastmond, 2006; Kelly et al., 2011). This effect is accentuated in the dark. Using seeds developed on mutant plants, we found that in the absence of exogenous sucrose, sex1-3 and pgm1-1 hypocotyls were significantly shorter than those of wild-type seedlings (Fig. 3A). This difference was abolished by the addition of sucrose. Growth of wild-type hypocotyls was largely unaffected by sucrose, whereas growth of sex1-3 and pgm1-1 hypocotyls was accelerated, and was similar to that of wild-type seedlings. These effects are consistent with the significant reduction in seed lipid content caused by the sex1-3 (Fig. 2D; Andriotis et al., 2010c) and pgm1-1 (Periappuram et al., 2000) mutations.

The experiment was repeated in the absence of exogenous sucrose with individually genotyped seedlings grown from seeds of selfed SEX1/sex1-3 plants. Regardless of genotype, hypocotyls of these seedlings were the same length as those of wild-type seedlings and longer than those of sexl seedlings grown from seeds of sexl plants (Fig. 3B). Thus hypocotyl growth was related to seed oil content rather than to genotype.

**sexl Mutants Have Reduced Silique Elongation**

Given that the presence of the sexl mutation in the maternal plant affects seed composition, we investigated whether inflorescence and fruit growth are also affected. When grown in a controlled environment with 12-h light, 12-h dark, sexl plants had more compact inflorescences, fewer, smaller flowers and shorter siliques than wild-type plants (Fig. 4, A and B). Silique elongation was much slower in sexl than in wild-type plants. Wild-type siliques achieved nearly double the length of mutant siliques. They reached their final length within four DAF, whereas sexl siliques continued to expand until five DAF.
To investigate differences in elongation patterns, siliques length was measured at the end of the day and the end of the night. Wild-type siliques elongated during both the day and the night. They exhibited a progressive decline in relative growth rate up to 4 DAF when elongation stopped. In marked contrast, sex1 siliques elongated very rapidly during the day, but exhibited almost no growth at night (Fig. 4).

**sex1 Mutants Exhibit Symptoms of Starvation in the Inflorescence and Siliques at Night**

To discover whether the lack of starch degradation in sex1 leaves at night affects carbohydrate availability in the inflorescence, we first made use of a “starvation-inducible” reporter gene. The reporter was produced by fusion of the promoter of the sugar-repressed gene At1g10070 to the firefly luciferase gene ($pAt1g10070_{1100}:LUC$; Graf et al., 2010). It is strongly and reproducibly induced in response to extended night periods, when carbohydrate availability is below the normal daily range (Graf et al., 2010; Barratt et al., 2011).

A sex1-3 mutant plant was crossed with the starvation-inducible reporter line and sex1-3 mutants carrying the reporter gene ($sex1-3/pAt1g10070_{1100}:LUC$; referred to as sex1 reporter plants) were selected in the F2 generation. Relative to the parental reporter line, three-week old sex1 reporter plants showed strong bioluminescence at the end of the night (Fig. 5A). Consistent with this, publicly-available microarray data show that At1g10070 transcript levels are 13-fold higher at the end of the night in 12-h light, 12-h dark conditions in sex1-3 than in wild-type plants (NASCarrays http://affymetrix.arabidopsis.info/narrays/experimentpage.pl?experimentid=411).

At the end of the night the apices of inflorescences and three-day old siliques showed strong bioluminescence in the sex1 reporter line, but not in the parental reporter line (Fig. 5, B and C). The reporter line cannot be used for study of starvation in older siliques because basal transcript levels of At1g10070 and several other sugar-repressed genes (Graf et al., 2010; see also Thimm et al., 2004; Bläsing et al., 2005; Osuna et al., 2007; Usadel et al., 2008) increase during wild-type fruit maturation (Supplemental Fig. S7). Therefore, to discover whether older (12 DAF) sex1 siliques also experience starvation during the night, we measured At1g10070
transcript levels by quantitative real-time (qRT) PCR on RNA from wild-type and sex1 siliques during the night. At1g10070 transcript abundance was similar in wild-type and sex1 siliques at the end of the day and remained almost constant during the night in wild-type siliques. However, transcript levels rose sharply in sex1 siliques after four hours in the dark such that by dawn they were much higher than in the wild-type (Fig. 5E). We obtained similar results for a further sugar-repressed gene, At3g59940 (Supplemental Fig. S7).

