EARLY CARBON MOBILIZATION AND RADICLE PROTRUSION IN MAIZE GERMINATION

Luis Sánchez-Linares, Marina Gavilanes-Ruiz, David Díaz-Pontones, Fernando Guzmán-Chávez, Viridiana Calzada-Alejo, Viridiana Zurita-Villegas, Viridiana Luna-Loaiza, Rafael Moreno-Sánchez, Irma Bernal-Lugo, Sobeida Sánchez-Nieto

SUPPLEMENTARY MATERIALS AND METHODS

**Determination of tryglicerides**

Lipid extraction from germinated embryos was carried out as described by Folch et al. (1957) with some modifications. Briefly, 100 mg of the frozen powdered specimens was homogenized with 5 ml of 2:1 chloroform-metanol (v/v). The homogenate was centrifuged at 1 000 g for 5 min. The pellet was re-homogenized, re-extracted and centrifuged. Supernatants were combined, heated at 70 °C and evaporated until dryness. The residue (total lipids) was weighed and dissolved in 300 μl of 2:1 chloroform-metanol (v/v) to be analyzed for triglycerides (TG). This was done by thin layer chromatography (TLC), applying 4 μl of each sample in a silica gel plate (10 x 20 cm), using hexane-ethylc ether-acetic acid (70:30:1, v/v/v) as developing solvent. TG were detected by iodine vapor, and vanillin sulfuric acid spray (Skipski et al., 1962). Triacetatine (synthesized as described in Macías et al., 2006) was used as internal standard to calculate the amount of extracted TG. TLC plates were scanned and analysed using the software Quantity One-4.6.3 from BIORAD (Hercules, CA, USA).

**ATP hydrolysis determination**

Enzymatic ATP hydrolysis was measured by spectrophotometric quantitation of the released inorganic phosphate, according to González-Romo et al. (1992). Reaction medium contained 250 mM sorbitol, 20 mM MOPS-BTP, pH 7.0, 5 mM ATP and 5 mM
MgCl₂. Reaction was started by adding plasma membrane vesicles (PMV) to a final concentration of 0.1 mg/ml, incubated at 30 °C and stopped by SDS addition (12% w/v, final concentration). Blanks to determine non-enzymatic ATP hydrolysis were included. Linearity of phosphate production was observed during 2 h (see Sánchez-Nieto et al., 2011). When indicated, 0.15 mM Na₃VO₄ or 0.015 mM erythrosin-B (to inhibit plasma membrane ATPase), or 2.0 mM NaN₃ or 0.01 mg/ml oligomycin (to inhibit mitochondrial ATPase) were added to the reaction medium. Erythrosin-B did not interfere with the colorimetric Pi assay.

**Determination of transmembrane ΔpH**

This was performed by ACMA (9-amine-6-chloro-2-methoxyacridine; Molecular Probes, Oregon, USA) fluorescence quenching in 2 ml of the reaction medium used for ATP hydrolysis activity supplemented with 1 µM ACMA and 10 µg/ml oligomycin. Reaction started with the addition of 100 µg of PMV protein into the reaction medium and then ACMA fluorescence quenching (excitation wavelength 415 nm; emission wavelength 480 nm) was recorded in a spectrofluorometer (Model RF5000U, Shimadzu Corp. Kyoto, Japan) at 20 ºC. Acidification rate was measured considering the negative slope at the end of a six-minute continuous fluorescence recording; after this time, the system was uncoupled by adding 20 µl of a KCl saturated solution and 20 µl of 1 mg/ml gramicidin solution. Fluorescence recovery caused by uncoupling was used to quantify the proton gradient formed according to a calibration curve. Standards for quenching ACMA fluorescence related to the transmembrane ΔpH were established for our experimental conditions using asolectin liposomes with an acidic interior (Rottenberg and Moreno-Sanchez, 1993). Fluorescence quenching was plotted as a function of the imposed transmembrane ΔpH and the resulting curve was used for estimating the ΔpH generated by the H⁺-ATPase activity in the PMV. A liposome system was used to calibrate the extent in fluorescence quenching as a result of the H⁺ pumping activity of
the plasma membrane vesicles. Proportionality was observed between the amount of
plasma membrane vesicles and their H⁺-pumping activity measured as ACMA
fluorescence in the interval from 100 to 300 μg of PMV protein (Fig. S4A). A linear
relationship between the signal and the imposed ΔpH to the liposomes was obtained in
the range from 0.1 to 1.0 pH units (Fig. S4B, 4C). Acidification rate (arbitrary
fluorescence units, f.u./min) was estimated by graphical calculation of the negative
slope after six-minute of continuous reaction (Fig. S4B, 4C). Then, the system was
uncoupled by adding gramicidin and KCl, and fluorescence recovery was attained,
which was used to quantify the proton gradient (ΔpH) formed according to the
calibration curve (Fig. S4).

