TITLE: Light dependent activation of the GCN2 kinase under cold and salt stress is mediated by the photosynthetic status of the chloroplast

AUTHORS:

Ansul Lokdarshi[1], Philip W. Morgan[1], Michelle Franks[1], Zoe Emert[1], Catherine Emanuel[2], Albrecht G von Arnim † [1,3]

Affiliations:

[1)] Department of Biochemistry & Cellular and Molecular Biology, The University of Tennessee, Knoxville, TN 37996-1939, USA

[2)] Department of Biology at Washington University in St. Louis, MO 63130-4899, USA

[3)] Graduate School of Genome Science and Technology, The University of Tennessee, Knoxville, TN 37996-1939, USA

† **Corresponding author:** Albrecht G. von Arnim (vonarnim@utk.edu)

Department of Biochemistry & Cellular and Molecular Biology

309 Ken and Blaire Mossman Bldg.

1311 Cumberland Avenue

University of Tennessee

Knoxville, TN 37996-1939, USA

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ORCID IDs: 0000-0003-3472-3357 (AGvA); 0000-0003-2264-2701 (AL), 0000-0001-5884-3543 (ZE), 0000-0003-0616-6987 (MF), 0000-0002-0439-8111(CE), 0000-0003-1669-4206 (PM)

Email addresses of all authors: alokdars@utk.edu, pmorgan4@vols.utk.edu, mfranks4@vols.utk.edu, zemert@vols.utk.edu, catherineemanuel@wustl.edu, vonarnim@utk.edu

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ABSTRACT

Regulation of cytosolic mRNA translation is a key node for rapid adaptation to environmental stress conditions. In yeast and animals, phosphorylation of the α-subunit of eukaryotic translation initiation factor eIF2 is the most thoroughly characterized event in regulating global translation under stress. In plants, the GCN2 kinase (*General Control Non-derepressible-2*) is the only known kinase for eIF2α. GCN2 is activated under a variety of stresses including reactive oxygen species. Here we provide new evidence that the GCN2 kinase in Arabidopsis is also activated rapidly and in a light dependent manner by cold and salt treatments. These treatments alone did not repress global mRNA ribosome loading in a major way. The activation of GCN2 was attenuated by inhibitors of photosynthesis and antioxidants, suggesting that it is gated by the redox poise or the reactive oxygen status of the chloroplast. In keeping with these results, *gcn2* mutant seedlings were more sensitive than wild type to both cold and salt in a root elongation assay. These data suggest that cold and salt stress may both affect the status of the cytosolic translation apparatus via the conserved GCN2-eIF2α module. The potential role of the GCN2 kinase pathway in the global repression of translation under abiotic stress will be discussed.

INTRODUCTION

The translation of mRNAs by cytosolic ribosomes into new proteins is dynamically regulated by abiotic environmental conditions such as temperature (Matsuura et al., 2010; Juntawong et al., 2013;Yanguez et al., 2013), oxygen (Branco-Price et al., 2008) and light (Juntawong and Bailey-Serres, 2012;Liu et al., 2012;Missra et al., 2015;Merchante et al., 2017). Both early and more recent studies have highlighted that redox poise and reactive oxygen species can also play important roles in regulating mRNA translation in global and mRNA sequence-specific ways (Tang et al., 2003;Branco-Price et al., 2008;Khandal et al., 2009;Benina et al., 2015). The mechanisms that regulate and coordinate mRNA ribosome loading across the plant transcriptome are generally only partially understood. Of the several mechanisms regulating global translation, phosphorylation of the α-subunit of the heterotrimeric eukaryotic initiation factor 2 (eIF2) is one of the best characterized translational control events in yeast and animals (Dever et al., 1992; Donnelly et al., 2013;Hinnebusch et al., 2016). In the unphosphorylated form, eIF2 bound to GTP delivers the initiator methionyl-tRNA to the ribosomal small subunit (40S) to initiate mRNA translation (Hinnebusch et al., 2016). Upon phosphorylation by one of several kinases, eIF2α becomes a poisoned substrate of the guanine nucleotide exchange factor for eIF2, named eIF2B (Kashiwagi et al., 2018) causing global translational repression, although some mRNAs escape the global repression by virtue of specific mRNA sequence elements (Harding et al., 2000;Liu and Qian, 2014).

General Control Non-derepressible 2 (GCN2) is the only known kinase in plants that phosphorylates eIF2α (Zhang et al., 2008;Lageix et al., 2008). In the well-studied vertebrate and yeast models, the GCN2 kinase can be activated by uncharged tRNA as a consequence of amino acid starvation (Wek et al., 1995;Dong et al., 2000;Anda et al., 2017). In plants the genetic elements of the GCN2 pathway appear to be substantially conserved, although not all biochemical details have been confirmed, and few of the biochemical steps have been investigated thoroughly. Specifically, GCN2 is encoded by a single gene in Arabidopsis that functionally complements a yeast *gcn2* mutant (Zhang et al., 2003) and can be activated by uncharged tRNA *in vitro* (Li et al., 2013). Accordingly, *in planta*, the kinase is activated by inhibitors of amino acid biosynthesis such as the herbicides chlorosulfuron, glyphosate and glufosinate (Lageix et al., 2008;Zhang et al.,

2008;Zhao et al., 2018), and the activation of GCN2 by herbicides can be suppressed by supplementation with amino acids (Zhang et al., 2008).

Aside from inhibitors of amino acid biosynthesis plant GCN2 kinase is activated by numerous other agents, including ultraviolet light, wounding, the ethylene precursor 1-aminocyclopropane carboxylic acid, the endogenous defense signals salicylic acid and methyl-jasmonate and bacterial infection (Lageix et al., 2008;Liu et al., 2019). What remains unclear is the nature of the biochemical signal that activates GCN2 under this variety of abiotic and biotic stresses. We recently described that GCN2 is activated by light-dependent reactive oxygen species (ROS) from the chloroplast. Even the stimulation of GCN2 by inhibitors of amino acid biosynthesis requires light and does not occur in darkness, suggesting that ROS are an essential requirement for GCN2 activation (Lokdarshi et al., 2019). A second conundrum surrounding plant GCN2 is that *gcn2* mutants have rather mild phenotypes under favorable lab conditions (Zhang et al., 2008;Lageix et al., 2008;Liu et al., 2015b) and a near-normal transcriptome (Faus et al., 2015; Lokdarshi et al., 2019). Moreover, among the various treatments that activate eIF2α phosphorylation, the herbicide chlorosulfuron is the only one that also results in a GCN2-dependent global translational repression (Lageix et al., 2008;Lokdarshi et al., 2019). In fact, the conditions that trigger eIF2α phosphorylation by the GCN2 kinase are not well correlated with the conditions under which *gcn2* mutant plants display maladaptive phenotypes.

Here we describe that the GCN2 kinase is activated by cold and salt stress in a light dependent manner. The activation of GCN2 by cold and salt can be suppressed by manipulating the status of the photosynthetic apparatus, suggesting that a chloroplastic signal contributes to the activation of GCN2. We also provide more evidence that eIF2α phosphorylation by different stresses does not always result in the same decline in polyribosome loading. However, *gcn2* mutant seedlings from two different ecotypes of Arabidopsis show reduced primary root growth under cold and salt stress, in keeping with a physiological role for the GCN2 kinase to adapt to these conditions. Taken together, these data suggest that the retrograde signaling from chloroplast to cytosol that targets protein synthesis may operate via the GCN2 kinase under cold and salt stress.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana ecotype Landsberg (Ler-0), Columbia (Col-0), and homozygous *gcn2-1* mutants of the GT8359 gene trap line (Zhang et al., 2008), and homozygous *gcn2-2* mutant seeds (Lokdarshi et al., 2019) were sterilized and stratified at 4°C for 2 days. Seeds were germinated on half-strength Murashige-Skoog (1/2X MS) plant media (MP Biomedicals, cat # 2633024) with 0.65% Phytoagar (Bioworld, cat # 40100072-2) and grown under a long-day period of 16 h light (80±10 μEin m^{-2} s^{-1})/8 h dark at 22 °C and 50% humidity. Unless stated, no sucrose was added to the medium.

