2012

Mechanosensitive channels protect plastids from hypoosmotic stress during normal plant growth

Kira M. Veley
Washington University in St Louis

Sarah Marshburn
Washington University in St Louis

Cara E. Clure
Washington University in St Louis

Elizabeth S. Haswell
Washington University in St Louis, ehaswell@wustl.edu

Mechanosensitive Channels

Protect Plastids from Hypoosmotic Stress

During Normal Plant Growth

Kira M. Veley, Sarah Marshburn, Cara E. Clure and Elizabeth S. Haswell*

Department of Biology, Box 1137

Washington University in St. Louis

Saint Louis, MO 63130

*Corresponding Author

Running head: MS Channels Protect Plastids From Osmotic Stress

SUMMARY

Cellular response to osmotic stress is critical for survival and involves volume control through the regulated transport of osmolytes [1-3]. Organelles may respond similarly to abrupt changes in cytoplasmic osmolarity [4-6]. The plastids of the *Arabidopsis thaliana* leaf epidermis provide a model system for the study of organellar response to osmotic stress within the context of the cell. An *Arabidopsis* mutant lacking two plastid-localized homologs of the bacteria mechanosensitive channel MscS (MscS-Like (MSL) 2 and 3) exhibits large round epidermal plastids that lack dynamic extensions known as stromules [7]. This phenotype is present under normal growth conditions and does not require exposure to extracellular osmotic stress. Here, we show that increasing cytoplasmic osmolarity through a genetic lesion known to produce elevated levels of soluble sugars, exogenously providing osmolytes in the growth media, or withholding water rescues the *msl2-1 msl3-1* leaf epidermal plastid phenotype, producing plastids that resemble the wild type in shape and size. Furthermore, the epidermal plastids in *msl2-1 msl3-1* leaves undergo rapid and reversible volume and shape changes in response to extracellular hypertonic or hypotonic challenges. We conclude that plastids are under hypoosmotic stress during normal plant growth and dynamic response to this stress requires MSL2 and MSL3.

RESULTS

In agreement with previously published results, confocal laser scanning microscopy (CLSM) of plants constitutively expressing a plastid-localized fluorophore (RecARED) revealed that the leaf epidermal plastids in wild type (WT) Columbia ecotype plants were small and ovoid, while those in *msl2-1 msl3-1* double mutant plants were enlarged and spherical (Figure 1A, [7]). MSL2 and MSL3 are plastid-localized *Arabidopsis* homologs of *E. coli* MscS, one of several bacterial mechanosensitive (MS) channels that open in response to increased membrane tension, allowing the efflux of osmolytes and preventing cellular lysis during extreme osmotic downshock [8]. These results led us to hypothesize that the osmolarity of the epidermal plastid stroma is higher than that of the cytoplasm, favoring the influx of water into the plastid. In WT plastids the resulting increase in envelope membrane tension would gate the MSL2 and MSL3 channels, allowing the efflux of osmolytes and maintaining normal plastid shape. In *msl2-1 msl3-1* plastids, osmolytes would not be released, water influx would be unrelieved, and large round plastids would result. If this hypothesis were correct, increasing the osmolarity of the cytoplasm relative to the plastid stroma should reduce water flux from the cytoplasm to the plastid stroma and suppress the round morphology of *msl2-1 msl3-1* leaf epidermal plastids.

Supplementing growth media with osmolytes suppresses the leaf epidermal plastid phenotype of the *msl2-1 msl3-1* mutant

To test the hypothesis that *msl2-1 msl3-1* leaf epidermal plastids are under hypoosmotic stress, we first investigated whether their large round phenotype could be suppressed by providing osmolytes to growing seedlings. Treatment of Arabidopsis seedlings with

NaCl, sucrose, or mannitol has been demonstrated to decrease leaf osmotic potential two- to three-fold [9, 10]. WT and *msl2-1 msl3-1* plants were grown on solid media containing 3% (166.5 mM) glucose or equiosmolar amounts of fructose, maltose, or NaCl and the morphology of leaf epidermal plastids examined by CLSM. While the leaf epidermal plastids of *msl2-1 msl3-1* plants grown on media without sugar were large and round, they were small and ovoid when grown on media supplemented with sugars or with salt (Figure 1A). A slightly higher concentration of sucrose (180 mM) was required to produce *msl2-1 msl3-1* leaf epidermal plastids that more closely resembled the WT in shape and size (Figure 1B). However, exogenous sorbitol (which cannot be taken up by plant roots and therefore should not affect the cytoplasmic osmolarity of leaf cells [11]) did not alter the large leaf epidermal plastid phenotype in *msl2-1 msl3-1* plants, even when provided at 180 mM (Figure 1B). WT leaf epidermal plastid morphology appeared unaffected by these treatments.

The *pgm-1* lesion suppresses the leaf epidermal plastid phenotypes of the *msl2-1 msl3-1* mutant

To further examine the role of cytoplasmic osmolarity in the round leaf epidermal plastid phenotype of *msl2-1 msl3-1* plants, we investigated whether it could be suppressed by a genetic lesion known to produce high levels of soluble sugars. The *PHOSPHOGLUCOMUTASE (PGM)/STARCH-FREE (STF1)* locus encodes a plastid-localized enzyme required for the conversion of photosynthate into starch [12]. *pgm/stf1* mutants contain very small amounts of plastidic starch [13-17] and exhibit high levels of soluble sugars in their leaves during the day [15, 17-24]. The concentration of soluble

sugars in *pgm-1* mutant cells is high enough to induce the expression of sugar-responsive genes [19, 20, 22, 25, 26].

The *pgm-1* allele was introduced into the *msl2-1 msl3-1 pRecARED* line by crossing, and *msl2-1 msl3-1 pgm-1 pRecARED* and *msl2-1 msl3-1 pgm-1 +/- pRecARED* siblings were identified in the F3 generation (see Experimental Procedures). As expected, WT and *pgm-1* plants exhibited small, ovoid leaf epidermal plastids, while *msl2-1 msl3-1* and *msl2-1 msl3-1 pgm-1 +/-* mutant lines had large, round leaf epidermal plastids (Figure 2A). However, the triple homozygous *msl2-1 msl3-1 pgm-1* mutant had small, oval-shaped leaf epidermal plastids that closely resembled the WT. Introduction of a *like sex4* mutant allele (*lsf1-1*), which causes the accumulation of high levels of plastidic starch [27], did not alter the leaf epidermal plastid phenotype of the *msl2-1 msl3-1* mutant.

