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The phytotoxic activity of the cerato-platanin BcSpl1 resides in a two-peptide motif on the protein surface

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SUMMARY

Cerato-platanin family proteins are secreted and have been found in both the fungal cell wall and the extracellular medium. They elicit defence responses in a variety of plants and have been proposed to be perceived as pathogen-associated molecular patterns (PAMPs) by the plant immune system, although, in the case of the necrotroph *Botrytis cinerea*, the cerato-platanin BcSpl1 contributes to fungal virulence instead of plant resistance. In this study, we report that BcSpl1, which was previously found in the secretome as an abundant protein, is even more abundant in the fungal cell wall. By fusion to green fluorescent protein (GFP), we also show that BcSpl1 associates with the plant plasma membrane causing rapid morphological changes at the cellular level, such as the disorganization of chloroplasts, prior to macroscopic necrosis in the treated tissue. By a combination of serial deletion studies, synthetic peptides and chimeric proteins, we mapped the eliciting activity to a two-peptide motif in the protein surface. The expression of a chimeric protein displaying this motif in *B. cinerea* mutants lacking BcSpl1 undoubtedly showed that the motif is responsible for the contribution of BcSpl1 to virulence.

INTRODUCTION

Botrytis cinerea is a widely distributed and highly successful plant pathogen causing devastating losses in crops worldwide (Dean *et al*., 2012). Early during infection, this fungus secretes a wide array of proteins involved in a variety of different functions (Espino *et al*., 2010), among which is the abundant necrosis-inducing cerato-platanin BcSpl1. Cerato-platanins constitute a widely distributed family of fungal secreted proteins whose founder member is cerato-platanin from *Ceratocystis platani* (formerly *C. fimbriata*) (Pazzagli *et al*., 1999), a pathogen of the European plane tree (*Platanus acerifolia*). The three-dimensional structure of this protein consists of a double psi-beta-barrel fold, similar to that of expansins and endoglucanases (Krieger *et al*., 2010; de Oliveira *et al*., 2011), a fold suggesting a role in polysaccharide metabolism, and the *C. platani* (de Oliveira *et al*., 2011) and *Trichoderma atroviride* (Frischmann *et al*., 2013) cerato-platanins have been shown to bind to oligosaccharides, although no hydrolysis has been reported.

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Cerato-platanins have been found bound to the cell wall (Boddi *et al*., 2004) and in the extracellular medium (Espino *et al*., 2010; Frías *et al*., 2011), and most are able to elicit defence responses in plants, including autofluorescence (Pazzagli *et al*., 1999; Wilson *et al*., 2002), synthesis of reactive oxygen species (Djonovic *et al*., 2006; Frías *et al*., 2011), induction of defence genes (Djonovic *et al*., 2007; Fontana *et al*., 2008; Frías *et al*., 2011) and cell death (Frías *et al*., 2011; Pazzagli *et al*., 1999; Yang *et al*., 2009), with markers and symptoms of the hypersensitive response (HR). These activities suggest that a putative perception of cerato-platanins by the plant immune system, as a pathogen-associated molecular pattern (PAMP), could have a role in the defence against fungal plant pathogens, and indeed the elicitor activity of the cerato-platanin BcSpl1 on *Arabidopsis thaliana* is partially dependent on the regulator of PAMP perception BAK1 (Frías *et al*., 2011). Nevertheless, BcSpl1 also contributes to the virulence of the fungus, as shown by the fact that *bcspl1* mutants produce lesions that are 20%–40% smaller than those of the wild-type (Frías *et al*., 2011), in agreement with the hypothesis that *B. cinerea* actively induces HR to generate dead tissue on which to grow (Govrin and Levine, 2000).

Here, we report that two small surface-exposed loops of BcSpl1 are necessary and sufficient for its necrosis-inducing ability, and also suffice for its contribution to the virulence of the fungus.

RESULTS

BcSpl1 is partially retained in the cell wall

BcSpl1 has been found consistently in the extracellular medium in cultures of *B. cinerea* of different ages and growth conditions (Espino *et al*., 2010; Girard *et al*., 2012; González-Fernández *et al*., 2013; Shah *et al*., 2009a, b), although the *C. platani* homologue has been found predominantly bound to the fungal cell wall (Boddi *et al*., 2004). In order to gather more information about the location of BcSpl1 in *B. cinerea*, a BcSpl1-green fluorescent protein (GFP) fusion was generated and expressed in the wild-type strain B05.10 under the control of the *Aspergillus nidulans OliC* promoter. Observation under a fluorescence microscope revealed that the *B. cinerea* cerato-platanin BcSpl1 is also bound to the cell wall, especially at the septa between adjacent cells (Fig. 1A). To obtain a quantitative estimation of the proportion of BcSpl1 which is

