Cell Lineage of Vein Formation in Variegated Leaves of the C₄ Grass

Stenotaphrum secundatum

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Received: 17 November 1999 Returned for revision: 26 January 2000 Accepted: 23 March 2000

Clonal analysis of variegated leaves of the C₄ grass, Stenotaphrum secundatum, indicates that invasions among meristematic layers occur during the organogenetic stage of leaf development, resulting in long, broad white and green stripes. These layer invasions cease prior to the second phase of leaf development when delimitation of leaf regions occurs. Vein precursors mostly arise during the second phase, so that procambial strand formation is superimposed on the lineage makeup of earlier-formed tissue. Anatomical evidence indicates that procambium arises through formative divisions within ground tissue of leaf primordia and that each strand is derived from a variable number (one–four) of ground meristem precursors. If a developing vein straddles the boundary between previously-formed green and white sectors, then the mature vein is half green and half white, reflecting its mixed cell lineage. In Stenotaphrum, 24.8% of the sectors observed were bounded by such ‘half veins’. The temporal relationship of layer invasion and tissue system delimitation in this species supports the view that positional signals are more important than lineage history in the determination of tissue type. However, analysis of planes of cell division in developing veins indicates, that, once formed, procambial strands are discrete lineage units that extend longitudinally by proliferative divisions. Thus, lineage restrictions may play an important role in the third stage of leaf development, differentiation of tissues and cells, which also includes the maintenance of cell identity.

Key words: C₄ photosynthesis, cell lineage, clonal analysis, leaf development, St. Augustine’s grass, Stenotaphrum secundatum, variegation, vein formation.

INTRODUCTION

The procambial precursors of the vascular tissue system become delimited early during leaf development (Nelson and Dengler, 1997). The development of leaves is characterized by three broadly defined, overlapping stages: (1) organogenesis; (2) establishment of leaf regional identity; and (3) tissue and cell differentiation (Sylvester et al., 1996). Precursors of all three tissue systems (dermal, ground and vascular) are delimited during stages 1 and 2. Precursors of the dermal and ground tissue systems are present at the time of leaf initiation from the flanks of the shoot apical meristem, since dermal and ground tissues are derived from the surface (L1) and subsurface (L2 and L3) layers of the meristem, respectively. In contrast, procambial strands, the first structural evidence of vascular patterning, are derived from ground tissue precursors well after leaf initiation. The underlying genetic mechanisms involved in the formation of leaf vascular pattern and the regulation of these processes are among the major unresolved issues of plant development.

Vascular pattern formation is also developmentally important because vascular tissue acts as a positional landmark that guides or influences the pattern of cell differentiation with adjacent tissue types (Cerioli et al., 1994; Freeling and Lane, 1994; Nelson and Dengler, 1997). Distinct cell types within the ground tissue system (such as fibres and photosynthetic parenchyma) and the dermal tissue system (such as trichomes and guard cells) are arranged in relation to the vascular system. Nowhere is this more apparent than in the leaves of grasses: specialized epidermal cell types and strands of fibres form long stripes that parallel the longitudinal array of veins. In C₄ grasses, each mesophyll cell is in direct contract with at least one of the parenchymatous bundle sheath cells surrounding the vascular tissue of the vein. The spatial arrangement of mesophyll and bundle sheath cells has functional significance since intermediate metabolites must diffuse rapidly between the two cell types for full operation of C₄ photosynthetic biochemistry (reviewed in Dengler and Nelson, 1999). This distinctive spatial relationship of mesophyll and bundle sheath tissue, along with the temporal pattern of cell-specific gene expression and enzyme accumulation in C₄ plants, led Langdale and Nelson (1991) to hypothesize that the developing vein is the source of an outwardly diffusing signal that induces specific developmental responses in adjacent bundle sheath and mesophyll tissues. Thus, knowledge of the lineage relationships between vascular and adjacent tissues may also be important for understanding cell to cell communication during tissue differentiation.

The leaves of maize (Zea mays L.) and other grasses are particularly well suited for addressing developmental questions about tissue pattern formation because of the longitudinal arrangement of cells in all tissues. Leaf organogenesis (stage 1) involves the formation of a primordium that encircles the shoot apical meristem before
undergoing elongation growth (Sharman, 1942; Sylvester et al., 1990; Timmermans et al., 1998). Cell divisions are first distributed uniformly throughout the primordium; then, as development proceeds, tissues at the tip of the leaf cease dividing, and cell division and elongation are gradually restricted to the leaf base (Sharman, 1942; Stein and Steffenson, 1959; Sylvester et al., 1990). Early in stage 1 of leaf development, the orientation of these cell divisions is primarily longitudinal, thus contributing to the increase in leaf width that accommodates the increasing diameter of the stem axis. These longitudinal divisions also play a formative role in producing the appropriate spatial arrangement of internal leaf tissues and their component cells (Dengler et al., 1985). During stages 2 and 3 of leaf development, cell divisions in the distal portion of the leaf are predominantly horizontal in orientation and serve to perpetuate cell files originating in the zone of formative divisions at the leaf base. Cell differentiation (stage 3), occurs basipetally along these cell files. Thus grass leaves display a longitudinal gradient of development in all three tissue systems, making it possible to relate mature cell position to the pattern of formative and proliferative divisions.

