Elicitin-like proteins Oli-D1 and Oli-D2 from *Pythium oligandrum* trigger hypersensitive response in *Nicotiana benthamiana* and induce resistance against *Botrytis cinerea* in tomato

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SUMMARY

The biocontrol agent *Pythium oligandrum* and its elicitin-like proteins oligandrins have been shown to induce disease resistance in a range of plants. In the present study, the ability of two oligandrins, Oli-D1 and Oli-D2, to induce an immune response and the possible molecular mechanism regulating the defence responses in *Nicotiana benthamiana* and tomato were investigated. Infiltration of recombinant Oli-D1 and Oli-D2 proteins induced a typical immune response in *N. benthamiana* including the induction of a hypersensitive response (HR), accumulation of reactive oxygen species and production of autofluorescence. *Agrobacterium*-mediated transient expression assays revealed that full-length Oli-D1 and Oli-D2 were required for full HR-inducing activity in *N. benthamiana*, and virus-induced gene silencing-mediated knockdown of some of the signalling regulatory genes demonstrated that NbSGT1 and NbNPR1 were required for Oli-D1 and Oli-D2 to induce HR in *N. benthamiana*. Subcellular localization analyses indicated that both Oli-D1 and Oli-D2 were targeted to the plasma membrane of *N. benthamiana*. When infiltrated or transiently expressed in leaves, Oli-D1 and Oli-D2 induced resistance against *Botrytis cinerea* in tomato and activated the expression of a set of genes involved in the jasmonic acid/ethylene (JA/ET)-mediated signalling pathway. Our results demonstrate that Oli-D1 and Oli-D2 are effective elicitors capable of inducing immune responses in plants, probably through the JA/ET-mediated signalling pathway, and that both Oli-D1 and Oli-D2 have potential for the development of bioactive formulae for crop disease control in practice.

Keywords: defence response/resistance, hypersensitive response, *Nicotiana benthamiana*, Oli-D1/Oli-D2, oligandrin, tomato.

INTRODUCTION

The innate immune system in plants contains two types of immune response: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Boller and He, 2009; Spoel and Dong, 2012). In addition to innate immunity, plants also develop and possess an inducible immunity that is activated on pathogen infection. The forms of inducible immunity are generally referred to as systemic acquired resistance (SAR) and induced systemic resistance (ISR), which are activated by different stimuli and regulated by distinct signalling pathways (Fu and Dong, 2013; Grant and Lamb, 2006; Kachroo and Robin, 2013; Loake and Grant, 2007; van Loon *et al.*, 1998; Shores *et al.*, 2010; van Wees *et al.*, 2008). Once activated, the inducible immunity in plants often confers a broad-spectrum resistance against a wide range of unrelated pathogens (Durrant and Dong, 2004; Grant and Lamb, 2006; van Wees *et al.*, 2008). This great advantage provides a promising strategy for disease control in practice (Walters *et al.*, 2013).

Plant inducible immunity can be triggered by the exogenous application of a number of elicitors (Walters *et al.*, 2013), which are capable of activating inducible immunity and protecting plants from disease damage. Among the elicitors identified so far, the microbe-derived proteinaceous elicitors show great potential for the development of environmentally friendly biopesticides and for the improvement of crop disease resistance by means of genetic engineering. For example, some of the harpin proteins, produced by Gram-negative phytopathogenic bacteria (Choi *et al.*, 2013), have been shown to be capable of controlling diseases and insects, as well as improving growth, yield and quality in many crop plants (Chen *et al.*, 2008a, b; Jang *et al.*, 2006; Strobel *et al.*, 1996), and the over-expression of harpin-encoding genes from phytopathogenic bacteria in tobacco and rice induces enhanced disease resistance against a number of fungal and bacterial pathogens (Miao *et al.*, 2010; Peng *et al.*, 2004; Shao *et al.*, 2008). Similarly, elicitins produced by a number of pathogenic oomycetes (Grant *et al.*, 1996; Kamoun, 2006; Yu, 1995) have been shown to induce SAR in plants (Bonnet *et al.*, 1996; Cordelier *et al.*, 2003; Kamoun *et al.*, 1993, 1998; Keller *et al.*, 1996), and transgenic plants over-expressing a *Phytophthora* elicitin gene display

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enhanced resistance to *Thielaviopsis basicola*, *Erysiphe cichoracearum* and *Botrytis cinerea*, three fungal pathogens that are unrelated to *Phytophthora* species (Keller *et al.*, 1999). With increasing demands for the development of environmentally friendly biopesticides, extensive studies have been performed using molecular, biochemical and proteomic approaches to screen and identify novel proteinaceous elicitors from microbes (Kulye *et al.*, 2012; Peng *et al.*, 2011; Qiu *et al.*, 2009; Yang *et al.*, 2009).

Pythium oligandrum, a non-pathogenic soil-inhabiting oomycete, has been reported to be an effective biocontrol agent that colonizes the rhizosphere of many crop species and reduces diseases caused by a number of soil-borne pathogens (Benhamou *et al.*, 2012). It has been reported recently that *P. oligandrum* can produce two types of proteinaceous elicitor that are distinct from those of the representative elicitors and elicitor-like proteins from oomycete species (Masunaka *et al.*, 2010; Takenaka *et al.*, 2006). One type of elicitor exists as a cell wall protein (CWP) in mycelial mats of *P. oligandrum* and has four members, including POD-1 and POD-2 (Takenaka *et al.*, 2006) and POS-1 and Oli-S1 (Masunaka *et al.*, 2010). POD-1 and POD-2 have been shown to induce defence responses in sugarbeet (Takenaka *et al.*, 2003; Takenaka and Tamagake, 2009), wheat (Takenaka *et al.*, 2003), tomato (Hase *et al.*, 2006, 2008; Takenaka *et al.*, 2011) and *Arabidopsis* (Kawamura *et al.*, 2009a). Another type of elicitor in *P. oligandrum*, called an oligandrin, is an extracellular protein that can induce resistance in tomato against *Phytophthora parasitica*, *Fusarium oxysporum* f. sp. *radicis-lycopersici* and *B. cinerea* (Benhamou *et al.*, 2001; Lou *et al.*, 2011; Mohamed *et al.*, 2007; Picard *et al.*, 2000; Wang *et al.*, 2011). However, the possible mechanism of oligandrins in regulating the immune response remains unclear. In the present study, we examined the ability of two *P. oligandrum* oligandrins, Oli-D1 and Oli-D2 (Masunaka *et al.*, 2010), to induce an immune response and their possible molecular mechanisms in regulating the defence responses in *Nicotiana benthamiana* and tomato. Our results demonstrate that Oli-D1 and Oli-D2 are potential proteinaceous elicitors that can be used to develop bioactive formulae for crop disease control in practice.

