Rapid activation of the *Arabidopsis thaliana* GCN2-eIF2α module by salicylic acid: Phenotypic and translational similarities between *gcn2* mutant and wild-type

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Abstract

Salicylic acid (SA) is a natural phytohormone involved in plant development and defense against biotrophic pathogens, acting as a potent stress signaling molecule to stimulate downstream pathways for cellular resource management. While most existing literature focusses on the role of SA in gene expression at the transcriptional level (mRNA synthesis), its role in translational regulation (protein synthesis) remains under-researched. While both are important, translational regulation serves as a fast method of regulating gene expression, allowing cells to rapidly adapt to quick changes in their environmental conditions. A protein kinase, General Control Nonderepressible 2 (GCN2), plays a major role in regulating protein synthesis by phosphorylating the alpha subunit of eukaryotic initiation factor 2 (eIF2), resulting in downregulation of global protein synthesis. This thesis investigates the role of SA in activating the GCN2-eIF2α module in *Arabidopsis thaliana* via chloroplastic reactive oxygen species (ROS). We find that SA treatment of Arabidopsis seedlings results in eIF2α phosphorylation, with dependence on GCN2, light, and photosynthesis. Surprisingly, increasing SA dosage does not correspond with increasing eIF2α phosphorylation. Additionally, wild-type and *gcn2-1* knockout mutant seedlings under prolonged SA stress display similar phenotypic parameters, including fresh weight and primary root length. These findings provide insight into the signaling mechanisms of the GCN2 protein under SA stress, and suggest that alternate mechanisms may be responsible for mediating phenotypic responses to SA. Still, further research is needed to understand the translational state of plant cells in response to SA stress, as well as the direct interaction of photosynthetic byproducts with GCN2 for its activation.

Introduction

Plants constantly face a wide range of environmental challenges that threaten their growth, development, and survival. Broadly, plant stress refers to exposure to non-ideal growth conditions that can impair development and, in extreme cases, lead to death. These stressors are typically classified as abiotic (e.g. heat, cold, light, drought, salt, flooding), biotic (e.g. bacteria, parasites, fungi, herbivores), and xenobiotic, meaning non-natural chemical agents such as herbicides (e.g., chlorosulfuron, methyl viologen, and glyphosate) (Anand et al., 2023; Chakraborty et al., 2023). Naturally, plants have evolved a diverse array of genetic, cellular, biomolecular, and physiological strategies to survive stressful conditions. A deeper understanding of the plant germplasm and the different biochemical and molecular mechanisms of the plant stress responses is crucial for enhancing agricultural productivity and promoting environmental sustainability.

At a molecular level, plant stress signaling involves adjustments to both transcription (DNA to mRNA) and translation (mRNA to protein) (Chiba et al., 2013; Muench et al., 2012; Sun et al., 2022). Translational control comprises the biochemical and cellular mechanisms that modulate the efficiency of protein synthesis by adjusting the initiation, elongation, and/or termination of ribosomes as they translate mRNA into proteins (Lokdarshi & von Arnim, 2022). In fact, translational control complements transcription as a more rapid, resource-efficient means of regulating gene expression at

the level of protein synthesis, capable of responding to rapidly fluctuating conditions (Merchante et al., 2017; Sonenberg & Hinnebusch, 2009). Thus, translational control has the potential to explain how plant cells nimbly adjust to the dynamic changes in their environment.

Translational control in plants is majorly regulated by three serine/threonine protein kinases: SNF1-related protein kinase 1 (SnRK1), Target of Rapamycin (TOR), and the general control of nonderepressible (GCN)2 (Camacho et al., 2020; Son & Park, 2023; Wu et al., 2024). While SnRK1 responds to low cellular energy and TOR to growth and anabolic processes under favorable environments, the GCN2 protein is primarily associated with stress signaling in plants (Camacho et al., 2020; Lokdarshi & von Arnim, 2022; Son & Park, 2023). Notably, the interplay between SnRK1, TOR, and GCN2 highlights the complex regulatory network required to manage translation as a major node in balancing growth and cellular homeostasis under a wide range of stresses.

The phosphorylation of eIF2α by the GCN2 protein represents a key translational control paradigm across different plant species (e.g., Arabidopsis, tobacco, barley, and wheat) (Halford, 2005; Li et al., 2018; Zhang et al., 2008). The eIF2 protein is a trimeric GTP-binding GTPase that charges the 40S small subunit of the ribosome with initiator methionyl-tRNA (tRNAiMet), a critical step in translation initiation (Wek, 2018). Under diverse stresses, eIF2α is phosphorylated (henceforth labelled as P-eIF2α) on a highly conserved N-terminal serine residue (S51) as part of an evolutionarily conserved homeostatic stress response program termed the integrated stress response (ISR) (Chang et al., 2024; Pakos-Zebrucka et al., 2016). In metazoans and yeast, P-eIF2α

then assumes an inhibitory role and binds to eIF2β, the guanine nucleotide exchange factor that converts inactive ternary complex (GDP-bound) to an active ternary complex (GTP-bound) (Wek, 2018). Thus, P-eIF2α results in the repression of translation initiation by reducing the availability of ternary complex (eIF2-GTP- tRNAiMet) (Wek, 2018). The ISR pathway is profoundly conserved among plants and metazoans with respect to core events: eIF2α phosphorylation and repression of general protein synthesis (Pakos-Zebrucka et al., 2016). The key highlight in plants, however, is the presence of only GCN2 that phosphorylates eIF2α under different stress stimuli versus other eukaryotes where P-eIF2α is catalyzed by up to four structurally related kinases that respond to specific stress inputs (Chang et al., 2024).

Activation of the GCN2-eIF2α module in plants has been shown previously in response to numerous stresses, including amino acid starvation by herbicides, bacterial pathogen, UV radiation, cold, wounding, and NaCl (Lageix et al., 2008; Liu et al., 2019; Llabata et al., 2019; Lokdarshi et al., 2020b). Specifically, studies in *Arabidopsis thaliana* have demonstrated that the GCN2-eIF2α module is rapidly activated (within 10 minutes) in response to reactive oxygen species (ROS) emanating from the chloroplast under diverse stress conditions (Lokdarshi et al., 2020a). Though the precise mechanism of AtGCN2 activation by ROS remains unclear, chloroplast-derived ROS serve as potential ligands for GCN2 activation under numerous stresses (Lokdarshi et al., 2020a). In addition, the loss-of-function *gcn2* mutant (*gcn2-1*) shows increased sensitivity towards numerous stresses, such as excess light, cold, salt, and herbicides, and exhibits changes in both mRNA and protein profiles, distinct from the wild-type (Lageix et al., 2008; Lokdarshi et al., 2020a, b). These findings - (1) highlight the role of

the AtGCN2-eIF2α module as a crucial missing signaling link between the chloroplast and cytosolic translation machinery, and (2) suggest the presence of a fast regulatory switch that modulates protein synthesis via ROS, complementing the conventional chloroplast-to-nucleus retrograde signaling pathway.

Building from this foundation, we want to investigate the role of salicylic acid (SA) in plant stress on a biochemical and physiological level. SA is a notable plant defense hormone involved in immune responses and systemic acquired resistance (SAR), and is known to activate GCN2 (Lageix et al., 2008; Spoel & Dong, 2024; Wang et al., 2017; Zhigailov et al., 2020). The role of SA as an important signaling molecule has been well documented, affecting the activities of both metabolic enzymes and transcriptional cofactors under immune responses (Pokotylo et al., 2019; Wang et al., 2007). In Arabidopsis, SA is primarily synthesized via the isochorismate synthase (ICS) pathway in response to biotrophic pathogen infection. Chorismate is converted to isochorismate (IC) in the chloroplast by ICS1/2 proteins. IC is then transported to the cytosol by EDS5 transporter and conjugated to glutamate by PBS3 to form the IC-9-Glu intermediate. This intermediate then spontaneously breaks down to form SA, in some cases aided by the protein EPS1 (Peng et al., 2021). Once SA is fully formed in the cytoplasm, it can subsequently aid in regulating cellular processes to mitigate effects of pathogen infection.

While SA is recognized as a vital signaling molecule, the role of SA in translational regulation remains underexplored. Our central hypothesis proposes that SA activates the AtGCN2-eIF2α module by leveraging chloroplastic ROS. We aim to determine: (1) the extent to which SA influences the activity of the cytosolic GCN2-

eIF2α module, (2) whether SA-induced stress aligns with existing findings of chloroplastic ROS, and (3) how these molecular interactions manifest in phenotypic changes in the Arabidopsis seedlings.

Literature Review

GCN2 protein and eIF2α kinase regulatory pathway

In eukaryotes, cytosolic protein synthesis under stress conditions is majorly regulated at the step of translation initiation. This key program is orchestrated by a group of structurally related protein kinases, known as eIF2α kinases. In metazoans, there are four eIF2α kinases that phosphorylate eIF2α at a conserved N-terminal serine residue in response to different types of stress: GCN2, Protein Kinase R (PKR), Protein Kinase R-like Endoplasmic Reticulum Kinase (PERK), and Heme-Regulated Inhibitor (HRI) (Fig. 1). However, *A. thaliana* and *S. cerevisiae* possess only the GCN2 protein for phosphorylating eIF2α at the conserved N-terminal serine (Baird & Wek, 2012; Lokdarshi & von Arnim, 2022).

GCN2's discovery and mechanisms

The GCN2 protein was first discovered and characterized in yeast (Wek et al., 1989), and further investigation led to the discovery that GCN2 activation most commonly involves recognition and binding of uncharged tRNA during amino acid starvation conditions (Dong et al., 2000) (Fig. 2). Structural analysis of GCN2 using cryo-EM revealed that the histidyl-tRNA synthetase-like (HIS RS) domain, which directly recognizes and interacts with uncharged tRNA molecules, initiates activity of the kinase domain (Solorio-Kirpichyan et al., 2024). In fact, downstream effects of GCN2 activation reflect direct responses to uncharged tRNA (i.e. amino acid starvation). For example, P-

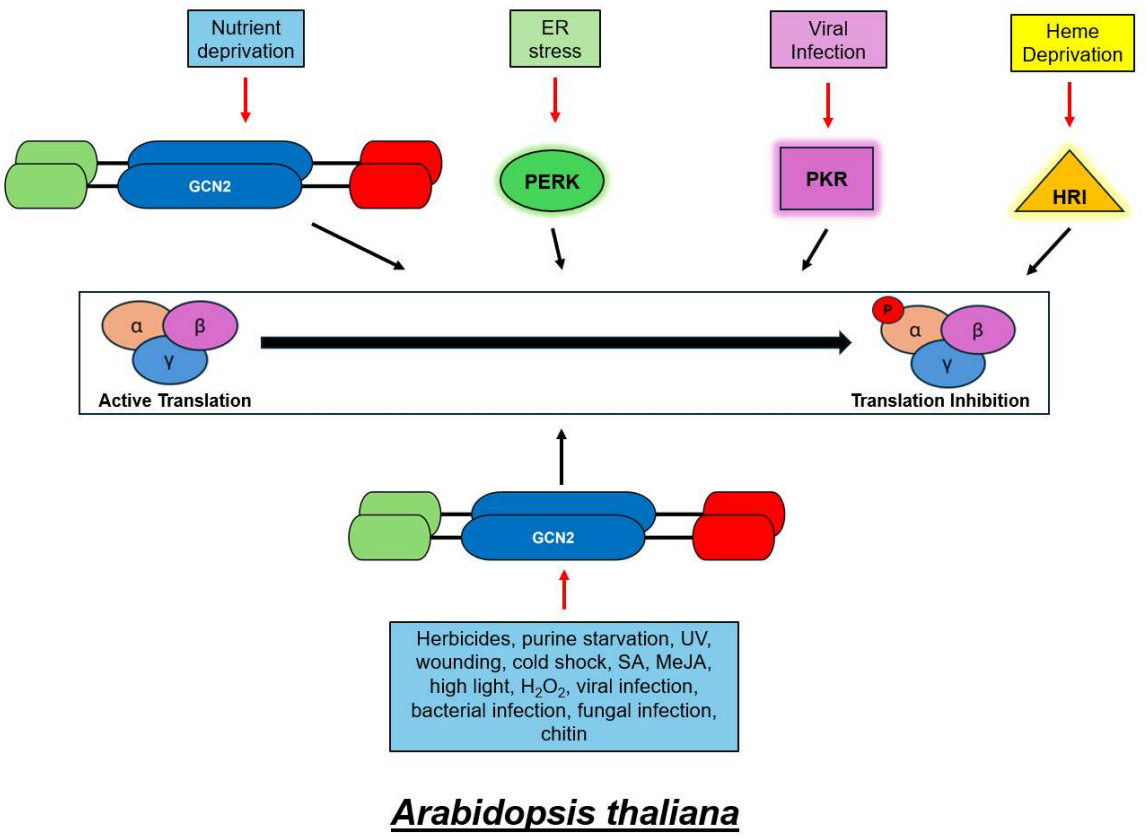

Figure 1. Comparative illustration of the major stress signaling kinases in mammals and *Arabidopsis thaliana*: Illustration of the current understanding of the GCN2-dependent P-eIF2α under different stresses. Red lines indicate stimuli activation of the respective kinase. Adapted and modified from Lokdarshi & von Armin, 2022.

