Methyl Jasmonate Stress Signaling in *Arabidopsis thaliana* Functions Via Reactive Oxygen to Activate the GCN2-eIF2α Module

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Abstract

Plant growth and productivity rely on rapid energy management strategies designed to cope with dynamic environmental conditions (e.g., fluctuating light intensities, temperature, humidity, and pathogen interactions). Previous work by Lokdarshi et al. (2020a), identified a novel fast-regulatory switch in *Arabidopsis thaliana* that functions at the nexus of two fundamental energy management programs, cytosolic translation and reactive oxygen species (ROS) signaling. The work showed that the General Control of Nonderepressible 2 (GCN2), a cytosolic serine/threonine protein kinase, is rapidly activated in response to ROS emanating under a variety of abiotic, biotic, and xenobiotic stresses. GCN2 then phosphorylates its target, α-subunit of the eukaryotic translation initiation factor (eIF)2, resulting in readjustments to the active protein synthesis, as a plausible mode for stress remediation. In the work presented here, we test the hypothesis that the biochemical, molecular and physiological responses of the Arabidopsis GCN2-eIF2α module towards the plant defense hormone, methyl jasmonate (MeJA) is regulated by light and ROS. We show that eIF2α phosphorylation (P-eIF2α) as proxy for GCN2 activation under MeJA stress requires light and this activation can be mitigated with antioxidants and photosynthetic inhibitors. At the physiological level, *gcn2* mutant seedlings show increased sensitivity towards MeJA stress in a primary root growth assay. Interestingly, the *gcn2* mutant shows a similar rate of protein synthesis as the wild-type under MeJA stress as evidenced by polysome profiling and puromycin incorporation assay. Taken together, we show the conservation of Arabidopsis GCN2-eIF2α activation by ROS during methyl jasmonate stress.

Introduction

Plants frequently encounter a wide range of environmental conditions that adversely affect their growth, development, and overall health. To adapt and/or survive, plants have evolved complex mechanisms that operate at the molecular, biochemical, and physiological levels (Nawaz et al., 2023; Zhang et al., 2020). One of the key metabolic signals that lies at the center of these adaptive responses is the synthesis of phytohormones (plant hormones) such as auxin, ethylene, methyl jasmonate, salicylic acid, and abscisic acid (Kumari et al., 2024; Verma et al., 2016). These naturally occurring compounds are critical for managing plant health under different types of environmental conditions (Verma et al., 2016). Additionally, they regulate a wide variety of growth and developmental processes such as seed germination, root growth, fruit ripening, and leaf senescence (Cheong & Choi, 2003; Li et al., 2018a; Yu et al., 2019).

Methyl jasmonate (MeJA) is a plant growth regulator hormone belonging to the jasmonate family (oxylipin jasmonic acid/JA), primarily known to mediate responses towards various abiotic (e.g., cold, high light, drought, salinity) and biotic stresses (e.g., insect-driven wounding, pathogen infection) (Cheong & Choi, 2003; Mohamed & Latif, 2017; Yu et al., 2019). MeJA is synthesized via the lipoxygenase pathway, which begins in the chloroplasts with the enzymatic oxidation of membrane-associated unsaturated α-ilenoic-acid 18:3 (α-LA). This process is followed by a series of β-oxidation reactions in the peroxisome, leading to the production of jasmonate. Finally, in the cytoplasm, different enzymes facilitate the methylation of jasmonate, producing the functional form of MeJA (Larrieu & Vernoux, 2016; Wasternack, 2007).

Given its volatile nature, MeJA can mediate both intra- and inter-plant communications to modulate plant defense responses under stress (Delgado et al., 2021). MeJA signaling involves the recognition of the hormone by cellular receptors and the activation of transcription factors that regulate the expression of genes related to defense responses. For example, some of the MeJA induced gene products include enzymes involved in the synthesis of antioxidants, phytoalexins, and secondary metabolites to protect against pathogens (Delgado et al., 2021; Larrieu & Vernoux, 2016). While the multifaceted role of MeJA in coordinating varied responses to abiotic and biotic factors remain extensively investigated at the level of transcription (Benevenuto et al., 2019; Cao et al., 2016; Pauwels et al., 2008; Shi et al., 2015; Ziosi et al., 2008), regulation at the level of translation (mRNA to protein synthesis) has received little attention (Son & Park, 2023).

Regulation of protein synthesis (translational control) is fundamental to plant growth and development, and the management of defense responses (Son & Park, 2023; Urquidi-Camacho et al., 2020; Wu et al., 2024).Translational control occurs primarily, but not exclusively, during the initiation phase, and involves the action of major signaling protein kinases, the General Control Non-derepressible 2 (GCN2), SNF-related kinase (SnRK), and the Target Of Rapamycin (TOR) (Urquidi-Camacho et al., 2020). Among these, GCN2 has received significant attention in regulating translation under a wide variety of abiotic, biotic and xenobiotic stresses (Cho et al., 2022; Lageix et al., 2008; Li et

al., 2018b; Liu et al., 2019; Llabata et al., 2019; Lokdarshi et al., 2020a; Lokdarshi et al., 2020b; Lokdarshi et al., 2022; Zhang et al., 2008).

The diverse stress sensing and signaling capabilities of the GCN2 protein aptly highlights its role as one of the most important “stress sensor” or, more appropriately, the “stress sentinel” kinases in plants (Lokdarshi & von Arnim, 2022; Sesma et al., 2017; Son & Park, 2023). In mammals and yeast, GCN2 protein is activated in response to amino acid deprivation and phosphorylates the α-subunit of the heterotrimeric eukaryotic translation initiation factor 2 (eIF2) on a conserved N-terminal serine residue (Dever et al., 1992). Phosphorylation of eIF2α (henceforth P-eIF2α) results in suppression of global translation while selective translation of stress-responsive mRNAs (e.g., *GCN4* in yeast). In plants, the GCN2-eIF2α is activated in response to abiotic (e.g., cold, excess light, salt), biotic (e.g., bacterial, fungal pathogens), and xenobiotic factors (e.g., herbicides, cadmium, hydrogen peroxide).

In the previous works, Lokdarshi (2020a, 2020b) identified reactive oxygen species (ROS) as a key activator of the *Arabidopsis thaliana* GCN2-eIF2α module. Their work demonstrated that stresses such as excess light, cold, salt, herbicides, and H_2O_2, all induce the accumulation of ROS, triggering rapid (within 10-30 minutes) activation of the GCN2 protein (Lokdarshi, 2020a, 2020b, 2022). Given the rapid response nature of the GCN2-eIFα module and the activation signal as ROS, their findings highlight the potential of translational control via the GCN2-eIFα module to explain how plant cells can nimbly adjust to dynamic environments.

In the study presented here, we address the central hypothesis: “Methyl Jasmonate signaling in *Arabidopsis thaliana* functions via Reactive Oxygen Species to activate the cytosolic GCN2-eIF2α module” with the following three aims:

Aim 1: Use a biochemical approach (immunoblotting) to detect activation of GCN2 in response to MeJA treatment and its dependence on chloroplast function.

Aim 2: Conduct a phenotypic assessment (primary root growth, fresh weight, anthocyanin levels) of the *gcn2* mutant under MeJA stress.

Aim 3: Evaluate the *in vivo* translation status of the *gcn2* mutant under MeJA stress.

We show that light is essential for the activation of Arabidopsis GCN2 in response to ectopic MeJA treatment, and the increase in P-eIF2α is attenuated by the application of photosynthetic inhibitors and ROS quenchers. *Loss-of-GCN2* mutants show severe growth defects under MeJA stress, which is rescued by complementing the *gcn2-1* mutant with a wild-type copy of the *GCN2*. Interestingly, both wild-type and *gcn2-1* mutant show similar rate of protein synthesis under MeJA stress as evidenced by polysome profiling and puromycin incorporation assay.

In summary, the current study highlights the rapid activation by ROS of the GCN2-eIF2α module (30 minutes) under MeJA stress, working in a dosage dependent manner, where higher P-eIF2α levels are expressed in plants with increasing MeJA concentration. Interestingly, *gcn2* mutants exhibit increased

sensitivity to prolonged MeJA exposure, resulting in lower fresh weight, shorter root length, and lower anthocyanin concentration compared to the wild type. These findings suggest that GCN2-eIF2α serves as an important early defense response mechanism regulating protein synthesis for better energy management under MeJA stress.

Materials and Methods

Plant materials and growth conditions

Seeds of the following *Arabidopsis thaliana* ecotypes and mutants were sterilized and stratified at 4°C for 2 days on ½-strength Murashige-Skoog (MS) salt plant medium (MP Biomedicals, Cat# 2633024) containing 0.65% Phytoagar (Bioworld, Cat # 40100072-2).

Wild-type Landsberg *erecta* (Ler-0), Columbia-0 (Col_0) and Columbia glabrous (Col (gl1); Homozygous *gcn2-1* mutant in Ler-0 (Genetrap line GT8359) (Lageix et al., 2008; Zhang et al., 2008) and *gcn2-1;GCN2* complementation with *GCN2* under native promoter (Lageix et al., 2008); Homozygous *gcn2-2* mutant in Col_0 (SALKseq_032196) (Faus et al., 2018). Seeds were germinated and grown under the standard long-day cycle of 16 h light (white light, 80±10 µE $m^{-2}s^{-}$) / 8 h dark at 22°C and 50% humidity.