Direct measurements of carbohydrate status revealed that sugar levels were comparable in wild-type and sex1 siliques at the end of the day, but much lower in sex1 siliques by the end of the night. In two independent experiments, levels of Suc at the end of the day differed by less than 50% between wild-type and sex1 siliques at 12 DAF. Levels in wild-type siliques fell by about 25% in the first two h of darkness, then remained almost constant. In contrast, levels in sex1 siliques declined exponentially during the night to very low levels by dawn. At this point, Suc levels in wild-type siliques were nearly 14 times greater than those in sex1 siliques (Fig. 6; Supplemental Fig. S8). Hexose levels at the end of the day were higher in sex1 siliques than the wild type. They declined during the night such that by dawn levels were lower in sex1 than in wild-type siliques.

**WRI1 expression is reduced in siliques of sex1 plants during the night**

The transcription factor WRINKLED1 (WRI1) has been implicated in the control of lipid accumulation in developing Arabidopsis embryos (Focks and Benning, 1998; Cernac and Benning, 2004; Masaki et al., 2005). wri1 seeds have greatly reduced lipid content, altered carbohydrate metabolism, and reduced gene expression and activity of several enzymes of glycolysis and fatty acid biosynthesis (Focks and Benning, 1998; Ruuska et al., 2002; Baud and Graham, 2006). We measured transcript abundance of WRI1 and several of its target genes in siliques at 12 DAF, the developmental point at which expression of WRI1 peaks (Baud et al., 2007a) and embryos enter the phase of lipid accumulation (Baud et al., 2002; Hills, 2004; Andriotis et al., 2010a). WRI1 transcript levels were similar in wild-type and sex1 siliques at the end of the day (Fig. 7). However, at the end of the night WRI1
transcript abundance in sex1 siliques was only half of that in wild-type siliques. Transcript levels for enzymes of fatty acid synthesis (BCCP2 and KAS1, a subunit of the heteromeric acetyl-CoA carboxylase and ketoacyl-ACP synthase 1 respectively) and glycolysis [PKp-α, PKp-β1 and ENO1, the α and β subunit of plastidial pyruvate kinase and the plastidial enolase respectively (Andre et al., 2007; Baud et al., 2007b; Andriotis et al., 2010a)] were the same in sex1 and wild-type siliques at the end of the day, and decreased during the night in sex1 but not in wild-type siliques (Fig. 7). Expression of BCCP2, KAS1 and PKp-β1 genes is directly activated by WRI1 (Maeo et al., 2009).

**Embryo Maturation is Delayed in sex1 Mutant Plants**

We established a developmental baseline with developing seeds from wild-type plants (Fig. 8). More than 90% of embryos had completed morphogenesis (heart stage of development) by six DAF. At eight DAF most embryos were at the walking-stick stage and the remainder had progressed to the subsequent early cotyledon-upturned U stage of development (Fig. 8). Wild-type embryos reached their final size by 12 DAF.

Early development of embryos on sex1 mutant plants was identical to that on wild-type plants; embryos reached the heart stage by six DAF (Fig. 8). However, after six DAF they progressed more slowly than the wild-type. At eight DAF, when most of the wild-type embryos were at the walking-stick stage, sex1 embryos were at the preceding torpedo stage of development. By the time wild-type embryos had completed development, sex1 embryos were still at the cotyledon stage of development (12 and 14 DAF, Fig. 8).

**Mutant Plants Defective in Stem Phloem Transport Have Reduced Seed Lipid Content**

To provide independent information about the relationship between availability of carbohydrate at night and seed lipid content, we examined seed lipid in gsl7 mutant plants in which carbohydrate supply to the inflorescence is restricted by impaired phloem transport rather than by defects in maternal starch metabolism. The gsl7
mutant lacks GLUCAN SYNTHASE-LIKE 7, an isoform of callose synthase that catalyzes the synthesis of the callose lining of phloem sieve plate pores (Barratt et al., 2011; Xie et al., 2011). The absence of the lining in the gsl7 mutant largely prevents the export of assimilates from leaves to inflorescences, which are smaller and contain less starch and sugars than those of wild-type plants. The most pronounced differences in carbohydrates are at night, and starvation reporter genes are activated at the end of the night in gsl7 inflorescences (Barratt et al., 2011). We reasoned that if the reduced lipid contents of sex1 and pgm1 mutant seeds were due to limited carbohydrate supply at night, seeds of the gsl7 mutant should also have a reduced lipid content. Indeed, mature seeds contained 9% less lipid than those of co-segregating wild-type plants grown at the same time in the same conditions (Table I).

