



Insight

# Constructing the bundle sheath towards enhanced photosynthesis

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**C<sub>4</sub> photosynthesis is a carbon-concentrating mechanism that alleviates photorespiratory losses by elevating the concentration of CO<sub>2</sub> around Rubisco. Extant genes evolved to form this CO<sub>2</sub>-concentrating mechanism through changes in cell-preferential enzyme activity and leaf morphology (termed Kranz anatomy). Relatively little is known about regulatory *trans*-factors that promoted these changes. Using an activation tagging screen in *C<sub>3</sub> Arabidopsis thaliana*, Rooijen *et al.* (2019) identified NAC052, a H3K4 demethylase, as a possible regulator of an increased number of bundle sheath cells and chloroplasts. This study helps to decipher the genetic basis behind the evolutionary neofunctionalization of the bundle sheath in *C<sub>3</sub>* species, which lead to morphological changes to facilitate *C<sub>4</sub>* photosynthesis.**

The enzyme Rubisco plays a central role in photosynthesis by capturing atmospheric carbon dioxide (CO<sub>2</sub>) in an organic form. This enzymatic step is the basis of *C<sub>3</sub>* photosynthesis. However, Rubisco also reacts with oxygen (O<sub>2</sub>) to generate a toxic by-product, which must be salvaged by an energy-intensive process known as photorespiration (Portis and Parry, 2007). Many lineages of plants have evolved means to prevent photorespiration by increasing the relative concentration of CO<sub>2</sub> around Rubisco. In plants, the *C<sub>4</sub>* cycle is the most prevalent of these mechanisms which involves spatial separation of the reactions of photosynthesis. CO<sub>2</sub> is initially fixed in mesophyll (M) cells as a four carbon (C<sub>4</sub>) intermediate, diffused deeper inside the leaf into bundle sheath (BS) cells where the C<sub>4</sub> intermediate is decarboxylated for refixation by Rubisco (Hatch and Slack, 1966). Therefore, *C<sub>4</sub>* photosynthesis requires BS cell-specific expression of Rubisco, but also several other enzymes that must be M or BS specific in order to operate the cycle. In conjunction, a unique cellular arrangement in most *C<sub>4</sub>* leaves (termed Kranz anatomy) has evolved to facilitate this molecular CO<sub>2</sub> pump (El-Sharkawy and Hesketh, 1965; Hatch, 1987). Compared with *C<sub>3</sub>* plants, *C<sub>4</sub>* Kranz anatomy generally comprises denser venation, increased BS cell size, number, and chloroplast content, a greater reliance on the BS for photosynthesis, fewer M cells, and more plasmodesmata connections

between the M and BS (Box 1). As Kranz anatomy is a multifaceted trait, identifying its genetic determinants has been a bottleneck in *C<sub>4</sub>* photosynthetic research.

