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Joint application of plant immunity-inducing elicitors and fungicides to control *Phytophthora* diseases

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Abstract

Phytophthora are destructive plant pathogens that pose a serious threat to crop production. Traditional control methods rely heavily on chemical fungicides, which are harmful to the environment and human health. Currently, effective green prevention and control methods for *Phytophthora* pathogens are lacking. Plants rely primarily on their innate immune system to resist pathogens. Plant cells perceive pathogen invasion and activate immune responses by recognizing specific pathogen-derived molecules, called elicitors, which mainly include pathogen-associated molecular patterns (PAMPs) and microbial effector proteins. PAMPs, which are conserved molecular features of microbes and recognized by plant cell surface-localized pattern-recognition receptors (PRRs), activate mild and broad-spectrum disease resistance. However, there are few reports on elicitor proteins that induce broad resistance against *Phytophthora* pathogens. In this study, we identified BcIEB1, a fungal-derived PAMP, which activated plant immune responses in a BAK1- and SOBIR1-dependent manner. BcIEB1 could induce plant resistance to various *Phytophthora* pathogens, including *P. capsici*, *P. infestans,* and *P. parasitica*. We further found that the combination of lower concentrations of BcIEB1 with fungicides, such as pyraclostrobin, azoxystrobin, and metalaxyl-M, could enhance the effect on *Phytophthora* disease control while reducing the dependence on fungicides, thereby reducing environmental pollution. This study identified a novel, less toxic strategy for controlling *Phytophthora* diseases.

Keywords *Phytophthora* diseases, Elicitor, PAMP, Plant immunity, Fungicide

Background

Phytophthora, a unique genus of the Oomycetes, is morphologically similar to filamentous fungi; however, they differ in many aspects, such as cell wall composition, biochemical metabolism, and reproductive methods (Jiang and Tyler 2012). *Phytophthora* species are highly destructive plant pathogens that significantly threaten crop production. With over 100 members reported, pathogenic *Phytophthora* species are responsible for diseases such as late blight in potatoes, which causes an estimated $6 billion in agricultural losses globally (Haverkort et al. 2008). For example, *P. capsici* is a highly damaging oomycete pathogen with a wide host range, infecting Solanaceous plants such as peppers and tomatoes, legumes, and

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members of the genus *Arabidopsis*, and causes economic losses exceeding $1 billion annually (Lamour et al. 2012). Similarly, *P. infestans* infects Solanaceous crops, such as potatoes, causing late blight outbreaks and severe losses (Judelson and Blanco. 2005). *P. parasitica* species are typical root pathogens that affect a broad range of hosts, causing diseases such as tobacco black shank and citrus root rot (Kamoun et al. 2015). *P. sojae* and *P. cinnamomi* have also caused substantial crop losses (Tyler. 2007). Therefore, preventing and controlling disease spread from *Phytophthora* pathogens requires urgent attention.

Scientists have long been working to control diseases caused by *Phytophthora*. Current strategies rely mainly on fungicides and the breeding of resistant cultivars (Kalyandurg et al. 2021). Fungicides, categorized into protective and systemic fungicides, are currently the most common, rapid, and effective prevention and control measures. Protective fungicides, however, are only effective before the occurrence of diseases and do not affect pathogens that have already invaded the plants. Meanwhile, systemic fungicides can be absorbed by plants (Kitchen et al. 2016). Commonly used systemic fungicides for preventing and controlling *Phytophthora* pathogens include various fungicides such as pyraclostrobin, azoxystrobin, and metalaxyl-M (Bartett et al. 2001). Among these, pyraclostrobin and azoxystrobin are methoxyacrylate fungicides, which are a class of efficient broad-spectrum fungicides that can control pathogens such as oomycetes, ascomycetes, and basidiomycetes (Rodrigues et al. 2013). Compared to azoxystrobin, pyraclostrobin has a stronger toxicity to fish (Wang et al. 2021). Meanwhile, metalaxyl-M is a phenylamide fungicide used to control *Phytophthora* and *Pythium* in vegetables, fruits, and nuts (Atmaca et al. 2018; White et al. 2019). Although using fungicides alleviates plant disease symptoms, excessive or improper use can pose potential risks to human health and the environment (Dong et al. 2012). For example, pyraclostrobin, which can influence human cell differentiation, has been detected in various fruits and vegetables (Dong et al. 2012; Luz et al. 2018). Azoxystrobin has potential toxicity to humans, birds, mammals, and bees (Rodrigues et al. 2013; Hu et al. 2022). Metalaxyl-M is toxic to aquatic and terrestrial organisms like algae, *Daphnia magna,* and zebrafish embryos (Liu et al. 2014; Ouyang et al. 2021). Besides, metalaxyl-M induces inflammation, necrosis, and vacuolization in mouse liver cells (Zhang et al. 2019). Therefore, the use of fungicides should be minimized to achieve sustainable and non-toxic disease prevention and control.

Improving plant disease resistance by breeding excellent disease-resistant varieties is the most economical and environmentally friendly way to prevent and control diseases. However, the lack of disease-resistant resources and the rapid evolution of pathogens severely limits the cultivation of disease-resistant varieties. Therefore, improving the broad-spectrum resistance of plants against *Phytophthora* is of great significance. Plant disease resistance is primarily dependent on the innate immune system. Plants have evolved two layers of immune responses: pathogen-associated molecular patterns (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl. 2006). Pathogen-derived molecules recognized by plant immune receptors are also known as elicitors. While cell surface-localized pattern recognition receptors (PRRs) recognize PAMPs and then activate the PTI, the NLR receptors activate the ETI by recognizing the effector proteins secreted by pathogens (Yuan et al. 2021).

Many PAMPs derived from bacteria, fungi, and oomycetes can be recognized by PRRs. For example, bacterial flagellin (or epitope flg22) is recognized by the FLS2 receptor in the presence of the co-receptor BAK1 (Gómez-Gómez and Boller. 2000; Zipfel et al. 2006). Chitin derived from fungal cell walls is recognized by *Arabidopsis* LYK4/5 or rice CEBiP receptors (Liu et al. 2012; Cao et al. 2014). Further, RcCDI1 is a PAMP found in fungi of the phylum Ascomycetes, which can induce cell death in Solanaceae plants. The cell death induced by RcCDI1 depends on NbBAK1, NbSOBIR1, and NbSGT1. However, the receptors that recognize RcCDI1 remain unknown (Franco-Orozco et al. 2017). BcIEB1 is a protein secreted by *Botrytis cinerea* whose conserved domain, ieb35, induces the expression of plant defense genes (Frías et al. 2016; Pérez-Hernández et al. 2020). Finally, XEG1, derived from *P. sojae,* is recognized by the RXEG1 receptor and activates plant immunity in *Nicotiana benthamiana* in the presence of BAK1 and SOBIR1(Ma et al. 2015).

PTI confers mild and broad-spectrum resistance to most pathogens, often with a lower growth penalty. Therefore, elicitor proteins could be used to improve plant disease resistance. Chitosan is a natural and safe biopolymer derived from chitin, which can induce plant defense; thus, it is widely used in agricultural production. Plants treated with chitosan exhibit high resistance to microorganisms such as fungi, bacteria, and viruses (Riseh et al. 2022) because chitosan enhances the expression of the rice endosperm kinase gene *REK* and activates signaling pathways mediated by jasmonic acid (JA) and salicylic acid (JA) (Jwa et al. 2002). Hypersensitivity proteins (harpins) are a class of elicitor proteins found in gram-negative bacteria that can induce plant resistance to pathogens, promote plant growth and development, and increase crop yields (Wei et al. 1992). VDAL is a protein secreted by *Dahlia verticillium* wilt that activates

plant disease resistance by regulating the auxin and ethylene signaling pathway (Ma et al. 2021; Jiang et al. 2022). Zhinengcong (ZNC) is a crude ethanol extract of the endophytic fungus *Paecilomyces variotii* that can induce reactive oxygen species (ROS) bursts and defense gene expression, thereby improving plant resistance to pathogens. In addition, ZNC can promote plant growth by enhancing nitrogen and phosphorus absorption and transport. Therefore, it is commonly used in agricultural production (Wang et al. 2022). However, only a few studies have used elicitors to improve the resistance of plants to *Phytophthora*.

