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# ***Fusarium* Phytotoxin Trichothecenes Have an Elicitor-Like Activity in *Arabidopsis thaliana*, but the Activity Differed Significantly Among Their Molecular Species**

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Phytopathogenic fungi such as *Fusarium* spp. synthesize trichothecene family phytotoxins. Although the type B trichothecene, deoxynivalenol (DON), is thought to be a virulence factor allowing infection of plants by their trichothecene-producing *Fusarium* spp., little is known about effects of trichothecenes on the defense response in host plants. Therefore, in this article, we investigated these effects of various trichothecenes in *Fusarium*-susceptible *Arabidopsis thaliana*. Necrotic lesions were observed in *Arabidopsis* leaves infiltrated by 1  $\mu$ M type A trichothecenes such as T-2 toxin. Trichothecene-induced lesions exhibited dead cells, callose deposition, generation of hydrogen peroxide, and accumulation of salicylic acids. Moreover, infiltration by trichothecenes caused rapid and prolonged activation of two mitogen-activated protein kinases and induced expression of both *PR-1* and *PDF1.2* genes. Thus, type A trichothecenes trigger the cell death by activation of an elicitor-like signaling pathway in *Arabidopsis*. Although DON did not have such an activity even at 10  $\mu$ M, translational inhibition by DON was observed at concentrations above 5  $\mu$ M. These results suggested that DON is capable of inhibiting translation in *Arabidopsis* cells without induction of the elicitor-like signaling pathway.

*Additional keywords:* DAS, MAP kinase cascade.

Phytopathogenic *Fusarium* spp. (e.g., *Fusarium graminearum*) are the etiological agents of Fusarium head blight (FHB) in monocotyledonous plants such as wheat and barley (Schroeder and Christensen 1963). These pathogenic fungi produce trichothecene phytotoxins, a group of sesquiterpenoid secondary metabolites (Desjardins et al. 1993). Trichothecenes commonly contain an epoxide at the C<sub>12,13</sub> position, which is responsible for their toxicological activity (Sudakin 2003). They are classified into four major groups by their chemical structures with varying degrees of cytotoxic potency. Type A (T-2 toxin, HT-2 toxin, and diacetoxyscirpenol) and type B (nivalenol and deoxynivalenol [DON]) often are detected in certain agricultural commodities and commercial foods. Trichothecenes

have been shown to interact with the peptidyl transferase site of the 60S ribosome subunit in eukaryotic cells and, consequently, block translational initiation or elongation (McLaughlin et al. 1977).

Phytopathogenic fungi produce a wide range of phytotoxic compounds, such as AAL-toxin and fumonisin B1 (Brandwagt et al. 2001; Jackson and Taylor 1996; Stone et al. 2000). Disease symptoms often result from the effects of these fungal toxins. Nonselective toxins affect a broad range of plant species, whereas host-selective toxins (HSTs) appear to be specific to an individual plant cultivar, and their effects are mediated by gene-for-gene interaction (Jackson and Taylor 1996). Trichothecenes are nonselective phytotoxins and are highly toxic to monocotyledonous plants, such as wheat and rye (Packa 1991; Wakulinski 1989). Trichothecenes inhibit seed germination and reduce root and shoot growth in wheat seedlings (Wakulinski 1989).

The phytopathogenicity of DON-producing *F. graminearum* is reduced by disruption of a trichothecene biosynthesis gene (Bai et al. 2002; Proctor et al. 1995). Thus, trichothecenes are thought to be virulence factors in the infection of plants by *Fusarium* spp. Recently, it has been reported that *Arabidopsis* is susceptible to *F. graminearum* and *F. culmorum* (Urban et al. 2002), and trichothecene production was detected in *Fusarium*-infected *Arabidopsis* flowers (Urban et al. 2002). In addition, trichothecene-induced growth inhibition also was reported in *Arabidopsis* (Poppenberger et al. 2003). Therefore, *Arabidopsis* is a useful model for studying the mode of action of trichothecenes in higher plants. In this study, we investigated the effects of trichothecenes on defense responses in *Arabidopsis thaliana*. Our results show that some type A trichothecenes might trigger cell death by mimicking an elicitor signal transduction pathway in *A. thaliana*. However, such an elicitor-like activity differed significantly among trichothecene molecular species.

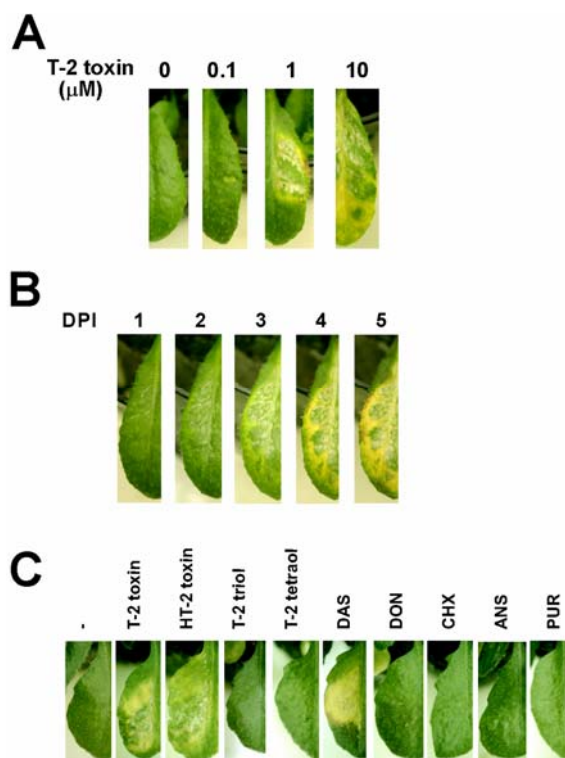
## **RESULTS**

### **Trichothecenes induce necrotic lesion formation and salicylic acid accumulation in *Arabidopsis* leaves.**

It has been known that pathogen-derived molecules often induce lesion formations in plant leaves (Jackson and Taylor 1996; Stone et al. 2000). Therefore, we investigated whether lesion formations were observed in *Arabidopsis* leaves infil-

