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Overexpression of an Endogenous Thionin Enhances Resistance of Arabidopsis against *Fusarium oxysporum*

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Thionins are antimicrobial proteins that are thought to be involved in plant defense. Concordant with this view, we have recently shown that the Arabidopsis thionin *Thi2.1* gene is inducible by phytopathogenic fungi. Here, we demonstrate that constitutive overexpression of this thionin enhances the resistance of the susceptible ecotype Columbia (Col-2) against attack by *Fusarium oxysporum* f sp *matthiolae*. Transgenic lines had a reduced loss of chlorophyll after inoculation and supported significantly less fungal growth on the cotyledons, as evaluated by trypan blue staining. Moreover, fungi on cotyledons of transgenic lines had more hyphae with growth anomalies, including hyperbranching, than on cotyledons of the parental line. No transcripts for pathogenesis-related *PR-1*, *PR-5*, or the pathogen-inducible plant defensin *Pdf1.2* could be detected in uninoculated transgenic seedlings, indicating that all of the observed effects of the overexpressing lines are most likely the result of the toxicity of the THI2.1 thionin. Our findings strongly support the view that thionins are defense proteins.

INTRODUCTION

Plants have evolved a variety of different mechanisms to cope with the constant threat by phytopathogenic microorganisms. They have developed physical barriers and antimicrobial compounds that are preformed in advance of pathogenic attack (Ride, 1985). After infection by pathogens, these constitutive defenses are supported by induced mechanisms. Cell walls can be reinforced, for example, by oxidative cross-linking (Brisson et al., 1994), and hypersensitive cell death is triggered to isolate the pathogens from the healthy part of the plant (Mittler and Lam, 1996). Antimicrobial compounds, such as phytoalexins (Dixon, 1986) and pathogenesis-related PR proteins (Linthorst, 1991), are then produced.

The systemic induction of PR proteins is thought to be involved in systemic acquired resistance (SAR), which can protect the plant against further pathogenic attacks. This view is supported by experiments showing that systemic protection of the plant, after the application of chemicals such as 2,6-dichloroisonicotinic acid (Métraux et al., 1991), salicylate (Ward et al., 1991), and benzothiadiazole (Lawton et al., 1996), coincides with the expression of PR proteins. Salicylate plays a key role in SAR (Gaffney et al., 1993), although it is still debated whether it is the transmitted signal. The proposed function of PR proteins as defense proteins has been tested by overexpression studies and has in some cases been shown to result in measurable resistance against certain pathogens (Broglie et al., 1991; Alexander et al., 1993; Liu et al., 1994). Considering that during pathogen attack, several PR proteins are induced coordinately, it is not surprising that better success has been achieved by combinatorial expression of PR proteins (Zhu et al., 1994; Jach et al., 1995; Jongedijk et al., 1995).

During the past several years, it has become clear that several families of small, basic, cysteine-rich antimicrobial proteins may also play a role in plant defense. These include plant defensins (Terras et al., 1995), lipid transfer proteins (Garcia-Olmedo et al., 1995), and thionins. The antimicrobial and toxic activities of thionins have been known for several decades (reviewed in Garcia-Olmedo et al., 1989; Bohlmann and Apel, 1991; Bohlmann, 1994; Florack and Stiekema, 1994). Based on the toxicity to phytopathogenic bacteria, Fernandez de Caleya et al. (1972) proposed a role for thionins in plant defense. Since then, experimental evidence has been obtained, especially with barley, that supports this view. Thionins from the leaves or the endosperm of barley have been shown to be toxic to plant pathogenic bacteria and fungi (Fernandez de Caleya et al., 1972; Bohlmann et al., 1988; Florack et al., 1993; Molina et al., 1993; Terras et al., 1993, 1996). An induction of barley leaf thionins has been documented in leaves as a response to mildew infection (Bohlmann et al., 1988; Boyd et al., 1994), in coleoptiles after infection by *Septoria nodorum* (Titarenko et al., 1993), and in roots after infection with *Drechslera graminea* (Valè et al., 1994).

