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Coordination of carbon supply and plant growth

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ABSTRACT

Plants must achieve a balance between carbon assimilation, storage and growth, but little is known about how this is achieved. We describe evidence for the existence of regulatory mechanisms that coordinate carbon supply and use, and the likely central role of sugar signalling. We propose the existence of both 'acute' and 'acclimatory' responses to alterations in carbon supply, the latter tuning the balance between carbon supply and demand to optimise the capacity for sustained growth. A full understanding of these responses requires new, systems-level approaches that integrate information from transcriptomic, enzyme activity, metabolomic and growth analyses. We illustrate the complexity of acute and acclimatory responses by consideration of the control of starch synthesis and degradation in leaves. Finally, we consider how carbon balance may be linked to growth, and the importance of these linkages for sustained plant growth in a changing environment.

Key-words: *Arabidopsis*; carbon allocation; enzyme activities; expression arrays; metabolites.

INTRODUCTION

Although it is obvious that plants must achieve a balance between carbon assimilation, carbon storage and growth, remarkably little is known about how this is achieved. Our ignorance stems partly from a lack of tools with which to tackle such a broad question, and partly from the traditional divisions within research in plant biology. Historically, researchers on plant physiology and metabolism have viewed the link between assimilation, storage and growth in terms of sink–source relationships – the complex interplay of availability of carbon in source (assimilatory) organs and the demand for carbon by sink (non-photosynthetic) organs – rather than examining directly if and how the carbon supply influences the rate of growth. Research on growth and development has tended to emphasize processes like the cell cycle, and assumes that assimilation and storage are adjusted to meet the demands of growth. A large sector of research on growth has addressed questions relating to the physical expansion of plants in terms of the movement of water rather than the supply of carbon. One of the possible ways in which assimilation, storage and growth may be linked is via sugar signalling. Sugar signalling has been an area of intensive research in the last 20 years, and many genes putatively involved in this process have been identified. However, signalling pathways that are firmly rooted in growth at one end and assimilation/storage at the other have yet to be established. In this review, we discuss the present evidence for the existence of regulatory mechanisms that balance the supply of carbon with its use within the plant. We argue that their importance lies in allowing plant growth to be sustained under a wide range of environmental conditions. We discuss how the mechanisms that coordinate carbon supply and use can be investigated, then describe new information derived from open-ended integrative approaches that combine transcriptomic, enzymatic and metabolomic measurements in contrasting physiological systems. We then describe current thinking on a key element in the acute and acclimatory responses – the control of the synthesis and the degradation of starch in leaves. Finally, we consider how and what level growth may be modulated in response to carbon availability.

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THE DIURNAL CARBON BALANCE

It is well established that although photosynthetic carbon assimilation occurs only in the light, growth and maintenance processes requiring carbon occur throughout the day–night cycle (Walter *et al.* 2002; Reddy *et al.* 2004; Walter & Schurr 2005; Nozue & Maloof 2006). Carbon is available at night because the rate of assimilation in the light is sufficient to support not only the immediate demand for growth but also the accumulation of storage compounds in the leaf, which are then mobilized to provide carbon for growth during the night. In *Arabidopsis* (see e.g. Caspar, Huber & Somerville 1985; Gibon *et al.* 2004a; Lu, Gehan & Sharkey 2005) and many other plant species (Geiger & Servaites 1994; Geiger, Servaites & Fuchs 2000), the immediate products of photosynthetic carbon assimilation in the light are partitioned between sucrose – immediately available for growth – and starch, which accumulates in the leaf through the day. At night, the starch is degraded to produce sucrose (Fig. 1). Starch is almost but not totally remobilized by the end of the night in plants growing with an adequate level of nutrients and favourable temperature. This basic pattern is echoed across many different groups of plants. In many grasses, for example, photosynthetic carbon assimilation during the day produces both sucrose for export and sucrose and fructans that are stored in the vacuoles of leaf cells during the day – these stores provide carbon for continued growth at night (Gordon *et al.* 1980). Because far less

is known about fructan- and sucrose-storing leaves than starch-storing leaves, this review concentrates on the latter, focusing largely on *Arabidopsis*.

The apparently simple pattern of carbon assimilation, storage and utilization over 24 h hides a wealth of complex and subtle flexibility and control. Firstly, it is obvious that carbon utilization for growth is not simply tuned to the immediate supply of assimilated carbon. Newly assimilated carbon provides both for the immediate demand and – via the accumulation of storage compounds – for an 'anticipated' demand during the following night. This implies that the rate of starch synthesis in the day is set by mechanisms that anticipate the amount of carbon required in the night. Secondly, the rate of starch degradation at night is essentially linear, and leads to almost complete utilization of the starch supply precisely at the end of the night. This implies that the rate of degradation is set by mechanisms that 'measure' the amount of starch in the leaf at the end of the day, and anticipate the length of the night, in order to maintain a constant supply of carbon from starch through the night. Thirdly, numerous studies show that changes in environmental conditions result in precise adjustments of assimilation and storage that serve to maintain this basic pattern. For example, a change in day length results in alterations in both the partitioning of photoassimilate between starch and sucrose during the day and the rate of starch degradation at night. The rate of starch synthesis is inversely related to day length so that the proportion of photoassimilate set aside for use at night is greater the longer the night is (Chatterton & Silvius 1979, 1980, 1981; Jablonski & Geiger 1987; Lorenzen & Ewing 1992; Matt *et al.* 1998; Gibon *et al.* 2004a). Thus, soybean plants grown with a 14 h light period partitioned 60% of their photoassimilate into starch whereas plants grown with a 7 h light period partitioned 90% of their photoassimilate into starch. *Arabidopsis* plants in a 6 h day accumulated 80% as much starch as in a 12 h day (Fig. 1). The rate of starch degradation at night is adjusted so that it continues to allow for the almost complete utilization of the starch supply precisely at the end of the night. *Arabidopsis* plants can adjust in this way to a remarkable range of day lengths: research in our labs shows that adjustment is possible under day lengths as short as 4 h. Adjustment is also seen in response to changes in light level. In soybean, partitioning of assimilate into starch during the day and the rate of starch mobilization at night both increase with increasing daytime light levels (Mullen & Koller 1988). Fourthly, genotypes vary in the tuning of their starch turnover. In a survey of 24 genotypically diverse *Arabidopsis* accessions, Cross *et al.* (2006) found that accessions that grew most quickly tended to accumulate smaller amounts of starch; in particular, they had lower levels of starch and sugars at the end of the night. This indicates that faster-growing genotypes operate in a less conservative manner, diverting a slightly larger proportion of the newly assimilated carbon into sucrose export and retaining less as starch to act as a reserve or buffer against changes in environmental conditions.

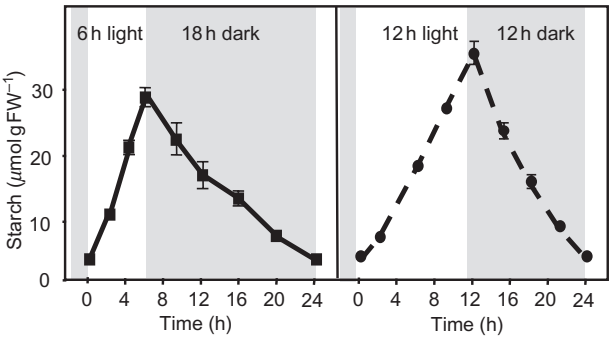

Figure 1. Diurnal changes of leaf starch content in rosettes of 5-week-old *Arabidopsis* (Col-0) plants. Plants were grown in a 6 h/18 h light–dark cycle (left) or a 12 h/12 h light–dark cycle (right). The data are from Gibon *et al.* (2004a). FW, fresh weight.

What is the function of this finely tuned pattern of assimilation, storage and utilization? We propose that it is of fundamental importance both in maintaining an appropriate carbon balance for sustained growth over a day–night cycle, and in establishing a new carbon balance and growth rate in response to alterations in carbon availability that are imposed by environmental perturbations. Evidence for this comes from examination of the effects of environmental perturbations that disrupt the pattern. In *Arabidopsis* growing in a 12 h light/12 h dark cycle, imposition of an extra 4 h of darkness at the end of the night – that is, continued darkness after the starch reserves have been used up – results in a complete cessation of root growth. Growth does not resume until several hours into the start of the next light period, resulting in a large accumulation of carbohydrate because the newly assimilated carbon is not used for growth (Gibon *et al.* 2004a; Yazdanbakhsh, Fisahn & Stitt unpublished data). If this switch to a 16 h dark/8 h light cycle is maintained for a few days, the rates of starch synthesis and degradation change and a new diurnal carbon balance is achieved, with a new, lower rate of growth and no cessation of root growth at night. Similarly, in maize growing in a 15 h light/9 h dark cycle, imposition of an additional 8 h of darkness leads to a complete inhibition of secondary and near-complete inhibition of primary root growth (Brouquisse, Gaudillière & Raymond 1998). An adjustment of diurnal carbon balance and a reduction in growth rate also occur when plants are subjected to less severe reductions in carbon availability, for example, reduced levels of light or of carbon dioxide during the day (see earlier discussion).

Our interpretation of these observations is as follows. A sudden, unprecedented depletion of carbohydrates (a condition we will call 'carbon starvation') sends signals to growth processes that trigger the cessation of growth on a very short timescale. Repetitions of the starvation condition, or sustained, less-severe episodes of altered carbon availability, trigger adjustments that optimize the use of available carbon so that carbon starvation and cessation of growth are avoided. Adjustments include a higher rate of starch synthesis during the day, an adjustment of the rate

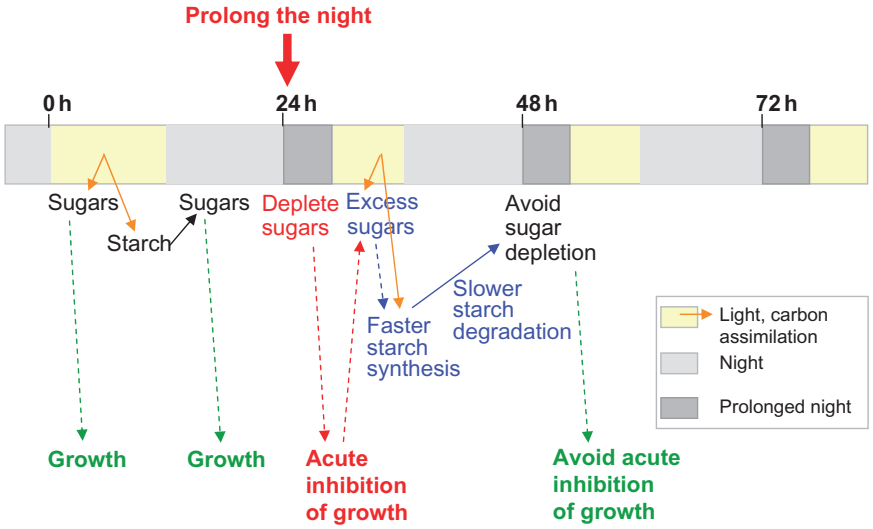

Figure 2. Schematic representation of the relation between carbon fixation, starch turnover and growth in response to prolongation of the night. In a regular light/dark cycle (exemplified here on the left-hand side of the scheme by a 12 h light/12 h dark cycle), starch reserves last until the end of the night, allowing a continuous supply of sugars. Growth continues through the day and night. When a lengthened night is imposed, starch is exhausted before the end of this extended dark period. This leads to acute depletion of sugars and an inhibition of growth by the end of the extended dark period. This inhibition is not reversed for several hours when the plants are re-illuminated. Newly assimilated carbon accumulates as sugars, because they are not being used for growth. High levels of sugar stimulate starch synthesis. During the following night, this stimulation together with a slower rate of starch degradation allows starch reserves to last until the end of the extended dark period, thus avoiding depletion of sugars and an acute inhibition of growth. This effect is accompanied over a period of time by a down-regulation of the growth rate, in accord with the reduced overall availability of carbon in the new long-night regime.

of starch mobilization through the night so that it is linear and precisely consumes all of the starch synthesized during the day, and a decreased rate of growth (Fig. 2).

