*Journal of Experimental Botany*, Vol. 61, No. 13, pp. 3799–3812, 2010
doi:10.1093/jxb/erq189 Advance Access publication 5 July, 2010
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

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

**Huajian Zhang†, Suomeng Dong, Meifang Wang, Wei Wang, Wenwen Song, Xianying Dou, Xiaobo Zheng and Zhengguang Zhang***

Department of Plant Pathology, College of Plant Protection, Nanjing Agricultural University, Key Laboratory of Monitoring and Management of Crop Diseases and Pest Insects, Ministry of Agriculture, Nanjing, 210095, China

† Present address: Department of Biology, University of Utah, Salt Lake City, Utah 84112-0840, USA.
* To whom correspondence should be addressed: E-mail: zhgzhang@njau.edu.cn

Received 7 May 2010; Revised 30 May 2010; Accepted 4 June 2010

## 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 $H_2O_2$ 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 (Nürnberger *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 laddering, and the release of cytochrome *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 coworkers (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 (Kuroyanagi *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 *VPE*s in *Nicotiana tabacum* (*NtVPE1a* and *NtVPE1b*), 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. AY196607) or *nep1* (GenBank accession no. MGG_08454) gene, 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 $OD_{600}$ 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, 0.4, 0.2, and 0.1% SDS at 15 °C. Finally, supernatants were 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*I (5′-CGGTCGACGACATTGCAAACAATGTAGAG-3′; *Sal*I site underlined) and the reverse primer NbVPE1a-R/*Cla*I (5′-CCATCGATCCTCAAATATACTACCAGACT-3′; *Cla*I site underlined). The PCR products were digested with *Sal*I and *Cla*I, 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*I (5′-CGGTCGACCCTACCGATCCGTACCTC-3′; *Sal*I site underlined) and the reverse primer NbVPE1b-R/*Cla*I (5′-CCATCGATGCATCCTTGCTGAGATGTAG-3′; *Cla*I site underlined). The PCR products were digested with *Sal*I and *Cla*I, 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/*Sph*I (5′-CCGCATGCGACATTGCAAACAATGTAGAG-3′; *Sph*I site underlined) and the

reverse primer NbVPE1a-R/*Cla*I, then digested with *Cla*I and *Sph*I. A 413 bp fragment of *NbVPE1b* was amplified with the forward primer NbVPE1b-U/*Sal*I and the reverse primer NbVPE1b-R/*Sph*I (5′-CCGCATGCGCATCCTTGCTGAGATG-TAG-3′; *Sph*I site underlined), digested with *Sph*I and *Sal*I. The 373 bp *NbVPE1a* and the 413 bp *NbVPE1b* fragments were ligated in tandem to the *Sal*I/*Cla*I-digested pgR107 to generate PVX-NbVPE1a/1b. 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 $H_2O_2$ 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 $MgCl_2$). 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 ran. 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 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 ($2^{-\Delta\Delta Ct}$).

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/1b*, 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 $CaCl_2$, 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 peer 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*

Dihydrorhodamine 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/1b*) *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/1b*-silenced plants. *NbVPE1b* expression also decreased

significantly in *NbVPE1b*-silenced and *NbVPE1a/1b*-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/1b*-silenced plants (Fig. 1).

Together, these data effectively demonstrate that *NbVPE1a*, *NbVPE1b*, and *NbVPE1a/1b* 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/1b*-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/1b*-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/1b*-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 $H_2O_2$ accumulation*

Although $H_2O_2$ 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 $H_2O_2$ accumulation is dependent on VPE. The contribution of *NbVPE1a* and *NbVPE1b* to $H_2O_2$ production in response to elicitors was examined. DAB polymerizes on contact with $H_2O_2$ in a reaction requiring peroxidase; thus, $H_2O_2$ 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

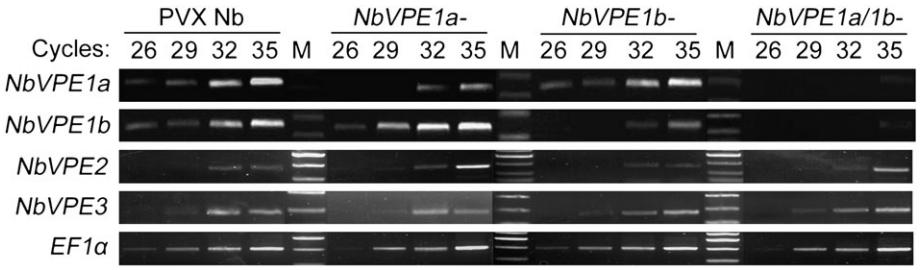


**Fig. 1.** Evaluation of *NbVPE1a* and *NbVPE1b* silencing in leaves of *N. benthamiana* infected with PVX, PVX-NbVPE1a, PVX-NbVPE1b, or PVX-NbVPE1a/1b. RT-PCR was performed with first-strand cDNA obtained from total RNA derived from various plants silenced for *NbVPE1a*, *NbVPE1b*, *NbVPE1a/1b*, and from controls. The *VPE* mRNA levels were estimated by RT-PCR with primer sets to each *VPE* homologue as follows: *NbVPE-1a* specific set (NbVPE1a-F and NbVPE1a-R), *NbVPE-1b* specific set (NbVPE1b-F and NbVPE1b-R), *NbVPE-2* specific set (NbVPE2-F and NbVPE2-R), and *NbVPE-3* specific set (NbVPE3-F and NbVPE3-R). After a 3-week inoculation, leaf samples were harvested from the third and fourth leaves above the inoculation site, and total RNA was isolated and used for RT-PCR. 7 μl PCR products were sampled from each cycle indicated, separated on an agarose gel and stained with ethidium bromide. Equal input of cDNA template for PCR was demonstrated by amplification of the constitutively expressed *EF1α* gene; Lane M shows DL2000 DNA molecular weight marker. The RT-PCR analysis was repeated for three sets of independently silenced plants in each experiment and in three independent experiments.

