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Scarecrow Plays a Role in Establishing Kranz Anatomy in Maize Leaves

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More than a quarter of the primary productivity on land, and a large fraction of the food that humans consume, is contributed by plants that fix atmospheric CO_2 by C_4 photosynthesis. It has been estimated that transferring the C_4 pathway to C_3 crops could boost yield by 50% and also increase water use efficiency and reduce the need for fertilizer, particularly in dry, hot environments. The high productivity of maize (*Zea mays*), sugarcane (*Saccharum* spp.) and several emerging bioenergy grasses is due largely to C_4 photosynthesis, which is enabled by the orderly arrangement, in concentric rings, of specialized bundle sheath and mesophyll cells in leaves in a pattern known as Kranz anatomy. Here we show that PIN, the auxin efflux protein, is present in the end walls of maize bundle sheath cells, as it is in the endodermis of the root. Since this marker suggests the expression of endodermal genetic programs in bundle sheath cells, we determined whether the transcription factor SCARECROW, which regulates structural differentiation of the root endodermis, also plays a role in the development of Kranz anatomy in maize. Mutations in the *Scarecrow* gene result in proliferation of bundle sheath cells, abnormal differentiation of bundle sheath chloroplasts, vein disorientation, loss of minor veins and reduction of vein density. Further characterization of this signal transduction pathway should facilitate the transfer of the C_4 trait into C_3 crop species, including rice.

Keywords: C_4 photosynthesis • Chloroplast structure • *Pin-formed* • Plasmodesmata • *Scarecrow* • *Zea mays*.

Abbreviations: BS, bundle sheath; M, mesophyll; SCR, Scarecrow; V, vein; YFP, yellow fluorescent protein.

Introduction

The C_4 photosynthetic pathway is an elaboration of the more common C_3 pathway characteristic of many important crops, including rice and wheat (Sage 2004, Langdale 2011). The advantage of C_4 photosynthesis is that it overcomes inherent limitations of Rubisco, the enzyme that fixes CO_2 in all plants and provides the substrate for carbohydrate synthesis (Sage 2004, Hibberd et al. 2008, Ghannoum et al. 2011, Sage and Zhu 2011, von Caemmerer et al. 2012). Rubisco carboxylation efficiency is limited by its slow turnover time and by a competing oxygenation reaction. A common way for C_4 species to overcome these problems is by assigning the key reductive step in photosynthesis to the bundle sheath (BS) cells surrounding the leaf veins where the CO_2 concentration is high. CO_2 is concentrated in maize BS cells in a two-step process. CO_2 is initially fixed in mesophyll (M) cells by phosphoenolpyruvate carboxylase to produce malate, a 4-C compound. Malate diffuses into the BS cells through plasmodesmata. In the BS cells, malate is decarboxylated and the released CO_2, now at elevated concentrations, is re-fixed by Rubisco to provide the reduced substrate for carbohydrate synthesis. BS chloroplasts in maize do not have appreciable PSII activity and as a result they lack grana (stacked thylakoid membranes).

The efficiency of intercellular metabolite shuffling, central to C_4 mechanism, is enhanced by the arrangement of BS and M cells into concentric rings around the veins, producing a wreath-like appearance known as Kranz anatomy. An important anatomical characteristic of C_4 grasses is that veins (V) are typically separated by only four cells (V–BS–M–M–BS–V) and vein density is higher than in C_3 grasses, which have additional interveinal M cells. Although the requisite enzymes for C_4 metabolism are present in C_3 plants, and a considerable amount is known about the differences in kinetic properties and expression patterns of these enzymes, the regulatory mechanisms underlying C_4 structure, including the differentiation of specialized BS chloroplasts and high vein density, are not understood (von Caemmerer et al. 2012). Screens for mutants with altered vein spacing or other aspects of C_4 anatomy have yielded limited information (Langdale 2011).