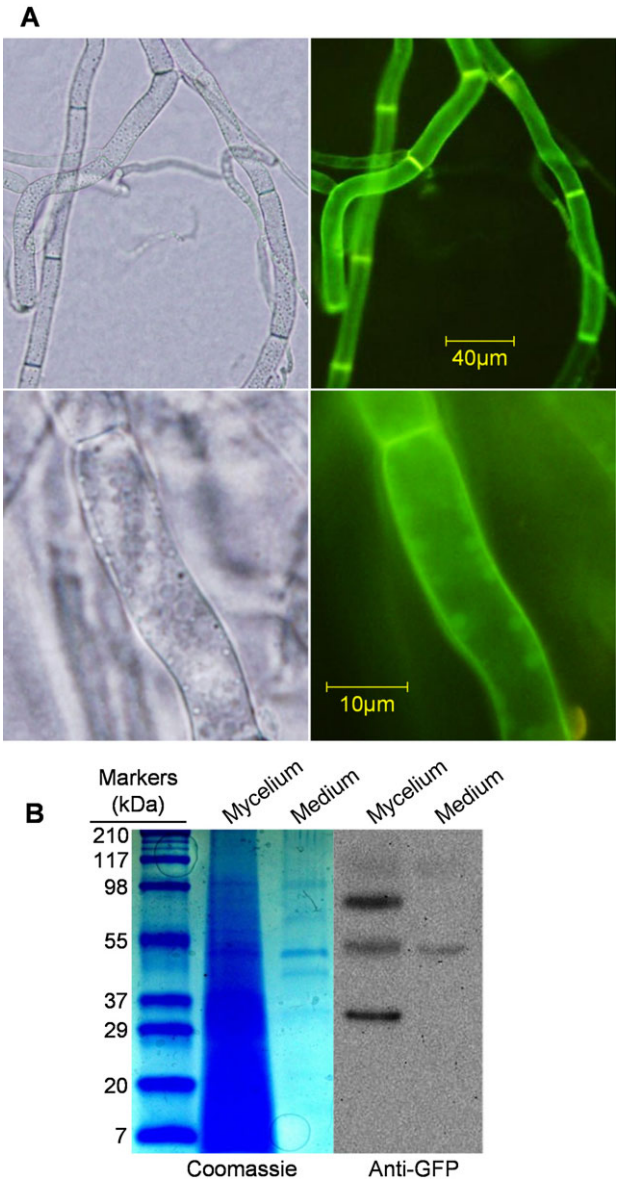

Fig. 1 Localization of BcSpl1 in *Botrytis cinerea* mycelium. (A) A homokaryotic *B. cinerea* transformant obtained with plasmid pCRP-GFP, and thus expressing a BcSpl1-GFP fusion protein, was grown for 3 days in YGG medium (0.5% yeast extract, 100 mM glucose and 0.3% Gamborg's B5) and observed under a visible (left) or fluorescence (right) microscope. (B) The same transformant was grown as in (A) and the whole mycelium and an aliquot from the culture medium were boiled with sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer; 40 μL (1/28 of total) of the former and 600 μL (1/33 of total, precipitated with methanol–chloroform) of the latter were electrophoresed and detected with Coomassie blue or anti-green fluorescent protein (anti-GFP) antibodies (in a Western blot) as indicated.

retained in the cell in comparison with the amount that ends up in the culture medium as a soluble protein, the amount of BcSpl1 in both compartments was estimated by Western blot with anti-GFP antibodies (Fig. 1B). Surprisingly, two extra bands above and below the band of the expected size were found in the mycelium fraction, which do not seem to be the result of cross-reaction of the anti-GFP antibody, as no signal was observed in *B. cinerea* strains not expressing GFP (not shown). One possible explanation for the large band could be covalent dimerization of BcSpl1, previously reported for other fungi (Vargas *et al*., 2008), and, for the small band, proteolysis at the BcSpl1-GFP linker region, given the highly abundant protease secretion in *B. cinerea* (ten Have *et al*., 2010). From the intensities of the bands obtained for four biological replicates of the same experiment, we estimated that, considering all bands for the calculation, 22.4% ± 4.2% of all BcSpl1-GFP synthesized ends up in the culture medium. We conclude that, similar to the cerato-platanin from *C. platani* (Boddi *et al*., 2004), BcSpl1 is basically a cell wall protein, although with the particularity that its binding to this structure is weak and about one-fifth can be found dissolved in the extracellular medium.

BcSpl1 binds to plant plasma membranes and causes cell shrinkage and chloroplast disorganization

Previous results from our group (Frías *et al*., 2011) have indicated that the necrosis-inducing activity of BcSpl1 on *A. thaliana* requires the plant signalling protein BAK1 for full activity. BAK1 has also been shown to be required for the transduction of the signal generated by the binding of several PAMPs, such as bacterial flagellin and elongation factor Tu, to their respective membrane receptors (Segonzac and Zipfel, 2011; Zipfel and Robatzek, 2010). This led to the idea that BcSpl1 could act as a PAMP and bind to a putative receptor in the plasma membrane to initiate a cascade of events leading to HR, thus resulting in the observed necrosis. To test this hypothesis, two fusion proteins containing the whole BcSpl1 sequence plus GFP at either its N- or C-terminal end were expressed in *Pichia pastoris*, as well as GFP alone as a control. The two fusion proteins were able to induce necrosis similarly to BcSpl1 in tomato, tobacco and *Arabidopsis* (Fig. S1, see Supporting Information), whereas GFP alone did not produce any effect. Incubation of tomato or tobacco protoplasts with either one of the two proteins resulted in GFP labelling of the plant plasma membrane (Fig. 2A), whereas no GFP signalling could be detected in the controls incubated with GFP alone, indicating that BcSpl1 indeed binds to plant plasma membranes. Attempts to observe, in a similar manner, the binding of BcSpl1 to *A. thaliana* leaf protoplasts were unsuccessful. This is probably because the leaves of this plant are much less susceptible to BcSpl1 than those of tobacco or tomato (not shown), and therefore the binding may not be sufficiently strong to be seen under the microscope. BcSpl1 binding to tomato or tobacco protoplasts also resulted in rapid

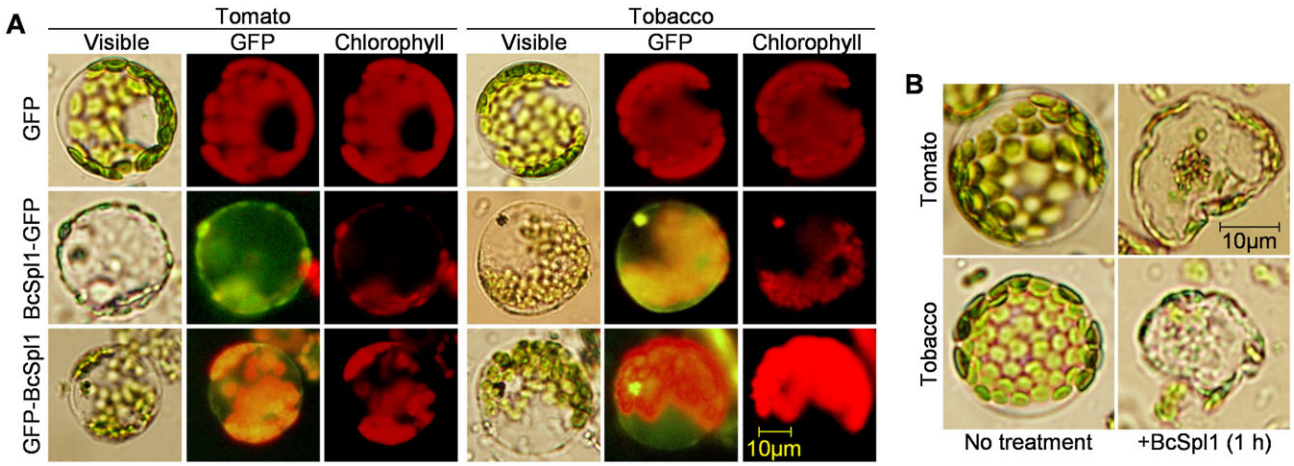

Fig. 2 BcSpl1 binds to plant plasma membranes. (A) Tomato or tobacco protoplasts were incubated with 3.4 μM BcSpl1-GFP, 3.4 μM GFP-BcSpl1 or no protein in the control, and then washed and observed under a microscope with visible light or appropriate filters for the fluorescence of green fluorescent protein (GFP) and chlorophyll. (B) Effect on protoplasts of a 1-h incubation with BcSpl1.

and clear morphological alterations, including cell shrinkage and chloroplast disorganization. These changes were especially evident at 60 min (Fig. 2B), whereas untreated cells, or cells treated with the control proteins GFP or bovine serum albumin (BSA) (not shown), did not show any alteration.

