Metabolic aspects of organogenesis in the shoot apical meristem

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Abstract
Research over the last decade has led to tremendous advances in the characterization of the transcriptional networks involved in the initiation and maintenance of the shoot apical meristem (SAM), as well as the factors involved in the formation and development of leaves by this organ. However, one aspect of the SAM that has received rather limited attention is the fact that it is characterized by being heterotrophic, in contrast to the majority of cells and tissues immediately derived from it which rapidly undergo differentiation to form photosynthetically active, autotrophic organs. This clear physiological and biochemical distinction of the SAM from the surrounding tissue raises interesting questions as to what controls the transition from heterotrophic to autotrophic growth, the nature and sequence of the metabolic events that must occur on this transition, as well as basic questions as to the potential intertwining of metabolic and developmental mechanisms during leaf initiation.

Key words: Biochemistry, carbohydrate, cell wall, development, photosynthesis.

Introduction
The shoot apical meristem (SAM) contains a group of pluripotent cells which undergo repeated rounds of division over an extended period of time to generate the daughter cells from which the main aerial bulk of the plant is produced. It has been the focus of extensive investigations and, with the advent of molecular genetic approaches, a detailed picture of the genetic and molecular interactions involved in the establishment, maintenance, and function of this specialized group of cells is beginning to emerge (reviewed in Veit, 2004). As a result, it is now common to view the SAM as an organ patterned by specific transcription factors whose combinatorial or exclusive expression dictates the fate of cells within it. In addition, it has become clear that precise yet complicated signalling processes occur both within the SAM and between the SAM and the surrounding tissue to reinforce or define the spatial boundaries of transcription factor activity. The nature of these signalling networks and how they feedback and feed-forward to the transcriptional networks is a major focus of current research (Hay et al., 2004). However, at the same time, the SAM has some unique metabolic and physiological attributes which have yet to be directly addressed by molecular genetic approaches. If the functioning of the SAM is to be fully understood, the nature of these target processes and their relationship with the transcriptional and signalling networks controlling meristem function must be unravelled. Indeed, some of the metabolic characters may be an essential aspect of meristem function. In this article, the focus is on defining this problem, providing a summary of some recent data in this area and a framework for future lines of research.

Metabolic events in the shoot apical meristem
Viewing the SAM under fluorescent illumination (Fig. 1A) reveals a basic aspect of meristem biology. The cells of the SAM are heterotrophic, i.e. they do not contain chlorophyll. Analysis of meristem cells at the TEM level backs up this simple observation, showing that the cells contain proplastids with no or very limited lamella structure (Fig. 1B). Similarly, analysis of transcript patterns for genes whose products are required for photosynthesis...
reveals that these genes are not expressed within the SAM but are highly expressed in regions just outside the meristem. Use of promoter::reporter gene constructs in transgenic plants supports this observation (Fig. 1D, E), indicating that transcriptional activation of photosynthetic target genes occurs within a very few cell diameters of leaving the meristem domain.

The inability of the SAM to perform photosynthesis, coupled with the rapid acquisition of photosynthetic capacity as cells leave the SAM domain, raises two basic questions. Firstly, what controls this transition and, secondly, what is the significance of this lack of photosynthetic capacity for the SAM?

Taking the question of the control of transition from non-photosynthetic to photosynthetic capacity, research over the last decade has led to the fundamental concept that the SAM is defined by a series of transcriptional networks whose spatial and temporal control defines SAM function (Veit, 2004). However, how easily the patterns of these transcriptional networks transpose onto the pattern of switching of photosynthetic capacity is questionable.

The SAM consists of a population of cells which, during embryogenesis, become committed to the formation of a group of initials (or stem cells) which undergo repeated rounds of proliferation. The positioning of these stem cells within the SAM is regulated by a set of genes including *WUSHEL*, *CLAVATA1*, and *CLAVATA3* which display spatially distinct patterns of expression (Brand *et al.*, 2000; Schoof *et al.*, 2000). However, none of these patterns easily links to those displayed in Fig. 1C, D or E by genes involved in the acquisition of photosynthetic capacity.

