Specification of bundle sheath cell fates during maize leaf development: roles of lineage and positional information evaluated through analysis of the *tangled1* mutant

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**SUMMARY**

In leaves of the maize *tangled1* (*tan1*) mutant, clusters of bundle sheath (BS)-like cells extend several cells distant from the veins, in association with the single layer of BS cells around the vein. We show that the BS-like cell clusters in *tan1* leaves result from the continued division of cells in the procambial/BS cell lineage that do not divide further in wild-type leaves. The ectopic BS-like cells accumulate the BS marker NADP-dependent malic enzyme but not the mesophyll cell marker phosphoenolpyruvate carboxylase, and exhibit thickened walls, suggesting that they differentiate as C4-type BS cells. We propose that bundle sheath cell fate can be conferred on some derivatives of procambial cell divisions in a manner that is heritable through multiple cell divisions and is position-independent.

Key words: Maize, *tangled1*, Bundle sheath cell, Cell fate

**INTRODUCTION**

The maize leaf vascular pattern consists of repeated longitudinal units of major veins that are separated by varying numbers of smaller minor veins (Sharman, 1942; Esau, 1943; Russell and Evert, 1985). Maize leaves exhibit a Kranz-type anatomy in which each vein is surrounded by a ring of photosynthetic bundle sheath (BS) cells and then by a ring of photosynthetic mesophyll (M) cells, a unit that is repeated laterally across the leaf, as is typical of C4-type grasses. BS and M cells have distinguishable histological features and accumulate distinct subsets of C4 photosynthetic enzymes. The compartmentalization of photosynthetic activities in these two cell types is essential for efficient fixation of CO2 and intercellular metabolic cooperation during C4 photosynthesis (Dengler and Nelson, 1999). In C3-C4 intermediate species of *Flaveria*, C4 photosynthesis is limited to the M cells immediately adjacent to veins (Edwards and Ku, 1987; Cheng et al., 1988; Moore et al., 1988). Surveys of C3, C4, and C3-C4 intermediate grass species revealed that interveinal distances range predictably from two M cells in C4 species to many M cells in C3 species, consistent with the requirement for vein-adjacency for C4-type M cells (Hattersley and Watson, 1975; Hattersley, 1987). These observations suggest that veins or their procambial precursors provide a spatial signal for the C4 differentiation of M cells that acts over a limited distance. According to this model, M cells in the maize leaf develop in a C3 pattern by default and in a C4 pattern only through the influence of closely neighboring veins (Langdale and Nelson, 1991).

The basis for BS cell determination is less clear. BS cells occur in a single layer around a vein, making it difficult to distinguish positional effects from lineage effects. Histological studies of vein ontogeny in NADP-ME type C4 grasses have demonstrated that BS cells surrounding a vein are predominantly, perhaps entirely, derivatives of procambial cells (Dengler et al., 1985; Nelson and Dengler, 1992; Bosabalidis...
Analyses of genetic clonal sectors in maize and in the C4 grass *Stenotaphrum secundatum* confirmed that the BS lineage is distinct from that which produces M cells, presumably from the time procambial strands are distinct from ground cells (Langdale et al., 1989; Sud and Dengler, 2000). In the maize study, however, rare clonal sectors were found in which a subset of the BS cells surrounding a vein was included in a sector with a neighboring M cell. One interpretation of such events is that an M cell was formed from the procambial lineage. Alternatively, a procambial cell (BS precursor) and a ground cell (M precursor) may have been derived from a division at the site of the sectoring event. A third interpretation, that the marked BS cells were generated from a non-procambial lineage, is unlikely because the sectors included, in cross section, several of the BS cells surrounding the vein, but only a single M cell.

Are the BS cells found in C4 plants influenced by their vein-adjacent position to become specialized for the C4 pathway, as is the case for M cells, or are they programmed entirely by their procambial lineage? The clonal studies cited above support the first possibility, suggesting that cells from the procambial lineage can assume an M cell fate if cell division pattern places them distant enough from the vein. However, this idea is challenged by the observation, investigated here, that in the maize tangled1 (*tan1*) mutant, clusters of BS-like cells extend various distances from veins. This aspect of the *tan1* phenotype provides a novel opportunity to investigate the roles of cell position and cell lineage in specification of BS cell fate.