Following from these interpretations, we propose that plants respond in two distinct ways to changes in carbon availability. An '*acute*' *response* is seen in conditions of sudden carbon starvation. Growth stops abruptly, and is not resumed for several hours after the relief of starvation. '*Acclimatory*' *responses* tune the balance between supply and demand to optimize the capacity for sustained growth. We envisage that these responses operate during normal day–night cycles to ensure that carbon starvation does not occur, and hence that growth is sustained. They coordinate the partitioning of the products of photosynthetic carbon assimilation during the day, the rate of utilization of starch at night and the rate of growth. Acclimatory responses also allow adjustments on a longer timescale to environmental changes that affect carbon availability – we envisage, for example, that acclimatory responses adjust growth and the carbon balance as the plant experiences seasonal changes in light level, day length, temperature and water availability.

This concept is supported by studies of mutant plants that are unable to synthesize starch. Mutants of *Arabidopsis* and tobacco that lack plastidial phosphoglucomutase (PGM), which is necessary for conversion of Calvin cycle intermediates to starch, have reduced growth rates except under very long days or continuous light. Detailed examination of growth patterns under 12 h light/12 h dark cycles shows that in these *pgm* mutants, growth ceases before the end of each night, and is not fully re-established until several hours into the next day. As a result, during the first 6 h of the light period, about 70% of the newly assimilated carbon accumulates as sugars (Gibon *et al.* 2004a; Yazdanbakhsh *et al.* unpublished data). Thus, on a daily basis, these plants without starch reserves are unable to adjust their carbon balance to avoid starvation. Each day, they behave like wild-type plants subjected to a single episode of acute carbon starvation.

Another important function of signalling responses to carbon availability may be to balance growth at different sites and times within the plant. A striking example is provided by reproductive growth. Avoidance of carbon starvation and the associated cessation of growth is particularly important during gamete formation and seed development. In maize and pea, seed abortion in response to a sudden episode of drought or heat stress is often a consequence of carbon starvation rather than a direct effect of these stresses on seed growth (Boyle, Boyer & Morgan 1991; Guilioni, Wery & Lecoeur 2003; McLaughlin & Boyer 2004a,b; Mäkelä, McLaughlin & Boyer 2005). This 'acute' response could have an adaptive value. Relatively few resources are invested during the formation of gametes or the first stages of seed development, compared to the amounts required during seed fill and maturation. Abortion of gametes or very young seeds would be a way of reducing carbon demand in the future, which will make more carbon available to complete the development of seeds that have already been established. This is illustrated in the

experiment shown in Fig. 3, in which a flowering *Arabidopsis* plant was subjected to 1 d of darkness, and then returned to a light/dark cycle. This treatment leads to abortion of flowers and very young seeds, leaving a bare segment on the floral stem. Seeds which were already established at this time recovered and continued to grow, shown by the fully developed siliques below the bare patch. The floral meristem also survived, allowing the formation of new flowers and the production of normal siliques above the bare patch. Thus, the ability to adjust patterns of assimilation, storage and utilization of carbon in response to changes in the environment may determine not only biomass production but also fitness in terms of reproductive success. Incidentally, this experiment also illustrates that the severe yield losses associated with periods of unfavourable conditions in some crops are partly a consequence of the determinant flowering response, which has been introduced by breeding.

Even more complicated interactions may occur during vegetative growth, when the carbon supply would interact with other signals including nutrients and light to determine the overall rate of growth and the sites at which growth occurs, and even to influence developmental events. For example, sugars exert a differential effect on petiole extension and leaf blade expansion, which is modified by the light regime in a phytochrome and cryptochrome-dependent manner (Kozuka *et al.* 2005). The possibility of systemic signalling of environmental cues between different parts of the plant, for example, mature and young growing leaves, introduces an additional level of complexity (Lake *et al.* 2001; Weaver & Amasino 2001; Coupe *et al.* 2006). There is some evidence that sugars may be one component of these complex responses (Lake, Woodward & Quick 2002). In the succeeding sections, we will concentrate on the contribution of sugar signalling to the coordination of carbon storage and growth during the diurnal cycle, because this provides a relatively well-defined experimental system in which to study these complex interactions.

Figure 3. Response of the floral shoot of *Arabidopsis thaliana* to 1 d of continuous darkness. *Arabidopsis* plants growing in a 12 h light/12 h dark cycle were subjected at the end of the night to a 24 h prolongation of the night, and then returned to a 12 h light/12 h dark cycle. The photograph was taken 2 weeks later. Flowers and very young siliques aborted during the 24 h extension of the night, resulting in a bare section of the stem. Below this region, the older siliques survived and continued to grow. The meristem survived, leading to formation of flowers and siliques above the bare patch (Bläsing & Stitt unpublished data).

THE ROLE OF SUGAR SENSING AND SIGNALLING

Both the acute and the acclimatory responses require mechanisms that sense the level of carbon availability in the plant. Such mechanisms would detect a change in a signal metabolite that reflects carbon availability, through binding of the metabolite to a sensor molecule. Binding would trigger a cascade of downstream events (a signalling pathway) culminating in the changes in growth and carbon balance seen in the acute and acclimatory responses. We can anticipate that there will be multiple sensing systems, and that these will feed into a complex signalling network in which they interact with further inputs from, for example, the circadian clock, nutrients, light and the developmental stage.

The most direct candidates for signal metabolites are sugars, including sucrose, glucose and fructose. By definition, low carbon availability equates to low levels of these sugars. There is abundant evidence from targeted analyses (see e.g. Sheen 1990; Koch 1996, 2004) and later from global studies with arrays (Contento, Kim & Bassham 2004; Price *et al.* 2004; Thimm *et al.* 2004; Thum *et al.* 2004; Bläsing *et al.* 2005; Li *et al.* 2006; Osuna *et al.* 2007) that expression of numerous plant genes responds to exogenously supplied sugars. Many of these genes encode enzymes of photosynthesis, sugar and starch metabolism (Smith *et al.* 2004), hence various feed-forward and feedback systems linking sugar availability with the capacity of the plant to assimilate, store, mobilize and utilize carbon have been proposed (Koch 1996; Stitt & Krapp 1999; Paul & Foyer 2001). The nature of the sensor(s) and signalling pathways for sugar levels has been the subject of intense research interest (Smeekens 2000; Koch 2004; Rolland, Baena-Gonzalez & Sheen 2006). Several different approaches have been adopted.

Genes putatively encoding sensors or downstream components of the signalling pathways have been identified in mutant screens in which phenotype or reporter gene responses to added sugars have been used in various ways to select mutant plants with altered responses. Mutants identified in this way include plants insensitive or hypersensitive to the inhibitory effects of high level of exogenous

sugar during germination (e.g. *glucose-insensitive* or *gin* mutants, and *sucrose insensitive* or *sis* mutants, and *glucose supersensitive* or *gss* mutants; Cheng *et al.* 2002; Moore *et al.* 2003; Yanagisawa, Yoo & Sheen 2003; Rolland & Sheen 2005), and plants showing increased or decreased activity in response to sugar of the highly sugar-inducible promoter of *APL3*, a gene encoding a large subunit of ADPglucose pyrophosphorylase, termed *impaired sugar induction* or *isi* mutants and *high sugar response* or *hsr* mutants (Rook *et al.* 2001, 2006a; Rook & Bevan 2003; Baier *et al.* 2004). Components of sugar-signalling pathways have also been sought via reverse genetics, using information on genes involved in this process in yeast to identify candidate genes in *Arabidopsis* (see e.g. Halford & Paul 2003; Francis & Halford 2006; Rolland *et al.* 2006). Sugar analogues that differ from endogenous sugars in uptake, transport or metabolism have been used to define sites of sugar sensing in various plants (Sheen 1990; Yu 1999).

The extensive and complex literature on sugar sensing and signalling has been comprehensively reviewed (Halford & Paul 2003; Rook & Bevan 2003; Koch 2004; Rolland *et al.* 2006; Rook *et al.* 2006b). The following major points have emerged. Firstly, there is more than one sensing mechanism for sugars. Elegant forward and reverse genetic approaches have identified the enzyme hexokinase as a site of glucose sensing in *Arabidopsis* (Moore *et al.* 2003), but there are at least two other glucose-sensing systems – not yet identified at a molecular level – that are independent of hexokinase (Sheen 1990; Xiao, Sheen & Jang 2000; Ho *et al.* 2001; Moore *et al.* 2003), as well as a distinct sucrose-sensing pathway (Wiese *et al.* 2004, 2005). Separate glucose-sensing and sucrose-sensing systems have been shown to exist in potato (Tiessen *et al.* 2003). Global transcript profiling reveals marked differences in the response to sucrose and glucose (Osuna *et al.* 2007; Pradesh & Lunn unpublished data). Secondly, the sugar sensors feed into a complex and interlinked network of signalling pathways. Many mutants identified by their abnormal response to exogenous sugars, and some of the *isi* mutants, prove to carry mutations in genes previously shown to be necessary for normal responses to the hormones abscisic acid and ethylene (Zhou *et al.* 1998; Rook *et al.* 2001, 2006a; Cheng *et al.* 2002; Leon & Sheen 2003; Yanagisawa *et al.* 2003; Gibson 2005; Li *et al.* 2006). However, some *isi* and *hsr* mutants appear to act independently of these hormones (Rook *et al.* 2001, 2006a; Rook & Bevan 2003; Baier *et al.* 2004). Thirdly, the sugar-signalling pathways of plants bear some resemblance to those in yeast. The sucrose non-fermenting (*SNF*) genes of yeast encode components of pathways that link sugar levels to the expression of genes and activities of enzymes of primary metabolism. Glucose-signalling pathways in yeast also involve a heterotrimeric G-protein complex (Lemaire *et al.* 2004). Coordination of carbon supply and utilization in yeast requires the presence of the signalling metabolite trehalose 6-phosphate and the enzyme responsible for its synthesis, trehalose 6-phosphate synthase (TPS) (Hohmann *et al.* 1996). In plants, reverse genetic approaches show that a homolog of the yeast SNF1, the protein kinase SNRK1, is involved in regulating gene expression and enzyme activity in primary metabolism, as part of a sugar-signalling pathway (Halford *et al.* 2003; Francis & Halford 2006). Plants lacking a component of a putative G-protein complex (GPA1) display altered sensitivity to exogenous glucose (Huang *et al.* 2006). The evidence for a central role for trehalose 6-phosphate in sugar signalling in plants (Rolland *et al.* 2006; Lunn 2007) is discussed in more detail later. Fourthly, sugars can potentially act at multiple levels to bring about changes in the metabolic capacity of the plant. The main research focus has been on the impact of exogenous sugars on gene expression, but exogenous sugars have also been shown to act selectively on RNA (Pradesh & Lunn unpublished data) and protein stability (Weiner & Kaiser 1999; Gibon *et al.* 2004b; Parrott *et al.* 2005; Monteoliva *et al.* 2006), on translation (Price *et al.* 2004; Wiese *et al.* 2004, 2005) and on post-translational modification of enzymes (Cotelle *et al.* 2000; Tiessen *et al.* 2002, 2003; Francis & Halford 2006). It is likely that the importance of these various post-transcriptional and post-translational regulatory mechanisms is seriously underestimated, because they are at present far more difficult to study than changes of transcript levels.

Although direct sensing of sugar levels may well be involved in the acute and acclimatory responses, it seems likely that there will be multiple inputs to the signalling pathways, not all of them sugars. Arguably, an ideal signal metabolite is not directly involved in the process to which a response occurs, but is present at low concentrations that can change dramatically and independently of the process concerned. A good example in plants of such a signalling metabolite is fructose 2,6-bisphosphate (Stitt 1990). The concentration of this signal metabolite is regulated by altering the activities of the enzymes that synthesize and degrade it; this involves modulation by metabolites like 3-phosphoglycerate, phosphate and fructose-6-P (Stitt 1990). It seems increasingly likely that trehalose 6-phosphate may fulfil an analogous role in modulating responses to falling carbon availability. Trehalose 6-P is present in miniscule amounts in higher plants, but its levels rise dramatically in response to carbon starvation (Lunn *et al.* 2006). It is synthesized from glucose 6-phosphate and UDP-glucose via TPS and degraded via trehalose phosphate phosphatase (TPP) (Cabib & Leloir 1958; Lunn 2007). The general importance of trehalose metabolism in modulation of primary metabolism, growth and development is illustrated by the dramatic phenotypes of mutants lacking particular TPS and TPPs. *tps1* knockout mutations are embryo lethal in *Arabidopsis* (Eastmond *et al.* 2002), and loss of a TPP from maize changes the branching pattern of the male inflorescence (the *ramosa3* mutant: Satoh-Nagasawa *et al.* 2006). Modulation of trehalose 6-P levels in *Arabidopsis* plants through introduction of heterologous trehalose 6-P synthesizing and degrading enzymes also affects both growth habit and starch accumulation (Schluepmann *et al.* 2003, 2004). The highly pleiotropic phenotypes in these mutants, however, mean that further

research is needed to understand the precise function of trehalose 6-P (see further discussion).

