Differential Induction of the *Arabidopsis thaliana* *Thi2.1* Gene by *Fusarium oxysporum* f. sp. *matthiolae*

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The *Arabidopsis thaliana* *Thi2.1* gene is inducible by necrotrophic fungi through a signal transduction pathway different from that for pathogenesis-related (PR) proteins. We have identified three ecotypes that are susceptible (Col-2, Ler, and Ws) and two ecotypes that are resistant (Mt-0 and Uk-4) to spray inoculation with *Fusarium oxysporum* f. sp. *matthiolae*. The *Thi2.1* transcript level after infection correlates with resistance, being 5 to 10 times higher in the resistant than in the susceptible ecotypes. The β -glucuronidase (GUS) expression of a *Thi2.1*-promoter-*uidA* fusion (with a promoter derived from Col-2) is on the average almost 10 times higher in the Uk-4 background than in the Col-2 background. This confirms the results obtained by Northern (RNA) blots and indicates that Uk-4, and probably other resistant ecotypes too, might have a more sensitive recognition system for *F. oxysporum* f. sp. *matthiolae* or might have a signal transduction system that gives a higher amplification of the original recognition signal. Our results suggest a role of the *Thi2.1* gene in resistance against *F. oxysporum* f. sp. *matthiolae* and perhaps other necrotrophic fungi.

Additional keywords: antimicrobial, cysteine-rich.

Plants have evolved different mechanisms against the constant threat by phytopathogenic microorganisms. These include preformed physical barriers and antimicrobial compounds (Ride 1985) as well as inducible defense compounds that are formed after infection by pathogens. Well-known examples of the latter group include phytoalexins (Dixon 1986) and pathogenesis-related (PR) proteins (Linthorst 1991). During recent years it has also become evident that different families of small, cysteine-rich, basic polypeptides play a role in the defense of higher eukaryotes against microorganisms, ranging from insects and vertebrates (Hoffmann 1995; Hancock et al. 1995; Nicolas and Mor 1995) to plants. One of these protein families consists of thionins. Thionins are antimicrobial polypeptides, with a molecular mass of 5 kDa, that are also basic and cysteine-rich. They have been known for several decades from different plant species (for a review see

Garcia-Olmedo et al. 1989; Bohlmann and Apel 1991; Bohlmann 1994; Florack and Stiekema 1994). In vitro toxicity of wheat purothionins against phytopathogenic bacteria led Fernandez de Caley et al. (1972) to propose a role of thionins in plant defense. Such in vitro effects on different phytopathogenic microorganisms have subsequently been demonstrated by several authors (Bohlmann et al. 1988; Florack et al. 1993; Molina et al. 1993; Terras et al. 1996). Concordant with a role in plant defense, an induction of thionin genes after pathogen attack has been documented in barley and *Arabidopsis thaliana* (Bohlmann et al. 1988; Titarenko et al. 1993; Boyd et al. 1994; Valè et al. 1994; Epple et al. 1995). The accumulation of barley leaf thionins after mildew infection was also investigated by immunogold labeling. In an incompatible interaction, higher concentrations of thionins were found in papillae and in the cell wall surrounding the infection peg. In a compatible interaction, only trace amounts of thionins could be detected in the corresponding regions (Ebrahim-Nesbat et al. 1989, 1993). Furthermore, it has been shown that the expression of a hordothionin in transgenic tobacco can lead to enhanced resistance against a phytopathogenic bacterium (Carmona et al. 1993). In similar experiments, however, Florack et al. (1994) did not find enhanced resistance in transgenic tobacco lines expressing alpha-hordothionin.

We have recently shown that inoculation of the *A. thaliana* ecotype Col-2 results in a strong induction of the *Thi2.1* gene (Epple et al. 1995) via a signal transduction pathway different from that for PR proteins. Here we report the identification of ecotypes that are either susceptible or resistant to this fungus. We observed that the induction of the *Thi2.1* gene is stronger in resistant ecotypes, suggesting a role of this gene in the resistance against *Fusarium oxysporum* f. sp. *matthiolae*. This supports our previous finding that overexpression of this thionin in the susceptible ecotype Col-2 gives enhanced resistance against *F. oxysporum* f. sp. *matthiolae* (Epple et al. 1997b).

