Biochemical and Biophysical Research Communications 457 (2015) 627–634

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

BcGs1, a glycoprotein from *Botrytis cinerea*, elicits defence response and improves disease resistance in host plants

Yi Zhang, Yunhua Zhang, Dewen Qiu, Hongmei Zeng, Lihua Guo, Xiufen Yang*

The State Key Laboratory for Biology of Plant Disease and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Science, No. 12 Zhongguan-cun South Street, Beijing 100081, China

ARTICLE INFO

Article history:
Received 5 January 2015
Available online 19 January 2015

Keywords:
Necrotrophic fungi
Necrosis-inducing activity
Defence response
Disease resistance
Systemic acquired resistance

ABSTRACT

In this study, a necrosis-inducing protein was purified from the culture filtrate of the necrotrophic fungus *Botrytis cinerea* BC-98 strain. Secreted proteins were collected and fractionated by liquid chromatography. The fraction with the highest necrosis-inducing activity was further purified. A glycoprotein named BcGs1 was identified by 2D electrophoresis and mass spectrometry. The BcGs1 protein consisted of 672 amino acids with a theoretical molecular weight of 70.487 kDa. Functional domain analysis indicated that BcGs1 was a glucan 1,4-alpha-glucosidase, a cell wall-degrading enzyme, with a Glyco_hydro_15 domain and a CBM20_glucoamylase domain. The BcGs1 protein caused necrotic lesions that mimicked a typical hypersensitive response and H_2O_2 production in tomato and tobacco leaves. BcGs1-treated plants exhibited resistance to *B. cinerea*, *Pseudomonas syringae* pv. tomato DC3000 and tobacco mosaic virus in systemic leaves. In addition, BcGs1 triggered elevation of the transcript levels of the defence-related genes *PR-1a*, *TPK1b* and *Prosystemin*. This is the first report of a *Botrytis* glucan 1,4-alpha-glucosidase triggering host plant immunity as an elicitor. These results lay a foundation for further study of the comprehensive interaction between plants and necrotrophic fungi.

1. Introduction

Plants live in complicated natural settings with a broad range of beneficial and harmful microbes. Plants have evolved a variety of mechanisms to defend against pathogen invasion, collectively known as the plant immune system [1]. Plants use pattern recognition receptors (PRRs) localised to the plasma membrane that respond to microbe (pathogen)-associated molecular patterns (M/PAMPs) to trigger PAMP-triggered immunity (PTI), which is a primary plant immune response [2]. However, successful pathogens can deliver effectors that interfere with PTI and consequently trigger plant immune responses based on effector recognition by NB-LRR proteins, which is known as effector-triggered immunity (ETI) [3]. M/PAMP-induced immune responses have been shown to promote basal resistance of host plants against pathogens and are critical for maintaining non-host resistance [4].

The breakdown products of the plant cell wall are known as endogenic signals and elicit plant immune responses besides M/PAMPs or effector-mediated non-self recognition [5]. These products of host cell wall fragments produced by cell wall-degrading enzymes (CWDEs) are regarded as damage-associated molecular patterns (DAMPs), and many of these P/DAMPs can induce PTI. Necrotrophic fungi secrete CWDEs that depolymerise the primary cell wall polysaccharides and absorb nutrients from dead cells during the course of infection. Many CWDEs play dual roles in plant–pathogen interactions as triggers of plant immune responses and virulence factors. The xylanase xyn11A and endopolygalacturonase 1 (BcPG1) trigger defence responses and are required for virulence in *Botrytis cinerea* [6].

Programmed cell death (PCD), which is commonly observed in ETI in both incompatible and compatible plant–pathogen interactions, is ubiquitous [7]. By direct or indirect interaction with plant PRRs, PAMPs can also cause PCD. As a type of PCD, hypersensitive response (HR)-associated cell death is considered to restrict the ability of pathogens to obtain nutrients. Regulation of cell death and host factors including hormones and reactive oxygen species (ROS) plays an important role in plant immune responses [8].

An increasing number of studies have demonstrated that the interaction between necrotrophic pathogens and plants is more complicated than initially believed. In particular, the mechanism by which a single purified CWDE induces host basal defence has not

* Corresponding author. Fax: +86 10 82109959.
E-mail address: yangxiufen@caas.cn (X. Yang).

http://dx.doi.org/10.1016/j.bbrc.2015.01.038
0006-291X/

yet been studied. Similarly to some other filamentous fungi, *B. cinerea* secretes metabolites and proteins to destroy the plant cell wall, which contributes to invasion and absorption of nutrients, such as the small compounds botrydial and botcinolide, oxalic acid, enzymes with endopolygalacturonase activity and Nep1-like proteins (NLPs). Twenty of 89 secretory proteins from *B. cinerea* participate in plant cell wall degradation [9]. One extracellular protein, BcSpl1, can cause necrosis, cell autofluorescence, cytoplasm shrinkage, electrolyte leakage and generation of ROS, as well as the induction of defence genes when infiltrated into tomato, tobacco and Arabidopsis leaves [10].

Here, we first report the purification and characterisation of a necrosis-inducing glycoprotein elicitor, BcGs1, from the culture filtrate of the necrotrophic fungus *B. cinerea*. This protein contains a Glyco_hydro_15 domain and a CBM20_glucoamylase domain, which are highly conserved in fungi, and belongs to the fungal CWDE family. BcGs1 induces typical HR symptoms and systemic acquired resistance (SAR), which follows the defence responses. This study provides a reference for the interaction between necrotrophic pathogenic fungi and plants.

