Histone H2B Monoubiquitination Is Involved in Regulating the Dynamics of Microtubules during the Defense Response to *Verticillium dahliae* Toxins in Arabidopsis

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Histone H2B monoubiquitination (H2Bub) is being recognized as a regulatory mechanism that controls a range of cellular processes in plants, but the molecular mechanisms of H2Bub that are involved in responses to biotic stress are largely unknown. In this study, we used wild-type and H2Bub loss-of-function mutations of Arabidopsis (*Arabidopsis thaliana*) to elucidate which of its mechanisms are involved in the regulation of the plant’s defense response to *Verticillium dahliae* (Vd) toxins. We demonstrate that the depolymerization of the cortical microtubules (MTs) was different in the wild type and the mutants in the response to Vd toxins. The loss-of-function alleles of HISTONE MONOUBIQUITINATION 1 and HISTONE MONOUBIQUITINATION 2 mutations present a weaker depolymerization of the MTs, and protein tyrosine phosphorylation plays a critical role in the regulation of the dynamics of MTs. Moreover, H2Bub is a positive regulator of the gene expression of protein tyrosine phosphatases. These findings provide direct evidence for H2Bub as an important modification with regulatory roles in the defense against Vd toxins and demonstrate that H2Bub is involved in modulating the dynamics of MTs, likely through the protein tyrosine phosphatase-mediated signaling pathway.

Ubiquitination is being recognized as a common regulatory mechanism that controls a range of cellular processes. Protein ubiquitination influences the ability of a target protein to interact with other proteins. Two types of protein ubiquitination, multiubiquitination and monoubiquitination, have been described previously. Proteins that have undergone multiubiquitination are often escorted to the 26S proteasome to undergo degradation, whereas monoubiquitination has several regulatory roles for the proteins it targets, such as changes in subcellular localization, conformation, activity, and protein interactions (Haglund et al., 2003; Hicke and Dunn, 2003). Monoubiquitination is usually associated with histone modification (Schnell and Hicke, 2003). Histone monoubiquitination primarily involves the histones H2A and H2B (Zhang, 2003). Histone H2B monoubiquitination (H2Bub) is mainly associated with transcriptional activation and regulates transcription elongation (Pavri et al., 2006; Shilatifard, 2006; Weake and Workman, 2008).

In Arabidopsis (*Arabidopsis thaliana*), histone H2B is monoubiquitinated by two RING E3 ligases (HISTONE MONOUBIQUITINATION 1 [HUB1] and HUB2) and three E2 conjugases (UBIQUITIN CONJUGATING ENZYME 1 [UBC1], UBC2, and UBC3). H2Bub has been reported to affect leaf and root growth and seed dormancy (Fleury et al., 2007; Liu et al., 2007), and H2Bub is also involved in regulating the expression of key flowering time genes for the control of flowering time and plant development (Cao et al., 2008; Gu et al., 2009; Xu et al., 2009; Schmitz et al., 2009). A recent study showed that the H2Bub-mediated regulation of gene expression played a role in the processes of photomorphogenesis and the circadian clock (Bourbousse et al., 2012; Himanen et al., 2012). Moreover, defense responses in plants have been demonstrated to require ubiquitination for both positive and negative regulation (Devoto et al., 2003; Trujillo and Shirasu, 2010). Arabidopsis HUB1 is a regulatory component of plant defense against necrotrophic fungal pathogens (Dhawan et al., 2009). These results suggest that H2Bub is involved in multiple developmental processes and responses to biotic stress in plants.

The plant cytoskeleton is essential for various types of antimicrobial defense (Schmidt and Panstruga, 2007), and its microfilaments and microtubules (MTs) are necessary for plants to block fungal penetration (Genre and Bonfante, 2002; Kobayashi and Hakuno, 2003). Recently, it was demonstrated that MTs play an important role in both the establishment of functional symbioses and the defense against invading pathogens (Hardham, 2013). The aggregation of subcellular components at the infection site has been shown to depend on the plant cytoskeleton (Lipka and Panstruga, 2005; Hardham et al., 2008). Moreover, the interaction between
Plant cells and pathogens triggers a range of highly dynamic plant cellular responses, including the reorganization of the cytoskeleton. In tobacco (*Nicotiana tabacum*), cell death induced by cryptogein coincides with the rapid disintegration of the MTs (Binet et al., 2001). Reduced MT dynamics have also been demonstrated to render plants less susceptible to *Tobacco mosaic virus* (Ouko et al., 2010). We have previously demonstrated that MTs play an important role in the defense response against *Verticillium dahliae* (*Vd*) toxins in Arabidopsis (Shi et al., 2009; Yao et al., 2011). However, the precise mechanisms regulating the dynamics and organization of the MTs remain poorly understood.