Stress treatments and phenotype characterization

For cold stress treatment in dark and light, plates with 14-days-old horizontally grown seedlings were acclimated in the dark for 24 h starting at Zeitgeber time 2 (ZT2), after which they were shifted to 4 ºC in the dark or light for the desired times. Dark-treated seedlings were harvested under green safe light. For salt stress treatment in the dark, plates with 9-days-old vertically grown seedlings were acclimated in darkness for 24 h starting at ZT2, after which seedlings were transferred to high salt or mock 1/2X MS salt media under green safe light and sampling was performed at the desired times. For salt stress treatment under light, seedlings were transferred to high salt (150mM NaCl), or control conditions (0.1% sucrose), or control conditions with equivalent osmolarity (300mM mannitol) starting at ZT2.

For chemical treatments with DCMU (Thermo-Fisher, cat# D2425) and DBMIB (Thermo-Fisher, cat# 271993) seedlings were sprayed with the desired amount of reagent and mock control (DMSO or water) under green safe light 30 minutes before the end of 24 hr dark acclimation. For antioxidant treatment, seedlings were germinated and grown for 10 days on 1/2X MS medium containing 0.5mM ascorbate and 0.5mM reduced glutathione.

For phenotype characterization under cold stress, 3-days-old vertically grown seedlings on 0.1% sucrose were transferred to media without sucrose and shifted to 4 ºC for 30 days. For salt stress, 3-days-old vertically grown seedlings without sucrose were transferred to media with 0.1% sucrose, or supplemented with 300mM mannitol, or 150mM NaCl. Photographs were taken with

a digital camera (Canon) and primary root length was measured using ImageJ (ver. 1.41; http://rsb.info.nih.gov/ij/index.html). Fresh weight measurements were performed by weighing seedlings per plate at the end of the stress treatment. Percent survival analysis for salt stress was performed by counting seedlings that showed bleached chlorophyll and no primary root growth from day 6 to day 9. All statistical analysis was performed using GraphPad Prism (ver. 8.1.2; GraphPad Software, Inc).

Protein extraction and immunoblot analysis

Sampling for total protein extraction was done by flash freezing 14-days-old seedlings in liquid nitrogen. Seedlings were ground using a plastic pestle in a 1.5 mL tube with extraction buffer containing 25 mM Tris-HCl (pH 7.5), 75 mM NaCl, 5% (v/v) glycerol, 0.05% (v/v) Nonidet P-40, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM DTT, 2% (w/v) insoluble PVP (Sigma P-6755), supplemented with 1X protease and phosphatase inhibitor cocktail (Thermo-Fisher; cat# PIA32959). Total protein content was quantified by Bradford assay (Thermo-Fisher, cat# 23236).

For eIF2α phospho-immunoblot analysis, 50 µg of total protein was separated on a 12% (w/v) SDS-PAGE gel and electroblotted onto polyvinylidene fluoride (PVDF) membrane. After 1 h of blocking at 22 ºC with TBST buffer (1X Tris-buffered saline [pH7.6], 0.1% Tween-20) with 10% non-fat dry milk and 0.2% BSA, the membrane was incubated overnight at 4 ºC with rabbit polyclonal phospho-eIF2α antibody (Cell Signaling, cat # 9712S) diluted to 1:5000 in 1X TBST with 0.5% BSA. Following washing with 1X TBST, 10 min each for three repeats, the membrane was incubated with horseradish peroxidase conjugated anti-rabbit IgG (Vector labs, cat# PI-1000) diluted to 1:2000 in 1X TBST with 1% non-fat dry milk for 1 h at room temperature. After washing with 1X TBST, 10 min each for six repeats, horseradish peroxidase was detected using chemiluminescence (WesternBright Quantum, Advansta) as per manufacturer's protocol. For immunoblot with rabbit polyclonal eIF2α antibody (a gift from Dr. Karen Browning, University of Texas, Austin), 5 µg of total protein was resolved by SDS-PAGE and electroblotted onto a polyvinylidene difluoride (PVDF) membrane. Blocking and incubation with antibodies was performed as described (Dennis et al., 2009) followed by chemiluminescent detection (Lokdarshi et al., 2016). Signal intensity on immunoblots was quantified with ImageJ (ver. 1.41; http://rsb.info.nih.gov/ij/index.html).

176

177 **Polysome profiling and protein fractionation**

178 Tissue for polysome profiling was harvested as described for total protein extraction. For polysome
179 profiling with cold stress tissue, seedlings were ground in liquid N_2 and 0.5g of tissue powder was
180 resuspended in 1 mL of polysome extraction buffer (200 mM Tris-HCl pH 8.4, 50 mM KCl, 25
181 mM $MgCl_2$, 1% deoxycholic acid, 2% polyoxyethylene 10 tridecyl ether, 50 µg/mL cycloheximide
182 and 40U/mL RNase inhibitor (Promega Cat# N2115)) and centrifuged at 13,000 x *g* for 5 min at
183 4 °C. One mL of the supernatant was layered onto a 10 mL 15-50% linear gradient prepared using
184 a Hoefer gradient maker and centrifuged at 35,000 rpm (Beckmann SW 41 Ti) for 3.5 hr at 4 ºC.
185 Absorbance at 254 nm was recorded using an ISCO UA 5 absorbance/fluorescence monitor and
186 individual data points were extracted using the DATA acquisition software (DATAQ instruments).
187 Polysome-to-monosome (P/M) ratios were calculated as described (Enganti et al., 2018). For
188 polysome profiling with salt stressed tissue, 150 mg of tissue powder was resuspended in 100µl of
189 polysome extraction buffer and centrifuged at 13,000 rpm for 5 minutes at 4°C. 100µl supernatant
190 was layered on a 2 ml 15-50% linear gradient prepared as above and centrifuged at 50,000 rpm
191 (Beckmann TLS55 rotor) for 1hr 10 minutes at 4°C. Absorbance was measured as described above.

192

193 **Hydrogen peroxide quantification**

194 H_2O_2 content in seedlings was measured using the Amplex Red kit (Thermo-Fisher, cat# A22188).
195 Briefly, 30 mg of 2-week-old seedlings were flash frozen in liquid N_2 and ground with a plastic
196 pestle to a homogeneous powder. Pulverized tissue was resuspended in 100 µl of sterile 1X
197 phosphate buffered saline (PBS) and centrifuged at 17000 x g at 4 ºC for 2 minutes and the
198 supernatant was used for H_2O_2 measurements as per manufacturer's protocol. Relative
199 fluorescence was measured on a POLARstar OPTIMA plate reader (BMG LABTECH) with an
200 excitation filter at 535 nm and emission filter at 600 nm.

201

202 **Photosynthetic efficiency measurement**

203 The maximum quantum yield of photosystem II [Qymax= F_v /F_m] was measured on a FluorCam
204 800MF (Photon Systems Instruments) as per manufacturer's instructions and modifications from
205 (Murchie and Lawson, 2013). Briefly, plants were dark adapted for 2 min (F_0) prior to applying a
206 saturating pulse of 1800 µEin m^{-2} s^{-1} for 0.8 sec (F_m). Variable fluorescence (F_v) was calculated as

the difference between F_o and F_m to get the maximum quantum yield [F_v/F_m]. For measurements under cold stress, pots with rosette stage wild-type and *gcn2* mutant plants on soil were shifted to cold (4°C) or left at 22°C (mock) and measurements were taken for the indicated times. Recovery from cold was done by moving the pot back to 22°C. For F_v/F_m under salt stress, 3-days-old seedlings grown on 0.1% sucrose were shifted to 1/2X MS plant media supplemented with 150mM NaCl or no salt as control (Mock) and F_v/F_m measurements were recorded as discussed above.

RESULTS

GCN2 kinase activation under cold stress is light dependent

Previous reports (Lageix et al., 2008;Wang et al., 2017) showed eIF2α phosphorylation as a read out of GCN2 activity under cold stress. Given that the response to cold stress is closely linked to photosynthesis (Crosatti et al., 2013;Adam and Murthy, 2014) we tested whether the activation of GCN2 under cold stress was light-dependent. In wild-type Arabidopsis seedlings subjected to 4 ºC cold in the light, phosphorylation of eIF2α increased gradually and remained high for at least 2 hours of cold treatment. As expected, eIF2α phosphorylation was mediated by GCN2 (Fig. 1A). In contrast, if the cold treatment was performed in dark-adapted plants, eIF2α remained unphosphorylated (Fig. 1C). Under regular temperature conditions in the light, eIF2α-P remained steady between ZT2 and ZT4 (Fig. 1B). Additionally, under all the test conditions the overall amount of eIF2α remained unchanged (Fig. 1A-C). These results show that GCN2-dependent eIF2α phosphorylation under cold stress is light dependent.

