Journal of Experimental Botany, Vol. 62, No. 9, pp. 3039–3048, 2011
doi:10.1093/jxb/err072 Advance Access publication 17 March, 2011

Journal of Experimental Botany
www.jxb.oxfordjournals.org

REVIEW PAPER

The grass leaf developmental gradient as a platform for a systems understanding of the anatomical specialization of C_4 leaves

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Received 2 December 2010; Revised 17 February 2011; Accepted 21 February 2011

Abstract

C_4 photosynthesis relies on spatial and quantitative specializations of common features of leaf anatomy, including venation pattern, bundle sheath cell and chloroplast differentiation, plasmodesmatal abundance, and secondary cell wall enhancement. It has thus far been challenging to dissect the molecular basis for these C_4-specific alterations in spatial and quantitative patterns of regulation. The target downstream networks of genes and protein interactions that produce these fundamental anatomical features in both C_4 and C_3 species are poorly understood. The developing leaves of monocot grasses provide a base-to-tip gradient of developmental stages that can provide the platform for comprehensive molecular and anatomical data that can yield a better understanding both of the regulators and the targets that produce C_4 patterns, through a variety of gene discovery and systems analysis strategies.

Key words: C_4 photosynthesis, grasses, leaf anatomy, leaf development, maize.

Introduction

Anatomical specializations for two-cell C_4 metabolism, in which primary carbon assimilation (PCA) and primary carbon reduction (PCR) occur in distinct cells, have evolved in at least 16 eudicot families and three monocot families (Kellogg, 1999; Sage *et al.*, 1999; Sage, 2004; Muhaidat *et al.*, 2007). Although details vary among these independent C_4 lineages, all provide four basic features: (i) high density of venation, (ii) sequestration of the PCR tissues (usually bundle sheath, BS) from the atmosphere via a diffusion barrier, (iii) optimized physical contacts and plasmodesmatal communication between PCR and PCA cells, and (iv) complementary photosynthetic/metabolic specialization of PCA and PCR cells and organelles, commonly through photosynthetic development of the BS as a PCR tissue (Dengler and Nelson, 1999; Dengler and Taylor, 2000; Muhaidat *et al.*, 2007). This convergence probably reflects the common selection pressures provided by the environments in which each lineage evolved (Sage, 2001, 2004).

How can we find the regulatory networks and downstream targets that produce C_4 anatomical features? The specialized anatomy of C_4 leaves appears to rely on spatial and quantitative variations in features that already exist in C_3 species, based on quantitative anatomical and developmental observations (Gutierrez *et al.*, 1974; Laetsch, 1974; Hattersley and Watson, 1976; Dengler *et al.*, 1994; Sinha and Kellogg, 1996; Dengler and Nelson, 1999; Dengler and Taylor, 2000; Sage, 2001; McKown *et al.*, 2005; McKown and Dengler, 2007; Muhaidat *et al.*, 2007; Nelson, 2010). Veins, plasmodesmata (PD), PCA and PCR functions, and gas diffusion barriers are likely to be produced by genetic and protein interaction networks that are similar among groups of higher plants. This implies that the recurring specialized patterns of these features, selected by C_4 evolution, map to genes that regulate common modules or networks of downstream genes. Thus far, conventional approaches such as genetic mutational analysis have revealed little of the regulatory networks, downstream

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genes, and gene product interactions that would constitute a mechanistic understanding of C_4 anatomy.

This review will describe the emerging opportunity to use systems approaches to understand how veins, PD, gas diffusion barriers, and BS are specialized into C_4 anatomical patterns. Comprehensive transcriptome, proteome, metabolome, and quantitative anatomical data are now available with developmental-stage and cell-type resolution from C_4 and C_3 species (Li *et al.*, 2010; Majeran *et al.*, 2010) providing the opportunity to reveal the regulatory and interaction networks responsible for each trait via coexpression analysis, systems modelling and testing, comparative expression analysis, and similar methods. This review will update the current understanding of C_4-optimized leaf anatomical features and will describe progress in using systems approaches to advance this understanding to a mechanistic level that explains the regulation of anatomy in C_4 patterns. More comprehensive descriptions of C_4-related anatomical features can be found in other reviews and surveys (Dengler and Nelson, 1999; Muhaidat *et al.*, 2007; Nelson, 2010).

