Ergosterol-targeting fusion antifungal peptide significantly increases the Verticillium wilt resistance of cotton

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Summary
Increasing the targeting ability of antifungal proteins towards specific components of fungal cells has the potential to improve their antifungal activity and reduce harmful effects to nontarget cells. To obtain effective disease resistance genes against cotton Verticillium wilt, we constructed several fusion genes, in which binding domains targeting chitin, sphingolipid or ergosterol in the fungal cell wall or membrane were individually fused to the antifungal peptide BbAFP1 from entomopathogenic fungus Beauveria bassiana. Transient expression of fusion genes in cotton cotyledons indicated that the BbAFP1::ErBD fusion peptide with an ergosterol binding domain exhibited better disease resistance against V. dahliae than wild-type BbAFP1 and other fusion genes. BbAFP1::ErBD and BbAFP1 transgenic cotton were obtained and verified by Southern and Western blotting. Compared with BbAFP1-expressing cotton, BbAFP1::ErBD-expressing cotton showed higher disease resistance against V. dahliae, with smaller lesion areas (0.07 cm2 vs. 0.16 cm2) on the leaves and a lower disease index (23.9 vs. 34.5). Overexpression of BbAFP1::ErBD by transgenic tobacco also showed enhanced disease resistance against V. dahliae compared with that of the wild-type gene. These results indicated that construction of fusion antifungal peptides that target fungal cells is a powerful strategy to obtain new anti-disease genes, and the obtained fusion gene BbAFP1::ErBD has the potential to defend against plant fungal diseases.

Keywords: fusion antifungal peptide, binding domain, transgenic plants, disease resistance, Verticillium dahliae.

Introduction
Verticillium wilt disease, caused by soil-borne Verticillium spp., has been found in hundreds of dicotyledonous plant species, including many economically important crops (Fradin and Thomma, 2006; Klosterman et al., 2009; Zhang et al., 2016a). In cotton, V. dahliae can cause severe disease, and the disease symptoms may include wilting, chlorosis, stunting, necrosis, vein clearing and brown vascular discoloration (Cai et al., 2009; Fradin and Thomma, 2006; Sink and Grey, 1999). Verticillium wilt caused an average of 12% annual cotton production loss in the USA (Rajasekaran et al., 2005). In China, approximately half of the cotton fields are infected by Verticillium wilt annually (approximately 2.5 million hectares), which has caused direct economic losses of 250–310 million U.S. dollars (Li et al., 2015). Presently, there are no fungicides available to cure plants once they are infected, and few germplasms of upland cotton are resistant to V. dahliae (Wang et al., 2016; Zhang et al., 2017a; Zhang et al., 2015).

