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Chloroplast membrane remodeling during freezing stress is accompanied by cytoplasmic acidification activating SENSITIVE TO FREEZING 2

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Short Title

Acidification is a functional response to freezing

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Article Title

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Rebecca L. Roston (rroston@unl.edu).

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Footnotes

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One Sentence Summary

Cytoplasmic acidification is a specific response to freezing; it contributes to activating freezing-tolerance responses including a lipid remodeling enzyme necessary for freezing tolerance.

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Abstract (175 words)

Low temperature is a seasonal abiotic stress which restricts native plant ranges and crop distributions. Two types of low temperature stress can be distinguished: chilling and freezing. Much work has been done on the mechanisms by which chilling is sensed, but relatively little is known about how plants sense freezing. Recently, SENSITIVE TO FREEZING 2 (SFR2) was identified as a protein which responds in a non-transcriptional manner to freezing. Here, we investigate the cellular conditions which allow SFR2 activation. Using a combination of isolated organelle, whole tissue and whole plant assays, we provide evidence that SFR2 is activated by changes in cytosolic pH and Mg^{2+}. Manipulation of pH and Mg^{2+} in cold acclimated plants is shown to cause changes similar to those of freezing. We conclude that pH and Mg^{2+} are perceived as intracellular cues as part of the sensing mechanism for freezing conditions. This evidence provides a specific molecular mechanism to combat freezing.

Introduction

Freezing is a distinct abiotic stress that adds to the stress experienced during chilling (low temperatures above 0°C). There are at least two, possibly related, types of damage during freezing; formation of ice crystals accompanied by cellular dehydration, and membrane leakage (Thomashow, 1999). In *Arabidopsis thaliana*, ice first nucleates outside the cell. The resulting change in the osmotic gradient across the plasma membrane swiftly and severely dehydrates the cell (Steponkus, 1984, Steponkus, 1980). Membrane damage occurs both as a direct response to temperature and as a secondary effect of cellular dehydration. The temperature directly affects membrane fluidity and therefore leakage (Xin and Browse, 2000, Hays et al., 2001). During dehydration, membrane damage is heightened because membranes become appressed as the cell shrinks (Steponkus, 1984). This enhanced proximity of membranes, lack of fluidity, and low hydration allow non-lamellar structures to form between membranes, fusing subcellular compartments and ultimately resulting in cell death after rehydration (Uemura et al., 1995, Webb et al., 1994). Multiple mechanisms have evolved in plants to avoid both dehydration and membrane fusion, including solute accumulation, cell wall modification, lipid desaturation, and lipid composition changes (Browse and Xin, 2001, Lineberger and Steponkus, 1980, Chen and Thelen, 2013, Degenkolbe et al., 2012, Ji et al., 2015). These changes typically occur during a period of "cold acclimation" or "cold hardening" in which plants are exposed to low, non-freezing temperatures prior to freezing and transcriptional changes accompany increased freezing tolerance.

An exception to this rule is the gene *SENSITIVE TO FREEZING 2 (SFR2*). It was discovered in an *A. thaliana* screen for freezing intolerance (Warren et al., 1996). Mutant plants (*sfr2*) lacking SFR2 are severely damaged by freezing, but they have no phenotypes under normal growth or a variety of other stress conditions (Fourrier et al., 2008), implying that SFR2's activity represents a specific adaptation to freezing tolerance. The *sfr2* mutation is unusual amongst freezing sensitive mutants because cells of *sfr2* remain intact during freezing, as evinced by their lack of ion leakage (Warren et al., 1996). This is likely due to the role of SFR2 in maintaining organellar rather than cellular integrity.

During freezing, SFR2 removes the galactose head-group from monogalactosyldiacylglycerol (MGDG) and adds it to a second MGDG. This activity is processive, generating oligogalactolipids (di-, tri-, and up to hexa-galactosyldiacylglycerol), and leaving diacylglycerol (DAG) as a byproduct (Moellering et al., 2010, Roston et al., 2014). SFR2 activity was initially discovered in isolated chloroplasts where it was referred to as galactolipid:galactolipid galactosyl transferase (Heemskerk et al., 1983, Heemskerk et al., 1986). During freezing conditions, the DAG is converted into triacylglycerol (TAG), and TAG and

oligogalactolipids derived from MGDG specifically increase in response to freezing (Moellering et al., 2010, Vu et al., 2014a). SFR2 is associated with the chloroplast outer envelope membrane (Heemskerk et al., 1986, Roston et al., 2014), where it is anchored by a single transmembrane domain facing the cytoplasm (Roston et al., 2014). The soluble portion of SFR2 is primarily composed of a single glycosyl hydrolase domain. The hydrolase domain was shown to be responsible for the MGDG-specific transferase activity, without measurable hydrolysis activity (Roston et al., 2014). Notably, in original reports of *SFR2*, its protein and mRNA levels did not change in response to cold (Thorlby et al., 2004), indicating that it may be post-transcriptionally regulated.

Currently, the mechanism by which freezing is sensed on a cellular level is unknown. This lack prevents further understanding of membrane freezing responses, which are separate from those of cold acclimation and critical for freezing tolerance (Li et al., 2008). Here, we take advantage of SFR2 activation to probe how an enzyme is specifically activated by freezing. We show that SFR2 is post-translationally activated, and probe which cellular responses to freezing activate it in isolated chloroplasts and whole tissues, determining that cytosolic pH and Mg^{2+} each are involved. We demonstrate that cytosolic acidification is occurring in intact plants in response to freezing. It is further shown that SFR2 is not substrate limited under normal conditions, and it has a consistently sized protein complex, implying that acidification may directly activate SFR2. Finally, we investigate whether cytosolic acidification can mimic freezing-like membrane changes in intact tissues.

Results

SFR2 is activated post-translationally in response to freezing. The SFR2 protein is present at all temperatures measured (Figure 1A, Thorlby et al., 2004). However, SFR2's specific oligogalactolipid reaction products tri- and tetra-galactolipid (TGDG, TeGDG) are only detectable after plants were incubated at or below -4°C overnight. No detectable accumulation occurred after one week of cold acclimation at + 6°C, and freezing at -2°C showed little or no oligogalactolipid accumulation (Fig. 1B).

SFR2 is activated by pH and Mg^{2+}. Because SFR2 is present inside the cell, we hypothesized that it may be activated by physical changes in the cell associated with freezing. To test this hypothesis, chloroplasts isolated from Arabidopsis (freezing tolerant) or *P. sativum* (freezing sensitive) were mixed with a radiolabeled precursor of galactolipid synthesis (UDP-Gal), and then incubated under conditions in which a single variable mimicked a possible cellular change in response to freezing. SFR2 activity was measured as production of radiolabeled TGDG. Reactive oxygen species can accumulate during many stresses including cold (Suzuki and Mittler, 2006), but addition of water soluble hydrogen peroxide, or lipid-soluble cumene peroxide had little effect on SFR2 activity (Fig. 1E). As lipids approach their transition temperatures, membrane leakage increases (Hays et al., 2001). The vacuole and extracellular space serve as reservoirs of protons, and cytoplasmic pH has been reported to change during cold conditions (Dietz et al., 2001). Low pH values had an activation effect on SFR2 in chloroplast preparations from either Arabidopsis or *P. sativum* (Fig. 1D). Notably, the effects were not identical, consistent with the two species' different response to freezing. The chloroplast stroma is a reservoir of Mg^{2+} ions, previously shown to activate SFR2 *in vitro* (Shaul, 2002, Roston et al., 2014). Increases in the Mg^{2+} concentration specifically affected SFR2 activity (Fig. 1F), and these effects were synergistic with low pH (Fig. 1G). Other cellular cations did not have strong effects when their biologically relevant levels were considered (Fig. 1F). Calcium levels are believed to be nano- or pico-molar (Monshausen et al., 2008), and potassium levels are near 60 mM (Halperin and Lynch, 2003). When 60 mM potassium was included, it did not prevent further activation by Mg^{2+} ions (Fig. 1G).

