The role of vacuolar processing enzyme (VPE) from *Nicotiana benthamiana* in the elicitor-triggered hypersensitive response and stomatal closure

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Abstract

Elicitors/pathogen-associated molecular patterns (PAMPs) trigger the plant immune system, leading to rapid programmed cell death (hypersensitive response, HR) and stomatal closure. Previous reports have shown that the vacuolar processing enzyme (VPE), a cysteine proteinase responsible for the maturation of vacuolar proteins, has caspase-1-like activity and mediates TMV- and mycotoxin-induced cell death. The role of VPE from *Nicotiana benthamiana* in the response to three elicitors: bacterial harpin, fungal Nep1, and oomycete boehmerin, is described here. Single-silenced (*NbVPE1a* or *NbVPE1b*) and dual-silenced (*NbVPE1a/1b*) *N. benthamiana* plants were produced by virus-induced gene silencing. Although *NbVPE* silencing does not affect H₂O₂ accumulation triggered by boehmerin, harpin, or Nep1, the HR is absent in *NbVPE1a*- and *NbVPE1a/1b*-silenced plants treated with harpin alone. However, *NbVPE*-silenced plants develop a normal HR after boehmerin and Nep1 treatment. These results suggest that harpin-triggered HR is VPE-dependent. Surprisingly, all gene-silenced plants show significantly impaired elicitor-induced stomatal closure and elicitor-promoted nitric oxide (NO) production in guard cells. Dual-silenced plants show increased elicitor-triggered AOS production in guard cells. The accumulation of transcripts associated with defence and cell redox is modified by VPE silencing in elicitor signalling. Overall, these results indicate that VPE from *N. benthamiana* functions not only in elicitor-induced HR, but also in elicitor-induced stomatal closure, suggesting that VPE may be involved in elicitor-triggered immunity.

**Key words:** AOS, elicitor, hypersensitive response, *Nicotiana benthamiana*, stomatal closure, virus-induced gene silencing.

Introduction

Plants have developed a complex immune system to resist pathogen attack, which includes rapid and localized cell death (hypersensitive response, HR), and stomatal closure (Dangl et al., 1996; Lam et al., 2001; Melotto et al., 2006; Mur et al., 2008). Basal plant defence response is induced by pathogen-associated molecular patterns (PAMPs; Hématy et al., 2009) and elicitors (Zhao et al., 2005; Garcia-Brugger et al., 2006).

Phytopathogens secrete a wide range of elicitors (including polypeptides, proteins, and oligosaccharides) into the plant cell wall, cell or extracellular space, such as elicitins (Tyler, 2002; Qutob et al., 2003; Lascombe et al., 2007), harpins (Wei et al., 1992; Miao et al., 2010), transglutaminases (Brunner et al., 2002), necrosis and ethylene-inducing peptide 1 (Nep1; Gijzen and Nürnberger, 2006; Motteram et al., 2009), other proteins (Nürnberg et al., 1994; Villaba Mateos et al., 1997; Fellbrich et al., 2002; Torto et al., 2003; Wang et al., 2003), and hepta-β-glucoside (Fliegmann et al., 2004; Daxberger et al., 2007).

The recognition of elicitors by the plant cell is followed by calcium influx and the production of active oxygen
species (AOS) and nitric oxide (NO). Subsequent signal transduction induces HR and stomatal closure (Torres et al., 2006; Melotto et al., 2008; Srivastava et al., 2009). A number of key players involved in HR have been identified (Greenberg and Yao, 2004; Gabriëls et al., 2006; Gan et al., 2009; EK-Ramos et al., 2010); these studies have revealed that mammalian and plant cell death mechanisms share common morphological and biochemical features, including cytoplasm shrinkage, nuclear condensation, DNA ladder- ing, and the release of cytchrome c from mitochondria (Sun et al., 1999; Sasabe et al., 2000; Kim et al., 2003; Ji et al., 2005). However, it remains unclear how signalling pathways lead to local HR, but not to whole-plant cell death, and how death occurs.

Many studies have shown that HR in plants is regulated by caspase-like activity. However, no caspase homologue has been found in the Arabidopsis genome (Cohen, 1997; Lam and Del Pozo, 2000; Woltering et al., 2002; Chichkova et al., 2004; Danon et al., 2004; Bonneau et al., 2008), although other proteinases (e.g. serine proteinases and metacaspase) have caspase-like activity and are involved in cell death in plants (Coffeen et al., 2004; Suarez et al., 2004; He et al., 2007). The vacuolar processing enzyme (VPE) is a cysteine proteinase responsible for the maturation of vacuolar proteins and has caspase-1-like activity. VPE homologues in Arabidopsis can be divided into two subfamilies: seed-type VPE and vegetative-type VPE (Kinoshita et al., 1995). Seed-type VPE is responsible for the conversion of proproteins of various vacuolar proteins into mature forms (Hara-Nishimura et al., 1993; Yamada et al., 1999; Wang et al., 2009), and the vegetative-type VPE may play a role in several types of cell death (Kinoshita et al., 1999; Rojo et al., 2004; Lam, 2005; Nakaune et al., 2005). Hatsugai and his co-workers (Hatsugai et al., 2004) have proved that VPE1a and VPE1b silencing in Nicotiana benthamiana inhibit hypersensitive cell death mediated by TMV/N via virus-induced gene silencing (VIGS). VPE deficiency suppresses vacuolar collapse, leading to mycotoxin-induced cell death (Kuroyayagi et al., 2005; Yamada et al., 2005); however, the role of VPE in elicitor-signalling remains unclear.