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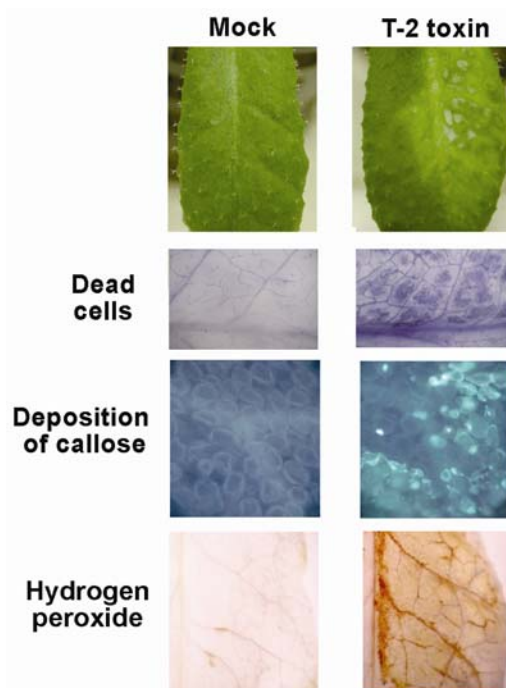
trated with trichothecenes. We found lesion formations at 4 days after *Arabidopsis* leaves were infiltrated with 1  $\mu\text{M}$  type A trichothecene, T-2 toxin (Fig. 1A). Such lesions spread out more rapidly in leaves injected with 10  $\mu\text{M}$  T-2 toxin. No lesions appeared at 0.1  $\mu\text{M}$  T-2 toxin after 4 days. Lesion formation in leaves injected with 10  $\mu\text{M}$  T-2 toxin at different time points is shown in Figure 1B. Small, scattered gray spots were observed at 2 days. These spots had spread over entire leaves by 4 days, and the leaf color had turned partially yellow or brown. Then, constriction of trichothecene-injected leaves was observed after 6 days or later (data not shown). When floral tissues of *Arabidopsis* were inoculated with *Fusarium* conidia, the main stem within the flower head constricted and turned gray and brown by 5 days (Urban et al. 2002). Developing siliques also exhibited gray or brown symptoms and appeared slightly shriveled by 5 days. In fact, both inter- and intracellular hypha colonization were observed in stem and silique tissues (Urban et al. 2002). Significant accumulation of the trichothecene was detected in *Fusarium*-invaded flower tissues at 8 days (Urban et al. 2002); therefore, it is likely that the trichothecene accumulated at least partially at 5 days. Thus, trichothecene-induced lesions shared some features of necrotic symptoms by infection of *Fusarium* spp. We also examined the effects of various trichothecenes at 1  $\mu\text{M}$  at 5 day. Lesions formed only when leaves were injected with type A trichothecenes (T-2 toxin, HT-2 toxin, and diacetoxyscirpenol [DAS]) (Fig. 1C). Lesion formations were not observed in DON-infiltrated leaves at this concentration. In addition, other translational inhibitors such as cycloheximide (CHX) did not induce lesion formations in *Arabidopsis* leaves. Therefore, trichothecene-induced lesion formations cannot be explained simply by translational inhibition.



**Fig. 1.** Trichothecene-induced lesion formation in *Arabidopsis* leaves. **A**, Dose response of T-2 toxin-induced lesion formation in *Arabidopsis* leaves at 4 days after injections. **B**, Time course of T-2 toxin-induced lesion formation in *Arabidopsis* leaves. Lower leaves of 4- to 5-week-old *Arabidopsis* plants were infiltrated with 10  $\mu\text{M}$  T-2 toxin and photographed 1 to 5 days later. **C**, Effects of various trichothecenes and other translational inhibitors on lesion formation in *Arabidopsis* leaves at 1  $\mu\text{M}$  after 5 days.

To characterize trichothecene-induced lesions, we examined leaves for cell death (trypan blue staining), deposition of callose (aniline blue staining), and generation of hydrogen peroxide (3,3'-diaminobenzidine [DAB] staining) (Conrath et al. 2002). Small, scattered gray spots were observed in T-2 toxin-infiltrated leaves after 3 days (Fig. 2, first row). These spots contained many dead cells that were stained with lactophenol-trypan blue (Fig. 2, second row). Callose deposition was barely visible in mock-injected leaves, whereas a high amount of callose was present in T-2 toxin-treated leaves (Fig. 2, third row). Staining of hydrogen peroxide by DAB was absent in the mock-infiltrated leaves but was apparent in T-2 toxin-injected leaves (Fig. 2, fourth row). Thus, trichothecene-elicited lesions exhibited dead cells, callose deposition, and generation of hydrogen peroxide. Then, we investigated the fragmentation of nuclear DNA in order to define features of the T-2 toxin-induced cell death. Fragmented DNA was not detected in these leaves even after 6 days. This feature of the type A trichothecene-induced cell death is different from that of apoptotic hypersensitive response (HR)-like cell death (Asai et al. 2000; Mittler and Lamb 1995).

Furthermore, we investigated whether salicylic acid (SA), an inducer of systemic acquired resistance (Alvarez 2000; Cornath et al. 2002), accumulates in T-2 toxin-treated *Arabidopsis* leaves (Fig. 3). The levels of both of free and total SA in T-2 toxin-treated leaves did not differ markedly from those observed in the mock-treated leaves at 6 h after injection. However, both free and total SA accumulated markedly in the T-2 toxin-injected leaves at 48 h after injection. Therefore, we inferred that trichothecene activates SA biosynthesis in *Arabidopsis* plants.



**Fig. 2.** Trichothecene-induced lesions exhibited many dying cells, callose deposition, and generation of hydrogen peroxides. First row, *Arabidopsis* leaves were infiltrated with 1  $\mu\text{M}$  T-2 toxin or carrier and photographed 3 days later. Small, scattered gray spots occurred in T-2 toxin-infiltrated leaves. Second row, dead cells in *Arabidopsis* leaves were stained with trypan blue. Third row, leaves stained with aniline blue, revealing callose deposition. Leaves were examined under UV epifluorescence. Fourth row, hydrogen peroxide was detected as a reddish-brown coloration by endogenous peroxidase-dependent histochemical staining using 3,3'-diaminobenzidine in T-2 toxin-injected leaves.

