# Investigating the leaf anatomy, biochemistry, and physiology of a $C_4$ photosynthetic plant


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## Introduction

- Global food demand is projected to grow 35% to 56% from 2010 to 2050[1].
- The objective of our research is to address food security under a changing climate by improving photosynthesis.
- Photosynthesis in plants converts light energy into food.
- Plants have evolved different photosynthetic pathways to succeed under different growth environments, such as the $C_4$ pathway[2].
- The $C_4$ pathway uses a carbon concentrating mechanism (CCM) to enhance photosynthesis under drought and high temperatures.
- While significant discoveries related to $C_4$ photosynthesis have been made, further research is needed to improve $C_4$ crops for future climate conditions.

## Hypothesis

Forward genetic screening of mutagenized $C_4$ plants without a functional CCM will identify genes influencing internal leaf $CO_2$ conductance.

## Material and methods

**Plant materials**: This study utilizes the $C_4$ plant *Setaria viridis with* the key $C_4$ photosynthetic enzyme phospho*enol*pyruvate carboxylase (PEPC) knocked via CRISPR-Cas9.

**Figure 1:** Scheme of the $C_4$ pathway for the study.

PEPC initiates the first committed step of the CCM during $C_4$ photosynthesis.

Plants lacking PEPC cannot survive in ambient $CO_2$ (0.04% ) and must be rescued at high $CO_2$ (1%).

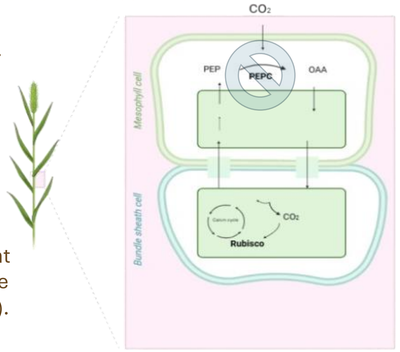
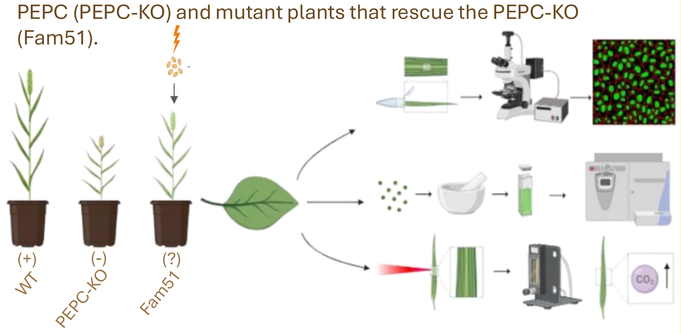



**Figure 2:** Workflow of characterizing the anatomy, biochemistry, and physiology of 3 genotypes: wild-type (WT), plants lacking PEPC (PEPC-KO) and mutant plants that rescue the PEPC-KO (Fam51).




## Results

### 1.Microscopic analysis of stomata

Leaves were cut from the plant and immediately placed in a tube with water for imaging using brightfield, fluorescence & confocal microscopy in the Franceschi Microscopy and Imaging Center. Initial measurements were done by hand to train a machine learning algorithm to label and measure stomata in tile sets and images.

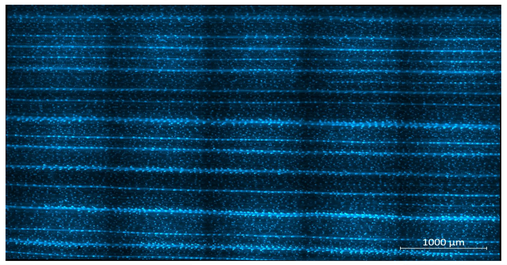



**Figure 1:** Tile set imaging of a WT plant.

- A tile set image captures microscopic images of different areas of the leaf.
- Individual images are combined to create a larger, higher resolution image in a grid-like structure.

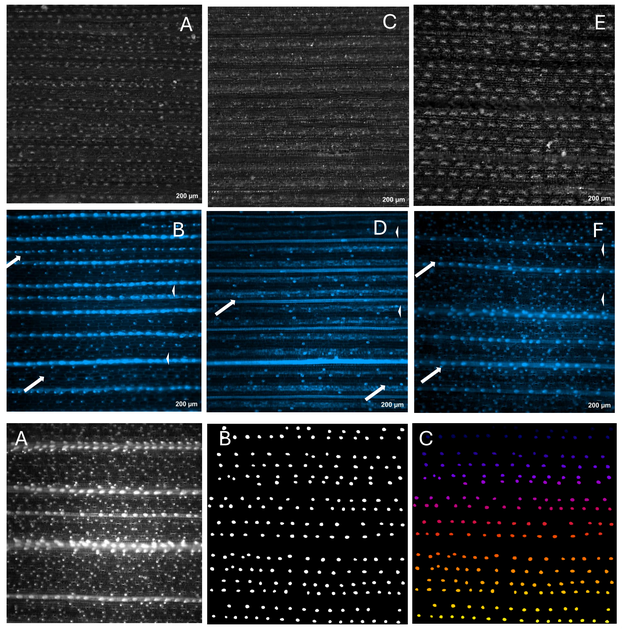



**Figure 2:** Brightfield (A, C, E) and fluorescence (B, D, F) imaging for Fam51 (A, B), PEPC-KO (C, D), and WT (E, F). In fluorescent images cutin autofluorescence is visible by using a UV filter cube. Cutin is concentrated over leaf veins (bright horizontal lines), trichomes (arrows), and stomata (triangles) which appear in rows parallel to veins



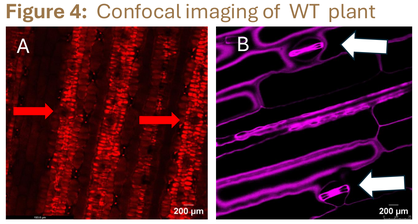

**Figure 3 & Table 1:** Microscopy Analysis: Fluorescent leaf images are used (A) and opened using ImageJ. All stomata are labeled for a new image (B). "3D Objects Counter" is used to analyze stomatal count and area alongside an overview image (C). This was used to calculate data shown in **Table 1** between abaxial (AB) and adaxial (AD) side of the leaf.

