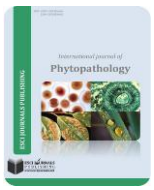

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# *ARABIDOPSIS THALIANA* AtGCN2 KINASE IS INVOLVED IN DISEASE RESISTANCE AGAINST PATHOGENS WITH DIVERSE LIFE STYLES

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**A B S T R A C T**

The ability of the plants to detect diverse stress conditions and initiate cellular responses is vital to their survival in a constantly changing environment. General regulatory molecules often play crucial roles in controlling a multitude of cellular processes throughout the life span of an organism. GCN2 (general control nonderepressible 2) is a serine/threonine-protein kinase that acts as a global translational regulator in all eukaryotes from yeast to mammals to plants. GCN2 plays universal roles in mitigating cellular stresses by directly binding with uncharged tRNAs and phosphorylating its target, eukaryotic initiation factor 2 alpha (eIF2α). Here, we demonstrate that *Arabidopsis thaliana* GCN2 (AtGCN2) serves as a general regulator of salicylic acid- and jasmonic acid-mediated immune responses triggered upon infection with biotrophic and necrotrophic pathogens. Intriguingly, we found examples of both positive and negative influence of AtGCN2 on plant immunity at different developmental stages. This effect is consistent with the variable amount of abscisic acid accumulation in plants lacking functional AtGCN2 at early stages of development. Finally, we illustrate that AtGCN2 positively contributes to water loss and might also be involved in the epidermis-mediated defense responses.

**Keywords**: AtGCN2, eIF2α, biotrophs, necrotrophs, *Hyaloperonospora arabidopsidis*, *Golovinomyces cichoracearum*, *Pectobacterium carotovorum* subsp. *Carotovorum*.

## INTRODUCTION

The lifestyles of plant pathogens vary considerably ranging from biotrophy to necrotrophy. Biotrophs establish intimate associations with the plant and require living host cells to complete their infection cycle (Glazebrook, 2005; Spoel *et al*., 2007). Typically, biotrophs acquire nutrients from the host cell, while residing and growing mainly in the extracellular matrix termed as apoplast. Through their virulent activities, the pathogens establish a nutrient sink at the infection site in such a way that the host is disadvantaged but not killed (Glazebrook, 2005; Spoel *et al*., 2007). In contrast, necrotrophs secrete cell wall-degrading enzymes and toxins to kill the host cells before colonizing them and subsequently extract nutrients from the dead tissues (Glazebrook, 2005; Spoel *et al*., 2007). In response to pathogen infection, plants deploy a tightly controlled multilayered defense program that is specifically tailored towards the offense strategy of their attacker. This response relies on a number of key regulatory factors as well as the activation of an intricate phytohormone signaling network. While the roles of primary hormones, salicylates (SAs), jasmonates (JAs) and ethylene (ET), in plant immune system have been undoubtedly well established (Spoel and Dong, 2008), recently other phytohormones such as abscisic acid (ABA), auxins (indole-3-acetic acid), gibberellins and cytokinins have been implicated in the complex hormone crosstalk (Argueso *et al*., 2012; De Torres Zabala *et al*., 2009; Kazan and Manners, 2009; Robert-Seilaniantz *et al*., 2011; Wang *et al*., 2007; Yang *et al*., 2012; Zheng *et al*., 2012). To the first approximation, SA is primarily involved in responses against biotrophic

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pathogens, while JA is usually employed as a defense signal against necrotrophs, and the two molecules exhibit mostly antagonistic effects (Boatwright and Pajerowska-Mukhtar, 2013; Loake and Grant, 2007; Spoel and Dong, 2008).

GCN2 (General Control Nonderepressible 2) is a multi-domain serine/threonine protein kinase that contains an N-terminal kinase domain and a C-terminal region homologous to histidyl-tRNA synthetase (HisRS) (Sood *et al*., 2000; Wek *et al*., 1995). GCN2 is conserved in virtually all eukaryotes both at the structural and functional levels. It coordinates translation initiation rates of the key regulatory factors by sensing amino acid starvation and thereby allowing the cells to effectively adapt to nutrient availability. In yeast and mammals, uncharged tRNAs bind with the HisRS domain under amino acid starvation conditions and activate GCN2 kinase activities (Hinnebusch, 2005; Wek *et al*., 2006). Subsequently, GCN2 phosphorylates eukaryotic initiation factor alpha (eIF2α) to derepress translation of downstream target mRNAs including mammalian Activating Transcription Factor 4 (ATF4) and yeast General Control Nonderepressible 4 (GCN4) (Hinnebusch, 2005; Wek *et al*., 2006). This translational derepression-mediated by GCN2-eIF2α is evolutionarily conserved across kingdoms (Castilho *et al*., 2014; Dever *et al*., 1992; Hinnebusch, 2005; Vattem and Wek, 2004; Wek *et al*., 2006).