### Trichothecenes induce MPK6 and p44 MAPK activation in *Arabidopsis* leaves.

Many pathogen-derived molecules are known to induce the activation of *Arabidopsis* 47- and 44-kDa mitogen-activated protein (MAP) kinases (Innes 2001). These MAP kinases were identified as MPK6 and MPK3, respectively. Both MPK6 and MPK3 play pivotal roles in defense signaling, including regulation of cell death in *A. thaliana* (Menke et al. 2004; Ren et al. 2002). We examined whether or not trichothecenes induce activation of these MAP kinases in *Arabidopsis* leaves by in-gel kinase assays using myelin basic protein as a substrate. Activities of two 47- and 44-kDa MAPKs started to increase by 1 h after 1  $\mu$ M T-2 toxin infiltration and peaked by 6 h (Fig. 4A); then they decreased within 12 h (data not shown). These results indicate that trichothecenes induce rapid and prolonged activation of these *Arabidopsis* MAPKs. In addition, we examined the kinase activity of MPK6 by in-gel kinase assay and immunoprecipitation with anti-MPK6 antibody (Desikan et al. 2001; Ichimura et al. 2000). MPK6 was activated by injection of T-2 toxin, suggesting that MPK6 is the T-2 toxin-activated 47-kDa MAPK (Fig. 4B). Furthermore, we investigated whether structural differences among individual trichothecenes influence their ability to activate these MAPKs in *Arabidopsis*

leaves. Type A trichothecenes (T-2 toxin, HT-2 toxin, and DAS) markedly activated these two MAPKs (Fig. 4C). This result closely concurs with that observed in lesion formation in *Arabidopsis* (Figs. 1C and 4C).

### Trichothecenes induce expression of the defense genes in *A. thaliana*.

We analyzed the mRNA levels of *PR-1* and *PDF1.2* genes as representative defense genes in T-2 toxin-treated *Arabidopsis* leaves to test whether trichothecenes induce expression of defense genes. The SA and jasmonic acid/ethylene (JA/ET) signaling pathways are involved in the regulation of *PR-1* and *PDF1.2*, respectively (Conrath et al. 2002; Glazebrook 2001). RNA gel blot analysis showed that the *PR-1* transcripts started to increase after 24 h, reached a maximum after 48 h, and began to decline 72 h after injection (Fig. 5A). This kinetics is consistent with those of the accumulation of SA in T-2 toxin-injected leaves (Fig. 3). *PDF1.2* mRNA accumulated between 48 and 72 h after injection (Fig. 5A). In addition, its level was high at 1 h and had diminished by 3 h after injection. To elucidate the signaling pathways involved in trichothecene-inducible expression of these defense genes, we analyzed the expression of these genes in SA-, JA-, and ET-related mutants or SA-depleted *NahG* transgenic plants 48 h after T-2 toxin application (Fig. 5B). Accumulation of *PR-1* mRNA by T-2 toxin was reduced significantly in the SA-insensitive mutants *npr1-2* and *npr1-5* and in the SA-depleted *NahG* plants. This result suggests that induction of the *PR-1* gene was regulated by accumulation of SA in response to T-2 toxin (Fig. 3). In contrast to these plants, the *PR-1* gene expression was higher in the *jar1* and ET-related mutants. The *PDF1.2* mRNA level in response to T-2 toxin was reduced significantly in these JA- and ET-related mutants (Fig. 5B), indicating that the induction of *PDF1.2* gene by T-2 toxin was depend on both JA- and ET-dependent signaling pathways. In addition, the induction of *PDF1.2* gene by T-2 toxin was enhanced in the two SA-insensitive mutants and the *NahG* plants (Fig. 5B). These results suggest that cross talk between these signaling pathways also was observed in trichothecene-inducible expression of defense genes (Kunkel and Brooks 2002).

T-2 toxin, HT-2 toxin, and DAS induced the expression of *PR-1* gene in *Arabidopsis* leaves (Fig. 5C). On the other hand, the *PDF1.2* gene was induced in leaves treated with T-2 toxin, T-2 triol, T-2 tetraol, and DAS. Therefore, induction of the *PR-1* gene by each trichothecene molecular species correlated well with the activation of MAP kinases and lesion formation. (Figs. 1C, 4C, and 5C).

### Induction of the elicitor-like signaling pathway by DON was observed only at higher concentrations.

A concentration of 1  $\mu$ M DON failed to induce the activities of two MAPKs and the expression of defense genes (Figs. 4 and 5). We investigated whether DON has the elicitor-like activity at higher concentration. Apparent activation of two MAPKs by DON occurred only at a concentration of 100  $\mu$ M, although 47-kDa MAPK was weakly activated by injection of 10  $\mu$ M DON (Fig. 6A). Similarly, induction of the *PR-1* gene was observed only in 100  $\mu$ M DON-infiltrated leaves (Fig. 6B). Correspondingly, DON did not cause lesion formation at 10  $\mu$ M but did so at 100  $\mu$ M (data not shown). Thus, the elicitor-like activity by DON was observed only at high concentration in *A. thaliana*.

### Trichothecenes inhibit protein synthesis in *Arabidopsis* cells.

We examined the effects of trichothecenes on protein synthesis in *Arabidopsis* suspension-cultured cells. Protein syn-