Transgenic engineering has achieved substantial success in cotton to improve insect resistance and fibre quality (Jin et al., 2018; Long et al., 2018; Ribeiro et al., 2017; Wei et al., 2015; Zhao et al., 2015). In insect-resistant cotton, a Bacillus thuringiensis (Bt) toxin gene was expressed effectively to eliminate the harm caused by cotton bollworm (Wu et al., 2008). In addition, cotton fibre quality was significantly improved through the precisely controlled production of auxin in fibre cells (Zhang et al., 2011). To increase disease resistance in cotton, resistance genes, such as chitinase, glucose oxidase, and genes involved in other signalling pathways, have been used (Gong et al., 2017; Jun et al., 2015; Murray et al., 1999; Song et al., 2018; Tohidfar et al., 2005; Yang et al., 2018). In these experiments, disease resistance towards V. dahliae was improved compared with that of wild-type; however, the increased resistance levels did not meet desired application needs, and in some cases, adverse effects were caused by the overexpressed gene. For example, a glucose oxidase gene from Talaromyces flavus was introduced into cotton. Transgenic cotton (with a root-specific construct) showed disease resistance against the root pathogen V. dahliae; however, the high-level expression of glucose oxidase in the cotton roots resulted in reduced height, seed set, seedling germination and lateral root formation (Murray et al., 1999). To date, transgenic cotton with broad-spectrum disease resistance has not been released, although many attempts have been made, and the lack of powerful resistance genes has been a possible factor hindering the progress of disease-resistant transgenic cotton.
The engineering of new AMPs (antimicrobial peptides) with desirable properties, such as specificity against pathogens, reduced toxicity against plant or animal cells, increased stability and/or improved action spectra (Marcos et al., 2008). For example, cecropins are well-characterized membrane-active antibacterial peptides but has shown toxicity to plant cells. Two cecropin analogs, SB-37 and Shiva-1, were engineered showing reduced toxicity to plant protoplasts (Jaynes et al., 1993; Nordeen et al., 1992). Increasing the ability of antimicrobial peptides to target cells is another effective strategy to enhance the antimicrobial activity. Peschen et al. (2004) constructed a fusion gene in which a Fusarium-specific antibody was fused to theAFP antifungal peptide from Aspergillus giganteus. Expression of the antibody fusion protein conferred A. thaliana and wheat high levels of resistance to Fusarium oxysporum, whereas plants expressing either the fungus-specific antibody or AFP alone exhibited only moderate resistance (Li et al., 2008; Peschen et al., 2004). Some specific components of the fungal cell wall and cell membrane, such as chitin in the cell wall and sphingolipid and ergosterol in the cell membrane, are also targets of antifungal treatments. We hypothesized that the increase in binding ability of antifungal peptides towards these components in the cell wall or cell membrane would improve their concentration around fungal cells, thereby improving the fungicidal activity. An antifungal peptide from the entomopathogenic fungus B. bassiana (BbAFP1, EJP62050.1) has been shown to display growth inhibitory activity against a number of phytopathogenic fungi, including F. oxysporum, M. oryzae, V. dahliae, B. cinerea and A. solani (Tong et al., 2020). Overexpression of BbAFP1 in tomatoes enhanced their disease resistance against B. cinerea and V. dahliae. No adverse effects on plant growth were observed in the transgenic tomatoes compared with nontransgenic plants (Tong et al., 2020). Here we have sought to increase the antifungal ability of BbAFP1 via construction of fusions with several different targeting domains, including those targeting binding to chitin, sphingolipids and ergosterol, termed ChBD, SpBD and ErBD, respectively (Broekaert et al., 1992; De Lucca et al., 1998; Thevissen et al., 2003; Thevissen et al., 2000). Among these binding domains, ErBD (originally named D4E1) is an artificial synthetic peptide that has been shown to be able to target ergosterol in fungal cells and shows direct fungicidal properties (De Lucca et al., 1998). Transient expression in cotton cotyledons indicated that BbAFP1 containing an ergosterol-targeting domain (ErBD) resulted in increased resistance against V. dahliae than the other constructs tested. Transgenic cotton and tobacco lines expressing BbAFP1::ErBD conferred higher resistance (than BbAFP1 alone) to plant pathogenic fungi. These data demonstrated that this targeted improvement strategy is feasible and can be applied to disease resistance genetic engineering in different plants.

Results

Construction of hybrid antifungal peptides with various tags targeting the fungal cell wall or cell membrane

BbAFP1 is an antifungal peptide from B. bassiana that shows antifungal activity against phytopathogenic fungi (Tong et al., 2020). To further improve the antifungal activity of BbAFP1, we constructed several fusion BbAFP1s with different tags that can bind to various targets in fungal cells, including the ChBD (30 aa) from Ac-AMP2 that binds chitin, the SpBD (50 aa) from DmAMP1 that binds sphingolipid and the ErBD (original name D4E1, 17 aa) that binds ergosterol (Figure 1a and Table S1). To evaluate the effects of adding various binding domains to BbAFP1, we attempted to express the fusion AFPs in Pichia pastoris. Like the wild-type BbAFP1, the BbAFP1::ChBD and BbAFP1::SpBD fusion peptides were successfully produced in P. pastoris; however, we were unable to obtain the BbAFP1::ErBD fusion peptide despite many attempts and optimized culture conditions (Figure 1b). Using an immunofluorescent probe, we found that BbAFP1::ErBD localized to the cell envelope of P. pastoris cells and was hard to elute (Figure 1c). To evaluate the disease resistance ability of the fusion genes, they were transiently expressed in cotton cotyledons. After inoculation with V. dahliae for 10 days, cotton cotyledons without antifungal peptide expression exhibited obvious disease symptoms including yellow and wilted cotyledons. In contrast, cotyledons expressing BbAFP1 had less extensive disease symptoms, and BbAFP1::ErBD expression resulted in the highest resistance level, with the following resistance order being exhibited: BbAFP1::ErBD > BbAFP1::SpBD > BbAFP1::ChBD = BbAFP1 (Figure 1d). Among cotyledons expressing BbAFP1::ErBD, those with grades of 0 and 1 accounted for 78%, which was significantly higher than that observed in those expressing BbAFP1::SpBD (65%) or BbAFP1 (40%) (Figure 1e). Real-time PCR analysis indicated that the biomass of V. dahliae in BbAFP1::ErBD-expressing cotton cotyledons was significantly less than those in cotyledons expressing the other AFP genes (Figure 1f).

