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BcIEB1, a *Botrytis cinerea* secreted protein, elicits a defense response in plants

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ABSTRACT

BcIEB1 is a very abundant protein in the secretome of *Botrytis cinerea* but it has no known function and no similarity to any characterized protein family. Previous results suggested that this protein is an elicitor of the plant defense system. In this work we have generated loss-of-function *B. cinerea* mutants lacking BcIEB1 and we have expressed the protein in yeast to assay its activity on plants. Analysis of the Δ*bcieb1* mutants did not result in any observable phenotype, including no difference in the virulence on a variety of hosts. However, when BcIEB1 was applied to plant tissues it produced necrosis as well as a whole range of symptoms: inhibition of seedling growth in *Arabidopsis* and tobacco, ion leakage from tobacco leaf disks, a ROS burst, cell death and autofluorescence in onion epidermis, as well as the expression of defense genes in tobacco. Moreover, tobacco plants treated with BcIEB1 showed an increased systemic resistance to *B. cinerea*. A small 35-amino acids peptide derived from a conserved region of BcIEB1 is almost as active on plants as the whole protein. These results clearly indicate that BcIEB1 elicits plant defenses, probably as a consequence of its recognition as a pathogen associated molecular pattern.

1. Introduction

The fungus *Botrytis cinerea* is a necrotrophic plant pathogen able to infect more than 1400 plant species causing major losses in agriculture worldwide [1], and has been rated second among the top ten fungal pathogens according to its economic and scientific importance [2]. The infection strategy of this pathogen is more complex than previously anticipated, and it is generally accepted that *B. cinerea* carries out subtle manipulations of the plant defense system. As a result of this interaction, plant cells undergo programmed cell death (PCD), facilitating the growth of this necrotrophic fungus in the dead plant tissue [3]. The molecular dialogue between *B. cinerea* and its host, necessary for these manipulations, is largely unknown, although some key factors have been identified especially on the plant side [3]. Meanwhile, the fungus is believed to secrete factors with the purpose of inducing cell-death in the plant, and to modulate this response so that it is not restricted to the site of infection but expands to neighbouring areas [4]. Although these factors are classically considered of proteinaceous nature, the recent finding that *B. cinerea* secretes small RNA molecules able to silence defense genes in plants [5] opens new possibilities.

PCD can occur in plants, among other things, as a defense mechanism against pathogens, and in that case it is known as the Hypersensitive Response (HR). *B. cinerea* induces HR in its hosts, but due to its necrotrophic nature, this plant response contributes to invasion by the pathogen rather than to plant defense [3,6]. This kind of response is usually considered part of the effector-triggered immunity (ETI), a second line of defense more specialized than pattern triggered immunity (PTI) and that is induced by the perception of pathogen-specific effectors. On the other hand, PTI is believed to be induced by the perception of pathogen/molecular associated molecular patterns (P/MAMPs) or markers of danger released from plant own molecules during an attack, that is, damage associated molecular patterns (DAMPs). However, PTI and ETI have been proposed to be not so distinct [7], so that PTI in response to PAMPs can be strong enough to result in cell death.

B. cinerea secretes a high number of proteins with diverse functions to the extracellular medium, about one third of them with unknown function [4]. Some of these proteins have been shown to produce necrosis when applied to plants in an isolated form, including the proteins BcNEP1 and 2 (*B. cinerea* Necrosis and Ethylene-inducing Proteins 1 and 2) [8], the xylanase Xyn11A [9], the cerato-platanin family protein BcSpl1 [10], several endopolygalacturonases [11], and the glucoamylase BcGs1 [12]. In some cases, symptoms triggered in plants by these proteins conform to the typical plant immune response and thus point to their

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recognition as PAMPs by plant pattern receptors. This is the case of NEPs [13], Xyn11A [9], BcSpl1 [10], and BcGs1 [12]. Indeed these receptors have been identified for NEPs [14], endopolygalacturonases [11] and xylanases [15]. Moreover, for two of these necrosis-inducing proteins a contribution to plant pathogenesis has been shown. The xylanase Xyn11A, on one hand, displays separate and independent xylan-degrading and necrotizing activities and has been shown to contribute to virulence with the latter [9]. On the other hand, the cerato-platanin family protein BcSpl1 is a protein with a putative role as extensin in the fungal cell wall [16], in which a small two-peptide motif in the protein surface accounts for the necrotizing activity and the contribution of this protein to *B. cinerea* virulence [17]. It seems therefore that these proteins are all able to induce PTI and cell-death in the host and could make a contribution to the infection strategy of *B. cinerea*. The abundance of such proteins makes it difficult to observe a decrease in the virulence of the corresponding single knock-out mutants, and could explain why only a very modest decrease has been observed in no more than a couple of these mutants [10,18]. To make things even more complicated, *B. cinerea* secretes two secondary metabolites, botrydial and oxalic acid, that have been proposed to elicit plant defenses culminating in HR [19,20] and could therefore also contribute to the generation of dead plant tissue during infection.

One interesting protein among the components of the *B. cinerea* secretome is BcIEB1 [21]. This protein has no known function so far and no similarity with any characterized protein family. It is the second most abundant protein in the early secretome [22] and has also been found often in other studies about the secretome composition [22–28]. BcIEB1 was also identified *in silico* as having two Ser/Thr-rich regions [29], which are presumably target sites for the *O*-glycosylation machinery, and was later shown experimentally to be actually glycosylated [21]. Overexpression of BcIEB1 in *B. cinerea* resulted in an increased ability of the culture medium to elicit plant defenses, assayed by seedling growth inhibition [21], pointing to a possible role of this protein as another elicitor of plant defenses.

