RESEARCH PAPER

Phosphorylation of ATG18a by BAK1 suppresses autophagy and attenuates plant resistance against necrotrophic pathogens

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ABSTRACT

Autophagy is critical for plant defense against necrotrophic pathogens, which causes serious yield loss on crops. However, the post-translational regulatory mechanisms of autophagy pathway in plant resistance against necrotrophs remain poorly understood. In this study, we report that phosphorylation modification on ATG18a, a key regulator of autophagosome formation in *Arabidopsis thaliana*, constitutes a post-translation regulation of autophagy, which attenuates plant resistance against necrotrophic pathogens. We found that phosphorylation of ATG18a suppresses autophagosome formation and its subsequent delivery into the vacuole, which results in reduced autophagy activity and compromised plant resistance against *Botrytis cinerea*. In contrast, overexpression of ATG18a dephosphorylation-mimic form increases the accumulation of autophagosomes and complements the plant resistance of *atg18a* mutant against *B. cinerea*. Moreover, BAK1, a key regulator in plant resistance, was identified to physically interact with and phosphorylate ATG18a. Mutation of *BAK1* blocks ATG18a phosphorylation at four of the five detected phosphorylation sites after *B. cinerea* infection and strongly activates autophagy, leading to enhanced resistance against *B. cinerea*. Collectively, the identification of functional phosphorylation sites on ATG18a and the corresponding kinase BAK1 unveiled how plant regulates autophagy during resistance against necrotrophic pathogens.

Abbreviations: 35s: the cauliflower mosaic virus 35s promoter; *A. thaliana*: *Arabidopsis thaliana*; *A. brassicicola*: *Alternaria brassicicola*; ABA: abscisic acid; ATG: autophagy-related; ATG18a: autophagy-related protein 18a in *A. thaliana*; ATG8a: autophagy-related protein 8a in *A. thaliana*; ATG8-PE: ATG8 conjugated with PE; *B. cinerea*: *Botrytis cinerea*; BAK1: Brassinosteroid insensitive 1-associated receptor kinase1 in *A. thaliana*; BiFC: biomolecular fluorescence complementation; BIK1: Botrytis-insensitive kinase 1 in *A. thaliana*; BKK1: BAK1-like 1 in *A. thaliana*; BR: brassinosteroid; Co-IP: coimmunoprecipitation; dai: days after inoculation; DAMPs: damage-associated molecular patterns; *E. coli*: *Escherichia coli*; ER: endoplasmic reticulum; ETI: effector-triggered immunity; GFP: green fluorescent protein; HA: hemagglutinin; IP: immunoprecipitation; LC-MS/MS: liquid chromatography-tandem mass spectrometry; LCI: luciferase complementation imaging; MPK3: mitogen-activated protein kinase 3 in *A. thaliana*; MPK4: mitogen-activated protein kinase 4 in *A. thaliana*; MPK6: mitogen-activated protein kinase 6 in *A. thaliana*; *N. benthamiana*: *Nicotiana benthamiana*; NES: nuclear export sequence; PAMP: pathogen-associated molecular pattern; PCR: polymerase chain reaction; PE: phosphatidylethanolamine; PRR: pattern recognition receptor; PtdIns(3,5)P₂: phosphatidylinositol (3,5)-biphosphate; PtdIns3P: phosphatidylinositol 3-biphosphate; PTI: PAMP-triggered immunity; qRT-PCR: quantitative reverse transcription PCR; SnRK2.6: SNF1-related protein kinase 2.6 in *A. thaliana*; TORC1: the rapamycin-sensitive Tor complex1; TRAF: tumor necrosis factor receptor-associated factor; WT: wild type plant; Yc: C-terminal fragment of YFP; YFP: yellow fluorescent protein; Yn: N-terminal fragment of YFP

ARTICLE HISTORY

Received 6 September 2019

Revised 29 July 2020

Accepted 3 August 2020

KEYWORDS

ATG18a; *A. thaliana*; autophagy; BAK1; *B. cinerea*; resistance

Introduction

Botrytis cinerea is a typical necrotrophic pathogen by killing plant cells to get nutrients [1–3]. It is one of the top ten fungal pathogens on plants because it can infect more than 200 plant species and cause serious damage to crops and fruits before and after harvest [3,4]. However, how plants defend against *B. cinerea* is still largely unknown. To fight against pathogen infection, plants depend on the two-branched innate immunity: PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) [5]. Pattern recognition receptors (PRRs) on plant plasma membrane detect conserved microbial molecules

(pathogen/microbe-associated molecular patterns, PAMPs/MAMPs) and plant-derived molecules (damage-associated molecular patterns, DAMPs) to activate PTI [6–8]. For successful infection, virulent pathogens secrete a battery of effector proteins, which get into plant cells and suppress PTI to help pathogen infection [9,10]. As a consequence of coevolution, plants generate different resistance (R) proteins to detect effector proteins directly or indirectly; and this interaction activates a strong resistance response, ETI, which is usually accompanied by localized cell death [11]. PTI and ETI have been used to explain plant resistance against biotrophic/hemi-biotrophic pathogens very well [12]. But ETI was found to be

hijacked by necrotrophs to induce cell death and promote disease development [2,13]. While, PTI plays positive roles in resistance against necrotrophs [14]. Based on the study on molecular genetics of *Arabidopsis*-necrotroph interaction, plant resistant system against necrotrophs is more complicated than that against biotrophs/hemibiotrophs.

Macroautophagy/autophagy is one of the major pathways required for resistance against necrotrophs. Blocking autophagy by knockout of *Arabidopsis* autophagy-related (ATG) genes such as *ATG5* and *ATG18a* or autophagy-regulating genes such as *TRAF1a/1b* causes hypersensitive responses to *B. cinerea* [15–18]. Autophagy is a conserved catabolic process in eukaryotes that removes dysfunctional proteins and organelles to vacuole for degradation to maintain cellular homeostasis [19,20]. Under stress conditions, the autophagy pathway is activated to engulf cytoplasmic damaged proteins or organelles into double-membrane structures termed autophagosomes; then, the outer membrane of autophagosomes fuses with the membrane of vacuoles, releasing the inner membrane including the enveloped cargo into vacuoles for degradation by proteases and hydrolases [21,22]. The autophagy pathway is processed by five systems organized by ATG proteins: ATG1 kinase complex, PtdIns3K complex, ATG9 complex, ATG8–PE conjugation system and ATG12–ATG5 conjugation systems [22,23]. While, the function of the ATG9 complex in plants including ATG2, ATG9 and ATG18, has not been extensively studied. In yeast, the ATG9 complex recruits lipid from endoplasmic reticulum (ER) and the Golgi to expand the phagophore membrane and ATG18 functions as a modulator of phosphatidylinositol (3,5)-biphosphate (PtdIns [3,5]P₂) and PtdIns3P [24–27]. In *Arabidopsis*, eight *ATG18* genes (*ATG18a*–*ATG18h*) have been identified and only *ATG18a* is induced in nutrient starvation and senescence [28]. Under stress conditions, ATG18a interacts with ATG9 and regulates the autophagosome initiation from ER [29]. Mutation of *ATG18a* gene blocks the formation of autophagosomes and enhances plant sensitivity to oxidative stress and nutrient starvation [28,30]. These observations highlight the importance of understanding how plants activate autophagy through ATG18a. But how plants regulate the function of ATG18a in autophagy and plant immunity is still unclear. In addition to its function in cytoplasm, ATG18a physically interacts with transcription factor WRKY33 in nuclei [17]. WRKY33 is a key resistance regulator against *B. cinerea* [31]. It positively regulates multiple resistance pathways such as JA/ET signaling pathway and camalexin biosynthesis and also suppresses SA and ABA signaling pathways, which negatively regulate resistance against *B. cinerea* [31–34]. So, ATG18a involves both of the autophagy-mediated resistance in cytoplasm and the WRKY33-mediated resistance in nuclei. But it is unknown whether the resistance function of ATG18a against *B. cinerea* is dependent on its cytoplasmic function or nuclear function.

Phosphorylation modification is an important post-translational regulation in autophagy. In yeast, the rapamycin-sensitive Tor complex 1 (TORC1) directly phosphorylates ATG13 to suppress the initiation of autophagy [35] and ATG1 kinase complex phosphorylates ATG9 to enhance its activity in recruitment of ATG8 and ATG18 to

autophagosome membrane [36,37]. In *Arabidopsis*, the ATG1-ATG13 protein kinase complex initiates autophagosome formation in response to stress [38]. But how the phosphorylation modification regulates the function of ATG18a in *Arabidopsis* is still unknown.

Here, we report that phosphorylation modification of ATG18a suppresses autophagosome formation during *B. cinerea* infection. By IP-LC-MS/MS, five phosphorylation sites (Thr241, Ser328, Ser344, Ser361 and Thr387) on ATG18a are identified. Overexpression of ATG18a dephosphomimetic form induces strong autophagy signals and enhances resistance against necrotrophic pathogens: *B. cinerea* and *A. brassicicola*, while overexpression of the phosphomimetic form of ATG18a only induces basal level of autophagy signal and cannot complement the susceptible phenotype of *atg18a* mutant against necrotrophs to the wild type completely. Further, BAK1 was identified to be responsible for phosphorylating ATG18a by direct interaction *in vivo*. Mutation of *BAK1* decreases the phosphorylation level of ATG18a and induces strong autophagy signals, resulting in enhanced resistance against necrotrophic pathogens.

