Chitinase-binding gene BvCHiB enhances resistance of Arabidopsis to verticillium wilt through JA and SA pathways

Yujing Liu

School of advanced manufacturing /School of ocean, Fuzhou University, 362251, China

Abstract. To date, no ideal effective method for controlling Verticillium wilt in upland cotton (*Gossypium hirsutum*) has been defined. There are few studies on *BvCHiB* gene from *B. velezensis* to improve plant disease resistance mechanism. The purpose of this study was to determine the effects and mechanism through which *BvCHiB* enhances plant disease resistance, and increases resistance to Verticillium wilt. The *BvCHiB* gene was cloned from an endophytic bacterium (*B. velezensis*) isolated from roots of the upland cotton cultivar Zhongzhimian 2. Transgenic *Arabidopsis* plants overexpressing BvCHiB showed significantly improved resistance to Verticillium wilt. The accumulation of resistance related substances such as lignin, PAL, POD, SOD, CAT and MDA increased significantly 48h after inoculation with verticillium wilt. These immune resistant substances can effectively resist the infection of verticillium wilt. In addition, the expression of related resistance genes in JA and SA pathway was significantly higher than that in control. Therefore, *BvCHiB* may enhance disease resistance of plants mainly through JA and SA signaling pathway. *BvCHiB* may be used as a resistance gene to improve resistance to *V. dahliae* in upland cotton.

Keywords: *B. velezensis*, upland cotton, disease resistance, *Verticillium dahliae*.

Abbreviations:

ETI, effector-triggered immunity; H_2O_2, hydrogen peroxide; JA, jasmonic acid; PAL, phenylalanine ammonia lyase; POD, peroxidase; PPO, polyphenol oxidase; PTI, pattern-triggered immunity; qRT–PCR, quantitative reverse transcription PCR; ROS, reactive oxygen species; SA, salicylic acid.

1. Introduction

Upland cotton (*Gossypium hirsutum*) is an oil and fiber crop with worldwide importance. Verticillium wilt seriously harms upland cotton production (Oktay et al., 2006; Zhang et al., 2013). Because *Verticillium dahliae* can form microsclerotia and exist in the soil for a long time, it is difficult to control Verticillium wilt in cotton. Breeding of disease resistant cotton varieties is limited due to a lack of upland cotton resources with immunity or high resistance to Verticillium wilt (Yang et al., 2007). Biological control is the most promising environmental strategy for controlling Verticillium wilt in cotton (Erdogan and Benlioglu, 2009; Liu et al., 2013). Biocontrol fungi such as *Trichoderma spp*. (Hanson, 2000), biocontrol bacteria such as *Bacillus spp*., *Pseudomonas spp*., and several endophytic bacteria (Erdogan and Benlioglu, 2009; Han et al., 2015; Li et al., 2012; Mansoori et al., 2013) are widely used for controlling Verticillium wilt. The products extracted from microorganisms can be used as an ideal substitute for chemically synthesized antifungal agents to improve the quality of agricultural products. Peptides and proteins extracted from microorganisms, such as iturins, fengycin and chitinase, β-1,3-glucanase can effectively inhibit the growth of *V. dahliae* (Zeng et al., 2018; Han et al., 2015; Gomaa, 2012; Guo et al., 2013; Sattarova and Konnova, 2000). Chitinases, which have the ability to digest chitin into N-acetylglucosamine, are produced by a wide range of organisms and can be potentially used in industry, medicines, scientific research, and agriculture (Khoushab and Yamabhai, 2010). *Bacillus thuringiensis* (Bt) also produces chitinases (Thamthiankul et al., 2001). Several studies have reported that chitinases generated by *Bacillus thuringiensis* are involved in its antifungal activity and can enhance the insecticidal activity of Bt strains (Gomaa, 2012). Many studies have reported the expression and application of chitinases in microorganisms (Bhattacharya et al., 2007). However, reports on the regulation mechanism of chitinase genes

improving resistance to *V. dahliae* are scarce. In this research, we found that the crude protein produced by B. velezensis fermentation broth has an inhibitory effect on V. dahliae strain Vd080. We identified the crude protein and obtained BvCHiB gene. The BvCHiB gene was isolated from the genomic DNA of B. velezensis, the BvCHiB transgene in Arabidopsis can induce the expression of key genes involved in the JA signaling pathways to improve plant disease resistance. Therefore, our research not only revealed a possible use of a new BvCHiB gene but also the effect and mechanism of BvCHiB improving resistance to *V. dahliae* in upland cotton.