To fill this gap, we collected 20 PAMPs with known immunity-inducing activities and transiently expressed them in *N. benthamiana* to analyze plant resistance to *P. capsici*. We found that BcIEB1 could induce plant resistance to *P. capsici* in a SOBIR1- and BAK1-dependent manner. Additionally, BcIEB1 significantly improved plant resistance to *P. capsici*, *P. infestans*, and *P. parasitica*. Further research has shown that the combined application of BcIEB1 with the fungicides azoxystrobin, pyraclostrobin, and metalaxyl-M can achieve sufficient disease control while reducing fungicide usage. This study provides new insights for the prevention and control of *Phytophthora* diseases.

Results

Transient expression of RcCDI1 and BcIEB1 in *N. benthamiana* enhanced plant resistance against *Phytophthora* pathogens

To screen for elicitors that could induce plant resistance to *Phytophthora* pathogens, we collected 46 elicitor protein sequences from published studies, most of which were PAMP molecules. We inserted these genes into the pCAMBIA1300-35S-HA-RBS vector, transiently expressed them in the leaves of *N. benthamiana*, and examined their protein expression levels by anti-HA immunoblotting. The results showed that 20 elicitors were typically expressed in *N. benthamiana* (Additional file 1: Figure S1a, b and Additional file 2: Table S1), and four elicitor proteins strongly induced plant cell death (Additional file 1: Figure S2).

We transiently expressed these elicitors in *N. benthamiana* leaves for 24 h, inoculated them with *P. capsici*, and recorded the disease phenotype 36–48 h later (Additional file 1: Figure S3). The results showed that RcCDI1 and BcIEB1, two fungal PAMPs, induced strong resistance against *P. capsici* infection (Fig. 1a). We further showed that RcCDI1 and BcIEB1 induced a strong resistance to *P. parasitica* (Fig. 1b). In addition, RcCDI1 and BcIEB1 induced plant resistance to the fungal pathogens *Sclerotinia sclerotiorum* and *B. cinerea* (Additional file 1: Figure S4).

RcCDI1 and BcIEB1 induced plant resistance against *Phytophthora* and fungal pathogens

To confirm the roles of RcCDI1 and BcIEB1 in promoting plant resistance to *Phytophthora* pathogens, we expressed these two proteins in *Escherichia coli* and purified the recombinant proteins (Fig. 2a). The *N. benthamiana* plants were infiltrated with 1 μM RcCDI1 or BcIEB1 proteins one day before the inoculation with *P. capsici* or *P. parasitica*. The results showed that RcCDI1 and BcIEB1 induced a strong resistance against these two *Phytophthora* pathogens (Fig. 2b, c). Similarly, RcCDI1 and BcIEB1 greatly enhanced plant resistance to the fungal pathogens *S. sclerotiorum* and *B. cinerea* (Fig. 2d, e). Thus, we identified two PAMP proteins, RcCDI1 and BcIEB1, that can induce plant resistance to *Phytophthora* and fungal pathogens.

RcCDI1 and BcIEB1 induced plant immunity in a BAK1- and SOBIR1-dependent manner

The roles of RcCDI1 and BcIEB1 in inducing plant immunity were studied. First, we examined the RcCDI1- and BcIEB1-induced production of ROS in *N. benthamiana* plants. 3,3'-diaminobenzidine (DAB) staining assays showed that RcCDI1 and BcIEB1 induced plant ROS accumulation (Additional file 1: Figure S5). Then, the RcCDI1- and BcIEB1-induced expression of *NbCYP71D20* and *NbPTI5*, two defense marker genes, were examined by qPCR analysis (Hu et al. 2020). The expression of *NbCYP71D20* and *NbPTI5* was significantly induced 3 h and 6 h after RcCDI1 and BcIEB1 treatments (Fig. 3a, b). Although the PRRs that recognize RcCDI1 and BcIEB1 are unknown, SOBIR1 and BAK1 have been suggested as co-PRRs that recognize many PAMPs (Heese et al. 2007; Domazakis et al. 2018). Therefore, we examined RcCDI1- and BcIEB1-induced cell death in *sobir1* mutants and *NbBAK1*-silencing plants. In line with the findings of previous studies, the occurrence of RcCDI1-induced cell death required BAK1 and SOBIR1 (Fig. 3c) (Franco-Orozco et al. 2017); however, no cell death was observed in BcIEB1-treated leaves (Fig. 3c). Quantitative real-time PCR analysis showed that the expression of *NbBAK1* was significantly reduced in the *NbBAK1*-silencing plants (Additional file 1: Figure S6). We then examined BcIEB1-induced defense gene expression in *sobir1* mutants and *BAK1*-silencing plants. Results showed that BcIEB1-induced expression of *NbPTI5* was significantly reduced in *sobir1* mutants and *NbBAK1*-silencing plants (Fig. 3d, e), suggesting that BcIEB1 functions in a BAK1 and SOBIR1-dependent manner. Further,

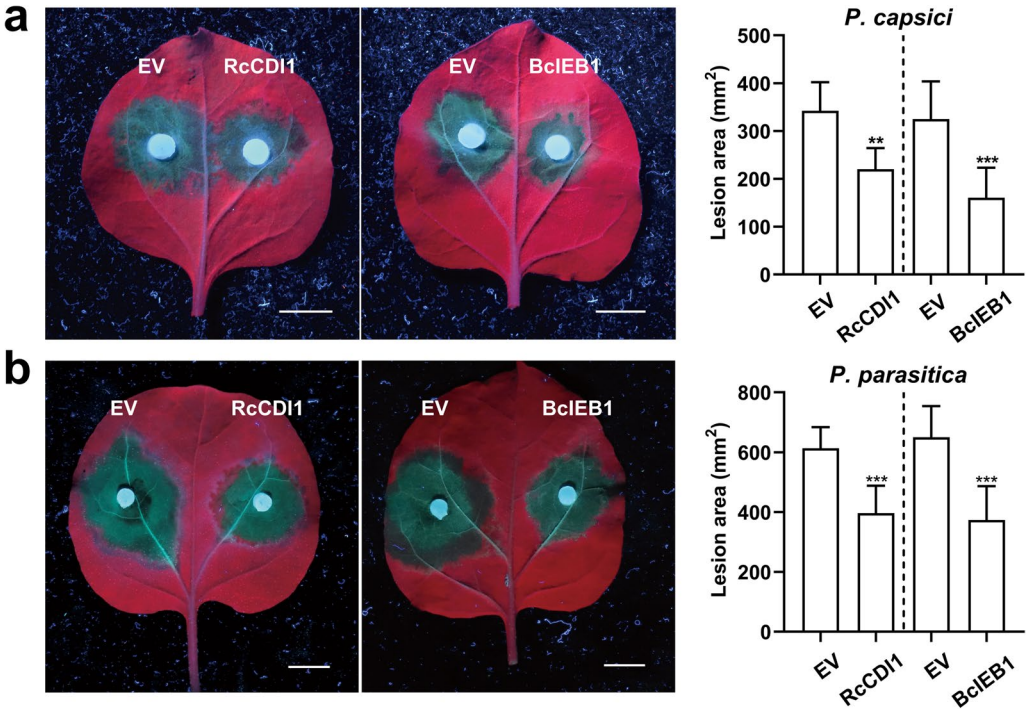


Fig. 1 Transient expression of RcCDI1 and BcIEB1 in *N. benthamiana* enhanced plant resistance to *P. capsici* and *P. parasitica*. **a** Transient expression of RcCDI1 and BcIEB1 in *N. benthamiana* enhanced plant resistance to *P. capsici* strain LT263. BcIEB1-HA, RcCDI1-HA, or an empty vector (EV) was expressed in *N. benthamiana* via *Agrobacterium*-mediated transient expression for 1 d. **b** Transient expression of RcCDI1 and BcIEB1 in *N. benthamiana* enhanced plant resistance to *P. parasitica* strain Pp025. The indicated constructs were transiently expressed in *N. benthamiana* as in **a**. Leaves were inoculated and photographed 36–48 h later under UV light. Lesion areas were measured and calculated (mean ± SD, n ≥ 6; Student's *t*-test; **, $P < 0.01$,***, $P < 0.001$). Scale bars: 1 cm. All experiments were performed three times with similar results