eIF2α has been shown to increase production of the transcription factor GCN4, leading to expression of genes encoding enzymes involved in amino acid biosynthesis (Hinnebusch, 1994). Further investigation into overexpression of the GCN2 protein revealed other important aspects of its downstream mechanisms in wheat (*Triticum aestivum*), where overexpression led to decreased levels of free amino acids and expression of sulfur deficiency-induced genes, revealing TaGCN2's important role in amino acid biosynthesis and sulfur deprivation (Byrne et al., 2012). Therefore, from a

conventional standpoint, uncharged tRNA binding under amino acid starvation is the widely accepted model of GCN2 activation.

GCN2 activity in mammals versus plants

While regulation of protein synthesis via P-eIF2α is a highly conserved node across all eukaryotes, the interplay between GCN2 and other translational regulatory mechanisms differs between organisms. For example, in embryonic fibroblast tissue, PERK and GCN2 have been shown to work in tandem to regulate P-eIF2α (Hamanaka et al., 2005). Additionally, Target of Rapamycin (TOR) is a serine/threonine protein kinase which has demonstrated a role in the promotion of protein synthesis, showing possible antagonistic effects to GCN2 and the entire eIF2α kinase family (Hofmann et al., 2012). However, looking specifically at *Arabidopsis thaliana*, silencing or overexpression of TOR does not affect P-eIF2α status under chlorosulfuron (CSF) stress, suggesting that GCN2 is independent of TOR activity in Arabidopsis (Lageix et al., 2008). Further, induction of P-eIF2α by GCN2 is also independent of MAP kinases in Arabidopsis, a group responsible for regulating signaling pathways involved in plant development and stress responses (Llabata et al., 2019).

Another way that plants differ from other organisms in this regulation is the extent to which P-eIF2α leads to translation inhibition. In plants, eIF2 does not bind GDP very strongly. This means that cycling of GDP to GTP may occur without any action from the β subunit, allowing for normal translation initiation even under P-eIF2α (Browning & Bailey-Serres, 2015). Further, in wheat, intense P-eIF2α signal only results in a 10-15% reduction in translation of certain mRNAs, with the plant remaining at a relatively high global translation state overall (Zhigailov et al., 2020). These findings highlight the key

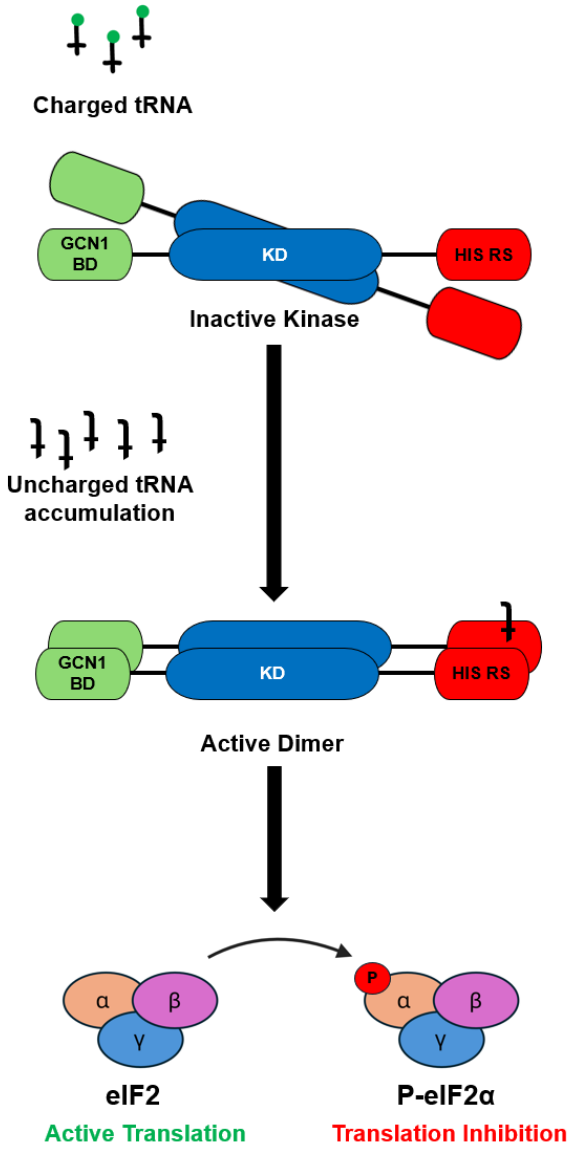

Figure 2. Conventional Model of GCN2 Activation: Illustration showing the general mechanism of GCN2 activation by uncharged tRNA. Amino acid starvation, leading to an accumulation of uncharged tRNAs, results in the direct binding of uncharged tRNA molecules to the histidyl-tRNA synthetase-like domain (HIS RS). Along with an association with GCN1 at the GCN1 binding domain (BD), the accumulation of uncharged tRNAs causes a parallel dimerization of the GCN2 protein monomers, allowing the kinase domain (KD) to become active. This allows for phosphorylation of eIF2 on its α subunit, resulting in downregulation of global protein synthesis.

differences in regulatory mechanisms employed across eukaryotic species and emphasize the importance of understanding plant stress responses in their own context.

Other Kinases Affecting eIF2α phosphorylation and translation in plants

Though GCN2 is credited as the sole kinase to phosphorylate eIF2α at S51 in plants, it is not the only kinase to phosphorylate eIF2α. A recent study has shown that Arabidopsis Suppressor of Phya-105 (SPA1) phosphorylates eIF2α under light conditions at its carboxyl terminal to promote translation initiation and binding of eIF2α with its complementary β and γ subunits (Chang et al., 2024). This activity is opposed to that of GCN2, as GCN2 is responsible for inhibiting translation at the step of initiation. This emerging evidence challenges the existing scope of research into plant translational regulation and opens avenues for investigating the antagonistic effects of these two proteins.

Complementary to the activity of GCN2, SnRK1 is an important kinase responsible for phosphorylating eIF4E and eIFiso4E in Arabidopsis to inhibit translation in response to energy needs for maintaining cellular homeostasis (Bruns et al., 2019). On this note, it is important to highlight that, while eIF2α plays a key role in translation, it is not the only factor capable of regulating this process, emphasizing the need for broader research in this area to understand the dynamic network of signaling molecules involved in regulating translation and energy efficiency under stress conditions.

GCN2 activity in diverse stress responses

Besides activation of GCN2 by uncharged tRNAs, many stressors induce P-eIF2α in Arabidopsis beyond amino acid deprivation (e.g. CSF, purine starvation, UV, wounding, cold shock, SA, methyl jasmonate (MeJA), high light, H_2O_2, viral infection,

bacterial infection, fungal infection, and chitin alone) (Berrocal-Lobo et al., 2020; Lageix et al., 2008; Liu et al., 2019; Zhang et al., 2008). Additional support to the hypothesis that GCN2 is required in mediating stress responses to a diverse set of stressors is provided by studies investigating Arabidopsis *gcn2*-knockout mutants. These mutants show a complete lack of P-eIF2α under purine starvation, UV, CSF, wounding, and viral pathogen infection (Lageix et al., 2008; Zhang et al., 2008). Additionally, *gcn2* mutants have demonstrated active translation states compared to wild-type plants under CSF stress, as well as a lack of photosynthetic inhibition under glyphosate treatment, which is usually observed in the wild-type (Faus et al., 2015; Lageix et al., 2008). From a phenotypic standpoint, mutants under drought stress have demonstrated increased fresh weight and reduced water loss compared to the wild-type, suggesting GCN2's role as a negative regulator of root growth and fresh weight under drought stress (Terry et al., 2015). From these investigations, GCN2 has emerged as a vital protein involved in a variety of stress signaling pathways, raising questions regarding its activation if not only by the conventional method of binding to uncharged tRNAs.

GCN2-GCN1 interaction

Two studies have demonstrated that, in *Arabidopsis thaliana,* GCN2 requires interaction with GCN1, a sensor of stalled ribosomes, for P-eIF2α to occur under several stress conditions, including SA, UV, cold shock, and amino acid starvation (Wang et al., 2017; Zheng et al., 2024). Supporting this mechanism, Arabidopsis *gcn1* mutants do not show a significant decline in protein translation under macronutrient deprivation (nitrogen or potassium) compared to wild-type, suggesting that GCN2 is not activated in the absence of GCN1 (Cui et al., 2025). Collectively, these findings

underline the critical role of GCN1 in the upstream activation of the GCN2-mediated stress response and open new avenues for exploring the broader network of ISR signaling pathways.