Regulation of the stomatal aperture in plants controls photosynthesis and the water status of the plant (Fan et al., 2004; Nadeau, 2008). Because mature guard cells lack plasmodesmata, all solute uptake and efflux must occur via the plasma membrane and vacuole (Pandey et al., 2007). Historically, stomata were considered as a passive portal for the entry of pathogenic bacteria (Pandey et al., 2007), but recent studies have suggested that stomata play an active role in the innate immune system (Melotto et al., 2006, 2008). Stomatal closure restricts bacterial invasion, although plant pathogenic bacteria can secrete specific virulence factors to effectively re-open stomata, and this is an important pathogenic strategy (Melotto et al., 2006). It is therefore necessary to study stomatal movement to understand bacterial pathogenesis, disease epidemiology, and phyllosphere microbiology.

Here, an attempt was made to suppress VPE1a and VPE1b, the most abundant VPEs in Nicotiana tabacum (NvVPE1a and NvVPE1b), in N. benthamiana via virus-induced gene silencing described by Hatsugai et al. (2004). This allowed detection of the phenotype of HR and stomatal movement after elicitor treatment with bacterial harpin, fungal Nep1, or oomycete boehmerin. NbVPE1a and NbVPE1b regulate elicitor-induced cell death differently. Moreover, NbVPE silencing affects elicitor-induced stomatal closure and NO production, and also dysregulates genes related to AOS accumulation and transcription. These results suggest that VPE plays an important role in elicitor signalling in plants.

### Materials and methods

#### Plant materials, elicitors, and treatment protocol

*N. benthamiana* plants were grown in a growth chamber under a 16/8 h light/dark cycle at 25 °C. A needleless syringe was used to inject 25 μl elicitor (50 nM) into tiny cuts on the underside of the leaf, thereby flooding the apoplastic space. To prepare Phytophthora boehmeriae boehmerin and Magnaporthe grisea Nep1, overnight cultures of *Escherichia coli BL21* cells which carried pET32b harbouring the boehmerin (GenBank accession no. AJ196607) or nep1 (GenBank accession no. MGG_08454) were diluted (1:100 v/v) in Luria-Bertani medium containing ampicillin (50 mg ml⁻¹) and incubated at 37 °C. To prepare *E. coli*-expressed harpin, overnight cultures of BL21 cells which carried pET30a harbouring the harpin gene (GenBank accession no. AY875714), were diluted (1:100 v/v) in Luria-Bertani medium containing kanamycin (50 mg ml⁻¹) and incubated at 37 °C. When the OD₆₀₀ of the cultures reached 0.6, boehmerin, harpin, or Nep1 secretion into the culture medium was induced via the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside for 6 h. The deposit was harvested by centrifugation, washed repeatedly, stored in 10 mM phosphate-buffered saline (PBS, pH 6.5), and then broken up by ultrasonification. Supernatants collected by centrifugation (12 000 g, 15 min, 4 °C) were dialysed successively against 0.8, 0.6, and 0.4% SDS at 15 °C (12 000 g for 3 h) and dialysed against 10 mM PBS (pH 6.5) and stored at −20 °C prior to use. Protein concentrations were determined using the Bradford reagent (Qutob et al., 2006) and concentrated stock solutions (500 nM) were prepared.

#### DNA constructs and seedling infection for virus-induced gene silencing

Virus-induced gene silencing for the NbVPE1a and NbVPE1b genes in *N. benthamiana* was performed using *Potato Virus X* (PVX), as described by Hatsugai et al. (2004). A 373 bp fragment of NbVPE1a (GenBank accession no. AB181187) was amplified with the forward primer NbVPE1a-U/Sal (5’-CGGTGCGAGCATTTGCAAACAATGTAGA-3’; SalI site underlined) and the reverse primer NbVPE1a-R/ClaI (5’-CCATGATCCTCAAAATATACTACAGACT-3’; Clai site underlined). The PCR products were digested with SalI and Clai, and ligated into the corresponding sites in the PVX vector pgR107, to generate PVX-NbVPE1a. A 413 bp fragment of NbVPE1b (GenBank accession no. AB181188) was amplified with the forward primer NbVPE1b-U/Sal (5’-CGGTGAGCATTCCTCAGGGTCGAG-3’; SalI site underlined) and the reverse primer NbVPE1b-R/ClaI (5’-CCATCGATCCTTGTAGATGTAG-3’; Clai site underlined). The PCR products were digested with SalI and Clai, and ligated into the corresponding sites in the PVX vector pgR107, to generate PVX-NbVPE1b. A 373 bp fragment of NbVPE1a was amplified with the forward primer NbVPE1a-U/SphI (5’-CCGCATGCGCATTTGCAAACAATGTAG-3’; SphI site underlined) and the
reverse primer NbVPE1a-R/ClaI, then digested with ClaI and SphI. A 413 bp fragment of NbVPE1b was amplified with the forward primer NbVPE1b-U/SalI and the reverse primer NbVPE1b-R/SphI (5'-CCCGATGGCCATCGTCTGAGTAGTAG-3'; SphI site underlined), digested with SphI and SalI. The 373 bp NbVPE1a and the 413 bp NbVPE1b fragments were ligated in tandem to the SalI/ClaI-digested pgR107 to generate PVX-NbVPE1a/b. The constructs containing the inserts were transformed into Agrobacterium tumefaciens strain GV3101. Bacterial suspensions were applied to the undersides of N. benthamiana leaves using a 1 ml needleless syringe. Plants exhibited mild mosaic symptoms 3 weeks after inoculation. The third or fourth leaf above the inoculated leaf, where silencing was most consistently established, was used for further analyses.