Despite the predictable arrangement of dermal, ground and vascular tissue systems, and the formation of conspicuous cell files in organs such as roots and grass leaves, clonal analyses have led to the conclusion that cell lineage is of limited importance in the determination of tissue patterns (e.g. Stewart, 1978; Langdale et al., 1989; Poethig and Szymkowiak, 1996; Brutnell and Langdale, 1998; Szymkowiak and Sussex, 1998). The distribution of genetically marked sectors in leaves of both dicotyledons and monocotyledons indicates that cells from one layer invade adjacent layers (Stewart, 1978; Stewart and Dermen, 1979; Poethig, 1984; Dawe and Freeling, 1991; Szymkowiak and Sussex, 1998). Invading cells change their fates to adopt the cellular identity characteristic of the new location, suggesting that positional signals override lineage relationships in determining cell fate. Clonal analyses have rarely been applied to vein formation, but in one study of six variegated maize mutants, Langdale et al. (1989) found evidence for mixed vein lineages, i.e. veins for which the bundle sheath cells on one side of the vein appeared to be more closely related clonally to adjacent mesophyll than to the rest of the bundle sheath. The lack of correspondence between lineage and tissue pattern contradicted results from an earlier analysis of planes of cell divisions in developing grass leaf veins in which Dengler et al. (1985) concluded that procambial strand lineages remained distinct from those of surrounding mesophyll tissue. This apparent contradiction has been partly resolved by examination of vein initiation within the zone of formative cell division of young maize leaves (Bosabalidis et al., 1994). These authors noted that some veins appeared to arise from groups of precursor cells; if precursors within this group were derived from distinct lineages, the resultant vein would represent a mixed lineage history.

In our study we have integrated the approaches of clonal analysis of mature leaves with an anatomical analysis of developing leaves in order to assess the role that cell lineage plays in leaf vein formation in the variegated C₄ grass *Stenotaphrum secundatum*. This species is native to subtropical and tropical America and is widely used in the southeastern United States as a turf grass (Jones, 1985). The variegated form of *Stenotaphrum secundatum* was included in an earlier survey of the clonal makeup of common variegated monocot species (Stewart and Dermen, 1979). The striping defect of the species is thought to be the result of a GWG (green L1, albino L2, and green L3 layers) periclinal chimera within the shoot apical meristem (Stewart and Dermen, 1979). Photosynthesis has been well studied in both the green and variegated forms (Jones, 1985; Suzuki et al., 1986). In the variegated form, albino tissue lacks ribulose-1,5-bisphosphate carboxylase activity, has reduced phosphoenolpyruvate activity, and displays rudimentary plastid ultrastructure (Suzuki et al., 1986). *Stenotaphrum* belongs to the same subfamily (Panicoidae) and possesses the same C₄ biochemical subtype (NADP-malic enzyme) as maize, thus allowing for a direct comparison to be made between a genetic model organism and a related species. Such comparisons can provide a test of generalizations made based on observations of the genetic model alone (Kellogg and Birchler, 1993). In this study we use clonal analysis of this GWG periclinal chimera to infer the lineage relationships between the precursors of vascular and adjacent ground tissue at the time of vascular pattern formation. We combine clonal analysis and developmental anatomy to investigate lineage relationships between vascular and adjacent tissues during the cell proliferation stage of growth. We also characterize phenotypes of green and white sectors to test for evidence of developmental interaction between lineages.

**MATERIALS AND METHODS**

**Plant material and growth conditions**

Plants of *Stenotaphrum secundatum variegatum* (Walter) Kunze (St. Augustine’s grass; Poaceae; subfamily Panicoidae) were maintained clonally under greenhouse conditions (approx. 21°C, 500 μmol m⁻² s⁻¹ natural and supplementary light, 16 h days) at the University of Toronto, Canada.

**Phenotypic characterization of clonal sectors**

In order to characterize the phenotypes of white and green sectors, transverse sections were made from the midregions of 25 fresh, unfixed leaf blades using a Hooker microtome. These sections were mounted in 50 % glycerol on glass slides and observed with a Reichert Polyvar microscope using both bright field and fluorescence (exciter filter 450–495 nm; barrier filter 520 nm) optics. Epidermis from white and green sectors was isolated from both the adaxial and abaxial sides of ten different leaves by scraping away underlying mesophyll tissue. These preparations were observed on a confocal laser scanning microscope (Zeiss LSM 510) to determine whether guard cell chloroplasts contained chlorophyll.

Leaf blade tissue from an additional ten leaves was prepared for further light and electron microscopy. Tissue was fixed in 4 % paraformaldehyde and 0-2 %
glutaraldehyde with 0.05% phosphate buffer and 0.1 M sucrose, post-fixed in 1% OsO₄ for 2 h, dehydrated through a graded acetone series, and embedded in Spurr’s resin (JB EM, Dorval). For light microscopy, semi-thin (4 μm) sections were made with a Sorvall Porter Blum MT-2 ultramicrotome and stained with 0.05% Toluidine Blue O in 0.1% sodium carbonate buffer. For electron microscopy, thin sections (70–80 nm) were made with a Reichert ultramicrotome, mounted on formvar-coated grids, stained with uranyl acetate and lead citrate, and observed using a Philips 201 transmission electron microscope.