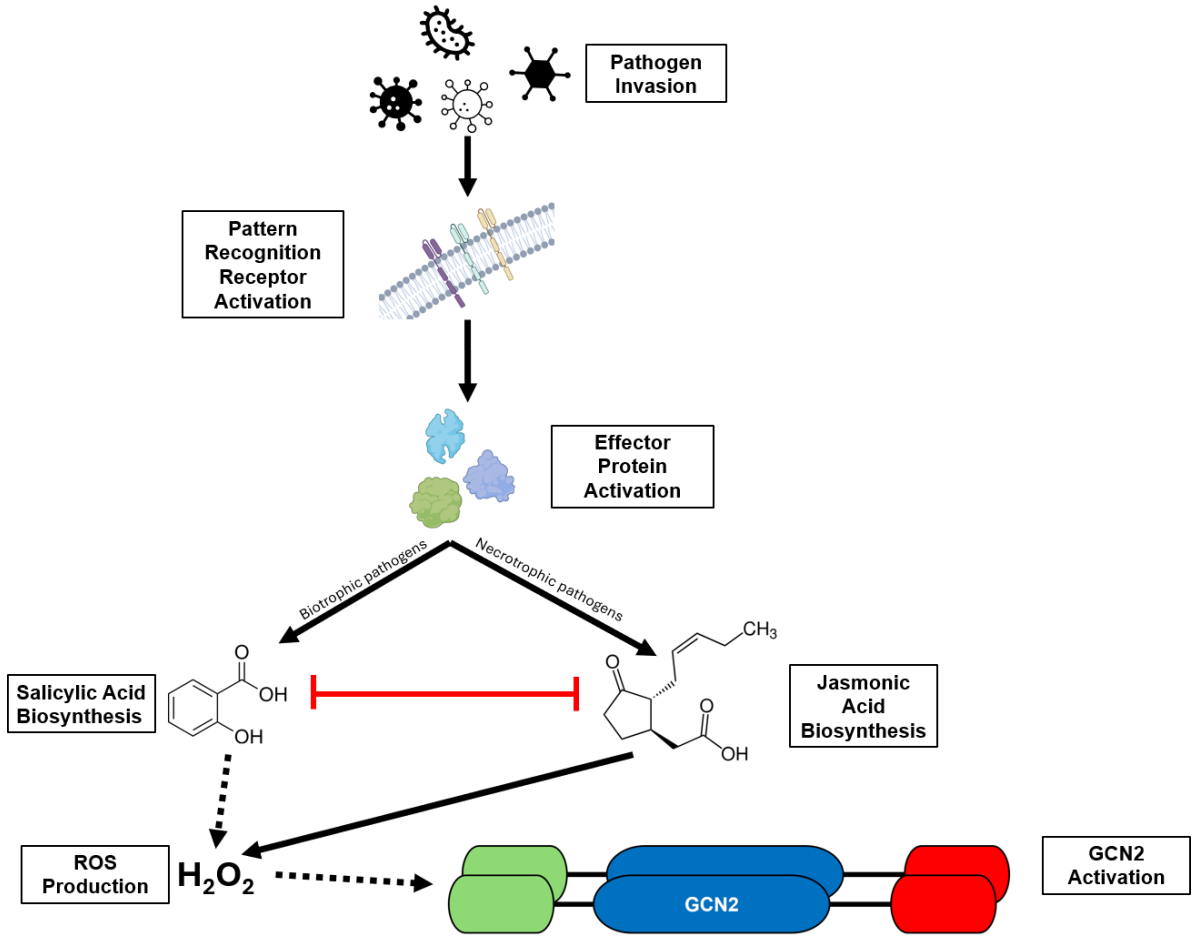

Figure 3. Proposed method of GCN2 activation through phytohormone biosynthesis: Illustration of the proposed model of pattern triggered immunity (PTI) and effector triggered immunity (ETI) for the synthesis of salicylic acid (SA) and jasmonic acid (JA) for GCN2 activation. Pathogen-associated molecular patterns (PAMPs) are recognized by cell membrane localized pattern recognition receptors (PRRs). The PRRs signal to effector proteins, which initiate pathways for phytohormone biosynthesis. JA has been shown to increase ROS accumulation and activate GCN2. The dotted lines represent the proposed methods by which SA leads to ROS production, and by which ROS accumulation leads to the activation of GCN2. Red line represents antagonistic effects.

Plant Immunity, Defense, and Phytohormones

Furthering the discussion of innate defense mechanisms, plants have developed several strategies that are fundamental to the conduction of stress responses to various signals. One of the biggest stressors plants often encounter is microbial invasion, which has fostered important evolutionary developments for activating molecular responses and controlling infection. Understanding these mechanisms is important for use in agricultural practices, as approximately $220 billion in global crop losses is attributed annually to diseases (Gula, 2023). Unraveling the mysteries behind plant immunity could reveal potential methods for enhancing disease resistance, even under stress conditions.

Pattern Recognition and Immune Activation

One of the first processes involved in plant recognition of pathogen infection is pattern-triggered immunity (PTI). This involves the recognition of pathogen-associated molecular patterns (PAMPs) by cell surface receptors, which activate pathogen effector proteins (Fig. 3). These effector proteins interact with nucleotide-binding domain leucine-rich repeat receptors (NLRs) to stimulate effector-triggered immunity (ETI). ETI caused by pathogen infection involves limiting the spread of pathogens via programmed cell death (Lukan & Coll, 2022).

Systemic Acquired Resistance (SAR) and Phytohormones

Systemic acquired resistance (SAR) is another mechanism of this process regarding plant immunity, which involves recognition of PAMPs in subsequent occurrences of infection. The process of SAR has demonstrated a significant reliance on several phytohormones known to be involved in immune responses. Firstly, pipecolic

acid, an amino acid not involved in protein synthesis, shows roles in SAR in Arabidopsis. It can be found in leaves distal to infection sites and can aid in priming defense pathways, even under defects of SAR (Návarová et al., 2012). Additionally, SA plays a notable role in ETI and aids in many processes involved in defense. Aided by Nonexpressor of Pathogenesis-Related 1 (NPR1) receptor for downstream responses, SA has demonstrated a central role in SAR, with SAR typically involving elevated SA levels and SA-responsive defense gene expression (Jung et al., 2009; Návarová et al., 2012; Peng et al., 2021). In fact, activation of NPR1 by SA is essential for activation of SAR through *N*-hydroxypipecolic acid biosynthesis (Liu et al., 2020b). Hence, biotrophic pathogen resistance has shown notable dependence on SA (Glazebrook, 2005). Interestingly, in tobacco, pathogen infection of transgenic roots unable to accumulate SA still showed signal transduction, but required the presence of SA in the non-transgenic, distant tissue (Vernooij et al., 1994). However, this also reveals that SA remains more localized and may not be the major signaling molecule used over long distances. ROS and calcium ions pose particular abilities to propagate cascading signals over much longer distances in plants through possible cell to cell interactions (Choi et al., 2017). More research is needed in this area to understand the exact mechanisms behind this signal transduction.

Interplay Between Phytohormones and Other Signaling Molecules

Furthering the discussion on the communication between hormones and signaling molecules, SA has been found to enhance ROS accumulation under wounding and high light stress, while jasmonic acid (JA) suppresses this same accumulation (Myers et al., 2023). This contrast is reasonable given that SA and JA typically exhibit

antagonistic signaling patterns, with one suppressing genes responsive to the other and vice versa (Koornneef et al., 2008) (Fig. 3). While SA is involved in response to biotrophic organisms, JA more so responds to necrotrophic organisms (Koornneef et al., 2008). However, synergistic effects of these two phytohormones have been observed in tobacco and Arabidopsis, but this is in regard to expression of specific genes and dependent on concentrations of each hormone (Mur et al., 2006). How these observed differences may relate to their respective effects on ROS accumulation remains to be explored.

Similar to SA, abscisic acid (ABA) and ethylene, two hormones involved in plant growth and development, are responsible for systemic ROS regulation under wounding stress (Myers et al., 2023), while putrescine, a polyamine, is required for defenses against pathogen infection. Interestingly, putrescine accumulates in response to bacterial infection, with a signaling mechanism somewhat dependent on SA and ROS, and usually leads to localized accumulation of SA (Liu et al., 2020a). Further, some microbes are capable of interfering with the auxin signaling pathway, a mechanism crucial in many aspects of plant development. SA has been found to interfere with the pathway of auxin signaling in Arabidopsis as part of the typical disease resistance response of the plant, contributing to the inhibition of pathogen growth (Wang et al., 2007). These intricate interactions between the various phytohormones and signaling molecules involved in plant defense open many areas for exploration in various stress conditions and plant varieties, allowing for broader understanding of the mechanisms underlying plant immunity.

Translational Control in Phytohormone Stress Response

Additionally, research has shown involvement of phytohormones in the translational control of plants under stress via the GCN2-eIF2α pathway. Firstly, ethylene plays a role in the activation of GCN2 under hypoxia stress, while GCN2 is also activated alongside ethylene insensitive 2 (EIN2) to manage translational responses (Cho et al., 2022). Compared to wild-type, ethylene and JA marker genes showed reduced expression in a *gcn2* mutant when undergoing infection with *B. cinerea,* a necrotrophic fungus (Berrocal-Lobo et al., 2020). This leads to the conclusion that GCN2 not only controls ethylene signaling through gene expression, but is regulated by ethylene itself, highlighting the intricate web of signaling involved in plant defense. Further, some evidence shows that GCN2 may be a negative regulator of JA signaling under immune responses to necrotrophic bacterium: Arabidopsis *gcn2* mutants showed higher transcripts of JA-induced plant defensin gene (*PDF1*) compared to wild-type under *P. carotovorum* infection (Liu et al., 2015). P-eIF2α has also been observed under *P. syringae* infection, which accompanies repressed transcription of TBF1, a regulator of some ABA signaling pathways involved in pre-invasion immunity (Liu et al., 2019).

Moreover, SA has been highlighted as a key component of the GCN2 pathway. In fact, treatment of wheat (*Triticum aestivum*) with SA has been shown to induce P-eIF2α (Zhigailov et al., 2020). Interestingly, under SA treatment compared to wild-type, Arabidopsis *gcn2* mutants have also shown higher induced levels of ICS1 transcripts, encoding a key enzyme involved in SA biosynthesis (Liu et al., 2015). This suggests that while SA acts as a GCN2 activator, GCN2 may also play a suppressive role in SA

signaling similar to that observed for JA. Further supporting this, Arabidopsis *gcn2* mutants displayed fewer conidiospores than wild-type under infection with the biotrophic pathogen *G. cichoracearum,* suggesting the potential for a stronger SA response when relieved of GCN2 function (Liu et al., 2015).

Overall, a very complex and sophisticated web of signaling exists within the plant cellular environment. This drives the major questions for how gene expression, specifically at translational regulation, can or will occur under a variety of conditions. This thesis focuses specifically on the role of SA in the activation of translational regulation pathways, but it is important to note that SA is far from acting alone in the grand scheme of plant stress regulatory mechanisms.

Chloroplast, light, and ROS function regarding translation

The importance of photosynthesis and chloroplast function in plant energy production, specifically in the many biochemical pathways responsible for plant growth and development, have been well established. However, in the context of this research, the focus of this discussion remains on the role of chloroplast function in the previously discussed translational regulatory pathways.

Regulation of GCN2 by Light Stress

Firstly, it is important to address the role of high light in GCN2 activity. Most notably, GCN2 activation has been demonstrated under excess light stress, while GCN2 has also been observed as a negative regulator of responses to UV-B exposure (Llabata et al., 2019; Lokdarshi et al., 2020a). UV stress in particular has demonstrated increases in uncharged tRNA levels, the major stress known to be directly recognized by the GCN2 protein (Misra et al., 2024). Whether this is the direct method by which

GCN2 activation occurs under UV stress is unclear. However, it has been well-established that GCN2 function is not only activated by, but dependent on light in many cases. Given GCN2's involvement in signals responsive to light conditions, it is important to discuss mechanisms and signaling pathways as they relate to photosynthesis.

GCN2 Activity Is Dependent on Photosynthesis

Firstly, GCN2 activity has shown significant dependence on light and photosynthetic activity. A variety of stressors and treatments demonstrated a complete lack in their abilities to cause P-eIF2α in dark-acclimated seedlings (e.g. water, CSF, glyphosate, MV, glufosinate ammonium, NaCl, ER stress, and mitochondrial stress) (Lokdarshi et al., 2020a, b). The establishment of this phenomenon led to a hypothesis regarding the involvement of not only light, but photosynthesis in GCN2 activation. It was similarly discovered that treatment of wild-type Arabidopsis seedlings with DCMU, a photosynthetic inhibitor, repressed P-eIF2α under CSF treatment. In fact, the only stress treatment found to activate GCN2 and result in P-eIF2α under dark conditions in Arabidopsis is H_2O_2, a relatively stable form of ROS (Lokdarshi et al., 2020a). ROS production, accompanied by P-eIF2α, has been shown to increase under macronutrient deprivation, including nitrogen, phosphorus, and potassium (Cui et al., 2025). This information has reinforced the hypothesis that the activity of ROS emanating specifically from the chloroplast due to photosynthesis activates the GCN2 protein. For example, seedlings treated with a ROS scavenger lack P-eIF2α, as do *gcn1*-knockout mutants (Lokdarshi et al., 2020a). Further supporting this, seedlings grown on media with

antioxidants before and during NaCl stress exhibited a reduction in P-eIF2α compared to control (Lokdarshi et al., 2020b).

Retrograde Signaling and ROS generation

Located on the thylakoid membrane within chloroplasts, the electron transport chain (ETC) acts as a major source of ROS generation within plant cells (Foyer & Hanke, 2022; Lokdarshi et al., 2020a). ROS molecules are known to participate in early stages of retrograde signaling under stress, referring specifically to messages emanating from organelles and travelling to the nucleus (Gläßer et al., 2014). Particularly, ROS signaling involves potential roles in regulating the three stages of translation (initiation, elongation, and termination) through interactions with their various system components (Moore et al., 2016). Beginning with this foundation highlights the key role of ROS as a mediator of cellular regulation and homeostasis. Understanding how ROS may function in combination with other signaling cascades remains an important area of study.

