Original Article

Carbon partitioning in *Arabidopsis thaliana* is a dynamic process controlled by the plants metabolic status and its circadian clock

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**ABSTRACT**

Plant growth involves the coordinated distribution of carbon resources both towards structural components and towards storage compounds that assure a steady carbon supply over the complete diurnal cycle. We used $^{14}$CO$_2$ labelling to track assimilated carbon in both source and sink tissues. Source tissues exhibit large variations in carbon allocation throughout the light period. The most prominent change was detected in partitioning towards starch, being low in the morning and more than double later in the day. Export into sink tissues showed reciprocal changes. Fewer and smaller changes in carbon allocation occurred in sink tissues where, in most respects, carbon was partitioned similarly, whether the sink leaf assimilated it through photosynthesis or imported it from source leaves. Mutants deficient in the production or remobilization of leaf starch exhibited major alterations in carbon allocation. Low-starch mutants that suffer from carbon starvation at night allocated much more carbon into neutral sugars and had higher rates of export than the wild type, partly because of the reduced allocation into starch, but also because of reduced allocation into structural components. Moreover, mutants deficient in the plant's circadian system showed considerable changes in their carbon partitioning pattern suggesting control by the circadian clock.

Key-words: *Arabidopsis*; carbon starvation; diurnal cycle; isotope labelling; photosynthesis; starch; source-sink relations.

**INTRODUCTION**

Plants perform photosynthesis, using light to drive the assimilation of carbon dioxide. The carbon taken up by the plant underpins the myriad of biosynthetic pathways needed for growth. The processes of photosynthesis and central carbon metabolism are quite well understood, and many studies have analysed leaf development and expansion (e.g. Blein et al. 2010, Poiré et al. 2010, Gonzalez et al. 2012). However, surprisingly little is known about the allocation of carbon resources and its regulation.

The amount of carbon resources available depends on the type of tissue and the developmental stage. While a mature leaf gains carbon exclusively from photosynthesis, younger developing leaves do not assimilate sufficient amounts of carbon and so import carbon in the form of sucrose. Thus, leaves that assimilate an excess of carbon are source tissues that support developing leaves and other sink tissues such as roots and seeds. During leaf ontogeny, a switch from sink to source takes place. The exact time of this transition is not precisely described for *Arabidopsis*, but it was shown to occur when leaves reach 30–60% of their final leaf area in sugar beet and *Curcubitacea* (Turgeon & Webb 1973; Fellows & Geiger 1974; Turgeon 1989). This implies that leaves continue growing even after becoming self-sustaining in terms of carbon assimilation.

The growth rate of a leaf obviously changes as it develops, but it also varies markedly over the diurnal cycle (Seneweera et al. 1995; Walter & Schurr 2005; Sulpice et al. 2014). Measurements of leaf expansion rates revealed periodic growth patterns with a defined phase and shape. *Arabidopsis thaliana* leaves show the highest growth rates at the beginning of the light period, followed by moderate growth over the remaining day. During the dark, low expansion growth is observed (Wiese et al. 2007). This diurnal pattern in expansion rate might simply be a result of hydraulic control, but could also be controlled by changes in carbon partitioning into structural components.

Leaf expansion is influenced by environmental conditions and by the metabolic state of the plant. Environmental fluctuations, like water availability, nutrient availability and air temperature, change the amplitude of growth, but the pattern remains unaltered (Schurr et al. 2000; Ainsworth et al. 2005). However, changes in carbohydrate availability influence both growth rate and growth pattern. For instance, the starchless mutant lacking plastidal phosphoglucomutase (*pgm* or *stf1*) has an overall reduction in growth compared with the wild type (Caspar et al. 1985). Like the wild type, its growth rate peaks at the beginning of the light period. Then, unlike the wild type, it has a second peak in the evening, while at night, it grows significantly less than the wild type (Wiese et al. 2007).

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The reduction and altered pattern of leaf expansion in *pgm* probably results from major changes in carbon availability – a surfeit during the day and starvation during the night. Only small amounts of sugar are stored in place of starch, and these are depleted early during the dark period. Night-time starvation can explain the low growth rates in the dark, but has also been shown to affect levels of the growth-promoting plant hormone gibberellic acid in the subsequent day, which can affect the rate, and presumably the patterns of leaf growth (Paparelli et al. 2013). Not surprisingly, the growth of other sink tissues is also affected in *pgm*. In *pgm* roots, growth is inhibited at the end of the night, but also at the beginning of the day despite the accumulation of the newly assimilated carbon as sugars (Gibon et al. 2004; Smith & Stitt 2007; Yazdanbakhsh et al. 2011).

An important regulator of plant growth and development is the circadian clock. It not only influences developmental transitions, such as flowering (Hayama & Coupland 2003; de Montaigu et al. 2010), but also growth processes like hypocotyl elongation (Nozue et al. 2007; Nusinow et al. 2011). According to recent modelling approaches, the Arabidopsis circadian clock can be described as three sequential negative feedback loops in which the components of the morning loop ([CIRCADIAN CLOCK-ASSOCIATED 1, (CCA10); LATE ELONGATED HYPOCHOTYL, (LHY)] are repressed by afternoon-expressed PSEUDO-RESPONSE-REGULATORS (PRRs), the PRRs are repressed by the evening complex (EC) comprising of ELF3, ELF4 and LUX, and the EC is repressed by LHY and CCA1 (Pokhilko et al. 2012; Hsu & Harmer 2013). The collective function of these feedback loops permits the plant to anticipate light and dark, and therefore to adjust growth and development effectively to the environment.

An ideal method to directly measure the incorporation of carbon into different metabolic compounds is isotopic labelling. Feeding with either radioactive (14C) or stable (13C) carbon isotopes can be performed to follow the incorporation of carbon in different compound classes like cell wall material, proteins and starch, or to measure carbon fluxes into specific metabolites, respectively (see Kölling et al. 2013 and references therein). Here, we used 14CO2 labelling to analyse carbon partitioning and to measure export of carbon from source to sink tissues. We reveal changes in carbon utilization at different stages of leaf development, over the light period and in mutants, which are altered in starch metabolism or the circadian clock. Together, these data suggest that there is control of carbon allocation via metabolic signalling and by the circadian clock.

**MATERIALS AND METHODS**

**Plant material and growth conditions**

Measurements were performed either with the wild-type accession Col-0 or Ws2 from *Arabidopsis thaliana*. All starch mutants and the clock mutants *gi-201, toc1* and *prr7-3/prr9-1* are in the Col-0 background. The clock mutants *lhy/ccal* and *elf3-4* are in the Ws2 background. All clock mutants were kindly provided by Prof Mark Stitt.

