Using evolution as a guide to engineer Kranz-type C\textsubscript{4} photosynthesis

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Kranz-type C\textsubscript{4} photosynthesis has independently and rapidly evolved over 60 times to dramatically increase radiation use efficiency in both monocots and eudicots. Indeed, it is one of the most exceptional examples of convergent evolution in the history of life. The repeated and rapid evolution of Kranz-type C\textsubscript{4} suggests that it may be a derivative of a conserved developmental pathway that is present in all angiosperms. Here, I argue that the Kranz-type C\textsubscript{4} photosynthetic system is an extension of the endodermis/starch sheath, that is normally only found in the roots and stems, into photosynthetic structures such as leaves. Support for this hypothesis was recently provided by a study that showed that the same genetic pathway that gives rise to the endodermis in roots, the SCARECROW/SHORT-ROOT radial patterning system, also regulates the development of Kranz anatomy and C\textsubscript{4} physiology in leaves. This new hypothesis for the evolution of Kranz-type C\textsubscript{4} photosynthesis has opened new opportunities to explore the underlying genetic networks that regulate the development and physiology of C\textsubscript{4} and provides new potential avenues for the engineering of the mechanism into C\textsubscript{3} crops.

Keywords: Kranz anatomy, C\textsubscript{4} photosynthesis, bundle sheath, endodermis, SCARECROW, SHORT-ROOT, phyllode, theory, evolution
more ancient C3 mechanism is best adapted for an environment that no longer exists. In contrast, the C4 mechanism overcomes the inherent limitations of Rubisco by dividing the photosynthetic process into two cell types. These cells are arranged into concentric circles around veins that produce a wreath-like appearance known as Kranz anatomy (Langdale et al., 1989). The bundle sheath (BS) cells comprise the inner circle attached to the vein and are responsible for the key reductive step in photosynthesis, carried out by Rubisco (Sage and Zhu, 2011; Sage et al., 2012; von Caemmerer et al., 2012). The mesophyll (M) cells encircle the BS and are responsible for the initial CO2 fixation by phosphoenolpyruvate carboxylase to produce the 4-C compounds malate or aspartate (Farquhar, 2011). Malate or aspartate then moves from the M to the BS cells through plasmodesmata where CO2 is then released and then re-fixed by Rubisco. This process concentrates CO2 around Rubisco while also excluding oxygen in the BS, thus eliminating the metabolic drag of photorespiration that is common in C3 photosynthesis (Sage et al., 2012).

Previously, it was proposed that there are five major phases of morphological and physiological adaptations that plants undergo in the evolutionary trajectory toward C4 photosynthesis (Sage et al., 2012). The first proposed step is preconditioning which includes increasing vein density and possible gene duplication. Second is modification of BS cells. This includes cell enlargement, production of more organelles, and altered localization of the chloroplasts and mitochondria. M cell volume is also reduced during this transition. Together these changes lead to a “Proto-Kranz” condition. Third is the installation of the basic photosynthetic CO2 pump which includes the reduction of the M-BS cell ratio, localization of the C4 cycle to the BS and activation of the basic C2 system. Fourth is the enhancement of C4 CO2 metabolic capture and pump cycle within the M cells, which includes up-regulation and M-specific expression of phosphoenolpyruvate carboxylase. Finally, in the optimization phase, anatomy and biochemistry are fine tuned to exploit the full efficiency of the C4 mechanism (Sage, 2004).

However, there are reasons to suggest that the evolutionary progression toward the C4 state has been rapid. Kranz-type C4 has independently evolved over 60 times, occurring throughout the angiosperms in both monocots and eudicots (Sage et al., 2011). Indeed, it is one of the most exceptional examples of convergent evolution in the history of life. Astonishingly, Kranz-type C4 appears almost “fully formed” in each of the evolutionary events where it has arisen (Langdale, 2011). There is little evidence that is a slow evolutionary progression toward the C4 state as C3-C4 intermediates are lacking for most of the extant C4 species. Many C4 species also have closely related C3 relatives suggesting recent and rapid appearance of the C4 syndrome within some families of plants (Sage et al., 2011). Indeed, Kranz-type C4 is a classic example of Goldschmidt’s “Hopeful Monsters” (Goldschmidt, 1933), in which spontaneous complexity rapidly appears in some branches of life, and cannot be easily explained in the Darwinian model of evolution. However, I interpret the repeated and rapid evolution of complete Kranz-type C4 differently. This “fully formed” phenomenon (Langdale, 2011) suggests that only simple changes in some of the innate genetic programs are required in order for C4 to arise from a C3 background (Westhoff and Gowik, 2010). Therefore, it is reasonable to hypothesize that Kranz-type C4 may be a modification or extension of a conserved morphogenetic pathway that is inherent to all of the angiosperms.