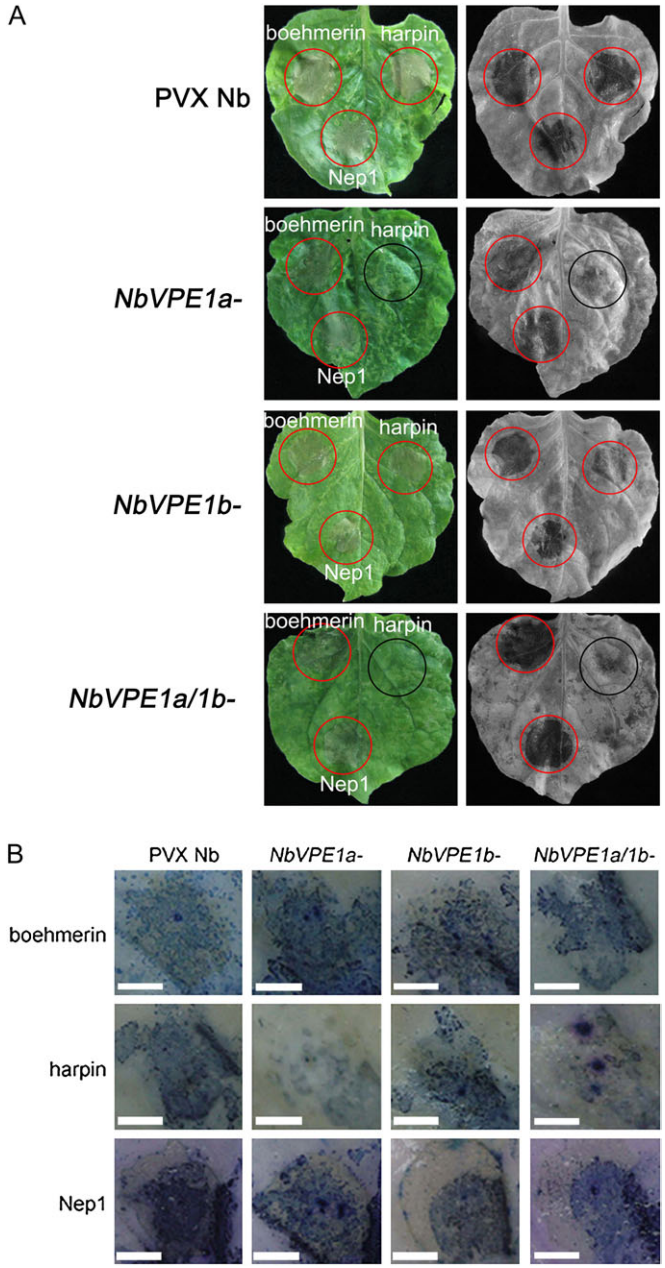


**Fig. 2.** Local induction of hypersensitivity responses with boehmerin (50 nM), harpin (50 nM), and Nep1 (50 nM). (A) Leaves (representative of three replicate treatments) from control PVX, *NbVPE1a*-, *NbVPE1b*-, and *NbVPE1a/1b*-silenced *N. benthamiana* were infiltrated with the elicitors simultaneously. 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.

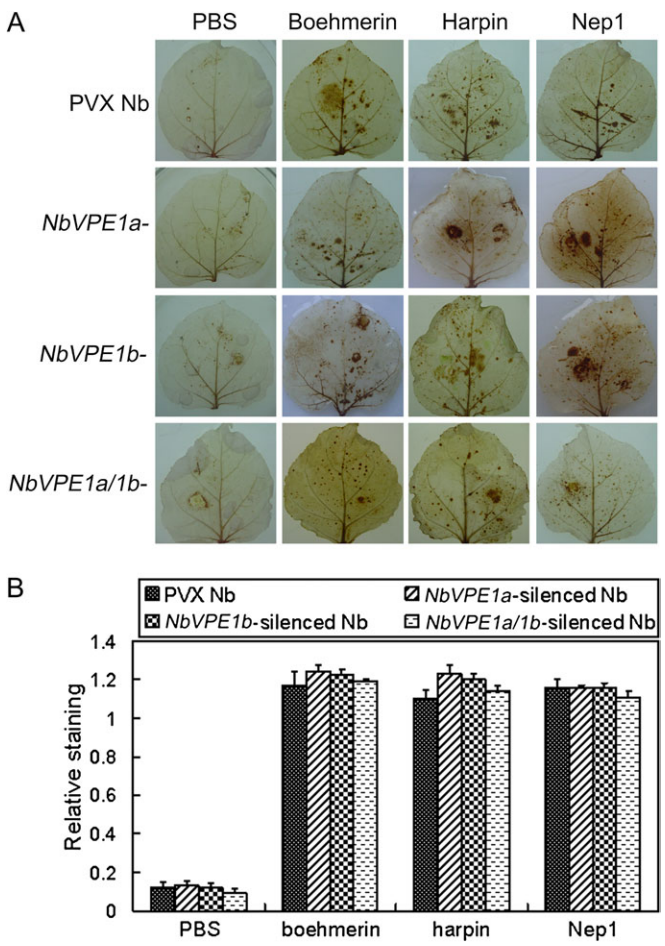


**Fig. 3.** *In situ* detection of hydrogen peroxide using DAB staining on control, *PVX-NbVPE1a*, *PVX-NbVPE1b*-, and *PVX-NbVPE1a/1b*-infected *N. benthamiana* leaves in response to elicitors. (A) Photographs of representative ethanol-bleached leaves from control and *NbVPE*-silenced plants 6 h after 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.

compared with control plants after boehmerin, harpin, and Nep1 infiltration (Fig. 3B). These data suggest that *NbVPE1a* and *NbVPE1b* contribute little to elicitor-induced $H_2O_2$ accumulation.

