Journal of Plant Protection Research eISSN 1899-007X

ORIGINAL ARTICLE

Identifying the key motif essential for enhancing plant defense against *Botrytis cinerea* by β-glucanase from *Bacillus velezensis* LJ02

Qi Sun[1], Tong Sun[1], Yujin Yuan[1], Ruokui Chang[2], Weiwei Yu[1], Xiaohui Qiu[1], Tianyi Wu[1], Anling Deng[1], Zhuoran Li[1], Yuanhong Wang[1]*

[1] Horticulture and Landscape Architecture, Tianjin Agriculture University, Tianjin, China

[2] College of Engineering and Technology Architecture, Tianjin Agriculture University, Tianjin, China

Vol. 64, No. 3: 253–262, 2024

DOI: 10.24425/jppr.2024.151258

Received: February 23, 2024
Accepted: April 10, 2024
Online publication: September 11, 2024

*Corresponding address:
wangyh@tjau.edu.cn

Responsible Editor:
Dzarifah Zulperi

Abstract

The glycosyl hydrolase β-glucanase elicits plant immune responses against pathogens and enhances plant immunity by activating signaling pathways. The specific functional domains responsible for disease prevention remain unclear. In this study, transient expression of β-glucanase significantly increased leaves resistance to *Botrytis cinerea* in *Nicotiana benthamiana* systemic leaves. Through sequence alignment and similarity analysis, five conserved motifs in the amino acid sequence of β-glucanase were identified, and five deletion mutants were generated to investigate its essential regions further. Notably, the N-terminal amino acid sites 5-54 deletion mutation of β-glucanase decreased resistance to *B. cinerea* infection. These results indicate that N-terminal amino acids 5-54 (N54) are crucial for β-glucanase induced *N. benthamiana* defense response and for enhancing resistance to *B. cinerea*. Further analysis using real-time quantitative fluorescent PCR (qRT-PCR) revealed a significant reduction in gene expression within the N54 region compared to that of unmutated β-glucanase. Additionally, there was a notable reduction in the relative expression levels of FRK, CYP71D20, WRKY7, WRKY8, ACRE31 and PTI genes. Therefore, the first 50 amino acids at positions 5-54 within the N-terminal domain were essential for triggering plant defense responses and enhancing resistance against *B. cinerea* infection. This study provides an important theoretical foundation for systematic investigation into key functional domains within β-glucanase that trigger defensive responses in plants against *B. cinerea*.

Keywords: *Bacillus velezensis*, *Botrytis cinerea*, deletion mutant, systemic acquired resistance, β-glucanase

Introduction

Botrytis cinerea is a serious pathogenic bacterium in the world, which causes gray mold after plant infection, resulting in huge economic losses (Dean *et al.* 2012). *Botrytis cinerea* is a necrotrophic fungus that commonly grows and infects damaged or aged tissues, eventually resulting in tissue death. Therefore, the prevention and control of *B. cinerea* are critical (Xiong *et al.* 2019). *Bacillus velezensis* can encode immune proteins that the plant senses in response to pathogens, influencing local responses and helping initiate systemic defenses that protect the plant from disease, thereby protecting the plant. *Bacillus velezensis* LJ02 produces elicitor proteins that enhance signaling pathways and increase plant resistance to pathogens (Hu *et al.* 2022; Li *et al.* 2022).

Plants use their immune systems to defend against microbial invasion. Pathogen-associated molecular patterns (PAMPs) can be recognized by pattern recognition receptors (PRRs), thereby initiating pattern-triggered immunity (PTI) (Ho *et al.* 2020). Generally, signaling mediated by PAMPs plays

a crucial role in combating pathogens through the activation of multiple defense-related genes (Heese *et al.* 2007). For example, CYP71D20 and ACRE31 were previously believed to be rapidly activated following PAMP treatment, which triggers PTI signaling pathways (Tunsagool *et al.* 2019). PTI is characterized by bursts of reactive oxygen species (ROS), protein kinase activation, expression of defense-related genes and mitogen-activated protein kinase (MAPK) activity (Pi *et al.* 2022). MAPKs have been identified as prime candidates for enhancing signaling pathways in plants (Xing *et al.* 2015; Buerstmayr *et al.* 2021). Specifically, WRKY transcription factors are regulated by MAPKs at both the transcriptional and post-transcriptional levels of defense-related signaling pathways (Dagvadorj *et al.* 2017). PTI is a basic mode to restrict the growth of pathogens. The release of pathogens effecting factors into host cells inhibits PTI reaction and promotes the growth of pathogens (Shoresh *et al.* 2010; Zhong *et al.* 2018). The occurrence of pathogen-induced systemic acquired resistance (SAR) is triggered by the local activation of the PTI response, accompanied by the cooperative activation of defense genes (Pieterse *et al.* 2014).

In the field of biological control, β-glucanase is produced as a result of rhizobia attacking plant pathogens, and it is considered to be an inducer of the plant immune response (Mishra *et al.* 2020). β-glucanase exhibits wide distribution in plants, insects, and microorganisms, with various associated applications (Shen *et al.* 2020; Sun *et al.* 2020). β-glucanase acts as an inducer that induces resistance in plants and reduces pathogen infection by promoting the production of a variety of defense-related chemicals (Prasannath 2017). Overexpression of key virulence factors of 1,3,4-β-D-glucanase in the endophytic bacterium *Bacillus* Y6 enhances resistance against verticillium wilt in cotton (Zhang *et al.* 2019). Immune function depends on conserved structural domains, which play an important role in the regulation of plant defense-related genes. It has been shown that wheat yellow rust resistance genes have atypical N-terminal BED structural domains that are important for resistance to wheat yellow rust (Marchal *et al.* 2018). Similarly, knocking down the PsCPK1 gene encoding the PKA conserved domain inhibited *Fusarium* infection, indicating that the PKA domain was resistant to wheat stripe rust at the genetic level of wheat (Qi *et al.* 2018). NBS-LRR domain protein is a kind of immune sensor which can play an important role in the process of plant disease resistance. The NBS-LRR novel gene ZmNBS25 of maize species responded to both pathogen inoculation and salicylic acid (SA) treatment, and caused HR response in *N. benthamiana*. ZmNBS25 is a valuable candidate gene for engineering resistance in breeding programs (Xu *et al.* 2018). However, the key domains for β-glucanase immune regulation are still unknown.

In this study, *B. velezensis* LJ02 produces β-glucanase, an inducer of plant immune response. To determine the key domain of β-glucanase, the amino acid sequences of β-glucanase were analyzed, and mutants with missing amino acid sequences were constructed. In addition, qRT-PCR was used to analyze the expression levels of resistance-related genes that express β-glucanase and mutants in the leaves of the *N. benthamiana* system to find key motifs that are critical for immune response.

Materials and Methods

Biological materials

Nicotiana benthamiana was planted in soil and cultured at 25°C for 4–6 weeks in a light/dark cycle of 16/8 h. *Botrytis cinerea* was routinely cultured on potato dextrose agar (PDA) at 25°C. *Botrytis velezensis* LJ02 was a strain left over from an earlier stage of our laboratory and was a source for the gene encoding β-glucanase (Li *et al.* 2015). The prokaryotic expression vector pET-28a, driven by the T7 promoter, is essential for efficient expression and purification of E. coli proteins.