**Diaminobenzidine (DAB) staining**

According to Lamb and Dixon (1997), and Zhang et al. (2009), leaves were harvested 6 h after elicitor treatment. Following the methods of Gan et al. (2009), samples were vacuum-infiltrated for 20 min with phosphate-buffered saline (PBS; pH 7.4) containing 0.5% (w/v) DAB. The leaves were placed in light for 10 h and then boiled for 20 min in 80% ethanol. The intensity and pattern of DAB staining was assessed visually. Quantitative scoring of H2O2 staining in leaves was analysed using Quantity One software (Leica DMR, Germany).

**RNA isolation, reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time PCR**

Total RNA was extracted following the Trizol extraction protocol (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase I (TaKaRa, Dalian, China). First-strand cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen) following the manufacturer’s directions. PCR was performed in 50 μl reactions using 1 μl of cDNA template, 1 μM of each gene-specific primer, 2 U of Taq polymerase, and the buffer provided by the manufacturer (containing 1.5 mM MgCl2). To ensure that similar amounts of cDNA were used for silenced and non-silenced plants, parallel reactions with elongation factor 1α (EF1α) primers as controls were run. Each PCR cycle included denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, and elongation at 72 °C for 30 s, as described in Zhang et al. (2004). The PCR products were analysed on a 1.2% agarose gel and stained with ethidium bromide. Details of RT-PCR-specific primers for NbVPE1a, NbVPE1b, NbVPE2, NbVPE3, and EF1α are given in Supplementary Table S1 at JXB online. NbVPE1a specific 413 bp primer set (NbVPE1a-F and NbVPE1a-R) and NbVPE1b specific primer set (NbVPE1b-F and NbVPE1b-R), which have been successfully applied by Hatsugai et al. (2004) were used to estimate the silencing specificity.

Quantitative real-time PCR was performed using the cDNA and gene-specific primers. Each cDNA was amplified by quantitative PCR using the SYBR® PrimeScript™ RT-PCR Kit (TaKaRa, Dalian, China) and the ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). N. benthamiana EF-1α expression was used to normalize the expression value in each sample, and relative expression values were determined against buffer or PVX-infected plants using the comparative Ct method (2-ΔΔCt).

Primers of quantitative real-time PCR are also described in Supplementary Table S1 at JXB online.

**Stomatal aperture measurements**

Stomatal apertures were measured as described by Chen et al. (2004) and Zhang et al. (2009). Leaves were derived from various plants silenced for NbVPE1a, NbVPE1b, NbVPE1a/b, and for controls. After a 3-week inoculation, leaf samples were harvested from the third and fourth leaves above the inoculation site. Abaxial (lower) epidermis was peeled off and floated in 5 mM KCl, 50 mM CaCl2, and 10 mM MES-Tris (pH 6.15) in light for at least 2 h to open the stomata fully before experimentation to minimize the effects of other factors in stomatal response, because the mesophyll signals can also significantly influence stomatal behaviour. The epidermal strips were then followed by elicitor treatment for 3 h to induce a stomatal response. The images of stomatal aperture in the epidermal strips were captured by a digital camera under a microscope. The maximum diameter of stomata was measured under an optical microscope. At least 50 apertures in each treatment were obtained. The experiments were repeated three times.

**NO measurement in guard cells**

NO accumulation was determined using the fluorophore 4,5-diaminofluorescein diacetate (DAF-2DA, Sigma-Aldrich) according to Ali et al. (2007). Epidermal strips were prepared from control and gene-silenced plants, and incubated in 5 mM KCl and 10 mM MES-Tris (pH 6.15) in the light for 2 h, followed by incubation in 20 μM DAF-2DA for 1 h in the dark at 25 °C, and finally rinsed three times with 10 mM Tris-HCl (pH 7.4) to wash off excess fluorophore. Images of guard cells were obtained 3 h after elicitor treatment under a fluorescence microscope (excitation wavelength, 470 nm; emission wavelength, 515 nm). Fluorescence emission from guard cells was analysed using Quantity One software.