What conserved tissue or genetic program in C3 plants could give rise to such a complex mechanism as Kranz-type C4 photosynthesis? If we take the view that cells such as the BS are derived from other cells that are already programmed with many of the underlying C4 biochemical programs, it is reasonable to hypothesize that the BS cells themselves confer the underlying properties of the C4 mechanism. The reasoning behind this hypothesis is that all living cells are programed with a specific “identity” that is determined at the time when ground meristem cells initiate differentiation. For example, cells that create the boundary between the outside environment and the internal organs all have a shared epidermis “identity.” They may have various characteristics depending on their location on the plant and specific function, but all share similar morphological and physiological properties as well as underlying developmental and genetic programs. Thus, root epidermal cells share identity with leaf or stem epidermal cells. Therefore, it is possible that C4 BS cells may share a similar identity with cells elsewhere in the plant. In the case of Kranz-type C4 cells, this shared identity may confer the underlying programs needed to establish or precondition the C4 metabolic mechanism within the context of photosynthetic tissues (Slewinski et al., 2012).

What other cells within all angiosperms could be similar to C4 BS cells? Katherine Esau may have already answered this question when she published her anatomical surveys in the 1940s and 1950s – before C4 was discovered (Esau, 1953). She described some atypical species of plants that had “starch sheaths” within the photosynthetic leaf blades. She also described all BS tissue in leaves as having properties of endodermal tissue. When we look back on these observations we find something striking. Many of the atypical plants that Esau (1953) described as having “starch sheaths” in the photosynthetic leaf blades, turned out to be Kranz-type C4 plants such as maize and sorghum. Indeed, we now know that the Kranz C4 BS in leaves share similarities with endodermal tissues in petioles, stems, and roots (Nelson and Dengler, 1997; Slewinski et al., 2012). In all of these tissues, the endodermis is comprised of a single cell layer that surrounds the vasculature, has suberized cell walls, and displays polar expression of the PIN-formed (PIN) effluxors, which conduct auxin through this cell layer (Slewinski et al., 2012). Based on Esau’s detailed observations of leaf anatomy and a plethora of recent reports on C4 physiology and development, I present a new hypothesis for the rapid and repeated evolution of C4 photosynthesis in the angiosperms.

HYPOTHESIS
The Kranz-type C4 photosynthetic mechanism arises when the endodermal/starch sheath program extends into photosynthetic structures, such as leaves, where it is normally repressed or underdeveloped. This leads to a synergistic interaction which can produce the novel C4 pathway from underlying components of both the C3 photosynthetic program and anatomical and metabolic features of the endodermis/starch sheath.

In other words, this suggests that the Kranz-type C4 mechanism is the context-specific manifestation of the endodermis in a
photosynthetic tissue. The C₄ condition arises when the endodermal/starch sheath program from stem and petiole into the leaf (Slewinski et al., 2012). A schematic of this hypothesis is presented in Figure 1. Thus, the inherent physiology of the endodermis may integrate into the photosynthetic program, resulting in a new synergistic physiology, which we know as C₄ photosynthesis.

In plants, the tissue in which a cell resides usually determines the cell’s function and physiological properties. This reasoning can be applied to the endodermis, which is a dynamic tissue that appears to have context-dependent functions. For example, in roots the endodermis encircles the vascular core of the root and acts as an internal barrier for solute transport from the cortex and epidermal cell layers that interact with the external soil environment (Alasimone et al., 2012). At the root tip, columnella cells have different properties than their adjacent stem cells, which are also part of the endodermal tissue (Welch et al., 2007; Ogasawara et al., 2011). Along the root length, endodermal cells do not accumulate starch whereas the endodermis in the stem and petiole does, and is thus termed the “starch sheath” (Wysocka-Diller et al., 2000). The starch sheath usually extends along the vascular core(s) from the base of the shoot–root junction to the petiole–leaf blade junction. The starch-filled amyloplasts within these cells act as statoliths – providing gravity cues to the cells in a similar manner to the columnella cells within the root tip (Morita et al., 2007). Within these cells, the amyloplasts display a polar localization at the base of the cell, in the direction of gravitational pull. Changes in amyloplast position in these cells trigger changes in auxin transport through the endodermal cell layer (Tanimoto et al., 2008). This results in differential cell expansion in the stem that properly orients the plant into the upright position, opposing the direction of gravity (Morita et al., 2007).

When comparing the many forms of endodermis that occur in plants, the C₄ BS is most similar to that of the starch sheath in petioles and stems (Hibberd and Quick, 2002; Tanimoto et al., 2008). Interestingly, C₄ chloroplasts are also similar to those found in the starch sheath of the stem and petiole in certain ways. First, the C₄ BS chloroplasts preferentially accumulate starch when compared to M chloroplasts (Lunn and Farquhar, 1987). Second, C₄ chloroplasts usually have a fixed location in the cells, either on the cell surface adjacent to the vascular core or adjacent to the M (Morita et al., 2007). Third, in many C₄ species, BS chloroplasts lack photosystem II and stacked thylakoid grana, similar to amyloplasts found in the starch sheath (Langdale, 2011). Although, there is great variation in all three of these characteristics in C₄ species, similarities suggest that chloroplasts within the starch sheath and the C₄ BS share components of their identity. Is it possible that chloroplasts in the C₄ BS are essentially photosynthetic-amyloplasts, i.e., plastids of hybrid identity? This may explain why dimorphic chloroplasts are frequently associated with the C₄ BS cells, because the BS cells have a mixed identity of both the starch sheath and photosynthetic cells. However, there is wide variation in BS chloroplast structure within the Kranz-type and single celled C₄ species, suggesting that a range of amyloplast-like features are compatible with C₄ BS, and that only a subset of associated starch sheath/amyloplasts mechanisms are required or sufficient to produce a functional C₄ photosynthetic system.