Salt stress activates GCN2 in a light dependent manner

eIF2α has been shown to get phosphorylated in response to salt stress in mammals (Lu et al., 2001) and yeast (Goossens et al., 2001). To determine this response in plants, Arabidopsis seedlings grown in long-day were shifted to 150mM sodium chloride or an osmotically matched control (300mM mannitol) (Fig. 2A). Similar to other eukaryotes, salt treatment triggered eIF2α phosphorylation within 2 hours only in the wild type but not in the *gcn2-1* mutant seedlings (Fig. 2A). In addition, mock transfer (to 0.1% sucrose) and transfer onto mannitol did not activate GCN2. Similar to cold stress, salt stress too has been linked to adverse effects on chloroplasts in terms of photosynthesis and ROS accumulation (Parida and Das, 2005;Suo et al., 2017;Robles and

Quesada, 2019). To test the role of light under salt triggered GCN2 activation, Arabidopsis seedlings were dark adapted for 24 h and shifted to salt or mock (0.1% sucrose) media. Salt treatment in the dark failed to activate GCN2 in wild-type seedlings, similar to the transfer control (Fig. 2B). Taken together, both cold and salt stress require light to activate GCN2.

Antioxidants and photosynthetic inhibitors alleviate GCN2 activity

In the light, low temperature and salt both affect the photosystem II (PS II), resulting in an increase in the PS II excitation pressure, which generates damaging reactive oxygen species, including hydrogen peroxide (Gray et al., 1996;Huner et al., 1998;Fowler and Thomashow, 2002;Murata et al., 2007). To test whether ROS may contribute to GCN2 activation under cold and salt stress, seedlings were grown in the light on media containing ascorbate and reduced glutathione before challenge with cold or salt stress. These antioxidants delayed the GCN2 activation, albeit weakly in the salt (Fig. 3A), possibly because antioxidants may be barely rate-limiting under these conditions. When eIF2-P was triggered with cold treatment, the presence of ascorbate and glutathione in the medium had only a minor effect (not shown) and there was no detectable boost in H_2O_2 levels (Supplemental Figure 1). To address the role of photosynthetic electron transport for GCN2 activity, herbicides that manipulate the plastoquinone (PQ)/ plastoquinol (PQH_2) pool, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) and 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) were applied shortly prior to the cold and salt treatments. DCMU keeps the plastoquinone pool more oxidized (PQ) and DBMIB more reduced (PQH_2) (Mateo et al., 2004;Kruk and Karpinski, 2006). Both these herbicides suppressed cold and salt stress triggered GCN2 activation (Fig. 3C, D). These results along with the light dependence of cold and salt stress on GCN2 activation support the notion that chloroplast generated ROS or redox signals may contribute to the activation signal for GCN2, leading to eIF2α phosphorylation.

***gcn2* mutant sensitivity towards cold and salt stress**

To determine the role of GCN2 specifically under cold and salt stress conditions at the whole plant level, an established *GCN2* mutant allele (*gcn2-1*) (Lageix et al., 2008;Zhang et al., 2008) in the Landsberg ecotype and a recently characterized homozygous T-DNA insertion allele of *GCN2* in the Columbia ecotype (*gcn2-2)* (Lokdarshi et al., 2019) were tested for phenotypic abnormalities.

Under normal growth conditions, *gcn2-1* mutants were indistinguishable from wild type in terms of both shoot and primary root growth (Fig. 4 A, B). However, after challenge with cold stress, *gcn2-1* mutants root lengths were retarded compared to wild type (Fig. 4A, B) as were *gcn2-2* mutants (Supplemental Fig. 2A, B). Of note, the defect in overall growth in the *gcn2* mutants could not be attributed to any defects in the photosynthetic quantum efficiency (Supplemental Figure 3A and B).

Similar to the root growth retardation in the cold, exposure of seedlings to 150 mM NaCl salt also retarded primary root growth in the *gcn2* mutants (Fig. 5A, B; Supplemental Fig. 4A, B). Additionally, *gcn2* mutants showed chlorosis and root growth arrest by day 6 and day 9 (Fig. 5A; Supplemental Fig. 4A: denoted by asterisks). These effects were specific to salt and not seen in the osmotic control (mannitol) and transfer control (0.1% sucrose) treatments. The growth defect of the *gcn2* mutant on salt was evident by Day 6 and resulted in a significant loss of fresh weight and percent survival by Day 9 (Fig. 6A, B). As previously seen for cold stress, the quantum efficiency of PS II declined similarly for *gcn2* and wild type under salt stress (Supplemental Figure 6). We conclude that the *GCN2* gene promotes adaptation of seedlings to cold and salt stress.

mRNA-ribosome loading under cold and salt stress

GCN2 activity has been implicated in the down-regulation of mRNA translation under a variety of stress conditions (Lageix et al., 2008;Zhang et al., 2008;Liu et al., 2015a;Wang et al., 2017). To test the role of GCN2 in global mRNA translation under cold and salt stress, *gcn2* mutant and wild-type seedlings were challenged with the respective stresses under light. Polysome profiles from sucrose density gradients revealed overall similar profiles and polysome-to-monosome ratios for wild-type and *gcn2* under both normal growth conditions (Fig. 7A) and after cold stress (Fig. 7B, C). Likewise, in response to salt stress, both wild-type and *gcn2* mutant displayed similar polysome profiles (Fig. 8). The trend towards slightly elevated ribosome loading in *gcn2-1*, while not uncommon, was not statistically significant. The lack of a clear effect on global polyribosome loading stands in contrast to data after herbicide treatment where ribosome loading declines in a GCN2-dependent manner (Lageix et al., 2008;Lokdarshi et al., 2019).

DISCUSSION

The GCN2-eIF2α module is an integral component of a pan-eukaryotic stress response program. In yeast and mammals, GCN2 is activated by binding to uncharged tRNAs via its C-terminal HisRS domain. In plants, GCN2 kinase is activated under a wide range of abiotic stresses (e.g., UV light, cold, wounding), synthetic agents (e.g., herbicides, purine starvation), hormones (e.g., methyl jasmonate, salicylic acid, abscisic acid) and live bacterial pathogen (e.g., *Pseudomonas syringae*). More recently, Arabidopsis GCN2 was found to be activated in response to H_2O_2 directly, as well as excess light stress and methyl viologen, treatments that produce ROS (Lokdarshi et al., 2019). In the present study, we show that both cold and salt challenge not only activate eIF2α-P but require light to do so, similar to our recent findings of GCN2 activation in response to herbicide. Taken together, our study suggests that the highly conserved GCN2-eIF2α module is activated in a common manner by different stresses, possibly by ROS, given that H_2O_2 is the only known signal to activate GCN2 in darkness (Lokdarshi et al., 2019). The precise biochemical mechanism remains to be determined.

Biochemically, the only known ligand to activate plant GCN2 *in vitro* are uncharged tRNAs, which presumably accumulate in the cell during amino acid starvation. Whether uncharged tRNAs are necessary and sufficient to activate GCN2 *in planta* under all stress conditions remains unclear. It is plausible that tRNA is bound to GCN2 as a coactivator but that additional signals are needed to boost kinase activity to physiologically relevant levels. Of note, recently Inglis and coworkers reported that mammalian GCN2 can be activated in a tRNA-independent mechanism by the ribosomal P-stalk protein complex (Sattlegger and Hinnebusch, 2000;Inglis et al., 2019). The mechanism of how GCN2 is activated *in planta* by tRNAs and ROS may also depend on the GCN2 interacting proteins GCN1 and GCN20 (Wang et al., 2017; Izquierdo et al., 2018), similar to yeast and mammals; however plastidic ROS as a GCN2 activation signal is unique to plants.

