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Expression and regulation of *Sclerotinia sclerotiorum* necrosis and ethylene-inducing peptides (NEPs)

ZAFER DALLAL BASHI[1,2], DWAYNE D. HEGEDUS[1,2], LONE BUCHWALDT[1], S. ROGER RIMMER[1] AND MOHAMMED H. BORHAN[1,*]

[1]*Agriculture and Agri-Food Canada, Saskatoon, SK, Canada*

[2]*Department of Food and Bioproduct Sciences, University of Saskatchewan, Saskatoon, SK, Canada*

SUMMARY

Successful host colonization by necrotrophic plant pathogens requires the induction of plant cell death to provide the nutrients needed for infection establishment and progression. We have cloned two genes encoding necrosis and ethylene-inducing peptides from *Sclerotinia sclerotiorum*, which we named *SsNep1* and *SsNep2*. The peptides encoded by these genes induce necrosis when expressed transiently in tobacco leaves. *SsNep1* is expressed at a very low level relative to *SsNep2* during infection. The expression of *SsNep2* was induced by contact with solid surfaces and occurred in both the necrotic zone and at the leading margin of the infection. *SsNep2* expression was dependent on calcium and cyclic adenosine monophosphate signalling, as compounds affecting these pathways reduced or abolished *SsNep2* expression coincident with a partial or total loss of virulence.

INTRODUCTION

Necrotrophic plant pathogens derive their nutrients from dead or dying host tissues. Host programmed cell death (PCD), which is associated with the hypersensitive response, is effective in restricting the growth of biotrophic pathogens, but is counteractive in the defence against necrotrophs (Dickman *et al*., 2001). *Sclerotinia sclerotiorum* is a necrotrophic fungus that infects many important crops, such as soybean, lentils, sunflower and oilseed rape (Boland and Hall, 1994). Its main pathogenicity determinants are considered to be oxalic acid and numerous polygalacturonases (PGs). Oxalic acid is particulary important as it lowers ambient pH, sequesters calcium in the middle lamellae, leading to a loss of cell wall integrity (Godoy *et al*., 1990), and suppresses the oxidative burst (Cessna *et al*., 2000). Indeed, oxalic acid-deficient mutants are non-pathogenic (Godoy *et al*., 1990). PGs are enzymes that degrade pectin in the plant cell wall and are highly active under acidic conditions. *Sclerotinia sclerotiorum* produces four different types of PG that are involved in infection establishment, lesion expansion and tissue maceration (Li *et al*., 2004a, b) In response, plants produce polygalacturonase inhibitor proteins (PGIPs) to inactivate these enzymes (Hegedus *et al*., 2008). Oxalic acid and lytic enzymes produced by necrotrophic pathogens have additional roles in pathogenesis, including the induction of PCD (Kim *et al*., 2008; Van Kan, 2006). *Botrytis cinerea* produces several PGs, some of which possess necrotizing activity (Kars *et al*., 2005), and a *S. sclerotiorum* PG has been shown to elevate cellular Ca^{+2} levels and induce PCD (Zuppini *et al*., 2005).

Another group of PCD elicitors found in many plant pathogens is the necrosis and ethylene-inducing peptides (NEPs) (Pemberton and Salmond, 2004). NEP1 was first isolated from *Fusarium oxysporum* culture filtrates as a 24-kDa protein that caused necrosis and induced ethylene production in the coca plant, *Erythroxylum coca* (Bae *et al*., 2006; Bailey, 1995). In the past few years, NEP1-like proteins (NLPs) have been identified in taxonomically diverse organisms, including bacteria, fungi and oomycetes. They have also been found in non-pathogenic organisms, such as *Vibrio pommerensis* (Jores *et al*., 2003), and are expressed in the hemibiotrophic fungus *Moniliophthora perniciosa* during the biotrophic stage (Garcia *et al*., 2007). Transformation of *Colletotrichum coccodes* with *F. oxysporum Nep1* increased virulence and the spectrum of hosts that could be infected (Amsellem *et al*., 2002). NLPs elevate internal Ca^{2+} levels, activate mitogen-activated protein (MAP) kinases and promote the formation of reactive oxygen species, ethylene and pathogenesis-related proteins (Fellbrich *et al*., 2002; Jennings *et al*., 2001; Keates *et al*., 2003; Qutob *et al*., 2006; Veit *et al*., 2001). In *Arabidopsis thaliana*, global gene expression patterns in response to the *Phytophthora parasitica* NLPs were similar to those observed in response to the bacterial flagellin (Qutob *et al*., 2006). The mechanism by which NLPs bring about these phenomenon is unknown, but they must associate with the outer surface of the plasma membrane to trigger PCD (Qutob *et al*.,

**Correspondence*: E-mail: borhanh@agr.gc.ca

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2006; Schouten *et al*., 2008). NLPs have also been reported to erode the host cuticle (Keates *et al*., 2003).

Common to all NLP proteins is a conserved GHRHDWE domain and an amino-terminal register of conserved cysteine residues. Based on the number of cysteine residues, NLPs can be classified as Type I or Type II, which possess either two or four cysteine residues, respectively. Both types of NLP are produced by fungi and bacteria; however, only Type I NLPs have been reported in oomycetes (Gijzen and Nürnberger, 2006). The conserved GHRHDWE domain is not solely responsible for activity (Schouten *et al*., 2008). Mutation of the conserved cysteine residues or other alterations that affect the tertiary structure abolish the ability to induce PCD in protoplasts or to generate necrotic lesions (Fellbrich *et al*., 2002; Qutob *et al*., 2006).

The majority of studies conducted on NLPs have focused on their interaction with their host. Little is known about factors governing the regulation of NLP gene expression. In *Erwinia carotovora*, *Nep* was expressed only when the bacteria were grown on solid medium (Mattinen *et al*., 2004). In this article, we report the characterization of two NLP genes from *S. sclerotiorum*, referred to as *SsNep1* and *SsNep2*. The peptides encoded by these genes cause necrosis, and *SsNep2* is expressed soon after contact of the mycelia with the leaf or other solid surfaces. We also investigated the role of cyclic adenosine monophosphate (cAMP) and Ca^{2+} in the regulation of *SsNep* expression. This study further illustrates the significance of having multiple effectors to initiate host PCD and tissue necrosis for successful infection by necrotic pathogens.

RESULTS

Identification of *SsNep1* and *SsNep2*

To identify *S. sclerotiorum* Nep (*SsNep*) genes, we conducted a BLASTX search of the *S. sclerotiorum* genome sequence (http://www.broad.mit.edu/annotation/genome/sclerotinia sclerotiorum/Home.html) with *F. oxysporum Nep1* (AF036580). Two *Nep1* homologues were identified with expectation values of 0 (SS1G_03080.1) and 1.06 e-31 (SS1G_11912.1), which were named *SsNep1* and *SsNep2*, respectively. The *SsNep1* gene contains two introns of 53 and 58 bp with the resultant 738-bp open reading frame encoding a 246-amino-acid protein with a predicted molecular weight of 26.7 kDa. The *SsNep2* gene contains two introns of 61 and 58 bp with a 735-bp open reading frame that encodes a 245-amino-acid protein predicted to have a molecular weight of 26.0 kDa.