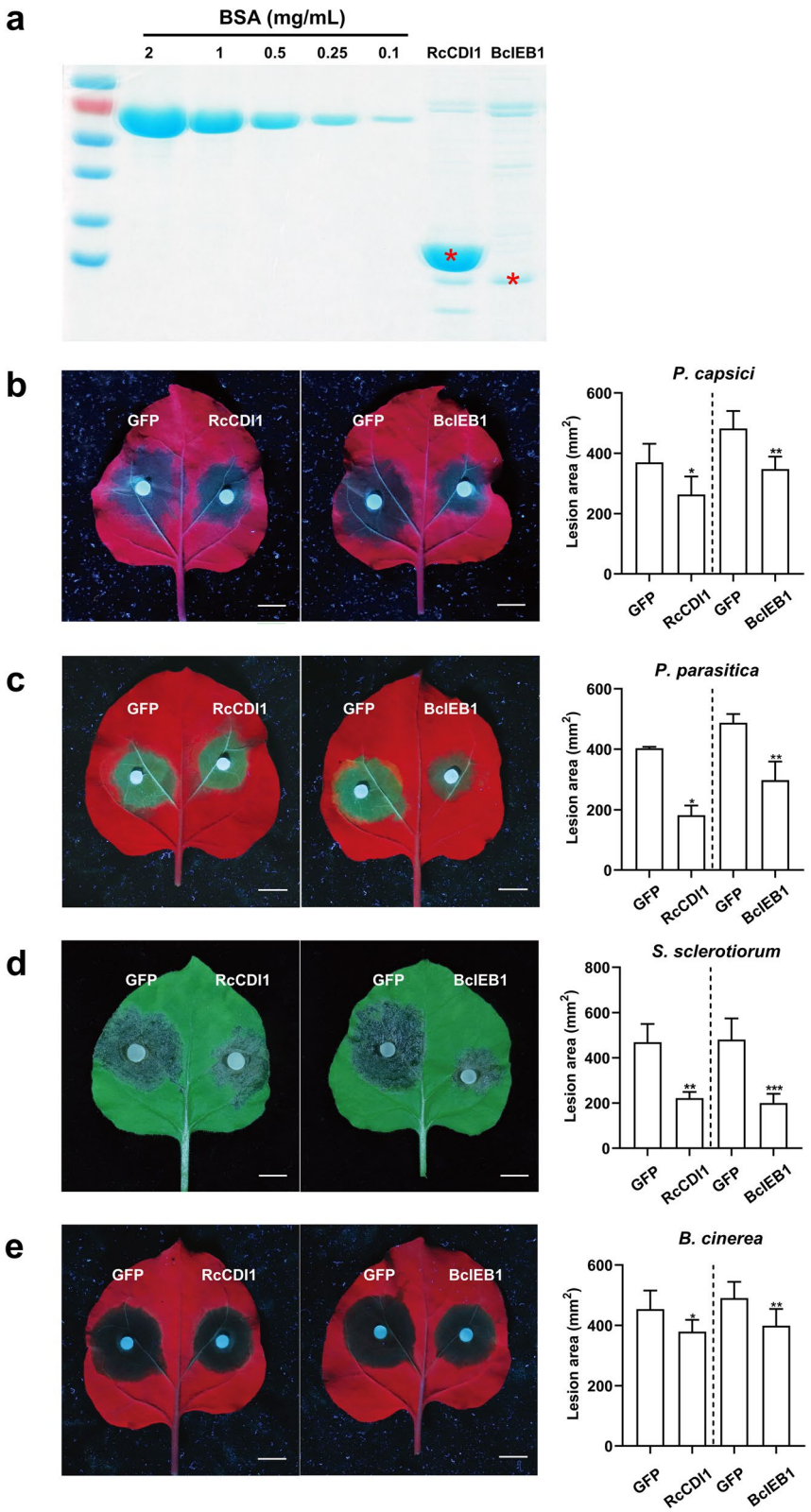
heteromeric G proteins function as key regulators of PTI. Therefore, we examined BcIEB1-induced defense gene expression in *Nbxlg3,5* and *Nbxlg4* mutants. The BcIEB1-induced expression of *NbCYP71D20* and *NbPTI5* was severely impaired in *Nbxlg4* and *Nbxlg3,5* mutants (Additional file 1: Figure S7a, b), indicating that G proteins are also required for the activation of BcIEB1-induced immunity. To further analyze whether BcIEB1 and RcCDI1 can be used to improve plant *Phytophthora* disease resistance under environmental conditions, we tested the immune-induction activity of these two proteins after 5 h of exposure at 25°C and 40°C. We found that BcIEB1 could still induce high expression of the *NbPTI5* gene after being placed at 25°C and 40°C for 5 h (Additional file 1: Figure S8a); however, the ability of RcCDI1 to induce PTI gene expression was significantly decreased (Additional file 1: Figure S8b), suggesting that the stability of BcIEB1 was better than that of RcCDI1. Therefore, we chose BcIEB1 to study its potential applications in improving plant resistance to *Phytophthora* diseases.

BcIEB1-derived ieb35 peptides induced a weaker plant immunity than BcIEB1

BcIEB1 is a protein secreted by *B. cinerea* with an N-terminal signal peptide and a conserved ieb35 motif from amino acids 84 to 118 (Fig. 4a), which can induce a series of plant immune responses (Frías et al. 2016). Compared to BcIEB1, the ieb35 peptide has a lower molecular

(See figure on next page.)

Fig. 2 RcCDI1 and BcIEB1 induced resistance to *P. capsici* and *P. parasitica* in *N. benthamiana* plants. **a** Purification of recombinant His-tagged RcCDI1 and BcIEB1 proteins from *E. coli*. The RcCDI1-His and BcIEB1-His proteins were marked with asterisk. **b** and **c** RcCDI1 and BcIEB1 induced plant resistance to *P. capsici* strain LT263 and *P. parasitica* strain Pp025 in *N. benthamiana*. **d** and **e** RcCDI1 and BcIEB1 induced plant resistance to fungal pathogen *S. sclerotiorum* and *B. cinerea* in *N. benthamiana*. Leaves of *N. benthamiana* were infiltrated with BcIEB1-His, RcCDI1-His, or GFP-His for 1 d. The leaves were inoculated with indicated pathogens and photographed 36–48 h later. Lesion areas were measured and calculated (mean ± SD, n ≥ 6; Student's *t*-test; *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$). Scale bars: 1 cm. All experiments were performed three times with similar results