Transient expression by *Agrobacterium tumefaciens*

Agrobacterium tumefaciformis GV3101 was used as competent cells. The target gene overexpression vector and the corresponding empty vector were introduced into *A. tumefaciformis* by the electric shock method (Li *et al.* 2019). The positive *Agrobacterium* carrying plasmids was cultured in LB medium supplemented with appropriate antibiotics on a shaking table at 28°C until reaching an OD600 of 1.5. Subsequently, the shaken bacterial solution was transferred to liquid LB medium containing antibiotics, and incubated at 220 rpm for 16 hours at 28°C. After centrifugation at 4000 rpm for 10 minutes, the collected *Agrobacterium* was resuspended in a solution of MgCl2 (10 mM). The OD value of suspension was measured using an ultraviolet spectrophotometer and adjusted to achieve a required concentration within an OD600 range of 0.6–0.8 before being left at room temperature for 2 h. *Nicotiana benthamiana* with similar growth patterns were selected, and their lower leaves were infiltrated with *Agrobacterium* suspensions while employing empty carrier control (Wang *et al.* 2016). Three days after inoculation, the upper systemic leaves of *N. benthamiana* were collected and inoculated with mycelial disks. Three days later, lesion diameter measurements were taken using a cross method

approach alongside photographic documentation (Ji *et al.* 2014).

In vitro evaluation for recombinant β-glucanase antifungal activity

The previously described (Li *et al.* 2022) plate confrontation method was used to determine the inhibitory effect of the recombinant β-glucanase on *B. cinerea*. A sterilized Oxford cup and *B. cinerea* mycelium plate were placed on the PDA solid medium, and β-glucanase purified protein was added to the Oxford cup. The Oxford cup with protein buffer was used as the control. If there is a bacteriostatic zone, a vernier truck to measure the diameter of the bacteriostatic zone in the PDA medium should be used.

Expression and purification of recombinant β-glucanase

The Bacterial Genomic DNA Extraction Kit (Solarbio) was used to extract the genomic DNA of *B. velezensis* LJ02. According to β-glucanase enzyme sequences primer design (Supplementary: Tables S1 and S2), the DNA fragment of β-glucanase was amplified by PCR. PCR reaction conditions were: 95°C for 5 min., 94°C for 15 s, 64°C for 15 s, 72°C for 1 min., 32 cycles, 72°C for 5 min. After introducing the pET-28a-β-glucanase plasmid into Escherichia coli *(E. coli)* BL21 (DE3), it was subsequently induced with isopropyl β-D-thiogalactoside (IPTG) at 16°C for a duration of 16 h, resulting in a spectrophotometer at 600 nm (OD600) value ranging from 0.8 to 1.0. The cells were placed in dissolved buffer and crushed by ultrasound. The supernatant was purified by Ni Sepharose 6 Fast Flow (Cytiva) after electrolysis with 12% sodium dodecyl sulphate-polyacrylimide gel and staining with Coomassie brilliant blue. Recombinant β-glucanase protein was stained with Solarbio on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

β-glucanase activity assay

At three separate time intervals (24, 48, 72 and 96 hours), the enzyme activity of recombinant protein β-glucanase was measured by quantifying the synthesis of reducing sugar from substrate hydrolysis using the 3,5-dinitrosalicylic acid (DNS) technique (Sun *et al.* 2020). The enzyme activity was determined by analyzing the reducing sugar produced in the solution following the catalytic action of the enzyme on β-glucanase (Amresco) as a substrate. At 50°C for 30 minutes, the substrate stock solution (0.3 ml, 10 mg · ml^{-1} β-glucan in 0.1 M Na-acetate buffer) was combined with 0.3 ml of the enzyme solution, after which DNS reagent (0.3 ml) was added. The color formed was monitored using a spectrophotometer at 540 nm. Enzyme activity was converted to units per milliliter (U · ml^{-1}). Three replications of each condition were used.

Multiple alignment and phylogenetic analysis of -glucanase

To further explore the key domains of β-glucanase in the control of *B. cinerea*, we obtained β-glucanase genes in several different species from the GenBank database using the NCBI-BLAST (https://www.megasoftware.net/) tool. MEGA alignment was used to create a phylogenetic tree of β-glucanase sequences (Sudhir *et al.* 2018). The conserved motifs of the β-glucanase sequences were analyzed using the MEME Suitev tool (http://meme.nbcr.net/meme).

Deletion mutant construction and agroinfiltration assay

To construct the transient expression of β-glucanase deletion mutant, the specific primers containing StuI enzyme cut site (Supplementary: Table S3) were designed by software, and the fusion gene fragments were amplified by fusion PCR. The fusion fragments were integrated into transient expression vector by homologous recombination reaction linkage. The vector was produced and converted into *Agrobacterium* GV3101.

Determination of resistance of *Nicotiana benthamiana* induced by β-glucanase to *Botrytis cinerea*

β-glucanase was injected with *N. benthamiana*. The experimental procedure was carried out according to Wang *et al.* (2016). After 3 days, the upper system leaves of *N. benthamiana* were carefully collected and placed in a sterile dish lined with sterile filter paper. The leaves were inoculated on a mycelium plate containing *B. cinerea* and sealed around the plate with 200 µl sterilized distilled water. The inoculated *N. benthamiana* was then stored in a moist incubator. After 3 days, the diameter of the *N. benthamiana* lesion was measured using the cross method and recorded by photography (Ji *et al.* 2014). Each of the 10 leaves were treated as a biological replicate and three biological replicates were performed.

RNA isolation and real-time quantitative PCR

The *N. benthamiana* leaves were treated with prepared recombinant *Agrobacterium* carrying the transient expression vector pKGlu (WT) and empty vectors as control (EV). qRT-PCR was used to detect the relative

transcriptional expression of PTI marker genes FRK, CYP71D20, WRKY7, WRKY8, ACRE31 and PTI1 in infected leaves. qRT-PCR normalization of *N. benthamiana* was performed using the actin gene, which is highly conserved and constitutionally expressed in *N. benthamiana*. The relative expression levels of some marker genes were detected 1 to 5 days after inoculation. qRT-PCR was used in 96-well plates using the iQ5 Multi color real-time Assay System (Bio-Rad) to analyze gene expression in infected *N. benthamiana* plants. The sequences of the qRT-PCR primers used in this study are shown in Supplemental Table S4. The 2-ΔΔCt method was used to calculate the relative gene expression (Livak and Schmittgen 2001).

Results

β-glucanase improved the protective effect of *Nicotiana benthamiana* to *Botrytis cinerea*

The leaves of *N. benthamiana* expressed with empty vector (Control) and β-glucanase overexpression vector for 3days were inoculated with *B. cinerea*. After 3 days of inoculation, the symptoms of the control group were obvious, and the symptoms of β-glucanase infiltrated leaves were mild (Fig. 1A), and there was significant difference in the lesion diameter (Fig. 1B).