## 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

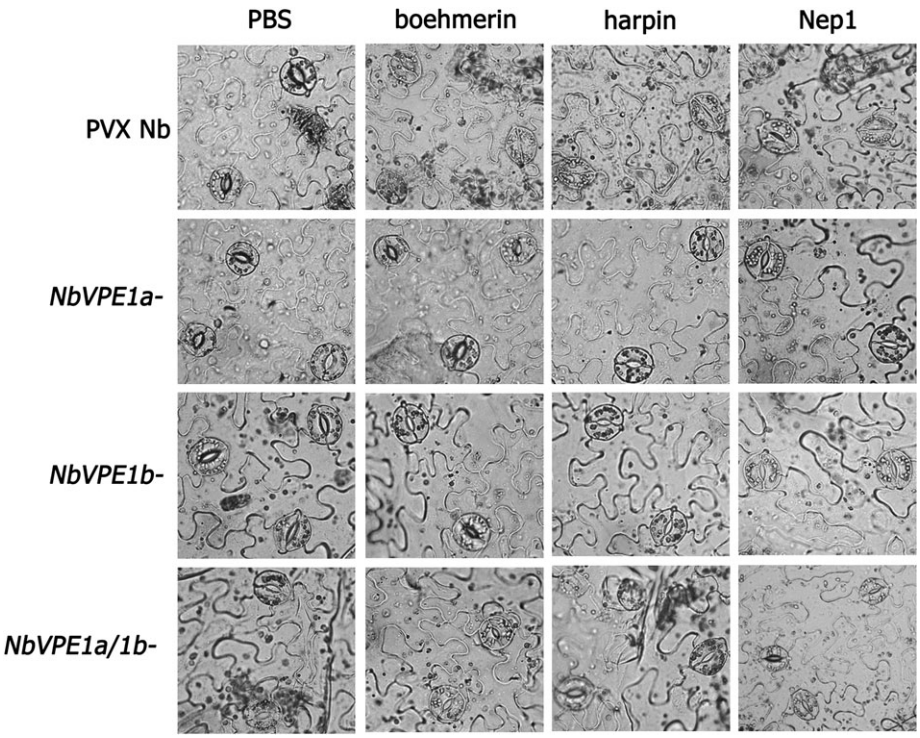


**Fig. 4.** *NbVPE* silencing shows insensitivity to elicitor-induced promotion of stomatal closure. Photograph of leaf epidermal peels from control and *NbVPE*-silenced plants were taken after 3 h of incubation in PBS (10 mM), boehmerin (50 nM), harpin (50 nM), and Nep1 (50 nM).

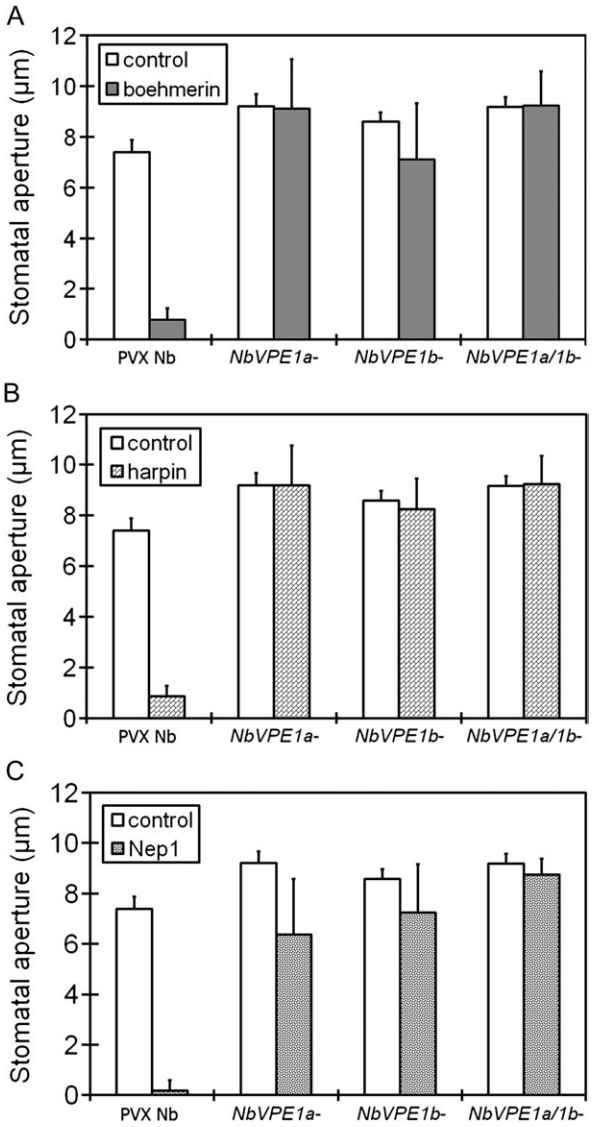


**Fig. 5.** Stomatal aperture measurements show that elicitor-induced stomatal closure is reduced in *NbVPE*-silenced *N. benthamiana*. Stomatal aperture was measured 3 h after incubation in PBS (10 mM), boehmerin (A) (50 nM), harpin (B) (50 nM), and Nep1 (C) (50 nM). Values represent means ±1 SE from three independent experiments; $n$=50 apertures per experiment. Data were compared using the Student's $t$ test at the 95% significance level.

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 *NbVPE1a/1b*-silenced plants after harpin treatment, and differences in the accumulation of $H_2O_2$, 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 *PR1a* (acidic pathogenesis-related protein, X06930), *NOA1* (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 *NbVPE1a/1b*-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 *NbVPE1a/1b*-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 *NbVPE1a/1b*-silenced plants, suggesting that NbVPE1a contributes to harpin-induced HR. However, HR is not impaired in gene-silenced 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