Venation pattern and C_4 anatomy

C_4 plants exhibit a high density of leaf venation and a correspondingly small interveinal distance and cell number (Hattersley and Watson, 1975; Prendergast *et al.*, 1987; Dengler and Nelson, 1999; Ueno *et al.*, 2006; Muhaidat *et al.*, 2007). The venation is the framework for Kranz anatomy (Hattersley, 1984; Dengler *et al.*, 1994; Dengler and Nelson, 1999; Dengler and Taylor, 2000). In most C_4 plants, PCA and PCR cells are organized in zones concentric with the veins, regardless of the clonal derivation of these cells in a particular species. The establishment of a vein centre precedes the specialized anatomical and physiological differentiation within that leaf region, making it likely that local cell division and differentiation are influenced by radial position relative to veins.

The organization of functions and cell types around C_4 veins may be homologous to the radial organization of the stem and root. The genetic effectors of the radial organization of xylem and phloem in the stem (e.g. Class III HD-zip transcription factors) appear to have evolved into the adaxial/abaxial polarity system of leaves (Emery *et al.*, 2003; Izhaki and Bowman, 2007). However, similar signals may be distributed radially around incipient vascular sites to guide the differentiation of BS and M cells in successively distant zones (Langdale *et al.*, 1988; Langdale and Nelson, 1991; Brutnell and Langdale, 1998). In this view, the leaf venation pattern is developmentally the first manifestation of a comprehensive local regulation that spatially organizes other aspects of C_4 leaf development. Local signals emanating from vascular centres might include hormones such as auxin, cytokinin, and brassinosteroids, as well as small RNAs, peptides, transcription factors, and metabolites. In the *Arabidopsis* root, the differentiation of radially arranged cell types such as the endodermis is guided by regulatory circuitry that includes auxin, small RNAs, and networks of transcription factors that produce radially distinct information at varying distance from the vascular centre (Birnbaum and Benfey, 2004; Petricka and Benfey, 2008; Iyer-Pascuzzi and Benfey, 2009). Given this similarity, it is intriguing to speculate that the regulatory circuitry that produces the leaf BS and its surrounding diffusion barrier of suberin in C_4 grasses is homologous to that producing the root endodermis and its surrounding suberin-rich Casparian strip.

Ontogeny of leaf venation patterns

The ontogeny of leaf venation has been described in several monocot and dicot C_4 species (Dengler and Dengler, 1990; Bosabalidis *et al.*, 1994; Dengler *et al.*, 1997; Nelson and Dengler, 1997; Sud and Dengler, 2000; Dengler and Kang, 2001; McKown and Dengler, 2007). Hierarchical leaf venation patterns appear progressively during leaf development, co-ordinated with leaf initiation and growth (Nelson and Dengler, 1997; Dengler, 2001). Successive vein orders are initiated in co-ordination with the increase in cell number and leaf area in the blade (Dengler and Kang, 2001; Kang and Dengler, 2002; Kang *et al.*, 2007). The pattern of venation appears to be influenced by the shape of the blade; treatments or mutations that limit vascular development are generally associated with a reduction in the blade (Mattsson *et al.*, 1999; Sieburth, 1999).

The control of the differentiation of tracheary elements, phloem cells, and other vascular cells in the continuous files that make up vascular bundles is understood with some mechanistic detail. Much experimental evidence supports the hypothesis proposed by Sachs (Sachs, 1991) that the formation of veins follows dynamic sink–source relationships that orient polar auxin transport (PAT) within a developing tissue. In this view, the ontogeny of venation pattern reflects the dynamic shifts in paths linking auxin sources and sinks during leaf growth (Aloni, 2001; Aloni *et al.*, 2003; Berleth *et al.*, 2000*b*). The paths become channelled in a self-reinforcing fashion that resists lateral spreading, through the induction of vascular cell differentiation by auxin (Sachs, 1991; Berleth and Mattsson, 2000; Berleth *et al.*, 2000*a*). The molecular agents that facilitate PAT and the vascular cell differentiation in its path, including PIN proteins, endomembrane traffic components, auxin-responsive transcription factors and target genes, and a variety of other factors, have been well described in several recent reviews (Fukuda, 2004; Caño-Delgado *et al.*, 2010; Hirakawa *et al.*, 2010).