Comparison of NLPs from several fungal pathogens revealed a diverse phylogenetic arrangement (Fig. 1B). SsNEP1 and SsNEP2 were most closely related to *Botrytis elliptica* Nep1 (ABB43266; 82% identity) and Nep2 (CAJ31369; 79% identity), respectively. SsNEP1 was also related to an *Aspergillus oryzae* NLP (XP_001824353), whereas SsNEP2 was related to an *Aspergillus fumigatus* NLP (EAL86241). The oomycete NLPs from *Phytophthora* and *Pythium* species formed a clade that was distinct from those including SsNEP1 and SsNEP2, although the former was more similar to the oomycete NLP sequences. SsNEP1 and SsNEP2 are highly divergent and exhibit only 35% identity at the amino acid level. The conserved GHRHDWE sequence is located between positions 103 and 110 in SsNEP1 and 109 and 116 in SsNEP2. Both appear to be secreted as they were predicted to possess signal peptides. Conserved cysteine residues define the type or class of NLP. Both SsNEPs are Type I with two conserved cysteine residues at positions 47 and 73 in the mature protein (Fig. 1A). SsNEP1 contains a third cysteine residue, and SsNEP2 contains three additional cysteines. The third cysteine in SsNEP1 and two of the three additional cysteines in SsNEP2 are conserved among the orthologues in *Botrytis* species, but not so in other NLPs.

In planta expression of SsNEPs

Recombinant *Potato virus X* (PVX) vectors allowing for the expression of *SsNep1* and *SsNep2* under the direction of the cauliflower mosaic virus (CaMV) promoter were introduced into *Nicotiana benthamiana* leaves. In each case, endogenous SsNEP signal peptides were included in the heterologous expression vectors, and therefore the proteins were expected to be secreted into the apoplast. Constructs expressing either *SsNep1* or *SsNep2* developed lesions within 10–12 days post-inoculation (dpi). The lesion started near the margin of the inoculation site and then proceeded to expand to the surrounding tissue by 14–16 dpi (Fig. 2). We did not observe any differences in the rate of lesion development or the severity of symptoms between the *SsNep1* and *SsNep2* constructs.

Fig. 1 *Sclerotinia sclerotiorum* necrosis and ethylene-inducing peptides (SsNEPs). (A) Alignment of SsNEP1 and SsNEP2 showing identical residues (white on black background), signal peptides (italics), conserved cysteine residues (!) indicative of Type I NEP1-like proteins (NLPs) and the GHRHDWE motif (*). (B) Phylogenetic relationship between NLPs from various fungi and oomycetes. The proteins used in the analysis are coded with the first two letters representing the organism followed by the GENBANK accession number. Af, *Aspergillus fumigatus*; As, *Aspergillus nidulans*; Ao, *Aspergillus oryzae*; Be, *Botrytis elliptica*; Fo, *Fusarium oxysporum*; Mg, *Magnaporthe grisea*; Pa, *Pythium aphanidermatum* NEP1; Pi, *Phytophthora infestans*; Pp, *Phytophthora parasitica*; Ps, *Phytophthora sojae*; Sn, *Stagonospora nodorum*; Ss, *Sclerotinia sclerotiorum*; Vd, *Verticillium dahliae*. Type I and Type II NLPs were classified according to Gijzen and Nürnberger (2006). Confidence values for each node are based on 100 bootstrap analyses.

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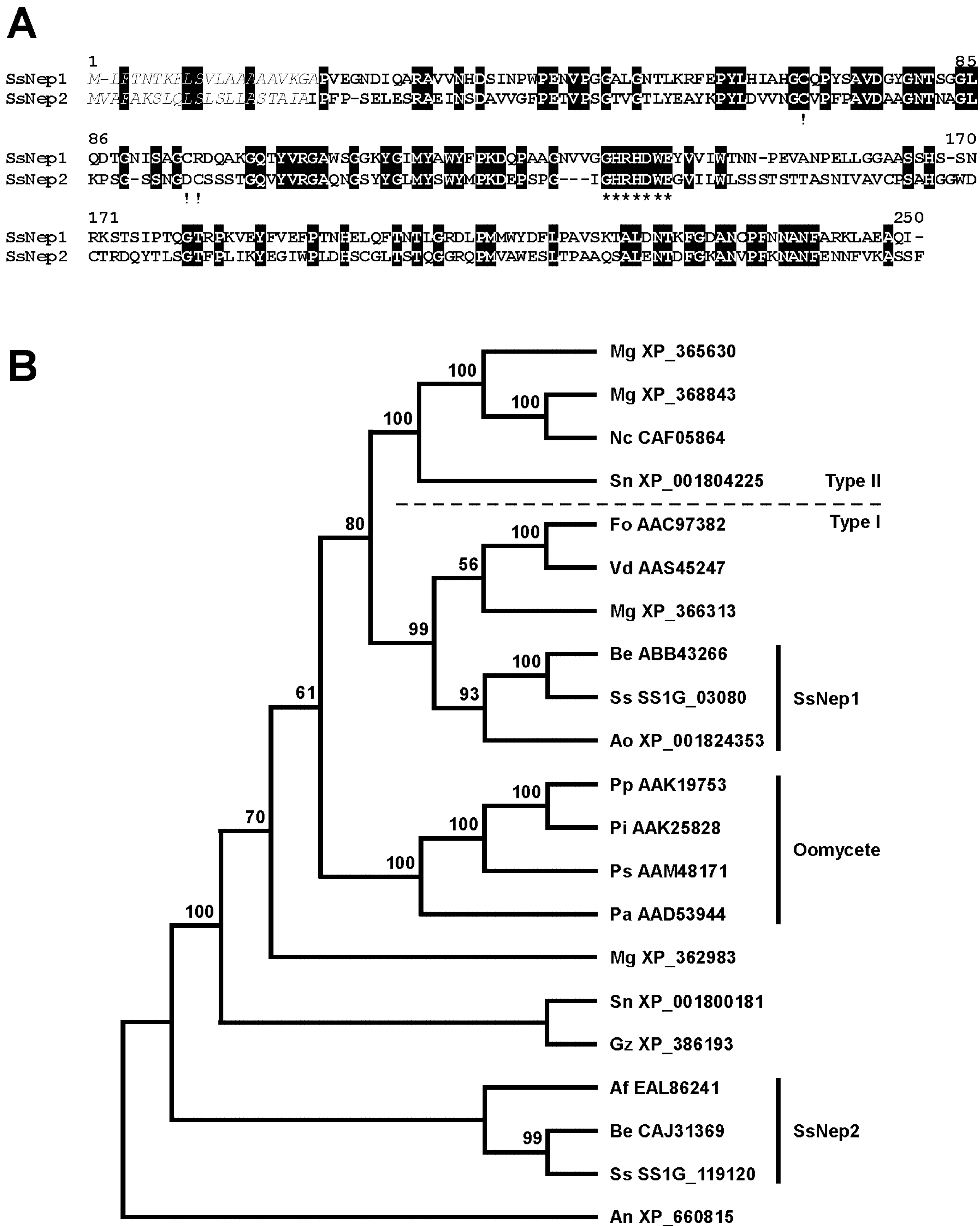

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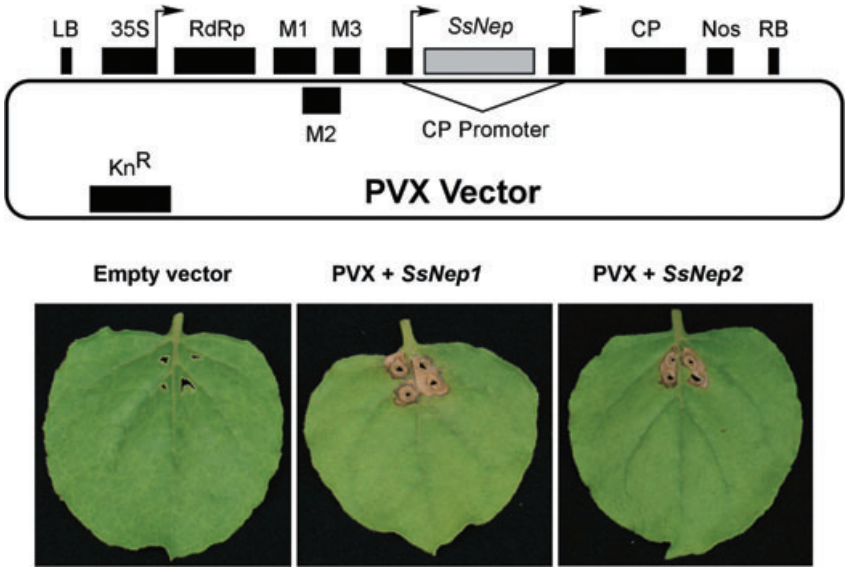