Protein kinases and protein phosphatases play central roles in signal transduction through the phosphorylation and dephosphorylation of proteins. Tyr phosphorylation has been recognized as a key regulatory event in the signaling pathways that underlie a broad spectrum of fundamental physiological processes. Moreover, it has been reported that the cytoskeleton is affected, directly or indirectly, by reversible Tyr protein phosphorylation (Luan, 2002, 2003; Tonks, 2006). The behavior of MTs in animal cells is known to be sensitive to protein phosphorylation at Tyr residues. In plant cells, Tyr protein kinase inhibitors and inhibitors of Tyr protein phosphatase have been reported to disorganize the cortical MT arrays (Blume et al., 2008; Yemets et al., 2008). This observation suggests that Tyr phosphorylation is involved in regulating the dynamics and organization of MTs. Furthermore, the inhibitors of Tyr kinases and phosphatases can be used as tools to investigate the dynamics and organization of MTs (Sheremet et al., 2012). In addition, the actin cytoskeleton has also been reported to be affected by reversible Tyr protein phosphorylation (Zi et al., 2007). The Tyr phosphorylation of actin is essential for actin cytoskeletal alterations (Kameyama et al., 2000).

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**Figure 1.** H2Bub is involved in regulating the defense response to *Vd* toxins. A, H$_2$O$_2$ production induced by *Vd* toxins was detected by fluorescence resulting from H$_2$DCF-DA in the leaves of wild-type and hub1-4, hub2-2, ubc1-1, and ubc1-1/ubc2-2 mutant Arabidopsis. B, H$_2$DCF-DA fluorescence intensities quantified using AxioVision Rel.4.8 software. Error bars indicate sd. C, Cell death induced by *Vd* toxins in the leaves of wild-type and hub1-4 mutant Arabidopsis. Leaves of 4-week-old plants were treated with *Vd* toxins and stained with trypan blue. D, Relative expression levels of the defense gene *PR1* after treatment with *Vd* toxins in wild-type and hub1-4, hub2-2, ubc1-1, and ubc1-1/ubc2-2 mutant Arabidopsis. The leaves untreated with *Vd* toxins were used as a control. Total RNA was extracted after 24 h of *Vd* toxin treatment for real-time PCR analysis. Error bars indicate sd.
**Vd** is a soil-borne pathogen that causes *Verticillium* spp. wilt in a variety of important plant species worldwide (Bhat and Subbarao, 1999). Although the physiology of plant defense against *Verticillium* spp. infection is well established, consisting of the production of pathogenesis-related (PR) proteins, phytoalexins, and phenolic compounds and the active expression of several disease response genes (McFadden et al., 2001; Fradin and Thomma, 2006), the molecular mechanisms and regulatory pathways involved in these plant defense responses to *Verticillium* spp. remain largely unknown. We have previously demonstrated that **Vd** toxins induce alterations in the cytoskeleton of an Arabidopsis cell suspension (Yuan et al., 2006) as well as that hydrogen peroxide (H$_2$O$_2$) and nitric oxide may act as upstream signaling molecules to regulate cortical MT depolymerization during the defense response to **Vd** toxins. The depolymerization of the cortical MTs plays a functional role in the signaling pathway that mediates the expression of defense genes (Shi et al., 2009; Yao et al., 2011). However, no direct evidence exists to demonstrate the functional significance of H2Bub on the dynamics and organization of MTs in the defense response.