It remains unclear whether and how the GCN2 mediated phosphorylation of eIF2α under various conditions drives global translational repression as seen at the level of polyribosome loading, and how this response supports plant growth and development. The clearest causal chain of events is observed with herbicides that inhibit amino acid synthesis, where activation of GCN2 kinase by herbicide in the presence of light-conditioned ROS causes eIF2α phosphorylation, followed by global translational repression, which is disrupted in the *gcn2* mutant (Lageix et al.,

2008;Lokdarshi et al., 2019). Moreover, the *gcn2* mutant is hypersensitive to herbicide (Zhang et al., 2008;Izquierdo et al., 2018), all in keeping with a simple, linear signaling pathway. However, it is much less clear how other GCN2-targeted abiotic stimuli affect translation, notwithstanding that it has been confirmed multiple times that eIF2α phosphorylation is always mediated by GCN2. Here we showed that upon cold treatment, eIF2α became phosphorylated by GCN2, but with no detectable translational repression by either cold or GCN2 kinase, although *gcn2* mutants were cold sensitive. We observed the same result for salt stress. Of note, salt stress at slightly higher intensity in rice (Ueda et al., 2012), but not cold stress in Arabidopsis (Juntawong et al., 2013) cause a drop in global ribosome loading. As for ROS, which we consider the most immediate activator of the GCN2 kinase, this stress represses translation as well as plant growth, but neither is detectably GCN2-dependent (Lokdarshi et al., 2019). The same pattern was seen in response to DTT and antimycinA (Izquierdo et al., 2018). Under high light, which is likely another relevant trigger of GCN2 in the natural environment, again, there is no GCN2-dependent translational repression, although *gcn2* mutants are sensitive to high light (Lokdarshi et al., 2019). For comparison, heat and hypoxia both rapidly repress global translation (Branco-Price et al., 2008;Matsuura et al., 2010;Yanguez et al., 2013), but without any apparent phosphorylation of eIF2α. Taken together, these observations clearly suggest that, despite the seemingly simple sequence of events in response to certain inhibitors of amino acid synthesis, not every instance of eIF2α phosphorylation causes global translational repression, and only some but not all instances of global translational repression are conditioned on eIF2α phosphorylation. These observations indicate that there must be additional translational control pathways that cooperate with GCN2-mediated eIF2α phosphorylation to organize the translatome under abiotic stress. Candidates are GCN1 and GCN20-mediated (Wang et al., 2017; Izquierdo et al., 2018), and autophagy-mediated processes (Zhao et al., 2018; Yoon and Chung, 2019) and processes involving SnRK-TOR signaling (Margalha et al., 2019) and stress granules (Chantarachot and Bailey-Serres, 2018). This conclusion is also in keeping with the emerging role of GCN2 in responses to plant pathogens. Under certain conditions, pathogens or effectors of immunity activate GCN2 or eIF2α phosphorylation (Pajerowska-Mukhtar et al., 2012;Liu et al., 2019) while in other conditions they do not (Zhang et al., 2008;Meteignier et al., 2017;Izquierdo et al., 2018). Certain pathogens do trigger translational reorganizations (Moeller et al., 2012;Xu et al., 2017) and GCN2 is involved in responses to bacterial pathogens (Liu et al., 2015b;Liu et al., 2019;Lokdarshi et al., 2019)

362 although the precise role of GCN2 kinase signaling in defense related translational control remains
363 to be defined.
364
365 Overall, the findings presented in this study add to a unified model of the regulation of the cytosolic
366 translation apparatus via the highly conserved GCN2-eIF2α module under a variety of abiotic
367 stresses, that may also extend to biotic stresses in plants. In summary, we show that activation of
368 GCN2 by cold and salt stress is dependent on the redox state of the chloroplast, and loss of *GCN2*
369 results in the increased sensitivity towards common abiotic stress inputs, cold and salt. In the
370 future, determining what biochemical and molecular events lead to GCN2 activation under these
371 natural stress inputs will shed light on the integrated stress response pathway in plants.
372 Additionally, the regulation of global translation versus specific mRNAs that fall under stress type
373 regulation is also a subject of further investigation.
374
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376
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379
380 **AUTHOR CONTRIBUTIONS**
381
382 AL, PM, MF, ZE and CE performed the experiments
383 AL, AVA – Analyzed the results and wrote the manuscript
384
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393

SUPPLEMENTARY MATERIAL

Supplemental Figure 1. Cold treatment barely modulates hydrogen peroxide levels.

Supplemental Figure 2. Loss of *GCN2* renders increased sensitivity towards cold stress in the Columbia ecotype.

Supplemental Figure 3. Effect of cold stress on photosynthetic efficiency of wild-type and *gcn2* mutants.

Supplemental Figure 4. Loss of *GCN2* renders increased sensitivity towards salt stress in the Columbia ecotype.

Supplemental Figure 5. Effect of salt stress on photosynthetic efficiency of wild-type and *gcn2.*

FIGURE LEGENDS

Figure 1. GCN2 kinase activation by cold is light dependent.

(A) Top - Schematic of the light regimen. Seedlings were grown in a 16 hr light 8 hr dark cycle and shifted to 4°C starting at zeitgeber time (ZT)2. The red arrow at ZT2 indicates the beginning of cold treatment and the start of sampling.

Bottom - Immunoblot showing the time course of eIF2*α* phosphorylation in 14-days-old wild-type Landsberg (Wt(Ler)) and *gcn2-1* mutant (*gcn2-1*) seedlings subjected to cold stress as described in panel A. Upper panel: Probed with phospho-specific antibody against eIF2*α*-P (38kDa). Middle panel: Rubisco large subunit (~ 55kDa) as a loading control after Ponceau S staining of the blot. Lower panel: Probed with antibody against eIF2*α* (38kDa). (+), arbitrary amount of total protein extract from glyphosate treated Wt seedlings indicating unphosphorylated (eIF2α) or phosphorylated (eIF2α-P) protein; (10, 30, 120) sampling time in minutes; (M) Molecular weight marker. Also shown on the right is the variation in eIF2α-P levels (percent intensity) across the tested time periods in Wt seedlings. Error bars represent Std. deviation from five biological replicates.

(B) Time course of eIF2*α* phosphorylation as in panel (A) but with Wt seedlings maintained at 22°C as a control. A partially cropped top band in the eIF2α-P blot indicates non-specific binding of the eIF2α-P antibody.

425 **(C)** eIF2α phosphorylation in Wt seedlings under 4°C in the dark. Seedlings were grown in a 16
426 hr light 8 hr dark cycle, dark-acclimated for 24 hr and shifted to 4°C in the dark. Time = 0 indicates
427 the beginning of the cold treatment and the start of sampling in dark.
428
429 **Figure 2. Salt stress activates GCN2 kinase in light.**
430 **(A)** Top - Schematic of growth regimen for seedlings under 16hr light and 8hr dark cycle. The red
431 arrow at ZT2=0 indicates the beginning of stress treatment and the start of sampling.
432 Bottom - eIF2*α* phosphorylation in 10-days-old wild-type Landsberg (Wt (Ler)) and *gcn2-1*
433 mutant (*gcn2-1*) seedlings shifted to media supplemented with 0.1% sucrose (Mock), 150mM
434 Mannitol (Mannitol) or, 150mM NaCl (NaCl). For details see legend to Fig. 1A. **(B)** Top -
435 Schematics of 24 hr dark acclimation starting at ZT2.
436 Bottom - eIF2*α* phosphorylation in Wt seedlings after shifting to 150mM NaCl in the dark. For
437 details see legend to Fig. 1C.
438
439 **Figure 3. Antioxidant and photosynthetic inhibitors mitigate GCN2 kinase activation under**
440 **cold and salt stress.**
441 **(A)** Time course of eIF2*α* phosphorylation in wild-type Landsberg (Wt) seedlings grown on media
442 supplemented with 0.5mM ascorbate and reduced glutathione for 10-days and shifted to with
443 150mM NaCl with either antioxidants (Asc + GSH) or mock control. Transfer and sampling of
444 seedlings was performed as described in Fig. 2A.
445 **(B, C)** eIF2*α* phosphorylation in Wt seedlings treated with either DMSO control (Mock), or 8µM
446 of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), or 16µM of 2,5-Dibromo-6-isopropyl-3-
447 methyl-1,4-benzoquinone (DBMIB) thirty minutes prior to **(B)** NaCl or **(C)** 4°C treatment. For
448 details see legend to Fig. 1, 2.
449
450 **Figure 4. Loss of *GCN2* renders increased sensitivity towards cold stress.**
451 **(A)** Top - Representative images of 3-days-old wild-type Landsberg (Wt (Ler)) and *gcn2-1* mutant
452 (*gcn2-1*) seedlings grown under a 16 hr light and 8 hr dark cycle (long day, LD) at 22°C. Seedlings
453 were grown on media with 0.1% sucrose for 3-days and transferred to no sucrose (Day 0).
454 Bottom - Same seedlings after 30 days of LD cycle at 4°C. Scale bars are 10mm.

(B) Primary root length of Wt and *gcn2*-1 mutant seedlings from panel A. Error bars indicate standard error of the mean from four biological replicates with n>80 per experiment. (Welch's t-test *P-value <0.05)

Figure 5. *gcn2* mutants are more sensitive to salt stress.