Several conserved regions of BcSpl1 are exposed on the protein surface and constitute potential necrosis-inducing motifs

Protein regions recognized as PAMPs by the plant immune system are usually very well conserved in the corresponding protein families, such as in the case of bacterial flagellin (Felix *et al*., 1999). To search for such regions in BcSpl1, an alignment of 146 cerato-platanin sequences was analysed in order to find parts of the sequences strongly conserved among all members of the family and among those cerato-platanins which have been shown to elicit plant defences (Fig. 3A). Among the regions with a degree of conservation above average in the two sets (regions 1–5 in Fig. 3A), there are clearly two groups. Regions 2 and 4 (red and blue, respectively, in Fig. 3B) correspond to two loops interacting with each other, forming a small protrusion in the protein surface (de Oliveira *et al*., 2011), stabilized by a disulphide bond. The C-terminal part of region 4, which is not as well conserved as the N-terminal part (Fig. 3A), extends into the beta strand β3, according to the nomenclature of de Oliveira *et al*. (2011). The other three conserved regions (1, 3, 5) correspond to three beta strands (β1, β2 and β5) forming one side of the beta barrel. These highly conserved regions nicely coincide with those parts of the *C. platani* cerato-platanin reported to be involved in binding to *N*-acetylglucosamine oligomers (de Oliveira *et al*., 2011).

A 40-amino-acid conserved BcSpl1 region induces necrosis by itself

In order to determine whether one or more of the conserved regions in the BcSpl1 sequence (Fig. 3) are necessary and sufficient for necrosis-inducing activity, bidirectional serial deletions were generated in the *bcspl1* gene, so that one or more of the five conserved regions were deleted from either the N- or C-terminus (Fig. 4A). The resulting truncated *bcspl1* genes were all expressed in *P. pastoris* as fusions to GFP, and the proteins were purified by nickel affinity chromatography. The necrosis-inducing ability of these protein variants is presented in Fig. 4B and shows that: (i) deletion of the conserved region 1 or 5 did not produce any effect on the necrosis-inducing activity of BcSpl1; (ii) deletion of regions 1–2 or 4–5 caused a significant reduction in the necrosis-inducing activity; and (iii) simultaneous deletion of regions 2–4 completely abolished the necrosis-inducing activity. These results indicate that the 40-amino-acid central region of the protein, comprising regions 2–4, is required for full activity. This was further confirmed by the analysis of the binding of all the truncated versions of BcSpl1 to tobacco (Fig. 4C) or tomato (Fig. S2, see Supporting Information) protoplasts. The deletion of regions 2–4 completely abolished binding to the membrane of the protoplasts of both plants, whereas regions 1 and 5 were dispensable. The deletion of region 1–2 seems to maintain sufficient binding capacity to produce green fluorescence in the membrane, and the same is true for the deletion of region 4–5. The comparison of the activity of these truncated proteins in the context of the three-dimensional structure predicted for the protein (Fig. 3B) indicates that the two surface loops comprising the conserved motif, marked as red and blue, seem to be required for the necrosis-inducing activity, whereas those deletions affecting the conserved regions in the beta barrel are not.