Therefore, in this study, we used wild-type plants and **hub1-4**, **hub2-2**, **ubc1-1**, **hub1-4/hub2-2**, and **ubc1-1/hub2-2** mutants of the Arabidopsis ecotype Columbia (Col-0) to elucidate which of its mechanisms are involved in regulating the dynamics of MTs during the defense response to **Vd** toxins. We present evidence that H2Bub plays an important role in regulating the dynamics of MTs during the defense response to **Vd** toxins. H2Bub is involved in modulating the dynamics of MTs, likely through the protein tyrosine phosphatase.

**Figure 2.** A, Sequential images of the cortical MT alterations induced by **Vd** toxins (150 μg mL$^{-1}$) in the leaf pavement cells of wild-type (Col-0) and **hub1-4**, **hub2-2**, and **hub1-4/hub2-2** mutant Arabidopsis expressing GFP-tubulin. Bar = 20 μm. B, Quantification of cortical MTs in the leaf pavement cells of wild-type (Col-0) and **hub1-4**, **hub2-2**, and **hub1-4/hub2-2** mutant plants using Image tool software ($n$ > 12 cells from three samples). The number of cortical MTs was determined by counting the MTs across a fixed line (approximately 50 μm) vertical to the orientation of most of the cortical MTs of the cell. Student’s $t$ tests compared the number of cortical MTs in the leaf pavement cells of the **hub1-4**, **hub2-2**, and **hub1-4/hub2-2** mutants with the number of cortical MTs in the wild type (Col-0) under the same conditions. **$P < 0.01$, *$P < 0.05$, by Student’s $t$ test. Error bars indicate sd.**
(PTP)-mediated signaling pathway, and it is a positive regulator of the expression of PTP genes in the response to \( Vd \) toxins. These results provide new insight into the mechanisms controlling the Arabidopsis defense response against \( Vd \) toxins.

RESULTS

H2Bub Is Involved in Regulating the Defense Response to \( Vd \) Toxins

The central features of the plant defense response are the generation of reactive oxygen species, the activation of defense genes, and rapid cell death, the combination of which is also known as the hypersensitive response (Dangl and Jones, 2001; Dixon, 2001; Greenberg and Yao, 2004; Torres and Dangl, 2005). Therefore, we analyzed the generation of \( \text{H}_2\text{O}_2 \), \( PR1 \) expression, and rapid cell death after treatment by \( Vd \) toxins in the wild type and in the \( hub1-4 \), \( hub2-2 \), \( ubc1-1 \), and \( ubc1-1/ubc2-2 \) mutants of Arabidopsis. The \( \text{H}_2\text{O}_2 \) was labeled with a permeable fluorescent probe, 2',7'-dichlorofluorescin diacetate (H2DCF-DA). We observed that the \( \text{H}_2\text{O}_2 \) level increased significantly in the wild type, whereas the mutants showed a reduction of \( \text{H}_2\text{O}_2 \) production (Fig. 1, A and B). We used the trypan blue staining method to detect cell death. In agreement with the observations of \( \text{H}_2\text{O}_2 \) content, relatively small areas of cell death were detected on the leaves of the \( hub1-4 \) mutant 24 h after \( Vd \) toxin treatment in comparison with the wild type (Fig. 1C). Furthermore, the relative expression levels of \( PR1 \) were monitored by real-time PCR. An increase in the expression level of \( PR1 \) was strongly induced in the wild type after \( Vd \) toxin treatment, whereas the expression level was reduced in the mutants (Fig. 1D).

Taken together, the mutants showed a reduction of \( Vd \) toxin-induced \( \text{H}_2\text{O}_2 \) production, cell death, and activation of defense genes, suggesting that H2Bub is an important modification with regulatory roles in the defense against \( Vd \) toxins in Arabidopsis.