DISCUSSION

pgm1 and sex1 Mutations Alter Seed Lipid Content via a Maternal Effect

To evaluate the importance of embryo starch metabolism in determining the final lipid content of the seed, we compared embryos defective in PGM1 or GWD1 that had developed either on phenotypically wild-type plants, or on mutant plants lacking PGM1 or GWD1. We found that embryos defective in either enzyme have defects in starch turnover, regardless of parental phenotype. Loss or reduction of PGM1 in the embryo leads to loss of at least 85% of the wild-type starch content at 12 DAF. Loss of GWD1 leads to reduced starch turnover in the embryo (Fig. 2; Andriotis et al., 2010c). By contrast, the final lipid contents and subsequent seedling growth of embryos defective in PGM1 or GWD1 are dependent on parental phenotype. If the parent plant lacks either PGM1 or GWD1, the lipid content of mature seeds is reduced (Fig. 2, Periappuram et al., 2000; Andriotis et al., 2010c). Growth of hypocotyls following germination is also reduced, almost certainly as a consequence of the low lipid content and hence limited carbon availability in seedlings. If the parent plant is phenotypically wild-type, seed lipid contents and rates of seedling
hypocotyl growth are like those of the wild-type, regardless of embryo genotype. The large reductions in seed lipid content seen in starch-excess and starch-deficient mutant plants (Periappuram et al., 2000; Andriotis et al., 2010c) are thus the result of altered starch turnover in the maternal plant rather than in the embryo itself.

Our results show that perturbations of embryo starch metabolism can affect other metabolic fluxes and developmental progression without affecting final seed weight and lipid content. Embryos with a reduced capacity for starch synthesis on phenotypically wild-type plants have delayed lipid accumulation (Fig. 1C). A similar effect was seen in oilseed rape plants with embryo-specific reductions in AGPase resulting in reduced embryo starch synthesis (Vigeolas et al., 2004). Lipid synthesis was delayed during embryo development but the final lipid content of the seed was unaffected. Similarly, large reductions in seed sucrose synthase activity (Angeles-Núñez and Tiessen, 2010) and loss of a sucrose transporter SUC5 (Baud et al., 2005) perturb carbohydrate and lipid metabolism and developmental progression in young embryos without altering the final lipid content of the seed. Thus the impact on lipid synthesis of early perturbations in embryo carbohydrate metabolism can be compensated for by higher rates and/or longer periods of lipid accumulation in the later stages of seed maturation.

**pgm1 and sex1 Mutations Cause Reduced Growth in Reproductive Structures due to Limited Carbohydrate Availability at Night**

As a first step to discover how maternal carbohydrate metabolism affects seed composition, we investigated inflorescence, silique and embryo development in *sex1* mutants. We found that both flowers and siliques are smaller in *sex1* mutants than in wild-type plants. The reduced length of siliques is specifically due to a failure to elongate at night. Embryo development is also seriously delayed in *sex1* mutants during the later stages when cell expansion and storage product deposition occur.

The reduced growth of reproductive structures is accompanied by indications of low carbohydrate availability at night. Starvation reporter genes are activated at night in floral apices and siliques of *sex1* but not wild-type plants. Whereas sugar
levels in mature siliques are maintained during the night in wild-type plants, they fall to very low levels by dawn in sex1 plants.