We considered the possibility that at least some aspects of C_4 structural specialization in maize are conferred by the signaling network that underlies endodermal cell identity in roots. The endodermis is a cell layer surrounding root vascular tissue, in the same way that the BS surrounds the leaf veins in C_4 plants. Esau (1953) considered the BS of angiosperm leaves to be an endodermis, based on anatomical considerations, and several lines of evidence support this view with respect to C_4

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BS cells. First, suberin, found in the cell walls of root endodermal cells (the Casparian strip), is also present in the walls of maize BS cells (Evert et al. 1977). Secondly, in maize, the *Scarecrow* (*Scr*) gene, which regulates endodermal cell identity in roots (Di Laurenzio et al. 1996), is expressed in developing leaf vascular tissue (Lim et al. 2005) and in the BS cells of mature leaf tissue (Li et al. 2010). Notably, *Scr* is also expressed in the starch sheath (endodermis) of the inflorescence stem in Arabidopsis (Wysocka-Diller et al. 2000), a cell type which, in tobacco, has characteristics of C_4 photosynthesis (Hibberd and Quick 2002). In Arabidopsis *scr* mutants, the starch sheath is missing (Fukaki et al. 1998, Wysocka-Diller et al. 2000). Taken together, these lines of evidence suggest that a conserved genetic pathway may be involved in the differentiation of both endodermis in roots and BS cells in leaves.

Results

Localization of PIN1a in BS cells

As a test for endodermal-like identity in maize BS cells, we localized the marker PIN1a–yellow fluorescent protein (YFP) (Gallavotti et al. 2008) in developing leaf tissue. PIN is an auxin transport protein that localizes in a polar manner to the cell boundaries across which auxin flows, including the developing endodermis and cortex in roots (Gälweiler et al. 1998, Paponov et al. 2005, Scarpella et al. 2006) and the endodermis (starch sheath) in stems (Friml et al. 2002).

In leaves of Arabidopsis and rice, both C_3 plants, PIN1 is highly expressed in cells that will give rise to vascular tissue, but it is not present in the BS (Scarpella et al. 2006, Wang et al. 2009). In contrast to this expression pattern in C_3 plants, we found that PIN1–YFP localizes at the transverse walls of vascular precursor cells, and in the newly formed BS (**Fig. 1A**).

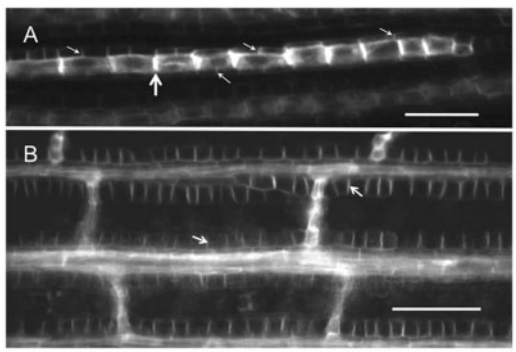

Fig. 1 Localization of PIN, the auxin efflux protein, in developing maize leaves. (A) In a newly differentiating minor vein, approximately 5 mm from the leaf base and within the zone of early differentiation, PIN1a–YFP is present in the end walls of cells in the vascular core of the vein (large arrow) and in the end walls of expanding bundle sheath cells (small arrows). (B) In slightly more mature veins, approximately 8 mm from the leaf base, PIN1a–YFP is clearly seen in the end walls of bundle sheath cells (arrows). Transverse veins are present between the longitudinal veins. Scale bars = 50 μm.

The protein persists as the BS expands and the vascular elements are formed (**Fig. 1B**). These data support the hypothesis that the BS in maize leaves shares elements of cell identity with the endodermis of roots and stems.

Scarecrow mutants

To test the involvement of *Scr* in C_4 structural specialization in maize leaves, we characterized two independently derived mutant alleles: *zmscr-m1* (unknown *Mutator* background) and *zmscr-m2* (W22 background). Gene functions in both alleles are disrupted by *Mutator* transposon insertion (Chandler and Hardeman 1992) in the first exon (**Supplementary Fig. S1**). The transposon sequence is also present in the mRNA of the mutant plants, as indicated by identically sized bands in PCR-amplified genomic DNA and cDNA derived from homozygous mutants (**Supplementary Fig. S2**). Sequence analysis verified that the transposons are incorporated into the RNA in the same locations as observed in the genomic DNA. In homozygous mutant plants, no wild-type transcripts were detected (**Supplementary Fig. 2B**), indicating that the transposons abolish the native function of the ZmSCR gene.

Since *Scr* is expressed specifically in the endodermis of maize roots (Lim et al. 2000), we predicted that mutations in this gene would alter endodermal differentiation. To test this prediction, we stained hand-cut sections of primary roots with berberine–aniline blue (Brundrett et al. 1988). In maize *scr* plants the root endodermal layer is present. However, Casparian bands are significantly reduced and only occasionally detected (**Fig. 2**).