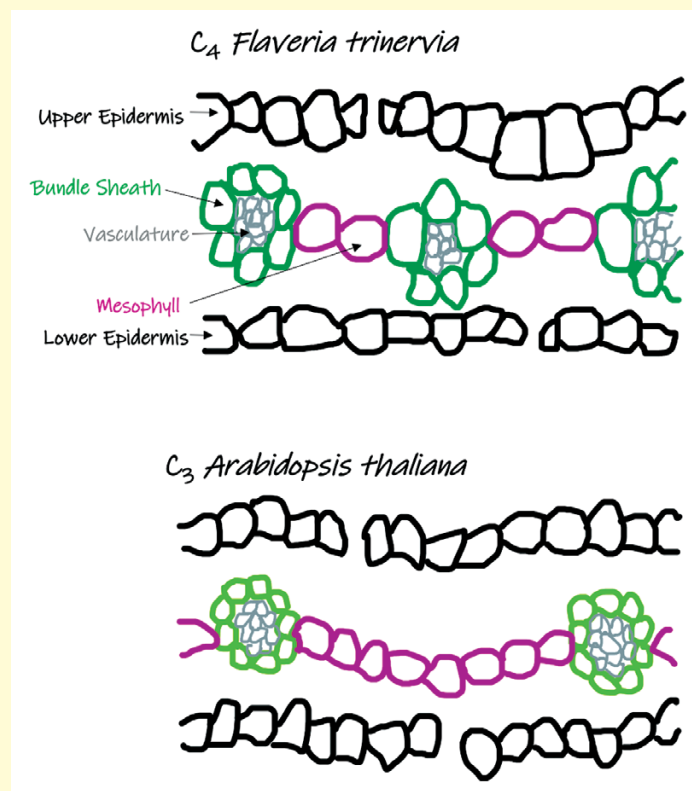
The transcription factors SCARECROW (SCR) and SHORTROOT (SHR) have been implicated with BS specification in *C<sub>4</sub>* maize and *C<sub>3</sub>* *Arabidopsis* (Slewinski *et al.*, 2012; Cui *et al.*, 2014). However, recent studies have shown that SCR/SHR regulate cell patterning in more broad contexts, such as root, epidermal, stomatal, and M cell patterning, suggesting that these two factors cannot fully account for the developmental changes in the BS to enable *C<sub>4</sub>* photosynthesis (Hughes *et al.*, 2019). Paralogues of the maize GOLDEN2 (G2) transcription factor family regulate dimorphic chloroplast differentiation in BS and M cells (Wang *et al.*, 2013). Overexpression of GOLDEN2-LIKE1 in *C<sub>3</sub>* rice led to increased chloroplast development in the vascular bundles of rice seedlings (Nakamura *et al.*, 2009). Thus, GOLDEN and GOLDEN-LIKE transcription factors play a role in plastid morphogenesis that probably aided in increasing the photosynthetic capacity of the BS. Beyond a few characterized regulatory steps that occur at multiple levels of gene expression, our current understanding of the steps required to engineer Kranz anatomy and cell-specific expression of *C<sub>4</sub>* cycle enzymes is limited (Reeves *et al.*, 2017; Sedelnikova *et al.*, 2018).

## NAC052, a H3K4 demethylase: identified as a novel genetic regulator of bundle sheath anatomy in *Arabidopsis*

In this issue of the *Journal of Experimental Botany*, van Rooijen *et al.* (2019) used activation tagging to identify a regulator influencing the number and chloroplast content of BS cells in *A. thaliana* (Box 2). In activation tagging, a promoter is randomly inserted into a reference genome, which results in transcriptional changes of genes in close proximity to the insertion site (Tani *et al.*, 2004). In this study, the authors used an *A. thaliana* reference line from Döring *et al.* (2019) which was transformed with the promoter of the *C<sub>4</sub>* *Flaveria trinervia* GLYCINE DECARBOXYLASE P-SUBUNIT gene (pGLDPA<sub>FL</sub>) to drive BS-preferential

### Box 1. Comparison of C<sub>3</sub> and C<sub>4</sub> leaf anatomy

A schematic of transverse cross-sections of mature C<sub>4</sub> *Flaveria trinervia* and C<sub>3</sub> *Arabidopsis thaliana* leaves. Cell outlines: upper and lower epidermis (black), vasculature (grey), bundle sheath (green), and mesophyll (pink). The middle layer of mesophyll cells (pink) highlights the difference in cell number between veins in C<sub>3</sub> and C<sub>4</sub> species. The dark green - color in C<sub>4</sub> plants represents higher photosynthetic capacity of BS cells.



expression of a chloroplast-targeted green fluorescent protein (pGLDPA<sub>Ft</sub>::RbcS.TP-sGFP). In order to identify regulators that influence the morphology of the BS, they used a second BS-preferential promoter from the *F. trinervia* GLYCINE DECARBOXYLASE T-SUBUNIT gene (pGLDT<sub>Ft</sub>) as an activation tag. Altered GFP fluorescence relative to the reference line allowed screening to find individual lines with altered BS-related phenotypes. Genomic analysis of one such line revealed that the reference promoter had inserted in the coding sequence of the gene encoding NAC052, a transcriptional repressor involved in H3K4 demethylation (Ning *et al.*, 2015).