RESULTS

Oli-D1 and Oli-D2 induce HR in *N. benthamiana*

Considering that *N. benthamiana* is a versatile host that can easily be infiltrated by foreign proteins or agrobacteria for the examination of the HR phenotype (Goodin *et al.*, 2008) and that the HR phenotype induced by Oli-D1 or Oli-D2 was not seen in tomato in our pilot experiments, *N. benthamiana* was used for our study. Infiltration with a preparation from bacteria carrying pET-32a empty vector did not show any HR symptoms during the experi-

mental period (Fig. 1A). Infiltration with Nep1_{Mo}, a necrosis- and ethylene-inducing peptide 1 (Nep1)-like protein in *Magnaporthe oryzae* that can produce a typical HR in *N. benthamiana* (Qutob *et al.*, 2006; Zhang *et al.*, 2010, 2012), rapidly induced a typical HR, and all the infiltrated areas were necrotic at 24 h post-inoculation (hpi) (Fig. 1A,B). Infiltration with Oli-D1 and Oli-D2 at concentrations of 1 and 3 µM also induced a typical HR, but the development of HR was much slower than that induced by Nep1_{Mo} (Fig. 1A,B). The HR was seen only at 48 hpi and approximately 50% of the infiltrated areas were necrotic when 1 µM of Oli-D1 or

Fig. 1 Recombinant Oli-D1 and Oli-D2 induce a hypersensitive response (HR) in the leaves of *Nicotiana benthamiana* plants. (A) Representative HR in leaves of *N. benthamiana* plants induced by recombinant Oli-D1 and Oli-D2 at 1 and 3 µM. Nep1_{Mo} and a preparation from bacteria carrying pET-32a empty vector were used as positive and negative controls, respectively. Photographs were taken at 48 h after infiltration. (B) Development of HR. Percentages of necrosis in the infiltration areas were estimated at different time points as indicated. (C) Expression of HR marker genes induced by Oli-D1 and Oli-D2. Infiltrated leaves were collected at the indicated time points and used to analyse the expression of *NbHIN1* and *NbHSR203J* by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Data presented are the mean ± SD from three independent experiments. Significant difference at **P* < 0.05 and ***P* < 0.01. hpt, hours post-treatment.

Oli-D2 was applied. In leaves infiltrated with 3 μ M of Oli-D1 or Oli-D2, the HR developed as small necrotic lesions at 24 hpi and eventually spread to nearly all the infiltrated areas at 72 hpi (Fig. 1B), indicating that Oli-D1 and Oli-D2 at higher concentrations induced a relatively more rapid and stronger HR. We further examined whether the expression of *HSR203J* and *harpin-induced 1 (HIN1)*, two HR-specific marker genes whose expression is induced during pathogen-triggered HR (Gopalan *et al.*, 1996; Pontier *et al.*, 1994, 1999; Takahashi *et al.*, 2004a), and is independent of the salicylic acid (SA)- or jasmonic acid (JA)/ethylene (ET)-mediated signalling pathways (Takahashi *et al.*, 2004b; Varet *et al.*, 2002; Zheng *et al.*, 2004), could be induced by Oli-D1 or Oli-D2. As shown in Fig. 1C, expression of *HSR203J* and *HIN1* was significantly up-regulated by Oli-D1 and Oli-D2 as early as 6 hpi. Taken together, these data indicate that Oli-D1 and Oli-D2 induce typical HR in *N. benthamiana*.

Oli-D1 and Oli-D2 activate HR biochemical responses in *N. benthamiana*

To examine whether Oli-D1 and Oli-D2 activated HR biochemical responses, we analysed the accumulation of reactive oxygen species (ROS), generated by plasma membrane-localized NADPH oxidase complex (Mur *et al.*, 2008), and the appearance of autofluorescence, probably resulting from the accumulation of phenolic compounds in and around the cells that had died (Dietrich *et al.*, 1994), in *N. benthamiana* leaves after infiltration with Oli-D1 or Oli-D2. In all leaves assayed, significant brown 3,3'-diaminobenzidine (DAB)-stained precipitates were easily and clearly observed in the areas infiltrated with 1 μ M Oli-D1 or Oli-D2 at 4 hpi, but only very weak brown DAB-stained precipitates were seen in the same leaves infiltrated with a preparation from bacteria carrying pET-32a empty vector (Fig. 2A). Similarly, Oli-D1 and Oli-D2 also induced the appearance of autofluorescence symptoms in *N. benthamiana* leaves (Fig. 2B). At 24 hpi, significant autofluorescence was seen in Oli-D1- and Oli-D2-infiltrated areas in *N. benthamiana* leaves and was comparable with that induced by Nep1_{Mo} (Fig. 2B). No autofluorescence was observed in negative control leaves (Fig. 2B). These results indicate that Oli-D1 and Oli-D2 can activate HR biochemical responses, such as the accumulation of H₂O₂ and the appearance of autofluorescence.