To investigate the effects of the *Scr* mutations, we stained fresh, mature leaf tissue with I–KI (**Fig. 3**). I–KI highlights the starch that preferentially accumulates in the chloroplasts of BS cells. In both *zmscr-m1* and *zmscr-m2* plants, veins are abnormal in many respects (**Fig. 3**). Extra BS cells are common (**Fig. 3B**, **C**, **F**; **Table 1**), with as many as four additional BS layers in some veins. Many veins merge (**Fig. 3B**, **D**; **Table 1**), and these merged veins may have extra BS cells between them. When veins merge, the vascular cores remain separate. Although merged veins exclude M cells ($V–BS_n–V$), in other cases there are more than two M cells between veins (**Fig. 4B**). Some minor veins have few cells and very small BS cells (**Figs. 3E**, **4C**), and other minor veins terminate abruptly (**Fig. 3B**, **E**).

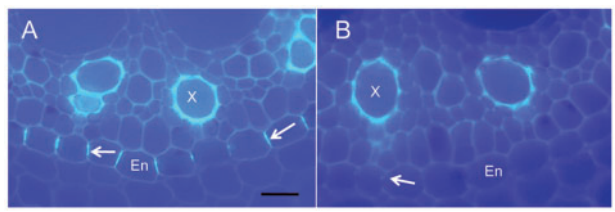

Fig. 2 Transverse hand-cutsections of root tissue approximately 8 cm from the root tip stained with berberine–aniline blue and viewed with UV light. (A) Wild-type root with pronounced Casparian bands (arrows) in the endodermis (En). (B) *zmscr-m1* root in which Casparian bands are small and visible in only some endodermal cells. Lignified walls of xylem (X) also stain.