The accumulation of thionins after mildew infection was also investigated by immunogold labeling. Thionins were

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found in papillae and in the cell wall surrounding the infection peg in an incompatible interaction. In a compatible interaction, only trace amounts of thionins could be detected in the corresponding regions (Ebrahim-Nesbat et al., 1989, 1993). Different chemical inducers are effective for barley leaf thionins: heavy metals (Fischer et al., 1989), jasmonic acid (Andresen et al., 1992), 2,6-dichloroisonicotinic acid (Wasternack et al., 1994), and salicylic acid (Kogel et al., 1995). All of these have been shown to induce defense reactions in other plant species (Dixon, 1986; Métraux et al., 1991; Gundlach et al., 1992; Uknes et al., 1992; Dempsey and Klessig, 1994).

In addition, it has been shown that the expression of a hordothionin in transgenic tobacco can give enhanced resistance against a phytopathogenic bacterium (Carmona et al., 1993). However, in similar experiments, Florack et al. (1994) did not find enhanced resistance in transgenic tobacco lines expressing α-hordothionin. The overexpression of a barley leaf thionin in tobacco also did not lead to enhanced resistance (B. Mollenhauer and K. Apel, unpublished results). Other antimicrobial peptides have similarly been tested in transgenic plants. Although the expression of a plant defensin conferred enhanced resistance to transgenic tobacco plants against *Alternaria longipes* (Terras et al., 1995), neither a knottin-type antimicrobial peptide from *Mirabilis jalapa* nor a hevein-type antimicrobial peptide from *Amaranthus caudatus* provided detectable resistance against *A. longipes* or *Botrytis cinerea* (De Bolle et al., 1996).

We have recently chosen Arabidopsis as an experimental system in which to study further the function of thionins. We identified two thionin genes that are regulated differently (Epple et al., 1995). Whereas the *Thi2.2* gene is expressed constitutively in seedlings, the *Thi2.1* gene is inducible by methyl jasmonate, silver nitrate, and pathogenic fungi. Salicylate and ethephon have no effect, indicating that the *Thi2.1* gene is inducible via a signal transduction pathway different from that for PR proteins. This situation is in contrast to barley leaf thionins, which are also inducible by salicylate (Kogel et al., 1995); however, because of the large number of leaf thionin genes, it is not clear whether the different inducers act on the same set of genes or whether certain genes are specialized for different inducers (Bohl and Apel, 1993; Holtorf et al., 1995a).

Among the different necrotrophic pathogens that induce the *Thi2.1* gene, the interaction with *Fusarium oxysporum* f sp *matthiolae* (CBS 247.61) has been studied in some detail, and susceptible and resistant Arabidopsis ecotypes have been identified. Whereas ecotype Columbia (Col-2) seedlings are susceptible when grown on Murashige and Skoog (MS) agar (Murashige and Skoog, 1962) and sprayed with an *F. o. matthiolae* spore suspension, ecotype Umkirch (Uk-4) is resistant under these conditions. This resistance correlates with a higher expression of the *Thi2.1* gene (P. Epple, A. Vignutelli, K. Apel, and H. Bohlmann, manuscript submitted), indicating that the *Thi2.1* gene might be a resistance factor against *F. o. matthiolae*. To test this hypothesis, we overexpressed THI2.1 in the susceptible ecotype Col-2 and compared the resistance of the transgenic lines with that of the ecotypes Col-2 and Uk-4. This enabled us to demonstrate in a homologous system that a thionin gene functions as a defense factor.

RESULTS

Generation of Transgenic Lines with High Constitutive Levels of THI2.1

The Arabidopsis *Thi2.1* gene is inducible in seedlings by silver nitrate, methyl jasmonate, and necrotrophic fungi (Epple et al., 1995). With *F. o. matthiolae*, the induction is much more pronounced in resistant ecotypes than in susceptible ecotypes (P. Epple, A. Vignutelli, K. Apel, and H. Bohlmann, manuscript submitted), indicating a function of the *Thi2.1* gene in the resistance against this fungus. As a further test for this proposed function, we overexpressed the coding sequence for the entire precursor under the control of a strong constitutive promoter in the susceptible ecotype Col-2.

For the overexpression of exogenous thionins in Arabidopsis, we had tested different promoters (Holtorf et al., 1995b) and finally used a cauliflower mosaic virus (CaMV) promoter with Ω element for a high-level constitutive expression. The promoter was further strengthened by doubling the enhancer. The coding region was amplified by polymerase chain reaction (PCR) from a *Thi2.1* cDNA without altering the sequence and, by triple ligation, was cloned together with a promoter fragment into pBIN19Ter, as described in Methods. The final construct is shown in Figure 1.