*Arabidopsis thaliana* (hereafter: Arabidopsis) GCN2 (AtGCN2) was demonstrated to bind uncharged tRNA molecules and exhibits its enzymatic activities on both Arabidopsis eIF2α homologs (Li *et al*., 2013). In addition, AtGCN2 was reported to complement the yeast strain lacking functional *GCN2* (Zhang *et al*., 2003). AtGCN2 is also involved in the chemical β-aminobutyric acid (BABA)-induced growth suppression, whereas BABA-induced resistance against virulent pathogen is AtGCN2-independent (Luna *et al*., 2014). Recently, AtGCN2 has been reported to function in the normal growth and development, including seed germination, chlorophyll accumulation and leaf shape (Liu *et al*., 2015; Merchant and Pajerowska-Mukhtar, 2015).Though the AtGCN2-eIF2α cascade can be activated under diverse stress conditions including herbicides, BABA, wounding and phytohormones such as SA, JA and ET (Lageix *et al*., 2008; Zhang *et al*., 2008), the involvement of AtGCN2 in pathogen-triggered immune responses remains unclear. We set out to comprehensively understand the role of GCN2 in defenses against various types of pathogens. Here, we demonstrate that AtGCN2 plays an essential role in disease resistance against both biotrophic and necrotrophic pathogens. We also present the evidence for differential basal ABA accumulation in loss-of-function *atgcn2* mutant plants at different stages of development. Finally, we show that AtGCN2-mediated immune responses can vary depending on the age and developmental stage of Arabidopsis plants.

## MATERIALS AND METHODS

**Biological materials and growth conditions:** *Arabidopsis thaliana* (L.) Heynh. accession Landsberg *erecta* (L*er*) plants were used in this study. *atgcn2* Genetrap insertion line GT8359 was obtained from Cold Spring Harbor Laboratory, New York. Seeds were sown on Super Fine Germination Mix soil and incubated at 4°C for 72h. Plants were grown under a 12h light/12h dark photoperiod at 21°C with 100 μmol/$m^2$/s light intensity and 40% relative humidity (standard conditions).

For *Golovinomyces cichoracearum* infections, plants were grown under controlled conditions in a growth chamber (Percival Scientific) at 22°C day/19°C night with under a 16h light/8h dark photoperiod and 50% relative humidity (RH). Inoculated plants were kept in growth chambers (Percival Scientific). For *Hyaloperonospora arabidopsidis* infections, plants were grown under controlled conditions in a growth chamber (Percival Scientific) at 19°C, under a 8h light/16h dark photoperiod and 50 RH. Inoculated plants were kept in growth chambers (Percival Scientific).

*Golovinomyces cichoracearum* var. *cichoracearum* (DC.) V. P. Heluta (strain UCSC1) was obtained from the laboratory of Dr. Shauna Somerville (University of California, Berkeley), cultured on cucumber and maintained at 22°C day/19°C night with 16 h of light per 24 h at a light intensity of 150 μmol $m^{-2}$ $s^{-1}$ and 85% RH. Fungal inoculums of *G. cichoracearum* were prepared and inoculations performed as previously described (Wilson *et al*., 2001). *Hpa* isolate Cala2 was obtained from the laboratory of Dr. Jeff Dangl (UNC-Chapel Hill), and propagated weekly on the susceptible ecotype L*er*.

**Pathogen infections and quantifications:** *Pectobacterium carotovorum* subsp. *carotovorum (Pcc)* infection was performed through syringe pressure infiltration with inoculum $OD_{600nm}$=0.0001 on four-week-old L*er* and *atgcn2* mutants.. In brief, two leaves per plant were marked and pathogen inoculum was delivered into the leaf through pressure infiltration. Two

days post inoculation, 12 infected leaves per genotype were detached and leaf discs were homogenized in 10mM $MgCl_2$. Leaf extracts were serially diluted and spotted onto a King's B agar plates for colony enumeration.

For determination of numbers of fungal conidia per colony, plants were lightly infected with *G. cichoracearum* conidia (3-4 conidia per leaf) and the infection was allowed to progress for 5 days. Infected leaves were harvested 5 dpi and cleared in 95% ethanol overnight. Leaves were then equilibrated in a 1:1:1 mixture of water: lactic acid: glycerol for 3 h and counter-stained in 200 mg/ml Trypan blue for 2 h to observe the fungal hyphae and conidiophores. Numbers of conidiophores per colony were then counted at 40X magnification using a light microscope.

*Hpa* Cala2 conidiospores (5 x $10^4$ spores/ml) were sprayed onto two-week-old plants using a pressurized sprayer (Preval). Inoculated plants were kept in growth chambers (Percival Scientific) (19°C, 8:16 hour light/dark) and covered with a transparent plastic dome to maintain high humidity. One day after the first appearance of conidiophores (5-6 dai) the first pair of true leaves was collected from three individual plants, and added to a previously weighed 1.5 ml microcentrifuge tube containing 300 µl of sterile water, for a total of six leaves per sample, and weighted again to determine fresh weight. Samples were vortexed for 1 minute to release spores. Spores were counted using a hemocytometer and normalized by plant fresh weight.