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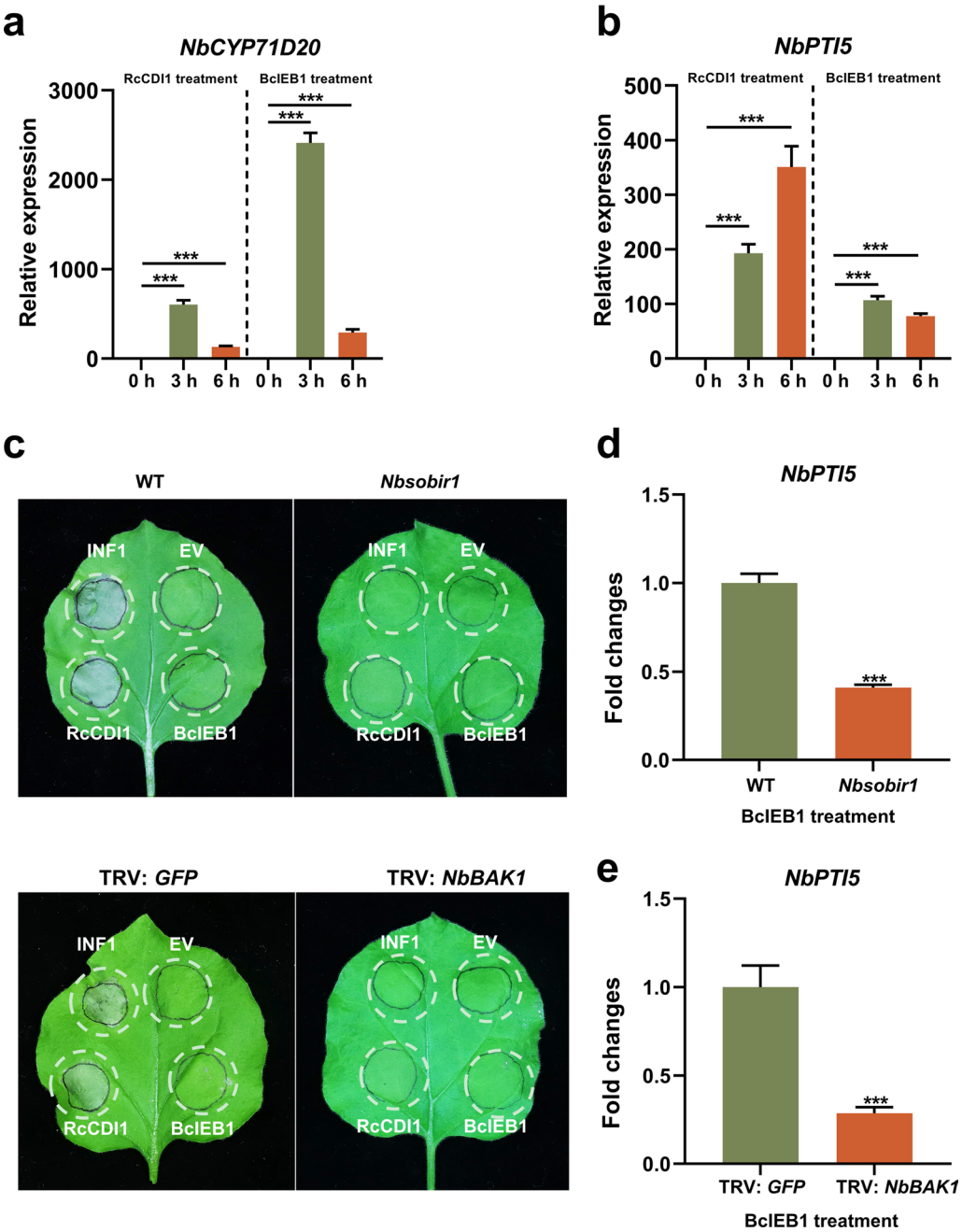

Fig. 3 RcCDI1- and BclEB1-induced plant immune responses required NbBAK1 and NbSOBIR1. **a** and **b** RcCDI1 and BclEB1 induced the expression of plant defense genes *NbCYP71D20* and *NbPTI5*. The 5-week old *N. benthamiana* plants were infiltrated with RcCDI1 and BclEB1 at the concentration of 1 μM. **c** RcCDI1 induced cell death in *N. benthamiana* in a NbBAK1- and NbSOBIR1-dependent manner. The indicated constructs were transiently expressed in leaves of *Nbsobir1* and *NbBAK1*-silencing plants. The cell death phenotype was visualized 4 d after. INF1 was used as a positive control. **d** BclEB1-induced expression of *NbPTI5* required NbSOBIR1. BclEB1 (1 μM) was infiltrated into the leaves of 5-week old WT and *Nbsobir1* mutant plants, and leaf tissues collected at 0 h and 6 h were used for the assay. **e** BclEB1-induced expression of *NbPTI5* required NbBAK1. BclEB1 (1 μM) was infiltrated into the 5-week old *TRV: GFP* and *NbBAK1*-silencing *N. benthamiana* plants, and leaf tissues collected at 0 h and 6 h were used for the assay. For gene expression analysis, total RNA was extracted and subjected to qRT-PCR assay, and fold changes before and after elicitor treatment were calculated (mean ± SD, Student's *t*-test; ***, $P < 0.001$). All experiments were performed three times with similar results

weight and thus may be more suitable for industrial production. Therefore, we compared the immune responses induced by BcIEB1 and ieb35. First, we observed that both BcIEB1 and ieb35 induced the expression of *NbPTI5* in *N. benthamiana*. However, BcIEB1 induced a much stronger *NbPTI5* expression than ieb35 (Fig. 4b). Next, we performed a PAMP-induced growth inhibition assay in 1/2 MS medium supplemented with different concentrations of BcIEB1 and ieb35 and examined the hypocotyl length of the seedlings. Results showed that BcIEB1 had a much stronger inhibitory effect on hypocotyl growth than did ieb35 (Fig. 4c), and BcIEB1-treated seedlings exhibited a much shorter hypocotyl length than ieb35-treated seedlings (Additional file 1: Figure S9). Consistent with this, BcIEB1-treated plants exhibited a much lower fresh weight than that of ieb35-treated plants (Fig. 4d). We then sought to determine whether, as seen in the application of BcIEB1, ieb35 induces plant resistance to *P. capsici*. We treated *N. benthamiana* with BcIEB1 (1 μM) and different concentrations of ieb35 and then infected with *P. capsici*. We found that lower concentrations of ieb35 (1 μM and 5 μM) only induced a slight resistance to *P. capsici* (Fig. 4e and Additional file 1: Figure S10), whereas ieb35 at 10 μM induced the same level of resistance as 1 μM BcIEB1 (Fig. 4e and Additional file 1: Figure S10). Hence, we showed that ieb35, like BcIEB1, can activate plant immunity, but its immune induction ability is much lower than that of BcIEB1. Therefore, we used BcIEB1 for further experiments.

Combined application of BcIEB1 with pyraclostrobin or azoxystrobin greatly alleviated plant disease symptoms caused by *P. capsici*

The above results indicated that BcIEB1 can induce plant resistance to *P. capsici*. However, high BcIEB1 concentrations severely inhibited plant growth (Fig. 4d), while lower BcIEB1 concentrations may make it difficult to ensure adequate disease control. In agricultural production, high concentrations of fungicides have good disease prevention and control effects but cause potential environmental pollution. Therefore, we attempted to use low-concentration fungicides in combination with BcIEB1. Pyraclostrobin is a fungicide that is commonly used to prevent *Phytophthora* disease. We observed that pyraclostrobin, at concentrations of 10 μg/mL or 15 μg/mL, significantly reduced the disease symptoms caused by *P. capsici*, but lower concentrations (1 μg/mL and 5 μg/mL) of pyraclostrobin only slightly reduced the disease symptoms (Fig. 5a, b). Notably, the use of low concentrations of BcIEB1 (1 μM) and pyraclostrobin (5 μg/mL) jointly reduced plant disease strongly (Fig. 5a, b). Similarly, using lower concentrations of BcIEB1 and azoxystrobin together could achieve disease prevention and control effects similar to those of higher concentrations of azoxystrobin (Fig. 5c, d). The disease prevention and control effect of using low concentrations of BcIEB1 and pyraclostrobin/azoxystrobin together was much better than that of using BcIEB1 alone, which was equivalent to the effect of using high concentrations of pyraclostrobin/azoxystrobin (Fig. 5b, d).