Results

The cytoplasm function of ATG18a is sufficient for resistance against *B. cinerea*

Previously, we reported that autophagy is required for disease resistance to necrotrophic pathogens: *B. cinerea* and *Alternaria brassicicola* and mutation of *ATG18a* enhances plant susceptibility to *B. cinerea* [17]. In order to confirm the function of *ATG18a* in resistance to *B. cinerea*, we generated an *ATG18a*-GFP construct and transformed it into *atg18a* mutant (GABI_651D08) to generate complementation lines. Two independent complementation lines (*ATG18a*-GFP/*atg18a*) expressing high level of ATG18a-GFP proteins were selected for disease assay (Figure S1A). Before inoculation with *B. cinerea*, no senescence phenotype was observed on the wild type, *atg18a* mutant and complementation plants (Figure S1B). At 4 dai (days after inoculation), *atg18a* mutant showed enhanced chlorosis and necrosis, but the two complementation lines and wild type plants showed limited disease symptoms (Figure 1A). Consistent with the disease phenotype, *atg18a* mutant accumulated more fungal biomass than wild type, measured by qRT-PCR amplification of *B. cinerea* *ActinA* mRNA and the two complementation lines accumulated the wild-type level of *B. cinerea* biomass (Figure 1B). Thus, expression of ATG18a-GFP restored the disease phenotype of *atg18a* mutant to the wild type level.

To investigate whether cytoplasm-localized ATG18a plays positive roles in resistance against *B. cinerea*, we fused a nuclear export sequence (NES) from HIV Rev protein [39] with *ATG18a*-GFP to generate *NES*-*ATG18a*-GFP construct. The construct was transformed into *atg18a* mutant to generate transgenic lines (*NES*-*ATG18a*-GFP/*atg18a*). Two independent lines expressing high protein level of ATG18a were picked up for further assays (Figure S1C). Confocal microscope assays showed that ATG18a-GFP, as the control, displayed tiny dots in both cytoplasm and nuclei, very similar to

Figure 1. Cytoplasmic function of ATG18a is sufficient to enhance resistance to *B. cinerea*. (A) Overexpressing ATG18a-GFP complemented *atg18a* mutant phenotype to resistance against *B. cinerea*. Four-week-old plants were spray-inoculated with *B. cinerea* at 2.5×10^5 spores/ml and the phenotype was checked at 4 dai. The experiment was repeated three times with similar results. WT, wild type. Scale bar: 2 cm. (B) Fungal biomass accumulation assay measured by qPCR revealed that overexpression of ATG18a-GFP suppressed *B. cinerea* growth. Fungal growth was determined by qRT-PCR amplification of the *B. cinerea* ActinA gene relative to *Arabidopsis* ACT2 gene. The data represent mean values $\pm$ SD ($n = 3$). The mean values following by different letters are significantly different from each other ($p < 0.01$, Student's *t*-test). The experiment was repeated three times with similar results. (C) Overexpressing NES-ATG18a-GFP suppressed the susceptibility of *atg18a* mutant to *B. cinerea*. Four-week-old plants were spray-inoculated with *B. cinerea* at 2.5×10^5 spores/ml and the phenotype was checked at 4 dai. The experiment was repeated three times with similar results. Scale bar: 2 cm. (D) Fungal biomass accumulation assay measured by qRT-PCR revealed that overexpression of NES-ATG18a-GFP in *atg18a* mutant suppressed *B. cinerea* growth. Fungal growth was determined by qPCR amplification of the *B. cinerea* ActinA gene relative to *Arabidopsis* ACT2 gene. The data represent mean values $\pm$ SD ($n = 3$). The experiment was repeated three times with similar results.

previous reports [17,29], while the NES-ATG18a-GFP protein was localized in cytoplasm but not in nuclei before and after *B. cinerea* infection (Figure S1D). This means the NES-ATG18a-GFP protein is localized in the cytoplasm, but not in nuclei. Next, we tested the disease phenotype of these transgenic plants against *B. cinerea*. At 4 dai, the NES-ATG18a-GFP/*atg18a* transgenic lines, similar to the ATG18a-GFP/*atg18a* transgenic lines, showed more resistance than *atg18a* mutant (Figure 1C). Biomass accumulation assay also showed that NES-ATG18a-GFP/*atg18a* transgenic lines accumulated the same level of fungal biomass as the ATG18a-GFP/*atg18a* transgenic lines (Figure 1D). These observations indicate that accumulation of ATG18a proteins in cytoplasm is sufficient to complement the defense phenotype of *atg18a* mutant in response to *B. cinerea* infection.

To further test whether the cytoplasmic function of ATG18a is sufficient for activating autophagy, we transiently co-expressed NES-ATG18a-3xHA construct and ATG18a-3xHA construct individually with GFP-ATG8a construct, an autophagy marker gene, into *atg18a* mutant protoplasts. Similar to ATG18a-3xHA, NES-ATG18a-3xHA induced the formation of autophagosome in *atg18a* mutant protoplasts (Figure S1E and S1F). We conclude that the function of ATG18a in cytoplasm is sufficient to induce autophagy and enhance resistance against *B. cinerea*.

ATG18a is phosphorylated in plant before and after *B. cinerea* infection

When we checked ATG18a-GFP protein level in transgenic plants by western blot, two clear bands were observed: a large band and a small band (Figure S1A). And similar phenotype was also observed when plants were infected with another necrotrophic pathogen *Alternaria brassicicola* (Figure S2A). Because phosphorylation is a common way to cause protein shift, we inferred that the large band might be the phosphorylated ATG18a protein. To examine this possibility, the protein samples from ATG18a-GFP/*atg18a* transgenic plants at 0, 1, 2, and 3 dai were treated with lambda-phosphatase. Compared with samples without lambda-phosphatase treatment, lambda-phosphatase removed the large band of ATG18a-GFP in the samples at 1, 2 and 3 dai (Figure 2A). This indicates that the large band in the samples after infection is the phosphorylated ATG18a-GFP protein. Surprisingly, lambda-phosphatase significantly reduced the level of the large band in the sample at 0 dai, but did not make it disappear (Figure 2A). So, in addition to phosphorylation modification, ATG18a protein contains other post-translational modifications under normal conditions; under *B. cinerea* infection conditions, the large band presented phosphorylated

Figure 2. ATG18a is phosphorylated before and after *B. cinerea* inoculation. (A) Mobility shift of ATG18a was reduced by lambda-phosphatase treatment. Total protein was isolated from the leaves of *ATG18a-GFP/atg18a* transgenic plants at 0, 1, 2, and 3 dai. Half of protein samples were loaded in the SDS-PAGE gel and half of them were loaded after lambda-phosphatase treatment. Those protein samples were separated and western-blotted by anti-GFP antibody. The protein loading control is shown by Ponceau staining. Ratio = large band signal:loading control. The ratio was used to present the relative level of phosphorylated ATG18a protein. The experiment was repeated three times with similar results. (B) ATG18a was phosphorylated at Ser344 before inoculation. To identify the phosphorylation sites on ATG18a before *B. cinerea* inoculation, the leaf samples of *35S:ATG18a-GFP/atg18a* transgenic plants were collected and the ATG18a-GFP protein immunoprecipitated by anti-GFP antibody was sent for LC-MS/MS. The MS analysis covered 78.35% of the total ATG18a protein and identified one monophosphorylated peptide: IAPDSTPS₅PSSSLSLFK. The experiment was repeated three times with the same results. (C) Thr241, Ser328, Ser344, Ser361 and Thr387 were phosphorylated after *B. cinerea* inoculation. To identification the phosphorylation sites on ATG18a after *B. cinerea* inoculation, the leaf samples of *35S:ATG18a-GFP/atg18a* transgenic plants were collected at 24 h after *B. cinerea* inoculation and the ATG18a-GFP protein immunoprecipitated by anti-GFP antibody was sent for LC-MS/MS. The MS analysis covered 76.71% of the total ATG18a protein and identified five monophosphorylated peptide: T-KFVMAHDSR, VNSG₅QVKDSSR, IAPDSTPS₅PSSSLSLFKGVLP, YF₅SEWSVAQFR, N₇VVILGMDGSFYR. The experiment was repeated three times with the same results.

ATG18a-GFP protein. It was also observed that *B. cinerea* infection reduced the amount of the large band (phosphorylated ATG18a-GFP) and enhanced the accumulation of the small band (dephosphorylated ATG18a-GFP) (Figure 2A). Thus, phosphorylation modification may regulate the function of ATG18a in resistance against *B. cinerea*.

To explore the phosphorylation sites on ATG18a *in vivo*, the leaf samples of the *ATG18a-GFP/atg18a* transgenic line before inoculation and those at 1 d after *B. cinerea* inoculation were collected. Then, ATG18a-GFP proteins immunoprecipitated by anti-GFP antibody were sent for liquid chromatography-tandem mass spectrometry (LC-MS/MS).

The results showed that only one serine residue (Ser344) on ATG18a protein was phosphorylated before inoculation (Figure 2B), while three serine residues (Ser328, Ser344, and Ser361) and two threonine residues (Thr241 and Thr387) were identified to be phosphorylated on ATG18a in *B. cinerea*-treated leaf samples (Figure 2C). This indicates that Ser344 on ATG18a is phosphorylated before and after infection and phosphorylation of Thr241, Ser328, Ser361, and Thr387 on ATG18a are induced by *B. cinerea* infection. Also, the phosphorylation pattern on ATG18a is different from the reported PpATG18 phosphorylation pattern (Figure S2B) [25].

