A novel elicitor protein from *Phytophthora parasitica* induces plant basal immunity and systemic acquired resistance

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SUMMARY

The interaction between *Phytophthora* pathogens and host plants involves the exchange of complex molecular signals from both sides. Recent studies of *Phytophthora* have led to the identification of various apoplastic elicitors known to trigger plant immunity. Here, we provide evidence that the protein encoded by *OPEL* of *Phytophthora parasitica* is a novel elicitor. Homologues of *OPEL* were identified only in oomycetes, but not in fungi and other organisms. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) revealed that *OPEL* is expressed throughout the development of *P. parasitica* and is especially highly induced after plant infection. Infiltration of *OPEL* recombinant protein from *Escherichia coli* into leaves of *Nicotiana tabacum* (cv. Samsun NN) resulted in cell death, callose deposition, the production of reactive oxygen species and induced expression of pathogen-associated molecular pattern (PAMP)-triggered immunity markers and salicylic acid-responsive defence genes. Moreover, the infiltration conferred systemic resistance against a broad spectrum of pathogens, including *Tobacco mosaic virus*, the bacteria wilt pathogen *Ralstonia solanacearum* and *P. parasitica*. In addition to the signal peptide, *OPEL* contains three conserved domains: a thaumatin-like domain, a glycine-rich protein domain and a glycosyl hydrolase (GH) domain. Intriguingly, mutation of a putative laminarinase active site motif in the predicted GH domain abolished its elicitor activity, which suggests enzymatic activity of *OPEL* in triggering the defence response.

Keywords: basal immunity, elicitor, laminarinase, *OPEL*, systemic acquired resistance.

INTRODUCTION

To combat pathogens, plants have developed two distinct layers of immunity: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Dodds and Rathjen, 2010; Jones and Dangl, 2006). PAMPs, also known as microbe-associated molecular patterns (MAMPs), are slowly evolving signature-pattern molecules derived from patho-

gens, such as flg22 and elf18 from bacteria, chitin, β -glucans and ergosterol from fungi, and cellulose-binding domains (CBDs), elicitors and Pep-13 from oomycetes (Ingle *et al.*, 2006). The perception of PAMPs by plant pattern recognition receptors rapidly activates PTI-associated responses, including mitogen-activated protein kinase cascades, the generation of reactive oxygen species (ROS), callose deposition, the induction of defence-related genes and, in some cases, cell death (Altenbach and Robatzek, 2007; Boller and Felix, 2009; Zipfel, 2009). In this way, plants exhibit the first layer of plant immunity to pathogen infection, also known as basal immunity (Jones and Dangl, 2006; Zipfel, 2008).

In addition to PAMPs, degradation products from plant components generated by microbial activity, known as damage-associated molecular patterns (DAMPs), can trigger plant immunity (Boller and Felix, 2009; Heil, 2012). Prominent examples of DAMPs include cell wall fragments generated by cell wall-degrading enzymes (CWDEs) of pathogens, such as oligogalacturonides (Ridley *et al.*, 2001).

To circumvent the activation of PTI, some microbial pathogens have developed the ability to produce effectors. As a countermeasure, plants have also evolved additional receptors, such as resistance (R) proteins, that specifically recognize cognate effectors or effector-mediated alteration of host targets. This interaction results in a rapid activation of strong plant resistance, known as ETI, which is often associated with the hypersensitive response (HR), to restrict pathogens to the infection site (Dodds and Rathjen, 2010; Jones and Dangl, 2006).

In addition to local resistance elicited by PTI and ETI, plants show systemic resistance responses in noninoculated portions of the plants. These responses are generally grouped into two categories: systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Grant and Lamb, 2006). The establishment of SAR involves systemic salicylic acid (SA) accumulation and the induction of a set of pathogenesis-related (PR) proteins together with other defence genes. Moreover, the resistance conferred by SAR is usually long-lasting and effective against a broad spectrum of pathogens, including viruses, bacteria, oomycetes and fungi (Fu and Dong, 2013). In contrast, ISR is induced in response to the colonization of plant roots by plant growth-promoting rhizosphere bacteria and involves both jasmonic acid (JA) and ethylene (ET) signalling (Pieterse *et al.*, 1998; Ton *et al.*, 2002).

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Oomycetes are a phylogenetically distinct group of eukaryotic microorganisms that resemble true fungi in morphological features and pathogenic lifestyle, yet belong to a different kingdom, the Chromista, with brown algae and diatoms as close relatives (Baldauf *et al.*, 2000). Members of the genus *Phytophthora* are of special interest because many cause devastating diseases in crops and ornamental plants, as well as producing environmental damage in natural ecosystems (Erwin and Ribeiro, 1996).

Phytophthora pathogens are hemibiotrophs, which acquire nutrients from living cells in the early stage of plant infection, thus as biotrophs, but later switch into necrotrophs, which trigger cell death in host plants to facilitate pathogen colonization. To promote infection and colonization of plant tissue, *Phytophthora* pathogens have evolved an arsenal of cytoplasmic effectors, such as the Avr-encoding RXLR effectors and crinkling- and necrosis-inducing proteins, which function to alter the host plant physiology and to suppress plant immunity (Hein *et al.*, 2009; Kamoun, 2006; Stassen and van den Ackerveken, 2011). In addition, *Phytophthora* pathogens secrete an array of elicitors that function in the plant apoplast to trigger PTI or similar defence responses; these elicitors include GP42 of *Phytophthora sojae* (Brunner *et al.*, 2002; Nürnberger *et al.*, 1994), cellulose-binding elicitor lectin (CBEL) of *P. parasitica* (Gaulin *et al.*, 2006; Mateos *et al.*, 1997), Nep1-like proteins (Gijzen and Nürnberger, 2006) and elicitors, such as INF1 of *P. infestans* (Kamoun *et al.*, 1997; Ponchet *et al.*, 1999).