The meristem domain itself is initially defined by expression of STM-like *KNOX* homeobox genes (Long *et al.*, 1996). Repression of STM-like gene expression within the SAM is associated with the determination of a subset of meristem cells to form a leaf primordium (Jackson *et al.*, 1994; Fig. 1F). However, this loss of STM-like gene expression (and concomitant gain of expression of ARP-like transcription factors, such as PHANTASTICA (Hay *et al.*, 2004)) occurs significantly before the overt acquisition of photosynthetic capacity, suggesting that these transcription factors are not simply linked to this physiological...
and metabolic switch. Moreover, in a number of SAMs
STM-like gene expression can extend into the sub-meristem
region of the stem where, for example, chlorophyll accu-
mulation is clearly occurring. These observations suggest
that the switch from heterotrophic to autotrophic growth is
not simply regulated by a KNOX gene-dependent mech-
anism. Other genes, such as AINTEGUMENTA, are ex-
pressed very early in the leaf primordium, but appear to be
absent from the subtending stem tissue. Since the acquisi-
tion of photosynthetic capacity occurs in both leaf and stem
tissues, it would seem unlikely that such genes are intim-
ately involved in this switching process. With respect
to genes belonging to the network of PHABULOSA,
KANADI and YABBY factors (Kerstetter et al., 2001;
McConnell et al., 2001; Eshed et al., 2004), altered
expression leads to clear changes in leaf polarity and
switching between adaxial and abaxial cell identity, but
the differentiation of the cells formed appears normal,
indicating that these networks control patterning rather than
the specific differentiation events. It is, thus, at present very
unclear how (or whether) the transcription factor net-
works so far identified as playing an essential role in
initiation and maintenance of the SAM play a direct role in
repressing the acquisition of the photosynthetic apparatus
in meristem cells. It is, however, intriguing that a recent
paper from the Weigel group has revealed that the ex-
genous supply of sucrose to plants mutated in a specific
WUS-related gene (STIMPY/WOX9) can rescue a wild-type
phenotype. In the absence of exogenous sucrose the mutant
displays an impaired ability to maintain a functional SAM
and is seedling lethal (Wu et al., 2005). The mutant
seedlings are green, and thus presumably can photosynthe-
size and generate sucrose. Either the transport, metabolism
or signalling of sucrose is impaired as a result of the loss of
the transcription factor activity, and this loss in carbohy-
drate metabolism or signalling is instrumental in the meri-
stem phenotype observed. Alternatively, the exogenous sucrose by-passes a pathway linked to the appropriate
expression of the STIMPY/WOX9 transcription network.
An input of sucrose on cell division is long established and
maintenance of cell division is a clear characteristic of the
SAM. It is therefore interesting to note that recent papers
have implicated CK-responsiveness in SAM function
(Leibfried et al., 2005). A linkage of cytokinin and sucrose
on cell proliferation is well established from work on tissue
cultures, and significant data indicate that at least some of
the transcription factors involved in meristem function lead
to altered hormone (CK and GA) levels (Jasinski et al.,
2005). A network is thus appearing in which sugars,
hormones, and transcription factors interact to regulate
meristem function.

A vast body of work shows that the acquisition of
photosynthetic capacity can be repressed by darkness,
leading to the formation of etiolated tissue. Thus, one
possibility is that the lack of photosynthetic capacity in the
SAM is a consequence of its position in the plant, i.e. that
its enclosed nature prevents access of light, thus leading
to an essentially etiolated tissue. However, simple exposure
of SAMs to light does not trigger either accumulation
of chlorophyll or the expression of light-regulated genes
such as RBCS (Fleming et al., 1996). These data indicate
that the specific pattern of metabolism in the SAM reflects
an intrinsic developmental state of the tissue rather than an
environmentally imposed limitation.