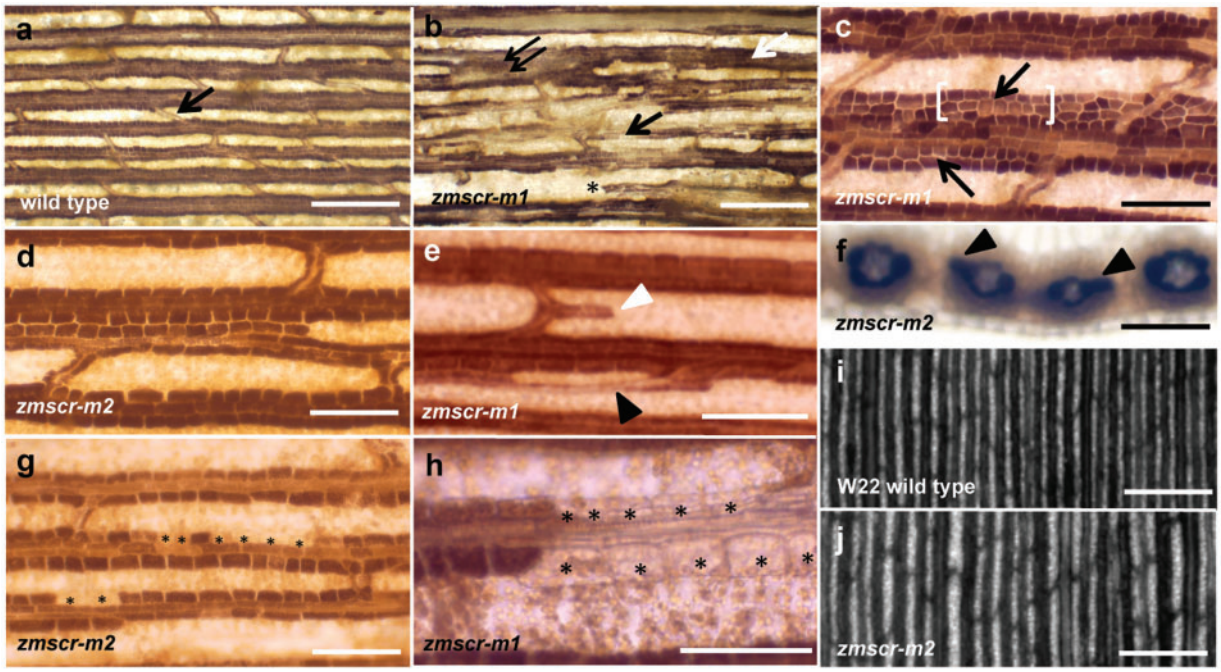

Fig. 3 Bundles sheath and vein defects in leaves of *zmscr* mutants. Starch in bundle sheath cells is stained dark brown to red; mesophyll cells are light brown or light red. All panels are paradermal (top) views except (F). (A) Wild-type plants with the same genetic background as *zmscr-m1*, with normal vein spacing and bundle sheath formation. Small transverse veins (arrow) connect longitudinal veins, which run from the tip to the base of the leaf. Scale bar = 400 μm. (B) Veins are highly disorganized in *zmscr-m1* leaves. Some veins merge (double arrows), others have additional bundle sheath cells (white arrow), some bundle sheath cells have no visible starch (black arrow) and some veins end blindly (asterisk). Scale bar = 400 μm. (C) Single vein in *zmscr-m1* with four bundle sheath layers (white brackets) on one side and two (arrow) on the other. Scale bar = 200 μm. (D) Merging veins in *zmscr-m2* exclude mesophyll cells. Scale bar = 100 μm. (E) Minor vein ends blindly (white arrowhead). Another vein, which is much reduced in size for a short distance, separates from, then merges with, a larger vein and has small bundle sheath cells (black arrowhead). A similar reduced vein is shown in **Fig. 4C**. Scale bar = 50 μm. (F) Transverse hand-cut section illustrating ectopic bundle sheath cell formation (black arrowheads). Scale bar = 100 μm. (G) Starchless bundle sheath cells (asterisks) in *zmscr-m2*. Scale bar = 100 μm. (H) Starchless bundle sheath cells in *zmscr-m1* retain normal cell shape. Scale bar = 50 μm. (I) Wild-type W22 tissue with normal vein density. Scale bar = 650 μm. (J) *zmscr-m2* tissue with reduced vein density. Scale bar = 650 μm.

Table 1 Vein and bundle sheath abnormalities

Trait	*zmscr-1*		*zmscr-2*	
	Wild type	Mutant	Wild type	Mutant
Leaf width (cm)	8.3 ± 0.3^a	4.6 ± 0.3*	10.8 ± 0.2^a	10.6 ± 0.02
Merged veins (cm^{-1})	6.7 ± 4.7^a	90.8 ± 16.1*	1.7 ± 0.2^b	4.8 ± 0.3*
Lateral vein number	19.0 ± 0.4^a	15.6 ± 0.9*	26.0 ± 0.6^a	21.4 ± 0.75*
Veins between lateral veins	14.4 ± 0.7^a	7.1 ± 0.4*	12.0 ± 0.4^b	8.2 ± 0.3*
Total vein density (cm^{-1})	73.2 ± 2.9^a	61.2 ± 2.2*	72.3 ± 1.3^b	66.1 ± 1.2*
Extra BS cells (cm^{-2})	31.9 ± 6.3^a	112.6 ± 26.3*	10.8 ± 0.2^b	122.6 ± 11.6*
Starchless BS cells (cm^{-2})	12.6 ± 1.9^a	111.8 ± 15.6*	11.9 ± 1.3^b	26.7 ± 2.4*

Veins were analyzed across transects across the leaf; bundle sheath (BS) cells were counted in randomly chosen areas in the same regions.
Values are the means ± SE.
*Significantly different from wild-type values at $P \leq 0.05$ using Student's *t*-test.
n is the same in the wild type and its corresponding mutant; $^a n = 5$, $^b n = 15$.

The number of lateral veins (the largest veins except the midrib) is reduced, as is the number of minor veins between them (**Table 1**). In some cases, the minor veins between laterals are entirely missing. The combined effect of these abnormalities is reduced vein density (**Fig. 3I**, **J**; **Table 1**).