For soil-grown plants, *Arabidopsis thaliana* seeds were sown on Einheitserde Typ ED 73. Seeds were stratified for three days at 4 °C before being transferred to a growth cabinet (12 h photoperiod, 150 μmol quanta m−2 s−1 light intensity, 20 °C constant temperature, 65% relative humidity).

For plants grown in hydroponic culture, seeds were sown on tubes filled with 0.65% (w/v) agar. The bottoms of the tubes were cut off and the tubes were placed in a rack filled with nutrient solution (Gibeaut et al. 1997). After stratification, the racks were transferred to a growth cabinet as specified earlier. The nutrient solution was changed weekly.

**14CO2 pulse-chase labelling**

Labelling experiments of whole plants were performed with 3-week-old, hydroponically grown plants introduced into a sealed Plexiglas chamber (17.2 L volume), illuminated with 150 μmol quanta m−2 s−1. 14CO2 with a specific activity of 29.5 mCi mmol−1 (300 μCi) was released in the sealed chamber by acidification of sodium 14C-bicarbonate, leading to an increase of the CO2 level within the chamber of 8 ppm. The ambient CO2 concentration of the air was typically 450 ppm. After 60 min, the Plexiglas chamber was opened and the plants were kept in normal air for a chase period of 60 min. During the labelling period, approximately 12% of the supplied CO2 was fixed by the plants. Therefore, CO2 levels in the chamber never decreased below 380 ppm. An increase in temperature of approximately 1 °C was recorded.

Labelling experiments on single leaves were performed with soil-grown plants on leaves having a comparable size and developmental stage as described in Kölling et al. (2013). In these experiments, less than 1% of the liberated CO2 was assimilated.

**Extraction and fractionation of 14CO2-labelled plant tissue**

Extraction and fractionation of the tissue was performed as described in Kölling et al. (2013).

**Perchloric acid extraction and starch measurements**

Starch measurements were performed on 3-week-old plants grown in hydroponic cultures. The extraction of the plant material and the starch measurements were performed as described in Hostettler et al. (2011).

**RESULTS**

**Changes in carbon partitioning depend on leaf developmental stage and carbon source**

While source leaves gain carbon solely via photosynthesis, sink leaves both photosynthesize and import sucrose from source leaves. Carbon derived from these two sources might be utilized differentially for metabolic processes. Moreover,
partitioning of available carbon might differ depending on leaf age and developmental status. To investigate this, a radio- 
labelling experiment was performed firstly to compare carbon partitioning in leaves at two different developmental 
stages (source and sink), and secondly to compare the partitioning of assimilated versus imported carbon in sink leaves.
In a labelled sink leaf, all 14C-containing compounds must derive from carbon assimilated by the leaf itself. If instead a 
neighbouring source leaf is labelled, then all 14C-containing compounds found in the sink leaf must derive from labelled 
compounds imported from the source leaf (predominantly as sucrose).

Plants for the labelling experiment were grown on soil in a growth chamber. In the middle of the photoperiod, a 
source leaf (leaf no. 8) was labelled for one set of plants, while a small developing leaf (leaf no. 13) was labelled for the 
second set of plants (Fig. 1a, Supporting Information Fig. S1). A 5 min pulse of 14CO2 was followed by a 60 min 
chase period in normal air. After the chase, the labelled leaf was harvested for both sets. For the first set of plants, leaf 13 
– a sink leaf previously determined to import carbon from the labelled leaf 8 (Kölling et al. 2013 and data not shown) 
– was also harvested (designated as unlabelled sink leaf). Subsequently, the amount of 14C partitioned to water-
soluble, ethanol-soluble and insoluble compounds was determined.

Differences in the partitioning of assimilated carbon were observed between source and sink leaves (Fig. 1b–d). In a 
source leaf, 55% of the assimilated carbon was present as water-soluble compounds, with 40% in insoluble compounds 
and 5% in ethanol-soluble compounds (e.g. lipids, pigments and waxes; Fig. 1b). Clearly, a fraction of the assimilated 
carbon will have been exported during the chase period, so the allocation into water-soluble compounds is an underesti-
mate (by as much as 20% – see later, Figs 2 & 3). In contrast, sink leaves partitioned carbon equally between water-soluble 
and insoluble compounds (45% each) and 10% into ethanol-soluble components. As sink leaves are unlikely to export 
much carbon, these data indicate a greater investment of assimilated carbon in insoluble compounds. Sub-
fractionating the insoluble compounds into starch, proteins and a remaining insoluble fraction (consisting primarily of 
cell wall material) revealed further differences between the source and sink leaves (Fig. 1c). Source leaves invested most 
carbon into starch (25%) and less into proteins and cell walls (7% and 9%, respectively). However, sink leaves partitioned 
assembled carbon equally between starch, protein and cell 
walls (15% each). No significant differences were observed for carbon partitioning within the soluble fraction 
upon sub-fractionation into neutral, acidic and basic compounds (Fig. 1d). Most of the carbon was found in the acidic 
fraction, containing organic acids and sugar phosphates. Less was observed in the neutral fraction (comprising sugars such 
as sucrose, glucose and fructose) and the basic fraction (mainly amino acids). These data show that assimilated 
carbon is invested into different metabolic processes depending on the developmental stage of the leaf. As expected, sink 
leaves invested more carbon resources into structural com-
pounds, while source leaves allocated more carbon into storage compounds (i.e. starch).

To investigate whether certain metabolic processes prefer-
entially use assimilated rather than imported carbon or vice 
versa, we compared the partitioning of photoassimilated 14C in the labelled sink leaf with the partitioning of 14C imported 
as sucrose into the unlabelled sink leaf from a labelled source leaf. Slight differences were apparent for the two carbon 
sources. While carbon was partitioned equally between soluble and insoluble compounds after assimilation, less 
imported carbon was found in the insoluble fraction (37.4%) than in soluble compounds (53.5%; Fig. 1b). Nevertheless,
sub-fractionation of the soluble compounds revealed that the distribution of label found among neutral, acidic or basic compounds was the same for assimilated and imported carbon (Fig. 1d). In contrast, partitioning within the insoluble fraction differed (Fig. 1c): the same proportion of assimilated and fixed carbon was channelled towards proteins and the remaining cell wall fraction, but significantly less imported carbon was channelled into starch. However, the fraction of imported carbon channelled into starch (9.3%) was still considerable. For both carbon sources, comparable proportions of carbon were channelled towards ethanol-soluble compounds (10.6 and 9.1%, respectively).

Taken together, carbon partitioning varied only slightly with the carbon source, indicating that assimilated and imported carbon can be used equally to fuel all major metabolic processes, presumably as both feed into major pools of metabolic intermediates such as the hexose-phosphates. However, it is important to note that there will be a time delay in the partitioning of imported carbon compared with assimilated carbon, which is difficult to account for in our partitioning data. Clearly, the assimilated carbon will reach metabolic end products or pools faster than carbon, which is assimilated elsewhere and then imported. Furthermore, even when assimilation in another leaf occurs during a short pulse, translocated carbon is likely to arrive more gradually via the phloem. Both of these factors might lead to small changes in the partitioning pattern.