H2Bub Is Involved in Modulating the Dynamics of MTs Induced by \( Vd \) Toxins

In the previous studies, we showed that MTs play an important role in the signaling pathway for mediating...
the defense response against *Vd* toxins in Arabidopsis (Shi et al., 2009; Yao et al., 2011). Therefore, we explored whether H2Bub and the dynamics of MTs are connected during the defense response to *Vd* toxins. We used wild-type Arabidopsis and the *hub1-4*, *hub2-2*, and *hub1-4/hub2-2* mutants to better understand the correlation of H2Bub and the dynamics of MTs with the defense response to *Vd* toxins. The MTs were observed by the fluorescence of GFP-tubulin using confocal microscopy. As shown in Figure 2, the depolymerization of the cortical MTs was induced after treatment with *Vd* toxins, and this trend increased with treatment time. Interestingly, it was observed that the depolymerization of the cortical MTs was different in the wild type and the mutants. After 30 min of *Vd* toxin treatment, the leaves from the wild type displayed a partial depolymerization of the cortical MTs but the leaves from the *hub1-4* and *hub2-2* mutants, especially those of the *hub1-4/hub2-2* double mutant, showed only a slight disassembly of the MTs. A more dramatic depolymerization of the cortical MTs was observed after 90 min of *Vd* toxin treatment in the wild type, whereas only a weaker depolymerization of the cortical MTs was seen in some pavement cells of the *hub1-4* and *hub2-2* mutants; only a slight depolymerization of the MTs was observed in the *hub1-4/hub2-2* double mutant. These data indicate that H2Bub is involved in modulating the dynamics of MTs induced by *Vd* toxins.

### The Dynamics of MTs Induced by *Vd* Toxins Are Regulated by Protein Tyr Phosphorylation

Tyr phosphorylation is known to be involved in the dynamics and organization of MTs (Yemets et al., 2008). We predicted that protein Tyr phosphorylation is involved in the modulation of the dynamics of MTs induced by *Vd* toxins. Thus, inhibitors of Tyr phosphorylation should have an effect on the dynamics of MTs induced by *Vd* toxins. To test this hypothesis, we analyzed the organization of MTs after treatments with *Vd* toxins plus inhibitors of protein tyrosine kinase (PTK) or PTP in the wild type and the *hub1-4* and *hub2-2* mutants. The depolymerization of the cortical MTs increased with treatment time, but it was significantly decreased when the leaves were induced by *Vd* toxins plus genistein (GN), a specific inhibitor of PTKs (Fig. 3, A and C), in comparison with induction by *Vd* toxins alone (Fig. 2); these results were similar in both the wild type and the mutants. In contrast to the results for the PTK inhibitor, the depolymerization of the cortical MTs was markedly increased when the leaves were induced by *Vd* toxins plus phenylarsine oxide (PAO), a specific inhibitor of PTPs (Fig. 3, B and D). This result indicated that the MT cytoskeleton disassembly induced by *Vd* toxins was inhibited in the presence of GN, while the presence of PAO stimulated the disruption of the cortical MTs.

The effect was particularly evident after 90 min of treatment with *Vd* toxins plus inhibitors (Fig. 4). GN was shown to cause a significant inhibition, while PAO led to the significant stimulation of the depolymerization of the cortical MTs.

Taken together, these results demonstrated that the GN treatment resulted in MT stabilization, whereas the PAO treatment resulted in the opposite effect on the dynamics of MTs. These results suggest that protein Tyr phosphorylation plays an important role in the regulation of the dynamics of MTs induced by *Vd* toxins.

### H2Bub Is Involved in Regulating the Expression of Key Protein Tyr Phosphatase Genes in the Response to *Vd* Toxins

To further determine whether Tyr phosphorylation is involved in the responses to *Vd* toxins, the gene