Taken as a whole, these data are consistent with the idea that the reduced growth of reproductive organs of sex1 plants is related to low carbohydrate availability at night. A relationship between reduction or cessation of growth and low carbohydrate availability has been observed previously for vegetative organs of Arabidopsis. In wild-type plants, an unexpected extension of the night beyond the normal dawn, when leaf starch reserves have been depleted, induces expression of sugar-repressed (starvation) genes (Gibon et al., 2004; Bläsing et al., 2005; Usadel et al., 2008) and results in rapid cessation of root growth (Yazdanbakhsh et al., 2011). In rosettes of both pgm and sex1 mutants, in which starch mobilization at night is low or absent, starvation genes are expressed during the normal night (Gibon et al., 2004; Thimm et al., 2004). Growth is retarded in sex1 and pgml rosettes and roots at night (Wiese et al., 2007; Smith and Stitt, 2007; Pantin et al., 2011; Yazdanbakhsh et al., 2011). Thus it seems likely that there is coordination between carbohydrate availability and the utilization of carbon for growth, brought about by a complex network of internal signals and external cues (Stitt and Zeeman, 2012).

It seems highly likely that the low lipid content of mature seeds of sex1 (and probably also pgm1 and gsl7) mutants is brought about by limited carbohydrate supply to the developing embryos at night, which affects both the rate of development and the expression of genes encoding enzymes necessary for lipid synthesis. First, the expression of WRII is markedly reduced at the end of the night in siliques of sex1 plants (Fig. 7). WRII is central in the control of lipid accumulation during Arabidopsis seed development (Focks and Benning, 1998) and acts as a transcriptional activator of a subset of genes encoding enzymes of glycolysis and fatty acid biosynthesis (Maeo et al., 2009; Baud et al., 2009). Consistent with this, we found that transcript levels of direct targets of WRII, PKp-\(\beta\), BCCP2, KAS1 and of other genes encoding glycolytic enzymes are reduced during the night in sex1 but not in wild-type siliques (Fig. 7). WRII is specifically expressed in the embryo and endosperm but not the seed coat and the silique walls (Baud et al., 2007a), thus our measurements of WRII transcript in siliques largely
reflect its expression in embryos. Second, the phenotype of the gsl7 mutant strongly supports the idea that reduced carbohydrate supply at night can lead to reduced lipid accumulation in the embryo (Table I). In this mutant, limited carbohydrate availability in the reproductive structures at night due to defective phloem transport is associated with symptoms of starvation and with a low lipid content of the mature seed.

Implications of Night-Time Starch Mobilization for the Seed Lipid Content of Oilseeds

Our work highlights the importance of night-time carbohydrate supply to the reproductive organs in determining the lipid content of oilseeds. Below we discuss how carbohydrate is supplied for seed filling during the growth of reproductive structures.

Carbohydrate can be supplied to the reproductive structures at night by mobilization of starch either in leaves or in the inflorescence itself. For young reproductive structures, starch mobilization in the leaves seems to make the more important contribution. We found that in the gsl7 mutant, which is defective in phloem transport in the stem, young reproductive structures have reduced growth, reduced carbohydrate content especially at night, and symptoms of starvation at the end of the night (Barratt et al., 2011). In wild-type plants the young inflorescence itself stores some starch, at the top of the stem and in silique walls (Barratt et al., 2009; 2011), probably derived in part from inflorescence photosynthesis. However, pulse-chase experiments with $^{14}$CO$_2$ reveal that most of the carbon for the growth of young reproductive structures is imported from the leaves (Robinson and Hill, 1999; Barratt et al, 2011). Overall, the available evidence suggests that in wild-type plants photosynthesis in the inflorescence may at least partially support inflorescence growth during the day, but carbohydrate imported from the leaves is important in maintaining growth at night.

The supply of carbohydrate for silique and seed growth changes as inflorescences and siliques mature. Depending on environmental conditions, rosette leaves often senesce while seeds are still filling. When this occurs, seed filling becomes more
dependent on photosynthesis in silique walls and cauline leaves (Robinson and Hill, 2002). Information on oilseed rape also indicates that carbohydrate availability for seed filling at night is limited after leaves have senesced. In this crop species, leaves usually senesce at an early stage of seed filling. Silique photosynthesis provides a large fraction of the carbon for seed filling (Major et al., 1978; Baud and Lepiniec, 2009; Hua et al., 2012; and references therein). However, relatively little of the silique photosynthate is stored as starch. In a greenhouse experiment, siliques partitioned a much smaller proportion of photosynthate into starch than did leaves. There was no significant increase in sugar or starch contents of siliques through the day, implying that photosynthate was used immediately for seed filling rather than to build up a reserve for use at night (King et al., 1997). Recent work reports a strong correlation between seed oil content and the starch content of the silique wall in oilseed rape (Hua et al., 2012). This observation is consistent with the idea that the availability of reserves for seed filling during the night can influence seed composition, but the authors did not investigate whether the silique starch was mobilized at night.