**RNA Extraction and q-RT-PCR:** Four Arabidopsis leaves were collected from individual plants and frozen in liquid nitrogen. Total RNA was extracted from each sample using TRIzol reagent (Invitrogen) and concentration were measured by BioPhotometer Plus (Eppendorf) as described previously (Pajerowska-Mukhtar *et al*., 2012). DNA contamination was removed by DNase I (Ambion) treatment. The cDNA were generated by reverse transcription through the SuperScript III first-strand RT-PCR kit (Invitrogen). The relative abundance of transcript was determined through quantitative RT-PCR using GoTaq qPCR Master Mix (Promega) in a RealPlex S MasterCycler (Eppendorf). Specific primers for *PDF1.2* (forward: 5' CTGCTCTTGTTCTCTTTGCTG 3', reverse: 5' CATGTTTGGCTCCTTCAAGG 3'), *SID2* (forward: 5' AATTGGCAGGGAGACTTACG 3', reverse: 5' GTCCCGCATACATTCCTCTATC 3'), *UBQ5* (forward: 5' GTAAACGTAGGTGAGTCC 3', reverse: 5' GACGCTTCATCTCGTCC 3') were used.

**Weight loss measurement:** Weight loss was determined by weighing 4-week-old rosettes of L*er* and *atgcn2*. Rosettes were detached from the root and placed on the weigh boat at room temperature. The weight was measured every 10 min for 70 min after the excision.

**Abscisic acid quantification:** Endogenous abscisic acid (ABA) concentration was measured in L*er* and *atgcn2* Arabidopsis seedlings. Plant tissues of L*er* and the *atgcn2* mutant were collected starting at 3 days post germination and every three days until 27-day-old. The extraction of ABA was performed as described previously (Arenas-Huertero *et al*., 2000). In brief, 0.1g of tissue was homogenized and extracted with 1ml buffer (10mM HCl, 1% PVPP in methanol) overnight. The extract was neutralized with 1M NaOH and dried under SpeedVac (Thermofisher). The dry residues were resuspended in water and quantified with Phytodetek ABA kit (AGDIA, Inc.).

**RESULTS**

**Plants lacking functional AtGCN2 exhibit enhanced disease resistance towards a necrotrophic pathogen:** Resistance against necrotrophic pathogens is primarily mediated by the stimulation of the JA signaling pathway. Given that AtGCN2-mediated phosphorylation of eIF2α is activated by application of methyl jasmonate as well as by mechanical wounding, a JA inducing treatment (Lageix *et al*., 2008), we hypothesized that AtGCN2 might participate in immune responses against necrotrophic pathogens. To test this hypothesis through genetic experiments, we used a Genetrap insertion line for AtGCN2 described previously (Liu *et al*., 2015).

To characterize the functions of AtGCN2 in the defense responses against a necrotrophic bacterial pathogen, we subjected the *atgcn2* loss-of-function mutant plants and L*er* wild type to infection with *Pectobacterium carotovorum* subsp. *carotovorum (Pcc,* also known as *Erwinia carotovora)*. We challenged the 4-week-old plants with *Pcc* through syringe inoculation and observed that the *atgcn2* mutant displays less severe disease phenotypes (fewer greasy, water-soaked lesions) compared to L*er* (Figure 1A). Pathogen quantification of infected leaf tissue revealed that the *atgcn2* mutant accumulated ~10 times lower bacterial load compared to the L*er* (Figure 1B), which is consistent with the visual disease symptoms phenotype. Given that JA-dependent immune responses are

essential for resistance against necrotrophs and this phytohormone positively influences the transcript levels of the plant defensin gene *PDF1. 2* (Glazebrook, 2005; Kariola *et al*., 2003), we investigated the accumulation of *PDF1.2* transcripts upon *Pcc* infection. As illustrated in Figure 1C, the *atgcn2* mutant exhibits ~40 times more *PDF1.2* transcript compared to L*er* upon *Pcc* challenge two days post infection, further corroborating the enhanced *Pcc* disease resistance phenotype. Taken together, our data suggest that AtGCN2 functions as a negative regulator of JA-mediated immune responses triggered by a necrotrophic bacterium.