2. Materials and methods

2.1. Plants, pathogens and culture conditions

B. cinerea strain BC-98 was originally isolated from diseased tomato tissues at the Beijing Region, PR China, and was preserved at the China General Microbiological Culture Collection of China (CGMCC no. 7057). *B. cinerea* was cultured on potato dextrose agar (PDA plate at 22 °C for 12 days. The conidia were collected and diluted to 3×10^5 conidia/ml with sterile water (with 0.1% Tween 20)) for inoculation. Phytopathogenic bacteria (*Pseudomonas. syringae* pv. tomato DC3000) were grown on a NYGA Kan^{25} Rif^{100} medium plate overnight at 28 °C. Add dipping solution (10 mM $MgCl_2$) into plate, gently swirl the plate until all the bacteria come off the media, take out the suspension, add more dipping solution, measure and adjust the $OD_{600} = 0.05$. The common tobacco mosaic virus (TMV) strain that was used for the bioassay was kindly provided by the Beijing Academy of Agriculture and Forestry Sciences, Beijing, China. Tomato (*Solanum lycopersicum*) cultivar Zhongza-9 and tobacco (*Nicotiana tabacum* cv. Samsun NN) plants were grown at 24–26 °C, with a day/night period of 16/8 h in a phytotron.

2.2. Protein purification

The *B. cinerea* strain BC-98 was cultured at PDA plate and grown for 8 days in the dark at 25 °C. Mycelial agar discs that were taken from the active colony edge of BC-98 were inoculated in a liquid glucose/asparagine medium and cultured for 5 days in the dark at 25 °C, 180 rpm as described by Mahesh et al. [11]. The culture filtrate was centrifuged at 15,000 *g* for 30 min at 4 °C. The supernatant was collected and then subjected to ammonium sulphate precipitation (80% saturation). The solution was centrifuged at 20,000 *g* for 15 min at 4 °C again. The precipitate was dissolved in 20 mM HEPES, pH 7.0 buffer, and dialysed (25,000 Da molecular weight cut-off) at 4 °C for 2 days. The concentrate that was collected and diluted was passed through a 0.22 μm filter (Millipore, SuZhou, China) and loaded onto an anion-exchange chromatography column (HiTrap™ DEAE FF; GE Healthcare, Niskayuna, NY, USA) that was first equilibrated with 5 ml of 20 mM HEPES, pH 7.0 buffer, using the AKTA protein purification system (GE Healthcare, Piscataway, USA). Then, the bound proteins were eluted with several indicated ionic gradient of NaCl in elution buffer at a flow rate of 2 ml/min. The fractions were collected and subjected to a desalting column (GE Healthcare, Uppsala, Sweden) for protein activity analysis.

2.3. Two-dimensional electrophoresis and mass spectrum analysis

After chromatography on an anion-exchange column, the peak fraction showing the necrotic activity was performed for further study referring to Mahesh et al. [11]. Then a mass spectrum analysis was conducted to identify the proteins from a 2D electrophoresis gel. Referring to Shevchenko et al. [12], an approximate 80-kDa spot was excised for following study. The results of some peptides were used to research the matching protein in NCBI.

2.4. Characteristics of the protein

The protein concentration was measured using the BCA™ Protein Assay Kit (Pierce, Inc., Appleton, WI, USA). The GelCode Glycoprotein Staining Kit (Pierce) was used for glycoprotein identification. Horseradish peroxidase and soybean trypsin inhibitor were, respectively, positive and negative controls. The 12% SDS–PAGE was placed in periodic acid solution (mixed 3% acetic acid) for 60 min at room temperature and then dipped in distilled water for 60 min. Then, the gel was decolorised with 1% sodium sulphite after staining with Schiff's reagent for 1 h.

2.5. Necrosis-inducing activities of BcGs1

5-6-week-old tomato and tobacco leaves were infiltrated with BcGs1 (1 μM, 20 μl) using a 1-ml syringe without a needle. After 24 h, necrotic symptoms were observed in the infiltrated area. To determine the minimum concentration for necrosis, a serially diluted protein solution (3 μM, 1 μM, 500 nM, 250 nM, 125 nM and 50 nM) was injected into tobacco and tomato leaves. Bovine serum albumin (BSA) and 20 mM HEPES buffer at the same concentration and/or volume were also included as controls. The experiments were repeated at least three times under the same conditions.

2.6. H_2O_2 production assays

According to Fitzgerald et al. [13], 5-week-old tobacco leaves were examined 6 h after infiltration with BcGs1 (1 μM, 20 μl) or buffer in the underside. The formation of H_2O_2 was assayed in BcGs1-infiltrated tobacco leaves with nitro 3,3′-diaminobenzidine (DAB).

2.7. Bioassay for BcGs1-induced systemic resistance in tobacco

5-6-week-old tobacco leaves were infiltrated with BcGs1 solution (250 nM, 20 μl), 3 days post-infiltration, the upper non-treated leaves were inoculated with the TMV by friction vaccination using a brush. Each treatment was performed with 15 plants and was repeated three times. Likewise, 20 mM HEPES buffer, which was used to the dissolve protein BcGs1, was included at the same volume as a control. The number of TMV lesions in every leaf was recorded on the third day post-TMV infection and the percentage of lesion inhibition was calculated [14].