**Preparation of liposomes**
Asolectin phospholipids (300 mg) from soybean (Sigma-Adrich, St. Louis MO, USA)
were washed with 1 ml ether; a 15 μl aliquot from this suspension was evaporated
followed by mixing with 0.9 ml of a buffered solution for each pH tested and sonicated
in an ice bath until opalescent appearance was obtained.

**Isolation of microsomal fraction**
Once imbibition time was finished, the embryos were frozen in liquid N₂ and kept at -70
°C. Afterwards, they were crushed and the fine powder was homogenized in the buffer
(250 mM sorbitol, 1 mM EDTA, 1 mM KCl, 1 mM DTT, 2 mM PMSF and 1 tablet of
protease inhibitor cocktail per 50 ml (Complete, Roche, Mannheim, Germany) in a ratio
of 2 volumes per 1 g of tissue, using a tissue tearor homogenizer (Biospec Products,
Daigger and Co. Inc. Vernon Hill, Il, USA) for 60 sec. Disulfide reducing reagents and
protease inhibitors were added just before use. The tissue was filtered through a four-
layero of pre-wet cheesecloth; the filtrate was centrifuged 5 min at 2 400 g at 4 °C. The
supernatant was centrifuged at 14 000 g for 10 min. The resultant supernatant was
centrifuged at 110 000 g for 45 min in a TLA-100.4 rotor in an Optima TL-100
Centrifuge (Beckman, Palo Alto, CA, USA) at 4 ºC. The microsomal pellet was resuspended in 100 µl of 250 mM sorbitol, 50 mM HEPES / KOH, pH 7.8, 10% glycerol, 2 mM DTT and 2 mM PMSF, and stored at -70 ºC until use.

**Isolation of plasma membrane vesicles**

Microsomal fraction was used for PMV purification by two-aqueous phase partition procedure essentially as described (Sánchez-Nieto *et al.* 1997). Briefly, 100 mg of microsomal protein were loaded to a 27 g two-aqueous phase system containing Dextran T-500 (Sigma-Aldrich, St. Louis MO, USA) and polyethylene glycol 3350 (PEG) at 6.4% (w/v) each in a solution of 330 mM sucrose and 5 mM KH₂PO₄ pH 7.8. After partitioning, PEG upper phase containing PMV was recovered and washed by dilution in a 2:1, v/v ratio with a solution of 250 mM sucrose, 20 mM MOPS-BTP, pH 7.0. PMV were obtained by centrifugation at 100 000 g for 4 h at 4 ºC and resuspended in a solution containing 250 mM sorbitol, 20 mM MOPS-BTP, pH 7.0, 4 mM DTT or DTE, 7 µg/ml chymostatin, 50 µg/ml TLCK and 100 µg/ml TPCK. This final PMV suspension was used for ATP hydrolysis and ΔpH assays. All steps were carried out at 4 ºC.

**Antibody preparation**

Polyclonal antibodies against the sucrose and hexose transporter were prepared in rabbit as described in the procedure of González-Halphen *et al.* (1988) using synthetic oligopeptides as antigens. In the case of the maize plasma membrane sucrose transporter (ZmSUT1), the oligopeptide used was TARWGRPRPFILIGC, which was conjugated to keyhole limpet hemocyanin (Quality Biochemicals, Maryland, USA). Analysis of the secondary structure of the ZmSUT1 showed that this oligopeptide is probably located in the first intracellular loop. The design of an antigenic oligopeptide for the plasma membrane hexose transporter considered an alignment of 27 plant hexose transporters which showed a soluble and conserved sequence, the
oligopeptide LGWLPSEIFPLEIRSA, which is located at the position 408 of the primary structure of the *Arabidopsis thaliana* (AtSTP1) transporter. The sixteen aminoacid sequence with an additional Cys at the carboxy terminal was conjugated to the keyhole limpet hemocyanin and synthesized by Genescript Corporation (Piscataway, NJ, USA). Antibody against the beet plasma membrane H\(^+\)-ATPase was a kind gift from Dr. Luis E. González de la Vara (CINVESTAV, Irapuato, México).