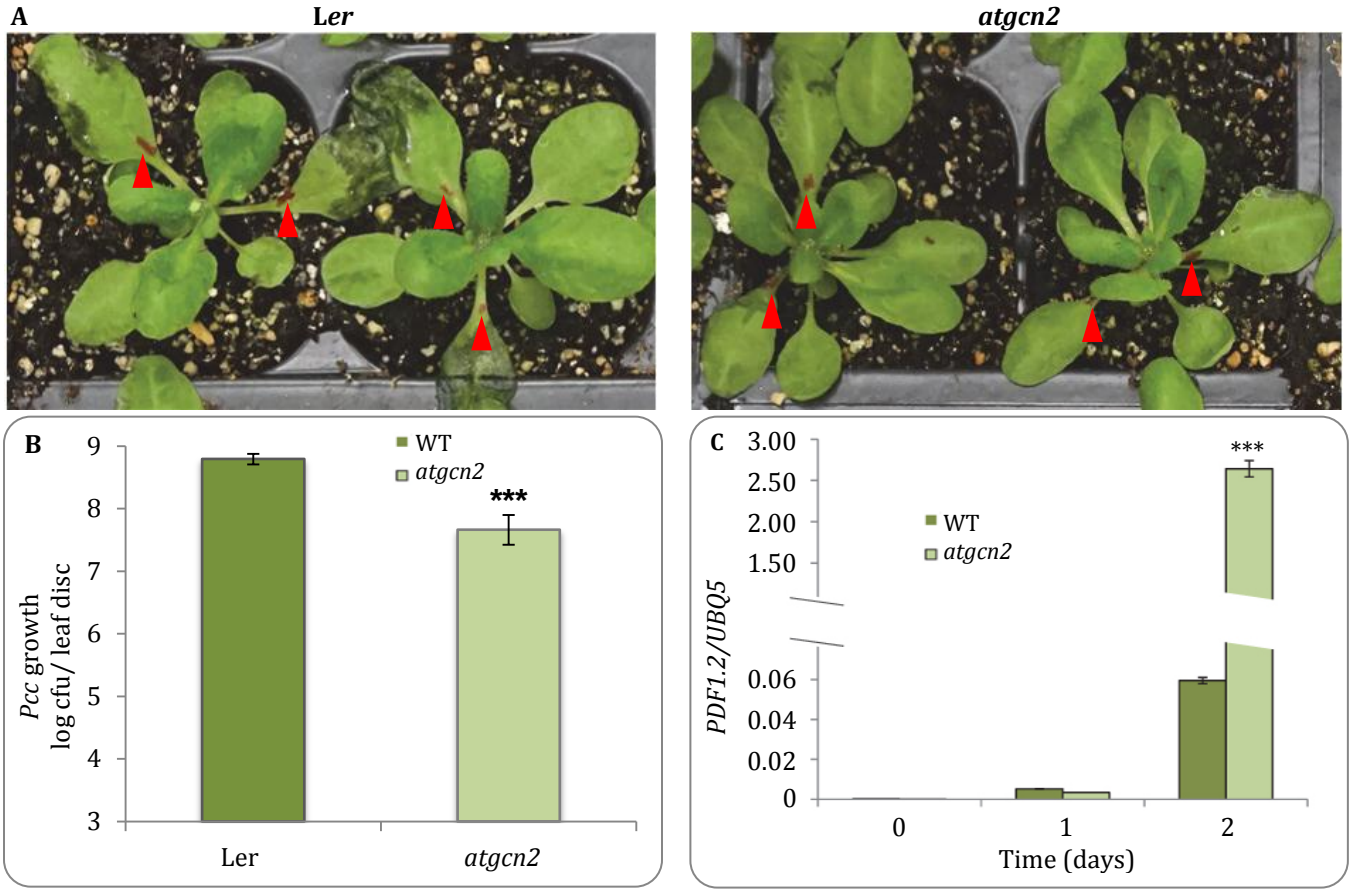


Figure 1. The *atgcn2* mutant displays enhanced disease resistance against *Pectobacterium carotovorum* subsp. *carotovorum (Pcc).*

(A) Representative disease symptoms on leaves from four-week-old plants infected with *Pcc.* Photographs were taken two days post inoculation with *Pcc* $OD_{600nm}$=0.0001. (B) *Pcc* growth (colony forming units – cfu/leaf disc, expressed on a log scale) was quantified in four-week-old plants two days post inoculation ($OD_{600nm}$=0.0001). Error bars represent 95% confidence intervals of the mean (n = 6) . Statistical analysis was performed with Student's t-test, *** p<0.001. (C) Transcript accumulation of *PDF1.2* upon *Pcc* infection was measured by real-time RT-PCR. Data represent means and standard errors of three technical replicates. Statistical analysis was performed with Student's t-test, *** p<0.0001. Experiments were conducted in three biological replications with similar results.

**Enhanced disease resistance towards a biotrophic pathogen in the *atgcn2* mutant:** The activation of SA signaling is required for resistance against biotrophic pathogens (Glazebrook, 2005). While the SA-JA antagonism is considered one of the dogmas in the field of plant immunity (Mur *et al*., 2006; Spoel and Dong, 2008), intriguingly AtGCN2-dependent phosphorylation of eIF2α can be activated by JA and SA (Lageix *et al*., 2008). Therefore, we asked whether AtGCN2 might also be involved in defenses against biotrophs. We subjected four-week-old *atgcn2* and L*er* plants to infection with an obligate biotrophic fungal pathogen *G. cichoracearum* and examined the disease progression over the course of five days. We observed a significant reduction of pathogen growth on the *atgcn2* mutant plants compared to L*er* (Figure 2A). Quantification of the *G. cichoracearum* growth five days post inoculation revealed that the *atgcn2* mutant exhibits 40% less conidiospores than L*er* (Figure 2B).