To confirm that the *pgm-1* allele resulted in the accumulation of high levels of soluble sugars, we measured the amounts of sucrose, D-glucose, and D-fructose in leaf tissue at the end of the light cycle using same plants used in Figure 2A. We observed increased levels of all three sugars in the homozygous triple *msl2-1 msl3-1 pgm-1* mutant, with an overall 2-fold increase in soluble sugars compared to *msl2-1 msl3-1 pgm-1 +/-* siblings (Figure 2B), similar to that previously reported for the *pgm-1* mutant under the same light conditions [20, 23].

msl2-1 msl3-1 leaf epidermal plastids exhibit a rapid and reversible change in morphology when intact leaves are subjected to extreme hyperosmotic shock

To characterize the dynamic nature of epidermal plastid morphology in the leaves of the *msl2-1 msl3-1* mutant, we subjected excised leaf tissue to hyperosmotic shock (Figure 3). Epidermal plastids from the first or second leaves of 2 week-old seedlings were imaged before and after submersion of the excised leaves in 30% sorbitol. As shown in Figure 3A, *msl2-1 msl3-1* leaf epidermal plastids lost their round shape and exhibited stromule-like extensions after 15 minutes of sorbitol treatment, but recovered a round shape and large size after a 5-minute incubation in distilled water. A time course revealed that *msl2-1 msl3-1* epidermal plastids began to lose their smooth edges 5 minutes after excised leaves were mounted in sorbitol, and stromule-like projections were visible by 10 minutes of treatment (arrows, Figure 3C). WT leaf epidermal plastid morphology remained unchanged throughout these treatments (Figure 3A, Supplementary Figure 1).

msl2-1 msl3-1 leaf epidermal plastid morphology dynamically responds to water availability in live plants

We also tested a physiological approach to increasing cytoplasmic osmolarity by withholding water from soil-grown plants, a treatment previously shown to decrease leaf osmotic potential two-fold [10]. In plants deprived of water for 9-12 days, *msl2-1 msl3-1* leaf epidermal plastids were small and no longer round (Figure 4A). 24 hours after re-watering, *msl2-1 msl3-1* leaf epidermal plastids were detected, but those that were

present had recovered their characteristic round shape. WT leaf epidermal plastids remained small and ovular throughout the drought and rehydration treatments.

msl2-1 msl3-1 leaf epidermal plastids lyse when subjected to extreme hypoosmotic shock

The low number of epidermal plastids in *msl2-1 msl3-1* mutant leaves recovering from dehydration stress lead us to speculate that they were lysing upon rehydration of the tissue in the same way that *E. coli* cells lacking MS channels lyse under hypoosmotic stress [8]. Rosette leaves from *msl2-1 msl3-1* plants were excised and allowed to dehydrate on the bench top for 45-60 minutes. This treatment resulted in the suppression of the large round plastid phenotype (Figure 4B and C). Dehydrated leaves were then mounted in water. Within a few minutes of rehydration, leaf epidermal plastids became large and round (Figure 4D and E, left-hand panels). After longer periods we frequently observed the lysis of leaf epidermal plastids and the subsequent appearance of dsRED signal in the cytoplasm; two examples are shown in the right-hand panels of Figure 4D and E. No lysis was observed in WT leaves subjected to the same regime.

DISCUSSION

Classic and contemporary studies have demonstrated that chloroplasts and nuclei are capable of volume regulation in response to extracellular osmotic challenges [4, 5, 28-31]. Here we add to these studies by addressing the extent to which organelles experience osmotic stress from within the cytoplasm during normal growth conditions, and by providing a molecular mechanism for volume control by organelles—the opening of MS ion channels under hypoosmotic stress. The large, round leaf epidermal plastid phenotype of *msl2-1 msl3-1* plants was reliably and reversibly ameliorated by a number of treatments that increase cytoplasmic osmolarity: growth on high levels of actively transported osmotica (Figure 1), a genetic lesion known to increase levels of soluble sugars (Figure 2), dehydration of soil-grown plants or excised leaves (Figure 4), and the immersion of excised leaves in a hypertonic solution (Figure 3). The leaf epidermal plastids of WT plants were not appreciably changed in size or shape by any of these treatments. Furthermore, *msl2-1 msl3-1* mutant plastids lysed inside leaf epidermal cells that were exposed to extreme hypoosmotic shock, while WT plastids were unaffected (Figure 4). Thus, the leaf epidermal plastid phenotype of the *msl2-1 msl3-1* mutant can be attributed to an abnormally high stromal osmolarity, leading to the influx of water, subsequent plastid swelling, and plastidic sensitivity to hypoosomotic shock.

MSL2 and MSL3 are likely to directly mediate the efflux of osmolytes when the plastid inner envelope is under tension. They are evolutionarily related to the bacterial MS channel MscS [32-34], and MSL3 can provide osmotic shock protection to an *E. coli* strain lacking three major MS ion channels [7]. Taken together, these data support the

conclusion that the stroma of leaf epidermal plastids has a lower osmotic potential (higher solute level) than the cytoplasm even under normal growth conditions and that MSL2 and MSL3 are required to relieve this hypoosmotic stress.

A high stromal solute level relative to the cytoplasm is likely to be generated through the active transport of energy and metabolites into leaf epidermal plastids [35]. Surprisingly, the chloroplasts found in the layer below the epidermis (the mesophyll) do not exhibit the same round phenotype as the plastids of the epidermis in *msl2-1 msl3-1* mutant leaves [7]. This may be explained by differences in the metabolic activity and membrane transport complement between chloroplasts and the nonphotosynthetic plastids of the epidermis. For example, during the day, sucrose is transported into the epidermal plastid, while chloroplasts produce sucrose and either store it as starch or export it to the cytoplasm [35]. Perhaps chloroplasts use the export or the storage of sugar to modulate stromal osmolarity, making MS channels superfluous. Another possibility is that the internal thylakoid membranes, which are specific to chloroplasts, provide a mechanism for raising the osmotic potential of the stroma, as has been proposed for the cristae of mitochondria [36].

The changes in plastid volume and morphology shown in Figure 3A and C are rapid, and can be attributed largely to the movement of water across the plasma membrane and plastid envelope. Figure 3B diagrams the expected movement of water in a leaf epidermal cell immediately after exposure to 30% sorbitol. While the osmotic potential of Arabidopsis leaf cells is -0.8 to -1.0 MPa [9, 10], a 30% sorbitol solution has an osmotic

potential of -4 MPa according to the van't Hoff equation. Extracellular sorbitol treatment thus creates the rapid efflux of water from the cytoplasm of the epidermal cell to the extracellular sorbitol solution (from higher to lower osmotic potential). As a result, the osmotic potential of the cytoplasm decreases, allowing water to move from the plastid stroma to the cytoplasm, and thereby reducing plastid volume. The transport of water across the plastid envelope could be facilitated by aquaporins, water transport channels found in all kingdoms of life [37]. Or, as is proposed for the many microbial species for which no aquaporin has been found, passive water transport may suffice [38].