Fig. 2 Map of the *Potato virus X*-based binary vector pgR107 used for the *in planta* expression of *Sclerotinia sclerotiorum* necrosis and ethylene-inducing peptide genes (*SsNep*). Elements shown are: LB, left border; 35S, cauliflower mosaic virus 35S promoter; RdRp, viral RNA-dependent RNA polymerase; M1–M3, viral movement proteins; CP, coat protein, Nos, *Agrobacterium tumefaciens* nopaline synthetase transcriptional terminator; RB, right border. Necrotic symptoms observed on *Nicotiana benthamiana* leaves 2 weeks after inoculation with PVX virus, PVX + *SsNep1* or PVX + *SsNep2*.

Expression of *SsNep1* and *SsNep2* during infection and in response to different surfaces

We examined the expression of *SsNep1* and *SsNep2* during the course of infection. Necrotic symptoms were visible within 6 h post-inoculation (hpi) of *Brassica napus* leaves with *S. sclerotiorum*. By 24 hpi, most of the area beneath the inocula had become necrotic and, by 48 hpi, the leaf tissue was completely macerated. *SsNep1* transcripts were detected at very low levels from 6 to 24 hpi (Fig. 3), but were undetectable at 48 hpi. *SsNep2* transcripts were also detected within 6 hpi and peaked at 24 hpi. The expression of *SsNep1* was always much less than that of *SsNep2*, with the former often being barely detectable using either Northern blotting or reverse transcriptase-polymerse chain reaction (RT-PCR).

We also examined the expression of *SsNep* genes in various infection zones, namely necrotic tissue, the lesion margin and uninfected or asymptomatic tissue (Fig. 3). *SsNep1* transcripts were not detected using Northern blotting in any of the zones tested, whereas *SsNep2* was expressed in the necrotic region and the expanding margin of the lesion.

Expression of *SsNep2* on different surfaces

A previous study found that the *E. carotovora Nep* gene was expressed only when the bacterium was grown on solid medium (Mattinen *et al*., 2004). Therefore, we examined the effect of different contact surfaces on *SsNep2* expression, the more highly expressed of the two *S. sclerotiorum Nep* genes. *SsNep2* transcripts were not detected in liquid medium, including minimal salts (MS) (starvation), MS–glucose or MS supplemented with finely granulated leaf wax (Fig. 4). Conversely, when mycelia were placed on solid or semi-solid surfaces (intact leaves, leaves stripped of wax, leaf wax on glass or glass), *SsNep2* expression was sharply induced within 24 h. Interestingly, *SsNep2* was not expressed in response to contact with the hydrophobic material Parafilm™ in either the presence or absence of glucose.

Role of calcium signalling and cAMP in *SsNep2* regulation

To study the role of calcium in *SsNep2* expression, we tested the effect of several compounds that affect calcium signalling. Caffeine increases cytosolic calcium levels by activating ryanodine receptors on the endoplasmic reticulum, leading to the release of internal calcium stores (Berridge, 1997). The treatment of mycelia with 20 mM caffeine resulted in a near-complete inability to penetrate the *B. napus* leaf cuticle, whereas 2.5 mM caffeine did not affect virulence (Fig. 5). Both concentrations of caffeine decreased *SsNep2* expression after 24 h on the leaf surface (Fig. 6). Lanthanum chloride, a plasma membrane calcium channel blocker, greatly reduced *S. sclerotiorum* virulence and expression of *SsNep2*. Ethylene Glycol Tetraacetic Acid (EGTA), which chelates extracellular calcium ions, had no effect, suggesting that mobilization of internal calcium stores is required for infection and *SsNep2* expression. Compounds 48/80 and U73122 inhibit phospholipase C activity, but were found to promote the release of calcium from intracellular stores (Mogami *et al*., 1997). Treatment with 48/80 slightly reduced virulence; however, both reduced *SsNep2* expression. None of the compounds tested above were found to inhibit mycelial growth at the concentrations used (Fig. 5). Calcineurin, a calcium- and calmodulin-dependent Ser/Thr protein phos-

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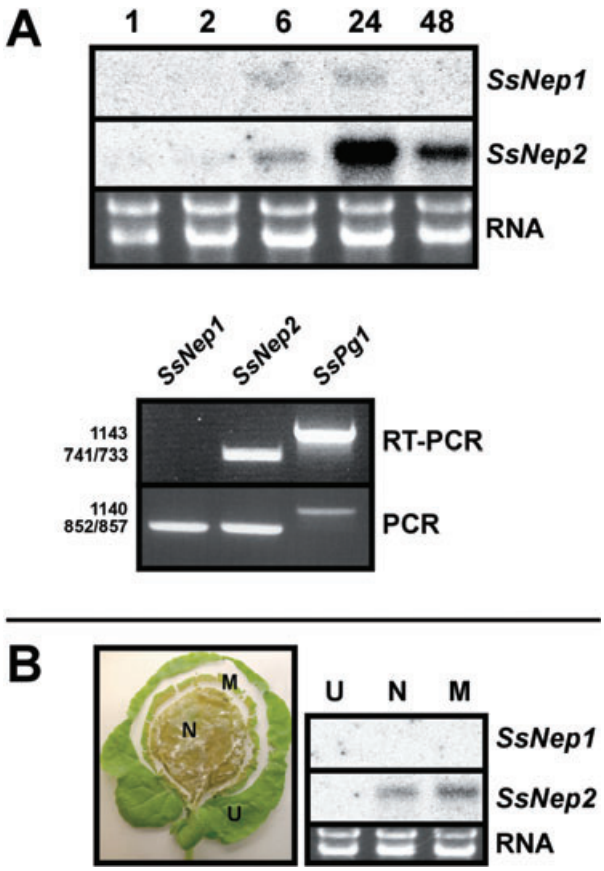

Fig. 3 Expression of *Sclerotinia sclerotiorum* necrosis and ethylene-inducing peptide genes (*SsNep*) during infection of *Brassica napus* leaves. (A) Northern blot analysis showing expression in mycelia at various times (hours) after inoculation. The total RNA loaded in each lane is also shown. Lower panel shows *SsNep1*, *SsNep2* and *SsPg1* reverse transcriptase-polymerase chain reaction (RT-PCR) products amplified from mycelia 24 h after inoculation. Amplification of the corresponding loci using genomic DNA was conducted as a positive PCR control. (B) Division of the lesion into uninfected (U), margin (M) and necrotic (N) zones and Northern blot analysis showing expression in each zone.