β-glucanase had no direct inhibitory effect on *Botrytis cinerea*

The expression vector pET-28a-β-glucanase was transformed into *E. coli* BL21 (DE3) for expression, and the cells were lysed and imidazole was imidazole eluted over a Ni-IDA column to obtain the fusion-expressed β-glucanase. The protein was then concentrated and eluted by dialysis (Fig. 2A). The enzymatic activity of the purified β-glucanase substrate was assessed at various incubation durations. The most suitable incubation time was 48 h for β-glucanase (10.3 ± 0.5 U · ml^{-1}) at 28°C (Fig. 2B).

To confirm that the purified β-glucanase protein had a direct inhibitory impact on *B. cinerea in vitro*, it was infected with *B. cinerea* and the effect of β-glucanase on *B. cinerea* growth and spore germination was determined using protein buffer as a control. The results showed that β-glucanase protein at the concentration of 0.05 g · l^{-1} did not form an inhibition ring near the Oxford cup. These results indicated that β-glucanase had no obvious inhibitory effect on the proliferation of Oxford cup mycelium *in vitro* (Fig. 2C).

Identification of conserved motifs in β-glucanase

Considering the biocontrol effect of β-glucanase on *B. velezensis* and the diversity of its sequences, NCBI was used to perform phylogenetic tree analysis of protein sequences and bacterial species (Fig. 3A). The conserved domain required for disease resistance action was selected using an alignment study of 22 amino acid sequences of β-glucanase. Comparative analysis of β-glucanase amino acid sequences using the MEME Suite tool to identify the five most conserved amino acid domains was required to identify disease resistance activity (Fig. 3B). We described five conserved amino acid domains, N5-54 N61-110, N61-110, N112-161, N164-213and N215-243 for the construction of transient expression vectors of deletion mutants (Fig. 3C).

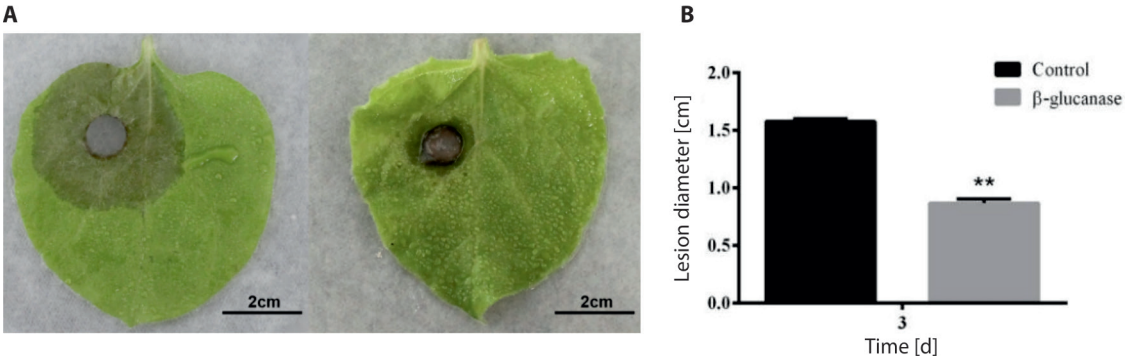

Fig. 1. Overexpression of β-glucanase enhanced the resistance of *Nicotiana* benthamiana. A – disease symptoms of isolated leaves after 3 days of inoculation with *Botrytis cinerea*; the leaves of *N. benthamiana* expressed with β-glucanase overexpression vector (β-glucanase), is shown on the right; the control, on the left; B – the diameter of the *N. benthamiana* lesion caused by *B. cinerea* at 3 days post inoculation (dpi).
The error line is the standard deviation of multiple biological replicates; results are mean ± standard deviation and the analysis of the significance of the data was performed using the t-test; $^{**}p < 0.01$

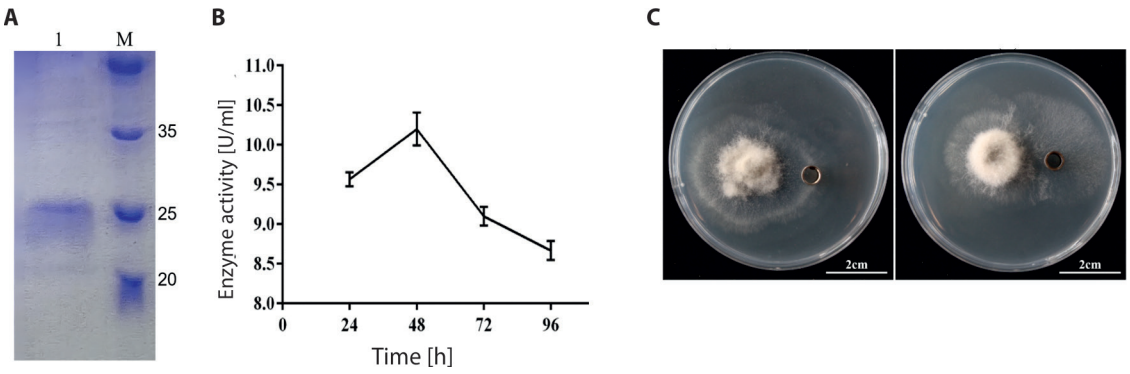

Fig. 2. Expression of β-glucanase and evaluation of its anti-fungal activity *in vitro*. A – SDS-PAGE analysis of the crude protein extract of the recombinant cell lysate and the fusion protein β-glucanase which migrated as a single band; lane M – protein molecular weight markers; lane 1 – purified protein. Molecular mass markers (M) indicated on the right from top to bottom were 35, 25, 20 kDa; B – time effect on enzyme activity of purified β-glucanase; C – anti-fungal effects of β-glucanase against pathogenic plant fungi *B. cinerea* under *in vitro* conditions. *Botrytis cinerea*, subjected to 100 µl purified protein treatment, is shown on the right, the control, on the left