Phosphorylation of ATG18a suppresses autophagy activity

In order to explore the function of ATG18a phosphorylation on autophagy and identify the important phosphorylation sites on ATG18a, we mutated the five phosphorylation sites to aspartate (D) on ATG18a as phosphomimetic form of ATG18a, or mutated them to alanine (A) as dephosphomimetic form of ATG18a. For simplicity, the five phosphorylation sites in ATG18a were designated with numbers, Ser344 as 1, Ser361 as 2, Thr241 as 3, Ser328 as 4 and Thr387 as 5. A series of Ser/Thr to Asp (D) mutant constructs of ATG18a: ATG18a^{1D}, ATG18a^{12D}, ATG18a^{2345D}, and ATG18a^{12345D} and a series of Ser/Thr to Ala mutant constructs of ATG18a: ATG18a^{1A}, ATG18a^{12A}, ATG18a^{2345A}, and ATG18a^{12345A} were generated. Same as previous reports, mutation of ATG18a compromised the accumulation of autophagosomes and overexpression of ATG18a can complement the phenotype of *atg18a* mutant to wild type (Figure S1E and S1F). So, we used protoplast system to detect the function of different mutated ATG18a in autophagosome formation. All of those ATG18a constructs were transiently expressed in *atg18a* mutant protoplasts with GFP-ATG8a construct, and the autophagosomes were detected by confocal microscope. Transient expression of ATG18a^{1A}, ATG18a^{1D}, ATG18a^{12A} and ATG18a^{2345A} in *atg18a* mutant protoplasts induced similar level of autophagy activity as transient expression of ATG18a; transient expression of ATG18a^{12D}, ATG18a^{2345D} and ATG18a^{12345D} induced significantly lower autophagy activity than that induced by transient expression of ATG18a (Figure 3A,B, S3A and S3B). Clearly, transient expression of ATG18a^{12345A} induced stronger autophagy activity than that induced by transient expression of ATG18a (Figure 3A,B). These observations suggest that dephosphorylation of ATG18a significantly enhances the accumulation of autophagosomes in protoplasts. Also, the induction of strong autophagy activity requires dephosphorylation of all five sites (Ser344, Ser361, Thr241, Ser328, and Thr387) on ATG18a protein.

In order to check whether ATG18a^{12345A} and ATG18a^{12345D} can induce different autophagy phenotype in plants, we generated GFP-ATG8a/ATG18a-3xHA/*atg18a*, GFP-ATG8a/ATG18a^{12345A}-3xHA/*atg18a* and GFP-ATG8a/ATG18a^{12345D}-3xHA/*atg18a* transgenic plants. Before *B. cinerea* infection, the basal level of autophagosomes in GFP-ATG8a/ATG18a^{12345A}-3xHA/*atg18a* was slightly stronger than that in GFP-ATG8a/ATG18a-3xHA/*atg18a* transgenic plants and the basal level of autophagosomes in GFP-ATG8a/ATG18a^{12345D}-3xHA/*atg18a* was slightly lower than that in GFP-ATG8a/ATG18a-3xHA/*atg18a* transgenic plants with or without concanamycin A (ConA) treatment (Figure 3C,D). At 2 d after *B. cinerea* infection, we observed the same pattern and only slightly more autophagosome accumulation was observed before ConA treatment, while much more autophagosome accumulation was detected after ConA treatment in *B. cinerea* infected plants and it was clear that GFP-ATG8a/ATG18a^{12345A}-3xHA/*atg18a* transgenic plants accumulated much more autophagosomes than GFP-ATG8a/ATG18a-3xHA/*atg18a* transgenic plants and GFP-ATG8a

/ATG18a^{12345D}-3xHA/*atg18a* transgenic plants accumulated much fewer autophagosomes than GFP-ATG8a/ATG18a-3xHA/*atg18a* transgenic plants (Figure 3C,D). This observation confirms that phosphorylation of ATG18a on the five sites suppresses autophagy activity in plants.

When autophagy is activated, ATG8 proteins are associated with phosphatidylethanolamine (PE) to form ATG8-PE [19,23] and the ATG8-PE level has been taken as a reliable tool to show the strength of autophagy activity [40,41]. In order to test the dynamic change of autophagy activity in resistance against *B. cinerea* infection, we conducted the lipidation assay to examine the ATG8-PE level at different time points before and after infection. ATG18a-GFP/*atg18a*, ATG18a^{12345A}-GFP/*atg18a* and ATG18a^{12345D}-GFP/*atg18a* transgenic plants were generated. The ATG8-PE level was weakly detected at 0 and 1 dai in those transgenic plants, but clear ATG8-PE accumulation was observed at 2 and 3 dai; interestingly, the level of ATG8-PE conjugates detected in ATG18a^{12345A}-GFP/*atg18a* plants was more than twice of that in ATG18a-GFP/*atg18a* plants at 2 and 3 dai, while the accumulated ATG8-PE conjugates observed on ATG18a^{12345D}-GFP/*atg18a* plants at 2 and 3 dai were only half of that detected on ATG18a-GFP/*atg18a* (Figure 3E). These data indicate that phosphorylation on ATG18a negatively regulates ATG8 lipidation, and dephosphorylation on ATG18a enhances ATG8 lipidation during resistance against *B. cinerea*. We also checked the GFP-ATG8a process before and after *B. cinerea* infection in those transgenic plants, which was used to measure the delivery of autophagosomes to the vacuole. Compared with GFP-ATG8a/ATG18a-3xHA/*atg18a* transgenic plants, there were more free GFP proteins detected in GFP-ATG8a/ATG18a^{12345A}-3xHA/*atg18a* transgenic plants and there were less free GFP proteins detected in GFP-ATG8a/ATG18a^{12345D}-3xHA/*atg18a* transgenic plants (Figure 3F). Further, we examined the protein level of NBR1, which is a substrate of autophagy degraded in the vacuole [42] and has been taken as a marker for detecting autophagy activity [43,44]. At 2 dai, the NBR1 protein level in GFP-ATG8a/ATG18a^{12345A}-3xHA/*atg18a* transgenic plants was less than that in GFP-ATG8a/ATG18a-3xHA/*atg18a* transgenic plants and that in GFP-ATG8a/ATG18a^{12345D}-3xHA/*atg18a* transgenic plants was more than that in GFP-ATG8a/ATG18a-3xHA/*atg18a* transgenic plants (Figure 3G). Taken together, our results indicate that phosphorylation of ATG18a on the five sites suppresses the autophagy activity induced by *B. cinerea* infection and dephosphorylation of ATG18a on the five sites enhances it.

Phosphorylation suppresses the resistance function of ATG18a against necrotrophs

Since ATG18a^{12345A} enhances autophagy activity and ATG18a^{12345D} suppresses autophagy activity, we assumed that ATG18a^{12345A} and ATG18a^{12345D} might have different function in defense against *B. cinerea*. Before inoculation, wild type plants, *atg18a* mutant plants, ATG18a-GFP/*atg18a*, ATG18a^{12345A}-GFP/*atg18a*, and ATG18a^{12345D}-GFP/*atg18a* transgenic plants did not show any senescence

Figure 3. *ATG18a*^{12345A} and *ATG18a*^{12345D} affect the induction of autophagy. (A) *ATG18a*^{12345A} induced strong autophagy signals in the protoplasts of *atg18a* mutant. Empty vector/*atg18a* (transient expression of empty vector in the protoplasts of *atg18a* mutant with GFP-ATG8a), *ATG18a*/*atg18a* (transient expression of *ATG18a* in the protoplasts of *atg18a* mutant with GFP-ATG8a), *ATG18a*^{12345A}/*atg18a* (transient expression of *ATG18a*^{12345A} in the protoplasts of *atg18a* mutant with GFP-ATG8a), *ATG18a*^{12345D}/*atg18a* (transient expression of *ATG18a*^{12345D} in the protoplasts of *atg18a* mutant with GFP-ATG8a). 24 h after transformation, the GFP signal was checked by confocal microscope and typical pictures were presented. The GFP signal was used to present the autophagy signal. The experiment was repeated three times with similar results. Bars: 10 μ m. (B) Autophagic activities were detected after empty vector, *ATG18a*, *ATG18a*^{12345A} and *ATG18a*^{12345D} were transferred into *atg18a* mutant protoplasts. The absolute GFP-ATG8a-labeled puncta per protoplast was counted. For each treatment, at least 30 cells were used to quantify autophagosomes and mean value is the statistical analysis based on more than 30 cells from an individual experiment. Different letter means a significant difference. The experiment was repeated three times with similar results. (C) Detection of autophagic activity in GFP-ATG8a/*ATG18a*-3xHA/*atg18a*, GFP-ATG8a/*ATG18a*^{12345A}-3xHA/*atg18a* and GFP-ATG8a/*ATG18a*^{12345D}-3xHA/*atg18a* transgenic plants before and after *B. cinerea* infection with or without ConA treatment. The experiment was

phenotype (Figure S3C). At 4 dai, *atg18a* mutant plants showed highly susceptible phenotype (clear necrosis and yellow lesion), *ATG18a*^{12345A}-GFP/*atg18a* transgenic plants, same as *ATG18a*-GFP/*atg18a* transgenic plants and wild type plants, showed limited phenotype on leaves, while the *ATG18a*^{12345D}-GFP/*atg18a* transgenic plants showed more necrosis on leaves than *ATG18a*-GFP/*atg18a* transgenic plants and wild type (Figure 4A). The fungal biomass accumulation results by qRT-PCR showed that *ATG18a*^{12345A}-GFP/*atg18a* transgenic plants and *ATG18a*-GFP/*atg18a* transgenic plants accumulated less amount of fungal biomass than wild type plants; while *ATG18a*^{12345D}-GFP/*atg18a* transgenic plants accumulated much more fungal biomass than wild type plants (Figure 4B). Thus, it is apparent that overexpression of *ATG18a*^{12345A} in *atg18a* mutant fully complemented the disease phenotype of *atg18a* mutant against *B. cinerea*, while overexpression of *ATG18a*^{12345D}-GFP in *atg18a* mutant only partially restored the disease phenotype of *atg18a* mutant. When challenged with *A. brassicicola*, the *atg18a* mutant displayed significantly larger lesion size than the wild type plants (Figure 4C). In addition, overexpression of *ATG18a*^{12345A} in *atg18a* mutant restored its disease resistance to the wild type, while overexpression of *ATG18a*^{12345D} in *atg18a* mutant did not (Figure 4C). These pieces of evidence suggest that phosphorylation of ATG18a represses plant resistance against necrotrophic pathogens; while, dephosphorylation on the five sites (Ser344, Ser361, Thr241, Ser328, and Thr387) of ATG18a enhances plant resistance against necrotrophic pathogens.

