Electronic Supplementary Material (ESI) for Molecular BioSystems


# Supplemental Methods

## 1. Maize cultivation and preparation of stem juice samples

***Water stress experiment***

The drought trial was done during the winter season in the CIMMYT field station located in Tlaltizapán, Morelos, México (18°41′ N latitude, 99°07′ O longitude, 945 masl altitude). The watering regime was performed according to the field management standards established by the physiology program of CIMMYT. The stress treatment (drought) consisted of suppressing irrigation for three weeks before flowering whereas the well watered treatment (control) was irrigated at the usual frequency (weekly watering). Six plants per genotype were sampled for each treatment (drought and control). Three plants per plot were harvested from each field experiment. The E-132 genotype (hybrid CLQ-RCWQ83/CML312SR) was harvested ~15 days after silking, at ~90 days after sowing for both control and drought treatment.

***Nitrogen stress experiment***

The low nitrogen trial (Low N) and control trial were done during the summer season in the CIMMYT field station located in El Batán, Texcoco, México state (19°30′ N latitude, 98°53′ O longitude, 2 247 masl altitude). The fertilization regime was performed according to the field management standards established by CIMMYT. The low nitrogen treatment (Low N) consisted of suppressing nitrogen fertilization for ~4 years whereas the control group was fertilized chemically at the usual frequency (3 times per growth cycle). We sampled six plants per genotype for each treatment (Low N and control). The CV-702 genotype (entry 3 from CIMMYT field trial HL2011-11-01-PVB-2) was harvested ~10 days after silking (~85 days after sowing). The entry 9 from CIMMYT field trial HL2011-11-01-PVB-1 was harvested ~12 days after silking (~85 days after sowing).

***Maize stem juice collection***

In all experiments, representative healthy plants from each genotype and treatment were selected in the field and processed within ~15 minutes after harvest. Morphological and physiological data was registered: total plant weight, plant height (up to the flag leaf), number of ears, ear weight and stem weight. The tassel and the leaf blades were removed and the supporting stem (including leaf-sheath) was cut into segments (~ 30 cm) for tissue homogenization. The stem juice (phloem sap + xylem sap + cellular extracts) was obtained using a mechanical extractor (Industrial scale juice extractor model EXS, International Co, Mexico). The stem juice (~80 ml) was collected in a plastic beaker, weighted accurately (±0.5 g), aliquoted and frozen in dry ice immediately (< 30 s). Stem fluid aliquots (1 mL) were stored at -80 °C for ~4 months in sealed deep well plates (96-well format). For analysis, the stem juice was defrozen and kept on ice for short time periods (~ 1 h). Samples were firstly centrifuged (10 min at 4000 RPM (~5000 g) at 4 °C) and the supernatant was used directly for protein analysis (see below). For DIESI-MS metabolite quantification, the

supernatant was filtered (0.4 µm mesh) and then appropriately diluted (1:10000) and acidified with formic acid (see 6. further below).

## 2. TCA/ acetone and ethanolic precipitation

***TCA/acetone precipitation***

Aliquots of 200 µL supernatant from the stem juice (see above) were mixed with 200 µL 10% (*w/v*) trichloroacetic acid (TCA) and incubated for one hour at 4 °C. TCA samples were centrifuged (eppendorf Centrifuge 5415R) for 10 minutes at 13,200 rpm at 4 °C; the supernatant was discarded and the protein pellet was washed twice with 500 µL acetone. Each time, the pellet was re-suspended in 200 µL acetone, vortexed thoroughly and re-centrifuged (see above). The acetone supernatant was discarded and the pellet was dried under vacuum at 25 °C. For MALDI measurements, the protein pellet was re-suspended in 200 µL of 50% acetonitril in 0.1% trifluoroacetic acid.

***Ethanolic precipitation***

Aliquots of 80 µL of maize stem juice supernatants were mixed with 320 µL of absolute ethanol and incubated overnight at -20 °C. Following the solution was centrifuged at 18,000 rpm for 30 min at 4 °C. The supernatant was concentrated down to 30 µL on a vacuum centrifuge, resulting in a dense solution, and stored at -20 °C until NALDI measurement. Prior to NALDI analysis, the sample was dried and re-suspended in absolute ethanol. The pellet was washed with 90% ethanol and centrifuged at 10,000 rpm for 10 min. This pellet was re-suspended in 200 µL 50% acetonitril in 0.1% trifluoroacetic acid (*v/v*) for MALDI analysis.

## 3. Protein quantification and Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein was quantified by acidic hydrolysis and subsequent colour reaction with ninhydrin, as previously described by Starcher.[1] The ninhydrin assay is largely independent of the protein composition and buffer compounds, additionally it exhibits superior sensitivity compared to other colorimetric assays.[2]

For SDS-PAGE 50 µL supernatant from the maize stem fluid with were mixed with 50 µL of 2x Laemmli buffer[3] and heated at 99 °C for 5 minutes. 3 µL molecular weight marker (BenchMark™ Protein Ladder, Cat. No. 10747-012, Invitrogen) or 10 µL of prepared sample were loaded into a 10% polyacrylamide gel. SDS-PAGE was run at constant voltage (150 V) for 120 min, gels were stained with Coomassie brilliant blue 0.25% (*w/v*) (0.25 g Coomassie, 90 mL methanol/water 1:1 (*v/v*), 10 mL acetic acid.