SFR2 activation by pH and Mg^{2+} is reproducible in whole tissues. To test if the pH and Mg^{2+}-based activation of SFR2 was generalizable to whole tissues, 3-week-old Arabidopsis rosettes or 2-week-old pea leaves grown at normal temperatures were excised and floated for 1 hour on top of various acids, and oligogalactolipid production was measured (Fig. 2). SFR2 activity occurred only when organic acids were

used, presumably because in their protonated form organic acids can carry protons across membranes to affect the cytosolic pH (Plieth et al., 1997). Consistent with this possibility, the membrane-permeable proton carrier 2,4-dinitrophenol had stronger effects at more neutral pHs, while hydrochloric acid had no measurable effect. SFR2 activity was further enhanced in the 2,4-dinitrophenol sample when 10 mM $MgCl_2$ was added (Fig. 2, lane 7M). Together, these data indicate that SFR2 can be activated in isolated organelles and in whole tissue by lowered pH and increased Mg^{2+} concentration.

Cytosolic pH changes in response to freezing and acetic acid treatment. To corroborate the hypothesis of SFR2 activation by pH and Mg^{2+}, physiologically relevant changes in cellular pH were measured during SFR2 activating conditions. This was done using two independent Arabidopsis lines stably expressing a pH-reporting fluorescent protein from sea pen (*Pt*GFP) shown to be located in the cytosol (Schulte et al., 2006, Geilfus et al., 2014). These plants were grown at room temperature, cold acclimated for a week, or cold acclimated and frozen at -6°C overnight, after which ratiometric fluorescence was measured by confocal microscopy (Fig. 3A). Low-temperature treated plants were measured at 4°C immediately after removal from their incubation temperature. A pH decrease was detected between normal and cold-acclimated plants, with a further decrease observable between cold-acclimated and frozen plants (Fig. 3C). The temperature of measurement did not appear to have a large effect on quantification, as ratiometric responses of purified *Pt*GFP buffered at multiple pH values and measured at 4°C and 22°C were nearly identical (Fig. 3B). To compare the level of cytosolic acidification during freezing with that during acid treatment as described in Figure 2, the *Pt*GFP transformed Arabidopsis lines were also treated with 20 mM acetic acid at pH 5.0. Plants were grown at room temperature, floated in acetic acid or water for one hour, and then measured precisely as above (Fig. 3D). After one hour of floatation on acetic acid, pH decreased significantly in both lines. It should be noted that using identical microscope parameters, we were able to measure a slight ratiometric response of wildtype plants not transformed with *Pt*GFP. This parameter was used to mathematically correct estimated pHs for all data.

SFR2 is not substrate limited. In addition to changes in the aqueous boundary layer that may occur in response to freezing and affect SFR2 activity, changes to the membrane itself may cause SFR2 activation. Specifically, it seemed possible that the substrate MGDG is not accessible to SFR2 in the outer chloroplast envelope membrane under normal conditions, but would become accessible following freezing-induced membrane disruption. To test this possibility, oligogalactolipids were quantified under phosphate-limited growth conditions known to induce additional MGDG synthases in the outer envelope membrane, the same sub-organellar location as SFR2 (Kobayashi et al., 2008). Plants were grown for 2 weeks then transferred to medium lacking phosphate for 10 days. If MGDG levels limit SFR2 activity, then oligogalactolipid levels would be expected to increase during phosphate deprivation, because of increased MGDG availability. However, increases in oligogalactolipids were not observed (Fig. 4A). In comparison, a positive control showed production of TGDG after a single hour of flotation on 20 mM acetic acid. Thus, substrate-availability is unlikely to play a major role in increasing SFR2 activity following freezing.

SFR2 does not have stable protein partners. In planta, SFR2 appears to form a complex of approximately 140 kDa, as determined by native gel electrophoresis (Fig. 4B). This complex does not appear to change with the activity level of SFR2, because the size of the complex did not shift in response to SFR2 activation at -6°C (Fig. 4C). If the complex represents stable association between SFR2 and other proteins, the other proteins could provide additional information about the mechanism of temperature sensing. To identify SFR2 interacting proteins, SFR2 antibodies were used to precipitate SFR2 in chloroplasts isolated from wild type or *sfr2* T-DNA insertion lines which lack SFR2 protein. A crosslinker was used to enhance complex stability during the process. Resulting eluates were analyzed by liquid chromatography tandem mass-spectrometry (LC-MS/MS). Proteins identified in the wild-type samples but not the *sfr2* samples in each of three replicates were few (Table 1). A full table of identified

peptides and proteins is provided as supplemental information. The most abundant as judged by numbers of identified spectra were further investigated. These included the Translocon at the outer membrane of chloroplasts, 64 kDa and a protein kinase family protein of unknown function. Arabidopsis insertion lines lacking all paralogues of these genes, *toc64* (Aronsson et al., 2007) and *prot. kin.*(Alonso et al., 2003), were obtained and confirmed to obtain genomic insertions by PCR (Supplemental Fig. S1A). These lines were tested for aberrant SFR2 complex formation and activity. The size of the SFR2-containing complex appeared normal (Fig. 4D), and plants did not have reduced freezing tolerance (Fig. 4E). SFR2 activation also appeared normal, as it could be activated in response to 20 mM acetic acid (Fig. 4E), and was not otherwise active during normal growth (Supplemental Fig. S1B). We concluded that SFR2 does not have stably interacting protein partners, and that the higher molecular weight complex visualized by native gel likely represents a homo-oligomer. Its size is consistent with a dimer.

Treatment with acetic acid mimics a freezing response. To determine whether treatment of tissues with acetic acid (to lower cytosolic pH) or Mg^{2+} mimicked freezing responses, lipid changes were measured in cold-acclimated wildtype and *sfr2* plants that were frozen or treated with 20 mM acetic acid (AcOH) pH 5.0 or acetic acid with 10 mM Mg^{2+} (AcOH+Mg, Fig. 5). To better mimic the cellular condition of cold-acclimated plants prior to freezing, all plants were cold-acclimated whether treated or frozen. Total fatty acid pools, MGDG, TAG, and phosphatidylglycerol (PG), were quantified. PG was included as a representative of a prominent chloroplast lipid without known SFR2-dependent effects. SFR2-dependence was determined by comparing wildtype Arabidopsis changes with those in *sfr2*. Note that acetic acid and acetic acid with Mg^{2+} treatments are expected to replicate the direction rather than the precise magnitude of changes due to freezing.

During freezing, a reduction in the amount of MGDG and a corresponding increase in the amount of TAG were observed in wild type plants as previously described (Moellering et al., 2010). The profile of TAG fatty acids changed in frozen wild-type plants to contain 16:3, a fatty acid contained primarily in MGDG. However, TAG from frozen *sfr2* did not contain significantly more 16:3 after freezing, making this change SFR2-dependent (Fig. 5A, B). In contrast, TAG levels increased in both wild type and *sfr2* plants, in an SFR2-independent change (Fig. 5A,B). Total fatty acid compositions were only slightly changed, MGDG fatty acid composition was unchanged, and changes to PG levels and composition were small (Fig. 5B). This is consistent with previous evidence that the species of fatty acids within MGDG do not change during freezing (Li et al., 2008). Treatment with acetic acid or acetic acid with Mg^{2+} caused increases in TAG levels and decreases in MGDG levels with relatively small changes to PG levels in wild type and *sfr2* (Fig. 5C). Few significant changes were observed in the total fatty acid profile or that of MGDG or PG (Fig. 5D). These patterns mimicked the direction and type of change seen during freezing. Notably, the increases in TAG levels in response to acidification were again independent of the *sfr2* genotype (i.e. occurred in wild type and *sfr2*), indicating that not only is SFR2 activated similarly in response to acetic acid and freezing, but at least one other lipid-remodeling enzyme is similarly activated by cytosol acidification. Fatty acid changes in TAG of wild-type plants included decreases in 16:0 and 18:0 and increases in 16:3 and 18:3, mimicking TAG fatty acid changes due to freezing (Fig. 5D).

To confirm that the increased levels of TAG depend on cytosolic acidification through an independent method, lipid droplets were observed by Nile Red staining of TAG droplets and subsequent confocal microscopy. Quantification of lipid droplets per cell show trends consistent with the total lipid changes observed for TAG (Fig. 5A, C, and E). This again demonstrates that TAG is accumulated and stored in similar ways during freezing and acetic acid or acetic acid with Mg^{2+} treatments.