Vein pattern formation and vascular cell differentiation have been characterized in several C_4 species, including the grasses, maize (Bosabalidis *et al.*, 1994), *Stenotaphrum secundatum* (Sud and Dengler, 2000), and *Arundinella hirta* (Dengler *et al.*, 1997) and several species of *Flaveria* (McKown and Dengler, 2007) and Cyperacea (Ueno *et al.*, 1989; Soros and Dengler, 1998, 2001). *Arundinella hirta* and certain other members of the genera *Arundinella, Garnotia, Microstegium*, and *Arthraxon* are of particular interest with

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regard to the influence of the vein patterning process on C_4 differentiation, since they lack the high density of minor veins found in most C_4 grasses, and instead form files of 'distinctive cells' (DC), PCR cells that co-operate with M cells but do not surround veins (Dengler *et al.*, 1990, 1995, 1996, 1997; Dengler and Dengler, 1990; Ueno, 1995; Wakayama *et al.*, 2003, 2006). Since files of DCs occupy sites that, in related species, develop into veins surrounded by photosynthetic BS cells, positional signals for vein formation may directly guide the formation of a provascular derivative cell type, the BS, in DC species.

Models of vein pattern formation

Most observed features of leaf venation patterns (closed loops, freely ending veinlets, parallel veins) and their ontogenetic sequence of appearance can be produced by computational models. Natural patterns can be self-organized by the properties of PAT in the context of the patterns of cell proliferation and expansion for the particular leaf and species. Most of these models assume that there exists feedback regulation between the flow of auxin (or other effector) and the localization of its carriers and facilitators (Meinhardt, 1996; Burton, 2004; Rolland-Lagan and Prusinkiewicz, 2005; Dimitrov and Zucker, 2006; Feugier and Iwasa, 2006; Fujita and Mochizuki, 2006*a*, *b*; Scarpella *et al.*, 2006; Berleth *et al.*, 2007). In *Arabidopsis* leaves, the observed patterns of PIN protein orientations and cell proliferation support the view that procambial differentiation follows the patterns of auxin flux or concentration (Donnelly *et al.*, 1999; Mattsson *et al.*, 1999, 2003; Sieburth, 1999; Scarpella *et al.*, 2006; Kang *et al.*, 2007).

High density of C_4 leaf venation

C_4 leaves exhibit a higher density of venation than their C_3 relatives, often producing a 1:1 ratio of surrounding BS and M cells that is favourable for 2-cell C_4 metabolism. Within taxonomic groups that include related C_4 and C_3 species, the C_4 species have a significantly smaller leaf interveinal distance and cell number than their C_3 relatives (Crookston and Moss, 1974; Hattersley and Watson, 1975; Kawamitsu *et al.*, 1985; Ueno *et al.*, 2006; McKown and Dengler, 2007; Muhaidat *et al.*, 2007). The correlation of C_4 biochemical properties with vein density among C_3, C_4, and C_3–C_4 intermediate species in genera such as *Flaveria* support the view that increased vein density is a 'precondition' for the evolution of other elements of C_4 physiology (McKown and Dengler, 2007; Sage, 2004). High vein density and reduced interveinal cell count are well correlated with degree of 'C_4 -ness' among the intermediate and C_4 species, and are predicted to provide physiological enhancements to C_4 species beyond a favourable BS/M ratio (Helliker and Ehleringer, 2000; Ogle, 2003). However, there are few experimental or genetic studies that demonstrate the consequence of altering vein density in C_4 species. C_4 species that achieve a high density of venation appear to do so via a heterochronic regulation of the existing machinery for vein formation: a persistence of the initiation of minor veins beyond the developmental time at which it ceases in their non- C_4 relatives. This occurs without a substantial difference in the blade shape or overall change in cell division patterns. Vein density is a plastic character that varies with environment, and light intensity in particular, in some species (Adams *et al.*, 2007).