Stress treatments

For stress treatments under light (eIF2α phosphorylation/GCN2 activation assay, polysome profiling and RNA analysis, hydrogen peroxide quantification, anthocyanin quantification, and puromycin assay), twelve-days-old horizontally (roots inside the media) grown seedlings were sprayed 10-15 times with the appropriate reagents from a distance of 2 inches. After 30 seconds, the excess reagent was drained off and plates were wrapped with 3M pore surgical tape (Fisher Scientific, Cat# 22-355-000). Stress treatments used: Mock – 0.1% (v/v) Dimethyl Sulfoxide (DMSO) (Fisher Scientific, Cat# D128-500) or double deionized sterile water, MeJA – Methyl Jasmonate (Millipore Sigma, Cat# 392707) in 0.1% (v/v) DMSO, CSF – Chlorosulfuron (Millipore Sigma, Cat#

34322) in 0.1% (v/v) DMSO, DCMU – (3-(3,4-dichlorophenyl)-1,1-dimethylurea) (Fisher Scientific, Cat# AAL0298618), and RdGSH – Reduced Glutathione (Millipore Sigma, Cat# 70-188).

For pre-treatment with DCMU or RdGSH, seedlings were sprayed at ZT1.5 (1.5 h after lights-on) and incubated for 30 minutes before stress treatment at ZT2 as described above. Stress treatments under dark were performed with seedlings after dark-acclimation for 24 h, i.e., starting two hours after lights-on (Zeitgeber time 2, ZT2). Treatment with various reagents was conducted as described for stress under light and sampling was performed under safe green light.

Phenotype characterization (Primary root length and fresh weight measurement)

To evaluate how wild-type and the *gcn2* mutant seedlings responded to prolonged MeJA stress, three-days-old vertically (roots on top of the media) grown seedlings grown on ½-strength MS medium were transferred on either 0.1% DMSO or µM MeJA containing media (Day of the transfer = Day 0) and grown under a long-day cycle for the period indicated in the respective figures.

Images of the seedlings were taken at 0, 3, 6, 9, and 12 days with a digital camera (Canon 60D). The primary root length was measured using ImageJ (ver.1.54g; http://rsb.info.nih.gov/ij/index.html) and fresh weight was analyzed by weighing the seedlings at the end of the stress treatments. All statistical analysis was performed using GraphPad Prism (Version 10.1.1 (270); GraphPad Software, Inc).

Total protein extraction and eIF2α phosphorylation/GCN2 activation assay

Protein extraction and eIF2α phosphor-immunoblot analysis was performed with the purpose of detecting P-eIF2α as a proxy of GCN2 activation, as described previously in Zhang et al. (2008) with slight modifications. Briefly, 50-150 mg of 12-days-old horizontally grown seedlings were stored in a pre-filled bead mill tube (Fisher Scientific, Cat# 15-340-151) and flash frozen in liquid N_2. Tubes were stored at -80°C prior to immunoblot analysis. For total protein extraction, 80-120 ml of freshly prepared extraction buffer containing 25 mM Tris-HCl (pH 7.5), 75 mM NaCl, 1 M Urea, 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 Polyvinylpyrrolidone (Sigma-Aldrich, Cat# P-6755), 2 mM β-mercaptoethanol (Sigma M-6250), supplemented with 1X Protease and Phosphatase inhibitor cocktail (Thermo-Fisher; Cat# PIA32959) was added into the tubes. Seedlings were ground using a bead mill (Fisher Scientific, Cat# 15-340-163) as per manufacturer's instructions and total protein content was quantified by Bradford assay (Thermo-Fisher, Cat# 23236).

The eIF2α phosphorylation/GCN2 activation assay was performed by immunoblot analysis as described in Lokdarshi (2020a, 2020b). P- eIF2α was detected with either the Cell Signaling Technology Phospho-eIF2α (Ser51) rabbit monoclonal antibody (Cat# 3597) or the polyclonal antibody (Cat# 9721S). All immunoblot signal intensities were quantified with the Image J program (ver.1.54g; http://rsb.info.nih.gov/ij/index.html). All immunoblots were repeated with at least three biological replicates for statistical test of significance.

Polysome Profiling and ribosomal RNA analysis

To check for the global translation status between wild-type and the *gcn2-1* mutant under MeJA stress , 12-days-old horizontally grown seedlings were flash frozen in liquid N_2 and stored at -80°C. Seedlings were ground in liquid N_2 using pre-chilled pestle and mortar and polysome profiling was performed as described previously in Missra et al. (2015) and Lokdarshi (2020a, 2020b). For fractionation, 900 ml of the gradient was manually aspirated and transferred into a fresh 2 ml tube for RNA extraction as described in Missra & von Arnim (2014). RNA quality/quantity was assessed by a Nanodrop Spectrophotometer (Thermo Scientific, Cat# 13-400-519) and statistical analysis of percent RNA recovery was performed with GraphPad Prism Version 10.1.1 (270).

Based on the agarose gel analysis of rRNA (18S and 25S) (Figure 7), three fractions of RNAs were generated: NP (non-polysomal) with no ribosomes, SP (small polysomal) with individual ribosomal subunits (40S, 60S) or considering one-two ribosomes (80S) per mRNA, and the LP (large polysomal) fractions representing multiple ribosomes per mRNA.

Puromycin Assay

To assess the rate of global protein synthesis, puromycin (PU) was performed as described previously by Llabata et al. (2019) with slight modifications. Briefly, 10-days-old horizontally grown seedlings were treated with either 0.1% DMSO or 20 µM MeJA for 2 h. At the end of the stress treatments, 15 ml of 65 µg ml^{-1} puromycin dihydrochloride (Fisher Scientific, Cat# AC227422500) was added to the plates and the seedlings were further incubated

for 2 h under the same growth conditions. Seedlings were transferred on a dry paper towel to remove excessive liquid before weighing and flash frozen in liquid N_2 and stored at -80ºC. Sample processing and the immunoblot experiment were performed as described in Llabata et al. (2019). The rate of translation was determined by measuring the signal intensities of all the dots from the dot blot assay using ImageJ (ver.1.54g; http://rsb.info.nih.gov/ij/index.html) and statistical analysis was performed using GraphPad Prism Version 10.1.1 (270).

Hydrogen peroxide quantification

Quantification of *in vivo* H_2O_2 levels was performed in order to demonstrate whether MeJA stress led to accumulation of H_2O_2 in wild-type seedlings, as described in Lokdarshi (2020a, 2020b) using the Amplex Red kit (Thermo-Fisher, Cat# A22188). Briefly, 30 mg of 12-days-old seedlings were flash frozen in liquid N_2 and ground with a plastic pestle (Fisher Scientific, Cat# 12-141-464) for 2 minutes until a homogenous powder was visible. Tissue powder was resuspended in 100 µl of 1X reaction buffer (Amplex Red kit) and centrifuged at 17000 x g 4ºC for 5 minutes. The supernatant was used for H_2O_2 measurements as per manufacturer's protocol. Absorbance at 560 nm was measured on the SpectrMax M5e (Molecular Devices, VWR Cat# 89212-400) plate reader.

Anthocyanin quantification

Quantification of anthocyanin was performed in wild-type, *gcn2-1*, and *gcn2-1;GCN2* complementation seedlings to test if the loss of *GCN2* would affect the anthocyanin production under MeJA stress, as a proxy of plant stress

resilience. Anthocyanin estimation was performed with seedlings at the Day 12 of the phenotype characterization experiments as described in Nakata & Ohme-Takagi (2014). Briefly, 30-50 mg of seedlings were flash frozen in liquid N_2 and grinded in a 1.5 ml tube using a plastic pestle (Fisher Scientific, Cat# 12-141-464). Pulverized tissue powder was resuspended in 5 volumes (based on fresh weight) of freshly prepared extraction buffer (45% (v/v) Methanol, 5% (v/v) Acetic acid). After centrifuging the tube at 12,000 x g for 5 minutes at room temperature, supernatant was transferred to a fresh 1.5 ml tube and recentrifuged to clear remaining cell debris. Anthocyanin content was measured by transferring 50 µl of the supernatant into a 96 well clear plate and recording the absorbance at 530 nm and 657 nm on the SpectraMax M5e (Molecular Devices, VWR Cat# 89212-400) plate reader.

Results

Methyl jasmonate induces GCN2-dependent phosphorylation of eIF2α.