Carbon partitioning changes over the diurnal cycle

Although the assimilation of CO₂ is fairly stable throughout the day, Arabidopsis leaf expansion varies over the light cycle (Wiese et al. 2007). To investigate if carbon partitioning shows changes over the light period as well, ¹⁴C-labelling experiments were performed at different time points throughout the day: just after dawn (0 h) and after 2, 6 and 10 h of light. At each time point, single-source leaves (leaf no. 8) were labelled for 2 min with ¹⁴CO₂, followed by a 60 min chase period. After the chase, the labelled leaf and the unlabelled rosette were harvested separately and carbon allocation between the different tissues and compound classes was determined. The export from leaf 8 to the sink leaves of the rosette (hereafter referred to as the ‘sink

Figure 2. Carbon export into sink leaves over the diurnal cycle. (a) Scheme of the labelling setup. (b) Leaf 8 of Col-0 and pgm plants was labelled with ¹⁴CO₂ for 2 min just after the plants were exposed to light (0 h) and after 2 h, 6 h and 10 h into the day. Following a chase period of 1 h the labelled leaf and the unlabelled rosette (sink leaves) were harvested separately and the incorporation of ¹⁴C was determined. The relative amount of ¹⁴C incorporated into the unlabelled leaves is shown as a percentage of the total label in the plant. Asterisks indicate a significant difference to wild type (t-test; significance levels: *, P < 0.05). Mean ± SE (n ≥ 5).

Figure 3. Carbon export in Col-0 and mutants altered in starch metabolism. (a) Scheme of the labelling setup. (b–c) Whole shoots of Col-0, adg2, pgi, pgm, pwd, sex4 and sex1 were labelled with ¹⁴CO₂ for 1 h just after the plants were exposed to light (b) and at the middle of the light period (c). Following a chase period of 1 h, the labelled shoot and the unlabelled root were harvested separately and the amount of ¹⁴C in the different tissues was determined. The relative amount of ¹⁴C incorporated into the root is shown as a percentage of the total label in the plant. Asterisks indicate a significant difference to wild type (t-test; significance levels: *, P < 0.05; ** P < 0.01). Mean ± SE (n ≥ 5).
leaves’) varied over the course of the day (Fig. 2b). The highest rates were observed in the morning. After 2 h of light, export was lower and decreased further towards the end of the day. It is important to note that the total exported carbon is underestimated here, given that roots were not harvested in this experiment.

Like export, carbon partitioning into the major metabolite fractions changed considerably within the first 2 h of light whereas later in the day it was more stable (Table 1). In general, less carbon was found in insoluble compounds in the labelled source leaf in the morning and more was found in water- and ethanol-soluble compounds. This pattern was reversed later in the day. Sub-fractionating the insoluble compounds into starch, proteins and remaining insoluble material revealed an increasing partitioning of $^{14}$C into starch over the light period (Table 1). At the onset of the day, only 14.1% of the carbon retained in the leaf was used for starch synthesis, while later, more than twice as much (32.8%) was used. In contrast, carbon allocation to proteins and the remaining insoluble fraction peaked early in the beginning of the light period. Labelling of neutral sugars in the source leaf was highest at the onset of light (43.9%; Table 1). After 2 h, only 16.2% was observed in this pool, but at the end of the day, partitioning into sugars increased again (23.0%). In contrast, carbon partitioning into acidic compounds was low at the beginning of the day compared with later time points, while label in basic compounds was more stable.

It is important to note that the partitioning data are presented as percentages of the label found in the source leaf, and do not consider exported carbon. For export, carbon would primarily be in the form of sucrose – a soluble, neutral sugar. Thus, if the differences in export shown in Fig. 2b are factored in, it considerably enhance the differences in allocation. However, as not all exported carbon was accounted for in this experiment (i.e. because the roots were not harvested), no correction is applied.

The time-dependent differences in carbon partitioning observed in the source leaf were not necessarily reflected in the sink leaves (Table 1). For example, while the high labelling of neutral sugars in the source leaf early in the day correlated with a high rate of export to the sink (Fig. 2), the pattern utilization of the exported carbon in the sink was similar for most of the day. Only at the end of the day were changes observed (even though export rates from the source were steady), with an increase in label in neutral sugars and a decrease in insoluble and ethanol-soluble compounds. Carbon found in acid compounds decreased steadily over the course of the day while that in basic compounds peaked in the middle of the day.

These data, like those of Fig. 1, indicate a higher allocation of carbon into structural components (e.g. ethanol-soluble compounds and cell wall material) in sink tissues than in source tissues, and less allocation into starch. Together, the data show that high rates of carbon export from source tissues and high rates of synthesis of cellular components in sink tissues accompany high leaf expansion rates reported for the beginning of the day.

**Table 1.** Carbon partitioning in Col-0 over the diurnal cycle

|                  | Col-0 0 h | Col-0 2 h | Col-0 6 h | Col-0 10 h |
|------------------|-----------|-----------|-----------|------------|
| **Labelled source leaf** |           |           |           |            |
| Water-soluble compounds | 59.8 ± 0.8 | 48.6 ± 2.7 | 47.5 ± 1.6 | 49.6 ± 1.9 |
| Neutral           | 43.9 ± 5.0 | 16.2 ± 1.4 | 16.2 ± 1.1 | 23.1 ± 2.0 |
| Acidic            | 12.1 ± 0.8 | 20.0 ± 2.6 | 21.2 ± 0.4 | 15.7 ± 1.0 |
| Basic             | 6.7 ± 0.4  | 9.3 ± 0.4  | 7.8 ± 0.5  | 8.2 ± 0.3  |
| Insoluble compounds | 29.8 ± 1.6 | 41.7 ± 2.5 | 45.1 ± 1.3 | 43.3 ± 1.6 |
| Starch            | 14.1 ± 2.1 | 26.1 ± 1.5 | 32.8 ± 1.1 | 30.8 ± 1.2 |
| Protein           | 5.1 ± 0.4  | 5.6 ± 0.9  | 3.6 ± 0.4  | 3.6 ± 0.3  |
| Remaining insoluble | 6.3 ± 0.4  | 8.1 ± 1.2  | 4.8 ± 0.5  | 4.8 ± 0.4  |
| Ethanol-soluble compounds | 10.4 ± 1.1 | 9.7 ± 1.5  | 7.5 ± 0.4  | 7.0 ± 0.4  |
| Sink leaves       |           |           |           |            |
| Water-soluble compounds | 60.3 ± 0.9 | 53.8 ± 3.1 | 55.8 ± 2.5 | 67.8 ± 1.3 |
| Neutral           | 20.2 ± 5.2 | 17.3 ± 1.4 | 17.4 ± 1.3 | 33.1 ± 2.0 |
| Acidic            | 19.9 ± 4.8 | 14.7 ± 1.1 | 15.4 ± 0.4 | 12.8 ± 0.4 |
| Basic             | 11.3 ± 2.2 | 17.6 ± 1.3 | 19.8 ± 1.5 | 17.0 ± 1.0 |
| Insoluble compounds | 29.4 ± 1.2 | 30.8 ± 2.0 | 34.5 ± 1.3 | 25.8 ± 0.6 |
| Starch            | 8.8 ± 0.7  | 9.1 ± 0.6  | 12.3 ± 1.0 | 8.3 ± 0.5  |
| Protein           | 5.5 ± 0.1  | 5.4 ± 0.2  | 5.4 ± 0.2  | 4.2 ± 0.2  |
| Remaining insoluble | 12.1 ± 0.8 | 12.1 ± 0.7 | 12.8 ± 0.9 | 10.4 ± 0.7 |
| Ethanol-soluble compounds | 10.3 ± 0.5 | 15.3 ± 4.0 | 9.7 ± 1.9  | 6.4 ± 1.0  |