Another notable feature of vein structure in *zmscr* mutant maize is the presence of BS cells that appear not to contain starch. These starchless cells may occur in isolation, but more commonly as a longitudinal series in the vein (**Fig. 3G**, **H**). In transverse sections, such cells may occur at any location around

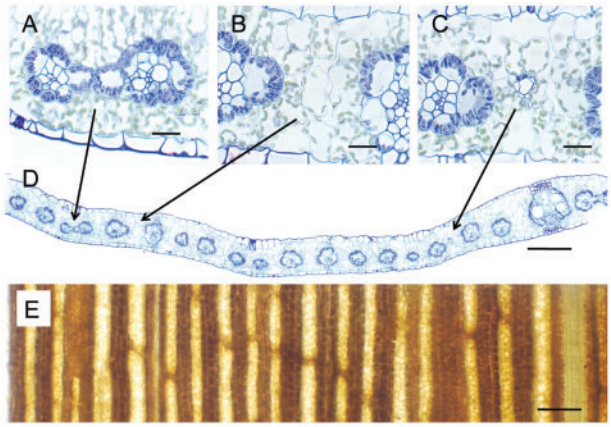

Fig. 4 Light microscope images of veins in maize mutant *zmscr-m2*. Images in (A–C) are enlargements of the image in (D), a transverse section obtained at the top edge of the corresponding starch-stained leaf tissue in (E). (A) Two minor veins with merged bundle sheath (BS) cells. Note in (E) that these two veins separate farther along their length. (B) Three mesophyll cells between the veins, rather than the normal two. (C) An abnormally small minor vein. This vein, separated from the adjacent vein by one mesophyll cell in (D), joins that vein in (E). Scale bars in A–C = 25 μm; D and E = 200 μm.

the circumference of the veins. There is considerable variation in expressivity at the cellular level, with starchless cells often abutting apparently normal cells (**Fig. 5A**). While they appear almost devoid of cytoplasm, the starchless cells are viable, as indicated by vital staining with carboxyfluorescein diacetate. In most cases, the plastids of these cells are round, rather than ellipsoidal, internal membranes appear as vesicles rather than flattened thylakoids, the interior of the plastids is occupied by a large vacuole and starch is absent (compare **Fig. 5C** and **D**). The presence of a vacuole suggests the accumulation of solute inside the plastid. Plastoglobuli may, or may not, be visible.

In some, but not all, of the starchless BS cells, the plasmodesmata have so-called 'sphincters' (Evert et al. 1977) (**Fig. 5E**, **F**). These structures, of unknown function, are composed of electron-dense material in the neck of the pore. In wild-type plants, sphincters are found only on the M cell side when the M cell abuts another cell type such as a BS cell (Evert et al. 1977) (**Fig. 4F**). However, at many interfaces between starch-free BS cells and M cells, sphincters are found on the BS side as well (**Fig. 5E**). The presence of sphincters on the BS cell side in starch-free BS cells suggests a confusion, rather than a simple loss, of identity in these cells. Although the chloroplasts in most affected BS cells are vacuolated, in others the chloroplasts do not have vacuoles but may contain semi-crystalline bodies, apparently of protein (**Fig. 5G**, **H**) or they may be very small, with minute starch grains (**Fig. 5B**). Abnormal chloroplasts are not distributed in the centrifugal pattern characteristic of wild-type BS cells (**Fig. 5A**, **B**). Importantly, all the plastids in a given cell have a similar structure, indicating that the effects of the mutation are conferred at the cell, rather than the plastid, level.

To be certain that the aberrant phenotypes described above are associated with the *Scr* mutations, we conducted a co-segregation analysis. Several families from both the *m1* and *m2* alleles were genotyped and phenotyped. For the *m1* allele, a total of 27 homozygous mutants, 79 heterozygous mutants and 43 wild-type plants were analyzed from nine independently segregating lines. For the *m2* allele, a total of 62 homozygous mutants, 22 heterozygous mutants and 50 wild-type plants were analyzed from seven independently segregating families. Within the *m1* lines, the transposon insertion co-segregated with the phenotype in both the heterozygous and the homozygous state. The presence of one copy of the *zmscr-m1* allele resulted in a less severe phenotype compared with homozygous mutants. Thus, the *zmscr-m1* allele appears to be a semi-dominant mutation. Within the *m2* families, the phenotype was correlated exclusively with homozygous mutant alleles, following the pattern of recessive inheritance. To ensure that the phenotypes observed were not due to background insertions unrelated to the transposon insertions in the *Scr* gene, homozygous wild-type plants were self-pollinated and progeny were grown under the same conditions as the *scr* mutants. Phenotypes associated with the *zmscr-m1* and *zmscr-m2* alleles were not observed in these families. Therefore, without exception, the abnormal phenotype correlated with the presence of the transposon insertions within the *scr* gene.