The BbAFP1::ErBD fusion peptide exhibited higher disease resistance than ErBD or BbAFP1 alone

To further evaluate whether the fusion gene BbAFP1::ErBD possesses higher antifungal activity than ErBD or BbAFP1 alone, we transiently expressed the individual peptides in cotton cotyledons and tobacco leaves. Although BbAFP1 and ErBD also conferred Verticillium wilt resistance in cotton cotyledons, the resistance level was significantly lower than that provided by the BbAFP1::ErBD fusion gene (Figure 2a). Compared to the disease indexes for BbAFP1 and ErBD, the disease index in cotton cotyledons expressing BbAFP1::ErBD decreased by 45.7% or 31.4%, respectively, after inoculation with V. dahliae for 10 days (Figure 2b). Similar results were obtained by transient expression in tobacco leaves (Figure 2c). Inoculation of B. cinerea onto leaves with BbAFP1::ErBD expression resulted in a 0.33 cm² lesion area, which was approximately 36% smaller than the lesions observed in tobacco leaves expressing BbAFP1 or ErBD (<0.52 cm²) (Figure 2d). These results indicated BbAFP1::ErBD fusion peptide has higher disease resistance ability than ErBD or BbAFP1 alone, which potentially resulted from an increased targeting ability of BbAFP1 towards fungal cells and accumulative antifungal effects from both BbAFP1 and ErBD.

Generation and selection of transgenic plants overexpressing BbAFP1 or BbAFP1::ErBD

To determine the disease resistance of BbAFP1 and BbAFP1:: ErBD in transgenic plants, we introduced these genes into cotton and tobacco, as described in the Methods. Transgenic plant lines were verified by GUS staining and PCR amplification. The expression levels of BbAFP1 and BbAFP1::ErBD were verified by real-time PCR and Western blotting (Figure 3). Compared with wild-type BbAFP1, BbAFP1::ErBD has an additional 17-AA ErBD domain, which causes the fusion protein to migrate slower.
than wild-type BbAFP1 on an SDS-PAGE gel. Several transgenic lines showing similar expression levels were selected for further analysis (BbAFP1-3/35/71 and BbAFP1::ErBD-37/53/61 in transgenic cotton and BbAFP1-13/19 and BbAFP1::ErBD-7/22 in transgenic tobacco). Southern blotting results verified that the six selected lines were independent transformation events (Figure S1a).

Resistance analysis of transgenic cotton

Resistance analysis of the six transgenic cotton lines (T0) selected above (based on real-time PCR) was performed by inoculating V. dahliae onto detached leaves. At 5 days postinoculation, the lesion areas on BbAFP1::ErBD transgenic leaves were significantly smaller than those on BbAFP1-expressing cotton leaves (0.03–
0.10 cm² vs. 0.11–0.18 cm²) (Figure S1b,c), which preliminarily indicated that BbAFP1::ErBD conferred higher V. dahliae resistance to cotton than BbAFP1. To test whether the disease resistance could be stably inherited, T2 generation transgenic cotton plants were obtained through continuous self-pollination of the original transformants. The disease resistance of T2 generation transgenic cotton lines (BbAFP1::ErBD-61 and BbAFP1-35) was first evaluated by inoculating the fungal pathogens V. dahliae and B. cinerea onto detached leaves. At 5 days postinoculation, V. dahliae caused a lesion area of approximately 0.5 cm² on wild-type leaves, while BbAFP1-expressing leaves had a lesion area of only approximately 0.16 cm². Compared to the BbAFP1 plant lesion area, the lesion area caused by V. dahliae on cotton leaves overexpressing BbAFP1::ErBD was decreased significantly by 56% (0.07 cm² vs. 0.16 cm²) (Figure 4a,b). BbAFP1::ErBD also conferred higher resistance levels to cotton fungal pathogens than BbAFP1 or ErBD.

Figure 2 Comparison of the disease resistance of the transient expression of the BbAFP1::ErBD hybrid gene, BbAFP1 and ErBD. (a) Disease symptoms of cotton cotyledons expressing antifungal peptides towards V. dahliae at 10 days postinoculation. (b) Disease indexes were calculated from cotton cotyledons at 10 days post-V. dahliae inoculation. (c) Disease symptoms of tobacco leaves expressing antifungal peptides towards B. cinerea at 4 days postinoculation. (d) Lesion areas of tobacco leaves at 4 days post-B. cinerea inoculation were measured using ImageJ software. Mock, inoculation with water. Error bars represent the standard deviation of three replicates. Asterisks indicate statistically significant differences, as determined by Student’s t-test (**P < 0.01, n > 30 cotton plants or tobacco leaves). All of the results indicated that the BbAFP1::ErBD hybrid peptide conferred higher resistance levels to cotton fungal pathogens than BbAFP1 or ErBD.