Leaf 8 of Col-0 plants was labelled with $^{14}$CO$_2$ for 2 min just after the plants were exposed to light (0 h) and after 2, 6 and 10 h of light. Following a chase period of 1 h, the labelled leaf and the unlabelled rosette (sink leaves) were harvested separately and the incorporation of $^{14}$C was determined. For the labelled leaf the relative amount of $^{14}$C in the different compound classes are expressed as a percentage of the total retained in that leaf. The amount of $^{14}$C exported to the sink leaves is given in Fig. 2. For the sink leaves, the relative amount of $^{14}$C in the different compound classes are expressed as a percentage of the total exported from the labelled leaf. Mean ± SE (n ≥ 5).

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Carbon partitioning in the starchless \(pgm\) mutant is severely altered

It was previously shown that plants deficient in starch synthesis have reduced overall growth, but also a changed growth pattern (Wiese et al. 2007). We analysed carbon partitioning over the day in the starchless \(pgm\) mutant as described earlier. Carbon export to the sink tissues in \(pgm\) was highest at the start of the day, and like the wild type, decreased during the day (Fig. 2b). However, significantly more carbon export was observed in \(pgm\) at the first two labelling time points compared with the wild type. Later in the day, \(pgm\) carbon export rates were comparable with those of the wild type.

As \(pgm\) is deficient in starch synthesis, the expected reduction of carbon partitioning towards starch, and therefore towards total insoluble compounds, was observed (Table 2). In labelled \(pgm\) source leaves, less than 0.1% of the \(^{14}\text{C}\) was in the starch fraction. The \(^{14}\text{C}\) found in sugars was considerably increased in \(pgm\) (two- to threefold higher than the wild type) and was the single largest labelled fraction, with labelling as high as 80% at the start of the day. At this time, \(^{14}\text{C}\) in all other fractions was much lower than in the wild type. However, this pattern changed during the course of the day. Interestingly, carbon partitioning into proteins was generally higher in \(pgm\) than in the wild type (except for the first time point). Partitioning towards the remaining insoluble fraction was initially low in \(pgm\) and increased during the day, while in the wild type it was initially high and then decreased. A gradual increase was also seen for the amounts of carbon in basic and ethanol-soluble compounds in \(pgm\), eventually reaching wild-type levels.

A very interesting situation was seen in the sink leaves of the \(pgm\) mutant. At every time point, the pattern of utilization of the exported carbon in the sink was similar. However, the distribution of carbon was very different from that in the wild type. Fewer label was found in all compound classes, with the exception of proteins and neutral sugars. This suggests that sink leaves of \(pgm\) are not using as much imported carbon for growth as wild-type sink leaves. This is the case whether the quantity of supplied carbon is similar to the wild type (e.g. in the second half of the day) or even higher (e.g. in the morning).

Effects of altered starch metabolism on carbon partitioning and growth

In the \(pgm\) mutant, reallocation into soluble compounds takes place as starch synthesis is impaired. However, this extent of reallocation exceeds the reduction in starch synthesis and is accompanied by numerous other changes in partitioning that cannot be directly linked to impaired starch synthesis. To reveal if this is a general pattern in carbon reallocation, further mutants altered in starch metabolism were analysed. The first mutant set comprised plants altered in starch synthesis in addition to \(pgm\). The phosphoglucoisomerase (\(pgi\)) and the ADP-glucose pyrophosphorylase (\(adg2\)) mutants are affected at different steps in the synthesis of the starch precursor ADP-glucose (Lin et al. 1988; Yu et al. 2000). These lines

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**Table 2.** Carbon partitioning in \(pgm\) over the diurnal cycle

| Percentage of \(^{14}\text{C}\) recovered in each fraction related to the total amount per tissue |
|---------------------------------------------------------------|
| **pgm 0 h** | **pgm 2 h** | **pgm 6 h** | **pgm 10 h** |
|---------------------------------------------------------------|
| **Labelled source leaf** | | | |
| Water-soluble compounds | 92.4 ± 0.8 | 84.5 ± 0.7 | 83.6 ± 0.5 | 82.2 ± 2.0 |
| Neutral | 80.0 ± 1.6 | 59.3 ± 1.5 | 54.2 ± 1.0 | 51.3 ± 3.0 |
| Acidic | 6.1 ± 0.7 | 18.6 ± 1.6 | 18.7 ± 1.5 | 19.4 ± 1.2 |
| Basic | 4.7 ± 0.7 | 3.9 ± 0.6 | 6.8 ± 1.2 | 9.4 ± 1.2 |
| Insoluble compounds | 4.6 ± 0.6 | 10.1 ± 0.4 | 10.3 ± 0.3 | 11.7 ± 1.4 |
| Starch | 0.1 ± 0.1 | 0.1 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Protein | 3.9 ± 1.1 | 6.5 ± 0.2 | 5.9 ± 0.3 | 5.9 ± 0.7 |
| Remaining insoluble | 0.9 ± 0.3 | 3.5 ± 0.5 | 4.4 ± 0.2 | 5.7 ± 1.1 |
| Ethanol-soluble compounds | 3.0 ± 0.3 | 5.4 ± 0.3 | 6.1 ± 0.3 | 6.1 ± 0.7 |
| **Sink leaves** | | | |
| Water-soluble compounds | 84.1 ± 2.6 | 82.9 ± 0.8 | 76.5 ± 2.0 | 79.8 ± 2.0 |
| Neutral | 67.7 ± 2.1 | 59.6 ± 3.4 | 52.8 ± 3.6 | 58.1 ± 3.4 |
| Acidic | 10.9 ± 1.0 | 13.5 ± 1.1 | 11.6 ± 0.4 | 12.9 ± 1.8 |
| Basic | 4.7 ± 0.7 | 4.7 ± 0.5 | 7.0 ± 1.4 | 7.8 ± 1.0 |
| Insoluble compounds | 14.8 ± 2.6 | 14.7 ± 0.7 | 21.1 ± 2.0 | 18.0 ± 2.0 |
| Starch | 2.0 ± 1.3 | 0.0 ± 0.0 | 4.7 ± 0.7 | 1.1 ± 0.7 |
| Protein | 8.1 ± 1.5 | 9.0 ± 0.4 | 13.2 ± 1.0 | 11.7 ± 1.4 |
| Remaining insoluble | 3.2 ± 0.8 | 5.7 ± 0.4 | 3.2 ± 0.5 | 5.3 ± 1.3 |
| Ethanol-soluble compounds | 1.1 ± 0.1 | 2.5 ± 0.5 | 2.4 ± 0.2 | 2.2 ± 0.1 |