The expression vector pPE13 was transformed into Agrobacterium strain C58, which subsequently was used for transformation of Arabidopsis by vacuum infiltration. Of the primary transformants, 15 were screened for *Thi2.1* transcripts in seedlings. As shown in Figure 2A, the internal *Thi2.1* transcripts were not detectable under these conditions, whereas several transgenic lines had a high *Thi2.1* transcript level. Lines with high transcript levels were further characterized by using DNA and protein gel blot analysis. The DNA gel blot shown in Figure 2B indicates that all lines were derived from independent transformation events and that most lines carry multiple insertions. No correlation

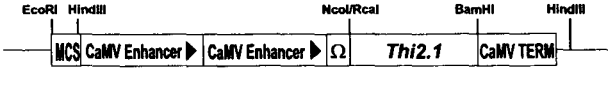

Figure 1. Construct for the Overexpression of THI2.1.

The coding sequence for THI2.1 was fused to a CaMV 35S promoter with double enhancer and Ω element and inserted into pBIN19 carrying the CaMV terminator (TERM). MCS, multiple cloning site.

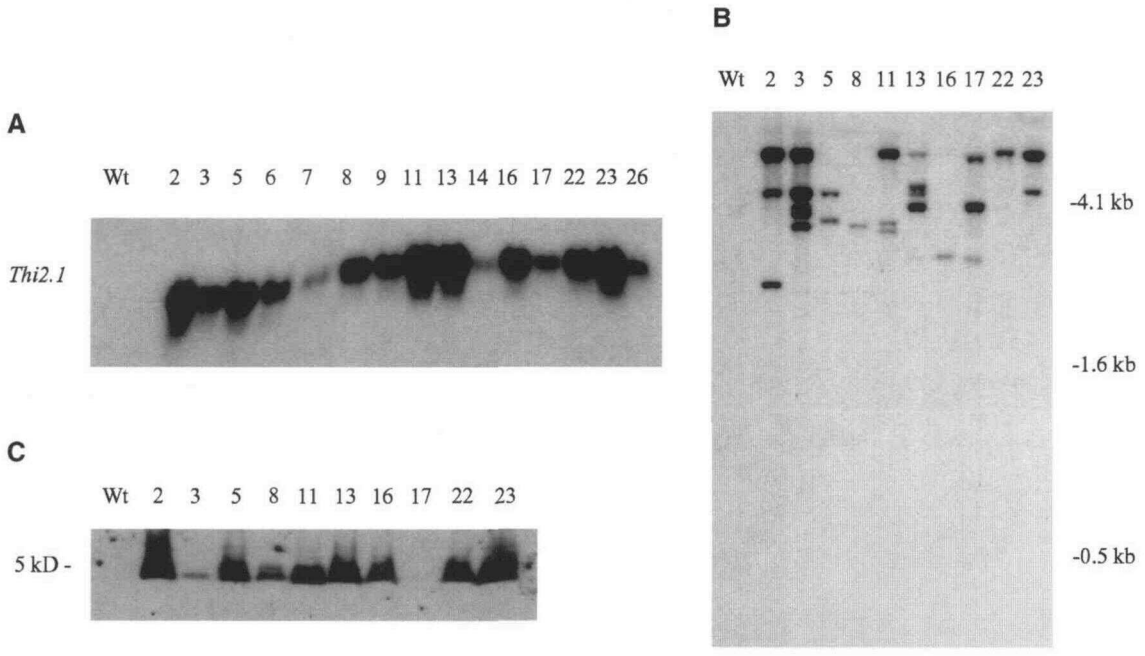

Figure 2. Molecular Characterization of the Transgenic Lines.

(A) RNA gel blot screening of independent transgenic lines. The blot was hybridized with a *Thi2.1*-specific probe.
(B) DNA gel blot. Genomic DNA was digested with HindIII, and the blot was hybridized with an *nptII*-specific probe to determine the copy number of the transgene. Positions of DNA standard fragments are indicated at right in kilobases.
(C) Protein gel blot showing the 5-kD band of the THI2.1 thionin in seedlings.
Lines 2, 5, 8, 16, 22, and 23 were used in resistance tests. Wt, wild type (Col-2).

could be found between the number of inserts and the transcript level or the protein level. According to the protein gel blot shown in Figure 2C, six lines with a high THI2.1 protein level were selected. Kanamycin-resistant T_2 seedlings from these lines were propagated to establish homozygous lines. From these, seed material was increased for additional experiments.