2.8. Induction of SAR in tomato against Pseudomonas syringae pv. tomato DC3000

Fully mature 4-5-week-old tomato plant leaves were infiltrated with BcGs1 (250 nM, 20 μl) at one side of the central vein and cultured in the phytotron for 3 days. Then upper three non-treated (systemic) leaves were soaked in *P. syringae* pv. tomato DC3000 suspension with gentle swirling for 30 s. At 3 days after challenge

with *P. syringae* pv. tomato DC3000, leaves were detached from the tomato and sterilised to remove epiphytic bacterial populations. Three samples were collected from each leaf with a sterilised hole punch and ground with a pestle in 100 μl sterilised water. The sample suspensions were vortexed completely and serially diluted to 10^{-4}. The bacteria were spread on a NYGA Kan^{25} Rif^{100} plate and grown for 48 h at 28 °C. The number of the colonies on each plate was counted, and the degree of infection was calculated.

$$\text{Degree of infection}(\%) = (\text{number of colonies on plate with treated leaves}) \times /\text{number of colonies on plant with control leaves}] \times 100.$$

2.9. Induction of disease resistance against grey mould in tomato

6-8 leaves of tomato plants were sprayed with 500 nM BcGs1 protein solution (5 ml), and HEPES buffer (20 mM) was used as a control. 3 days post-BcGs1 treatment, plants were sprayed with a *B. cinerea* spores suspension at a concentration of 3×10^5 conidia/ml, and then incubated in 100% humidity chambers for 48 h. The disease intensity was recorded on the 7th day post-inoculation, and the induced disease resistance was calculated. Fifteen plants were used for each treatment, and each experiment was repeated three times. The disease reduction was calculated using the following formulas:

$$\text{Disease index} = [\Sigma(\text{disease score} \times \text{number of infected leaves for each score}) \times /\text{total number of inoculated leaves} \times \text{highest score}] \times 100.$$

$$\text{Disease reduction}(\%) = [(\text{Disease index of control} - \text{Disease index of treatment}) \times /\text{Disease index of control}] \times 100\%.$$

2.10. Quantitative real-time PCR

5-week-old tomato plants were infiltrated with BcGs1 and control plants were infiltrated with buffer. The total RNA was extracted from upward-facing systemic leaves at the indicted time using a plant RNA Kit (TransGen Biotech, Beijing, China). First-strand cDNA was synthesised from 0.5 μg of total RNA using SuperMix for qPCR (TransGen). The synthesised cDNA was used as a template for qPCR. The amplification was performed in an iQ5 Real-Time PCR system with SYBR Green supermix (the primers are listed in Table 1) using the same method and some primers as reported previously [15,16]. Actin was used as an internal reference to normalise the amount of RNA in each reaction. Triplicates were necessary for all of the biological replicate reactions to calculate the average values for quantification. Then, the relative mRNA quantities were calculated from the average values using the ΔΔCt method [17].

Table 1
Primer sequences that were used for qPCR in this study.

Names	Forward primers	Reverse primers
PR-1a	5′-GAGGGCAGCCGTGCAA-3′	5′-CACATTTTTCCACCAACACATTG-3′
TPK1b	5′-GAATGGCTGGCAGAAGTTA-3′	5′-TTCGTCCTCCAAGCAGTA-3′
Prosystemin	5′-GATGGAAGGGGAGGGTG-3′	5′-CGCTTTGATGGAGGTTTTG-3′
Actin	5′-GGTGTGATGGTGGGTATGG-3′	5′-GCTGACAATTCCGTGCTC-3′

3. Results

3.1. Purification of BcGs1, 2D electrophoresis and detection of partial amino acid sequences

The pre-processed *B. cinerea* supernatant was fractionated with an anion exchange chromatography HiTrap™ DEAE FF 5-ml column. The column was previously equilibrated with 20 mM HEPES (pH 7.0) and then eluted with five different NaCl gradients in 20 mM HEPES (pH 7.0). The peaks were collected and injected into tobacco and tomato leaves for the necrosis assay. Peak D1 (Fig. 1A) was selected for further study because it had necrosis activity and showed a single band on SDS-PAGE with a relative apparent molecular weight of 80 kDa (Fig. 1B). We designated this protein BcGs1. To identify the BcGs1 protein, two-dimensional (2D) electrophoresis was performed, which generated only a single spot on the gel (Fig. 1C). Liquid chromatography-mass spectrometry analysis was performed to determine the amino acid sequence of BcGs1, the spot that was excised from the gel. A battery of polypeptides was generated by MALDI-TOF and de novo sequencing. Three primary amino acid sequences, SVDSFIATESPIAFR, VSPDYFYTWTR and SAFTGSWGRPQR, were obtained. The protein showed high identity with the conserved hypothetical protein BC1G_04151 (GenBank accession no. XM_001557491) from *B. cinerea* B05.10. The hypothetical protein consisted of 672 amino acids encoded by 2019 bp, with a calculated molecular weight of 70.487 kDa and isoelectric point of 5.39. Functional domain analysis indicated that BcGs1 was a glucan 1,4-alpha-glucosidase and belonged to the glycosyl hydrolase family 15 of protein hydrolases, with a Glyco_hydro_15 domain and a CBM20_glucoamylase domain (Fig. 1D).

3.2. Characteristics of the BcGs1 protein

Glycoprotein identification was performed by glycoprotein-specific staining. All of the samples produced bands upon Coomassie brilliant blue staining and SDS-PAGE (Fig. 2A). However, in the SDS-PAGE with glycoprotein-specific staining, only the tested protein BcGS1 produced a band that was the same as the positive control (Fig. 2B), demonstrating that this 80-kDa protein was a glycoprotein.