**AOS measurement in guard cells**

Dihydroorhodamine 123 (DHR, Merck, Whitehouse Station, NJ) was used to analyse elicitor-induced AOS production in guard cells. Epidermal strips were incubated in 20 μM DHR for 2 h in the dark at 37 °C, and then rinsed three times with PBS (pH 7.4) to remove excess fluorophore. Subsequently, 3 h after elicitor treatment, guard cell images were obtained using Adobe Photoshop 5.5 (Mountain View, CA) during a 2 s short ultraviolet (UV) exposure (one exposure per sample) under a fluorescence microscope equipped with a digital camera. Fluorescence emission of guard cells was analysed using Quantity One software.

**Results**

**Silencing of the NbVPE1a and NbVPE1b genes**

VPE genes in N. benthamiana have been specifically silenced via VIGS, and it has been proven that VPE deficiency prevents virus-induced hypersensitive cell death in tobacco plants (Hatsugai et al., 2004). To clarify whether VPE was essential for the elicitor-induced cell death, the VIGS method was used to produce single-silenced (NbVPE1a or NbVPE1b) and dual-silenced (NbVPE1a/b) N. benthamiana plants described by Hatsugai et al. (2004). Plants were separately or simultaneously silenced using two DNA fragments from the NbVPE1a and NbVPE1b coding regions. No difference in development or growth was observed between the NbVPE-silenced and control PVX-infected plants (data not shown). A common positive control is silencing of the phytoene desaturase (PDS) gene, which results in photobleaching of the silenced regions and is a readily visible phenotype. When photobleaching was apparent (see Supplementary Fig. S1 at JXB online), the efficiency of virus-induced NbVPE-silencing was evaluated by semi-quantitative RT-PCR. NbVPE1a and NbVPE1b transcripts were detected in control plants, but the accumulation of NbVPE1a transcripts was reduced in NbVPE1a-silenced and NbVPE1a/b-silenced plants. NbVPE1b expression also decreased...
significant in NbVPE1b-silenced and NbVPE1a/b-silenced plants (Fig. 1).

To test the possibility that closely related gene(s) can also be silenced in our experiments, Blast analysis and RT-PCR were used to examine the silencing specificity. Blast results showed that both NbVPE1a and NbVPE1b shared significant homology with N. tabacum VPE2 (NtVPE2) and N. benthamiana VPE3 (NbVPE3) [AB075949 and TC11831, The Institute for Genomic Research (TIGR), version 9.0]. Semi-quantitative RT-PCR was performed using unique primers to distinguish each of these homologues to check the transcript of these homologues. Our analysis suggests that NbVPE2 and NbVPE3 transcript accumulation is not affected in NbVPE1a-, NbVPE1b-, and NbVPE1a/b-silenced plants (Fig. 1).

Together, these data effectively demonstrate that NbVPE1a, NbVPE1b, and NbVPE1a/b have been specifically silenced, respectively. Consistent with our results, these genes were also found to be silenced in N. benthamiana (Hatsugai et al., 2004). Thus, the three gene-silenced plants were deemed appropriate for further analyses.

**NbVPE1a plays a role in harpin-mediated HR, but is not involved in boehmerin and Nep1-induced HR**

Typical hypersensitive cell death occurred in the leaves 24 h after the elicitor (i.e. boehmerin, harpin, or Nep1) was infiltrated into control PVX-infected N. benthamiana leaves. As shown in Fig. 2A, typical hypersensitive cell death was also observed in the gene-silenced plants after boehmerin or Nep1 infiltration. However, typical hypersensitive cell death did not occur in NbVPE1a- or NbVPE1a/b-silenced leaves after harpin infiltration. Although ethanol bleaching can be used to enhance the visualization of HR in leaves (Schornack et al., 2004; Weber et al., 2005; Gan et al., 2009), HR remained undetectable in NbVPE1a- and NbVPE1a/b-silenced leaves 72 h after harpin treatment (Fig. 2A). Cell death was further investigated in situ using trypan blue, which accumulated in dead cells. The application of boehmerin, harpin, and Nep1 induced blue staining that was localized to treated tissues, whereas leaves of NbVPE1a- and NbVPE1a/b-silenced plants infiltrated with harpin remained unstained, with a negligible number of blue spots (Fig. 2B). These results indicate that NbVPE1a, but not NbVPE1b, is required for harpin-mediated cell death, but not for the boehmerin- or Nep1-triggered cell death response. The results also suggest that the molecular basis of hypersensitive cell death triggered by harpin may differ from that after boehmerin or Nep1 treatment.