In an insightful paper by Hibberd and Quick (2002), it was shown that the starch sheath in aerial parts of the plant, especially petioles, is involved in internal CO₂ recycling (Hibberd and Quick, 2002). Respiring tissues such as roots produce abundant CO₂ as a waste product. However, not all of the CO₂ is released into the soil environment that surrounds the roots (Bloemers et al., 2013). Much of the respired carbon migrates into the xylem stream that flows from the roots toward the leaves. A study using mature poplar trees shows that a significant portion of the
Kranz/C4-like anatomical features but with C3 physiological program in the C3–C4 intermediates is just as likely.抑制 remnants of the C3 photosynthetic pathway that are unneeded or redundant, while concurrently enhancing the more dominant features of the C4 metabolic pathway. This is not to say that C3–C4 intermediates always represent a transitional stage; they may be fully adapted in their current form in many cases (Sage et al., 2011). It can be argued that selection against the C4/starch sheath physiological program in the C3–C4 intermediates is just as likely (Vicentini et al., 2008; Langdale, 2011). A full reversal of C4 to C3 is also possible and has already been reported in some of the C3 grasses (Vicentini et al., 2008). As a result, plants could arise that possess Kranz/C4-like anatomical features but with C3 photosynthetic metabolism. Another possibility is that C3–C4 intermediates, arising from either selection for or against the Kranz-type C4 pathway, could have some of the advantageous characteristics of full C4 plants in hot and dry environments (Sage et al., 2011). Thus, it can also be argued that development of C4-like traits can confer fitness on their own, implying that the C3–C4 intermediate state is an evolutionary trajectory (Langdale, 2011; Sage et al., 2011). Overall, this new view of C4 evolution suggests that only small changes are required to rapidly produce dramatic diversity in anatomy and physiology. This diversity is then subject to selection for or against the C4 mechanism based on the environmental pressures of the organism. Selection for enzymatic cell specificity may also be necessary to increase the CO2 metabolic pump from the M to the BS, while also concurrently enriching Rubisco in the BS. Presumably, these two processes would evolve in parallel because sequestration of Rubisco to the BS without the CO2 pump would reduce carbon fixation in free air and lead to an evolutionary disadvantage. Interestingly, only phosphoenolpyruvate carboxylase is common to all of the decarboxylation types of C4 (Sage, 2004; Furbank, 2011). Extrapolation of the underlying endodermal physiology may occur differently with each independent evolutionary event—leading to variations in the CO2 metabolic pump, i.e., Nicotinamide adenine dinucleotide phosphate-malic enzyme (NADP-ME), Nicotinamide adenine dinucleotide-malic enzyme (NAD-ME), or phosphoenolpyruvate carboxykinase (PEPCK) types (Sage et al., 2011). However, recent evidence suggests that these three decarboxylation types may not be distinct, but are flexible depending on environmental and developmental conditions (Furbank, 2011; Pick et al., 2011). Within the grasses, switching of decarboxylation types within a species has been reported (Vicentini et al., 2008). However, if the three decarboxylation types are extrapolations of the underlying physiology of the endodermal/starch sheath program, then it is reasonable to hypothesize that each type is simply a dominate enzymatic pathway within a larger physiological context that includes subtle forms of the other two types. Section pressures on a recently evolved C4 species then determines which of the three decarboxylation types become dominant. The other pathways are most likely not eliminated in this selection but left in their original and more subtle "housekeeping" roles or suppressed to lower levels. Thus under this new hypothesis, significant plasticity and flexibility in the C4 mechanism would also be conferred by the underlying endodermal/starch sheath program. Following these arguments, it is important to also highlight that, in both roots and stems, the endodermis functions as a high-capacity auxin conducting tissue (Alasoinne et al., 2012). In both C3 and C4 plants, vein patterning is regulated by auxin gradients.
Auxin produced in the epidermis drains toward preexisting veins why C4 plants reduce M cell counts to the extent that they match their development, architecture, and physiology. This may explain identity on the already present photosynthetic M cells, modifying endodermal developmental program may also confer a cortex-like function. Thus, the proximity and intercellular interactions from the BS. BS cells then generate signals that determine M specifica-

nals generated from the vascular core – which first determines the development and identity of the cells is determined by the sig-

als that establish endodermis identity. Thus, it is reasonable to hypothesize that if Kranz-type BS tissue is just an extension of the endodermal program, they should also be subject to mutations in the essential endodermal patterning and development genes SCR and SHR. Indeed, support for this reasoning was recently provided. It was shown that the maize ortholog of SCR plays a role in BS development in maize leaves (Slewinski et al., 2012). Mutations in the ZmSCR gene result in proliferation of BS cells, altered differentiation of BS chloroplasts, vein distortion, and reduction in minor vein formation and overall vein density. zmscr mutant plants also produce starch-less BS cells that closely resemble starch-

less stem endodermal cells in the shr mutant of Arabidopsis called endodermal amyloplasts less or edl (Mitra et al., 2007). In the scr mutant of maize, some of these starch-less cells also have altered plasmodesmata within the cell walls that separate the BS and M cells (Slewinski et al., 2012), suggesting that their specialization is also linked to the endodermal program. Thus, this provides for the first time, genetic evidence that the endodermal development pathway underlies C4 BS development. This study also suggests, though does not directly prove, that SHR also plays a critical role in the development of the BS and underlying metabolism in C4 plants.