RESULTS

We have previously shown that inoculation of *A. thaliana* seedlings with *F. oxysporum* f. sp. *matthiolae* induces expression of the *Thi2.1* gene in the susceptible ecotype Col-2 (Epple et al. 1995). It was therefore of interest to compare the induction of this gene in other susceptible and resistant ecotypes. In addition to the originally used Col-2, Ws and Ler were also found to be susceptible, whereas Uk-4 and Mt-0

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turned out to be resistant against *F. oxysporum* f. sp. *matthiolae*. As shown in Figure 1 (left column, magnifications in the middle column), the susceptible ecotypes allowed considerable fungal growth that is macroscopically detectable and were visibly damaged whereas the resistant ecotypes were much more healthy without fungal growth being visible to the naked eye. To quantify the fungal growth on the different ecotypes, seedlings were harvested 8 days post infection (dpi) and stained for fungal hyphae (Keogh et al. 1980). Figure 1 (right column) shows representative trypan blue stainings from cotyledons of the different ecotypes 8 dpi. Cotyledons were evaluated for the presence of fungal hyphae and assigned to different infection classes. Their distribution correlates with the resistance of the ecotypes as shown in Figure 2. The cotyledons harvested from the susceptible ecotypes are usually totally covered by fungal hyphae and most of them belong to the infection class 3. Many of these cotyledons are heavily damaged by the fungus (data not shown). The cotyledons of the resistant ecotypes support much less fungal growth and are mostly assigned to infection class 1. The disease index calculated from these data is almost 3 for the susceptible ecotypes and less than 2 for the resistant ecotypes.

The three susceptible ecotypes Col-2, Ler, and Ws and the resistant ecotypes Mt-0 and Uk-4 were further investigated for the expression of thionin genes and other putative defense related genes in response to infection with *F. oxysporum* f. sp. *matthiolae*. Total RNA was prepared from seedlings harvested 1, 2, 3, 4, and 5 dpi. Figure 3A shows representative Northern blots hybridized with a *Thi2.1* probe and the corresponding quantifications from a PhosphoImager. It is evident that the *Thi2.1* gene is expressed earlier in the resistant ecotype Mt-0, already at 1 dpi. Furthermore, the expression level is also significantly higher in the resistant ecotypes than in the susceptible ecotypes. While resistance correlates with the expression of the *Thi2.1* gene, the same does not hold for the expression of other defense-related genes, as shown in Figure 3B. *PR-1* and *PR-5* transcript levels are higher post infection but no correlation with resistance is evident. The same is true for the plant defensin gene *Pdfl.2*, which is inducible by necrotrophic fungi via a signal transduction pathway that is also salicylate independent (Penninckx et al. 1996; Eppe et al. 1997a), as in the case of *Thi2.1*. Mock inoculations showed no induction of the genes under investigation (data not shown).

There could be several reasons for the enhanced expression of the *Thi2.1* gene in the resistant ecotypes. One possibility could be that these ecotypes have multiplied the *Thi2.1* gene. This was tested by Southern blots with DNA from the different ecotypes. With three different restriction enzymes only single bands were detected in all cases, as shown in Figure 4. Thus, all ecotypes under study in this work have most probably only single copies of the *Thi2.1* gene.

Differences in the expression of the *Thi2.1* gene could also originate from mutations in the promoter resulting in enhanced expression in the resistant ecotypes. This could be tested by cloning and sequencing the *Thi2.1* genes from these ecotypes. We have chosen a different approach to address this problem. The promoter of the *Thi2.1* gene (derived from the susceptible ecotype Col-2) was isolated and fused to a *uidA* reporter gene (A. Vignutelli, C. Wasternack, K. Apel, and H. Bohlmann, *manuscript submitted*). This construct was used to establish transgenic lines of the susceptible ecotype Col-2 and