Full-length Oli-D1 and Oli-D2 are required for HR-inducing activity

The Oli-D1 and Oli-D2 proteins are 120 amino acids (aa) in size and contain a signal peptide (SP) (1–18 aa) and an elicitor superfamily domain (20–116 aa) (Masunaka *et al.*, 2010). To examine whether there is a region/motif responsible for the HR-inducing activity in the Oli-D1 and Oli-D2 proteins, five deletion mutants were generated (Fig. 3A) and analysed for their ability to induce

Fig. 2 Recombinant Oli-D1 and Oli-D2 activate the defence response in *Nicotiana benthamiana* plants. (A) Accumulation of H₂O₂ in Oli-D1- and Oli-D2-infiltrated areas. Recombinant Oli-D1 and Oli-D2 at 1 μ M were infiltrated into *N. benthamiana* leaves and *in situ* detection of H₂O₂ using 3,3'-diaminobenzidine (DAB) staining was performed on leaves at 4 h post-inoculation (hpi). (B) Autofluorescence in *N. benthamiana* leaves induced by Oli-D1 and Oli-D2. Recombinant Oli-D1 and Oli-D2 at 1 μ M were infiltrated into *N. benthamiana* leaves and autofluorescence was visualized at 48 hpi. Photographs were taken in dark field for green and red fluorescence, in bright field for the morphology of the cells and in combination.

HR in *N. benthamiana*. When transiently expressed in leaves of *N. benthamiana* plants, full-length Oli-D1 and Oli-D2 induced typical HR (Fig. 3B), similar to the HR induced by the purified recombinant Oli-D1 and Oli-D2 proteins (Fig. 1A), and the green fluorescent protein (GFP) negative control did not induce HR, indicating that the transient expression system worked properly in *N. benthamiana*. However, no HR was observed when the deletion mutants for Oli-D1 or Oli-D2 were transiently expressed (Fig. 3B). To rule out the possibility that an error may have been introduced during sequence amplification and vector construction, we examined through Western blotting the detection of the FLAG tags that had been fused to the C-termini of the proteins of the deletion mutants in *N. benthamiana* leaves. As shown in Fig. 3C, all the

Fig. 3 Full-length Oli-D1 and Oli-D2 are required for hypersensitive response (HR)-inducing activity in *Nicotiana benthamiana* plants. (A) Diagram of the deletion mutants for Oli-D1 and Oli-D2 proteins. Amino acid (aa) positions in Oli-D1 and Oli-D2 are indicated above the diagram. SP, signal peptide. (B) HR induced by Oli-D1 and Oli-D2 and their deletion mutants when transiently expressed in *N. benthamiana* leaves. Photographs were taken 3 days after infiltration. GFP, green fluorescent protein. (C) Western blotting of Oli-D1 and Oli-D2 and their deletion mutant proteins in *N. benthamiana* leaves. Leaf samples were collected at 36 h post-inoculation and soluble proteins were extracted for Western blotting analysis. Top row: detection of target proteins with anti-FLAG antibody; bottom row, Coomassie blue staining showing equal loading of the samples.

deletion mutant proteins and the full-length Oli-D1 and Oli-D2 were detected at 24 hpi in *N. benthamiana* leaves infiltrated with the corresponding constructs, indicating that all the deletion mutants were expressed correctly in *N. benthamiana* leaves. Collectively, these results demonstrate that full-length Oli-D1 and Oli-D2 are required for HR-inducing activity and the deletion of any part of Oli-D1 or Oli-D2 abolishes their HR-inducing activity.

NbSGT1 and NbNPR1 are required for Oli-D1- and Oli-D2-induced HR

To gain an insight into the molecular mechanism involved in Oli-D1 and Oli-D2 function, we examined the requirements of some important regulatory components in the defence signalling pathways for Oli-D1- and Oli-D2-induced HR using a standard virus-induced gene silencing (VIGS) procedure (Liu *et al.*, 2002). In our experiments, the silencing effectiveness for a single target gene was >70%, as revealed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using gene-specific primers (Fig. 4A). Infiltration with pGR106-GFP (as a negative control) did not cause HR in all the plants; however, infiltration with pGR106-Nep1_{Mo} (as a positive control) induced HR in *NbLRK1*-silenced and pTRV2-GUS-infiltrated plants, but did not cause HR in *NbSGT1*-, *NbNPR1*-, *NbHSP90.2*-, *NbCOI1*- and *NbTGA2.2*-silenced plants (Fig. 4B). Typical HR was observed in *NbLRK1*-, *NbHSP90.2*-, *NbCOI1*- and *NbTGA2.2*-silenced plants after infiltration with

pGR106-Oli-D1 or pDR106-Oli-D2, as in pTRV2-GUS-infiltrated plants (Fig. 4B). However, infiltration with pGR106-Oli-D1 or pGR106-Oli-D2 did not induce HR in *NbSGT1*- and *NbNPR1*-silenced plants (Fig. 4B). These results indicate that *NbSGT1* and *NbNPR1* are required for Oli-D1- and Oli-D2-induced HR in *N. benthamiana*.

Oli-D1 and Oli-D2 are targeted to the plasma membrane

Subcellular localization assays in *N. benthamiana* revealed that, when transiently expressed, the GFP fluorescence from pGFP-Oli-D1- or pGFP-Oli-D2-infiltrated and pGFP-Oli-D1-d1- or pGFP-Oli-D2-d2-infiltrated leaves was observed mainly on the plasma membrane of the cells, whereas GFP fluorescence in pFGC-Egfp empty vector-infiltrated leaves was observed throughout the entire cells, including the plasma membrane and nucleus, without specific compartmental localization (Fig. 5). These data indicate that not only the full-length, but also the SP-deleted mutants of Oli-D1 and Oli-D2 proteins, are targeted to the plasma membrane of the cells.