Analysis of the large scutellar node (lm) mutant of maize also supports the endodermal development model for C4 BS in leaves. The lm mutant phenotype mimics the abnormalities generated by both synthesis and transport (Scarpella et al., 2010). Auxin produced in the epidermis drains toward preexisting veins within the developing tissues. When larger veins form, they also produce auxin gradients within the adjacent ground meristem tissue by depleting auxin from the surrounding cells. This creates an auxin minima that initiates the formation of smaller vein orders that form after the larger orders of veins have been established and are undergoing differentiation (Scarpella et al., 2010; Gardiner et al., 2011). The formation pattern of minor veins between established major and intermediate veins in maize is shown in Figure 3.

The extension of the endodermal layer into the vascular tissue in the developing leaf may enhance the depletion of auxin from the ground tissue in C4 leaves when compared to developing C3 leaves. Under these assumptions, it is reasonable to hypothesize that the increased vein density observed in C4 plants is, at least in part, due to the increased auxin depletion associated with the developing endodermal layer. This would presumably create more or stronger auxin minima, thus initiating more minor veins.

Unlike non-Kranz species, each vein initiation confers the formation of entire Kranz units (vascular core, BS, and surrounding M cells; Nelson and Dengler, 1997). It is unlikely that veins can get closer than one vascular Kranz unit because of the nature of the underlying endodermal developmental program. In this model, the development and identity of the cells is determined by the signals generated from the vascular core – which first determines BS. BS cells then generate signals that determine M specification. Thus, the proximity and intercellular interactions from the endodermal developmental program may also confer a cortex-like identity on the already present photosynthetic M cells, modifying their development, architecture, and physiology. This may explain why C4 plants reduce M cell counts to the extent that they match BS cells, ultimately ending in a 1:1 ratio. In contrast, veins in C3 plants do not form such units. Rather they form in a pool of ground meristem cells – defining cells that will become part of the vasculature and excluding the cells that will give rise to the M. Thus, the C3 mode of vascular development leads to more variable numbers of M cells between vascular strands.

The shift in plasmodesmata density and specialization at the M–BS interface in the leaves of C4 plants may also be a pleiotropic effect of the endodermal program in the leaf. In other parts of the plant, the endodermis is coated with suberin and other hydrophobic compounds that create an apoplastic barrier that limits cell-to-cell flow of water and solutes through the cell wall (Goldner, 2013). Therefore most transport between the endo-

dermal cells and the surrounding cortex or parenchyma cells is restricted to the symplastic route – through abundant plas-

modesmata that connect the cytosolic domains of adjacent cells. Although the suberized apoplastic barrier is only sometimes associated with the C4 BS (Sage, 2004), increased intercellular sym-

plastic transport between BS and M cells appears to be necessary for an efficient CO2 metabolic pump between M and BS cells. As with the other traits associated with C4 specialization mentioned above, the intercellular transport mechanism utilized by the C4 BS and M cells in the leaf may be an extension and modification of the system found in the endodermal tissue in the roots and stems. In Arabidopsis the genes that underpin endodermis formation, Scarecrow (SCR) and Short-root (SHR), are expressed in roots, stems, and leaves (Wysocka-Diller et al., 2000; Gardiner et al., 2011). The SHR gene is expressed in cells within the vascular core (Helariutta et al., 2000), except for the phloem initial cells (Yu et al., 2010). The SHR protein moves out from the vascular core cells and activates the Scx gene within the cells that are in contact with the vascular core (Koizumi et al., 2012). SCR protein binds to SHR and sequesters the protein in the nucleus, preventing further movement (Wu and Gallagher, 2012). This mechanism deliminates a single cell layer as well as initiates the cascade of sig-

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observed when auxin transport inhibitors are applied to developing leaves (Landoni et al., 2000). These abnormalities include vein distortions, vascular hypertrophy, and disorganized vascular core structure (Figures 4A,B). What is interesting in the lsn mutant is the formation of normal BS and M, both structurally and physiologically, around the distorted vascular core in the leaves (Figures 4C,D). Lsn BS cells preferentially accumulate starch like wild type plants (Figures 4E,F), and both BS and M plastids appear normal in transmission electron microscopy (TEM) analysis (Figures 4G,H). This finding conflicts with the cell lineage models that have previously been proposed for the development of the C₄ BS which suggested that BS and M cells arose from organized cell division patterns (Langdale et al., 1989; Sud and Dengler, 2000). However, BS formation in lsn more closely resembles the endodermis that surrounds distorted veins in Arabidopsis plants grown in the presence of auxin transport inhibitors (Wysocka-Diller et al., 2000), suggesting that organized and coordinated cell division is not essential for the development of Kranz anatomy. Although, analysis of lsn does fit within the framework of the endodermis/starch sheath developmental model (Helariutta et al., 2000). Additionally in Arabidopsis, the SCR::GFP construct is still only expressed in a single cell layer of endodermal cells when internal vascular hypertrophy or distortion occurs (Wysocka-Diller et al., 2000). This again suggests that both the development of the endodermis and C₄ BS are regulated by a non-cell autonomous signal that radiates from the internal vascular core, most likely the SHR protein.