So, if our knowledge of the transcriptional networks
involved in switching non-photosynthetic meristem cells to
photosynthetic non-meristem cells is very much incom-
plete, what of our understanding of the consequences of this
metabolic status? What is the metabolic character of the
SAM? What changes occur as a meristem cell undergoes
differentiation to form a leaf or stem cell? If meristem cells
possess a specific metabolism, does this in any way have an
impact on their specific developmental function, i.e. is
meristem-cell function dependent on a particular metabolic
state? Is it possible to form a green SAM, or are these two
attributes exclusive?

Early work on the SAM indicated that specific enzyme
activities (e.g. acid phosphatase, succinic dehydrogenase)
showed specific patterns within the SAM, however, the
functional significance of these patterns of enzyme activity
remained open to speculation (Fosket and Miksche, 1966;
Gifford and Corson, 1971). Following these initial studies,
interest in the metabolic status of the SAM waned but was
reinvigorated by the work of Kerk and Feldman studying
the root apical meristem (RAM). They showed that the
quiescent centre of the RAM (which forms part of the stem
cell niche) contains very low levels of ascorbate and high
activities of the enzyme ascorbate oxidase (AOO). More-
over, they could demonstrate that the gene encoding AOO
was auxin-inducible. Since the available evidence indi-
cates that auxin transport is channelled down the root
waters the RAM, these authors proposed that the flux of
auxin towards the QC induced AOO, leading to low levels
of ascorbate and (in addition) metabolism of auxin by
AOO. Since low levels of ascorbate had previously been
correlated with blockage of the cell cycle, they proposed
that the altered oxidative environment in the QC invoked
by auxin was the mechanism by which cell proliferation
was blocked (Kerk and Feldman, 1995; Jiang et al., 2003).
Indeed, feeding roots with low levels of ascorbate did
promote cell proliferation. Whether a similar situation
exists in the SAM (where auxin flux plays a key role in
leaf initiation) remains unknown. It is also worth noting
that at least some animal stem cell populations are
characterized by high activities of aldehyde dehydrogenase
(ALDH) (Storms et al., 1999). Although it has been
proposed that this activity functions in a defence-related
mode to destroy cytotoxic compounds, it has also been
suggested that ALDH plays a role in the metabolism of
retinal, a vitamin A-derived growth factor implicated in
various aspects of animal developmental biology. It is interesting that lines of investigation in both animals and plants suggest that stem cell niches are characterized by oxidizing enzyme activities which have been proposed to play a role in the metabolism of growth factors. Although many aspects of stem cell identity are distinct between plant and animals (Sablowski, 2004), some aspects (such as cell cycle regulation) appear highly conserved. Is a distinct metabolic status a unifying theme in stem cells? The advent of novel methods for metabolite analysis in very small tissue samples provides a way of testing some of these ideas, as will be discussed later in this article.

Metabolic events during the earliest stages of leaf initiation

As outlined above, despite significant advances in our understanding of the transcriptional networks controlling leaf development, none of the genes so far characterized seem to play an obvious role in the differentiation of meristem to non-meristem (leaf) cell. Certainly, some of these transcriptional changes impinge on hormone (notably GA) metabolism (Sakamoto et al., 2001; Hay et al., 2002), however, how or to what degree the resulting changes in hormone action are related to the differentiation of, for example, leaf tissues is unclear. As also outlined above, the switch from meristem to non-meristem cell appears to be entwined with plastid differentiation and the acquisition of photosynthetic capacity, suggesting that a switch in carbohydrate metabolism, which must accompany this plastid differentiation, might be involved in this change of cell identity. Some data from this laboratory have provided intriguing data that altered carbohydrate metabolism is indeed linked with the earliest phase of commitment by meristem cells to form a leaf (Pien et al., 2001a).