Summary. We have previously described an *Arabidopsis* mutant lacking two plastid-localized MS channels [7]; in this study we used this mutant as a sensitized background for the analysis of plastidic osmotic stress within the context of the cell cytoplasm. We used genetic, physiological, and media manipulations to show that under normal growth conditions the epidermal plastids of the leaf experience hypoosmotic stress. Furthermore, the effects of extreme osmotic stresses on mutant plastid morphology are rapid and reversible, occurring within a few minutes of treatment with hypertonic or hypotonic solutions. Finally, our data show that MS channels are critical for maintaining the wild-type responses of leaf epidermal plastids to changing cellular osmolarity, offering a likely molecular mechanism by which these endosymbiotic organelles respond to hypoosmotic stress. Given the evolutionary relationship between MSL2, MSL3, and MS channels found in bacteria, these results also provide an opportunity to study how this survival mechanism may allow other organelles and intracellular pathogens to respond to the dynamic osmotic challenges of the cytoplasm.

ACKNOWLEDGEMENTS

This work was supported by NSF MCB0816627 (to E.S.H). We acknowledge the Arabidopsis Biological Resource Center for *pgm-1* and *lsf1-1* mutant lines. We are also grateful to the Washington University in Saint Louis greenhouse staff for their assistance and David Braun for advice regarding soluble sugar quantification.

FIGURE LEGENDS

Figure 1. Supplementing growth media with osmolytes suppresses the leaf epidermal plastid phenotype of *msl2-1 msl3-1* mutant plants. Representative confocal laser scanning microscopy (CLSM) images taken from plants harboring the *pRecARED* plastid marker (pseudocolored red). Leaf epidermal plastids from 12-day old WT and *msl2-1 msl3-1* seedlings were imaged after growth on solid media supplemented with the indicated amounts of (A) glucose, fructose, maltose, NaCl, or (B) sucrose or sorbitol. Images were taken from the lower right-hand quadrant of the first or second true leaf. Size bar is 10 microns.

Figure 2. The *pgm-1* lesion leads to increased levels of soluble sugars and suppresses the leaf epidermal plastid phenotype of the *msl2-1 msl3-1* mutant. (A) Representative CLSM images of leaf epidermal plastids in plants of the indicated genotypes harboring the *pRecARED* plastid marker. Images were taken from the lower right-hand quadrant of fully expanded cauline leaves of 3-week old soil-grown plants. At least six plants from each genotype were characterized. Size bar is 10 microns. (B) Soluble sugar levels were significantly increased in *msl2-1 msl3-1 pgm-1* plants compared to *msl2-1 msl3-1 pgm-1 +/-* siblings ($P = 0.02$, Student's *t* test). Leaf tissue was collected at the end of a 16-hour day. Error bars indicate standard deviation between three biological replicates for each genotype.

Figure 3. Intact *msl2-1 msl3-1* leaves exhibit rapid and reversible changes in epidermal plastid morphology when subjected to hyperosmotic shock. CLSM images taken from plants harboring the *pRecARED* plastid marker. (A) Epidermal plastid morphology was imaged in WT and *msl2-1 msl3-1* leaves mounted in water (top), after 15 minutes of treatment with 30% sorbitol (middle), and after washing and re-mounting in water (bottom). Size bar is 10 microns. (B) Cartoon depicting the immediate effects of immersing a single mutant epidermal leaf cell in 30% sorbitol. Red arrows indicate the flow of water from compartments with higher osmotic potential (dark blue) to compartments with lower osmotic potential (light blue). (C) Changes in *msl2-1 msl3-1* leaf epidermal plastid morphology over the course of a 30-minute treatment with 30% sorbitol. Arrows indicate stromules. Size bar is 50 microns.

Figure 4. *msl2-1 msl3-1* leaf epidermal plastids shrink in plants and intact leaves experiencing drought, then swell and lyse upon rehydration. Representative CLSM images taken from plants harboring the *pRecARED* plastid marker. (A) WT and *msl2-1 msl3-1* plants were grown in the same pot for three weeks and leaf epidermal plastids imaged before and after indicated dehydration and rehydration treatments. (B-C) Leaf epidermal plastid morphology was imaged before (B) and after (C) 60 minutes of dehydration on the bench top. (D) and (E) Epidermal cells from excised, dehydrated, and rehydrated leaves before (left-hand panels) and after (right-hand panels) plastid lysis. Size bars are 50 microns in (A-C) and 10 microns in (D-E).

Supplemental Figure 1. WT leaf epidermal plastid morphology does not appreciably change in intact cells experiencing hyperosmotic shock. CLSM images taken from excised leaves of WT plants harboring the *pRecARED* plastid marker over the course of a 30-minute on-slide incubation in 30% sorbitol. Arrow indicates a stromule. Bar is 50 microns.

REFERENCES

1. Pedersen, S.F., Kapus, A., and Hoffmann, E.K. (2011). Osmosensory mechanisms in cellular and systemic volume regulation. J Am Soc Nephrol *22*, 1587-1597.
2. Hohmann, S., Krantz, M., and Nordlander, B. (2007). Yeast osmoregulation. Methods Enzymol *428*, 29-45.
3. Zhu, J.K. (2002). Salt and drought stress signal transduction in plants. Annu Rev Plant Biol *53*, 247-273.
4. Finan, J.D., Chalut, K.J., Wax, A., and Guilak, F. (2009). Nonlinear osmotic properties of the cell nucleus. Ann Biomed Eng *37*, 477-491.
5. McCain, D.C. (1995). Combined effects of light and water stress on chloroplast volume regulation. Biophysical Journal *69*, 1105-1110.
6. Boustany, N.N., Drezek, R., and Thakor, N.V. (2002). Calcium-induced alterations in mitochondrial morphology quantified in situ with optical scatter imaging. Biophys J *83*, 1691-1700.
7. Haswell, E.S., and Meyerowitz, E.M. (2006). MscS-like proteins control plastid size and shape in Arabidopsis thaliana. Curr Biol *16*, 1-11.
8. Levina, N., Totemeyer, S., Stokes, N.R., Louis, P., Jones, M.A., and Booth, I.R. (1999). Protection of Escherichia coli cells against extreme turgor by activation of MscS and MscL mechanosensitive channels: identification of genes required for MscS activity. EMBO J *18*, 1730-1737.

9. Inan, G., Zhang, Q., Li, P., Wang, Z., Cao, Z., Zhang, H., Zhang, C., Quist, T.M., Goodwin, S.M., Zhu, J., et al. (2004). Salt cress. A halophyte and cryophyte Arabidopsis relative model system and its applicability to molecular genetic analyses of growth and development of extremophiles. Plant Physiol *135*, 1718-1737.

10. Dejardin, A., Sokolov, L.N., and Kleczkowski, L.A. (1999). Sugar/osmoticum levels modulate differential abscisic acid-independent expression of two stress-responsive sucrose synthase genes in Arabidopsis. Biochem J *344 Pt 2*, 503-509.

11. Gibson, S.I. (2000). Plant sugar-response pathways. Part of a complex regulatory web. Plant Physiol *124*, 1532-1539.

12. Zeeman, S.C., Smith, S.M., and Smith, A.M. (2007). The diurnal metabolism of leaf starch. Biochem J *401*, 13-28.

13. Fettke, J., Malinova, I., Albrecht, T., Hejazi, M., and Steup, M. (2010). Glucose-1-phosphate transport into protoplasts and chloroplasts from leaves of Arabidopsis. Plant Physiol *155*, 1723-1734.