It was reported that ATG18a interacts with a key resistance protein WRKY33 in resistance against necrotrophic pathogens. So, we assumed that phosphorylation on ATG18a might affect its interaction with WRKY33. At first, the subcellular localization of *ATG18a*^{12345A}-GFP and *ATG18a*^{12345D}-GFP proteins in *Arabidopsis* was checked. *ATG18a*^{12345A}-GFP was found in the cytoplasm and the nuclei, while *ATG18a*^{12345D}-GFP was only found in the nuclei. This indicates that phosphorylation on ATG18a changes its subcellular localization (Figure S3D). Next, we check their interaction with WRKY33 in BiFC assay. The results showed that both *ATG18a*^{12345A} and *ATG18a*^{12345D} proteins interacted well with WRKY33, similar to ATG18a protein (Figure S3E).

To analyze the function of different ATG18a forms against hemibiotrophic pathogen, those transgenic plants were challenged with *Pseudomonas syringae* pv. *tomato* strain DC3000

(*Pst* DC3000). Similar to the previous report [16], *atg18a* mutant was more resistant against *Pst* DC3000 than wild type plants and overexpression of ATG18a complemented the disease phenotype of *atg18a* mutant to wild type plant (Figure S3F). Overexpression of *ATG18a*^{12345A} partially complemented the disease phenotype of *atg18a* mutant, but *ATG18a*^{12345D}-GFP/*atg18a* plants showed the same phenotype as *atg18a* mutant (Figure S3F). The results were consistent with the previous reports that autophagy pathway negatively regulates disease resistance against biotrophic and hemibiotrophic pathogens [16,45,46].

BAK1 physically interacts with and phosphorylates ATG18a

Since phosphorylation modification regulates the resistance function of ATG18a, it prompted us to identify the kinase responsible for phosphorylating ATG18a. It was reported that MPK3, MPK4, MPK6, SnRK2.6, BIK1, BKK1 and BAK1 play important roles in plant stress responses [14,47–51]. So, we took them as the candidate kinases responsible for phosphorylating ATG18a protein. First, we tested whether these kinases could interact with ATG18a by BiFC assay. These kinases were individually fused to the C-terminal domain of the yellow fluorescent protein (YFP) to generate *MPK3-Yc*, *MPK4-Yc*, *MPK6-Yc*, *SnRK2.6-Yc*, *BIK1-Yc*, *BKK1-Yc* and *BAK1-Yc* constructs; and ATG18a was fused with N-terminal domain of YFP to get *ATG18a-Yn* construct. When *MPK3-Yc*, *MPK4-Yc*, *MPK6-Yc*, *SnRK2.6-Yc*, *BIK1-Yc* and *BKK1-Yc* were individually co-expressed with *ATG18a-Yn* in *Nicotiana benthamiana* leaves, no YFP signal was detected (Figure 5A and S4). Only co-infiltration of *BAK1-Yc* with *ATG18a-Yn* showed clear YFP signals in cytoplasm, which were not merged with the DAPI signal (Figure 5A), indicating that BAK1 interacted with ATG18a in cytoplasm. This conclusion was further consolidated with the luciferase complementation imaging (LCI) assay showing a strong signal with co-expression of *ATG18a-cLuc* and *BAK1-nLuc* in *N. benthamiana* (Figure 5B). Next, we crossed *ATG18a-3xHA/atg18a* with *Pro_{native}:BAK1-GFP/Col-0* [52] to generate *Arabidopsis* transgenic plants expressing both *ATG18a-3xHA* and *BAK1-GFP* and performed co-immunoprecipitation (Co-IP) assay using GFP-conjugated beads followed by anti-HA immune blot to test BAK1 and ATG18a interaction *in vivo*. As shown in Figure 5C, *ATG18a-3xHA* was precipitated with BAK1-GFP, while the control experiment where BAK1-GFP was co-expressed with an empty

repeated three times with similar results. Bars: 40 μ m. (D) Quantification of autophagic activity in *GFP-ATG8a/ATG18a-3xHA/atg18a*, *GFP-ATG8a/ATG18a*^{12345A}-3xHA/*atg18a* and *GFP-ATG8a/ATG18a*^{12345D}-3xHA/*atg18a* transgenic plants before and after *B. cinerea* infection with or without ConA treatment. The absolute GFP-ATG8a-labeled puncta per protoplast was counted. For each treatment, at least 20 cells were used to quantify autophagosomes. The mean value is the statistical analysis based on more than 20 cells from an individual experiment. The experiment was repeated three times with similar results. Different letter means significant difference. (E) Western blot to detect the ATG8 lipidation level in four-week old *ATG18a*-GFP/*atg18a*, *ATG18a*^{12345A}-GFP/*atg18a* and *ATG18a*^{12345D}-GFP/*atg18a* plants before and after *B. cinerea* inoculation. Four-week old *ATG18a*-GFP/*atg18a*, *ATG18a*^{12345A}-GFP/*atg18a* and *ATG18a*^{12345D}-GFP/*atg18a* plants were spray-inoculated with *B. cinerea* at 2.5×10^5 spores/ml and leaf samples were collected at 0, 1, 2, and 3 dai, respectively. Membrane fractions were blotted with ATG8 antibody. Western blotting with *Arabidopsis* CFBP antibody was used as the loading control. Relative intensity of ATG8-PE normalized to the loading control was shown below. The relative level of ATG8-PE was also shown to present the autophagy accumulation. The experiment was repeated three times with similar results. (F) Western blot to detect the vacuolar delivery of GFP in *GFP-ATG8a/ATG18a-3xHA/atg18a*, *GFP-ATG8a/ATG18a*^{12345A}-3xHA/*atg18a* and *GFP-ATG8a/ATG18a*^{12345D}-3xHA/*atg18a* transgenic plants before and 2 d after *B. cinerea* infection. Relative intensity of GFP was normalized to the level of GFP-ATG8a to detect the vacuolar delivery of GFP. The experiment was repeated three times with similar results. (G) Detection of the accumulation of NBR1 protein in *GFP-ATG8a/ATG18a-3xHA/atg18a*, *GFP-ATG8a/ATG18a*^{12345A}-3xHA/*atg18a* and *GFP-ATG8a/ATG18a*^{12345D}-3xHA/*atg18a* transgenic plants before and after *B. cinerea* infection. The ratio (NBR1 protein level divided by *Arabidopsis* ACT2 protein level) is used to show the relative protein levels of NBR1 in plants. The experiment was repeated three times with similar results.

Figure 4. *ATG18a*^{12345A} and *ATG18a*^{12345D} affect plant defense against necrotrophic fungi. (A) *ATG18a*^{12345A}-GFP complement the disease phenotype of *atg18a* mutant to wild type against *B. cinerea*. Four-week-old plants were spray-inoculated with *B. cinerea* at 2.5×10^5 spores/ml and the phenotype was checked at 4 dai. Scale bar: 1 cm. (B) Fungal biomass accumulation on plants at 4 dai. Fungal growth was determined by qPCR amplification of the *B. cinerea* ActinA gene relative to *Arabidopsis* ACT2 gene. The data represent mean values $\pm$ SD ($n = 3$). The mean values following by different letters are significantly different from each other ($p < 0.01$, Student's *t*-test). The experiment was repeated three times with similar results. (C) The disease phenotype of *ATG18a*^{12345A}-GFP/*atg18a* and *ATG18a*^{12345D}-GFP/*atg18a* plants to *Alternaria brassicicola*. Four-week-old plants were drop-inoculated with *A. brassicicola* at 2.5×10^5 spores/ml and the phenotype was checked at 4 dai. The experiment was repeated three times with similar results. WT, wild type. Scale bar: 1 cm.

vector expressing the 3xHA epitope alone did not show any interaction signal. Together, the results indicate that ATG18a indeed interacts with BAK1 *in vivo*.

To confirm the function of BAK1 on ATG18a phosphorylation, we generated recombinant constructs encoding GST-ATG18a, 6xHis-BAK1 kinase domain (KD) and 6xHis-BAK1^{D416N} kinase domain (a kinase dead variant). Those constructs were expressed in *E. coli*, and the purified recombinant proteins were used for *in vitro* phosphorylation test. As shown in Figure 5D, BAK1, but not BAK1^{D416N}, directly phosphorylated ATG18a in the presence of [³²P]-gamma-ATP.

Phosphorylation level of ATG18a is regulated by BAK1

Next, we tried to figure out the phosphorylation level and phosphorylation sites on ATG18a regulated by BAK1. *ATG18a-GFP/WT* and *ATG18a-GFP/bak1-4* transgenic plants were generated and treated with *B. cinerea*. Leaf samples at 0, 1, and 2 dai were collected individually. ATG18a-GFP proteins immunoprecipitated from those leaf samples by anti-GFP antibody were western blotted by anti-phosphate antibody. The ratio (anti-phosphate signal:total ATG18a-GFP signal) was used to present the relative phosphorylation level. Interestingly, two clear bands in *ATG18a-GFP/WT* and *ATG18a-GFP/bak1-4* transgenic plants were detected by anti-phosphate antibody, indicating both of the two bands in Figure 2A contained phosphorylated ATG18a proteins. In *ATG18a-GFP/WT* plants, the relative phosphorylation level of ATG18a protein was increased at 1 dai and then reduced at 2 dai (Figure 5E). Compared with wild type plants, mutation of BAK1 does not significantly change the relative phosphorylation level of ATG18a before infection. But the relative phosphorylation level of ATG18a protein in *bak1-4* plants was only a half of that detected in wild type plants at 1 and 2 dai (Figure 5E). This data indicates that BAK1 is a major kinase responsible for the phosphorylation level on ATG18a after *B. cinerea* infection.