3.3. Necrotising activity on tomato and tobacco leaves

To check whether BcGs1 was able to induce necrosis on host plants, 1 μM BcGs1 solution (20 μl) was infiltrated into tobacco and tomato leaves and its effect was recorded for several days. An apparent necrotic zone appeared in the infiltration area at 24 h post-infiltration (Fig. 3A), while there was no necrosis on leaves that were infiltrated with buffer or the control protein BSA at the same concentration. The earliest necrotic symptoms in tobacco and tomato leaves appeared 1 h after infiltration (data not shown). To determine the lowest protein concentration that induced necrosis in the plants, different BcGs1 concentrations were infiltrated into tobacco and tomato leaves. This demonstrated that 250 nM and 125 nM concentrations could induce obvious necrosis 24 h post-infiltration, while 125 nM and 50 nM were the lowest doses that

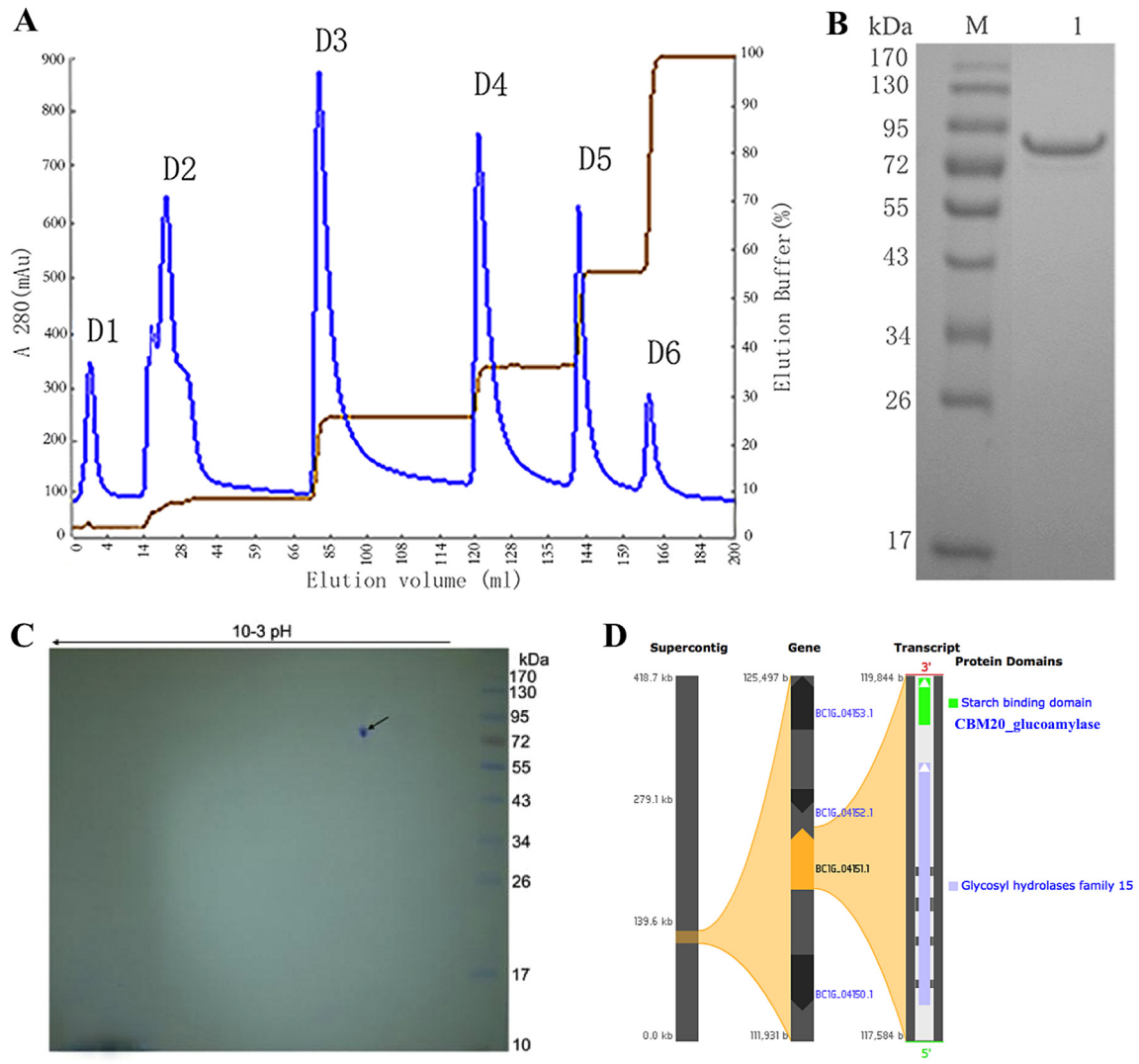

Fig. 1. Purification of the protein from the culture filtrates of *B. cinerea* and identification of the purified protein BcGs1. (A) Elution profile of the protein extracts with necrosis-inducing activity that were applied to a HiTrap™ DEAE FF column. Six peaks were obtained, of which peak D1 induced necrosis activity. (B) SDS-PAGE of the purified protein. M. protein molecular weight marker, 1. Protein BcGS1, showing a single band stained with Coomassie Brilliant Blue R-250. (C) 2-D electrophoresis of the purified protein as visualised by Coomassie brilliant blue staining. The arrow shows the positions of the single protein spot. (D) Protein domain Info was searched from the *Botrytis cinerea* database (http://www.broadinstitute.org/annotation/genome/botrytis_cinerea).

induced appreciable necrosis 3 days post-infiltration in tobacco and tomato, respectively (Fig. 3B and C).

The ability of BcGs1 to induce the production of ROS in the infiltrated leaves was also studied by determining the production of hydrogen peroxide (H_2O_2) with DAB, since H_2O_2 is a hallmark of the HR. Histological imaging showed dark brownish-red precipitate staining (indicated by arrows) in BcGs1-treated leaves but not in non-infiltrated or water-infiltrated leaves (Fig. 3D).