All of the transgenic lines looked phenotypically normal (data not shown). The only difference from the wild type was that the overexpressing lines did not germinate on MS agar without sucrose, whereas the wild type did (data not shown). Germination was normal with 0.5% sucrose or in soil.

Resistance Tests

We noted that *F. o. matthiolae* is a strong inducer of the *Thi2.1* gene, even in the compatible interaction with Col-2 (Epple et al., 1995). However, in the resistant ecotype Uk-4, the induction is up to 10 times stronger (P. Epple, A. Vignutelli, K. Apel, and H. Bohlmann, manuscript submitted), indicating that the *Thi2.1* gene might be part of the resistance mechanism against this fungus. Consequently, we tested the THI2.1 overexpressing lines against *F. o. matthiolae.* For comparison, we always included the Col-2 wild type and the resistant Uk-4 ecotype.

Seedlings were grown in Petri dishes on MS agar and challenged with a spore suspension (10^5 spores mL^{-1}) when they were 12 days old. Approximately 6 to 8 days later, the susceptible ecotype Col-2 was visibly damaged by the fungus, with hyphae growing on the leaves, whereas seedlings of the resistant ecotype Uk-4 appeared to be much healthier. For the THI2.1 overexpressing lines, we observed that they did not appear as damaged as did the wild type. An example is shown in Figure 3, in which the phenotypic appearance of the transgenic line 2 is compared with that of the resistant ecotype Uk-4 and the parental line Col-2. To quantify the reaction of the different lines, we determined the chlorophyll content in untreated seedlings as well as in seedlings at 4, 6, and 8 days postinoculation (dpi). Figure 4 shows that after inoculation, the chlorophyll content of the susceptible wild type decreased to ~60% of its initial value, whereas the content of the resistant ecotype Uk-4 increased. Similar results have been obtained in other, independent experiments. All of the transgenic lines had an intermediate chlorophyll content, in agreement with the visible appearance.

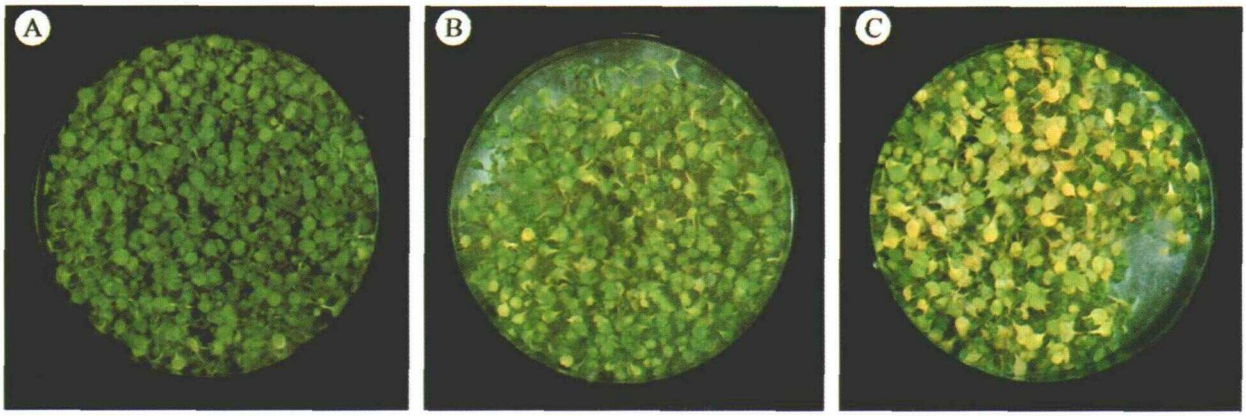

Figure 3. Enhanced Resistance of THI2.1 Overexpressing Lines.