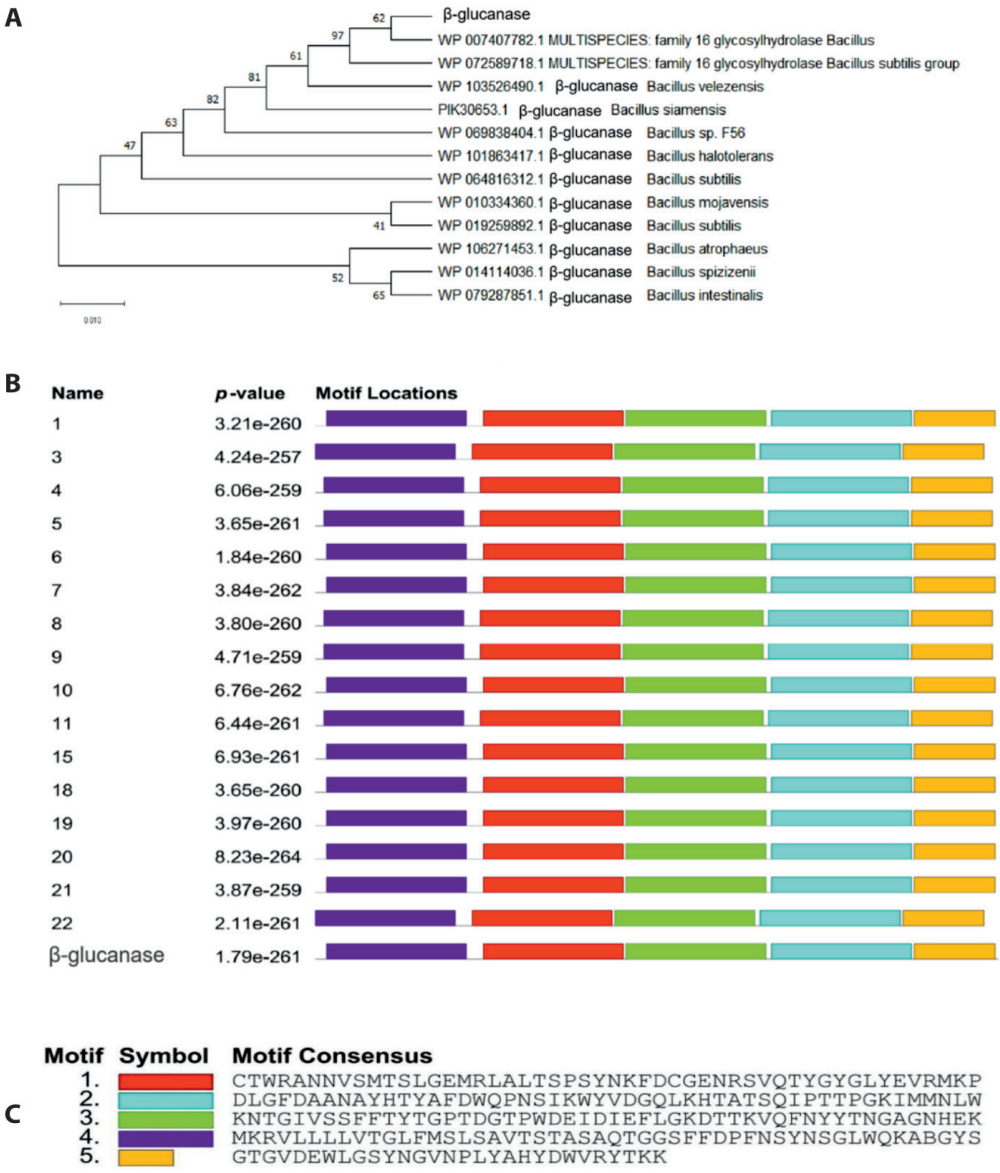

Fig. 3. Functional domain analysis for β-glucanase. A – gene phylogenetic tree analysis of β-glucanase amino acid sequences; B – comparison of the amino acid sequence of β-glucanase with those of its homologues in other species, analyzed using the MEME Suite, different colors represent conserved residues in different regions; C – all five motifs are composed of long residues

N54 is the key domain of β-glucanase against *Botrytis cinerea* of *Nicotiana benthamiana*

To completely study the biological significance of β-glucanase in the infection process, we created five deletion mutants of β-glucanase and produced the vectors created in N. benthamiana (Fig. 4A). Those mutants, empty vector control (EV) and wild-type pKGlu (WT) were transformed in *N. benthamiana* by agroinfiltration. The ability to develop resistance was discovered by inoculating *B. cinerea* on systemic leaves, which allowed us to assess the biocontrol efficacy of β-glucanase mutants against *B. cinerea*. The resistance of the N54 deletion mutation to *B. cinerea* differed less from that of *N. benthamiana* expressing the control (EV), and it was significantly lower than that of *N. benthamiana* expressing WT. In addition to N54, the resistance of the of the delecion mutations of *N. benthamiana* was not significantly different from WT but it was smaller than that of EV (Fig. 4B, C). Hence, N5-54 identified as the functional peptide of β-glucanase was indispensable to resist *B. cinerea* in *N. benthamiana*.

N54-induced expression of PTI marker genes was significantly inhibited

To further verify that the leaves of the *N. benthamiana* system trigger an immune response under β-glucanase treatment (WT), we examined the expression of PTI marker genes. Empty vector (EV) treatment was used as a control. The expression of the genes WRKY7, WRKY8, ACRE31, CYP71D20 and FRK in leaves treated with β-glucanase was significantly up-regulated, compared to the expression levels in EV at 3 and 4 dpi (Fig. 5).

Moreover we analyzed the expression levels of WRKY7, WRKY8, FRK, PTI1, CYP71D20 and ACRE31 in control, N54, WT. qRT-PCR study found that the gene expression level of N54 with deletion of WRKY7, WRKY8, FRK, PTI1, CYP71D20 and ACRE31 was significantly lower than that of WT on the 3rd and 4th day (Fig. 5).

Discussion

The β-glucanase in *B. velezensis* LJ02 is an extracellular protein, which activates host defense mechanisms to influence the spread of pathogenic bacteria (Ky *et al.* 2012; Yang *et al.* 2018). In this paper, the lession diameter of β-glucanase was significantly lower than that of control group at 3 dpi (Figs. 1A, B). In addition, β-glucanase protein was purified in vitro (Fig. 2A), and the purified protein inoculation did not produce an inhibitory circle near the Oxford cup (Fig. 2C).

The immune systems of plants have evolved to activate immune responses, known as pathogen-triggered immunity (PTI), in response to pathogen-related molecular patterns (PAMPs) (Chisholm *et al.* 2006). This enables effective protection against infection by