The role of SHR in recruiting C₄ BS may also explain some of the diversity seen in Kranz anatomy. As noted above, phloem initial cells do not express the Shr gene and therefore must symplastically

**FIGURE 4 | Vascular development and bundle sheath formation in the lsn mutant of maize.** Panel showing wild type (A,C,E,G) and lsn mutant (B,D,F,H) maize leaf sections. (A) Section of iodine potassium iodide (IKI) stained wild type leaf showing regular and uniform vascular patterning. (B) Section of IKI stained lsn mutant leaf showing distorted vascular patterning. (C) Cross section of wild type leaf under UV light showing canonical Kranz anatomy (red represents chlorophyll autofluorescence, blue represents autofluorescence of the cell walls). (D) Cross section of the lsn mutant under UV light, showing distorted veins with internal vascular hypertrophy and irregular internal differentiation surrounded by single layers of bundle sheath and mesophyll cells. (E,F) Cross sections of IKI stained leaves, respectively, showing normal starch accumulation in the BS cells and absence of staining in the M cells. (G,H) Transmission electron micrographs of wild type (G) and lsn mutant (H) BS and M cells, showing normal C₄ plastid differentiation and identity in both. BS, bundle sheath cell, M, mesophyll cell, scale bars: (A,B) 600 μm; (C–F) 50 μm; (G,H) 5 μm.
import the SHR protein to proceed through normal phloem differentiation (Yu et al., 2010). Thus, the developing phloem presumably acts as a SHR protein sink, rather than a source of the signal. Usually, the phloem is localized within the vascular core – completely surrounded by cells that produce the SHR protein signal (Väätänen et al., 2011). However, there are some C4 species like Atriplex rosea which develop phloem bundles close to the edge of the vascular core in leaves (Dengler et al., 1995). In this species, the C4 BS only encircles part of the vein, and is absent where the phloem bundle protrudes from the vascular bundle. Indeed, many other C4 species show a similar arrangement, where C4 BS are either absent or converted to sclerenchyma cells in the regions adjacent to the phloem bundles (Edwards and Vosnesenskaya, 2011). Therefore it is reasonable to hypothesize that the internal vascular structure influences the dynamics of non-cell autonomous developmental signaling of the endodermal/BS program which could lead to the wide variations in Kranz-type structures seen in many C4 species (Edwards and Vosnesenskaya, 2011).

**ENGINEERING A NOVEL FUNCTION FOR A CONSERVED TISSUE**

Esau (1953) suggested that all BS in angiosperms have some endodermis-like features. But the extent to which these endodermal features are manifested in the BS varies greatly. Therefore it is likely that in C4 plants, full Kranz anatomy arises when the underlying endodermal framework becomes enhanced – leading to the more dominant features that are associated with full endodermal/starch sheath identity. Following this reasoning, C4 species like Atriplex rosea which develop phloem bundles close to the edge of the vascular core in leaves (Dengler et al., 1995). This suggests that other SHR/SCR interacting proteins modulate the SCR/SHR complex in the C4 BS cells and that the endodermal program is most likely regulated on the protein level. These proposed SCR/SHR interacting proteins could either confer a specific cell identity (Welch et al., 2007; Ogasawara et al., 2011), or suppress the pathway as in the case of C4 leaves. The SCR/SHR pathway has been extensively studied in Arabidopsis, and yet very little has been reported on the function of these proteins in leaves (Wysocka-Diller et al., 2006; Yu et al., 2010; Gardiner et al., 2011; Ogasawara et al., 2011; Cui et al., 2012). From these data, it is reasonable to hypothesize that there may be a negative feedback loop to repress the endodermal developmental pathway in leaves. This may be why when either SHR or SCR are knocked out or over-expressed in Arabidopsis, major structural aspects of leaves are for the most part, unaltered (Cui et al., 2007). These reports also support the hypothesis that C3 plants may have functional repressors in the leaves that mediate the down-regulation of the key genes needed for Kranz and C4 differentiation irrespective of the amount of SCR or SHR protein present during development. Other cell types may share many of the developmental signaling cascades with the endodermis in the stems and petals, but their tissue specificity may be controlled by other SHR/SCR interacting proteins that function in their respective feed-forward differentiation pathways. This may also explain why SHR and SCR are found in developing stomata and in the case of SCR, in the L1 layer of the shoot meristem (Wysocka-Diller et al., 2006; Kamiya et al., 2003; Lim et al., 2005), tissues not associated with the endodermis. This suggests that although SHR and SCR are essential for the patterning and formation of the endodermis and other cell types, they do not individually confer cell specificity or “identity.” Analogous to the ARCE model of floral development (Bowman et al., 2012), specific variants of the endodermis may be under the combinatorial control of multiple factors that form a functional protein complex that regulates differentiation. In other words, SHR and SCR are essential base, or “E” type functions (Bowman et al., 2012) in the endodermal developmental program.