ROS and Phytohormone Dynamics

As mentioned previously, plant hormones like SA and JA are generally more localized to areas of immediate stress response. This emphasizes the potential importance of ROS as a long distance stress signaling molecule and its link to phytohormones (Choi et al., 2016). In fact, a great deal of evidence has emerged concerning ROS regulation by phytohormones, and vice versa. For example, Arabidopsis mutants for high accumulation of SA displayed increases in H_2O_2 levels in low light conditions, while reduced levels of SA led to a reduction in H_2O_2 (Mateo et al., 2006). Interestingly, SA also seems to have a positive effect on the biosynthesis of

glutathione, a ROS scavenger, highlighting its important role in regulating a delicate balance of ROS molecules (Mateo et al., 2006).

To summarize, chloroplast-derived ROS have demonstrated a critical function in the regulation of translation in plant stress responses. Both direct and indirect interactions between ROS, GCN2, and phytohormones reinforce the complex interplay between signaling pathways and regulatory mechanisms. Further research in these areas will provide valuable information to be used for developing crops with strengthened stress resilience and disease immunity, with wide implications for improving agronomic productivity. Therefore, our research offers key insights into the intersection of SA and ROS in regulating GCN2 activity.

Materials and Methods

Experimental Design

Methods for Arabidopsis seedling growth were conducted to ensure a sterile and controlled environment for all seedlings to minimize potential extraneous factors affecting P-eIF2α status, except for the intended mock, stress, and pre-treatments. Seedlings undergoing the various treatments were collected for immunoblotting to assay for P-eIF2α status as a proxy for the level of GCN2 activity. Hydrogen peroxide quantification was additionally performed on seedlings to estimate endogenous ROS levels under control versus SA treatments. Lastly, a phenotypic assay was conducted to assess the physiological effects of prolonged SA stress on wild-type and *gcn2-1* knockout mutant seedlings, including growth parameters of root length and fresh weight. Treatments used in this study were as follows:

(1) 0.1% (v/v) dimethyl sulfoxide (DMSO) in deionized water as control

(2) 1 mM SA in 0.1% DMSO

(3) 0.1 mM SA in 0.01% DMSO

(4) 30 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in 0.1% DMSO

(5) 1 μM chlorsulfuron (CSF) in deionized water as positive control

(6) 200 μM SA in 0.1% DMSO

For immunoblotting experiments, treatments 1-5 were used. Three to four independent experiments (biological replicates) were conducted per blot, as indicated in figure legends. For H_2O_2 quantification experiments, treatments 1 and 2 were used for

quantification from four biological replicates. For phenotype analysis, treatments 1 and 6 were used for analysis from four biological replicates.

Plant growth, stress treatments, and sample collection for immunoblots

Wild-type seeds of *Arabidopsis thaliana* ecotype Landsberg erecta (Ler-0), a homozygous *gcn2-1* mutant gene trap line (GT8359), and a complementation line (*gcn2-1;GCN2)* were sterilized and spotted on ½-strength Murashige-Skoog (MS) media plates (Sigma Aldrich, cat# M5524-10L, lot# SLCN6516) containing 0.65% Phytoagar (bioWORLD, cat# 40100072-2, lot# V19021100). Approximately 50 seeds were spotted per plate, then plates were placed in dark at 4°C for 48 h before being shifted into a growth chamber. Germination and growth occurred under conditions of a 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.

All seedling treatments began at Zeitgeber time 2 (ZT2) time point (two h into the light period). Seedlings receiving no treatment were collected at ZT2 to obtain a baseline phosphorylation level. For immunoblot experiments comparing wild-type and *gcn2-1* seedlings, 12-day-old wild-type seedlings were treated with 0.1% DMSO (VWR, cat# 0231-500ML, lot# 20L0756022) as control and 1 mM SA (Thermo Scientific, cat# A12253, lot# 10236572) in 0.1% DMSO, and *gcn2-1* seedlings were treated with 1 mM SA in 0.1% DMSO. For immunoblot experiments comparing various concentrations of SA, wild-type seedlings were treated with either 0.1 mM or 1 mM SA in 0.1% DMSO. For immunoblot experiments comparing SA stress in light versus dark conditions, plates of wild-type seedlings were transferred to continuous darkness at ZT2 for 24 h, with 1 mM SA in 0.1% DMSO treatment and sampling conducted under dim green light to minimize photosynthetic activity. Wild-type seedlings receiving 1 mM SA in 0.1% DMSO

treatment in standard light conditions were also collected. For immunoblot experiments comparing SA stress under control and photosynthetic inhibition, wild-type seedlings either received no pretreatment (mock) or were pretreated with 30 μM DCMU (Sigma Aldrich, cat# D2425-100G, lot# 061M0130V) 30 minutes prior to ZT2 (ZT1.5), followed by 1 mM SA in 0.1% DMSO application at ZT2. In all cases, seedlings were removed from the growth chamber, sprayed approximately 20 times with the treatment solution from approximately 20 cm away, incubated for 2 minutes, and excess liquid was then decanted. Plates were then placed back into the growth chamber until seedlings were collected. Whole seedlings were promptly harvested in 2 mL screw cap homogenizer tubes containing 2 mm metal beads at 10, 30, and 120 minute time points and flash-frozen in liquid N_2. For positive control samples, wild-type seedlings were treated with 1 μM CSF and samples were collected at 120 minutes.

Protein Extraction and Immunoblotting

Protein extraction and eIF2α immunoblot analysis were performed as previously described by Lokdarshi et al. (2020b), with some modifications. Total protein was extracted by grinding whole seedlings using a bead mill homogenizer (Fisher Scientific, Cat# 15340163) in lysis buffer containing 1 M urea (IBI Scientific, Cat# IB72064, Lot# 15A1250), 25 mM Tris-HCl (pH 7.5), 75 mM NaCl (J.T. Baker, Cat# 4058-01, Lot# G43726), 5% (v/v) glycerol (Fisher Scientific, Cat# G33-500, Lot# 090956), 0.5 mM EGTA (Sigma Aldrich, Cat# E3889-25G, Lot# 095K5447), 0.5 mM EDTA (Fisher Scientific, Cat# S311-100, Lot# 161094), 2% (w/v) insoluble polyvinylpyrrolidone (PVP) (Sigma Aldrich, Cat# PVP-40, Lot# 101H0426), and 2 mM β-mercaptoethanol (Sigma Aldrich, Cat# M-6250, Lot# 58H0066). The buffer was supplemented with protease and

phosphatase inhibitors (Thermo Scientific, Cat# A32961, Lot# YG3916414). Total protein concentrations were determined using the Bradford assay to standardize gel loading across all samples (Thermo Scientific, Cat# 1856209, Lot# WF320332).

For immunoblot experiments, 50 µg of total protein was separated on a 12% (w/v) SDS-PAGE acrylamide gel and electroblotted onto a 0.45 µm polyvinylidene difluoride (PVDF) membrane (Immobilon, cat# IPVH00010, lot# 0000160396) for 16 h at 4°C in a 1× transfer buffer [20 % (v/v) methanol, 25 mM tris base, 190 mM glycine, 0.01% (w/v) SDS]. Prior to transfer, SDS-PAGE gels were equilibrated in transfer buffer with gentle shaking for 30 minutes at room temperature. After the transfer, membranes were blocked for 1 hour at room temperature in 1× TBST buffer [20 mM Tris-buffered saline (pH 7.6), 0.07% Tween-20 (Fisher Scientific, Cat# BP337-500, Lot# 181617)] supplemented with 10% non-fat dry milk (Cat# M17200, Lot# 174340-193727) and 0.2% bovine serum albumin (BSA) (Fisher Scientific, Cat# BP9706-100, Lot# 198742). Following blocking, membranes were washed eight times for 10 minutes each in 1× TBST and incubated overnight at 4 °C with a monoclonal anti-eIF2S1 (phospho-S51) antibody (Abcam, Cat# ab32157) diluted 1:2500 in 1× TBST containing 5% (w/v) BSA. After primary antibody incubation, membranes were again washed eight times for 10 minutes in 1× TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen, Cat# 32260, Lot# YL386239) diluted 1:5000 in 1× TBST containing a 1:10 dilution of the original blocking buffer for 1 hour at room temperature. After a final eight washes with 1× TBST for 10 minutes, chemiluminescent detection was performed using the WesternBright Quantum (Advansta) as per the manufacturer’s protocol. For eIF2α protein blots, the immunoblot analysis was

conducted as described in Lokdarshi et al. (2020b). Blots were imaged using an ImageQuant LAS 4000 biomolecular imager and band intensities were quantified using ImageJ (ver. 1.5).

Hydrogen Peroxide Quantification

An Amplex Red kit (Thermo-Fisher, Cat# A22188) was used to quantify *in vivo* H_2O_2 level as described in Lokdarshi et al. (2020a). 12-day-old wild-type seedlings were treated with either 0.1% DMSO or 1 mM SA in 0.1% DMSO. Thirty mg of tissue from each condition was collected at 10, 30, and 120 minutes of treatment in a 1.5 mL microcentrifuge tube, flash frozen in liquid N_2, and ground in liquid N_2 with a plastic pestle for 2 minutes to form a homogenous powder. Samples were resuspended in 100 µL of 1× reaction buffer (Amplex Red kit) and centrifuged at 17000 x g at 4 ºC for 2 minutes. The resulting supernatant was used to measure absorbance as per manufacturer's protocol. Absorbance at 590 nm was measured using a SpectraMax M5e (Molecular Devices, VWR Cat# 89212-400) plate reader. H_2O_2 concentration was determined by a standard curve analysis.

Phenotype assessment: Primary root length and fresh weight measurements

For phenotype characterization under SA stress, 3-day-old seedlings, initially grown vertically on ½ strength MS-agar medium, were transferred to fresh ½ strength MS-agar plates supplemented with either 200 µM SA or 0.1% DMSO (mock). Images of seedlings were captured on day 0 (transfer day), day 6, and day 12 using Canon EOS 60D digital camera. On day 12, seedlings were harvested and weighed for fresh biomass determination. Primary root length was measured using ImageJ (version 1.4).

Statistics

All bar graphs and statistical analyses were done using RStudio (version 4.5). Immunoblot images were analyzed using Image J (ver 1.5) to quantify relative signal intensity. Intensities were individually measured and totaled to calculate percent intensities within each immunoblot image. Averages were determined from 3-4 biological replicates per experiment. Welch's t-tests and one-way ANOVA were performed in RStudio for densitometric analysis of the eIF2α phospho and alpha blots, respectively. Welch's t-tests were individually performed to compare two treatment conditions (mock vs SA, 0.1 mM SA vs 1 mM SA, light vs dark, or SA vs DCMU + SA) at the same time points, as well as SA treatment compared to ZT2 for figure 4. Two-way ANOVA was conducted to test for differences in total eIF2α protein level across genotypes and treatment conditions.

For H_2O_2 quantification, H_2O_2 concentrations were averaged from four biological replicates and Welch's t-tests were performed using RStudio. Statistical tests were done comparing mock and SA treatments at each of their respective time points, as well as comparing SA treatment at each of the listed time points to ZT2.

For root length and fresh weight analyses, measurements were averaged from four biological replicates and Welch's t-tests were performed using RStudio. Statistical tests were conducted comparing *gcn2-1* to wild-type and *gcn2-1* to complementation line seedlings within each of the days and treatments indicated for root length analysis, and within each of the treatments listed for fresh weight analysis.