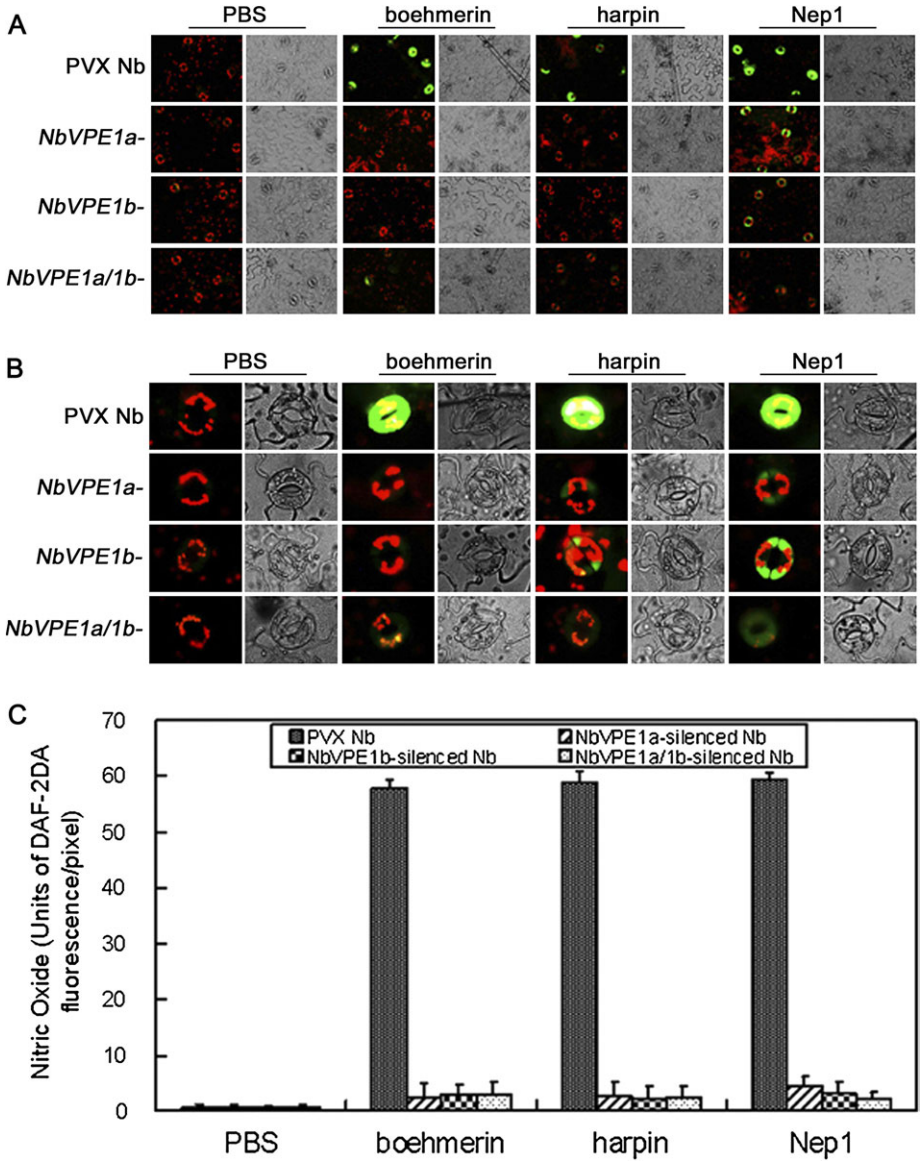


**Fig. 6.** Elicitor activation of NO is reduced in guard cells of *NbVPE*-silenced plants. In all cases, NO-sensitive dye DAF-2DA was loaded into cells of the epidermal peels, and fluorescence was measured after the addition of PBS (10 mM), boehmerin (50 nM), harpin (50 nM), and Nep1 (50 nM). For each 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) 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 ($n \geqslant 3$) fluorescence intensity per pixel.

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

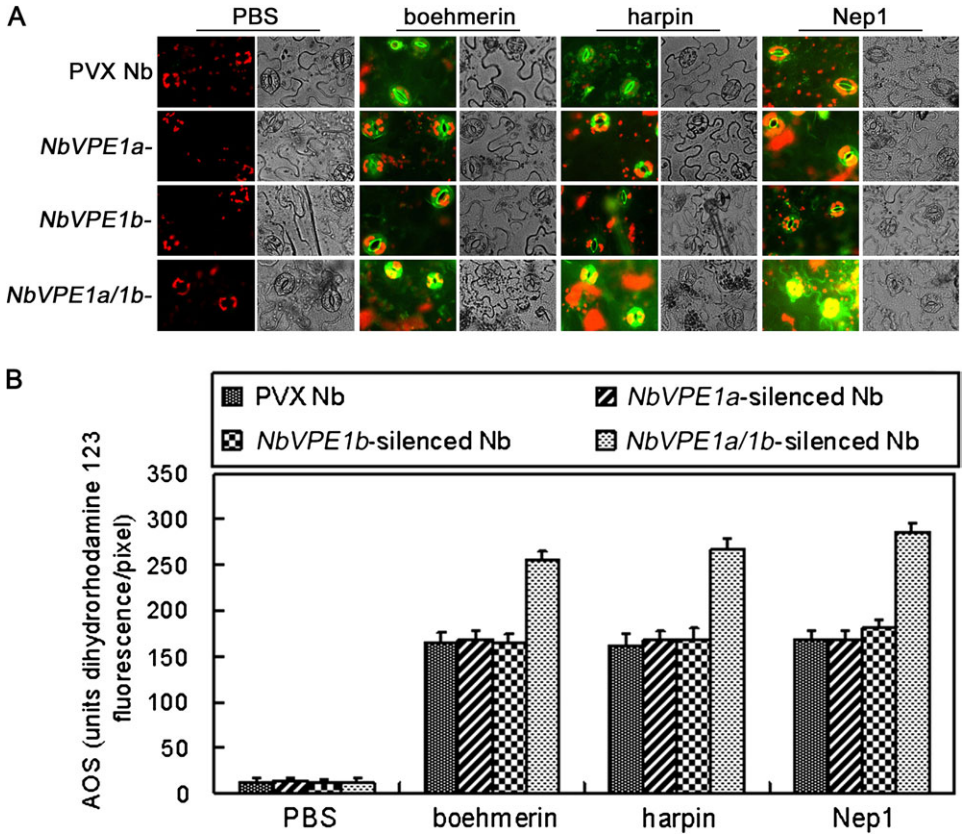


**Fig. 7.** Elicitor-induced AOS increase in guard cells of the control and *NbVPE*-silenced plants. In all cases, AOS dye DHR was loaded into cells of epidermal peels, and fluorescence was detected after incubation in PBS (10 mM), boehmerin (50 nM), harpin (50 nM), and Nep1 (50 nM). For each treatment, 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 ($n \geqslant 3$) fluorescence intensity per pixel.