If major metabolizable sugars and compounds derived from them (such as trehalose 6-P) prove to be important classes of signal metabolite in the acute and acclimatory responses, we expect that at least some of the sensors will be proteins related to those directly involved in sugar metabolism. The only widely accepted example of a sugar sensor thus far described is hexokinase, the enzyme that phosphorylates glucose and fructose. Loss of a specific isoform of hexokinase AtHXK1 affects plant growth and development as well as the response of seedlings to exogenous glucose and light, yet mutant plants (*gin2* mutants) are not defective in the conversion of hexoses to hexose phosphates (Moore *et al.* 2003). They retain 50% of the wild-type hexokinase activity (*Arabidopsis* has at least three functional isoforms of hexokinase) and have normal levels of sugar phosphates. The mutant phenotype can be complemented by expression of forms of HKX1 that cannot catalyse the transfer of phosphate groups to glucose and fructose, implying that the function of HKX1 as a sugar sensor relies on its ability to recognize hexoses and not on its catalytic capacity (Moore *et al.* 2003). It has recently been shown that a small proportion of the protein of HXK1 is present in the nucleus, where it forms a regulatory complex with a subunit of the vacuolar ATPase (VHA-B1) and a subunit of the proteosome (RPT-5B) (Cho, Yoo & Sheen 2006). These authors present evidence that one of the targets of this complex is a member of the *CAB* gene family, encoding a photosynthetic protein that is known to be repressed by glucose. They propose that HXK1 may modulate the binding of transcription factors to the VHA-B1/RPT-5B complex in a glucose-dependent manner. Thus, there seems to have been a differentiation of function within the hexokinase family, with at least one member not only catalysing an important metabolic reaction but also acting as a sensor for levels of the substrate of that reaction.

Analogous situations have been proposed for sucrose and for trehalose 6-P sensing. Use of non-transported sucrose analogues shows that sucrose sensing can occur at the plasma membrane. It has been suggested that a member of the sucrose transporter family, SUT2, might be the sensor (Barker *et al.* 2000) because this protein does not show transport activity when it is heterologously overexpressed in yeast cells, and possesses a cytoplasmic loop that may be analogous to loops in yeast glucose sensors that are related to glucose transporters. TPS and TPP families in *Arabidopsis* consist of 11 and 10 members, respectively (Lunn 2007). The functions of most of these enzymes are not yet known, and it is entirely feasible that some act as sensors that link changes in major sugars to changes in trehalose 6-P or trigger signalling pathways when trehalose 6-P levels change. It is already clear that some of these enzymes are subject to complex post-translational modification (Glinski & Weckwerth 2005; Harthill *et al.* 2006). It will be interesting to discover whether large gene families encoding other central enzymes of primary metabolism – for example, beta-amylases and neutral invertases, both of which exist as nine-member families in *Arabidopsis* – include some sensor proteins. It is also intriguing that some individual members of the large multi-enzyme families for enzymes in other central metabolic pathways (e.g. phosphoglycerate mutase) show large changes in expression in response to many different inputs (see e.g. AtGenExpress; http://web.uni-frankfurt.de/fb15/botanik/mcb/AFGN/atgenex.htm).

Research on sensing and signalling systems based on sugars and related metabolites has produced a wealth of candidate components for these systems, and identified some tantalizing glimpses of the complexity of the network, but it is thus far of limited value in understanding acute and acclimatory responses to reduced carbon availability. One limitation is that many studies have focused on responses of seedlings to high levels of sugars, rather than the physiologically more important situation of limiting carbon availability, and few have subjected the mutants to a detailed analysis in more physiological conditions to define the role of the gene. A second problem is that the identification of mutants with impaired sugar responses reveals proteins that are necessary for the response, but does not give information about whether they are directly or indirectly involved, or at what point on the pathway(s) they act. Classic genetic dissection is a powerful tool to analyse linear pathways, but can have limitations when applied to complex and potentially redundant signalling networks. The problem is particularly acute when there is not enough molecular or physiological knowledge to allow further and more specific screens, which address defined parts of the system. The challenge is made apparent by recent microarray experiments in which the response to added sugars of a large proportion of the *Arabidopsis* transcriptome has been examined (Contento *et al.* 2004; Price *et al.* 2004; Thimm *et al.* 2004; Thum *et al.* 2004; Bläsing *et al.* 2005; Li *et al.* 2006; Osuna *et al.* 2007). Such experiments show that levels of thousands of transcripts respond to sugars. A high proportion encodes proteins of unknown function, or proteins likely to be part of signalling pathways not previously implicated in the response to sugars (transcription factors, protein kinases, proteasome components and so on). These results suggest that the components of sugar sensing and signalling pathways identified thus far may represent a small piece of a rather large jigsaw.

Microarray experiments cannot by themselves define sensing and signalling pathways. They do not, for example, allow direct and indirect effects to be distinguished, transcripts for many important regulatory proteins cannot be assayed accurately, and changes in transcript cannot be assumed to result in changes in the levels of the encoded proteins. However, transcriptome analysis does provide a much more open and systems-level view of plant responses than is provided by purely genetic and inhibitor-based approaches. It is currently the broadest and most sensitive means of viewing plant responses available to us. We believe that integrative approaches of this sort are an essential first step towards understanding the acute and acclimatory responses to carbon availability. They also open up the possibility of an iterative strategy that combines in-depth

molecular phenotyping and forward genetics; existing mutants can be analysed in a much wider range of conditions and for a wider range of parameters, and a new generation of forward screens can be devised to obtain more mutants. In the next section, we describe how such integrative approaches can be applied, present the emerging picture of the acute and acclimatory responses, then describe the radically new light these results shed on the way in which the plant maintains a carbon balance appropriate for the level of carbon availability.

'SYSTEMS-LEVEL' CHARACTERIZATION OF THE ACUTE AND ACCLIMATORY RESPONSES TO CARBON AVAILABILITY

There are two main requirements for a systems-level understanding of responses to changing carbon availability. Firstly, responses must be examined at multiple levels. Examination of the transcriptome alone cannot give information about levels of signalling metabolites, changes in metabolism, the carbon balance of the plant, or the rate or quality of growth. Levels of proteins and metabolites, fluxes through pathways of primary metabolism and rates of cell division and expansion are determined by a host of complex post-transcriptional and translational control mechanisms as well as by levels of gene expression (Greenbaum *et al.* 2003; Gibon *et al.* 2004b, 2006; Hirai *et al.* 2004; Lafaye *et al.* 2005; Tohge *et al.* 2005; Fan, Higashi & Lane 2006; Thomas & Ganji 2006). One very relevant illustration of the problem of interpreting changes in the transcriptome is provided by recent studies of transcripts for genes encoding the set of enzymes believed to be responsible for starch degradation. Levels of these transcripts change dramatically on a diurnal basis, and are subject to strong circadian control. Starch degradation in the leaf occurs only at night, and it would be tempting to suggest that changes in the transcriptome drive this diurnal pattern. However, levels of the proteins, and of their extractable activities, show essentially no diurnal or circadian change (Smith *et al.* 2004; Lu *et al.* 2005).

Secondly, direct aspects of the responses must be distinguished from indirect effects of the experimental treatments used to induce changes in carbon availability. This requires careful choice of experimental systems. Earlier, we described two circumstances in which *Arabidopsis* plants undergo carbon starvation: during an extended night, and during the normal night in mutants that lack the capacity for starch synthesis. Both of these circumstances are complex. In both cases, changes due to carbon starvation will be layered onto gene expression and physiological changes due to circadian and other inputs that change during diurnal rhythms. The extended night may – for example – cause abnormal accumulation or depletion of nitrogen as well as carbon metabolites, because these follow strong diurnal patterns (Scheible *et al.* 1997; Matt *et al.* 2001). In the *pgm* mutant, sink as well as source capacity for starch synthesis is compromised (Caspar *et al.* 1985), so patterns of sucrose export from the leaf and signals from sink organs may be abnormal. Further, any signals that are generated during starch degradation will be absent in this mutant. Changes in carbon availability and carbon starvation can also be induced in seedlings grown in liquid culture – but this too is a complex and rather non-physiological situation. The biological system can be simplified by using cell suspensions (e.g. Contento *et al.* 2004), but the physiological state of such cells can be debated, and non-cell-autonomous signals and responses are probably lost.

To investigate responses to changes in carbon availability, high-throughput platforms were used that provide information about global transcript levels, a wide range of metabolites and enzyme activities, and growth parameters. These platforms were applied to many different experimental systems, in which carbon availability is changing. Common features of the systems that are likely to be directly related to carbon availability are then distinguished from indirect and unrelated features of the systems by statistical methods. The high-throughput platforms have been described previously: these include qRT-PCR for transcription factors and other putative regulatory proteins (Czechowski *et al.* 2004, 2005), high-throughput methods for assay of 23 enzymes (Gibon *et al.* 2004b) and over 130 metabolites of primary metabolism (Gibon *et al.* 2006), and the MapMan tool for display and analysis of different types of data set within a common framework centred on metabolism (Thimm *et al.* 2004; Usadel *et al.* 2005, 2006). The main experimental systems used were seedlings grown for 7 d in liquid culture with sucrose, subjected to 2 d in sucrose-free medium, then re-supplied with sucrose (at physiologically meaningful levels: 15 mM or about 0.5%); wild-type plants harvested at different times during a 12 h light/12 h dark cycle, or after transfer to prolonged darkness, or after 4 h illumination in the presence of compensation point concentrations of CO_2; and *pgm* mutant plants harvested at different times during a 12 h light/12 h dark cycle. Next, we summarize the critical features of the acute and acclimatory responses that emerge from these studies. The reader is referred to a more specific review of these experiments (Stitt *et al.* 2007) and to the original papers (Gibon *et al.* 2004a,b, 2006; Thimm *et al.* 2004; Bläsing *et al.* 2005; Osuna *et al.* 2007) for further details. We start by describing the acute response: this is arguably easier to define and study than the acclimatory response.