Figure 3 Overexpression of BbAFP1 and BbAFP1::ErBD in cotton and tobacco. (a) Schematic representation of the pLGN-BbAFP1 and pLGN-BbAFP1::ErBD expression vectors. RB and LB are the right and left borders of the T-DNA region, respectively. NOS-T, NOS terminator; 35S, CaMV 35S promoter. The expression levels of the antifungal peptide genes were analysed by real-time PCR after reverse transcription and Western blotting in transgenic cotton (b) and tobacco (c). For Western blotting analyses, an antibody against BbAFP1 was used.
transgenic cotton plants, with the latter BbAFP1::ErBD-61 V. dahliae with BbAFP1-35 while no or only slight symptoms appeared in on wild-type plants, including defoliation, necrosis and wilting, postinoculation, typical symptoms of Verticillium wilt appeared than BbAFP1 and BbAFP1::ErBD-61 area and almost no browning were observed in the straight-cut wild-type shoots. In contrast, a smaller browning postinoculation, obvious browning of the xylem was observed in appearing to have symptoms of wilt (Figure 4e). At 15 days inoculation, obvious browning of the xylem was observed in the straight-cut wild-type shoots. In contrast, a smaller browning area and almost no browning were observed in the BbAFP1-35 and BbAFP1::ErBD-61 cotton, respectively (Figure 4f), indicating that BbAFP1::ErBD conferred stronger resistance to V. dahliae than BbAFP1.

Wild-type and transgenic cotton plants were also inoculated with V. dahliae using the root dipping method. At 15 days postinoculation, typical symptoms of Verticillium wilt appeared on wild-type plants, including defoliation, necrosis and wilting, while no or only slight symptoms appeared in BbAFP1-35 or BbAFP1::ErBD-61 transgenic cotton plants, with the latter showing a better level of resistance (Figure 5a). The disease indexes of the wild-type, BbAFP1-35 and BbAFP1::ErBD-61 plants were 81.25, 34.52 and 23.91, respectively (Figure 5b). To detect hyphae in V. dahliae-infected cotton leaves, lactophenol-trypsin blue staining was performed on the leaves of diseased plants. The results showed that BbAFP1::ErBD-61 transgenic cotton leaves had the smallest stained area, which was approximately 30% and 50% of the stained areas in nontransgenic and BbAFP1-expressing leaves, respectively (Figure 5c). Moreover, the xylem of BbAFP1-35 transgenic cotton stems exhibited more severe browning than BbAFP1::ErBD-61, while wild-type stems showed the darkest coloration (Figure 5d). We also inoculated transgenic plants with a low concentration of fungal conidia (one third of previously used, 3 × 10^5 vs. 10^6 conidia/mL). Under this condition, V. dahliae still resulted in severe disease symptoms in wild-type cotton at 30 days postinoculation, and almost all plants died at 60 days postinoculation. However, BbAFP1-35 and BbAFP1:: ErBD-61 transgenic cotton grew well under this inoculation amount, and the latter showed larger biomass (Figure S3).

The mechanism underlying improved disease resistance ability of BbAFP1::ErBD

The binding of BbAFP1::ErBD and BbAFP1 towards V. dahliae cells was analysed by immunofluorescence method. Total proteins of plant root were extracted and used for the binding assay. After V. dahliae cells were treated for 1 h, the fluorescent signal for BbAFP1 was found to mainly localize in the cell surface of V. dahliae, while the signal for BbAFP1::ErBD was concentrated

![Figure 4](image_url) Disease resistance analysis of detached leaves or shoots from T2 generation transgenic cotton. (a) Disease symptoms of cotton leaves inoculated with V. dahliae. (b) Lesion areas of cotton leaves inoculated with V. dahliae. (c) Disease symptoms of cotton leaves inoculated with B. cinerea. (d) Lesion areas of cotton leaves inoculated with B. cinerea. The phytopathogenic fungi V. dahliae and B. cinerea were individually inoculated onto cotton leaves at points where three small pinholes were created using the needle of the syringe, and the inoculated leaves were kept for 5 days at 26 °C. Lesion areas were measured using ImageJ software. (e) Disease symptoms of cotton shoots at 3 days post-V. dahliae inoculation. (f) Longitudinal sections of cotton shoots were photographed at 15 days postinoculation. Mock, inoculation with water; WT, wild-type cotton; BbAFP1, BbAFP1-35 transgenic cotton line; BbAFP1:: ErBD, BbAFP1::ErBD-61 transgenic cotton line. Error bars represent the standard deviation of three replicates. Asterisks indicate statistically significant differences, as determined by Student’s t-test (**P < 0.01). More than 20 cotton leaves or 15 individual cotton shoots were used for inoculations in each experiment.
Engineering of an antifungal peptide