3.4. Induction of disease resistance and expression of defence-related genes

BcGs1 strongly induced SAR in the BcGs1-treated tobacco and tomato plants. The number of TMV lesions in the SAR leaves of BcGs1-treated tobacco plants was significantly lower than that of buffer-treated tobacco plants. The number of TMV lesions was reduced by 46.99% in BcGs1-treated leaves (Fig. 4A_1 and A_2). BcGs1-treated plants showed a significant reduction in the disease index of tomato grey mould caused by *B. cinerea* on tomato leaves; the reduction in the disease index was 61.42% in BcGs1-treated plants (Fig. 4B). In addition, BcGs1 also improved resistance to the phytopathogenic bacteria *P. syringae* pv. tomato DC3000. The degree of infection of plants that were treated with BcGs1 solution was as low as 45.1% (Fig. 4C). To characterise the defence mechanism of tomato, plants that were infiltrated with BcGs1 were assayed for the induction of three genes: (1) the well-known SAR marker *PR-1a*, a pathogenesis-related gene; (2) *TPK1b*, which plays a signalling role in an ET-mediated defence mechanism for resistance to necrotrophic fungi and herbivorous insects and is required for resistance to *Botrytis* independent of JA biosynthesis; and (3) *Prosystemin*, which produces the precursor of the polypeptide systemin, an elicitor of JA signalling in tomato. BcGs1 application resulted in the up-regulation of all three genes. A 51-fold increase in *PR-1a* expression was observed in the treated plants (Fig. 4D_1). The maximum level of the *TPK1b* gene increased by 4-fold at 6 h

post-infiltration (Fig. 4D$_2$). In addition, the *Prosystemin* mRNA level increased by 2-fold 6 h after infiltration in BcGs1-treated plants compared with untreated plants (Fig. 4D$_3$).

4. Discussion

In interactions between plants and pathogens, the plant cell wall plays a significant role in protecting plants from pathogen attack. *B. cinerea*, like other phytopathogenic fungi, secretes many enzymes including CWDEs such as pectinases, cellulases, and xylanases, as well as cutinases, lipases, and proteases [18], to degrade the plant's defencive barriers during the infection process. Here, we report the purification and identification of an 80-kDa glycoproteinaceous elicitor from the culture medium of *B. cinerea* that triggers plant immunity in tobacco. Three peptide micro sequences obtained after trypsin digestion led to its identification as glucan 1,4-alpha-glucosidase (BcGs1). BcGs1-treated tomato and tobacco cells showed rapid production of ROS and accumulation of defence gene transcripts. These results indicate that BcGs1 activates signal transduction events involved in plant responses to elicitors. Although there are a variety of reports on diverse secretory proteins and enzymes from *B. cinerea* that activate defence responses in host plants, this is the first investigation of a glucan 1,4-alpha-glucosidase inducing host plant resistance.

BcSpl1 is one of the most abundant proteins in the *B. cinerea* secretome and elicits the hypersensitive response in hosts. A minimum dose of 17 μM BcSpl1 induces observable necrosis 4 h after infiltration of tobacco leaves, while 1 μM BcGs1 protein induces obvious necrosis 1 h post-infiltration (data not shown); even concentrations as low as 125 nM could induce appreciable necrosis 3 days post-infiltration (Fig. 3B). These results imply that BcGs1 induces necrosis more efficiently and rapidly than the elicitor BcSpl1 [10] in tobacco.

The elicitor activity of CWDEs is not only due to the release of plant cell wall breakdown products through enzyme activity, but also the recognition of specific motifs of the CWDEs. BcPG1 is a well-known elicitor and virulence factor participating in *B. cinerea* pathogenicity. Instead of its enzyme activity, the elicitor activity comes from the BcPG1 protein itself, although elicitor activity can be derived from oligogalacturonides that are released by the enzyme [19]. The BcGs1 protein we identified in this study belongs to the glycosyl hydrolase family 15 and contains a CBM20_glucoamylase. Which domain or specific motif of BcGs1 is involved in defence responses and whether BcGs1 activity is required for the infection process of *B. cinerea* is still unclear. In addition, how plants recognise BcGs1 and how BcGs1 triggers defence signal transduction cascades is also unknown.

Our laboratory has isolated elicitors from *Magnaporthe oryzae*, *Alternaria tenuissima* and *Verticillium dahliae*, such as Mohrip1, Hrip1 and PevD1. All of these elicitors can induce host plant SAR against broad-spectrum diseases. We report here that BcGs1-infiltrated tobacco and tomato plants exhibited systemic resistance to TMV, *B. cinerea* and *P. syringae* pv. tomato DC3000. Thus, BcGs1 appears to induce host plant broad-spectrum resistance.

Salicylic acid (SA) and jasmonic acid (JA) are secondary messengers in the regulation of signalling networks involved in PTI and ETI. The SA and JA signalling pathways can be either antagonistic or synergistic, resulting in negative or positive functional outcomes. In our study, a well-established SAR marker, *PR-1a*, which is a downstream defence response gene that is indirectly activated by SA, showed a 51-fold increase in transcript level in BcGs1-treated tomato plants compared with untreated plants. BcGs1 also increased the *Prosystemin* transcript level. *Prosystemin* encodes a 200-amino acid precursor of systemin, a known elicitor of JA signalling in tomato. More interestingly, the expression of the gene *TPK1b*, which plays a signalling role in an ET-mediated defence mechanism for resistance to necrotrophic fungi and is independent of JA biosynthesis, also increased to a certain degree. These results indicate that BcGs1 triggers the host plant defence response through a multi-signal pathway. However, which signalling pathway is more important and the exact relationships among the SA, JA and ET signalling remain unclear and require further study.