During an extended night, the available carbon reserves in *Arabidopsis* leaves are very rapidly used up (Fig. 4). This is followed by rapid changes in metabolism and growth (Thimm *et al.* 2004; Usadel *et al.* unpublished data). Within 2–4 h, starch essentially disappears and the levels of soluble sugars collapse. There is a twofold decrease in glucose 6-phosphate, a key intermediate at the interface between sucrose and starch metabolism and respiration. This acute carbon starvation is accompanied by a very rapid cessation of root extension growth, which stops within 2–4 h. Within 4–6 h, there are changes in metabolites consistent with a switch to catabolism of proteins, cell walls and lipids – for example, there are increases in many amino acids, and decreases in some classes of lipids. Catabolism partially replenishes the pools of respiratory intermediates – the

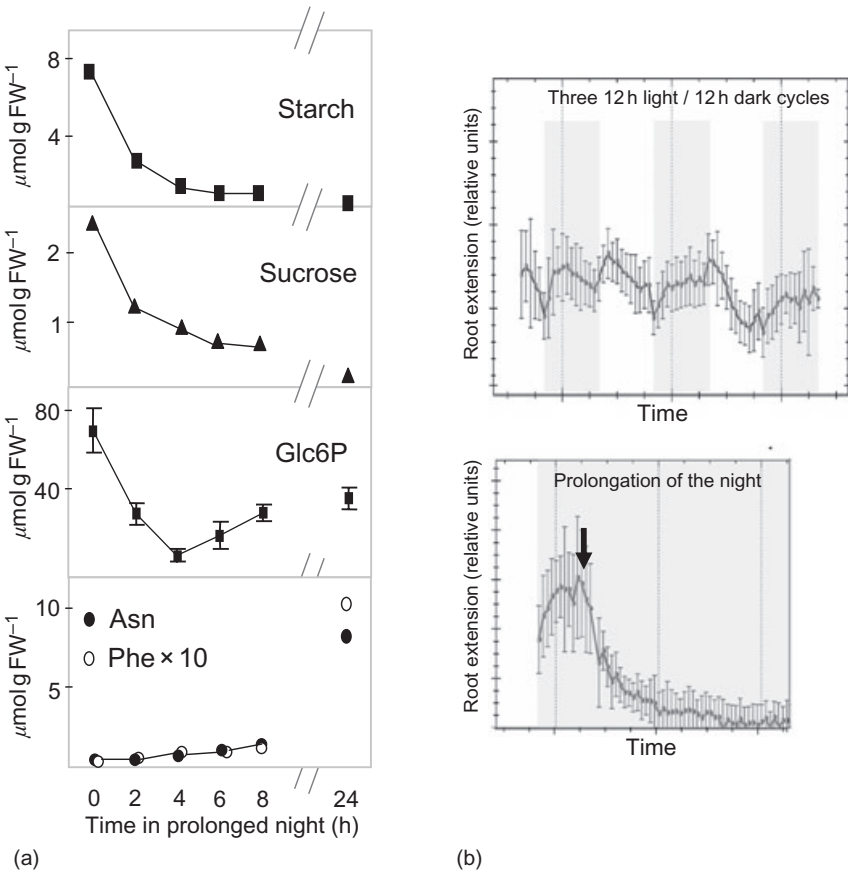

Figure 4. Changes of metabolites and root extension growth in a prolonged night. (a) Changes in metabolites during a 24 h prolongation of the night. Five-week-old plants growing in a 14 h/10 h light–dark cycle were transferred to continuous dark at the end of the night (time 0). Starch content was already low at this point (see Fig. 1). The panels show the time course of the final depletion of starch, the collapse of sucrose, the decrease and partial recovery of glucose 6-phosphate (Glc6P) and, as examples of metabolites formed during catabolism of alternative substrates, the increase of asparagine (Asn) and phenylalanine (Phe) (Gibon & Usadel, unpublished data). (b) The impact on root extension in 9-day-old seedlings growing on vertical agar plates on full nutrient medium without sucrose. Root growth rate was analysed by image analysis, with measurements at 1 h intervals. Each curve shows the mean and SE for at least 10 separate seedlings. The upper panel shows that root growth continues through the light and dark period in wild-type seedlings. The bottom panel shows that root growth is rapidly inhibited after prolongation of the night (Yazdanbakhsh & Fisahn, unpublished data). FW, fresh weight.

level of glucose 6-phosphate recovers to pre-starvation levels by 6 to 8 h – but there is no resumption of growth.

These basic features of the starvation response are seen in *pgm* mutant plants during a normal 12 h night (Gibon *et al.* 2004a). Hexose and sucrose levels fall within 4 h to values comparable with those seen during carbon starvation in a wild-type plant. Extension growth of roots at night is only 10% of that of wild-type plants (Yazdanbakhsh *et al.* unpublished data). These large, coordinated changes serve to emphasize the fine balance between carbon reserves and growth in normal conditions – either a reduction in the capacity for carbon storage or an extension of the night by only a few hours places the plant under a serious carbon deficit that impacts upon its growth rate. A similar sequence of events has been reported during an extended night in maize plants growing in a 15 h light/9 h dark cycle (Brouquisse *et al.* 1998). Imposition of an additional 8 h of darkness leads to a complete inhibition of secondary and near-complete inhibition of primary root growth, induction of protease activities and a perceptible decrease of total protein, and increase of amino acids.

Carbon starvation in wild-type plants during an extended night is accompanied by major changes in the transcriptome. Within 6 h, there are changes in levels of transcripts for hundreds of genes (Thimm *et al.* 2004). Levels of transcripts encoding enzymes involved in synthesis of amino acids, nucleotides, proteins and lipids generally fall, and levels of transcripts encoding enzymes involved in amino acid, cell wall and lipid catabolism generally increase. However, examination of more than 20 enzymes of primary metabolism shows that the rapid changes in transcripts are not reflected in parallel changes in enzyme activities (Gibon *et al.* 2004b). Striking examples are ADPglucose pyrophosphorylase (AGPase; starch synthesis), sucrose phosphate synthase (SPS; sucrose synthesis) and glutamate dehydrogenase (GDH; amino acid metabolism). Transcripts for all of the isoforms of SPS and subunits of AGPase fall within 4–6 h of the onset of an extended night, and those for GDH rise. The activities of these enzymes show essentially no change by 6 h, indeed all three show very little change after a 24 h extension of the night in spite of dramatic changes in transcript levels. Overall, it appears that the metabolic switch to catabolism within 4–6 h of the onset of carbon starvation does not require changes in expression of the genes encoding the enzymes involved. Changes in enzyme activities do occur if starvation continues for more than 2 d, but these are of a much smaller magnitude than the faster changes in the corresponding transcripts. Thus, the response to carbon starvation in terms of metabolic capacity is damped and delayed dramatically relative to the response at the transcript level.

A complementary picture emerges from study of the transcriptome of the *pgm* mutant (Gibon *et al.* 2004a; Bläsing *et al.* 2005; Gonzali *et al.* 2006). At the start of the night, the transcript profile of a *pgm* mutant resembles that of a wild-type plant (Thimm *et al.* 2004; Bläsing *et al.* 2005).

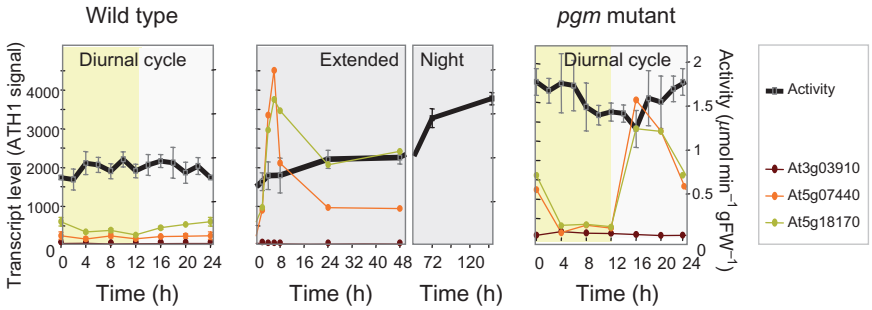

Figure 5. Changes of glutamate dehydrogenase activity and transcripts. Changes are compared during a diurnal light–dark cycle in wild-type plants (Col-0, left panel), during a prolonged night in wild type (central panel, time 0 is the end of the normal 12 h night), and during a diurnal light–dark cycle in the starchless *pgm* mutant (right panel). Activity measured in optimal conditions is shown as a thick black line, and levels of transcripts for *GDH1* (At5g18170), *GDH2* (At5g07440) and *GDH3* (At3g03910) as determined in ATH1 arrays by thinner coloured lines (see legend panel in figure). For the time points from 72 h into a prolonged night, only enzyme activity was measured. All results are the mean and SE of three biological replicates. The data are from Gibon *et al.* (2004b) and Bläsing *et al.* (2005), and the diagram is modified from Stitt *et al.* (2007). FW, fresh weight.

By the end of a normal 12 h night, when the *pgm* mutant is experiencing carbon starvation, its transcript profile more closely resembles that of a wild-type plant after a 4–8 h extension of the night. In contrast, activities of enzymes of primary metabolism in the *pgm* mutant show relatively little diurnal change, and resemble those of a wild-type plant after several days of carbon starvation (Gibon *et al.* 2004b). It appears that the nightly episodes of 'starvation' patterns of gene expression are integrated over time to produce a sustained, systematic shift in enzyme activities. This conclusion was further tested by investigating the metabolite profile, which will integrate changes in the activities of hundreds of enzymes. Sugars show much larger diurnal changes in the *pgm* mutant than in wild-type plants (see earlier discussion). However, most of the other 137 metabolites investigated showed smaller diurnal changes in the *pgm* mutant than in wild-type plants, and the levels in the mutant resembled those found in wild-type plants after several days in the dark (Gibon *et al.* 2006). These results are consistent with the idea that the nightly episodes of carbon starvation in the *pgm* mutant clamp this genotype in a starvation mode.

A comparison of the responses to acute starvation of GDH illustrates these points (Fig. 5). GDH recycles carbon from glutamate that is formed during protein degradation and amino acid catabolism. In wild-type plants, the two major transcripts (*GDH1*, *GDH2*) and GDH activity are low throughout the diurnal cycle. When the night is prolonged, the transcript levels rise sharply during the first 4–6 h, whereas GDH activity rises slowly, over several days. In the *pgm* mutant, there is a repeated peak of *GDH1* and *GDH2* transcript every night. GDH activity shows only small diurnal changes, but is maintained at a twofold high level than in wild-type plants. A further detail in Fig. 5 is that, following the initial rapid rise of *GDH1* and *GDH2* transcript in a prolonged night, the transcript levels decrease about twofold. This adjustment clearly does not require significant changes of GDH expression at the protein level. It occurs in the same time frame as the switch from anabolism to catabolism and the partial recovery of glucose 6-P levels (see earlier discussion; Fig. 4). One explanation for this would be that acute carbon starvation triggers metabolic signals that lead to the change in *GLU1* and *GLU2* transcript levels, and that these signals relax after a new metabolic state has been established by post-translational or other regulatory mechanisms that do not require changes in the levels of GDH protein. This example illustrates that changes of transcripts provide a powerful readout of the operational state of signalling pathways but the interpretation of these changes will often require a wealth of additional information about changes of protein levels, post-translational mechanisms, metabolite levels and fluxes.

To summarize, changes in gene expression occur rapidly in response to starvation, presumably triggered by changing levels of one or several sugars or dedicated signal metabolites. The altered levels of transcripts position the plant to make major changes in metabolic capacity, but change only occurs if the adverse conditions persist. This delayed response ensures that major investment of scarce resources in new enzymatic machinery does not occur in response to short-lived adverse conditions, but is made only in response to sustained starvation.

SIGNALLING MECHANISMS UNDERLYING THE ACUTE AND ACCLIMATORY RESPONSES TO CARBON AVAILABILITY

How is carbon starvation sensed, and what signalling pathways trigger the short-term transcriptional, post-translational and growth responses, and the longer-term adjustments in metabolism and growth? How is information about mid- or longer-term changes in the carbon supply identified and extracted from the 'background noise' caused by short-term and transient changes in the environmental conditions (e.g. fluctuations of the light intensity) and the recurring 24 h light/dark cycle? Important clues about these processes come from our improving

understanding of the acclimatory responses that balance assimilation, partitioning of assimilate into starch, starch mobilization at night, and the rate and pattern of growth of the plants under normal growth conditions. We consider acclimatory responses next, then summarize our view of sensing and signalling events involved in acute and acclimatory responses.

The acclimatory response acts over the diurnal cycle to prevent carbon starvation. Accordingly, in wild-type plants under 12 h light–dark cycles, carbon availability (taken as levels of sucrose and hexoses) and the rate of growth (taken as root extension) show only relatively small diurnal fluctuations (Gibon *et al.* 2004a; Yazdanbakhsh *et al.* unpublished data). Sucrose and hexoses are roughly twofold higher at the end of the day than at the end of the night, for example. Underlying these 'buffered' parameters are huge diurnal changes in the transcriptome of the leaf (Bläsing *et al.* 2005). There are statistically significant diurnal changes in transcript levels for more than 30% of the genes expressed in the rosette. Strong, coordinated patterns of diurnal change occur in transcripts for many enzymes of primary metabolism. A good example is the set of enzymes of starch degradation on the pathway between the starch granule in the chloroplast and the hexose phosphate pool from which sucrose is synthesized in the cytosol (Smith *et al.* 2004; Lu *et al.* 2005). Levels of transcripts for most of these enzymes peak towards the end of the day and fall to low levels at the end of the night (Fig. 6). However, as already discussed for the acute response, these diurnal changes of transcripts are not for the most part reflected in changes in activity or protein levels of the encoded enzymes of primary metabolism (Gibon *et al.* 2004b; Smith *et al.* 2004; Lu *et al.* 2005). The large changes in flux are likely to be brought about by post-translational control mechanisms, rather than by changes in the amounts of enzymes.