Previous work has established the importance of light intensity during the day in determining the final lipid content of Arabidopsis and oilseed rape seeds (Li et al., 2006). Our results suggest that the supply of carbon to the seed at night can also have a major impact on lipid content. We suggest that better understanding of daily patterns of carbohydrate availability during oilseed filling will provide important new information about the determination of seed lipid content.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Mutant and transgenic plants were in the Col-0 wild-type background. Plants were grown in compost at 20°C, in 12 h light, 12 h dark unless otherwise stated, at 60-75% relative humidity and 250 μmol quanta photosynthetically-active radiation m⁻² s⁻¹. Unless otherwise stated, reproductive growth was restricted to the primary
flowering shoot. gsl7-1 and gsl7-2 mutants were as described by Barratt et al. (2011).

**Generation of Poleosin::asPGM1, Poleosin::uidA and sex1-3/pAt1g10070;1100:LUC Plants**

The *B. napus* oleosin BN-III promoter (*Poleosin*; EMBL accession X61937) was excised with *Bgl*II and *Xho*I from vector SLJOP (Keddie et al., 1994) and introduced into vector pGreen0029 (Hellens et al., 2000). A 1.1 kb fragment of the *B. napus* *PGM1* coding region (EMBL accession number AJ250771; Harrison et al., 2000) was amplified from *B. napus* cDNA with primers SS1A and SS1B (Supplemental Table S1) and was used to create two plasmids. Plasmid pGreenSS0 contained the fragment in the reverse orientation downstream of the *Poleosin* in vector pGreen0029. For plasmid pGreenSS1, the CaMV 35S terminator sequence from vector pJIT117 (Guerineau et al., 1988) was excised with *Kpn*I and *BamHI*, and introduced into pGreenSS0 downstream of the *BnPGM1* antisense construct. Digestion of pGreenSS0 with *Kpn*I and *BamHI* deleted a 177 bp fragment of *BnPGM1*, thus the pGreenSS1 binary vector carries a 0.9 kb fragment of the *BnPGM1* coding region. Plasmids pGreenSS0 and pGreenSS1 were introduced into *Agrobacterium tumefaciens* GV3101 and used to transform *Arabidopsis* plants by floral dipping (Clough and Bent, 1998).

For the generation of *Poleosin::uidA* reporter lines, the expression cassette from vector SLJOP was excised with *Bgl*II and *Pst*I and introduced into vector pGreen0029. This plasmid was used to transform Arabidopsis plants as above.

For transfer of the starvation reporter construct *pAt1g10700;1100::LUC* (Graf et al., 2010) into the *sex1-3* background, F2 seed from a cross between *sex1-3* and a plant expressing *pAt1g10700;1100::LUC* were sown on medium containing glufosinate. Resistant seedlings were transferred to soil, screened for starch-excess and bioluminescence phenotypes, and used to establish homozygous lines. For bioluminescence screening plants were treated with luciferin according to Graf et al. (2010).
Identification of the sex1-3 Mutant Allele

Genomic DNA was extracted with the DNeasy Plant Mini Kit (Qiagen; www.qiagen.com) or the Extract-N-Amp™ Plant PCR Kit (Sigma-Aldrich; www.sigmaaldrich.com) and screened by PCR using primers SEX1-14 and SEX1-15 (Supplemental Table S1), as described by Andriotis et al. (2010a).

RNA Preparation, Reverse Transcription and qRT-PCR

Preparation of total RNA from siliques and first strand cDNA synthesis were as described by Andriotis et al. (2010b). qRT-PCR analysis was performed with a CFX96 Touch™ cycler (Bio-Rad; www.bio-rad.com) in reactions containing LightCycler® 480 SYBR Green I (Roche; www.roche-applied-science.com). Cycling conditions were according to Barratt et al. (2011). Primers are listed in Supplemental Table S1.