If SHR and SCR are not direct targets for engineering Kranz-type C4, then what genes are? From a variety of published reports, the most likely candidates to function with SHR and SCR are the interacting proteins which include, but may not be restricted to, the indeterminate-domain family of transcription factors (IDDs; Levesque et al., 2006; Welch et al., 2007; Tanimoto et al., 2008; Ogasawara et al., 2011). Within the roots and stems, different combinations of these factors promote the formation of root and stem endodermal identity, quiescent cells, and stem cells. For example, in Arabidopsis roots a combination of AtIDD10 and AtIDD3 maintains stem cell identity (Welch et al., 2007).
In stems, AtIDD15/SHOOTGRAVITROPISM5 (SGR5) functions with ASKHR and ASKCR to promote starch sheath identity (Tan-imoto et al., 2008). But the most interesting and tantalizing evidence for the involvement of the IDDs in BS development comes from IDD over-expression studies. For example, when AtIDD5/NitraTracker, a target and interacting protein of ASKHR and ASKCR in the root endodermis (Levesque et al., 2006), was over-expressed in Arabidopsis (Seo et al., 2011), photosynthesis and plastid structures and were both dramatically altered. Most notable of these alterations is that M chloroplasts displayed reduced granal stacking – similar to what is seen in the BS of C4 plants (Levesque et al., 2008). This finding is reminiscent to the conversion of leaves into petioles when chromoplasts developed instead of chloroplasts in the petaloid-like structures (Pelaz et al., 2001) showing that physiology can be controlled by developmental programming.

Only one of the IDD genes has been characterized in a C4 plant thus far. In maize, loss of function of Indeterminate growth1 (ZmID1), the founding member of the gene family, results in altered growth and flowering time (Colasanti et al., 1998). Interestingly, all mutants also have altered expression of many of the genes involved in C4 biochemistry, suggesting there may be a broader role for ID1 in leaf development and physiology (Connera et al., 2007). ZmID1 is only expressed at the base of developing leaves and decreases as the leaf matures, suggesting a role in leaf development (Wong and Colasanti, 2007). In this region it is expressed in all cells. Therefore, based on overlapping expression with both ZmScr and ZmIdh genes, and the altered expression of C4-related genes in the mutant, it is likely that ZmID1 plays a role in the development of the C4 pathway in maize. Many of the other IDD, SHR-like and SCR-like genes in maize are also expressed at the base of the developing leaf and have either BS- or M-specific expression patterns (Li et al., 2010), suggesting potential roles in establishing C4 BS or M cell identity and cell-specific organization of physiology. However, more research is needed to elucidate these proposed roles for ZmID1 and other IDD genes in either the SCR/SHR or C4 developmental pathways in leaves.

The IDD class of genes may also have the potential to act as negative regulators of endodermal development and identity. Recently it was found that some of the members of the IDD gene family contain ethylene-responsive element binding factor-associated amphiphilic repression (EAR) domains (Wu et al., 2013), which have been shown to act as strong transcriptional repressors. Might these be the factors that keep the endodermis/Kranz program suppressed in C3 leaves as hypothesized earlier? Overall, the published data on the IDD class of genes suggests they may play a significant role in Kranz-type C4 regulation and development, both as potential positive and negative regulators. However, much more research is needed to explore the hypotheses presented here.

**EVOLUTION OF KRANZ-TYPE C4 MECHANISM IN MONOCOTS: REVISITING THE PHYLLODE HYPOTHESIS**

The emergence of C4 in monocots appears to be ancient, arising with the grasses and sedges as they began to diverge from the other monocots (Sage et al., 2011). Can the hypothesis stated above, that Kranz-type C4 is a synergistic interaction between the photosynthetic tissue and the endodermis, also shed light on the evolution of C4 in grass leaves? In order to explore this question, it is essential to compare eudicot and monocot leaf blade anatomy. Most important is to recognize the theory that monocot’s leaves may not be true “leaf blade” tissue when compared to the eudicots (Arber, 1918; Kaplan, 1973).

It has been hypothesized that monocots evolved in an aquatic environment (Arber, 1918). This dramatically shifted the morphology of the shoot organs, such as leaves and stems. It is presumed that when these plants became submergent, their petioles or lower leaf blades became greatly extended in order to keep the leaf blades above or on the surface of the water. Over time, the upper leaf blade became greatly reduced, resulting in the petiole/lower leaf zone becoming the primary photosynthetic organ of the plant (Arber, 1918). The petiole/lower leaf zone then expanded and extrapolated into a new leaf blade (Tsiantis et al., 1999; Nardmann et al., 2004). The phylloide theory is illustrated in Figure 5.

Whether the monocot leaf blade is derived from either the petiole as argued by Arber (1918) or the lower leaf zone (base including stipules) as argued by Kaplan (1973) is still unclear and highly debated. However, in either case, the extrapolation of either the lower leaf zone or the petiole into a new leaf base would support the arguments presented below.

