Supplemental methods

High throughput microscopy

After 7 weeks, one fully-expanded intact adult leaf (one of the largest leaves that developed after leaf 4) was selected on each plant for microscopic analysis. From each leaf, two discs were cut mid-way along the length of the leaf on both sides of the main vein, using a 6mm hole punch. If the leaf was too small to cut two discs, an additional leaf was collected. The discs were loaded onto an array of 80 spring mounted stamps with the abaxial side up and fixed on a thin layer of dental adhesive cream (blend-a-dent Super-Haftcreme). The leaf discs were stained using 25µl of a 100µg/ml propidium iodide solution for specific staining of stomata and cell walls (Fitzgibbon et al., 2013). A Zellkontakt 96-well glass-bottom plate was then put on top of the stamp array and fixed using four screws. To infiltrate the leaf discs with the stain, the plate was put under vacuum three times for one minute. Microscopic images were taken using the Opera High Content Screening System from Perkin Elmer. The following settings were used: excitation wavelength 561nm; laser power 11000µW; magnification 20x; camera filter 600/40 nm; dichro filter 568 nm; exposure time 200ms; binning 1. Images were taken in 15 fields (0.15 mm^2) per well/sample. For each field 11 images were taken along the z-axis with 3µm distance to acquire image stacks. In total, we acquired 341,000 microscopic images of abaxial leaf epidermises from 31,000 image fields.

Image processing and analysis

Maximum projection of stacks

The first step of image processing was performed using Acapella, the image analysis software designed specifically for the Opera by Perkin Elmer. The aim was to project the image stacks acquired for each field into single 2D images using maximum projection. First, a sliding parabola transformation was applied to each image layer in to reduce background noise. Second, the maximum projection function was applied to each stack. Third, the resulting image was saved to

Bitmap format. The output folder was named after the plate number and the date of capturing of the images. The filename contained the following information separated by "_": Plate number, plant ID, plate well coordinates and image field within well. The Plant-ID contained additional information separated by '-': genotype, tray number, tray position and leaf ID (for corresponding leaf size measures). The bitmap images were further processed in MATLAB.

Pre-processing

In MATLAB, as in most other programming languages, images are read as 2D matrices with each pixel represented as one value in the matrix. For grayscale images the value is an integer between 0 (black) and 255 (white). On these matrices different mathematical operations can be performed to transform or analyze the images.

Images were enhanced using a histogram expansion function. This function stretches the pixel intensity values over the whole grayscale range, thus increasing brightness and contrast. Next, images were divided in 3x3 fields and for each field entropy and thresholding effectiveness of Otsu's method (Otsu, 1975) were calculated. Based on these values the quality of each image part was determined. Only if at least 7 of 9 parts of the imaged matched the criteria the image was further analyzed. The critical values were determined on sets of manually selected high and low-quality images. This step is crucial as it not only decreases computing time by eliminating of low quality images before complex analysis, but also because automated feature detection works more reliably if the images are relatively uniform. High quality images were then analyzed to detect stomata.

Stomata detection

Stomata appeared in images as bright, small and mostly elliptic objects with a gap in their center. These morphological features were used for stomata detection. Thresholds for all parameters were determined using training datasets and manual curation and would likely have to be adjusted for use with a different dataset.

First noise was removed by applying a Gaussian filter. Then the image contrast was strongly increased using histogram expansion and eliminating the darkest 60% of all pixels. The image was then converted from grayscale to logical by setting all pixels with value of 255 to one and all

others to zero to mask the brightest objects in the images. The result was an image of foreground objects (connected 1-pixels) and background (all 0-pixels). Because stomata were among the detected objects, but not exclusively, the following filters were applied to the initial detection image:

- Objects located closely together were merged using image dilation
- Objects smaller than 700px or larger than 2000px were removed
- If afterwards the number of objects was still higher than 60, the image was discarded
- Holes in objects were filled

Upon detection of very large objects e.g. trichomes the image was discarded as this often lead to inaccurate stomata recognition. Objects were also filtered based on their eccentricity as stomata are usually ellipsoid. Stomata with eccentricity below 0.4 were removed instantly. Major axis length of the ellipse had to be shorter than 80px. Moreover, the area of the ellipse with the same second moment as the presumed stomata was calculated. Out of this area and the actual object area a ratio was calculated ("area ratio") to determine how well the object fit into an elliptic shape. Furthermore, the intensity profile through the minor axis of the object and two offset parallels was analyzed to detect the characteristic stoma gap. The gap was showing up in the profile by two large peaks separated by a low intensity minimum. Based on whether the gap was detected in each of the three lines a gap score was calculated for each object. Because stomata were not necessarily uniformly shaped (e.g. open and closed stomata look different) various combinations of thresholds on these criteria were allowed for true stomata. For example, if an object is too round for a typical stoma it was still considered if the gap is very prominent. The following combinations were allowed for stomata:

- Area ratio > 0.9 & eccentricity > 0.8
- Profile score > 3 & eccentricity > 0.4 & area ratio > 0.8
- Profile score > 0 & eccentricity > 0.7 & area ratio > 0.85
- Profile score > 2 & eccentricity > 0.6 & area ratio > 0.8

Stomata detected by the different combinations of filters were then joined. The minimum convex area spanned by stomata an additional quality criterion. If this area was smaller than 50% of the

image size, the result was discarded. Finally, the median of all images for each sample was calculated. If the median was smaller than 33 the image was discarded, as such a low value was never observed in manual controls and likely caused by low quality images which passed pre-filtering.

Pavement cells could not be accurately quantified by the algorithm, so that stomata indices, which describe the rate of epidermal cell differentiation (Salisbury, 1928), were not measured. As a result, the genetic diversity of cell differentiation processes was not evaluated. Nevertheless, our approach provides a complete view of the amount of stomata diversity displayed on the leaf surface. This morphological variance is indeed the one that should ultimately have an impact on the stomatal conductance required for photosynthesis and growth.

Leaf size measurement

For leaf size measurements, each hole-punched leaf was fixed on a gridded A4 paper sheet (8 per page) using transparent tape. Possible gaps in the edge of leaves were closed using a pen. Paper sheets were then digitized using a common flatbed scanner.

The gridded A4 paper sheets with the fixed leaves were enumerated and scanned. Images were manually checked for closed leaf borders. This step was important as closed borders were necessary to fill the holes from disc cutting during image processing. The image analysis was also performed in MATLAB:

- Splitting of image into 8 leaf fields and the reference field (black 2cm^2 square) using relative coordinates
- For each sub-image:
    - Inversion of grayscale values
    - Conversion to logical image by intensity thresholding
    - Removal of small objects by area opening
    - Calculation of leaf area: number of white pixels in image/pixelarea in mm^2
    - Pixelarea was calculated using the reference field of known size

In case two leaves were used from one plant, these were detected by the algorithm as two objects and the mean was calculated.

Carbon isotope discrimination measurements

The rosettes of block 1 were placed in individual paper bags after microscopic imaging was completed and dried at 70 °C for 3 weeks. Plant material was then ground to fine powder using a 25mm steel bead and a mixer mill (Retsch, MM 301). Isotope composition was determined using an ISOTOPE cube elemental analyzer coupled to an Isoprime 100 isotope ratio mass spectrometer (both from Elementar, Hanau, Germany) according to (Gowik, Bräutigam, Weber, Weber, & Westhoff, 2011). The carbon isotope ratio is expressed as ‰ against the Vienna Pee Dee Belemnite (VPDB) standard.

References

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