**Table 1**:

| | WT_AB | PEPC-KO_AB | Fam51_AB | WT_AD | PEPC-KO_AD | Fam51_AD |
|---|---|---|---|---|---|---|
| Stomatal Count/mm² | 150.55 | 96.89 | 107.04 | 191.99 | 101.42 | 161.6 |
| Average Stomata Area (µm²) | 319.31 | 405.42 | 336.51 | 229.96 | 259.79 | 277.38 |
| Standard Error | 3.69 | 12.94 | 3.46 | 2.71 | 4.40 | 3.90 |

### 1.1 Supplemental Microscopical analysis of guard cell

**Figure 4:** Confocal imaging of WT plant




A: Imaging chlorophyll autofluorescence using UV laser and 10x objective showing chloroplasts in mesophyll cells below the leaf surface. Stomata are located above the dark spots within the vertical lines of chloroplasts (red arrows).
B: Cutin autofluorescence using UV laser and 63x objective. Showing the cuticle and highlighting stomata (white arrows).

## Results

### 2.Leaf-level physiology analysis of photosynthesis rates

The Li-COR Li-6800 was used to measure the photosynthetic rate (*A*) in response to intercellular $CO_2$ ($C_i$) concentration and photosynthetic photon flux density (PPFD), (0-2100 mmol $m^{-2}$ $s^{-}$ for $C_i$) and (0-800 mmol $m^{-2}$ $s^{-}$ for PPFD).

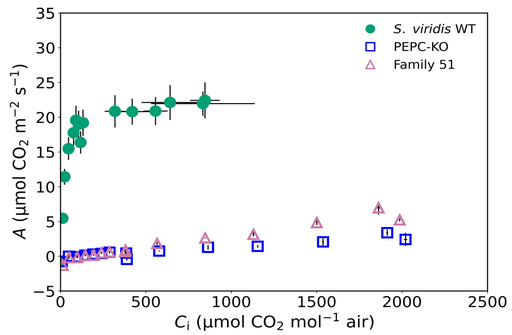



**Figure 5:** Photosynthetic rate (*A*) to $CO_2$ response ($C_i$).

- Fam51 shows higher *A* at high internal concentrations of $CO_2$ compared to PEPC-KO.
- This may suggest higher internal $CO_2$ conductance in Fam51.

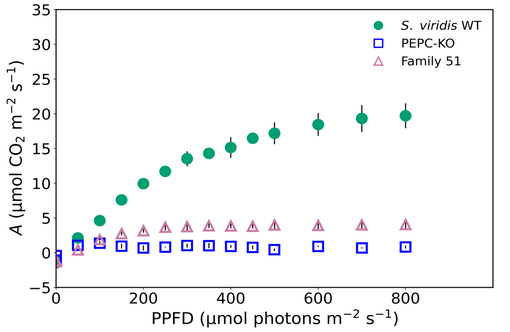



**Figure 6:** Photosynthetic rate (*A*) to light (PPFD).

- Fam51 shows higher *A* at light levels above 200PPFD.
- This may suggest improved photosynthetic capacity in Fam51 compared to PEPC-KO.

### 3. Biochemical analysis of enzyme activity

Leaf discs were ground by chilled mortar and pestle with extraction buffer. PEPC and Rubisco activity were measured via a coupled enzyme assay to visualize NADH consumption on the spectrophotometer.

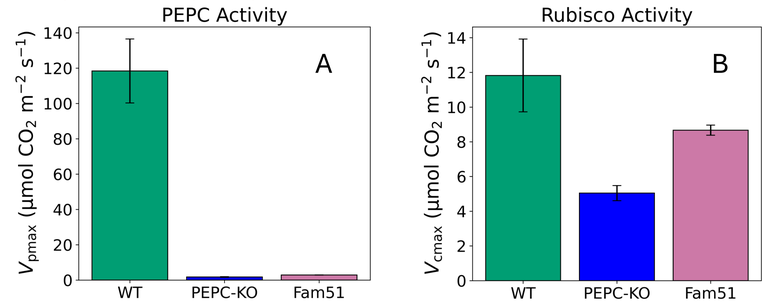



**Figure 7:** Enzyme activity for 3 genotypes. Lack of PEPC activity in Fam51 and PEPC-KO (A) suggests PEPC activity does not contribute to higher *A* in Fam51. Rubisco assays (B) show varying levels of Rubisco activity across genotypes.

## Conclusion

-We learned that Fam51 has higher *A* than PEPC-KO under increasing Ci and PPFD, and that this is not due to increased PEPC activity.
-Stomatal size and density vary between genotypes and may play a role in Fam51's rescue phenotype, but more analysis of anatomical imaging is needed.
-Future work will utilize machine learning to quantify microscopy images (1) and investigate other photosynthetic parameters obtained from physiological measurements (2).


[1] Van Dijk, et al., (2021). Nature food, 2(7), 494–501.
[2] Yamori et al., (2014). Photosynthesis research, 119(1-2), 101–117.