on some specific patches in the cell envelope and exhibited stronger intensity than BbAFP1 (Figure 6a). In addition, more fluorescent signals were observed inside the BbAFP1::ErBD-treated fungal cells (Figure 6a). Propidium iodide (PI) staining verified that more V. dahliae conidia were disrupted after treatment with the total protein from BbAFP1::ErBD transgenic cotton than after treatment with BbAFP1 (Figure 6b). Fungal growth in transgenic plants was observed by inoculating a GFP-expressing V. dahliae strain V991-GFP. At 6 days postinoculation, a large number of fluorescent hyphae were found in wild-type cotton roots; however, fewer hyphae were observed in BbAFP1::ErBD-61 transgenic cotton (Figure 6c). These results indicated that ErBD improved the ability of BbAFP1 to bind fungal cells and the fusion peptide exhibited greater fungicidal activity than that of wild-type BbAFP1.

The expression of several plant endogenous PR genes, including GhPR1, GhPR2 (β-1,3-glucanase gene), GhPR3 (chitinase gene) and GhPR5, was detected in transgenic cotton by real-time PCR before and after V. dahliae inoculation. The results showed that BbAFP1 and BbAFP1::ErBD had no significant effects on the expression of these PR genes with or without fungal inoculation (Figure S4). Phenotypic analysis showed that BbAFP1 and BbAFP1::ErBD had no negative impact on the growth and development of transgenic cotton (Figure S5).

BbAFP1::ErBD confers high disease resistance to V. dahliae in tobacco

We further evaluated the disease resistance of BbAFP1::ErBD in transgenic tobacco. After V. dahliae was inoculated onto tobacco leaves for 15 days, the leaves of nontransgenic plants became yellow. In contrast, the BbAFP1-expressing tobacco exhibited high disease resistance with a few yellow patches in leaves, and even higher disease resistance was observed in BbAFP1::ErBD-expressing tobacco, which showed only small patches of yellow or no obvious symptoms (Figure 7a). The disease indexes of BbAFP1::ErBD-7 and BbAFP1::ErBD-22 were 25.2 and 22.4, respectively, which were significantly lower than those of BbAFP1-13 (40.2), BbAFP1-19 (36.8), and the wild-type (76.3) (Figure 7b), demonstrating that the constitutive expression of BbAFP1::ErBD also conferred higher disease resistance in tobacco to V. dahliae than BbAFP1.

Discussion

Transgenic strategies have the potential to improve cotton disease resistance; however, only a few genes have been reported to show antifungal activity in this crop. In the present study, we constructed three fusion genes containing binding domains with predicted binding ability towards chitin in the fungal cell wall and sphingolipid and ergosterol in the cell membrane, respectively (Broekaert et al., 1992; De Lucca et al., 1998; Thevissen et al., 2003). Disease resistance analyses, including transient expression of antifungal genes in cotton cotyledons and in transgenic cotton or tobacco, indicated that the fusion gene BbAFP1::ErBD with the ergosterol binding domain exhibited higher resistance to Verticillium wilt than wild-type BbAFP1. This fusion gene increases the resistance gene reservoir that can be used for constructing transgenic plants with improved disease resistance.

Ergosterol is the major membrane sterol in fungi that regulates membrane fluidity, plasma membrane biogenesis and function (Zhang and Rao, 2010). Due to the importance of ergosterol on fungal cell membrane functions, it is a vital target for some fungicidal treatments. ErBD (originally named D4E1) is an artificial synthetic peptide based on the cecropin B peptide toxin from Cecropia, and it consists of 17 amino acids: FKLRAKVKRLRAKIKL. Physicochemical analysis indicated that ErBD complexes with ergosterol in fungal cells and shows fungicidal properties against a diverse group of fungi (De Lucca et al., 1998). Although ErBD or BbAFP1 alone exhibited antifungal activity, the antifungal ability of the fusion peptide containing both was higher. We attempted to produce these fusion peptides in P. pastoris and analyse their
protein properties and detailed action mechanism. The BbAFP1 and fusion peptides BbAFP1::ChBD/SpBD were successfully produced in P. pastoris. Unfortunately, we were unable to obtain sufficient active BbAFP1::ErBD for further functional analysis. BbAFP1::ErBD was found bound to the yeast cell membrane by immunofluorescent analysis and was difficult to elute. We performed the binding assay of BbAFP1::ErBD to fungal cells using an immunofluorescence assay with the total proteins
extracted from transgenic plants. The results indicated that more fusion peptide was bound to V. dahliae, with some concentrated in specific sites of the cell envelope. The increased ability of BbAFP1::ErBD to destroy cell membranes was verified by PI staining. Based on these results, we could conclude that the addition of ErBD increased the ability of BbAFP1 to target fungal cells. However, considering the antifungal activity of ErBD, the higher resistance to Verticillium wilt in cotton and tobacco plants conferred by BbAFP1::ErBD might derive from at least two aspects: the higher concentration of antifungal peptide (BbAFP1) on fungal cells and the additive antifungal effects from BbAFP1 and ErBD. In addition, although providing less resistance than BbAFP1::ErBD, the fusion genes BbAFP1::SpBD, which contains the sphingolipid binding domain, also exhibited greater resistance than wild-type BbAFP1. The protein properties of this fusion gene deserve further study in future work.