In order to further characterize the phenotype of white and green sectors, cellular localization of bundle sheath-specific ribulose-1,5-bisphosphate carboxylase (RuBPCase) and mesophyll-specific phosphoenolpyruvate carboxylase (PEPCase) was determined by immunolocalization experiments. Leaf blade tissue was fixed in ethanol : glacial acetic acid (3 : 1), dehydrated through an ethanol–tertiary butyl alcohol series, and embedded in Paraplast (Fisher Scientific, Toronto). Thick sections (7 μm) were made using a Spencer AO rotary microtome and mounted on poly-L-lysine coated slides. The antisera used were raised to RuBPCase and PEPCase. RuBPCase was isolated and prepared as described in Dengler et al. (1995), and the PEPCase antibody, provided by Dr. J. Berry (State University of New York, Buffalo), was prepared as described by Wang et al. (1992). The reagents used for immunolocalization were from the Immunoselect® Immunocytochemistry, ELISA, and Immunoblotting System (Gibco BRL, Burlington, ON, USA) and the protocol followed was that described in Dengler et al. (1995). A total of ten leaves were examined in four replicate experiments. Control slides were included with each experiment: for RuBPCase localization, control preimmune serum was used in place of the primary antibody, and the PEPCase localization, the primary antibody (but not secondary antibody or colour reagents) was omitted from control slides.

Clonal analysis

Transverse sections of 25 leaves were observed to characterize the type and extent of clonal sectors. Presence or absence of chlorophyll was scored for bundle sheath cells and adjacent mesophyll cells for each of the approx. 100 longitudinal veins within the leaf blade. Sector types were defined when the same pattern of chlorophyll distribution was associated with three or more adjacent veins. Patterns that spanned fewer than three veins were very diverse and probably represented latitudinal invasions within layers. The width of each sector (number of veins spanned) was recorded for each leaf. Sectors from an additional ten leaf blades were analysed at 10 equidistant points from base to apex and the longitudinal and latitudinal extent of each sector was mapped.

Leaf development and vein ontogeny

In order to relate clonal sector patterns to early events in leaf development, shoot apices were examined by scanning electron microscopy (SEM) and serial sections were observed by light microscopy. For SEM, tissue was dissected, fixed in 70% FAA (formalin : 70% ethanol : glacial acetic acid; 1 : 18 : 1), dehydrated through an alcohol series, critical point dried, coated with gold, and observed using a Hitachi S2500 scanning electron microscope. For serial sections, six shoot tips were fixed and embedded in Spurr’s resin as described above. The number, arrangement, and appearance of vascular bundles were determined from serial sections of each shoot tip. Inferences about cell division patterns were based on the orientation of mitotic figures and on the relative thickness of shared cell walls: i.e. two cells sharing a thin common wall were judged to be more closely related clonally than two cells sharing a thicker wall (Dengler et al., 1985; Sylvester et al., 1990; Bosabalidis et al., 1994). Mitotic figures were counted in serial sections of the three youngest leaves from the six shoot tips and scored according to whether the two derivatives were in the procambium, the ground meristem, or one in each.

RESULTS

Leaf variegation

White and green stripes of varying widths extended for the entire length of the leaf blade; in addition, some leaves had macroscopically visible, narrow pin stripes that extended less than half the blade length (Fig. 1). Most leaves had a broad white stripe that included the midvein region. Occasionally shoots reverted to all green leaves, while shoots with all white leaves were never observed. Stripping also occurred in the leaf sheath and in stem internodes, but was more difficult to discern, so clonal analysis focused on leaf blades only. Leaf stripping differed between successive leaves on an individual shoot, indicating that sectors arose independently in each leaf.

Phenotypes of green and albino sectors

*Stenotaphrum* leaf blades displayed the distinctive features of Kranz anatomy typical of species utilizing C₄ photosynthesis: close spacing of longitudinal veins, specialized bundle sheath cells with large, conspicuous chloroplasts surrounding each vein, and a spatial arrangement of photosynthetic tissues in which most mesophyll cells are in direct contact with a bundle sheath cell (Figs 2, 3; Dengler and Nelson, 1999). In this study, we recognized two categories of longitudinal veins: (1) major veins with conspicuous metaxylem vessels (equivalent to maize midvein and laterals; Sharman, 1942; Fig. 2); and (2) minor veins without conspicuous metaxylem vessels (equivalent to maize small and intermediate veins; Sharman, 1942; Figs 2, 3). Typically, three layers of chloroplast-containing mesophyll tissue were present, but the layered arrangement was partially obscured by cell expansion patterns (Figs 2, 3). In addition, a fourth ground tissue layer of non-chlorenchymatous hypodermal cells was present on the abaxial side of the leaf (Figs 2–5). Some cells of the abaxial mesophyll differentiated as hypodermal cells, thus bringing hypodermal-like cells in direct contact with bundle sheath cells.
FIGS 1–7. Leaf variegation in *Stenotaphrum secundatum*. Fig. 1. Leaf blades showing wide median white stripe and several narrow stripes that extend the entire length of the blade (left arrow) or extend through only part of the blade (right arrow). Bar = 1 cm. Fig. 2. Transverse section of fresh leaf blade showing a major and several minor longitudinal veins and the boundary between green and white sectors (arrow). Fig. 3. Transverse section of fixed leaf blade showing boundary between white and green sectors passing through minor vein (unlabelled arrow). Note chloroplasts in bundle sheath and mesophyll cells of green sector and osmiophilic bodies in middle mesophyll layer in both green and white sectors. Fig. 4. Transverse section of fresh leaf blade with type V sector (between arrows). Fig. 5. Chlorophyll fluorescence of same tissue. Fig. 6. Confocal image of stomata from abaxial epidermis of green sector. Fig. 7. Confocal image of stoma from abaxial epidermis of white sector. Bars = 25 μm (Figs 2–5); 1 μm (Figs 6, 7). BS, Bundle sheath; C, chloroplast; H, hypodermis; M, mesophyll; O, osmiophilic body; S, stomate; X, major vein.
Clonal analysis of vein formation