## 4. Protein profiles by matrix assisted laser desorption/ionization (MALDI) – time of flight (ToF) mass spectrometry

20 µl of sample were dried in a vacuum centrifuge and re-suspended in 20 µl of 0.1% trifluoroacetic acid (TFA) (*v/v*). In order to remove non-proteic contaminants, the sample was bound to a C4 ZipTip column, washed three times with 0.1% TFA and eluted with 5 µl

50% acetonitril in 0.1% TFA (*v/v*).
Aliquots of the eluted protein solution (1 µl) were spotted on a ground steel target (Bruker Daltonics, Germany) and mixed with 1 µl sinapinic acid matrix directly on the target plate. The matrix was prepared as 10 mg/mL solution with 50% acetonitrile in 0.5% trifluoroacetic acid (*v/v*). Samples were allowed to dry before MALDI-TOF MS analysis. Spectra were obtained on a Bruker Daltonics autoflex III MALDI-ToF/ToF mass spectrometer, using FlexControl V 3.0 as control software. The instrument was operated at a pulse rate of 200 Hz and with an ion extraction delay of 160 ns. Measurements were carried out in positive liner mode using an acceleration voltages of 20 kV (ion source 1) and 18.45kV (ion source 2). The lens voltage was set to 7.5 kV. Mass spectra were recorded in the *m/z* range between 4 and 20 kDa. Insulin (5,734.51 Da), Cytochrome C (12,360.97 Da), Ubiquitin (8,565.76 Da) and Myoglobin (16,952.30 Da) were used as external calibration reference standards.

## 5. Metabolic profiles by nano-assisted laser desorption/ionization (NALDI) – time of flight (ToF) mass spectrometry

One micro-litre of the sample aliquots were spotted directly on the NALDI plates (Bruker Daltonics, part number 252248). Samples were allowed to dry before introduction into the mass spectrometer. Spectra were obtained on a Bruker Daltonics autoflex III MALDI-ToF/ToF mass spectrometer, using FlexControl V 3.0 as control software. The instrument was operated at a pulse rate of 200 Hz. Measurements were carried out in positive reflector mode, using acceleration voltages of 19 kV (ion source 1) and 16.7 kV (ion source 2). The lens voltage was 8.43 kV. Reflectors were adjusted to 21.05 kV (reflector 1) and 9.73 kV (reflector 2). Data were collected in a *m/z* range of 40-1,400 amu.

## 6. Direct injection electron spray mass spectrometry (DIESI-MS)

***Mass spectrometry conditions***

In order to avoid the saturation of the capillary and cone soiling, the stem juice supernatant (see 1) was filtered through a 0.45 µm PVDF filter and diluted with de-ionized water to obtain a final dilution of 1:100. To improve ionization, 25 µl pure formic acid was added to 475 µl of diluted sample, to a final concentration of 5% (v/v) formic acid. The diluted and acidified samples were analysed by direct injection electro spray ionization (DIESI) in positive mode. A mass spectrometer (MS) Micromass ZQ 2000 with a quadrupole analyzer and MassLynx 4.0 as control software was employed. The volumetric flow rate of the direct infusion was 10 $\mu L \cdot min^{-1}$ using a mechanically driven syringe loaded with 100 µL sample. The mass spectrometer was operated with capillary voltage set to 3 kV, the cone voltage to 60 V and the extractor voltage to 3 V. The RF lens was left at 0.5 V. A source temperature of 80 °C and a desolvation temperature of 150 °C were used at a desolvation gas flow of 250 $L \cdot h^{-1}$ and a cone gas flow of 50 $L \cdot h^{-1}$. In the Analyzer section, LM and HM resolution of 15.0 and ion energy of 0.5 were set. The multiplier was adjusted to a value of 650.
Continuous spectra were collected in a range of 15-2000 m/z, with a total run duration of 1 min, a scan time of 10 s and a inter scan time of 0.1 s, producing 6 spectra per sample. The files .raw spectra were converted to mzXML using masswolf V 1.4.
The analysis of mass spectra was performed using the OpenMS/TOPP suite, version 1.8.0.[45] First, a pipeline was written for TOPPAS, executing the following tasks for all spectra: File conversion to mzML, Spectra Merger with a block method, NoiseFilter sgolay with a frame

length of 21 and polynomial order of 4, followed by a PeakPicker with a Signal to Noise of 1 and a peak width of 0.15. Finally, data were converted into DTA2D text format.
File names, *m/z* values and corresponding signal intensities of the individual sample files were written into a single data file called `massR.dat` for further processing with the free software R (http://www.r-project.org).

## 7. Statistical analysis of DIESI-MS data with R

For statistic analyses, dendograms and heatmaps, R version 2.14.1 was employed with the libraries `vegetarian` and `pheatmap`. The R script is provided as supplementary data. Mass spectrometry data are imported, sorted into bins with an interval of 1 *m/z* and organized in a matrix. Following, the data are normalized with respect to signal intensity of the spectra. The hierarchical clustering is limited to the 100 mass bins which show the highest signal intensities in any of the rows. Finally the hierarchical clustering is performed with scaled rows, using the Euclidean algorithm for both, rows and columns.

## 8. Data and script availability

The R script file together with the massR.dat data file, which was used for this study, as well as the *.mzXML mass spectrometry raw data files and data preparation scripts were submitted to the PANGAEA data repository (http://www.pangaea.de) and can be accessed at doi:10.1594/PANGAEA.776064.

**References**

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