In order to check whether knockout of BAK1 also affects phosphorylated sites on ATG18a, we did IP-LC-MS/MS to check the phosphorylation sites on ATG18a-GFP in *ATG18a-GFP/bak1-4* transgenic plants. Leaf samples of *ATG18a-GFP/bak1-4* transgenic plants were collected before and after *B. cinerea* infection. Then, ATG18a-GFP proteins were immunoprecipitated by anti-GFP antibody and were sent for LC-MS/MS. Interesting, only phosphorylated Ser344 on ATG18a was detected in *ATG18a-GFP/bak1-4* transgenic plants before and after *B. cinerea* infection (Figure 5F,G). These observations suggest that phosphorylation on ATG18a at Thr241, Ser328, Ser361, and Thr387 is completely dependent on BAK1 after *B. cinerea* infection, and phosphorylation of Ser344 on ATG18a may be independent on BAK1.

BAK1 negatively regulates autophagy during resistance against *B. cinerea*

The relationship between ATG18a and BAK1 proteins indicated that BAK1 might regulate autophagy pathway. To test this possibility, we checked the autophagy phenotype of *bak1* mutant through protoplast assay. When GFP-ATG8a was expressed in the protoplasts of *bak1-4* mutant, a lot of

Figure 5. BAK1 interacts with and phosphorylates ATG18a. (A) BiFC assay to screen ATG18a interacting proteins in *N. benthamiana*. ATG18a-Yn was co-infiltrated with BAK1-Yc, BKK1-Yc, and Yc in *N. benthamiana*. Yellow fluorescence signal was checked at 48-h after infiltration, and DAPI staining was used to detect nuclei. The experiment was repeated three times with similar results. Bars: 25 μ m. (B) Split luciferase assay to check the interaction between ATG18a and BAK1. The *Agrobacterium* carrying the indicated constructs were co-infiltrated and transiently expressed in *N. benthamiana*. The positive luminescence monitored by a CCD camera indicates interaction. The experiment was repeated three times with similar results. Scale bar: 1 cm. (C) Coimmunoprecipitation assay to check the interaction of ATG18a with BAK1. Transgenic plants carrying *BAK1_{pro}:BAK1-GFP* and *35s:ATG18a-3xHA* constructs and transgenic plants carrying *BAK1_{pro}:BAK1-GFP* and *35s:3xHA* constructs were used for Co-IP. Proteins isolated from 7-d old seedlings were immunoprecipitated by anti-GFP beads and western-blotted by anti-GFP and anti-HA antibodies. The experiment was repeated three times with similar results. (D) *In vitro* phosphorylation of ATG18a by BAK1. GST-ATG18a, GST-BAK1 and GST-BAK1^{D416N} were expressed and purified in *E. coli* and GST tag of those purified proteins were digested by the enzyme. The experiment was repeated three times with similar results. (E) Mutation of *BAK1* suppressed the phosphorylation level of ATG18a. Four-week old *ATG18a-GFP/WT* and *ATG18a-GFP/bak1-4* transgenic plants were inoculated with *B. cinerea* and leaf samples were collected at 0, 1 and 2 dai. ATG18a-GFP proteins were immunoprecipitated by using anti-GFP beads, then they were

autophagosomes were observed; but, when *GFP-ATG8a* and *BAK1-3xHA* were co-expressed in the protoplasts of *bak1-4* mutant, only a few autophagosomes were detected (Figure S5A). In order to confirm the results, we also tested *bak1-5* mutant and got the same results (Figure S5B). Those data suggest that BAK1 functions as a suppressor of autophagy pathway. In order to confirm the kinase activity of BAK1 is required for suppressing autophagy, the BAK1 kinase dead variant *BAK1^{D416N}-3xHA* was coexpressed with *GFP-ATG8a* in the protoplasts of *bak1-4* and *bak1-5* mutants, and the same level of autophagosome accumulation as that in *bak1* mutants was observed (Figure S5A-S5C). It indicates that the kinase activity of BAK1 is required for inhibiting autophagy.

Next, we tried to test the function of BAK1 in inducing autophagy during *B. cinerea* infection. The wild type plant, *bak1-4* mutant and *bak1-5* mutant plants were treated with *B. cinerea* and the ATG8 lipidation assay was used to detect the ATG8-PE level on leaf samples collected before and after *B. cinerea* infection. As Figure 6A shows, the ATG8-PE levels in *bak1-4* and *bak1-5* mutants at all time points were higher than that in wild type plants. Also, the protein levels of NBR1 in *bak1-4* and *bak1-5* mutants were much lower than that in wild type plants before and after *B. cinerea* infection (Figure 6B). Taken together, those observations indicate that BAK1 negatively regulates autophagy activity.

BAK1 negatively regulates resistance against *B. cinerea*

In order to validate the function of BAK1 in plant immunity against *B. cinerea*, we challenged the wild type, *bak1-4* and *bak1-5* mutant plants with *B. cinerea*, by taking *ATG18a* and *rbold* mutant plants as controls. Since *BAK1* is critical for activating PTI and mutation of *BAK1* significantly suppresses PTI [53], we included another PTI-deficient mutant, *rbold* mutant in this assay as the control. Before inoculation, all plants grew well and no senescence phenotype was observed; although no macroscopic spots were observed on the leaves of *bak1-4* mutant, microscopic spots cannot be excluded (Figure S5D). Interestingly, *bak1-4* and *bak1-5* mutants showed more resistant response than the wild type plant at 4 dai, while *atg18a* and *rbold* mutants showed a more susceptible response than the wild type plant (Figure 6C). The fungal biomass assay tested by qRT-PCR also supported the results (Figure 6D). These pieces of evidence indicate that BAK1 negatively regulates plant resistance against *B. cinerea*. The susceptible phenotype of *rbold* mutant supported the previous report that PTI positively regulates resistance against *B. cinerea* [2,14]. The enhanced resistance phenotype of *bak1*

mutants to *B. cinerea* indicates that the increased autophagy activity in *bak1* mutants is strong enough to fight against *B. cinerea* even PTI is deficient in *bak1* mutants. Next, we inoculated *bak1* mutants with *A. brassicicola*, and it was observed that the wild type plant leaves showed yellow lesions, while the leaves of both *bak1-4* and *bak1-5* mutants did not show yellow necrosis and the lesion size on *bak1* mutants were significantly smaller than that on the wild type plants (Figure 6E,F). Thus, our results indicate that BAK1 negatively regulates resistance against necrotrophic pathogens.

ATG18a acts genetically downstream from BAK1 in plant defense and autophagy

In order to understand the genetic relationship between BAK1 and ATG18a, the *atg18a* mutant was crossed with *bak1-4* mutant to generate *atg18a; bak1-4* double mutant and those plants were challenged with *B. cinerea*. Before inoculation, none of those plants showed senescence phenotype (Figure S5E). As shown in Figure 7A, *atg18a* mutant was more susceptible and *bak1-4* was more resistant than wild type plant; while, the double mutant is susceptible to *B. cinerea*, similar to the phenotype of *atg18a* mutant. The fungal biomass assay tested by qRT-PCR also showed that the fungal biomass accumulated on *atg18a* mutant and *atg18a; bak1-4* double mutant was much more than that on wild type plants and the fungal biomass on *atg18a; bak1-4* double mutant was similar to that on *atg18a* mutant (Figure 7B). After inoculation with *A. brassicicola*, a similar disease phenotype was observed: *atg18a; bak1-4* double mutant, similar to *atg18a* mutant, was more susceptible to *A. brassicicola* than the wild type plant (Figure 7C,D). It indicates that ATG18a functions in the downstream of BAK1 in resistance against necrotrophic pathogens. In order to check their relationship in the autophagy pathway, *GFP-ATG8a/atg18a; bak1-4* transgenic plants were generated. And the GFP-ATG8a processing assay showed that the free GFP protein level in *GFP-ATG8a/atg18a; bak1-4* transgenic plants were similar to that in *GFP-ATG8a/atg18a* transgenic plants (Figure 7E). Moreover, the NBR1 protein levels in WT, *atg18a*, *bak1-4* and *atg18a; bak1-4* double mutant were checked by using anti-NBR1 antibody. The NBR1 protein level in the *atg18a; bak1-4* double mutant was similar to that in *atg18a* mutant plants and was higher than that in *bak1-4* mutant and wild type plants at 2 dai (Figure 7F). Together, our results indicate that ATG18a acts genetically

western blotted by using anti-GFP and anti-phosphate antibodies. The signal gotten by anti-GFP was used to present the amount of total ATG18a protein. The signal gotten by using anti-phosphate antibody was used to present the amount of phosphorylated ATG18a protein. Ratio = phosphate signal:total ATG18a, and the ratio was used to present the relative level of phosphorylated ATG18a protein. The experiment was repeated three times with similar results. (F) LC-MS/MS assay showed that only Ser344 on ATG18a was phosphorylated before *B. cinerea* inoculation in *ATG18a-GFP/bak1-4* transgenic plants. To identify the phosphorylation sites on ATG18a in *bak1-4* mutant before *B. cinerea* inoculation, the leaf samples of 35s:*ATG18a-GFP/bak1-4* transgenic plants were collected and the ATG18a-GFP protein immunoprecipitated by anti-GFP antibody was sent for LC-MS/MS. The MS analysis covered 60% of the total ATG18a protein and identified one monophosphorylated peptide: IAPDSTPS₅PSSSLSLFK. (G) LC-MS/MS assay showed that only Ser344 on ATG18a was phosphorylated at 24 h after *B. cinerea* inoculation in *ATG18a-GFP/bak1-4* transgenic plants. To identify the phosphorylation sites on ATG18a in *bak1-4* mutant after *B. cinerea* inoculation, the leaf samples of 35s:*ATG18a-GFP/bak1-4* transgenic plants at 24 h after *B. cinerea* inoculation were collected and the ATG18a-GFP protein immunoprecipitated by anti-GFP antibody was sent for LC-MS/MS. The MS analysis covered 66.61% of the total ATG18a protein and identified one monophosphorylated peptide: IAPDSTPS₅PSSSLSLFK.