A previous report by Lageix et al. (2008) showed P-eIF2α as a read out of GCN2 activity after 4 h of MeJA treatment. Given that P-eIF2α levels increase significantly within 30-120 minutes under a wide variety of stresses (Lokdarshi, 2020a, 2020b, 2022) we tested whether the activation of GCN2 under MeJA stress also followed a rapid activation model. Upon MeJA treatment as indicated in the growth regimen (Figure 1A), P-eIF2α levels increased significantly within 30 minutes and showed even higher levels at 120 minutes in the wild-type Arabidopsis seedlings (Figure 1B). The homozygous *gcn2* mutant failed to show P-eIF2α as evidenced in several previous reports (Lageix et al., 2008; Lokdarshi, et al., 2020a; Lokdarshi, et al., 2020b; Zhang et al., 2008), supporting our hypothesis of the rapid GCN2-dependent eIF2α phosphorylation under MeJA stress. In addition to the dependence on time, the GCN2 activity was responsive to increasing MeJA concentrations (Figure 1C). Treatment with 200 µM MeJA showed higher P-eIF2α versus 20 µM and 2 µM MeJA at the 30- and 120-minute time intervals respectively. In all the treatments above, the total eIF2α levels remain unchanged suggesting phosphorylation of eIF2α as a signaling mode for MeJA in Arabidopsis.

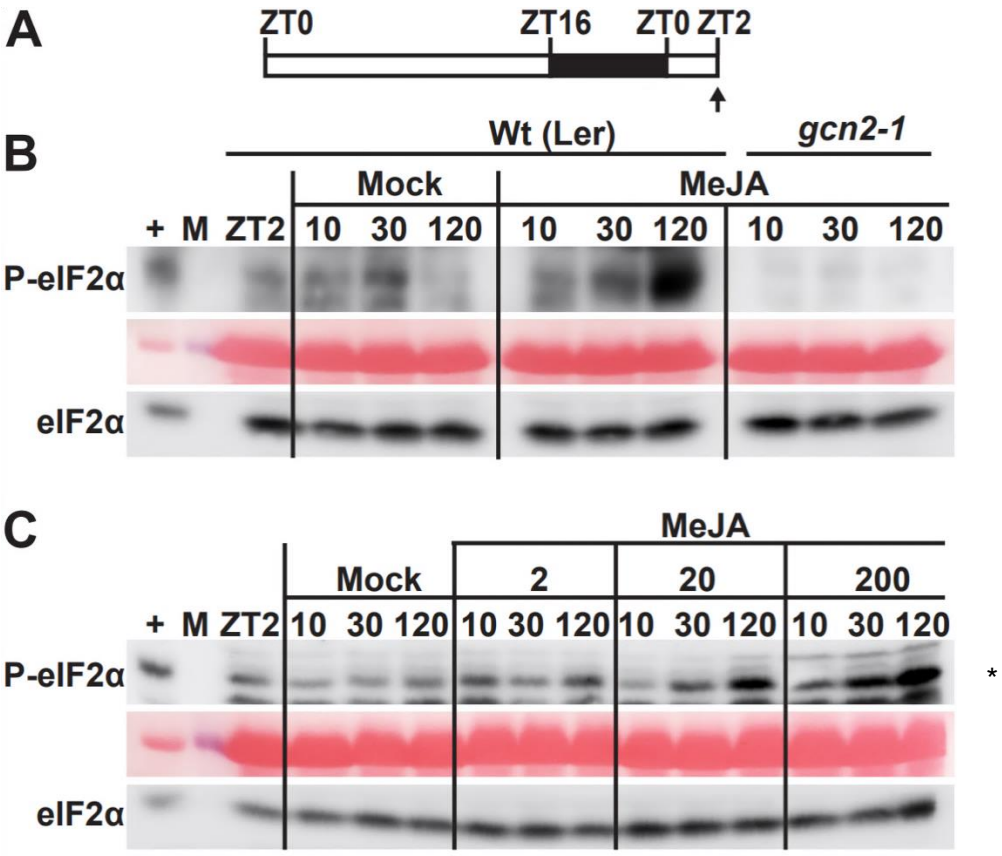


Figure 1: Methyl Jasmonate (MeJA) triggers GCN2-dependent eIF2α phosphorylation in a dose dependent manner. (A) Schematic of the 16 h light (Zeitgeber time ZT0 – ZT16) and 8 h dark (ZT16 – ZT0) cycle. The black arrow indicates the time of sampling at ZT2 (before treatment) and the start of the treatments. A faint signal of slightly higher molecular weight than expected ~38 kDa of P-eIFα in the *gcn2-1* mutant represents nonspecific binding of the P-eIF2α antibody **(B)** Immunoblot showing the time course of eIF2α phosphorylation in 12-days-old wild-type Landsberg (Wt(Ler)) and *gcn2-1* mutant (*gcn2-1)* seedlings treated with either 0.1% DMSO (Mock) or 20 mM MeJA (MeJA). **(C)**. Dosage treatment of wild-type seedlings with 2, 20, and 200 µM MeJA as described in panel (A, B). For the panels B and C: The middle panel shows the Rubisco large subunit (~ 55kDa) in the total protein sample after Ponceau S staining as a loading control; (+) arbitrary amount of total protein extract from hydrogen peroxide treated wild-type seedlings indicating unphosphorylated (eIF2α) or phosphorylated (eIF2α-P) protein (~38kDa); (10, 30, 120) sampling time in minutes; (M) Molecular weight marker. A partially cropped band at the bottom represents nonspecific binding of the P-eIF2α antibody.

Dark and ROS quenchers mitigate GCN2 activity under MeJA stress.

Phosphorylation of eIF2α is dependent on light and the treatment of seedlings with antioxidants or photosynthetic inhibitors alleviates GCN2 activation under stress (Lokdarshi et al., 2020a; Lokdarshi et al., 2020b) .To investigate whether MeJA triggered GCN2 activation is under the control of light, we dark acclimated the wild-type Arabidopsis seedlings for 24 h (Figure 2 A). Exposure of the dark-adapted seedlings to MeJA failed to illicit significant P-eIF2α signal versus treatment under light (Figure 2 B), supporting the role of light in the activation of GCN2 under MeJA stress. Additionally, to test the role of ROS in GCN2 activation, we pre-treated wild-type seedlings with the antioxidant reduced glutathione or the photosynthetic inhibitor DCMU. Interestingly, both DCMU and reduced glutathione suppressed GCN2 activation significantly in response to MeJA compared to the mock pre-treatment (Figure 2 D-E). Taken together, these observations are consistent with previous report by Lokdarshi et al. (2020a), highlighting the importance of light and ROS in GCN2 activation under MeJA stress.

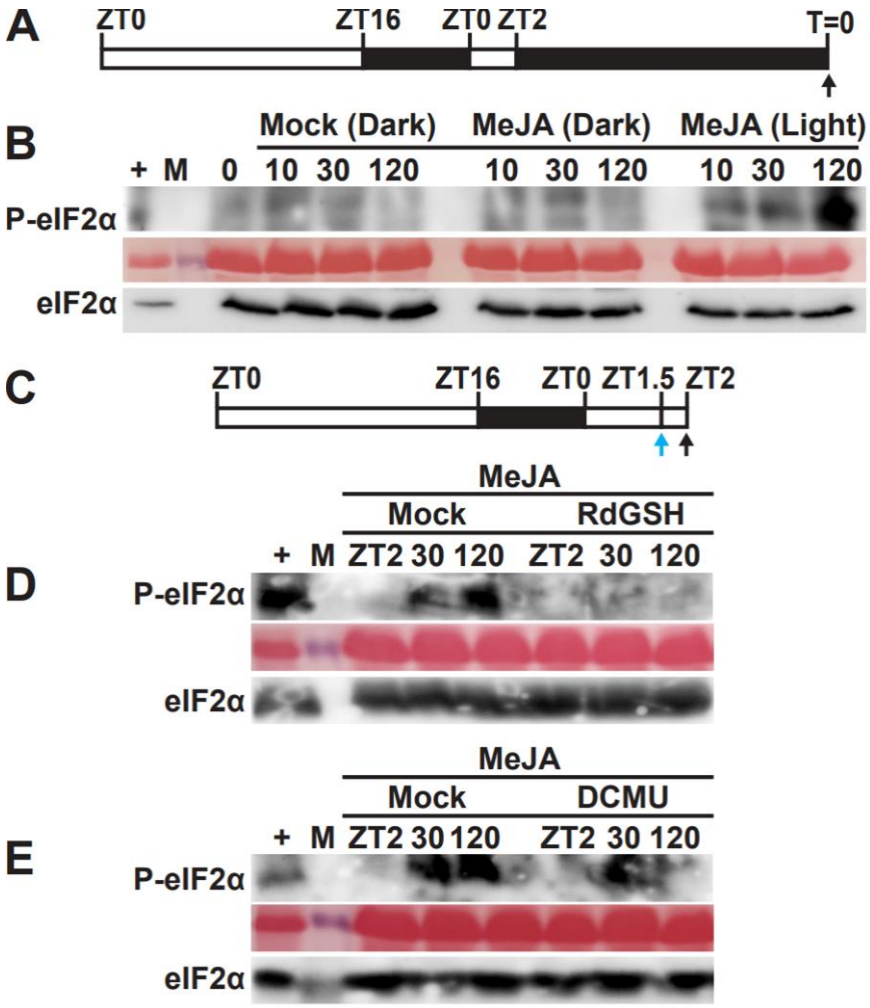


Figure 2: Dark and ROS quenchers attenuate GCN2 activation by MeJA. (A) Schematic of the 24 h dark acclimation to 12-days-old wild-type (Ler) seedlings. Black arrow indicates the time of samples at T=0 (at the end of 24 h dark period) and represents the time of reagent application. **(B**) eIF2α phosphorylation in wild-type seedlings treated with 0.1% DMSO (Mock (Dark)) or 20 µM MeJA (MeJA (Dark)) as described in panel A. MeJA treatment under light (MeJA (Light)) performed as described in Figure 1. **(C)** Growth regimen showing the time of treatment with either mock or ROS quenchers, thirty minutes prior to MeJA treatment (ZT1.5/blue arrow). Black arrow indicates the time of sampling at ZT2 (end of ROS quencher treatment) and represents the time of MeJA application. **(D-E)** eIF2α phosphorylation in wild-type seedlings treated with either water (Mock), or 1 mM reduced glutathione (RdGSH), or 30 µM (3-(3,4-dichlorophenyl)-1,1-dimethylurea) (DCMU) thirty minutes prior to 20 µM MeJA treatment under light as described in panel C. For detailed legend to panel B, D, and E, please see Figure 1.