**NbVPE does not regulate elicitor-triggered H2O2 accumulation**

Although H2O2 production triggered by an elicitor is strongly correlated with the early defence response preceding HR (Garcia-Brugger et al., 2006; Pitzschke and Hirt, 2006), it is unclear whether elicitor-triggered H2O2 accumulation is dependent on VPE. The contribution of NbVPE1a and NbVPE1b to H2O2 production in response to elicitors was examined. DAB polymerizes on contact with H2O2 in a reaction requiring peroxidase; thus, H2O2 can be visualized in situ as a reddish-brown precipitate (Thordal-Christensen et al., 1997). A heavy staining was observed in control plants 6 h after boehmerin, harpin, or Nep1 treatment (Fig. 3A), which was consistent with the late peak of oxidative production during incompatible plant–pathogen interactions (Baker and Orlandi, 1995; Allan and Fluhr, 1997; Lamb and Dixon, 1997). Light staining was observed after PBS injection. Similar analyses were conducted with NbVPE-silenced plants. No change in DAB staining intensity was observed in NbVPE-silenced plants.
compared with control plants after boehmerin, harpin, and Nep1 infiltration (Fig. 3B). These data suggest that NbVPE1a and NbVPE1b contribute little to elicitor-induced H2O2 accumulation.

Fig. 2. Local induction of hypersensitivity responses with boehmerin (50 nM), harpin (50 nM), and Nep1 (50 nM). (A) Leaves (representative of three replicate treatments) from control PVX, NbVPE1a-, NbVPE1b-, and NbVPE1a/1b-silenced N. benthamiana were infiltrated with the elicitors simultaneously. The red and black circles indicate cell death and no cell death, respectively. Leaves were removed from plants after 3 d of treatment (left panels) and bleached in ethanol (right panels). (B) Trypan blue staining of leaves from control and NbVPE1a-, NbVPE1b-, and NbVPE1a/1b-silenced plants in response to boehmerin, harpin, and Nep1. Responses in the inoculated leaf are shown. Pictures were taken from plants after 24 h of treatment. The experiments were performed in triplicate. Bars, 1 cm.

Fig. 3. In situ detection of hydrogen peroxide using DAB staining on control, PVX-NbVPE1a, PVX-NbVPE1b-, and PVX-NbVPE1a/1b-infected N. benthamiana leaves in response to elicitors. (A) Photographs of representative ethanol-bleached leaves from control and NbVPE-silenced plants 6 h after PBS (10 mM), boehmerin (50 nM), harpin (50 nM), or Nep1 (50 nM) treatment; elicitation with the elicitor was conducted on plants by infiltrating an equivalent elicitor solution of 25 μL. (B) Quantitative scoring of staining in leaves of the control and NbVPE-silenced plants with elicitor treatment. The analysis was repeated for three sets of independently silenced plants in each experiment; the values shown were the means ± SD of duplicate assays. The experiment was repeated twice with similar results.

NbVPE silencing impairs elicitor-activated stomatal closure

VPE is localized in the vacuole and is involved in provoking the disintegration of vacuolar membranes, leading to hypersensitive cell death after exposure to TMV or mycotoxins (Hatsugai et al., 2006). Stomatal closure is driven by the reduction of intracellular solutes, and large amounts of cell solutes are stored in the vacuole. It has previously been reported that elicitors including boehmerin, harpin, and Nep1 can induce stomatal closure (Zhang et al., 2009), but it remains unclear whether NbVPE1a and NbVPE1b contribute to elicitor-induced stomatal closure. Stomatal responses to boehmerin, harpin, and Nep1 were
observed in NbVPE-silenced leaves. Elicitors induced stomatal closure in control leaves, but closure was clearly impaired in NbVPE-silenced leaves (Fig. 4). Consequently, elicitor-induced stomatal aperture analyses were performed with NbVPE-silenced plants, and it was found that boehmerin-induced stomatal closure was significantly inhibited compared with control plants (Fig. 5A). Similarly, silenced plants showed a markedly reduced response to harpin and Nep1 (Fig. 5B, C). Compared with control PVX-infected plants, neither single- nor dual-silenced plants showed any alteration in stomata after treatment with PBS. These results suggest that NbVPE silencing compromises elicitor-induced stomatal closure.

NbVPE silencing decreases NO production in guard cells in response to elicitors

NO co-ordinates the HR and plant innate immunity, serving as a cellular signalling molecule in a wide range of organisms, including plants, and especially in stomatal guard cells (Dangl, 1998; Ali et al., 2007). To determine the effect of VPE silencing on NO accumulation, NO generation was compared in guard cells isolated from control and NbVPE-silenced plants 3 h after treatment with boehmerin, harpin, or Nep1. Elicitor treatment evoked NO generation in the guard cells of control plants (Fig. 6), but this response was reduced in both single- and dual-silenced plants. PBS-treated guard cells showed almost no fluorescence in control and gene-silenced plants. Quantification of NO fluorescence demonstrated that NbVPE-silenced plants showed levels of fluorescence comparable with those of control plants after boehmerin, harpin, or Nep1 infiltration. NO generation in NbVPE-silenced plants decreased markedly after elicitor treatment compared with the control plants (Fig. 6C). NO is known to be involved in stomatal closure induced by these elicitors (Zhang et al., 2009). Collectively, these data suggest that elicitor-induced stomatal closure is mediated by NO accumulation, and NbVPE is relative to elicitor-induced NO accumulation in guard cells.