Discussion

SFR2 catalyzes a lipid headgroup transfer reaction which is critical to plant survival of freezing. Because the protein is present in all chloroplasts under all conditions (Fig. 1), it must be activated in a non-transcriptional manner. How plants sense temperature or freezing conditions, is unknown. Here, we explored a molecular freezing sensing mechanism at the level of SFR2 activation. To understand the regulation of SFR2 activity through a post-translational mechanism requires understanding physical changes under freezing conditions inside the cell. We have shown that acidification causes SFR2 activation, and this activation is heightened by addition of Mg^{2+} in either isolated chloroplasts or whole shoot tissues (Figs. 1 and 2). In fact, a decrease in cytosolic pH is apparent during both cold and freezing, to the extent consistent with activating SFR2 (Fig. 3). It is likely that this activation occurs through a direct mechanism, as stable interactions of SFR2 with other proteins were not detected, and SFR2 is not substrate limited (Fig. 4). Using whole-tissue assays, changes to the levels and fatty acid profiles of MGDG, TAG, and lipid droplet formation seen during freezing could be mimicked by pH changes. Together, these data provide evidence that pH changes provide a critical link to activation of SFR2, and this finding can be taken as paradigm for a molecular mechanism by which plants sense freezing within cells.

Interestingly, SFR2 response to pH is not due to direct pH manipulation of its glycosyltransferase activity. Yeast-produced SFR2 has a pH optimum of ~ 7.5, though it responds similarly to magnesium ions (Roston et al., 2014). Thus, the need for Mg^{2+} can be directly attributed to a requirement for catalysis, while the pH change required for activation *in situ* cannot. SFR2 activation by decreased pH does not coincide with the previously observed pH optimum of 7.5 *in vitro* for SFR2 (Roston et al., 2014). Hence proper sensing of freezing by SFR2 must require it being in its natural local environment, within the outer envelope membrane. It is possible that pH changes affect the properties of the membrane or its constituents, and thus affect SFR2.

We did not identify stable protein partners that interact with SFR2 by immunoprecipitation using SFR2-specific antisera in wild-type plants (Fig. 4, Supplemental Table S1). Because SFR2 produced heterologously in yeast is always active (Roston et al., 2014), we consider it likely that transient protein interactions or post-translational modifications play a role in SFR2 activation. Recently, an association of SFR2 with Open Stomata1 (OST1) was reported using tagged OST1 overproduced under control of the ubiquitin promoter (Waadt et al., 2015). We did not detect OST1 as even a minor component in any of our immunoprecipitations, and it should be noted that SFR2 interaction with OST1 was only reported after abscisic acid (ABA) treatment (Waadt et al., 2015). ABA levels are known to increase in response to chilling (Mantyla et al., 1995), and OST1 is active during cold-acclimation in Arabidopsis (Ding et al., 2015). Thus, it is unlikely that OST1 is directly involved in the response of SFR2 to below-freezing conditions. However, we cannot rule out activation of SFR2 by other mechanisms in addition to those described here.

Specifically, production of oligogalactolipids independent of freezing conditions has been observed in distinct genetic backgrounds or conditions. The trigalactolipid (TGD) genes were named for the constitutive production of TGDG in their Arabidopsis mutants (Hurlock et al., 2014). The TGD proteins have been shown to enhance transport of lipids from the endoplasmic reticulum to the chloroplast, and the respective *tgd* mutants have altered outer envelope membrane compositions which could contribute to SFR2 activation. Additionally, oligogalactolipid production appears to increase in response to oxidative stress, including ozone fumigation (Sakaki et al., 1990). It is clear that SFR2 does not respond directly to oxidative stress (Fig. 1), but it is unclear if ozone fumigation affects SFR2 activity through changes in pH, Mg^{2+}, or additional factors.

Activation of SFR2 by pH and Mg^{2+} is relevant to freezing because they likely represent the sensing of membrane damage. Membrane leakage increases when membranes approach phase transition temperatures of their lipid constituents (Hays et al., 2001), which has been measured in *P. sativum*

chloroplasts to begin at 10°C and continue until -10°C (Leheny and Theg, 1994). Further, membrane damage increases after freezing as cellular dehydration contracts the cell and osmotic potential increases (Steponkus, 1984). The vacuole and extracellular spaces of plant cells are highly acidic, while the chloroplast stroma has a high Mg^{2+} ion concentration, which increases during the day up to 10 mM. As the cells chill and the membranes become partially damaged, leakage of small ions including protons and Mg^{2+} could provide a convenient mechanism for rapidly activating membrane protective machinery beginning with the activation of SFR2 at the outer chloroplast envelope membrane. Consistent with this hypothesis, wounding by a crushing force was observed to cause SFR2 activity (Vu et al., 2015, Vu et al., 2014b). Wounded tissue allows cytoplasmic mixing with acidic apoplastic fluid, and possibly through this simple mechanism, SFR2 activation. Similarly, SFR2 is activated during isolation of intact chloroplasts (Heemskerk et al., 1983). Both wounding and chloroplast isolation provide stresses that are independent of cold acclimation. During either, tissues are broken and multiple forces act on the isolated chloroplasts in ways which may mimic membrane environments during freezing.

The majority of cold and freezing tolerance studies have identified transcriptionally controlled genes (Fowler and Thomashow, 2002). SFR2 mRNA levels show little or no response to low temperature (Thorlby et al., 2004), though enzymatic activity increases dramatically below freezing (Fig. 1). The pH changes which activate SFR2 also appear to activate SFR2-independent changes to TAG and MGDG (Fig. 5), which mimic those which occur during freezing (Fig. 5). Changes to cytosolic pH are unlikely to be the only changes that act as signals during freezing, but they appear to play an important role in chloroplast membrane lipid remodeling.

Conclusion

Freezing tolerance is a necessary resilience mechanism for plants native to temperate climates. Unlike many proteins required for cold or freezing tolerance, ubiquitous SFR2 is not increased in abundance, but activated to combat freezing stress. This provides plants with a rapid response mechanism during fluctuating weather conditions, which are more frequently encountered as global weather patterns become more unstable and extreme. Here we showed that SFR2 is activated by cytosolic pH and ionic changes, and that these changes can mimic other plant responses to freezing. Specifically, SFR2 activation by relatively moderate pH and ionic changes are supported at the organelle and whole tissue levels in two species, while pH changes are observed to occur by pH-sensitive GFP responses during freezing of whole Arabidopsis plants. Tissue-level activation of cold-adapted Arabidopsis by pH or pH and Mg^{2+} is observed to promote freezing-like lipid changes. We conclude that cytoplasmic acidification is a molecular mechanism through which freezing conditions are communicated throughout the plant cell.

Materials and Methods

Plant material Wild-type *A. thaliana* was of the Columbia ecotype. The Arabidopsis Biological Resource Center supplied a T-DNA insertion in At3g06510, herein referred to as the *sfr2* mutant, also published as *sfr2-3*, SALK_106253 (Moellering et al., 2010) and the protein kinase At4g32250 with a T-DNA inserted in the last exon of the gene, SALK_051823 (Alonso et al., 2003). Toc64 has three homologs in Arabidopsis with possible functional redundancy. Arabidopsis with insertions causing loss of all three Toc64 full-length transcripts was kindly donated by Dr. Paul Jarvis and Sean Maguire. Presence of transgenes was confirmed using primers given in (Aronsson et al., 2007, Moellering et al., 2010) or for *prot. kin.*, 5'- AGAACATGGATGTGCCAGAAG-3', 5'- CGCTGCATATACCATGTGATG -3', and T-DNA specific primer LB3.1 (Salk institute).

Plant growth Seeds were sterilely planted on Murashige-skoog medium (Caisson Laboratories, Inc.) containing 1% sucrose and 0.5% MES, pH 5.7 solidified with 6% AgarGel (Sigma). Seeds are exposed to 4°C for 2 days in the dark and then grown in 16 h day, 8 h night conditions at a constant 22°C. Plants used to test phosphate-stress activation of SFR2 were transferred to another plate of the media described

above, or similarly prepared media lacking phosphate 10 days after germination (Caisson Laboratories, Inc). Whole shoot tissues were sampled after 11 days of growth on the new medium.