(A) Representative images of wild-type Landsberg (Wt (Ler)) and *gcn2-1* mutant (*gcn2-1*) seedlings grown under 16 hr light and 8 hr dark period on plant media supplemented with 150mM NaCl (salt treatment), 300mM Mannitol (osmotic control), or 0.1% sucrose (transfer control) . On the day of transfer (Day 0) seedlings were 3 days old on 0.1% sucrose. Scale bar is 10mm.

(B) Primary root length of Wt and *gcn2-1* mutants from panel (A). Error bars indicate standard error of the mean of four biological replicates with n>36 per experiment (Welch's t-test *P-value <0.05; ** P-value <0.005).

Figure 6. *gcn2* mutants accumulate less fresh weight and exhibit low survival under salt stress.

(A) Left panel - Fresh weight (grams) of wild-type Landsberg (Wt(Ler)) and *gcn2-1* mutant (*gcn2-1*) seedlings after 9-days of growth on 0.1% sucrose or, 300mM Mannitol or, 150mM NaCl. Right panel - Percent survival of Wt and *gcn2-1* mutant seedlings at day 6 and day 9 on 150mM NaCl. Analysis performed on seedlings from Fig. 5

(B) Fresh weight and percent survival of wild-type Columbia (Wt (Col)) and *gcn2-2* mutant (*gcn2-2*) seedlings as described in panel A. Analysis performed on seedlings from Supplemental Fig. 4

Error bars indicate standard error of the mean of four biological replicates with n>36 per experiment (Welch's t-test *P-value <0.05; **P-value <0.005; *** P-value <0.0005).

Figure 7. Ribosome-RNA profile of wild-type and *gcn2-1* under standard growth conditions and cold stress.

Top - **(A, B)** Schematic of light regimen showing seedling growth in long day period (16 hr light and 8 hr dark) indicating the beginning of cold (4°C) treatment starting at ZT2 (blue arrow) and the sampling time at ZT4 (red arrow).

Bottom - UV Absorbance profile at 254nm of 14-days-old wild-type Landsberg (Wt(ler)) and *gcn2-1* mutant (*gcn2-1*) seedlings at (A) 22°C at ZT4 (Mock) or subjected to cold at 4°C **(B)** for 2 hr, or **(C)** for 24 hr under a long day period.

The positions of the 40S, 60S, 80S and the polysomes are indicated on the profiles. The ratio of polysomes (P) to monosomes (M) is indicated with standard error from 3 replicates.

Figure 8. Ribosome-RNA profile of wild-type and *gcn2* mutant under salt stress.

Representative UV absorbance (A254) profile of 10-days-old Wild-type Landsberg (Wt(Ler)) and *gcn2-1* mutant (*gcn2-1*) seedlings after 2 hr of treatment with **(A)** 0.1% sucrose (Mock) or **(B)** NaCl. Seedling transfer was performed as described in Figure 2. Positions of the 40S, 60S, 80S and the polysome are indicated on the profile.