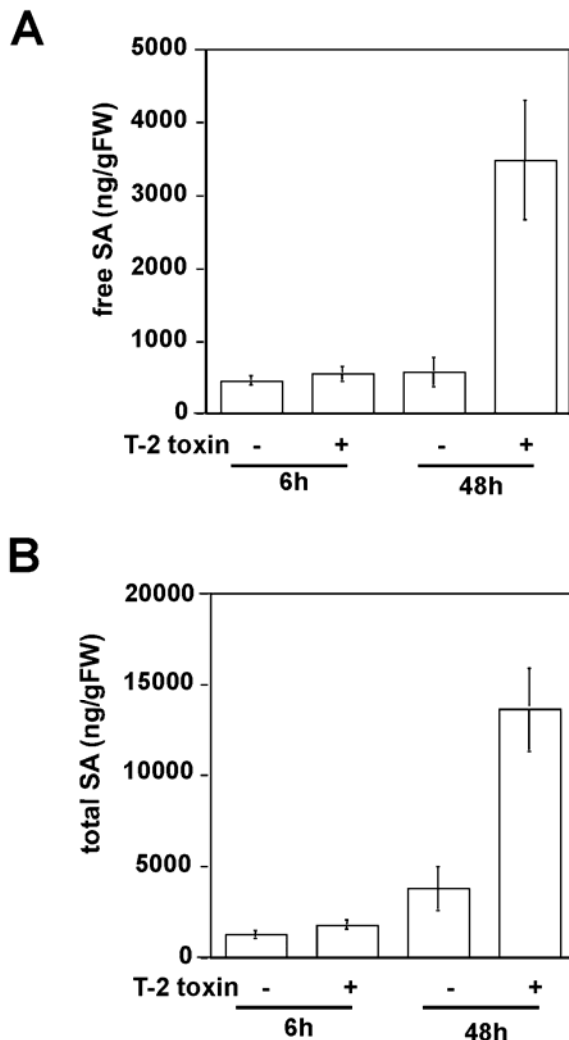


Fig. 3. Trichothecene activates the biosynthesis of salicylic acid (SA) in *Arabidopsis* leaves. *Arabidopsis* leaves were harvested at the indicated times after treatment with 1  $\mu$ M T-2 toxin or carrier. **A**, Free SA and **B**, total SA levels were quantified by high-performance liquid chromatography (mean  $\pm$  standard deviation,  $n = 4$ ).

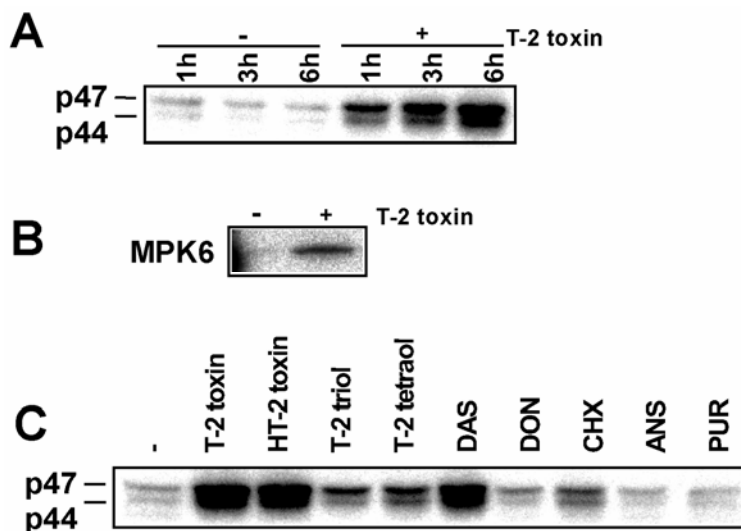
thesis was estimated as incorporation of [<sup>3</sup>H]-leucine into protein extracted from *Arabidopsis* cells (Kodama et al. 1991). We first examined the effects of T-2 toxin (type A) and DON (type B). T-2 toxin decreased protein synthesis to approximately 30% of that in control cells at 1 μM, to approximately 10% at 10 μM, and to 0% at 100 μM (Fig. 7A). On the other hand, DON did not inhibit protein synthesis at 1 μM, but did so at concentrations above 5 μM (Fig. 7A). Because the elicitor-like activity by DON was not observed at a concentration of 10 μM in *Arabidopsis* leaves (Fig. 6), DON is capable of inhibiting translation in *Arabidopsis* cells without induction of the elicitor-like signaling pathway. We also investigated the effects of six trichothecene molecular species on translation in *Arabidopsis* cells at 1 μM. T-2 toxin, HT-2 toxin, and DAS effectively inhibited protein synthesis at this concentration in *Arabidopsis* cells (Fig. 7B). These results indicate that structural differences between individual trichothecenes can markedly influence their ability to inhibit translation in *Arabidopsis* cells at low concentrations. The effects of other translational inhibitors at concentrations of 1 μM in *Arabidopsis* cells also are shown in Figure 7B (Iordanov et al. 1997). CHX apparently inhibited protein synthesis (Fig. 7B), whereas it failed to induce the elicitor-like signaling pathway (Figs. 1C, 4C, and 5C). On the other hand, both anisomycin and puromycin had only minor effects on translation at this concentration.

## DISCUSSION

In this study, we showed that pure trichothecenes have an elicitor-like activity, including activation of MAPKs, induction of defense genes, accumulations of SA and reactive oxygen species, and lesion formations (Figs. 1 to 5). Among various trichothecenes, some type A trichothecenes were potent in activating the elicitor-like signaling pathway at low concentrations. Type A-producing fungi such as *F. sporotrichioides* often were observed in various cereal crops and processed grains. In addition, Perkowski and associates (1997) analyzed natural contamination of barley kernels colonized by *F. sporotrichioides* with type A trichothecenes. Among 24 samples analyzed, 12 were T-2 toxin positive in a range of contamination from 0.02

to 2.4 ppm (average 0.45). In this study, the amounts of T-2 toxin-infiltrated leaves at 1 μM correspond to approximately 0.6 ppm. Therefore, it seems reasonable to suppose that the amount of trichothecene being injected in this study mimics that which is found in the host plant infected by *Fusarium* spp. Type A trichothecenes caused cell death within 3 days after infiltration (Fig. 2A). However, DNA fragmentation was not observed even after 6 days (data not shown). Although light illumination is required for formation of HR-like lesions (Genoud et al. 1998; Stone et al. 2000), type A trichothecene-induced lesions also were observed in the dark (data not shown). Taken together, cell death elicited by type A trichothecenes similar to cell death in compatible interaction rather than cell death associated with the HR. Asai and associates (2000) reported that HR-like cell death elicited by fumonisin B1 phytotoxins required JA-, ET-, and SA-dependent signaling pathways in *Arabidopsis* protoplasts. In addition, leaves infiltrated with fumonisin B1 in SA-depleted *NahG* plants produced fewer lesions than the corresponding wild-type plants. In contrast, trichothecene-inducible lesions also were formed in all mutants examined here or in the SA-depleted *NahG* transgenic plants (data not shown). This result implied that trichothecene-induced lesion formations occurred apart from these defense-signaling pathways. Therefore, it is at least possible that type A trichothecene-induced cell death could have very little to do with host defense pathways mediated by SA, JA, and ET, and is contributing directly to virulence of necrotrophic phytopathogens.