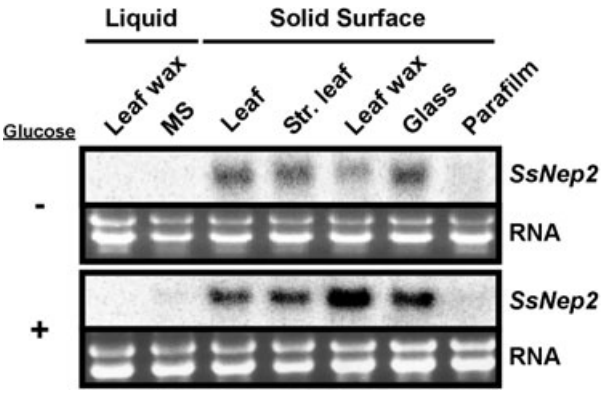

Fig. 4 Northern blot analysis showing the expression of *Sclerotinia sclerotiorum* necrosis and ethylene-inducing peptide 2 gene (*SsNep2*) in mycelia in various liquid media or after transfer to solid surfaces in the presence or absence of glucose. The bottom panel shows the total RNA loaded in each lane.

phatase, has been shown to play a central role in fungal pathogenesis (Fox and Heitman, 2002). The calcineurin inhibitor cyclosporin A reduced virulence and strongly reduced the expression of *SsNep2*, although mycelial growth was also reduced. Northern blot analysis was conducted with similar amounts of total RNA from each sample; however, to determine the extent to which the treatments affected housekeeping gene expression, the same blots were probed with the *SsActin* gene. The intensity of the hybridization signal in the control lane for both *SsNep2* and *SsActin* was similar. In all cases in which *SsNep2* expression was reduced, the level of actin expression was similar to the control or was affected to a far lesser degree than *SsNep2*, indicating that the compounds had a greater effect on *SsNep2* than *SsActin* expression.

Caffeine also elevates internal cAMP levels by inhibiting cAMP-phosphodiesterase activity, which converts cAMP to a non-cyclic form (Berridge, 1997). Several studies have reported that cAMP signalling plays a role in many aspects of fungal biology, including virulence and development (D'Souza and Heitman, 2001; Rollins and Dickman, 1998). To test the effect of cAMP on the colonization of *B. napus* by *S. sclerotiorum*, we monitored lesion formation and *SsNep2* expression after application of exogenous cAMP (Fig. 7). At 0.1 and 1 mM concentrations, cAMP had no effect on disease development or *SsNep2* expression; however, at 10 mM, lesion formation was severely reduced. *SsNep2* expression also decreased; however, the expression of *SsActin* also declined with increasing cAMP levels, indicating that the loss of virulence may be a result of factors in addition to reduced *SsNep2* expression. The *S. sclerotiorum* adenylate cyclase deletion mutant, *sac1*, which is unable to generate cAMP, was also non-pathogenic (Jurick and Rollins, 2007). This mutant grew very slowly and, although mycelial rRNA levels were similar to those of the wild-type strain, we were unable to detect transcripts from *SsNep2* or *SsActin*, indicating that the mutation has broad pleiotrophic effects. Furthermore, the application of exogenous cAMP to the *sac1* mutant failed to restore pathogenicity or *SsNep2* expression, although *SsActin* expression was not detected in the *sac1* mutant.

DISCUSSION

The induction of host cell death is crucial for *S. sclerotiorum* pathogenicity (Dickman *et al*., 2001) and its genome was found to contain two *NLP* genes. The *Phytophthora sojae* and *Phytophthora ramorum* genomes contain 50–60 *NLP* genes and pseudogenes, whereas the *Magnaporthe grisea* and *Giberella zeae*

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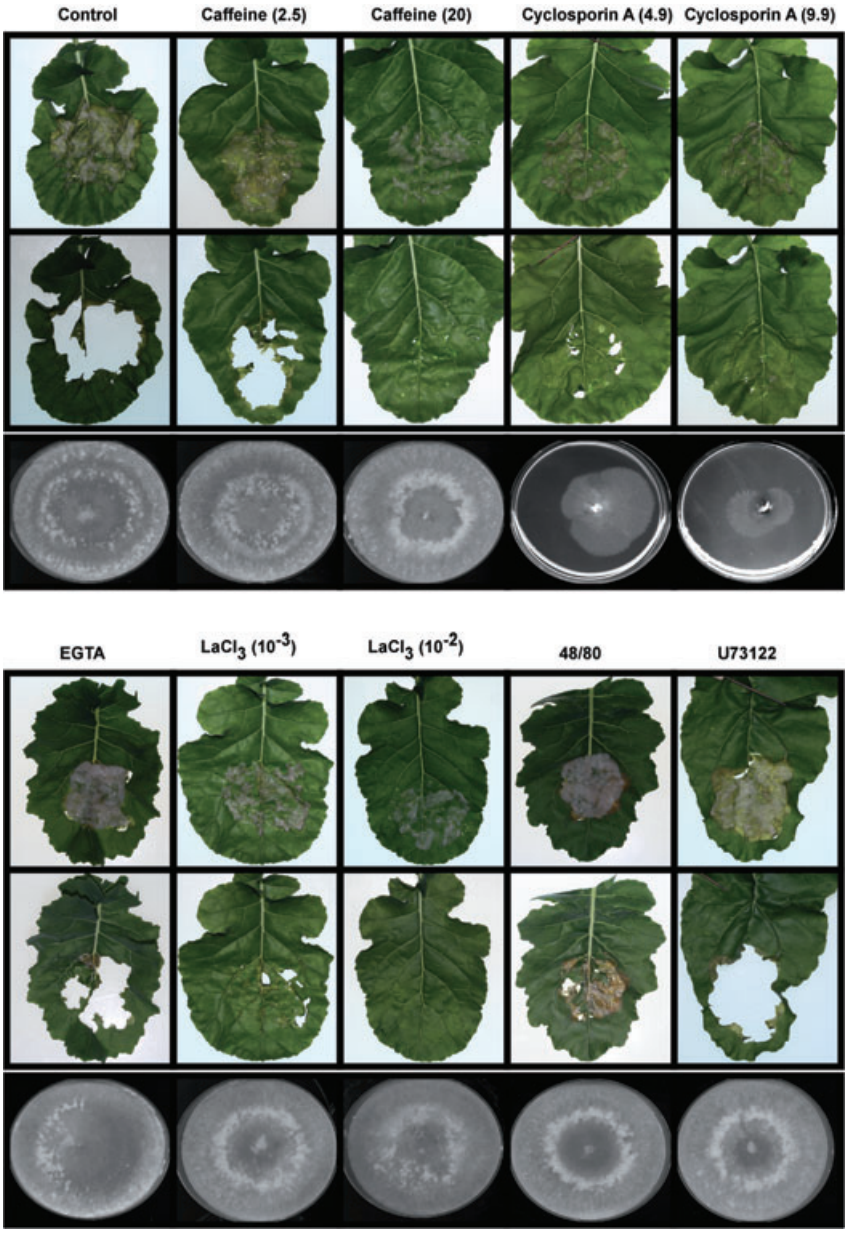

Fig. 5 The effect of compounds affecting cyclic adenosine monophosphate (cAMP) levels (caffeine) and calcium signalling on *Sclerotinia sclerotiorum* infection of *Brassica napus* leaves and expression of *S. sclerotiorum* necrosis and ethylene-inducing peptide 2 gene (*SsNep2*). The top panels show leaves 16 h after inoculation (upper frame), as well as the lesions beneath (lower frame), for mycelia that were untreated (control) or treated with caffeine (2.5 and 20 mM), cyclosporin A (CspA; 4.9 and 9.9 nM), [ethylenebis(oxonitrilo)]tetraacetic acid (EGTA) (10 mM), lanthanum chloride ($LaCl_3$; 1 and 10 mM), compound 48/80 (5 μM) or compound U73122 (10 mM). The bottom panels show the effect of these compounds on new mycelial growth. A sample of the mycelial preparation used to inoculate the leaves was also placed in the centre of a minimal salts–glucose (MS-Glu) plate and radial growth was assessed after 5 days.

genomes contain only four (Gijzen and Nürnberger, 2006). The *S. sclerotiorum* NEP proteins were most similar to those from *Botrytis elliptica* (Staats *et al*., 2007), the genus closest to *Sclerotinia*. Although SsNEPs are highly divergent, each possessed features highly conserved among NLPs, and were effective in causing necrosis when introduced into *B. napus* leaves using the PVX expression system.