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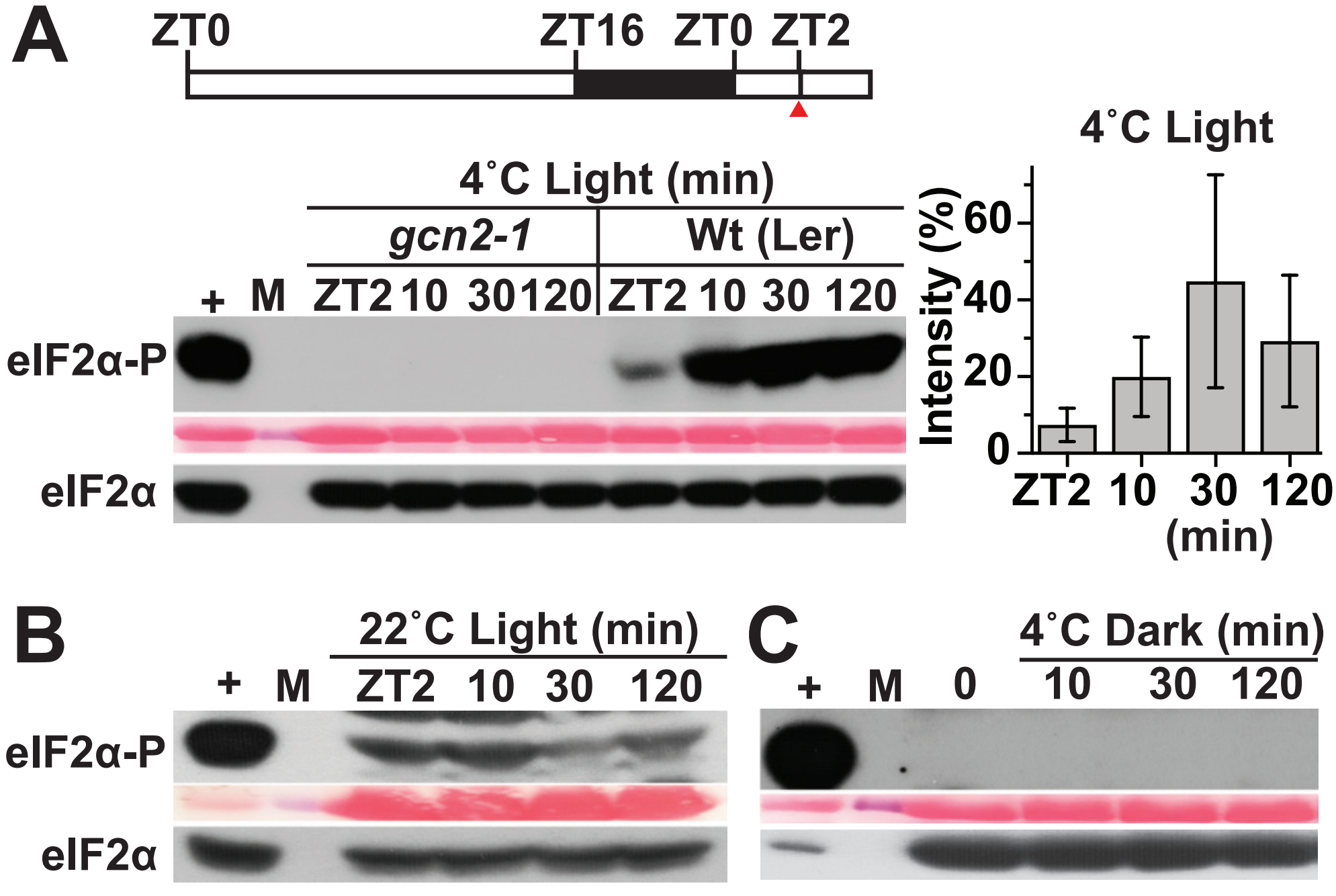
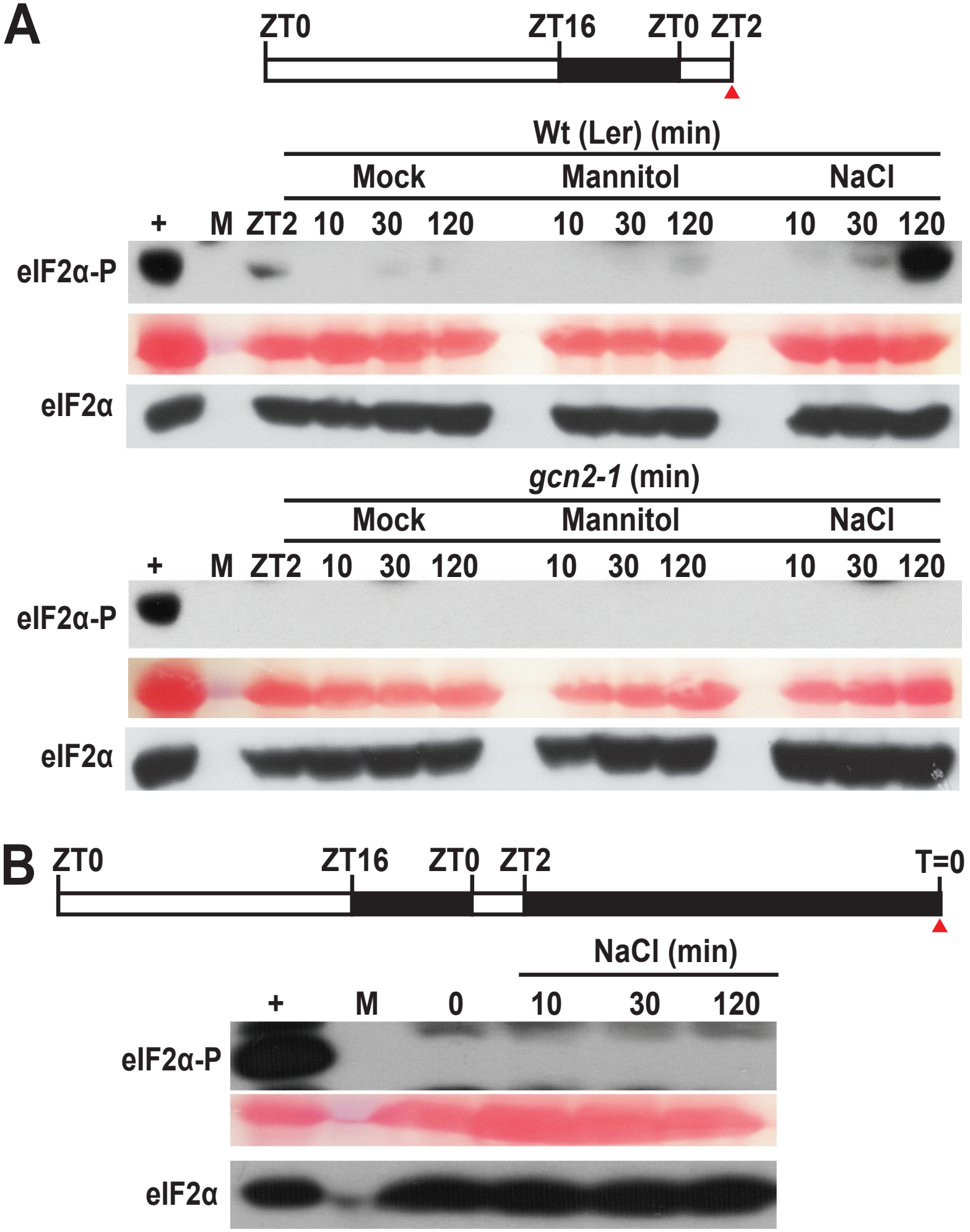