(A) Phenotypic appearance of the resistant ecotype Uk-4.
(B) Phenotypic appearance of transgenic line (line 2).
(C) Phenotypic appearance of the parental line Col-2.
Seedlings are shown 8 dpi with *F. o. matthiolae.*

Resistance results from the ability of the plant to restrict the growth of a pathogen either in time or space or both. Therefore, it was necessary to quantify the fungal biomass of the seedlings from the different lines. We decided to evaluate the different lines microscopically. Seedlings from the controls and from the overexpressing lines were harvested randomly at 4, 6, and 8 dpi and were stained for fungal hyphae (Keogh et al., 1980). Three independent experiments were performed. Cotyledons were mounted on slides and evaluated for fungal growth. We assigned the fungal growth of a cotyledon to four arbitrary infection classes. Examples are shown in Figure 5 and include no visible infection (class 0; Figure 5A), very few (1 to 20) hyphae (class 1; Figure 5B), 20 to 100 hyphae (class 2; Figure 5C), and cotyledons densely covered with hyphae (class 3; Figure 5D). To minimize the subjective bias in such methods, all cotelydons from one experiment were evaluated directly one after the other.

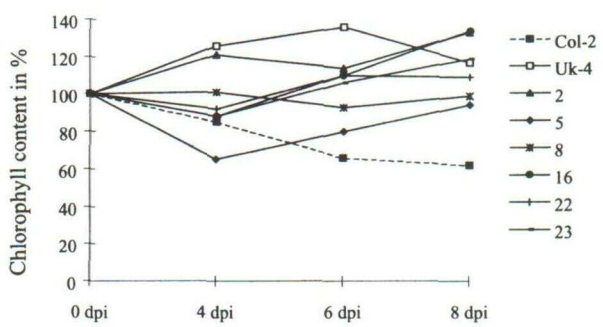

Figure 4. Chlorophyll Content.

Shown is the chlorophyll content of wild-type line Col-2, several transgenic lines, and the resistant ecotype Uk-4 at 0, 4, 6, and 8 dpi inoculated with *F. o. matthiolae.* The chlorophyll content of the untreated control was set as 100%.

At 4 dpi, small differences in the disease index between the resistant and the susceptible ecotypes could be observed (data not shown). At 6 and 8 dpi, the differences among Col-2, Uk-4, and the transgenic lines could be clearly observed. Table 1 shows the results obtained in one experiment at 8 dpi. The results of all three experiments are added up in Figure 6. These results clearly indicate that all transgenic lines overexpressing the THI2.1 thionin at a high level are significantly more resistant than is the wild type, although they are not as resistant as the ecotype Uk-4. A transgenic line expressing a *Thi2.1* promoter–*uidA* fusion from the same pBIN19 vector was as susceptible as the Col-2 wild type in control experiments (data not shown).

In addition, during evaluation of the cotyledons, we noticed hyphae having a hyperbranching morphology as shown in Figure 7C instead of the normal growth as shown in Figure 7A. As documented in Table 2, these are often found on the transgenic lines but not on the control lines Col-2 and Uk-4. Similar structures have been found in vitro after treatment of fungal cultures with antimicrobial proteins, including a radish thionin (Terras et al., 1996). Other growth anomalies, especially coilings like the one shown in Figure 7B, can also be found. Again, as is evident from Table 2, these are more often found on the overexpressing lines. Thus, the overexpression of THI2.1 disturbs and suppresses the growth of *F. o. matthiolae.*

It has been demonstrated before that overexpression of different proteins that somehow disturb the homeostasis of

a plant cell can lead to expression of defense-related proteins (Takahashi et al., 1989; Mittler et al., 1995; Herbers et al., 1996). Thus, one might argue that the enhanced resistance that was observed in the THI2.1 overexpressing lines is not due to a direct effect of the thionin but rather is a secondary effect due to the expression of other defense-related proteins. Therefore, we tested the transgenic lines for expression of *PR-1*, *PR-5*, *Thi2.1*, *Thi2.2*, and the pathogen-inducible plant defensin *Pdf1.2*.

Seedlings were grown with 0.5% sucrose under the same conditions that were employed during the resistance tests. Inoculated seedlings and control seedlings were harvested at 4 dpi. At that time, induction of *PR-1*, *PR-5*, *Thi2.1*, and *Pdf1.2* is clearly detectable in inoculated seedlings (Epple et al., 1995; P. Epple, A. Vignutelli, K. Apel, and H. Bohlmann, manuscript submitted). As is evident from the RNA gel blots shown in Figure 8, expression of these genes, except for *Thi2.2*, could not be detected in control seedlings that were not inoculated. *PR-1*, *PR-5*, and the defensin gene were only detectably expressed in the wild type, in transgenic lines, and in the resistant ecotype Uk-4 after inoculation with *F. o. matthiolae*. The internal *Thi2.1* gene was also induced. *Thi2.2* was not induced but was downregulated (Epple et al., 1995; P. Epple, A. Vignutelli, K. Apel, and H. Bohlmann, unpublished results).