Patterns of cell proliferation, expansion and differentiation in C_4 leaves

C_4 species that localize PCA and PCR functions in separate cell types (e.g. M and BS) have evolved a variety of ways of organizing and producing these cells, summarized in a number of reviews (Dengler and Nelson, 1999; Soros and Dengler, 2001; Muhaidat *et al.*, 2007). In maize, the PCR functions are specialized in the BS immediately surrounding the vascular bundles. The BS specializations include increased cell volume, increased chloroplast size and number, asymmetric arrangement of cytoplasmic contents, and a surrounding extracellular barrier to gas diffusion (e.g. suberin). Mutations have been described that influence plastid differentiation in the BS (Langdale and Kidner, 1994; Roth *et al.*, 1996; Hall *et al.*, 1998; Brutnell *et al.*, 1999; Cribb *et al.*, 2001), but none that affect formation of the BS itself. Numerous PD link adjacent BS and M cells, facilitating the intercellular diffusion of metabolites. Alternatives to the Kranz scheme have evolved to support C_4 metabolism, such as the occurrence of single-cell compartmentalization of PCA and PCR functions in the Chenopodiaceae (Edwards *et al.*, 2004), the formation of BS-like 'distinctive cell' files in certain grass species (Dengler *et al.*, 1996) and the radially extended files of BS cells in the maize *tangled* mutant (Jankovsky *et al.*, 2001). This suggests that a broad range of cellular arrangements can provide anatomies that confer C_4 advantages as long as the PCA and PCR functions are sufficiently compartmentalized.

Barriers and connections: suberin lamellae and plasmodesmata

In two-cell C_4 schemes, the PCR cells (usually BS) are surrounded by a diffusion barrier that enables cells to exclude oxygen and to retain carbon dioxide, and are joined to neighbouring PCA (mesophyll) and vascular cells by abundant PD. Mechanically isolated BS strands from C_4 plants are capable of limiting gas diffusion and of selective permeability to metabolites (Weiner *et al.*, 1988; Furbank *et al.*, 1989, 1990; Jenkins *et al.*, 1989). The diffusion barrier surrounds the cell wall and can consist of lamellae of suberin, the complex three-dimensional polyester that acts as an apoplastic water/solute barrier in roots, and in a variety of other roles (Franke and Schreiber, 2007; Graca and Santos, 2007; Kolattukudy, 2001). Recently, a suberin

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biosynthetic pathway based on products of numerous candidate genes, encoding membrane-associated P450 and fatty acid elongation complexes and other enzymes, was proposed for *Arabidopsis* (Franke and Schreiber, 2007), providing a basis for further genetic/genomic analysis of suberin biosynthesis and its regulation. The apparent similarity of the suberin-surrounded leaf BS and root endodermis is striking and suggests that these features may have common regulatory and biochemical origins.

The PD of C_4 leaves appear to be qualitatively similar to those present in C_3 leaves and elsewhere in the C_4 plant, based on appearance and range of size-exclusion-limits (Evert *et al.*, 1977, 1996; Robinson-Beers and Evert, 1991*a*, *b*; Botha, 1992; Botha *et al.*, 1993). At the BS/M junction they are abundant enough to facilitate the metabolite fluxes of the C_4 pathway (Weiner *et al.*, 1988; Robinson-Beers and Evert, 1991*b*; Sowiński *et al.*, 2008). Although an increasing number of functions of PD in plant development and physiology have been studied in detail, including selective intercellular traffic of viruses, transcription factors, RNAs, and other signals (Haywood *et al.*, 2002; Cilia and Jackson, 2004; Gallagher and Benfey, 2005; Kim, 2005; Hofmann *et al.*, 2007; Kim *et al.*, 2007; Bayer *et al.*, 2008; Maule, 2008; Lucas *et al.*, 2009; Amari *et al.*, 2010; Ehlers and van Bel, 2010; Xu and Jackson, 2010), it has proved exceedingly difficult to analyse the biogenesis and regulation of PD in more than a descriptive manner. Although it has long been clear that PD include a desmotubule core continuous with the ER of the adjoined cells, cytoplasmic sleeve, and a plasma membrane continuous with that of adjoined cells, only recently have specific proteins been associated with PD (exclusive of viral movement proteins), including a RabGTPase, centrin, calreticulin, and others (Cilia and Jackson, 2004; Maule, 2008; Lucas *et al.*, 2009; Xu and Jackson, 2010). The formation, location, and selectivity of PD is highly regulated during the development of embryos and seedling organs, and it is reasonable to assume that the same or similar systems regulate the PD-producing gene/protein networks to produce the abundance, location and qualities of BS/M PD for C_4 metabolism. The abundance of PD appears to be a developmentally regulated and plastic character that responds to environmental factors such as light intensity at the time of leaf development (Ormenese *et al.*, 2000; Roberts *et al.*, 2001; Amiard *et al.*, 2005; Adams *et al.*, 2007).