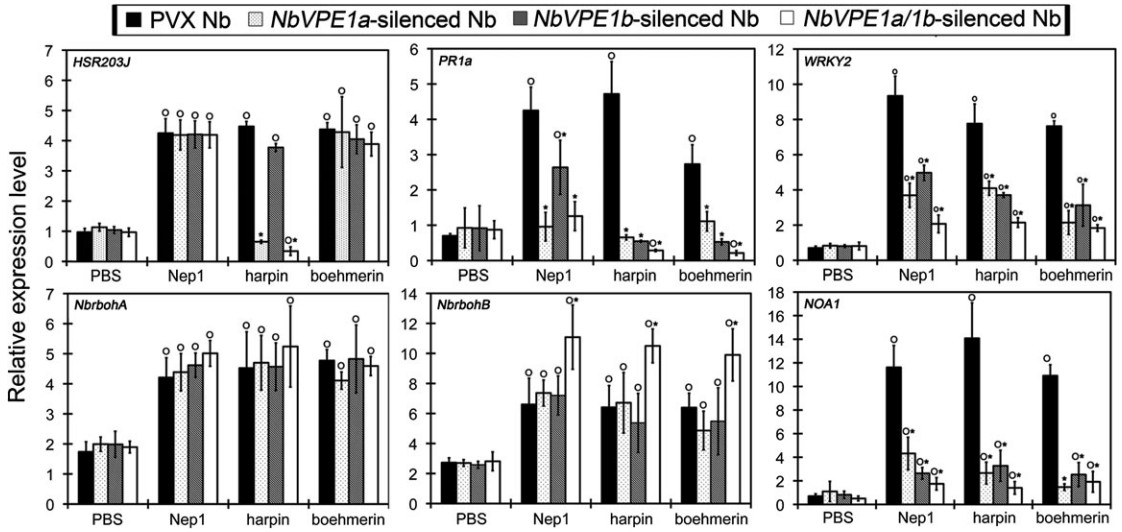


**Fig. 8.** Expression analysis of genes associated with redox-control and transcription in *NbVPE*-silenced plants by qRT-PCR. 6 h after treatment with or without boehmerin (50 nM), harpin (50 nM), and Nep1 (50 nM), leaf samples were harvested from the inoculation site, the lower and the upper leaves; 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, *rboh* 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/1b* 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 capase-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 NtrbohD protein (Kwak *et al.*, 2003), G protein-coupled receptors (GPCRs; Pandey and Assmann, 2004), fast vacuolar (FV) 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; Hatsugai *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 NO 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/1b*-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 NbrbohB 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.*, 2006*a*, *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.

## Acknowledgements

This research was supported in part by the National 863 project of China (No. 2008AA10Z410, ZG Zhang), the National Natural Science Foundation of China (Grant No. 30871605 and 30471123, ZG Zhang), New Century Excellent Scholar Project of Ministry of Education of China (NCET-07-0442 to ZG Zhang), and the Commonweal Specialized Research Fund of China Agriculture (3-20). We thank David Baulcombe (Sainsbury Laboratory, John Innes Centre, Norwich, UK) for the gift of PVX vector and *Agrobacterium* strains, Gongyou Chen (Department of Plant Pathology, Nanjing Agricultural University, Nanjing, China) for the gift of the *harpinXoo* gene, Xueping Zhou (College of Agriculture and Biotechnology, Zhejiang University, Hangzhou, China) for the gift of *Nicotiana benthamiana* seeds.

## References

**Ali R, Ma W, Lemtiri-Chlieh F, Tsaltas D, Leng Q, von Bodman S, Berkowitz GA.** 2007. Death don't have no mercy and neither does calcium: Arabidopsis cyclic nucleotide gated channel2 and innate immunity. *The Plant Cell* **19,** 1081–1095.

**Allan AC, Fluhr R.** 1997. Two distinct sources of elicited reactive oxygen species in tobacco epidermal cells. *The Plant Cell* **9,** 1559–1572.

**Allen GJ, Sanders D.** 1995. Calcineurin, a type 2B protein phosphatase, modulates the $Ca^{2+}$-permeable slow vacuolar ion channel of stomatal guard cells. *The Plant Cell* **7,** 1473–1483.

**Asai S, Ohta K, Yoshioka H.** 2008. MAPK signalling regulates nitric oxide and NADPH oxidase-dependent oxidative bursts in *Nicotiana benthamiana*. *The Plant Cell* **20,** 1390–1406.

**Baker CJ, Orlandi EW.** 1995. Active oxygen in plant pathogenesis. *Annual Review of Phytopathology* **33,** 299–321.

**Bonneau L, Ge Y, Drury GE, Gallois P.** 2008. What happened to plant caspases? *Journal of Experimental Botany* **59,** 491–499.

**Bright J, Desikan R, Hancock JT, Weir IS, Neill SJ.** 2006. ABA-induced NO generation and stomatal closure in *Arabidopsis* are dependent on $H_2O_2$ synthesis. *The Plant Journal* **45,** 113–122.

**Brunner F, Rosahl S, Lee JJ, Geiler C, Kauppinnen S, Rasmussen G, Scheel D, Nürnberger T.** 2002. Pep-13, a plant defence-inducing pathogen associated pattern from *Phytophthora* transglutaminases. *EMBO Journal* **21,** 6681–6688.

**Chen YL, Huang RF, Xiao YM, Lu P, Chen J, Wang XC.** 2004. Extracellular calmodulin-induced stomatal closure is mediated by heterotrimeric G protein and $H_2O_2$. *Plant Physiology* **136,** 4096–4103.

**Chichkova NV, Kim SH, Titova ES, Kalkum M, Morozov VS, Rubtsov YP, Kalinina NO, Taliansky ME, Vartapetian AB.** 2004. A plant caspase-like protease activated during the hypersensitive response. *The Plant Cell* **16,** 157–171.

**Coffeen WC, Wolpert TJ.** 2004. Purification and characterization of serine proteases that exhibit caspase-like activity and are associated with programmed cell death in *Avena sativa*. *The Plant Cell* **16,** 857–873.

**Cohen GM.** 1997. Caspases: the executioners of apoptosis. *Biochemical Journal* **326,** 1–16.

**Cona A, Rea G, Angelini R, Federico R, Tavladoraki P.** 2006*a*. Functions of amine oxidases in plant development and defence. *Trends in Plant Science* **11,** 80–88.

**Cona A, Rea G, Botta M, Corelli F, Federico R, Angelini R.** 2006*b*. Flavin-containing polyamine oxidase is a hydrogen peroxide source in the oxidative response to the protein phosphatase inhibitor cantharidin in *Zea mays* L. *Journal of Experimental Botany* **57,** 2277–2289.

**Crawford NM.** 2006. Mechanisms for nitric oxide synthesis in plants. *Journal of Experimental Botany* **57,** 471–478.

**Dangl JL.** 1998. Innate immunity: plants just say NO to pathogens. *Nature* **394,** 525–527.

**Dangl JL, Dietrich RA, Richberg MH.** 1996. Death don't have no mercy: cell death programs in plant–microbe interactions. *The Plant Cell* **8,** 1793–1807.

**Danon A, Rotari VI, Gordon A, Mailhac N, Gallois P.** 2004. Ultraviolet-C overexposure induces programmed cell death in *Arabidopsis*, which is mediated by caspase-like activities and which can be suppressed by caspase inhibitors, p35 and Defender against apoptotic death. *Journal of Biological Chemistry* **279,** 779–787.

**Daxberger A, Nemak A, Mithöfer A, Filegmann J, Ligterink W, Hirt H, Ebel J.** 2007. Activation of members of a MAPK module in beta-glucan elicitor-mediated non-host resistance of soybean. *Planta* **225,** 1559–1571.