*tan1* was originally described in reference to the altered cell division orientations and frequencies that were observed in the leaves (Smith et al., 1996). These are associated with changes in the spatial orientation of cytoskeletal arrays in dividing cells (Cleary and Smith, 1998). *tan1* mutants exhibit abnormally oriented divisions and a reduction in normally oriented divisions in both the transverse and longitudinal directions. The resulting plants are reduced in stature and have leaves with a crepe paper-like surface, although with normal overall shape (Smith et al., 1996). Here we show that in *tan1* leaves, abnormally late divisions within the procambial lineage give rise to BS-like cells in aberrant locations. Despite their non-vein-adjacent positions, these cells differentiate as C4-type BS cells. This is a rare example of lineage-dependent cellular differentiation in plants, where cell fates are generally dictated by positional information.

**MATERIALS AND METHODS**

**Stocks**

*tan1-py/tan1-py* stocks were obtained from the Maize Coop Stock Center. A stock homozygous for the *wd* mutation and carrying a ring chromosome with a portion of chromosome 9S including the wild-type *Wd* locus was a gift from Dr Sarah Hake (USDA Plant Gene Expression Center, Albany, CA). *tan1-py1* homozygous mutants were crossed to the *wd/wd* 9S *Wd* ring chromosome stock; progeny from this cross were selfed to recover individuals homozygous for both *wd* and *tan1-py1* and carrying the 9S *Wd* ring chromosome. As discussed previously (Langdale et al., 1989), occasional loss of the ring chromosome during somatic cell divisions uncovers the *wd* mutation, producing white clonal sectors in a *tan1-py1* mutant background.

**Histology**

Leaf sections were cleared by dehydrating 5×5 mm sections of fresh leaf tissue through an ethanol series to 100% ethanol. The sections were maintained in 100% ethanol until clear, then stained for 15 minutes with a 0.1% aqueous Toluidine Blue solution. Material for plastic and paraffin wax embedding was prepared by cutting 1-2 mm wide sections of fresh tissue and fixing in 4% paraformaldehyde in Sorenson’s buffer under vacuum for 1 hour (Sylvester and Ruzin, 1994). The fixative was replaced and the tissue was fixed overnight at 4°C, then dehydrated through an ethanol series to 100% ethanol. Sections for embedding paraffin wax were put through a Hemo-De (Fisher Scientific):ethanol series and then embedded in Paraplast Plus (Fisher Scientific) for 4 days at 60°C, with 4 changes of Paraplast. 8 μm sections of the embedded material were made using a rotary microtome. Sections were de-waxed in Hemo-De (Fisher Scientific), rehydrated, stained in aqueous 0.1% Toluidine Blue for 10 minutes, dehydrated and mounted. Sections for plastic-embedding were rinsed 3 times in 100% polypropylene oxide after ethanol dehydration and infiltrated overnight at room temperature in a 1:1 mixture of polypropylene oxide:Spurr’s resin (Electron Microscope Sciences). The tissue sections were then embedded in Spurr’s resin at 65°C overnight. 2 μm sections of the plastic-embedded material were made on a Sorvall MT-2 ultramicrotome using a glass knife. Sections were dried on slides at 60°C, then stained with 1% Toluidine Blue, 1% borax at 60°C for 3 minutes. Microscope observations were made and photographs taken using a Zeiss Axioshot light microscope. Sites and planes of recent cell division were identified by locating pairs of cells with relatively thin separating walls, in transverse sections.

**Immunolocalizations**

Tissue from the fourth leaf of 2-week-old seedlings was fixed and embedded in Paraplast Plus as described above. Immunolocalizations were performed as previously described (Smith et al., 1992), with the following modifications: the proteinase step was omitted, a dilution of 1:50 was used for all primary antibodies, alkaline phosphatase-conjugated goat anti-rabbit-IgG (Boehringer Mannheim) diluted 1:600 was used as a secondary antibody and the signal was visualized by staining for 2 hours in the substrate 5-bromo-4-chloro-3-indolyphosphate, p-toluidine salt/nitroblue tetrazolium chloride. ME antibodies were as described previously (Rothermel and Nelson, 1989) and PEPCase antibodies were a gift from Dr James Berry (SUNY; Buffalo).

**RESULTS**

**BS-like cells extend in clusters from veins in *tan1* leaves**

Vascular strands in *tan1* are disorganized and irregularly spaced (compare Fig. 1B with 1A). Both phloem and xylem are present in vascular bundles, although the number and orientation of the vascular elements is variable compared to the regular pattern in wild-type leaves. Chains or clusters of BS-like cells are adjacent to the veins, in positions not limited to the vascular sheath (Fig. 1D compared with 1C). The BS-like cells exhibit centrifugally arranged chloroplasts and thick walls that resemble those of BS cells in wild-type leaves. The more peripheral BS-like cells are generally considerably smaller in diameter than those more adjacent to veins. The cell clusters are randomly distributed, usually 10-15 cells long and 2-5 cells wide in paradermal view (Fig. 1D), and include at least one contact with the nearest vein. In cross section, these cell clusters emanate radially from the veins (Fig. 2C-H). Of 523 minor veins scored in cross section at comparable positions in...
the leaf, 62 (12%) had more than 10 associated ectopic BS-like cells, 178 (34%) had 5-10 such cells, 94 (18%) had 2-5 such cells, and 189 (36%) exhibited no ectopic BS-like cells.