Chitin is one of the main components in the fungal cell wall, which plays an important role in maintaining the cell wall integrity. We originally hypothesized that addition of a chitin-binding domain to BbAFP1 would increase the concentration of antifungal peptides in fungal cells, thus improving the fungicidal activity. However, our results indicated that ChBD addition to BbAFP1 did not improve its disease resistance. In our previous study, the chitin-binding ability of BbAFP1 was confirmed, and a putative chitin-binding domain (CKFKGQ) was identified in the amino acid sequences. Mutation of phenylalanine in this domain into alanine affected the binding ability of BbAFP1 to chitin and the fungal cell wall and significantly decreased antifungal activity (Tong et al., 2020). Therefore, as BbAFP1 possesses chitin-binding ability itself, adding an additional chitin-binding domain to BbAFP1 cannot further increase the fungal cell targeting and activity of BbAFP1.

It is notable that compared with nontransgenic plants, transgenic cotton and tobacco plants expressing wild-type BbAFP1 exhibited disease resistance against Verticillium wilt, although this effect was less pronounced than that provided by the fusion gene BbAFP1::ErBD. BbAFP1 derives from B. bassiana, which has been widely used for the biocontrol of agricultural, forest and urban pests (Ortiz-Urquiza and Keyhani, 2013). In addition to parasitizing the insect host, B. bassiana can also antagonize phytopathogenic growth and provides protection for plants when applied to the seeds of tomatoes and cotton (Owensley et al., 2008). Although the detailed mechanisms are unknown, several genes with disease resistance have been isolated from B. bassiana. A chitinase from B. bassiana, Bbchit1, exhibits antifungal activity, and expression of the encoding gene in Chinese white poplar (Populus tomentosa Carr.) increases disease resistance to Cytospora chrysosperma (Jia et al., 2010). These results suggest that B. bassiana may be a good source to explore plant disease resistance genes.

The results presented in this study indicate that using protein engineering to increase antifungal activity or target cell binding ability of AFPs is a powerful technique to obtain new disease resistance genes. The BbAFP1::ErBD fusion gene is an effective antifungal peptide gene that can be used to improve resistance to Verticillium wilt in cotton. In addition, this fusion gene also has the potential to increase disease resistance in other plants.

**Methods**

**Strains and media**

V. dahliae and B. cinerea used for inoculation were routinely grown in/on potato dextrose broth/agar (PDB/PDA) at 26 °C. E. coli DH5α used for gene cloning and vector construction was grown in/on LB broth/agar at 37 °C. P. pastoris GS115 used for the heterologous expression of antifungal peptides was grown in/on YPD/YPDA (yeast extract/peptone/dextrose) at 30 °C.

**Plant material and growth conditions**

Gossypium hirsutum L. cv. Jimian14 used in this study was kindly provided by Hebei Agricultural University, China. Nicotiana benthamiana (for transient expression) and Nicotiana tabacum (for genetic transformation) used in these experiments were preserved in our laboratory. Transgenic cotton and tobacco were grown in a greenhouse under a photoperiod of 14 h light and 10 h dark at 28–34 °C during the day and 24–27 °C at night.

**Protein expression and purification**

Hybrid antifungal peptide genes with various tag sequences were artificially synthesized (GeneCreate, Wuhan, China) and inserted into pUC57 cloning vectors. For the heterologous expression of BbAFP1 and hybrid antifungal peptides, sequences with flanking EcoRI and NotI restriction sites were amplified by PCR using the synthetic sequences as templates and cloned into pPIC9K for transformation and expression in P. pastoris. The primer pairs used for vector construction are shown in Table S2. To facilitate purification, a 6 × His tag was fused at the C-termini of the antifungal peptides. P. pastoris transformation and colony screening were performed according to the manufacturer’s recommendations (Invitrogen, Carlsbad, California). Protein expression and purification were carried out as described in our previous study (Fan et al., 2007). In addition, the individual ErBD was synthesized by GeneCreate.