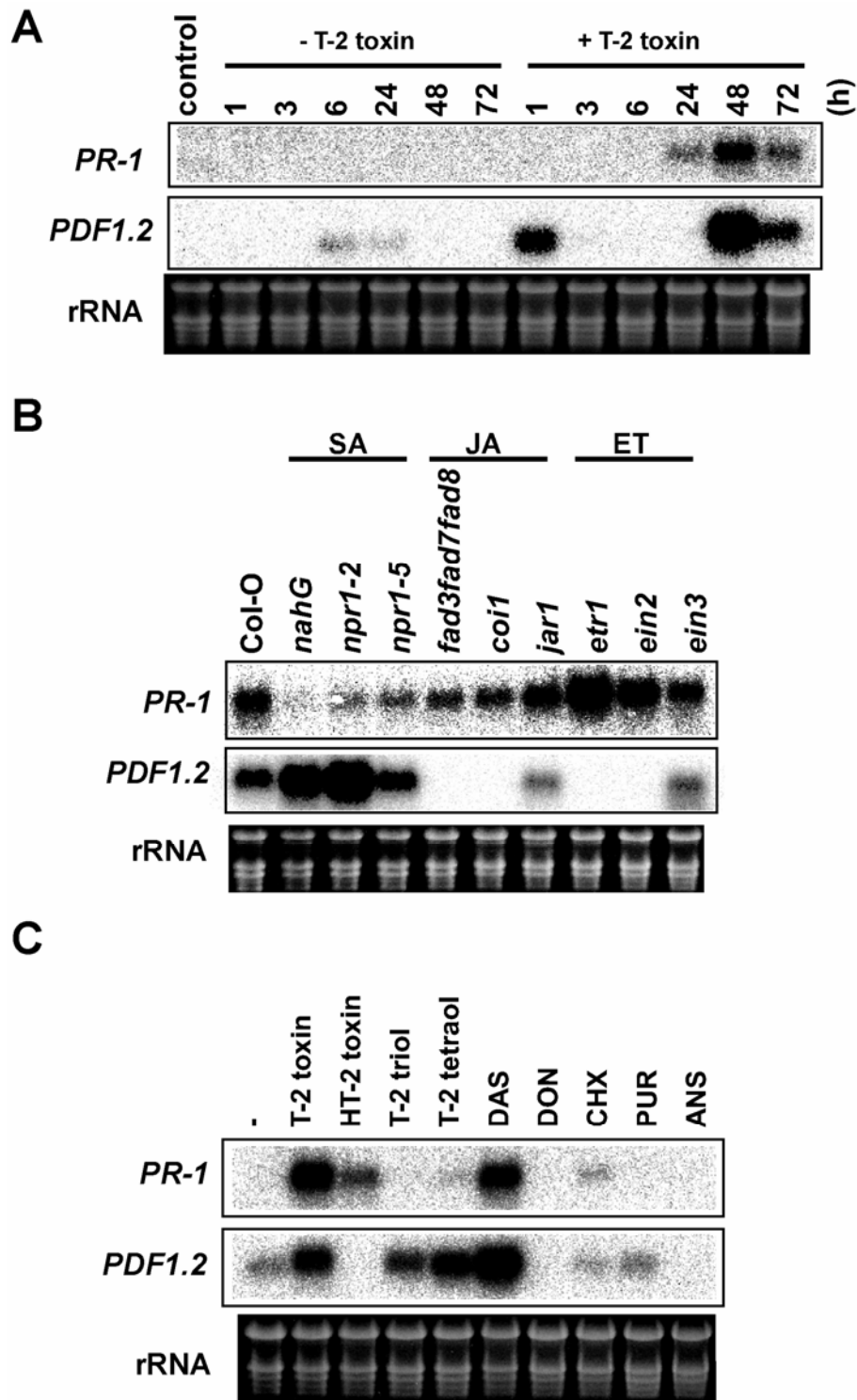
Urban and associates (2002) reported that DON was detected in *F. graminearum*- or *F. culmorum*-invaded flower tissues at 8 days postinoculation. On these *Fusarium*-infected flowers, the recovered values of DON ranged between 1.21 and 2.27 ppm, corresponding to between 3.2 and 6.0 μM in DON-injected leaves of this study. In this article, we showed that 5 μM DON apparently inhibited protein synthesis in *Arabidopsis* cells (Fig. 7A). On the other hand, 10 μM DON failed to induce the elicitor-like signaling pathway (Fig. 6). Therefore, DON-producing *Fusarium* spp. may affect translational systems in host plants without induction of the defense response. On the contrary, defense-related genes such as patho-



**Fig. 4.** Trichothecenes activate MPK6 and p44 MAPK in *Arabidopsis* leaves. **A**, Treatment of *Arabidopsis* with T-2 toxin induces rapid and prolonged activation of 47- and 44-kDa mitogen-activated protein (MAP) kinases. Protein extracts were prepared from leaves treated with 1 μM T-2 toxin (+) or EtOH (-). The resulting protein (20 μg) was assayed for kinase as described in Experimental Procedures. **B**, MPK6 was activated by T-2 toxin. Protein extracts were prepared from *Arabidopsis* leaves at 6 h after treatment with 1 μM T-2 toxin or EtOH. Protein kinase activity of MPK6 was assayed by in-gel kinase assay and immunoprecipitation with anti-MPK6 antibody. **C**, Type A trichothecenes (T-2 toxin, HT-2 toxin, and diacetoxyscirpenol [DAS]) markedly activated these two MAPKs in *Arabidopsis* leaves. Leaves were collected at 6 h after incubation at 1 μM in each inhibitor treatment. DON = deoxynivalenol; CHX = cycloheximide; ANS = anisomycin; and PUR = puromycin.