Based on chlorophyll distribution patterns, six sector types were observed, although the frequency and extent of these varied considerably. Type I sectors, in which all three mesophyll layers were green, were the most frequent sector type (42.0% of all sectors scored in 25 leaves; Figs 17, 18, 25, 26A). Type I sectors spanned an average of 13 longitudinal veins (range three–32 veins wide) and typically extended the full length of the leaf blade (mean = 82% of blade length, n = ten leaves) (Figs 26B, C). Type II sectors, in which all three mesophyll layers were albino, were also frequent (27.8%) and formed the widest sectors, spanning a mean of 23.9 veins (range three–57) (Figs 17, 18, 25, 26A, B). Type II sectors tended to extend the full length of the blade, with only a few extending less than 50% (Fig. 26C), Sector type III (adaxial mesophyll layer albino) occurred at a lower frequency (7.7%) and tended to be narrow (mean of 4.4 veins spanned) and short (mean of 16.7% of total leaf length) (Figs 19, 25, 26). Sector types IV (abaxial mesophyll layer albino, Figs 20, 25) and V (both adaxial and abaxial mesophyll layers albino, Figs 4, 5, 25) occurred at low frequencies and were narrow, but tended to extend the full length of the leaf (Fig. 26). Type VI sectors, in which both adaxial and middle mesophyll layers were albino (Figs 21, 25), occurred at a higher frequency (17.7%), and were narrow (mean span of four veins) and long (mean of 70.3% of leaf length) (Fig. 26). Interestingly, we never observed the reverse of type VI (adaxial layer green), while Langdale et al. (1989) found that, in maize mutants, this pattern (adaxial layer green) was four-times more common than our type VI (abaxial layer green).

Leaf blade anatomy of white and green sectors was identical, except that white sectors lacked detectable chloroplasts (e.g. Figs 2, 4, 5, 11, 12, 17–24). The presence or absence of chloroplasts with appreciable amounts of chlorophyll was evident for mesophyll and bundle sheath cells using both bright field and fluorescence microscopy (Figs 4, 5, 17, 18). The intensity of the green colour of a stripe was roughly correlated with the number of chlorophyll-containing layers, but considerable variation occurred. For instance, green adaxial and middle mesophyll layers in combination with an albino abaxial layer resulted in a darker green stripe than the reverse. All the epidermal guard cells examined had normal chloroplasts. Plastids of guard cells overlying broad white stripes had reduced amounts of chlorophyll in comparison to those overlying green stripes (Figs 6, 7). This may explain earlier observations that Stenotaphrum guard cells from white stripes did not open and close in response to light (Li and Nothnagel, 1988).

In green sectors, both bundle sheath and mesophyll cells had large ovoid chloroplasts with extensive thylakoids, uniform stroma density, chloroplast ribosomes, and lipid bodies (Figs 9, 10). Thylakoid membranes formed granal stacks in mesophyll cells, but in bundle sheath cells this stacking was highly reduced; this is the characteristic pattern for the NADP-malic enzyme C₄ biochemical subtype (Dengler and Nelson, 1999). In white sectors, chloroplast development was arrested: plastids were small and flattened, and possessed few internal membranes, as previously reported by Suzuki et al. (1986). Both bundle sheath and mesophyll cell plastids contained dispersed vesicles, had irregular stromal density (often with large lightly stained areas), lacked chloroplast ribosomes, but did possess lipid bodies (Figs 11, 12). The albino plastids of Stenotaphrum closely resembled those described for the iiojap mutant of maize (Shumway and Weier, 1967; Walbot and Coe, 1979; Thompson et al., 1983). All cells examined were homoplasic, i.e. cells contained either all normal plastids or all mutant plastids.

The accumulation pattern of bundle sheath RuBPCase mirrored the distribution of chlorophyll: RuBPCase was present in bundle sheath cells of green sectors and was not detectable in white sectors (Figs 13, 15). The pattern for mesophyll PEPCase was less affected by the white striping: PEPCase accumulated in mesophyll throughout the leaf blade, but at lower levels in white compared with green sectors of the same leaf (Figs 14, 16). This may account, in part, for the reduced PEPCase enzyme activity observed by Suzuki et al. (1986) for white sectors of Stenotaphrum leaves.

### Fig. 8. Mean (± s.e.) stomatal frequency (A) guard cell length (B) in the adaxial and abaxial epidermis of green and white sectors.
Sector type V was observed only in association with minor veins, while all other types spanned both major and minor veins. Midveins were either all white (sector type II) or formed a ‘half vein’ between sector types I and II.