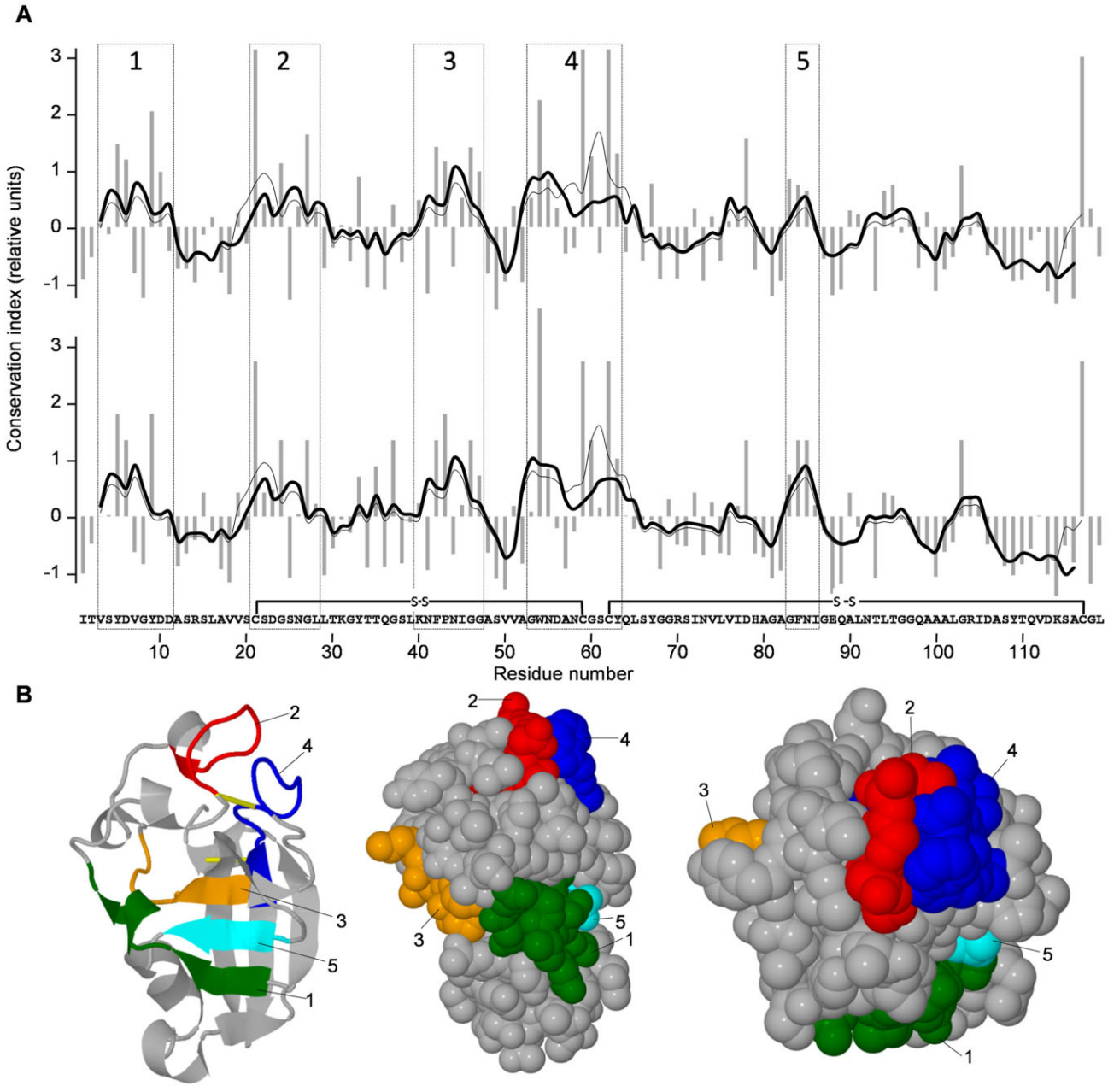

Fig. 3 Identification of evolutionarily conserved regions in BcSpl1. (A) Conservation indices (grey bars) were calculated with AL2CO for every BcSpl1 residue from the alignment of either 146 complete cerato-platanin family sequences downloaded from Pfam (top panel) or the eight cerato-platanins known (Buensanteai *et al*., 2010; Comparini *et al*., 2009; Frías *et al*., 2011; Pazzagli *et al*., 1999; Vargas *et al*., 2008; Wilson *et al*., 2002; Yang *et al*., 2009; Zaparoli *et al*., 2009) to elicit plant defences (bottom panel). Zero corresponds to the average conservation index for the whole protein. Average conservation indices were also calculated for windows of five residues for the whole protein (thin line) or after deleting the four cysteines (thick line) to avoid the effect of its absolute conservation. Broken line boxes indicate the five regions with conservation indices well above average (zero) in the two alignments. (B) Position of the five conserved regions in the structure of BcSpl1 predicted by SWISS-MODEL in three different views.

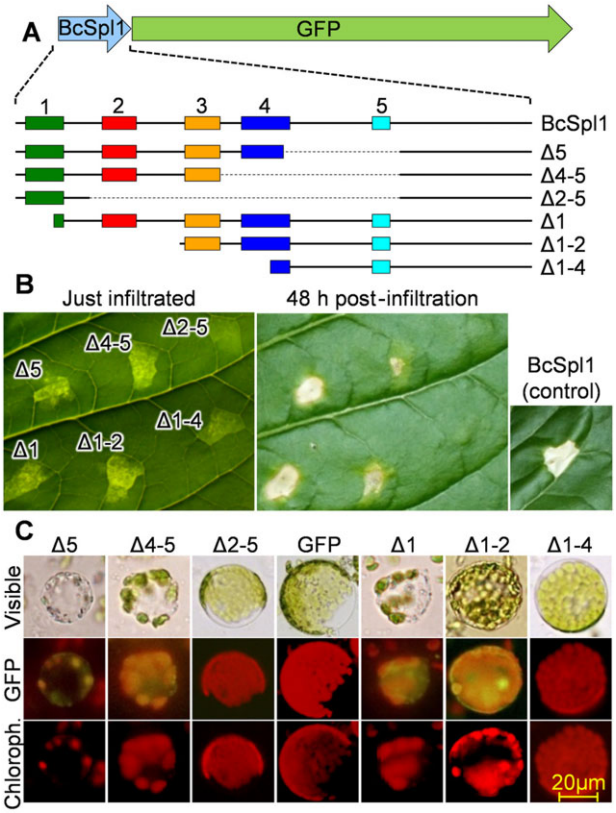

Fig. 4 Determination of necrosis-inducing regions in BcSpl1. (A) Scheme showing the different serial deletions that were made in the BcSpl1 part of the BcSpl1-GFP fusion protein. The five numbers and different colours identify the five conserved regions displayed in Fig. 3. Broken lines indicate internal regions deleted from the gene. (B) Necrosis-inducing activity of the different truncated versions of BcSpl1, at a concentration of 34 μM, on tobacco leaves. (C) Binding of the truncated proteins to tobacco protoplasts (see legend to Fig. 2A for details). GFP, green fluorescent protein.

Two short BcSpl1 fragments cooperate to induce necrosis

To analyse the hypothesis that the two conserved loops in the BcSpl1 surface may constitute the motif responsible for the necrosis-inducing activity, two 10-amino-acid peptides were synthesized with the sequence of the most exposed and conserved parts of the loops: PepA (VSCSDGSNGL) and PepB (VVAGWNDANC) (Fig. 3). The sequences of the two peptides include the two cysteine residues which form a disulphide bond connecting the two loops in the predicted BcSpl1 structure (Fig. 3A). Infiltration of either one of the two peptides was able to produce necrosis in tobacco leaves, in a concentration-dependent manner, but less efficiently than the whole BcSpl1 (Fig. 5A). Higher concentrations were needed to induce necrosis in the treated area, and even very high concentrations (136 μM) did not reproduce the effect observed with 34 μM BcSpl1. Moreover, as expected for the binding of PepA and PepB to a putative BcSpl1 receptor, the two peptides