In *S. sclerotiorum* strain 1980, *SsNep2* was expressed at a much higher level than *SsNep1* during the infection of *B. napus* leaves, which was confirmed by both Northern blot and RT-PCR analysis. The differential expression of genes encoding pathogenicity factors has been shown elsewhere. For example, in *Fusarium solani* f. sp. *pisi*, the pectate lyase gene *pelD* was expressed only in infected pea tissue, whereas *pelA* expression

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was undetectable (Guo *et al*., 1996; Rogers *et al*., 2000). We also noted that *SsNep2* was expressed soon after mycelial inoculation and in both the necrotic region and at the expanding margin of the lesion. In the hemibiotrophic pathogen *P. sojae*, the highest level of *Nep* gene expression coincided with the transition from the biotrophic to the necrotrophic stage (Qutob *et al*., 2002). There is an accumulating body of evidence that NLPs contribute to virulence. Transgenic *C. coccodes* expressing *Nep1* from *F. oxysporum* was more virulent on the original host and was able to infect tobacco and tomato lines that were resistance to wild-type *C. coccodes* (Amsellem *et al*., 2002). Similarly, expression of the *E. carotovora Nip* gene, which encodes an NLP, resulted in larger lesions and more rotting of host tissues (Mattinen *et al*., 2004).

The expression of *SsNep2* was also found to be regulated by the physical properties of the contact surface. *SsNep2* transcripts were not detected in MS-based liquid cultures, but were induced by mycelial contact with a solid or semi-solid surface. Many studies have examined the effect of physical properties and chemical signals associated with the leaf surface on pathogen development and the differentiation of infectious structures. Appressoria formation by *Colletotrichum* species was related to leaf topography (Kolattukudy *et al*., 1995), whereas, in *Blumeria graminis* f. sp. *hordei*, these were formed in response to barley leaf wax (Tsuba *et al*., 2002). The induction of *SsNep2* expression in response to contact with a solid surface is in accordance with the observation that it is expressed at very early stages (detectable within 6 h by Northern blot analysis) of the infection. Interestingly, NLPs may affect the structure of the host cuticle, as application of *F. oxysporum* NEP1 to leaves of several plant

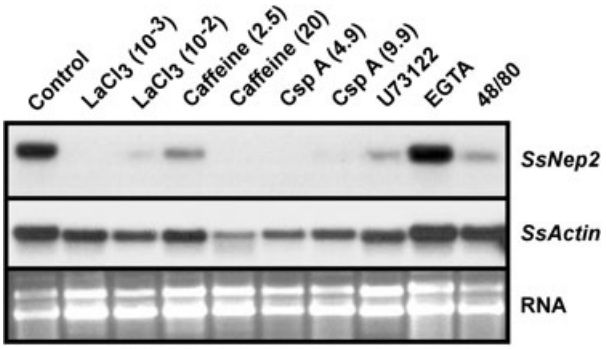

Fig. 6 Northern blot analysis showing the expression of *Sclerotinia sclerotiorum* necrosis and ethylene-inducing peptide 2 (*SsNep2*) and *SsActin* genes in mycelia treated with compounds affecting cyclic adenosine monophosphate (cAMP) levels (caffeine) and calcium signalling and placed on *B. napus* leaves for 16 h. The bottom panel shows the total RNA loaded in each lane.

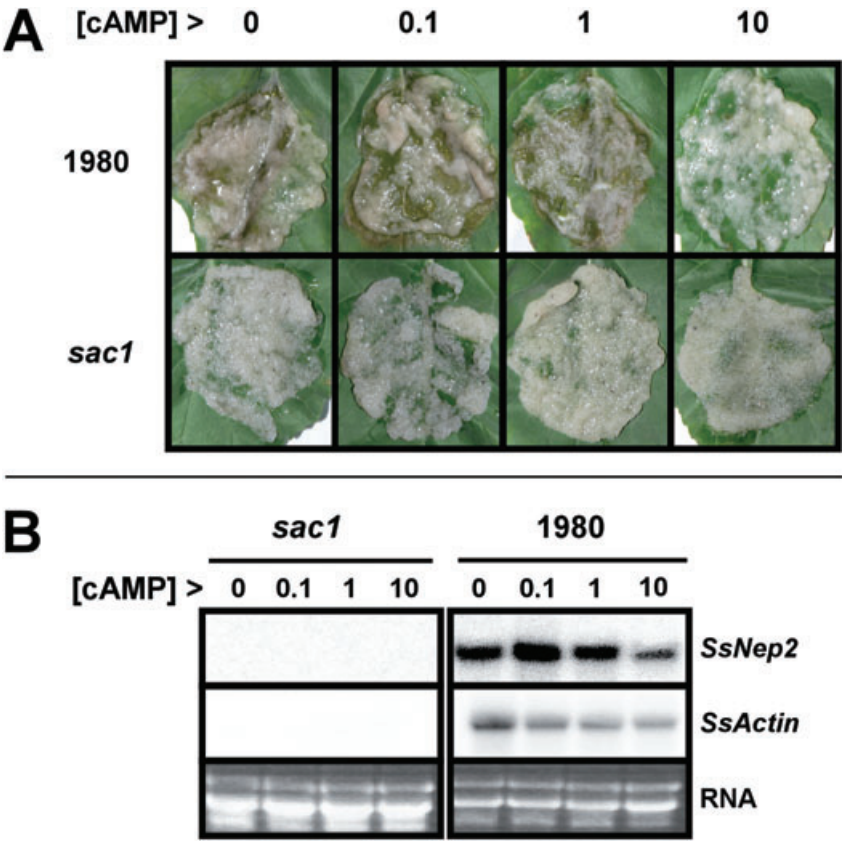

Fig. 7 The effect of exogenous cyclic adenosine monophosphate (cAMP) application on *Sclerotinia sclerotiorum* infectivity and *SsNep2* expression. (A) Lesions forming on *Brassica napus* leaves when mycelia from *S. sclerotiorum* strain 1980 and the *sac1* adenylate cyclase mutant were treated with increasing levels of cAMP (mM) prior to inoculation. (B) Northern blot analysis showing *SsNep2* expression 24 h after inoculation of *B. napus* leaves with mycelia treated with cAMP. The bottom panel shows the total RNA loaded in each lane.

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species results in a thinning and/or sloughing of the cuticle (Keates *et al*., 2003). This may be a result of the direct interaction of NEP1 with lipophilic compounds in the cuticle, or to chloroplast disruption and reprofiling of gene expression that occurs soon after NEP1 application (Keates *et al*., 2003). Regardless of the underlying mechanism, the rapid induction of NLP gene expression by contact with the leaf surface and the ability to erode the cuticle probably allows NLPs to work in concert with cutinases and plant cell wall-degrading enzymes to breach this primary plant defence.