**Electrophoresis and immunodetection by Western Blot**

Proteins were separated by SDS-PAGE according to Shagger and von Jagow (1987) in the case of the H\(^+\)-ATPase or to Laemmli (1970) in the cases of the sucrose and hexose transporters. Twenty \(\mu\)g of protein or 6 \(\mu\)l of Precision Plus Protein Standard Protein (Bio-Rad, Hercules, CA, USA) were loaded per lane. Gels were stained with Coomassie Blue or used for Western blotting. Transfer of proteins was made in a wet chamber (C.B.S. Scientific Co., Del Mar, CA, USA) at 250 mA, for 2 h at 4 °C. Proteins in nitrocellulose or PVDF membrane were blotted and exposed against the sucrose transporter or the hexose transporter antibody both in a 1:3000 dilution. Antibody for the H\(^+\)-ATPase (directed against the red beet plasma membrane H\(^+\)-ATPase, was done with a diluted antibody 1:4 000 in PBS. The second antibody, an anti-IgG conjugated with alkaline phosphatase was diluted 1:10 000 using the conditions of the manufacturer. Specificity of these antibodies was as follows: Single bands for the two transporters were obtained and a main single band for the H\(^+\)-ATPase (see Fig. S1).
SUPPLEMENTARY REFERENCES

Laemmli LK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.
Macías F, Marin D, Oliveros-Bastidas A, Molinillo JMG. 2006. Optimization of benzoxazinones as natural herbicide models by lipophilicity enhancement. Journal of Agriculture and Food Chemistry 54, 93578-9365.
Rottenberg H, Moreno-Sanchez R. 1993. The proton pumping activity of H⁺-ATPases: an improved fluorescence assay. Biochimica et Biophysica Acta 1183, 161-170.
Schägger H, von Jagow G. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Analytical Biochemistry 166, 368-379.
Skipski VP, Peterson RF, Sanders J, Barclay M. 1962. Thin-layer chromatography of phospholipids using silica gel without calcium sulphate binder. Journal of Lipid Research 4, 227-228.
SUPPLEMENTARY RESULTS

Table S1.

Equivalence between the quenching of ACMA fluorescence and the $\Delta$ pH formed in the plasma membrane vesicles obtained from embryos imbibed at different times. Values were calculated according to the calibration curves shown in Fig. S4B, C. $\%Q_{\text{max}}$ was calculated from the trace with the uncoupling of every plasma membrane vesicles preparation with KCl/gramicidin addition. $\Delta p\text{H}_{\text{max}}$ was calculated from the calibration curve with liposomes (Fig. S4C) by interpolation of the $\%Q_{\text{max}}$ value.

| Measurement          | Imbibition time (h) |
|----------------------|---------------------|
|                      | 6       | 12      | 18      | 24      |
| a.u.f./min           | 0.104   | 0.386   | 0.417   | 0.417   |
| $\%Q/\text{min}$    | 0.063   | 0.234   | 0.256   | 0.256   |
| $\Delta p\text{H}/\text{min}$ | 0.004 | 0.015   | 0.016   | 0.016   |
| $\%Q_{\text{max}}$  | 2.1     | 2.6     | 3.0     | 3.1     |
| $\Delta p\text{H}_{\text{max}}$ | 0.14  | 0.17    | 0.19    | 0.20    |
Figure S1. Characterization of the plasma membrane sucrose and hexose transporter antibodies. Molecular mass standards and 20 µg microsomal proteins were separated in SDS-PAGE and subjected to Western analyses. (A) Coomassie blue stained gel as a loading control. (B) Reaction of microsomal membranes with the sucrose transporter antibody. (C) Reaction of microsomal membranes with the hexose transporter antibody. (D) Reaction of microsomal membranes with the H⁺-ATPase antibody.
**Figure S2.** Levels of immunodetected sucrose and hexose transporters and plasma membrane H⁺-ATPase during embryo germination. Proteins of microsomal fractions from embryos imbibed at the indicated times were separated on SDS-PAGE, electrotransferred and detected with specific antibodies (Figs. 3C, S1). Then, densitometric analysis of each band was done. The values reported were calculated considering the intensity of the band and compared to the band at 0 h (100%). n= 5
Figure S3. Negative controls of the sucrose transporter immunolocalization. Two different controls were made where no specific staining was detected. (A) Embryo sections were incubated with preimmune rabbit antibodies. (B) Embryo section incubated with the specific sucrose transporter antibody but omitting the incubation with the second antibody. Vascular bundle of the scutellum-nodal zone with a tracheidal element are shown. (C) Embryo section showing the plumule region. t, tracheidal elements; v, vascular bundle. Bar in A-C, 160 μm.
Figure S4. Determination of medium acidification by quenching of ACMA fluorescence. (A) Quenching of ACMA fluorescence dependent on the plasma membrane protein concentration. (B) ΔpH formed in liposomes under several imposed pH differences. (C) Calibration curve of the fluorescence signal and the ΔpH formed in liposomes. See Supplementary Materials and Methods for experimental details.