Oli-D1 and Oli-D2 induce disease resistance against *B. cinerea* in tomato

Considering that Oli-D1 and Oli-D2 trigger immune responses in *N. benthamiana*, we examined whether they had the ability to

Fig. 4 NbSGT1 and NbNPR1 are required for the Oli-D1- and Oli-D2-induced hypersensitive response (HR) in *Nicotiana benthamiana*. Leaves of *N. benthamiana* plants at the five- to six-leaf stage were infiltrated with a mixed suspension of agrobacteria carrying pTRV1 and pTRV2-GUS, pTRV2-SGT1, pTRV2-NPR1, pTRV2-LRK1, pTRV2-HSP90.2, pTRV2-COI1, pTRV2-MEK2 or pTRV2-TGA2.2, and the upper leaves were infiltrated with agrobacteria carrying pGR106-D1, pGR106-D2, pGR106-Nep1_{MO} (as a positive control) or pGR106-GFP (as a negative control) 3 weeks after virus-induced gene silencing (VIGS) infiltration. (A) Silencing effectiveness of the target genes in corresponding VIGS-silenced plants. Leaf samples were collected 2 weeks after VIGS infiltration and the transcript levels of the target genes were analysed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Relative expression was expressed as the percentages of the transcript levels of the target gene-silenced plants vs. that in pTRV-GUS-infiltrated plants. (B) Development of HR induced by transiently expressed Oli-D1 or Oli-D2 in the target gene-silenced plants. GUS, β -glucuronidase; SGT1, suppressor of G2 allele of *skp1*; NPR1, nonexpresser of PR genes 1; LRK1, lectin-like receptor kinases 1; HSP90.2, heat shock protein 90.2; MEK2, mitogen-activated protein kinase kinase 2; COI1, CORONATINE INSENSITIVE 1; TGA2.2, TGACG sequence-specific binding protein 2.2.

induce disease resistance in other plants, such as tomato. As shown in Fig. 6, *B. cinerea*-induced lesions developed and expanded much more slowly in leaves treated with either recombinant or transiently expressed Oli-D1 and Oli-D2 than in corresponding control plants (Fig. 6A,D). At 3 days post-inoculation (dpi), the lesion sizes in leaves treated with either recombinant or transiently expressed Oli-D1 and Oli-D2 were markedly reduced by ~30% when compared with those in corresponding control plants (Fig. 6B,E). Consistent with the disease phenotypes observed in Oli-D1- and Oli-D2-treated plants, *in planta* growth of *B. cinerea*, as measured by the transcript level of the *BcActinA* gene (Benito *et al.*, 1998), in either recombinant or transiently expressed Oli-D1- and Oli-D2-treated leaves, was significantly reduced, showing a reduction of 55–65% when compared with that in corresponding control plants (Fig. 6C,F). These results suggest

that either recombinant or transiently expressed Oli-D1 and Oli-D2 can induce resistance against *B. cinerea* in tomato, resulting in decreased disease severity and *in planta* growth of *B. cinerea*.

Oli-D1 and Oli-D2 activate the defence response in tomato plants

As Oli-D1 and Oli-D2 induced resistance against *B. cinerea* in tomato, we examined whether they could activate the defence response. Similar to the observations in *N. benthamiana*, Nep1_{MO} induced HR in tomato leaves, but a preparation from bacteria carrying the pET-32a empty vector did not (Fig. 7A). In contrast with Nep1_{MO}-induced HR, however, a weak HR phenotype was observed at 72 hpi in leaves infiltrated with recombinant Oli-D1 and Oli-D2, and HR was evident in leaves infiltrated with a

Fig. 5 Oli-D1 and Oli-D2 are targeted to the plasma membrane. Agrobacteria carrying pGFP-Oli-D1, pGFP-Oli-D1-d1, pGFP-Oli-D2, pGFP-Oli-D2-d1 or pFGC-Egfp empty vector were infiltrated into *Nicotiana benthamiana* leaves for transient expression, and leaf samples were collected at 16 h post-inoculation for observation under a confocal laser scanning microscope. Photographs were taken in dark field for green fluorescence (left), in white field for the morphology of the cells (middle) and in combination (right). GFP, green fluorescent protein.

relatively high concentration of Oli-D1 and Oli-D2 (Fig. 7A). The expression levels of *SILapA1*, *SIPin2*, *SILOX-E* and *SIERF2*, which are considered to be involved in the JA/ET-mediated signalling pathway (Kawamura *et al.*, 2009b; Kawazu *et al.*, 2012), were significantly increased at 12 and 24 hpi in Oli-D1- and Oli-D2-treated plants, giving increases of three- to seven-fold (Fig. 7B) when compared with the expression levels at 0 h after treatment. The expression levels of *SIPR1a* and *SIPR-P2*, two SA-dependent defence genes (Kawazu *et al.*, 2012), were not induced in Oli-D1- and Oli-D2-treated tomato plants (Fig. 7C); however, the expression of *SIPR1b*, another SA-dependent defence gene (Kawazu *et al.*, 2012), was increased by two- to three-fold when compared with the level at 0 h after treatment (Fig. 7C). These results indicate that Oli-D1 and Oli-D2 can activate the expression of a set of

genes associated with the JA-mediated signalling pathway in tomato.

DISCUSSION

In the present study, we have shown that Oli-D1 and Oli-D2 proteins, two elicitor-like elicitors produced by *P. oligandrum*, can trigger typical immune responses in *N. benthamiana* and induce resistance in tomato against *B. cinerea*. We also found that full-length Oli-D1 and Oli-D2 are required for HR-inducing activity, which is dependent on the functions of NbSGT1 and NbNPR1. The data presented in this study not only expand our understanding of the molecular mechanism for the action of oligandrins in the regulation of the plant immune response, but also provide

Fig. 6 Oli-D1 and Oli-D2 induce resistance in tomato against *Botrytis cinerea*. (A–C) Resistance induced by recombinant Oli-D1 and Oli-D2 proteins. (D–F) Resistance induced by transiently expressed Oli-D1 and Oli-D2. (A, D) Representative phenotypes of disease caused by *B. cinerea* in Oli-D1- and Oli-D2-induced plants. (B, E) Size of lesions caused by *B. cinerea* in detached leaves from Oli-D1- and Oli-D2-induced plants at 3 days post-inoculation (dpi). (C, F) *In planta* growth of *B. cinerea* in detached leaves from Oli-D1- and Oli-D2-induced plants. One half of the leaf was infiltrated with 1 µM Oli-D1, Oli-D2 or a preparation from bacteria carrying pET-32 empty vector (A–C) or with agrobacteria carrying pGR106-Oli-D1, pGR106-Oli-D2 or pGR106-GFP (D–F) and the opposite half of the same leaf was inoculated by dropping 5 µL of spore suspension of *B. cinerea* at 24 h after infiltration. Photographs, lesion sizes and *in planta* fungal growth were taken or analysed at 3 dpi. Data presented in (B), (C), (E) and (F) are the means ± SD from three independent experiments. Significant difference at ***P* < 0.01.