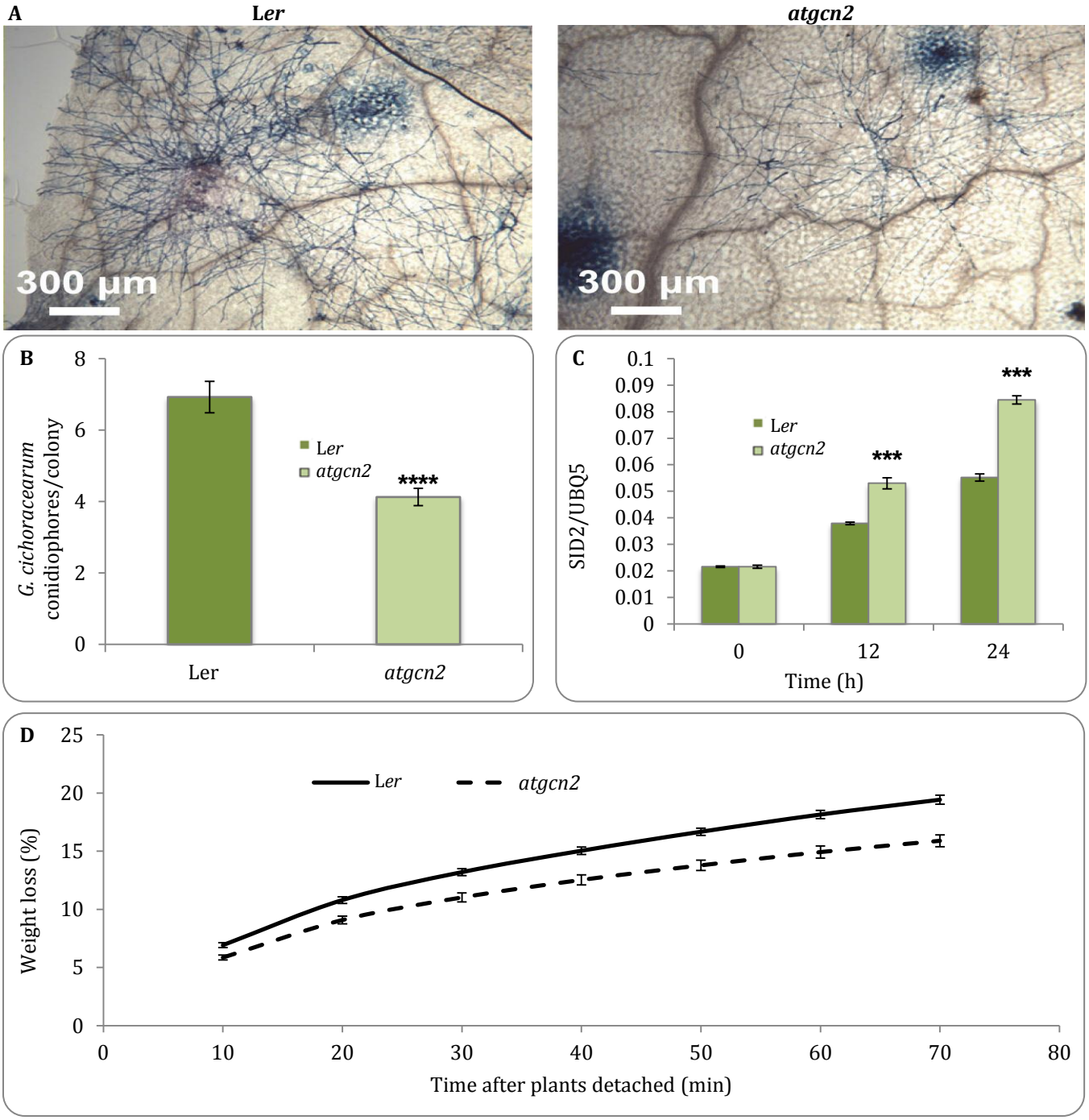


Figure 2. The *atgcn2* mutant displays enhanced disease resistance against a biotrophic fungal pathogen *Golovinomyces cichoracearum*

(A) Representative disease symptoms on leaves infected with *G. cichoracearum* on four-week-old plants. Trypan blue staining was used to visualize fungal structures. (B) The four-week-old *atgcn2* mutant is more resistant against *G. cichoracearum.* Data represent means of *G. cichoracearum* conidiophores per colony and error bars represent standard error. Statistical analysis was performed with Student's t-test, **** $p<0.0001$. Experiments were repeated in two independent biological replications with similar results. (C) The transcript accumulation of *SID2* at 12 and 24 hours after 1mM SA spray was measured by real-time RT-PCR. Data represent means and standard errors of three technical replicates. Statistical analysis was performed with Student's t-test, *** $p<0.001$. Experiments were repeated three times with similar results.

Cont…

(D) The *atgcn2* mutant displays a slower rate of water weight loss upon excision. Data represent means of the weight loss and error bars represent standard error. Experiments were repeated in two independent biological replications with similar results.

To determine whether the enhanced resistance against a biotroph in the *atgcn2* mutant is associated with altered SA-mediated defenses, we quantified the transcript accumulation of an SA-responsive gene *SID2* upon external SA application. SID2, also known as ICS1, is the key SA biosynthesis enzyme responsible for 95% SA biosynthesis (Garcion *et al*., 2008; Wildermuth *et al*., 2001). Consistent with the enhanced disease resistance phenotypes against *G. cichoracearum*, we demonstrated that the induced but not the basal *SID2* expression levels are elevated in the *atgcn2* mutant compared to L*er* and this trend increases over time (Figure 2C).

In addition to the induced resistance, plants are also equipped with constitutive defense responses, mainly through the existence of physical barriers. Since most fungi need to penetrate through the epidermis of the leaf to achieve host colonization, the epidermis and cuticle play an important role in limiting the pathogen entrance (Javelle *et al*., 2011). Recently, genes responsible for cuticle synthesis have been implicated in defense responses against both biotrophs and necrotrophs (Mang *et al*., 2009). To investigate whether AtGCN2 is involved in epidermis permeability, we performed the weight loss assay that quantifies the rate of dehydration in a detached rosette. We observed a significantly reduced transpiration rate in the *atgcn2* mutant compared to L*er* suggesting a positive role of AtGCN2 in regulating epidermal permeability (Figure 2D). Collectively, our data indicate that AtGCN2 serves as a negative regulator of plant immune responses by inhibiting the preformed and induced disease resistance against a biotrophic pathogen.