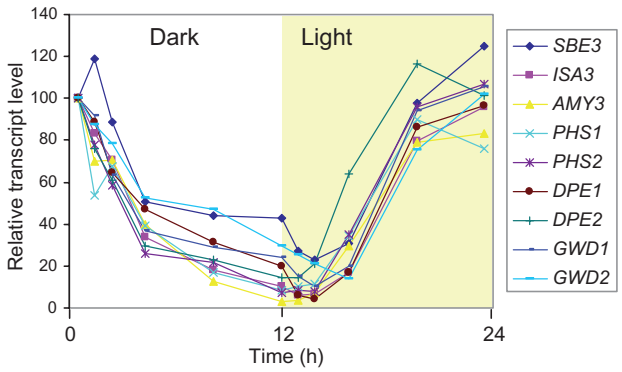

Figure 6. Diurnal pattern of transcript abundance in leaves of 4-week-old plants for genes encoding nine enzymes of starch metabolism. Transcript abundance was determined in ATH1 arrays. *SBE3*, starch-branching enzyme 3 (At2g36390); *ISA3*, isoamylase 3 (At4g09020); *AMY3*, α-amylase 3 (At1g69830); *PHS1*, *PHS2*, glucan phosphorylases (At3g29320, At3g46970); *DPE1*, disproportionating enzyme (At5g64860); *DPE2*, transglucosidase (At2g40840); *GWD1*, *GWD3*, glucan water dikinases (At1g10760, At5g26570). Data are from Smith *et al.* (2004).

What signals drive the large-scale diurnal changes in the transcriptome, and how are they linked to the mechanisms that coordinate carbon balance with growth over the day? Strong candidates as signals are light, the circadian clock and sugars. The expression of large sets of genes responds to light and/or the circadian clock, and these sets of genes overlap extensively with the set undergoing diurnal change. For example, patterns of expression of genes encoding many of the enzymes of starch degradation are under circadian control (Harmer *et al.* 2000; Lu *et al.* 2005). As described earlier, there is a wealth of evidence for the existence of sugar sensing and signalling mechanisms. The correlation between falling sugar levels and rapid, dramatic changes in the transcriptome at the onset of carbon starvation also lends support to the idea that sugar levels could directly influence gene expression in normal growth conditions (Thimm *et al.* 2004; Bläsing *et al.* 2005).

To distinguish between these possible signals, Bläsing *et al.* (2005) and Osuna *et al.* (2007) examined metabolic and transcriptional changes in three different situations in which normal carbon balances and growth rates are being established after a period of carbon starvation: wild-type plants in the light period following an extended night, *pgm* mutants in the light period in normal 12 h light/12 h dark cycles, and seedlings in the period following the re-supply of sucrose after growth in liquid culture in carbon-starved conditions. Sugar levels are increasing in all three systems, but the systems differ with respect to light and circadian signals, enabling the relative importance of sugar levels in driving transcriptional changes to be dissected statistically.

Important features of these data sets are as follows. Following both provision of light and CO_2 to carbon-starved mature plants and provision of sugar to carbon-starved seedlings, there is a large increase in endogenous levels of available carbon (sugars and – for wild-type plants – starch) in the first few hours but no immediate resumption of growth or of biosynthesis of, for example, proteins. Direct measurements of the rate of protein synthesis are still needed, but indirect evidence (including a decrease of amino acid levels and a perceptible increase of total protein) indicates that protein synthesis starts to increase after about 3–8 h (Osuna *et al.* 2007). The significance of this lag between increased carbon availability and its utilization for growth is discussed further in the next section. In wild-type plants, carbon accumulates as both sugars and starch; in the *pgm* mutant, the increase is solely as sugars and the levels of accumulation during the day are up to 10-fold higher than in wild type plants after either a normal night or starvation. Within 4 h of provision of light to carbon-starved mature plants (Bläsing *et al.* 2005) and provision of sugar to carbon-starved seedlings (Osuna *et al.* 2007), there is a coordinated induction of expression of hundreds of genes involved in biosynthesis and energy production, including genes encoding enzymes of carbohydrate, amino acid, DNA and protein synthesis, and protein folding. There is a decrease in levels of transcripts encoding enzymes of catabolism of lipids and amino acids. Large numbers of transcripts change in the same direction in all three systems,

enabling candidate lists for sugar-activated and sugar-repressed genes to be drawn up.

Do the genes that respond to increases in carbon availability after starvation also change in a normal day–night cycle? Inspection of the diurnal patterns of change of sugar-responsive genes in relation to endogenous sugar levels in rosettes showed that this is the case (Bläsing *et al.* 2005). About half of the genes showed diurnal patterns consistent with regulation by endogenous sugar levels in a normal day–night cycle. Transcript levels for genes listed as sugar inducible rose during the day and fell at night; those for genes listed as sugar repressible had the opposite pattern. Independent evidence for correlations between sugar levels and gene expression patterns was obtained by application of principal component analysis to transcriptome data gathered at multiple points across a normal light–dark cycle. The first component accounted for 40% of the diurnal variation. It separated samples from the end of the light period from samples from the end of the dark period. Most sugar-responsive genes had high weightings in this component – in other words, the separation was strongly influenced by the patterns of expression of genes that are either induced or repressed by sugars. Genes known to have strong circadian regulation of expression also had high weightings, but genes induced/repressed by light, water deficit and nitrogen had much lower weightings. Overall, these analyses suggested that sugar levels and the circadian clock are major inputs that drive diurnal changes in transcript levels in *Arabidopsis* leaves.

Further evidence for the importance of sugar levels in influencing the transcriptome comes from comparison of the relationship between sugar levels and transcriptional changes in the *pgm* mutant. As we describe earlier, this mutant goes through a feast-to-famine cycle on a daily basis. Following starvation in the latter part of the night, when sugar levels are well below those encountered by wild-type plants in normal day–night cycles, sugar levels rise at the start of the day to levels several times higher than those seen in wild-type plants (Gibon *et al.* 2004a, 2006). If endogenous sugar levels are important in driving changes in transcript levels, the *pgm* mutant would be expected to display much larger diurnal changes in levels of many sugar-responsive transcripts than are seen in wild-type plants. This proves to be the case. There are accentuated diurnal changes in more than 4000 transcripts in *pgm* plants, across most sectors of metabolism and cellular growth processes (Bläsing *et al.* 2005). In particular, most of the sugar-responsive genes show greater diurnal changes in *pgm* than in wild-type plants (see also Gonzali *et al.* 2006).

Comparison of wild-type and *pgm* mutant transcriptomes through a day–night cycle also reveals that the transcriptome is much more sensitive to low and falling levels of sugars than to high and rising levels (Bläsing *et al.* 2005). The global transcript profiles of the two sorts of plants are much more similar during the day, when levels of sugars are maximal in both sorts of plants, than during the night when sugar levels are low. Further, the difference between the levels of transcripts in *pgm* and wild-type plants in the dark is strikingly similar to the response to carbon depletion; transcripts of genes that are induced by sugars are lower and transcripts of genes that are repressed by sugars are higher during the night in *pgm* than in wild-type plants (Bläsing *et al.* 2005). This contrasts with the situation in the light, when transcript levels in *pgm* and wild-type plants are rather similar, even though sugars are much higher in *pgm*. The fact that the transcriptional changes *in vivo* in *pgm* occur at the time when sugars are depleted, rather than when they are higher, re-emphasizes the importance of studying how low sugar levels are sensed, rather than studying sensing of high levels of sugars.

What function can be attributed to the large diurnal changes in transcripts for enzymes of primary metabolism, given that the enzymes undergo little or no diurnal change? We believe that they are central to the acclimatory response, allowing adjustments to the carbon balance in response to sustained environmental change. Environmental changes that alter carbon availability during a single day will alter the precise pattern of diurnal transcriptional change, but this will not be translated into alterations in the metabolic capacity of the leaf. If the environmental change is sustained over a longer period, the repeated shift in the diurnal changes of transcript levels will lead to a gradual adjustment in enzyme levels towards a new carbon balance optimal for sustained growth in the new conditions. For example, when *Arabidopsis* is grown in very short days, the activities of enzymes like GDH and invertase rise relative to activities of enzymes from glycolysis and starch and sucrose synthesis (Gibon & Stitt unpublished data). This is reminiscent of the situation after several days of darkness, or in the *pgm* mutant. However, it should be stressed that many signalling events operate at the post-transcriptional and post-translational level; dissection of the biological function of changes of transcripts for signalling pathway components will require quantitative information about the levels of the encoded protein and discovery of the ways in which the turnover and biological activity of the protein are regulated. This is nicely illustrated by a response that has already been intensively researched: the photoperiod regulation of floral induction. The CONSTANS-1 protein promotes flowering by activating transcription of FT, a floral activator. Circadian regulation of *CONSTANS-1* transcription leads to an increase of CONSTANS-1 protein in the middle of each 24 h cycle (Suarez-Lopez *et al.* 2001). However, CONSTANS-1 protein is subject to rapid turnover, being stabilized in the light but rapidly degraded in the dark (Valverde *et al.* 2004). When days are long, CONSTANS-1 protein can accumulate over several hours, but when days are short, the newly synthesized CONSTANS-1 protein is rapidly degraded. This interplay between transcription and protein stability thus allows CONSTANS-1 to promote flowering in long-day but not in short-day conditions.

So far we have considered acute and acclimatory response in terms of what is sensed. We have shown that carbon availability is of paramount importance, and that changes in levels of sucrose and hexoses are directly linked

to transcriptional changes. Sudden declines in sugar content to very low levels trigger an acute response, less strong declines provide signals that drive acclimatory responses. We now consider briefly the nature of signalling pathways that link direct or indirect sensing of sugar levels to responses in terms of changes in transcript levels, growth and enzyme activities.

The lists of sugar-responsive genes from experiments in which carbon is re-supplied to starved plants include not only those encoding enzymes of primary metabolism but also genes encoding proteins that could be key players in signalling and signal transduction. In experiments with seedlings, levels of transcripts encoding many such proteins respond very rapidly (within 30 min) to re-supply of carbon (Osuna *et al.* 2007). They include 20 transcription factors, 15 proteins involved in protein turnover via the proteasome and four trehalose phosphate synthases. Within 3 h, there are changes in levels of many more transcripts encoding putative regulatory proteins including over 60 protein kinases and several protein phosphatases, receptor kinases and wall-associated kinases. Importantly, many of these transcripts also undergo changes in leaves of mature plants. In wild-type plants under normal day–night cycles, the changes are not synchronous but phased, implying that different sets of genes encoding regulatory proteins respond to different levels of sugars (Osuna *et al.* 2007).

The roles of most of these regulators are not yet known. They may be involved in post-transcriptional or post-translational regulation of metabolic pathways crucial to the diurnal carbon balance of the leaf. The roles of possible regulators in controlling flux through pathways of starch synthesis and degradation are discussed later. Others may be part of the medium-term acclimatory response, adjusting levels of metabolic enzymes in response to sustained shifts in the diurnal patterns of transcript abundance. Reverse-genetic approaches to uncover functions are underway. It will also be important to understand whether the proteins encoded by these genes undergo large diurnal changes in abundance or whether the response of proteins levels is strongly damped as it is for metabolic enzymes.

In the next two sections, we consider in more detail emerging information about the regulation of two central aspects of the carbon balance of the plant, the control of partitioning of newly assimilated carbon into starch during the day and the control of mobilization of carbon from starch at night.