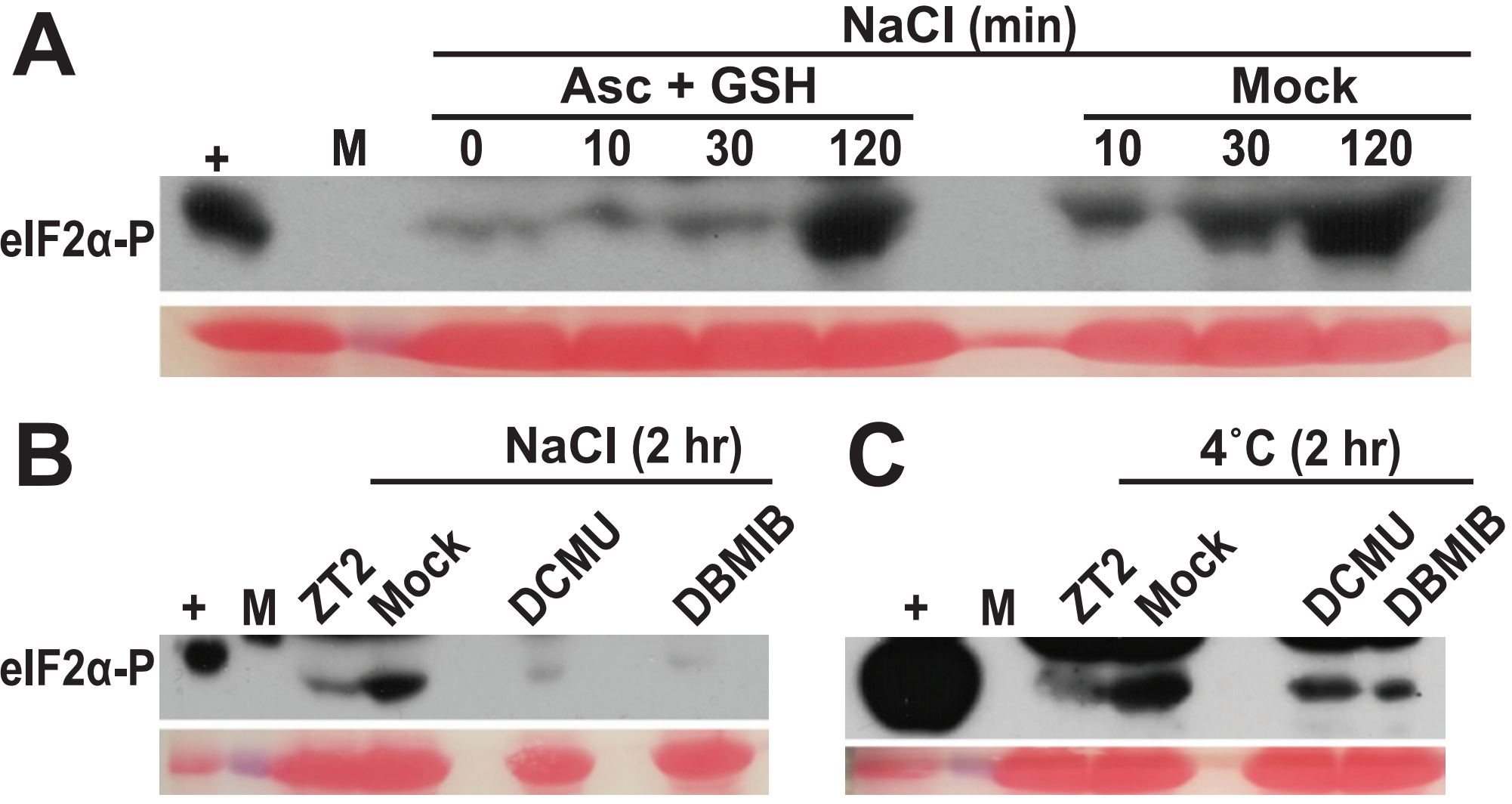
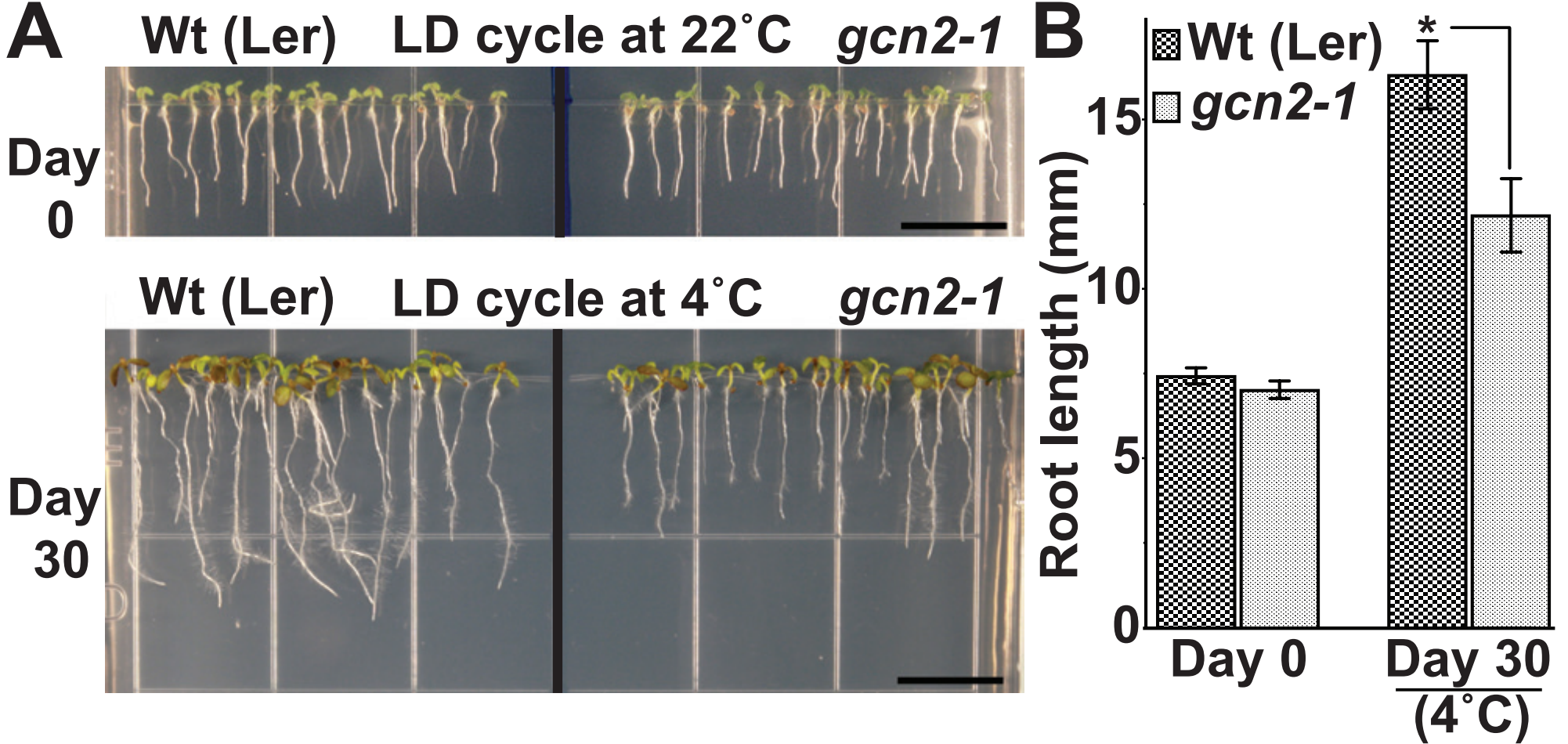
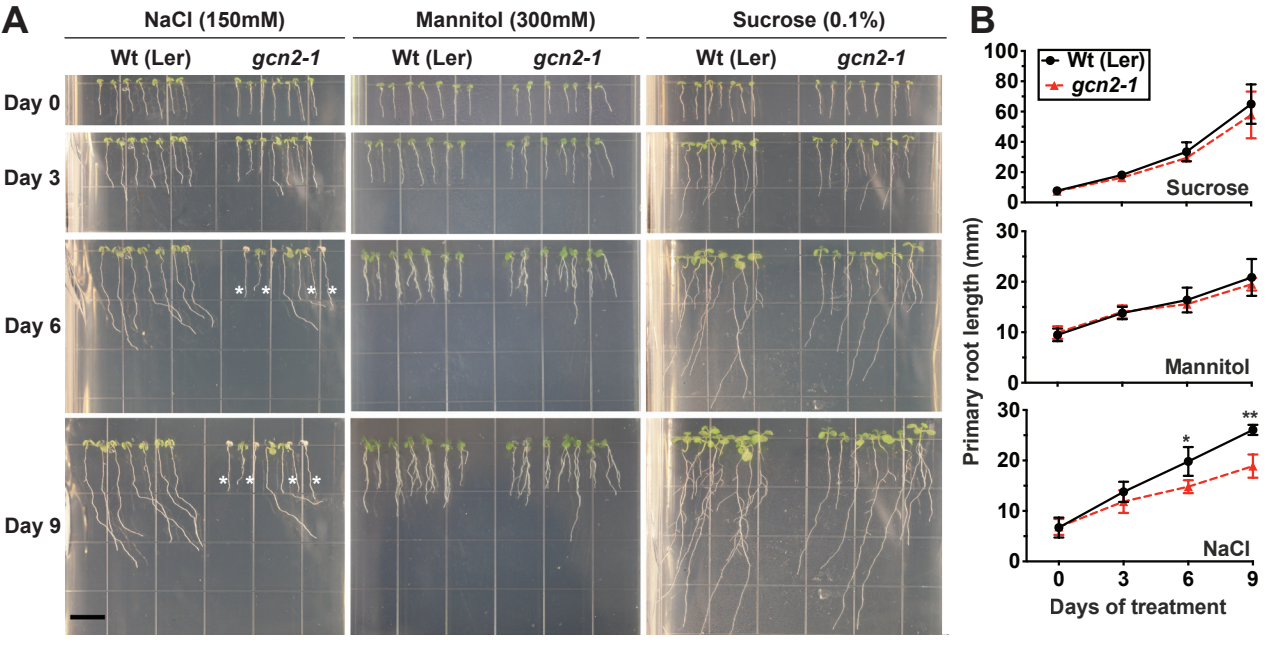
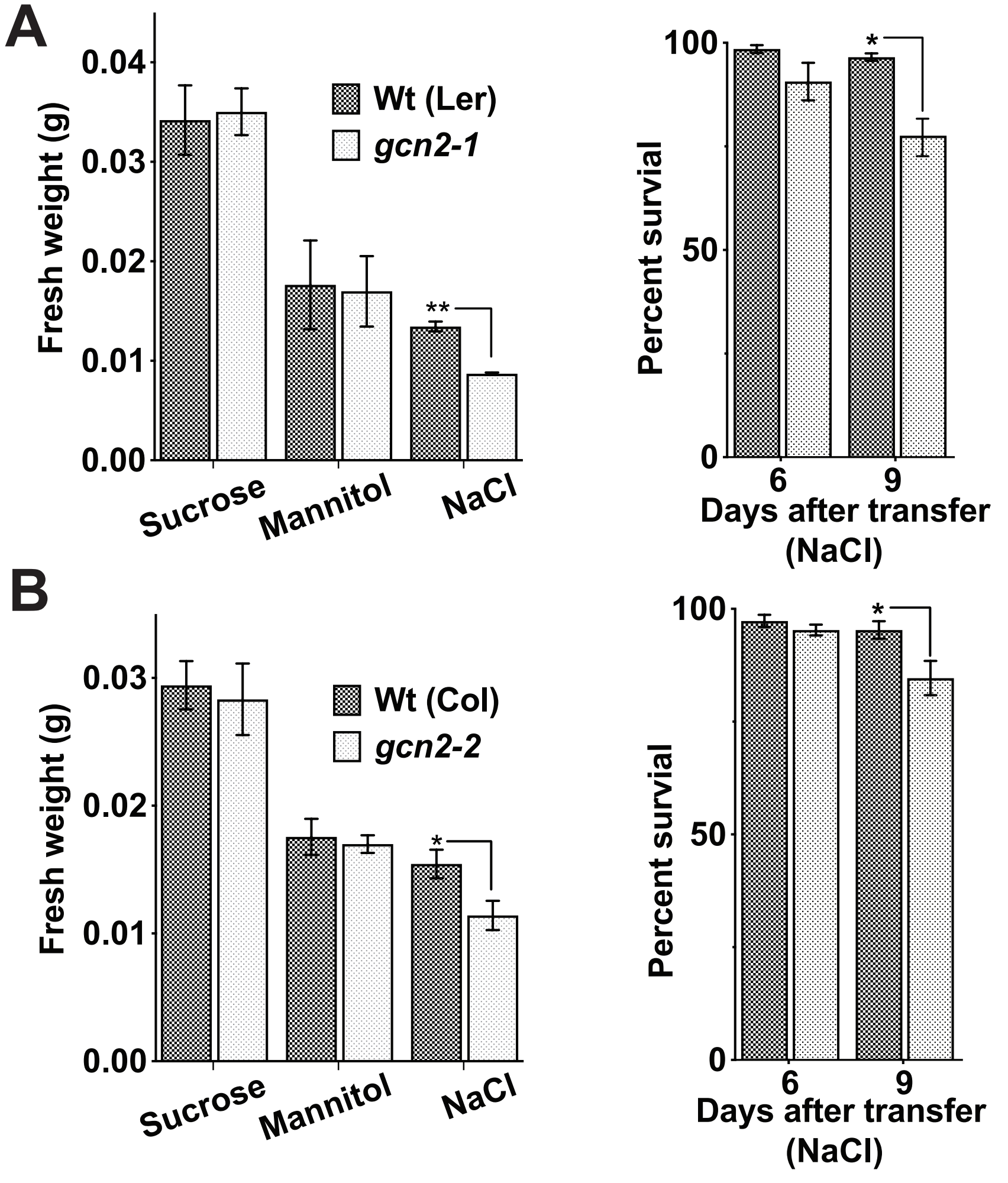