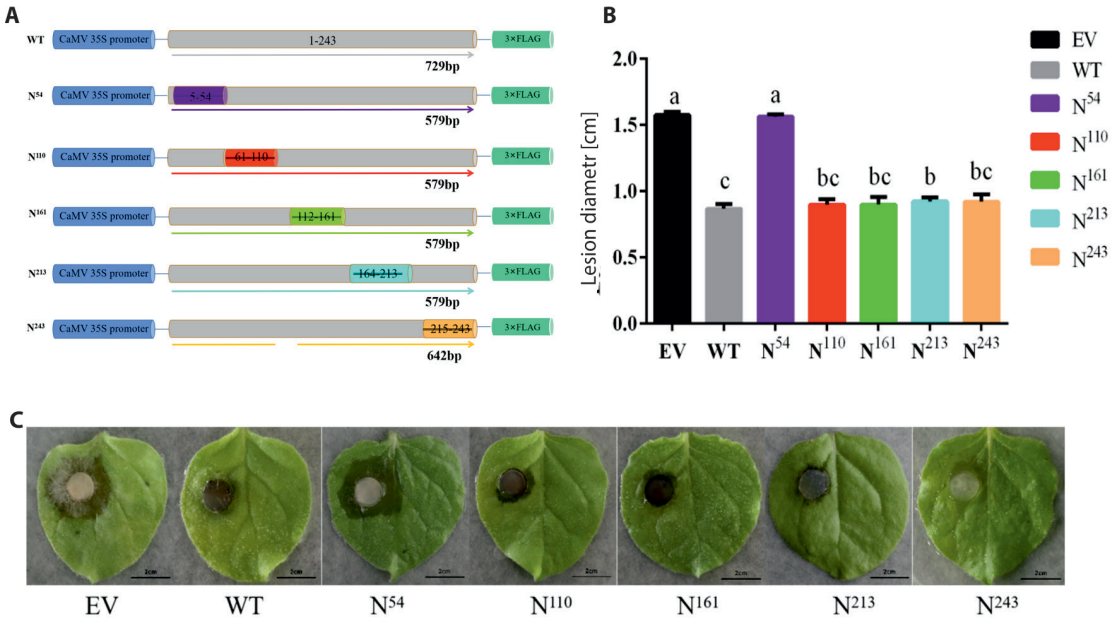

Fig. 4. Characterization of β-glucanase mutants in transient assays. A – line drawing of the deletion mutant context in WT, including N54, N110, N161, N213, N243; B – the diameter of the lesion 48 h after inoculation with *Botrytis cinerea* in WT and transiently expressed β-glucanase in *Nicotiana benthamiana*; C – disease symptoms of *N. benthamiana* caused by *B. cinerea* at 3 dpi. Columns with different letters indicate significant differences according to Duncan's multiple tests ($p < 0.05$)

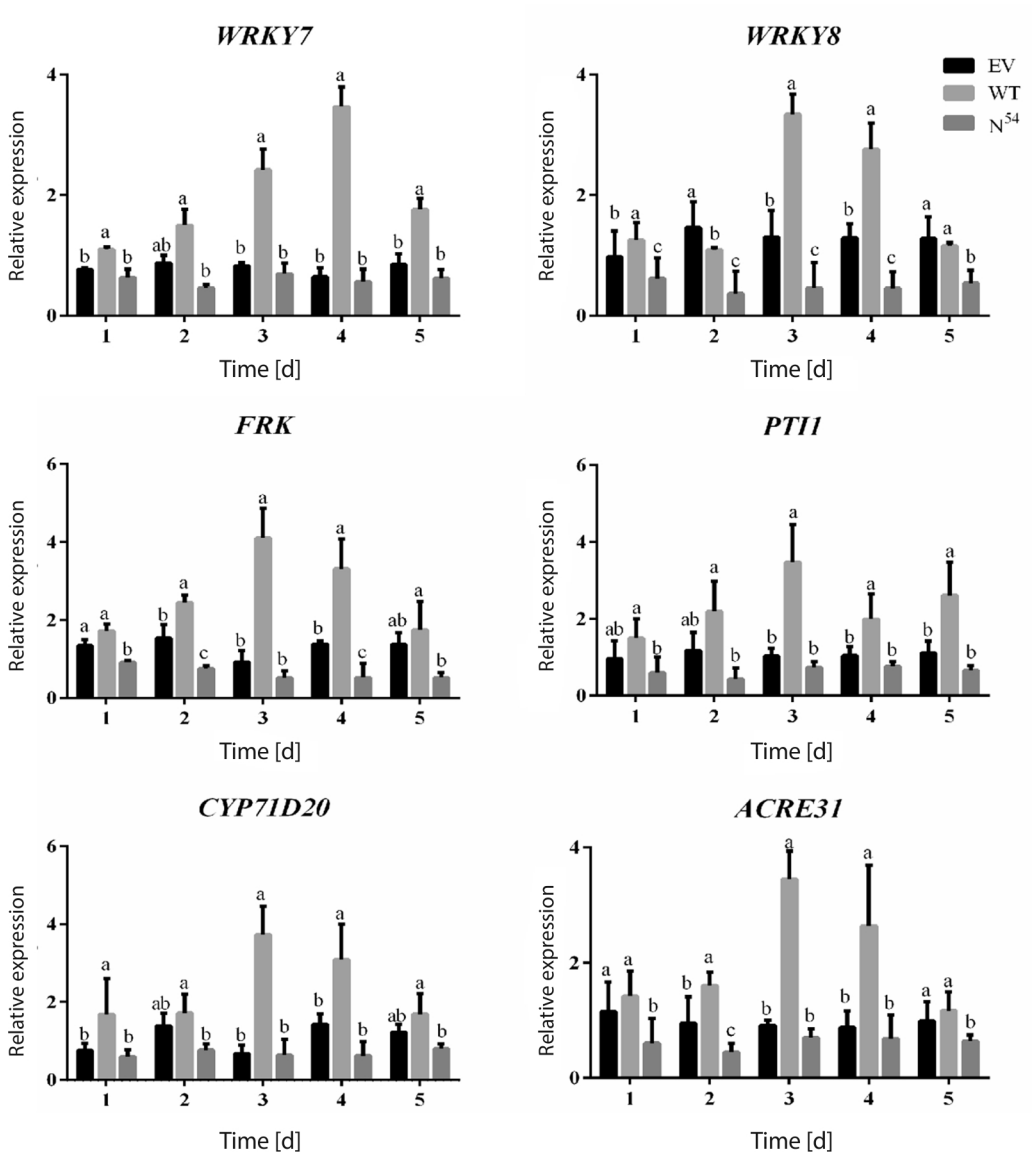

Fig. 5. qRT-PCR analysis was performed on transcription levels of WRKY7, WRKY8, FRK, PTI1, CYP71D20, and ACRE31 genes was performed on RNA isolated from leaves harvested at 1, 2, 3, 4 and 5 dpi. Empty vector was used as a control. The qRT-PCR data were reported and calculated on the basis of the actin gene using the 2-ΔΔCt method. Quantitative data are given as mean ± SEM (n = 3). Columns with different letters indicate significant differences according to Duncan's multiple tests ($p < 0.05$)

various pathogenic microorganisms. When confronted with external pathogens, plants' transmembrane pattern recognition receptors (PRRs) can identify them and initiate immune responses that are stimulated by the presence of PAMPs (Zhai *et al.* 2022). β-glucanase can elicit an immune response to SAR and PAMP defensive responses. To prevent further infection and spread of pathogens, plants will stimulate cellular immune responses such as MAP kinase signaling and activation of defense genes (Zhou *et al.* 2021). Also, it is an important factor in triggering SAR induction. We studied some PTI-related expression levels in the process of SAR induced by β-glucanase to investigate the relationship between them. β-glucanase elevated the expression of the gene ACRE31 and the putative cytochrome P450 CYP71D20 in *N. benthamiana*. Their expression levels have been shown to increase considerably following PAMP treatment (Mishina and Zeier 2007; Xin *et al.* 2012). PTI1 is a serine/threonine protein kinase with significantly increased gene expression at 3 dpi and is involved in disease-resistant pathways (Fig. 5). In addition, WRKY7 and WRKY8 relative gene expression was considerably greater in leaves treated with β-glucanase than in control at 3 dpi (Fig. 5), which is essential for inducing defense-related signaling pathways by interacting with the MAPK cascade. PAMP is a hydrolyzed protein (Ma *et al.* 2015) and β-glucanase can be involved in PAMP-triggered immunity. SAR is fundamental to the control of cellular defense responses in plants during