Freeze testing. All freeze tested plants were cold acclimated for 1 week at 6°C prior to freezing. During cold acclimation, they were on a 12 hour day/12 hour night cycle. Freeze tolerance tests were performed as previously (Moellering et al., 2010) with the following exceptions: All freeze testing was performed at the end of the day / beginning of the night cycle. After ice nucleation at -2°C, temperatures were lowered within one hour to the reported freezing temperature. Post-freezing recovery was performed at 22°C under bench light for 3 days before return to normal growth conditions.

SFR2 assays in isolated chloroplasts Arabidopsis wild type was grown for 3 to 4 weeks on medium as described above, or *Pisum sativum* (garden pea) variety "Little Marvel" was grown for approximately 2 weeks. The plants were not cold acclimated. All shoot tissue was harvested, and chloroplasts were isolated essentially as described previously (Bruce et al., 1994). 100 µg of chlorophyll equivalent chloroplasts were pelleted and resuspended in 98 µl of buffer. The buffer content varied by experiment, but included 44 mM Hepes at pH 7.5 unless specified, 300 mM sorbitol or as specified, 0.5 mM glycerol-3-phosphate, 0.3 mM monobasic potassium phosphate, 0.2 mM Coenzyme A, and 4 mM magnesium chloride unless specified otherwise. As indicated in the text, specific experiments included one or more of the following: 0.1 – 10 mM hydrogen peroxide, 0.1 – 1 mM cumene hydroperoxide, 0 to 10 mM total magnesium chloride, 8 – 60 mM potassium chloride, 4 mM calcium chloride, pH of 6.8 to 8.3. Immediately after resuspension, 2 µl of 0.1 mCi/ml Uridine 5'-diphosphate galactose [14C] (American Radiolabeled Chemicals) was added and mixed by gentle agitation. The chloroplasts were allowed to react for 30 minutes at room temperature in low bench-top lighting. Following incubation, intact chloroplasts were re-isolated on top of a 35% Percoll (Sigma), 330 mM sorbitol, 50 mM Hepes pH 7.5 cushion, washed once in buffered sorbitol without Percoll, then extracted with 200 µl of methanol:chloroform (2:1, v/v). Because of the variance in recovery of intact chloroplasts from many of the experimental conditions, levels were equalized using chlorophyll fluorescence prior to loading onto a silica gel 60 plate (Merck) and separating as described above. MGDG, DGDG, and TGDG bands were identified by comparison to standards purchased or generated using SFR2 expressed in yeast (Roston et al., 2014). Radioactivity in the bands was quantified by scintillation counting. Presented data express the level of radioactivity in TGDG as a percentage of all radioactivity in the sum of MGDG, DGDG, and TGDG as a method to rule out control of MGDG synthesis, a prerequisite to TGDG radioactivity caused by SFR2.

SFR2 assay in whole tissue 20 mM of hydrochloric acid, acetic acid, propionic acid, butyric acid or 2,4-dinitrophenol were adjusted to pH 4, 5, 6, or 7 $\pm$ 0.01 with dibasic potassium phosphate. 2,4-Dinitrophenol was not pHed to 4 because when dissolved it was already too basic (pKa = 4.09). As indicated, 10 mM magnesium chloride was added. 5 ml of each solution was used to float either whole, plate-grown Arabidopsis rosettes or 2 fully expanded *P. sativum* leaves. The plants were grown under normal conditions (see above), and were not cold acclimated. The thick waxy cuticle of *P. sativum* leaves was bypassed by cutting 5 slits across the epidermis of each pea leaf with a fine razor blade. Plants were incubated at room temperature for 1 hour, then gently patted dry and analyzed for lipid content.

Lipid Analysis Plant tissue as described in the texts were extracted with a modified Bligh and Dyer protocol to isolate lipids, as described (Wang and Benning, 2011). Sampling of frozen plants was done carefully, to minimize thawing. Frozen plants were protected from thawing during sampling by harvesting with chilled forceps into pre-chilled tubes and immediately immersing in liquid nitrogen. Comparisons between direct extraction of whole leaf samples by vigorous shaking and extraction of tissues crushed in liquid N_2 did not show noticeable changes in oligogalactolipid levels, therefore whole leaf extraction was primarily used. Thin-layer chromatography analysis of oligogalactolipids was performed on silica gel 60

TLC plates (Millipore) in resolving solvent composed of chloroform:methanol:acetic acid:water (85:20:10:4, v/v/v/v). Thin-layer chromatography isolation of lipids prior to quantification by gas chromatography was performed on silica G plates with a preadsorbent zone (SiliCycle) plates in resolving solvent composed of acetone:toluene:water:acetic acid (91:30:7:2, v/v/v/v), dried and additionally separated in petroleum ether:diethyl ether:acetic acid (80:20:1, v/v/v). Otherwise, TLCs were performed as described (Wang and Benning, 2011). Silica retaining separated lipids was scraped from the plates, pentadecanoic acid was added as a standard, all lipids were derivatized to fatty acid methyl esters, and quantified by gas chromatography coupled to a flame ionization detector. Derivatization and gas chromatography were essentially as described, however hydrogen was used as the carrier gas for a 30 m capillary HP-Innowax column (Agilent) set at 90°C for 1 min, ramped at 30°C per min to 235°C and held for 5 min. Statistical analysis was by the student's t-test.

Protein analysis Blue-native PAGE was done essentially as described previously (Kikuchi et al., 2006). Samples were prepared by extracting leaf tissue by homogenization on ice in ice-cold native sample buffer containing 2% (w/v) digitonin. Particulates were removed by centrifugation at 21,000 x g for 10 min at 4°C. 1% dodecylmaltoside, decylmaltoside, and Triton X-100 were also screened but did not resolve a single complex species. Immunoblotting using the SFR2 antisera was as described (Roston et al., 2014). For immunoprecipitation experiments, freshly desalted, 400 µl of SFR2N and SFR2C antisera mixed in a 1:1 ratio were coupled to AminoLinkPlus coupling resin (ThermoScientific, Pierce). The resulting resin was split into two microcolumns. Identical amounts of chloroplasts freshly prepared from wild-type or *sfr2* plants as described above were crosslinked by incubation with 10 mM dithiobis(succinimidyl propionate) for 2 minutes at room temperature and 20 minutes on ice. 50 mM (final concentration) Tris-HCl at pH 7.5 was used to quench the cross-linker reaction by incubation at room temperature for 15 min. Crosslinked chloroplasts were precipitated and resuspended to 2 mg chlorophyll/ml in 50 mM Hepes pH 7.4, 150 mM sodium chloride, 1% dodecylmaltoside, and complete protease inhibitor without EDTA (Roche). After 30 min, insoluble material was precipitated by ultracentrifugation at 100,000 x g for 10 min at 4°C. This was used as the starting material for immunoprecipitation which was performed essentially as per AminoLinkPlus instructions. Binding to resin occurred overnight at 4°C in the dark, the column was washed with 80 column volumes of chloroplast solubilization buffer containing 0.1% dodecylmaltoside, then eluted with 90°C non-reducing, SDS-PAGE loading buffer. Mass spectrometry was essentially as described (Roston et al., 2012), except peptides were re-suspended in 2% acetonitrile/0.1% TFA to 25uL. From this, 5uL was automatically injected by a Thermo EASYnLC 1000 onto a Thermo Acclaim PepMap RSLC 0.075mm x 150mm C18 column and eluted over 60min with a gradient of 2% B to 30% B in 49min, ramping to 100%B at 50min and held at 100%B for the duration of the run (Buffer A = 99.9% Water/0.1% Formic Acid, Buffer B = 99.9% Acetonitrile/0.1% Formic Acid) at a constant flow rate of 0.3 nL/min. Eluted peptides were sprayed into a ThermoFisher Q-Exactive mass spectrometer using a FlexSpray spray ion source. Survey scans were taken in the Orbi trap (35000 resolution, determined at m/z 200) and the top ten ions in each survey scan are then subjected to automatic higher energy collision induced dissociation (HCD) with fragment spectra acquired at 17,500 resolution.