The cellular localization of the mutant phenotypes reported here, in BS cells, is consistent with expression patterns of the *Scr* gene. *Scr* transcripts are most abundant at the base of the growing maize leaf, where early developmental events take place (Li et al. 2010). In situ hybridization studies (Lim et al. 2005) demonstrate that the transcripts are localized at the base of the leaf, and RNA-Seq analyses (Li et al. 2010) indicate specific expression in BS cells as veins mature. Furthermore, in fully mature maize leaves, transcripts for the SHORTROOT protein, which activates the transcription of *Scr* in the root endodermis (Wysocka-Diller et al. 2000), are also highly enriched in BS cells (Li et al. 2010).

Discussion

The C_4 syndrome has evolved >60 times in similar ways (Kajala et al. 2012). This exceptional example of convergent evolution would be even more remarkable if it were shown that the requisite regulatory mechanisms arose de novo with each initiation. However, the identification of SCR involvement in several aspects of C_4 structural development suggests instead that this syndrome evolved repeatedly from an established repertoire of signals that govern endodermal differentiation in roots and shoots. In other words, C_4 identity is a projection of endodermal identity into leaves.

A number of lines of evidence are consistent with this hypothesis. At the genetic level, the *Scr* gene, which is a key factor in endodermal development in roots (Di Laurenzio et al. 1996), is