Fig. 7 The hypersensitive response (HR) and expression of defence genes induced by Oli-D1 and Oli-D2 in tomato plants. (A) Representative HR in tomato leaves induced by recombinant Oli-D1 and Oli-D2 at 1 and 3 µM. Nep1_{Mo} and a preparation from bacteria carrying pET-32a empty vector were used as positive and negative controls, respectively. Photographs were taken at 48 h after infiltration. (B, C) Expression of jasmonic acid/ethylene (JA/ET)-responsive (B) and salicylic acid (SA)-responsive (C) genes in Oli-D1- and Oli-D2-infiltrated tomato leaves. Leaf samples were collected at different time points as indicated after infiltration with 1 µM Oli-D1 or Oli-D2, and expression of defence genes was analysed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Data presented in (B) and (C) are the means ± SD from three independent experiments. *Significant difference at *P* < 0.05. hpt, hours post-treatment. *LapA1*, leucine aminopeptidase A1; *Pin2*, proteinase inhibitor 2; *ERF2*, ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 2; *LOX-E*, lipoxygenase-E; *PR1a*, pathogenesis-related protein 1a; *PR1b*, pathogenesis-related protein 1a; *PR-P2*, pathogenesis-related protein P2.

experimental evidence for the development of potential bioactive formulae with Oli-D1 and Oli-D2 for crop disease control.

It is well known that some elicitors from *Pythium* and *Phytophthora* spp. can induce HR in *Nicotiana* species (Ponchet *et al.*, 1999); for example, 100 nM INF1, an elicitor from *Phytophthora infestans*, can induce HR-like cell death in tobacco (Ponchet *et al.*, 1999). By contrast, it has been reported previously that both CWPs (POD-1 and POD-2) and oligandrin elicitors from *P. oligandrum* are unable to induce HR in tobacco and tomato leaves (Kawamura *et al.*, 2009b; Picard *et al.*, 2000; Takahashi *et al.*, 2006; Takenaka *et al.*, 2006). Unlike these observations, however, we found, in this study, that recombinant Oli-D1 and Oli-D2 can induce visible HR in both *N. benthamiana* and tomato leaves, as verified by the appearance of rapid cell death, expression of HR marker genes (Fig. 1) and activation of the defence response, such as the accumulation of ROS and the production of autofluorescence (Fig. 2). Several reasons may explain the differences in induction of HR by oligandrins and CWP elicitors from *P. oligandrum*. First, the induction of HR requires appropriate concentrations of CWP and oligandrins (Oli-D1 and Oli-D2). For example, it was found that CWP at 300 nmol/mL and 1 μ M did not cause visible HR-like necrosis in leaves of *Arabidopsis* and tobacco plants, respectively (Kawamura *et al.*, 2009a; Takenaka *et al.*, 2006). Indeed, our results imply that the HR-inducing activity of Oli-D1 and Oli-D2 is dosage dependent, as Oli-D1 and Oli-D2 at relatively higher concentrations triggered a more rapid and stronger HR (Fig. 1). This feature of dosage-dependent HR-inducing activity for Oli-D1 and Oli-D2 is similar to the function of quercinin, an elicitor from *Phytophthora quercina*, which induces a dose-dependent cell death and oxidative burst in tobacco cell suspension cultures (Koehl *et al.*, 2003, 2007). Second, Oli-D1 and Oli-D2, comprising 120 aa with an elicitor domain (Masunaka *et al.*, 2010), are structurally similar to class I elicitors, whereas CWPs, comprising 145–151 aa containing an elicitor domain and an additional C-terminus with glycosylation sites, are structurally similar to class III elicitors (Kamoun *et al.*, 1997; Masunaka *et al.*, 2010). Our analyses of the different deletion mutants demonstrated that full-length Oli-D1 and Oli-D2 proteins are required for HR-inducing activity in *N. benthamiana* (Fig. 3). This is reasonable as Oli-D1 and Oli-D2 are small proteins (<10 kDa) and deletion of any part could destroy their high-order structures. Third, Oli-D1 and Oli-D2 are phylogenetically distinct from CWPs, showing approximately 52% identity at the amino acid level (Masunaka *et al.*, 2010), and thus may have different activity in HR induction. Based on the speed and degree of HR development in *N. benthamiana* and tomato leaves, Oli-D1 and Oli-D2 seem to be weak inducers of HR when compared with Nep1_{Mo} (Figs 1 and 7) (Zhang *et al.*, 2010, 2012). However, this should be an advantage for the development of bioactive formulae for disease control because significant HR or necrosis induced by the application of strong HR inducers may not be acceptable for practical use.

Both Oli-D1 and Oli-D2 belong to the oligandrin type of elicitors in *P. oligandrum* and contain typical SPs at N-termini (Masunaka *et al.*, 2010), suggesting that Oli-D1 and Oli-D2 might be extracellularly secreted proteins. This is supported by the fact that oligandrin was first identified and purified from a culture filtrate of *P. oligandrum* (Picard *et al.*, 2000). In our subcellular localization experiments, we found that Oli-D1 and Oli-D2 proteins were targeted to the plasma membrane when transiently expressed in *N. benthamiana* leaves (Fig. 4), implying that receptors for Oli-D1 and Oli-D2 may be localized on the plasma membrane of plant cells. This is consistent with common knowledge from the identification of plasma membrane surface-localized pattern recognition receptors for some well-known PAMPs, such as FLS2 for flg22 and EPR for Tu-EF (Boller and Felix, 2009; Greeff *et al.*, 2012). It has also been shown that some oomycete-secreted elicitors, such as cryptogin and INF1, have binding sites on the plasma membranes (Kanzaki *et al.*, 2008; Wendehenne *et al.*, 1995). Thus, it is likely that similar but distinct plasma membrane-localized receptors might be involved in the perception of the diverse range of elicitors and elicitor-like elicitors produced by oomycetes (Kanzaki *et al.*, 2008). However, silencing of *NbLRK1*, encoding for a lectin-like receptor kinase that interacts with INF1, did not affect the development of HR induced by Oli-D1 and Oli-D2 (Fig. 4B), indicating that *NbLRK1* is not a shared receptor for both INF1 and oligandrins. Considering that plant genomes encode a large number of receptor-like kinases (Shiu *et al.*, 2004), further identification of the Oli-D1 and Oli-D2 receptors or receptor complex and its associated components will broaden our understanding of the molecular action mechanism of these elicitor-like elicitors, especially the clarification of the earliest events and downstream signalling pathway involved in Oli-D1- and Oli-D2-induced HR.