Peptide and Protein Identification Tandem mass spectra without charge state deconvolution or deisotoping were extracted by Mascot Distiller version v2.4, and analyzed with Mascot version 2.5.0 and X! Tandem version CYCLONE (2010.12.01.1). Both Mascot and X! Tandem were set up to search the version 10 TAIR database supplemented with common contaminants of the cRAP3 database assuming trypsin digestion. Searches had a fragment ion mass tolerance of 0.30 Da and a parent ion tolerance of 10.0 ppm. Allowed fixed modifications included only carbamidomethyl of cysteine. Allowed variable modifications were deamidated asparagine and glutamine, oxidized methionine and thioacylated lysine. X! Tandem variable modifications additionally included N-terminal pyro-Glutamate and N-terminal ammonia-loss. Scaffold version 4.4.8 was used to validate peptide and protein identifications. Peptide

identifications were accepted if they could be established at greater than 7.0% probability to achieve a false discovery rate (FDR) of less than 0.1%. Peptide Probabilities from X! Tandem were assigned by the Peptide Prophet algorithm (Keller et al., 2002) with Scaffold delta-mass correction. Peptide Probabilities from Mascot were assigned by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 97.0% probability to achieve an FDR less than 1.0% and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins were grouped to satisfy the principles of parsimony if they contained similar peptides and could not be differentiated. If significant peptide evidence was shared, proteins were grouped into clusters.

***Pt*GFP measurement of cytosolic pH** Use of cytosolic *Pt*GFP to measure cytosolic pH was essentially as described (Schulte et al., 2006, Geilfus et al., 2014), with the following exceptions: excitation was with a blue diode laser at 405 nm or an Argon gas laser at 488 nm with emission recorded from 505 to 530 nm. Measurement of tissue was done in unbuffered water for all untreated samples on standard microscope slides, and in 20 mM acetic acid, pH 5.0 for the acetic acid treated samples. Measurement of purified *Pt*GFP donated by Christoph Plieth was done in 0.5 M buffers used within their pH range in microslides, precision rectangular capillaries (Vitro Dynamics Inc.). This study was duplicated at Michigan State University and the University of Nebraska Lincoln. A Zeiss 10 meta ConfoCor 3 Confocal microscope fitted with a PE100-ZAL cooling stage (Linkham Scientific Instruments) at the Michigan State University Center for Advanced Microscopy was used for *Pt*GFP imaging. A similar process to that described was used at the University of Nebraska-Lincoln Morrison Microscopy Core, microscope description in the following section. Image J software with the FIJI plugin package was used for processing raw data (Schindelin et al., 2012). For testing pH changes during cold treatments, cold-acclimated and frozen plants were processed as follows: A temporary incubator made of a thick-walled Styrofoam container filled with ice was kept at the same temperature as the plants during the overnight temperature treatment. The plants were kept in these temporary incubators enroute to the microscope. Individual plants were removed from the container as quickly as possible, placed on a pre-chilled slide, and then onto the cooling stage at 4°C. A maximum of two images were taken within a minute of placing the plant onto the slide. All images were collected within 1 hour. Plants used to measure cytosolic pH during cold treatment were 4 weeks old and grown at 22°C; 22°C for 3 weeks and 6°C for 1 week; or 22°C for 3 weeks, 6°C for 1 week, and -6°C overnight. Plants used to measure cytosolic pH during acetic acid treatment were 3 weeks old and were not cold acclimated.

Lipid Droplet Quantification Wild-type and *sfr2* plants were grown for three weeks at 22°C and then cold acclimated precisely as described for freeze-treatments. Plants were then subsequently frozen at -6°C overnight or floated on 20mM acetic acid pH5, 20mM acetic acid pH 5 with 10 mM magnesium chloride or water for three hours. After treating, leaves were removed from the rosette and cut into slices for all treatments except freezing, which were left whole. The leaf sections were soaked in 0.1mg/mL Nile Red stain with 8% DMSO for one hour on ice. Leaf sections were then rinsed with deionized water three times before transport to the University of Nebraska-Lincoln Morrison Microscopy Core in deionized water. Measurement of tissue was done in deionized water for all samples on standard microscope slides. Images were taken on a Nikon Eclipse 90i upright fluorescence microscope with excitation at 561.4 and emission from 570-620 nm for Nile Red stain and with excitation at 640.6 nm and emission from 663-738 nm for chloroplast autofluorescence. Images were acquired sequentially and with a Z-step of 1µm. Image J software with the FIJI plugin package was used for processing raw data. Cells were manually cropped by their dimensions and converted into two-dimensional images using Z projections of maximum intensity. Droplets were then hand counted on a per cell basis. Hand counts were statistically analyzed by ANOVA PROC GLIMMIX analysis using SAS Version 9.4 (SAS Institute Inc). Assumptions were satisfied using a Gaussian response distribution with the response variable recorded as the per cell number of lipid droplets. A completely randomized experimental design was implemented, with treatments considered as fixed effects.

Accession Numbers

The following genes referred to in the text are listed with their accession numbers. *SFR2*, At3g06510, *PROTEIN KINASE*, At4g32250, *TOC64* has three homologs *TOC64-I*, At1g08980, *TOC64-III*, At3g17970, and *TOC64-V*, At5g09420.

Tables

Table 1. Proteins identified as potential interactors of SFR2.

Protein Names	Accession No.	*sfr2*[1]	WT[1]	*sfr2*[2]	WT[2]	*sfr2*[3]	WT[3]
SFR2, 71 kDa	AT3G06510	0	49	0	55	0	55
Translocon at the outer membrane of chloroplasts, 64 kDa	AT3G17970	0	11	0	10	0	9
Protein kinase superfamily protein, 68 kDa	AT4G32250	0	5	0	7	0	5
Outer Membrane Protein of 24 kDa	AT3G52230	0	4	0	3	0	2
Translocon at the inner envelope membrane of chloroplasts 214 kDa	ATCG01130	0	8	0	8	0	1
Translocon at the outer membrane of chloroplasts, 132 kDa	AT2G16640	0	5	0	4	0	1
ABC-2 type transporter family protein, 79 kDa	AT2G01320	0	6	0	2	0	1
Plastid division 2, 34 kDa	AT2G16070	0	2	0	3	0	1
Dephospho-CoA kinase family protein, 26 kDa	AT2G27490	0	3	0	2	0	1

1-3. Numbers of spectra associated with each protein in each sample from the first, second, or third biological replicates are given. Peptide identifications were accepted to achieve a false discovery rate of less than 0.1%. Protein identifications were accepted to achieve a false discovery rate of less than 1.0%

Figure Legends

Fig. 1. SFR2 is post-translationally activated by pH and Mg ions. Wild-type Arabidopsis were cold-acclimated at 6°C for 1 week, incubated overnight at temperatures indicated above, and then sampled for lipids and proteins. A) Immunoblot detecting SFR2 protein levels. B) Thin-layer chromatogram separating lipids identified at right visualized with a sugar-specific stain. Images shown are representative of three separate plant growth trials. (C-G) Isolated chloroplasts were incubated with radiolabeled UDP-Galactose in 300mM Sorbitol, 50 mM Hepes pH 7.5, or modified buffers as indicated below the graph axis. Radiolabel in oligogalactolipid product TGDG is quantified as percent of total radiolabeled lipids. Error bars represent standard deviation of at least three separately grown trials. An asterisk represents significance (p less than or equal to 0.05) between the treatment and the condition most closely mimicking normal cytoplasm (300 mM Sorbitol, pH 7.4, 0 mM H_2O_2, 0 mM Cumene hydroperoxide, no divalent cations (F), or 0.4 mM $MgCl_2$ (G)).

Fig. 2. pH and Mg changes activate SFR in whole tissues. Thin layer chromatogram separating lipids from extracts of Arabidopsis (*A.t.*) shoots or pea (*P.s.*) leaves floated on 20 mM of the acid indicted at left adjusted to the pH indicated above with dipotassium phosphate for 1 hour. 7 M indicates pH 7 with additional 20 mM $MgCl_2$. TGDG is indicated by an arrowhead. Images shown are representative of three separate plant growth trials.