**del Pozo O, Lam E.** 1998. Caspases and programmed cell death in the hypersensitive response of plants to pathogens. *Current Biology* **8,** 1129–1132.

**EK-Ramos MJ, Avila J, Cheng C, Martin GB, Devatenne TP.** 2010. The T-loop extension of the tomato protein kinase AvrPto-dependent Pto-interacting protein 3 (Adi3) directs nuclear localization for suppression of plant cell death. *Journal of Biological Chemistry* doi:10.1074/jbc.M110.117416

**Fan LM, Zhao Z, Assmann SM.** 2004. Guard cells: a dynamic signalling model. *Current Opinion in Plant Biology* **7,** 537–546.

**Fellbrich G, Romanski A, Varet A, *et al*.** 2002. NPP1, a *Phytophthora*-associated trigger of plant defence in parsley and Arabidopsis. *The Plant Journal* **32,** 375–390.

**Fliegmann J, Mithöfer A, Wanner G, Ebel J.** 2004. An ancient enzyme domain hidden in the putative β-glucan elicitor receptor of soybean may play an active part in the perception of pathogen-associated molecular patterns during broad host resistance. *Journal of Biological Chemistry* **279,** 1132–1140.

**Gabaldón T.** 2010. Peroxisome diversity and evolution. *Philosophical Transactions of the Royal Society B: Biological Sciences* **365,** 765–773.

**Gabriëls SEJ, Takken FLW, Vossen JH, *et al*.** 2006. cDNA-AFLP combined with functional analysis reveals novel genes involved in the hypersensitive response. *Molecular Plant–Microbe Interactions* **19,** 567–576.

**Gan YZ, Zhang LS, Zhang ZG, Dong SM, Li J, Wang YC, Zheng XB.** 2009. The LCB2 subunit of the sphingolip biosynthesis enzyme serine palmitoyltransferase can function as an attenuator of the hypersensitive response and Bax-induced cell death. *New Phytologist* **181,** 127–146.

**Garcia-Brugger A, Lamotte O, Vandelle E, Bourque S, David L, Benoit P, Lecourieux D, Poinssot B, Wendehenne D, Pugin A.** 2006. Early signalling events induced by elicitors of plant defences. *Molecular Plant–Microbe Interactions* **19,** 711–724.

**Garcia-Mata C, Lamattina L.** 2002. Nitric oxide and abscisic acid cross talk in guard cells. *Plant Physiology* **128,** 790–792.

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

**Greenberg JT, Yao N.** 2004. The role and regulation of programmed cell death in plant–pathogen interactions. *Cell Microbiology* **6,** 201–211.

**Guo FQ, Okamoto M, Crawford NM.** 2003. Identification of a plant nitric oxide synthase gene involved in hormonal signalling. *Science* **302,** 100–103.

**Hara-Nishimura I, Takeuchi Y, Nishimura M.** 1993. Molecular characterization of a vacuolar processing enzyme related to a putative cysteine proteinase of *Schistosoma mansoni*. *The Plant Cell* **5,** 1651–1659.

**Hatsugai N, Kuroyanagi M, Nishimura M, Hara-Nishimura I.** 2006. A cellular suicide strategy of plants: vacuole-mediated cell death. *Apoptosis* **11,** 905–911.

**Hatsugai N, Kuroyanagi M, Yamada K, Meshi T, Tsuda S, Kondo M, Nishimura M, Hara-Nishimura I.** 2004. A plant vacuolar protease, VPE, mediates virus-induced hypersensitive cell death. *Science* **305,** 855–858.

**He R, Drury GE, Rotari VI, Gordon A, Willer M, Tabasum F, Woltering EJ, Gallois P.** 2007. Metacaspase-8 modulates programmed cell death induced by UV and $H_2O_2$ in Arabidopsis. *Journal of Biological Chemistry* **283,** 774–783.

**Hématy K, Cherk C, Somerville S.** 2009. Host–pathogen warfare at the plant cell wall. *Current Opinion in Plant Biology* **12,** 406–413.

**Hetherington AM, Woodward FI.** 2003. The role of stomata in sensing and driving environmental change. *Nature* **424,** 901–908.

**Hosy E, Vavasseur A, Mouline K, *et al*.** 2003. The Arabidopsis outward $K^+$ channel GORK is involved in regulation of stomatal movements and plant transpiration. *Proceedings of the National Academy of Sciences, USA* **100,** 5549–5554.

**Ji R, Zhang ZG, Wang XB, Zheng XB.** 2005. *Phytophthora* elicitor PB90 induced apoptosis in suspension cultures of tobacco. *Chinese Science Bulletin* **50,** 435–439.

**Kim M, Ahn JW, Jin UH, Chai D, Poek KH, Pai HS.** 2003. Activation of the programmed cell death by inhibition of proteasome function in plant. *Journal of Biological Chemistry* **278,** 19406–19415.

**Kinoshita T, Nishimura M, Hara-Nishimura I.** 1995. Homologues of a vacuolar processing enzyme that are expressed in different organs in *Arabidopsis thaliana. Plant Molecular Biology* **29,** 81–89.

**Kinoshita T, Yamada K, Hiraiwa N, Kondo M, Nishimura M, Hara-Nishimura I.** 1999. Vacuolar processing enzyme is up-regulated in the lytic vacuoles of vegetative tissues during senescence and under various stressed conditions. *The Plant Journal* **19,** 43–53.

**Kuroyanagi M, Yamada Kenji, Hatsugai N, Kondo M, Nishimura M, Hara-Nishimura I.** 2005. Vacuolar processing enzyme is essential for mycotoxininduced cell death in *Arabidopsis thaliana*. *Journal of Biological Chemistry* **280,** 32914–32920.

**Kwak JM, Mori IC, Pei ZM, Leonhardt N, Torres MA, Dangl JL, Bloom RE, Bodde S, Jones JDG, Schroeder JI.** 2003. NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in Arabidopsis. *EMBO Journal* **22,** 2623–2633.

**Lam E.** 2005. Vacuolar proteases livening up programmed cell death. *Trends in Cell Biology* **15,** 124–127.

**Lam E, Kato N, Lawton M.** 2001. Programmed cell death, mitochondria and the plant hypersensitive response. *Nature* **411,** 848–853.

**Lam E, del Pozo O.** 2000. Caspase-like protease involvement in the control of plant cell death. *Plant Molecular Biology* **44,** 417–428.