Elicitins are a family of structurally related proteins secreted by *Phytophthora* spp. that induce HR and other defence responses in a limited range of dicot plants, including *Nicotiana* spp. (Kamoun *et al.*, 1993a, b, 1997; Ricci *et al.*, 1989). Moreover, elicitors from *P. cryptogea* and *P. capsici*, known as cryptogein and capsicein, respectively, can trigger SAR in tobacco (Keller *et al.*, 1996; Ricci *et al.*, 1989). Cryptogein can bind to sterols, such as dehydroergosterol, and thus may function as sterol carrier proteins to help with the uptake of sterols by *Phytophthora* (Boissy *et al.*, 1999; Mikes *et al.*, 1997; Vauthrin *et al.*, 1999). GP42 of *P. sojae* encodes a calcium-dependent transglutaminase, an abundant cell wall glycoprotein that induces elicitor activity by binding to a plasma membrane receptor in parsley cells (Brunner *et al.*, 2002; Nürnberger *et al.*, 1994). The elicitor activity of GP42 can be narrowed to a fragment with only 13 amino acid residues, known as Pep-13, which, in parsley, is necessary and sufficient to stimulate defence responses, including ion efflux, ROS generation, phytoalexin synthesis and the induction of defence-related genes (Brunner *et al.*, 2002). Thus, Pep-13 was recognized as a PAMP of *Phytophthora* (Brunner *et al.*, 2002). CBEL is a cell wall protein, first isolated from *P. parasitica* var. *nicotianae*, and is highly conserved in the genus *Phytophthora* (Mateos *et al.*, 1997; Séjalon-Delmas *et al.*, 1997). It is required for polysaccharide deposition in the cell wall

and for adhesion of the mycelium to cellulosic substrates, but features no hydrolytic activity (Gaulin *et al.*, 2002; Mateos *et al.*, 1997). Sequence analysis indicated that CBEL contains two CBDs that belong to carbohydrate-binding module 1 and are sufficient to stimulate defence responses in plants (Gaulin *et al.*, 2006). Accordingly, CBD has been defined as a novel molecular pattern in oomycetes that might act by interacting with the cell wall (Gaulin *et al.*, 2006).

In this article, we demonstrate that OPEL of *P. parasitica* (= *P. nicotianae*) can elicit basal defence responses, including callose deposition, ROS accumulation and cell death, in *Nicotiana tabacum* (cv. Samsun NN). In addition, it induces SAR against different pathogens. Homologues of OPEL were identified in oomycetes, but not in fungi or other organisms. Sequence analysis revealed the presence of a putative active site motif in the C-terminal glycosyl hydrolase (GH) domain of OPEL. Mutations on this motif abolished the elicitor activity of OPEL. These results demonstrate that OPEL is a novel elicitor of *Phytophthora* and the enzymatic activity of OPEL may be essential for its elicitor activity.

RESULTS

OPEL encodes a secretory protein in *P. parasitica*

OPEL (GenBank accession no. AAP85258.1) was first identified as a secretory protein in the culture filtrate of *P. parasitica* (Yan, 2001). To clone this gene, we determined the N-terminal amino acid sequence of OPEL by Edman degradation, which resulted in 'ETVNFINKXSFPIELYXS'. One set of degenerated oligonucleotides, PGSA (Table S1, see Supporting Information), was then designed and used as gene-specific primer for cloning of the partial cDNA sequence by 3'-rapid amplification of cDNA ends (3'-RACE). Subsequently, 5'-RACE was performed with the primer PGR1 (Table S1), which was designed from the newly obtained cDNA sequence, to obtain the full-length cDNA sequence of OPEL.

Sequence analysis revealed that OPEL encodes a polypeptide of 556 amino acid residues with a predicted molecular mass of 58 kDa and pI of 4.8. Conserved domain analysis revealed that OPEL contains a signal peptide in the N-terminus (amino acids 1–23), followed by a thaumatin-like domain (amino acids 24–220), which contains three repeats of DQTQQQ, a glycine-rich protein domain (amino acids 240–315; DUF2403; Pfam accession code PF10290) and a GH domain (amino acids 316–556; DUF2401; Pfam accession code PF10287), which includes two putative *N*-glycosylation sites (³¹⁷NGSW³²⁰ and ³³⁵NLTF³³⁸) (Figs 1a and S1, see Supporting Information). BLAST search of public databases identified TOS1 (NP_009720) of the budding yeast *Saccharomyces cerevisiae*, a cell wall protein with a two-domain structure consisting of an N-terminal glycine-rich protein

Fig. 1 Conserved domain prediction and phylogenetic analysis of OPEL. (a) Conserved domains and a putative laminarinase active site motif found in OPEL (AAP85258.1). Numerals indicate the positions of amino acid residues. (b) Phylogenetic analysis of OPEL and its homologues. The phylogenetic tree was constructed by the neighbour-joining algorithm implemented in MEGA 5 with 1000 bootstrap replicates. The accession number of each gene is indicated after the pathogen name.

domain (DUF2403) and a C-terminal GH domain (DUF2401). The latter shows 47% similarity and 33% identity to the GH domain of OPEL. But, Tos1 lacks the thaumatin-like domain. Therefore, it does not seem to be a homologue of OPEL. Recently, TOS1 and other members of the DUF2401 family have been found to contain a signature active site motif of laminarinases (1,3- β -glucanase; EC 3.2.1.6), ExDxxE (x, any amino acid residue) (Steczkiewicz *et al.*, 2010). The same motif was identified in OPEL at positions 473–478 (Figs 1a and S1).

Homologues of OPEL exist only in oomycetes, but not in fungi

OPEL exists as a single-copy gene in *P. parasitica*, as shown by Southern blot analysis (Yan, 2001). To determine the presence of OPEL homologues in other organisms, we performed a BLAST search of the National Center for Biotechnology Information (NCBI) and genome databases for various oomycetes. Homologues of OPEL were identified only in oomycetes, including *P. capsici*, *P. infestans*, *P. ramorum*, *P. sojae*, *Hyaloperonospora arabidopsidis*, *Pythium ultimum* and *Albugo laibachii*, but not in fungi or other organisms. Furthermore, we performed genomic polymerase chain reaction (PCR) using the degenerated primers for OPEL (Table S1) and found DNA fragments of the expected

size from *P. botryosa*, *P. colocasiae*, *P. meadii*, *P. nemorosa* and *P. pseudosyringae*. Analyses of their sequences confirmed the presence of OPEL homologues in various *Phytophthora* species (Fig. S1). Alignment of OPEL homologues demonstrated a high conservation of OPEL among different species of oomycete (Fig. S2, see Supporting Information). Phylogenetic analysis revealed that OPEL and its homologues from other *Phytophthora* spp. form a clade distinct from those of other oomycetes, with the homologue from *H. arabidopsidis* as the closest relative (Fig. 1b).