In *Colletotrichum*, several genes are induced in response to contact with solid surfaces, including *Colletotrichum* Hard Surface Induced Protein 1 (*CHIP1*), which encodes a ubiquitin-conjugating enzyme, *CHIP2*, encoding a transcription factor, and *CHIP3*, which has been predicted to encode a membrane-localized protein. The expression of these genes under these circumstances is mediated by calcium and calmodulin signalling pathways (Kim *et al*., 1998, 2000; Liu and Kolattukudy, 1998). We showed that compounds affecting intracellular calcium levels (caffeine, compounds 48/80 and U73122) also affect *SsNep2* expression, although the last two compounds had little if any effect on virulence at the concentrations tested. Conversely, the downregulation of *SsNep2* expression after treatment with the general calcium channel blocker lanthanum chloride coincided with a reduction in virulence. Such compounds are known to have pleiotrophic effects and the expression of the housekeeping gene *SsActin* was also affected, although not to the same degree as *SsNep2*. The importance of calcium-dependent signalling on *SsNep2* expression was also indicated by the inhibition of calcineurin, a calcium- and calmodulin-dependent Ser/Thr protein phosphatase, by cyclosporin A, which resulted in a reduction in virulence and the expression of *SsNep2*. Harel *et al*. (2006) showed that inhibition of calcineurin impairs sclerotial development without affecting the secretion of oxalic acid, which is another important virulence factor.

cAMP was also found to be involved in the regulation of *SsNep2* expression and *S. sclerotiorum* pathogenicity; however, this may be related to the fine control of cAMP levels. The *S. sclerotiorum sac1* mutant, in which the adenylate cyclase gene was deleted, is cAMP deficient and exhibits altered mycelial development and is non-pathogenic (Jurick and Rollins, 2007). We observed that the elevation of cAMP levels by the application of exogenous cAMP also reduced pathogenicity. Some of the developmental abnormalities associated with the adenylate cyclase mutant were corrected by the application of exogenous cAMP (Jurick and Rollins, 2007), however, this was not the case for pathogenicity in our study. Interestingly, the expression of *SsNep2* or *SsActin* was not observed in the *sac1* mutant under any conditions, suggesting that the mutation may have far-reaching pleiotrophic effects.

In summary, we have shown that *S. sclerotiorum* NEPs are able to cause necrosis when expressed *in planta*, but are differentially expressed during infection. The initial expression of, at least, *SsNep2* is dependent on the interaction with solid surfaces, and calcium and cAMP signalling are probably involved. Although the lack of *SsNep2* expression often coincided with reduced virulence or a loss of pathogenicity, some pharmacological treatments reduced *SsNep2* expression without any apparent affect on virulence. Interestingly, expressed sequence tags generated from cDNA libraries made from infected *B. napus* (Li *et al*., 2004a) or infection cushions (Sexton *et al*., 2006) failed to identify genes encoding either SsNEP. As such, further studies, such as the testing of *SsNep* mutants, are required to determine their overall contribution to pathogenesis.

EXPERIMENTAL PROCEDURES

Cultivation of fungi

Sclerotinia sclerotiorum strain 1980 (ATCC18683) was propagated on minimal salts–glucose (1% w/v) (MS-Glu) agar according to Li *et al*. (2004a). The adenylate cyclase deletion mutant, *sac1*, was obtained from J. Rollins (Jurick and Rollins, 2007). The inoculum for all experiments was prepared by grinding 2 g of sclerotia in 200 mL of MS-Glu in a Waring blender for 4 min. The volume was increased to 500 mL in a 1 L baffled flask, and the culture was incubated at 20 °C with shaking (60 r.p.m.) for 3 days.

Isolation and analysis of *SsNep1* and *SsNep2*

RNA was extracted from *B. napus* leaves infected with *S. sclerotiorum* as described below. cDNA was synthesized using an oligo dt20 primer and Superscript 2 reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Using the cDNA as template, the *SsNep1* and *SsNep2* open reading frames were amplified using SsNep1-F (5′-ATCA*GGATCC*ATGCTTTTCACCAACACA-3′) and SsNep1-R (5′-ATAAGAAT*GCGGCCGC*TTAGATTTGTGCCTCTGCGAG-3′) or SsNep2-F (5′-TACTGAATTCGTCGACGGATCCATGGTTGCCTTTGC CAAATCTC-3′) and SsNep2-R (5′-ACAA*CTCGAG*GCGGCCGC CTAGAAACTACTAGCCTTCAC-3′), respectively. As a result of the low levels of *SsNep1* transcripts, the product from the first PCR (50 cycles) was purified and re-amplified to generate sufficient material for cloning. *SsPg1* was amplified using SsPg1-F (5′-CTCGAGAAGAGAGCTCCAGCACCAGCACCA-3′) and SsPg1-R (5′-GGAGGAAGAGACGGTACCGTGAACG-3′).

Signal peptides were identified using SignalP and *N*-linked or *O*-linked glycosylation sites employing NetNGlyc 1.0 and NetOGlyc 2.0 (http://www.expasy.org), respectively. Intron–exon boundaries were inferred using NNSPLICE 0.9 (http://

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www.fruitfly.org/seq_tools/splice.html). Dendograms arising from phylogenetic analysis were constructed according to the neighbour-joining method, and confidence values for the branches were determined using bootstrap analysis, where 100 trees were generated from randomly resampled data obtained by CLUSTALW.16 as provided in PHYLIP version 3.5C (distributed by J. Felsenstein at http://www.cbr.nrc.ca/cgi-bin/WebPhylip/index.html). The CLUSTALW alignment was performed using the Blosum scoring matrix with the following gap penalties; opening gap (10), end gap (10), extending gap (0.05) and separation gap (0.05).

In planta production of SsNEPs via the PVX system

The amplified *SsNep1* fragment was digested with *Bam*HI and *Not*I and cloned into the GATEWAY entry vector pTK172 (a modified GATEWAY entry 1A vector with zeocin resistance; a gift from K. Rozwadowski, AAFC, Saskatoon). To convert pGR107 (Chapman *et al*., 1992) into a GATEWAY destination vector, the GATEWAY cassette A was cloned into the *Sma*I site of pGR107 as a blunt end fragment. This vector was named pGR107-GWY. The *SsNep*1 open reading frame was transferred from pTK172 to pGR107-GWY by recombination according to the GATEWAY protocol (Invitrogen). The *SsNep2* open reading frame was amplified and cloned into pGEM-T Easy (Promega, Madison, WI, USA). The insert was excised from the pGEM-T Easy vector by digestion with *Sal*I and *Xho*I and cloned into the *Sal*I site of the PVX vector pGR107 (Chapman *et al*., 1992). The PVX constructs were transferred to *Agrobacterium tumefaciens* Gv3101 and grown on Luria broth agar. Leaves were inoculated by piercing the base of the leaf with a bacteria-laden toothpick near the main vein of 4–6-week-old *N. benthamiana* plants.

Expression of *SsNep* genes during infection

To study the expression of *SsNep1* and *SsNep2* in different infection zones, 1 g of mycelia (wet weight) was spread over a 5-cm-diameter circle of a *B. napus* cv. Westar leaf surface and incubated in a humidified chamber. Newer leaves of approximately 7 cm in diameter from 45-day-old plants were used. Tissue was collected from several regions, including unaffected tissues beyond the lesion, a 0.5-cm zone at the edge of the lesion and necrotic tissue. In cases in which the leaf was degraded, the lesion was collected and mycelia were separated from any remaining leaf tissue in a Petri dish. A time course study was also conducted in which the samples were collected and processed for transcript analysis.

Expression of *SsNep2* in response to surface contact

Mycelia were spread over the surface of intact *B. napus* leaves, leaves stripped of surface wax, Parafilm™ and glass Petri dishes with or without a coating of leaf wax. Leaf wax was isolated by dipping *B. napus* leaves in a chloroform bath for 30 s and evaporating the chloroform under a stream of liquid nitrogen. Wax was applied by dissolving the crystals in a small volume of chloroform, spreading it to form a thin film on the Petri dish and allowing the chloroform to evaporate. Samples were incubated on the various surfaces in a humidified chamber at 20 °C, after which the mycelia were harvested, frozen in liquid nitrogen and RNA was extracted for Northern blot analysis.

