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Balancing supply and demand: the spatial regulation of carbon metabolism in grass and cereal leaves

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Abstract

Leaf primary metabolism responds to changes in both supply of inputs and demand for products. Metabolic control in leaves changes both spatially and temporally. Using leaves of C_3 temperate Gramineae, the spatial control of carbohydrate metabolism has been studied using a range of approaches. Single-cell sampling and subsequent analysis of metabolites, proteins and transcripts has indicated significant differences between epidermal, mesophyll and parenchymatous bundle sheath cells. These differences correlate with differentiated function as heterotrophic, autotrophic and transport pathway components of the leaf. The review emphasizes the key role of sucrose and discusses its catabolism to hexoses and its anabolism to fructans as mechanisms for the preservation of sucrose gradients within the leaf.

Key words: Cell types, fructan, leaf, sucrose, transcripts.

Introduction

There is a significant body of work dealing with the short- and medium-term partitioning of carbon within photosynthetic cells (for a review see Stitt, 1996). Essentially, the current view is that effective operation of the photosynthetic carbon reduction cycle under fluctuating environmental conditions is based around optimizing the regeneration of RUBP and the utilization of ATP and NADPH. Failure to do this not only reduces the rate of carbon fixation but also increases the likelihood of photo-damage. Carbon fixed in excess of that required to regenerate RUBP is available for sucrose synthesis in the cytosol. In leaves of many species, failure to export this sucrose is associated with an increased accumulation of plastidic starch, whereas in other species sucrose or its polymerization products (eg fructans or raffinose-family oligosaccharides) accumulate in the vacuole (Pollock *et al*., 1999).

There is now good evidence that longer-term imbalances in supply and demand can lead to the down-regulation of photosynthesis that is manifest at the level of enzyme or transcript abundance (Jang and Sheen, 1994; Farrar *et al*., 2000). It is also known that treatments such as root restriction that reduce demand can induce premature senescence (Herold and McNeil, 1979), leading to a reduction in photosynthetic capacity. One can postulate, therefore, that flux control of carbohydrate metabolism resides at three levels: short-term (fine) control that optimizes flux between competing pathways; medium to long-term control of protein abundance that alters capacity and the control of organ development that modulates total light interception.

However, this model ignores the spatial distribution of function within the leaf. Analysis by Jellings and Leech (1982), for example, demonstrates that only 55% of the cells in wheat leaves are photosynthetic mesophyll cells. Many, but not all the other cell types, are heterotrophic, leading to internal gradients of supply and demand as well as to external ones. Histochemical analysis has shown that diel starch accumulation differs between cell types in barley leaves (Williams *et al*., 1989) and sugar-metabolizing enzymes are not distributed evenly within the leaf (Kingston-Smith and Pollock, 1996). Thus, in the authors' view, it is not possible to extrapolate hypotheses about the control of carbon flux within mesophyll cells to explain all the elements of carbon partitioning in leaves or between leaves and distant sinks.

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Techniques have, however, been developed to study the spatial distribution of metabolism. Outlaw and co-workers (see Outlaw and Zhang, 2001, and references therein) have developed techniques for the isolation, extraction and assay of metabolites from intact single cells mechanically isolated from freeze-dried leaves. Some elements of carbon metabolism including metabolites, proteins and mRNA species can also be detected histochemically (Kingston-Smith *et al*., 1999, and references therein). Enzyme digestion can be used to separate cells, although the process takes some time to achieve and metabolic status may be altered as a result. More recently, techniques for sampling cell contents directly through microcapillaries have been developed (Tomos *et al*., 1994) and these techniques have been used to study spatial differences in partitioning in cereal leaves.

There are three good reasons for the use of temperate C_3 grasses in studies of this kind. Firstly, soluble sugars are the major products of photosynthesis and this makes single-cell sampling more effective. Secondly, physiological alterations in the balance between supply and demand produce qualitative rather than quantitative changes in metabolism. Leaves maintained under conditions where demand equals or exceeds supply do not have the capacity to synthesize fructan polymers from sucrose. However, when sucrose is forced to accumulate by any one of a number of physiological treatments, the leaves are induced to produce the enzymes that synthesize fructan from sucrose. This induction can be blocked by inhibitors of gene expression (Pollock and Cairns, 1991). Finally, in many of these species, including barley, there is a clear distinction between photosynthetic mesophyll cells and photosynthetic parenchymatous bundle sheath cells (Farrar *et al*., 2000). These, together with epidermal cells, can be sampled easily using single-cell analysis and provide a good model for investigating both internal and external partitioning.