In summary, our results demonstrate that the glycoprotein BcGs1 produced by *B. cinerea* has elicitor activity. Purified BcGs1 induces necrosis in tobacco and tomato leaves, improves host disease resistance, and elevates the expression of defence genes. Our data provide a significant advance in our understanding of the

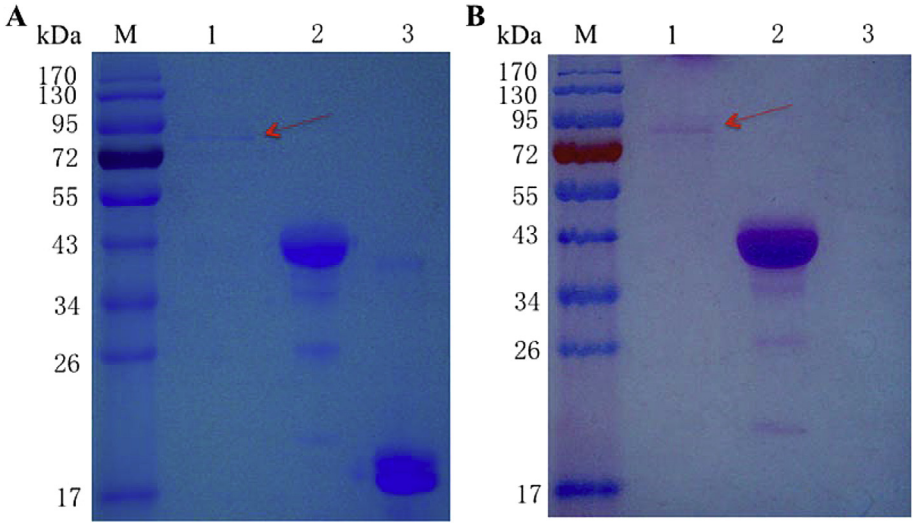

Fig. 2. Characteristics of the protein BcGs1. (A) SDS-PAGE of BcGs1 illustrating the bands of all of the samples with Coomassie brilliant blue staining. M. protein molecular weight marker, 1. protein BcGs1, 2. horseradish peroxidase, 3. Soybean trypsin inhibitor. (B) The gel from (A) was treated with periodic acid solution and then dipped in distilled water. Then, the gel was decolorised with 1% sodium sulphite after staining with Schiff's reagent.