**Immunofluorescence localization assay**

The immunofluorescence localization in P. pastoris was performed as previously described (Wang et al., 2018). Briefly, P. pastoris strains induced with methanol for 4 days were incubated with normal goat serum (final concentration of 5%, Solarbio, Beijing, China) at 4 °C overnight. After washing three times with PBS (10 mM, pH 6.0), the samples were incubated with anti-BbAFP1 rabbit antibody (1:1000 (v/v) dilution) for 3 h and then incubated with FITC-conjugated goat anti-rabbit IgG (1:500 (v/v) dilution, Proteintech, Chicago, Illinois) for 2 h. The green fluorescence on the cell surfaces of P. pastoris was visualized using confocal microscopy (FV1000; Olympus, Tokyo, Japan).

**Expression of BbAFP1 and BbAFP1::ErBD in cotton**

To express BbAFP1 and BbAFP1::ErBD in cotton, the synthetic sequences cleaved from pUC57-BbAFP1 and pUC57-BbAFP1::ErBD by EcoRI/BamHI were cloned into the plGN vector with the constitutive CaMV 35S promoter and a terminator sequence derived from the nopaline synthase gene (NOS) (Zeng et al., 2019). The plant expression constructs were transformed into cotton using A. tumefaciens strain LBA4404 (Luo et al., 2007). The expression levels of BbAFP1 and BbAFP1::ErBD were monitored by real-time PCR with the cotton GhHis gene (Table S2) as the reference as previously described (Yan et al., 2018). The total RNA was extracted using the EASYspin rapid extraction kit (Biomed, Beijing, China) and converted to cDNA using the NovoScript Plus All-in-one 1St Strand cDNA Synthesis SuperMix (gDNA Purge) (Novoprotein, Shanghai, China). Southern blotting analysis was conducted as previously described (Zhang et al., 2016b). Thirty micrograms of DNA from the leaves of wild-type and transgenic plants were digested with HindIII. Probes were
prepared from the purified PCR products of the NPTII gene. The labelling of probes, hybridization and detection were performed according to the protocol supplied with the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche, Mannheim, Germany). The primer pairs used for Southern blotting are shown in Table S2. Protein production in transgenic cotton was confirmed by Western blotting using anti-BbAFP1 as the primary antibody and goat anti-rabbit IgG (Thermo, Waltham, Massachusetts) as the secondary antibody. Total proteins were extracted as previously described (Zhang et al., 2016b). For immunofluorescence localization analysis, *V. dahliae* conidia were preincubated at 26 °C for 12 h in PDB. After washing two times with PBS (10 mM, pH 6.0), the samples were incubated with 1 mg/mL cotton total proteins for 1 h at 26 °C and then used for the immunofluorescence experiment as described above. The PI staining of *V. dahliae* conidia was performed as described (Zhou et al., 2018).

The disease resistance of transgenic cotton was assessed using detached leaf bioassays as previously described (Gao et al., 2015). In brief, small holes were created in the leaves with a syringe needle, and 5 μL of a conidial suspension of *V. dahliae* (10^2 conidia/mL) or a plug (5 mm in diameter) from *B. cinerea* were inoculated onto the leaves. Lesion areas were measured with ImageJ software at 5 days postinoculation. The *V. dahliae* infection assays using the root dipping method were performed with a conidial concentration of 3 × 10^5 or 1 × 10^6 conidia/mL as previously described (Zhang et al., 2017b). The disease index (DI) was calculated according to the following formula: DI = \[ \frac{\sum_{n}^{\text{disease grades × number of infected plants}}}{\text{total checked plants × 4}} \] × 100 (Fang, 1998), and the disease grades were classified as previously described (Xu et al., 2012). Leaves infected with fungi were stained with lactophenol-trypsin blue by vacuum infiltration for 3 min and then destained overnight with chloral hydrate (2.5 g/mL) as previously described (Gao et al., 2013). To observe the growth of *V. dahliae* in plants, cotton seedlings were inoculated with a GFP-expressing strain V991-GFP as previously described (Zhao et al., 2014). Briefly, cotton seedlings with only a taproot were dipped into the conidial suspension of V991-GFP strain (10^6 conidia/mL) for 24 h and then transferred to water for 5 days. The cross sections of taproots were stained with FM4-64, and fungal hyphae with GFP fluorescence were observed under a confocal microscope.