14. Streb, S., Egli, B., Eicke, S., and Zeeman, S.C. (2009). The Debate on the Pathway of Starch Synthesis: A Closer Look at Low-Starch Mutants Lacking Plastidial Phosphoglucomutase Supports the Chloroplast-Localized Pathway. Plant Physiology *151*, 1769-1772.

15. Caspar, T., Huber, S.C., and Somerville, C. (1985). Alterations in Growth, Photosynthesis, and Respiration in a Starchless Mutant of Arabidopsis thaliana

(L.) Deficient in Chloroplast Phosphoglucomutase Activity. Plant Physiol *79*, 11-17.

16. Neuhaus, H.E., and Stitt, M. (1990). Control analysis of photosynthate partitioning. Planta *182*, 445-454.
17. Kofler, H., Hausler, R.E., Schulz, B., Groner, F., Flugge, U.I., and Weber, A. (2000). Molecular characterisation of a new mutant allele of the plastid phosphoglucomutase in Arabidopsis, and complementation of the mutant with the wild-type cDNA. Mol Gen Genet *263*, 978-986.
18. Corbesier, L., Lejeune, P., and Bernier, G. (1998). The role of carbohydrates in the induction of flowering in Arabidopsis thaliana: comparison between the wild type and a starchless mutant. Planta *206*, 131-137.
19. Solfanelli, C., Poggi, A., Loreti, E., Alpi, A., and Perata, P. (2006). Sucrose-specific induction of the anthocyanin biosynthetic pathway in Arabidopsis. Plant Physiol *140*, 637-646.
20. Gonzali, S., Loreti, E., Solfanelli, C., Novi, G., Alpi, A., and Perata, P. (2006). Identification of sugar-modulated genes and evidence for in vivo sugar sensing in Arabidopsis. J Plant Res *119*, 115-123.
21. Antonio, C., Larson, T., Gilday, A., Graham, I., Bergstrom, E., and Thomas-Oates, J. (2007). Quantification of sugars and sugar phosphates in Arabidopsis thaliana tissues using porous graphitic carbon liquid chromatography-electrospray ionization mass spectrometry. J Chromatogr A *1172*, 170-178.
22. Blasing, O.E., Gibon, Y., Gunther, M., Hohne, M., Morcuende, R., Osuna, D., Thimm, O., Usadel, B., Scheible, W.R., and Stitt, M. (2005). Sugars and

circadian regulation make major contributions to the global regulation of diurnal gene expression in Arabidopsis. Plant Cell *17*, 3257-3281.

23. Gibon, Y., Blasing, O.E., Palacios-Rojas, N., Pankovic, D., Hendriks, J.H., Fisahn, J., Hohne, M., Gunther, M., and Stitt, M. (2004). Adjustment of diurnal starch turnover to short days: depletion of sugar during the night leads to a temporary inhibition of carbohydrate utilization, accumulation of sugars and post-translational activation of ADP-glucose pyrophosphorylase in the following light period. Plant J *39*, 847-862.

24. Lunn, J.E., Feil, R., Hendriks, J.H., Gibon, Y., Morcuende, R., Osuna, D., Scheible, W.R., Carillo, P., Hajirezaei, M.R., and Stitt, M. (2006). Sugar-induced increases in trehalose 6-phosphate are correlated with redox activation of ADPglucose pyrophosphorylase and higher rates of starch synthesis in Arabidopsis thaliana. Biochem J *397*, 139-148.

25. Usadel, B., Blasing, O.E., Gibon, Y., Retzlaff, K., Hohne, M., Gunther, M., and Stitt, M. (2008). Global transcript levels respond to small changes of the carbon status during progressive exhaustion of carbohydrates in Arabidopsis rosettes. Plant Physiol *146*, 1834-1861.

26. Gibon, Y., Blaesing, O.E., Hannemann, J., Carillo, P., Hohne, M., Hendriks, J.H., Palacios, N., Cross, J., Selbig, J., and Stitt, M. (2004). A Robot-based platform to measure multiple enzyme activities in Arabidopsis using a set of cycling assays: comparison of changes of enzyme activities and transcript levels during diurnal cycles and in prolonged darkness. Plant Cell *16*, 3304-3325.

27. Comparot-Moss, S., Kotting, O., Stettler, M., Edner, C., Graf, A., Weise, S.E., Streb, S., Lue, W.L., MacLean, D., Mahlow, S., et al. (2010). A putative phosphatase, LSF1, is required for normal starch turnover in Arabidopsis leaves. Plant Physiol *152*, 685-697.

28. Gupta, A.S., and Berkowitz, G.A. (1988). Chloroplast osmotic adjustment and water stress effects on photosynthesis. Plant Physiol *88*, 200-206.

29. Robinson, S.P. (1985). Osmotic Adjustment by Intact Isolated Chloroplasts in Response to Osmotic Stress and Its Effect on Photosynthesis and Chloroplast Volume. Plant Physiology *79*, 996-1002.

30. Santakumari, M., and Berkowitz, G. (1991). Chloroplast volume: cell water potential relationships and acclimation of photosynthesis to leaf water deficits. Photosynthesis Research *28*, 9-20.

31. McCain, D.C., and Markley, J.L. (1992). In vivo study of chloroplast volume regulation. Biophys J *61*, 1207-1212.

32. Pivetti, C.D., Yen, M.R., Miller, S., Busch, W., Tseng, Y.H., Booth, I.R., and Saier, M.H., Jr. (2003). Two families of mechanosensitive channel proteins. Microbiol Mol Biol Rev *67*, 66-85, table of contents.

33. Haswell, E.S. (2007). MscS-Like Proteins in Plants. In Mechanosensitive Ion Channels, Part A, Volume 58, O.P. Hamill, ed.

