Hydrogen peroxide modulates the dynamic microtubule cytoskeleton during the defence responses to *Verticillium dahliae* toxins in *Arabidopsis*

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ABSTRACT

The molecular mechanisms of signal transduction of plants in response to infection by *Verticillium dahliae* (VD) are not well understood. We previously showed that NO may act as an upstream signalling molecule to trigger the depolymerization of cortical microtubules in *Arabidopsis*. In the present study, we used the wild-type, and *atrbohD* and *atrbohF* mutants of *Arabidopsis* to explore the mechanisms of action of H₂O₂ signals and the dynamic microtubule cytoskeleton in defence responses. We demonstrated that H₂O₂ may also act as an upstream signalling molecule to regulate cortical microtubule depolymerization. The depolymerization of the cortical microtubules played a functional role in the signalling pathway to mediate the expression of defence genes. The results indicate that H₂O₂ modulates the dynamic microtubule cytoskeleton to trigger the expression of defence genes against *V. dahliae* toxins (VD-toxins) in *Arabidopsis*.

Key-words: cortical microtubule depolymerization; defence gene expression; H₂O₂.

INTRODUCTION

One of the earliest events in the plant defence response against pathogen attack is the production of reactive oxygen species (ROS), including hydrogen peroxide (H₂O₂) (Lamb & Dixon 1997). The spatial and temporal fluctuations of ROS levels are interpreted as a signal required for plants to respond to pathogens. ROS plays a critical role in induced plant resistance by activating or inducing mitogen-activated protein kinases (MAPKs), ROS-responsive transcription factors, antioxidant enzymes and pathogenesis-related (PR) proteins (Gechev et al. 2006). The rapid generation of H₂O₂ has been documented in many plant–pathogen interactions (Grant et al. 2000). H₂O₂ signals have been shown to induce large transcriptional changes and cellular reprogramming that can either protect the plant cell or induce programmed cell death (Gadjev et al. 2006). H₂O₂ appears to act as an intercellular or intracellular secondary messenger, resulting in gene expression in plant defence; however, the effect of H₂O₂ on defence response appears to be diverse. H₂O₂ generation has been correlated with programmed cell death (Levine et al. 1994), and H₂O₂ acted as a secondary signal in the activation of plant programmed cell death (Overmyer, Brousch & Kangarji 2003; Gechev & Hille 2005). In contrast, pathogen-induced NADPH oxidase-derived ROS were demonstrated to play a role in suppressing the spread of cell death in *Arabidopsis* (Torres, Jones & Dangl 2005). Additionally, in *Nicotiana benthamiana*, silencing NADPH oxidase *NbrbohB* reduced the hypersensitive reaction (HR) and decreased resistance to *Phytophthora infestans*, but not to *Colletotrichum orbiculare* (Yoshioka et al. 2003; Asai, Ohta & Yoshioka 2008). These findings indicate that the effect of H₂O₂ on the defence response varies depending on the plant–pathogen interaction.

Plasma membrane NADPH oxidase, as well as cell wall-associated peroxidase, is the main ROS-producing enzyme (Pogány et al. 2009; Miller et al. 2010). *AtbohD* and *AtbohF* encode two major NADPH oxidases expressed in guard and mesophyll cells in *Arabidopsis*. They have been shown to be responsible for apoplastic ROS generation (Torres & Dangl 2005).

The plant cytoskeleton is essential for various types of antimicrobial defence (Schmidt & Panstruga 2007), with microfilaments and microtubules necessary for plants to block fungal penetration (Genre & Bonfante 2002; Kobayashi & Hakuno 2003). The interaction between plant cells and pathogens triggers a range of highly dynamic plant cellular responses including reorganization of the cytoskeleton. It has been shown that aggregation of subcellular components at the infection site depends on the plant cytoskeleton (Lipka & Panstruga 2005; Hardham, Takemoto & White 2008). Dynamic actin cytoskeleton rearrangements are regulated by various biotic and abiotic responses (Staiger & Blanchon 2006), and the actin cytoskeleton is intimately associated with the activation of defence responses in plants (Day & Graham 2007; Hardham, Jones & Takemoto 2007). However, reported changes in cytoskeletal microtubules in response to infection are more varied than those observed for actin, and...
their role remains unclear (Takemoto & Hardham 2004). In tobacco, cell death induced by cryptogein strictly coincides with a rapid disintegration of the microtubules (Binet et al. 2001). Recently, it was demonstrated that the reduced microtubule dynamics renders plants less susceptible to tobacco mosaic virus (TMV) (Ouko et al. 2010). These results indicate that microtubules may play an important part in the mobilization of the plant defence response. However, details of the contribution of microtubules are not clear, especially in terms of the molecules that signal and bring about the dramatic reorganizations that are often observed.