A
ZT0
ZT16
ZT0
ZT2
4˚C Light (min)
gcn2-1
Wt (Ler)
+
M
ZT2
10
30
120
ZT2
10
30
120
eIF2α-P
eIF2α
4˚C Light
Intensity (%)
60
40
20
0
ZT2
10
30
120
(min)
B
22˚C Light (min)
+
M
ZT2
10
30
120
eIF2α-P
eIF2α
C
4˚C Dark (min)
+
M
0
10
30
120

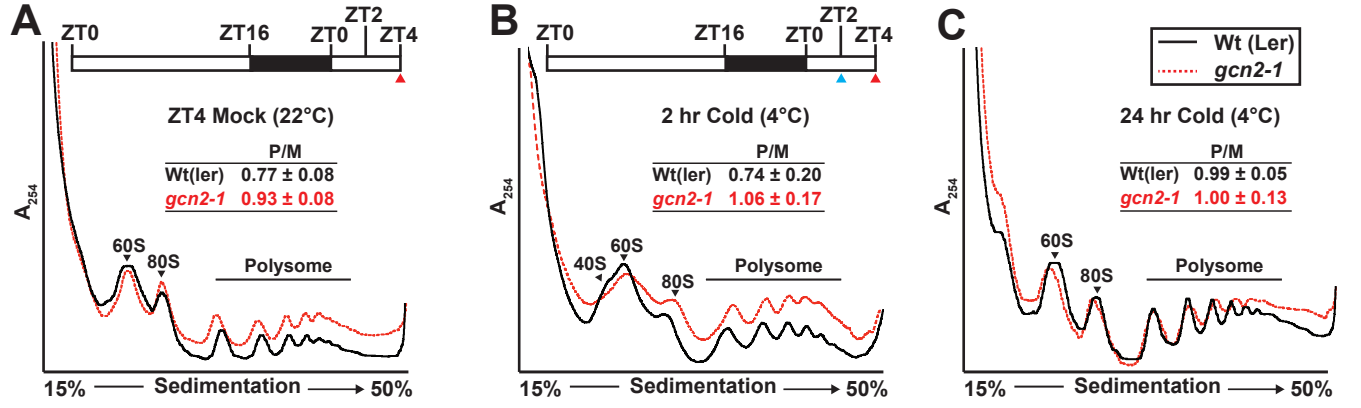

A
ZT0
ZT16
ZT0
ZT2
Wt (Ler) (min)
Mock
Mannitol
NaCl
+
M
ZT2
10
30
120
10
30
120
10
30
120
eIF2α-P
eIF2α
gcn2-1 (min)
Mock
Mannitol
NaCl
+
M
ZT2
10
30
120
10
30
120
10
30
120
eIF2α-P
eIF2α
B
ZT0
ZT16
ZT0
ZT2
T=0
NaCl (min)
+
M
0
10
30
120
eIF2α-P
eIF2α

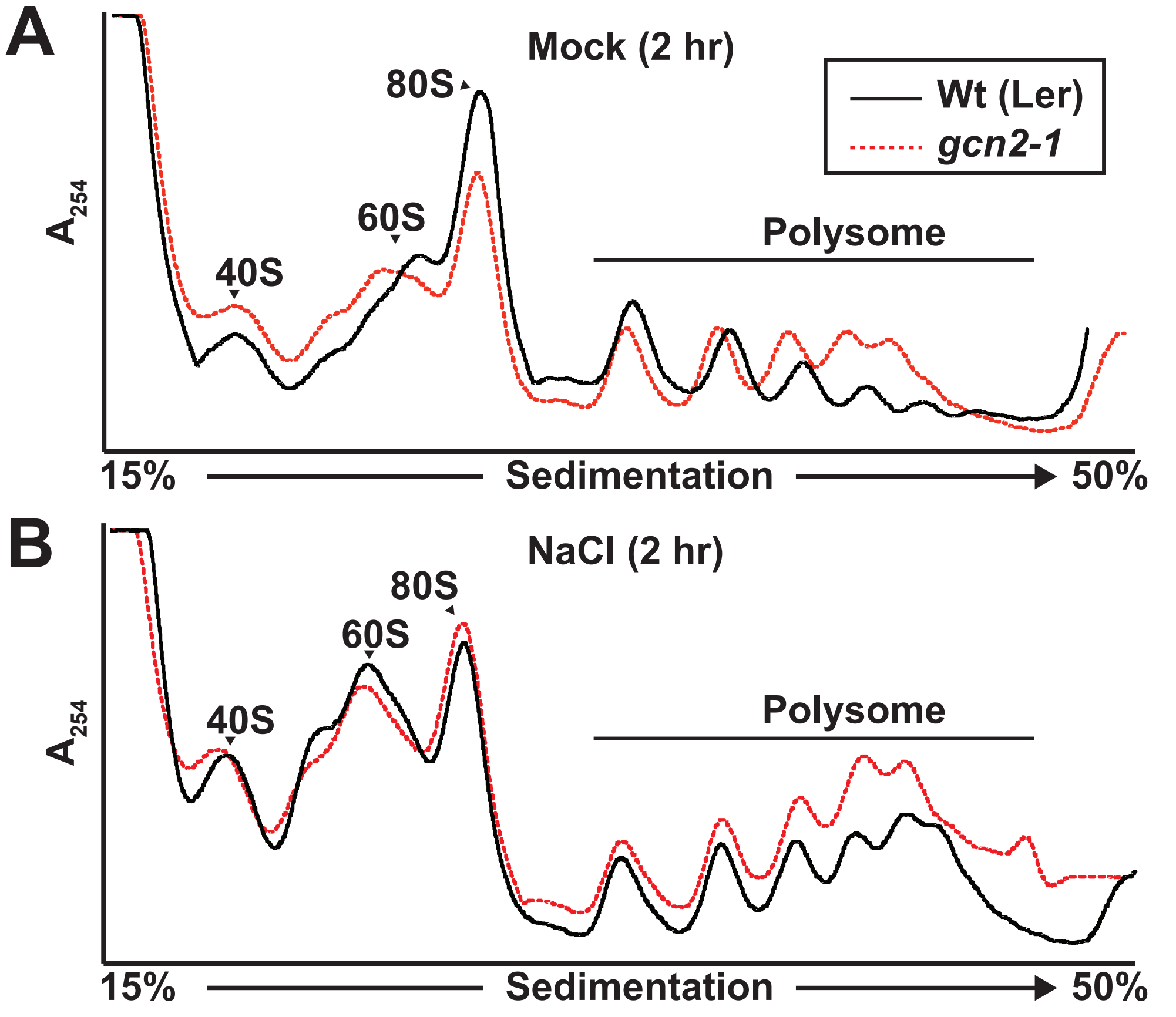

A
NaCl (min)
Asc + GSH
Mock
+
M
0
10
30
120
10
30
120
eIF2α-P
B
NaCl (2 hr)
+
M
ZT2
Mock
DCMU
DBMIB
eIF2α-P
C
4˚C (2 hr)
+
M
ZT2
Mock
DCMU
DBMIB


A
Wt (Ler)
LD cycle at 22˚C
gcn2-1
Day 0
Wt (Ler)
LD cycle at 4˚C
gcn2-1
Day 30
B
Wt (Ler)
gcn2-1
*
Root length (mm)
0
5
10
15
Day 0
Day 30
(4˚C)


A
NaCl (150mM)
Mannitol (300mM)
Sucrose (0.1%)
Wt (Ler)
gcn2-1
Day 0
Day 3
Day 6
Day 9
B
Wt (Ler)
gcn2-1
Sucrose
Mannitol
NaCl
Primary root length (mm)
Days of treatment
0
3
6
9
100
80
60
40
20
30
10
*
**


A
Fresh weight (g)
0.04
0.03
0.02
0.01
0.00
Wt (Ler)
gcn2-1
**
Sucrose
Mannitol
NaCl
Percent survial
100
50
0
*
6
9
Days after transfer
(NaCl)
B
Fresh weight (g)
0.03
0.02
0.01
0.00
Wt (Col)
gcn2-2
*
Sucrose
Mannitol
NaCl
Percent survial
100
50
0
*
6
9
Days after transfer
(NaCl)


A
ZT0
ZT16
ZT0
ZT2
ZT4
ZT4 Mock (22°C)
P/M
Wt(ler) 0.77 ± 0.08
gcn2-1 0.93 ± 0.08
A254
60S
80S
Polysome
15%
Sedimentation
50%
B
ZT0
ZT16
ZT0
ZT2
ZT4
2 hr Cold (4°C)
P/M
Wt(ler) 0.74 ± 0.20
gcn2-1 1.06 ± 0.17
A254
40S
60S
80S
Polysome
15%
Sedimentation
50%
C
Wt (Ler)
gcn2-1
24 hr Cold (4°C)
P/M
Wt(ler) 0.99 ± 0.05
gcn2-1 1.00 ± 0.13
A254
60S
80S
Polysome
15%
Sedimentation
50%


A
Mock (2 hr)
80S
60S
40S
Polysome
Wt (Ler)
gcn2-1
A254
15%
Sedimentation
50%
B
NaCl (2 hr)
80S
60S
40S
Polysome
A254
15%
Sedimentation
50%