Figure 6. BAK1 suppresses autophagy and defense against *B. cinerea*. (A) Western blot to detect the ATG8 lipidation level in wild type and *bak1-4* and *bak1-5* mutant plants before and after *B. cinerea* inoculation. Four-week old wild type, *bak1-4* mutant and *bak1-5* mutant plants were spray-inoculated with *B. cinerea* at 2.5×10^5 spores/ml and leaf samples were collected at 0, 1, 2, and 3 dai, respectively. Membrane fractions were blotted with ATG8 antibody. Western blotting with *Arabidopsis* CFBP antibody was used as the loading control. Relative intensity of ATG8-PE normalized to the loading control was shown below to present autophagy accumulation. The experiment was repeated three times with similar results. (B) Western blot to detect the accumulation of NBR1 protein in wild type plants and *bak1-4* and *bak1-5* mutant plants before and after *B. cinerea* infection. Four-week old wild type, *bak1-4* mutant and *bak1-5* mutant plants were spray-inoculated with *B. cinerea* at 2.5×10^5 spores/ml and leaf samples were collected at 0, 1, 2, and 3 dai, respectively. Total protein was blotted with NBR1 antibody and *Arabidopsis* ACT2 antibody. The ratio (the protein level of NBR1 divided by the protein level of *Arabidopsis* ACT2) was used to show the relative protein levels of NBR1. The experiment was repeated three times with similar results. (C) *bak1* mutant phenotype to resistance against *B. cinerea*. Four-week-old plants were spray-inoculated with *B. cinerea* at 2.5×10^5 spores/ml and the phenotype was checked at 4 dai. The experiment was repeated three times with similar results. WT, wild type. Scale

downstream from BAK1 in plant defense and autophagy during defense against *B. cinerea*.

Discussion

Autophagy plays critical functions in senescence, nutrient recycling and abiotic stress [20]. Also, it was reported to play positive roles in resistance against necrotrophic pathogens and ATG18a is required for this defense process [17]. But little is known about how plants regulate ATG18a protein to activate autophagy during resistance against necrotrophic pathogens. In this study, we reported that phosphorylation modification on ATG18a plays negative roles in regulating its function in autophagy and defense against *B. cinerea* and five amino acids (Ser344, Ser361, Thr241, Ser328, and Thr387) on ATG18a were found to be phosphorylated during the early stage of *B. cinerea* infection. Moreover, BAK1 was identified to physically interact with and directly phosphorylate ATG18a and mutation of *BAK1* blocks phosphorylation on four (Ser361, Thr241, Ser328, and Thr387) of the five sites on ATG18a and enhances plant resistance against necrotrophs through increasing autophagy activity. So, it is possible that necrotrophic pathogens hijack BAK1 to suppress autophagy pathway to help disease development.

Protein phosphorylation is a common mode for regulating autophagy. In yeast *Pichia pastoris*, PpATG18 protein is regulated by phosphorylation and the phosphorylation sites (Ser388, Ser391, Ser492 and Ser495) are in the blades 6 and 7 [25]. Although *PpATG18a* mutant is defective of autophagy, phosphorylated PpATG18a does not affect its autophagy activity. In this study, we found that phosphorylation modification on ATG18a inhibits its function in activating autophagy. Dephosphorylated form ATG18a^{12345A} enhances ATG8 lipidation and promotes the accumulation of autophagosomes, while phosphorylated form ATG18a^{12345D} suppresses autophagy activity (Figure 3A–G). Also, five phosphorylation sites (Thr241, Ser328, Ser344, Ser361 and Thr387) on ATG18a *in vivo* after *B. cinerea* infection are in blades 4, 6 and 7, different from the PpATG18 phosphorylation pattern in blades 6 and 7 (Figure S2B). This indicates that plants and yeasts may use different mechanisms to regulate the function of ATG18 in autophagy. Another possibility is that the different phosphorylation patterns on ATG18 proteins may regulate different functions of phosphorylated ATG18a and PpATG18 proteins.

ATG18a is critical for resistance against *B. cinerea* [17]. Since it localizes both in cytoplasm and nuclei, two different resistance mechanisms of ATG18a has been proposed: 1) ATG18a enhances resistance through activating autophagy

in cytoplasm, 2) ATG18a activates the expression of resistance gene through interaction with transcription factor WRKY33 in nuclei, which is the key positive regulator in resistance against *B. cinerea* [17,31–34,54]. In this study, we observed that NES signal peptide fused on the N terminus of ATG18a could efficiently trap ATG18a protein in the cytoplasm. Disease assay showed that NES-ATG18a is sufficient to complement the disease phenotype of *atg18a* mutant to wild type against *B. cinerea* (Figure 1C,D). And NES-ATG18a can effectively induce autophagy when expressed in *atg18a* mutant (Figure S1E and S1F). Those pieces of evidence confirm that ATG18a regulates resistance response mainly in cytoplasm. We also tried multiple ways to trap ATG18a in the nuclei, but none of them can efficiently keep the ATG18a in the nuclei. So, we cannot exclude the possibility that ATG18a may activate other pathways to defense against *B. cinerea*. Since it was observed that reduced autophagy activity by expression of phosphorylated ATG18a in *ATG18a* mutant plants also showed attenuated plant resistance against *B. cinerea*, we conclude that the cytoplasm activity of ATG18a plays critical roles in resistance.

Several lines of evidence indicate that BAK1 plays critical roles in regulating plant growth, cell death and plant immunity [53]. Initially, BAK1 was identified as the co-receptor for BRI1, the receptor of brassinosteroid (BR) and regulates BR signaling [55,56]; the cell death suppressed by BAK1 involves with protein glycosylation pathway [57]; also, BAK1 positively regulates the initiation of PAMP/DAMP-triggered immunity (PTI) by physically interacting with FLS2 (flagellin sensing 2), EFR (elongation factor [EF]-Tu receptor) and Pep receptors (PEPR1 and PEPR2), which recognize bacteria-derived PAMPs (flagellin and EF-Tu) and plant-derived DAMP (Pep), respectively [58–61]. Here, we show a new function of BAK1 that suppresses autophagy. ATG8–PE assay and the protein level of NBR1 indicates that mutation of *BAK1* enhances autophagy activity (Figure 6A,B); overexpression of *BAK1* in *bak1* mutant protoplasts suppresses the autophagy signal (Figure S5A–S5C). Interestingly, autophagy has been proved by multiple groups to positively enhances resistance against necrotrophic pathogens [16–18]. It indicates BAK1 plays negative roles in resistance against necrotrophs, and the disease assay also confirmed that *bak1* mutant showed enhanced resistance against *B. cinerea* and *A. brassicicola* (Figure 6C–F). It is inconsistent with the previous report that *bak1* mutants were more susceptible to *B. cinerea* than wild type plants [62]. The inconsistent results might be caused by the age of plants or different growth conditions. Normally, older plants are more susceptible than younger plants, which is caused by the enhanced senescence in older plants. Under

bar: 1 cm. (D) Fungal biomass accumulation assay measured by qRT-PCR revealed that *bak1* mutants suppressed *B. cinerea* growth. Fungal growth was determined by qRT-PCR amplification of the *B. cinerea* *ActinA* gene relative to *Arabidopsis* *ACT2* gene. The data represent mean values $\pm$ SD ($n = 3$). The mean values following by different letters are significantly different from each other ($p < 0.01$, Student's *t*-test). The experiment was repeated three times with similar results. (E) The disease phenotype of *Arabidopsis* *bak1* mutant to *A. brassicicola*. Four-week-old plants were drop-inoculated with *A. brassicicola* at 2.5×10^5 spores/ml and the phenotype was checked at 4 dai. The experiment was repeated three times with similar results. WT, wild type. Scale bar: 1 cm. (F) The diameter of lesion size of inoculated *bak1* mutant leaves by *A. brassicicola*. Four-week-old plants were drop-inoculated with *A. brassicicola* at 2.5×10^5 spores/ml and the phenotype was checked at 4 dai. The experiment was repeated three times with similar results. WT, wild type.

Figure 7. ATG18a functions on the downstream of BAK1 in response to *B. cinerea*. (A) The disease phenotype of *atg18a; bak1* double mutant to *B. cinerea*. Four-week-old plants were spray-inoculated with *B. cinerea* at 2.5×10^5 spores/ml and the phenotype was checked at 4 dai. The experiment was repeated three times with similar results. WT, wild type. Scale bar: 1 cm. (B) Fungal biomass accumulation assay measured by qRT-PCR revealed that *bak1* mutants suppressed *B. cinerea* growth. Fungal growth was determined by qRT-PCR amplification of the *B. cinerea* ActinA gene relative to *Arabidopsis* ACT2 gene. The data represent mean values $\pm$ SD ($n = 3$). The mean values following by different letters are significantly different from each other ($p < 0.01$, Student's t-test). The experiment was repeated three times with similar results. (C) The disease phenotype of *atg18; bak1* double mutant to *Alternaria brassicicola*. Four-week-old plants were drop-inoculated with *A. brassicicola* at 2.5×10^5 spores/ml and the phenotype was checked at 4 dai. The experiment was repeated three times with similar results. WT, wild type. Scale bar: 1 cm. (D) The diameter of lesion size of inoculated leaves by *A. brassicicola*. Four-week-old plants were drop-inoculated with *A. brassicicola* at 2.5×10^5 spores/ml and the phenotype was checked at 4 dai. The experiment was repeated three times with similar results. WT, wild type. (E) The GFP-ATG8a processing assay. GFP-ATG8a/WT, GFP-ATG8a/*atg18a*, GFP-ATG8a/*bak1-4* and GFP-ATG8a/*atg18a; bak1-4* transgenic plants were inoculated with *B. cinerea*. Leaf samples were taken at 0 and 2 dai for western blot to check the protein levels of GFP-ATG8a and GFP. The protein level of *Arabidopsis* ACT2 detected by western blot was used as the loading control. (F) The NBR1 protein accumulation level in WT, *atg18a*, *bak1-4*, *bak1-5* and *atg18a; bak1-4* double mutant before and 2 d after *B. cinerea* inoculation. Four-week old wild type *atg18a*, *bak1-4*, *bak1-5* and *atg18a; bak1-4* double mutant plants were spray-inoculated with *B. cinerea* at 2.5×10^5 spores/ml and leaf samples were collected at 0 and 2 dai, respectively. Total protein samples were blotted with NBR1 antibody and ACT2 antibody. Relative intensity of NBR1 normalized to the loading control (the intensity of ACT2) was shown below to present the relative NBR1 protein level. The experiment was repeated three times with similar results.