Combined application of BcIEB1 with metalaxyl-M greatly alleviated plant disease symptoms caused by oomycete and fungal pathogens

We sought to examine joint effects with fungicides such as metalaxyl-M. We observed that using low concentrations of BcIEB1 (1 μM) and metalaxyl-M (5 μg/mL) to treat plants jointly could achieve the same disease prevention and control effect as a high concentration of metalaxyl-M (10 μg/mL) (Fig. 6a, b). Therefore, adding BcIEB1 could reduce the use of fungicides in preventing and controlling *Phytophthora* diseases. The fungicides we used can act on various pathogenic microorganisms, and BcIEB1 is a fungal-derived PAMP. Therefore, we investigated whether this approach could control fungal diseases. We showed that the combined application of lower concentrations of BcIEB1 with pyraclostrobin or metalaxyl-M could achieve a similar resistance level against *S.*

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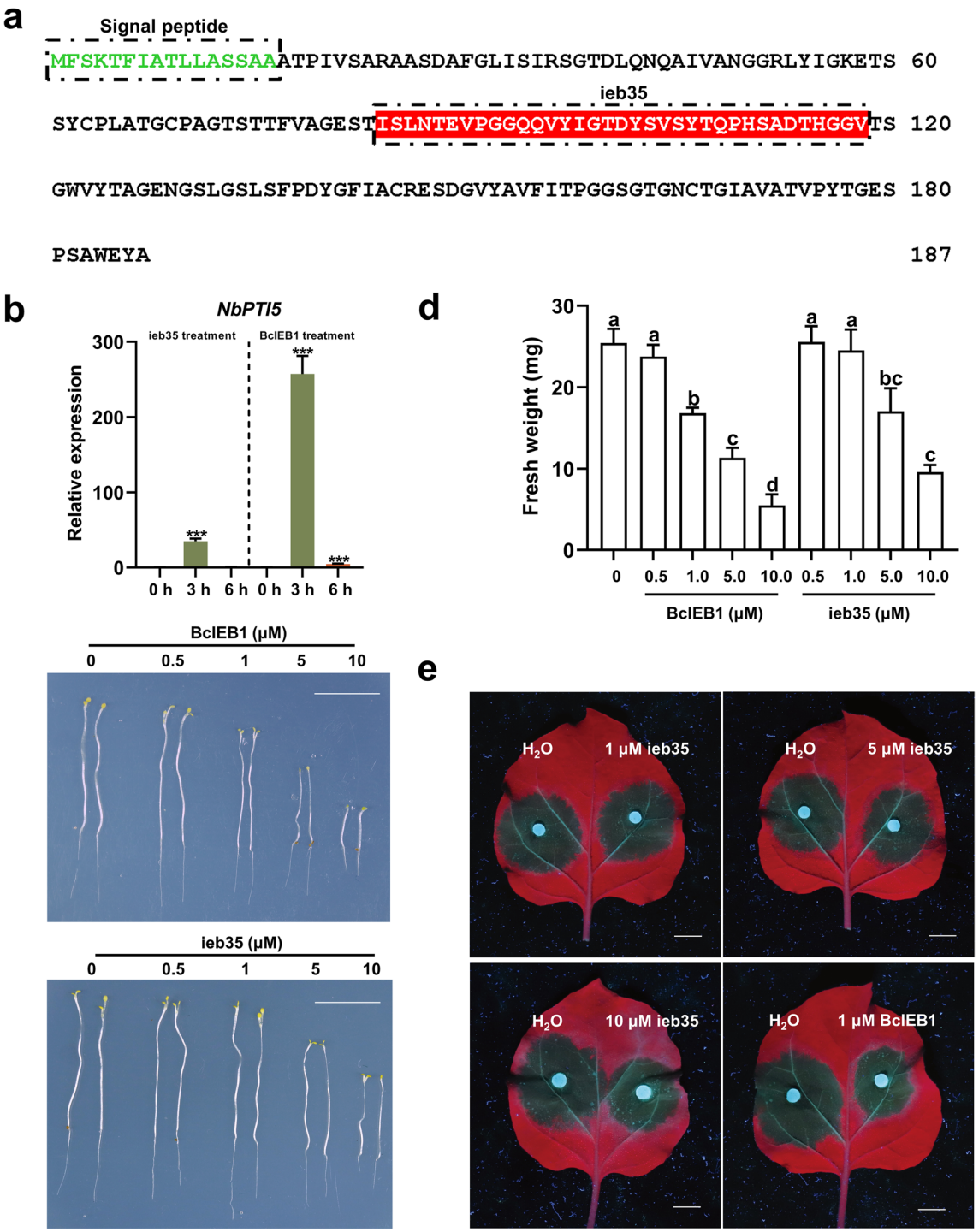
Fig. 4 ieb35 peptide induced a weaker plant immune response than that of BcIEB1. **a** A chart showing the ieb35 motif in BcIEB1 protein. **b** ieb35 induced a lower level of *NbPTI5* expression than BcIEB1. The 5-week old *N. benthamiana* plants were infiltrated with ieb35 and BcIEB1 at the concentration of 1 μM. Total RNA was extracted and subjected to qPCR assay to analyze the expression of *NbPTI5* (mean ± SD, Student's *t*-test; ***, $P < 0.001$). **c** BcIEB1 induced a stronger hypocotyl growth inhibition effect than ieb35 on *Arabidopsis* seedlings. Wild-type Col-0 plants were vertically grown on 1/2 MS agar plates in the dark with addition of different concentrations of BcIEB1 or ieb35, and hypocotyl length were photographed 6–7 days later. Scale bars: 1 cm. **d** BcIEB1 induced a stronger plant growth inhibition than ieb35. Wild-type Col-0 plants were germinated on 1/2 MS agar plates and transfered to liquid 1/2 MS medium with the addition of different concentrations of BcIEB1 or ieb35. Fresh weight was measured and calculated 5 d later (mean ± SD, $n \geq 6$, different letters indicate significant difference at $P < 0.05$, one-way ANOVA followed by Tukey's post hoc test). **e** BcIEB1 induced stronger resistance to *P. capsici* than ieb35 in *N. benthamiana*. Leaves of *N. benthamiana* were infiltrated with different concentrations of ieb35 or BcIEB1-His (1 μM) for 1 d. The treated leaves were inoculated by *P. capsici* strain LT263 and photographed under UV light 36–48 h later. Scale bars: 1 cm. The experiments were performed three times with similar results



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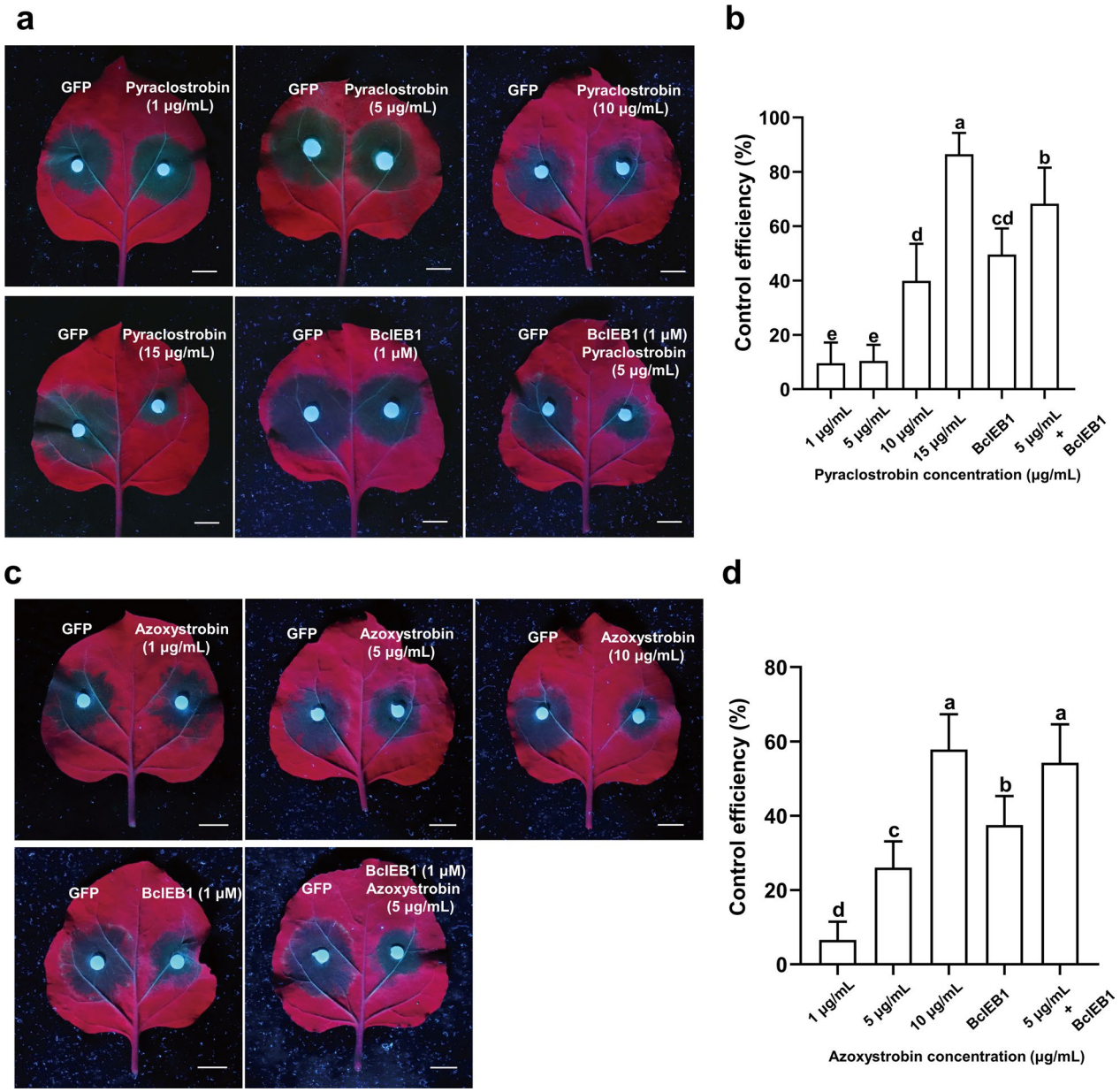