**Differential ABA accumulation at early developmental stages influences *atgcn2* responses to biotrophic pathogens:** While SA and JA are predominantly responsible for plant's reactions to pathogen-mediated biotic stresses, they also engage in an intricate phytohormonal cross-talk involving other signals including ABA. ABA has been previously shown to close stomata and limit pathogen entrance (Cao *et al*., 2011; Javelle *et al*., 2011; Melotto *et al*., 2006; Ton *et al*., 2009), thus participating in epidermal defenses. Given that ABA synthesis is activated by water stress, exogenous application of ABA represses water loss in detached Arabidopsis leaves and ABA signaling differentially contributes to both pre-invasive and post-invasive phases of plant defense (Melotto *et al*., 2008; Shinozaki and Yamaguchi-Shinozaki, 1997; Ton *et al*., 2009) , we hypothesized that AtGCN2 might be involved in ABA biosynthesis and/or accumulation. Towards this, we quantified endogenous ABA accumulation at various developmental stages. We observed an increased concentration of basal ABA in three-day-old *atgcn2* mutant compared to L*er* (Figure 3A).

This is consistent with our previous report illustrating that the *atgcn2* mutant shows a delayed seed germination phenotype (Liu *et al*., 2015). Intriguingly, the *atgcn2* mutant accumulates ~30% less ABA compared to L*er* in six- and nine-day-old plants. However, ABA levels were indistinguishable between *atgcn2* and L*er* starting at 12 days old and throughout the remainder of the time period assayed. This differential accumulation of ABA in the early plant development and its role in modulating pre-invasive and post-invasive defenses prompted us to investigate the responses of the young *atgcn2* mutant to the biotrophic pathogens.

We challenged young *atgcn2* mutant seedlings with *G. cichoracearum* and quantified the pathogen growth at five days post inoculation. In contrast to our results obtained with older plants, we observed that the *atgcn2* plants exhibited 31% more pathogen growth than L*er* as determined by conidiospores enumeration (Figure 3B). To corroborate our observations, we subsequently subjected the young *atgcn2* mutant seedlings to an infection with another obligate biotroph, oomycete *Hyaloperonospora arabidopsidis* (*Hpa*) isolate Cala2 (compatible with L*er*). Consistent with our previous results, the *atgcn2* mutant seedlings supported a 78% higher spore formation compared to L*er* (Figure 3C). Collectively, these data demonstrate that young *atgcn2* plants display a reversed trend of enhanced susceptibility to obligate biotrophs, and this effect may be explained by increased ABA accumulation leading to altered epidermal defenses.