A model for the regulation of sugar metabolism in different cell types

The model under test in these studies is summarized in Fig. 1. It proposes that there are at least three distinct patterns of carbon metabolism and storage in leaves. In heterotrophic cells that are terminal sinks, such as the epidermis, there is a need to catabolize sucrose for metabolism and also to generate a gradient of sucrose metabolism that is independent of the flow of sugars into the phloem. In autotrophic mesophyll cells, there is a need to maintain sucrose concentrations below the level that would lead to down-regulation of photosynthesis; this is achieved by the synthesis of fructan once a critical sucrose concentration is reached. Finally, in cells involved in the supply and loading of sucrose into the long-distance transport pathway, there is a need to maintain a gradient of sucrose concentration between the mesophyll and site of active loading. Once again, sucrose concentrations can be modulated by the synthesis of fructan. However, the threshold for fructan synthesis would need to be different if the gradient between mesophyll and vasculature is to be maintained. Although there are a number of different cell types within leaves, it is only possible at present to sample directly in intact barley leaves the epidermal, mesophyll and parenchymatous bundle sheath (PBS) cells. The PBS cells do contain chloroplasts, but are thought to play an important role in the transport of assimilates into the phloem (Williams *et al*., 1989).

Tissue distribution of carbohydrates

The model was tested by measuring the tissue distribution of water-soluble sugars in cells from mature barley leaves with different carbohydrate abundance. Previous physiological studies have shown that sucrose and fructan accumulation in temperate grasses and cereals is stimulated by treatments that increase photosynthesis (increased irradiance, photoperiod or CO_2 concentration) or reduce sink activity (root cooling, root pruning or excision of the leaves). The exogenous administration of sucrose or sugars that can be metabolized to sucrose also leads to fructan accumulation (Pollock *et al*., 1999). In these studies root cooling, increased irradiance and sugar feeding were used to induce sucrose and fructan accumulation (Koroleva *et al*., 1998, 2000). Cell sap samples were taken from epidermal, mesophyll and bundle sheath cells of control plants held at 500 μmol m^{-2} s^{-1} PAR and compared with samples taken from plants given either 1000 μmol m^{-2} s^{-1} PAR or held at the lower irradiance with root cooling. The results obtained are summarized in Table 1. Significant differences between the three cell types were observed. Epidermal cells contained low concentrations of sucrose, glucose and fructose, and no fructan. The experimental treatments that promoted sucrose and fructan accumulation did not affect the sugar contents of these cells. The majority of the soluble carbon in epidermal cells was in the form of malate (Koroleva *et al*., 2000). By contrast, mesophyll and bundle sheath cells accumulated sucrose after both environmental treatments, together with significant amounts of fructan. Both the absolute sucrose concentrations and the ratio between sucrose and fructan differed markedly between the two cell types. Sucrose was lower and the fructan to sucrose ratio was much higher under both treatments for bundle sheath cells, which is consistent with a gradient of sucrose concentration between the mesophyll and the site of active vein loading in the phloem parenchyma (Koroleva *et al*.,1998, 2000). $^{14}C0_2$ feeding to the leaves, followed by analysis of the radioactivity in single-cell samples using accelerator mass spectrometry (Koroleva *et al*., 2000) indicated that the sucrose pool in mesophyll cells rapidly reached isotopic