Pythium oligandrum, as a biocontrol agent, can protect a variety of plants from disease damage through induced resistance (Benhamou *et al.*, 2012). For instance, direct application of *P. oligandrum* can trigger not only local resistance, but also systemic induced resistance, against fungal, oomycete and bacterial pathogens in tomato and grapevine plants (Le Floch *et al.*, 2003; Masunaka *et al.*, 2009; Mohamed *et al.*, 2007). The *P. oligandrum*-triggered induced resistance might result from the action of the elicitors, because CWP and oligandrin elicitors induced resistance in sugarbeet, wheat, tomato and *Arabidopsis* against a range of pathogens (Hase *et al.*, 2006, 2008; Kawamura *et al.*, 2009a; Lou *et al.*, 2011; Mohamed *et al.*, 2007; Picard *et al.*, 2000; Takenaka *et al.*, 2003, 2011; Wang *et al.*, 2011). In the present study, we found that either the recombinant or transiently expressed Oli-D1 and Oli-D2 induced resistance to *B. cinerea* by reducing disease severity and decreasing *in planta* fungal growth (Fig. 7). The latter phenomenon is somewhat similar to the observation that the growth of *Ph. parasitica* was restricted to the outermost tissues and its viability was decreased in oligandrin-treated tomato plants (Picard *et al.*, 2000). It is worth noting that, in our study, the

Oli-D1- and Oli-D2-induced resistance to *B. cinerea* was observed in the untreated half of the tomato leaves, which were treated on one half with recombinant or transiently expressed Oli-D1 or Oli-D2 (Fig. 7). This is consistent with previous results indicating that foliar application or soil drenching with oligandrin induced resistance against *Ph. parasitica* and *B. cinerea* (Lou *et al.*, 2011; Picard *et al.*, 2000). These observations indicate that oligandrins, such as Oli-D1 and Oli-D2, trigger the generation of signal(s) in the treated tissues and the signal(s) are then transmitted to the untreated parts, where they activate effective multiple defence responses, including the accumulation of ROS and autofluorescence, increased activity of defence-related enzymes and up-regulated expression of defence and signalling regulatory genes (Figs 2 and 7) (Lou *et al.*, 2011). However, there is still a possibility that Oli-D1 and Oli-D2 may have a direct effect on *B. cinerea* within the plants, as oligandrin was found to be translocated within tomato plants through the vascular system (Picard *et al.*, 2000).

Recent studies have demonstrated that the JA/ET-mediated signalling pathway plays an important role in the signalling network involved in *P. oligandrum* CWP-mediated induced resistance (Hase *et al.*, 2006, 2008; Kawamura *et al.*, 2009a). Our VIGS-based analyses indicate that the HR-inducing activity in *N. benthamiana* plants requires the functions of NbSGT1 and NbNPR1, but not NbCOI1, NbMEK2, NbTGA2.2 and NbHSP90.2 (Fig. 4). This is in agreement with the action of CWP elicitors in Arabidopsis, in which the CWP-induced defence response is dependent on AtSGT1, AtRAR1 and AtNPR1 (Kawamura *et al.*, 2009a). Although SGT1 and NPR1 are known to be critical regulators in R protein-triggered disease resistance (Shirasu, 2009) and SA signalling-mediated SAR (Dong, 2004; Durrant and Dong, 2004), the requirements for SGT1 in nonhost resistance and the JA response (Meldau *et al.*, 2011; Peart *et al.*, 2002), and for NPR1 in JA/ET signalling-mediated ISR (Pieterse and van Loon, 2004), have also been documented. Surprisingly, the silencing of *NbCOI1* did not affect the development of HR induced by Oli-D1 and Oli-D2 (Fig. 4), differing from the observation that AtCOI1 and AtJAR1 are required for the CWP-induced defence response in Arabidopsis (Kawamura *et al.*, 2009a). Consistent with the fact that INF1-induced cell death is independent of TGA2.2 and MEK2 (Kanneganti *et al.*, 2006), our results also revealed that both NbTGA2.2 and NbMEK2 are not involved in Oli-D1- and Oli-D2-induced HR (Fig. 4). However, treatment with recombinant Oli-D1 and Oli-D2 activated the expression of some selected JA/ET signalling-responsive genes, such as *SILpa1*, *SIPin2*, *SILox-E* and *SIERF2* (Kawamura *et al.*, 2009b; Kawazu *et al.*, 2012), but did not affect the expression of two SA signalling-responsive genes, *SIPR1a* and *SIPR-P2* (Kawazu *et al.*, 2012) (Fig. 7), indicating that the JA/ET signalling pathway is involved in the Oli-D1- and Oli-D2-induced defence response in tomato. This is similar to previous observations indicating that CWP or INF1 up-regulated the expres-

sion of JA/ET-responsive genes, but not SA-responsive genes (Hase *et al.*, 2008; Kawamura *et al.*, 2009b; Takahashi *et al.*, 2006), and that CWP-induced resistance is dependent on the JA/ET signalling pathway, but not the SA signalling pathway (Hase *et al.*, 2008; Kawamura *et al.*, 2009b). Collectively, our data indicate that the JA/ET signalling pathway and the functions of SGT1 and NPR1 are involved in the regulation of Oli-D1- and Oli-D2-induced HR and disease resistance. Further genetic studies are required to identify key regulatory components and to elucidate the detailed signalling pathway regulating the Oli-D1- and Oli-D2-induced defence response.