Verticillium dahliae is a soil-borne pathogen that causes Verticillium wilt in a variety of important plant species worldwide (Bhat & Subbarao 1999). Although the physiology of plant defence against Verticillium infection is well established, comprising the production of the PR proteins, phytoalexins and phenolic compounds, and active expression of some disease response genes (McFadden et al. 2001; Fradin & Thomma 2006), the regulatory signal and pathways involved in plant defence responses to Verticillium remain largely unknown. It has been reported that V. dahliae produces or releases toxic or elicitor-like substances (Fradin & Thomma 2006), and this toxin complex is required for pathogenicity (Nachmias, Buchner & Burstein 1985; Jiang, Fan & Wu 2005; Liu 2005), and it has been demonstrated that V. dahliae toxins (VD-toxins) act as a virulence factor to induce cytoskeleton alteration and defence gene expression in Arabidopsis (Yuan et al. 2006; Jia, Yuan & Li 2007; Hou, Shi & Li 2008). The Arabidopsis–VD-toxins interaction is an excellent model plant–pathogen system to study defence signalling (Shi & Li 2008). Recently, we used this system to demonstrate that NO production and cortical microtubule dynamics appeared to be parts of an important signalling system, and regulated the defence mechanisms to VD-toxins in Arabidopsis (Shi et al. 2009).

In the present study, we used wild-type, and atrbohD and atrbohF mutants of Arabidopsis to explore the mechanisms of action of H2O2 signals and the dynamic microtubule cytoskeleton in the defence responses.

**MATERIALS AND METHODS**

**Plant material and tissue culture conditions**

The seeds of wild-type Arabidopsis (Col-0), and atrbohD and atrbohF mutants (background Col-0) were used in the experiment. The atrbohD and atrbohF mutant seeds were obtained from Dr M.A. Torres (University of North Carolina at Chapel Hill). The mutants are knock-out lines containing a T-DNA insertion in the AtatrbohD and AtatrbohF gene, respectively (Torres, Dangl & Jones 2002). The seeds were sterilized and incubated in Petri dishes containing MS medium (Murashige & Skoog 1962), 3% sucrose (w/v) and 0.7% agar (w/v) at 4 °C in darkness for 4 d. Then, the plates were placed at 21 ± 2 °C under 14 h light (100 μmol m−2 s−1) and 10 h darkness, and 70% relative humidity in a growth cabinet for 7 d.

**Preparation of crude VD-toxins from V. dahliae**

A highly infectious and non-defoliating strain of V. dahliae Kleb (V229) was used for extraction of VD-toxins. The Verticillium culture filtrate was purified as described previously (Jia et al. 2007; Shi & Li 2008).

**H2O2 assays**

For the 2′,7′-dichlorofluorescin diacetate (H2DCF-DA) staining assay, the 7-day-old seedlings were incubated in 10 mm MES–KCl buffer (pH 6.0) supplemented with VD-toxins (150 μg mL−1) and 20 μM H2DCF-DA for 20 min at room temperature. They were then washed three times with 10 mm MES–KCl buffer (pH 6.0) to remove the excess H2DCF-DA. The seedlings incubated in heat-inactivated VD-toxins were used as controls. Fluorescence was detected with a confocal laser scanning microscope (CLSM) (Zeiss LSM 510, Oberkochen, Germany). The CLSM working conditions were as follows: power 70%, excitation at 488 nm and emission at 505–530 nm. This experiment was independently repeated at least three times.