genesis-related genes were induced in wheat spikes infected by *F. graminearum* (Li et al. 2001; Pritsch et al. 2000). It may be that DON production was not the cause for the induction of these defense genes in wheat spikes during *Fusarium* infec-

tion. It was known that non-DON-producing *F. graminearum* lines are less virulent (Proctor et al. 1995). Bai and associates (2002) reported that DON production is not necessary for initial infection by *F. graminearum*, but is important for disease



**Fig. 5.** Treatment of *Arabidopsis* with trichothecenes induces expression of *PR-1* and *PDF1.2* genes. **A**, Time-course of induction of *PR-1* and *PDF1.2* by 1  $\mu$ M T-2 toxin in *Arabidopsis* leaves. Total RNA (5  $\mu$ g) was prepared from *Arabidopsis* leaves at indicated times after treatment with 1  $\mu$ M T-2 toxin or carrier. RNA blots were hybridized to  $^{32}$ P-labeled probes specific to *PR-1* and *PDF1.2*. Ethidium bromide staining of rRNA confirmed the equivalence of RNA loading. **B**, Effects of various defense-related mutants on T-2 toxin-inducible expression of *PR-1* and *PDF1.2*. Total RNA (5  $\mu$ g) was isolated from wild-type (Col-0) or mutant plants at 48 h after T-2 treatment. Accumulation of *PR-1* mRNA in response to T-2 toxin was reduced markedly in the salicylic acid (SA)-insensitive mutants (*npr1-2* and *npr1-5*) and SA-depleted *NahG*. On the other hand, the *PDF1.2* mRNA level in response to T-2 toxin was reduced markedly in a jasmonic acid (JA)-deficient mutant (*fad3fad7fad8*), two JA-insensitive mutants (*jar1-1* and *coi1-1*), and three ethylene (ET)-insensitive mutants (*etr1-1*, *ein2-1*, and *ein3-1*). **C**, Effects of various trichothecenes on the induction of *PR-1* and *PDF1.2*. Total RNA (5  $\mu$ g) was prepared from *Arabidopsis* leaves at 48 h after treatment with various trichothecenes or translational inhibitors at 1  $\mu$ M. DON = deoxynivalenol.

spread in wheat spike. Therefore, *Fusarium* spp. might utilize DON as a non-defense-inducing translational inhibitor during disease spread in host plants. Trichothecene-producing *Fusarium* spp. have strain-specific trichothecene metabolite profiles (Ward et al. 2002). It is thought that trichothecene chemotype may play a role in the phytopathogenicity of individual *Fusarium* strains. This study suggests that the role of type B trichothecene in a virulence of their producing fungi is different from that of type A trichothecene.

We also showed that structural differences between individual trichothecenes markedly influence their ability to inhibit translation and to activate an elicitor-like signaling pathway in *Arabidopsis* cells (Figs. 1C, 4C, 5C, and 7B). Among T-2 toxin derivatives, the HT-2 toxin's structure is very similar to that of T-2 triol, but the effects of these two molecules on translational inhibition differ distinctly (Fig. 7B). HT-2 toxin and T-2 triol are distinguished by the presence and absence, respectively, of an acetyl group at the C<sub>15</sub> position, suggesting that that acetyl group is important for the binding of T-2 toxin derivatives to eukaryotic ribosomes. On the other hand, acetylation at the C<sub>3</sub> position of trichothecene acts as a detoxification between yeast and fungi (Kimura et al. 1998). The *Tri101* gene, encoding trichothecene 3-*O*-acetyltransferase, is responsible for this acetylation step in *F. graminearum* (Kimura et al. 1998; Muhitch et al. 2000). In fact, expression of *Tri101* in *Arabidopsis* plants abolished trichothecene cytotoxicity (*unpublished results*).

We revealed that application of T-2 toxin engenders marked accumulation of SA and SA glucoside (SAG) in *Arabidopsis* leaves after 48 h (Fig. 3). Correspondingly, the maximum induction of the *PR-1* occurred in the T-2 toxin-infiltrated leaves after 48 h (Fig. 5A). We also showed that induction of the *PR-1* gene was enhanced in the ET-related mutants (Fig. 5A). Therefore, it may be that trichothecene-inducible expression of the *PR-1* gene is controlled by negative interaction between the SA and ET signaling pathways. Similarly, Lawton and associates (1994) reported that the *PR-1* expression was significantly elevated in the *ein2* mutant. It is possible that JA- and ET-dependent signaling pathways regulate the induction of *PDF1.2* by T-2 toxin (Fig. 5B). In addition, T-2 toxin-inducible expression of *PDF1.2* may be enhanced in *npr1* mutants and *NahG* plants (Fig. 5B). Many studies provide evidence for an antagonistic effect of SA on JA signaling (Kunkel and Brooks 2002). Therefore, the induction of *PDF1.2* by trichothecenes also regulated a negative interaction of SA and JA signaling.

Trichothecenes act as translational inhibitors; therefore, it generally has been thought that trichothecenes suppress the defense response in host plants. However, this study has revealed that some type A trichothecenes such as T-2 toxin induced lesion formation, callose deposition, generation of hydrogen peroxide, and accumulation of SA. In addition, these trichothecenes activate defense-related MAPKs and the expression of defense genes. Thus, these type A trichothecenes induce an elicitor signal transduction pathway in *A. thaliana*. It has been known that diverse pathogen-derived molecules induce the highly conserved signaling pathway mediating plant defense response (Nürmberger et al. 2004). These elicitors include peptides, glycoprotein, lipids, oligosaccharides, and so on. Here, this article indicated that trichothecenes also act as such an elicitor in *A. thaliana*. Inhibition of protein synthesis by CHX is equivalent to that observed by T-2 toxin in *Arabidopsis* cells at the same concentration, but CHX did not elicit lesion formation, activation of MAPKs, or induction of *PR-1* (Figs. 1C, 4C, 5C, and 7B). A plausible explanation for these observations is that other effects occurring along with translational inhibition by trichothecenes might activate an elicitor-like signaling pathway