Pharmacological studies

Mycelia were grown in MS-Glu liquid medium as described above, washed with MS medium and then resuspended in MS-Glu with compounds known to affect calcium signalling and cAMP levels. The mycelia were incubated with the compounds for 18 h at 20 °C in the dark prior to use. Compound U73122 was dissolved in 95% ethanol and used at a final concentration of 10 mM. EGTA was used at 10 mM, caffeine at 2.5 and 20 mM, compound 48/80 at 5 μM and lanthanum chloride at 1 and 10 mM dissolved in water. Cyclosporin A was dissolved in dimethylsulfoxide (DMSO) and used at final concentrations of 4.9 and 9.9 nM. Mycelia were transferred to *B. napus* leaves and maintained under humid conditions for 16 h, at which time mycelia were collected and used for Northern blot analysis.

The effect of these compounds on mycelial growth was tested by placing mycelia, treated in the same manner as *B. napus* leaves, on MS-Glu agar and incubating at 20 °C.

RNA isolation, Northern blotting and RT-PCR analysis

To isolate total fungal RNA, 150 mg of mycelia (wet weight) was ground in liquid N_2 and dispensed into a 1.5-mL microcentrifuge tube containing 600 μL of extraction buffer [0.1 M NaCl, 2% sodium dodecylsulfate (SDS), 50 mM Tris–HCl (pH 9.0), 10 mM ethylenediaminetetraacetic acid (EDTA)] and 600 μL phenol–chloroform–isoamyl alcohol (25 : 24 : 1). Samples were mixed for 30 s using a vortex and centrifuged for 10 min at 15 000 ***g***. The aqueous phase was extracted again with an equal volume of chloroform, transferred to a new microcentrifuge tube and the RNA was precipitated using lithium chloride (2 M final concentration) overnight at 4 °C. The pellet was washed once with 2 M lithium chloride and once with 75% ethanol. RNA was air dried for 10 min and dissolved in 35 μL diethylpyrocarbonate (DEPC)-treated double-distilled H_2O. Northern blotting was performed as described in Li *et al*. (2004b). *SsNep* cDNA was amplified using the same primers as mentioned above, labelled with ^{32}P-CTP and used as a probe. A segment of the *SsActin* gene (GENBANK accession no. XP_001589969) was amplified using SsActinF (5′-CAGCGTTCTACGTCTCTATC-3′) and SsActinR (5′-CGAACATC AACATCACAC-3′) primers.

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For RT-PCR analysis, mRNA was converted to cDNA using the Superscript First Strand Synthesis System (Invitrogen), followed by PCR, employing the primer pairs described above. The PCR mixture contained 2.5 μL of 10 × buffer, 2 μL of deoxynucleoside triphosphates (dNTPs) (5 mM), 2 μL of forward primer (2.5 mM), 2 μL of reverse primer (2.5 mM), 0.5 μL of rTaq polymerase I, 2 μL of a 1/20th dilution of cDNA and 14 μL of distilled H_2O. A 2-μL aliquot of genomic DNA (40 ng/μL) was used as a control. The PCR conditions used were as follows: initial denaturation at 94 °C for 5 min, followed by 27 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension of 72 °C for 7 min.

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REFERENCES

Amsellem, Z., Cohen, B.A. and Gressel, J. (2002) Engineering hypovirulence in a mycoherbicidal fungus for efficient weed control. *Nat. Biotechnol.* **20**, 1035–1039.

Bae, H., Moon, S.K., Richard, C.S., Bae, H.-J. and Bailey, B. (2006) Necrosis and ethylene inducing peptide from *Fusarium oxysporum* induces a complex cascade of transcripts associated with signal transduction and cell death in Arabidopsis. *Plant Physiol.* **141**, 1056–1067.

Bailey, B.A. (1995) Purification of a protein from culture filtrate of *Fusarium oxysporum* that induces ethylene and necrosis in leaves of *Erythroxylum coca*. *Phytopathology*, **85**, 1250–1255.

Berridge, M.J. (1997) Elementary and global aspects of calcium signaling. *J. Physiol.* **499**, 291–306.

Boland, G.J. and Hall, R. (1994) Index of plant hosts of *Sclerotinia sclerotiorum*. *Can. J. Plant Pathol.* **16**, 93–100.

Cessna, S.G., Sears, V.E., Dickman, M.B. and Low, P.S. (2000) Oxalic acid, a pathogenicity factor for *Sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. *Plant Cell*, **12**, 2191–2200.

Chapman, S., Kavanagh, T.A. and Baulcombe, D.C. (1992) Potato virus X as a vector for gene expression in plants. *Plant J.* **2**, 549–557.

Dickman, M.B., Park, Y.K., Oltersdorf, T., Li, W., Clemente, T. and French, R. (2001) Abrogation of disease development in plants expressing animal antiapoptotic genes. *Proc. Natl. Acad. Sci. USA*, **98**, 6957–6962.

D'Souza, C.A. and Heitman, J. (2001) Conserved cAMP signalling cascades regulate fungal development and virulence. *FEMS Microbiol. Rev.* **25**, 349–364.

Fellbrich, G., Romanski, A., Varet, A., Blume, B., Brunner, F., Enelhardt, S., Felix, G., Kemmerling, B., Krzymowska, M. and Nurnberger, T. (2002) NPP1, a *Phytophthora*-associated trigger of plant defense in parsley and Arabidopsis. *Plant J.* **32**, 375–390.

Fox, D.S. and Heitman, J. (2002) Good fungi gone bad: the corruption of calcineurin. *BioEssays*, **24**, 894–903.

Garcia, O., Macedo, J., Tiburcio, R., Zaparoli, G., Rincones, J., Bittencourt, L., Ceita, G., Micheli, F., Gesteira, A., Mariano, A., Schiavinato, M., Medrano, F., Meinhardt, L., Pereira, G. and Cascardo, J. (2007) Characterization of necrosis and ethylene-inducing proteins (NEP) in the basidiomycete *Moniliophthora perniciosa*, the causal agent of witches' broom in *Theobroma cacao*. *Mycol. Res.* **3**, 443–455.

Gijzen, M. and Nürnberger, T. (2006) Nep1-like proteins from plant pathogens: recruitment and diversification of the NPP1 domain across taxa. *Phytochemistry*, **67**, 1800–1807.

Godoy, G., Steadman, J.R., Dickman, M.B. and Dam, R. (1990) Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on *Phaseolus vulgaris*. *Physiol. Mol. Plant Pathol.* **37**, 179–191.

Guo, W., Gonzalez-Candelas, L. and Kolattukudy, P.E. (1996) Identification of a novel *pelD* gene expressed uniquely *in planta* by *Fusarium solani* f. sp. *pisi* (*Nectria haematococca*, mating type VI) and characterization of its protein product as an endo-pectate lyase. *Arch. Biochem. Biophys.* **332**, 305–312.

Harel, A., Bercovic, S. and Yarden, O. (2006) Calcineurin is required for sclerotial development and pathogenicity of *Sclerotinia sclerotiorum* in an oxalic acid-independent manner. *Mol. Plant–Microbe Interact.* **19**, 682–693.

Hegedus, D., Li, R., Buchwaldt, L., Parkin, I., Whitwill, S., Coutu, C., Bekkaoui, D. and Rimmer, R. (2008) *Brassica napus* possesses an expanded set of polygalacturonase inhibitor protein genes that are differentially regulated in response to *Sclerotinia sclerotiorum* infection, wounding and defence hormone treatment. *Planta*, **228**, 241–253.