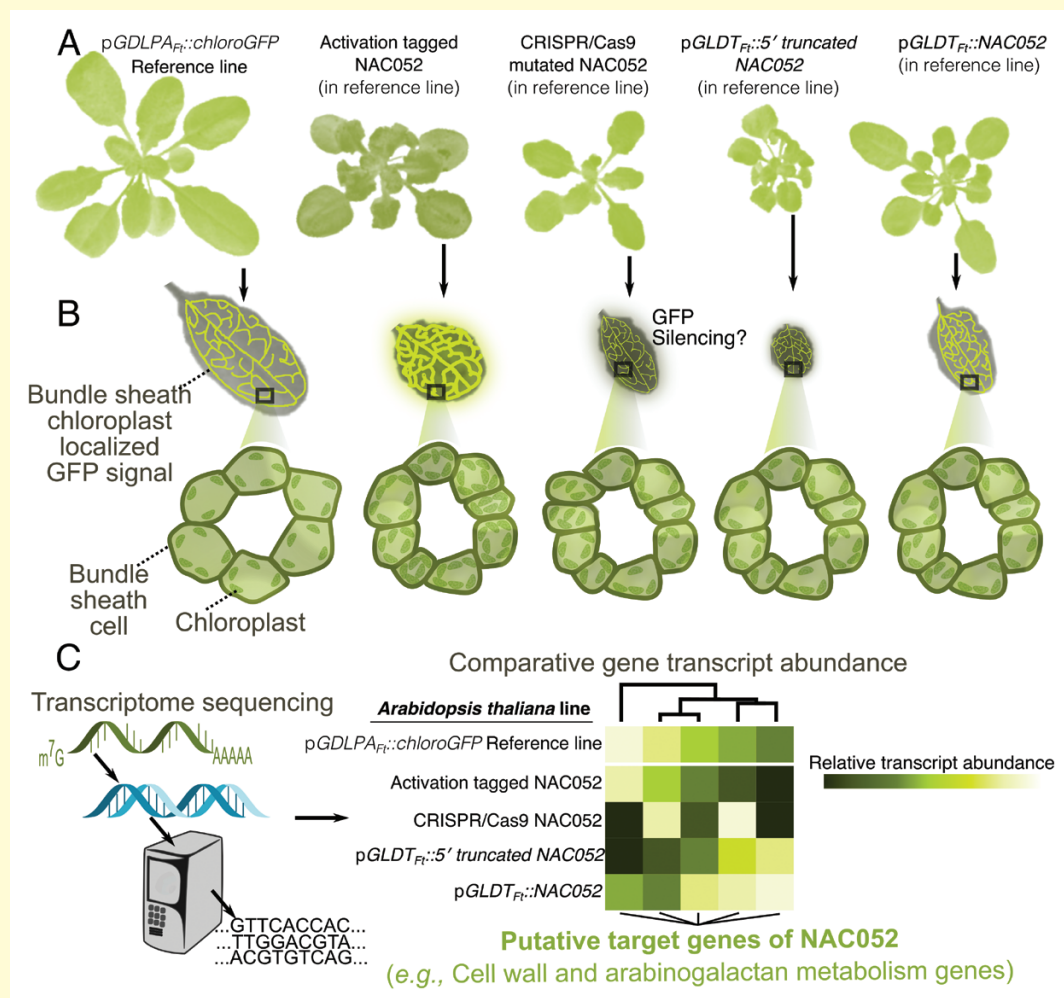
The insertion event resulted in a 5'-truncated transcript variant of NAC052 leading to a partial deletion of its DNA-binding domain. The mutation led to changes in GFP fluorescence as well as changes in BS anatomy and leaf and whole-plant morphology, such as a greater number of BS cells and chloroplasts as compared with the reference line. The JM14-NAC052 module is involved in post-transcriptional gene silencing by acting as a H3K4 demethylase which promotes transgene transcription by preventing DNA methylation (Butel *et al.*, 2017). Furthermore, the activation tagging mutation event was reconstructed by expressing pGLDT<sub>Ft</sub>::5'-truncatedNAC052 in the pGLDPA<sub>Ft</sub>::RbcS.TP-sGFP reference line. This recapitulated