Leaf 8 of \(pgm\) plants was labelled with \(^{14}\text{CO}_2\) for 2 min just after the plants were exposed to light (0 h) and after 2, 6 and 10 h of light. Following a chase period of 1 h, the labelled leaf and the unlabelled rosette (sink leaves) were harvested separately and the incorporation of \(^{14}\text{C}\) was determined. For the labelled leaf the relative amount of \(^{14}\text{C}\) in the different compound classes are expressed as a percentage of the total retained in that leaf. The amount of \(^{14}\text{C}\) exported to the sink leaves is given in Fig. 2. For the sink leaves, the relative amount of \(^{14}\text{C}\) in the different compound classes are expressed as a percentage of the total exported from the labelled leaf. Mean ± SE (\(n \geq 5\)).

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display different degrees of starch accumulation between wild-type levels and almost unmeasurable amounts of starch in pgm (Supporting Information Table S1). A second mutant set contained plants with altered starch degradation. Two of these mutants lack phosphoglucan, water dikinase (PWD) and glucan water dikinase [GWD, encoded at the STARCH EXCESS 1 (SEX1) locus], respectively. These enzymes are both involved in phosphorylation of starch, a process thought to disrupt the semi-crystalline structure of starch, making it accessible for amylolytic degradation (Yu et al. 2001; Kötting et al. 2005). The third mutant lacks a phosphoglucan phosphatase (encoded by the STARCH EXCESS 4 [SEX4] locus) that removes phosphate groups from the glucan chains during amylolysis to allow for complete degradation (Kötting et al. 2009). Loss of any of these enzymes reduces starch degradation, leading to a starch excess phenotype, which is mild in pwd and most severe in sex1.

To supply $^{14}$CO$_2$ to so many plants at the same time, whole-plant labelling was performed rather than single leaf labelling. Three-week-old hydroponically grown rosettes were used, which also allowed us to measure carbon export to the roots. $^{14}$CO$_2$ was supplied for 1 h, either at the start or in the middle of the day, followed by a 1 h chase in normal air. The rosettes and the roots were harvested separately and the $^{14}$C in the different tissues and compound classes was determined. Wild-type plants partitioned carbon similarly as for the single leaf labelling (Figs 3–5); in the morning, export to the root was high, as was carbon allocation into structural components (remaining insoluble and ethanol-soluble fractions) and proteins. Later in the day, carbon allocation into starch increased.

As expected, the starch synthesis mutants had lower carbon allocation into starch than the wild type (Figs 4 & 5), correlating with the measured starch levels (Supporting

|                  | Col-0 | adg2 | pgi | pgm | pwd | sex4 | sex1 |
|------------------|-------|------|-----|-----|-----|------|------|
| Insoluble        |       |      |     |     |     |      |      |
| % of $^{14}$C    |       |      |     |     |     |      |      |
|                  |       |      |     |     |     |      |      |
| Starch           |       |      |     |     |     |      |      |
| % of $^{14}$C    |       |      |     |     |     |      |      |
|                  |       |      |     |     |     |      |      |
| Protein          |       |      |     |     |     |      |      |
| % of $^{14}$C    |       |      |     |     |     |      |      |
|                  |       |      |     |     |     |      |      |
| Remaining        |       |      |     |     |     |      |      |
| % of $^{14}$C    |       |      |     |     |     |      |      |
|                  |       |      |     |     |     |      |      |

**Figure 4.** Carbon partitioning at the beginning of the day in Col-0 and mutants altered in starch metabolism. Whole shoots of Col-0, adg2, pgi, pgm, pwd, sex4 and sex1 were labelled with $^{14}$CO$_2$ for 1 h just after the plants were exposed to light. Following a chase period of 1 h, the labelled shoot and the unlabelled root were harvested separately and the incorporation of $^{14}$C into the different fractions was determined. The relative amount of $^{14}$C incorporated into the different fractions of the labelled rosette leaves as a percentage of the total label in the plant is shown. Asterisks indicate a significant difference to wild type ($t$-test; significance levels: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Mean ± SE ($n ≥ 4$).
Interestingly, $^{14}$C incorporation into starch was higher at midday compared with the morning, even in the lines with very low starch levels (e.g. 3.1% and 10.2% in the morning and 13.1% and 28% at midday in pg$_{i}$ and adg$_{2}$, respectively). This suggests that an increase in starch synthesis over the light period is determined by a regulatory system independent from the enzymes of the starch synthesis pathway. The decreased carbon flow towards starch in the mutants was accompanied by several changes in carbon partitioning, as for pg$_{m}$ in the previous experiment. However, it is important to note that the different design of the experiments means that the results cannot be directly compared. The wild type transported 8.7% of its assimilated carbon to the root at the start of the day, while pg$_{m}$ exported 19.4% (Fig. 3b,c). Export to the root in the other starch synthesis mutants was between the wild-type and pg$_{m}$ values.