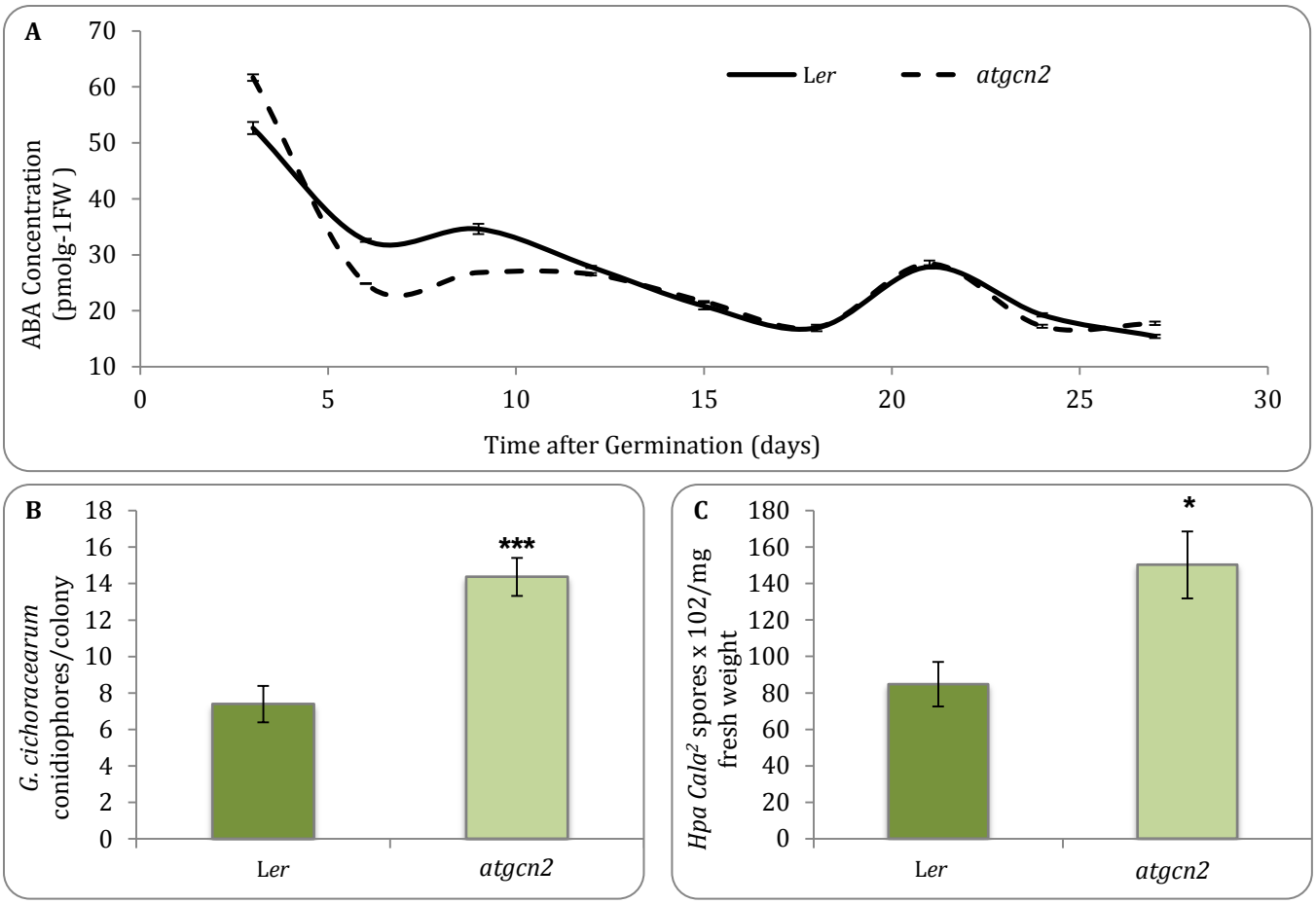


Figure 3. The *atgcn2* mutant displays enhanced disease susceptibility towards biotrophic pathogens during early developmental stages.

(A) The *atgcn2* mutant accumulates less endogenous ABA during early developmental stages. Data represent the ABA concentration per fresh weight and error bars represent standard error. Experiment was repeated in two independent biological replications with similar results. (B) The two-week-old *atgcn2* mutant is more susceptible towards *G. cichoracearum.* Data represent means of *G. cichoracearum* conidiophores per colony and error bars represent standard error. Statistical analysis was performed with Student's t-test, ****p<0. 0001. Experiments were repeated in three independent biological replications with similar results. (C) The two-week-old *atgcn2* mutant is more susceptible towards *Hpa* Cala2. Data represent means of *Hpa* Cala2 spores per fresh weight and error bars represent standard error. Statistical analysis was performed with Student's t-test, * p<0.05. Spore counts from at least four samples per genotype were determined. Experiments were repeated in three independent biological replications with similar results.

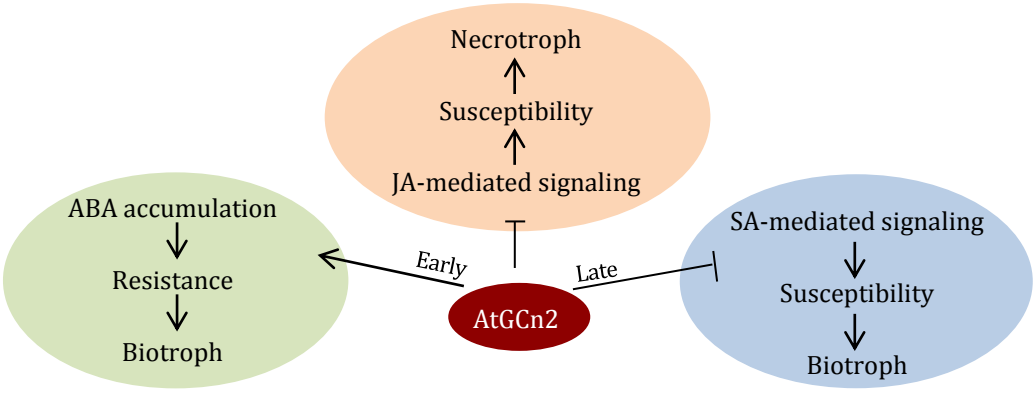


Figure 4. AtGCN2 contributes to plant immune responses against pathogens with different life styles.

AtGCN2 negatively regulates JA- and SA-mediated immune responses to confer disease susceptibility towards necrotrophic and biotrophic pathogens, respectively. In addition, AtGCN2 is required for endogenous ABA accumulation to promote disease resistance against biotrophic pathogens during early developmental stages.

**DISCUSSION**

The consistently enhanced disease resistance phenotypes of four-week old *atgcn2* plants to both biotrophic and necrotrophic pathogens (Figure 4) are somewhat unexpected and intriguing because examples of opposite outcomes linked to contrasting pathogen lifestyles are prevailing in the literature (Li *et al*., 2006; Mang *et al*., 2009; Murmu *et al*., 2014; Veronese *et al*., 2006). However, it is entirely plausible that both necrotrophs and biotrophs, regardless of their lifestyles, feed on the host and induce amino acid starvation leading to the accumulation of uncharged tRNAs. Consequently, this process results in the activation of AtGCN2 followed by eIF2α phosphorylation and might lead to the translational depression of downstream susceptibility target transcripts. It has been demonstrated that the GCN2 kinase is essential for intact immune responses in mice and fruit fly (Bunpo *et al*., 2010; Chakrabarti *et al*., 2012) and recently, a study in humans showed a link between this ancient starvation signaling pathway and response following immunization with yellow fever vaccine (Ravindran *et al*., 2014). Thus, it seems plausible that AtGCN2 might act as a universal immune regulator in plants, negatively controlling both SA- and JA-mediated defenses.