**Lamb C, Dixon R.** 1997. The oxidative burst in plant disease resistance. *Annual Review of Plant Physiology and Plant Molecular Biology* **48,** 251–275.

**Lascombe MB, Retailleau P, Ponchet M, Industri B, Blein JP, Prangé T.** 2007. Structure of sylvaticin, a new alpha-elicitin-like protein from *Pythium sylvaticum*. *Acta Crystallographica D* **63,** 1102–1108.

**Lavrik IN, Golks A, Krammer PH.** 2005. Caspases: pharmacological manipulation of cell death. *Journal of Clinical Investigation* **115,** 2665–2672.

**Li JH, Liu YQ, Lü P, Lin HF, Bai Y, Wang XC, Chen YL.** 2009. A signalling pathway linking nitric oxide production to heterotrimeric G protein and hydrogen peroxide regulates extracellular calmodulin induction of stomatal closure in *Arabidopsis. Plant Physiology* **150,** 114–124.

**Lim TS, Chitra TR, Han P, Pua EC, Yu H.** 2006. Cloning and characterization of *Arabidopsis* and *Brassica juncea* flavin-containing amine oxidases. *Journal of Experimental Botany* **57,** 4155–4169.

**MacRobbie EAC.** 2006. Control of volume and turgor in stomatal guard cells. *Journal of Membrane Biology* **210,** 131–142.

**MacRobbie EAC.** 1998. Signal transduction and ion channels in guard cells. *Philosophical Transactions of the Royal Society B* **353,** 1475–1488.

**Melotto M, Underwood W, He SY.** 2008. Role of stomata in plant innate immunity and foliar bacterial diseases. *Annual Review of Phytopathology* **46,** 101–122.

**Melotto M, Underwood W, Koczan J, Nomura K, He SY.** 2006. Plant stomata function in innate immunity against bacterial invasion. *Cell* **126,** 969–980.

**Miao WG, Song CF, Wang Y, Wang JS.** 2010. HpaXm from *Xanthomonas citri* subsp. *malvacearum* is a novel harpin with two heptads for hypersensitive response. *Journal of Microbiology and Biotechnology* **20,** 54–62.

**Montillet JL, Chamnongpol S, Rustérucci C, Dat J, Van De Cotte B, Agnel JP, Battesti C, Inzé D, Van Breusegem F, Trianphylidès C.** 2005. Fatty acid hydroperoxides and $H_2O_2$ in the execution of hypersensitive cell death in tobacco leaves. *Plant Physiology* **138,** 1516–1526.

**Motteram J, Küfner I, Deller SM, Brunner F, Hammond-Kosack KE, Nürnberger T, Rudd JJ.** 2009. Molecular characterization and functional analysis of MgNLP, the sole NPP1 domain-containing protein, from the fungal wheat leaf pathogen *Mycosphaerella graminicola. Molecular Plant–Microbe Interactions* **22,** 790–799.

**Mur LA, Kenton P, Lloyd AJ, Ougham H, Prats E.** 2008. The hypersensitive response; the centenary is upon us but how much do we know? *Journal of Experimental Botany* **59,** 501–520.

**Nadeau JA.** 2008. Stomatal development: new signals and fate determinants. *Current Opinion in Plant Biology* **12,** 1–7.

**Nakaune S, Yamada K, Kondo Mi, Kato T, Tabata S, Nishimura M, Hara-Nishimura I.** 2005. A vacuolar processing enzyme, δVPE, is involved in seed coat formation at the early stage of seed development. *The Plant Cell* **17,** 876–887.

**Negi J, Matsuda O, Nagasawa T, Oba Y, Takahashi H, Kawai-Yamada M, Uchimiya H, Hashimoto M, Iba K.** 2008. $CO_2$ regulator SLAC1 and its homologues are essential for anion homeostasis in plant cells. *Nature* **452,** 483–486.

**Neill SJ, Desikan R, Hancock JT.** 2003. Nitric oxide signalling in plants. *New Phytologist* **159,** 11–35.

**Neill SJ, Desikan R, Clarke A, Hancock JT.** 2002. Nitric oxide is a novel component of abscisic acid signalling in stomatal guard cells. *Plant Physiology* **128,** 13–16.

**Nürnberger T, Nennstiel D, Jabs T, Sacks WR, Hahlbrock K, Scheel D.** 1994. High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defence responses. *Cell* **78,** 449–460.

**Pandey S, Assmann SM.** 2004. The Arabidopsis putative G protein-coupled receptor GCR1 interacts with the G protein a subunit GPA1 and regulates abscisic acid signalling. *The Plant Cell* **16,** 1616–1632.

**Pandey S, Zhang W, Assmann SM.** 2007. Roles of ion channels and transporters in guard cell signal transduction. *FEBS Letters* **581,** 2325–2336.

**Pitzschke A, Hirt H.** 2006. Mitogen-activated protein kinases and reactive oxygen species signalling in plants. *Plant Physiology* **141,** 351–356.

**Qutob D, Kemmerling B, Brunner F, *et al*.** 2006. Phytotoxicity and innate immune responses induced by Nep1-like proteins. *The Plant Cell* **18,** 3721–3744.

**Qutob D, Huitema E, Gijzen M, Kamoun S.** 2003. Variation in structure and activity among elicitins from *Phytophthora sojae. Molecular Plant Pathology* **4,** 119–124.

**Rojo E, Martin R, Carter C, Zouhar J, Pan S, Plotnikova J, Jin H, Paneque M, Sanchez-Serrano JJ, Baker B.** 2004. VPE gamma exhibits a caspase-like activity that contributes to defence against pathogens. *Current Biology* **14,** 1897–1906.

**Rustérucci C, Montillet JL, Agnel JP, *et al*.** 1999. Involvement of lipoxygenase-dependent production of fatty acid hydroperoxides in the development of the hypersensitive cell death induced by cryptogein on tobacco leaves. *Journal of Biological Chemistry* **274,** 36446–36455.

**Sasabe M, Takeuchi K, Kamoun S *et al*.** 2000. Independent pathways leading to apoptotic cell death, oxidative burst and defence gene expression in response to elicitin in tobacco cell suspension culture. *European Journal of Biochemistry* **267,** 5005–5013.