For quantitative measurement of H2O2, the 7-day-old seedlings were treated as described earlier. H2O2 content was determined by the chromogenic peroxidase-coupled assay (Veljovic-Jovanovic, Novtor & Foyer 2002). The leaves (0.1 g) were ground to a fine powder in liquid nitrogen and the powder extracted in 2 mL 1 m HClO4. Where indicated, extraction was performed in the presence of insoluble polyvinyl pyrrolidine (PVP) (5%). Homogenates were centrifuged at 12 000 g for 10 min and the supernatant was neutralized with 5 m K2CO3 to pH 5.6 in the presence of 100 μL 0.3 m phosphate buffer (pH 5.6). The homogenate was centrifuged at 12 000 g for 1 min to remove KClO4. Where indicated, the sample was incubated prior to assay for 10 min with 1 U ascorbate oxidase (Sigma, St Louis, MO, USA) to oxidize ascorbate. The reaction mixture consisted of 0.1 m phosphate buffer (pH 6.5), 3.3 mM 3-dimethylaminobenzoic acid (DMAB), 0.07 mM 3-methyl-2-benzothiazolinonohydrazone (MBTH) and 0.1 U mL−1 peroxidase (Sigma). The reaction was initiated by the addition of an aliquot (200 μL) of the sample. The absorbance change at 590 nm was monitored at 25 °C. Three independent experiments were performed, each with three replicates, and similar results were obtained.

To determine the extent to which H2O2 production induced by VD-toxins is NADPH oxidase dependent, the seedlings were pretreated with 50 μM diphenylene iodonium (DPI, a potent inhibitor of NADPH oxidase; Sigma) or 15 mM dimethylthiourea (DMTU, a H2O2 scavenger; Sigma) for 2 h, respectively. The seedlings were then transferred into an Eppendorf tube containing VD-toxins plus DPI, or VD-toxins plus DMTU to co-treat for 60 min. The leaves were observed using CLSM. Experiments were repeated at least three times, with at least 10 seedlings observed in each experiment.
Visualization of cortical microtubules

The cortical microtubules in leaf pavement cells in Arabidopsis seedlings expressing GFP–tubulin were observed using the CLSM as described previously (Shi et al. 2009). The 7-day-old seedlings were treated with VD-toxins (150 µg mL⁻¹) at room temperature. The organization and dynamics of the cortical microtubules were observed at various times after the treatment. Images of GFP fluorescence are projections of optical sections taken at 1.5 µm intervals from the outer epidermal wall, through to immediately above the cortical cytoplasm adjacent to the inner periclinal wall of the epidermal cell. Images were captured using Zeiss LSM 510 software, converted to TIFF for export and processed in Adobe Photoshop 5.0.

To investigate the interaction between the depolymerization of cortical microtubules and H₂O₂ production, the leaves were treated with VD-toxins (150 µg mL⁻¹) alone, VD-toxins supplemented with 50 µM DPI, VD-toxins supplemented with 15 mM DMTU, or 1, 5 or 10 mM exogenous H₂O₂, respectively. The leaves were observed using CLSM. The observations reported arise from three separate experiments and images presented are representative of >30 similar images collected for each phenomenon being illustrated.

Arabidopsis seedlings expressing GFP–tubulin were crossed with atrbohD and atrbohF mutants, and the homozygous lines produced were used for the observation of the dynamic of cortical microtubules in atrbohD and atrbohF mutants.

Extraction of total RNA and real-time PCR

Total RNA was extracted from the control and samples using TRIzol reagent (Bio Basic Inc, Markham, ON, Canada) following the manufacturer’s instructions. The concentration of RNA was accurately quantified by spectrophotometric measurements, and 2 µL of total RNA was separated on 0.8% agarose gel to check the concentration and to monitor integrity. For real-time PCR analysis, first-strand cDNA was synthesized from 2 µL of total RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Quantitative PCR was performed using SYBR Premix Ex Taq Kit (Takara, Dalian, China) on an ABI 7500 Sequence Detector (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocol. Gene-specific primer pairs were as follows: PRI forward (AGCTCTGTAGGTGCTTGTGTTT) and reverse (GTGCTTGGTTGGAACCCTTA); PR2 forward (ATC TCCTTGTCTGGAATCCT) and reverse (TGGAGATTTCGTCGAATAG); PR5 forward (CTCTTCTCGTGT TCATCACA) and reverse (TCAATTCAATCTCTCC ATCG); and actin2 forward (TACAGTTGCTGAGATG GTGGTT) and reverse (GGGCTTGGGAGATCCACAT). Samples of 2 µL of cDNA were amplified in 25 µL reaction volume containing SYBR premix Ex Taq, PCR forward primer, PCR reverse primer and ROX reference dye, according to the manufacturer’s instructions (Takara). The PCR was carried out at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s (denaturation), and annealing at 60 °C for 34 s. Data analysis, such as the determination of the threshold cycle that represents the starting point of the exponential phase of PCR, and graphic presentation were carried out using the Sequence Detection Software v.1.07 (Applied Biosystems). Quantification of the transcript level of cDNA fragments was normalized to the expression of the actin2 gene in Arabidopsis at 48 h with VD-toxins (150 µg mL⁻¹) treatment. Three independent experiments were performed, each with three replicates.