Fig. 3. Cytosolic pH changes during freezing and acetic acid treatment. Arabidopsis plants stably transformed with *Pt*GFP were grown under control conditions (22°C), or grown and cold acclimated at 6°C for 1 week, or cold acclimated and frozen overnight at -6°C. A cold stage (4°C) was used to measure chilled plants. Ratiometric fluorescence was measured in hypocotyls, with excitation at 488 nm divided

by excitation at 405 nm with detection constantly between 505-530 nm. Scale bar = 22 μm (B) Pure *Pt*GFP protein was measured identically to (A) in microcapillaries at 22°C or on the cold stage (4°C) to provide a pH scale. (C) Ratiometric fluorescence images of two independent lines of *Pt*GFP including those shown in (A) were transformed into pH as described in the methods and are graphed according to most recently exposed temperature. Statistical significance values are as follows: 22°C vs 6°C (all samples) $p = 0.0325$, 6°C vs -6°C (all samples) $p = 5 \times 10^{-8}$. Line 1 individually: 22°C vs 6°C $p = 0.215$, 6°C vs -6°C $p = 0.0006$. Line 2 individually: 22°C vs 6°C $p = 0.0661$, 6°C vs -6°C $p = 9 \times 10^{-8}$. (D) The same two independent lines of *Pt*GFP used in (C) and (A) were untreated or floated on water or 20 mM acetic acid at pH 5.0 for 1 hour, mimicking treatments in Figure 2. Statistical significance values are as follows: acetic acid vs water (all samples) $p = 1.21 \times 10^{-16}$, acetic acid vs untreated (all samples) $p = 1.3 \times 10^{-24}$, water vs untreated (all samples) $p = 1.09 \times 10^{-9}$. Line 1 individually: acetic acid vs water $p = 0.0052$, acetic acid vs untreated $p = 2.5 \times 10^{-10}$, water vs untreated $p = 4.44 \times 10^{-10}$. Line 2 individually: acetic acid vs water $p = 5.22 \times 10^{-19}$, acetic acid vs untreated $p = 2.54 \times 10^{-16}$, water vs untreated $p = 0.0023$.

Fig. 4. SFR2 is not substrate limited and does not stably interact with other proteins. (A) 10 day old wild-type or *sfr2* Arabidopsis were transferred to regular medium or medium lacking phosphate for 10 days, and then lipids were extracted. Resulting lipids were analyzed by thin-layer chromatography for presence of TGDG (arrowhead). The location of DGDG is indicated by a white arrowhead. (B) Immunoblot of 40 μg of chlorophyll equivalent wild-type (top) or *sfr2* chloroplasts solubilized with 2% digitonin separated in 2D, 4-14 % blue native PAGE in the first dimension, 7.5 % denaturing PAGE in the second dimension, detected with the SFR2 antiserum. An arrowhead indicates SFR2-specific signal while asterisks identify non-specific signal. (C) Comparisons of SFR2 leaf protein 2D immunoblots of plants grown at 22°C, cold acclimated for one week (6°C), or cold acclimated and frozen overnight at -6°C. (D) Comparisons of SFR2 2D immunoblots as in B for mutants and controls identified at left. (E) Wildtype or mutant Arabidopsis as indicated above were tested for the ability to produce TGDG (arrowhead) in response to 1 hour incubation in 20 mM acetic acid, pH 5, or to withstand freezing at -6°C (lower panel). All portions of the figure are representative of at least 3 separately grown biological replicates.

Fig. 5. pH and Mg^{2+} treatments mimic lipid changes due to freezing

Plants were grown at 22°C for three weeks and cold acclimated at 6°C for one week for all treatments (cold). They were subsequently frozen at -6°C overnight (frozen), or floated on 20 mM acetic acid pH 5 (AcOH), 20 mM acetic acid pH 5 with 10 mM magnesium chloride (AcOH + Mg) or water for three hours. All plants were sampled as rosettes with roots removed. Molar percentage (A, C) of monogalactosyldiacylglycerol (MGDG), phosphatidylglycerol (PG), and triacylglycerol (TAG) relative to total lipid amount and fatty acid profiles of each lipid species relative to total fatty acids for each individual fatty acid were quantified (B, D). Values are biological replicate means ±SD. Each biological replicate consists of an average of 3 or 4 technical replicates. Lipid droplets were visualized with confocal microscopy after Nile Red staining, and quantified as the number of lipid droplets per cell (E). The box encompasses the interquartile range, with the central line representing the median. Whiskers represent maximum and minimum counts, respectively. For all data, significance (p less than or equal to 0.05) between control and treatment is represented by a double dagger and an asterisk represents significance (p less than or equal to 0.05) between wild type and *sfr2*.

Supplemental Materials

Figure S1. Confirmation of *toc64* and *protein kinase* disruption lines and test of SFR2 activity during normal growth.

Table S1. Proteins and peptides identified by SFR2 immunoprecipitation. Table S1A includes all identified proteins broken down per experiment. Table S1B and S1C provide the minimum information about proteomics experiments for each protein and peptide identification, respectively.

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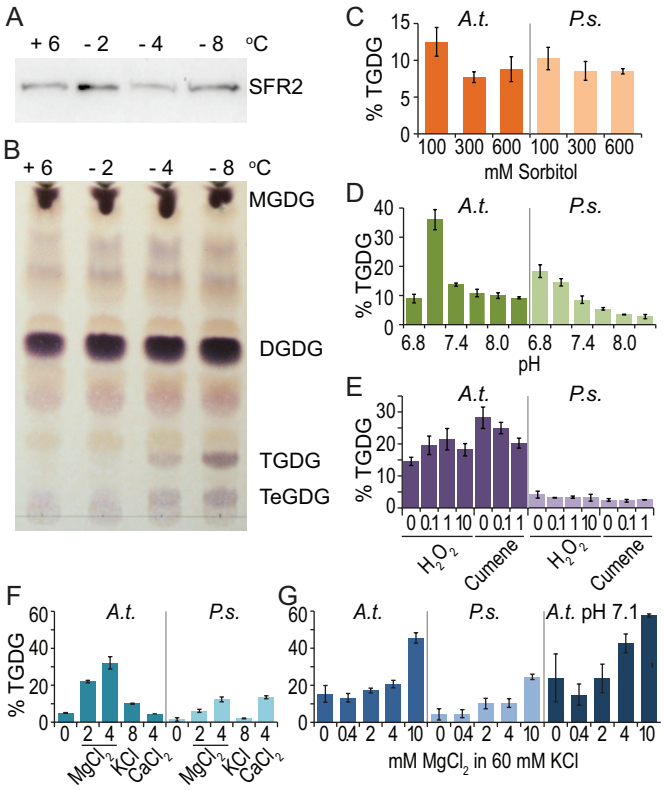

Figure 1. SFR2 is post-translationally activated by pH and Mg ions. Wild-type Arabidopsis were cold-acclimated at 6°C for 1 week, incubated overnight at temperatures indicated above, and then sampled for lipids and proteins. A) Immunoblot detecting SFR2 protein levels. B) Thin-layer chromatogram separating lipids identified at right visualized with a sugar-specific stain. Images shown are representative of three separate plant growth trials. (C-G) Isolated chloroplasts were incubated with radiolabeled UDP-Galactose in 300mM Sorbitol, 50 mM Hepes pH 7.5, or modified buffers as indicated below the graph axis. Radiolabel in oligogalactolipid product TGDG is quantified as percent of total radiolabeled lipids. Error bars represent standard deviation of at least three separately grown trials.