In most ways, the differences in labelling patterns between the wild type and in the starch-deficient mutants was similar to that seen for pg$_{m}$ in the previous experiment. Compared with the wild type, label found in neutral sugars was higher in the mutants, while label in structural components was lower. This trend was especially clear at the start of the day (Fig. 4), but less so in the middle of the day (Fig. 5), when a higher incorporation of carbon into structural compounds was observed in the mutants. The extent of the differences to the wild type was inversely correlated to the severity of the starch deficiency. For example, while 13% of the label was in the neutral compounds in the morning, the values were 21%, 27% and 43% for the starch mutants adg$_{2}$, pg$_{i}$ and pg$_{m}$, respectively. The corresponding amounts incorporated into starch were 16% for the wild type, and 10, 3 and 0% for the mutants. Again, at the start of the day, the extent

Figure 5. Carbon partitioning in the middle of the light period in Col-0 and mutants altered in starch metabolism. Whole shoots of Col-0, adg$_{2}$, pg$_{i}$, pg$_{m}$, pwd, sex$_{4}$ and sex$_{1}$ were labelled with $^{14}$CO$_{2}$ for 1 h in the middle of the light period. Following a chase period of 1 h the labelled shoot and the unlabelled root were harvested separately and the incorporation of $^{14}$C into the different fractions was determined. The relative amount of $^{14}$C incorporated into the different fractions of the labelled rosette leaves as a percentage of the total label in the plant is shown. Asterisks indicate a significant difference to wild type ($t$-test; significance levels: *, $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Mean ± SE ($n \geq 4$).
of the increase in carbon allocation to the neutral fraction in the mutants exceeded the decrease in labelling of starch, suggesting broader changes in partitioning. At midday, partitioning into neutral compounds was still increased relative to the wild type in the mutants, but less so compared with the morning.

In mutants impaired in starch degradation, partitioning was generally more similar to the wild type than the starch synthesis mutants (Figs 4 & 5). Yet some changes were consistent between the two groups, although less pronounced in the starch degradation mutants and only evident at the start of the day. A decrease in label was seen in structural components and a slight increase was observed in water-soluble compounds and the export to the roots, compared with the wild type. The higher labelling in water-soluble compounds was attributable to an increase in the neutral fraction. Label in the acidic and basic fractions was generally decreased. The more severe the starch excess phenotype, the more carbon was found in the neutral fraction (up to 31% in sex1). Label in the insoluble fraction was slightly reduced in the mutant lines compared with the wild type. Carbon flow into starch did not change significantly, but carbon flow into proteins and the remaining insoluble compounds was reduced. Partitioning into the ethanol-soluble compounds mostly resembled the wild-type levels. The fact that a similar redistribution of carbon is seen in starch synthesis and starch degradation mutants at the beginning of the light period probably reflects the fact that both experience carbon starvation at night, which increases with the severity of the starch phenotype. However, at midday, the partitioning pattern in the starch degradation mutants closely resembled the wild type, while the starch synthesis mutants still exhibited differences.

Here again, it should be noted that the values given – in this case for the rosettes – do not take into account the label exported. Therefore, the differences in allocation patterns between mutants and time points in Figs 4 & 5 are, in many cases, underestimates. Even though, we have data for the amount of 14C in the root in this experiment, a fraction of the exported sugars will have been respired and lost (Zeeman & ap Rees 1999). Therefore, we have not attempted to correct the rosette data.

**Effects of a shortened or elongated night on carbon partitioning**

To investigate whether the strong alterations in partitioning and export in starch metabolism mutants at the beginning of the day is due to carbon starvation and/or changes in the carbon demand of sink tissues, we labelled wild-type plants after an unexpectedly short night (4 h less darkness) and an unexpectedly long night (4 h more darkness, Fig. 6). Single source leaves (leaf no. 8) of soil-grown plants were labelled for 2 min and the labelled leaf and rest of the rosette were harvested separately after a 1 h chase in normal air.

Interestingly, after a short night, the export of carbon from the source leaf to the rest of the rosette was decreased compared with the end of the normal night (the control plants), possibly because of a reduced sink strength from the rest of the plant (Fig. 6b). Apart from the altered export, carbon partitioning within the source leaf exposed to a shortened night was the same as in the control plants (Fig. 6c–e). After an elongated night, however, there were more changes. Carbon partitioning into neutral sugars in the source leaf was strongly increased, as was observed for the starch mutants suffering from carbon starvation at the end of a normal night. However, carbon export after an elongated night was reduced compared with the control plants, despite the increased partitioning into neutral sugars. Interestingly, partitioning into starch was also higher compared with after a
normal night. Thus, starvation leads to (1) carbon accumulating in sugars; (2) reduced carbon export; (3) less carbon being used for growth (i.e. low carbon incorporation into proteins, the remaining insoluble fraction and the ethanol-soluble fraction); and (4) an increase in carbon partitioning towards starch.

**Carbon partitioning in circadian clock mutants**

Our data show that carbon starvation influences carbon partitioning, but this cannot fully explain the allocation patterns seen in starch mutants, nor those observed in wild-type plants during the day in normal growth conditions. To evaluate whether carbon partitioning is also influenced by the plants circadian clock, we measured the allocation patterns in the clock mutants *lhy/cca1* and *elf3-4* (both Ws background), and in *prr7/prr9, gi* and *toc1* (all Col-0 background). Eighteen-day-old hydroponically grown plants were subjected to whole-plant labelling for 30 min just after the light was switched on and after 2, 4, 6, 8 and 10 h of light. Following a 1 h chase in normal air, the rosettes and the roots were harvested separately, three of each were pooled (because of the small size of some of the mutant lines) and the distribution of $^{14}$C was determined.

Both wild-type accessions (WS and Col-0) had similar partitioning patterns (Fig. 7 and Supporting Information).
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Fig. S2), resembling that already seen for Col-0 plants (Table 1). However, Ws partitioned slightly more carbon into starch, consistent with previous measurements of starch content (Sulpice et al. 2009). The lhy/cca1 mutant channelled similar amounts of carbon into the respective compound classes, but had a shifted partitioning pattern. Partitioning into neutral compounds was high just after the light was switched on, but was markedly decreased 2 h later, as did export to the root. Thereafter, partitioning into neutral compounds and export increased again for most of the rest of the day. At the end of the day, allocation into the neutral fraction increased to a greater extent than the wild type, but without an increase in export. Partitioning towards starch showed reciprocal changes; while it was highest after 6 h of light in wild-type plants, it peaked 2 h after the onset of light in lhy/cca1, coinciding with the minimum allocation to sugars and export. Allocation to starch in lhy/cca1 then decreased steadily during the rest of the day.

The elf3 mutant had smaller differences in the pattern of carbon partitioning compared with the wild type, but marked changes in the absolute amount of carbon channelled into certain compound classes, especially starch. While Ws partitioned 30% of the assimilated carbon into starch on average, elf3 partitioned only 17%. This led to an increased partitioning into other compound classes (mainly neutral compounds).

Both gi and tocl had similar partitioning patterns and amounts to the Col-0 wild type. However, these patterns were lost in prr7/prr9, where carbon partitioning into neutral compounds and starch remained stable throughout the day. In this line, slightly more carbon overall was allocated into neutral and acidic compounds and less into starch and proteins compared with the wild type.

