**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-Seilanianzt 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...
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 (Ler) 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/m²/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 Ler.

Pathogen infections and quantifications: Pectobacterium carotovorum subsp. carotovorum (Pcc) infection was performed through syringe pressure infiltration with inoculum OD₆₀₀nm=0.0001 on four-week-old Ler 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 MgCl2. 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 PDDF1.2 (forward: 5’ GTAAACGTTAGGTAGTCC 3’, reverse: 5’ GACGCTTCATCTCGTCC 3’) were used.

**Weight loss measurement:** Weight loss was determined by weighing 4-week-old rosettes of Ler 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 Ler and atgcn2 Arabidopsis seedlings. Plant tissues of Ler 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 Ler 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 Ler (Figure 1A). Pathogen quantification of infected leaf tissue revealed that the atgcn2 mutant accumulated ~10 times lower bacterial load compared to the Ler (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 Ler 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.

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 Ler 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 Ler (Figure 2A). Quantification of the G. cichoracearum growth five days post inoculation revealed that the atgcn2 mutant exhibits 40% less conidiospores than Ler (Figure 2B).

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.
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.
(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 Ler 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 Ler 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 Ler (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 Ler in six- and nine-day-old plants. However, ABA levels were indistinguishable between atgcn2 and Ler 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 Ler 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 Ler). Consistent with our previous results, the atgcn2 mutant seedlings supported a 78% higher spore formation compared to Ler (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.
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. arabidopsis. 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 Ler 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-Seilianiitz 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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