the resistant ecotype Uk-4 (see Materials and Methods). Twenty-four kanamycin-resistant Col-2 lines and 23 Uk-4 lines were obtained. The T₂ generation was grown again on MS agar plates supplemented with 50 µg of kanamycin per ml for 14 days. Twenty Col-2 and 16 Uk-4 lines remained kanamycin resistant and were tested in parallel for β-glucuronidase (GUS) activity after inoculation with the same *F. oxysporum* f. sp. *matthiolae* spore suspension. As shown in Figure 5, several Uk-4 lines have very high GUS activities after inoculation, much higher than those of well-responding Col-2 lines. The mean value of GUS activity for all transgenic Uk-4 lines is compared with the mean value from the corresponding Col-2 lines in Table 1. The GUS activity on average is almost nine times higher in Uk-4 lines than in Col-2 lines carrying the same *Thi2.1* promoter-GUS construct. The difference in promoter activity is similar to the different levels of *Thi2.1* transcripts in the two ecotypes. Thus, the observed differences in the expression level of the *Thi2.1* gene after infection with *F. oxysporum* f. sp. *matthiolae* are most probably due to differences in perception of the pathogen and/or differences in the signal transduction pathway connecting this perception with the expression of the *Thi2.1* gene.

DISCUSSION

In the work presented in this paper we have identified susceptible and resistant *A. thaliana* ecotypes for *F. oxysporum* f. sp. *matthiolae*. Seedlings grown on MS agar were about 12 days old when they were infected by spraying with a fungal spore suspension. Under our growth conditions seedlings at this point had only small, developing primary leaves. We therefore evaluated the cotyledons for fungal growth by performing a trypan blue staining. This procedure (Keogh et al. 1980) stains fungal hyphae and necrotic plant cells. Fungal growth was grouped into four different infection classes. Evaluation of randomly harvested cotyledons gave a measure for the resistance of the different lines (Fig. 2) and confirmed the visual appearance (Fig. 1). Mauch-Mani and Slusarenko (1994) have also identified resistant and susceptible *A. thaliana* ecotypes to leaf inoculation with an *F. oxysporum* strain that they isolated from naturally infected *A. thaliana* plants. It is not known how their isolate is related to the strain that we have used in the present work.

The Northern blot studies revealed a clear correlation between resistance and the expression of the *Thi2.1* gene (Fig. 3). The expression in resistant ecotypes was 5 to 10 times higher than that in susceptible ecotypes. Expression of *PR-1* and *PR-5* genes, on the other hand, showed no correlation with resistance against this fungus. The *PR-5*-specific probe detected two bands on the Northern blots. An explanation for this might be that, in addition to the original *PR-5* gene, other related genes are also induced by *F. oxysporum* f. sp. *matthiolae*. The expression of the *Pdfl.2* gene also showed no correlation with resistance. *Pdfl.2* (Penninckx et al. 1996; Eppe et al. 1997a) and *Thi2.1* (Eppe et al. 1995) are both inducible by jasmonate and necrotrophic fungi via signal transduction pathways that are salicylate independent. It is currently unknown to what extent these pathways overlap.

Southern blots indicated a single *Thi2.1* gene in all ecotypes. This excludes the possibility that the higher expression level in the resistant ecotypes is simply due to gene amplifi-

Fig. 1. Fungal growth on resistant and susceptible ecotypes. Col-2 (A–C) , Ler (D–F) , Ws (G–I) , Uk-4 (K–M) , and Mt-0 (N–P) 8 days after inoculation with *Fusarium oxysporum* f. sp. *matthiolae*. Left column, macroscopic appearance of plates. Middle column, close-up. Right column, representative trypan blue stainings of the fungal hyphae. Bar in C, F, I, M, and P represents 100 μ m.

cation and indicates that the differences must originate from the regulation of the *Thi2.1* gene. A comparison of the Col-2 and Uk-4 transgenic lines carrying the same *Thi2.1*-promoter-*uidA* construct (with a *Thi2.1* promoter from Col-2) clearly shows that, on the average, the induction of the promoter by *F.*

oxysporum f. sp. *matthiolae* was nine times higher in the Uk-4 background than in the Col-2 background. This correlates with the expression levels of the *Thi2.1* gene that were determined by Northern blots. Thus, Uk-4, and probably other resistant ecotypes too, might have a more sensitive recognition system for *F. oxysporum* f. sp. *matthiolae* or might have a signal transduction system that gives a higher amplification of the original recognition signal. It will be interesting to see if the induction of the *Thi2.1* gene depends on an elicitor from the fungus or if the induction is due to a wound signal originating from attack of the plant with lytic enzymes.