The reduction of loss of a true leaf blade still occurs in some dicots. For example, the amphibious plant Ranunculus fuscatus has different phenotypes when plants develop in dry or submerged conditions (Burkhardt, 1977). When grown on dryer soil, the plants develop similar to normal eudicots. They have broad and fully expanded leaves and are compact. However, under submerged conditions, plants reduce or eliminate the upper leaf blade tissue, and extend and expand the petiole/lower leaf zone and stems into string-like structures (Osborne, 1984), similar to leaf structures in early monocots (Arber, 1918). Indeed, these plants show extensive plasticity in their ability to dramatically shift their shoot-specific morphology and physiology. In this submerged state, the stems and petioles take over the primary role of photosynthetic organ (Buchnera and Ntidual, 2009). This raises the question: what if successive generations of such amphibious plants experience the flooded situation throughout the majority of their lifecycle? Could the plants permanently fix the flooded pheno-type – leading to a grass like appearance due to the reduction of the upper leaf blade and an extrapolation of the petiole/lower leaf zone and the stem (Figure 5)? This morphological shift reduces many of the dicot leaf blade anatomical features, the most profound being the elimination of reticulate vein patterning. The formation of parallel veins in monocots is presumed to be derived from the merger of two sides of a previously radial-organized veins in the stems and petioles (Figure 6, Arber, 1918; Kaplan, 1973). Alternating phloem and xylem polarity within adjacent parallel veins of some of the monocot leaves supports this view of leaf blade evolution (Arber, 1918).

This may also explain why all of the C4 grasses and sedges use the Kranz-type mechanism (Langdale, 2011). As argued above, the petiole and lower leaf zone contains most, if not all, of the necessary anatomical and biochemical elements to establish the C4 photosynthetic syndrome (Hübner and Quick, 2002; Brown et al., 2010; Siewinski et al., 2012). Thus, a new leaf structure extrapolated from this area of the leaf would inherently contain all of the necessary underlying components of Kranz-type C4.
However, most of the monocots utilize the C₃ photosynthetic mechanism. Another look at monocot anatomy may explain why. In both C₄ petiole/lower leaf blades and in early monocots, the endodermis and the outer layer of photosynthetic cells (beneath the epidermis) are usually separated by one or many layers of non-photosynthetic parenchyma cells (Figure 6A; Arber, 1918). These parenchyma cells block direct interaction between the starch sheath and the active site of photosynthesis. But as monocots evolved, and the grass and sedge clade emerged, leaf structures became flattened and thinner (Figures 6B,C). The surrounding photosynthetic layers, one on either side of the leaf, start to invade the region of the central vascular strands (Arber, 1918), most likely through the progressive elimination of parenchyma cells. In many of the grasses and sedges, these parenchyma cells are entirely absent in the leaf blade and are usually only found in the large central mid-vein (Figure 6D). This anatomical adjustment would also bring the outer photosynthetic layer of cells in direct contact with the endodermis/starch sheath, allowing the two programs to interact. Thus, the morphological shifts that lead to the emergence of the grasses could also have been the events that pre-conditioned Kranz-type C₄ within these clades.

This raises another important question. Why is rice C₃ instead of C₄? Under the phyllode theory of monocot evolution, the ancestors of rice may have been pre-conditioned for C₄ metabolism in the same manner as other C₄ grasses and sedges. However, it is important to remember that when compared to C₄ photosynthesis, the C₃ mechanism is energetically more expensive. It takes 18 ATP to fix one CO₂ molecule in the C₃ mechanism and 30 ATP in the C₄ system (Langdale, 2011). It is possible that the C₄ preconditioning event in the grasses did not confer an advantage within the environment in which the ancestors to domestic rice evolved. Thus the ancestors of rice and other C₃ grasses may have either repressed or allowed the degradation of the C₄ metabolic pathway in the leaf tissue. Under these assumptions, it can be argued that the vascular BS in rice may be a remnant of the endodermal and the mestome sheath may be a remnant of the pericycle (Figure 7; Martins and Scatena, 2011). It is interesting to point out that it only took one mutation in the SCR gene of maize to produce many of the anatomical features that are seen in rice. Most notably, the starch-less BS cells reported in the scarecrow mutant of maize (Slewinski et al., 2012) have a striking resemblance to the vascular BS in rice (Langdale, 2011). Both cell types form a non-photosynthetic BS with undifferentiated plastids. Additionally, there are many other monocots that followed the same evolutionary trajectory as the grasses, producing flattened leaf blades that lack non-photosynthetic parenchyma cells, but retaining the C₃ photosynthetic mechanism.

This may be why in oat-maize addition lines, the addition of individual maize chromosomes do not confer a functional C₄ photosynthetic mechanism (Tolley et al., 2012), because the oat C₄ program may have been suppressed or undergone degradation in leaves. However, in these studies, it is also important to take into consideration the caveats of the experiment itself. For example, in wheat, maize chromosomes are destroyed after fertilization (Lehbrecht and Devos, 1996), a phenomenon that is exploited in the production of double haploid wheat and oat. Thus, the addition of individual chromosomes may not represent a true “addition”
FIGURE 6 | Simplified schematic representation of cross sections through monocot “leaf blades” along the evolutionary trajectory toward the grasses. (A) Simplified model of leaf structure in the early monocots (note: the early monocot leaves are depicted as radial structures in order to simplify the concepts presented). The vasculature encased in endodermal starch sheath tissue is separated from the outer photosynthetic layer by non-photosynthetic parenchyma cells. (B) The leaf structure begins to flatten and compress the vascular cores toward the center of the leaf, leading to a parallel vein patterning seen in (C). In grasses and sedges (D) the non-photosynthetic parenchyma cells are reduced or completely absent, bringing the outer photosynthetic layers in contact with the endodermis/starch sheath layer that surrounds the vasculature (model simplified and extrapolated form Arber, 1918).