Overall, we examined sector patterns in 35 leaves. We scored a total of 241 sectors that spanned approx. 3070 veins and observed a total of 85 ‘half veins’ in which one half of the bundle sheath was white and the other half green (Figs 3, 22–24; Langdale et al., 1989). Some sectors were associated with two ‘half veins’ (one at each boundary). Overall, a strikingly large proportion of sectors (24.8%) were bounded by at least one ‘half vein’. In each case, the green half of the bundle sheath was adjacent to (mostly) green mesophyll and white bundle sheath to (mostly) white mesophyll, a pattern indicating that a closer ontogenetic relationship exists between bundle sheath and adjacent mesophyll cells than between bundle sheath cells on opposite sides of the vein. In Stenotaphrum, such ‘half veins’ were observed in leaf blade midribs, major (lateral) veins, and minor veins. All other sector boundaries passed between bundle sheath and adjacent mesophyll; sector boundaries that passed between two adjacent mesophyll cells were never observed.

Leaf development and vein ontogeny

The procambial strands of the leaf midvein and eight to ten major longitudinal veins were formed sequentially during leaf organogenesis (stage 1). Leaves first arose as a bulge on one side of the shoot apical meristem and then extended to encircle the stem (Fig. 27). The leaf primordium then elongated, and the conduplicate folding of the blade region became more conspicuous (Fig. 28). The first procambium to appear was that of the midvein, followed by the first set of major veins which were formed equidistantly between the midvein and leaf margin (Fig. 30A). Next, a second and third set of procambial strands were intercalated between pre-existing veins, forming a total of ten–12 major veins (Fig. 30A). Major vein procambial strands were initially discontinuous with the procambial strands of the stem below the level of leaf attachment; subsequent basipetal extension connected leaf vasculature with the stem strands, as previously described for maize (Sharman, 1942), barley (Hordeum vulgare L.; Trivett and Evert, 1998) and Arundinella hirta (Thunb.) Tanaka (Dengler et al., 1997).

During stage 2 of development, leaves became longitudinally differentiated into a proximal thickened
sheath region and a distal thinner blade region (Fig. 29). Longitudinal cell files that had extended by proliferative divisions were conspicuous by this stage (Fig. 29). Formation of minor vein procambial strands occurred early during this stage. Eight to ten small veins were formed sequentially between each pair of adjacent large lateral veins in *Stenotaphrum*. Leaf width expansion occurred simultaneously with vein formation, so that new procambial strands were intercalated equidistantly between pre-existing strands (Fig. 30B). Minor veins fused with adjacent minor veins at the apex and base of the blade and did not extend basipetally into the nascent leaf sheath region; thus the leaf blade was distinguished internally from the leaf sheath region by a higher vein density.

In *Stenotaphrum*, major vein procambial strands could be distinguished from surrounding ground tissue by the absence of osmiophilic bodies. In leaf cross sections, major veins appeared to be derived from either one (Fig. 31), two (Fig. 31), three (Fig. 32), or four (Fig. 33) precursor cells. Derivation from two precursor cells was the most common configuration. Additional formative divisions within procambial strands rapidly obscured the presumed lineage relationships of the first-formed cells. Some of these divisions also led to the delineation of a peripheral layer around a major vein, the future bundle sheath (Fig. 34; Dengler *et al.*, 1985). Minor vein procambial strands arose within the middle layer of leaf tissues when the distal portion of the primordium consisted of five tissue layers (Figs 35, 36). Minor vein procambium appeared to be derived from either one (Fig. 35) or two (Figs 35, 36) precursor cells. Further formative divisions gave rise to a distinct peripheral layer of procambial tissue (Fig. 36).

Analysis of the planes of formative cell divisions associated with major and minor vein procambial strands indicated that, once formed, the lineages of vascular tissue and bundle sheath on one hand, and of mesophyll cells on the other remained distinct. Divisions that gave rise to two procambial precursors were most frequent (67 %, \( n = 112 \)) and those that gave rise to two ground meristem cells were less frequent (33 %). No mitotic divisions that straddled the procambium/ground meristem boundary were observed. Similar observations were previously obtained for a larger sample of grass species (Dengler *et al.*, 1985; Nelson and Dengler, 1992). The periclinal divisions that give rise to the abaxial hypodermal layer generally occur after the formation of minor veins (Fig. 35).
FIGS 17–24. Transverse sections of sector types in variegated leaves of *Stenotaphrum secundatum*. Fig. 17. Type I sector (left arrow) and type II sector (right arrow). Fig. 18. Same leaf viewed with fluorescence microscopy. Fig. 19. Type III sector (between arrows). Fig. 20. Type IV sector (between arrows). Fig. 21. Type VI sector (between arrows). Continuity of the sector is interrupted by differentiation of hypodermal-like cell in abaxial mesophyll layer. Fig. 22. Boundary between type I (left) and type II (right) sectors passing through a major vein, forming a green ‘half vein’ and a white ‘half vein’. Fig. 23. Boundary between type IV (left) and type VI (right) sectors passing through a minor vein (arrow). Fig. 24. Boundaries passing through minor veins at arrows. These sectors span fewer than three veins and were not included in the analysis of sector types. H, Hypodermal cell. Bar = 40 µm.
Clonal sectors in *Stenotaphrum secundatum variegatum* are formed by invasions between genetically different layers of the shoot apical meristem. This process occurs early in leaf development, generally prior to the formation of leaf vein procambial strands. Procambial strands are formed sequentially in the nascent leaf primordium and arise from one to four laterally adjacent ground tissue precursor cells. A surprisingly high proportion (approx. 25%) of procambial strands straddle the boundary between green and white sectors, while the remainder (approx. 75%) lie adjacent to the clonal boundary. Once formed, procambial strands elongate through proliferative divisions that appear to respect lineage boundaries. Cell to cell communication across these boundaries has been postulated to be important in cell differentiation, as photosynthetic and other cell types differentiate according to their position in relation to the veins (Langdale and Nelson, 1991). Here we discuss the albino phenotype in relation to cell communication, the results of our clonal analysis, our conclusions about the role of cell lineage in vein formation, and the role of vein cell lineage in the differentiation of C₄ photosynthetic tissues.