The expression of OPEL is highly induced in planta

To determine whether OPEL is induced during invasion by *P. parasitica*, we inoculated 5-week-old tobacco (*Nicotiana benthamiana*) with zoospores of the pathogen and analysed OPEL expression. We also analysed RNA samples from different life stages of *P. parasitica*. Data were normalized to the expression of WS21, which encodes a ribosomal protein and shows a constant expression across different life stages of *P. parasitica* (Yan and Liou, 2006). Compared with the mRNA level of OPEL in mycelium, that in sporangia, zoospores, cysts and germinating cysts was low, but high in inoculated plants at 12 h post-inoculation (hpi) and even greater at 24 hpi, and then declined at 48 and 72 hpi (Fig. 2).

Fig. 2 Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of *OPEL* expression. RNA samples were prepared from different life stages of *Phytophthora parasitica*, including mycelium (MY), sporangia (SP), zoospores (ZO), cysts (CY) and germinated cysts (GC), as well as *Nicotiana benthamiana* plants (5 weeks old) inoculated with zoospores of *P. parasitica*. The expression of *OPEL* is presented as the fold change relative to that of mycelium, and values are means $\pm$ SD from three independent experiments. Different letters indicate significant differences among samples by Fisher's protected least-significant difference test at $P < 0.001$. hpi, hours post-inoculation.

OPEL induces cell death, callose deposition and ROS accumulation

To investigate the biological function of OPEL, we expressed it as a glutathione transferase (GST) fusion protein in *Escherichia coli*. After treatment of GST-OPEL fusion protein with thrombin to remove GST, we isolated OPEL and GST recombinant proteins by gel filtration, and confirmed their purity by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. S3, see Supporting Information). We then infiltrated the OPEL recombinant protein [or 2-(*N*-morpholino)ethanesulphonic acid (MES) and the GST recombinant protein as a control] into leaves of 5-week-old *N. benthamiana* and inspected them daily for symptoms, but found no changes. We then performed similar experiments on *N. tabacum*. At 2 days post-treatment, leaves of control plants remained green and healthy, but infiltration with OPEL conferred symptoms of necrosis (Fig. 3a, top panel). Moreover, cell death was increased with increasing concentration of OPEL from 0.1 to 0.3 μ M. At 7 days post-treatment, leaves infiltrated with 0.3 μ M OPEL showed the characteristic HR-like cell death, which was further confirmed by staining with trypan blue (Fig. 3a, bottom panel).

To determine whether OPEL triggers the plant defence response, we examined callose deposition and ROS accumulation

in *N. tabacum* leaves after infiltration with OPEL. OPEL increased callose deposition relative to MES or GST treatment (Fig. 3b). Treatment with Pep-13 or flg22, representing PAMPs of *Phytophthora* and bacteria and positive controls, also induced callose deposition (Fig. 3b). Callose deposition induced by treatment with 0.1 μ M OPEL was similar to that induced by 0.3 μ M flg22 (Fig. 3c). Treatment with 0.3 μ M OPEL increased callose deposition to an even greater level, which differed significantly from that with 0.1 μ M OPEL (Fig. 3c). Staining with 3,3'-diaminobenzidine (DAB) at 6 h post-treatment showed that treatment with OPEL (0.1 or 0.3 μ M) induced prominent accumulation of H_2O_2 relative to treatment with MES, GST, Pep-13 or flg22 (Fig. 3d). The intensity of signals with OPEL at 0.1 or 0.3 μ M differed significantly from that with MES, GST, Pep-13 or flg22 (Fig. 3e). Therefore, OPEL could induce cell death, callose deposition and ROS accumulation in *N. tabacum*.

OPEL enhances plant resistance against *P. parasitica*

To determine whether OPEL indeed induces plant resistance, we infiltrated *Agrobacterium tumefaciens* (strain C58C1) harbouring pk7WG2::OPEL-GFP into *N. tabacum* to express the OPEL-green fluorescent protein (OPEL-GFP) fusion protein. However, transfected leaves turned yellow 2 days after agroinfiltration. As an alternative, the experiments were performed with *N. benthamiana* leaves, with one-half of the leaf expressing the OPEL-GFP fusion protein and the other side expressing GFP as a control. Two days after agroinfiltration, when OPEL proteins were detectable by immunoblot analysis (Fig. S4, see Supporting Information), we inoculated both sides of the leaves with mycelial discs of *P. parasitica* and inspected them daily for disease symptoms. Compared with GFP-pretreated leaves, OPEL-GFP-pretreated leaves showed significantly reduced disease severity in terms of symptoms (Fig. 4a) and infected area (Fig. 4b). Therefore, OPEL induced plant resistance against *P. parasitica*.