Systems analysis and the grass leaf developmental gradient

The leaf traits supporting C_4 biology appear to result from quantitative and spatial adjustments during leaf development in the regulation of traits that exist in C_3 plants. That is, C_4 biology evolved primarily through the re-regulation of existing gene networks and traits, rather than through the evolution of novel genes and traits. This view is consistent with the numerous independent lineages in which the conditions or stepwise 'preconditions' for C_4 physiology were achieved in evolution, since relatively few regulatory factors can re-pattern entire downstream networks producing the traits. The efficiency of C_4 biochemistry has been enhanced by the evolution of optimized isoforms of C_4 enzymes such as PEPC (Akyildiz *et al.*, 2007; Gowik *et al.*, 2004; Wang *et al.*, 2009), but the repeated evolution of C_4 schemes is likely to have relied on altered patterns of the regulators farther upstream (Westhoff and Gowik, 2010).

Recently, systems approaches have become feasible for the analysis of quantitative traits such as C_4 anatomy (Long *et al.*, 2008; Moreno-Risueno *et al.*, 2010). This includes the gathering of systems inventories at the whole transcriptome, proteome, metabolome, and phenome levels, and computational approaches that model these data within networks of regulation, interaction, expression, and metabolism (Hartwell *et al.*, 1999; Barabasi and Oltvai, 2004; Yu *et al.*, 2006; Zhu *et al.*, 2007). This approach is promising for features such as C_4 anatomical specializations, for which the genetic basis is hierarchical and/or redundant (Yu *et al.*, 2008). With system-wide molecular inventories, the limited number of genes and proteins now associated with anatomical traits can serve as starting points for gene discovery, molecular interactions and modelling that will eventually explain the regulation and production of the trait. A recent transcriptome analysis that compares leaves of mature *Cleome* (C_4) with *Arabidopsis* (C_3) provides RNA inventories of this type that can be correlated with features of C_4 metabolism in mature leaves (Bräutigam *et al.*, 2011).

For systems approaches to be fruitful for gene discovery and regulatory modelling for C_4 anatomy, at least four criteria must be met: (i) samples must include the developmental stages during which anatomical features are produced, (ii) the datasets must be comprehensive in coverage and depth to provide an accurate inventory of all interacting components, (iii) biological sampling must be rigorous and reproducible in its attention to developmental, circadian, metabolic, and environmental status, and (iv) the inventories of different systems (RNAs, proteins, metabolites, anatomical phenotypes) must be derived from biological materials with the exactly corresponding state for each of these 'data channels'. The developing grass leaf provides a base-to-tip gradient of successive developmental stages that can serve as a source of rigorously comparable biological systems data. Along this gradient, one can observe the appearance of cellular, physiological, and molecular events at distinct and reproducible sites that correspond to distinct developmental stages. From base to tip are arranged successive cellular zones of division, expansion/elongation, and differentiation. These physiological zones indicate the transition from respiratory (sink) to photosynthetic (source) metabolism and the sequential appearance or disappearance of other anatomical features and biochemical activities that correspond to stages in the development of the mature leaf. This makes it possible to sample and compare leaf regions or even specific cell types that are at distinct stages of development and activity. This developmental gradient in young grass leaves has been exploited since the 1970s to characterize the succession of

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cellular events and photosynthetic components and activities that produce the mature autotrophic leaf (Leech *et al.*, 1973; Robertson and Laetsch, 1974).