Results

Effect of Salicylic Acid on GCN2 activity

Salicylic acid triggers rapid P-eIF2α in a GCN2-dependent manner

A prior study in *A. thaliana* showed that SA induces P-eIF2α at 4 h and 12 h in the wild-type Landsberg seedlings (Lageix et al., 2008). In contrast, more recent studies demonstrated numerous stress conditions can elicit a much more rapid P-eIF2α response, within 10-30 minutes (Lokdarshi et al., 2020a, b). To test whether SA can also trigger a rapid P-eIF2α in a GCN2-dependent manner, we performed total protein extraction and followed by immunoblotting to assay P-eIF2α as a proxy for GCN2 activity.

Upon SA treatment, P-eIF2α levels increased within 30 minutes and remained elevated up to 120 minutes, compared to ZT2 baseline, in the wild-type Arabidopsis seedlings ($p = 0.01$) (Fig. 4B-C). Conversely, the homozygous *gcn2-1* mutants failed to show P-eIF2α at any time points (Fig. 4B). Total eIF2α protein levels remained consistent across all genotypes ($p = 0.220$) and treatments ($p = 0.608$), confirming that the observed changes were due to phosphorylation rather than altered protein expression. Combined, our results demonstrate that SA-mediated P-eIF2α is dependent on GCN2, and reveal that GCN2 activation by SA occurs much earlier than previously reported at 4 h post-treatment (Lageix et al., 2008).

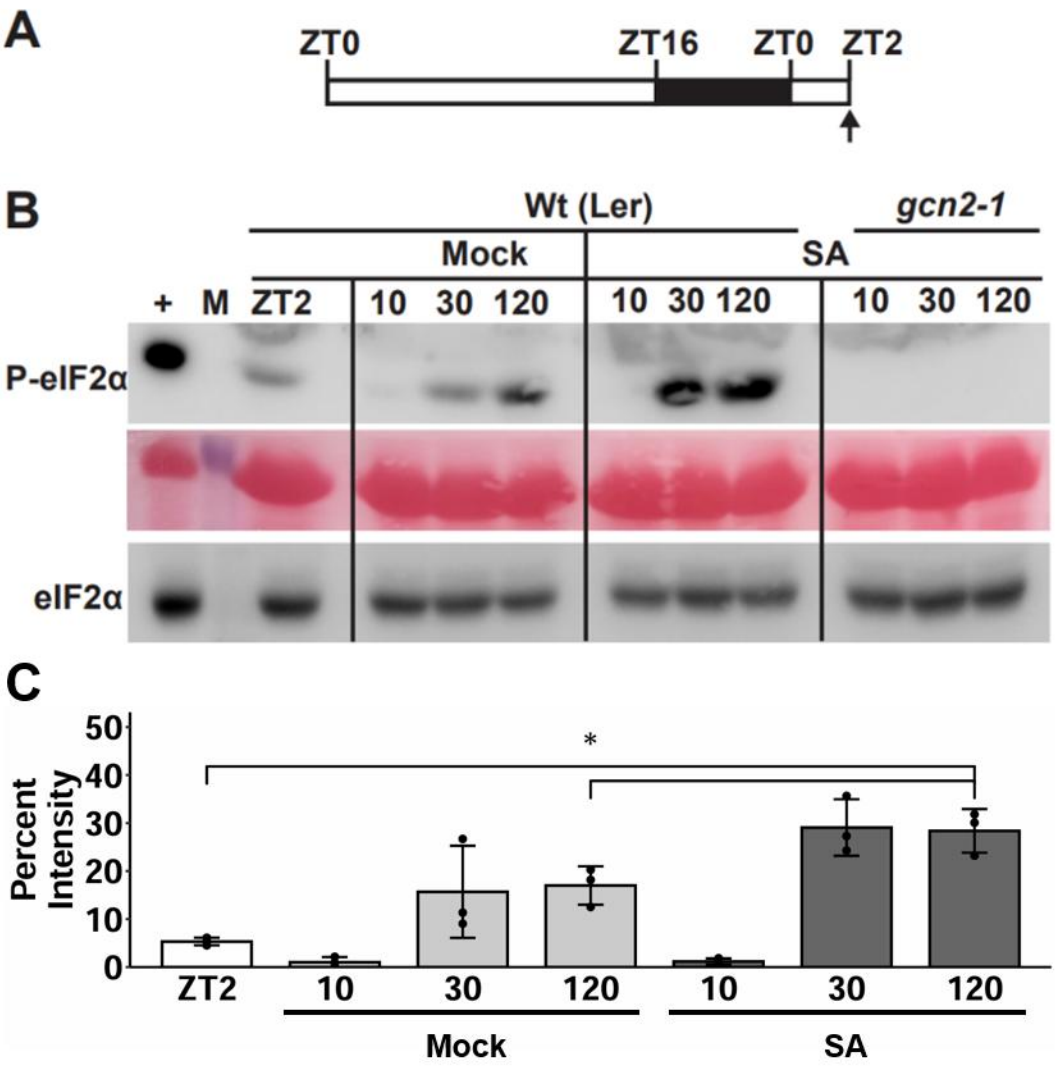

Figure 4. Salicylic acid induces P-eIF2α in a GCN2-dependent manner: (A) Schematic of seedling growth in 16 h light and 8 h dark. Black arrow at ZT2 (2 h into the light period) denotes the time of sampling before treatment and reagent application. **(B)** Top: Immunoblot showing P-eIF2α (~38 kDa) in wild-type and *gcn2-1* mutant seedlings at ZT2 and in response to either mock (0.1% DMSO) or 1 mM salicylic acid (SA) at time points 10, 30, and 120 minutes post-treatment. Middle: Ponceau stained P-eIF2α blot showing rubisco large subunit indicating consistent total protein loading. Bottom: Immunoblot showing total eIF2α (~38kDa). (+) represents a positive control for P-eIF2α (wild-type seedlings collected 120 minutes after treatment with 1 µM chlorosulfuron). (M) represents a molecular weight marker. **(C)** Bar graph showing mean percent intensity quantification of P-eIF2α signal in wild-type Landsberg seedlings under mock and 1 mM SA treatment. Error bars display standard deviation of the mean from three biological replicates. (Mock 120 vs SA 120: *$p = 0.032$) (ZT2 vs SA 120: *$p = 0.010$).

P-eIF2α Remains Constant Across Salicylic Acid Dosages

Given that GCN2 activity under SA stress is time-dependent (Fig. 4B), we next tested whether GCN2 also responds to varying SA concentrations. We hypothesized

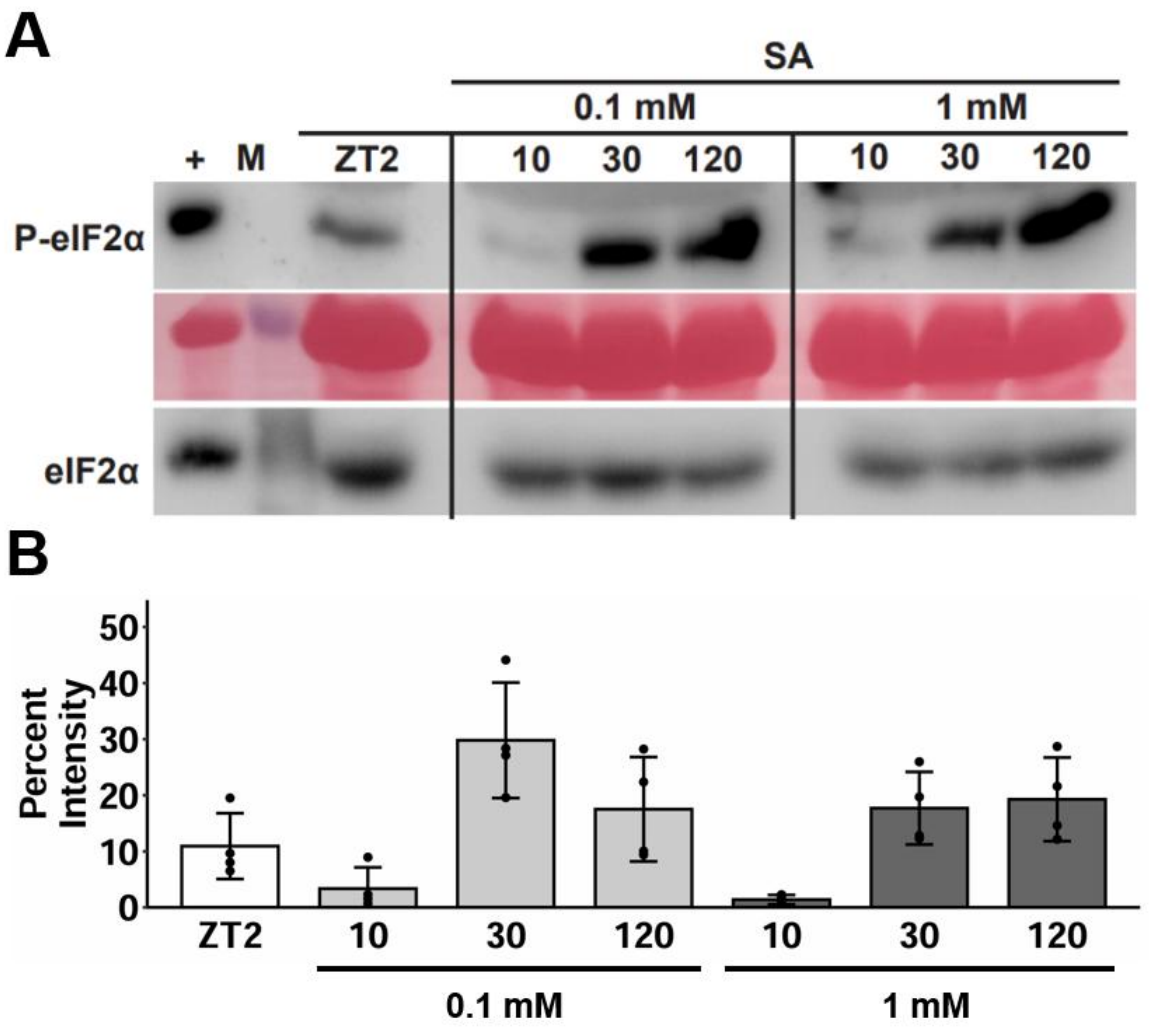

Figure 5. Increasing salicylic acid dosage does not increase P-eIF2α beyond threshold: (A) Top: Immunoblot showing P-eIF2α in wild-type seedlings treated with 0.1 mM or 1 mM SA at time points 10, 30, and 120 minutes. Middle: Ponceau stain. Bottom: Immunoblot showing total eIF2α protein levels (~38kDa). Please see figure 4B for details in legend. **(B)** Bar graph showing mean percent intensity quantification of P-eIF2α signal in wild-type Landsberg seedlings under 0.1 mM and 1 mM SA treatment. Error bars display standard deviation of the mean from four biological replicates.

that the level of P-eIF2α in response to SA dosage would reflect a cellular stress response proportional to the intensity of the stress. To test this, we treated wild-type seedlings with two concentrations of SA differing by an order of magnitude: 0.1 mM and 1 mM.

Interestingly, the extent of P-eIF2α induction was similar between the two SA concentrations (Fig. 5A). This finding contradicts our original hypothesis that higher levels of exogenous SA would elicit a proportionally greater GCN2 response, suggesting that GCN2 activation may reach a plateau or threshold level that is already saturated at lower SA concentrations.

Salicylic Acid Triggers P-eIF2α Only in the Presence of Light

The photosynthetic electron transport chain (PETC) in chloroplasts is the major contributor of ROS in plant cells (Foyer & Hanke, 2022). Previous work demonstrated a lack of P-eIF2α in Arabidopsis seedlings at the end of a 24-hour dark acclimation period, suggesting that light modulates P-eIF2α accumulation (Lokdarshi et al., 2020b). In support of this, activation of GCN2 by inhibitors of amino acid biosynthesis (e.g. chlorosulfuron and methyl viologen) requires light, implicating a crucial role of chloroplast-derived ROS in this process (Lokdarshi et al., 2020a). Taken together, these findings support the hypothesis that chloroplastic ROS is necessary for the activation of GCN2, and that the absence of P-eIF2α under prolonged darkness is due to the inhibition of photosynthetic light reactions (Lokdarshi et al., 2020a). To test if this hypothesis also applies to the SA-induced AtGCN2 activation, we dark acclimated wild-type seedlings for 24 h prior to treatment (Fig. 6A).