Fig. 5 Combined application of BcIEB1 with pyraclostrobin or azoxystrobin significantly alleviated plant disease symptoms caused by *P. capsica*. **a** and **b** Leaves of *N. benthamiana* were infiltrated with BcIEB1 (1 μM), different concentrations of pyraclostrobin, or BcIEB1(1 μM) plus pyraclostrobin (5 μg/mL) for 1 d. The leaves were inoculated by *P. capsici* strain LT263 and photographed 36–48 h later under UV light. The lesion areas were measured and calculated (mean ± SD, n ≥ 6, different letters indicate significant difference at $P < 0.05$, one-way ANOVA followed by Tukey's post hoc test). Scale bars: 1 cm. **c** and **d** Joint application of BcIEB1 with azoxystrobin significantly reduced plant susceptibility to *P. capsici*. The assays were performed as in **a** (mean ± SD, n ≥ 6, different letters indicate significant difference at $P < 0.05$, one-way ANOVA followed by Tukey's post hoc test). Scale bars: 1 cm. All experiments were performed three times with similar results

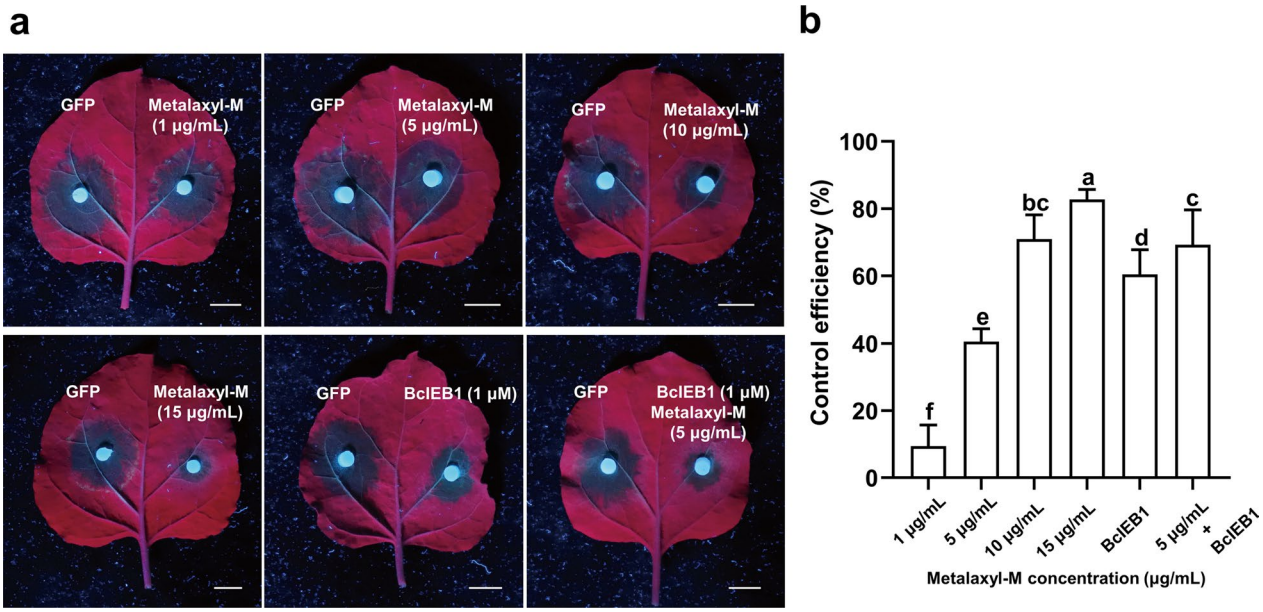

Fig. 6 Combined application of BcIEB1 with metalaxyl-M or azoxystrobin alleviated plant disease symptoms caused by *P. capsica*. **a** and **b** Combined application of BcIEB1 with metalaxyl-M significantly reduced plant susceptibility to *P. capsici*. Leaves of *N. benthamiana* were infiltrated with BcIEB1 (1 μM), different concentrations of metalaxyl-M, or BcIEB1(1 μM) plus metalaxyl-M (5 μg/mL) for 1 d. The leaves were inoculated by *P. capsici* strain LT263 and photographed 36–48 h later under UV light. The lesion areas were measured and calculated (mean ± SD, n ≥ 6, different letters indicate significant difference at $P < 0.05$, one-way ANOVA followed by Tukey's post hoc test). Scale bars: 1 cm. All experiments were performed three times with similar results

sclerotiorum infection as higher concentrations of fungicides used alone (Additional file 1: Figure S11).

Joint application of BcIEB1 and pyraclostrobin alleviated plant disease symptoms caused by *Phytophthora* pathogens with less usage of fungicide in tomato and potato

Finally, we aimed to determine whether this strategy applies to other plants, especially crop plants, in agricultural production. We treated potato plants with BcIEB1 (1 μM), pyraclostrobin (10 μg/mL), and BcIEB1 (1 μM) plus pyraclostrobin (5 μg/mL) one day before inoculation with *P. infestans*. Plants co-treated with pyraclostrobin and BcIEB1 showed the best control effect on *P. infestans* (Fig. 7a). We then treated tomato plants with BcIEB1, pyraclostrobin, or BcIEB1 plus pyraclostrobin for one day before inoculation with *P. capsici*. Similarly, combining pyraclostrobin and BcIEB1 significantly improved the effect of disease control (Fig. 7b).

Discussion

Members of the *Phytophthora* genus cause devastating diseases in numerous plants, making them among the most important pathogens affecting dicots. There is a lack of efficient strategies for controlling *Phytophthora* diseases in agricultural production. In the present study, we identified the elicitor protein BcIEB1, capable of inducing plant resistance to various *Phytophthora* species, including *P. capsici*, *P. infestans,* and *P. parasitica*. When combined with fungicides, such as pyraclostrobin, metalaxyl-M, or azoxystrobin, BcIEB1 effectively controlled *Phytophthora* diseases while reducing fungicide usage, thus offering new insights into less toxic and more sustainable *Phytophthora* disease control.