our growth conditions, *bak1-4* mutant starts to show senescence phenotype at 40-d old and autophagy-deficient mutants start to show senescence phenotype at 45-d old. The disease phenotypes of autophagy-deficiency mutant against biotrophs and necrotrophs were reported to be related to the early senescence phenotype [18,46,63]. In order to alleviate the effect of senescence, 30-d old plants were used to test the disease phenotype and autophagy phenotype; and no senescence phenotype was observed on all tested plants before infection (Figure S3C, S5D and S5E). Our results indicate that the function of BAK1 and ATG18a in plant resistance against necrotrophs is not mainly related to senescence. Recently, it is reported that disruption of BAK1 enhances PEP2-mediated growth inhibition and the expression level of

PEP2-induced defense genes; moreover, BAK1 suppresses AtPROPEP peptides (PEP epitopes)-mediated resistance to the fungal pathogen *Colletotrichum higginsianum* [64]. Thus, BAK1 suppresses at least two resistance pathways in defense against pathogens: autophagy and PEP epitopes-mediated resistance.

In vivo assay showed that four of the five phosphorylation sites on ATG18a are dependent on BAK1, and mutation of BAK1 dramatically reduces the phosphorylation level on ATG18a (Figure 5E). It indicates that BAK1 is the major kinase responsible for the phosphorylation modification on ATG18a. While Ser344 site on ATG18a protein is detected in *bak1-4* mutant background before and after *B. cinerea* infection. This means some unknown kinases are responsible for

phosphorylating ATG18a on Ser344 site. BKK1, the homolog of BAK1 protein, was chosen as the best candidate but it fails to interact with ATG18a in the BiFC assay (Figure 5A). It indicated that BKK1 might not be the kinase responsible for the Ser344 phosphorylation in *bak1-4* mutant. Another possibility is that BKK1 phosphorylates ATG18a through transient interaction, and this interaction cannot be detected by BiFC. Since those kinases targeting ATG18a genetically upstream, identification of the unknown kinases responsible for Ser344 phosphorylation will help to explain what kind of signals from outside regulates autophagy through ATG18a.

Since two bands were detected by anti-phosphate antibody (Figure 5E), both of the two bands in Figure 2A actually contained phosphorylated ATG18a protein and some phosphorylated sites did not cause band shift. Although only Ser344 phosphorylated site on ATG18a protein in *ATG18a-GFP/bak1-4* was detected by LC-MS/MS, two phosphorylated bands were identified by anti-phosphate antibody. So, there are more phosphorylated sites unidentified on ATG18a protein, and Ser344 site was not the only phosphorylated site on ATG18a protein in *bak1-4* mutant. So, the function of ATG18a is regulated by more phosphorylation sites, and more kinases play important roles in regulating the function of ATG18a. Also, dephosphorylation on ATG18a is critical for activating autophagy; therefore, identification of phosphatases responsible for dephosphorylating ATG18a will also give more details on how plants regulate the function of autophagy in defense in the future.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana lines used were in Columbia-0 (Col-0) background. The mutants are *atg18a* (GABI_651D08), *bak1-4* (SALK116202) and the *atg18a; bak1-4* double mutant was generated by genetic crossing. *ATG18a* coding sequence (CDS), *ATG18a* (241,328, 344, 361, 387A), *ATG18a* (241,328 344, 361, 387D) were PCR amplified and fused to *GFP*, then it was driven by the CaMV 35s promoter in wild type ecotype Col-0 and *atg18a* mutant plants, resulting in *35s:ATG18a-GFP*, *35s:ATG18a(241,328,344,361,387A)-GFP*, *35s:ATG18a(241,328 344, 361, 387D)-GFP* transgenic plants. Multiple independent transgenic lines were obtained, and two representative lines were assayed in parallel. *Agrobacterium*-mediated *Arabidopsis* plant transformation was performed by a modified floral dip method [65]. *A. thaliana* plants were grown in growth chambers at 22°C and 120 $\mu\text{E m}^{-2} \text{s}^{-1}$ light on a photoperiod of 12 h light and 12 h dark [17].

Pathogen inoculation

Growth of *B. cinerea* strain B05-10 and *A. brassicicola* on 2xV8 agar, conidia collection, and spray inoculation using a Preval sprayer were as previously described [31]. Inoculated plants were covered with a plastic dome to maintain high humidity, and symptom development was observed at 4 dai (days after inoculation).

Growth of *P. syringae* pv. *tomato* DC3000 strain on King'B medium (Sigma-Aldrich, 60786) supplemented with 100 $\mu\text{g/ml}$ rifamycin (Sigma-Aldrich, R3501). The infiltration inoculation method was as previously described [66].

Phosphatase treatment

Arabidopsis transgenic seedlings were ground to a fine powder in liquid nitrogen. Proteins were extracted in buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl; 2 mM EDTA (Sigma-Aldrich, V900106); 1 mM NaF (Sigma-Aldrich, S7920); 1 mM NaVO_3 (Sigma-Aldrich, 590088); 1 mM PMSF (Roche, 10837091001); 10 mM beta-glycerol phosphate (Sigma-Aldrich, 50020); 0.5% (v/v) Triton X-100 (Sigma-Aldrich, X100); and 1xprotease inhibitor cocktail (Sigma-Aldrich, P8340). Extracts were incubated 10 min at 4°C and centrifuged for 10 min at 12,000 g at 4°C. Add 10 mg sample, 5 μl of 10 x NEB Buffer, 5 μl of 10 mM MnCl_2 , H_2O_2 and 1 μl of Lambda Protein Phosphatase (NEB, 0753) to make a total reaction volume of 50 μl , incubate at 30°C for 30 min, add 6xSDS loading buffer to stop reaction.

ATG8 lipidation assay

The detection of ATG8 and ATG8-PE was conducted according to the previous report [67]. The cell membrane fraction was isolated by 45-min centrifugation at 100,000 g. Then the pellets were separated on 15% SDS-PAGE gel with 6 M urea (Sigma-Aldrich, V900119). Western blot assays were done with antibodies against *Arabidopsis* ATG8 (Agrisera, AS14 2811) and *Arabidopsis* CFBP (Agrisera, AS04 043).

BiFC assay

DNA sequences of the N-terminal, 173-amino acid, enhanced YFP (N-YFP) and C-terminal, 64-amino acid (C-YFP) fragments were PCR amplified and cloned into pFGC5941 (TAIR, CD3-447) to generate pFGC-N-YFP and pFGC-C-YFP, respectively [66]. The *ATG18a* coding sequences were inserted into pFGC-N-YFP to generate the N-terminal in-frame fusions with N-YFP, whereas *BAK1*, *BKK1*, *BIK1*, *MPK3*, *MPK4*, *MPK6* and *SnRK2.6* coding sequences were introduced into pFGC-C-YFP to form N-terminal in-frame fusions with C-YFP. The resulting clones were verified through sequencing, then the plasmids were introduced into *Agrobacterium tumefaciens* (strain GV3101), and infiltration of *N. benthamiana* was performed as described previously [66]. Infected tissues were analyzed at ~48 h after infiltration. Fluorescence and DAPI were visualized using a Leica SP8 confocal microscope; YFP was excited with a 489 nm laser line and detected from 500–580 nm.

Luciferase complementation imaging assays

The CDS of *ATG18a* were inserted into the pCAMBIA1300-nLuc vector [68] using the *Sall* and *Bam*HI restriction enzyme sites. The CDS sequence of *BAK1* was inserted into the pCAMBIA1300-cLuc vector using the *Kpn*I and *Bam*HI sites. *Agrobacterium* strain GV3101 carrying the indicated

constructs were cultured to OD₆₀₀ 0.5 and incubated at room temperature for 3 h before being infiltrated into *N. benthamiana* leaves. Luciferase activity was detected 2 d after infiltration through the NightSHADE LB 985 imaging system (Berthold Technologies), and ten minutes before detection, 0.1 mM luciferin (Promega, E1602) was sprayed on the infiltrated leaves.

IP-Ms-spec

The *ATG18a-GFP* fusion sequences were subcloned into the pFGC5941 vectors, and the expression constructs were driven by the CaMV 35s promoter and transferred into the *Arabidopsis* Col-0 and *bak1* mutant. *Arabidopsis* transgene seedlings were ground to a fine powder in liquid nitrogen. Proteins were extracted in buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl; 2 mM EDTA (Sigma-Aldrich, V900106); 1 mM NaF (Sigma-Aldrich, S7920); 1 mM NaVO₃ (Sigma-Aldrich); 1 mM PMSF (Roche, 10837091001); 10 mM beta-glycerol phosphate (Sigma-Aldrich, 50020); 0.5% (v/v) Triton X-100 (Sigma-Aldrich, X100); and 1x protease inhibitor cocktail (Sigma-Aldrich, P8340). Extracts were incubated 40 min at 4°C and centrifuged for 15 min twice at 13,000 rpm at 4°C. The supernatant was incubated for 3 h at 4°C with GFP-Trap Agarose (ChromoTek, gta-100), and was washed 3–4 times with extraction buffer without protease inhibitor cocktail (Sigma-Aldrich, P8340). For GFP-Trap immunoprecipitation, beads were eluted by pH 2.0 glycine buffer (Thermo Fisher Scientific, BP381-500) to release proteins. Proteins were loaded into SDS-PAGE gel, then the gel was stained with Coomassie Brilliant Blue R 250 (Sigma-Aldrich, 1154440025), and send target bands to Center of biomedical Analysis, Tsinghua University for Ms-Spec analysis.