Moreover, our data indicate that GCN2 is also required for ABA biosynthesis and/or accumulation during early developmental stages. A growing body of evidence suggests that ABA levels can have impact on biotic stress responses (Achuo *et al*., 2006) but the specific contribution varies widely depending on the type of pathogen, its nutritional strategy and the host tissue infected (Fan *et al*., 2009; Mauch-Mani and Mauch, 2005; Ton *et al*., 2009). It was proposed that ABA positively contributes to early stages of immune response, promoting stomatal closure that helps restrict entry of bacterial, fungal and oomycete pathogens alike (Ton *et al*., 2009). Consequently, the deficiency in endogenous ABA accumulation in the young *atgcn2* mutant could explain the enhanced susceptibility to penetration by *H. arabidopsidis*. At the later stages of infection, the role of ABA becomes more complex as it engages in cross-talk with other immune hormones such as SA, JA and ET (Ton *et al*., 2009). Thus, the contrasting disease resistance patterns to obligate biotrophs, manifested at diverse developmental stages of *atgcn2* plants could be caused by the differential ABA production and/or accumulation.

The connection between modified ABA concentration and/or signaling and defenses against necrotrophs has been reported previously. High concentrations of ABA were shown to promote tomato susceptibility to *Dickeya dadantii* (previously known as *Erwinia chrysanthemi*, a necrotrophic bacterial pathogen related to *Pcc*) (Asselbergh *et al*., 2008) and a recent study, exploring the mechanistic roles of novel ABA signaling components HAS1 and HAS2, demonstrated a connection between disrupted stomatal responses and resistance to *D. dadantii* (Plessis *et al*., 2011). Our findings describing the *atgcn2* immune phenotypes are overall consistent with the previously shown correlation between ABA concentration and levels of susceptibility.

Although the differences in endogenous ABA accumulation offer a plausible explanation for the observed immune phenotypes in the *atgcn2* plants, it is also conceivable that additional differences exist in the ABA signal transduction pathway. To explore this possibility, we compared basal transcript levels of several well-known ABA signaling genes: *HAB1*, *ABI1*, *ABI2* and *PP2CA* (Cutler *et al*., 2010) in L*er* and *atgcn2* plants and found no significant differences in their expression profiles under the conditions tested (data not shown). However, it cannot be ruled out that factors other than ABA accumulation contribute to disease phenotypes of the *atgcn2* mutant plants.

The relationship between plant age and its responses to infection has been an intriguing problem in plant-microbes interactions. While it has been generally acknowledged that younger plants are more susceptible, it is clear that the mechanistic underpinnings of this response are complex and multifaceted (Develey-Riviere and Galiana, 2007). The phenomenon of age-regulated resistance is of paramount importance in agriculture, but remains underexploited, largely due to a lack of understanding of the genetic, molecular and cellular mechanisms underlying this response (Panter and Jones, 2002; Whalen, 2005). Several studies showed the resistance (R) proteins and SA signaling components contribute to this response (Century *et al*., 1999; McDowell *et al*., 2005; Panter *et al*., 2002) but the contributions of other phytohormones remain unexplored. Given that every aspect of plant development is hormonally controlled and extensive crosstalk between all hormone signaling pathways exist (Robert-Seilaniantz *et al*., 2011; Spoel and Dong, 2008), it seems plausible that additional hormones could

contribute to the age-regulated resistance phenomenon. Our genetic data provide evidence that AtGCN2 contributes to age-dependent defense responses and suggest that, in addition to changes in the expression of SA and JA signaling genes, ABA accumulation might contribute to the immune differences observed between young and mature *atgcn2* mutant plants.

The favorably enhanced disease resistance against unrelated pathogens with contrasting invasion styles and feeding strategies in the loss-of-function *atgcn2* plants makes AtGCN2 a potentially attractive target for manipulation in crop plants. At the first glance, additional benefits exist, such as increased chlorophyll levels and overall larger leaves (Liu *et al*., 2015; Merchant and Pajerowska-Mukhtar, 2015). However, the *atgcn2* mutant plants also display a range of undesirable phenotypes, such as delayed germination and disruption of gibberellic acid signaling pathway (Liu *et al*., 2015) as well as enhanced pathogen susceptibility in young plants (this report). If AtGCN2 were to be manipulated in crop plants, the interventions would need to include custom-tailored mutations that induce favorable phenotypes but prevent the onset of any detrimental responses.

**CONCLUSION**

Overall, we demonstrated that AtGCN2 acts as a general negative regulator of SA- and JA-mediated immune response against biotrophic and necrotrophic pathogens in adult Arabidopsis plants (Figure 4). Beside induced resistance, AtGCN2 may also play a role in the epidermis-mediated defense response. Moreover, AtGCN2 is also required for endogenous ABA accumulation during early developmental stages. We conclude that AtGCN2 is implicated in the immune responses against phytopathogens with diverse life styles and contributes to developmentally-regulated ABA accumulation.

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