Elicitor proteins include many PAMPs, which are conserved and recognized by plant cell-surface PRRs, leading to relatively mild disease resistance responses. These PAMPs are ideal candidates for enhancing broad-spectrum plant disease resistance. Therefore, we screened PAMP molecules that induce resistance against various *Phytophthora* species. Using a screening system based on the *P. capsici-N. benthamiana* interaction, we found that the fungal proteins RcCDI1 and BcIEB1 induced strong resistance against multiple *Phytophthora* species in *N. benthamiana*. However, the receptors that recognize RcCDI1 and BcIEB1 in plants remain unclear. Further, RcCDI1 is conserved among different ascomycete fungi and triggers cell death in Solanaceous plants but not in monocots (Franco-Orozco et al. 2017). We found that RcCDI1 induces cell death in *N. benthamiana*, indicating that it triggers a strong immune response, which may affect plant growth and development and limit its

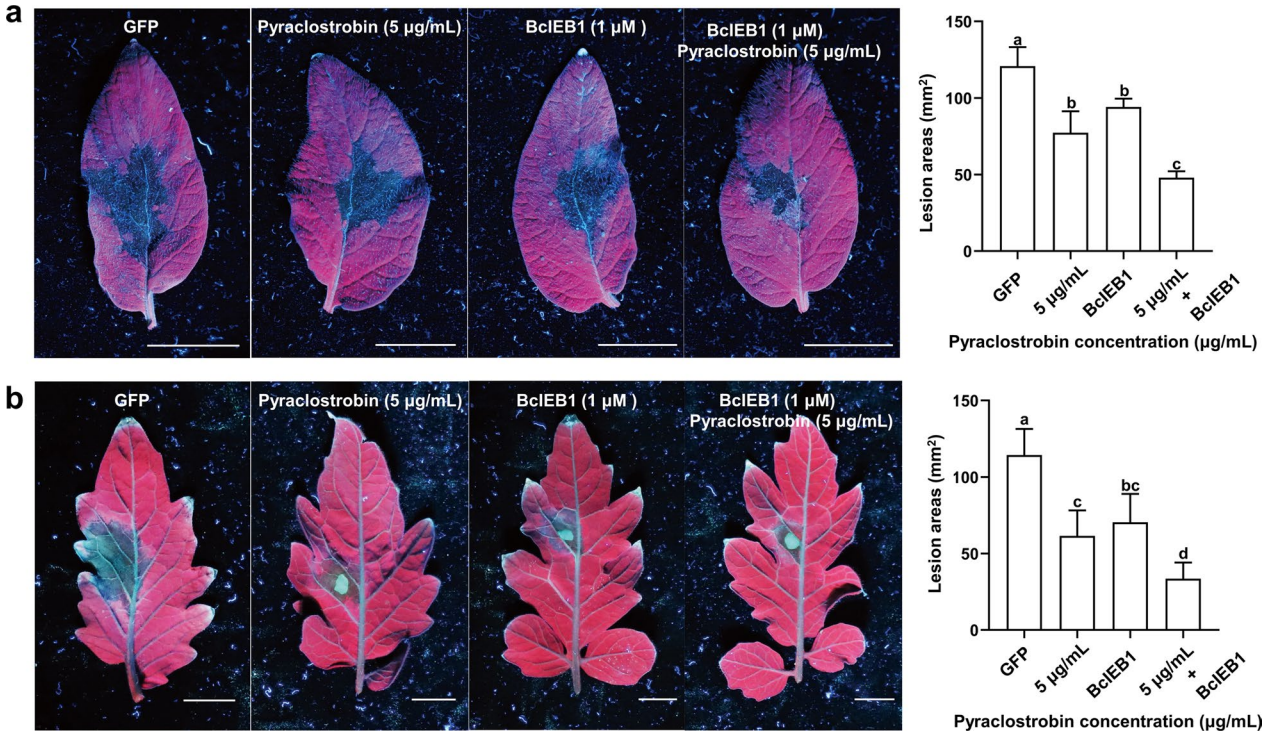

Fig. 7 Combined application of BcIEB1 and pyraclostrobin alleviated plant disease symptoms caused by *P. capsici* and *P. infestans* and allows lower usage of fungicide in tomato and potato plants. **a** Combined application of BcIEB1 and pyraclostrobin reduced potato susceptibility to *P. infestans.* Leaves of potato were infiltrated with BcIEB1 (1 μM), pyraclostrobin (10 μg/mL), or BcIEB1(1 μM) and pyraclostrobin (5 μg/mL) for 1 d. The leaves were inoculated by *P. infestans* strain pd21355-Hap1 and photographed under UV light 7 d later. The lesion areas were measured and calculated (mean ± SD, n ≥ 6, different letters indicate significant difference at $P < 0.05$, one-way ANOVA followed by Tukey's post hoc test). Scale bars: 1 cm. **b** Combined application of BcIEB1 and pyraclostrobin reduced tomato susceptibility to *P. capsici*. Leaves of tomato were infiltrated with BcIEB1 (1 μM), Pyraclostrobin (10 μg/mL), or BcIEB1(1 μM) plus Pyraclostrobin (5 μg/mL) for 1 d. The leaves were inoculated by *P. capsici* strain LT263 and photographed under UV light 2 d later. The lesion areas were measured and calculated (mean ± SD, n ≥ 6, different letters indicate significant difference at $P < 0.05$, one-way ANOVA followed by Tukey's post hoc test). Scale bars: 1 cm. All experiments were performed two times with similar results

application in disease control. BcIEB1 is the second most abundant protein in *B. cinerea* secretome, indicating that it may be more suitable for mass production. BcIEB1 induces defense responses in monocot and dicot plants such as onions, *Arabidopsis*, tobacco, and tomato plants. BcIEB1 has been shown to induce cell death in tobacco cv. Havana (Frías et al. 2016). However, we did not observe cell death in *N. benthamiana*, tomato, or potato plants after BcIEB1 treatment, likely due to variations in the BcIEB1 recognition in different plant species (Kessens et al. 2014).

To explore the ability of BcIEB1 to induce plant immune responses, we purified the recombinant protein of BcIEB1 in *E. coli*. We found that BcIEB1 triggered ROS accumulation and defense gene expression. Further studies revealed that BcIEB1-induced immune responses rely on the co-receptors BAK1 and SOBIR1, as well as key immune signal transduction regulators, such as heterotrimeric G proteins.

The BcIEB1 treatment significantly enhanced plant resistance to *P. capsici*, *P. infestans,* and *P. parasitica*. Studies have shown that ieb35, a conserved key motif of BcIEB1 consisting of 35 amino acids, induces plant immune responses. However, its immunity-inducing capacity was significantly lower than that of the full-length BcIEB1 protein, suggesting that plants may also recognize other motifs or structural features of BcIEB1. We further discovered that BcIEB1 retained its ability to induce defense gene expression after six hours of treatment at 25°C and 40°C, indicating good protein stability under different temperatures.

Fungicides are widely used for *Phytophthora* control in agriculture. Azoxystrobin, pyraclostrobin, and metalaxyl-M are fungicides commonly used to control *Phytophthora* diseases. Despite their effectiveness, excessive fungicide application can cause potential damage to the environment and human health. Our study revealed that the combined use of BcIEB1 and fungicides significantly improved resistance against *Phytophthora* compared to

that of BcIEB1 alone. This combination achieved a similar level of control efficiency while substantially reducing fungicide usage. BcIEB1 also induces resistance against fungal pathogens such as *S. sclerotiorum* and *B. cinerea*. The joint application of fungicides with BcIEB1 reduced fungicide usage and achieved consistent effectiveness against *S. sclerotiorum*, indicating that BcIEB1, when used in combination with various fungicides, may effectively control multiple pathogenic microorganisms and decrease dependence on fungicides.

This study developed a novel approach for the environmentally friendly control of *Phytophthora* diseases by combining immune-eliciting elicitor proteins with fungicides. This approach can reduce fungicide use and enhance the effectiveness of disease control. Future research should focus on identifying more efficient and stable elicitor proteins and establishing scalable production methods to reduce costs. In addition, exploring how to combine elicitor proteins efficiently and stably with fungicides will be a crucial focus of future studies. Whether this approach applies to control other plant diseases also present an interesting avenue of research.