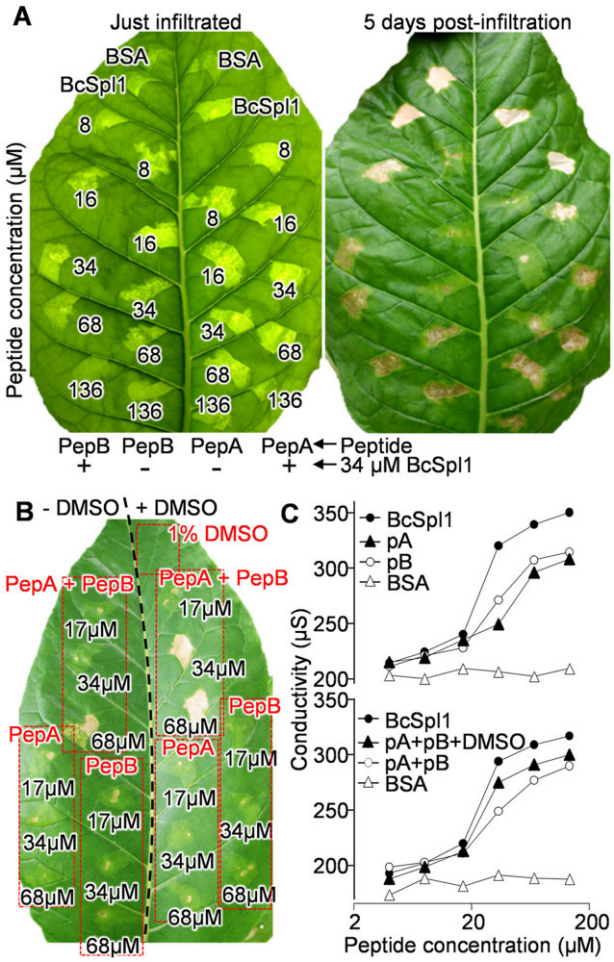

Fig. 5 Eliciting activity of peptides PepA and PepB. (A) Necrosis-inducing activity of different concentrations of the two peptides on tobacco leaves, either alone or mixed with BcSpl1. Controls were made with bovine serum albumin (BSA) or with BcSpl1 at a concentration of 34 μM. (B) Necrosis-inducing activity of peptides PepA, PepB and their mixture, with or without preincubation with 1% dimethylsulphoxide (DMSO). The indicated peptides or peptide mixture (in red) were infiltrated at the indicated concentration (in black). Photographs were taken at day 3 after infiltration. (C) Ion leakage caused on tobacco leaf discs by infiltration with the indicated proteins and peptides. pA, PepA; pB, PepB. The experiment was repeated three times with similar results.

were able to act as inhibitors of the necrosis-inducing activity of BcSpl1 (Fig. 5A), presumably by competition for the receptor. At a molar ratio of 1 : 4 between BcSpl1 and either one of the two peptides (34 μM BcSpl1 and 136 μM PepA or PepB), the necrosis produced had an appearance similar to that produced with the same concentration of the peptides alone, indicating that, at these concentrations, BcSpl1 is not contributing significantly to necrosis and is therefore inhibited, i.e. out-competed, to a high degree.

Contrary to our initial expectation, the infiltration of tobacco leaves with a mixture of PepA and PepB was not much more effective in causing necrosis than the infiltration of either peptide

alone; the necrotic areas were clear only at a high concentration of the peptides in the mixture (Fig. 5B). One possible explanation for this result is that the disulphide bridge, which putatively binds the two peptides in the native BcSpl1, does not form in the peptide mixture, preventing the two peptides from reconstituting the whole motif. This possibility was explored by incubating the mixture with dimethylsulphoxide (DMSO) prior to infiltration, as this compound has been reported to encourage disulphide bond formation between peptides in aqueous solutions (Tam *et al*., 1991). Indeed, DMSO had a stimulating effect on the necrosis-inducing activity of the peptide mixture (Fig. 5B), which was especially evident at 34 μM, but not of each peptide alone. DMSO alone at the same concentration, 1%, did not produce any effect on tobacco leaves or stimulate the effect of the whole BcSpl1 or of the unrelated elicitor EIX (Ron and Avni, 2004) (Fig. S3, see Supporting Information). Therefore, it seems that PepA and PepB contribute synergistically to the production of necrosis, in conditions of disulphide bond formation, in accordance with the hypothesis that the protrusion of BcSpl1, marked red and blue in the predicted structure (Fig. 3B), may constitute the necrosis-inducing motif. A more quantitative determination of the eliciting ability of the different peptides or mixture was obtained by assaying the electrolyte leakage produced as a consequence of elicitation (Fig. 5C), and the results confirmed those of necrosis. PepA, PepB and their mixture (without DMSO) all have about the same effect, which was lower than that of BcSpl1, and the incubation of the mixture of the two peptides with DMSO had a stimulating effect on the activity.

A chimeric protein displaying the two-peptide eliciting motif exhibits BcSpl1 necrosis-inducing activity

To better confirm the participation of the PepA and PepB in the building of the elicitor motif, a chimeric protein was designed in which the two peptides were displayed on the surface of the small, highly stable DNA-binding protein Sso7d (Baumann *et al*., 1994) from the hyperthermophilic archaeon *Sulfolobus solfataricus*, in an orientation designed to reproduce the putative eliciting motif (marked blue and red in Fig. 3B). For this purpose, a gene was synthesized *de novo* that coded for an Sso7d variant in which the N- and C-terminal amino acids were substituted by PepA and PepB, respectively (Fig. 6A). The fusion was performed in such a way that the distance between the C-terminus of PepA and the N-terminus of PepB in the chimeric protein, assuming that it folds as the native Sso7d (protein data bank id. 1SSO), was the same as in the structure predicted for BcSpl1. The new protein (named Sso7d-PepAB), together with a negative control without the BcSpl1 sequence (Sso7d), was expressed in *P. pastoris*, purified and assayed for its necrosis-inducing activity. The necrosis observed for Sso7d-PepAB was almost identical to that caused by the infiltration of BcSpl1, with a marginally lower necrosis-inducing activity that was clear only at the lowest concentrations