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**Figure 4. A.** Effect of GN or PAO on the disruption of MTs induced by *Vd* toxins in the leaf pavement cells of wild-type (Col-0) and *hub1-4* and *hub1-4/hub2-2* mutant Arabidopsis expressing GFP-tubulin. Photographs were taken 90 min after treatment. Bar = 20 μm. **B.** Quantification of cortical MTs in the leaf pavement cells of the wild type (Col-0) and the *hub1-4*, *hub2-2*, and *hub1-4/hub2-2* mutants using Image tool software (*n* > 12 cells from three samples). The number of cortical MTs was determined by counting the MTs across a fixed line (approximately 50 μm) vertical to the orientation of most of the cortical MTs of the cell. Student’s *t* tests compared the number of cortical MTs in the leaf pavement cells of the *hub1-4* and *hub2-2* mutants with the number of cortical MTs in the wild type (Col-0) under the same conditions. **P < 0.01, *P < 0.05, by Student’s *t* test. Error bars indicate s.e.**
expression of PTPs, including \textit{AtPTP1} and \textit{AtPFA-DSP5} (for \textsc{Arabidopsis thaliana} Plant and \textsc{fungi} Atypical Dual-Specificity Phosphatase5), in response to \textit{Vd} toxins in wild-type and mutant Arabidopsis seedlings was confirmed by real-time PCR. As shown in Figure 5, the relative levels of gene expression of \textit{AtPTP1} and \textit{AtPFA-DSP5} are significantly different in the wild-type and mutant plants. The transcript levels of \textit{AtPTP1} and \textit{AtPFA-DSP5} were induced strongly in the wild type, whereas the transcript level was increased slightly in the \textit{hub1-4}, \textit{hub2-2}, and \textit{hub1-4/hub2-2} mutants (Fig. 5). These results suggest that H2Bub is involved in regulating the expression of key protein Tyr phosphatase genes in the response to \textit{Vd} toxins, that H2Bub may be a positive regulator of the expression of PTP genes, and that \textit{AtPTP1} and \textit{AtPFA-DSP5} are important in the response to \textit{Vd} toxins.

\textbf{DISCUSSION}

H2Bub is a key modification that plays roles in gene transcriptional regulation in various cellular processes. In this study, we provide several lines of evidence showing that H2Bub is crucial to the modulation of the plant defense response against \textit{Vd} toxins. We found that the accumulation of H$_2$O$_2$, hypersensitive response-like cell death, and the activation of defense gene expression, all of which are altered in the plant defense response, were eliminated in the \textit{hub1-4}, \textit{hub2-2}, \textit{ubc1-1}, and \textit{ubc1-1/ubc2-2} mutants after the \textit{Vd} toxin treatment (Fig. 1), suggesting that H2Bub is an important modification and apparently has a positive regulatory role in the defense response against \textit{Vd} toxins.

Previous studies have demonstrated that the depolymerization of MTs plays a functional role in triggering the defense response to \textit{Vd} toxins in Arabidopsis (Shi et al., 2009; Yao et al., 2011). It has been demonstrated that the depolymerization of cortical MTs may act as a sensor and mediator for monitoring defense gene expression against \textit{Vd} toxins in Arabidopsis. However, it is still unclear whether H2Bub is involved in regulating the depolymerization of cortical MTs during the defense response. The results of this study have established that a more dramatic depolymerization of the cortical MTs was induced by \textit{Vd} toxins in the wild type than in the mutants (Fig. 2). The loss-of-function alleles of \textit{HUB1} and \textit{HUB2} were shown to lead to the stabilization of the cortical MTs. These data indicated that H2Bub was involved in modulating the dynamics of

\begin{figure}[h]
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\caption{Relative expression levels of \textit{AtPTP1} and \textit{AtPFA-DSP5} after treatment with \textit{Vd} toxins in the wild type and the \textit{hub1-4}, \textit{hub2-2}, and \textit{hub1-4/hub2-2} mutants. Leaves untreated with \textit{Vd} toxins were used as a control. Total RNA was extracted after 24 h of \textit{Vd} toxin treatment for real-time PCR analysis. Error bars indicate SD.}
\end{figure}
MTs induced by Vd toxin. However, the possible functional role of H2Bub in the depolymerization of the cortical MTs remains unclear.

MT cytoskeletal organization and function depend on the presence of microtubule-associated proteins (MAPs) and their regulatory kinases and phosphatases; that is, the stability of MTs is regulated in part by the reversible phosphorylation of MAP (Sedbrook, 2004; Tian et al., 2004). The phosphorylation of a MAP by the mitogen-activated protein kinase (MAPK) pathway is conserved among various species (Sasabe and Machida, 2012). MTs are cytoskeletal targets of activated MAPK (Komis et al., 2011). MAP65-1 is phosphorylated by MAPK to reduce its MT-bundling activity, leading to the destabilization of MT organization (Sasabe and Machida, 2006; Smertenko et al., 2006).