Co-immunoprecipitation

Arabidopsis transgene seedlings, which grow in MS medium for two weeks, were ground to a fine powder (~0.5 g) in liquid nitrogen. Total proteins were extracted with 1 ml protein extraction buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl; 2 mM EDTA [Sigma-Aldrich, V900106]; 1 mM NaF; 1 mM NaVO₃ [Sigma-Aldrich, 590088]; 1 mM PMSF [Roche, 10837091001]; 10 mM beta-glycero phosphate [Sigma-Aldrich, 50020]; 0.5% (v/v) Triton X-100 [Sigma-Aldrich, X100]; and 1xprotease inhibitor cocktail [Sigma-Aldrich, P8340]). Extracts were incubated 40 min at 4°C and centrifuged for 15 min twice at 13,000 rpm at 4°C. Supernatants were incubated for 3 h at 4°C with GFP-Trap (ChromoTek, gta-100) and washed 3–4 times with extraction buffer lacking protease inhibitor cocktail; beads were eluted by pH 2.0 glycine buffer (Thermo Fisher Scientific) to release proteins. Twenty percent of the eluted proteins were used as immunoprecipitation control, anti-GFP (Genscript, A00185-40) and anti-HA antibodies (NewEast Biosciences, 28003) were used for the immunological detection of BAK1-GFP and ATG18a-HA fusion proteins, respectively.

Recombinant protein and in vitro phosphorylation assay

In order to generate a 6xHis N-terminal fusion of the BAK1 kinase domain, the sequence coding for the BAK1 kinase domain (255 aa-615 aa) was introduced into the pET-28b expression vector. To produce an inactive kinase fusion protein of BAK1 kinase domain, a point mutation at the kinase active site (D416N) was introduced into the pET-28b (Sigma-Aldrich, 59865) expression vector. The 6xHis-BAK1KD and 6xHis-BAK1^{D416N} fusion proteins were expressed in the *E. coli* strain BL21 (NEB, C2527H). After overnight induction at 16°C with 0.2 mM IPTG (Sigma-Aldrich, I6758), the bacteria were pelleted by centrifugation, resuspended in 50 ml lysis buffer (50 mM Tris-HCl pH 7.5, 0.4 M NaCl, 10% glycerol [Sigma-Aldrich, V900122], 0.1% PMSF [Roche, 10837091001] and 0.02% mercaptoethanol [Sigma-Aldrich, M6250]), and sonicated it. The soluble His-tagged proteins were affinity purified using a HisTrap column (Cytiva, 17524701) according to the manufacturer's instructions, purified 6xHis-BAK1KD and 6xHis-BAK1^{D416N} were detected by western blot.

To generate GST-tagged ATG18a, the sequence coding for the ATG18a, ATG18a(344A), ATG18a(344D), ATG18a(344A, 361A), ATG18a(241A, 328A, 361A, 387A), ATG18a(241A, 328A, 344A, 361A, 387A) were introduced into the PGEX-28b (GE Healthcare, 28-9545-49) expression vector, using the *E. coli* strain BL21 (NEB, C2527H) and purified using GST Trap column (Cytiva, 17513002) following the manufacturer's instructions. Finally, GST-tagged of purified protein were digested and get target protein of ATG18a, ATG18a(344A, 361A), ATG18a(241A, 328A, 361A, 387A), ATG18a(241A, 328A, 344A, 361A, 387A).

In vitro kinase assay was performed as described previously [69]. Briefly, kinase reactions were performed in 30 µl of kinase buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 5 mM EDTA [Sigma-Aldrich, V900106], 100 mM NaCl, and 1 mM DTT [Sigma-Aldrich, 10197777001]) containing 10 µg of fusion proteins with 0.1 mM cold ATP and 5 µCi of [³²P]-gamma-ATP (Perkinelmer, BLU502A500UC) at 30°C for 1 h with gentle shaking. The reactions were stopped by adding 4xSDS loading buffer. The phosphorylation of fusion proteins was analyzed by autoradiography after separation through SDS/PAGE.

Transient expression in arabidopsis protoplasts and microscopy analyses

Protoplasts were prepared from *Arabidopsis* rosette leaves, each transformed using 10–20 µg of plasmid DNA via the PEG method [70]. Transformed protoplasts were incubated in darkness for 20 h, concanamycin A (Sigma-Aldrich, L7647) or dimethyl sulfoxide (DMSO) (Sigma-Aldrich, D8418) was added 9 h before visualization by confocal microscopy. Confocal microscopy was performed with a Leica (Leica Microsystems) SP8 confocal microscope. Excitation and detection wavelengths were the same as those described for BiFC colocalization assays.

For autophagy activity, the absolute number of GFP-ATG8a-labeled autophagic puncta per cell or per protoplast was quantified. More than 20 cells per leaf sample or more than 30 protoplasts for each treatment were checked. Mean value is the statistical analysis based on more than 20 leaf cells or 30 protoplasts from an individual experiment. Every experiment was repeated at least three times.

RNA extraction and qRT-PCR analysis

Total RNA was isolated with Trizol reagent according to the manufacturer's instructions (Sigma-Aldrich, 93289). DNase treatment (NEB, B0303) and complementary DNA synthesis (NEB, M0277) were performed according to the manufacturer's instructions (New England Biolabs) using gene-specific primers with *Arabidopsis* ACT2 gene as an endogenous reference for normalization. Real-time PCR was conducted using the SYBR PCR Master mix (Takara, RR820) and run on the BIO-RAD CFX96 Real time system according to the manufacturer's instructions. Quantitative real-time PCR was performed using gene-specific primers.

Protein isolation and immunoblot analysis

Arabidopsis transgene seedlings were ground to a fine powder in liquid nitrogen. Proteins were extracted in buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl; 2 mM EDTA (Sigma-Aldrich, V900106); 1 mM NaF (Sigma-Aldrich, S7920); 1 mM NaVO₃ (Sigma-Aldrich, 590088); 1 mM PMSF (Roche, 10837091001); 10 mM beta-glycerol phosphate (Sigma-Aldrich, 50020); 0.5% (v/v) Triton X-100 (Sigma-Aldrich, X100); and 1xprotease inhibitor cocktail (Sigma-Aldrich, P8340). Extracts were incubated 10 min at 4°C and centrifuged for 10 min at 12,000 g at 4°C, and add 6xSDS loading buffer to supernatant, and boiled for 5 min.

For immunoblotting, 10% SDS-PAGE gels were run at 80 to 150 V for 2 h before electroblotting on nitrocellulose membrane (Millipore, HATF00010) at 24 V for 1–1.5 h (according to molecule weight of protein). Membranes were rinsed in TBS-Tween 0.1% (v/v) and blocked in 5% (w/v) nonfat milk powder (Sangon Biotech, A600669) in TBS-Tween for 1 h. Primary antibodies were diluted in TBS-Tween solution to the following ratios: mouse anti-GST (Abmart, M20007) 1:5000; mouse anti-GFP 1:5000 (Genscript, A00185-40); mouse anti-HA (NewEast Biosciences, 28003) 1:5000, and incubated overnight. Membranes were washed two times in TBS-Tween before 1 h incubation with secondary antibodies anti-mouse-HRP (CWBio, CW0102S) (1:10000). Signals were visualized using an enhanced chemiluminescence system (Biosharp, BL520A) and a GeneGnome XRQ (Syngene) scanner following the manufacturer's instructions.

Statistical analysis

In this study, Student's t-test was used to analyze statistical significance. Data is presented as mean $\pm$ SD. Different letters are presented as $p < 0.01$.

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL database or *Arabidopsis* Genomic Initiative under the following accession numbers: ATG18a (AT3G62770), ATG8a (AT4G21980), ATG5 (AT5G17290), ATG7 (AT5G45900), BAK1 (AT4G33430), BKK1 (AT2G13790), BIK1 (AT2G39660), CFBP (AT1G43670), MPK3 (AT3G45640), MPK4 (AT4G01370), MPK6 (AT2G43790), RbohD (AT5G47910), SnRK2.6 (AT4G33950), NBR1 (AT4G24690).

Acknowledgments

We thank Dr. Jia Li at Lanzhou University for *Pro_{native}:BAK1-GFP/Col-0* transgenic plants, Dr. Jianmin Zhou at Institute of Genetics and Developmental Biology, Chinese Academy of Science for *bak1-4* mutant, Dr. Yangrong Cao at Huazhong Agricultural University for providing *Pst* DC3000 strain and *rbohD* mutant seeds, Dr. Fengming Song at Zhejiang University for providing *B. cinerea* strain B05-10 and *A. brassicicola*, Dr. Mingqiu Dai at Huazhong Agricultural University for providing *Agrobacterium tumefaciens* strain GV3101, Dr. Feng Li at Huazhong Agricultural University for providing *N. benthamiana* seeds, Dr. Shunping Yan at Huazhong Agricultural University for providing pCAMBIA1300-nLuc vector and pCAMBIA1300-cLuc vector, Dr. Yueyun Hong at Huazhong Agricultural university for providing ultracentrifuge, Dr. Xia Li at Huazhong Agricultural University and Dr. Zuyu Zheng for critical reading of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by a Thousand Talents Plan of China-Young Professionals Grant (to Zhibing Lai), the Fundamental Research Funds for the Central Universities Grant [2662014PY054] and [2662015PY185], University Student Research Fund [2016090] and Innovation Training Plan of University Student Fund [201510504023].

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