EXPERIMENTAL PROCEDURES

Plants and growth conditions

Plants of *N. benthamiana* and tomato (*Solanum lycopersicum* cv. Suhong 2003) were grown in compost soil mix (perlite : vermiculite : plant ash, 1:6:2) in a growth room under fluorescent light (200 $\mu\text{E}/\text{m}^2/\text{s}$) at 22–24 °C with 60% relative humidity in a 14 h light/10 h dark regimen. Four- to five-week-old plants were used for all experiments.

Cloning of the *Oli-D1* and *Oli-D2* genes

The open reading frames (ORFs) of the *Oli-D1* and *Oli-D2* genes, encoding for the oligandrins of *P. oligandrum*, were synthesized by Invitrogen, Shanghai, China according to the sequences of GenBank accessions AB474242 and AB474243, and cloned into pMD19, yielding pMD19-Oli-D1 and pMD19-Oli-D2. The sequences of Oli-D1 and Oli-D2 in plasmids pMD19-Oli-D1 and pMD19-Oli-D2 were confirmed by sequencing and used as templates for further study. Nep1_{Mo} (MGG_08454) was cloned from *M. oryzae* and used as a positive control (Zhang *et al.*, 2010).

Preparation of recombinant Oli-D1 and Oli-D2 proteins

The ORF sequences of *Oli-D1* and *Oli-D2* were amplified from plasmids pMD19-Oli-D1 and pMD19-Oli-D2 using pairs of specific primers (Table S1, see Supporting Information) and cloned into pET-32a, yielding plasmids pET32a-Oli-D1 and pET32a-Oli-D2, which were then transformed into *Escherichia coli* rosetta strain. The *E. coli* strains carrying pET32a-Oli-D1 and pET32a-Oli-D2 were grown at 28 °C with shaking (220 rpm) in Luria–Bertani (LB) liquid medium to an optical density at 600 nm (OD_{600}) = 0.6 and were induced for 4 h by the addition of isopropyl β -D-1-thiogalactopyranoside to a final concentration of 0.1 mM. Bacteria were collected by centrifugation and the recombinant proteins were purified using Ni-NTA-Agarose (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Protein concentration was determined with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis with a 6 $\times$ histidine (6 $\times$ His) tag antibody revealed that the purified Oli-D1 and Oli-D2 proteins with 6 $\times$ His tags were homogeneous with a single band and migrated with the same molecular weights as calculated from protein sequences (data not shown).

A similar procedure was used to purify the recombinant Nep1_{Mo} protein. All the purified Oli-D1, Oli-D2 and Nep1_{Mo} proteins were stored at -70 °C and used for further experiments.

Construction of vectors

Five deletion mutants of Oli-D1 and Oli-D2 proteins were designed. The d1 mutant deleted the SP, but kept the entire elicitor domain. The d2 and d3 mutants kept the SP, but deleted 20 and 40 aa from the C-terminus, respectively, which disrupted the integrity of the elicitor domain. The d4 and d5 mutants deleted both the SP and 20 or 40 aa from the C-terminus, respectively. For transient expression assays, the Oli-D1 and Oli-D2 ORFs without stop codons and five deletion mutants (d1–d5) for Oli-D1 and Oli-D2 were amplified using specific primers (Table S1) from plasmids pMD19-Oli-D1 and pMD19-Oli-D2 and cloned into pFLAG-CTS to add FLAG tags at the C-termini. The FLAG-tagged target fragments were then amplified and inserted into vector pGR106, yielding pGR106-Oli-D1, pGR106-Oli-D1-d1–d5, pGR106-Oli-D2 and pGR106-Oli-D2-d1–d5. For subcellular localization analysis, the ORFs and d1 deletion mutants of Oli-D1 and Oli-D2 were amplified from plasmids pMD19-Oli-D1 and pMD19-Oli-D2 and cloned into vector pFGC-Egfp, yielding pGFP-Oli-D1, pGFP-Oli-D2, pGFP-Oli-D1-d1 and pGFP-Oli-D2-d1. For VIGS assay, cDNA fragments for *NbSGT1.1* (AF516180), *NbHSP90.2* (AY368905), *NbMEK2* (AB360636), *NbLRK1* (AB247455), *NbCOI1* (*NtCOI1*, AB433899), *NbTGA2.2* (*NtTGA2.2*, AF031487) and *NbNPR1* (*NtNPR1*, DQ837218) were amplified from cDNAs of *N. benthamiana* using primers designed on the basis of corresponding *N. benthamiana* or tobacco sequences. After confirmation of the sequences of interest of the genes by cloning and sequencing, the cDNA fragments were amplified and cloned into the pTRV2 vector (Liu *et al.*, 2002), resulting in pTRV2-NbSGT1.1, pTRV2-NbHSP90.2, pTRV2-NbMEK2, pTRV2-NbLRK1, pTRV2-NbCOI1, pTRV2-NbTGA2.2 and pTRV2-NbNPR1. All of these constructed vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation using a GENE PULSER II Electroporation System (Bio-Rad Laboratories, Hercules, CA, USA). *Agrobacterium* carrying different constructs were grown in YEP medium (50 µg/mL rifampicin, 50 µg/mL kanamycin and 25 µg/mL gentamicin) for 24 h with continuous shaking at 28 °C. Cells were centrifuged and resuspended in infiltration buffer [10 mM MgCl₂, 10 mM 2-(*N*-morpholino)ethanesulphonic acid (MES), 200 µM acetosyringone, pH 5.7].

HR-inducing activity assays

The purified Oli-D1 and Oli-D2 at concentrations of 1 and 3 µM were infiltrated into leaves of 4-week-old *N. benthamiana* plants using 1-mL needleless plastic syringes. A preparation from empty vector pET-32a and the recombinant Nep1_{Mo} protein at a concentration of 1 µM were used as negative and positive controls, respectively. Leaves were harvested at the indicated time points to monitor the development of HR and to analyse the expression of marker genes.