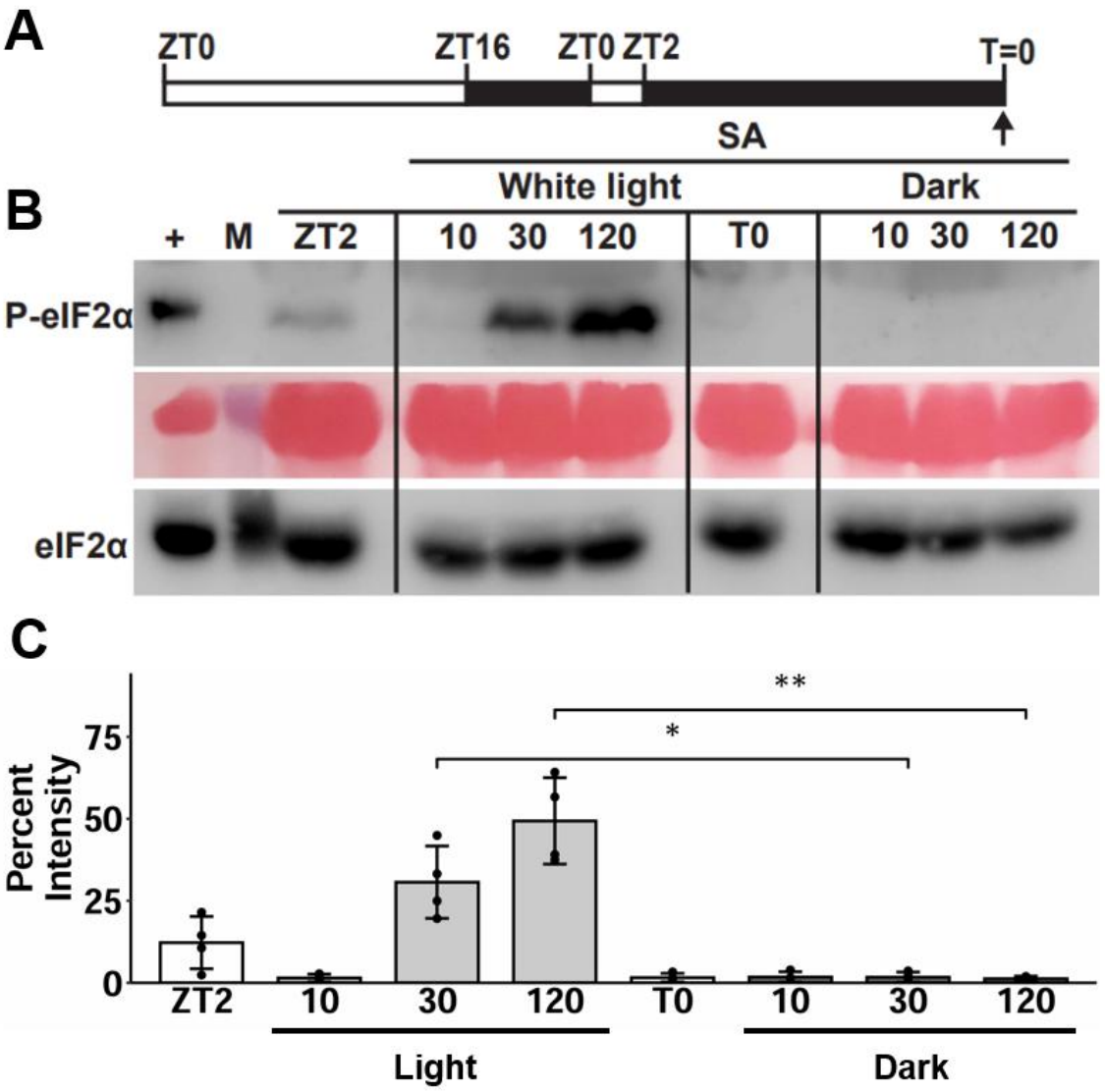

Figure 6. P-eIF2α is dependent on light under salicylic acid stress: (A) Growth regimen of wild-type seedlings and period of dark acclimation. Black arrow at T=0 indicates end of 24-hour dark acclimation and application of reagents. **(B)** Top: Immunoblot showing P-eIF2α in wild-type seedlings treated with 1 mM SA in light versus dark conditions at time points 10, 30, and 120 minutes. Middle: Ponceau stain. Bottom: Immunoblot showing total eIF2α protein levels (~38kDa). T0 denotes 24-hour dark-acclimated seedlings collected before treatment with SA. Please see figure 4B for details in legend. **(C)** Bar graph showing mean percent intensity quantification of P-eIF2α signal in wild-type Landsberg seedlings under light versus dark conditions and 1 mM SA treatment. Error bars display standard deviation of the mean from four biological replicates. (Light 30 vs Dark 30: *$p = 0.012$) (Light 120 vs Dark 120: **$p = 0.005$).

We show that wild-type seedlings treated with SA under normal white light show

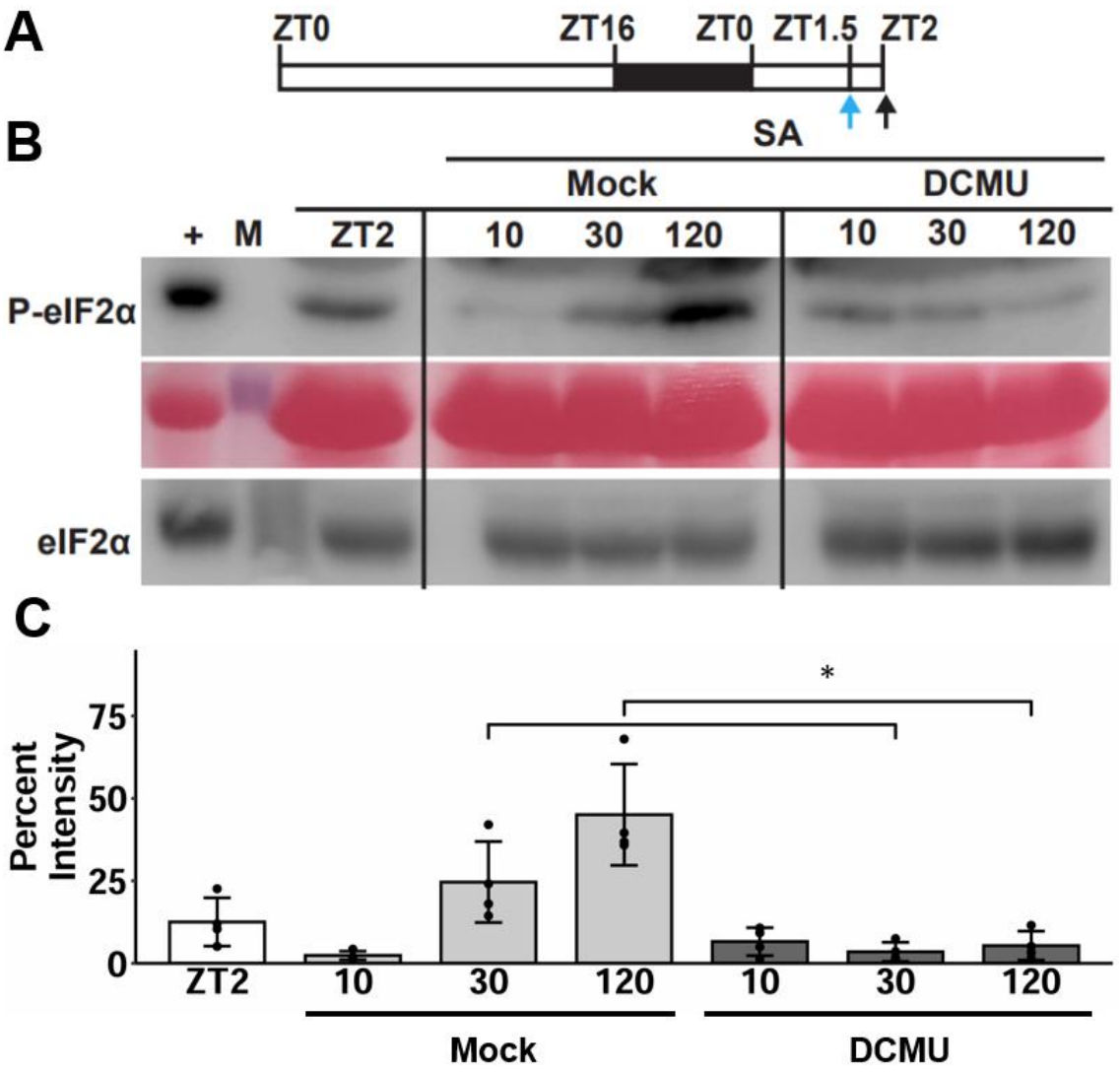

Figure 7. P-eIF2α is dependent on photosynthetic function under salicylic acid stress: (A) Growth regimen of wild-type seedlings and time of treatments. Blue arrow denotes application of DCMU at ZT1.5 followed by SA treatment at ZT2 (black arrow). **(B)** Top: Immunoblot showing P-eIF2α in wild-type seedlings that received no pretreatment (Mock) or 30 μM DCMU prior to treatment with 1 mM SA. Middle: Ponceau stain. Bottom: Immunoblot showing total eIF2α protein levels (~38kDa). Please see figure 4B for details in legend. **(C)** Bar graph showing mean percent intensity quantification of P-eIF2α signal in wild-type Landsberg seedlings under mock versus DCMU pretreatment. Error bars display standard deviation of the mean from four biological replicates. (Mock 30 vs DCMU 30: *$p = 0.037$) (Mock 120 vs DCMU 120: *$p = 0.010$).

a time-course increase in P-eIF2α (Fig. 6B), consistent with our earlier observation (Fig. 4B). In contrast, seedlings that were dark-acclimated for 24 h showed no detectable P-eIF2α signal (T0) and failed to respond to SA treatment at any time point (Fig. 6B). These findings indicate that AtGCN2 activity requires light, implicating a role for the photosynthetic electron transport chain (PETC) in SA-mediated activation of GCN2.

Photosynthetic Inhibition Mitigates GCN2 Activation Under Salicylic Acid Stress

Given the absence of P-eIF2α signal in 24-hour dark-acclimated seedlings (Fig. 6B), we further investigated the role of active photosynthesis and the PETC in GCN2 activation. To test this hypothesis, we pretreated the wild-type seedlings with DCMU 30 minutes prior to ZT2, followed by SA application at ZT2 (Fig. 7A). DCMU is a photosystem II (PSII) inhibitor that blocks electron transfer from PSII to plastoquinone, thereby lowering the production of chloroplastic ROS, ATP, and NADPH (Haydon et al., 2013; Lokdarshi et al., 2020a).

Here, we show that seedlings pretreated with DCMU exhibit reduced P-eIF2α compared to mock-treated controls across all time points (Fig. 7B, C). This finding, together with our previous results indicating light dependence, suggests that photosynthesis plays a key role in GCN2 activation under SA-induced stress.

Effect of SA on H_2O_2 level in wild-type seedlings

Given that GCN2 activation is dependent on light and photosynthetic activity (Fig 6B, 7B), we next tested the hypothesis that SA treatment under light conditions leads to the overaccumulation of ROS. A previous study demonstrated that high SA concentrations induce H_2O_2 accumulation in tomato leaves (Takács et al., 2016). Since ROS are primarily produced in the chloroplast during photosynthesis (Foyer & Hanke,

2022), we measured ROS levels in seedlings treated with either DMSO (mock) or 1 mM SA under standard light conditions. Our goal was to determine whether the SA-induced ROS accumulation observed in tomato leaf is conserved in Arabidopsis.