pathogen challenge (Mishina and Zeier 2007). Bacterial production of PAMP can potentially activate FRK expression, which increases when the SAR response is activated (Hu *et al*. 2022). These findings support the possibility of a close relationship between SAR and PTI activation. It was found that qRT-PCR further verified the reduction in the expression of several host defense-related genes due to N54-induced impaired resistance of *N. benthamiana* to *B. cinerea* (Fig. 5). PAMP signaling plays a key role in resistance to pathogens by activating multiple defense-related genes (Ma *et al*. 2015). To explore the biological control effect of β-glucanase against *B. cinerea*, we studied key domains of β-glucanase conserved sequences to reveal the immune function mechanism of this enzyme. In the amino acid sequence of β-glucanase, five conserved motifs were discovered, and five deletion mutants with conserved amino acid domains were constructed. Compared to WT, *N. benthamiana* treated with N54 in β-glucanase showed reduced resistance to *B. cinerea* (Fig. 4B, C). These findings show that N54 loses the resistance of plants to *B. cinerea* diseases.

MAPK is a key element of the PTI response, and it is involved in the convergence signaling cascade in plant immunity. In defense-related signaling networks, MAPKs regulate WRKY transcription factors at the transcriptional and post-transcriptional levels (Ishihama *et al*. 2012). In N54-induced systemic leaves, WRKY8 gene expression decreased at 2, 3, and 4 dpi (Fig. 5). Furthermore, PTI is considered a potent inducer (Mishina and Zeier 2007; Dempsey and Klessig 2012). In the current understanding of the plant immune system, the PTI response can initiate a long-lasting and widespread SAR in unvaccinated plant tissues against subsequent pathogens, which is referred to as organism-triggered immunity against microorganisms (Mishina and Zeier 2007; Dempsey and Klessig 2012). Following stress-induced responses, the resistance response is systematically enhanced through induction by infectious agents (Verma and Satyanarayana 2012). Pathogen or elicitor induced SAR involves localized activation of the PTI response accompanied by synergistic up-regulation of defense genes. Early defense signals and SAR activation in plant leaves were detected, along with the up-regulation of the Flg22-induced Receptor Kinase (FRK) defense gene (Asai *et al*. 2002; Block *et al*. 2014). Pathogen or elicitor induced SAR is a local activation of the PTI response, accompanied by a synergistic activation of defense genes. Along with the up-regulation of defense gene FRK, early defense signals and SAR activation of plant leaves were detected (Asai *et al*. 2012). We found that N54 reduced FRK expression at 2, 3, 4, 5 (dpi) (Fig. 5), and SAR activation was blocked due to reduced expression of defense-related genes. The disease resistance defense response may not be triggered. As a protein elicitor released by LJ02, β-glucanase can effectively stimulate host defense mechanisms and prevent pathogen growth. This study provides an important theoretical basis for the systematic study of the key functional domains necessary for β-glucanase to develop defensive response resistance to *B. cinerea* in plants.

Conclusions

β-glucanase stimulates plant immunity, thereby enhancing the resistance of *N. benthamiana* against *B. cinerea*. *In vitro* experiments revealed that β-glucanase did not directly exhibit antibacterial effects on *B. cinerea*. Additionally, β-glucanase can activate PTI, ETI, and SAR pathways while also upregulating the expression of defense-related genes. Notably, the absence of 5–54 amino acids at the N-terminal reduced the disease-inducing resistance of β-glucanase to *N. benthamiana*. qRT-PCR further confirmed that N54 induced PTI marker gene expression was significantly inhibited, suggesting that N54 expressed *N. benthamiana* could not trigger the typical PTI response, including the expression of defense genes. This study provides fundamental insights for further analysis on how β-glucanase activates plant defense responses and highlights the value of our research framework in identifying key functional motifs for β-glucanase.

Acknowledgements

We wish to thank the Science and technology projects of Gansu (22CX8NH226); Science and technology projects of Gansu (23CXNH0014) for supporting this work.

References

Asai T., Tena G., Plotnikova J., Willmann M.R., Chiu W., Gomez-Gomez L., Boller T., Ausubel F.M., Sheen J. 2002. MAP kinase signalling cascade in Arabidopsis innate immunity. Nature 415: 977–983. DOI: https://doi.org/10.1038/415977a

Block A., Toruño T.Y., Elowsky C.G., Zhang C., Steinbrenner J., Beynon J., Alfano J.R. 2014. The *Pseudomonas syringae* type III effector HopD1 suppresses effector-triggered immunity, localizes to the endoplasmic reticulum, and targets the *Arabidopsis transcription* factor NTL9. New Phytologist 201: 1358–1370. DOI: https://doi.org/10.1111/nph.12626

Buerstmayr M., Wagner C., Nosenko T., Omony J., Steiner B., Nussbaumer T., Mayer K.F.X., Buerstmayr H. 2021. Fusarium head blight resistance in European winter wheat: insights from genome-wide transcriptome analysis. BMC Genomics 22: 470. DOI: https://doi.org/10.1186/s12864-021-07800-1

Chisholm S.T., Coaker G., Day B., Staskawicz B.J. 2006. Host-microbe interactions: shaping the evolution of the plant immune response. Cell 124: 803–814. DOI: https://doi.org/10.1016/j.cell.2006.02.008

Dagvadorj B., Ozketen A.C., Andac A., Duggan C., Bozkurt T.O., Akkaya M.S. 2017. A *Puccinia striiformis* f. sp. *tritici* secreted protein activates plant immunity at the cell surface. Scientific Reports 7 (1): 1141. DOI: 10.1038/s41598-017-01100-z

Dean R., Van K. J.A.L., Pretorius Z.A., Hammond-Kosack K.E., Pietro A.D., Spanu P.D., Rudd J.J., Dickman M., Kahmann R., Ellis J., Foster G.D. 2012. The Top 10 fungal pathogens in molecular plant pathology. Molecular Plant Pathology 13: 414–430. DOI: https://doi.org/10.1111/j.1364-3703.2011.00783.x

Dempsey D.A., Klessig D.F. 2012. SOS – too many signals for systemic acquired resistance? Trends in Plant Science 17: 538–545. DOI: https://doi.org/10.1016/j.tplants.2012.05.011

Heese A., Hann D. R., Gimenez-Ibanez S., Jones A. M. E., He K., Li J., Schroeder J. I., Peck S. C., Rathjen J. P. 2007. The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. Proceedings of the National Academy of Sciences of the United States of America 104: 12217–12222. DOI: https://doi.org/10.1073/pnas.0705306104

Hu J., Chang R., Yuan Y., Li Z., Wang Y. 2022. Identification of key residues essential for the activation of plant immunity by subtilisin from *Bacillus velezensis* LJ02. Frontiers in Microbiology 13: 869596. DOI: https://doi.org/10.3389/fmicb.2022.869596