Kinases and phosphatases can be grouped by substrate specificity into Ser/Thr, Tyr, and dual-specificity classes. While Ser/Thr phosphorylation has been widely accepted as a predominant modification of plant proteins, the function of Tyr phosphorylation in plants had been largely neglected. Protein Tyr phosphorylation is controlled through the coordinated actions of PTKs and PTPs. However, typical Tyr kinases have not been predicted in any plants; only protein kinases with dual specificity have been reported in plant species (Sugiyama et al., 2008; Yu and Kim 2012; Nito et al., 2013). PTPs have been characterized from higher plants and are critical regulators of signal transduction in plant cells. PTP1 is the only member of the Tyr-specific protein phosphatases in Arabidopsis (Bartels et al., 2009). AtPTP1 gene expression has been found to be altered in response to different environmental stresses (Xu et al., 1998). DSPs are the most abundant enzymes within the PTP family. PFA-DSPs are a group of atypical DSPs present in plants and fungi. In Arabidopsis, there are five PFA-DSPs (Romá-Mateo et al., 2007, 2011). However, little is known about the function of PFA-DSPs, especially that of PFA-DSP5, in plants. It has been reported that SIW14 (for synthetic interaction with wh2), a PFA-DSP in yeast (Saccharomyces cerevisiae), is involved in the response of the actin cytoskeleton to stress conditions (Care et al., 2004).

PTPs are critical regulators of signaling in their own right, playing an important role under normal and pathophysiological conditions (Tonks, 2013). The major functional theme of PTPs is the regulation of signaling by MAPK, which acts in a concerted manner to determine both physiological and pathological responses to a wide variety of extracellular and intracellular stimuli (Luan, 2002; Caunt and Keyse, 2013). Furthermore, the disruption of PTP function has been implicated as an underlying cause of human diseases (DeLong, 2006; Tonks, 2006). Tau is a major brain MAP and is a key player in human disease, the physiological function and MT-binding activity of tau are controlled by phosphorylation, and the MT disruption and deregulation of MT-based functions have been implicated in the disease process (Nunbhakdi-Craig et al., 2007; Sontag et al., 2012). It has also been reported that PTP participates in the disease resistance signaling pathway in plants (Bretz et al., 2003). In this study, we have demonstrated that the depolymerization of the cortical MTs was induced by Vd toxins and was significantly decreased when the leaves were induced by Vd toxins plus a PTK inhibitor, while it was markedly increased when the leaves were induced by Vd toxins plus a PTP inhibitor (Figs. 3 and 4). These data indicated that the specific inhibition of PTPs increased the depolymerization of the cortical MTs and, conversely, that the specific inhibition of PTKs increased the MT stabilization. Our results suggest that protein Tyr phosphorylation plays a critical role in the regulation of the dynamics of MTs during the defense response to Vd toxins in Arabidopsis. Therefore, we speculate that PTP may be the critical regulator of signaling, and the PTP-mediated signaling pathway was inferred to be a crucial mechanism for regulating the dynamics of MTs. Activated PTPs may function to dephosphorylate MAP and increase its MT-bundling activity, leading to the stabilization of MT organization. The dynamics of MTs plays an important part in the defense response against Vd toxins.

Ubiquitination is crucial to the regulation of numerous cellular processes. H2Bub acts as a polyvalent histone mark that plays different roles in the regulation of gene expression (Hammond-Martel et al., 2012). This study showed that the transcript levels of PTP genes, including AtPTP1 and AtPFA-DSP5, were strongly increased in the wild type after Vd toxin treatment, whereas a slight increase was observed in the mutants (Fig. 5). This result indicated that loss-of-function alleles of Hub1 and Hub2 affected the expression of the AtPTP1 and AtPFA-DSP5 genes. Our results suggest that H2Bub is a positive regulator of the expression of PTP genes. Therefore, we speculate that the PTP genes could be regulated or act as the target genes of H2Bub.

On the basis of our results, we demonstrated that H2Bub is an important modification with a regulatory part in the defense response to Vd toxins and involved in modulating the dynamics of MTs. H2Bub is a positive regulator of the expression of PTP genes. Activated PTPs are involved in regulating the dynamics of MTs, suggesting that it plays a functional role in the signaling pathway for mediating the expression of defense genes in response to Vd toxins (Shi et al., 2009; Yao et al., 2011).