Using bisected tomato SAMs, the RNA profiles in meristem tissue just undergoing commitment to leaf formation was compared with meristem tissue that would not undergo this process for approximately another 48 h. Five clones were obtained that showed increased RNA expression (as revealed by in situ hybridization analysis) in tissue associated with leaf formation (the I1 position in the SAM). One of these genes encoded an ARP transcription factor, i.e. the expression pattern of a gene expected to be specifically expressed in I1 tissue (Hay et al., 2004). A second gene encoded a P450 enzyme implicated in the biosynthesis of brassinolide, a hormone implicated in various aspects of plant growth. More recent data also indicate that brassinolide synthesis plays a role during the early stages of leaf formation (Montoya et al., 2005). The fact that both GA and brassinolide metabolism may be specifically altered at the site of presumptive leaf formation, along with the data indicating that altered auxin flux at this site is a key step in leaf initiation (Reinhardt et al., 2003, reviewed in Fleming, 2005), demonstrates the importance of concerted hormone metabolism and flux at the site of presumptive leaf formation. However, the remaining three I1 markers encoded proteins involved in various aspects of carbohydrate metabolism (sucrose synthase (SuSy), ADP glucose pyrophosphorylase (AGPase) and a SNF1-like kinase). The potential roles of these gene products will be discussed below, but it is perhaps worth considering first why genes involved in carbohydrate metabolism might be identified by the screen performed here.

The anatomy and function of the SAM present the plant with an intriguing set of problems. The SAM is among the most metabolically active regions of the plant, generating new cells to supply the rest of the plant. However, all carbon must be imported into the SAM from the vasculature which, moreover, terminates at a distance from the base of the SAM. It is, therefore, not unexpected that the SAM might have a distinct pattern of carbohydrate metabolism from the surrounding tissue which is derived from it. However, the nature of this metabolism and its control remains unclear. For example, the pattern of AGPase at sites internal to where leaf initiation occurs (Fig. 1G) suggests that starch accumulation is occurring, and TEM analysis of starch grain density supports this view (Pien et al., 2001a). However, why starch should accumulate at this site is open to speculation. One possibility is that a temporary storage of C ensures that during the initial stages of leaf formation a sufficient supply of C and energy is available to ensure that the process proceeds. The differential expression of an SNF1-like protein kinase suggests that the response of meristem cells to sugar signals might be distinct from that of the surrounding tissue. SNF1-like protein kinases are thought to be necessary for the transduction of signals regulating the response of a tissue to specific sugar signals (Halford et al., 2000). Moreover, activity of an SNF1-like kinase has been implicated in the control of AGPase activity, suggesting that the overlapping expression domains observed in the analysis in the SAM may have functional significance. Certainly, meristem cells respond to exogenous supplied sucrose by altered patterns of gene expression (Pien et al., 2001a), but the significance of this is at present unclear since meristem function does not appear to be affected. Finally, the local expression of SuSy at the presumptive site of leaf formation is intriguing since the activity of this enzyme has often been correlated with sink activity (i.e. regions of high sucrose utilization) (Zrenner et al., 1995; Koch, 2004). Furthermore, it has also been postulated that SuSy might function in regions where low oxygen or low adenylate energy charge preclude or limit alternative means of sucrose breakdown (e.g. via invertase) (Ricard et al., 1998). The situation in the SAM is unclear since invertases are certainly expressed in this tissue, however, the relative in vivo activity of the two metabolic pathways is unknown.

A recurring problem with the data described above is that of interpreting metabolism on the basis of transcript data alone. Transcript patterns provide an indication of where
Metabolite profiling is a mass spectrometry-based technique whereby molecular components of a complex sample are volatilized and passed through a mass spectrometer and separated according to their charge/mass ratio. In the simplest scenario, each ionized molecule within a tissue will possess a distinct charge/mass ratio which allows its facile identification by comparison with known standards. In reality, the extremely complex nature of most tissues, coupled with differences in ease of volatilization, molecular breakdown pattern, and various other factors, makes identification of all compounds in one sample virtually impossible. Nevertheless, in most samples a number of common metabolites can be identified relatively easily and, moreover, in a semi-quantifiable fashion. In addition, the exquisite sensitivity of the methods now available means that it is feasible to analyse samples taken from extremely small samples, indeed down to the level of the single cell.