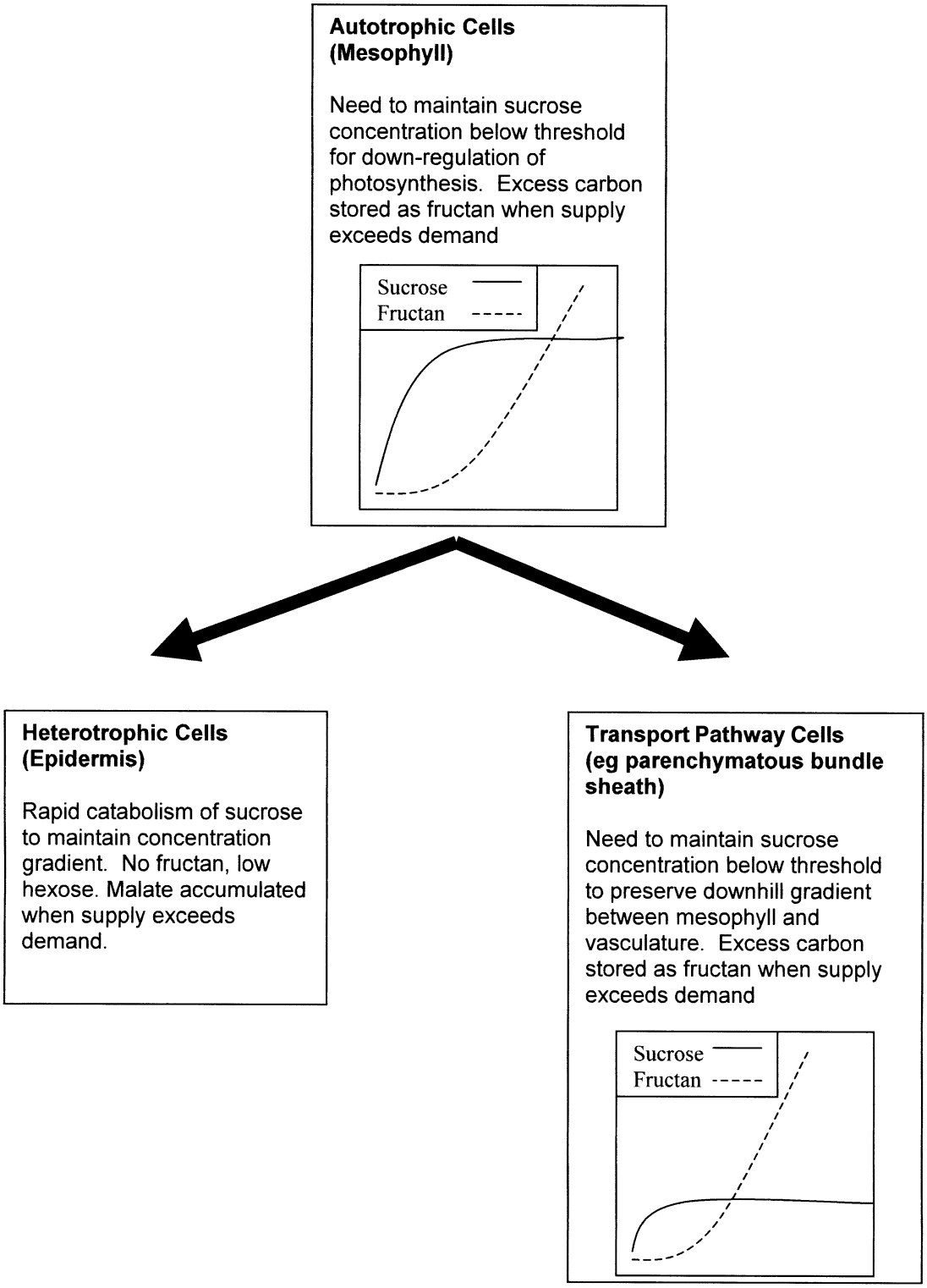

Fig. 1. Model for assimilate partitioning in cell types of temperate C_3 Gramineae. The inset graphs show sugar and fructan concentrations (*y*-axis) as a function of time after initiation of sugar accumulation (*x*-axis).

equilibrium with the external $^{14}CO_2$. By contrast, bundle sheath cells had a lower specific activity, suggesting that there were significant unlabelled pools of sucrose, and perhaps that the cells were involved in the fixation of carbon dioxide released by respiration from within the vascular bundles (Koroleva *et al.*, 2000). It has been

Table 1. *Carbohydrate concentrations (mM ±sd) in sap samples from single cells of barley leaves*

Cell type	Sugar	Control	After 4 d sink cooling	After 2 d high light
Epidermis	Glucose	4±5	5±3	nm[c]
	Fructose	5±4	nd	nm
	Sucrose	8±3	8±1	nm
	Fructan[a]	nd[b]	nd	nm
Mesophyll	Glucose	7±4	35±17	34±4
	Fructose	6±8	6±10	nm
	Sucrose	90±20	169±47	128±13
	Fructan[a]	nd	194±36	148±27
Bundle sheath	Glucose	nd	7±6	27±10
	Fructose	nd	1±2	nm
	Sucrose	16±1	96±17	107±27
	Fructan[a]	nd	177±77	420±152