MeJA stress induces an increase in ROS accumulation in wild-type seedlings

Ectopic treatment of plants with MeJA induces ROS accumulation as part of the defense response signaling (Zhang & Xing, 2008). Given that stress triggered GCN2 activation in Arabidopsis is dependent on ROS accumulation as reported by Lokdarshi et al. (2020a). We measured the levels of H_2O_2 (most predominant ROS) in response to mock (0.1% DMSO) and 20 µM MeJA treatment in wild-type seedlings. As expected, mock treatment did not induce significant changes in the H_2O_2 in comparison to the ZT2 time point (before MeJA treatment) (Figure 3). Interestingly, both 30 minutes and 120 minutes after MeJA treatment triggered a significant increase in the H_2O_2 in comparison to the ZT2 time point, supporting the previous reports of MeJA induced ROS accumulation in plants.

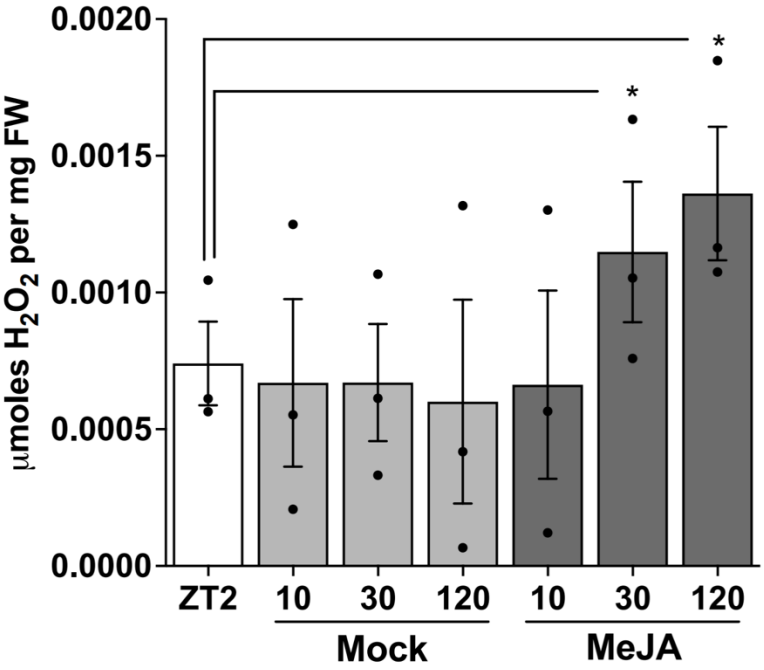
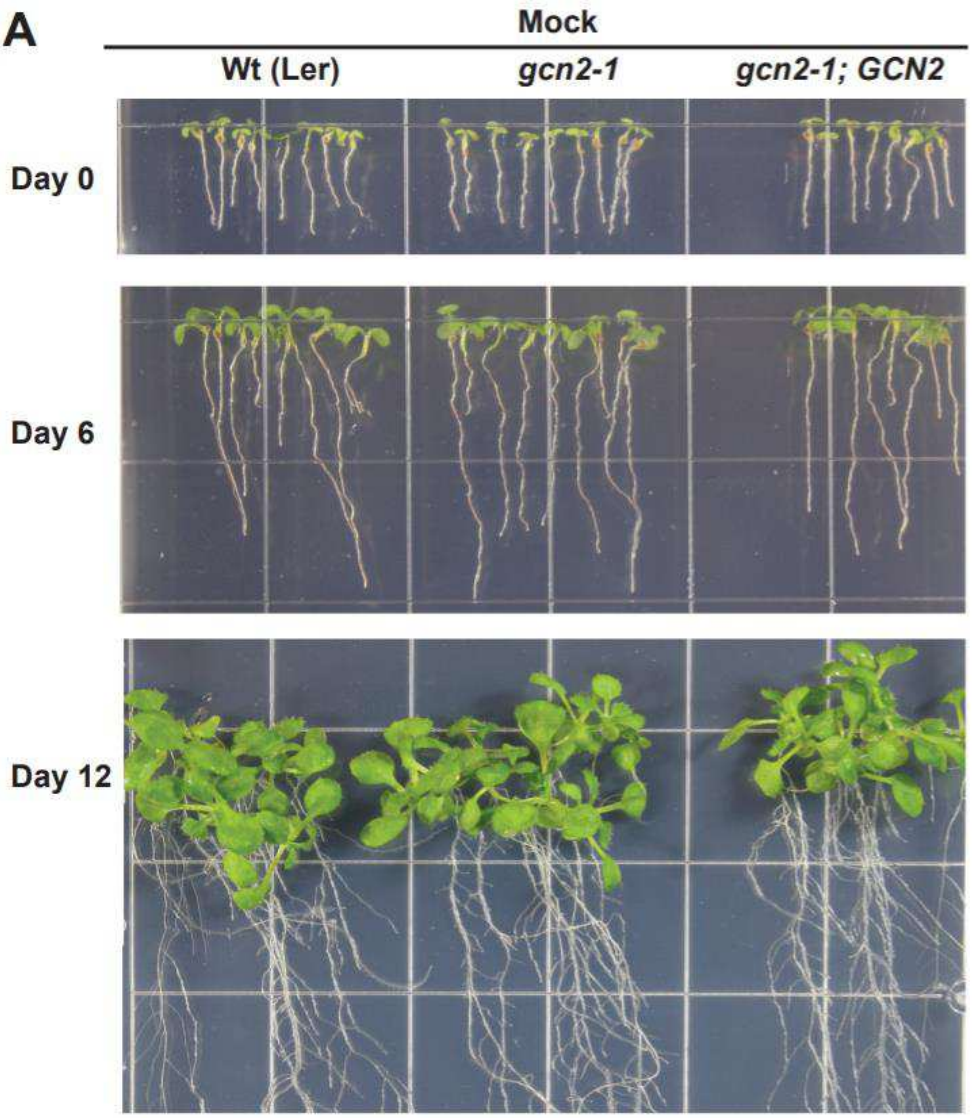


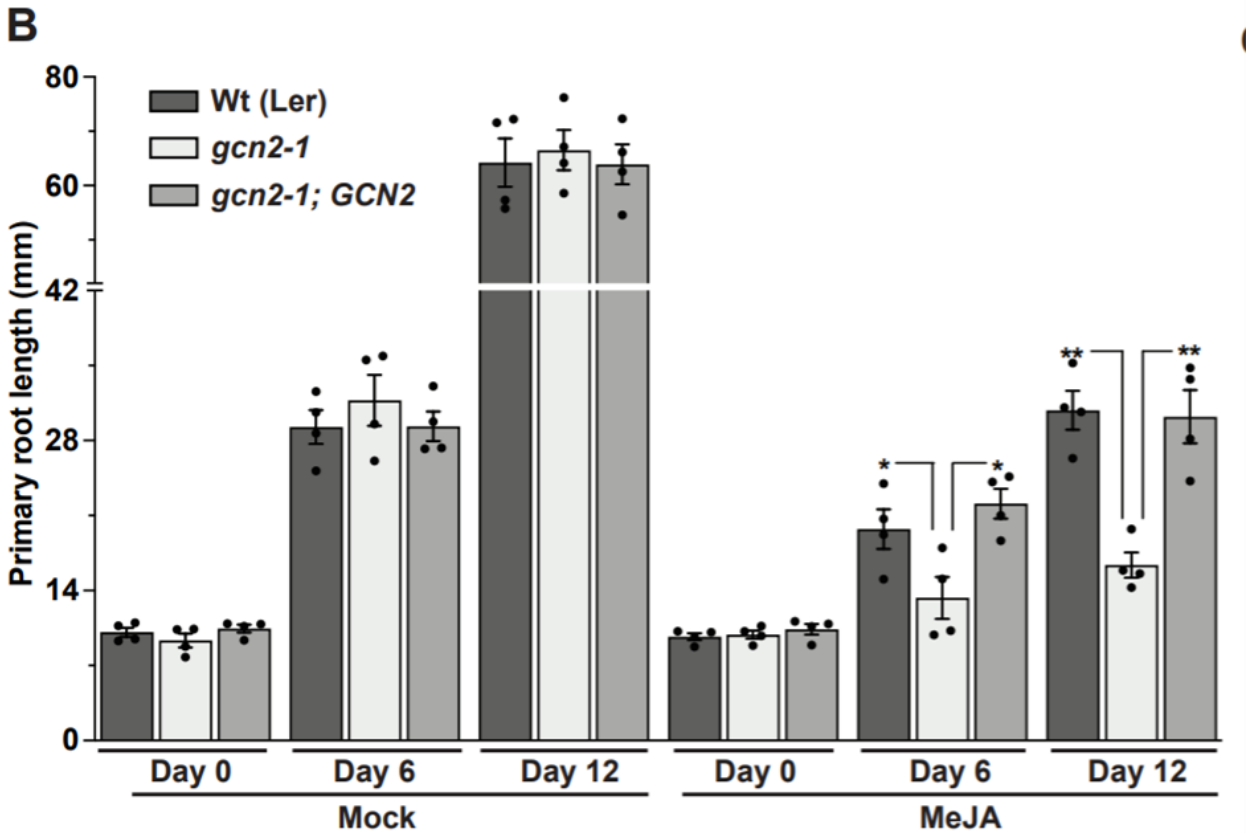
Figure 3: MeJA stress induces an increase in H_2O_2 levels. Amplex red assay showing relative H_2O_2 levels in wild-type (Ler) seedlings at the indicated time points. ZT2 = Zeitgeber Time (ZT)2; Mock = 0.1% DMSO; MeJA = 20 µM MeJA. Errors bars indicate standard error mean of three biological replicates. Welch's t-test P-value *<0.05.