NbVPE1a and NbVPE1b dual-silencing regulates elicitor-induced AOS accumulation in guard cells

It was found that VPE silencing compromised elicitor-induced stomatal closure via the suppression of NO accumulation in guard cells. To evaluate further whether VPE silencing affected the generation of other AOS in elicitor-induced stomatal closure, peroxide and peroxynitrite levels were analysed via incubation with DHR that is oxidized to form the fluorochrome rhodamine 123 in the presence of AOS (Schulz et al., 1996). Neither control nor gene-silenced plants showed AOS fluorescence after PBS treatment. As shown in Fig. 7A, treating guard cells with one of the indicated elicitors resulted in obvious AOS fluorescence. Fluorescence in response to boehmerin in NbVPE1a- and NbVPE1b-silenced plants did not differ from the response in control plants. However, boehmerin-induced AOS fluorescence in NbVPE1a/1b-silenced plants increased markedly compared with that in control plants. Similar results were obtained with harpin and Nep1.

Fig. 4. NbVPE silencing shows insensitivity to elicitor-induced promotion of stomatal closure. Photograph of leaf epidermal peels from control and NbVPE-silenced plants were taken after 3 h of incubation in PBS (10 mM), boehmerin (50 nM), harpin (50 nM), and Nep1 (50 nM).
treatment (Fig. 7B). These results suggest that NbVPE1a and NbVPE1b show an overlap in the negative regulation of elicitor-induced AOS production in guard cells.

NbVPE1a/1b-silenced plants show altered gene expression related to AOS homeostasis and transcription

Compromised cell death in NbVPE1a- and NbVPE1al/b-silenced plants after harpin treatment, and differences in the accumulation of H$_2$O$_2$, NO, and AOS between control and silenced plants may be caused by the dysregulation of genes associated with defence-related redox control and transcription. To address this possibility, the kinetics of the expression of six selected genes after elicitor infiltration was examined by qRT-PCR in gene-silenced and control plants (Fig. 8). The transcript level of PRIa (acidic pathogenesis-related protein, X06930), NOAI (NO associated protein, AB303300), and WRKY2 (AF096299) showed no significant difference ($P>0.05$) in NbVPE-silenced and control plants with PBS treatment, but it was 1.5- to 15-fold lower in NbVPE-silenced plants than in control plants upon elicitor treatment. The transcript level of HSR203J (AB091430), an HR marker gene, was also not influenced ($P>0.05$) with PBS treatment, but was appropriately 7- and 13-fold lower after harpin treatment in NbVPE1a- and NbVPE1al/b-silenced plants than in control plants, which was coincident with necrotic lesion formation (Fig. 3). In NbVPE-silenced and control plants, the expression of NbrbohA (encoding an AOS-inducible NADPH oxidase, AB079498) was induced by elicitor compared with PBS treatment, and had no marked difference between NbVPE-silenced and control plants. Compared with the control plants, NbrbohB (AB079499) expression showed no obvious difference in NbVPE-silenced plants with PBS treatment. It was approximately 1.5-fold higher in NbVPE1al/b-silenced plants than in control plants after elicitor treatment, and had no obvious difference among NbVPE1a-, NbVPE1b-silenced, and control plants. In summary, an overlapping set of genes related to AOS accumulation and transcription were identified to show significant changes in expression in NbVPE-silenced plants, compared with control plants.

Discussion

The roles of VPE in plant cell death have been intensively reported (Kinoshita et al., 1999; Hatsugai et al., 2004; Kuroyanagi et al., 2005; Nakaune et al., 2005), but whether these genes are involved in the plant response to pathogen elicitors is unclear. In this study, a VIGS system was used to elucidate the function of NbVPE in elicitor-signalling. Our results suggest that only NbVPE1a silencing compromises harpin-mediated HR, but not HR triggered by fungal and oomycete elicitors. To our knowledge, this is the first report that NbVPE silencing impairs elicitor-induced stomatal closure via the suppression of NO accumulation in guard cells.

NbVPE1a silencing compromises harpin-induced HR, but not boehmerin- and Nep1-induced HR

In this paper, harpin, Nep1, and boehmerin were selected as representative bacterial, fungal, and oomycete elicitors, respectively, to compare the patterns recognized by plants. After infiltration with one of the three elicitors, only harpin-triggered HR is compromised in NbVPE1a- and NbVPE1al/b-silenced plants, suggesting that NbVPS1a contributes to harpin-induced HR. However, HR is not impaired in genesilenced or control plants in response to boehmerin or Nep1. This is similar to a previous report that animal programmed cell death (PCD) may be dependent or independent of
caspase activity (Lavrik et al., 2005). The data in this paper indicate that pathogen elicitors-triggered cell death can also be dependent or independent of VPE, which is the first plant protease showing caspase-1-like activity, although plants do not encode a caspase homologue (Hatsugai et al., 2004).

Hypersensitive cell death triggered by INF1 from Phytophthora infestans and Nep1-like protein from Phytophthora sojae does not require caspase activity (Sasabe et al., 2000; Qutob et al., 2006), and no caspase-like catalytic activity is detected in INF1-treated tobacco cells (Sasabe et al., 2000). Furthermore, Arabidopsis hypersensitive cell death triggered by Nep1-like protein could not be blocked by several caspase-specific peptide inhibitors, suggesting that this kind of cell death is not mediated by caspase activity (Qutob et al., 2006). Unlike previous strategies, the role of VPE in elicitor-signalling was studied using targeted gene silencing. The data again demonstrate that VPE is not required in boehmerin or Nep1-mediated HR.