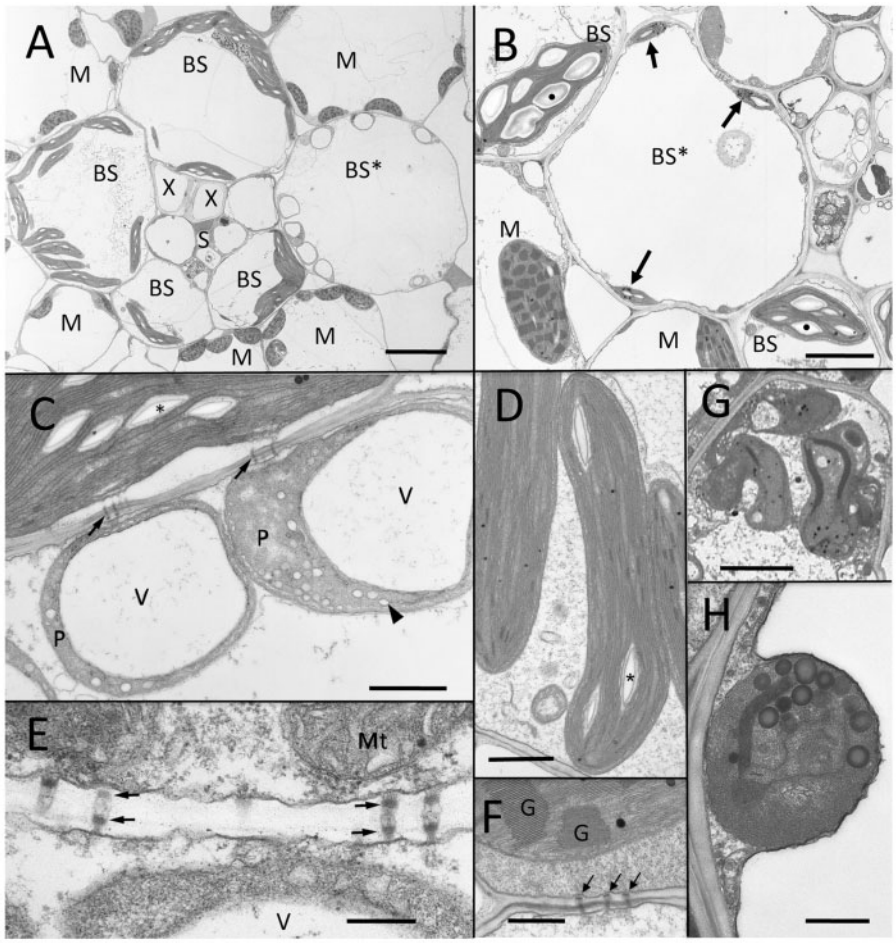

Fig. 5 Transmission electron micrographs of veins, plastid types and plasmodesmata in *zmscr-m1* (A, B, C, E, G, H) and in the wild type (D, F). (A) Small vein with eight undifferentiated, vacuolated plastids visible in one of the bundle sheath cells (BS*). This is a low magnification image of the cell shown in C. Note the similar appearance of each of the plastids in this cell. The plastids are not centrifugally arranged as they are in the other bundle sheath cells. Scale bar = 7.0 μm. (B) Bundle sheath and mesophyll cells in layers around a small vein. Chloroplasts in two of the bundle sheath cells are normal in appearance, with large starch grains (●). The randomly distributed plastids (arrows) in one bundle sheath cell (BS*) are much smaller, with plastoglobuli (small, dense spots) and diminutive starch grains. Chloroplasts in mesophyll cells are normal in appearance, with stacked thylakoids (grana). Scale bar = 3.0 μm. (C) Plastids in two adjacent bundle sheath cells. Plastids in the cell below are undifferentiated, with large central vacuoles and small vesicles (arrowhead), while the plastid in the adjacent bundle sheath cell (above) has normal structure, including starch grains (asterisks). Note plasmodesmata (arrows) traversing the common wall. Scale bar = 1.0 μm. (D) Plastids without grana in bundle sheath cells. Scale bar = 1.0 μm. (E) Plasmodesmata in the common wall of a mesophyll cell (top) and a bundle sheath cell (bottom). Note the dense 'sphincters' on both sides of the plasmodesmata (arrows). The plastid in the bundle sheath cell is relatively undifferentiated, with a large vacuole. Scale bar = 0.25 μm. (F) Plasmodesmata in the common wall of a mesophyll cell (top) and a bundle sheath cell (bottom) of a wild-type plant with sphincters only on the mesophyll cell side (arrows). Scale bar = 0.5 μm. (G) Abnormal plastids in a bundle sheath cell with semi-crystalline bodies, apparently of protein. Scale bar = 3.0 μm. (H) A plastid of the same type as in (D), at higher magnification. Note the semi-crystalline protein body and circular plastoglobuli. Scale bar = 0.5 μm. BS, bundle sheath cell; G, granum; M, mesophyll cell; Mt, mitochondrion; S, sieve element; V, plastid vacuole; X, xylem.

also expressed in the starch sheath (endodermis) in the inflorescence stem in Arabidopsis (Wysocka-Diller et al. 2000) and is required for the development of that cell type (Fukaki et al. 1998, Wysocka-Diller et al. 2000). The starch sheath also has characteristics of C_4 photosynthesis (Hibberd and Quick 2002). Morphologically, the BS of angiosperm leaves forms a ring enclosing the vascular tissue, just as the endodermis encircles the vascular tissue in roots, and *Scr* is expressed in the BS of maize (Li et al. 2010), which also synthesizes suberin (Evert et al. 1977).

In the studies reported here, we add correlative evidence supporting shared gene expression profiles in endodermis and BS by demonstrating that PIN, which transports auxin in vascular and endodermal cells in the root, is also present in developing vascular and BS cells of maize leaves. PIN1a–YFP localizes to the transverse walls of BS cells as it does in root endodermal cells.

If maize BS development is indeed regulated by the same underlying genetic pathways that produce the endodermis, it

should be subject to mutations in the *Scr* gene. As predicted, in leaves of these mutants, the veins and BS cells are abnormal in several respects. The veins meander off course and merge with one another, some minor veins terminate blindly, extra layers of BS cells are produced, BS chloroplasts are highly atypical and BS plasmodesmata sometimes have 'sphincters', present only in M cell plasmodesmata in wild-type plants.

Co-segregation analysis indicates that there is a causal relationship between the *Scr* mutation and the structural abnormalities described above. All aspects of the abnormal phenotype segregate together, and the phenotype requires at least one copy of a mutant allele. *zmscr-m1* is more severe than *zmscr-m2* and is semi-dominant, whereas *zmscr-m2* is recessive. The reason for the semi-dominant nature of *zmscr-m1* is not known at present, but dominant suppression due to transposon insertion has been noted, for example in the loss of anthocyanin coloration in maize kernels (Singer et al. 1998). Whatever the reasons for differences between the two mutations, the central fact remains that the absence of *zmscr-m1* and *zmscr-m2* correlates faithfully with the absence of the mutant phenotype.