Loss-of-*GCN2* mutant seedlings are sensitive to prolonged MeJA stress.

GCN2 is essential to the plant survival/adaption responses and the loss of *GCN2* function renders plant sensitive toward different types of stresses such as continuous light, excess light, salt, cold, and herbicides (Berrocal-Lobo et al., 2020; Lokdarshi et al., 2020a; Lokdarshi et al., 2020b; Zhang et al., 2008). To determine the role of *GCN2* under MeJA stress, we tested the phenotypic response of GCN*2* homozygous mutants: *gcn2-1* (Lageix et al., 2008; Zhang et al., 2008) in the Landsberg ecotype. On the normal growth (DMSO) medium, *gcn2-1* mutant seedlings appear phenotypically similar to both the wild-type and the *GCN2* complementation line, *gcn2-1; GCN2* (Lageix et al., 2008) (Figure 4 A). However, when seedlings are challenged to MeJA containing medium, *gcn2-1* mutant seedlings exhibit overall stunted growth, characterized by significantly reduced primary root length (at day 6 and day 12) and lower fresh weight (day 12) compared to the wild-type (Figure 4 B-C). Complementation of the *GCN2* gene under the native promoter (*gcn2-1; GCN2*) is able to rescue the growth defects observed in *gcn2-1* mutant seedlings, restoring both primary root length and fresh weight, suggesting GCN2 as an essential component of the MeJA stress signaling in Arabidopsis.


A
Mock
Wt (Ler)
gcn2-1
gcn2-1; GCN2
Day 0
Day 6
Day 12


MeJA
Wt (Ler)
gcn2-1
gcn2-1; GCN2


B
Wt (Ler)
gcn2-1
gcn2-1; GCN2
Primary root length (mm)
80
60
42
28
14
0
Day 0
Day 6
Day 12
Day 0
Day 6
Day 12
Mock
MeJA
*
*
**
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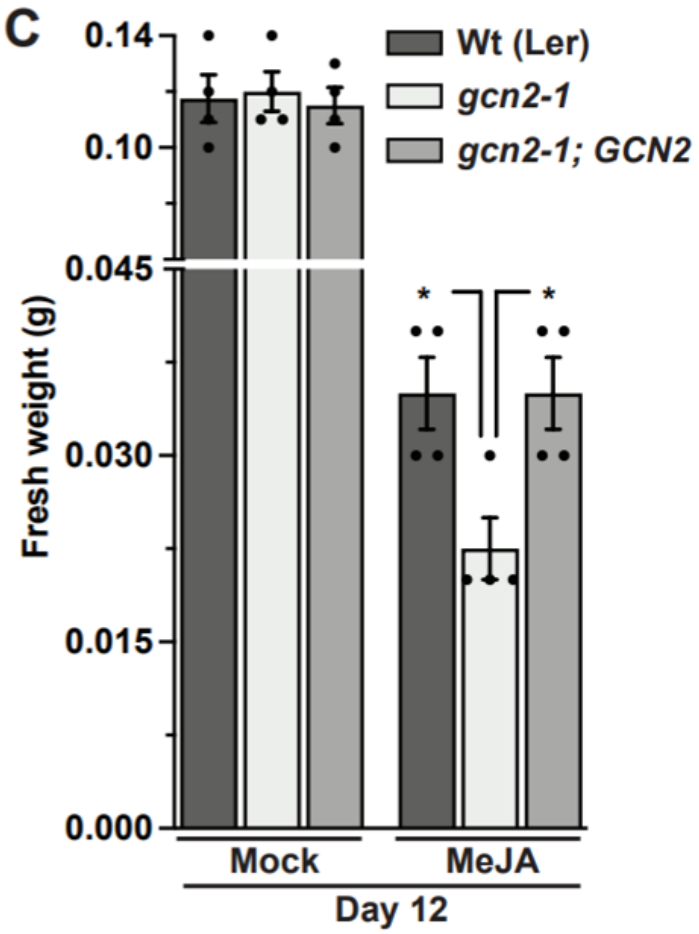


Figure 4. Loss of *GCN2* renders increased sensitivity towards MeJA stress. **(A)** Top panel: Representative images of 3-days-old wild-type (Wt(Ler)), *gcn2-1* mutant and GCN2 complementation (*gcn2-1*; GCN2) seedlings grown under standard long-day cycle of 16 h light (80$\pm$ 10mEin m^{-2} s^{-1}) and 8 h dark at 22°C with 50% humidity. Seedlings were transferred on plant media with DMSO (Mock) or 20 µM MeJA on Day 0. Middle and Bottom panel: Same seedlings after six (Day 6) and twelve days (Day 12) of growth. **(B)** Primary root length in millimeters (mm) of all seedlings on Day 0, 6 and 12 on Mock or MeJA supplemented media. **(C)** Fresh weight in grams (g) of all seedlings on Day 12 on Mock or MeJA supplemented media. Error bars represent standard error mean of at four biological replicates (Welch's t-test * P-value <0.05; ** P-value <0.005).

Sensitivity towards to prolonged MeJA stress in *gcn2* mutants is conserved across ecotypes

To assess whether GCN2 sensitivity to prolonged MeJA stress is consistent across ecotypes, we analyzed the phenotype of the GCN2 homozygous mutant, *gcn2-2*, in the Columbia ecotype (Faus et al., 2018). Similar to the Landsberg ecotype (Figure 4), *gcn2-2* mutants exhibited no significant phenotypic changes compared to the wild-type under normal growth conditions (DMSO medium) (Figure 5). However, when seedlings were exposed to a MeJA containing medium, the *gcn2-2* seedlings displayed a significant reduction in primary root length and fresh weight (at day 6 and day 12) compared to wild-type seedlings. These results support the idea of the conserved role of GCN2 as an essential component of Arabidopsis MeJA stress response signaling.