Ho T.-H., Chuang C.-Y., Zheng J.-L., Chen H.-H., Liang Y.-S., Huang T.-P., Lin Y.-H. 2020. *Bacillus amyloliquefaciens* strain PMB05 intensifies plant immune responses to confer resistance against bacterial wilt of tomato. Phytopathology® 110: 1877–1885. DOI: https://doi.org/10.1094/PHYTO-01-20-0026-R

Ishihama N., Yoshioka H. 2012. Post-translational regulation of WRKY transcription factors in plant immunity. Current Opinion in Plant Biology 15: 431–437. DOI: https://doi.org/10.1016/j.pbi.2012.02.003

Ji M., Wu X., Yao K., Chen H., Yang J., Wang L., Zhuang Y. 2014. Identification of strawberry anthracnose pathogens and screening of germicides. Journal of Agricultural Science and Technology 15: 94. DOI: https://doi.org/10.16175/j.cnki.1009-4229.2014.01.014

Ky I., Lorrain B., Jourdes M., Pasquier G., Fermaud M., Gény L., Rey P., Donèche B., TEISSEDRE P.L. 2012. Assessment of grey mould (*Botrytis cinerea*) impact on phenolic and sensory quality of Bordeaux grapes, musts and wines for two consecutive vintages. Australian Journal of Grape and Wine Research 18: 215–226. DOI: https://doi.org/10.1111/i.1755-0238.2012.00191.x

Livak K.J., Schmittgen T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25: 402–408. DOI: https://doi.org/10.1006/meth.2001.1262

Li Y., Gu Y., Li J., Xu M., Wei Q., Wang Y. 2015. Biocontrol agent *Bacillus amyloliquefaciens* LJ02 induces systemic resistance against cucurbits powdery mildew. Frontiers in Microbiology 6: 883. DOI: https://doi.org/10.3389/fmicb.2015.00883

Li Z., He Y., Luo T., Zhang X., Wan H., Ur R.A., Bao X., Zhang Q., Chen J., Xu R., Deng Y., Zeng Y., Xu J., Hong N., Li F., Cheng Y. 2019. Identification of key residues required for RNA silencing suppressor activity of p23 protein from a mild strain of Citrus tristeza virus. Viruses 11: 782. DOI: https://doi.org/10.3390/v11090782

Li Z., Hu J., Sun Q., Zhang X., Chang R., Wang Y. 2022. A novel elicitor protein phosphopentomutase from *Bacillus velezensis* LJ02 enhances tomato resistance to Botrytis cinerea. Frontiers In Plant Science 13: 1064589. DOI: https://doi.org/10.3389/fpls.2022.1064589

Ma Z., Song T., Zhu L., Ye W., Wang Y., Shao Y., Dong S., Zhang Z., Dou D., Zheng X., Tyler B.M., Wang Y. 2015. A Phytophthora sojae glycoside hydrolase 12 protein is a major virulence factor during soybean infection and is recognized as a PAMP. Plant Cell 27: 2057–2072. DOI: https://doi.org/10.1105/tpc.15.00390

Marchal C., Zhang J., Zhang P., Fenwick P., Steuernagel B., Adamski N.M., Boyd L., McIntosh R., Wulff B.B.H., Berry S., Lagudah E., Uauy C. 2018. BED-domain-containing immune receptors confer diverse resistance spectra to yellow rust. Nature Plants 4: 662–668. DOI: https://doi.org/10.1038/s41477-018-0236-4

Mishra P., Mishra J., Dwivedi S., Arora N.K. 2020. Microbial enzymes in biocontrol of phytopathogens. p. 259–285. In: "Microbial Enzymes: Roles and Applications in Industries". Springer. DOI: https://doi.org/10.1007/978-981-15-1710-5_10

Mishina T.E., Zeier J. 2007. Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in Arabidopsis. Plant Journal 50: 500–513. DOI: https://doi.org/10.1111/j.1365-313X.2007.03067.x

Pieterse C.M., Pierik R., Van Wees S.C. 2014. Different shades of JAZ during plant growth and defense. New Phytologist 204 (2): 261–264. DOI: https://doi.org/10.1111/nph.13029

Pi L., Yin Z., Duan W., Wang N., Zhang Y., Wang J., Dou D. 2022. A G-type lectin receptor-like kinase regulates the perception of oomycete apoplastic expansin-like proteins. Journal of Integrative Plant Biology 64: 183–201. DOI: https://doi.org/10.1111/jipb.13194

Prasannath K. 2017. Plant defense-related enzymes against pathogens: a review. AGRIEAST: Journal of Agricultural Sciences 11: 38–48. DOI: https://doi.org/10.4038/agrieast.v11i1.33

Qi T., Zhu X., Tan C., Liu P., Guo J., Kang Z., Guo J. 2018. Host-induced gene silencing of an important pathogenicity factor PsCPK1 in *Puccinia striiformis* f. sp. *tritici* enhances resistance of wheat to stripe rust. Plant Biotechnology Journal 16: 797–807. DOI: https://doi.org/10.1111/pbi.12829

Romanazz G., Smilanick J. L., Feliziani E., Droby S. 2016. Integrated management of postharvest gray mold on fruit crops. Postharvest Biology and Technology 113: 69–76. DOI: https://doi.org/10.1016/j.postharvbio.2015.11.003

Shen L., Yang S., Yang F., Guan D., He S. 2020. CaCBL1 Acts as a positive regulator in pepper response to *Ralstonia solanacearum*. Molecular Plant-Microbe Interactions 33: 945–957. DOI: https://doi.org/10.1094/mpmi-08-19-0241-r

Shoresh M., Harman G.E., Mastouri F. 2010. Induced systemic resistance and plant responses to fungal biocontrol agents. Annual Review of Phytopathology 48: 21–43. DOI: https://doi.org/10.1146/annurev-phyto-073009-114450

Sudhir K., Glen S., Michael L., Christina K., Koichiro T. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution 35: 1547–1549. DOI: https://doi.org/10.1093/molbev/msy096

Sun Z.B., Xu J.L., Lu X., Zhang W., Ji C., Ren Q. 2020. Directed mutation of β-glucanases from probiotics to enhance enzymatic activity, thermal and pH stability. Archives of Microbiology 202: 1749–1756. DOI: https://doi.org/10.1007/s00203-020-01886-z

Tunsagool P., Jutidamrongphan W., Phaonakrop N., Jaresitthikunchai J., Roytrakul S., Leelasuphakul W. 2019. Insights into stress responses in mandarins triggered by *Bacillus subtilis* cyclic lipopeptides and exogenous plant hormones upon *Penicillium digitatum* infection. Plant Cell Reports 38: 559–575. DOI: https://doi.org/10.1007/s00299-019-02386-1

Verma D., Satyanarayana T. 2012. Molecular approaches for ameliorating microbial xylanases. Bioresource Technology 117: 360–367. DOI: https://doi.org/10.1016/j.biortech.2012.04.034