the previous chlorotic and wrinkled leaf edge phenotypes and caused a greater accumulation of BS cells as compared with the reference line. Further validation of the involvement of NAC052 in leaf development was confirmed by expressing the full reading frame of the NAC052 transcript under the control of the GLDT<sub>Ft</sub> promoter in the pGLDPA<sub>Ft</sub>::RbcS.TP-sGFP reference background (pGLDT<sub>Ft</sub>::NAC052). The lines showed enhanced GFP signal intensity and more BS cells. As an additional line of evidence of NAC052 function, the endogenous NAC052 was mutated with clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9). The CRISPR/Cas9 mutant line was small and had chlorotic leaf edges, but it did not show the wrinkled leaf edges. However, in contrast to the activation-tagged mutant, the GFP signal intensity was decreased in the CRISPR/Cas9 mutant as compared with the reference line.

To assess the genetic impact of ectopic NAC052 expression, mRNA sequencing of the reference and all the transgenic lines was undertaken to identify its downstream gene regulatory targets. Comparative transcriptomics of the lines showed differential transcript abundance of genes involved in leaf cell wall organization and arabinogalactan metabolism, which are mediators between the cell wall, the plasma membrane, and

## Box 2. Activation tagging of NAC052 alters leaf anatomy and the expression of its target genes

(A) Whole-plant morphology of transgenic lines transformed with various versions of NAC052, a H3K4 demethylase, into a reference background containing a bundle sheath-localized GFP signal. (B) Screening lines for differences in GFP intensity allowed detection of the enhanced number of chloroplasts in the bundle sheath and overall number of bundle sheath cells. (C) Comparative transcriptomics identified putative regulatory targets of NAC052.



the cytoplasm. In summary, [van Rooijen \*et al.\* \(2019\)](#) associate NAC052 with leaf developmental patterns that alter anatomy specifically related to Kranz-like features. This opens up exploration into the role of other *trans*-factors that may have arisen from existing regulatory networks to transition from an ancestral C<sub>3</sub> state to a derived C<sub>4</sub> photosynthetic state.

## Future perspectives

The study from [van Rooijen \*et al.\* \(2019\)](#) is an advancement in our current understanding of BS anatomical regulation and furthers investigation into the role of post-transcriptional gene silencing in leaf development. Incorporation of bisulfite sequencing and methylome data sets might uncover underlying epigenetic patterns affecting BS anatomy and function across C<sub>3</sub> and C<sub>4</sub> species.

Here, [van Rooijen \*et al.\*](#) found that NAC052 had a transcriptionally repressive role in C<sub>3</sub> *A. thaliana*, which caused a boost in BS number and chloroplast content when ectopically expressed. Extension of their methodology to a C<sub>4</sub> species would allow association of NAC052 with traits of Kranz anatomy. Coupled with putative regulators of cell and plastid division genes, misexpression of NAC052 might shed insights into a mechanistic understanding of gene-regulatory networks that enhance the photosynthetic capacity of the BS. It would be particularly interesting to see if this could lead to trait stacking for efforts to engineer C<sub>4</sub> photosynthesis in C<sub>3</sub> crops, such as increased vein density or metabolic flux between M and BS cells from more plasmodesmata connections.

To sum up, [van Rooijen \*et al.\* \(2019\)](#) highlight how high-throughput phenotyping of transgenic activation-tagged lines can uncover novel gene-regulatory networks. This expands

knowledge on how to manipulate the role and structure of the BS in  $C_3$  species. Use of forward genetics like this seems to be a promising approach to unravel the complexity of Kranz anatomy. Hopefully this will lead to further reports that identify genetic determinants underpinning the regulation of Kranz traits in  $C_4$  photosynthesis.

**Keywords:** Activation tagging, bundle sheath cells,  $C_4$  photosynthesis, Kranz anatomy, NAC052, transcriptional regulation.