2. Materials and methods

2.1. Plant materials, culture conditions, and treatments

Seeds of Arabidopsis thaliana were sterilized in 75% ethanol and 3% NaClO for 30min and then washed three times with sterilized water. The Arabidopsis seeds were sown on 1/2-strength Murashige and Skoog (MS) medium containing 3% sucrose and 0.8% (w/v) agar for 2weeks. Once they reached the three-leaf stage, the Arabidopsis seedlings were transplanted into nutrient-enriched soil and cultivated in a growth chamber at 22/20°C (night/day) with 60% relative humidity and a 16-h day/8-h night photoperiod. The roots of Arabidopsis thaliana plants were inoculated via irrigation with spore suspensions adjusted to a concentration of 1×10^7 spores/ml (Feng et al., 2021).

2.2. Measurement of lignin, phenylalanine ammonia lyase, polyphenol oxidase, peroxidase, catalase activities, superoxide dismutase and malondialdehyde

Forty-eight hours after the Arabidopsis thaliana were inoculated with Vd080, The Leaves of each group were selected to detect the content of lignin according to the method descried by Xiong et al. (2021), and SOD activity was measured using the procedure descried by Hou et al (2022). PAL activity was measured using the method described by Chen et al. (2009), and MDA content was measured as described by Ma et al. (2019). POD activity was measured using the method described by Hou et al (2022), and CAT activity was measured as described by Hou et al (2022). Three independent biological and technical repeats were performed.

2.3. Measurement of resistance-related gene expression by quantitative reverse transcription PCR

qRT-PCR was carried out with the SYBR Green PCR Master Mix system. Three biological duplications were performed with independently isolated RNA in all qRT-PCR. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Jin et al., 2019).

2.4. Arabidopsis transformation

The open reading frame (ORF) of BvCHiB was cloned and inserted into a pBI121 (Cambia) plant binary vector together with the 35S promoter. The resulting pBI121-BvCHiB plasmid was introduced into Agrobacterium tumefaciens strain GV3101. The transformants (T0, T1, and T2 seeds) were screened for survival on half-strength MS medium supplemented with 50mg/L kanamycin. T3 transgenic lines were used to identify disease resistance.

2.5. Statistical analysis

All the experiments used to obtain the data described in this paper were repeated at least three times. The resulting data were subjected to ANOVA using SPSS 19.0 software (SPSS, Inc.). Student's t-test and one-way ANOVA ($p<0.05$) followed by

Duncan tests were used for multiple comparisons.

3. Results and discussion

3.1. Mass spectrum of BvCHiB inhibitory protein from Bacillus Velezensis

In order to systematically analyze the bacteriostatic active components of Bacillus Velezensis crude protein, the protein of Bacillus Velezensis was identified by LC-MS/MS analysis with in-line nappen ion source. The peptides were isolated and identified by QExactive HF mass spectrometer technique in series EASY-nanoLC 1200, and annotated with mass spectrometry database information. B. velezensis antibacterial substance was found to contain BvCHiB protein (Fig.1).