Recently, successive base-to-tip regions of developing leaves of maize (C_4) have been sampled with stage-specific and cell-type resolution to provide transcriptomic, proteomic, anatomic, metabolic, and physiological data that can be rigorously compared and modelled (Li *et al.*, 2010; Majeran *et al.*, 2010). These studies share the same biological source material and staged samples, making possible the correlation of gene regulation, protein accumulation, modification and interaction, anatomical output, metabolic consequences or accompaniment for each state. Although these channels of exactly corresponding RNA, protein, metabolic, anatomical, and physiological data have not yet been fully exploited by detailed mining, correlation, and modelling, they already provide a remarkably detailed view of the ontogeny of the leaf and have produced some striking observations related to C_4 anatomical features.

(i) The maize leaf transcriptome exhibits dramatic developmental dynamics (Li *et al.*, 2010). Of the ~80% of the annotated genome that is expressed at some time during leaf development, 64% is dynamic in expression from base to tip; 56% of transcripts with introns are alternatively spliced through the developmental gradient. The observed classes of developmental kinetics provide a coexpression and correlation resource for associating related genes. The study employed laser microdissection to obtain developmentally staged transcriptome data from isolated BS and M cells, so further cell-specific coexpression correlation is possible. At each stage, BS and M cells express ~15 000 genes, of which ~1000 are exclusively expressed in one cell type or the other. The genes for 938 transcription factors (TFs) are differentially expressed during maize leaf development, many of them with cell-type specificity. The transcripts for many putative regulatory and pattern-forming TFs with potential roles in C_4 anatomy are only expressed early in development and are not present in the transcriptome of the mature C_4 state, while genes associated with photosynthetic differentiation and C_4 carbon fixation are only expressed late in development (Fig. 1). A striking observation is that regulatory hierarchies appear to be resolved in the developmental space represented by the leaf gradient. The putative master regulators of pathways such as those for suberin synthesis are first expressed at positions nearer the leaf base than are their regulatory TF and downstream enzyme-coding targets. Current published data only resolve four representative stages in depth; work is underway to provide transcriptome data for the full developmental gradient in 15 steps (T Brutnell, personal communication).

(ii) The proteomic views of the same developmental stages are largely consistent with the transcriptome data for the proteins that can be detected, both with regard to timing and dynamics of accumulation (Majeran *et al.*, 2010). The proteomic data are particularly rich in information about the developmental onset of metabolic pathways. A striking observation is that Calvin Cycle and C_4 pathway proteins accumulate co-ordinately; there is no evidence of an early C_3 state followed by a mature C_4 state. There are many unknown proteins that share the detailed expression dynamics of known C_4-related proteins, making them logical candidates for further study.