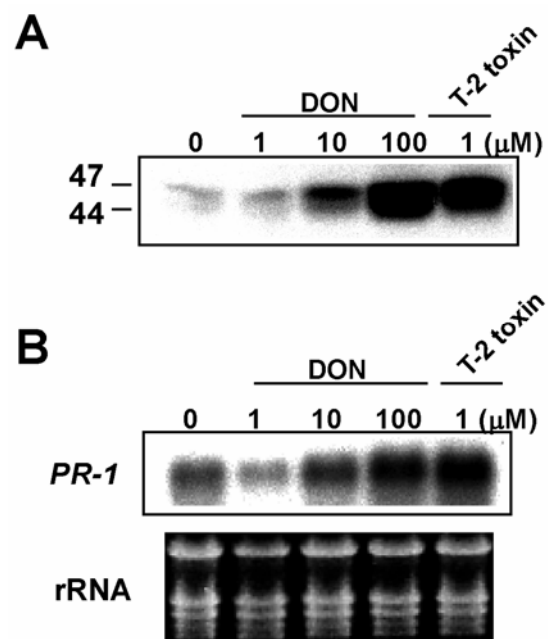
in *Arabidopsis*. For instance, unidentified trichothecene-binding receptors might function upstream of a trichothecene-inducible signaling pathway in *Arabidopsis*.

Among these signaling events, it is well known that many elicitors commonly activate MPK6 and MPK3 in *Arabidopsis* (Innes 2001). In fact, this MAPK signaling cascade plays a key role of these defense responses. In this study, we showed that trichothecene also induced rapid and prolonged activation of both MPK6 and a 44-kDa MAPK (probably MPK3) in *Arabidopsis* leaves (Fig. 4). Asai and associates (2002) identified a complete MAP kinase cascade (MEKK1, MKK4/MKK5, and MPK3/MPK6) and WRKY29 transcription factors that function downstream of flagellin receptor FLS2, a leucine-rich repeat (LRR) receptor kinase in *Arabidopsis*. Similarly, our microarray analysis revealed that *WRKY29* responds early to trichothecene application (T. Nishiuchi and K. Yamaguchi, *unpublished data*). For this reason, signaling components that function downstream of these MAPK signaling cascades might at least partially overlap flagellin and trichothecene. On the other hand, some trichothecenes also act as potent activators of MAPK (JNK and p38 MAPK) signaling and apoptotic cell death in vertebrate cells (Nagase et al. 2001; Shifrin and Anderson 1999; Yang et al. 2000). Therefore, trichothecenes are capable of activating the MAPK cascade and leading to cell death in both plants and animals (Figs. 1, 2, and 4). Furthermore, recent studies suggest the functional similarity between animal JNK/p38 MAPK and *Arabidopsis* MPK3/MPK6 or their plant orthologs (Caffrey et al. 1999; Ren et al. 2002).

## MATERIALS AND METHODS

### Plant materials and growth conditions.

*A. thaliana* Heynh. plants were grown in soil with supplementary light on a cycle of 16 h of light and 8 h of dark at 22°C. All plants used in this study were ecotype Columbia (Col-0) and its derivatives, except for the *npr1-5* mutant (ecotype Nössen). The wild-type (Nössen) also expressed defense



**Fig. 6.** Induction of the defense signal transduction pathway by deoxynivalenol (DON) was observed only at the concentration of 100  $\mu$ M. **A**, Dose response of DON-induced MAPK activation in *Arabidopsis* leaves at 6 h after injections. **B**, Dose response of DON-induced *PR-1* expression in *Arabidopsis* leaves at 48 h after injections.

genes in response to trichothecene (data not shown). *Arabidopsis* Col-0 *NahG* transgenic plants (Lawton et al. 1996) were obtained from L. Friedrich (Syngenta-Biotechnology, Raleigh, NC, U.S.A.). Similar results also were obtained with other Col-0 *NahG* transgenic lines obtained from F. M. Ausubel (data not shown). Lines *npr1-2* (Glazebrook et al. 1996), *npr1-5* (Shah et al. 1997), *jar1-1* (Staswick et al. 1992), *etr1-1* (Bleeker et al. 1988), *ein2-1* (Guzman and Ecker 1990), and *ein3-1* (Roman et al. 1995) were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, U.S.A.). The *coil-1* mutant was obtained from J. G. Turner (Feys et al. 1994). The *fad* triple (*fad3-2 fad7-2 fad8*) mutant was obtained from J. Browse (McConn and Bowse 1996).

#### Treatment with trichothecenes.

Each solution containing a trichothecene or other protein synthesis inhibitor was infiltrated into lower leaves of 4- to 5-

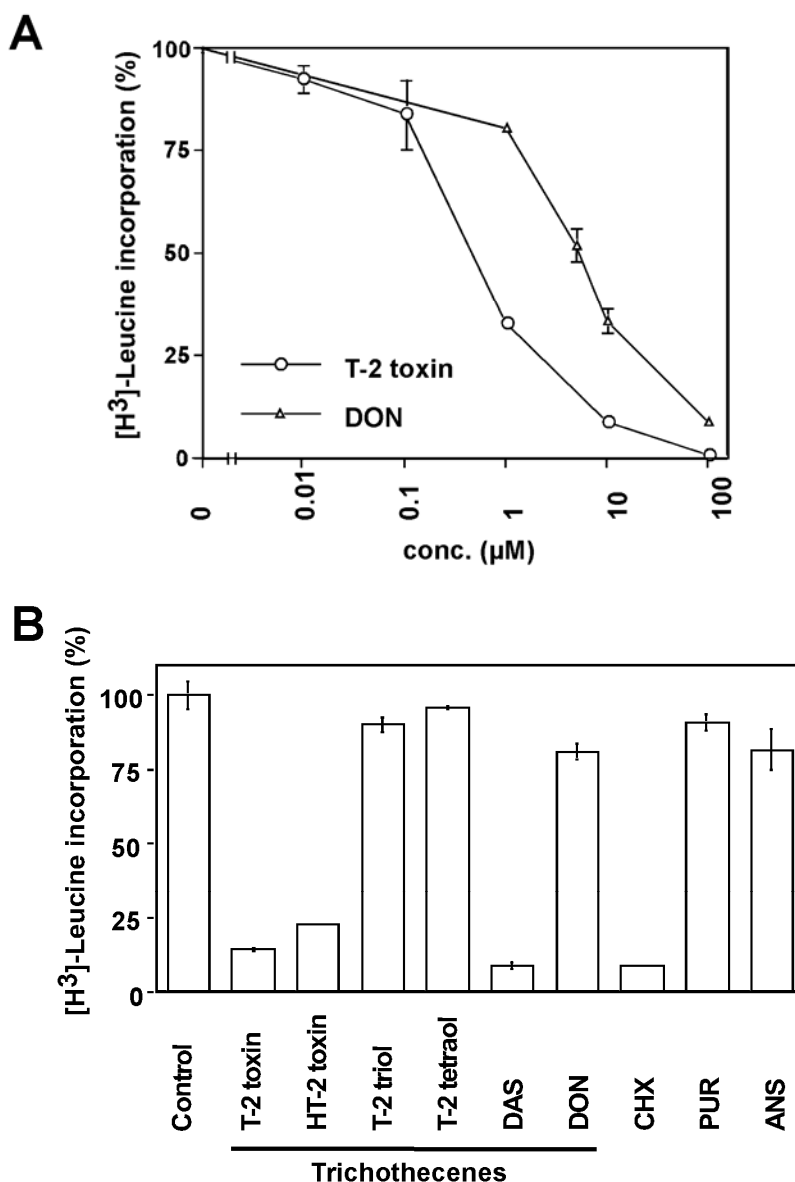
week-old *Arabidopsis* plants with a 1-ml syringe. As a negative control, we used solutions containing ethanol solvent alone.