Methods

Plant growth conditions

N. benthamiana and tomato cultivar Moneymaker were grown and maintained in plant growth chambers at 25°C with 60% relative humidity and a 14 h light/10 h dark photoperiod. The plantlets of potato cultivar E3 were grown in a growth chamber with a 16 h light/8 h dark photoperiod at 26°C. The *Nbsobir1* mutant used in this study was generated using the CRISPR-Cas9 approach, as described previously (Pi et al. 2022).

Plasmid construction

The sequences of the indicated elicitors were obtained from corresponding publications (Additional file 1: Figure S1). Coding sequences were synthesized and cloned into the pCAMBIA1300-35S-HA-RBS vector (Sangon, Beijing). For construction of the pET28 vector, the coding sequences of BcIEB1 and RcCDI1 were amplified by PCR and cloned into pET28a using the ClonExpress II One-Step Cloning Kit (Vazyme, Nanjing, China). The primers used in this study are listed in Additional file 2: Table S2.

Transient expression in *N. benthamiana*

A. tumefaciens GV3101 strains, carrying the indicated vectors, were cultured in liquid Luria-Bertani (LB) medium at 28°C for 8–12 h and collected by centrifugation. The bacteria were washed and resuspended in a 10 mM $MgCl_2$ solution, and the concentration of the bacterial solution was adjusted to an OD_{600} of 0.5. Then, 4 to 5-week old *N. benthamiana* plants were exposed to *A. tumefaciens* for 24–36 h.

Phytophthora inoculation assays

For the *P. capsici* and *P. parasitica* inoculation assays, *P. capsici* strain LT263 or *P. parasitica* strain Pp025 was cultured at 25°C on 2.5% (vol/vol) vegetable juice (V8) medium in the dark for 2–3 d. For mycelium inoculation, mycelial plugs were taken using a 5 mm diameter cork borer and then inoculated on *N. benthamiana* leaves for 24–30 h. For the zoospore inoculation on tomato, the mycelial plugs were cultured in V8 liquid medium for 2 d at 25°C, washed five times with sterilized water, and incubated in sterilized water for 1–2 d until the formation of sporangia. The cultures were incubated at 4°C for 30 min, followed by incubation at 25°C for 30 min to release the zoospores (Liang et al. 2021). Detached leaves were inoculated with approximately 100 zoospores and kept in plastic boxes at high humidity for 24–48 h. The lesion areas were measured using IMAGE J software (https://imagej.nih.gov/ij).

For the *P. infestans* inoculation assay, *P. infestans* strain pd21355-Hap1 was cultured on rye agar medium at 18°C in the dark for 8–12 d. An appropriate amount of precooled sterile water was added to the plates and scraped with a glass rod to collect the sporangia. *N. benthamiana* and potato leaves were inoculated with approximately 350 sporangia and placed in plastic boxes in the dark at high humidity for 7 d. The inoculated leaves were photographed under UV light, and the lesion areas were measured using IMAGE J software.

Fungal inoculation assays

For the *S. sclerotiorum* inoculation assay, the fungal strain was cultured for 2–3 d at 25°C on potato dextrose agar (PDA) medium in the dark. The fresh mycelial plugs were obtained using a sterile 5 mm diameter cork borer and then inoculated on *N. benthamiana* leaves for 24 h (Li et al. 2022). For the *B. cinerea* inoculation assay, fungi were cultured on a PDA plate at 23°C for 5–7 d. *N. benthamiana* leaves were inoculated with 5 mm mycelial plugs for 24 h. The inoculated leaves were photographed under UV light, and the lesion areas were measured using IMAGE J software.

Purification of recombinant BcIEB1 and RcCDI1 proteins

The coding sequences of *BcIEB1* or *RcCDI1* were cloned into a pET28a vector with a C-terminal 6×His-tag and then introduced into *E. coli* strain BL21. *E. coli* was cultured in liquid LB medium containing kanamycin at 28°C to an OD_{600} value of 0.6. Subsequently, 0.3 mmol/L isopropyl β-D-1-thiogalactopyranoside (IPTG) was added

to the liquid LB medium to induce recombinant protein expression at 16°C for 20 h. *E. coli* cells were harvested by centrifugation and disrupted by sonication. Recombinant BcIEB1 and RcCDI1 were purified using a Ni-NTA resin affinity chromatography kit (Thermo Scientific) according to the manufacturer's instructions.

Electrolyte leakage measurement

Five leaf disks (9 mm diameter) from *N. benthamiana* were collected in a 10 mL test tube containing 5 mL of distilled water at 23°C for 3 h. Then, the electrical conductivity was measured and denoted as A. Samples were boiled for 25 min and cooled to room temperature for electrical conductivity measurement, and denoted as B. The final conductivity (A/B) was calculated (Pi et al. 2023).

DAB staining

The DAB staining solution was prepared by mixing DAB (0.5 g DAB, 0.1% Tween-20, 500 mL of distilled water), and the pH was adjusted to 3.8 by gradually adding hydrochloric acid. The *N. benthamiana* leaves infiltrated by BcIEB1 protein (1 μM) were incubated in DAB staining solution and gently shaken for 8 h in the dark. The leaves were then de-stained with 95% ethanol at 100°C for 5 min to remove the chlorophyll. Leaves were equilibrated with 30% (v/v) glycerol for photography (Wang et al. 2020).

RNA isolation and quantitative RT-PCR

The *N. benthamiana* leaves were harvested and ground into powder after freezing in liquid nitrogen. Total RNA was isolated from the leaves using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The first-strand cDNA was synthesized using M-MLV RNA transcriptase (Takara, Tokyo, Japan). Finally, qRT-PCR was performed using the 2×RealStar Fast SYBR qPCR Mix (GeneStar, Beijing, China) and specific primers.

Abbreviations

ETI	Effector-triggered immunity
hpi	Hours post-inoculation
LB	Luria–bertani
LRR	Leucine-rich repeat
PAMPs	Pathogen-associated molecular patterns
PDA	Potato dextrose agar
PRR	Pattern-recognition receptor
PTI	Pattern-triggered immunity
qRT-PCR	Quantitative real time PCR
RLK	Receptor-like kinase
ROS	Reavtive oxygen species
VIGS	Virus-induced gene silencing

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-024-00233-0.

Additional file 1: Figure. S1. A list of the 20 elicitor proteins used in this study. **Figure. S2.** Examination of elicitor-induced cell death in *N. benthamiana*. **Figure. S3.** Examination of elicitor-induced resistance to *P. capsici* in *N. benthamiana*. **Figure. S4.** BcIEB1 and RcCDI1 induced plant resistance to *S. sclerotiorum* and *B.cinerea* in *N. benthamiana*. **Figure. S5.** BcIEB1 and RcCDI1 induced the accumulation of reactive oxygen species (ROS) in *N. benthamiana*. **Figure. S6.** Examination of *NbBAK1* expression in TRV: *GFP* and TRV: *NbBAK1* plants. **Figure. S7.** BcIEB1-induced expression of *NbCYP71D20* and *NbPTI5* were compromised in *Nbxlg4* and *Nbxlg3,5* mutants. **Figure. S8.** BcIEB1 has better protein stability than RcCDI1 at ambient temperature. **Figure. S9.** BcIEB1 induced a much stronger hypocotyl growth inhibition effect than ieb35 on *Arabidopsis* seedlings. **Figure. S10.** BcIEB1 induced a much stronger resistance to *P. capsici* than that of ieb35 in *N. benthamiana*. **Figure. S11.** Combined application of BcIEB1 with pyraclostrobin or metalaxyl-M greatly reduced plant susceptibility to *S. sclerotiorum*.

Additional file 2: Table S1. CDS sequences of all the elicitors. **Table S2.** Primer list used in this study.

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Author contributions

DD (Daolong Dou) and XL coordinated the research and wrote the paper. RC performed the majority of the experiments. DM, YB, and DD (Dandan Du) contribute to pathogen infection assays. WW contributed to plasmid constructions. XC contributed to the growth inhibition assays and improvement of the language.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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