However, previous data show that caspase is involved in plant virus- and mycotoxin-induced cell death. Caspase inhibitors can affect TMV-induced HR in tobacco (del Pozo and Lam, 1998), and VPE is responsible for both mycotoxin-induced cell death in Arabidopsis and TMV-N interactions (Hatsugai et al., 2004; Kuroyanagi et al., 2005). Thus, it is proposed that harpin-, mycotoxin-, and N gene-mediated cell death may share a similar PCD mechanism, which depend on VPE but differ from those of boehmerin and Nep1. Accordingly, these data suggest that the PCD

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**Fig. 6.** Elicitor activation of NO is reduced in guard cells of NbVPE-silenced plants. In all cases, NO-sensitive dye DAF-2DA was loaded into cells of the epidermal peels, and fluorescence was measured after the addition of PBS (10 nM), boehmerin (50 nM), harpin (50 nM), and Nep1 (50 nM). For each treatment, fluorescence and bright-field images were shown. Results from several experiments were compiled in this figure. Experiments were repeated at least three times, and representative images were shown in (A) and (B) (enlarged image). Green indicates NO burst. (C). Quantitative analysis of in vivo NO generation monitored using DAF-2DA fluorescence as shown in (B). Results were presented as the mean (n = 3) fluorescence intensity per pixel.
Fig. 7. Elicitor-induced AOS increase in guard cells of the control and NbVPE-silenced plants. In all cases, AOS dye DHR was loaded into cells of epidermal peels, and fluorescence was detected after incubation in PBS (10 mM), boehmerin (50 nM), harpin (50 nM), and Nep1 (50 nM). For each treatment, fluorescence and bright-field images were shown. Results from several experiments were compiled in this figure. Experiments were repeated at least three times, and representative images were shown in (A). Green indicates AOS burst. (B) Quantitative analysis of in vivo AOS generation monitored using DHR fluorescence as shown in (A). Results were presented as mean (n > 3) fluorescence intensity per pixel.

Fig. 8. Expression analysis of genes associated with redox-control and transcription in NbVPE-silenced plants by qRT-PCR. 6 h after treatment with or without boehmerin (50 nM), harpin (50 nM), and Nep1 (50 nM), leaf samples were harvested from the inoculation site, the lower and the upper leaves; Bars represent mean (three technical replicates) ± standard deviation. Circles indicate a significant difference (P < 0.05) between elicitor-non-inoculated and elicitor-inoculated plants, and asterisks indicate a significant difference (P < 0.05) between the NbVPE-silenced plants and corresponding control PVX plants within the same treatment.
patterns involved in plant-microbe interactions have diversified throughout evolution.

Cell death in NbVPE-silenced plants differs in response to various elicitors, but with a similar \( H_2O_2 \) accumulation

In this study, VPE deficiency affects harpin-induced cell death, but not \( H_2O_2 \) accumulation, suggesting that hypersensitive cell death and \( H_2O_2 \) accumulation triggered by harpin are independent. This is consistent with a previous report that INF1-treated tobacco cells involve independent signalling pathways leading to cell death and oxidative burst (Sasabe et al., 2000). Similarly, \( rbhoh \) silencing attenuates elicitor-induced \( H_2O_2 \) accumulation, but not HR in \( N. benthamiana \) (Zhang et al., 2009). Our results also show that NbVPE-silenced and control plants have a similar DAB staining intensity and cell death in response to boehmerin and Nep1, indicating that VPE is not involved in \( H_2O_2 \) production or cell death after boehmerin and Nep1 treatment. Other studies failed to find a strict correlation between \( H_2O_2 \) production and cell death; for example, although \( AtrbohD \), a plant NADPH oxidase, contributes to \( H_2O_2 \) production to a greater degree than \( AtrbohF \), plant cell death is more strongly inhibited in an \( AtrbohF \) mutant than in an \( AtrbohD \) mutant after treatment with avirulent \( Pseudomonas syringae DC3000 \) expressing the AvrRpm1 elicitor (Torres et al., 2002). Similarly, in cryptogein-treated tobacco plants, \( H_2O_2 \) plays an essential role in plant cell death by provoking AOS-mediated lipid peroxidation in the light (Montillet et al., 2005), but is \( H_2O_2 \)-independent in the dark (Rustérucci et al., 1999). Further study is required to confirm whether \( H_2O_2 \) accumulation is involved in boehmerin- or Nep1-induced cell death. Overall, \( H_2O_2 \) plays different roles in cell death induced by various elicitors.