RESULTS

VD-toxins induce H₂O₂ production in Arabidopsis

To determine whether H₂O₂ was involved in VD-toxin-induced stress responses, the H₂O₂ in Arabidopsis leaves was labelled using a fluorescent probe of H₂DCF-DA. The fluorescent intensity in wild-type Arabidopsis leaves significantly increased after treatment with VD-toxins, and the H₂O₂ level displayed a time-dependent increase and reached a steady level after 60 min (Fig. 1a,b). We also measured the H₂O₂ content in the leaves by the chromogenic peroxidase-coupled assay and obtained similar results (Fig. 1c).

We then investigated the source of H₂O₂ production after treatment with VD-toxins. To determine whether H₂O₂ production induced by VD-toxins was dependent on NADPH oxidases, we measured H₂O₂ changes in wild type and NADPH oxidase mutants of atrbohD and atrbohF after treatment with VD-toxins. VD-toxins induced a dramatic increase in H₂O₂ level as early as 0.5 h and stabilized at 1 h, and remained at this level for >5 h in wild type (Fig. 2), whereas H₂O₂ accumulated to a much smaller extent in atrbohD and atrbohF mutants, and was only about one-third of that in the wild type. This suggests that AtrbohD and AtrbohF were necessary for the VD-toxin-induced H₂O₂ production.

In addition, we also treated wild-type plants with DPI, a potent inhibitor of NADPH oxidase, and DMTU, a H₂O₂ scavenger. VD-toxin-induced H₂O₂ production was sensitive to DPI and DMTU (Fig. 3); DPI almost completely prevented the VD-toxin-induced H₂O₂ production, and DMTU had a similar effect. These results indicate that VD-toxin-induced H₂O₂ production in Arabidopsis was NADPH oxidase dependent.

H₂O₂ modulates VD-toxin induction of the dynamic microtubule cytoskeleton

Previous experiments indicated that VD-toxins can induce the depolymerization of cortical microtubules in Arabidopsis (Yuan et al. 2006; Shi et al. 2009). To determine whether H₂O₂ was involved in VD-toxin induction of the dynamic microtubule cytoskeleton in Arabidopsis expressing GFP–tubulin, the wild type, and atrbohD and
atrbohF mutants were used to visualize microtubules in living leaf cells. The microtubules were observed by fluorescence of GFP–tubulin using CLSM. The fluorescence of the microtubules was caused by the fluorescence of GFP–tubulin that was polymerized into the microtubules, and disorganized GFP–tubulin formed faintly fluorescent small nodes or filaments (Ueda, Matsuyama & Hashimoto 1999). When seedlings were treated with VD-toxins, the

Figure 1. H$_2$O$_2$ production induced by Verticillium dahliae toxins (VD-toxins) in the leaves of wild-type (Col-0) Arabidopsis. (a) H$_2$O$_2$ was detected by fluorescence resulting from 2',7'-dichlorofluorescin diacetate (H$_2$DCF-DA), as described in Materials and methods. Image a was taken 2 min after treatment by VD-toxins, and images a to t were taken at 4 min intervals. (b) H$_2$DCF-DA fluorescence intensities in the leaves of wild-type Arabidopsis. Confocal data are displayed as estimated mean pixel intensities and associated 95% confidence intervals. (c) Quantitative measurement of H$_2$O$_2$ content in the leaves by the chromogenic peroxidase-coupled assay.
The organization of cortical microtubules remained normal and all three plant types appeared similar after 15 min (Fig. 4). However, at 45 min, there was a partial disappearance of the cortical microtubules in the wild type, but only slight disassembly of microtubules in atrbohD and atrbohF mutants. At 75 min, there was a more dramatic depolymerization of cortical microtubules in the wild type, and a weaker depolymerization in some pavement cells of atrbohD and atrbohF mutants. Thus, it was clear that loss of AtrbohD and AtrbohF led to a slow and decreased depolymerization of cortical microtubules in response to VD-toxins.