The correlation of the induction of the *Thi2.1* gene with resistance against *F. oxysporum* f. sp. *matthiolae* suggests a role for this thionin gene in plant defense, at least against this fungus. Direct evidence for such a role has recently been obtained in our laboratory. Overexpression of *THI2.1* in the susceptible ecotype Col-2 resulted in significantly enhanced resistance against *F. oxysporum* f. sp. *matthiolae* (Epple et al. 1997b).

Fig. 2. Distribution of infection classes for susceptible and resistant ecotypes. Sixty cotyledons from infected seedlings of each ecotype were stained at 8 days post infection and assigned to different infection classes. The number of cotyledons in each infection class is shown.

MATERIALS AND METHODS

Growth and treatment of plants.

The following *A. thaliana* ecotypes were used: Col-2, Ler, Mt-0, Uk-4, and Ws. For seed production and for the isolation

Fig. 3. Expression of the *Thi2.1* gene correlates with resistance. Northern (RNA) blots showing the induction of defense-related genes. Seedlings of the ecotypes Col-2, Ler, Ws, Uk-4, and Mt-0 were inoculated with *Fusarium oxysporum* f. sp. *matthiolae*. Total RNA was prepared from seedlings harvested at the indicated times after inoculation. Twenty micrograms was separated on gels and Northern blots were hybridized. **A**, *Thi2.1*-specific probe. Northern blots hybridized with the *Thi2.1* probe were quantified with a PhosphorImager. **B**, blots were stripped and rehybridized with *PR-1*, *PR-5*, and *Pdf1.2*-specific probes. Exposure time was 3 h in the case of the *Thi2.1* probe and 3 days for the other probes.

of genomic DNA, plants were grown in soil in a greenhouse. For treatment with the *Fusarium* sp., seeds were sterilized, sown on MS plates (Murashige and Skoog 1962) supplemented with vitamins (glycine 2 mg/liter, nicotinic acid 0.5 mg/liter, pyridoxine/HCl 0.5 mg/liter, thiamine/HCl 0.1 mg/liter), 2% sucrose, and 0.8% agar, stored at 4°C for 2 days, and grown in a growth chamber (16 h light, 20°C, 8 h dark, 18°C) for 12 days.

F. oxysporum f. sp. *matthiolae* (strain 247.61, Centraalbureau voor Schimmelcultures, Baarn - Delft, The Netherlands) was grown on potato dextrose agar at room temperature for 2 to 3 weeks. Spores were taken up in sterile tap water, filtered through sterile Miracloth, and counted with a Fuchs/Rosenthal chamber. Spore suspensions were diluted to 5×10^5 spores ml^{-1} . Seedlings were grown as described and sprayed with a spore suspension (1 ml per 9-cm petri dish) under sterile conditions. Petri dishes were closed and incubated again in the growth chamber (the first 24 h in the dark) until harvest of the infected plants.

Trypan blue staining (Keogh et al. 1980) was performed according to Mauch-Mani and Slusarenko (1996) in order to quantify the fungal growth. Seedlings were harvested randomly and boiled in the staining solution for 1 min. Afterward, they were left in the solution for 4 h at room temperature. They were de-stained in chloral hydrate twice for 4 and 14 h. Seedlings were stored and viewed in 50% glycerol. Microscopy was done with a Zeiss Axiophot (Zeiss, Oberkochen, Germany). The fungal growth on a cotyledon was assigned to four arbitrary infection classes: no visible infection (class 0); very few (1 to 20) hyphae (class 1); 20 to 100 hyphae (class 2); and densely covered cotyledons (class 3). A disease index (DI) was calculated as follows: $\text{DI} = \sum i \times j / n$, where i = infection class, j = number of cotyledons in each class, and n = total number of cotyledons.

Northern blots.

Plants were treated as described above. Plants grown on MS medium in petri dishes were harvested by pouring liquid nitro-

gen onto the plates. Plant material was ground in liquid nitrogen and RNA was prepared as described by Melzer et al. (1990).

Twenty micrograms of total RNA was separated on denaturing 1.0% agarose gels as described by Ausubel et al. (1994). Ethidium bromide was included to verify equal loading of the RNA. After transfer to Gene Screen membranes (NEN, Ghent, Switzerland) filters were hybridized with 10^6 cpm ml^{-1} oligolabeled ^{32}P probes (Feinberg and Vogelstein 1983) in HYBSOL (Yang et al. 1993). A *Thi2.1* probe was

Fig. 4. All ecotypes have a single *Thi2.1* gene. Southern blots of the ecotypes Col-2 (1), Ler (2), Mt-0 (3), Uk-4 (4), and Ws (5) were probed with a *Thi2.1*-specific probe. Genomic DNA was digested with *Bam*HI, *Eco*RV, and *Hind*III.