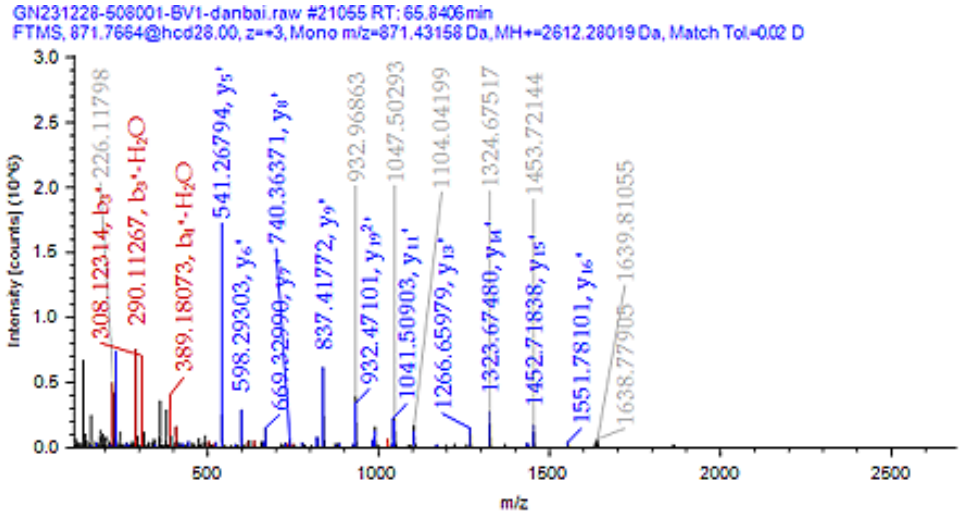

Figure 1. Mass spectrum of BvCHiB inhibitory protein from Bacillus Velezensis

3.2. Three-dimensional structure and phylogenetic tree of BvCHiB

In order to further analyze the three-dimensional structure of BvCHiB and its evolutionary relationships among different species, SWiSS-Model and MEGA5.0 were used to construct the three-dimensional structure and phylogenetic tree of BvCHiB. It was found that BvCHiB protein was closely related to Bacillus siamensis and far related to other species (Fig.2)

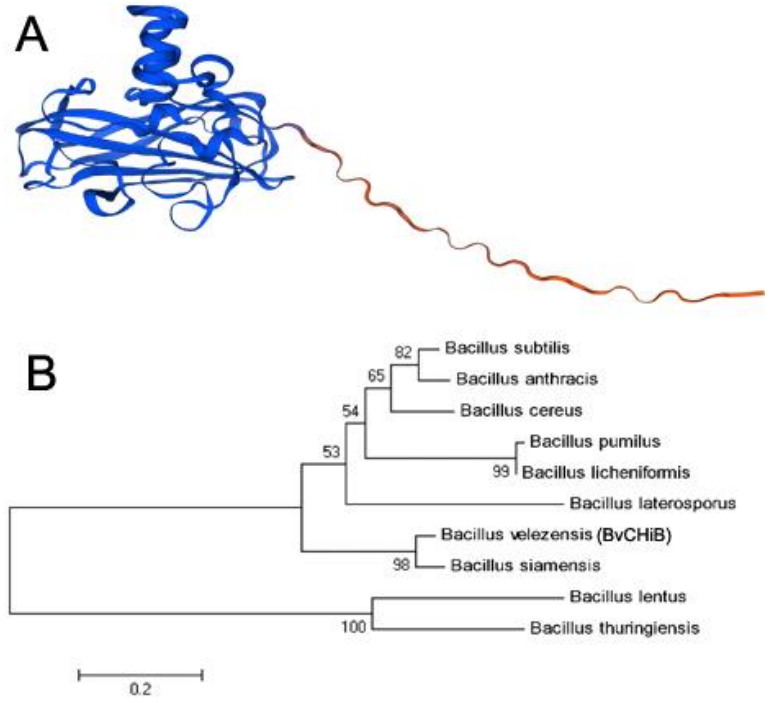

Figure 2. Three-dimensional structure and phylogenetic tree of BvCHiB
A, Three-dimensional structure of BvCHiB; B, phylogenetic tree of BvCHiB