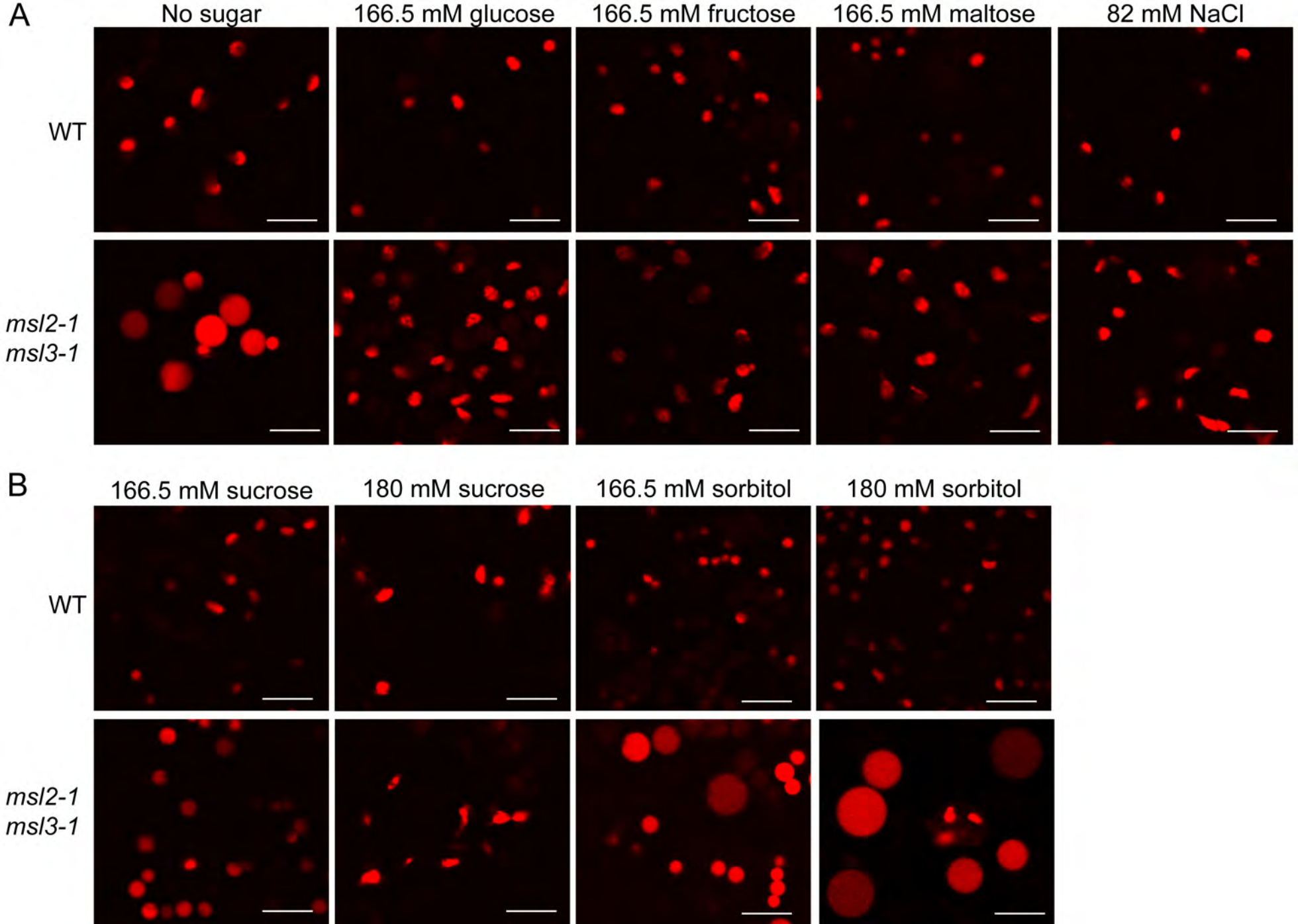
34. Balleza, D., and Gomez-Lagunas, F. (2009). Conserved motifs in mechanosensitive channels MscL and MscS. Eur Biophys J *38*, 1013-1027.

35. Weber, A.P., and Linka, N. (2011). Connecting the plastid: transporters of the plastid envelope and their role in linking plastidial with cytosolic metabolism. Annu Rev Plant Biol *62*, 53-77.

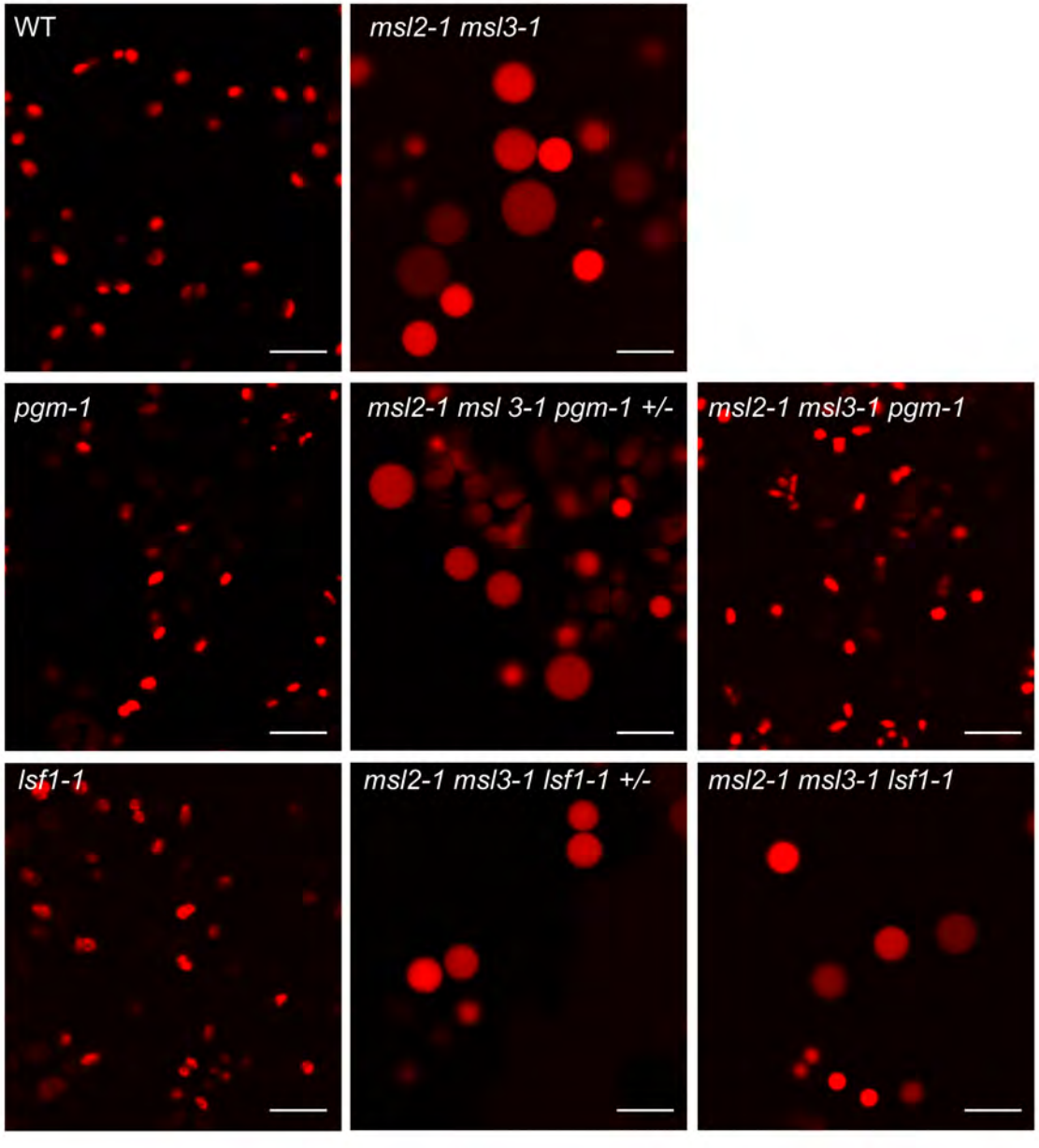
36. Mannella, C.A., Pfeiffer, D.R., Bradshaw, P.C., Moraru, II, Slepchenko, B., Loew, L.M., Hsieh, C.E., Buttle, K., and Marko, M. (2001). Topology of the mitochondrial inner membrane: dynamics and bioenergetic implications. IUBMB Life *52*, 93-100.