DISCUSSION

The possible function of thionins has been debated for a long time. The toxic activity of purothionins was discovered soon after purification (Stuart and Harris, 1942), and the antimicrobial activity of several thionins in vitro against phytopathogenic bacteria and fungi indicates a role in plant defense. Furthermore, several thionin genes can be induced

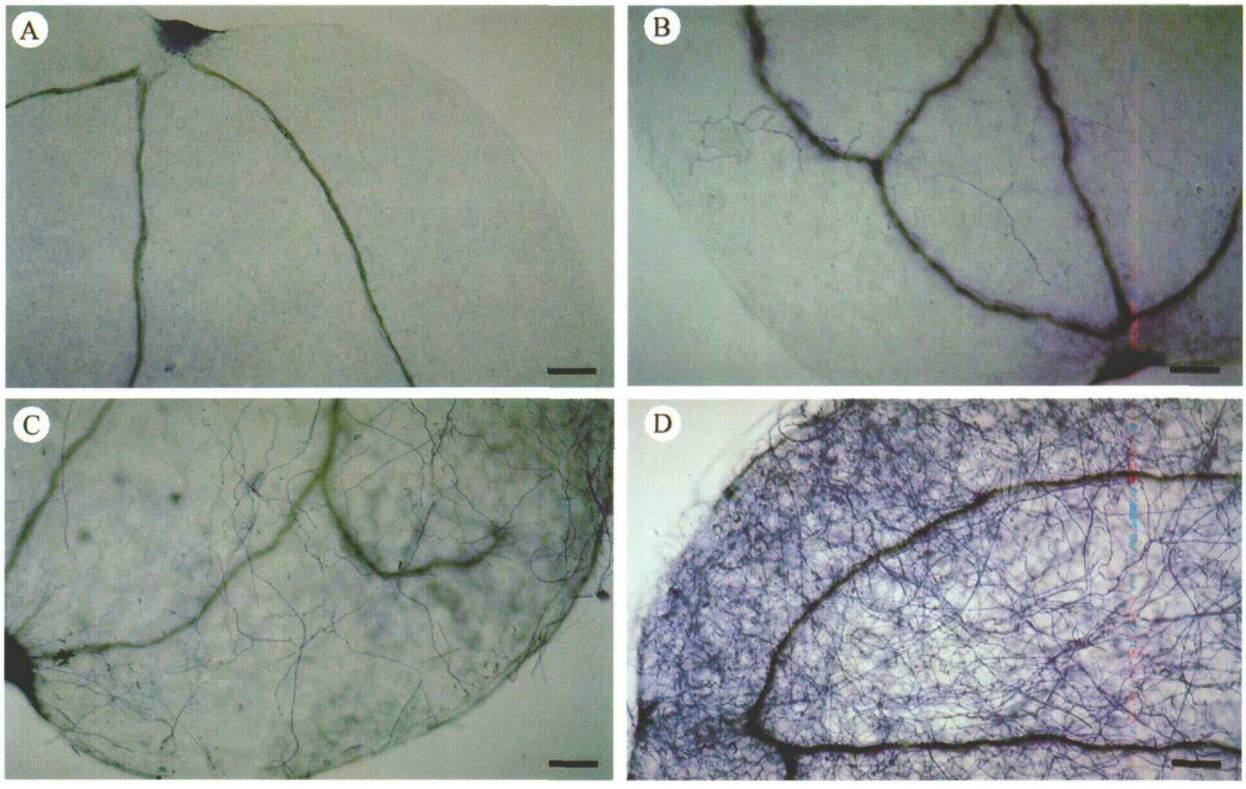

Figure 5. Infection Classes.

Shown are classes of hyphal growth of *F. o. matthiolae* on Arabidopsis cotyledons (trypan blue staining).

(A) Class 0.
(B) Class 1.
(C) Class 2.
(D) Class 3.
For each class, a typical example is shown. Bars = 100 μm.