REGULATION OF STARCH SYNTHESIS IN LEAVES

Acclimatory responses that adjust carbon supply to demand over the diurnal cycle require complex control of the partitioning of assimilate between sucrose and starch. Partitioning must provide sufficient sucrose for the immediate demands of the plant during the day, and sufficient starch to meet 'anticipated' demands during the following night, and must do so in a manner responsive to changes in day length and other factors that alter the amount of carbon fixed in the 24 h light–dark cycle. Until relatively recently, the need for and importance of these longer-term requirements for the control of partitioning were not appreciated, and starch synthesis was regarded simply as an 'overflow' mechanism that corrects short-term imbalances between carbon supply and demand in the leaf during the day. The fact that starch also provides a supply of carbon during the night was regarded as a fortuitous secondary consequence of this overflow mechanism. Research during the 1980s revealed that starch synthesis in the chloroplast is activated by accumulation of sucrose or intermediates of its synthesis in the cytosol. Briefly, accumulation of sucrose or hexose phosphates in the cytosol reduces the rate at which carbon in the form of triose phosphates can be exported from the chloroplast via the triose phosphate-phosphate transporter (Stitt, Huber & Kerr 1987; Stitt 1990; Huber & Huber 1996). This leads to an accumulation of 3-phosphoglycerate (3-PGA), which is the initial product of photosynthetic carbon assimilation, and a depletion of phosphate in the chloroplast. The decrease in the phosphate:3-PGA ratio activates the first committed enzyme of starch synthesis, ADPglucose pyrophosphorylase (AGPase) (Preiss 1988; Ballicora *et al.* 2000) leading to increased starch synthesis.

Although this model is undoubtedly correct in many respects, it provides only short-term mechanisms for control of starch synthesis. Many observations of the rates of starch and sucrose synthesis in leaves over a whole light period reveal a complex relationship that cannot be explained simply on the basis of an overflow model. In particular, the overflow model fails to explain how partitioning of carbon into starch is matched to the 'anticipated' length of the night, and how partitioning is adjusted in response to changes in day length (see previous discussion for references). It also fails to explain why phosphorylated intermediates often remain unaltered or decrease when starch synthesis is stimulated by addition of sucrose, or by inhibition of phloem export (for a review, see Stitt 1991; also Krapp & Stitt 1995; Geiger, Geigenberger & Stitt 1998).

Important clues about further mechanisms that may be involved in the control of starch synthesis came from the discovery that AGPase is also sensitive to redox state. The recombinant enzyme from potato was shown to have a disulphide bridge linking the two small subunits of the enzyme (a tetramer of two large and two small subunits) under oxidizing conditions (Fu *et al.* 1998; Ballicora, Iglesias & Preiss 2004). Addition of dithiothreitol reduced this S–S bond and substantially increased the activity of the enzyme lowering its affinity for its substrates and increasing its sensitivity to activation by 3-PGA (Tiessen *et al.* 2002, 2003). Subsequent studies of the redox state of the enzyme *in planta* show that it is reduced – and hence activated – in response to light and to exogenous sugars (Tiessen *et al.* 2002, 2003; Hendriks *et al.* 2003), and under conditions when sugars are increasing *in vivo* (see further discussion). It seems likely that redox regulation of AGPase plays a major role in the control of starch synthesis *in vivo*. This mechanism permits adjustments to the rate of starch synthesis independently of changes in levels of 3-PGA

and phosphate in the chloroplast and phosphorylated intermediates in the cytosol.

Insights into the signalling pathways that link sugar levels to redox activation of AGPase have come from the experiments designed to elucidate plant responses to changing carbon availability. As we described earlier, there is a very rapid accumulation of sugar and – in wild-type plants – starch, following provision of light to carbon-starved mature plants or provision of sugar to carbon-starved seedlings. These increases are accompanied by rapid redox activation of AGPase (Gibon *et al.* 2004a; Lunn *et al.* 2006): it seems likely that this activation is in response to the rise in sugar levels and is responsible for the high rate of starch synthesis.

Several lines of evidence suggest that changes in trehalose 6-P levels during starvation and recovery are part of the signalling pathway linking sugar levels to redox activation of AGPase. Indirect evidence for this hypothesis is provided by transgenic plants overexpressing either *TPS* or *TPP*: redox activation of AGPase is elevated in the former and decreased in the latter (Kolbe *et al.* 2005). Trehalose 6-P levels correlate with AGPase activation in several different experimental systems (Lunn *et al.* 2006). In wild-type plants, trehalose 6-P levels fall threefold during an extended night and rise 12-fold upon re-illumination. Similar falls and 'overshoot' recoveries of trehalose 6-P levels are seen during carbon starvation and recovery in seedlings, and in *pgm* mutant plants. An important step towards understanding events downstream of trehalose 6-P was provided by the observation that treatment of isolated, intact pea chloroplasts with micromolar concentrations of trehalose 6-P leads to the reductive activation of AGPase in the dark (Kolbe *et al.* 2005). This system will permit further dissection of the mechanisms involved in triggering AGPase activation. Unpublished results indicate that chloroplasts possess a high-affinity trehalose 6-P transport system and that micromolar concentrations of trehalose 6-P promote activation of AGPase in a cell-free system (Michelski *et al.* unpublished data). It should be noted that there is at present no evidence that trehalose 6-P is involved in the regulation of starch synthesis in response to light/dark transitions per se. Trehalose 6-P levels do not change greatly between the light and the dark in wild-type *Arabidopsis* growing in a regular 12 h light/12 h dark cycle (Lunn *et al.* 2006). Changes in the rate of starch synthesis in response to illumination and darkening may be achieved by changes of allosteric effectors 3-PGA and Pi, and light-dependent changes in the redox activation of AGPase (Hendriks *et al.* 2003; Kolbe *et al.* 2005).

Further research is required to elucidate the link between sugar levels and levels of trehalose 6-P. The contributions of transcriptional, translational and post-translational regulation to increases in trehalose 6-P levels are under investigation. Levels of transcripts for several trehalose 6-P synthases are low during carbon starvation and increase rapidly and strongly in response to increases in sucrose, suggesting that sucrose activation of *TPS* gene expression underlies the changes in trehalose 6-P levels (Bläsing *et al.* 2005; Osuna *et al.* 2007). There is also evidence that some trehalose 6-P metabolizing enzymes are subject to phosphorylation and can bind 14-3-3 proteins (Glinski & Weckwerth 2005; Harthill *et al.* 2006). Indirect evidence for involvement of the protein kinase SnRK1 in the link between sucrose and redox activation of AGPase has been obtained in potato (Tiessen *et al.* 2003). These investigations are complicated by uncertainties about the actual function of many of the trehalose 6-P metabolizing enzymes. Of the large *TPS* gene family, only *TPS1* has been demonstrated to have catalytic activity (Leyman, van Dijck & Thevelein 2001; Lunn 2007). Several genes in the *Arabidopsis* genome encode proteins with both TPS- and TPP-like domains that are predicted to be catalytically inactive: it has been proposed that these proteins may modulate the activities of TPS or TPP proteins or act as trehalose 6-P sensors (Lunn 2007).

Taking these observations as a whole, we suggest that trehalose 6-P plays a central role in linking the rate of starch synthesis to demand for carbon and to growth. This role is most simply illustrated by the sequence of events we envisage during an extended night and the subsequent light period. Carbon starvation during the extended night triggers a cessation of growth, which is not reversed for several hours after re-illumination (see earlier discussion). During this first few hours, newly assimilated carbon accumulates as sugars because it is not being used for growth, and sugar levels are much higher than at the start of a normal day. Trehalose 6-P levels rise from very low levels to levels much higher than normal during the first few hours of illumination (Lunn *et al.* 2006). This overshoot leads to higher than normal activation of AGPase (Lunn *et al.* 2006), and a higher rate of starch synthesis than during a normal day (Gibon *et al.* 2004a; Lunn *et al.* 2006). The elevated levels of starch provide extra reserves for the following night – if the night is again extended, there is a reduced likelihood of carbon starvation. A similar mechanism might account for more subtle, acclimatory responses to gradual changes in environmental conditions such as day length, and in growth rate. We envisage that the rate of starch synthesis during any given day is adjusted in response to levels of sugars at the end of the night and the start of the following day. These levels are determined by a host of interacting environmental and internal factors. They in turn determine the extent of change in trehalose 6-P levels during the day–night transition, and this sets the level of activation of AGPase and hence the proportion of newly assimilated carbon that is partitioned into starch.

REGULATION OF STARCH DEGRADATION IN LEAVES

In order to avoid carbon starvation and optimize the supply of carbon available for growth through the night, the rate of starch degradation must allow for the almost complete utilization of the starch supply precisely at the end of the night. We described previously how depletion of starch before the end of the night gives rise to carbon starvation: symptoms of starvation are also seen at the end of the night in a mutant with a strongly reduced capacity for starch degradation (the

sex1 mutant: Weise & Smith unpublished data). It is highly likely that control within a single diurnal cycle is exerted primarily at a post-translational level. Levels of transcripts encoding many of the enzymes of starch degradation show strong diurnal and circadian rhythms, but – as for other enzymes of primary metabolism – enzyme activities and proteins change very little over the course of the day (Smith *et al.* 2004; Lu *et al.* 2005). We will discuss first the points in the pathway of starch degradation at which control may be exerted, then suggest what information is used to set the rate of starch degradation at night so that carbon balance is maintained.

Important clues about how starch degradation may be controlled come from the recent elucidation of the pathway in *Arabidopsis* leaves (reviewed in Smith, Zeeman & Smith 2006; Zeeman *et al.* 2007a; Zeeman, Smith & Smith 2007b). This reveals two distinct points at which control might be exerted: the initial attack on the granule, and the conversion of maltose to hexose phosphates in the cytosol.

The initial attack on the starch granule is most likely catalysed by beta-amylase and a debranching enzyme (isoamylase3 and possibly also limit dextrinase), acting in concert and producing primarily maltose and maltotriose (Delatte *et al.* 2006; Zeeman *et al.* 2007a). Beta-amylases are encoded by a family of nine genes, and the isoforms differ considerably in predicted structure. Several different beta-amylases are known to be located in the chloroplast (Lao *et al.* 1999; Scheidig *et al.* 2002; Kaplan & Guy 2005). Although their transcript levels show marked diurnal changes (Smith *et al.* 2004; Lu *et al.* 2005), it is not yet known whether levels of any of the encoded proteins follow similar patterns. Functional analyses *in vivo* and *in vitro* suggest that different isoforms may play distinct roles in starch degradation, and may be subject to post-translational modifications (Smith & Zeeman personal communication).

Beta-amylase is an exoamylase, hence conversion of the semi-crystalline granule matrix to malto-oligosaccharides is likely to proceed rather evenly across the granule surface. Leaf starch granules are generally thin and disc-shaped, presenting the maximum surface area for degradation and potentially minimizing the impact of decreasing surface area on the rate of starch degradation through the night. Almost nothing is known about the control of granule shape: mutations affecting a starch synthase (Roldan *et al.* 2007) and a protein phosphatase (SEX4 – see further discussion; Zeeman *et al.* 2001) result in larger, rounder granules but there is no evidence that either protein exerts significant control over granule shape in normal conditions.

Normal rates of starch granule degradation require the presence of two glucan water dikinases (GWDs); enzymes that add phosphate groups from ATP onto either the 3- or the 6-position of glucose residues within starch polymers (Ritte *et al.* 2002, 2004; Baunsgaard *et al.* 2005; Kötting *et al.* 2005). These enzymes do not themselves catalyse degradation, and much remains to be discovered about why they are required. One possibility is that beta-amylase and isoamylase3 cannot readily attack the ordered, semi-crystalline surface of the granule. The addition of phosphate groups to starch polymers may disrupt the organization of the granule matrix and make it a better substrate for exoamylolytic attack (Engelsen *et al.* 2003). GWDs are subject to post-translational modifications that affect both activity and affinity for starch (Mikkelsen, Baunsgaard & Blennow 2004; Mikkelsen *et al.* 2005). This raises the possibility that the rate of starch degradation might be controlled via modulation of the activity of these enzymes. However, there is at present no evidence that GWDs exert significant control over flux through the pathway of starch degradation during a normal night.