37. Gomes, D., Agasse, A., Thiebaud, P., Delrot, S., Geros, H., and Chaumont, F. (2009). Aquaporins are multifunctional water and solute transporters highly divergent in living organisms. Biochim Biophys Acta *1788*, 1213-1228.

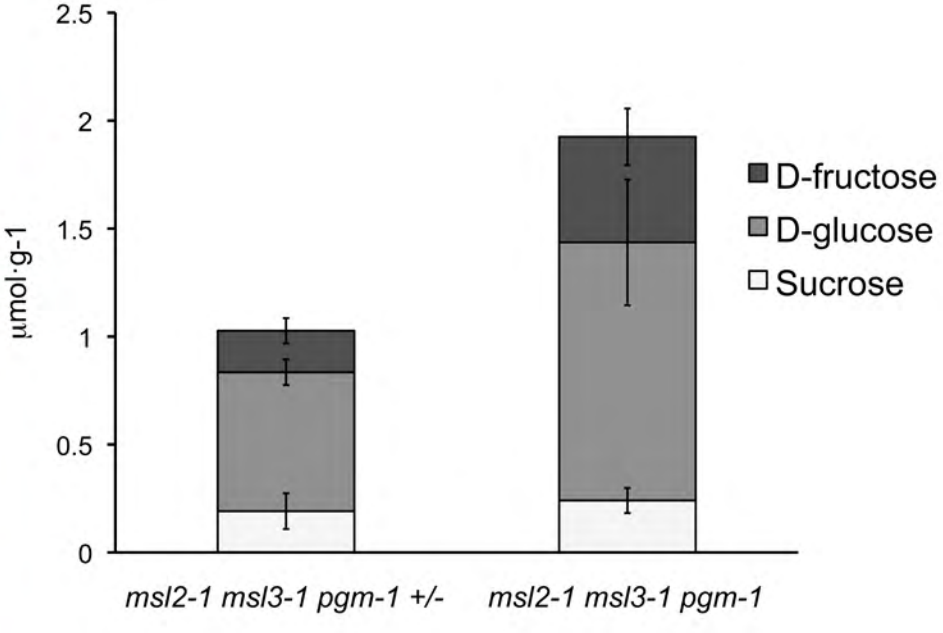
38. Tanghe, A., Van Dijck, P., and Thevelein, J.M. (2006). Why do microorganisms have aquaporins? Trends Microbiol *14*, 78-85.

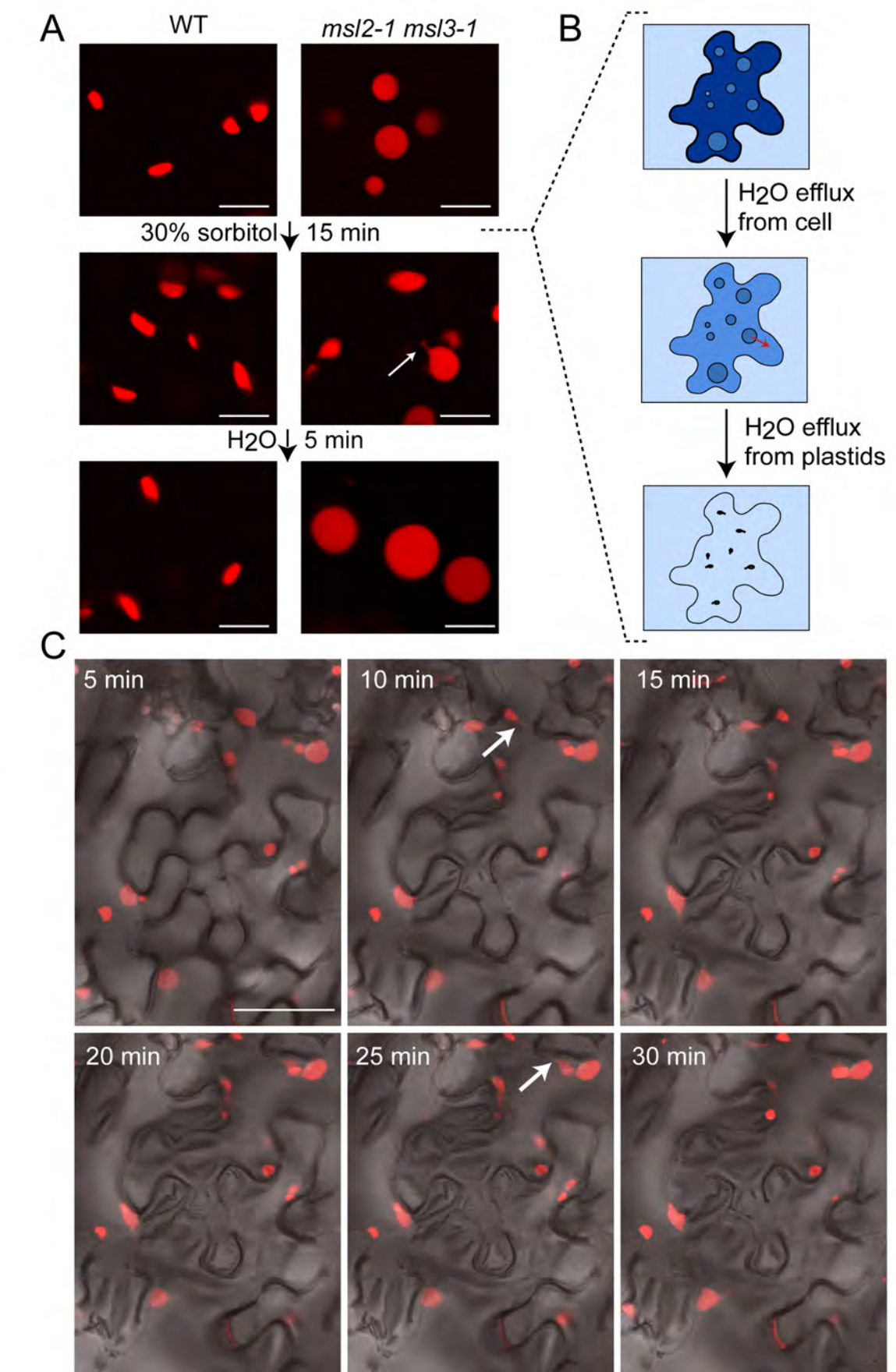

A
No sugar
166.5 mM glucose
166.5 mM fructose
166.5 mM maltose
82 mM NaCl
WT
msl2-1
msl3-1
B
166.5 mM sucrose
180 mM sucrose
166.5 mM sorbitol
180 mM sorbitol
WT
msl2-1
msl3-1