DISCUSSION

Similar utilization of assimilated and imported carbon

In sink leaves, only small differences in carbon partitioning were observed between photo-assimilated carbon and imported carbon. Less imported carbon was found in the insoluble fraction and more in the water-soluble fraction compared with fixed carbon, but the distribution across subfractions of the soluble compounds (e.g. neutral, acidic and basic) was the same. Of course, further fractionation of these large compound classes might reveal differences such as the labelling pattern of amino acids within the basic compounds. Partitioning towards proteins and other insoluble compounds was similar for imported and assimilated carbon, but less imported carbon was used for starch synthesis. To some extent this is not surprising, as starch is only made in the chloroplast. Subcellular fluxes estimated using dynamic 13C labelling suggest that ADPglucose (ADPGlc) – the precursor for starch synthesis – is rapidly labelled during photosynthesis (Szecowka et al. 2013). Perhaps more surprising is that such a large fraction of the imported carbon still ends up in starch. This is likely due to equilibration between the cytosol and the stroma of intermediates (e.g. triose-, pentose- and hexose-phosphates) derived from sucrose metabolism and does not necessarily represent a net uptake of carbon from the cytosol.

Interestingly, carbon partitioning into starch of pgm sink leaves was considerably higher than in source leaves (Table 2). Starch is synthesized from hexose-phosphates, which are converted to ADPGlc. Import of either ADPGlc or glucose-1-phosphate would provide substrates for starch synthesis in pgm chloroplasts (Streb et al. 2009). Evidence for transport of glucose-1-phosphate into plastids was shown recently in photosynthetic tissues (Fettke et al. 2011), but the transporter responsible is not known. A transporter for ADPGlc has been described in amyloplasts from cereal endosperm. However, plastidial transporters from the same class from Arabidopsis appear unable to transport ADPGlc (Kirchberger et al. 2007).

Carbon partitioning changes during the day

Plants labelled with 14CO2 at different times of day showed striking differences in carbon export from source to sink tissues and partitioning within the source, even though photosynthetic carbon supply is essentially constant. Particularly large changes occurred in the first hours of the day, with more subtle changes seen later. Changes in partitioning during the day were much less pronounced in sink leaves.

Incorporation of 14C into neutral sugars, organic acids and starch changed most, while incorporation into other pools (e.g. basic compounds and proteins) was more stable. Just after dawn, labelling of sugars was high and was accompanied by high rates of carbon export (the majority of which must also have passed through neutral sugars). This could reflect the source ‘pushing’ carbon into the phloem via high rates of sucrose synthesis or a high sink strength, which ‘pulls’ sugars from the phloem, promoting export from source tissues. However, it is also possible that export is also influenced by other factors (e.g. regulation of the sugar transporters, see later). Carbon flow to structural compounds like proteins, the remaining insoluble fraction (cell walls), and ethanol-soluble compounds (lipids, pigments and waxes), reflecting the growth and/or maintenance of the leaf, was also relatively high at the beginning of the light period.

Carbon flow into starch increased during the light period from 14% at the beginning of the day to more than 30% later on. Even though starch synthesis is generally described as being linear over the day, our data show that this most likely is not the case. Also other studies with frequent sampling suggested a lag in starch accumulation at the beginning of the day (Zeeman et al. 2004). This trend is more pronounced in longer photoperiods, where much of the starch is synthesized in the second half of the light period (Zhang et al. 2005, 2008; Sulpice et al. 2014). We predict that carbon partitioning measurements in long-day conditions using 14C would reveal even higher fluctuations than shown here.
Carbon starvation strongly effects carbon partitioning and utilization

Changes in carbon partitioning occurred in all mutants affected in starch synthesis, increasing with the severity of the starch deficiency. Reduced carbon flow into starch was accompanied by changes in partitioning towards nearly all other compound classes, particularly at the beginning of the day. Large amounts of carbon were channelled into sugars and more carbon was exported to sink regions. Plants altered in starch degradation displayed a similar carbon partitioning phenotype at the beginning of the day, with increased allocation into sugars and export to sink tissues. This is interesting as these plants are not deficient in starch biosynthesis and allocation into starch at the start of the day was comparable with the wild type. The increased allocation into sugars was due to re-allocation from other metabolite pools or end products. What starch synthesis and starch degradation mutants have in common is that they suffer from starvation at night; the former deplete their carbon reserves too early and the latter cannot degrade their reserve efficiently (Messerli et al. 2007; Usadel et al. 2008).

Carbon starvation brings about a complex response in Arabidopsis. Exposure of wild-type plants to an extended night induces genes involved in lipid and protein degradation. Free amino acid levels increase, and Rubisco-containing bodies derived from the chloroplast stroma appear in the vacuole – all evidence for the recycling of proteins (Usadel et al. 2008; Izumi et al. 2010). Comparable gene expression, metabolite and cellular changes could be seen for pgm mutants during a normal night after sugars are depleted (Gibon et al. 2006; Usadel et al. 2008). This triggering of the catabolism of cellular components to enable the plant to survive the night is accompanied by decreases in growth. Analysis of sink tissues such as root tips shows that growth stops at night in pgm plants, whereas wild-type roots grow continuously. Furthermore, at the start of the day, sugars hyper-accumulate in pgm root tips, but growth only recommences after a delay of several hours (Gibon et al. 2004; Yazdanbakhsh et al. 2011). Gibon et al. (2004) estimated that 75% of the newly assimilated carbon accumulates as sugars at the start of the day in pgm, which is substantiated by our measurements (at dawn, 80% of the assimilated carbon is introduced into neutral compounds). Our measurements also reveal a reduced allocation of carbon into most pools representative of growth (e.g. cell wall biosynthesis, ethanol-soluble compounds) in both sink and source tissues (Table 1). Interestingly, although, in many cases, carbon partitioning into proteins was actually higher in starch synthesis mutants than in the wild type. This was especially the case in sink leaves of pgm, where more than twice as much carbon was introduced into proteins compared with the wild type throughout the day (Table 2). This might be due to the need to resynthesize large amounts of protein that were degraded at night simply to survive. The increase in labelling of proteins in pgm was accompanied by decrease in label in basic compounds. This could reflect an increased utilization of amino acids and flux through these compounds, rather than a decreased allocation per se. Much energy is consumed for the biosynthesis of proteins (4.7–7.9 ATP per amino acid, Zerihun et al. 1998) compared with the synthesis and degradation of other compounds such as starch. Thus, the apparent necessity for starchless plants to use proteins as a carbon source at night is associated with high costs for the plants. This helps to explain why less carbon is used for the synthesis of structural components and the decreased growth rates observed in such mutants.