[a] Fructan concentrations as mM hexose equivalents. (From Koroleva *et al*., 1998).
[b] nd: Not detectable.
[c] nm: Not measured.

suggested (Labhart *et al*., 1983) that fructan synthesis in leaves serves to stabilize the sucrose concentration at levels that would prevent adverse effects such as inhibition or down-regulation of photosynthetic enzymes. If this is so, then the observations summarized above indicate that the process must also be considered in conjunction with sequestration of sucrose, both within the vasculature and within individual cell types.

There is evidence to suggest that this former process may be significant. It has been calculated that the mean sucrose content of cell sap from cell types that can easily be sampled by single cell analysis (comprising about 70% of tissue volume). When this was compared with the sucrose content of whole leaf extracts from replicate leaves, it was only possible to account for some 60% of the total leaf sucrose, suggesting that the remainder is accumulated within the vasculature (comprising only about 6% of tissue volume) (Koroleva *et al*., 1998). These observations suggest that there is extensive compartmentation of primary carbon metabolism within leaves of temperate gramineae. Epidermal cells appear to be buffered from environmentally induced changes in assimilate abundance, with mesophyll and bundle sheath cells showing differential responses for starch, sucrose and fructan metabolism in a manner that is broadly consistent with the model outlined in Fig. 1.

Are differences in substrate abundance associated with differences in protein abundance?

It is possible to challenge sap samples from individual cells adsorbed onto membranes with specific antisera. Strongly positive signals proportional to the amount of cell sap bound to the membrane were observed for the abundant, strongly antigenic protein, Rubisco, using sap extracted from mesophyll and PBS cells. Sap from epidermal cells did not bind the antiserum (Koroleva *et al*., 2000).

Table 2. *Distribution of sucrose cleavage activity in samples from different cell types within barley leaves (from Koroleva* et al.*, 1997)*

Sample	Sucrose cleavage activity (μmol g^{-1} fr. wt. h^{-1})
Whole tissue	30
Epidermal cells	2
Mesophyll cells	4
Bundle sheath cells	2

Unfortunately, the enzymes associated with the polymerization of sucrose to fructan are neither abundant nor strongly antigenic. They belong to a family of acid β-fructofuranosidases that are ubiquitous in higher plants and which are highly glycosylated. (Pollock *et al*., 1999). Proteins of this type cannot be detected antigenically in single cell samples. However, they can be detected by tissue printing (Kingston-Smith and Pollock, 1996) and display a strong association with the vasculature in leaves of barley.

The precise location of these proteins cannot be identified using tissue printing. However, time-dependent release of hexose from sucrose added directly to sap samples can be used to assess enzyme activity, and this can be compared with whole tissue estimates (Table 2). Only some 25% of the total soluble activity could be accounted for in sap samples of epidermis, mesophyll and PBS, suggesting that the bulk of the activity was within the vasculature (Koroleva *et al*., 1997). *In vivo,* acid β-fructofuranosidases can catalyse sucrose hydrolysis, fructan synthesis or fructan breakdown dependent upon the sucrose and the enzyme protein concentration (Cairns *et al*., 2000). It is not, therefore, possible to use non-specific activity assays or antibody methods to distinguish between 'catabolic' and 'anabolic' enzymes from the same family and such measurements cannot be used directly to