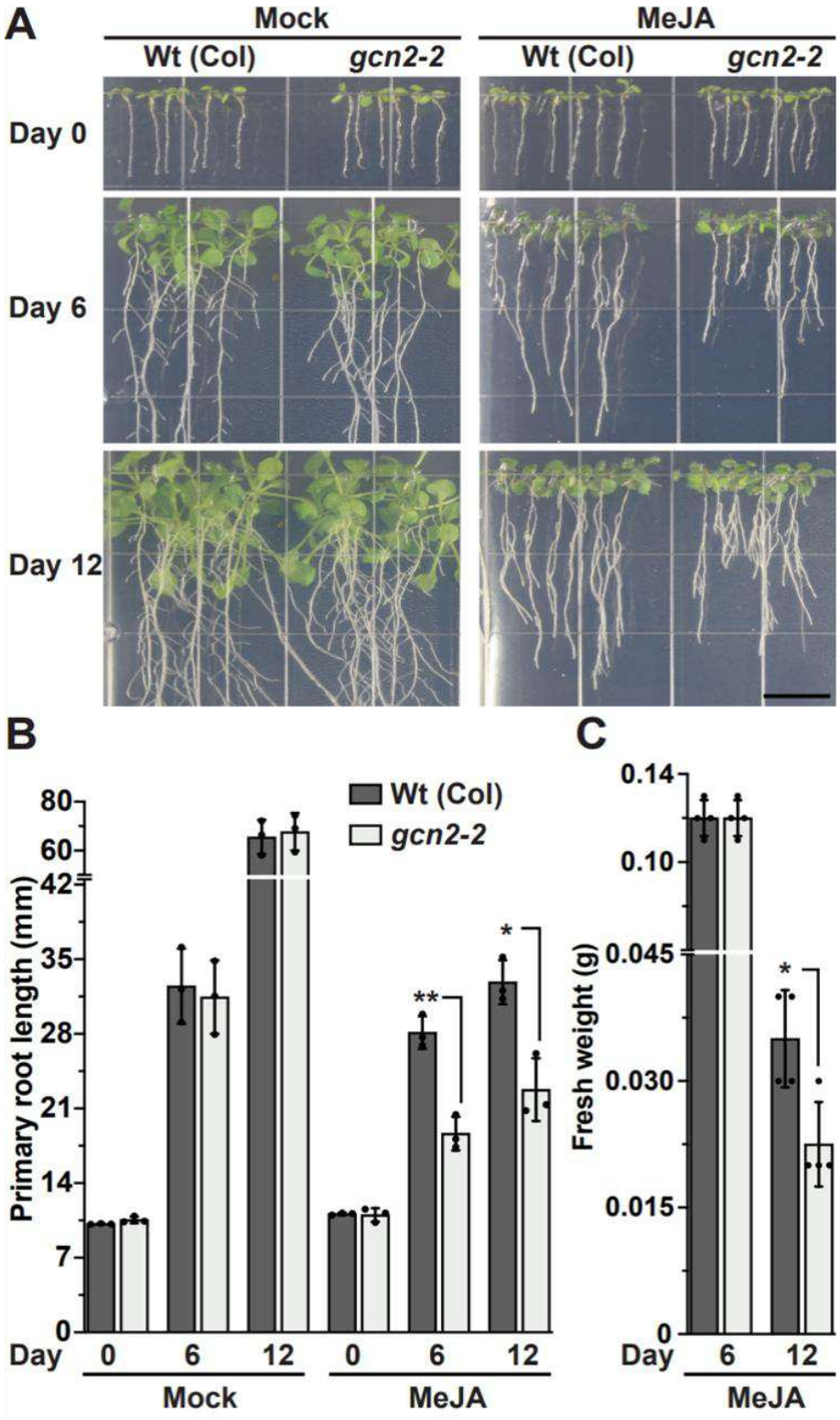


Figure 5. Loss-of-function *gcn2* mutant shows increased sensitivity towards prolonged MeJA stress versus wild-type (Columbia). (A) Wild-type (Col) and *gcn2-2* mutant were grown under standard long-day cycle of 16 h light (80± 10

mEin m^{-2} s^{-1}) and 8 h dark at 22°C with 50% humidity. At day 3, seedlings were transfer to new MS plates with either 0.1% DMSO or 20 µM MeJA. Root length was recorded at day 0, 6, and 12. **(B)** Primary root length of wild-type (Col) and *gcn2-2* mutant from panel (A) at day 12. **(C)** Fresh weight (grams) of wild-type (Col) and *gcn2-2 mutant* from panel (A) at day 12. Error bars represent standard error of the mean of four biological replicates. (Welch's t-test *P < 0.05; **P < 0.005)

Wild-type seedlings accumulate higher anthocyanin levels versus *gcn2* mutant under MeJA stress

Anthocyanins are flavonoid pigments that give red, violet, and purple coloration to plants based on the excitation wavelengths (Hatier & Gould, 2008) and share different functions, e.g., attractants of pollinators, and ROS quenchers (Agati et al., 2012; Kovinich et al., 2015). Given that MeJA treatment induces accumulation of ROS (Zhang and Xing, 2008), and a function of anthocyanin is to limit ROS accumulation as part of the stress resilience program, we quantified the level of anthocyanin in wild-type and *gcn2-1* mutant after 12 days of MeJA treatment. As expected, wild-type seedlings showed significantly higher levels of anthocyanin versus *gcn2-1* mutant after 20 µM of MeJA treatment (Figure 6). The high anthocyanin level in wild-type is further increased with 100 µM MeJA treatment compared to the *gcn2-1* mutant, suggesting that wild-type seedlings may have better ROS management. This could be due to the antioxidant function of anthocyanin, which could limit the accumulation of hydrogen peroxide, superoxide anion radical, and hydroxyl radical preventing oxidative damage. Ultimately, this potentially could help to better energy management in the wild-type seedlings under prolonged MeJA stress.

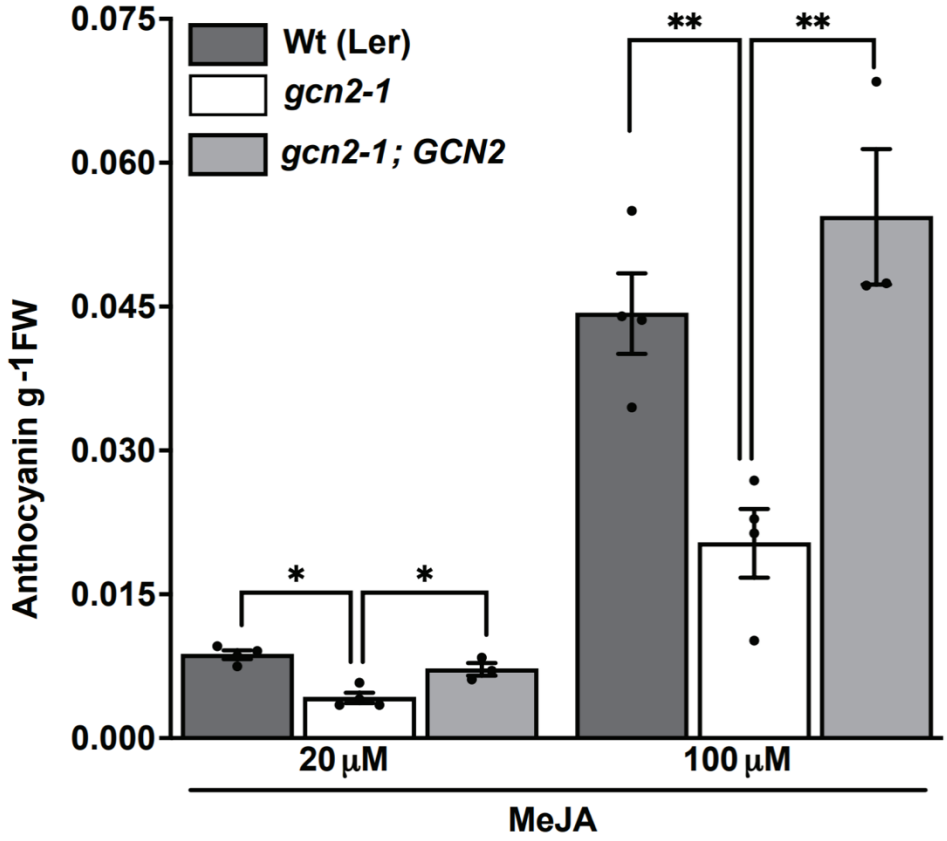


Figure 6. Wild-type seedlings accumulate higher anthocyanin versus *gcn2* mutant under prolonged MeJA stress. Relative anthocyanin levels in 12-days-old wild type (Wt(Ler)), *gcn2-1* mutant and GCN2 complementation (*gcn2-1*; GCN2) seedlings challenged to 20 µM MeJA, and 100 µM MeJA. Error bars represent standard error of the mean of four biological replicates. (Welch's t-test *P < 0.05; **P < 0.005)

Rate of global protein synthesis appears similar in wild-type and *gcn2* mutant in response to MeJA stress

In yeast and mammals, different environmental conditions such as herbicides, light, salt, and wounding, have been shown to down-regulate cytosolic protein synthesis through the activation of the GCN2 protein (Lageix et al., 2008; Li et al., 2018b; Wang et al., 2017; Zhang et al., 2008). This phenomenon appears to be conserved to some extent in plants, as demonstrated by Lageix et al. (2008) and Lokdarshi et al. (2020a) showing that seedlings sprayed with chlorsulfuron (CSF), led to a statistically significant difference in the large polysome fraction content between *gcn2-1* mutant and wild-type, suggesting *gcn2-1* exhibits higher ribosome loading. We hypothesize that under MeJA stress, a difference in the polysome profile will be observed as reported previously with CSF.

To investigate the role of GCN2 in regulating global translation under MeJA stress, we assessed the percentage of rRNA recovery (18S and 25S) from the sucrose gradient fractions using agarose gel electrophoresis (Figure 7). Both wild-type and *gcn2-1* seedlings showed similar rRNA recovery of the non-polysomal (NP), small polysomal (SP), and large polysomal (LP) fractions under standard growth conditions (DMSO) and MeJA treatment (Figure 8).

Interestingly, we found a similar trend *in vivo* translation status between the wild-type and *gcn2-1* mutant by performing a puromycin assay (Figure 9 A-C). There was no statistically significant difference in the anti-Puromycin

hybridization signal between wild-type and *gcn2-1* for both normal (Mock) conditions and MeJA stress, indicating a similar profile of translating ribosomes. These results identified MeJA stress as a condition that activates the GCN2-eIF2α module without inducing a detectable global translational repression in the seedlings.