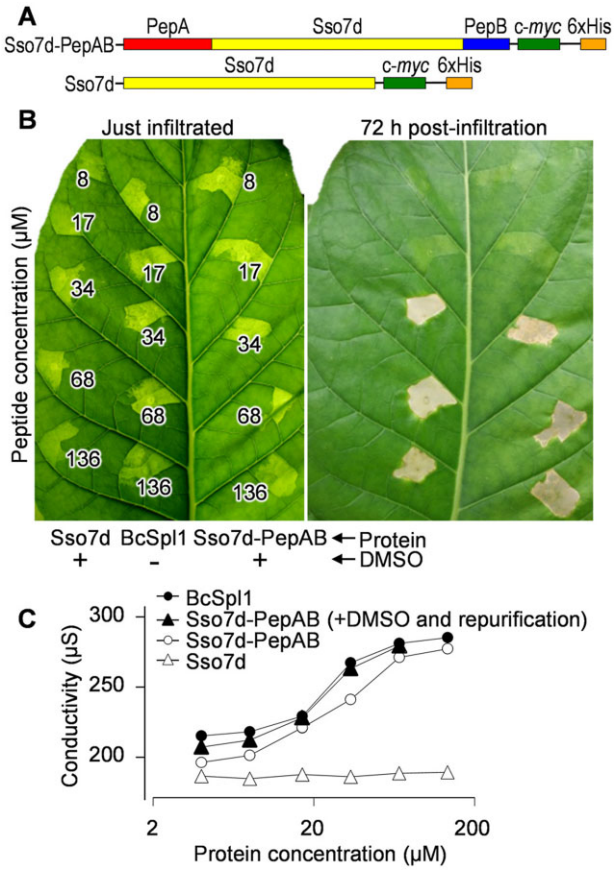

Fig. 6 Eliciting activity of peptides PepA and PepB on a different protein backbone. (A) Scheme of the chimeric protein displaying the two peptides on the protein Sso7d from *Sulfolobus solfataricus* (Sso7d-PepAB), as well as the control protein lacking the two peptides (Sso7d). (B) Necrosis-inducing activity of the two chimeric proteins in comparison with BcSpl1. Sso7d-PepAB and Sso7d were treated with dimethylsulphoxide (DMSO) and infiltrated without further processing. (C) Ion leakage on tobacco leaf discs caused by infiltration with the indicated proteins. Where indicated, Sso7d-PepAB was treated with DMSO and re-purified.

(Fig. S3). As happened with the mixture of PepA + PepB, incubation of Sso7d-PepAB with DMSO to restore the disulphide bond had a stimulating effect, taking the activity to a level not distinguishable from that of BcSpl1 (Fig. 6B). The infiltration of the negative control (Sso7d), with (Fig. 6B) or without (not shown) DMSO, did not produce any visible effect in the leaves. In order to exclude any possible artefactual effect of the infiltration of DMSO together with the proteins, we carried out a re-purification of the DMSO-treated Sso7d-PepAB protein, taking advantage of the 6 × histidine (6 × His) tag. The infiltration of the re-purified protein, dissolved in water, also resulted in necrosis in the tobacco leaves not distinguishable from that of BcSpl1 (Fig. S3). These results were confirmed by assaying the ability of Sso7d-PepAB to produce electrolyte leakage, which was the same as that of BcSpl1 for the DMSO-treated protein (Fig. 6C).

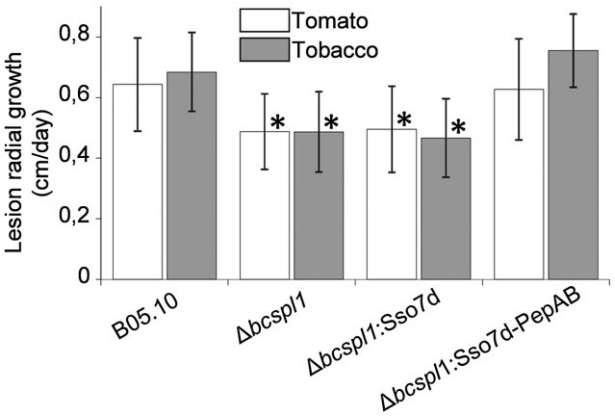

Fig. 7 Contribution of Sso7d-PepAB to *Botrytis cinerea* virulence. The strains Δ*bcspl1*:Sso7d and Δ*bcspl1*:Sso7d-PepAB were generated by transformation of the *bcspl1* mutant Δ*bcspl1*.1 with the genes coding for the chimeric proteins Sso7d-PepAB and Sso7d, respectively (see Fig. 6). Virulence for these three strains and the wild-type (B05.10) was assayed on tomato and tobacco leaves by measuring the rate of expansion of the lesions, and is represented as mean values and standard deviation. Asterisks indicate a statistically significant difference from the wild-type in the same plant, by Student's *t*-test ($n \geq 30$, $P < 0.01$).

The two-peptide eliciting motif suffices for the BcSpl1 contribution to *B. cinerea* virulence

As *bcspl1* mutants are about 20%–40% less virulent on tomato or tobacco leaves, when compared with the wild-type (Frías *et al*., 2011), we wondered whether the necrosis-inducing activity located in the two-peptide motif constitutes the contribution of BcSpl1 to infection. To answer this question, the chimeric protein Sso7d-PepAB was tested for its capacity to complement the *bcspl1* mutation in *B. cinerea*. The genes coding for the chimeric proteins Sso7d-PepAB and Sso7d (Fig. 6A) were expressed in *B. cinerea* under the control of the *bcspl1* promoter and signal peptide. One of the two previously characterized Δ*bcspl1* mutants (Frías *et al*., 2011) was transformed with the new genes and the secretion of the two chimeric proteins was corroborated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot with anti c-*myc* antibodies (Fig. S4, see Supporting Information). When assayed for its virulence on tobacco and tomato leaves, the strain expressing the chimeric protein with the two-peptide eliciting motif (named Δ*bcspl1*-Sso7d-PepAB) recovered the virulence of the wild-type in both plants (Fig. 7), unequivocally showing that the two-peptide motif accounts for the contribution of BcSpl1 to virulence. The expression of the control protein Sso7d did not increase the virulence (Fig. 7).

DISCUSSION

We have shown here that the cerato-platanin BcSpl1 is able to bind to plant protoplasts, causing a rapid cell shrinking and disorganization of the chloroplast (Fig. 2), which macroscopically results in tissue necrosis. Serial deletion experiments (Fig. 4), chemically synthesized peptides (Fig. 5) and the expression of the suspected regions in a different protein backbone (Fig. 6) showed that two conserved regions (Fig. 3), interacting with each other in the protein surface, are necessary and sufficient for the phytotoxic activity of BcSpl1. Moreover, the expression of this two-peptide motif in a Δ*bcspl1* mutant restores its virulence to wild-type levels (Fig. 7), therefore accounting for the *bcspl1* contribution to virulence. This is the first example, to our knowledge, of a plant eliciting motif composed of different peptides. These results corroborate previous observations obtained with two longer BcSpl1 peptides (Frías *et al*., 2011), which, in practice, were extended versions of PepA and PepB comprising about one-third of the protein each. The longer peptides behaved similarly to PepA and PepB in producing necrosis when infiltrated in plant leaves, i.e. infiltration of either one of the peptides produced chlorosis in the leaves but no necrosis, whereas infiltration of the mixture produced necrosis similar to that produced by the whole BcSpl1 protein.