We used BS and M cell-specific antibodies to test whether the BS-like cells present in these ectopic cell clusters have other characteristics of BS cells. Immunolocalizations were performed using antibodies against NADP-dependent malic enzyme (ME) and phosphoenolpyruvate carboxylase (PEPCase). In wild-type leaves of C4 plants, ME accumulates only in chloroplasts of mature BS cells (Fig. 3A), and PEPCase accumulates only in cytosol of mature M cells (Fig. 3B). All cells in the aberrant cell clusters in tan1 mutant leaves accumulate ME but not PEPCase (Fig. 3C,D). ME accumulation was independent of the distance of the cell from the vein. This suggests that the cells in the ectopic cell clusters are functional C4-type BS cells and that C4 enzyme accumulation in these cells is independent of cell position relative to the vein.

**BS-like cells are clonally related to adjacent BS cells**

In order to determine whether clusters of BS-like cells represent cell clones, we visualized the clonal relationships among BS cells in both wild-type leaves and tan1 leaves using wd sector analysis. Sporadic loss of a ring chromosome uncovering the wd mutation produces clonally derived and non-revertable albino sectors in a green background that can be evaluated in all subepidermal layers of the leaf by conventional microscopy. To ensure that neighboring albino cells are clonally related and not due to successive independent sectoring events, we considered only sectors that spanned at least two minor veins, and are likely due to a relatively early sectoring event. We estimate that sectors of width greater than or equal to 2 minor veins represent single-cell events that occurred at least four plastochrons prior to the procambial cell divisions that subsequently established the minor veins within that sector. On the basis of their boundaries, sectors were divided into two classes. Class I lateral boundaries encompass a complete ring of BS cells or terminate among M cells (Fig. 4A,B). Class II lateral boundaries fractionate a BS ring or (rarely) encompass an entire sheath plus a single adjacent M cell (Fig. 4C), Class II boundaries can reveal lineage relationships among BS cells at the boundary site.

As shown in Table 1 and Fig. 4, all sectors in both mutant (tan1-py) and wild-type (Tan1+) leaves fell into either class I (64% and 67%) or II (36% and 33%). This confirms previous observations in maize and *Stenotaphrum secundatum* that it is relatively common for the procambial/BS lineage to be derived from multiple ground cells (Dengler et al., 1985; Langdale et al., 1989; Nelson and Dengler, 1992; Bosabalidis et al., 1994; Sud and Dengler, 2000). A similar distribution of sectors was observed in the tan1 leaves (Table 1; Fig. 4D,E), suggesting that the underlying procambial lineage patterns of *Tan*+ and tan1 leaves are comparable. Among the 35 sectors observed that spanned an aberrant cell cluster in tan1, the ectopic BS-like cells were without exception clonally related to at least one of the normal BS cells surrounding the adjacent vein. In addition, no cases were observed in which a sector boundary fractionated such a cell cluster. This demonstrates that the ectopic BS cells in tan1 leaves are more closely related to normally positioned BS cells than they are to the M cells with which they share a vein-distal location.

**Determined BS cells continue to divide in tan1 leaves**

The observation that in tan1, ectopic clusters of BS-like cells appear to represent BS cell clones suggests that BS cells or their precursors proliferate aberrantly, yet the BS fate is preserved in a position-independent manner. To examine this,
we compared cell division patterns in young leaf primordia of tan1 mutants and wild-type siblings by light microscopy. Recent planes of cell division can be identified by the thin walls separating the daughter cells. Cell division patterns in the second subepidermal layer of young tan1 leaf primordia are less regular than those seen in wild-type leaf primordia, due to an increased number of abnormally oriented cell divisions, consistent with previous observations (Smith et al., 1996 and Fig. 5C,D). Comparisons of wild-type and tan1 veins at various stages of development indicate that although cells in complete BS rings of wild-type rarely divide further (Fig. 5A,B), irregular late cell divisions frequently occur in complete BS rings of tan1 veins (Fig. 5C,D). This suggests that aberrant BS cell clusters in tan1 leaves result from continued division of already determined BS cells. Thus, it appears that formation of BS cell clusters is lineage-dependent. This apparent prolongation of cell divisions produces supernumerary populations of several other cell types in tan1 leaves, including midrib clear cells, vascular cells and epidermal cells (data not shown), with late cells generally being smaller than normal.