Figure 2. H₂O₂ production induced by Verticillium dahliae toxins (VD-toxins) in the leaves of wild type (Col-0), and atrbohD and atrbohF mutants of Arabidopsis. (a) H₂O₂ was detected by fluorescence resulting from 2′,7′-dichlorofluorescin diacetate (H₂DCF-DA). (b) The H₂DCF-DA fluorescence intensities in the leaves of Arabidopsis. Confocal data are displayed as estimated mean pixel intensities and associated 95% confidence intervals. Error bars indicate standard deviations. Values of each group with the same letters were not significantly different (P < 0.05) [one-way analysis of variance (ANOVA) and Student–Newman–Keuls (SNK) test].

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To investigate the interaction between the depolymerization of cortical microtubules and H$_2$O$_2$ production, the wild-type seedlings were co-treated with VD-toxins plus DPI or DMTU. The microtubule cytoskeleton disassembly induced by VD-toxins was inhibited in the presence of DPI or DMTU (Fig. 5). To further examine the role of H$_2$O$_2$ in microtubule cytoskeleton destabilization, we used different concentrations of exogenous H$_2$O$_2$ to treat the wild-type seedlings. The depolymerization of cortical microtubules increased with increasing concentrations of exogenous H$_2$O$_2$ (Fig. 6), confirming that H$_2$O$_2$ was involved in the regulation of microtubule depolymerization. Thus, our results indicate that H$_2$O$_2$ was required for VD-toxin-induced microtubule depolymerization.

H$_2$O$_2$ and cortical microtubule alterations correlate with the activation of defence response to VD-toxins

ROS were proposed to orchestrate the establishment of plant defences following successful pathogen recognition (Torres, Jones & Dangl 2006). To test whether the defence gene expression induced by VD-toxins was dependent on H$_2$O$_2$ production, we treated wild type, and $\text{atrbohD}$ and $\text{atrbohF}$ mutants of Arabidopsis seedlings with VD-toxins. At 48 h after treatment, the leaves were collected and subjected to real-time RT-PCR analysis. VD-toxins induced an up-regulated expression of $\text{PR1}$, $\text{PR2}$ and $\text{PR5}$ genes in wild type (Fig. 7). In contrast to the wild type, $\text{PR1}$ expression was strongly reduced in $\text{atrbohD}$ and $\text{atrbohF}$ mutants, whereas $\text{PR2}$ and $\text{PR5}$ were partially reduced. These results indicate that VD-toxin-inducible expressions of $\text{PR1}$, $\text{PR2}$ and $\text{PR5}$ were regulated by H$_2$O$_2$-dependent signals, while expressions of $\text{PR2}$ and $\text{PR5}$ were also regulated by H$_2$O$_2$-independent signals.