**Expression of BbAFP1 and BbAFP1::ErBD in tobacco**

To express *BbAFP1* and *BbAFP1::ErBD* in tobacco, the constructs used in transgenic cotton were transformed into tobacco using *A. tumefaciens* strain LBA4404 (Liu et al., 2013). The expression of *BbAFP1* and *BbAFP1::ErBD* was analysed by real-time PCR with the tobacco NTEF-1x gene (Table S2) as the reference as well as by Western blotting using plant extracts. The disease resistance of transgenic tobacco was also assessed using detached leaf bioassays, which were different from the assays described above. Briefly, the petioles of the tobacco leaves were wrapped in a piece of absorbent paper, and a 500 μL conidial suspension (3 × 10^7 conidia/mL) was added to the absorbent paper. Then, the treated leaves were placed in a plastic basket and covered with transparent plastic film to maintain a high relative humidity. After treatment for 15 days, the disease grades of the infected leaves were calculated.

**Transient expression in planta**

Transient expression of the antifungal genes in *N. benthamiana* leaves (30-day-old) or cotton cotyledons (10-day-old) was performed with *A. tumefaciens* GV3101 strains carrying the related constructs (Gao et al., 2019; Zhou et al., 2012). In brief, small pinholes were created in the tobacco leaves or cotton cotyledons using a syringe needle, and then, the resuspended *A. tumefaciens* cultures (OD_600 = 1.0) were infiltrated into the leaves/cotyledon with a 1-ml syringe without a needle. One day post-treatment, *V. dahliae* was inoculated onto cotton cotyledons, and *B. cinerea* was inoculated onto tobacco leaves. The relative amount of *V. dahliae* was detected by real-time PCR as described previously (Ellendorff et al., 2009) using the total genomic DNA extracted from cotton cotyledons at 10 days postinoculation as a template. The reference gene primers used were PGHHis-1 and PGHHis-2, and the fungal-specific primers used were IT51-F and ST-VE1-R (Table S2).

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**Conflicts of interest**

The authors declare no conflicts of interest.

**Author contributions**

YF and ST designed the research; ST, MY, DL, DJ, HL, DY, AM and YW performed the research; YF, YL, XC and YP analysed the data; ST wrote the original draft; and YF wrote, reviewed and edited the paper.

**Data availability statement**

All relevant data are within the manuscript and its Supporting Information files.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** Disease resistance analysis of detached leaves from T0 generation transgenic cotton lines. (a) Southern blotting analysis of *BbAFP1* or *BbAFP1::ErBD* insertions in transgenic lines. Genomic DNA was digested with *Hin*dIII and hybridized with a 740 bp *NPTII* fragment. P: positive control (2650 bp fragment including the *NPTII* gene from pLGN). Disease symptoms (b) and lesion area statistics (c) of detached leaves from different transgenic cotton lines at 5 days post-*V. dahliae* inoculation. *V. dahliae* was inoculated at sites on cotton leaves in which three small pinholes were created using the needle of a syringe, and the inoculated leaves were kept for 5 days at 26 °C. Lesion areas were measured using ImageJ software. Error bars represent the standard deviation of three replicates, and more than 20 cotton leaves were used for inoculation in each experiment.

**Figure S2** Trypan blue staining of *B. cinerea* hyphae on T2 generation cotton leaves at 5 days postinoculation. The experiment was performed in triplicate and more than 6 stained leaves were used for observation in each experiment. Scale bars: 100 μm.

**Figure S3** Disease resistance analysis in cotton plants after *V. dahliae* inoculation. (a) Disease grades of cotton plants at 30 days post-*V. dahliae* inoculation. (b) Disease grades of cotton plants at 60 days post-*V. dahliae* inoculation. (c) Disease symptoms of cotton plants at 60 days post-*V. dahliae* inoculation. The concentration of *V. dahliae* used for inoculation above was 3 × 10⁵ conidia/mL. WT, wild-type cotton; *BbAFP1*, transgenic cotton line *BbAFP1*-35; *BbAFP1::ErBD*, transgenic cotton line *BbAFP1::ErBD*-61. All experiments were performed in duplicate, and more than 30 corresponding cotton plants were used in each experiment, respectively.

**Figure S4** Detection of PR gene expression levels in cotton after inoculation with *V. dahliae*. The total RNAs were extracted from roots at 0, 1 and 3 days postinoculation. Expression levels were determined by real-time PCR using *GhHis* as the internal reference gene. Error bars represent the standard deviation of three replicates.

**Figure S5** Phenotypic analysis of wild-type and transgenic cottons. *BbAFP1* and *BbAFP1::ErBD* have no negative impact on the growth and development of transgenic cottons.

**Table S1** Information about the three binding domains.

**Table S2** Primers used in this study.