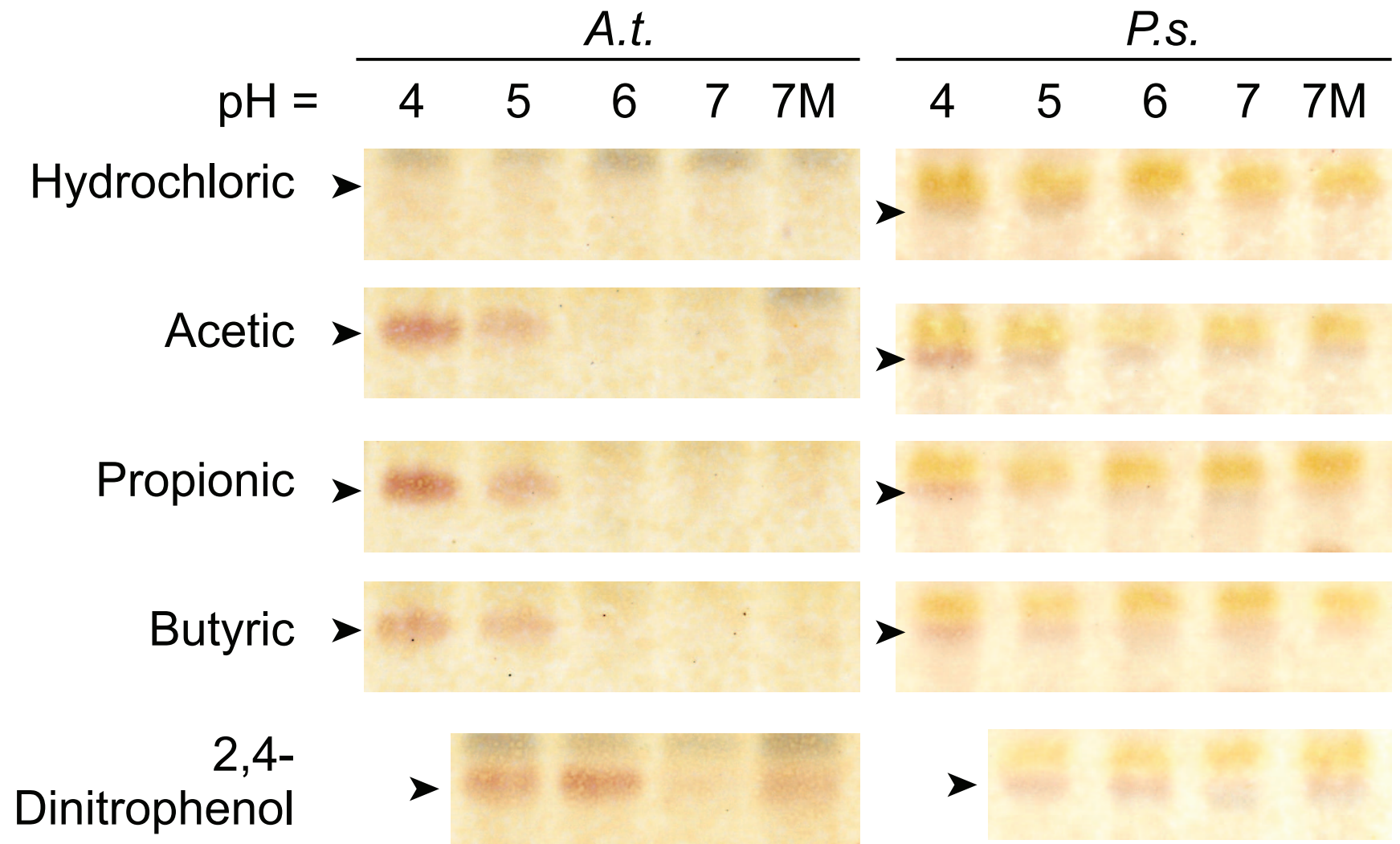

Figure 2. pH and Mg changes activate SFR in whole tissues. Thin layer chromatogram separating lipids from extracts of Arabidopsis (A.t.) shoots or pea (P.s.) leaves floated on 20 mM of the acid indicted at left adjusted to the pH indicated above with dipotassium phosphate for 1 hour. 7 M indicates pH 7 with additional 20 mM MgCl2. TGDG is indicated by an arrowhead. Images shown are representative of three separate plant growth trials.

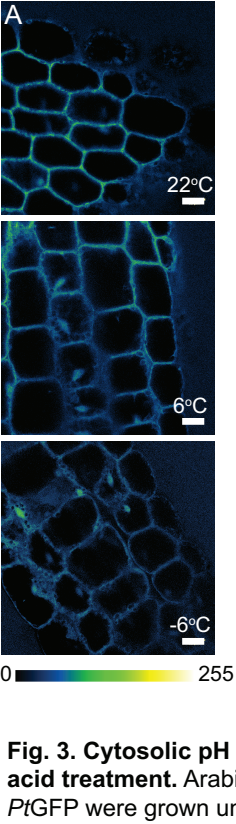

Fig. 3. Cytosolic pH changes during freezing and acetic acid treatment. Arabidopsis plants stably transformed with *Pt*GFP were grown under control conditions (22°C), or grown and cold acclimated at 6°C for 1 week, or cold acclimated and frozen overnight at -6°C. A cold stage (4°C) was used to measure chilled plants. Ratiometric fluorescence was measured in hypocotyls, with excitation at 488 nm divided by excitation at 405 nm with detection constantly between 505-530 nm. Scale bar = 22 µm (B) Pure PtGFP protein was measured identically to (A) in microcapillaries at 22°C or on the cold stage (4°C) to provide a pH scale. (C) Ratiometric fluorescence images of two independent lines of *Pt*GFP including those shown in (A) were transformed into pH as described in the methods and are graphed according to most recently exposed temperature. Statistical significance values are as follows: 22°C vs 6°C (all samples) p = 0.0325, 6°C vs -6°C (all samples) p = 5×10^{-8}. Line 1 individually: 22°C vs 6°C p = 0.215, 6°C vs -6°C p = 0.0006. Line 2 individually: 22°C vs 6°C p = 0.0661, 6°C vs -6°C p = 9×10^{-8}. (D) The same two independent lines of *Pt*GFP used in (C) and (A) were untreated or floated on water or 20 mM acetic acid at pH 5.0 for 1 hour, mimicking treatments in Figure 2. Statistical significance values are as follows: acetic acid vs water (all samples) p = 1.21×10^{-16}, acetic acid vs untreated (all samples) p = 1.3×10^{-24}, water vs untreated (all samples) p = 1.09×10^{-9}. Line 1 individually: acetic acid vs water p = 0.0052, acetic acid vs untreated p = 2.5×10^{-10}, water vs untreated p = 4.44×10^{-10}. Line 2 individually: acetic acid vs water p = 5.22×10^{-19}, acetic acid vs untreated p = 2.54 x 10-16, water vs untreated p = 0.0023.

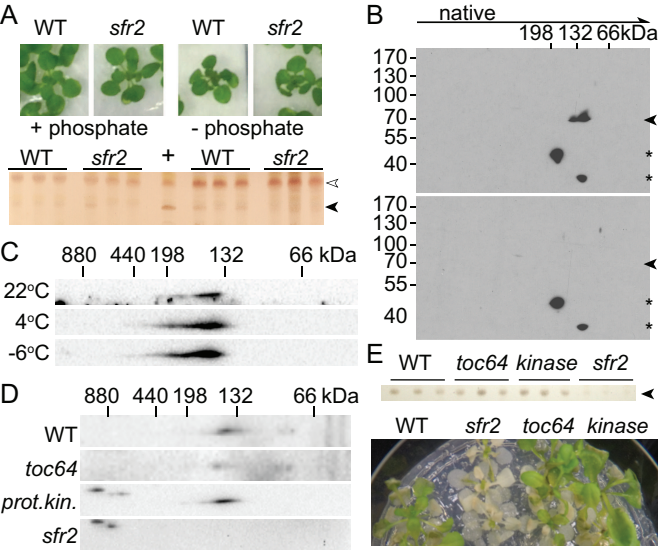

Figure 4. SFR2 is not substrate limited, may have transient interactions

Fig. 4. SFR2 is not substrate limited and does not stably interact with other proteins. (A) 10 day old wild-type or *sfr2* Arabidopsis were transferred to regular medium or medium lacking phosphate for 10 days, and then lipids were extracted. Resulting lipids were analyzed by thin-layer chromatography for presence of TGDG (arrowhead). The location of DGDG is indicated by a white arrowhead. (B) Immunoblot of 40 µg of chlorophyll equivalent wild-type (top) or *sfr2* chloroplasts solubilized with 2% digitonin separated in 2D, 4-14 % blue native PAGE in the first dimension, 7.5 % denaturing PAGE in the second dimension, detected with the SFR2 antiserum. An arrowhead indicates SFR2-specific signal while asterisks identify non-specific signal. (C) Comparisons of SFR2 leaf protein 2D immunoblots of plants grown at 22ºC, cold acclimated for one week (6ºC), or cold acclimated and frozen overnight at -6ºC. (D) Comparisons of SFR2 2D immunoblots as in B for mutants and controls identified at left. (E) Wildtype or mutant Arabidopsis as indicated above were tested for the ability to produce TGDG (arrowhead) in response to 1 hour incubation in 20 mM acetic acid, pH 5, or to withstand freezing at -6ºC (lower panel). All portions of the figure are representative of at least 3 separately grown biological replicates.