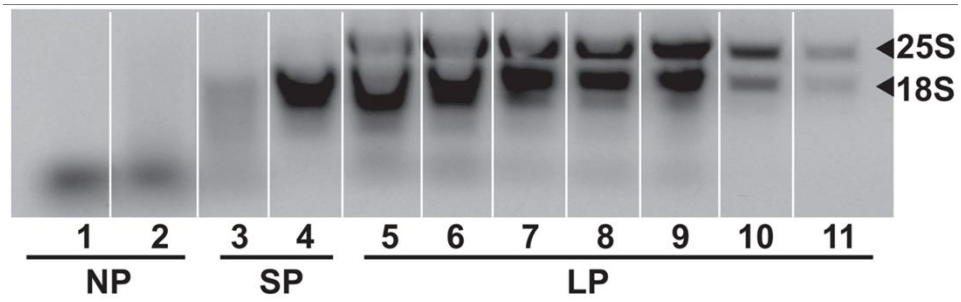
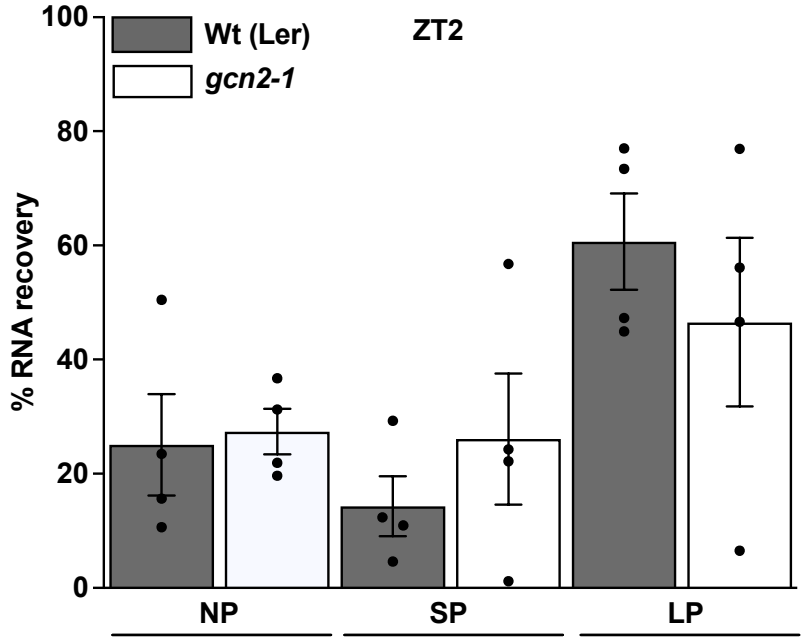
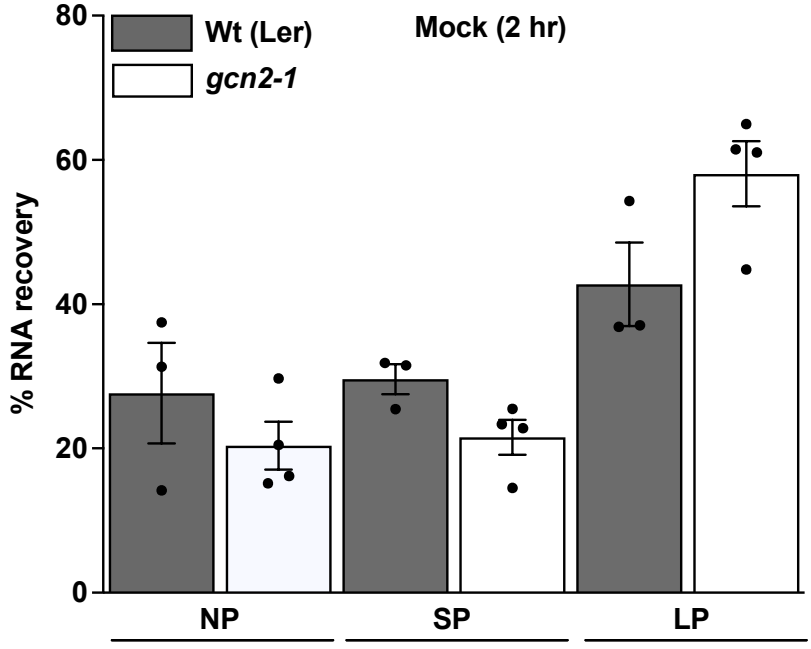


Figure 7. Agarose gel analysis of ribosomal RNA in wild-type and *gcn2-1* mutant. Representative image of ethidium bromide-stained agarose gel (1% w/v) showing the distribution of 18S (40S subunit) and 25S (60S subunit) ribosomal RNA in the 11 fractions. Fraction numbers 1-2 correspond to non-polysomes (NP), 3-4 to small polysomes (SP), and 5-11 to large polysomes (LP).


ZT2
Wt (Ler)
gcn2-1
% RNA recovery
100
80
60
40
20
0
NP
SP
LP


Mock (2 hr)
Wt (Ler)
gcn2-1
% RNA recovery
80
60
40
20
0
NP
SP
LP

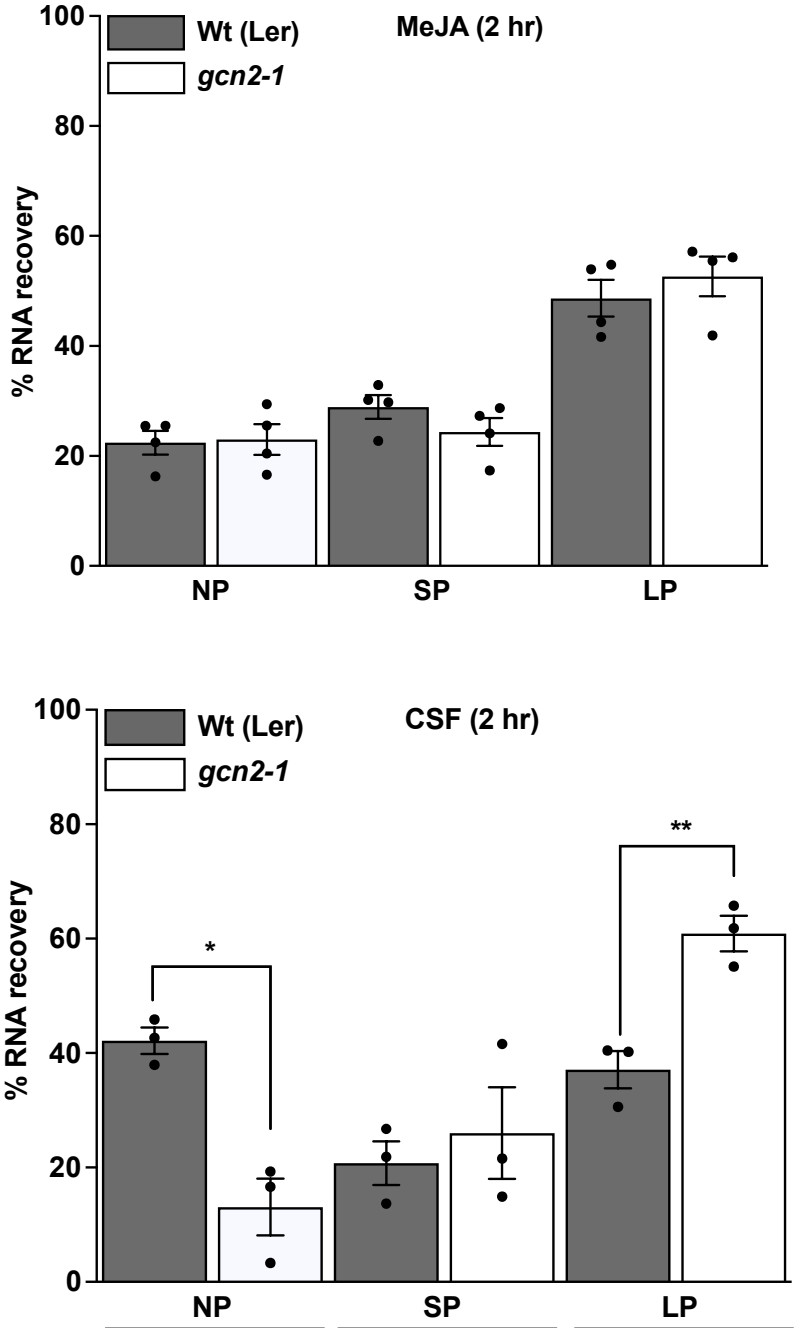


Figure 8. Translational state in wild-type and *gcn2-1* mutant. Histogram showing percent RNA recovery from sucrose gradient fractions of 12-days-old wild-type Landsberg (Wt(Ler)) and *gcn2-1* mutant seedlings at ZT2 and after two hour (2 hr) of treatment with 0.1% DMSO (Mock (2 hr)), or 20 µM (MeJA (2 hr)), or 0.6 µM chlorosulfuron (CSF (2 hr)). NP = Non Polysomal; SP = Small Polysomal; LP = Large polysomal. Error bars represent standard error mean of four biological replicates for ZT2, Mock, MeJA, and three biological replicates for CSF treatment (Welch's t-test *P < 0.05; **P < 0.005).