**The albino phenotype in Stenotaphrum**

Albino plastids in both bundle sheath and mesophyll cells are small, have few internal membranes, lack chlorophyll, and lack chloroplast ribosomes. White bundle sheath chloroplasts do not accumulate RuBPCase holoenzyme at detectable levels, although C₄ mesophyll cells do not accumulate PEPCase in both white and green sectors. While the nature of the albino striping defect in *Stenotaphrum* is unknown, phenotypic characters of the plastids are similar to those of *iojap* in maize (Shumway and Weier, 1967; Walbot and Coe, 1979; Gadal et al., 1983; Thompson et al., 1983; Coe et al., 1988; Han et al., 1992, 1995) and to *albostriains* in barley (Hess et al., 1994). Both *iojap* and *albostriains* are recessive nuclear mutations that result in the loss of both chloroplast ribosomes and the ability to synthesize chloroplast DNA-encoded components of the photosynthetic apparatus (Wu et al., 1999). Certain other mutants, such as *bundle sheath defective2* (Roth et al., 1996) and *leaf permease1* (Schultes et al., 1996) of maize and

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**DISCUSSION**

**FIG. 25.** Diagram of sector types in variegated leaves of *Stenotaphrum secundatum*. Three layers of mesophyll tissue with vein in middle layer are shown.

**FIG. 26.** Variation among sector types I–VI in: frequency as a percentage of all sectors (A), mean width expressed as number of longitudinal veins spanned (B), and mean length as a percentage of total leaf blade length (C). *n* = 25 leaves, 169 sectors (A, B) or *n* = 10 leaves, 72 sectors (C).
immutans of Arabidopsis thaliana (L.) Heynh. (Wu et al., 1999) have pigment-deficient phenotypes that are more strongly expressed under high light conditions or that are alleviated with leaf age, suggesting that plastid phenotypes are the secondary consequence of photooxidation.

Although we did not carry out controlled experiments on the effects of differing irradiance levels on albino striping in Stenotaphrum, we never observed striping patterns that suggested environmental effects of greening of individual leaves, indicating that this albino mutation has a primary effect on chloroplast development.

Whatever its nature, the albino defect in Stenotaphrum has no effect on leaf anatomy; vein spacing and appearance, guard cell size and density, and organization of bundle sheath and mesophyll tissue are identical in white and green stripes. This is also the case for other albino mutants such as iojap, chloroplast mutator, argentia, and leaf permease1 of maize and ghost of tomato (Lycopersicon esculentum L.) (Thompson et al., 1983; Scolnik et al., 1987; Langdale et al., 1988; Schultes et al., 1996). These mutants illustrate that the developmental ‘decisions’ that determine tissue pattern and cell differentiation are uncoupled from those that regulate plastid differentiation. In contrast, there are other mutations that result in albino or reduced chlorophyll phenotypes and also have effects on cellular morphogenesis. For example, in the differentiation and greening mutant of Antirrhinum (Chatterjee et al., 1996), the defective chloroplasts and leaves—mutable mutant of tomato (Keddie et al., 1996), and the pale cress mutant of Arabidopsis (Reiter et al., 1994), mesophyll cell expansion and intercellular space formation are disrupted in sectors with aberrant chloroplasts, indicating that chloroplast-derived developmental signals are required for normal mesophyll cell differentiation.

In Stenotaphrum, the homoplastidic albino defect behaves in a cell-autonomous manner: boundaries between green and white tissues are sharp, with no intermediate cells. Three exceptions to this general observation are: (1) the reduced levels of chlorophyll in guard cells overlying a broad white stripe; (2) the reduced levels of PEPCase in mesophyll cells of white stripes; and (3) slightly reduced chlorophyll fluorescence observed in green mesophyll cells adjacent to albino bundle sheath cells, similar to that reported for certain striping mutants of maize (Langdale et al., 1987, 1989). These results indicate that, although cellular differentiation may be largely autonomous after cell

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**Figures 27–29.** Scanning electron micrographs of developing leaves of *Stenotaphrum secundatum*. Fig. 27. Shoot apical meristem with leaf primordia at plastochron 1 (P1) and plastochron 2 (P2), both representing ‘stage 1’ of leaf development. Fig. 28. Older plastochron 2 leaf (P2). Fig. 29. Plastochron 3 leaf (P3) representing ‘stage 2’ of development. Note longitudinal files of cells. Arrow indicates boundary between leaf sheath and blade. Bar = 100 μm.

**Fig. 30.** Diagrams representing the sequence of vein initiation in developing leaves of *Stenotaphrum secundatum*. A, Major veins, including midvein (1), first set of laterals (2), and subsequent sets of major vein procambial strands (3, 4). B, Three sequential sets of minor veins (a, b, c). Box in A represents region shown in B.
fate is determined, signalling between adjacent tissues is required for full expression of a functional phenotype in mature cells.