#### Histochemistry and microscopy.

We used a microscope (BX-50; Olympus Optical, Tokyo) with a built-in BX-FLA epifluorescent unit. Trypan blue (Bowling et al. 1997) stained dead cells blue. Hydrogen peroxides were detected as a reddish-brown coloration by endogenous peroxidase-dependent histochemical staining using DAB (Ren et al. 2000). Callose was stained with aniline blue as described by Stone and associates (2000). The stained leaves were observed under epifluorescence illumination (excitation at 395 nm, emission at 495 nm).

#### SA measurement.

SA and SAG levels in trichothecene-treated or mock-treated leaves were measured as described previously (Nakashita et al. 2002; Yasuda et al. 2003).



**Fig. 7.** Inhibition of protein synthesis by various trichothecenes in *Arabidopsis* cells. **A**, Inhibition of protein synthesis by type A trichothecene (T-2 toxin) and type B trichothecene (deoxynivalenol [DON]). Protein synthesis was assayed by measuring the incorporation of [<sup>3</sup>H]leucine into proteins of *Arabidopsis* suspension-cultured cells, as described in Experimental Procedures. Values are mean  $\pm$  standard deviation (SD) ( $n = 3$ ). **B**, Effects of various trichothecenes and other translational inhibitors on protein synthesis in *Arabidopsis* cells at 1  $\mu$ M. Data are mean  $\pm$  SD ( $n = 3$ ). DAS = diacetoxyscirpenol; CHX = cycloheximide; ANS = anisomycin; and PUR = puromycin.



### In-gel kinase assay.

Crude extracts were prepared from trichothecene-treated or mock-treated leaves and subjected to in-gel kinase assay, as described by Romeis and associates (1999). The concentration of protein in the extracts was estimated with a protein assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) with  $\gamma$ -globulin as the standard. Aliquots of crude extract were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in an 11.25% polyacrylamide gel that had been polymerized with MBP at 0.25 mg ml<sup>-1</sup> (Sigma-Aldrich, St. Louis). After the sample was electrophoresed, the SDS was removed. The proteins in the gel were denatured, then renatured. The gel then was incubated for 90 min in a solution containing 200 nM ATP (1.85 MBq [ $\gamma$ -<sup>32</sup>P]ATP) (Amersham Biosciences, Piscataway, NJ, U.S.A.). The gel was washed extensively, dried, and autoradiographed. The molecular mass of the protein kinase detected on SDS-polyacrylamide gels was estimated with a Rainbow Marker kit (Amersham Biosciences). The protein kinase activity of MPK6 was assayed by in-gel kinase assay coupled with immunoprecipitation, as described previously (Ichimura et al. 2000).

### RNA gel blot analysis.

Total RNA was prepared from *Arabidopsis* leaves by guanidine hydrochloride-phenol-chloroform extraction as described previously (Fukuda et al., 1991). Total RNA (5  $\mu$ g) was denatured, separated by electrophoresis on formaldehyde-agarose gels, and then transferred to a Hybond-N+ nylon membrane (Amersham Biosciences). RNA blots were hybridized to <sup>32</sup>P-labeled probes specific for *PR-1* (Rogers and Ausubel 1997) and *PDF1.2* (Penninckx et al. 1996). Probes were prepared by amplification of appropriate sequences from genomic DNA by polymerase chain reaction, purification of the products on agarose gels, and labeling them by use of Ready-To-Go DNA labeling beads (Amersham Biosciences). Primers used for amplification of *PDF1.2* were KGTP5 (sense, 5'-ACG-CAC-CGG-CAA-TGG-TGG-AAG-CAC-AGA-AGT-3') and KGTP6 (anti-sense, 5'-AAT-ACA-CAC-GAT-TTA-GCA-CCA-AAG-ATTA-3'); those for amplification of *PR-1* were described by Rogers and Ausubel (1997).

### Inhibition of protein synthesis by trichothecenes in *Arabidopsis* suspension cells.

Protein synthesis was assayed by measuring the amount of [<sup>3</sup>H]leucine (Amersham Biosciences) incorporated into cellular protein, essentially as described by Kodama and associates (1991). *Arabidopsis* suspension-cultured cells (1 ml, cell line T87) (Axelos et al. 1992) were transferred to 12-well plates, incubated for 60 min at 22°C, and then treated in triplicate with protein synthesis inhibitors for 60 min. Then, [<sup>3</sup>H]leucine (37 kBq) was added to 1 ml of suspension culture, which was incubated for 60 min at 22°C. Proteins in these suspension cells were solubilized using hot 1 M NaOH. Then, incorporation of [<sup>3</sup>H]leucine into these cellular proteins was measured with a liquid scintillation counter.

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