DISCUSSION

Our observations indicate that products of abnormally late divisions in tan1 mutant leaves differentiate as C4-type BS cells in a lineage-dependent manner. How is BS cell fate in wild-type and mutant leaves initially specified? Histological and

Fig. 3. Immunolocalizations of BS and M cell-specific antibodies. BS cell-specific localization of the ME antibody in sections of (A) wild type and (C) tan1, including localization in the ectopic cell clusters. (B) M cell-specific localization of the PEPCase antibody in sections of wild type and (D) tan1. Arrows indicate ME localization in wild-type BS cells (A), tan1 mutant ectopic BS cell clusters (C) and PEPCase localization in wild-type BS cells (B) and tan1 mutant ectopic BS cell clusters (D). All tissue was embedded in Paraplast Plus for this experiment. Bar, 10 μm.
clonal studies of leaf ontogeny in maize and other NADP-ME-type C4 grasses show that the majority of BS cells are derived from cells formed from procambial strand precursors, and that the strands themselves are derived from one or more adjacent ground cells (Dengler et al., 1985; Langdale et al., 1989; Nelson and Dengler, 1992; Bosabalidis et al., 1994; Dengler et al., 1996; Dengler et al., 1997; Sud and Dengler, 2000). We found that the majority of sectors analyzed in both wild-type (67%) and tan1 mutant leaves (64%) belong to a class (class I) consistent with the formation of a complete BS ring from a single marked provascular cell. The remaining sectors in both wild-type and mutant leaves (class II) encompassed a fraction of the sheath plus one or more M cells, consistent with the marking of a cell that gave rise to both provascular and ground

Fig. 4. Examples of sector classes. Class I sectors have lateral boundaries either among M cells (A) or include a complete ring of BS cells (B). Class II sectors have boundaries that fractionate the BS cell ring (C). BS cells in an aberrant cell cluster in tan1 were without exception clonally related to at least one of the normal BS cells surrounding the adjacent vein (D, E). Neither D nor E depicts a section through a transverse vein, as determined by examination of serial sections.

Fig. 5. Cell division patterns in tan1 vein formation. Cell divisions present in the formation of wild-type minor veins (A,B) and tan1 minor veins (C,D), where divisions appear much later than in comparable wild-type veins. Arrows indicate procambial strands in wild-type (A,B) and late divisions within procambial strands in the tan1 mutant (C,D). Bar, 10 μm.
precursors, as observed in an earlier maize clonal study (Langdale et al., 1989). Since we observed no difference in the average size of class I and class II sectors, they appear to represent alternative ways that procambial/BS lineages can arise. The formation of veins and BS rings from multiple ground cell precursors suggests that some early positional cell-cell information coordinates their differentiation to produce regular patterns of BS cells and other procambial derivatives. Our observations here suggest that once the procambial lineage is established, its most peripheral derivatives always differentiate as BS cells. They also suggest that the peripheral derivatives have a BS cell fate that is stable through multiple cell divisions, even with regard to C4 photosynthetic metabolism.

The formation of veins and their associated bundle sheaths from non-clonal groups of ground cell precursors that can also give rise to M cells suggests that positional information initially specifies the fates of BS cells and other procambial derivatives. BS fate may be conferred on the cells immediately surrounding the vein cells by a positional signal, either from less peripheral procambial cells or from more peripheral non-procambial cells. Alternatively, positional information may act only to set aside the procambial lineage from the mesophyll, whereas the fates of BS cells and other procambial derivatives are subsequently determined by lineage. In this case, the regular pattern of procambial divisions might determine that certain derivatives become BS cells, and might assure that they are in a peripheral position relative to vascular tissue. However, the observation that tan1 veins are surrounded by a continuous bundle sheath, despite irregular patterns of division of the procambial strands, is more consistent with the former possibility that positional information confers BS cell fate on the cells immediately surrounding the vein.

Regardless of the mechanism by which BS fate is initially specified within the procambial lineage, this fate appears to be inherited by the products of abnormally late divisions that occur in tan1 mutant leaves. In mutant leaves, BS cells are formed as many as 6 cells distal to the vascular sheath, but are clonally related to sheath cells. It is possible that the lineage-based mechanism by which these cells differentiate as BS might also depend on positional information. That is, these cells might become BS cells through the action of a positional cue for which only procambial derivatives are primed, signaling either an adjacency to M cells or a distance from the vascular tissue. However, the simplest interpretation is that, once specified as BS, cells remain committed to the BS fate while they continue to divide, and therefore differentiate as BS cells regardless of their final positions. Cells of the bundle sheath are formed 3-4 plastochron intervals from the vein-initiating procambial cell divisions in maize (Nelson and Dengler, 1992). Our observations on tan1 mutants suggest that BS cells and any subsequent daughter cells are committed to BS fate at this time.