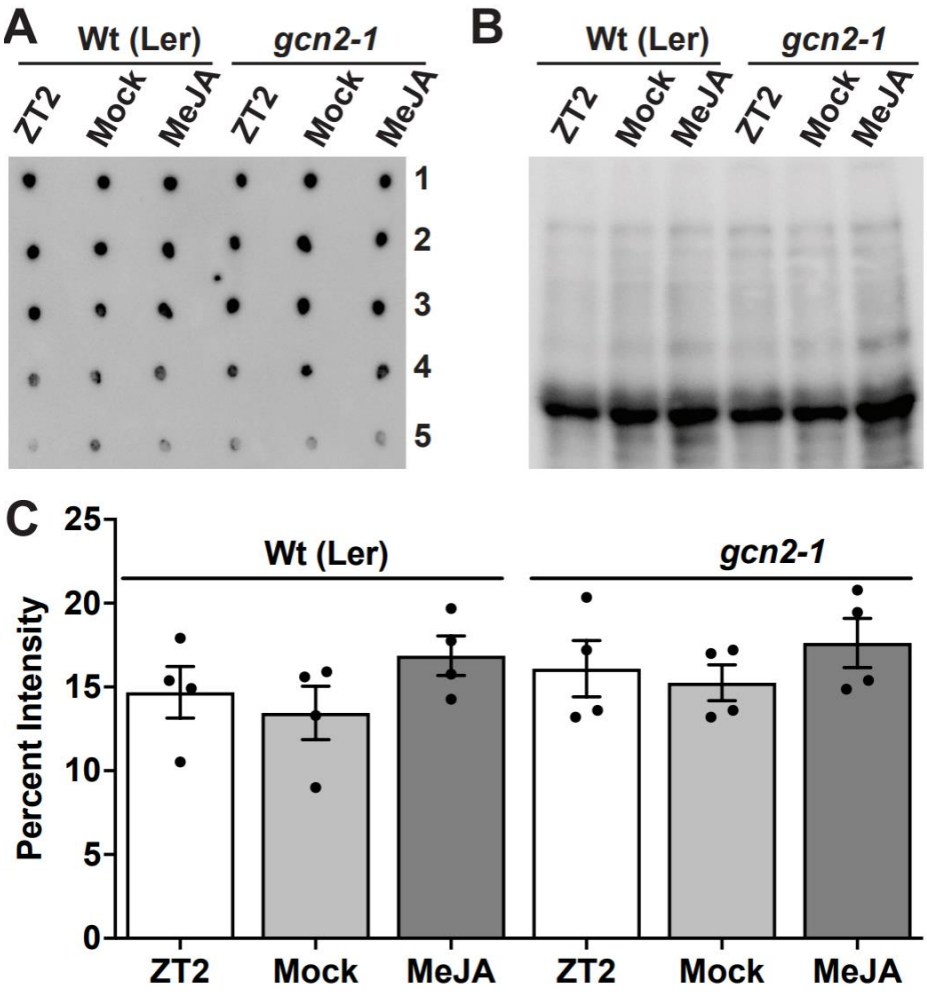


Figure 9. Wild-type and *gcn2-1* mutant show similar rate of protein synthesis under MeJA stress. (A) Dot blot analysis with total protein extracts of puromycin (PU) treated wild-type Landsberg (Wt(Ler)) and *gcn2-1* mutant (*gcn2-1*) seedlings at ZT2 and after 2 h of treatment with either 0,1% DMSO (Mock), or 20 µM methyl jasmonate (MeJA). The numbers on the right (1-5) represent a serial dilution of the total protein extract. PU incorporation was detected with anti-PU antibody. **(B)** Anti-PU immunoblot showing PU incorporation in wild-type and *gcn2-1* total protein extract after separation on 10% (w/v) sodium dodecyl sulfate polyacrylamide gel and transfer to Polyvinylidene difluoride (PVDF) membrane. **(C)** Quantification of dot blot signal intensity shown in panel A.

Discussion

The pan-eukaryotic GCN2 plays a pivotal role as a "stress sentinel kinase" in plants, efficiently mediating a wide spectrum of stresses, including abiotic, biotic, and xenobiotic challenges (Cho et al., 2022; Lokdarshi et al., 2022; Lokdarshi & von Arnim, 2022a; Wang et al., 2023). While the activation mechanism of GCN2 in yeast and mammals via uncharged tRNA binding is well established (Masson, 2019), the signals responsible for GCN2 activation in plants have remained elusive. Previous studies from have provided compelling evidence of chloroplastic reactive oxygen species (ROS) as rapid activators of cytosolic GCN2 protein under various stress conditions, such as exposure to herbicides, cold, excess light, and salinity (Lokdarshi & von Arnim, 2022). In the present study, we explored the conservation of the chloroplast ROS-GCN2-eIF2alpha signaling pathway in response to the plant hormone MeJA in *Arabidopsis thaliana*.

Lageix et al. (2008) demonstrated eIF2α phosphorylation at 4 h and 12 h post-MeJA treatment, while our current study further elucidates that MeJA-induced stress not only rapidly triggers eIF2α phosphorylation (within 30 minutes) but also requires the presence of light for this activation. In this study, we observed that the GCN2-eIF2α module responds to MeJA stress in a dose-dependent manner. We found that there is an increase in eIF2α phosphorylation levels when higher concentrations of MeJA are applied to Arabidopsis seedlings. Moreover, the reduction of P-eIF2α signal in seedlings pre-treated with photosynthetic inhibitor DCMU, or the ROS quencher reduced

glutathione, are consistent with our earlier findings regarding rapid Arabidopsis GCN2 activation in response to various stressors and the dependence on chloroplast function (Lokdarshi et al., 2020a; Lokdarshi et al., 2020b). Considering the chloroplast's role as the primary producer of ROS during photosynthesis and its transmission of redox and ROS signals to the cytosol for rapid adjustments in transcription and translation (Dietz, 2015; Dietz et al., 2016), our findings support the involvement of chloroplastic ROS in GCN2 activation.

Although the exact biochemical mechanism(s) underlying this common activation pathway remain to be fully elucidated, starvation of amino acids upon exposure to MeJA, resulting in the accumulation of uncharged tRNA within 10-30 minutes are unlikely to be the primary causes of rapid GCN2 activation. The early activation of the kinase following MeJA application likely stems from either direct activation by ROS or other GCN2 activators that are responsive to rapid ROS signaling. While we do not discount the conventional model of GCN2 activation by uncharged tRNA, we propose an alternative scenario where ROS serves as a quick activation ligand followed by the involvement of both ROS and uncharged tRNA. Dissecting these hypotheses will offer deeper insights into the activation mechanisms of GCN2 under MeJA stress and other ROS-related stresses.

Previous studies have demonstrated a clear sequence of events with herbicides inhibiting amino acid synthesis, where GCN2 kinase activation by the synthetic non-natural agent/herbicides such as chlorosulfuron in the presence of light leads to eIF2α phosphorylation, subsequent global translational repression, and hypersensitivity in *gcn2* mutants (Lokdarshi et al., 2020a; Lokdarshi et al.,

2020b). However, the translation response to natural GCN2-targeted abiotic stimuli like cold, salt, excess and high light appears less straightforward. Our research indicates that while GCN2 protein rapidly phosphorylates eIF2α in response to MeJA treatment, there's no detectable translational repression within the 2 h time frame, although *gcn2* mutants exhibit sensitivity to prolonged MeJA stress treatment. Similar patterns are observed for other ROS related stresses, where GCN2 activation doesn't correlate directly with translational repression (Lokdarshi et al., 2020a; Lokdarshi et al., 2020b). Moreover, high light stress doesn't induce GCN2-dependent translational repression despite *gcn2* mutants' sensitivity to it.

These observations suggest that not every instance of eIF2α phosphorylation leads to global translational repression, and only some instances of translational repression are dependent on eIF2α phosphorylation. Given that ROS accumulation is localized in Arabidopsis leaves exposed to high light or herbicide stress (Lokdarshi et al., 2020a; Lokdarshi et al., 2020b), it is plausible that retrograde control of translation may be a potent mechanism for immediate spatial and temporal adjustment of the cytosolic translation and chloroplast proteome. Furthermore, it is hypothesized that retrograde control of translation under high light could allow for spatially heterogeneous protein synthesis (Dietz, 2015). This molecular arrangement would mean preferential loading of transcripts that directly code for repair and photosynthesis related proteins thereby supporting a faster translational retrograde circuit. How does the GCN2-

eIF2α module function within the proposed spatiotemporal translation control remains to be tested.

Taken together, our study reveals new information that MeJA signaling in plants involves the highly conserved GCN2-eIF2α module working under the command chloroplast ROS. Despite *gcn2* mutant displaying similar *in vivo* translation status as the wild type under short MeJA challenge, it exhibits heightened sensitivity to prolonged MeJA stress. These findings bolster the proposed model of chloroplastic ROS triggering rapid activation of cytosolic GCN2, regulation of translation under diverse abiotic stresses and long-term adjustments to energy management. The accumulation of ROS in chloroplasts induced by other stressors like UV radiation (Kataria et al., 2014) and *Botrytis cinerea* (Rossi et al., 2017) infection suggests the evolution of a highly conserved mechanism wherein the GCN2-eIF2alpha module responds to chloroplastic ROS cues to restore cellular homeostasis. Future research should elucidate the biochemical and molecular events leading to GCN2 activation under MeJA and ROS related stresses, shedding light on the elusive integrated stress response pathway in plants. Furthermore, investigating the regulation of global translation versus stress-specific mRNA targets warrants further exploration for deeper understanding of the role of GCN2 as a stress sentinel kinase in plants.

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