Here we report a more detailed study of this protein in which we have generated *B. cinerea* mutants lacking BcIEB1 and we have expressed the protein in *Pichia pastoris*. We provide evidence, for the first time, that this protein elicit plant defenses, probably because of the recognition of a small conserved peptide as a PAMP by the plant immune system.

2. Materials and methods

2.1. General methods, organisms, and growth conditions

B. cinerea strains used in this work were the wild-type strain B05.10, the BcIEB1-overexpressing strain B05.10 (BcIEB1), and the Δ*bcieb1* knock-out mutants described below. These strains were maintained as conidial suspensions in 15% glycerol at −80 °C for long storage. For routine use, fungal strains were maintained at 4 °C in silica gel [30]. *B. cinerea* was grown in YGG medium (0.5% Yeast Extract, 2% glucose, 0.3% Gamborg B5 from Duchefa biochemie), 1% Malt Extract (Fluka), 0.3% Gamborg B5 (0.3% Gamborg B5, 10 mM Sucrose, 10 mM KH_2PO_4), or 25% tomato (25% ripe tomato fruits in water), as indicated. When necessary, agar was added at a concentration of 1.5%. Plants (tobacco cv. Havana, tomato cv. Moneymaker, and *Arabidopsis thaliana* Col-0 and *bak1-5*) were maintained in a growth chamber at 22 °C, 70% humidity, and with a light/dark cycle of 14 h light/10 h dark.

PCR amplifications were made with Phusion High-Fidelity DNA Polymerase (New England Biolabs, USA) when the DNA product was to be used in cloning experiments, and *Taq* polymerase (GenScript, China) was used in any other case. All oligonucleotides used (Table S1 in the Supplementary material) were from Life Technologies (USA). The 35-aa peptide ieb35 was chemically synthetized by GenScript.

Statistical analysis were carried out with the statistical analysis package SPSS 17.0 (IBM, Armonk, NY, USA), details of the tests applied are described in figure legends for each experiment.

2.2. Generation of Δbcieb1 mutants

In order to obtain the mutant strains deleted in the *bcieb1* gene, a plasmid was constructed carrying the hygromycin resistance cassette flanked by the 5′ and 3′ regions of *bcieb1* (Fig. S1 in the Supplementary material). The oligonucleotides IGE-IN5-FW-HIND and IGE-IN5RV-RV (Table S1 in the Supplementary material) were used to amplify a 534-bp 5′ region of the gene from *B. cinerea* B05.10 genomic DNA, and the product was cloned in the *Hind*III and *Eco*RV sites of pLOB1, a plasmid that contains a hygromycin resistance cassette (GenBank accession no. AJ439603) in a pUC18 background. On the other hand, the oligonucleotides IGE-3END-FW-XBA and IGE-3END-RV-SMA were used to amplify a 558-bp 3′ region of *bcieb1* and the product was cloned in the *Xba*I and *Sma*I sites, at the other end of the resistance cassette. *B. cinerea* B05.10 protoplasts were transformed with the resulting plasmid, pLOB-IgE-INTR. To get homokaryons, some of the hygromycin resistance transformants obtained were purified by the isolation of single germinating conidia. To ensure the site-directed integration of transforming DNA, a PCR reaction was done on genomic DNA from all transformants using primers CHECK RV, which binds to the hygromycin resistance cassette, and IGE-INT-DIR, which binds to a genomic region in *bcieb1* not included in the transforming DNA. A second PCR reaction was performed to ensure homokaryosis with primers IGE-IN5-FW-HIND and IGE-3END-RV-SMA, which bind to *bcieb1* at both sides of the integration site for the hygromycin resistance cassette. Two bona fide mutants, Δ*bcieb1*-2P2 and Δ*bcieb1*-2P3, showed only the expected bands (Fig. S1 in the Supplementary material). The disruption of *bcieb1* in these two mutant strains was also confirmed by Southern blot analysis, and no additional ectopic integrations were detected by hybridization with a probe specific for the *bcieb1* gene (Fig. S1 in the Supplementary material). Southern blots were carried out with digoxigenin labeled probes using the DIG DNA Labeling and Detection kit (Roche Applied Science, Germany).

2.3. Pathogenicity tests and phenotypic study

To assay virulence of mutant *B. cinerea* strains, plant tissues were inoculated with 5 μl of a suspension containing 5×10^6 conidia/ml in TGGK (60 mM KH_2PO_4, 10 mM glycine, 0.01% Tween 20, 0.1 M glucose) or with mycelia in agar plugs (0.2-cm YGG-agar cubes). In the case of plugs, 20 μl of sterile water were added over each plug to facilitate adherence. The infected plant material was incubated at 22 °C under conditions of high humidity on water-soaked filter paper in closed containers, and lesions at different timepoints were photographed. Where possible, quantitative results are presented as the growth rate of the lesion radius (cm per day), calculated for each individual infection from three measures taken at different days. To make these measurements, the shape of the lesions was approximated to an ellipse for which the two radii were measured, and lesion sizes were calculated as the geometrical mean of these two radii, that is, as the radius of a hypothetical circle with the same area as the ellipse.

Growth rates on different media, adhesion force to plant surfaces, production of conidia and sclerotia, and amount of extracellular matrix were assayed as previously described [31]. Microscope observations were carried out with an Olympus BX-50 fluorescence microscope, equipped with a U-MWIB filter if plant autofluorescence was to be detected.