**Schornack S, Ballvora A, Gurlebeck D, Peart J, Baulcombe D, Ganal M, Baker B, Bonas U, Lahaye T.** 2004. The tomato resistance protein Bs4 is a predicted nonnuclear TIR-NB-LRR protein that mediates defence responses to severely truncated derivatives of AvrBs4 and overexpressed AvrBs3. *The Plant Journal* **37,** 46–60.

**Schulz JB, Weller M, Klochgeter T.** 1996. Potassium deprivation-induced apoptosis of cerebellar granule neurons: a sequential requirement for new mRNA and protein synthesis, ICE-like prorease activity, and reactive oxygen species. *Journal of Neuroscience* **16,** 4696–4706.

**Srivastava N, Gonugunta VK, Puli MR, Raghavendra AS.** 2009. Nitric oxide production occurs downstream of reactive oxygen species in guard cells during stomatal closure induced by chitosan in abaxial epidermis of *Pisum sativum. Planta* **229,** 757–765.

**Suarez MF, Filonova LH, Smertenko A, Savenkov EI, Clapham DH, von Arnold S, Zhivotovsky B, Bozhkov PV.** 2004. Metacaspase-dependent programmed cell death is essential for plant embryogenesis. *Current Biology* **14,** 339–340.

**Sun Y, Zhao Y, Hong X, Zhai Z.** 1999. Cytochrome *c* release and caspase activation during menadione-induced apoptosis in plants. *FEBS Letters* **42,** 317–321.

**Tamás L, Mistrík I, Huttová J, Halusková L, Valentovicová K, Zelinová V.** 2010. Role of reactive oxygen species-generating enzymes and hydrogen peroxide during cadmium, mercury and osmotic stresses in barley root tip. *Planta* **231,** 221–231.

**Tao Z, Liu H, Qiu D, Zhou Y, Li X, Xu C, Wang S.** 2009. A pair of allelic WRKY genes play opposite roles in rice–bacteria interactions. *Plant Physiology* **151,** 936–948.

**Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB.** 1997. Subcellular localization of $H_2O_2$ in plants. $H_2O_2$ accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *The Plant Journal* **11,** 1187–1194.

**Tisi A, Angelini R, Cona A.** 2008. Wound healing in plants: cooperation of copper amine oxidase and flavin-containing polyamine oxidase. *Plant Signaling and Behavior* **3,** 204–206.

**Torres MA, Jones JDG, Dang JL.** 2006. Reactive oxygen species signalling in response to pathogens. *Plant Physiology* **141,** 373–378.

**Torres MA, Dangl JL, Jones JDG.** 2002. *Arabidopsis* gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in plant defence response. *Proceedings of the National Academy of Sciences, USA* **99,** 517–522.

**Torto TA, Li S, Styer A, Huitema E, Testa A, Gow NAR, van West P, Kamoun S.** 2003. EST mining and functional expression assays identify extracellular effector proteins from the plant pathogen. *Phytophthora. Genome Research* **13,** 1675–1685.

**Tyler BM.** 2002. Molecular basis of recognition between *Phytophthora* pathogens and their hosts. *Annual Review of Phytopathology* **40,** 137–167.

**Vahisalu T, Kollist H, Wang Y, *et al*.** 2008. SLAC1 is required for plant guard cell S-type anion channel function in stomatal signalling. *Nature* **452,** 487–491.

**Villaba Mateos F, Rickauer M, Esquerré-Tugayé MT.** 1997. Cloning and characterization of a cDNA encoding an elicitor of *Phytophthora parasitica* var. *nicotianae* that shows cellulose-binding and lectin-like activities. *Molecular Plant–Microbe Interactions* **10,** 1045–1053.

**Wang Y, Zhu S, Liu S, Jiang L, Chen L, Ren Y, Han X, Liu F, Ji S, Liu X, Wan J.** 2009. The vacuolar processing enzyme OsVPE1 is required for efficient glutelin processing in rice. *The Plant Journal* **58,** 606–617.

**Wang YC, Hu DW, Zhang ZG, Ma ZC, Zheng XB, Li DB.** 2003. Purification and immunocytolocalization of a novel *Phytophthora boehmeriae* protein inducing the hypersensitive response and systemic acquired resistance in tobacco and Chinese cabbage. *Physiological and Molecular Plant Pathology* **63,** 223–232.

**Weber E, Ojanen-Reuhs T, Huguet E, Hause G, Romantschuk M, Korhonen TK, Bonas U, Koebnik R.** 2005. The type III-dependent Hrp pilus is required for productive interaction of *Xanthomonas campestris* pv. *vesicatoria* with pepper host plants. *Journal of Bacteriology* **187,** 2458–2468.

**Wei ZM, Laby RJ, Zumoff CH, Bauer DW, He SY, Collmer A, Beer SV.** 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora. Science* **257,** 85–88.

**Woltering EJ, van der Bent A, Hoeberichts FA.** 2002. Do plant caspases exist? *Plant Physiology* **130,** 1764–1769.

**Yamada K, Shimada T, Nishimura M, Hara-Nishimura I.** 2005. A VPE family supporting various vacuolar functions in plants. *Physiologia Plantarum* **123,** 369–375.

**Yamada K, Shimada T, Kondo M, Nishimura M, Hara-Nishimura I.** 1999. Multiple functional proteins are produced by cleaving Asn-Gln bonds of a single precursor by vacuolar processing enzyme. *Journal of Biological Chemistry* **274,** 2563–2570.

**Zemojtel T, Frohlich A, Palmieri MC, *et al*.** 2006. Plant nitric oxide synthase: a never-ending story? *Trends in Plant Science* **11,** 524–525.

**Zhang HJ, Fang Q, Zhang ZG, Wang YC, Zheng XB.** 2009. The role of respiratory burst oxidase homologs in elicitor-induced stomatal closure and hypersensitive response in *Nicotiana benthamiana*. *Journal of Experimental Botany* **60,** 3109–3122.

**Zhang ZG, Wang YC, Li J, Ji R, Shen G, Wang SC, Zhou X, Zheng XB.** 2004. The role of SA in hypersensitive response and systemic acquired resistance induced by the elicitor PB90 of *Phytophthora boehmeriae. Physiological and Molecular Plant Pathology* **65,** 31–38.

**Zhao JT, Davis LC, Verpoorte R.** 2005. Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnology Advances* **23,** 283–333.

**Zhao MG, Tian QY, Zhang WH.** 2007. Nitric oxide synthase-dependent nitric oxide production is associated with salt tolerance in Arabidopsis. *Plant Physiology* **144,** 206–217.