The Tan1 gene encodes a highly basic protein with microtubule-binding activity, and with a localization pattern consistent with its inferred role in the orientation of cytoskeletal structures in dividing cells (Smith et al., 2001). The observed prolongation of cell division relative to commitment or differentiation in tan1 mutant leaves indicates that Tan1 is required for division arrest in differentiating cells, and suggests that the schedule of cellular differentiation can be controlled independently of the timing of cell division. It is possible that in tan1 leaves, cell division occurs on a normal schedule while cellular differentiation occurs precociously. Alternatively, cellular differentiation may occur on schedule while cell division is prolonged. If this is the case, then Tan1 might play a role in cell cycle regulation in addition to its role in the spatial regulation of cytokinesis (Cleary and Smith, 1998). This possibility might be investigated by examining the expression in tan1 leaves of cell cycle regulators such as cyclin-dependent kinases, which reflect the competency of the cell to divide (Hemerly et al., 1993; Shaul et al., 1996) and mitotic cyclins, which appear to be expressed only in actively dividing cells (Ferriera et al., 1994; Shaul et al., 1996). Alternatively, Tan1 may be indirectly required for the proper timing of cell division because of its role in the orientation of cell division. Following misorientation of subsidiary mother cell divisions in maize by centrifugation (Galatis et al., 1984) or by mutations perturbing these divisions (Gallacher and Smith, 1999; Gallacher and Smith, 2000), aberrant divisions are often ‘corrected’ by additional, normally oriented divisions. Cell volume and shape may be important factors that influence cell cycle activity (Jacobs, 1997). It may be that when a cell is stimulated to divide but daughter cells of appropriate shape or volume are not produced because the new cell wall is misoriented, one or both daughters can respond again to the same stimulus and re-enter the cell cycle.

Examples in which a cell whose fate is already committed or restricted transmits that state to its progeny are more common in the animal developmental biology literature than in the plant literature. For example, cell lineages leading to germline precursors in C. elegans are progressively restricted through five cell divisions; those to body muscles through more (Sulston and Horvitz, 1977). In contrast, similar studies of plant development have almost universally indicated that cell fates are dictated by their positions within the tissue. For example, clonal analyses of maize leaf development have shown that although patterns of cell division are variable, the final arrangement of various cell types within the leaf is highly predictable (Langdale et al., 1989; Cerioli et al., 1994; Poethig and Szymbkowiak, 1995; Hernandez et al., 1999). In some plant organs, patterns of cell division are sufficiently regular that cell fates can be accurately predicted on the basis of lineage, such as in the Arabidopsis root (Dolan et al., 1993; Dolan et al., 1994). Nevertheless, when an individual epidermal or cortex-endodermis initial cell in the Arabidopsis root meristem is laser-ablated, a neighboring cell belonging to a different lineage divides so that one of its daughters occupies the position of the ablated cell, and differentiates according to its new position rather than its lineage (van den Berg et al., 1995).

The formation of BS cells at vein-distal positions in the tan1 mutant provides a relatively rare example in plant development of a lineage-committed state that is transmitted to daughter cells. Another such example occurs during stomatal development in dicots. Guard cell pairs are formed from meristemoids, which are produced through asymmetric cell divisions. Before forming a guard cell pair, the meristemoid may undergo additional asymmetric divisions to form non-stomatal cells. However, regardless of how many times it divides, only one guard cell pair is formed by each meristemoid (Kagan et al., 1992; Larkin et al., 1997). This suggests that the meristemoid is determined to form guard cells and passes this state to one of its daughters each time it divides. The transmission of a lineage-committed state from a mother cell to one or both of its daughters may be uncommon in plants because of the nature of the plant body.
Unlike animal cells, which can migrate extensively during embryogenesis, plant cells are constrained by their walls to remain at the site where they are initially formed. If cell fate commitments were ‘hard-wired’ in cells that are still dividing, irregularities in cell division pattern could not be corrected and would therefore perturb the pattern of cellular differentiation. Such mistakes could have deleterious consequences, particularly at early stages of development. For this reason, reliance on positional information for cell fate specification is a strategy that appears to be well suited to plants.

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REFERENCES