Clonal analysis of Stenotaphrum leaves

The pattern of clonal sectors in mature leaves of *Stenotaphrum* indicates that layer invasions occur primarily during, or before, leaf organogenesis (stage 1). In this study, we have followed Stewart and Dermen (1979) in designating the *Stenotaphrum* periclinal chimera as GWG (layers L1, L2 and L3 are green, white and green, respectively). Although the diversity of sector types indicates that sectors could have arisen from other kinds of periclinal chimeras (i.e. GWW, WGW, etc.), we found that the epidermal layer was uniformly genetically green, providing additional support for the GWG designation. If correct, sector types I, III and IV could arise from replacement of the white L2 layer by the green L1 layer or from displacement by the green L3 layer (Marcotrigiano and Bernatsky, 1995; Marcotrigiano, 1997). In this study, ‘sectors’ were arbitrarily defined as patterns that spanned three or more veins. Average sector widths ranged from 3.4 veins (approx. 3.4 % of total leaf width) for sector type V to 23.9 veins (approx. 23.9 % of leaf width) for sector type II. The distribution of sector widths in *Stenotaphrum* leaf blades...

**FIGS 31–36.** Transverse sections of developing leaves illustrating early stages of procambial strand formation. **Figs 31–33.** Formation of major vein procambium from one (Fig. 31, right arrow), two (Fig. 31, left arrow), three (Fig. 32, arrow), or four (Fig. 33, arrow) precursor cells. **Fig. 34.** Later stage of major vein procambium development, after formation of bundle sheath layer (BS). **Figs 35–36.** Formation of minor vein procambium from one (Fig. 35, right arrow) or two (Fig. 35, left arrow; Fig. 36, arrow) precursor cells. Note formation of bundle sheath layer (BS) in earliest-formed minor vein procambial strand and evidence of periclinal division in abaxial mesophyll layer that gives rise to leaf hypodermis (*). **Bar = 10 μm.**
seemed too variable to infer the number of founder cells that give rise to a leaf primordium as has been possible for maize using X-ray irradiation of seedlings heterozygous at a pigmentation locus (Stein and Steffenson, 1959; Poethig, 1984; Poethig and Szymkowiak, 1996).

In *Stenotaphrum*, almost all leaves have a broad median white stripe (sector type I) that includes the midrib and can extend up to approx. 50% of leaf width. One explanation for the extent of this stripe is that the periclinal GWG chimera may exist as a mericlinal chimera for periods of time in the shoot apical meristem, giving rise to a broad sector of albino tissue (Marcotrigiano, 1997); however, the absence of this large albino sector in the internode argues against this explanation. An alternative explanation is that broad white sectors arise very early during leaf initiation, and that these tissues at the initial locus of leaf initiation contribute more to the growth in width of the primordium than do marginal tissues. In *Stenotaphrum*, most narrow sectors occur near the blade margin, indicating that layer invasion events in this position occur later than for the median stripe and/or that cells near the margin contribute less to blade tissues, as observed for maize (Poethig, 1984).

Because the distinctive striping patterns of leaf blades do not extend over successive leaves on the same axis, sectors probably arise *de novo* within each leaf after delimitation of leaf and associated internode progenitors, as inferred for the striped phenotypes of the *iojap* mutation of maize (Walbot and Coe, 1979). The majority of *Stenotaphrum* sectors extend for the full length of the leaf blade, although most type III sectors and a small proportion of each of the other sector types extend for less than 100% of leaf length. Stripes that extend the full length of the leaf blade presumably arise at or before leaf initiation and extend via the polarized pattern of proliferative cell divisions. Our sampling method could not detect sectors shorter than 10% of leaf blade length, but, as sectors that extended 10% of leaf length were rare, we conclude that most invasions between layers occur before leaf primordia reach ten cells in height. At this stage of *Stenotaphrum* leaf development, organogenesis (stage 1) is complete, and the delimitation of the first procambial strands from ground meristem precursors is underway. The boundaries between sector types range from simple to very irregular, indicating that cell proliferation may follow different patterns in adjacent layers. Thus, when minor vein procambial strands are formed during stage 2 of leaf development, strands formed within an irregular sector boundary may have very complex patterns of green and white cells.

Evidence for developmental modules within maize leaves has been obtained by both Cerioli et al. (1994) and Scanlon et al. (1996). Cerioli et al. (1994) observed a striking pattern in which boundaries of spontaneous revertant sectors in *glossy* (reduced wax) mutants of maize tended to coincide with the positions of large lateral (major) or intermediate (largest minor) veins. Although they examined epidermal features only, they hypothesized that these spontaneous sectors were indicative of the modular construction of all tissues within the maize leaf. Scanlon et al. (1996) found that leaves of *narrow sheath* mutant plants were missing a large domain that included the leaf margin. Since other regions of the leaf were more or less normal, they argued that *narrow sheath* mutant phenotype represented a deletion of this developmental domain, or module. Our study of sectors caused by layer invasions within a periclinal chimera provides evidence that the putative developmental modules observed in surface layers by Cerioli et al. (1994) also extend to internal tissues. Although there is considerable variability in sector patterns in *Stenotaphrum* as in maize (Cerioli et al., 1994; Poethig and Szymkowiak, 1996), we found that all sector boundaries passed either directly through the centre of a vein (Fig. 37A) or between bundle sheath and adjacent mesophyll tissue (Fig. 37B), thus supporting the idea of developmental modules within the leaf (Cerioli et al., 1994).