Wang N., Liu M., Guo L., Yang X., Qiu D. 2016. A novel protein elicitor (PeBA1) from *Bacillus amyloliquefaciens* NC6 induces systemic resistance in *N. benthamiana*. International Journal of Biological Sciences 12: 757. DOI: https://doi.org/10.7150/ijbs.14333

Xin D.W., Liao S., Xie Z.P., Hann D.R., Steinle L., Boller T., Staehelin C. 2012. Functional analysis of NopM, a novel E3 ubiquitin ligase (NEL) domain effector of Rhizobium sp.

strain NGR234. PLoS Pathogens 8: e1002707. DOI: https://doi.org/10.1371/journal.ppat.1002707

Xing Y., Chen W. H., Jia W., Zhang J. 2015. Mitogen-activated protein kinase kinase 5 (MKK5)-mediated signalling cascade regulates expression of iron superoxide dismutase gene in Arabidopsis under salinity stress. Journal of Experimental Botany 66: 5971–5981. DOI: https://doi.org/10.1093/jxb/erv305

Xiong F., Liu M., Zhuo F., Yin H., Deng K., Feng S., Liu Y., Luo X., Feng L., Zhang S., Li Z., Ren M. 2019. Host-induced gene silencing of BcTOR in Botrytis cinerea enhances plant resistance to grey mould. Molecular Plant Pathology 20: 1722–1739. DOI: https://doi.org/10.1111/mpp.12873

Xu Y., Liu F., Zhu S., Li X. 2018. The Maize NBS-LRR gene ZmNBS25 enhances disease resistance in rice and arabidopsis. Frontiers in Plant Science 9: 1033. DOI: https://doi.org/10.3389/fpls.2018.01033

Yang Y., Yang X., Dong Y., Qiu D. 2018. The *Botrytis cinerea* xylanase BcXyl1 modulates plant immunity. Frontiers in Microbiology: 2535. DOI: https://doi.org/10.3389/fmicb.2018.02535

Zhang L., Li W., Tao Y., Zhao S., Yao L., Cai Y., Niu Q. 2019. Overexpression of the key virulence factor 1,3–1,4-β-d-Glucanase in the endophytic bacterium *Bacillus halotolerans* Y6 to improve *Verticillium* resistance in cotton. Journal of Agricultural and Food Chemistry 67: 6828–6836. DOI: https://doi.org/10.1021/acs.jafc.9b00728

Zhai K., Liang D., Li H., Jiao F., Yan B., Liu J., Lei Z., Huang L., Gong X., Wang X. 2022. NLRs guard metabolism to coordinate pattern- and effector-triggered immunity. Nature 601: 245–251. DOI: https://doi.org/10.1038/s41586-021-04219-2

Zhong C., Ren Y., Qi Y., Yu X., Wu X., Tian Z. 2018. PAMP--responsive ATL gene StRFP1 and its orthologue NbATL60 positively regulate *Phytophthora infestans* resistance in potato and *Nicotiana benthamiana*. Plant Science 270: 47–57. DOI: https://doi.org/10.1016/j.plantsci.2018.01.016

Zhou L., Song C., Muñoz C.Y., Kuipers O.P. 2021. *Bacillus cabrialesii* BH5 protects tomato plants against *Botrytis cinerea* by production of specific antifungal compounds. Frontiers in Microbiology 12: 707609. DOI: https://doi.org/10.3389/fmicb.2021.707609

Journal of Plant Protection Research eISSN 1899-007X

ORGINAL ARTICLE

Identifying the key motif essential for enhancing plant defense against *Botrytis cinerea* by β-glucanase from *Bacillus velezensis* LJ02

Qi Sun[1], Tong Sun[1], Yujin Yuan[1], Ruokui Chang[2], Weiwei Yu[1], Xiaohui Qiu[1], Tianyi Wu[1], Anling Deng[1], Zhuoran Li[1], Yuanhong Wang[1]*

[1] Horticulture and Landscape Architecture, Tianjin Agriculture University, Tianjin, China
[2] College of Engineering and Technology Architecture, Tianjin Agriculture University, Tianjin, China

Vol. 64, No. 3: 263–264, 2024

DOI: 10.24425/jppr.2024.151258

Received: February 23, 2024
Accepted: April 10, 2024
Online publication: September 11, 2024

*Corresponding address:
wangyh@tjau.edu.cn

Responsible Editor:
Dzarifah Zulperi

SUPPLEMENTARY MATERIAL

The authors are fully responsible for both the content and the formal aspects of the supplementary material. No editorial adjustments were made.

Table S1. pET-28a(+)-β-glucanase cloning primers

Primer ID	Sequence
pET-28a(+)-β-glucanase-F	GGGTCGCGGATCCGAATTCATGTTTTATCGTATGAAACGAGTGC
pET-28a(+)-β-glucanase-R	GTGGTGGTGGTGGTGCTCGAGTTATTTTTTTGTATAGCG

Table S2. β-glucanase transient vector construction primers

Primer ID	Sequence
β-glucanase-F	ATATAACATTACGCCGAGGCCTATGTTTTATCGTATGAAAC
β-glucanase-R	CTTGTAATCATAGGGAAGAGGCCTTTATTTTTTTGTATAGCG

Table S3. β-glucanase mutants vector construction primers

Primer ID	Sequence
β N^{54}-F	CTTGCCGTCACTGGATTGTTTT
β N^{54}-R	CTCAAAAACAATCCAGTGACGGCAAGCAGCAGCACTCGTTTCA
β N^{110}-F	CCAATGGAGATATGTTCAACGCTAAAAACACAGGGATCGT
β N^{110}-R	ACGATCCCTGTGTTTTTAGCGTTGAACATATCTCCATTGGAA
β N^{161}-F	GTCAGAATGAAACCGGCTGTTGCGGATCTCGGATTT
β N^{161}-R	ATCCGAGATCCGCAACAGCCGGTTT
β N^{213}-F	CCATGAGAAGGTTGCGAATGGGATAGGTGTCGATGACTGGCT
β N^{213}-R	ACACCTATCCCATTCGCAACCTTCTC
β N^{243}-R	GTAATCATAGGGAAGAGGCCTTTAATTCCACAAGTTCATCATGAT

Table S4. The sequences of RT-qPCR primers

Primer ID	Sequence
PTI1-F	GACAGGTCAAGTGCGCTTCTTCG
PTI1-R	CAATAGTTGGTGCGCTCGTGGG
WRKY7-F	CAGCGCGTAAACATGTGGAAAGAG
WRKY7-R	CCAAAGAAGATCTGAGCCGTGGAG
WRKY8-F	CACAACCATCCAAAGCCGACTCAG
WRKY8-R	GGGTCTGGTTCTTTTGAGTCTGGTT
ACRE31-F	CCAGCCATCCACGATCCATTCATA
ACRE31-R	GAAGAAGACTTAAGTTTGCCCCTGG
CYP71D20-F	AAGGTCCACCGCACCATGTCCTTAGAG
CYP71D20-R	AAGAATTCCTTGCCCCTTGAGTACTTGC
FRK-F	AGCAGTTGACGGACAAAAGTG
FRK-R	TGTGTAGTTTTGCCCATTGG