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 CO2 by C4 photosynthesis. It has been estimated that transferring the C4 pathway to C3 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 C4 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 C4 trait into C3 crop species, including rice.

Keywords: C4 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 C4 photosynthetic pathway is an elaboration of the more common C3 pathway characteristic of many important crops, including rice and wheat (Sage 2004, Langdale 2011). The advantage of C4 photosynthesis is that it overcomes inherent limitations of Rubisco, the enzyme that fixes CO2 in all plants and provides the substrate for carbohydrate synthesis (Sage 2004, Hibberd et al. 2008, Ghannoun 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 C4 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 CO2 concentration is high. CO2 is concentrated in maize BS cells in a two-step process. CO2 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 CO2, 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 C4 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 C4 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 C3 grasses, which have additional interveinal M cells. Although the requisite enzymes for C4 metabolism are present in C3 plants, and a considerable amount is known about the differences in kinetic properties and expression patterns of these enzymes, the regulatory mechanisms underlying C4 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 C4 anatomy have yielded limited information (Langdale 2011).

We considered the possibility that at least some aspects of C4 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 C4 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 C4...
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 C₄ 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 C₃ 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 C₃ 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](https://example.com/image1.png)

**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 C₄ 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–BSn–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](https://example.com/image2.png)

**Fig. 2** Transverse hand-cut sections 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.
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...
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 C₄ 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 C₄ 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, C₄ 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...
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 C₄ 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 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 C4 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 C4 structural specialization have not been successful. This is often cited as the most challenging aspect of the ambitious, worldwide project to introduce C4 photosynthesis to C3 crops (Covshoff and Hibberd 2012). However, the results presented here cast a more optimistic light on these objectives. Recapitulating the evolution of C4 structure in C3 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'-CAACGCCTCATTTCTGAAATCC-3'), and the gene-specific primer ZmSCR F6 (5'-CTCACAACCTTGATCATCAGG-3'). The wild-type allele was identified by using the gene-specific primer set ZmSCR F6 and ZmSCR RA1 (5'-GTCGTAAGCCGAGTGTGACCCAGTGGACG-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, Majoran 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 replica. Measurements of leaf width and lateral vein density were
conducted on leaves 7, 9 and 11 for five zmcr-m2 mutants and on comparable leaves of five wild-type plants grown concur-
rently under the same conditions.

**Supplementary data**

Supplementary data are available at PCP online.

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