Jennings, J.C., Apel-Birkhold, P.C., Mock, N.M., Baker, C.J., Anderson, J.D. and Bailey, B.A. (2001) Induction of defense responses in tobacco by the protein Nep1 from *Fusarium oxysporum*. *Plant Sci.* **161**, 891–899.

Jores, J., Appel, B. and Lewin, A. (2003) Cloning and molecular characterization of a unique hemolysin gene of *Vibrio pommerensis* sp. nov.: development of a DNA probe for the detection of the hemolysin gene and its use in identification of related *Vibrio* spp. from the Baltic Sea. *FEMS Microbiol. Lett.* **229**, 223–229.

Jurick, W.M. and Rollins, J.A. (2007) Deletion of the adenylate cyclase (*sac1*) gene affects multiple developmental pathways and pathogenicity in *Sclerotinia sclerotiorum*. *Fungal Genet. Biol.* **44**, 521–530.

Kars, I., Krooshof, G.H., Wagemakers, L., Joosten, R., Benen, J.A.E. and Van Kan, J.A.L. (2005) Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. *Plant J.* **43**, 213–225.

Keates, S.E., Kostman, T.A., Anderson, J.D. and Bailey, B.A. (2003) Altered gene expression in three plant species in response to treatment with Nep1, a fungal protein that causes necrosis. *Plant Physiol.* **132**, 1610–1622.

Kim, Y.K., Li, D. and Kolattukudy P.E. (1998) Induction of Ca^{2+}-calmodulin signalling by hard-surface contact primes *Colletotrichum gloeosporioides* conidia to germinate and form appressoria. *J. Bacteriol.* **180**, 5144–5150.

Kim, Y.K., Liu, Z.M., Li, D. and Kolattukudy, P.E. (2000) Two novel genes induced by hard-surface contact of *Colletotrichum gloeosporioides* conidia. *J. Bacteriol.* **182**, 4688–4695.

Kim, K.S., Min, J.-Y. and Dickman, M.B. (2008) Oxalic acid is an elicitor of plant programmed cell death during *Sclerotinia sclerotiorum* disease development. *Mol. Plant–Microbe Interact.* **21**, 605–612.

Kolattukudy, P.E., Rogers, L.M., Li, D., Hwang, C.S. and Flaishman,

© 2009 HER MAJESTY THE QUEEN IN RIGHT OF CANADA

M.A. (1995) Surface signaling in pathogenesis. *Proc. Natl. Acad. Sci. USA*, **92**, 4080–4087.

Li, R., Rimmer, R., Buchwaldt, L., Sharpe, A., Séguin-Swartz, G., Coutu, C. and Hegedus, D.D. (2004a) Interaction of *Sclerotinia sclerotiorum* with a resistant *Brassica napus* cultivar: expressed sequence tag analysis identifies genes associated with fungal pathogenesis. *Fungal Genet. Biol.* **41**, 735–753.

Li, R., Rimmer, R., Buchwaldt, L., Sharpe, A., Seguin-Swartz, G. and Hegedus, D.D. (2004b) Interaction of *Sclerotinia sclerotiorum* with *Brassica napus*: cloning and characterization of endo- and exo-polygalacturonases expressed during saprophytic and parasitic modes. *Fungal Genet. Biol.* **41**, 754–765.

Liu, Z.M. and Kolattukudy, P.E. (1998) Identification of a gene product induced by hard-surface contact of *Colletotrichum gloeosporioides* conidia as a ubiquitin-conjugating enzyme by yeast complementation. *J. Bacteriol.* **180**, 3592–3597.

Mattinen, L., Tshuikina, M., Mae, A. and Pirhonen, M. (2004) Identification and characterization of Nip, Necrosis-Inducing Virulence Protein of *Erwinia carotovora* subsp. *carotovora*. *Mol. Plant–Microbe Interact.* **17**, 1366–1375.

Mogami, H., Lloyd Mills, C. and Gallacher, D.V. (1997) Phospholipase C inhibitor, U73122, releases intracellular Ca^{2+}, potentiates Ins(1,4,5)P_3-mediated Ca^{2+} release and directly activates ion channels in mouse pancreatic acinar cells. *Biochem. J.* **324**, 645–651.

Pemberton, C.L. and Salmond, G.P.C. (2004) The Nep1-like proteins—a growing family of microbial elicitors of plant necrosis. *Mol. Plant Pathol.* **5**, 353–359.

Qutob, D., Kamoun, S. and Gijzen, M. (2002) Expression of *Phytophthora sojae* necrosis-inducing protein occurs during transition from biotrophy to necrotrophy. *Plant J.* **32**, 361–373.

Qutob, D., Kemmerling, B., Brunner, F., Kufner, I., Engelhardt, S., Gust, A.A., Luberacki, B., Seitz, U.H., Stahl, D., Rauhut, T., Glawischnig, E., Schween, G., Lacombe, B., Watanabe, N., Lam, E., Schlichting, R., Scheel, D., Nau, K., Dodt, G., Hubert, D., Gijzen, M. and Numberger, T. (2006) Phytotoxicity and innate immune responses induced by Nep1 like proteins. *Plant Cell*, **18**, 3721–3744.

Rogers, L.M., Kim, Y.K., Guo, W., Gonzalez-Candelas, L., Li, D. and Kolattukudy, P.E. (2000) Requirement for either a host- or pectin-induced pectate lyase for infection of *Pisum sativum* by *Nectria haematococca*. *Proc. Natl. Acad. Sci. USA*, **97**, 9813–9818.

Rollins, J.A. and Dickman, M.B. (1998) Increase in endogenous and exogenous cyclic AMP levels inhibits sclerotial development in *Sclerotinia sclerotiorum*. *Appl. Environ. Microbiol.* **64**, 2539–2544.

Schouten, A., Van Baarlen, P. and Van Kan, J. (2008) Phytotoxic Nep1 like proteins from the necrotrophic fungus *Botrytis cinerea* associate with membranes and the nucleus of plant cells. *New Phytol.* **177**, 493–505.

Sexton, A.C., Cozijnsen, A.J., Keniry, A., Jewell, E., Love, C.G., Batley, J., Edwards, D. and Howlett, B.J. (2006) Comparison of transcription of multiple genes at three developmental stages of the plant pathogen *Sclerotinia sclerotiorum*. *FEMS Microbiol. Lett.* **258**, 150–160.

Staats, M., Van Baarlen, P., Schouten, A. and Van Kan, J. (2007) Functional analysis of NLP genes from *Botrytis elliptica*. *Mol. Plant Pathol.* **8**, 209–214.

Tsuba, M., Katagiri, C., Takeuchi, Y., Takada, Y. and Yamaoka, N. (2002) Chemical factors of the leaf surface involved in the morphogenesis of *Blumeria graminis*. *Physiol. Mol. Plant Pathol.* **60**, 51–57.

Van Kan, J. (2006) Licensed to kill: the lifestyle of a necrotrophic plant pathogen. *Trends Plant Sci.* **11**, 247–253.

Veit, S., Worle, J.M., Nurnberger, T., Koch, W. and Seitz, H.U. (2001) A novel protein elicitor (PaNie) from *Pythium aphanidermatum* induces multiple defence responses in carrot, *Arabidopsis*, and tobacco. *Plant Physiol.* **127**, 832–841.

Zuppini, A., Navazio, L., Sella, L., Castiglioni, C., Favaron, F. and Mariani, P. (2005) An endopolygalacturonase from *Sclerotinia sclerotiorum* induces calcium-mediated signalling and programmed cell death in soybean cells. *Mol. Plant–Microbe Interact.* **18**, 849–855.

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