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Identification of bundle sheath cell fate factors provides new tools for C3-to-C4 engineering

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Spatial compartmentation of the photosynthetic process between bundle sheath (BS) cells and mesophyll cells is one of the features that increase the productivity of C4 plants. To introduce C4 photosynthesis into C3 plants therefore calls for the identification of factors that control BS cell fate and promoter sequences that confer gene expression specifically in the BS and mesophyll cells. We recently demonstrated that 3 GRAS family transcription factors, SHORT-ROOT (SHR), SCARECROW (SCR) and SCR-LIKE 23 (SCL 23), are required for BS cell fate specification in *Arabidopsis thaliana*. Homologs to these genes are present in other plant species, C3 and C4, suggesting a conserved mechanism for BS cell fate specification. Interestingly, initially SCR and SCL23 are expressed uniformly in BS cells, but at later stages of leaf development SCR expression becomes restricted to the BS cells associated with the phloem, whereas SCL23 is preferentially expressed in the BS cells abutting the xylem. Characterization of the functions and expression patterns of *SHR*, *SCR* and *SCL23* homologs in other plants, especially C3 crops, will not only advance the knowledge about BS cell development but also provide new tools for manipulating the number and physiology of BS cells, a critical prerequisite for C3-to-C4 engineering.

Keywords: SCARECROW, SHORT-ROOT, SCL23, Bundle sheath cell, C3-to-C4 engineering, *Arabidopsis*

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Since the emergence of terrestrial plants, the global atmosphere has undergone many remarkable changes including CO_2 depletion and increase in O_2 concentration. As a result of acclimation, some flowering plants evolved C4 photosynthetic mechanisms, resulting in increase in carbon assimilation and reduction in photorespiration.[1,2] Studies have shown that transferring the C4 pathway into C3 crops could increase water-use efficiency, reduce the need for fertilizer, and boost yields significantly, particularly in dry and hot environments.[3] As a consequence, there has been considerable interest in C4 engineering to increase photosynthetic efficiency in C3 crops.[4-6]

Bundle sheath (BS) cells are a leaf cell type that forms a tightly packed layer surrounding the veins. In 2-cell C4 plants, the division of the photosynthetic process into the BS and mesophyll cells is one of the most significant features that make photosynthesis more efficient. Therefore, to achieve C4 photosynthesis in C3 plants requires a good understanding of the mechanisms that control BS cell fate and physiology.

BS cells are considered analogous to endodermis, because they both surround the veins.[7] It is known that SHR and SCR are necessary for endodermis cell fate specification in root.[8-10] It is therefore likely that these 2 genes also play a role in BS cell fate specification. Supporting this hypothesis, it has been shown that SCR is expressed specifically in the BS cells.[11,12] SHR and SCR are also required for the formation of the starch sheath cell layer, which is also positioned around the vascular tissue in the hypocotyl and inflorescence stem.[12] Although the mechanisms underlying BS cell fate

specification remain elusive, it has been suggested that signals derived from the vascular tissue play a pivotal role in determining the position of the BS cell layer.[13,14] SHR could be the signaling molecule, because it is expressed in the vascular tissue and the SHR protein moves into the neighboring cells.[15,16] Taken together, these observations suggest that similar mechanisms are used to determine the cell fate of the endodermis, starch sheath and BS cells, in which SHR and SCR are central players.

To determine whether SHR and SCR control BS cell fate, we examined the cell pattern in the leaves of *shr* and *scr* mutants. As expected, the small, compact, and rectangular-shaped BS cell layer was missing in the *shr* mutant. However, the BS cells remained largely intact in the *scr* mutant. This surprising result has led to our further finding that *SCL23*, the closest paralog to *SCR*, was also required for BS cell fate determination. Another surprising finding is that the cell pattern in the *shr* and *scr scl23* mutants was still normal, which suggests that SHR and SCR function differently in the leaves than in other organs. Remarkably, although SCR and SCL23 are expressed uniformly in the BS cells in young leaves and act redundantly in determining the BS cell fate, their expression and function become diverged at later stages of leaf development. While SCR is preferentially expressed in the phloem-associated BS cells and plays a role in sugar transport, SCL23 is more strongly expressed in the xylem-associated BS cells and is involved in mineral transport. This result also suggests that there are 2 types of BS cells with distinct functions.

In maize, mutations in *SCR* results in proliferation of BS cells as well as abnormal differentiation of BS chloroplasts.[17] Because maize is a C4 monocot, whereas *Arabidopsis* is a C3 dicot, this result suggests different mechanism may be used in C3 and C4 plants in BS cell fate specification. However, multiple copies of homologs to *SHR* and *SCR* have been identified in other plants, including maize.[18-20] It is therefore equally possible that BS cell fates are controlled by the same set of genes but individual homologs function differently or redundantly. To distinguish these possibilities, we need to identify and functionally characterize *SHR*, *SCR* and *SCL23* homologs in other plants. Gene functional analysis with organisms with a large genome has been hindered by the lack of efficient gene targeting methods, but this has been changed with the development of several powerful genome-editing technologies, such as the TALENs method and the CRISPER-Cas system.[21-25]

Our identification of genes that control BS cell fate in *Arabidopsis* not only has advanced the understanding of the mechanisms underlying BS cell development, but also provides new tools for C3-to-C4 engineering. As transcription factors, these BS cell fate determination factors can be used to increase the number of BS cells, which is also critical to C4 photosynthesis. To introduce C4 photosynthesis into C3 crops, some genes involved in the Calvin-Benson cycle need to be silenced in mesophyll cells but expressed specifically in BS cells.[26] Moreover, because the 2 populations of BS cells associated with the phloem or xylem have different functions, as suggested by our study, they need to be modified separately. This calls for cell-type specific promoters and the SCR and SCL23 promoters are the only regulatory sequences known so far that have such specificity. Unlike the BS cell-type specific promoters previously isolated, the promoters of *SCR* and *SCL23* or their homologs are unlikely to be affected by cellular metabolites and thus expected to confer similar and robust expression pattern across species.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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