OPEL induces the expression of SA-responsive genes and PTI marker genes

To investigate the mechanism underlying OPEL-triggered plant resistance, we infiltrated leaves of 6-week-old *N. tabacum* with OPEL recombinant proteins from *E. coli* (or GST as a control) and analysed the expression of several plant defence-related genes. *PR1*, *PR5* and *phenylalanine ammonia lyase* (*PAL*), all SA responsive, were significantly induced in response to OPEL when compared with the control (Fig. 5). *Pto-interacting 5* (*Pti5*) and *Gras* (*GAI*, *RGA*, *SCR*) 2 (*Gras2*), both PTI markers (Nguyen *et al.*, 2010), were also significantly induced. In contrast, OPEL treatment did not alter the expression of *CORONATINE INSENSITIVE 1* (*COI1*), *ethylene insensitive 2* (*EIN2*) or *plant defensin gene 1.2* (*PDF1.2*)

Fig. 3 OPEL induces plant basal defence response on *Nicotiana tabacum* (cv. Samsun NN). (a) OPEL induces cell death. Tobacco leaves (6 weeks old) were infiltrated with 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer (as a control), recombinant proteins of glutathione transferase (GST; 0.3 μM) or OPEL (0.1 or 0.3 μM). Photographs were taken at 2 or 7 days post-infiltration (dpi) directly under bright light (top panel) or after staining with trypan blue (bottom panel). (b) OPEL induces callose deposition. Two days after infiltration with MES buffer, flg22, Pep-13, recombinant protein of OPEL or GST into leaves of 6-week-old tobacco plants, leaves were stained with aniline blue, and photographs were taken under a microscope. Bar, 25 μm . (c) Callose deposition quantified as the number of individual depositions per square millimetre microscopic field. Values are means $\pm$ SD from three independent experiments. Different letters indicate significant difference between treatments by Fisher's protected least-significant difference tests at $P < 0.05$. (d) OPEL causes hydrogen peroxide (H_2O_2) accumulation. Six hours after the infiltration of 6-week-old tobacco leaves with MES buffer, recombinant OPEL protein, GST, flg22 or Pep-13, leaves were stained with 3,3'-diaminobenzidine (DAB) and then photographed. (e) Quantification of DAB staining. Values are means $\pm$ SD from three independent experiments. Different letters indicate significant difference among treatments as revealed by Fisher's protected least-significant difference test at $P < 0.001$.

Fig. 4 OPEL induces plant resistance against *Phytophthora parasitica*. (a) Transient expression of OPEL enhances plant resistance against *P. parasitica*. After agroinfiltration, OPEL-green fluorescent protein (OPEL-GFP) and GFP (as a control) were expressed transiently on opposite sides of *Nicotiana benthamiana* (5-week-old) leaves. After 2 days, both sides of the leaves were inoculated with mycelial discs of *P. parasitica* and examined for disease symptoms. The photographs were taken at 60 h post-inoculation (hpi) under UV light. (b) Measurement of the infected areas. Values are means $\pm$ SD from three independent experiments. * $P < 0.05$ by Student's *t*-test.

Fig. 5 OPEL induces the expression of defence genes. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of gene expression in leaves of 6-week-old *Nicotiana tabacum* (cv. Samsun NN) infiltrated with OPEL recombinant protein (0.3 μ M; OPEL) or glutathione transferase (0.3 μ M; GST) at 6 h post-infiltration. Values are means $\pm$ SD fold change relative to the transcript level of GST-treated plants from three independent experiments. ** $P < 0.001$ by Student's *t*-test compared with GST. PAL, phenylalanine ammonia lyase; PR1, pathogenesis-related 1; PR5, pathogenesis-related 5; COI1, CORONATINE INSENSITIVE 1; PDF1.2, plant defensin gene 1.2; EIN2, ethylene insensitive 2; Pti5, Pto-interacting 5; Gras2, (GAI, RGA, SCR) 2.

(Fig. 5). *EIN2* is ET responsive, *COI1* is JA responsive and *PDF1.2* is responsive to both ET and JA (Bari and Jones, 2009). Thus, OPEL induced the expression of PTI markers as well as SA-responsive defence genes.

OPEL induces plant systemic resistance against Tobacco mosaic virus (TMV), *P. parasitica* and *Ralstonia solanacearum*

SA plays a central role in SAR. To determine whether OPEL triggers SAR, we infiltrated the OPEL recombinant protein (or MES as a control) into leaves of 6-week-old *N. tabacum* plants. After 3, 7 and 14 days, we inoculated systemic leaves with TMV and examined them for local lesions. The size of local lesions caused by TMV infection was similar in OPEL-pretreated and control plants at 3 dpi, with diameters of 2.17 ± 0.46 mm (OPEL) and 2.15 ± 0.45 mm (MES) (Fig. 6a). However, the number of local lesions was lower for OPEL-pretreated plants than control plants with TMV inoculation at 3, 7 and 14 days after OPEL infiltration (Fig. 6b). Hence, OPEL can induce plant systemic resistance against TMV, and the effect could last for at least 14 days.

To determine whether SAR triggered by OPEL is effective for other pathogens, after treatment of *N. tabacum* with OPEL, systemic leaves were detached and inoculated with mycelial discs of *P. parasitica*. Severe disease symptoms developed on control leaves at 7 dpi (Fig. 6c), whereas the infection area on systemic leaves of OPEL-pretreated plants was significantly reduced (Fig. 6c,d). In addition, OPEL-pretreated tobacco plants were inoculated with the bacterial wilt pathogen *R. solanacearum*. The wilting symptom characteristic of *R. solanacearum* infection was first detected at 5 dpi in both OPEL-pretreated and control plants (Fig. 7a). Subsequently, disease symptoms in the control plants became more severe, with most plants showing a high wilting score of 4 at 10 dpi (MES; Fig. 7a). In contrast, OPEL pretreatment conferred plant systemic resistance against infection with *R. solanacearum*, with about one-half of the inoculated plants remaining symptomless at 10 dpi (Fig. 7a,b). Therefore, OPEL pretreatment conferred plant systemic resistance against a broad spectrum of pathogens. To determine whether the genes involved in long-distance signalling of SAR are induced by OPEL, we analysed the expression of a tobacco *DEFECTIVE IN INDUCED RESISTANCE 1 (DIR1)* homologue and found that it was highly induced in systemic leaves at 12 h after OPEL treatment (Fig. 7c).