Hypocotyl and Silique Measurements

For hypocotyl measurements seeds were germinated on half-strength MS agar medium with or without 2% (w/v) sucrose. Plates were chilled at 4°C for three days in the dark, then transferred to 20°C in a vertical orientation. Hypocotyl length was measured after 7 d and individual seedlings were then genotyped by PCR using primers SEX1-14 and SEX1-15. Hypocotyl and silique length was measured using ImageJ software (http://rsbweb.nih.gov/ij/).

Enzyme assays

For assays of β-glucuronidase (GUS), developing embryos of Polesin::uidA plants were rapidly dissected and homogenized in 50 mM NaPO₄ (pH 7), 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% (v : v) sodium lauryl sarcosine, 0.1% Triton X-100 (v : v) and protease inhibitors (plant cocktail, Sigma-Aldrich) on ice then centrifuged at 15,000g at 4°C for 30 min. Assays were according to Bevan et al. (1989) and the histochemical detection of GUS activity in developing embryos was according to Andriotis et al. (2010b).
PGM and PGI activity measurements were on leaves were harvested 8 h into the light period from three-week-old rosettes, and on developing embryos at 10-12 DAF. Extracts were prepared according to Andriotis and Rathjen (2006). Soluble proteins were subjected to non-denaturing PAGE on gels containing 1% (w/v) potato starch, and stained for activity according to Vriet et al. (2010).

**Metabolite analysis**

Starch was assayed according to Smith and Zeeman (2006). Soluble sugars in developing siliques were measured on samples each of five siliques pooled from five plants, according to Critchley et al. (2001). FAME analysis of individual embryos was done on cotyledon tissue remaining after genotyping, according to Larson and Graham (2001). Most of the lipid accumulation in embryos is in the cotyledons rather than the axis (Li et al., 2006). Lipid content of mature seeds was assayed by NMR (Hobbs et al., 2004).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Expression of *PGM1* and *OLEOSIN* genes during seed development and specificity of the *B. napus* oleosin promoter.

**Supplemental Figure S2.** Analysis of *P.oleosin::asPGM1* transgenic plants.

**Supplemental Figure S3.** Starch in embryos of *P.oleosin::asPGM1* transgenic plants.

**Supplemental Figure S4.** The *sex1-3* mutation.

**Supplemental Figure S5.** Starch in mature *sex1* seeds.

**Supplemental Figure S6.** Transcript levels for sugar-repressed genes during wild-type fruit development.

**Supplemental Figure S7.** Transcript levels of two sugar-repressed genes in wild-type and *sex1* siliques.

**Supplemental Figure S8.** Carbohydrate contents of wild-type and *sex1* siliques.

**Supplemental Table S1.** Primers used in this study.
ACKNOWLEDGEMENTS

We thank Alastair Skeffington and Doreen Feike (JIC) for help with bioluminescence measurements and useful suggestions, Dr Matthew Hills (JIC) for the kind gift of vector SLJOP, Dr Heike Kofler for sequencing the sex1-3 allele, Baldeep Kular for measuring starch on pgml embryos, Andrew Davis for photography and Sheila Mitchell and Damian Alger for expert horticultural support. VMEA is indebted to Prof Ian Graham (York).