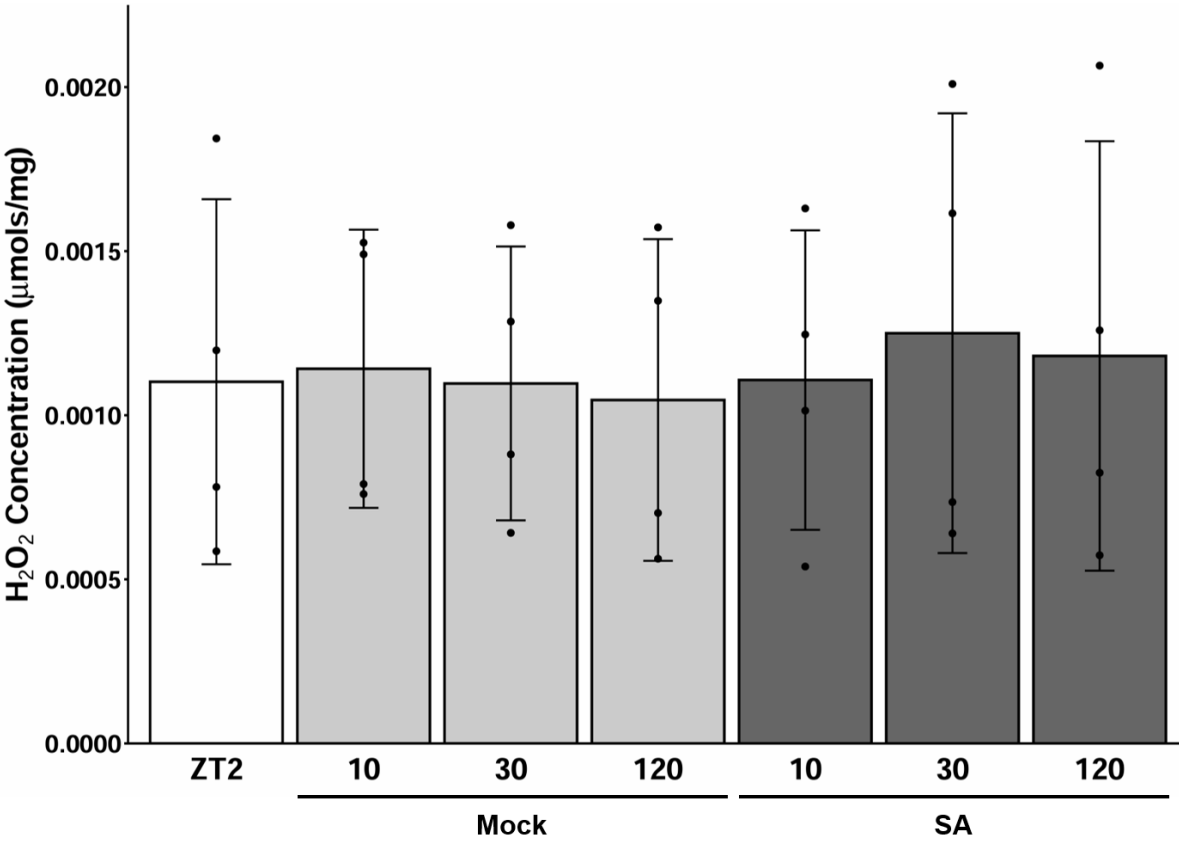

Figure 8. Salicylic acid does not directly impact endogenous H_2O_2 accumulation: Bar graph showing H_2O_2 concentration in µmols/mg of tissue at ZT2 (pre-treatment) and after the application of either 0.1% DMSO (mock) or 1mM SA treatment at 10, 30, and 120 minutes. Error bars indicate standard deviation from four independent experiments.

Our results showed no significant difference in H_2O_2 concentration between ZT2 and SA-treated seedlings, nor between DMSO and SA treatments (Fig. 8). These findings suggest that SA treatment does not directly affect total H_2O_2 levels in Arabidopsis seedlings, but may instead contribute to maintaining optimal ROS homeostasis under stress conditions.

Effect of prolonged SA stress on the growth of wild-type and gcn2-1 seedlings

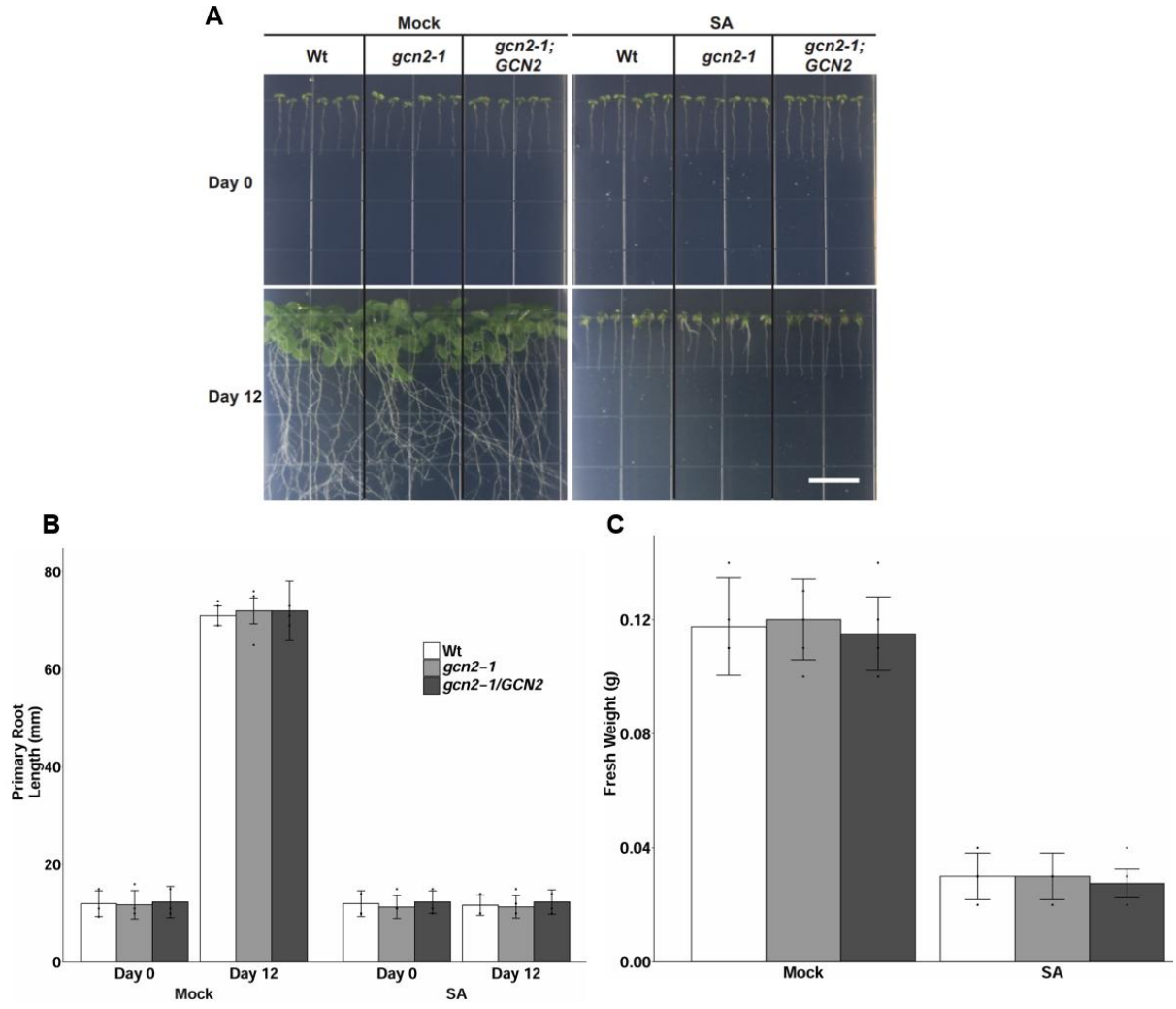

Figure 9. Wild-type and *gcn2-1* mutant show phenotypic similarities under salicylic acid stress: (A) Representative images of day 0 and 12 photographs of Wt (wild-type, Ler), *2-1* (*gcn2-1* mutant), and *gcn2-1/GCN2* complementation line seedlings on vertical plates with 0.1% DMSO (Mock) or 200 µM SA. **(B)** Bar graph showing primary root length of Wt, *gcn2-1*, and *gcn2-1/GCN2* seedlings at day 0 and day 12 of growth on media containing either 0.1% DMSO (Mock) or 200 µM SA. Error bars display standard deviation from three biological replicates with n>30 seedlings per biological replicate. **(C)** Bar graph showing fresh weight of 12-day-old Wt, *gcn2-1*, and *gcn2-1/GCN2* seedlings grown on media containing either Mock (0.1% DMSO) or 200 µM SA. Error bars display standard deviation from four biological replicates with n>30 seedlings per biological replicate.

Our findings in the biochemical realm of translational regulation show that SA triggers rapid GCN2 activation (Fig. 4B), consistent with the previous reports under various abiotic and xenobiotic stressors (Lageix et al., 2008; Lokdarshi et al., 2020b). To extend these observations, we investigated how these internal responses to SA stress manifest in phenotypic changes in Arabidopsis seedlings.

We subsequently tested the hypothesis that *AtGCN2* function is critical for the survival responses towards SA stress by assessing the phenotypic differences between wild-type and *gcn2-1* seedlings. Surprisingly, we found similar phenotypic behavior between the wild-type, *gcn2-1* mutant, and a complementation line (*GCN2/2-1*) in a primary root length and fresh weight assay across all treatments and time points (Fig. 9). These results suggest that GCN2 may not be the sole or even primary signaling component involved in mediating growth responses under SA stress.

Discussion

GCN2 and Photosynthesis: Dual Requirements for SA Signaling

The results presented support the prevailing hypothesis that GCN2 plays a central role in regulating cellular responses to a wide variety of stresses via a rapid signaling mechanism in plants. While previous reports indicate that SA activates the GCN2-eIF2α module within 4 h of treatment (Lageix et al., 2008), we demonstrate this activation occurs as early as 30 minutes post-SA application (Fig. 4B). Additionally, the time-course increase in P-eIF2α signal observed in this study mirrors the response observed with other stressors (e.g. excess light, H_2O_2, chlorosulfuron, cold, and NaCl) (Lokdarshi et al., 2020a, b).

Further, we observed a greater P-eIF2α signal in wild-type seedlings under SA stress compared to mock treatment (Fig 4B, C). The increase in baseline P-eIF2α detected in the mock condition may be attributed to the presence of 0.1% DMSO used as a solvent. This is consistent with findings in *Artemisia annua* shoot cultures, where 0.25%-2% (v/v) DMSO was shown to induce the generation of ROS (Mannan et al., 2010). Notably, the DMSO-induced ROS production was inhibited by the addition of vitamin C, a well-known antioxidant. These findings support the notion that DMSO can trigger oxidative stress responses, even at low concentrations, which may underlie the basal activation of the GCN2-eIF2α module observed under mock conditions in our study. Nonetheless, our results clearly show a substantial increase in P-eIF2α signal

following SA treatment, underscoring the specific and robust activation of the GCN2-eIF2α module by SA.

Additionally, we show a complete lack of P-eIF2α in *gcn2* mutant seedlings, emphasizing the dependence of SA signaling on GCN2 function. This lack of P-eIF2α signal in *gcn2* mutants under SA stress aligns with previous findings involving other stresses as well (e.g. purine starvation, UV, chlorosulfuron, wounding, cold shock, excess light, and NaCl), further reinforcing GCN2's essential role as the "stress sentinel kinase" in plants (Lageix et al., 2008; Lokdarshi et al., 2020a, b). Importantly, our findings extend these observations by offering fresh insights into the involvement of phytohormones as endogenous signaling mediators in plant defense and immunity. The activation of the GCN2-eIF2α signaling module by SA introduces a compelling component in the complex regulatory network governing plant defense responses, particularly against those targeting biotrophic pathogens (Spoel & Dong, 2024). Therefore, our results position GCN2-eIF2α as a key regulator within the defense signaling pathway, opening new avenues for future research on stress signaling related to plant immunity.