The GH domain alone can induce callose deposition and ROS accumulation

To investigate which of the conserved domains plays a key role in the induction of plant resistance, we expressed recombinant proteins corresponding to different segments of OPEL in *E. coli*: the thaumatin-like domain (amino acids 24–220; Thau), the glycine-rich domain (amino acids 240–315; GR), the GH domain (amino acids 316–556; GH) and one encompassing both the glycine-rich and GH domains (amino acids 240–556; GR-GH),

Fig. 6 OPEL induces systemic resistance against *Tobacco mosaic virus* (TMV) and *Phytophthora parasitica* on *Nicotiana tabacum* (cv. Samsun NN). (a) Disease symptoms caused by TMV infection. Three days after infiltration with the OPEL recombinant protein or 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer (as a control) on leaves of 6-week-old tobacco plants, systemic leaves were inoculated with TMV. Photographs were taken at 3 days post-inoculation (dpi). (b) Systemic resistance conferred by OPEL can last for at least 14 days. At 3, 7 and 14 days after infiltration with the OPEL recombinant protein or MES buffer on leaves of 6-week-old tobacco plants, systemic leaves were inoculated with TMV. At 3 dpi, the number of local lesions caused by TMV infection was counted. Values are means $\pm$ SD from three independent experiments. $**P < 0.001$ by Student's *t*-test. (c) Disease symptoms caused by *P. parasitica* infection. Three days after infiltration with the OPEL recombinant protein or MES buffer on leaves of 6-week-old tobacco plants, systemic leaves were detached and inoculated with mycelial discs of *P. parasitica*. Photographs were taken at 60 h post-inoculation (hpi) under UV light. (d) Measurement of the infected areas. Values are means $\pm$ SD from three independent experiments. $*P < 0.05$ by Student's *t*-test.

and analysed their activity at a concentration of 0.3 μ M. All four truncated proteins appeared as a single band of the expected size when analysed by SDS-PAGE (Fig. S5, see Supporting Information). Only GH and GR-GH could induce callose deposition (Fig. 8a). Moreover, the extent of callose deposition caused by OPEL and GR-GH did not differ significantly, nor did that of GR-GH and GH (Fig. 8b), which indicates that GH plays a key role in inducing callose deposition by OPEL. Infiltration with GH or GR-GH significantly increased the production of ROS (Fig. 8c,d). In contrast, infiltration with GR or Thau caused only a slight increase in ROS accumulation (Fig. 8c,d). Treatment with GH, GR or GH-GR did not cause necrosis on leaves of *N. tabacum*. Therefore, although not equivalent to OPEL, GH represents an essential part of OPEL for its elicitor activity.

The laminarinase signature active site motif is essential for the elicitor activity of OPEL

OPEL contains a laminarinase signature active site motif in the C-terminal GH domain (473-ExDxxE-478) (Fig. 1a). However, when using OPEL recombinant proteins from *E. coli* and laminarin or 1,3- β -glucan as substrates, we could not detect any enzymatic activity. To determine whether this site is essential for the elicitor activity of OPEL, we performed site-directed mutagenesis and generated two OPEL mutant proteins, E473Q and E478Q, which contain a single point mutation at positions E473 and E478, respectively. These residues are indispensable for the enzymatic activity of a laminarinase from *Rhodothermus marinus* (Krah *et al.*, 1998). Callose deposition (Fig. 9a,b) and ROS accumulation

Fig. 7 OPEL induces systemic resistance against *Ralstonia solanacearum* and expression of DEFECTIVE IN INDUCED RESISTANCE 1 (*DIR1*) on *Nicotiana tabacum* (cv. Samsun NN). (a) Wilting scores of plants inoculated with *R. solanacearum* (Pss4). Three days after infiltration with the OPEL recombinant protein or 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer (as a control) on tobacco leaves (6 weeks old), plants were inoculated with *R. solanacearum* and scored for wilting symptoms. The experiment was repeated three times, each with 12 plants. (b) Statistical analysis. Values are means $\pm$ SD of wilting scores. * $P < 0.05$ by Student's *t*-test compared with MES. (c) *DIR1* is induced by OPEL. Tobacco leaves (6 weeks old) were infiltrated with recombinant proteins of OPEL (0.3 μ M) or glutathione transferase (GST) (0.3 μ M), followed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of gene expression at 3, 6 and 12 h post-infiltration (hpi). Values are mean $\pm$ SD fold change relative to the transcript level of GST-treated plants from three independent experiments. ** $P < 0.001$ by Student's *t*-test compared with GST.

(Fig. 9c,d) on *N. tabacum* leaves were significantly lower with 0.3 μ M of mutant proteins than with OPEL treatment. Moreover, these two mutant proteins did not induce defence gene expression (Fig. 10). These results demonstrate the importance of E473 and E478 as key residues for the elicitor activity of OPEL.

DISCUSSION

To protect against attack by pathogens, plants have developed a system to perceive molecular signatures derived from microbes or

danger signals from hosts, such as endogenous molecules and cell wall fragments, and thereby to trigger plant immunity (Boller and Felix, 2009). In this study, we demonstrated that OPEL is a novel elicitor of *P. parasitica* and can induce plant basal defence responses and also SAR against a variety of pathogens. Furthermore, a putative active site motif in the C-terminal GH domain of OPEL is essential for its elicitor activity.

OPEL was originally identified as a secretory protein from the culture filtrate of *P. parasitica* (Yan, 2001). Sequence analysis indicated that OPEL indeed contains an N-terminal signal peptide and

Fig. 8 The glycosyl hydrolase (GH) domain plays a key role for OPEL to induce callose deposition and hydrogen peroxide (H_2O_2) accumulation on leaves of *Nicotiana tabacum* (cv. Samsun NN). (a) The GH domain induces callose deposition. Two days after infiltration with 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer, OPEL recombinant protein, thaumatin-like domain (Thau), glycine-rich domain (GR), GH domain or GR-GH into leaves of 6-week-old tobacco plants, leaves were stained with aniline blue, and photographs were taken under a microscope. Bar, 25 μ m. (b) Callose deposition quantified per square millimetre microscopic field. Values are means $\pm$ SD from three independent experiments. Different letters indicate significant difference by Fisher's protected least-significant difference tests at $P < 0.001$. (c) The GH domain causes H_2O_2 accumulation. Six hours after infiltration of 6-week-old tobacco leaves with MES buffer, the recombinant protein of OPEL, Thau, GR, GH or GR-GH, leaves were stained with 3,3'-diaminobenzidine (DAB), and then photographed with a digital camera. (d) Quantification of DAB staining. Values are means $\pm$ SD from three independent experiments. Different letters indicate significant difference among treatments as revealed by Fisher's protected least-significant difference test at $P < 0.001$.