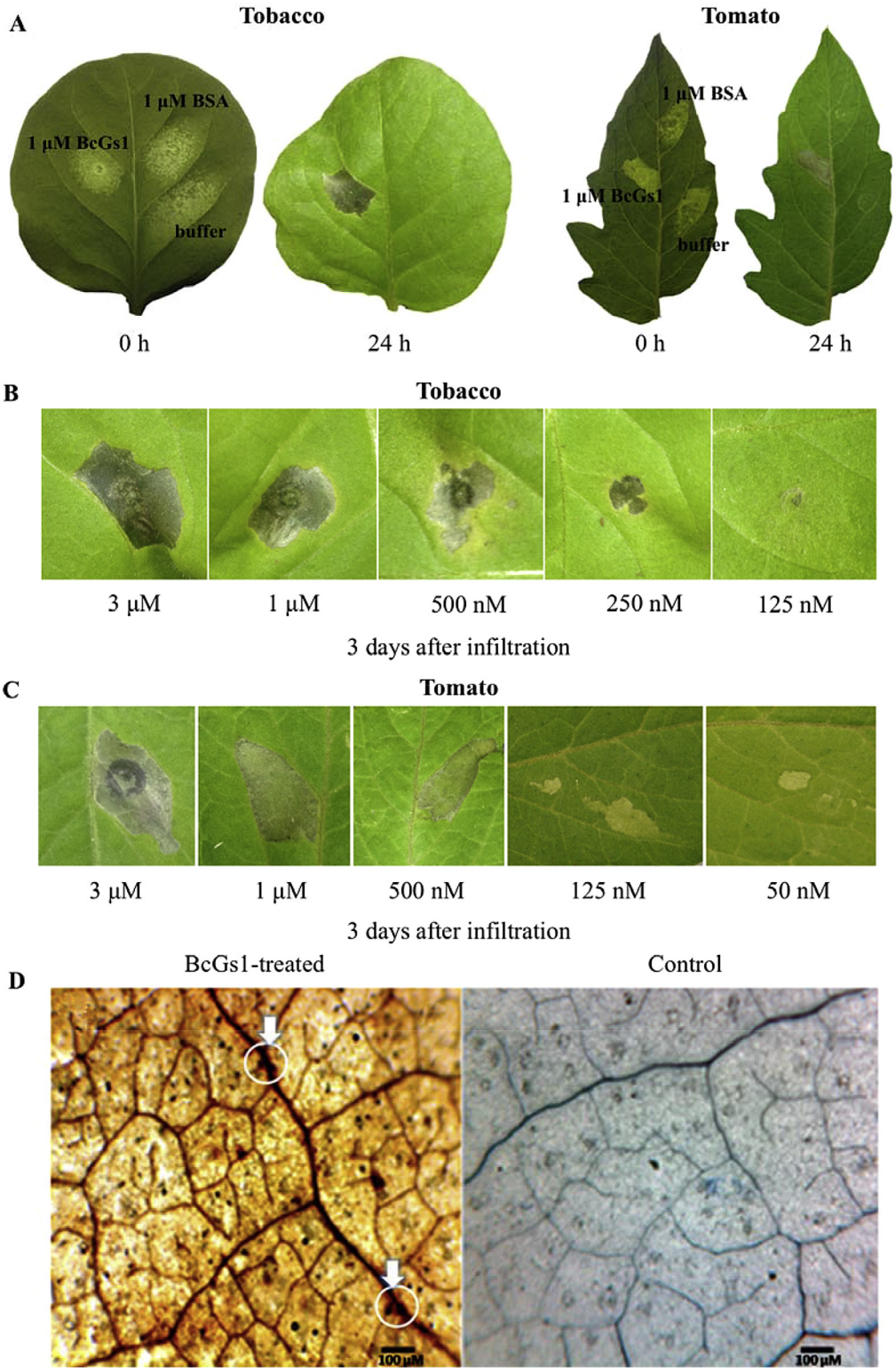

Fig. 3. Induction of necrosis and accumulation of H_2O_2 as induced by BcGs1 in plant leaves. (A) Tobacco and tomato leaves were infiltrated with the indicated protein solutions, and BSA as controls. Pictures were taken with back illumination to illustrate the infiltration area (0 h). After 24 h, pictures were taken to illustrate the effect in the leaves. (B) and (C) Effect of the infiltration of different BcGs1 concentrations on tobacco and tomato leaves. The pictures were taken 3 days post-infiltration, depending on the different protein concentrations. (D) The accumulation of H_2O_2 in tobacco leaf. The treated tissues were harvested 6 h after treatment, and the excised leaf sections were infiltrated with a solution of DAB. In the presence of H_2O_2, DAB polymerised, forming a brownish-red precipitate staining the leaf vines.

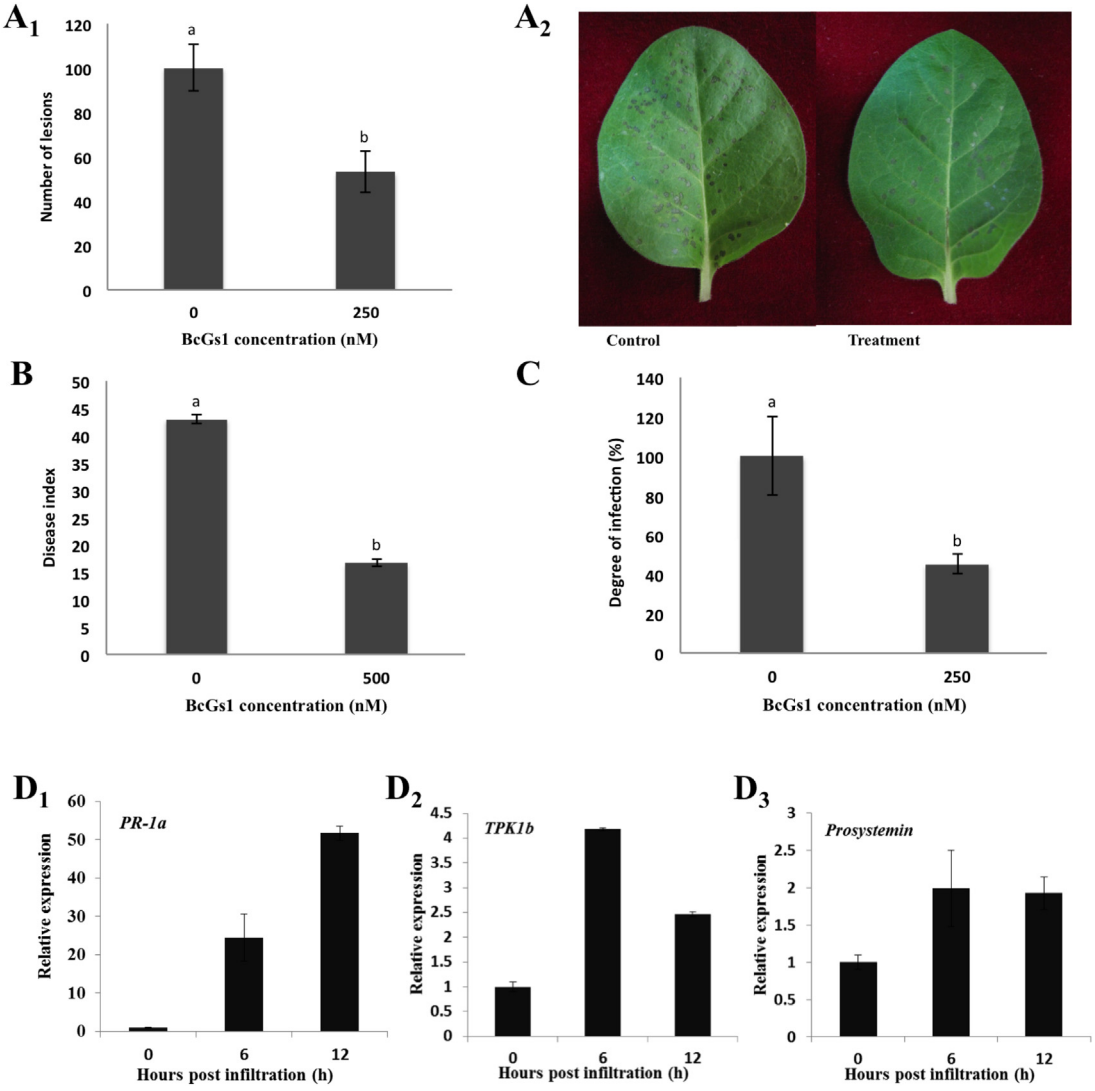

Fig. 4. BcGs1 induces significant resistance in tobacco against TMV, in tomato against *B. cinerea*, and *P. syringae* pv. tomato DC3000 and expression levels of tomato *PR-1a*, *TPK1b* and *Prosystemin* relative to *Actin*. (A_1) Tobacco plants were infiltrated with BcGs1 solution in one of the leaves and, the upper non-treated leaves were inoculated with TMV. (A_2) Systemic acquired resistance to TMV infection in BcGs1-treated tobacco plants. One of leaves per plant were infiltrated with the BcGs1 or buffer, the upper leaves were inoculated with TMV 3 days later. SAR leaves were photographed 3 d after inoculation of TMV. (B) Induced tomato plants resistance to *B. cinerea* in different concentration BcGs1 solution. (C) The degree of infection of *P. syringae* pv. tomato DC3000 to tomato plants that were infiltrated with BcGs1 solution. (D_1), (D_2) and (D_3) show the relative expression of *PR-1a*, *TPK1b* and *Prosystemin,* respectively.