3.3. Transgenic BvCHiB Arabidopsis thaliana showed increased resistance to Verticillium wilt

The transgenic *BvCHiB* Arabidopsis thaliana and the wild type were inoculated with Vd080 at the 8-leaf stage. After 15 days, the wild type showed yellow leaf edges, and the *BvCHiB* transgenic lines only had a few small areas of yellow leaves (Fig. 3). The disease index of transgenic plants (35 %) decreased to a significant level compared with that of wild type (85 %), and the biomass of VD in transgenic plants decreased to a very significant level compared with that of wild type (Fig. 3). These results indicated that transgenic *BvCHiB* Arabidopsis thaliana significantly increased the resistance to Verticillium wilt.

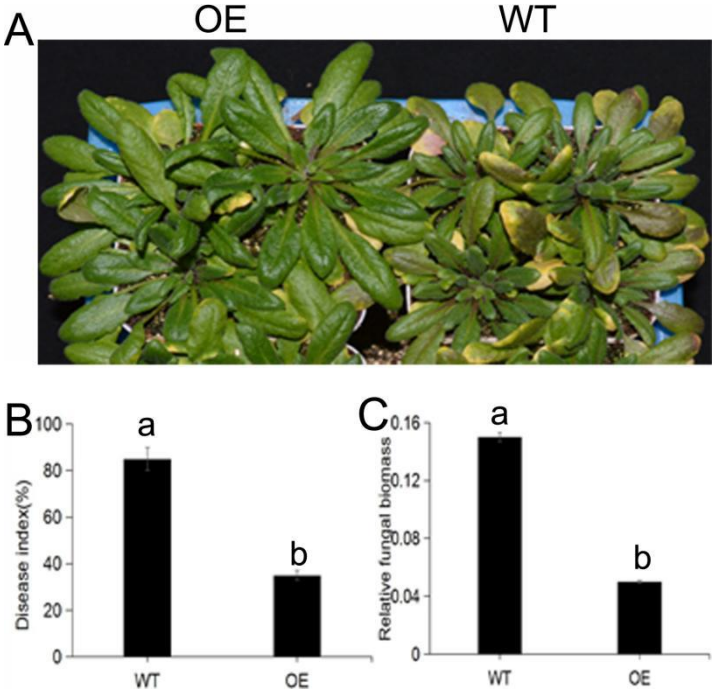

Figure 3. Resistance detection of transgenic *BvCHiB* a*rabidopsis thaliana*. A, resistance detection of transgenic *BvCHiB* Arabidopsis thaliana; B, the disease index of overexpressed lines OE was investigated; C, detection of the biomass of VD in OE overexpression lines; One-way ANOVA ($P < 0.05$) followed by Duncan tests was used for multiple comparisons. The different letters indicate significance at the 0.05 probability level.

3.4. The enzyme activity of transgenic lines was increased to enhance disease resistance

In order to test whether the improvement of disease resistance of overexpressed transgenic lines is related to the improvement of enzyme activity. After inoculation with Vd080 for 48h, the leaves of OE and WT plants were detected, and it was found that the accumulation of disease-resistant substances such as lignin, PAL, POD, SOD, CAT and MDA significantly increased compared with the control (Fig. 4). These resistant substances can effectively resist the infection of verticillium wilt. These results indicated that overexpressed plants might enhance disease resistance by increasing enzyme activity.