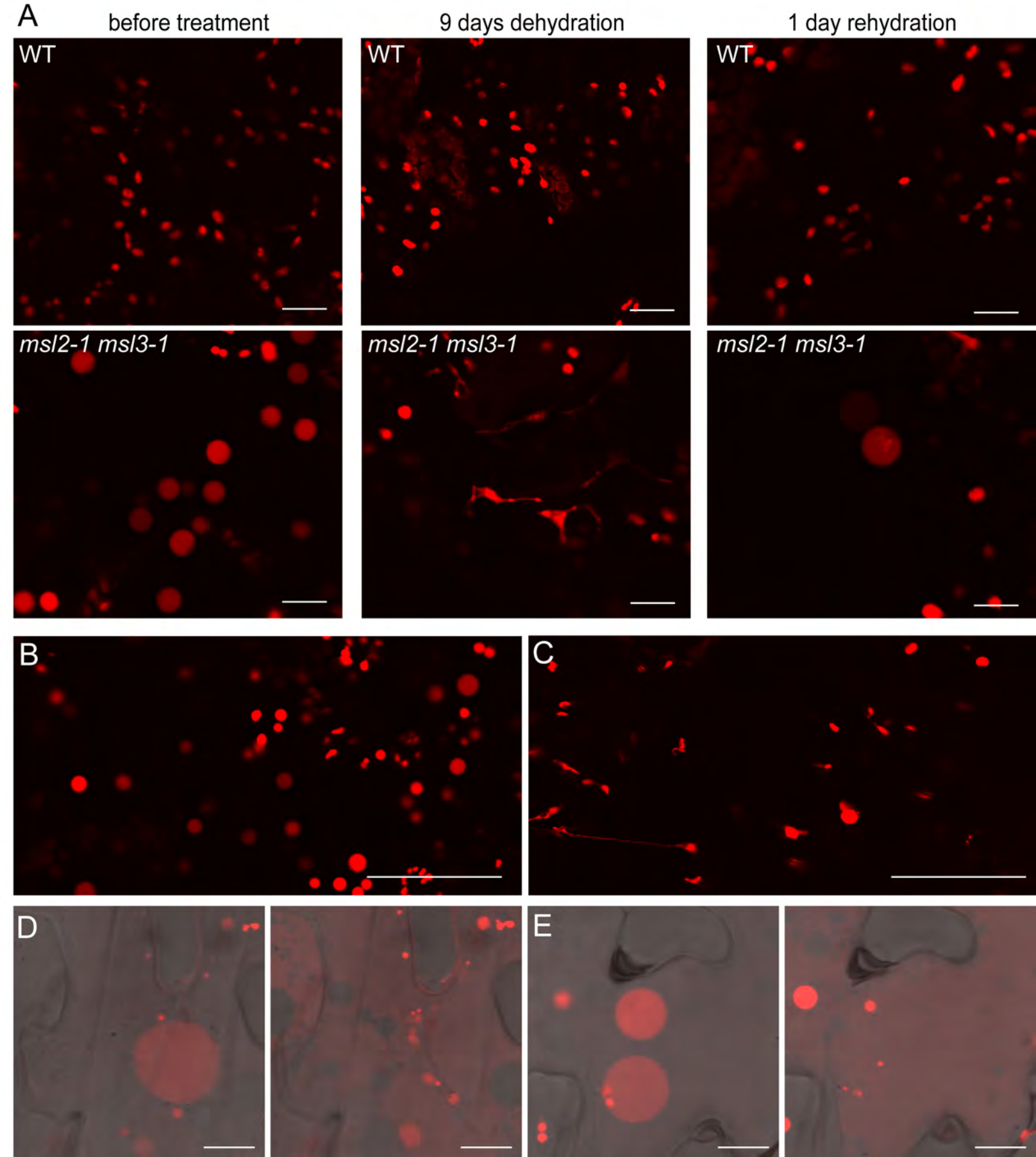
A


WT
msl2-1 msl3-1
pgm-1
msl2-1 msl 3-1 pgm-1 +/-
msl2-1 msl3-1 pgm-1
lsf1-1
msl2-1 msl3-1 lsf1-1 +/-
msl2-1 msl3-1 lsf1-1

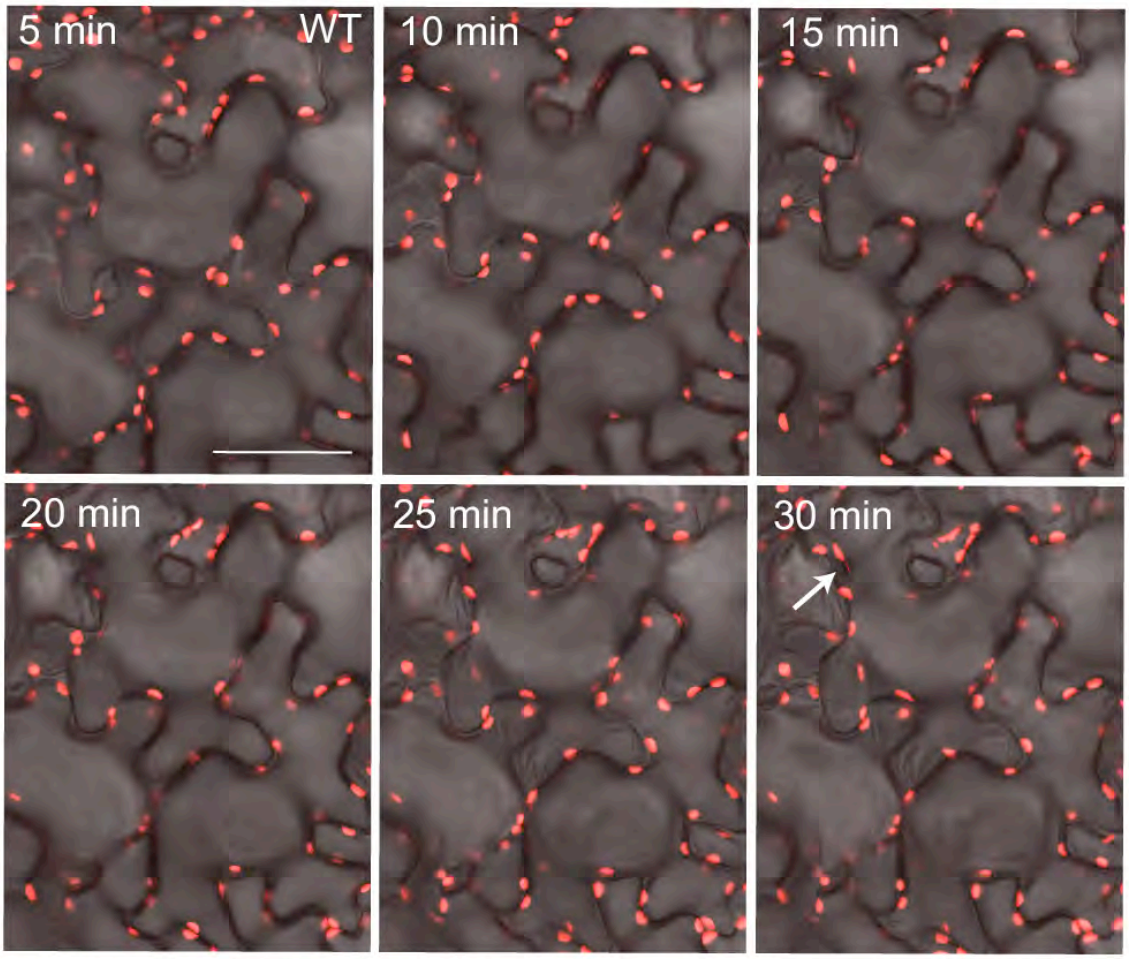
B


2.5
2
1.5
1
0.5
0
µmol·g-1
D-fructose
D-glucose
Sucrose
msl2-1 msl3-1 pgm-1 +/-
msl2-1 msl3-1 pgm-1