A project has been initiated aimed at analysing the metabolite profiles of single tomato meristems, focusing on small metabolites closely associated with carbohydrate metabolism. By comparison with the transcript patterns described above, the relevance of the observed patterns of gene expression to actual metabolism within the meristem and young developing leaves is being investigated.

In a first series of experiments, individual SAMs were dissected from tomato plants and analysed by TOF mass spectrometry. For comparison, young leaf primordia (P3-P4) were also analysed, as were SAMs which had been pretreated with 1 μM IAA. Previous data have indicated that local treatment with auxin is sufficient to induce leaf formation on tomato SAMs and reflects an endogenous function of auxin in determining the site of leaf initiation (Reinhardt et al., 2003). As a control, tomato fruit tissue was analysed since data for this organ were already available (Overy et al., 2005) and it represents a clearly differentiated tissue from both the SAM and leaf primordia. The preliminary results, shown in Fig. 2, indicate that, firstly, metabolite profiling of individual SAMs is feasible. Clean, reproducible traces were obtained using the minute amounts of starting material. Secondly, metabolite profiles from untreated SAMs were distinct from auxin-treated SAMs and from leaf primordia and fruit tissue, i.e. distinct tissues show distinct profiles and the SAM displays a distinct response to auxin, a known modulator of SAM activity.

Analysis of the individual metabolites within the different samples is still in progress, but the initial data are already intriguing. Of the top 50 metabolites showing a significant relative difference in level between SAM and leaf primordia tissue, eight were provisionally identified as either dNTPs or dNDPs. In particular, in all cases there is an elevated ratio of dNDP to dNTP in the SAM relative to leaf tissue. Interestingly, one of the earliest reports on a gene specifically expressed in the SAM described the expression pattern of a dUTPase whose transcripts were elevated in the SAM (Pri-Hadesh et al., 1992). This analysis provides the first indication that the specific
transcript pattern (described 14 years ago!) has relevance at the metabolite level. This, of course, raises the question of the functional significance of this metabolite pattern. Bearing in mind the specific transcript pattern for SuSy observed in the tomato SAM, it is worth noting that SuSy catalyses the production of fructose and UDPGlc from sucrose and UDP. The accepted hypothesis is that, under most circumstances, SuSy catalyses the reaction in this direction although (based on delta G values) the reaction is readily reversible (Geigenberger and Stitt, 1993). Sucrose is thought to reach the SAM from the sub-adjacent phloem (i.e. is present is excess), but its catalysis requires an appropriate supply of UDP. Maintenance of a relatively high ratio dNDP/dNTP ratio might facilitate this.

This still, of course, leaves open the question of why SuSy expression should be up-regulated at the site of presumptive leaf formation. SuSy is not the only option for sucrose breakdown in the SAM since data indicate that invertase genes are expressed at reasonable levels within this tissue, at least at the transcript level (Pien et al., 2001a). Various ideas have been proposed that under certain physiological conditions (e.g. hypoxia, low adenylate energy charge) utilization of SuSy might be preferable to invertase (Ricard et al., 1998; Koch, 2004). What the situation is in the SAM is unclear. SuSy has also been implicated in metabolite channelling, in particular with respect to a role of providing UDP-glucose precursors for cell wall biosynthesis (Amor et al., 1995). As described in the next section, altered cell wall characteristics are likely to play a key role in the initial events of leaf formation, thus localization of SuSy at the site of leaf initiation might have a role in channelling UDP-glucose towards cell wall biosynthesis.