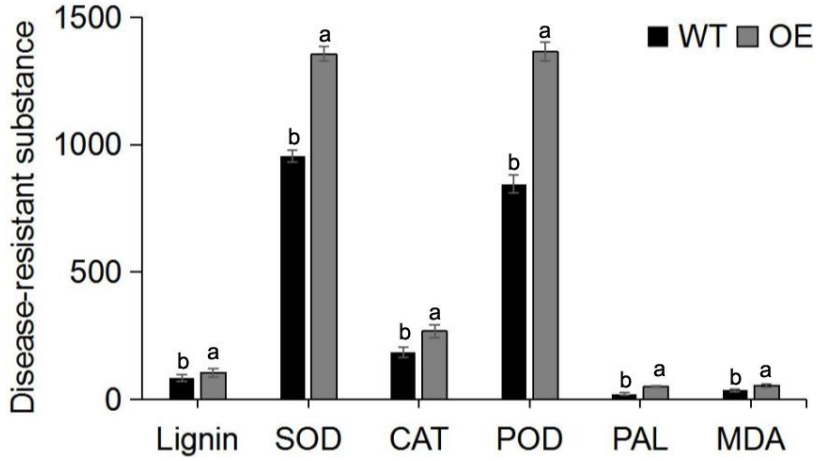

Figure 4. The enzyme activity of transgenic lines was increased to enhance disease resistance, One-way ANOVA ($P < 0.05$) followed by Duncan tests was used for multiple comparisons. The different letters indicate significance at the 0.05 probability level.

3.5. Transgenic Arabidopsis overexpressing the BvCHiB gene exhibits increased expression of resistance genes

Arabidopsis plants overexpressing the *BvCHiB* gene exhibited enhanced disease resistance to *V. dahliae* compared with WT Arabidopsis plants. To verify that Arabidopsis plants overexpressing the *BvCHiB* gene exhibit enhanced resistance to *V. dahliae*, the expression levels of LOX, PAL, PR1 and VSP genes were measured after inoculated with *V. dahliae* for 48 hours, and the results showed that the expression of the four disease resistance genes were significantly increased. Therefore, overexpression of the *V. dahliae* gene in Arabidopsis can effectively increase the expression of disease resistance genes related to the salicylic acid (SA) and jasmonic acid (JA) signaling pathways and enhance resistance to *V. dahliae*. Therefore, in transformed Arabidopsis plants, the *BvCHiB* gene can effectively upregulate the expression of SA and JA signal-related genes in leaves and can enhance resistance to *V. dahliae* (Fig. 5).

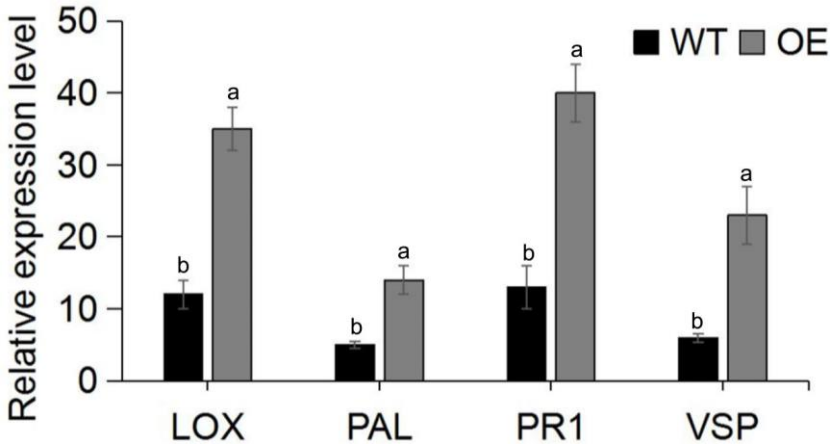

Figure 5. Transgenic Arabidopsis overexpressing the *BvCHiB* gene exhibits increased expression of resistance genes, One-way ANOVA ($P < 0.05$) followed by Duncan tests was used for multiple comparisons. The different letters indicate significance at the 0.05 probability level.

4. Conclusion

BvCHiB protein was closely related to Bacillus siamensis and far related to other species. Transgenic *Arabidopsis* plants overexpressing *BvCHiB* improves disease resistance by increasing disease-resistance related substances. *BvCHiB* may enhance disease resistance of plants mainly

through JA and SA signaling pathway. *BvCHiB* may be used as a resistance gene to improve resistance to *V. dahliae* in upland cotton.

CRediT authorship contribution statement

Yujing Liu: Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Writing – original draft.

Data availability

Data will be made available on request.

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