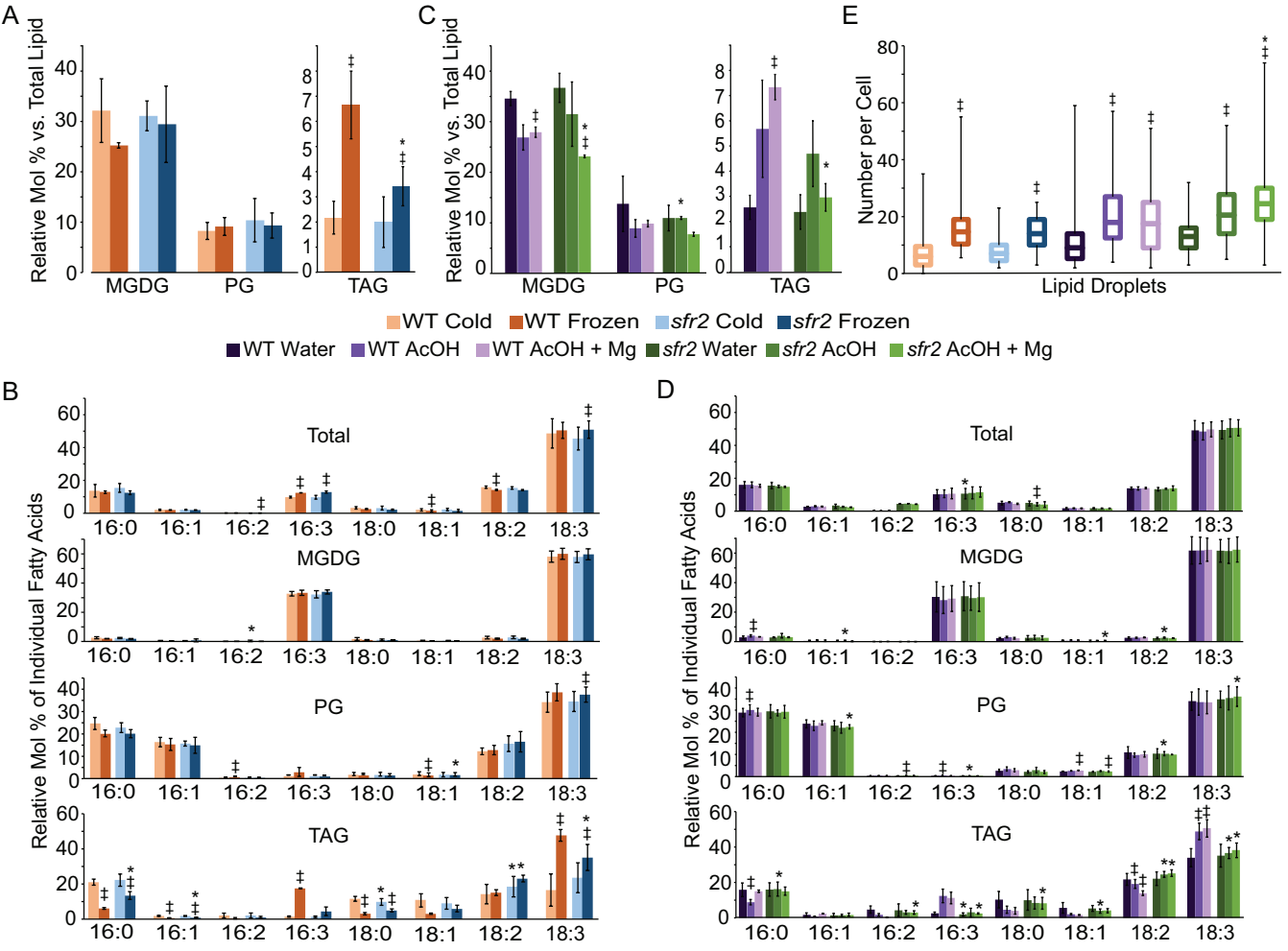

Figure 5. pH and Mg2+ treatments mimic lipid changes due to freezing. Plants were grown at 22°C for three weeks and cold acclimated at 6°C for one week for all treatments (cold). They were subsequently frozen at -6°C overnight (frozen), or floated on 20 mM acetic acid pH 5 (AcOH), 20 mM acetic acid pH 5 with 10 mM magnesium chloride (AcOH + Mg) or water for three hours. All plants were sampled as rosettes with roots removed. Molar percentage (A, C) of monogalactosyldiacylglycerol (MGDG), phosphatidylglycerol (PG), and triacylglycerol (TAG) relative to total lipid amount and fatty acid profiles of each lipid species relative to total fatty acids for each individual fatty acid were quantified (B, D). Values are biological replicate means ±SD. Each biological replicate consists of an average of 3 or 4 technical replicates. Lipid droplets were visualized with confocal microscopy after Nile Red staining, and quantified as the number of lipid droplets per cell (E). The box encompasses the interquartile range, with the central line representing the median. Whiskers represent maximum and minimum counts, respectively. For all data, significance (p less than or equal to 0.05) between control and treatment is represented by a double dagger and an asterisk represents significance (p less than or equal to 0.05) between wild type and *sfr2*.

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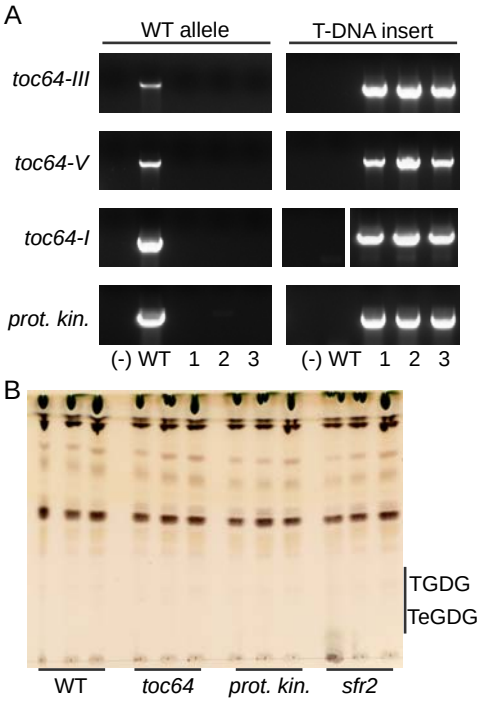

Supplemental Figure 1. Confirmation of toc64 and protein kinase disruption lines and test of SFR2 activity during normal growth. (A) DNA extracted from wild-type Arabidopsis (WT) or three individual *toc64* or *protein kinase* disruption lines (1, 2, 3) was amplified to test for the presence of specific alleles. The gene-specificity of the allele amplified is given on the left. The presence of a band in any given lane indicates the presence of the wild-type allele or the T-DNA insertion, as indicated above. The lack of a wild-type allele in *toc64* for *TOC64-III*, *TOC64-V* or *TOC64-I*, and the presence of a T-DNA insertion in each confirms that *toc64* lacks uninterrupted alleles of any TOC64 paralog. Similarly, the lack of a wild-type allele and the presence of a T-DNA insertion in the protein kinase (prot. kin.), indicates the lack of an uninterrupted allele of the protein kinase family protein. (B) A thin-layer chromatogram visualized with a sugar-specific stain separates lipids from three individual wildtype, *toc64*, *prot. kin.*, or *sfr2* plants grown under normal conditions. The location at which oligogalactolipids would appear is labeled at right (TGDG, TeGDG).