Placing wild-type plants into an extended dark period resulted in some, but not all of the changes in carbon allocation seen in starch mutants. A decreased utilization of carbon for growth and an increased allocation of carbon into neutral sugars were seen, but surprisingly, this was associated with a decrease rather than an increase in export to the sink tissues. This suggests that high sugars do not necessarily force export and that export rate is to some extent controlled independently of the current sugar levels. This is consistent with the observation that later in the day, pgm mutants have export rates comparable with the wild type, despite still having higher allocation into sugars. This is important to consider when designing strategies to alter sink source relations for crop improvement. After an extended dark period, wild-type plants made more starch than after a normal night. This supports previous measurements where both sucrose accumulation and starch synthesis were elevated after exposure to an extended night. This effect on starch synthesis was shown to correlate with a greater degree of redox-activation of ADP-glucose pyrophosphorylase and could serve to avoid starch depletion in the subsequent night (Gibon et al. 2004; Lunn et al. 2006).

Carbon partitioning is under the control of the circadian clock

Marked changes in the pattern of carbon partitioning were observed in the clock mutants lhy/cca1 and prr7/prr9. LHY and CCA1 are the two components of the morning loop. They are abundant in the morning and repress the expression of the EC genes. In the lhy/cca1 double mutant, the clock runs too fast, leading to a period length of only 20 h (Mizoguchi et al. 2002; Lu et al. 2009). The mis-timing of the clock leads to premature exhaustion of starch reserves at the end of the night (Graf et al. 2010). Not surprisingly therefore, carbon partitioning at the onset of light resembles to a large extent what we observed already for wild-type plants exposed to an extended night. Increased partitioning into neutral compounds and starch is accompanied by a decreased partitioning into most of the other compound classes especially structural components. Even more interesting is the temporal pattern of carbon partitioning in lhy/cca1. Partitioning into neutral compounds and export differs to the wild type. Instead of a steady decrease over the light period, lhy/cca1 displays a strong increase in the morning followed by an increase throughout the rest of the day. Furthermore, while partitioning of carbon into starch in the wild type peaks after 6 h of light, it peaks already after 2 h of light in lhy/cca1. A 4h shift is also seen for the expression of several clock genes in
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Although our data show that variations in carbon partitioning accompany the fluctuations in leaf expansion reported in previous studies, other factors, such as hydraulic control and controlled changes in cell wall extensibility probably contribute significantly to the pattern of leaf expansion (Pantin et al. 2012). It remains to be elucidated how tightly linked are the partitioning of carbon into structural compounds and the phases of expansion growth, and to understand the extent to which the underlying molecular regulators are co-regulated (e.g. by the circadian clock). It would be interesting in this context to measure the diurnal leaf expansion patterns of the clock mutants analysed in this study.

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**Carbon partitioning into structural compounds and its relation to growth**

Growth at the metabolic level equates to high biosynthetic activity, particularly of proteins and structural components such as lipids for membranes and cell wall materials. Our experiments give insight into overall rates of protein, lipid and cell wall synthesis. Not surprisingly, our data revealed significant allocation of carbon into these pools in sink leaves, where a high biosynthetic activity would be expected as the cells proliferate and expand. Nevertheless, in source leaves, which continue to grow, primarily by cell expansion rather than proliferation, we could still measure significant incorporation into all three fractions. For lipids and cell walls, this is not altogether surprising as these are needed for expansion growth as well as cell wall regeneration (Gibeaut & Carpita 1991). For protein, this level of allocation could reflect a continued increase in net protein content of the leaf, or the extent of protein turnover that occurs during the normal housekeeping processes within the leaf cells.

Wild-type plants display a peak in expansion growth at the start of the day (Wiese et al. 2007), which correlates with the period when we observed the highest allocation of carbon into ethanol-soluble compounds and the cell wall containing fraction. The *pgm* mutant, which starves at night, displayed altered temporal expansion growth patterns in addition to having reduced overall growth rates (Wiese et al. 2007). Expansion still peaks at the beginning of the day, but is dampened. From midday, relative growth rate increases again, reaching a second peak at or shortly after dusk. This change in expansion growth was to some extent reflected in the partitioning pattern of the mutant. At the start of the day, there is a low allocation of carbon into structural components, while after 2 h and later in the day allocation increased.

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**lhycla1** such as TOC1, LUX, GI and ELF3 (Locke et al. 2005; Dixon et al. 2011; Pokhilkov et al. 2012), suggesting that the clock is 4 h too early in the morning. We propose that this causes the changes in the carbon partitioning pattern.

The pseudo-response regulator proteins PRR7 and PRR9, together with the homologs PRR5 and TOC1, form a cascade of clock components peaking throughout the day. The expression of PRR9 early in the morning is followed by PRR7, PRR5 and TOC1. PRR5, PRR7 and PRR9 repress the morning genes LHY and CCA1 (Nakamichi et al. 2010). In the long-period double-mutant *prr7/prr9*, LHY and CCA1 both show a broader expression peak, while the expression of the evening-specific genes is delayed (Farré et al. 2005). It is interesting that the partitioning pattern is strongly disturbed in this line, with alterations in carbon flow into most compound classes. This suggests a function for PRR7 and/or PRR9 in regulating either carbon partitioning directly, or growth processes that utilize assimilates. The metabolic phenotype of this double mutant is particularly interesting given that PRR7 has also been proposed to mediate metabolic signals to the clock itself (Haydon et al. 2013). Further analyses will be needed to show how PRRs mediate the clock output to control metabolism and vice versa.
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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Figure S1.** Chambers for the single leaf labelling with carbon isotopes. Cartoons (A, B) and photographs (C, D) of the labelling chambers attached to individual source (A, C) and sink (B, D) leaves of *Arabidopsis*.

**Figure S2.** Carbon partitioning in circadian clock mutants. Whole shoots of Ws, lhy/cca1, elf3, Col, prr7/prr9, gi and toc1 were labelled with $^{14}$CO$_2$ in a parallel experiment for 30 min just after the plants were exposed to light (0 h) and after 2, 4, 6, 8 and 10 h into the light. Following a 1 h chase period, the labelled shoots and the unlabelled roots were harvested separately. Because of the small size of the plants, tissues from three individual plants were pooled for each sample. The incorporation of $^{14}$C into different fractions was determined. The relative amount of $^{14}$C exported (A) and the relative amount of $^{14}$C incorporated into different fractions of the labelled rosette leaves (B) are given as a percentage of the total in the pooled plants. Values are the mean ± SE ($n = 3$). Col-0 in the left and middle panel is the same data plotted twice for comparability reasons.

**Table S1.** Starch levels of Col-0 and mutants altered in starch metabolism at the end of the light period (EOD) and the end of the dark period (EON). Asterisks indicated significant differences to the wild type (*t*-test; significance levels: *, $P < 0.05$; ***, $P < 0.01$; ***, $P < 0.001$). Mean ± SE ($n \geq 5$).