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FIGURE LEGENDS

Figure 1. Effects of embryo-specific down-regulation of the activity of PGM1. A, Detection of PGM (top panel) and PGI (bottom panel) activity after native PAGE. Extracts were prepared from embryos at 12 DAF, on plants grown in 16 h light, 8 h dark. The asterisk indicates plastidial PGM activity. Each lane contained 20 μg protein. B, Starch accumulation in developing embryos from wild-type plants (left panel) and Poleosin::asPGM1 lines 144 (middle panel) and 51 (right panel). Embryos at 12 DAF were stained with Lugol’s iodine solution. The asterisk indicates a zone of starch accumulation above the radicle tip of embryos from transgenic plants. The bar represents 200 μm and applies to all panels. C, Fatty acid accumulation (measured as fatty acid methyl esters, FAME) during embryo development. Black bars, wild-type; grey bars, Poleosin::asPGM1 line 51; white bars, Poleosin::asPGM1 line 144. Values are means ± SE of measurements made on three replicates, each of 50 seeds pooled from five plants.
Figure 2. Effect of the maternal genotype on the lipid content of Arabidopsis seeds. A, Detection of starch in leaves of Col-0, sex1-3 and SEX1/sex1-3 plants at the end of the day (EOD) and the end of the night (EON). Leaves from three-week old rosettes were stained with Lugol’s iodine solution. B, Starch content of leaves from three-week old Col-0, sex1-3 and SEX1/sex1-3 plants at the end of the day (white bars) and the end of the night (black bars). Values are means ± SE of measurements on three biological replicates. C, Mature seed of sex1-3 (left) and SEX1/sex1-3 (middle), and a mature SEX1/sex1-3 embryo (right), stained with Lugol’s iodine solution. The presence of starch in a zone above the radicle tip (asterisked) was exhibited by one quarter of the mature embryos on the same plant. The scale bar represents 200 μm. D, Total fatty acid methyl ester (FAME) content of wild-type (+/+), SEX1/sex1-3 (+/-) and sex1-3 (-/-) mature embryos from a selfed SEX1/sex1-3 plant and of mature embryos from wild-type and sex1-3 maternal plants. Measurements were on 65 individual, genotyped embryos (segregation ratio: 13 wild-type, 31 SEX1/sex1-3, 20 homozygous sex1 mutants). Similar results were obtained for a second, independent SEX1/sex1-3 plant. The solid line inside the box represents the average FAME content, the box represents the values between the 1st and 3rd quartile of the dataset, and the whiskers show the range of values.

Figure 3. Effect of altered starch turnover in the maternal plant on seedling growth. A, Hypocotyl length of seven-day-old dark-grown seedlings from seed of wild-type, sex1-3 and pgm1-1 maternal plants. Seeds were germinated on agar in the absence (black bars) or the presence (white bars) of 2% (w/v) sucrose. Values are means of measurements on 15 seedlings, bars are SE. Similar results were obtained with seed of independently-grown plants. B, Hypocotyl length of seven-day-old dark-grown seedlings from seed of a selfed SEX1/sex1-3 plant. For comparison, seeds from wild-type and sex1-3 maternal plants grown at the same time were included. Values for SEX1/sex1-3 progeny are means of measurements on 67 individual, genotyped seedlings. Values for wild-type and sex1-3 means of measurements on 16 and 10 seedlings, respectively, bars are SE.
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Figure 5. Luciferase (LUC) activity in plants expressing luc under control of the promoter of the sugar-repressed gene At1g10070. A, LUC activity (visualized by bioluminescence, in pseudocolour) at the end of the night in three-week-old wild-type (left) and sex1-3 (right) plants expressing pAt1g10070:1000:LUC. The scale at the top represents bioluminescence intensity (black = low, red = high). B, LUC activity at the end of the night in the apex of the primary flowering stem of a wild-type (left) and a sex1-3 (right) plant expressing pAt1g10700:1000:LUC. The images are at the same magnification and were taken at the same time. C, LUC activity at the end of the night in developing siliques at three DAF from wild-type (top) and sex1-3 (bottom) plants expressing pAt1g10070:1100:LUC. D, Quantification of LUC activity in siliques of pAt1g10070:1100:LUC (black bars) and sex1-3/pAt1g10070:1100:LUC (white bars) plants at the end of the night. Values are means ± SE (bars) of measurements on 8-10 siliques pooled from five plants. E, Expression of the sugar-repressed gene At1g10070 during a 12-h night in siliques from wild-type (diamonds) and sex1-3 (squares) plants at 12 DAF. RNA was extracted from five siliques pooled from five plants. Transcript levels were measured by quantitative real-time PCR and normalised against a UBIQUITIN10 control. Values are means of three technical replicates, and are expressed relative to the wild-type at the end of the day (0 h). Bars are SE.
Figure 6. Carbohydrate contents of siliques from wild-type and sexl mutant plants during the night. Each value is for a pool of five siliques (12 DAF) from five plants, assayed in triplicate. Bars are SE. Diamonds: wild-type, squares: sexl. Similar results were obtained in an independent experiment (see Supplemental Fig. S8).