two putative *N*-linked glycosylation sites characteristic of secretory proteins. However, studies by Grenville-Briggs *et al.* (2010) and Meijer *et al.* (2006) indicated that homologues of OPEL are present in the cell wall of *P. ramorum* and *P. infestans*, which suggests that OPEL is probably a component of the cell wall of *P. parasitica*. The reason it is found in the culture fluid of *P. parasitica* may be because of its loose binding with the cell wall. Recombinant proteins of OPEL obtained from *E. coli* elicit plant defence responses, including cell death (Fig. 3a), callose deposition (Fig. 3b,c), ROS accumulation (Fig. 3d,e) and defence gene expression (Fig. 5), and so OPEL is an elicitor, and *Phytophthora*-specific post-translational modifications are not required for its activity. In this respect, OPEL is analogous to elicitors, such as parA1 of *P. parasitica* and INF1 of *P. infestans*, both displaying elicitor activity independent of previous modification by glycosylation (Kamoun *et al.*, 1993a, 1997).

Close homologues of OPEL were identified only in oomycetes, with those from *Phytophthora* highly conserved in the thaumatin-like domain, glycine-rich protein domain and GH domains

(Fig. S1). Thaumatin-like proteins, first identified from the African berry *Thaumatococcus daniellii* Bennett (van der Wel and Loeve, 1972), yet thaumatin-like proteins of plants are now classified as PR protein family 5, with expression induced by environmental stresses, such as cold and drought, as well as attack by pathogens and pests (Liu *et al.*, 2010). The function of the glycine-rich protein domain is currently unknown. Domain analysis revealed that the GH domain, rather than the thaumatin-like and glycine-rich domains, is the key domain required for OPEL-induced defence responses, including callose deposition and ROS production (Fig. 8). The GH domain shows significant similarity to TOS1 of *Saccharomyces cerevisiae*. The exact function of TOS1 is unknown, but yeast *Tos1* deletion mutants show reduced accumulation of glycogen and become highly resistant to treatment with 1,3- β -glucanase (Wilson *et al.*, 2002; Yin *et al.*, 2005). Thus, TOS1 may be involved in cell wall modification, probably through the alteration of the 1,3- β -glucan network (Yin *et al.*, 2005). TOS1 and other members of the DUF2401 family were found to contain a signature active site motif (ExDxxE) of

Fig. 9 Mutations in the putative active site of the glycosyl hydrolase (GH) domain abolish the elicitor activity of OPEL. (a) OPEL mutants E473Q and E478Q lost activity to induce callose deposition. Two days after infiltration with 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer, flg22, recombinant protein of glutathione transferase (GST), OPEL, E473Q or E478Q into leaves of 6-week-old tobacco plants, leaves were stained with aniline blue, and photographs were taken under a microscope. Bar, 25 μm. (b) Callose deposition quantified as the number of individual depositions per square millimetre microscopic field. Values are means ± SD from three independent experiments. Different letters indicate significant difference by Fisher's protected least-significant difference tests at $P < 0.001$. (c) OPEL mutants E473Q and E478Q lost the activity to induce hydrogen peroxide (H₂O₂) accumulation. Six hours after infiltration of 6-week-old tobacco leaves with MES buffer, flg22, recombinant protein of GST, OPEL, E473Q or E478Q, leaves were stained with 3,3'-diaminobenzidine (DAB) and then photographed with a digital camera. (d) Quantification of DAB staining. Values are means ± SD from three independent experiments. Different letters indicate significant difference among treatments as revealed by Fisher's protected least-significant difference test at $P < 0.001$.

laminarinases, which suggests their enzymatic activity involved in the cleavage of 1,3-β-glucans (Steczkiewicz *et al.*, 2010). Bacterial 1,3-β-glucanases, such as the laminarinases from the thermophilic eubacterium *R. marinus*, are polysaccharide endohydrolases classified as members of glycoside hydrolase family 16 (GH16) (Henrissat, 1991; Henrissat and Bairoch, 1993). Moreover, two residues in the active site of laminarinase from *R. marinus* (E129 and E134) are essential for its enzymatic activity (Krah *et al.*, 1998). The signature active site motif of laminarinases (ExDxxE) was also identified in OPEL (Figs 1a and S1). Remarkably, mutagenesis of E473 or E478 abolished the elicitor activity of OPEL (Figs 9 and 10). These results highlight the importance of this active site motif for the elicitor activity of OPEL and suggest that OPEL may function as an enzyme. However, we could not characterize any enzymatic activity of OPEL using laminarin or 1,3-β-glucan as a substrate. Enzymes of the GH16 family show extreme substrate diversity and share little sequence similarity (Steczkiewicz *et al.*, 2010). More effort is required to verify

the enzymatic activity of OPEL by using a suitable substrate and to identify its cognate substrates, which are probably polysaccharides of the cell wall.