VPE-dependent NO accumulation is involved in elicitor-induced stomatal closure

NO, as a signalling molecule, is a key regulator of plant responses to a range of endogenous signals and stimuli such as auxin, abscisic acid (ABA), and elicitors (Neill et al., 2003; Asai et al., 2008). Stomatal closure occurs in response to physiological and stress stimuli (MacRobbie, 1998; Hetherington and Woodward, 2003). It was found that VPE-silenced plants showed stomatal closure to ABA treatment (data not shown). It suggests that different mechanisms may regulate stomatal response in biotic and abiotic stress. Previous reports have also shown that NO is a key mediator of ABA-induced stomatal closure in pea (Neill et al., 2002), \( Vicia faba \) (Garcia-Mata and Lamattina, 2002), and Arabidopsis (Bright et al., 2006). In this report, \( NbVPE1a, NbVPE1b \), and \( NbVPE1a/b1b \) silencing suppresses NO accumulation in guard cells triggered by elicitors, suggesting that VPE is involved in NO accumulation. Elicitor-induced stomatal closure is also compromised in these silenced plants. These data indicate that VPE mediates elicitor-triggered stomatal closure via NO signalling and caspase-1-like activity may be involved in NO generation. To our knowledge, this is the first report that VPE with caspase-1-like activity is involved in NO-mediated stomatal closure triggered by elicitors.

Other studies have shown that membrane-associated proteins are involved in guard cell signal transduction (Hosy et al., 2003; Negi et al., 2008; Vahisalu et al., 2008). The NbrbohD protein (Kwak et al., 2003), G protein-coupled receptors (GPCRs; Pandey and Assmann, 2004), fast vacuolar (VF) channels, \( K^- \)-selective vacuolar (VK) cation channels (Allen and Sanders, 1995), and slow vacuolar (SV) channels are localized to the membrane and function during ABA responses in guard cells (MacRobbie, 2006). Stomatal movement is dependent on solute content in guard cells and vacuoles (Pandey et al., 2007). VPE is localized in plant cell vacuoles (Lam, 2005; Hatusgai et al., 2006), and VPE-mediated cell death is associated with vacuolar membrane disintegration (Kuroyanagi et al., 2005). Thus, it is suggested that VPE silencing may affect vacuolar membrane function and subsequently inhibit elicitor-induced stomatal closure.

It is reported that harpin, a bacterial PAMP, elicited VPE-dependent responses, including HR and stomatal closure, in \( N. benthamiana \) leaves. HR and stomatal closure are known to be important in plant immunity to bacterial invasion (Melotto et al., 2006). Therefore, the data presented here suggest that VPE or a VPE-dependent pathway may play important roles in plant PAMP-triggered immunity (PTI). The evidence suggests that bacteria inject a group of effectors into the plant cell using a type III secretion system (TISS) to suppress PTI, which inhibits stomatal closure. Thus, VPE and its dependent pathway may be a target for TISS effectors, and deserve future exploration.

\( NbVPE \) plays a role in elicitor-signalling

NO and AOS are important signalling molecules in elicitor signalling (Garcia-Brugger et al., 2006; Asai et al., 2008). Our results show that the compromised stomatal closure in NbVPE-silenced plants is accompanied by decreased NO accumulation, suggesting that NbVPE is a positive regulator of elicitor-triggered stomatal closure. NO can be synthesized in plants via a reduction in nitrite by nitrate reductase, oxidation of Arg to citrulline by NOS, and a non-enzymatic generation system (Crawford, 2006). NOA1 protein has a sequence similar to NOS, but no NOS activity (Zemojtel et al., 2006). The \( NOA1 \) Arabidopsis mutant shows impaired stomatal closure and less NO accumulation in response to ABA, salt, and extracellular calmodulin (Guo et al., 2003; Zhao et al., 2007; Li et al., 2009). \( NOA1 \) silencing compromises INF1-mediated NO bursts in \( N. benthamiana \) (Asai et al., 2008). Here, qRT-PCR analyses show that \( NbVPE \) silencing appears to inhibit the expression of \( NOA1 \) after elicitor treatment. These results suggest that NbVPE is involved in elicitor-induced stomatal closure, mainly via NOA1-dependent NO accumulation.

The expression of \( NbrbohA \) is not influenced by \( NbVPE \) silencing. Thus, the elicitor-signalling pathway silenced by
NbVPE is an NbrbohA-independent pathway. In NbVPE1a/b-silenced plants, the up-regulation of NbrbohB is not consistent with the observed increase in AOS fluorescence in guard cells in response to elicitors, suggesting that NbVPE may not be the key contributor to additional AOS accumulation in guard cells triggered by elicitors. Other enzymes (such as cell wall peroxidases, amine oxidase, oxalate oxidase, and flavin-containing oxidase) may account for AOS production (Cona et al. 2006a, b; Lim et al., 2006; Tisi et al., 2008; Gabaldón, 2010; Tamás et al., 2010).

WRKY plays an important role in regulating the expression of defence-responsive genes, including PR1a, in disease resistance (Tao et al., 2009). PR1a and WRKY2 show a similar expression pattern in both NbVPE-silenced and control plants after elicitor treatment, suggesting that NbVPE may positively regulate WRKY activity, resulting in the activation of PR1a in elicitor-signalling. Further study is needed to analyse whether WRKY2 plays an important role in elicitor-signalling.

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