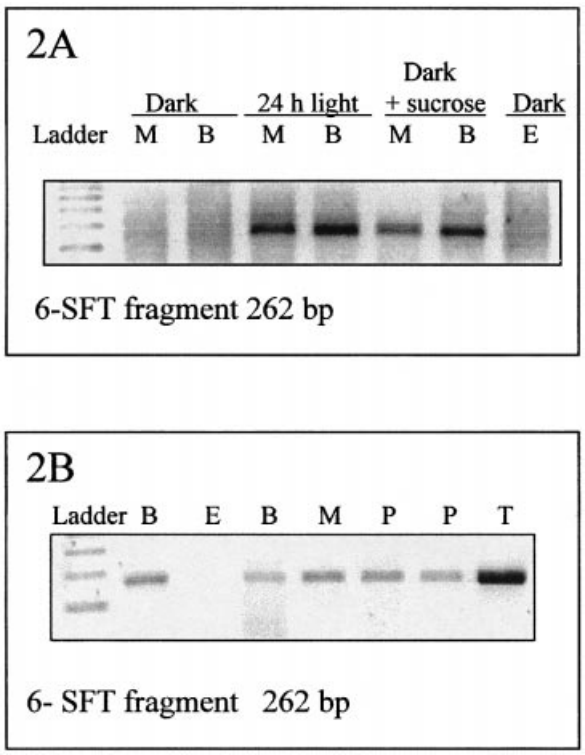

Fig. 2. 6-SFT expression in single cells or whole extracts of barley leaves. (A) Samples taken from leaves after an 8 h dark period; cut leaves illuminated for 24 h and cut leaves fed 200 mM sucrose in the dark for 24 h. (B) Samples taken 8 h into the photoperiod from attached leaves maintained at 500 µmol m^{-2} s^{-1} PAR. B, parenchymatous bundle sheath; E, epidermis; M, mesophyll; P, phloem parenchma; T, total leaf extract.

test the model. However, the data are wholly consistent with the strong spatial localization of elements of sucrose metabolism in leaves.

Can measurements of gene expression changes be used to test the model?

In principle, measurements of transcript abundance can be made more sensitive (by PCR) and more specific (by careful primer selection) than can measurements of protein abundance. In practice, there are limitations to such approaches, together with significant challenges that need to be addressed if the measurements are to be made wholly or approximately quantitative. Two approaches have been adopted. In the first, cDNA libraries were constructed, giving access to the whole expression profile (Karrer *et al*., 1995; Gallagher *et al*., 2001); in the second, RT-PCR was used to amplify specific sequences, dependent upon the primers used (Brandt *et al*., 1999; Koroleva *et al*., 2001). In both cases magnetic beads surfaced with oligo$(dT)_{25}$ were used to bind mRNA and facilitate the removal of DNA.

To study fructan synthesis in different cell types, primers were used to amplify a 262 bp fragment from a gene from barley coding for a fructosyl transferase (6-SFT; Sprenger *et al*., 1995). Although this is an acid β-fructofuranosidase closely related to invertase, there is evidence to suggest that its primary function *in vivo* is anabolic, and transcript abundance in whole leaf extracts is strongly increased in the presence of sucrose (Müller *et al*., 2000).

These findings are summarized in Fig. 2, and represent comparative, qualitative observations. Transcript abundance is low in tissues maintained in the dark, but is clearly visible in both mesophyll and bundle sheath cells after 24 h illumination of excised leaves (high sucrose contents and a significant induction of fructan synthesis; Pollock *et al*., 1999; Fig. 2a). Administration of exogenous sucrose via the transpiration stream also induces fructan accumulation (Koroleva *et al*., 2000, 2001) and again leads to an increase in transcript abundance, In both cases the PBS shows a greater abundance, suggesting a lower threshold for induction consistent with the model. Transcript cannot be detected in samples from epidermal cells harvested in the light, but is apparently detectable in samples from phloem parenchyma harvested from the cut surfaces of leaves (Fig. 2b). This observation suggest that fructan metabolism may also occur within the vasculature, offering further potential for the control of sucrose gradients within the leaf as an essential component of the regulation of carbon export.

Conclusions

The evidence summarized in this brief review is consistent with the model shown in Fig. 1, but this is still highly speculative. There is a need to be able to determine quantitatively the relationship between changes in gene expression and changes in flux. This requires both a quantitative assessment of transcript abundance and the ability to 'poise' leaves at different steady-states where export rates and sugar contents can both be measured. However, it is clear from the data presented here that carbohydrate metabolism in leaves is highly discontinuous, showing both qualitative and quantitative differences between cell types. Single-cell sampling offers a precise and adaptable method for studying this discontinuity, but a full understanding of the processes involved will need to involve measurements from within the vasculature. There is indirect and circumstantial evidence that this compartment has a significant involvement in sugar storage and metabolism as well as in long-distance transport, but, at present, its quantitative significance cannot be assessed. The patterns of carbon metabolism in temperate C_3 cereal leaves make them excellent experimental systems to study these processes.

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