of C4 genes that can be expected to function normally. The additional alien chromosome may undergo inactivation when taken out of the context of it native genomic and cellular context. This is commonly the case when exotic chromosomes are added into animal cell lines. It is likely, as mentioned above, the underlying factors that give rise to the endodermis and starch sheath, the SHR and SCR proteins, are already present within the nuclei of cells that comprise the C3 BS (Wysocka-Diller et al., 2000; Gardiner et al., 2011). Analogous to the C3 BS, it is possible that the negatively regulating interacting factors are in place within rice leaves – suppressing the development of full or sufficient endodermal identity.

Suppression of the C4 pathway in hybrids between closely related C3 and C4 species has also been well documented (Brown and Bouton, 1993), supporting the hypothesis that C3 plants repress the activation of sufficient endodermal program in the leaves. Thus, if the full genome complement fails to activate a C4-like state in C3–C4 hybrids, it is unlikely that an individual chromosome, a partial genomic component, can initiate the C4 program within the C3 context.

The mechanism of C4 suppression or down-regulation could also be argued for many of the C3 grasses such as bamboo, oat, and wheat. Intriguingly, five independent reversals from C4 to C3 have been reported in the grasses (Vicentini et al., 2008). It is possible that the ancestor at the base of the Pooidae (containing oat, barley, and wheat), Ehrhartoidea (containing rice), and Bambusoidae (containing bamboo) families underwent a C4 to C3 reversal early in its evolution! These three families contain only C3 species, unlike the majority of the other grass families such as Panicaceae, Andropogoneae, and Centothecoideae in which some or all of its members contain C4 species (Vicentini et al., 2008; Sage et al., 2011). It is tempting to speculate that it is harder to re-evolve the C4 mechanism from a C4 to C3 reversal species than it is to newly evolve from a basic C3 species.

However, there does seem to be some hope for rice. When photosynthesis was surveyed in diverse rice species, considerable variation in photosynthetic rates was found (Ito et al., 1994). None of the rice species were shown to employ the C4 mechanism, but some varieties had unusually low photorespiration rates, as well as increased phosphoenolpyruvate carboxylase activity and photosynthetic rates that are comparable to reported C3–C4 intermediate species. Thus, similar to the arguments for the origin of the vascular BS, some physiological aspects of the ancient C4 preconditioning event in the grasses may persist in a few rice species.

FUTURE PERSPECTIVES

How can we transfer the Kranz-type C4 syndrome into C3 crops such as soybean and rice? From the hypothesis describe in this paper, the conversion of dicot species such as soybean may be easier than previously envisioned. Isolation of both the positive and negative regulators that control endodermal development would be the first step in engineering C4 by recapitulating
Maize

Endodermis becomes photosynthetic BS - C4 metabolism

Selection for C4

Grasses/preconditioning event

Disruption in the endodermal program

Rice

Endodermis becomes vascular BS sheath – C3 metabolism

Mestome sheath may be a remnant of the pericycle tissue

remnants of the endodermis/starch sheath remain after the endodermal program is either disrupted or suppressed. The endodermis/starch sheath becomes the non-photosynthetic vascular bundle sheath in rice, whereas remnants of the pericycle tissue may be adapted to form the mestome sheath layer within the vascular core.

In maize (top), the endodermis/starch sheath becomes incorporated into the photosynthetic system – giving rise to the bundle sheath (Kranz anatomy) and synergistic interaction that underlies C4 photosynthesis. In rice (bottom), the synergistic interaction is selected against, thus maintaining a C3 photosynthetic mechanism. However, structural

Evolution. In the case of C4 rice, the hypotheses and arguments made in this manuscript suggest that there may be alternative paths that might achieve this goal. Here I suggest that there might be two potential engineering trajectories. The first is to completely overhaul the physiology of the rice leaf with transgenic constructs that target the metabolism directly. The second is to try to reawaken the hypothesized C4-like state of rice’s distant past.

MATERIALS AND METHODS

PLANT MATERIALS, GROWTH CONDITIONS, AND TISSUE PREPARATION

Stocks containing the lsn mutation were kindly provided by Giuseppe Gavazzi at the Università degli Studi di Milano, Milan, Italy. Plants heterozygous for the mutation were self-pollinated to produce segregating families of mutant and wild type plants for analysis. lsn mutant and wild type plants were grown until the sixth leaf emerged. Leaves four and five were used for the analysis. Plants were grown and tissue processed, fixed, and stained for light and electron microscopy as described in Slewinski et al. (2012). Maize lines containing the pin-formed1A-Yellow Fluorescent Protein (Pin1A-YFP) transgene were grown, prepared, and visualized as described in Slewinski et al. (2012).

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