The presence of additional BS layers in both the *zmscr-m1* and *zmscr-m2* homozygous mutants suggests, though does not prove, the involvement of SHORTROOT in establishing BS identity. In Arabidopsis roots, SCR restricts the movement of SHORTROOT and concentrates it in the endodermis (Cui et al. 2007, Koizumi et al. 2012). When *Scr* function is compromised with *Scr-RNAi* (RNA interference) constructs, SHORTROOT moves beyond the endodermal cells and ectopically activates cell division and differentiation in adjacent cortex/ground tissue. It is possible that *ZmScr* acts in a similar manner in developing leaf primordia, restricting the movement of the maize ortholog of SHORTROOT to the cells that will become the BS. However, more work is needed in maize to support the role of SHORTROOT in bundle sheath formation and identity, and its function in the establishment of the C_4 photosynthetic mechanism.

The phenotypic abnormalities are not uniform throughout the entire vein network. The reasons for irregular expressivity at the cellular level are not known, but this is a common feature of mutant phenotypes in maize. For example, the *warty-1* mutation, that results in excessive cell enlargement (Reynolds et al. 1998), and the *globby1-1* mutation, that disrupts nuclear and cell division (Costa et al. 2003), are both patchy in distribution. It is possible that genetic redundancy plays a role in the ability of some veins to establish normal Kranz anatomy. ZmSCARECROW is part of a family of GRAS-type transcription factors in maize (Lim 2005), and it is possible that one of the SCR-like genes is functionally redundant with *ZmSCR*.

In this regard, it is notable that BS cells without starch are often arranged in linear files, indicating a clonal origin. This suggests that whatever genetic disturbance is responsible for disrupting normal differentiation of a nascent BS cell, the determined state is carried forward through subsequent cell divisions.

Given the opacity of genetic mechanisms responsible for the development of complex structural traits, it has been generally assumed that engineering Kranz anatomy will be exceptionally difficult. Indeed, long-term efforts to screen for the regulatory elements underlying C_4 structural specialization have not been successful. This is often cited as the most challenging aspect of the ambitious, worldwide project to introduce C_4 photosynthesis to C_3 crops (Covshoff and Hibberd 2012). However, the results presented here cast a more optimistic light on these objectives. Recapitulating the evolution of C_4 structure in C_3 plants is likely to be a much more manageable goal if the underlying regulatory components are already in place in roots and stems.

Materials and Methods

Genetics stocks and genotyping

The *zmscr-m1* allele was obtained from the Barkan lab Mu-Illumina collection, and the *zmscr-m2* allele from the Maize Genetics Stock Center–Uniform Mu collection. Plants carrying the Mutator alleles were identified by genotyping using an outward facing primer in the TIR of *Mutator*, Mu3456 (5′-CAACGCCTCCATTTCGTCGAATCC-3′), and the gene-specific primer ZmSCR F6 (5′-CTCCACTACCTCCATCA GCTAGACCACC-3′). The wild-type allele was identified by using the gene-specific primer set ZmSCR F6 and ZmSCR RA1 (5′-GTCGTAGAGCGAGTGGTGCAGCCAGTGGACG-3′). PCR conditions were 95°C for 30 s, 56°C for 30 s, 72°C for 1 min, repeated for 35 cycles with 3% dimethylsulfoxide and 6% glycerol.

Plant growth conditions

Plants were grown in Percival PGW-40-HID growth chambers under 16 h day (27°C, 1,000 μmol m^{-2} s^{-1} light) and 8 h night (25°C) or in a greenhouse supplemented with sodium vapor lamps at 900 μmol m^{-2} s^{-1} under 16 h (approximately 30°C) day and 8 h (approximately 20°C) night.

Tissue preparation and microscopy

Transmission electron microscopy and starch staining were performed as previously described (Slewinski et al. 2009, Li et al. 2010, Majeran et al. 2010). Sections for light microscopy were cut from the Spurr-embedded blocks, 1.5 μm thick, and stained with 0.5% (w/v) toluidine blue in 1% (w/v) sodium borate.

Vein and bundle sheath structural analysis

Quantification of abnormalities in small and intermediate veins was conducted on randomly selected 10 cm long × 20 cm wide leaf segments taken from the middle of leaf 8 on 6-week-old plants, counted at 0, 10 and 20 cm along the proximal–distal axis for each segment, then averaged for each biological replicate. Measurements of leaf width and lateral vein density were

conducted on leaves 7, 9 and 11 for five *zmscr-m2* mutants and on comparable leaves of five wild-type plants grown concurrently under the same conditions.

Supplementary data

Supplementary data are available at PCP online.

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