In *Arabidopsis thaliana*, applications of flagellin or lipopolysaccharide increased the levels of SA and PR gene expression in treated and also distant leaves (Mishina and Zeier, 2007). Elicitors of *Phytophthora*, such as cryptogein of *P. cryptogea* and capsicein of *P. capsici*, can trigger SAR in tobacco plants (Keller *et al.*, 1996; Ricci *et al.*, 1989). In this study, we provide evidence that OPEL enhances the expression of several SA-responsive genes, including *PAL*, *PR1* and *PR5* (Fig. 5) and induces systemic resistance against a broad spectrum of pathogens, including TMV, the oomycete pathogen *P. parasitica* and the soil-borne bacterial wilt pathogen *R. solanacearum* (Figs 6 and 7), in addition to plant basal immunity (Fig. 4). With TMV, the protection effect lasts for at least 14 days (Fig. 6b). The occurrence of SAR is generally accompanied by an increase in SA level and that of its derivative SA-glucoside, as well as elevated expression of SA-responsive

Fig. 10 The active site mutants of OPEL do not induce the expression of defence genes. Leaves of 6-week-old *Nicotiana tabacum* (cv. Samsun NN) plants were infiltrated with recombinant proteins of OPEL (0.3 μ M; OPEL), OPEL mutants with a point mutation at the putative active site (E473Q and E478Q) or glutathione transferase (GST) (0.3 μ M), followed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of gene expression at 6 h post-infiltration. Values are means $\pm$ SD fold change relative to the level of the GST-treated plant from three independent experiments. ** $P < 0.001$ by Student's *t*-test. PAL, phenylalanine ammonia lyase; PR1, pathogenesis-related 1; PR5, pathogenesis-related 5; COI1, CORONATINE INSENSITIVE 1; PDF1.2, plant defensin gene 1.2; EIN2, ethylene insensitive 2; Pti5, Pto-interacting 5; Gras2, (GAI, RGA, SCR) 2.

defence genes (Dempsey and Klessig, 2012; Shah and Zeier, 2013; Sticher, 1997). In addition, SAR involves long-distance communication between the primary pathogen-infected or elicitor-treated organs and the rest of the plant. Several candidates for this long-distance signalling have been identified and include the methyl ester of SA, the abietane diterpenoid dehydroadietinal, the dicarboxylic acid azelaic acid and a glycerol-3-phosphate-dependent factor (Dempsey and Klessig, 2012; Shah and Zeier, 2013). Long-distance signalling by some of these factors requires the lipid transfer protein DIR1 (Liu *et al.*, 2011; Maldonado *et al.*, 2002). The expression of *DIR1* was induced by OPEL 12 h after OPEL treatment (Fig. 7c), which suggests its involvement in the triggering of SAR. Further investigation is required to identify the signals involved in long-distance communication for OPEL-inducing SAR.

The cell wall provides the first barrier to prevent attack by plant pathogens. To facilitate invasion, pathogens have evolved an arsenal of CWDEs, including endopolygalacturonases (PGs), for the decomposition of cell walls and the maceration of plant tissues (van den Brink and de Vries, 2011). Cell wall fragments generated by CWDEs may serve as DAMPs that trigger defence signalling pathways (Nühse, 2012). A prominent example is 1,4- α -linked oligogalacturonides, degradation products of PGs, which act as DAMPs via their perception by wall-associated kinase1 to induce plant innate immunity (Brutus *et al.*, 2010; D'Ovidio *et al.*, 2004). However, as shown for PGs of *Botrytis cinerea*, some CWDEs can trigger plant defence responses even when they bear mutations in

the active site, and so CWDEs themselves may serve as MAMPs (Poinssot *et al.*, 2003; Zhang *et al.*, 2014). Moreover, despite a lack of hydrolytic activity, CBEL of *P. parasitica* can elicit plant defence by interacting with cellulose, and so cellulose might be part of a sensing machinery to monitor cell wall integrity (Gaulin *et al.*, 2006). With regard to OPEL, although its enzymatic activity remains to be verified, the GH domain was essential for elicitor activity, and mutations in a putative active site motif in this domain abolished the elicitor activity, which suggests that degradation products generated by OPEL in the apoplast may serve as DAMPs to trigger plant immunity. A comprehensive analysis of global changes in proteomes, transcriptomes and metabolomes will help to identify tentative DAMPs generated by OPEL and to elucidate the mechanisms underlying OPEL-induced plant defence responses. Moreover, whether or not OPEL plays an essential role in the life cycle, especially pathogenesis, of *P. parasitica* is of interest.

EXPERIMENTAL PROCEDURES

Growth of *P. parasitica* and plant materials

Phytophthora parasitica (isolate 94069) was provided by Dr. P. J. Ann (Taiwan Agricultural Research Institute, WuFeng, Taiwan) and routinely grown on 5% V8 juice agar (5% Campbell's V8 juice, 0.02% CaCO_3 and 2% agar) at 25 $^{\circ}\text{C}$ in the dark. *N. benthamiana* and *N. tabacum* (cv. Samsun NN) were grown in a mixture of peat moss, perlite and vermiculite at 25 $^{\circ}\text{C}$ under a 12-h light/dark regime.

Molecular cloning and sequence analysis of OPEL

The full-length cDNA sequence of OPEL was cloned by 5'- and 3'-RACE using the SMART RACE cDNA amplification kit (Clontech, Mountain View, CA, USA), with PGR1 and PGSA (Table S1) as gene-specific primers. We used BLAST for homology searches of the US NCBI and genome websites for several oomycetes. Conserved protein domains and putative *N*-glycosylation sites were predicted using the Phyre2 Protein Fold Recognition Server and NetNGlyc 1.0 Server. Alignment of OPEL and its homologues from other organisms involved CLUSTAL X. Phylogenetic trees were generated by the neighbour-joining algorithm implemented in MEGA 5 with the default parameters. Nodal support of the tree was estimated by bootstrapping with 1000 pseudoreplicate datasets.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from different life stages of *P. parasitica* and inoculated plant samples was prepared as described by Yan and Liou (2006) with some modifications. RNA was isolated using the Plant Total RNA Extraction Kit (Viogene-Biotek, Taipei, Taiwan). To avoid DNA contamination, residual DNA present in the RNA solution was removed using a Turbo-DNA free kit (Ambion, Huntingdon, UK). First-strand cDNA was synthesized by the SuperScript III reverse transcriptase method (Life Technologies-Invitrogen, Carlsbad, CA, USA) with 1 μ g of total RNA as the template and 25 μ M

oligo(VdT) (V: A, C or G at the 3' end) as the primer. Quantitative PCR involved the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The reaction mixture (20 µL) contained 10 µL of Power SYBR Green PCR Master Mix (Applied Biosystems), 0.25 µM primers (Table S1) and 1 µL of cDNA product diluted 10 times. PCR conditions were 95 °C/10 min and 40 cycles of 95 °C/15 s, 60 °C/1 min, followed by melting curve analysis. For the analysis of gene expression in *P. parasitica*, the level of *WS21* was used as an internal control for normalization, with β-actin as the internal control for *N. benthamiana* and *N. tabacum*.