Previous experiments indicated that depolymerization of cortical microtubules in Arabidopsis induced expression of the $\text{PR1}$ gene (Shi et al. 2009). To investigate additional defence gene expressions as results of the dynamics of the microtubule cytoskeleton, we analysed the expression of $\text{PR1}$, $\text{PR2}$ and $\text{PR5}$ genes induced by VD-toxins in wild-type Arabidopsis in the presence of microtubule-targeting drugs (Fig. 8). Co-treatment of VD-toxins with microtubule-targeting drugs significantly affected the expression of $\text{PR}$ genes. Expression of $\text{PR1}$, $\text{PR2}$ and $\text{PR5}$ genes was strongly inhibited by treatment with taxol, a drug that inhibits the depolymerization of microtubules, whereas their expression was increased by...
Figure 4. (a) Sequential images of cortical microtubule alterations induced by *Verticillium dahliae* toxins (VD-toxins) (150 μg mL⁻¹) in the leaf pavement cells of the wild type (Col-0), and *atrbohD* and *atrbohF* mutants expressing GFP–tubulin of *Arabidopsis*. Bars = 20 μm. (b) GFP–tubulin fluorescence intensities in leaves treated with VD-toxins in wild type, and *atrbohD* and *atrbohF* mutants. Confocal data are displayed as estimated mean pixel intensities and associated 95% confidence intervals. Error bars indicate standard deviations. Values of each group with the same letters were not significantly different (*P* < 0.05) [one-way analysis of variance (ANOVA) and Student–Newman–Keuls (SNK) test].
treatment with oryzalin, a drug that inhibits the polymerization of microtubules. These results indicate that the dynamic microtubule cytoskeleton plays a functional role in the signalling pathway to mediate the expression of defence genes.

**DISCUSSION**

Previous studies have demonstrated that NO acts as an upstream signalling molecule to trigger the depolymerization of cortical microtubules (Shi et al. 2009). The present study provides evidence that H$_2$O$_2$ may also act as an upstream signalling molecule to modulate the dynamic microtubule cytoskeleton during the defence responses to VD-toxins in *Arabidopsis*. The results suggest a relation linking the H$_2$O$_2$ signal with dynamic microtubule cytoskeleton in plant defence responses.

H$_2$O$_2$ plays an important role in regulating the VD-toxin-induced defence gene expression

Rapid production of ROS has been implicated in diverse physiological processes including resistance to biotic and abiotic stress (Torres et al. 2006). ROS were proposed to orchestrate the establishment of plant defences and HR following successful pathogen recognition. However, the requirement for ROS appears to be different for resistance to different pathogens (Asai et al. 2008; Torres 2010). ROS also play a critical role as signalling intermediates during the defence responses to bacterial pathogens (Choi et al. 2007). In contrast, ROS produced by *AtrbohD* and *AtrbohF* did not have dramatic effects on defence genes against *Alternaria brassicicola* (Pogány et al. 2009), and the *AtrbohD*-mediated H$_2$O$_2$ generation is not required for the activation of defence responses against *Botrytis cinerea* (Galletti et al. 2008). In the present study, H$_2$O$_2$ production...
was one early response to VD-toxins in Arabidopsis, and H2O2 production was impaired by the inhibitor of NADPH oxidase or H2O2 scavenger, or the atrbohD and atrbohF mutations (Figs 1–3). These data suggest that NADPH oxidases appear to be important in H2O2 production induced by VD-toxins in Arabidopsis.

Moreover, the expressions of PR were simultaneously decreased in atrbohD and atrbohF mutants (Fig. 7). The induction of the PRI gene by VD-toxins was completely blocked, and PR2 and PR5 genes were partially blocked by atrbohD and atrbohF mutations. These results suggest that the expressions of PRI, PR2 and PR5 were regulated by H2O2-dependent signals, while the expressions of PR2 and PR5 were also regulated by H2O2-independent signals. The results of the present study provide convincing evidence that H2O2 plays an important role in the regulation of VD-toxin-induced defence gene expression in Arabidopsis. It is possible that H2O2 is necessary, but not sufficient to trigger the defence gene expression against VD-toxins in Arabidopsis.

Microtubule depolymerization is mediated by H2O2-dependent signalling

Plant interactions with pathogens are known to stimulate cytoskeleton reorganization (Takemoto & Hardham 2004). The plant cytoskeleton readily remodels in response to various intracellular and external stimuli. Cortical microtubules are intimately associated with the plasma membrane and are implicated as targets of signalling networks (Gilroy & Trewavas 2001; Wasteneys & Galway 2003). It is therefore not surprising that certain signalling pathways are interconnected and are used to regulate the dynamic microtubule cytoskeleton simultaneously. However, specific knowledge about upstream signalling pathways that regulate cortical microtubule dynamics is limited.