Figure 7. Daily pattern of gene expression in siliques of wild-type and sexl plants. RNA was extracted from five siliques pooled from five plants at 12 DAF, at the end of the day (EOD) and the end of the night (EON). Transcript abundance was measured by quantitative real-time PCR with UBIQUITIN10 as a control. Values are means of three technical replicates, bars are SE. Similar results were obtained in a separate experiment. Black: wild-type, white, sexl. WRI1, WRINKLED1. KAS1, KETOACYL-ACP SYNTHASE 1. BCCP2, subunit of the heteromeric acetyl-CoA carboxylase. PKp-β1 and PKp-α, subunits of pyruvate kinase. ENO1, plastidial enolase.

Figure 8. Development of embryos on wild-type and sexl maternal plants. Seeds were viewed under differential interference contrast optics at the indicated DAF. Results are per cent of embryos examined. Numbers of embryos examined were: 6 DAF wild-type 153, sexl 125; 8 DAF wild-type 160, sexl 158; 10 DAF wild-type 159, sexl 105; 14 DAF wild-type 92, sexl 82. The developmental stages were: 1, globular; 2, transition stage; 3, heart; 4, torpedo; 5, walking stick; 6, early cotyledon; 7, cotyledon; 8, fully grown embryo. Black bars: wild-type plants. White bars: sexl plants.
Table I. Seed lipid content in transgenic and mutant lines.

<table>
<thead>
<tr>
<th>Line</th>
<th>Starch (ng embryo⁻¹)</th>
<th>Lipid (% dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>83.0±10</td>
<td>45.7±0.05</td>
</tr>
<tr>
<td>pgm1-1</td>
<td>0.02±0.008 a</td>
<td>nd</td>
</tr>
<tr>
<td>P_oleosin::asPGM1 222</td>
<td>24.2±8.8 b</td>
<td>45.5±0.11</td>
</tr>
<tr>
<td>P_oleosin::asPGM1 21</td>
<td>24.7±1 c</td>
<td>45.2±0.11 d</td>
</tr>
<tr>
<td>P_oleosin::asPGM1 144</td>
<td>17.8±6 e</td>
<td>44.8±0.16 f</td>
</tr>
<tr>
<td>P_oleosin::asPGM1 51</td>
<td>13.3±1 g</td>
<td>44.3±0.11 h</td>
</tr>
<tr>
<td>gsl7-1 (-/-)</td>
<td>nd</td>
<td>37.03±0.41 i</td>
</tr>
<tr>
<td>gsl7-1 (+/+</td>
<td>nd</td>
<td>40.85±0.29</td>
</tr>
<tr>
<td>gsl7-2 (-/-)</td>
<td>nd</td>
<td>37.16±0.36 j</td>
</tr>
<tr>
<td>gsl7-2 (+/+</td>
<td>nd</td>
<td>41.16±0.39</td>
</tr>
</tbody>
</table>

Starch was determined on embryos at 12 DAF. Values are means ± SE of measurements on three replicates, each analysed in triplicate. Replicates were pools of 30 embryos from five plants. Lipid content was determined on mature seed of plants grown as for starch determinations. Values are means ± SE of measurements on nine pools of seeds (for wild-type, pgm1-1, and transgenic lines 222, 21, 144, 51) and four pools of seeds (for gsl7-1 and gsl7-2 mutants (-/-) and their co-segregating wild-type plants (+/+), each from one pot of five plants, each analysed in triplicate. gsl7 mutant and their co-segregating wild-type plants were grown independently from the pgm1 and P_oleosin::asPGM1 transgenic plants. Roman numerals denote
significant differences (Student’s t-test) from wild-type values. \(^a\), \(P = 0.008\); \(^b\), \(P = 0.015\); \(^c\), \(P = 0.05\); \(^d\), \(P = 0.004\); \(^e\), \(P = 0.007\); \(^f\), \(P = 0.0002\); \(^g\), \(P = 0.011\); \(^h\), \(P = 0.0002\); \(^i\), \(P = 0.0015\); \(^j\), \(P = 0.0007\). nd, not determined.
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