The three-dimensional structure of the cerato-platanin from *C. platani* (de Oliveira *et al*., 2011) showed a conserved region in its surface, which was proposed to have an important role in oligosaccharide binding, as shown by the fact that chemical shift perturbation of the amide proton, caused by the binding of short N-acetylglucosamine oligomers, was high in the regions 17–29 and 51–54 of the *C. platani* protein (de Oliveira *et al*., 2011). The corresponding regions in BcSpl1 (18–30 and 53–56, respectively), according to an alignment of the whole family (de Oliveira *et al*., 2011), are the two regions shown here to be involved in the necrosis-inducing activity. In addition, as shown here (Fig. 3) and by de Oliveira *et al*. (2011), these two regions are among the most evolutionarily conserved parts of the protein. This opens up interesting questions regarding the oligosaccharide-binding activity and necrosis-inducing activity of cerato-platanins. The coincidence of both activities on the same protein region may indicate, on the one hand, that the second is just a consequence of the first. One may speculate, for example, that the binding of cerato-platanins to a glycosylated receptor in the plant plasma membrane is the first step in the elicitation process. However, the coincidence may be just because the plant immune system has, by evolution, developed recognition of the most conserved, and thus functionally important, parts of the protein.

We have also shown here that the two-peptide motif is responsible for the contribution of BcSpl1 to virulence. One reason why this may be so is because the necrosis-inducing activity of BcSpl1 could generate dead tissue appropriate for the growth of *B. cinerea*. This necrotroph has indeed been proposed to benefit from HR (Choquer *et al*., 2007; Govrin and Levine, 2000; van Kan, 2006; Williamson *et al*., 2007), a form of programmed cell death in plants (Mur *et al*., 2008), and BcSpl1 has been shown previously

to induce symptoms and markers of HR in plant leaves (Frías *et al*., 2011). An alternative, not contradictory, explanation may be that BcSpl1 could soften plant cell walls with its putative expansin activity (Baccelli *et al*., 2013), and therefore contribute to virulence by facilitating plant invasion by the fungus. As discussed above, the necrosis-inducing and polysaccharide-binding activities are located in the same protein region, making it difficult to set up experiments to discriminate between the two hypotheses. Finally, we cannot rule out possible defects in the fungal cell wall, caused by the lack of cerato-platanin, as contributing factors in the lower virulence of *bcspl1* mutants. Although no phenotypic difference, especially no alteration in the growth rate, was observed for the *bcspl1* mutants in axenic culture (Frías *et al*., 2011), it is not possible at this point to exclude cell wall defects that become apparent only *in planta*.

The analysis of cerato-platanins is further complicated because no clear biological function has yet been assigned to this protein. Its expansin-like fold (de Oliveira *et al*., 2011), ability to bind polysaccharides (de Oliveira *et al*., 2011; Frischmann *et al*., 2013) and to weaken cellulosic material (Baccelli *et al*., 2013), and the fact that it remains mostly bound to the cell wall on secretion (Fig. 1) (Boddi *et al*., 2004), suggest some expansin-like role in the fungal cell wall, in addition to its ability to induce necrosis in plants. This hypothesis also agrees with the fact that cerato-platanins are widely distributed in fungi which are not plant pathogens, but disagrees with the fact that mutants lacking cerato-platanin show no phenotype other than a decreased virulence (Frías *et al*., 2011; Frischmann *et al*., 2013; Jeong *et al*., 2007).

EXPERIMENTAL PROCEDURES

Organisms, strains and culture conditions

Botrytis cinerea strain B05.10 (Büttner *et al*., 1994), one of the two strains with a sequenced genome (Amselem *et al*., 2011; Staats and van Kan, 2012), was used as wild-type strain. *bcspl1* mutants have been generated previously (Frías *et al*., 2011). Fungal strains were kept as conidia in 15% glycerol at −80 °C for long-time storage, or in silica gel at 4 °C for routine use (Delcan *et al*., 2002). The silica stock was used to inoculate tomato-agar plates (25% tomato fruit extract, 2% agar, pH 5.5) to obtain conidia, as described by Benito *et al*. (1998). The YGG medium used for *B. cinerea* contains 0.5% yeast extract, 100 mM glucose and 0.3% Gamborg's B5 (Duchefa Biochemie, http://www.duchefa-biochemie.nl). Plants (*Nicotiana tabacum* cv. Havana, *Lycopersicon esculentum* cv. Moneymaker and *A. thaliana* Col-0) were maintained at controlled temperature, humidity and photoperiod in a phytotron. Tobacco leaves were infiltrated in the necrotizing assays with the help of a 1-mL syringe, without a needle, filled with the indicated proteins dissolved in water, except where otherwise indicated. Virulence assays were performed as described elsewhere (Frías *et al*., 2011) on tomato and tobacco leaves, and the lesion expansion rates were calculated between days 2 and 3 after inoculation, with a minimum of 30 inoculations per assay.

Standard techniques

SDS-PAGE and Western blots were carried out according to standard procedures with Bio-Rad (http://www.bio-rad.com) precast gels and polyvinylidene fluoride (PVDF) membranes. Monoclonal anti-GFP or anti-c-*myc* primary antibodies were diluted 1:1000 and detected with a 1:3000 dilution of antimouse IgG conjugated to horseradish peroxidase (HRP) (Sigma-Aldrich, http://www.sigmaaldrich.com). The peroxidase was detected with Immobilon Western Chemiluminescent HRP Substrate (Millipore, http://www.millipore.com). Quantification of Western blot bands (Fig. 1B) was performed with the software Quantity One (Bio-Rad) on the chemiluminescence signal recorded with a Chemidoc system (Bio-Rad). Ion leakage was assayed as explained elsewhere (Frías *et al*., 2011). Protein sequence alignments were performed with Clustal Omega (Sievers *et al*., 2011) and processed with AL2CO (Pei and Grishin, 2001) to generate conservation indices for every amino acid, by making use of the score matrix BLOSUM62.

Plasmid constructions and the generation of new *B. cinerea* strains

Polymerase chain reaction (PCR) amplifications were made with *Phusion* polymerase (New England Biolabs, http://www.neb.com) when the DNA product was to be cloned, and *Taq* polymerase (GenScript, http://www.genscript.com) was used in all other cases. All oligonucleotides used (Table S1, see Supporting Information) were from Life Technologies (http://www.lifetechnologies.com). *Botrytis cinerea* transformations were carried out as described by Hamada *et al*. (1994), with the modifications introduced by van Kan *et al*. (1997).