A further point at which starch degradation may be controlled occurs in the cytosol. Maltose produced from starch granule degradation is exported from the chloroplast to the cytosol, where it is converted to hexose phosphates via a pathway that requires a transglucosidase, DPE2 (Fig. 7; Chia *et al.* 2004; Lu *et al.* 2006). In the absence of DPE2, maltose accumulates to levels over 40-fold greater than in wild-type plants and starch granule degradation is inhibited. The precise reaction catalysed by DPE2 *in vivo* and steps between this and hexose phosphates are not yet understood. In the test tube, DPE2 releases one of the glucosyl residues of maltose as free glucose, and transfers the other to an acceptor molecule. It will use a variety of acceptors, including mammalian glycogen and various hexose and pentose sugars and oligomers (Fettke *et al.* 2006), but the nature of the acceptor *in vivo* is unknown. It has been proposed that this acceptor may be a glucose-containing heteroglycan found in the cytosol (Fettke *et al.* 2006). DPE2 will use purified heteroglycan as a substrate, and the heteroglycan undergoes diurnal changes in mass and glucose content *in vivo* (Fettke *et al.* 2005), consistent with a direct relationship with primary C metabolism. Importantly, glucose residues on the heteroglycan are also a substrate for a ubiquitous cytosolic glucan phosphorylase (PHS2), which converts them to glucose 1-phosphate. *In*

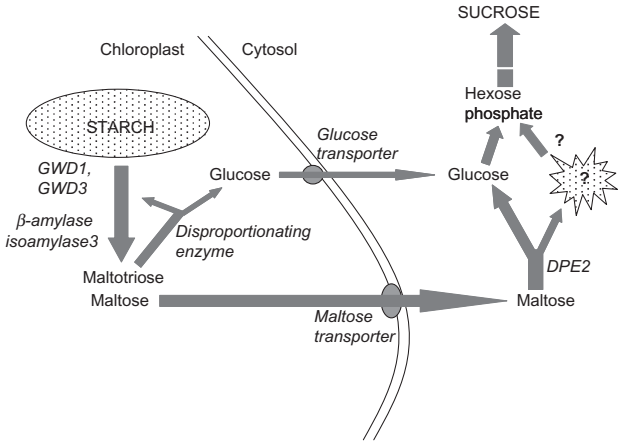

Figure 7. Current view of the pathway of starch degradation in *Arabidopsis* leaves at night. *GWD1*, *GWD3*, glucan water dikinases. *DPE2*, transglucosidase. Question marks denote the unknown cytosolic acceptor for glucose residues from maltose, and the unknown enzyme(s) that catalyses the conversion of these glucosyl residues to hexose phosphate.

vitro, glucose from maltose can be transferred via DPE2 onto purified heteroglycan, then metabolized to glucose 1-phosphate via PHS2 (Fettke *et al.* 2006). Evidence that this pathway may operate *in vivo* comes from the recent demonstration that a *phs2* mutant accumulates maltose at night to higher levels than do wild-type plants (Lu *et al.* 2006).

This hypothetical DPE2–PHS2 pathway is potentially reversible, raising the interesting possibility that the heteroglycan acts as a 'glucose buffer' between starch degradation and sucrose synthesis (Lu & Sharkey 2004). Elevation of hexose phosphate levels would reduce the rate of conversion of maltose to hexose phosphate. The free glucose produced from maltose via DPE2 is presumably metabolized to hexose phosphate via hexokinase, an enzyme shown to be a central player in sugar sensing and signalling. The conversion of maltose to hexose phosphates thus presents several possible mechanisms for fine-tuning the rate of starch degradation to match the requirements for sugars in the plant.

Screens for mutants with elevated starch contents have recently identified two, related proteins predicted to be protein phosphatases that are required for normal rates of starch degradation at night. SEX4 (Niittylä *et al.* 2006) and PTPKIS2 (Fordham-Skelton *et al.* 2002); Comparot & Smith unpublished data) both possess predicted carbohydrate-binding modules and SEX4 has been shown to bind to starch polymers and granules (Niittylä *et al.* 2006). Intriguingly, these proteins are closely related in structure to laforin, a protein required for normal glycogen metabolism in mammals. Human laforin can remove phosphate groups from starch as well as from peptides (Worby, Gentry & Dixon 2006), and it has been suggested that it may act as a glucan phosphatase rather than a protein phosphatase *in vivo*. This raises the interesting possibility that the plant enzymes may be involved together with the GWDs in a cycle of starch phosphorylation and dephosphorylation necessary for the degradation of the granule surface. The rate of incorporation of phosphate into starch actually increases in the dark (Ritte *et al.* 2004), indicating that there may be a cycle of starch phosphorylation and dephosphorylation during starch degradation. However, it also remains possible at this stage that SEX4 and PTPKIS2 are part of a regulatory pathway that modulates the activities of enzyme(s) of starch degradation.

How is starch degradation initiated at the onset of darkness, and how is the rate set and maintained through the night? Triggers for the initiation of degradation could include the changes in redox potential, pH, sugars and cations that occur at the onset of darkness (Buchanan 1980), or changes of specific metabolites in the stroma, but specific information on this crucial point is lacking. The rate of degradation, appears to be set by mechanisms that 'measure' the amount of starch in the leaf at the end of the day, and anticipate the length of the night, as discussed earlier. The evidence for this is as follows. Firstly, the rate of starch degradation is adjusted after changes in day length or light intensity so that it remains essentially linear and consumes almost all of the starch precisely at the end of the night (see e.g. Chatterton & Silvius 1979, 1980, 1981; Fondy & Geiger 1985; Gibon *et al.* 2004a; Smith *et al.* 2004; Lu *et al.* 2005). Secondly, mutations that directly reduce the rate of starch synthesis also reduce the rate of starch degradation. The *adg2* mutant of *Arabidopsis* lacks a large subunit of AGPase and makes only about 40% of the normal amount of starch during the day. The rate of starch degradation is also only 40% of the wild-type rate, so that the supply of starch lasts for the full length of the night (Lin *et al.* 1988). Thirdly, when darkness is imposed several hours prior to the start of a normal night, the rate of starch degradation is reduced relative to that in a normal night so that the supply of starch lasts for the full length of the extended night (Lu *et al.* 2005; Graf & Smith unpublished data).

One obvious candidate for a mechanism that anticipates the length of the night is the circadian clock. Two recent observations support this possibility. To test whether the clock participates in setting the rate of starch degradation at night, plants grown under normal conditions were exposed to various treatments in which the light period was shortened or the dark period lengthened, and the rate of starch degradation was monitored during the next dark period. These results show that the rate of starch degradation is adjusted so that starch content is reduced to normal end-of-night levels exactly 24 h after the start of the immediately preceding light period. If plants are grown under 28 h rather than 24 h days, the rate of starch degradation during the night is still adjusted in this way, so that starch content is reduced to normal end-of-night levels 4 h before the end of each and every night. Conversely, if plants are grown in 20 h days, the rate of starch degradation is appropriate for a night 4 h longer than the actual night and considerable amounts of starch remain in the leaf at the end of each night (Graf & Smith unpublished data).

Independent evidence that the clock contributes to the regulation of starch degradation is provided by studies of the transcript levels of genes involved in starch degradation. The transcripts show very marked diurnal changes; most increase during the day and decrease during the night (Smith *et al.* 2004; Bläsing *et al.* 2005; Lu *et al.* 2005). Lu *et al.* (2005) and Weise *et al.* (2006) have shown that the diurnal responses of these transcripts adjust to alterations of the light period, and that the clock contributes to these changes. A more detailed examination of 14 genes for which there is experimental evidence for a role in starch degradation was made by combining information from the large microarray data set for diurnal changes in wild-type plants, a detailed time series during a prolongation of the night, responses to illumination with compensation point concentrations of CO_2, diurnal changes in the *pgm* mutant, responses to added sucrose and glucose, and publicly available data sets for free-running circadian responses (Usadel & Stitt unpublished data). Many of these genes are under circadian control, with a peak in the first part of the subjective light period. This peak is still seen, although more weakly, when the night is prolonged. However, the timing and amplitude of the increase after dawn are modulated by light and/or carbon fixation. These

act to bring forward and amplify the increase of the transcripts, with the precise impact varying from gene to gene. This reveals a close cross-talk between the clock and light and sucrose signalling, which could potentially affect many processes in addition to gene transcription. Together these data are consistent with a central role for the circadian clock in setting the rate of starch degradation at night, although the mechanisms that link the clock to starch degradation remain to be discovered.

The setting of the rate of degradation at night requires not only a clock but also a mechanism that directly or indirectly senses and signals the amount of starch available for degradation. There is no information at present about how this is achieved, but several intriguing possibilities can be envisaged. One is that starch granules – volume or surfaces – can be directly sensed by mechanisms in the chloroplast. Another possibility is that an unknown signalling molecule is quantitatively sequestered into starch granules when they are synthesized, and released during their degradation.

LINKS BETWEEN THE CARBON BALANCE AND GROWTH PROCESSES

In this section, we consider how the mechanisms that control the carbon balance of the plant may be linked to the control of growth. Understanding of plant growth is being revolutionized by sophisticated kinematic analyses (e.g. Beemster *et al.* 2005) and by molecular understanding arising from microarray analyses and elegant transgenic experiments (Maizel & Weigel 2004; Castellano & Sablowski 2005; Wellmer *et al.* 2006). The impact on growth of factors such as carbon availability can now be described in terms of the relationship between cell division, cell expansion and morphogenesis (Cookson, van Lijsebettens & Granier 2005; Fleming 2006; Horiguchi *et al.* 2006; Tsukaya & Beemster 2006), providing a basis for generation of testable hypotheses. The next few years will yield major advances in this field. We have chosen to deal with two aspects that hold great promise for experimental elucidation in the near future, rather than attempt an exhaustive analysis. These aspects are the impact of carbon availability on cell division or expansion and proliferation, and the clear similarities between control of plant growth by carbon availability and by environmental perturbations.

Little is known about the level(s) at which changes in carbon availability impact on the growth of whole plants. Root extension – the parameter we have used thus far – is influenced by both cell extension and cell proliferation and we do not yet know which of these is affected by carbon starvation. Models of the interaction of cell proliferation and extension in determining organ expansion and morphogenesis will allow more sophisticated analyses in the future. However, it is already apparent from analyses of transcriptional and translational changes during carbon starvation and re-supply that growth may be affected at several levels (Price *et al.* 2004; Thimm *et al.* 2004; Osuna *et al.* 2007). Firstly, during starvation there are general decreases in the levels of transcripts encoding proteins necessary for biosynthesis of proteins (amino acid activation, ribosomal proteins, rRNA), cell division, the cell cycle, and DNA synthesis and repair. There is a general decrease of transcripts encoding proteins for cell wall synthesis and proteins that are believed to modify the cell wall during expansion growth (expansins, xyloglucan endotransglycosylases, etc.). There is also a general repression of transcripts encoding proteins involved in cell cycle and cell growth and in the control of transcriptional activity in the nucleus. Recovery from starvation in mature plant and seedling systems involves very rapid increases (within 30 min to 2 h) in expression of many of the biosynthesis- and growth-related genes that are down-regulated during starvation, including ribosomal proteins (Li *et al.* 2006; Osuna *et al.* 2007). Regulation of the number and the activity of ribosomes may play an important role in linking carbon availability to growth. The global regulation of ribosome synthesis is mediated by nucleolins (Pontvianne *et al.* 2007). Kojima *et al.* (2007) recently showed expression of nucleolin gene *AtNUC-L2* is repressed by carbon starvation and induced by sucrose or glucose addition. Further, disruption of *AtNUC-L2* attenuates the sucrose-dependent increase of transcripts for ribosome components, and leads to decreased growth. *AtNUC2* transcript shows diurnal changes in *Arabidopsis* rosettes, which are accentuated in the *pgm* mutant (data available in Bläsing *et al.* 2005).

Additional regulatory mechanisms determine the extent to which ribosomes are active in protein synthesis. Translational control has been studied by analysis of total and polysomal RNA in *Arabidopsis* cell cultures. Cell proliferation stops on transfer to sugar-free medium, and this is accompanied by a differential decrease in the polysomal relative to the total fraction of transcripts related to growth and the cell cycle and to histone modification (Nicolaï *et al.* 2006). Strikingly, there is a strong decrease of polysomes relative to free ribosomes by the end of the night in *Arabidopsis* plants growing in a normal 12 h light/12 h dark cycle, showing that even moderate changes of the carbon supply may exert major effects on the rate of protein synthesis (Piques & Stitt unpublished data).