A
WT
msl2-1 msl3-1
30% sorbitol
15 min
H2O
5 min
B
H2O efflux
from cell
H2O efflux
from plastids
C
5 min
10 min
15 min
20 min
25 min
30 min


A
before treatment
9 days dehydration
1 day rehydration
WT
WT
WT
msl2-1 msl3-1
msl2-1 msl3-1
msl2-1 msl3-1
B
C
D
E

SUPPLEMENTAL FIGURE 1



Supplemental Figure 1. WT leaf epidermal plastid morphology does not appreciably change in intact cells experiencing hyperosmotic shock. CLSM images taken from excised leaves of WT plants harboring the pRecARED plastid marker over the course of a 30-minute on-slide incubation in 30% sorbitol. Arrow indicates a stromule. Bar is 50 microns.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plant growth and genotyping. Plants were grown on soil or on solid media at 21°C under a 16-hour light regime, except for the plants in Figure 1, which were grown under 24 hours of light. For the analysis of exogenously provided sugar and salt, plants were grown on agar-solidified MS medium (2 g/L Murashige and Skoog salts (Caisson Labs)), pH 5.7 and 0.8% agar (Caisson Labs). NaCl was added before autoclaving, while filter-sterilized 10X glucose, sucrose, maltose, fructose, and sorbitol stocks were added after autoclaving.

The *pgm-1* and *lsf1-1* alleles have been previously characterized [1-3]; seed was obtained from the Arabidopsis Biological Resource Center (stock numbers CS210 and SALK_053285, respectively). All WT controls, *pgm-1*, and *lsf-1* mutants are in the Columbia background, while the *msl2-1; msl3-1* mutant is in the Ws background. Primer sequences used for genotyping can be found in Supplementary Table 1. T-DNA insertion mutants in *MSL2* (At5g10490) and *MSL3* (At2g58200) were identified as in [4]. The *lsf1-1* T-DNA insertion (in At3g01510) was amplified using LB.a (TGGTTCACGTAGTGGGCCATCG) and LSF.F (AGTAAGAGGAGCTCGCCGAC) while the WT allele was amplified using LSF.F and LSF.R (TTCGAGAGCTCCTAAACCGG). A derived cleaved amplified polymorphic sequence (dCAPS) strategy [5] was used to genotype the *pgm-1* allele (a point mutation in aa 191 of At5g51820, changing a TGG codon to TGA [3]), using pgm-3 (CCACTTTGTTGACATATCGAG) and pgm-11 (GTGTACCAGAGACGAGACC) followed by digestion with Bsr1 (only the WT product is digested). The *msl2-1; msl3-1; pgm-1* triple mutant was generated by crossing *msl2-1 msl3-1 pRecARED* to *pgm-1*. Next, *msl2-1 msl3-1 pgm-1 pRecARED* plants were

crossed to *msl2-1 msl3-1 pRecARED* plants to obtain a line that was homozygous for *msl2-1* and *msl3-1*, but segregating the *pgm-1* allele (*msl2-1; msl3-1; pgm-1+/-; pRecARED* plants). The offspring of this line were analyzed in Figure 2.

Sugar quantification. Sugars were extracted from rosette leaves of plants grown to stage 6.0 as defined by [6] using a protocol modified from [7]. Briefly, tissue was frozen in liquid nitrogen after 14 hours of light (under a 16-h light/8-h dark photoperiod). Frozen tissue was ground into a powder with a mortar and pestle. 1 ml chilled buffer (500 mM MOPS pH 7.5, 5 mM EDTA, 10% ethylene glycol) was added to 100 mg powder and the entire homogenate filtered through a 0.22 µm filter, boiled for 10 minutes, then centrifuged for 5 minutes at 11500 x g. Sucrose, D-fructose, and D-glucose content in the supernatant were then determined using a kit (Megazyme) according to the manufacturer's instructions.

Microscopy. Confocal laser scanning microscopy (CLSM) was performed using a Fluoview FV-1000 (Olympus), and images were captured with FVIO-ASW software (Olympus). The *pRecARED* signal was excited at 543 nm and emissions collected with a 560- to 620-nm band-pass filter. DIC images were collected using a transmitted light detector.

SUPPLEMENTAL REFERENCES

1. Caspar, T., Huber, S.C., and Somerville, C. (1985). Alterations in Growth, Photosynthesis, and Respiration in a Starchless Mutant of Arabidopsis thaliana (L.) Deficient in Chloroplast Phosphoglucomutase Activity. Plant Physiol *79*, 11-17.
2. Comparot-Moss, S., Kotting, O., Stettler, M., Edner, C., Graf, A., Weise, S.E., Streb, S., Lue, W.L., MacLean, D., Mahlow, S., et al. (2010). A putative phosphatase, LSF1, is required for normal starch turnover in Arabidopsis leaves. Plant Physiol *152*, 685-697.
3. Periappuram, C., Steinhauer, L., Barton, D.L., Taylor, D.C., Chatson, B., and Zou, J. (2000). The plastidic phosphoglucomutase from Arabidopsis. A reversible enzyme reaction with an important role in metabolic control. Plant Physiol *122*, 1193-1199.
4. Haswell, E.S., and Meyerowitz, E.M. (2006). MscS-like proteins control plastid size and shape in Arabidopsis thaliana. Curr Biol *16*, 1-11.
5. Neff, M.M., Neff, J.D., Chory, J., and Pepper, A.E. (1998). dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in Arabidopsis thaliana genetics. Plant J *14*, 387-392.
6. Boyes, D.C., Zayed, A.M., Ascenzi, R., McCaskill, A.J., Hoffman, N.E., Davis, K.R., and Gorlach, J. (2001). Growth stage-based phenotypic analysis of Arabidopsis: a model for high throughput functional genomics in plants. Plant Cell *13*, 1499-1510.

7. Dinges, J.R., Colleoni, C., James, M.G., and Myers, A.M. (2003). Mutational analysis of the pullulanase-type debranching enzyme of maize indicates multiple functions in starch metabolism. Plant Cell *15*, 666-680.