To express BcSpl1-GFP and GFP-BcSpl1 fusion proteins in *P. pastoris*, two different plasmids, pCP-GFP and pGFP-CP, were designed as follows: the *mgfp4* gene (Haseloff *et al*., 1997) was amplified from the nos-GFP cassette (Hellens *et al*., 2000) with the primer pair GFPCP-FW/GFPCP-RV or CPGFP-FW/CPGFP-RV, and cloned at the *Eco*RI site at the 5' end or the *Xba*I site at the 3' end of *bcspl1*, respectively, in plasmid pCP (Frías *et al*., 2011).

Plasmid pCRP-GFP, used to express the BcSpl1-GFP fusion protein in *B. cinerea*, is derived from plasmid pOptGFP, which contains a GFP gene codon optimized for *B. cinerea* under the strong *Aspergillus nidulans OliC* promoter (Leroch *et al*., 2011) in a plasmid backbone previously designed for site-directed integration in the *B. cinerea* genome (Noda *et al*., 2007). pCRP-GFP was constructed by cloning in the *Sma*I site of pOptGFP the *bcspl1* gene amplified by PCR from plasmid pCP (Frías *et al*., 2011). The primer pair used, SS-CRP-SmaI/CRP-Sma1-REV, restored the native signal sequence to the *bcspl1* gene and amplified it together with the c-*myc* and 6 × His epitopes originally from pPICZαA (Invitrogen, Carlsbad, CA, USA). The circular pCRP-GFP plasmid was then transformed into *B. cinerea* B05.10 and the transformants were subjected to several rounds of single spore isolation. A transformant (CRP-GFP8.1.1) producing only fluorescent conidia, and thus putatively homokaryotic, was selected.

Bidirectional deletions in the *bcspl1* gene were carried out by PCR amplifying the whole pCP-GFP plasmid described above, except the *bcspl1* region to be deleted. The two primers facing outward from the intended deletion introduced *Kpn*I sites in the borders of the amplicons, and these were then cut with *Kpn*I and ligated with each other to generate a plasmid

in which a *bcspl1* region was substituted by the two amino acids coded by the *Kpn*I site (glycine-threonine). The resulting plasmids were then transformed into *P. pastoris* to express the truncated proteins.

Plasmids pSsoCP and pSso, designed to express the chimeric proteins Sso7d-PepAB and Sso7d in yeast, were constructed by cloning the corresponding *de novo*-synthesized genes (GenScript) in the *P. pastoris* expression vector pPICZαA. The sequences of the genes (Fig. S5, see Supporting Information) were optimized according to the codon usage in *P. pastoris* highly expressed genes (Bai *et al*., 2011) and in *B. cinerea* (Leroch *et al*., 2011), and included *EcoR*I and *Xba*I sites at the ends to clone them in the *P. pastoris* vector. The two chimeric proteins Sso7d-PepAB and Sso7d were expressed in *B. cinerea* Δ*bcspl1.1* by transformation with the plasmids pNR2-SsoCP and pNR2-Sso. These plasmids are derived from plasmid pNR2-CP, which contains the whole *bcspl1* gene with promoter and terminator and a nourseothricin resistance cassette for selection (Frías *et al*., 2011). The whole pNR2-CP plasmid, except the *bcspl1* open reading frame (ORF), was amplified by inverse PCR with primers Pro-cp-Rv and Ter-cp-Fw, and ligated to the synthetic genes for Sso7d-PepAB and Sso7d (Fig. S5) obtained with the primer pairs Ssocp-Fw/Sso-rv and Sso-Fw/Sso-rv, respectively. pNR2-SsoCP and pNR2-Sso were then transformed into the *B. cinerea bcspl1* mutant Δ*bcspl1*.1.

Peptides and recombinant proteins

Peptides were from GenScript. The EasySelect *Pichia* Expression Kit (Life Technologies) was used to express recombinant proteins in *P. pastoris* according to the manufacturer's instructions. Proteins were purified from the supernatants of cultures of *P. pastoris* induced for 2 days with 0.5% methanol in BMMH (100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin) medium with HisTrap FF prepacked minicolums (GE Healthcare Life Sciences, http://www.gelifesciences.com), as explained elsewhere (Frías *et al*., 2011). The formation of disulfide bridges in purified proteins, or in peptide mixtures, was encouraged when necessary by incubating them overnight, with slow shaking at room temperature, with 1% DMSO (Tam *et al*., 1991). When indicated, proteins were then re-purified by binding to magnetic nickel beads (GenScript) for 2 h, washing four times with washing buffer and eluting with water, using the same reagents as those employed in the column purification (Frías *et al*., 2011).

Binding assay of GFP-tagged proteins to plant protoplasts

Tomato, tobacco and *A. thaliana* protoplasts were prepared from leaf material equilibrated for 30 min in 10 mM MES–KOH, pH 5.7, 0.5 M mannitol. The protoplast solution contained 2.5 mM MES–KOH, pH 5.7, 200 mM mannitol, 5 mM KCl, 5 mM $CaCl_2$, 2% cellulase from *Trichoderma viride* (Sigma-Aldrich C1794) and 0.5% pectinase from *Rhizopus* sp. (Sigma-Aldrich P2401). This solution was first infiltrated into plant tissue by applying a vacuum for 3 min, and the mixture was then incubated for 1 h at 20 °C with slow shaking. After carefully washing with 5 mM MES–KOH, pH 5.7, 400 mM mannitol, 5 mM KCl and 5 mM $CaCl_2$, protoplasts were incubated with a 0.34 μM concentration of the appropriate protein, washed again with the same buffer and examined with an Olympus BX-50 fluorescence microscope equipped with a U-MWIB filter to detect GFP and a U-MWIG filter to detect chlorophyll.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Necrosis-inducing activity of the two fusion proteins between BcSpl1 and green fluorescent protein (GFP) (BcSpl1-GFP and GFP-BcSpl1) in leaves from three different plants.

Fig. S2 Binding of the truncated BcSpl1 proteins to tomato protoplasts.

Fig. S3 Effect of dimethylsulphoxide (DMSO) on the necrosis-inducing activities of Sso7d-PepAB, BcSpl1 and EIX.

Fig. S4 Secretion of the chimeric proteins Sso7d and Sso7d-PepAB in the *Botrytis cinerea* mutant Δ*bcspl1*.

Fig. S5 Nucleotide sequences of the chemically synthesized genes *Sso7d-PepAB* and *Sso7d*. The amino acid translation is shown below the DNA sequence and the relevant regions are indicated.

Table S1 Oligonucleotides used in this study.