In addition to the dependence on GCN2, P-eIF2α under SA also showed a significant reliance on chloroplast function. When deprived of light or PSII function, wild-type seedlings displayed either absent or reduced P-eIF2α signal, respectively (Fig 6B, 7B). Our findings are consistent with previous research showing many other stressors with a strong dependency on light/photosynthesis for GCN2 activation, as they show no detectable P-eIF2α after dark acclimation (e.g. NaCl, cold shock, excess light, hypoxia, glufosinate, and chlorosulfuron) (Cho et al., 2022; Lokdarshi et al., 2020a, b; Takano et

al., 2019). These observations suggest that photosynthetic byproducts may serve as intermediate signals linking SA accumulation to GCN2 activation.

Earlier studies have shown that inhibiting photosynthesis with DCMU reduces ROS accumulation in algae, implicating ROS as a major byproduct of the PETC and a crucial signaling molecule in this pathway (Irihimovitch & Shapira, 2000; Marshall et al., 2002). This supports the hypothesis that light-dependent ROS production and accumulation may be a critical intermediate step required for GCN2 activation. In fact, Arabidopsis seedlings treated directly with H_2O_2 in dark conditions retain significant levels of P-eIF2α at both 30 and 120 minutes post-treatment (Lokdarshi et al., 2020a). Additionally, SA itself has been known to function as a major regulator of ROS, capable of promoting initial ROS accumulation under stress conditions and subsequently acting as an antioxidant during overaccumulation (Saleem et al., 2021). Taken together, these findings suggest that, in the absence of photosynthesis-derived ROS, SA and other stress signals may be unable to effectively trigger downstream components of the stress response pathway. As to the exact mechanism by which SA may lead to ROS accumulation, and how ROS may directly interact with GCN2 for its activation, remain to be explored.

Excess Salicylic Acid Does Not Enhance GCN2 Activation Beyond Threshold

Having established that SA rapidly activates GCN2 and triggers P-eIF2α, we hypothesized that decreasing SA concentrations would result in a corresponding reduction in phosphorylation signal. This dose-dependent GCN2 activation has been previously documented under various stress conditions (e.g. H_2O_2, chlorosulfuron, NaCl) (Lokdarshi et al., 2020a, b). However, in our study, 1 mM SA-treated seedlings

did not exhibit a significantly greater P-eIF2α signal compared with the 0.1 mM treatment group at 30 and 120 minutes (Fig 5A). This lack of linear response may reflect the existence of an optimal phytohormone concentration range required for efficient signaling in Arabidopsis seedlings.

Several studies have shown this concentration-dependent hormonal effect in plants. For instance, low concentrations of SA have been shown to promote seedling germination, whereas higher concentrations reduced germination in wheat, cucumbers, and carrots (Bahrani & Pourreza, 2012; Dolatabadian et al., 2009; Rajasekaran et al., 2002; Singh et al., 2010). Similarly, 0.5 mM SA enhanced root length in pearl millet, while higher doses caused root growth inhibition (Ngom et al., 2017). These responses are known to be species-specific, and Arabidopsis itself possesses mechanisms that maintain SA levels via negative regulators, such *CPR5* (Constitutive expresser of Pathogenesis-Related genes-5*)* and *DND1* (Defense No Death 1) (Bowling et al., 1997; Clough et al., 2000). Remarkably, an Arabidopsis hybrid with parental lines harboring suboptimal and supraoptimal SA levels exhibited enhanced root growth, suggesting that a physiological balance of SA is critical for optimal growth responses (Zhang et al., 2016). Hence, the 1 mM SA treatment in our study may exceed the endogenous optimum in Arabidopsis, thereby disrupting the GCN2-dependent signaling mechanism.

Although P-eIF2α levels did not increase further with 1 mM treatment, SA has been shown to suppress protein synthesis in a dose-dependent manner. For example, incorporation of a tagged methionine analogue was lower in 1 mM SA-treated seedlings compared to 0.1 mM treatment (Li et al., 2023). One explanation for the observation in our study is that GCN2 activity reaches a maximum (V_{max}) at 0.1 mM SA, beyond which

additional SA does not enhance P-eIF2α levels. Interestingly, the same study identified eIF2α as a SA-binding protein (Li et al., 2023). This suggests that SA may play a more direct role in regulating the activity of eIF2α in addition to its indirect role via GCN2 activation. At elevated concentrations, such as 1 mM, it is possible that excess SA interferes with any additional phosphorylation by GCN2. However, there is no evidence to suggest that SA binds directly with the critical, N-terminal S51 residue in eIF2α, which is the sight of phosphorylation by GCN2.

Taken together, these observations highlight the need for further investigation into the dose-dependent relationship between SA and GCN2-eIF2α signaling. Future work should aim to dissect how SA concentration influences both GCN2 activation and potential direct modulation of eIF2α activity.

SA treatment does not necessarily increase endogenous ROS levels

It is well established that ROS accumulate in response to numerous stresses, including high light, drought, salt, cold, nutrient deficiency, and pathogen attack (Lokdarshi et al., 2020a, b; Tripathy & Oelmüller, 2012). As previously mentioned, prior studies noted increased ROS production in tomato plant leaves following SA treatment (Poór et al., 2017; Takács et al., 2016). However, in our study, 12-day-old Arabidopsis seedlings undergoing two h of 1 mM SA treatment did not show elevated H_2O_2 levels compared to ZT2 or mock control (Fig 8). This observation could be due to several factors, which will be discussed further.

Firstly, in addition to H_2O_2, other ROS, such as superoxide ($O_2^{\bullet -}$), as well as reactive nitrogen species (RNS) may contribute to early stress signaling triggered by SA. RNS, including nitric oxide, are generated from multiple organelles including the

mitochondria, chloroplasts, and cytosol, making them a potential player in the complex signaling web under many stressors (Mandal et al., 2022). For example, in tomato leaf, nitric oxide production significantly increased approximately 6 h after onset of 1 mM SA treatment in light conditions (Takács et al., 2016). Moreover, recent studies suggest that $O_2^{\bullet -}$ production occurs rapidly after SA exposure. For instance, 1 mM SA induces a strong oxidative burst in tomato plant leaves, with early peaks of both $O_2^{\bullet -}$ and H_2O_2 production in a light-dependent manner, largely mediated by NADPH oxidase activity and inhibition of antioxidant enzymes such as catalase and APX (Poór et al., 2017). These findings suggest that our H_2O_2-focused measurements may have missed early or compartmentalized RNS and ROS activity, particularly $O_2^{\bullet -}$ bursts. Supporting this, a prior study demonstrated that plant peroxidases can catalyze $O_2^{\bullet -}$ production in a H_2O_2-independent manner upon SA exposure, challenging the traditional view that H_2O_2 is the initiating ROS signal (Kimura & Kawano, 2015). Taken together, although our study did not detect a significant increase in H_2O_2 following SA treatment, it remains plausible that rapid production of $O_2^{\bullet -}$ or RNS could contribute to the early activation of GCN2 and phosphorylation of eIF2α observed within 30 minutes (Fig. 4B). Thus, broader profiling of ROS types beyond just H_2O_2 is necessary to accurately characterize the early signaling dynamics induced by SA.

Secondly, the temporal dynamics of SA accumulation may explain the observed delay in ROS induction. As implied earlier, in tomato plants, SA levels do not peak until 24 h after application of 1 mM SA (Poór et al., 2017), suggesting that exogenous SA may not induce a rapid ROS burst within the two hour experimental window. In the case of this study, exogenous treatment with SA may not accurately mimic pathogen-

triggered SA biosynthesis at infection sites, which typically involves localized, rapid accumulation of SA associated with ETI and SAR. Given that ROS production is typically elevated during pathogen attack, it is plausible that endogenous accumulation of SA during infection triggers a concurrent increase in ROS, whereas exogenous application of SA alone may not elicit the same response (Tripathy & Oelmüller, 2012).

Thirdly, it is possible that the spatial and temporal characteristics of ROS accumulation contributed to the lack of detectable increase in our assay. For instance, two studies have shown that in Arabidopsis leaves, ROS accumulation occurs predominantly within the chloroplasts and is not uniformly distributed throughout the leaf (Kim et al., 2012; Li & Kim, 2021). Rather, ROS tend to accumulate in discrete zones in response to various abiotic stresses (Lokdarshi et al., 2020a). Because our experimental approach involved collecting whole leaves, this may have diluted localized ROS signals, masking transient or spatially restricted increases in ROS concentration.

Finally, SA may provide increased photosynthetic activity during stress periods to meet heightened energy demands for fighting pathogen infection, while simultaneously triggering antioxidant biosynthesis pathways to maintain redox homeostasis and prevent oxidative damage. In wheat, SA treatment enhances both net photosynthetic rate and antioxidant enzyme activity simultaneously under salt stress (Alam et al., 2022). Similarly, superoxide dismutase activity rises under 0.5 mM SA treatment in the algae *Haematococcus pluvialis,* and enhanced ROS scavenging has been reported in SA-treated mungbean and *Dianthus superbus* (Ma et al., 2017; Nazar et al., 2011; Raman & Ravi, 2010). These findings support the idea that although SA may drive ROS production indirectly via increased photosynthesis, elevated antioxidant activity may

mask this by keeping ROS levels in check. Indeed, our results suggest that a basal or optimal ROS level is essential for proper translational regulation, as GCN2 activation does not occur under conditions lacking photosynthesis, even in the presence of SA (Fig. 6B, 7B).

In summary, while our results show no significant ROS increase under short-term SA treatment, multiple lines of evidence point toward ROS as key signaling intermediates in SA-mediated GCN2 activation. Further research is needed to fully dissect the spatial, temporal, and redox dynamics of SA-ROS interactions in the context of the GCN2-eIF2α signaling module.

Phenotypic Convergence Suggests Partial GCN2 Independence in SA Signaling

Loss-of-GCN2 mutants display a range of phenotypes, particularly under stress conditions (Lokdarshi & von Arnim, 2022). These phenotypes range from growth retardation under prolonged cold and salt stress (Lokdarshi et al., 2020b), to growth patterns indistinguishable from wild-type after infection with viral pathogens (Zhang et al., 2008), bacterial pathogens, or xenobiotic agents (Izquierdo et al., 2018). At the molecular level, *gcn2* mutants also exhibit altered levels of P-eIF2α and corresponding changes in translational repression across different stress conditions. For example, two h of chlorosulfuron treatment induces P-eIF2α and downregulation of global translation only in the wild-type seedlings, whereas the *gcn2* mutants maintain elevated translation rates (Lokdarshi et al., 2020a). In contrast, under two h of cold or salt stress, both wild-type and *gcn2* mutants show similar translation profiles, despite the induction of P-eIF2α in the wild-type (Lokdarshi et al., 2020b). These findings suggest that *gcn2* mutants lack a distinct molecular/physiological and phenotypic conservation across all stress types.

Instead, the *GCN2* gene appears to function in a context-dependent manner, modulating responses based on the specific environmental cue or stressor encountered. This variability in phenotypic outcome likely reflects the differential biochemical and molecular compensatory mechanisms that operate in the absence of *GCN2* in the mutant plants. One such compensatory pathway could involve the low-energy sensing protein kinase, SnRK1, which may assume a regulatory role in modulating translation during prolonged stress conditions (Bruns et al., 2019; Peixoto & Baena-González, 2022).

Overall, our results align with previous findings and support the hypothesis that rapid activation of Arabidopsis GCN2 (assessed by monitoring the P-eIF2α status) under SA may not be the sole or even the primary mechanism driving the long-term phenotypic outcomes under SA stress. Future investigations integrating alternative kinase pathways and extended time-course analyses of P-eIF2α and translational repression will be essential to fully understand the phenotypic behavior of *gcn2* mutants during prolonged SA exposure.

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