Microtubule destabilization occurs independently of the production of ROS in tobacco cells in response to cryptogein (Binet et al. 2001). In contrast, it has been suggested that the actin cytoskeleton is a central signalling component that couples the accumulation of ROS to programmed cell death in yeast (Farah & Amberg 2007; Leadsham & Gourlay 2008). Recently, it was reported that NO acts as an upstream signal and subsequently modulates cortical microtubule dynamics in Arabidopsis (Shi et al. 2009) and also the actin cytoskeleton dynamics in maize (Kasprowicz et al. 2009). It has been demonstrated that NO cooperates with ROS to activate HR in plants (Delledonne et al. 2001; Polverari et al. 2003; Torres et al. 2006; Leitner et al. 2009; Yoshioka et al. 2009). However, to date, very little is known about the relationship between ROS and cytoskeleton dynamics in plants. To the authors’ knowledge, there are no reports linking H2O2 signalling with the dynamic microtubule cytoskeleton in plant defence responses.
Figure 7. Relative expression levels of defence genes after treatment with *Verticillium dahliae* toxins (VD-toxins) in wild type, and *atrbohD* and *atrbohF* mutants. Total RNA was extracted at 48 h with VD-toxin treatment for real-time PCR analysis. *Actin2* was used as internal control. Error bars indicate standard deviations. Values of each group with the same letters were not significantly different ($P < 0.05$) [one-way analysis of variance (ANOVA) and Student–Newman–Keuls (SNK) test].

Figure 8. Relative expression levels of defence genes after treatment with *Verticillium dahliae* toxins (VD-toxins) and oryzalin or taxol in wild-type *Arabidopsis*. Total RNA was extracted at 48 h with VD-toxin treatment for real-time PCR analysis. *Actin2* was used as internal control. Error bars indicate standard deviations. Values of each group with the same letters were not significantly different ($P < 0.05$) [one-way analysis of variance (ANOVA) and Student–Newman–Keuls (SNK) test].
The present study suggests that a stronger H$_2$O$_2$ production was induced by VD-toxins in wild-type compared to the atrbohD and atrbohF mutants, especially at the later stages (Fig. 2). Most importantly, at this phase, a dramatic depolymerization of cortical microtubules was observed in the wild type compared with the atrbohD and atrbohF mutants (Fig. 4). Furthermore, scavenging of H$_2$O$_2$ accumulation by DMTU or inhibition of the VD-toxin-induced H$_2$O$_2$ synthesis by DPI reduced the depolymerization of cortical microtubules (Figs 3 & 5). In addition, the depolymerization of cortical microtubules increased with increasing concentrations of exogenous H$_2$O$_2$ (Fig. 6). Taken together, these results reveal that the presence of H$_2$O$_2$ affected the dynamic microtubule cytoskeleton. H$_2$O$_2$ may act as an upstream signalling molecule to regulate the depolymerization of cortical microtubules. Microtubule-associated proteins (MAPs) have been shown to be targets for H$_2$O$_2$ (Zhang et al. 2003; Landino et al. 2004). MAPs serve a wide variety of functions and control microtubule organization and dynamics (Sedbrook 2004); therefore, we speculate that the depolymerization of cortical microtubules may be the consequence of the inactivation of MAPs regulated by H$_2$O$_2$.

**Microtubule depolymerization plays a functional role in VD-toxin-induced defence responses**

Depolymerization of microtubules has been reported in parsley–Phytophthora and soybean–Phytophthora interactions, and in elicitor-treated tobacco cells (Gross et al. 1993; Binet et al. 2001; Cahill et al. 2002). Thus, biotic interactions involve specific alterations to the microtubule cytoskeleton. However, a direct connection between microtubule depolymerization and triggering of defence responses remains to be elucidated.

The present study showed that the disruption of cortical microtubules affected the expression of PR1, PR2 and PR5 genes during the response against VD-toxins (Fig. 8). Stabilization of cortical microtubules using taxol reduced these VD-toxin-induced expressions of genes, whereas depolymerization of cortical microtubules using oryzalin strongly increased the expression of these genes. This indicates that the cortical microtubule dynamics plays an important role in mediating the plant defence response. Cortical microtubules may be an earlier and specific sign to induce the expression of defence genes against VD-toxins in *Arabidopsis*. Whether other signalling pathways are also activated requires further investigation.

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**References**


H$_2$O$_2$ modulates MT dynamics in the defence responses


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