**Cell lineage of vein formation**

Prior to vascular pattern formation, precursors of both dermal and ground tissues are present in the leaf primordium. In *Stenotaphrum* and other grasses, procambial strands arise *de novo* through formative divisions in ground tissue precursor cells (Dengler et al., 1985; Bosabalidis et al., 1994; Smith, 1996; Trivett and Evert, 1998). Procambial precursors are identified by cytoplasmic properties and the distinctive pattern of formative divisions within them; at present the molecular markers known are specific only for later stages of procambial development (Nelson and Dengler, 1997). In maize (Bosabalidis et al., 1994) and in barley (Trivett and Evert, 1998), most incipient procambial strands can be first recognized in transverse section as ‘assemblages’—groups of two to four laterally adjacent cells derived from a common precursor. Less often, procambial strands appeared to arise from two or
more cells that did not share an immediate precursor (Bosabalidis et al., 1994). Bosabalidis et al. (1994) recognized that if a procambial strand arose in a position that straddled a sector boundary in a variegated plant, the mature vein would form a ‘half vein’ (e.g. Fig. 37A). ‘Half vein units’ were previously described by Langdale et al. (1989) as one half a vein (bundle sheath and vascular tissue) plus a single adjacent mesophyll cell, all derived from a common single precursor cell; two such units would form a whole vein and one adjacent mesophyll cell on each side. However, this was a rare finding (four cases in >800 sectors observed). Although we did see numerous half veins in Stenotaphrum, we did not find evidence for the single adjacent mesophyll cell. The early timing of periclinal chimera layer invasions might not reveal a late event such as the formation of a putative ‘half vein unit’. In contrast, studies using spontaneous excision of a transposon (e.g. Cerioli et al., 1994) of x-irradiation (e.g. Poethig and Szymkowiak, 1996) were able to induce sector formation over a longer period of leaf development, and thus might detect comparatively late events.

Taken together, clonal analyses (Langdale et al., 1989; this study), anatomical studies (Bosabalidis et al., 1994; this study), and characterization of planes of cell division (Dengler et al., 1985; Nelson and Dengler, 1992; this study) indicate that the vascular tissue pattern in grass leaves develops within ground tissue precursors that already represent mixed cell lineages. Vascular pattern is thus superimposed on the previous clonal makeup of these ground tissue precursors. If a procambial strand arises from two (or more) precursors, each from a different clonal sector, ‘half veins’ will be formed (Fig. 37A). If a procambial strand arises from a single precursor (or from multiple precursors on one side of the clonal boundary), the sector boundary will lie between vein bundle sheath and adjacent mesophyll tissue (Fig. 37B). These observations indicate that a single underlying mechanism may influence both the location of sector boundaries and the position of new vascular strands.

Both clonal analyses and anatomical studies also indicate that, once veins are delimited within the ground tissue, the procambial lineage remains distinct from that of the surrounding mesophyll. This lineage restriction may play a role in determining vascular tissue identity and in the maintenance of this identity during cell differentiation, much as has been suggested for lineage restrictions between organ whorls in the flower of Antirrhinum (Vincent et al., 1995). The combination of putative lineage restrictions and the polarized pattern of proliferative cell divisions within developing grass leaves means that all cells within a longitudinal vascular strand are more closely related to each other than to adjacent mesophyll tissue if the original procambial strand arose within a single sector (Fig. 37B). Even if the original procambial strand straddled a boundary between sectors, procambial cells within each proliferating half vein are more recently related to each other than to the adjacent mesophyll of the same sector (Fig. 37A). Signalling within these tissues, especially along longitudinal files may be highly significant for coordinated tissue development (Nelson and Dengler, 1992).

Differentiation of C₄ photosynthetic tissues

In all C₄ plants, mesophyll and bundle sheath cells carry out complementary portions of the full C₄ photosynthetic pathway (primary carbon assimilation and photosynthetic carbon reduction, respectively). Coordination between the two cell types requires intercellular signalling in mature leaves, and it is likely that signalling is also required for their coordinated development. In grasses that are the ‘classical’ C₄ NADP-malic enzyme biochemical subtype and belong to the subfamily Panicoideae, such as Stenotaphrum and maize, bundle sheath cells are derived from procambium and mesophyll cells from ground meristem (Dengler et al., 1985). Although it appears that accumulation of cell-specific mRNAs and proteins occurs first in bundle sheath cells and then in adjacent mesophyll cells (Langdale et al., 1988; Dengler et al., 1995), on the whole cellular differentiation of the two cell types is temporally coincident (Dengler et al., 1995, 1996). Communication between differentiating bundle sheath and mesophyll cells must therefore occur across a lineage restriction boundary that has been in place from early vein development. The mechanisms of communication across this boundary are currently unknown (Hall and Langdale, 1996), but identification of such mechanisms should be important in understanding the roles that lineage restrictions play in tissue pattern formation and in cell differentiation.

ACKNOWLEDGEMENTS

We thank Connie Soros and two anonymous reviewers for helpful comments on the manuscript, Petra Donnelly for preparation of illustrations, Kathy Saul for sectioning for electron microscopy and Gillian Bower for assistance in data collection.

LITERATURE CITED