VIGS in *N. benthamiana*

A standard VIGS procedure was performed according to the method described previously (Liu *et al.*, 2002). The recombinant plasmids carrying

the inserts of the genes of interest or pTRV2-GUS (as a control) were transformed into *A. tumefaciens* strain GV3101. Bacterial suspensions were infiltrated into leaves of 3-week-old *N. benthamiana* plants using 1-mL plastic needleless syringes. Plants infiltrated with VIGS constructs were allowed to grow in a glasshouse at 22 °C for another 2 weeks, and leaf samples were collected for the analysis of silencing efficiencies for each gene of interest by qRT-PCR. Only when the silencing efficiency in an independent experiment was >70% were the VIGS-infiltrated plants used for further experiments.

Transient expression assays in *N. benthamiana*

Fully expanded leaves of 4-week-old or VIGS-infiltrated *N. benthamiana* plants were infiltrated using 1-mL plastic needleless syringes with suspensions of *agrobacteria* carrying different constructs (pGR106-Oli-D1, pGR106-Oli-D1-d1–d5, pGR106-Oli-D2, pGR106-Oli-D2-d1–d5) or pGR106-GFP (as a control), and leaf samples were collected at 48 h after infiltration for the visualization of HR and for Western blotting analyses.

SDS-PAGE and Western blotting

Leaf samples were ground in 200 µL of extraction buffer [4 M urea, 100 mM dithiothreitol (DTT)], followed by the addition of 100 µL of loading buffer. After 5 min of boiling and subsequent 10 min of centrifugation at 10 000 × *g*, 20 µL of the supernatant were separated on a 15% SDS-PAGE gel and transferred onto nitrocellulose by wet electroblotting. The detection of FLAG tags was performed using a mouse monoclonal antibody (1:1000 dilution) (Huaan Company, Hangzhou, China) and a peroxidase-conjugated antimouse antibody (1:8000 dilution) (Huaan Company) according to the manufacturer's instructions. Proteins in the SDS-PAGE gel were detected by an ECL Plus detection system (Huaan Company).

Subcellular localization

The subcellular localization of Oli-D1 and Oli-D2, as well as the mutants (Oli-D1-d1 and Oli-D2-d1) with deletion of the SP, was examined. *Agrobacteria* carrying pGFP-Oli-D1, pGFP-Oli-D1-d1, pGFP-Oli-D2 and pGFP-Oli-D2-d1, or pFGC-Egfp empty vector, were infiltrated into *N. benthamiana* leaves using 1-mL needleless syringes. Leaves were collected at 24 h after agroinfiltration and GFP was detected with a confocal laser scanning microscope (Zeiss LSM 510 META; argon laser excitation wavelength, 488 nm) (Zeiss, Jena GmbH, Jena, Germany).

Disease assays

Inoculation of *B. cinerea* was performed using a spore suspension at a spore density of 4 × 10⁵ spores/mL according to a previously reported procedure (AbuQamar *et al.*, 2008). One half of the leaves of 4-week-old tomato plants were infiltrated with recombinant Oli-D1 or Oli-D2 proteins, or with *agrobacteria* carrying pGR106-GFP (as a negative control), pGR106-Oli-D1 or pGR106-Oli-D2. Two days later, the *agrobacteria*-infiltrated leaves were detached and inoculated by dropping 5 µL of a spore suspension on the opposite half of the same leaves. The inoculated

leaves were kept in a high-humidity chamber and disease severity was recorded by measurement of the lesion size. The inoculated leaves were collected at 0 and 3 dpi, and the fungal growth *in planta* was measured by qRT-PCR, analysing the transcript of the *B. cinerea ActinA* gene as an indicator of fungal growth (Benito *et al.*, 1998) using a pair of primers (BcActin-F and BcActin-R; Table S1) and with a tomato *Actin* gene as an internal control. Relative growth was expressed as the fold change of *BcActinA* transcripts/tomato *SlActin* transcripts.

qRT-PCR analysis of gene expression

Total RNA was extracted from leaf samples using TRIZOL reagent (Invitrogen) and the first-strand cDNA was synthesized from 1 µg of total RNA with a SuperScript III Kit (Invitrogen) according to the manufacturer's instructions. qRT-PCR was performed with SYBR Premix Ex Taq (Takara, Dalian, China) on a CFX96 Real-time System (Bio-Rad Laboratories). Tomato and *N. benthamiana Actin* genes were used as internal controls for normalization of the data obtained. Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method. The primers used are listed in Table S1.

Detection of H₂O₂ and autofluorescence

Detection of H₂O₂ *in planta* was performed using the DAB staining method as described previously (Thordal-Christensen *et al.*, 1997). *Nicotiana benthamiana* leaves infiltrated with 1 µM of recombinant Oli-D1 or Oli-D2 were collected 4 h after infiltration, dipped into DAB solution (1 mg/mL, pH 3.8) and incubated for 10 h in the dark at room temperature. After incubation with DAB, the leaves were removed, placed into acetic acid–glycerol–ethanol (1:1:1, v/v/v) and boiled for 5 min, followed by several changes of the solution. Brownish-red precipitates formed in the presence of H₂O₂ were visualized using a digital camera. Examination of autofluorescence was performed according to Dietrich *et al.* (1994) with slight modifications. Detached leaves were fixed in an autofluorescence-fixing solution (10% formaldehyde, 5% acetic acid and 45% ethanol). Samples were cleared in alcoholic lactophenol, rinsed with distilled water and then equilibrated in 50% glycerol before mounting on slides. Autofluorescence was visualized under an EGFP filter set (excitation, 488 nm; emission, 500/600 nm; dichroic, 495 nm) with a confocal laser scanning microscope (Zeiss LSM 510 META).

Statistical analysis

All experiments were repeated independently three times and at least six plants were included in each treatment in an independent experiment. Data obtained from three biological replications were subjected to statistical analysis by Student's *t*-test using DPS software (<http://www.chinadps.net/>) and the results are presented as mean ± standard deviation. Probability values of *P* < 0.05 were considered as significant between different treatments.

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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 Primers used in this study.