## References

- Butel N, Le Masson I, Bouteiller N, Vaucheret H, Elmayan T.** 2017. *sgs1*: a neomorphic *nac52* allele impairing post-transcriptional gene silencing through SGS3 downregulation. *The Plant Journal* **90**, 505–519.
- Cui H, Kong D, Liu X, Hao Y.** 2014. SCARECROW, SCR-LIKE 23 and SHORT-ROOT control bundle sheath cell fate and function in *Arabidopsis thaliana*. *The Plant Journal* **78**, 319–327.
- Döring F, Billakurthi K, Gowik U, Sultmanis S, Khoshravesh R, Das Gupta S, Sage TL, Westhoff P.** 2019. Reporter-based forward genetic screen to identify bundle sheath anatomy mutants in *A. thaliana*. *The Plant Journal* **97**, 984–995.
- El-Sharkawy M, Hesketh J.** 1965. Photosynthesis among species in relation to characteristics of leaf anatomy and  $CO_2$  diffusion resistances. *Crop Science* **5**, 517–521.
- Hatch MD.** 1987.  $C_4$  photosynthesis: a unique blend of modified biochemistry, anatomy and ultrastructure. *Biochimica et Biophysica Acta* **895**, 81–106.
- Hatch MD, Slack CR.** 1966. Photosynthesis by sugar-cane leaves. A new carboxylation reaction and the pathway of sugar formation. *The Biochemical Journal* **101**, 103–111.
- Hughes TE, Sedelnikova OV, Wu H, Becraft PW, Langdale JA.** 2019. Redundant SCARECROW genes pattern distinct cell layers in roots and leaves of maize. *Development* **146**, 1–12.
- Nakamura H, Muramatsu M, Hakata M, Ueno O, Nagamura Y, Hirochika H, Takano M, Ichikawa H.** 2009. Ectopic overexpression of the transcription factor OsGLK1 induces chloroplast development in non-green rice cells. *Plant & Cell Physiology* **50**, 1933–1949.
- Ning YQ, Ma ZY, Huang HW, Mo H, Zhao TT, Li L, Cai T, Chen S, Ma L, He XJ.** 2015. Two novel NAC transcription factors regulate gene expression and flowering time by associating with the histone demethylase JMJ14. *Nucleic Acids Research* **43**, 1469–1484.
- Portis AR Jr, Parry MA.** 2007. Discoveries in Rubisco (Ribulose 1,5-bisphosphate carboxylase/oxygenase): a historical perspective. *Photosynthesis Research* **94**, 121–143.
- Reeves G, Grangé-Guermente MJ, Hibberd JM.** 2017. Regulatory gateways for cell-specific gene expression in  $C_4$  leaves with Kranz anatomy. *Journal of Experimental Botany* **68**, 107–116.
- van Rooijen R, Schulze S, Petzsch P, Westhoff P.** 2019. Targeted misexpression of *NAC052*, acting in H3K4 demethylation, alters leaf morphological and anatomical traits in *Arabidopsis thaliana*. *Journal of Experimental Botany* **71**, 1434–1448.
- Sedelnikova OV, Hughes TE, Langdale JA.** 2018. Understanding the genetic basis of  $C_4$  Kranz anatomy with a view to engineering  $C_3$  Crops. *Annual Review of Genetics* **52**, 249–270.
- Slewiniski TL, Anderson AA, Zhang C, Turgeon R.** 2012. Scarecrow plays a role in establishing Kranz anatomy in maize leaves. *Plant & Cell Physiology* **53**, 2030–2037.
- Tani H, Chen X, Nurmberg P, Grant JJ, Santa Maria M, Chini A, Gilroy E, Birch PR, Loake GJ.** 2004. Activation tagging in plants: a tool for gene discovery. *Functional & Integrative Genomics* **4**, 258–266.
- Wang P, Fouracre J, Kelly S, et al.** 2013. Evolution of GOLDEN2-LIKE gene function in  $C_3$  and  $C_4$  plants. *Planta* **237**, 481–495.