interaction between necrotrophic fungi and plants. Knowledge of BcGs1, as an elicitor, may lead to strategies for the control of necrotrophic pathogens in crop production.

Conflict of interest

The authors including Yi Zhang, Yunhua Zhang, Dewen Qiu, Hongmei Zeng, Lihua Guo and Xiufen Yang have no competing interests to declare.

Acknowledgments

This research was supported by the National High Technology Research and Development Program (“863” Program) of China (2011AA10A205).

References

[1] F. Katagiri, K. Tsuda, Understanding the plant immune system, Mol. Plant. Microbe Interact. 23 (2010) 1531–1536.
[2] P.N. Dodds, J.P. Rathjen, Plant immunity: towards an integrated view of plant–pathogen interactions, Nat. Rev. Genet. 11 (2010) 539–548.
[3] B.P. Thomma, T. Nurnberger, M.H. Joosten, Of PAMPs and effectors: the blurred PTI-ETI dichotomy, Plant Cell. 23 (2011) 4–15.
[4] C. Zipfel, K. Kunze, D. Chinchilla, et al., Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts agrobacterium-mediated transformation, Cell 125 (2006) 749–760.
[5] S. Vorwerk, S. Somerville, C. Somerville, The role of plant cell wall polysaccharide composition in disease resistance, Trends Plant Sci. 9 (2004) 203–209.
[6] A. Brutus, L.B. Recall, S. Herga, et al., A family 11 xylanase from the pathogen *Botrytis cinerea*is inhibited by plant endoxylanase inhibitors XIP-I and TAXI-I, Biochem. Biophys. Res. Commun. 337 (2005) 160–166.
[7] O.J. Desmond, J.M. Manners, A.E. Stephens, et al., The *Fusarium* mycotoxin deoxynivalenol elicits hydrogen peroxide production, programmed cell death and defence responses in wheat, Mol. Plant Pathol. 9 (2008) 435–445.
[8] F.R. Rossi, A. Garriz, M. Marina, et al., The sesquiterpene botrydial produced by *Botrytis cinerea* induces the hypersensitive response on plant tissues and its action is modulated by salicylic acid and jasmonic acid signaling, Mol. Plant. Microbe Interact. 24 (2011) 888–896.

[9] P. Shah, A. James, I.I.I. Atwood, et al., Comparative proteomic analysis of *Botrytis cinerea* secretome, J. Proteome Res. 8 (2008) 1123–1130.
[10] M. Frías, C. González, N. Brito, BcSpl1, a cerato-platanin family protein, contributes to *Botrytis cinerea* virulence and elicits the hypersensitive response in the host, New. Phytol. 192 (2011) 483–495.
[11] K. Mahesh, L. Hua, Z. Yuliang, et al., Hrip1, a novel protein elicitor from necrotrophic fungus, *Alternaria tenuissima*, elicits cell death, expression of defence-related genes and systemic acquired resistance in tobacco, Plant Cell Environ. 35 (2012) 2104–2120.
[12] A. Shevchenko, H. Tomas, J. Havlis, et al., In-gel digestion for mass spectrometric characterization of proteins and proteomes, Nat. Protoc. 1 (2006) 2856–2860.
[13] H.A. Fitzgerald, M.S. Chern, R. Navarre, et al., Overexpression of (At) NPR1 in rice leads to a BTH- and environment induced lesion-mimic/cell death phenotype, Mol. Plant. Microbe Interact. 17 (2004) 140–151.
[14] J. Mao, Q. Liu, X. Yang, et al., Purification and expression of a protein elicitor from *Alternaria tenuissima* and elicitor-mediated defence responses in tobacco, Ann. Appl. Biol. 156 (2010) 411–420.
[15] M.E. Oirdi, T.A.E. Rahman, L. Rigano, et al., *Botrytis cinerea* manipulates the antagonistic effects between immune pathways to promote disease development in tomato, Plant Cell. 23 (2011) 2405–2421.
[16] M.A.B. Herman, S. Restrepo, C.D. Smart, Defense gene expression patterns of three SAR-induced tomato cultivars in the field, Physiol. Mol. Plant Pathol. 71 (2008) 192–200.
[17] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative C(T) method, Nat. Protoc. 3 (2008) 1101–1108.
[18] B. Blanco-Ulate, A. Morales-Cruz, K.C.H. Amrine, et al., Genome-wide transcriptional profiling of *Botrytis cinerea* genes targeting plant cell walls during infections of different hosts, Front. Plant Sci. (2014), http://dx.doi.org/10.3389/fpls.2014.00435.
[19] B. Poinssot, E. Vandelle, M. Bentéjac, et al., The endopolygalacturonase 1 from *Botrytis cinerea* activates grapevine defense reactions unrelated to its enzymatic activity, MPMI 16 (2003) 553–564.