These observations are consistent with the idea that a global inhibition of protein synthesis contributes to conservation of resources when carbon availability is limited. Inhibition can occur rapidly via inhibition of translation, and more slowly via changes in the levels of ribosomes, potentially contributing to both rapid, 'acute' inhibition of growth and acclimatory adjustments of the growth rate. For example, a general inhibition of protein synthesis could contribute to the 'acute' cessation of growth. In addition, translational regulation can potentially lead to changes in the spectrum of proteins that are synthesized. This would allow swift changes in the proteome, which might directly contribute to the cessation of growth. A restriction of protein synthesis during part of the 24 h light–dark cycle (see earlier discussion) could, over a longer period, contribute to a lower rate of growth. The resumption of protein synthesis upon re-supply of carbon lags behind large

transcriptional changes, and it is tempting to speculate that some of the up-regulated genes may be directly involved in, and necessary for, this resumption. In this context, it is noteworthy that the set of transcripts that respond rapidly to sucrose includes several E3-ligases (Osuna *et al.* 2007). Analyses of transcriptional and translational changes during recovery from starvation and during normal diurnal cycles in multiple systems (see previous discussion) will permit the identification of candidate genes that may mediate acclimatory changes in protein synthesis in response to changes in carbon availability.

The best understood link between carbon availability and growth is in control of the G1/S transition in the cell cycle. The role of sugars in promoting cell division has been known for many years. Cultured cells starved of sugars arrest at the G1 control point of the cell cycle, a step at which nutritional, hormonal and developmental signals are integrated to determine whether the cell undergoes DNA replication and commits to a further division (Menges & Murray 2002). Recently, the effect of sucrose in promoting G1 to S transition has been pinpointed to a D-cyclin, CYCD3;1. This protein determines the rate at which the transition occurs in cultured *Arabidopsis* cells (Menges *et al.* 2006). Expression of the *CYC3;1* gene is strongly up-regulated by sucrose, and because the protein turns over rapidly (Planchais, Samland & Murray 2004), the presence of sucrose is essential to maintain protein levels and hence the rate pf G1/S transition. (Riou-Khamlichi *et al.* 2000; Menges *et al.* 2006). The G1/S transition is also strongly influenced by hormonal status, and sugars and hormones act interactively (Riou-Khamlichi *et al.* 1999). It seems likely that a complex network of signalling pathways lies between the sensing of carbon availability and the control of the cell cycle. The potential relevance to whole plants of the results from this model system is shown by the observation that genes involved in the cell cycle and cell division belong to the set of genes whose transcript levels decrease during the night and show diurnal changes (see earlier discussion).

The aforementioned studies are starting to uncover how growth is regulated in response to acute changes of the carbon supply and how acclimatory responses may moderate the rate of growth in anticipation of a shortfall of carbon. Evidence is emerging that the relation between growth rates and the levels of carbon and other central metabolites is not passive. In a study of 24 *Arabidopsis* ecotypes, Cross *et al.* (2006) showed that the rate of growth is inversely related to the levels of sugars and starch, including the amount of starch left in leaves at the end of the night. This finding has recently been confirmed in a larger study of more than 100 *Arabidopsis* ecotypes (Sulpice, Gibon & Stitt unpublished data). In these studies, levels of carbohydrates and especially of starch were negatively correlated with growth, while the activities of large numbers of enzymes showed a positive correlation with growth. A recent study in a Col-0 × C24 recombinant inbred population showed that the levels of a combination of 40 metabolites were strongly predictive of the rate of growth, with most of these metabolites showing a negative rather than a positive correlation (Meyer *et al.* 2007). Taken together, these results indicate that faster-growing genotypes have higher activities of enzymes, which drive faster fluxes and deplete the levels of central metabolites. This implies that the genotypes differ in the fine-tuning of the regulatory network that coordinates growth with the supply of carbon and other central metabolites. It also raises questions about the selective pressures that maintain this natural variation in the balance between growth rates and carbon supply.

A WIDER PERSPECTIVE ON ACUTE AND ACCLIMATORY RESPONSES

This article has proposed that plants respond to low carbohydrate by increasing storage and decreasing growth, and that acclimatory mechanisms exist to do this before acute carbon depletion occurs. This has some interesting implications. One is that plants grow somewhat slower than they might in a given condition. The advantage of this 'conservative' response emerges in changing conditions, when it allows the plant to avoid carbohydrate depletion and acute carbon starvation, except when there are relatively large or sudden changes in the environment. It will obviously be important to design experiments to test this hypothesis, both in laboratory conditions and in the field.

This hypothesis also has an interesting implication for the design of experiments that search for phenotypes in sugar-sensing mutants or transformants altered in the expression of candidate genes that may be involved in sugar responses. Screens typically use one or more *fixed* growth regimes. If one of the important functions of carbon sensing is to maintain a balance between carbon supply, allocation and growth in a *changing* world, it may be more informative to study the response to repeated changes of the carbon supply, generated, for example, by repeatedly changing the day length or the light intensity. There are precedents, unrelated to sugar sensing, for transformants showing a phenotype in fluctuating but not in fixed growth conditions. For example, Ganeteg *et al.* (2004) showed that mutants lacking particular members of the light-harvesting complex (LHC) protein family do not show phenotypes in a standard light/dark cycle in growth chambers, but do show a marked inhibition of growth when grown in the field. The inhibition can be reproduced in growth chambers when the light intensity is allowed to fluctuate. This point can be made more generally; an impaired network that is less stable or that responds more slowly may still be able to approach optimal function in stable conditions, but not in changing or fluctuating conditions. The frequency of the changes needed to disrupt the network will depend on the response under study. Information about the frequency will not only be useful for experimental design but may also provide insights into the regulatory network. This can be illustrated by the *pgm* mutant; it grows as well as the wild type in continuous light or very long days but growth is increasingly strongly inhibited when the length of the night is increased (Caspar *et al.* 1985; Gibon *et al.* 2002). As argued in this review, this is a consequence of the depletion of sugars during the night.

Intriguingly, this inhibition is progressively reversed if *pgm* is given a fixed number of hours of light per day, but is switched at increasing rates between light and dark (Gibon & Stitt unpublished data). The frequency at which this recovery occurs provides information about how rapidly carbon depletion triggers changes in metabolism and physiology that lead to a restriction of growth. This resembles approaches in physics and engineering, in which regular or irregular perturbations are applied to disturb and analyse complex systems.

Evidence is accumulating for analogous 'acclimatory' responses to other challenges in addition to carbon depletion. One striking example is a recent breakthrough in understanding the control of cell proliferation and expansion in response to abiotic stresses. Genetic dissection of the signalling pathway that links the hormone gibberellin to plant growth led to the discovery of a class of negative regulators of growth called DELLA proteins. These proteins act to repress levels of transcription factors that mediate the growth-promoting effects of gibberellins, in part at least through influencing levels of a specific microRNA (Achard *et al.* 2004). Levels of DELLA proteins are themselves controlled by gibberellin. Rising gibberellin levels cause DELLAs to be targeted for destruction by the proteasome (Alvey & Harberd 2005). It is becoming apparent that the DELLA proteins are central integrators of independent signals from a wide range of hormonal inputs, which modulate the effects of gibberellin on DELLA levels. The growth-regulating effects of auxin, for example, are attributable to the modulation by auxin of gibberellin-mediated destruction of DELLAs (Fu & Harberd 2003). Remarkably, DELLA proteins are also involved in the down-regulation of growth in response to salt stress (Achard *et al.* 2006). *Arabidopsis* plants lacking DELLA proteins show greater root elongation, leaf production and biomass accumulation under salt stress than wild-type plants. In wild-type plants, salt treatment leads to reduced levels of gibberellin and elevated levels of the hormone abscisic acid, which act together to increase levels of DELLA protein and thus to down-regulate growth.

Resistance to other abiotic stresses is also closely linked with changes in growth. For example, transfer of tolerant species to lower temperatures leads to major changes in gene expression and metabolism, which allow survival even at sub-zero temperatures. Much of this response is mediated by the *CBF* genes, which are rapidly induced by a decrease of the temperature and trigger widespread changes in transcription and metabolism (Gilmour *et al.* 2000; Cook *et al.* 2004; Vogel *et al.* 2005). Even small changes of the temperature induce *CBF* expression (Zarka *et al.* 2003) and lead to changes of global transcript levels, enzyme activities and metabolism that are similar to but less marked than those seen at lower temperatures (Bläsing, Poree & Stitt unpublished data). Decreased temperatures also inhibit growth. It might seem intuitively obvious that lower temperatures will decrease growth. However, overexpression of *CBF* genes leads not only to increased freezing tolerance but also to a strong inhibition of growth (Gilmour *et al.* 2000). This indicates that the decrease in growth at lower temperatures is an integral component of a larger regulatory response.

These integrated responses to abiotic stress are strikingly analogous to the acclimatory response to carbon availability. In both cases, the plant has the capacity to sense changes in conditions – salt, low temperature or carbon availability – which, if they became more extreme or persisted, would directly inhibit or damage central growth and metabolic processes. Detection of potentially deleterious conditions at an early stage triggers a down-regulation of growth that reduces the impact of any further changes. In describing the role of DELLA proteins, Achard *et al.* (2006) suggest that they permit 'flexible and appropriate modulation of plant growth to changes in natural environments'. Similarly, we suggest that carbohydrate sensing and signalling mechanisms permit a flexible and appropriate modulation of plant growth to changes in carbohydrate availability.

The acclimatory response may also prove to be a valuable framework for re-examining the concept of the cost of defence in plant–pathogen interactions. Disease resistance is generally assumed to be costly in terms of plant growth and/or fitness, because specific resistance mechanisms are usually induced only when the plant is challenged with a disease-causing organism (Heil & Baldwin 2002; Brown 2003). In a few cases, induction of resistance mechanisms has been shown to be directly associated with reduced growth or fitness (Heil *et al.* 2000; Tian *et al.* 2003). Costs of resistance have often been discussed in metabolic terms. It is suggested that allocation of resources to resistance mechanisms (synthesis of specific toxins, proteins, cell wall structures and so on) reduces resources available for growth (Heil & Baldwin 2002). A corollary of this idea is the growth-differentiation balance hypothesis, which holds that slow-growing plant parts will be more resistant to pathogens than faster-growing parts because more resources are available for defence responses (Herms & Mattson 1992). We suggest that the interaction between resistance and growth should not be viewed as a straight competition for resources, in which reductions in growth are due to direct carbon limitation. We propose instead that a reduction in carbon availability caused by rapid up-regulation of biosynthesis associated with defence will trigger an acclimatory response, resulting in a downward adjustment of growth to a level that can be sustained at the new level of carbon availability. The acclimatory response can potentially be viewed as part of the defence response. Firstly, failure of this response could result in carbon starvation, and thus cessation of growth, when resources are diverted to defence. Secondly, hijacking of plant carbon by pathogens will trigger down-regulation of growth via the acclimatory response, reducing the risk of carbon starvation and conserving carbon for use in defence responses. Thirdly, the levels of defence metabolites and the mounting of an appropriate defence response may in any case depend on adequate levels of central metabolites (Henkes *et al.* 2001; Matt *et al.* 2002; Fritz *et al.* 2006).

CONCLUSION

In this review, we have argued that plants possess acute and acclimatory responses to carbon deprivation, and that the latter allow plant growth to be sustained under a wide range of environmental conditions. We have discussed how open-ended integrative approaches – combining transcriptomic, enzymatic and metabolomic measurements in contrasting physiological systems – allow these responses to be defined and characterized, and provide information about mechanisms involved in carbohydrate sensing. This approach identifies many new candidate genes, whose role in carbohydrate sensing can now be tested. Detailed information about the physiological and molecular responses to carbon deprivation will allow a much more meaningful analysis of mutants and transformants than was previously possible, and will also provide new avenues to explore in studying natural diversity with respect to carbohydrate sensing and allocation. Finally, our analysis emphasizes the importance of temporal dynamics in plant carbon responses; time delays are central in the response to and the recovery from carbon depletion, are implicit in the concept of an 'acclimatory' response, and may be a central feature in ultimately understanding how plants cope with and adjust to a changing environment.

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