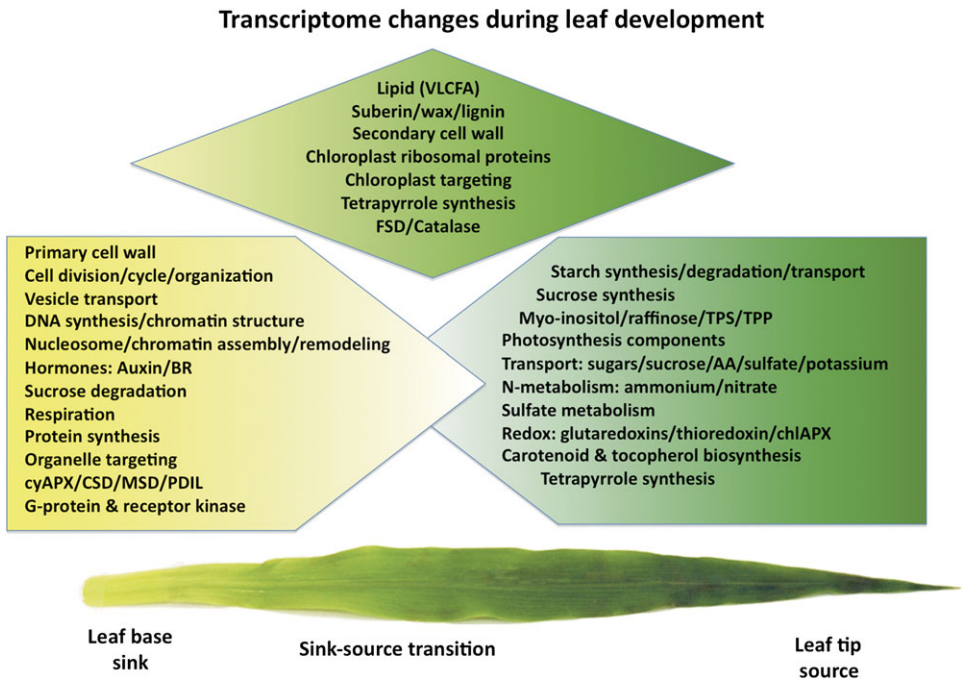

Fig. 1. Developmental sequence of basic processes in plant development, metabolism and physiology along the maize leaf gradient, based on dynamics of gene transcripts detected in stage-specific transcriptomes obtained by the Illumina RNA-seq method. Adapted from Li *et al.*, 2010 with kind permission from the Nature Publishing Group; ©The author.

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(iii) The anatomical analysis of the same developmental stages by light and electron microscopy provides the means to correlate the comprehensive RNA and protein data with specific anatomical features (Majeran *et al.*, 2010). Evaluation of the leaf gradient revealed no evidence of a 'C_3' anatomical state early in development. Kranz anatomy, with a well-developed bundle sheath, diffusion barrier, and BS-M plasmodesmata, is evident early. The site of the sink–source transition is an inflection point for numerous developmental processes. Prior to this point, most cellular 'infrastructure' is complete, including cell division and expansion, the formation of PD, an increase in plastid number, and other anatomical features. In the region of the sink–source transition, the formation of the suberin diffusion barrier and the dimorphic specialization of BS and M chloroplasts accelerates, reaching the mature anatomical state near the leaf tip. Secondary PD appear between BS and M in the more distal regions.

(iv) All of the inventories (RNA, proteins, anatomy) exhibit a clearly resolved developmental dynamic in young maize leaves (Li *et al.*, 2010; Majeran *et al.*, 2010). From base to tip are displayed the transcripts and proteins for cellular infrastructure and pattern formation (base region, respiratory metabolism), followed by synthesis of plastid components and secondary wall features (sink–source transition region), followed by building of photosynthetic function and capacity (distal maturing region) (Fig. 1). This developmental stage resolution for molecular inventories, when combined with cell-type resolution, provides a means for sorting out members of regulatory, signalling, biosynthetic, and metabolic pathways from candidates provided by often redundant gene families based on their coexpression with other pathway members. For C_4 anatomy, it provides the means to associate features such as vein density with a limited number of candidate TFs and structural genes.

Additional resources and tools that will accelerate the analysis of C_4 anatomy are appearing rapidly. Correlated developmental-stage and cell-type specific molecular and anatomical inventories are being collected for rigorously sampled rice (C_3) leaf gradients. The comparison of maize and rice inventories from the same stages and cell types should highlight common networks of genes that are distinctly regulated in the C_3 and the C_4 grasses. This should provide candidates for genes and proteins responsible for anatomical features that differ quantitatively or qualitatively at corresponding developmental times. As additional correlated 'channels' of information are obtained from the maize and rice leaf gradients, including small RNAs, various classes of metabolites and additional subcellular proteomic fractions, the potential for associating more processes and traits with detailed molecular mechanisms increases. For regulatory and metabolic models to be validated, their predictions must be tested in a C_4 plant. The NADP-ME-type C_4 plant *Setaria viridis* has numerous potential advantages for this, since its genome is sequenced and it shares the leaf developmental patterns and C_4 scheme of maize, yet it can be transformed and regenerated more rapidly (Brutnell *et al.*, 2010).

Acknowledgements

The author is grateful for many discussions with Drs Thomas Brutnell, Klaas van Wijk, Robert Turgeon, Qi Sun, Peng Liu, Pankaj Jaiswal, and the members of their laboratories, as well as with Drs S Lori Tausta and Neeru Gandotra. The author's work on C_4 biology and vein patterning is supported by US National Science Foundation awards DBI-0701736 and IOS-102118, respectively

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