Fig. 5. β -Glucuronidase (GUS) induction of transgenic lines transformed with a *Thi2.1* promoter-*uidA* construct. Seedlings of independent transgenic lines were grown on MS-agar plates containing 50 μg of kanamycin per ml and sprayed with a *Fusarium oxysporum* f. sp. *matthiolae* spore suspension or with water (control). GUS activities were determined at 3 days post infection and are given in $\text{nmol MU min}^{-1} \text{mg protein}^{-1}$. The diagrams for the Col-2 background (top) and the Uk-4 background (bottom) have different scales.

Table 1. *Fusarium* inoculation gives higher β -glucuronidase (GUS) induction of a *Thi2.1*-promoter-*uidA* fusion in Uk-4 versus Col-2

Ecotype	Control ^a	Infected ^b	Σ^c
Col-2	147	5,979	20
Uk-4	745	52,526	16

^a GUS expression in water-treated seedlings.

^b Average values of GUS induction in transgenic Col-2 and Uk-4 lines transformed with a *Thi2.1*-promoter-*uidA* construct after *Fusarium oxysporum* inoculation. GUS activities are in $\text{nmol MU min}^{-1} \text{mg protein}^{-1}$.

^c Total number of independent transgenic lines.

prepared from the corresponding cDNA (Epple et al. 1995). *PR-1*- and *PR-5*-specific probes were amplified with specific primers according to the published sequences (Uknes et al. 1992). A *Pdf1.2* probe was isolated from an EST clone (37F10T7) with *SalI* and *NotI* and gel purified.

Filters were washed for 15 min at 60°C with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS (sodium dodecyl sulfate) and then twice for 15 min at 60°C with 0.5× SSC, 0.1% SDS. Filters were exposed to X-Omat-AR (Kodak) films at -80°C for 1 to 4 days. Probes were stripped off the membrane by boiling in 0.2% SDS according to the manufacturer's instructions.

Quantification was done with a PhosphoImager (Molecular Dynamics, Sunnyvale, CA), using protocols and software supplied by the manufacturer.

Southern blots.

Genomic DNA from 2-week-old plants was isolated according to the method of Tai and Tanksley (1990). Three micrograms of DNA was digested with restriction enzymes (Boehringer, Mannheim, Germany) according to the manufacturer's instructions and separated on 0.8% agarose gels. Afterward, the DNA was transferred to Pall Biodyne A membranes (PALL, Muttentz, Switzerland) and blots were hybridized (Sambrook et al. 1989) with a ³²P-labeled *Thi2.1*-specific probe. Filters were washed twice with 2× SSC, 0.1% SDS at 45°C, once with 0.1× SSC, 0.1% SDS at 65°C, and exposed for 4 days.

Generation of transgenic *A. thaliana* lines.

The *A. tumefaciens* strain C58 was transformed as described by Holsters et al. (1978) with a *Thi2.1*-promoter-*uidA* fusion (A. Vignutelli, C. Wasternack, K. Apel, and H. Bohlmann, *manuscript submitted*). Briefly, this construct contained a 1.5-kb fragment of the *Thi2.1* promoter fused to the *uidA* gene at the start codon. Promoter-*uidA* cassettes were cloned into pBin19 (Bevan 1984) to give pHB2202.

In planta transformation of *A. thaliana* ecotypes was performed as described by Bechtold et al. (1993) with the following modifications: Plants were grown under short day conditions for 8 weeks. Bolting was induced by long day treatment and the plants were used when the shoots were 10 to 15 cm long. Only the shoots were infiltrated in the *A. tumefaciens* suspension. Transformants were selected on MS agar containing 1% sucrose, 250 µg of timenten per ml, and 50 µg of kanamycin per ml.

GUS assays.

GUS activity was determined according to Jefferson et al. (1987) with methylumbelliferone as a standard, and is expressed as nmol MU min⁻¹ mg protein⁻¹. Protein concentrations were measured as described by Bradford (1979).

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