Expression of recombinant proteins in *E. coli* and protein infiltration into plants

A DNA fragment corresponding to the coding sequence of OPEL without the signal peptide was subcloned into pGEX 4T-1 with GST fused in frame to its N-terminus. Site-directed mutagenesis at positions 473 and 478 of OPEL was performed as described by Wu *et al.* (2008). After transformation of the construct into the *E. coli* strain RIL, expression of the recombinant protein was induced with the addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in Luria-Bertani (LB) broth, and bacteria were grown at 16 °C for 3 days. Recombinant proteins were purified using Glutathione Sepharose 4B (GE Healthcare Life Sciences, Pittsburgh, PA, USA), followed by treatment with thrombin to remove GST and further purification with a Sephacryl S-200 high-resolution column (GE Healthcare Life Sciences). To express truncated versions of OPEL recombinant protein, nucleotide sequences corresponding to amino acid residues 24–220 (Thau), 240–315 (GR), 316–556 (GH) and 240–556 (GR-GH) were amplified by PCR with the primers in Table S1 and subcloned into pET28a(+). After transformation into the *E. coli* strain BL21(DE3), expression of the recombinant protein was induced with the addition of 1 mM IPTG in LB broth, and bacteria were grown at 26 °C for 3 h. The recombinant protein was purified using Ni-NTA agarose (Qiagen, Valencia, CA, USA). The concentration of the protein was determined by the method of Bradford (1976) with the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). SDS-PAGE and immunoblot analysis were performed according to standard procedures. Pep-13 and flg22 were synthesized by Genomics (New Taipei City, Taiwan). For the infiltration experiment, proteins were resuspended in MES buffer and infiltrated into tobacco leaves until the intercellular space was filled with the sample fluids.

Staining and quantification for callose deposition

Callose staining of tobacco leaf discs was performed as described by Luna *et al.* (2011). Callose deposition at the adaxial surface was examined under a Leica DM LB microscope (Buffalo Grove, IL, USA) equipped with filter cube A (bandpass (BP) filter = 340–380 nm, longpass (LP) filter = 425 nm) and a Canon (Ohta-ku, Tokyo, Japan) digital camera EOS 550D. The number of callose deposits on digital photographs was counted using ImageJ and shown as the number of depositions per square millimetre microscopic field. For each treatment, photographs from 10 leaf discs were analysed and the experiment was repeated 3 times.

Staining with DAB and trypan blue

DAB staining was performed as described by Bindschedler *et al.* (2006), and images were captured using a Canon digital camera EOS 550D. DAB

staining intensity on photographs was quantified using ImageJ and is shown as the number of dark-brown DAB pixels relative to total pixels corresponding to plant material. Trypan blue staining was performed according to the method described by Wilson and Coffey (1980).

Agrobacterium-mediated transient expression of OPEL

The sequence of OPEL-GFP was amplified from pBI::OPEL-GFP with O_F_BamHI and O_R_NotI (Table S1) employed as primers and subcloned into pK7WG2 using the Gateway LR Clonase II enzyme mix (Life Technologies-Invitrogen) to generate pK7WG2::OPEL-GFP. Culture of *Agrobacterium* strain C58C1 carrying this construct and infiltration into leaves of 5-week-old *N. benthamiana* were performed as described by Wu *et al.* (2008).

Inoculation of TMV

TMV, which was provided by Dr Y. C. Chang (National Taiwan University, Taipei, Taiwan), was inoculated evenly over the upper surface of *N. tabacum* leaves with carborundum used as an abrasive. Inoculated plants were maintained at 25 °C under a 12-h light/dark regime and inspected daily for disease symptoms.

Inoculation of *P. parasitica*

Mycelia discs of *P. parasitica* (isolate 94069) were transferred to detached *N. benthamiana* or *N. tabacum* leaves, and inoculated leaves were kept at 25 °C in a moisture box and inspected daily for disease symptoms. For the evaluation of disease severity, photographs were taken under UV light and infected areas were measured using ImageJ.

Inoculation of *R. solanacearum*

Ralstonia solanacearum strain Pss4 (phylotype I, biovar3) was provided by Dr C. P. Cheng (National Taiwan University, Taipei, Taiwan) and grown in 523 medium (1% sucrose, 0.8% casein hydrolysate, 0.4% yeast extract, 0.2% K₂HPO₄, 0.03% MgSO₄·7H₂O and 1.5% Bacto agar). For inoculation, 20 mL of *R. solanacearum* (Pss4) (2×10^7 colony-forming units/mL) suspension was drenched around the stem base, and inoculated plants were maintained at 28 °C under a 12-h light/dark regime. Symptom development was inspected daily and the degree of wilting was scored as: 0, no symptoms; 1, one leaf wilted; 2, two leaves wilted; 3, three leaves wilted; 4, all leaves wilted.

Statistical analysis

Statistically significant differences were analysed by Student's *t*-test or analysis of variance (ANOVA) followed by Fisher's protected least-significant difference test. Significance was set at $P < 0.05$ or $P < 0.001$ and determined by PASW Statistics 18 (SPSS Inc., Chicago, IL, USA).

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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:

Table S1 Oligonucleotide primers used in this study.

Fig. S1 Alignment of partial sequences of OPEL from different *Phytophthora* species.

Fig. S2 Sequence alignment of OPEL and its homologues.

Fig. S3 Analysis of OPEL and glutathione transferase (GST) recombinant proteins by sodium dodecylsulphate-polyacrylamide gel electrophoresis.

Fig. S4 Immunoblot analysis to detect the expression of OPEL-green fluorescent protein (OPEL-GFP) fusion protein after agroinfiltration.

Fig. S5 Analysis of the truncated forms of OPEL recombinant proteins by sodium dodecylsulphate-polyacrylamide gel electrophoresis.