Molecular events in the cell wall during leaf initiation

A substantial body of evidence supports the hypothesis that control of cell wall structure and architecture plays a key role in the initial events of leaf formation (Cosgrove, 2000). Plant cell walls act to restrain expansion, counteracting the turgor force generated within the cell. For controlled growth to occur, a temporary imbalance in these forces must occur. The majority of data indicate that plants control this process by regulation of cell wall extensibility, i.e. local loosening of the cell wall allows growth to occur in this region until a new biophysical equilibrium is established between tensile force in the cell wall and the internally generated turgor pressure. This growth via altered cell wall looseness would, if integrated over time, lead to a gradual thinning of the cell wall as its perimeter increased to contain the increased volume of the enclosed material. Such thinning of the cell wall would tend to weaken the cell wall, decreasing the resistance to expansion, thus leading to a potentially cataclysmic cycle of events leading to eventual tearing apart of the wall. The fact that this does not happen is due to two co-ordinated processes. Firstly, the evidence suggests that the alterations in cell wall architecture that occur during expansion growth occur in a highly controlled fashion. Secondly, observations on most growing tissues (with hypocotyls being an exception) indicate that cell wall thinning does not occur, implying that the process of cell wall synthesis and turnover is tightly coupled to the process of cell wall loosening.

With respect to cell wall loosening, most attention has focused on a family of proteins termed expansins (Cosgrove, 2000; Lee et al., 2001). These proteins are localized in the cell wall, are found in very wide range of plants, and their expression pattern frequently correlates with tissue undergoing rapid expansion. In vitro assays show that purified expansins can promote cell wall elongation and, moreover, manipulation of expansin gene expression in transgenic plants leads to phenotypes consistent with the proposed function of these proteins as regulators of cell wall extensibility (Cho and Cosgrove, 2000; Pien et al., 2001b; Choi et al., 2003). However, the mechanism of expansin action is still somewhat shrouded in mystery. The data indicate that the optimal substrate for these proteins is a mixture of cellulose and xyloglucans (Whitney et al., 2000). Since the non-covalent interaction of these two macromolecules is thought to form a lattice-work providing resistance to extension, a site of action of a cell wall loosening protein at the interface of this lattice seems very reasonable. However, how expansin acts at this interface to loosen the lattice in a controlled fashion is unknown. It certainly appears that expansin does not possess any of the classical enzymatic activities associated with cell wall synthesis and metabolism. Immunocytochemistry data indicate that expansins accumulate at the middle lamella, but whether this reflects their site of action is unknown, i.e. it might indicate where expansins end up but does not necessarily show where they act in the cell wall matrix. If expansins were to be closely associated with controlling the architecture of newly synthesized cellulose and xyloglucans, a localization towards the inner side of the cell wall close to the plasmalemma-localized cellulose synthase complexes would seem more likely. It should also be pointed out that expansins are not the only potential player in controlling cell wall extensibility and evidence exists that other proteins, such as lipid transfer proteins, also influence cell wall architecture (Nieuwland et al., 2005). Furthermore, enzymatic functions that lead to the modification of cell wall macromolecules (such as XETs) and to the generation of hydroxyl radicals have also been proposed as endogenous regulators of cell wall function (Schopfer, 2001). A tight co-ordination and channeling of new wall material at the plasma membrane/cell wall interface, coupled with a mechanism for controlling how the cell wall constituents interact would seem eminently
sensible. However, knowledge of the dynamics of cell wall architecture during synthesis of the cell wall and, indeed, the process by which new cell wall material is integrated into the existing matrix is so limited (Somerville et al., 2004) that any mechanisms put forward must be treated as speculative. Progress in this area is essential if the integration of catabolic events associated with growth and differentiation in the SAM is to be understood.

Conclusions

The SAM is an organ of fundamental importance in plant biology. It is clear that the SAM is metabolically distinct from the adjacent tissue derived from it. The mechanism controlling the switch of metabolism that must occur as cells leave the SAM domain is unclear. Whether the SAM displays a unique or special metabolic character is suggested by available data, but is essentially not documented. Whether such a metabolic character is intrinsic to the special function that the SAM performs is similarly untested. With so many unknowns, it is clear that the field is open for essential progress in this area. The advent of highly sensitive and powerful tools for the analysis of metabolism in extremely small samples means that at last it is possible at least to attempt to answer these fundamental questions. Coupled with the broad range of molecular genetic tools now available, correlations and indications revealed by such metabolic analyses can now be realistically put to the test. Although such work will obviously be technologically challenging, it is feasible, and if the goal of gaining a meaningful understanding of all plant gene function is to be accomplished, such challenges must be met.

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