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Treatments

Wild-type (Col-0) Arabidopsis (Arabidopsis thaliana) seeds, as well as hub1-4, hub2-2, ubc1-1, hub1-4/hub2-2, and ubc1-1/ubc2-2 mutants (background Col-0), were used in the experiment. The hub1-4, hub2-2, ubc1-1, hub1-4/hub2-2, and ubc1-1/ubc2-2 mutants have been described previously (Cao et al., 2008). The seeds were sterilized with 0.2% sodium hypochlorite for 20 min, rinsed with water, and germinated on Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 3% Suc (w/v) and 0.7% agar (w/v) in petri dishes, and the seedlings were grown vertically at 21°C ± 2°C under 14 h of light (100 μmol m⁻² s⁻¹) and 10 h of darkness and 70% relative humidity in a growth cabinet for 4 d. The seedlings were then transplanted onto plates supplemented with Verticillium dahliae toxins and returned to the growth chamber.
Preparation of Crude Vd Toxins

A highly infectious and defoliating strain of Vd (Vd991) was used for the extraction of Vd toxins. The *Verticillium* spp. culture filtrate was purified as described previously (Jia et al., 2007; Shi and Li, 2008).

**H₂O₂ Assays**

For the H₂DCF-DA staining assay, 7-d-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. The seedlings were then washed three times with 10 mM MES-KCl buffer (pH 6.0) to remove the excess H₂DCF-DA. Seedlings incubated in heat-inactivated Vd toxins were used as controls. Fluorescence was detected with a confocal laser scanning microscope (CLSM; Zeiss LSM 510). The CLSM working conditions were as follows: power, 70%; excitation at 488 nm; and emission at 505 to 530 nm. This experiment was independently repeated at least three times.

Detection of Cell Death in Leaves

Cell death in the leaves was measured by staining with trypan blue according to the method described previously (Browning et al., 1997). All experiments were repeated at least three times.

Visualization of Cortical MTs

The cortical MTs in the leaf pavement cells of Arabidopsis seedlings expressing GFP-tubulin were observed using the CLSM as described previously (Shi et al., 2009). Seven-day-old seedlings were treated at room temperature with Vd toxins (150 µg mL⁻¹) alone, Vd toxins plus GN (20 µmol L⁻¹), or Vd toxins plus PAO (40 µmol L⁻¹). The organization and dynamics of the cortical MTs were observed at several time points after treatment. The images of GFP fluorescence (Figs. 2–4) are projections of optical sections taken at 1.5-µm intervals from the outer epidermal wall 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.

The reported observations are based on three separate experiments, and the images presented are representative of more than 30 similar images collected for each phenomenon being illustrated.

Arabidopsis seedlings expressing GFP-tubulin were crossed with hub1-4, hub2-2, and hub1-4/hub2-2 mutants, and the homozygous lines produced were used for observation of the dynamics of the cortical MTs in the hub1-4, hub2-2, and hub1-4/hub2-2 mutants.

Extraction of Total RNA and Real-Time PCR

Total RNA was extracted from the controls and samples using Trizol reagent (Bio Basic) following the manufacturer’s instructions. The concentration of RNA was accurately quantified by spectrophotometric measurements, and 2 µL of total RNA was separated on a 0.8% agarose gel to check the concentration and to monitor integrity. For real-time PCR analysis, first-strand complementary DNA (cDNA) was synthesized from 2 µL of total RNA using Moloney murine leukemia virus reverse transcriptase (Promega). Quantitative PCR was performed on an ABI 7500 Sequence Detector (Applied Biosystems) using the SYBR Premix Ex Taq Kit (Takara). PCR was conducted at 95°C for 3 min, followed by 40 cycles of 95°C for 5 s (denaturation) and annealing at 60°C for 34 s. Data analysis, including the determination of the threshold cycle that represents the starting point of the exponential phase of PCR, and graphic presentation were conducted using the Sequence Detection Software version 1.07 (Applied Biosystems). The quantification of the transcript levels of the CDNA fragments was normalized to the expression of the ACTIN2 gene in Arabidopsis at 24 h under the Vd toxin (150 µg mL⁻¹) treatment. Three independent experiments were performed, each with three replicates.

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LITERATURE CITED


Microtubule Dynamics Are Regulated in the Defense Response