Table 1. THI2.1 Overexpressing Lines Support Less Fungal Growth[a]

Line	Class 0	Class 1	Class 2	Class 3	Σ[b]	Disease Index
Uk-4	0	58	2	0	60	1.03
Col-2	0	14	12	34	60	2.33
2	0	48	4	8	60	1.33
5	0	37	18	5	60	1.46
8	0	43	7	10	60	1.45
16	0	46	10	4	60	1.30
22	0	42	11	7	60	1.41
23	0	32	18	10	60	1.63

[a] Evaluation of *F. o. matthiolae* growth on cotyledons of the susceptible wild-type Col-2, several transgenic lines overexpressing THI2.1, and the resistant ecotype Uk-4 at 8 dpi. Given are the number of cotyledons in each infection class and the disease index.
[b] Σ, total number of cotyledons.

after pathogen attack. Our finding that Arabidopsis contains thionin genes opened the way to investigate further the proposed role as defense proteins. The Arabidopsis *Thi2.1* gene is inducible by pathogenic fungi (Epple et al., 1995), and this induction is faster and more intense in resistant ecotypes than in susceptible ecotypes (P. Epple, A. Vignutelli, K. Apel, and H. Bohlmann, manuscript submitted). Although we have not yet shown that the THI2.1 thionin has in vitro antimicrobial activity, the protein has the characteristics that have been discussed as being important for toxicity (Bohlmann, 1994). It is basic and possesses a tyrosine residue at position 13 (Epple et al., 1995). More importantly, in this article, we demonstrate a defense function of an Arabidopsis thionin in vivo in a homologous system.

Overexpression of the endogenous THI2.1 thionin results in clearly enhanced resistance against *F. o. matthiolae*. The resistance of the transgenic lines was discernible by a slower decrease of the chlorophyll content after inoculation (Figure 6) and, more directly, by determining the fungal growth. The ratings of the transgenic lines always gave a disease index below that of the susceptible parental ecotype Col-2, as shown for the 8 dpi time point of one of the experiments, with results being shown in Table 1. Two other independent experiments gave similar results (Figure 6). In all three experiments, the transgenic lines were significantly more resistant than the parental line.

It has been shown previously that disturbing the homeostasis of plant cells by expression of a bacterial proton pump (Mittler et al., 1995), by ectopic expression of invertase (Herbers et al., 1996), or by expression of the CaMV VI gene (Takahashi et al., 1989) can lead to constitutive expression of PR proteins. Therefore, we checked for constitutive expression of other defense-related proteins in the THI2.1 overexpressing lines. Transcripts for *PR-1*, *PR-5*, and a pathogen-inducible plant defensin were not detectable on RNA gel blots in untreated seedlings (Figure 8) but were always induced 4 dpi in the transgenic lines as well as in the wild-type controls.

The *Thi2.2* gene is not induced but suppressed after inoculation with *F. o. matthiolae* (Figure 8), which is in line with previous observations (Epple et al., 1995; P. Epple, A. Vignutelli, K. Apel, and H. Bohlmann, unpublished results). The suppression of the *Thi2.2* gene is less pronounced in the resistant ecotype Uk-4 compared with the susceptible ecotype Col-2. This indicates that the *Thi2.2* gene might also play a role in the resistance against *F. o. matthiolae*. First results with transgenic Arabidopsis lines overexpressing THI2.2 seem to support this view (P. Epple, K. Apel, and H. Bohlmann, unpublished results).

Overexpression of the THI2.1 thionin causes a sucrose requirement during germination (data not shown). The reason for this is not known. Such an effect has not been observed for THI2.2 overexpressing lines (P. Epple, K. Apel, and H. Bohlmann, unpublished results).

Further strong support that the enhanced resistance that we observed is due to a direct effect of the overexpressed THI2.1 thionin comes from growth anomalies of the fungal hyphae on the transgenic lines. On cotyledons of the THI2.1 overexpressing lines, we found more hyphae with growth anomalies than on the parental line (Table 2). Most impressive is the hyperbranching growth of the hyphae that we found only on the transgenic lines (Figure 5C and Table 2). Similar effects on fungal growth have also been reported for fungicides (Robson et al., 1989; Wiebe et al., 1990) and for plant defensins in vitro (Terras et al., 1995). Recently, a similar in vitro effect, exerted by a purified thionin from radish

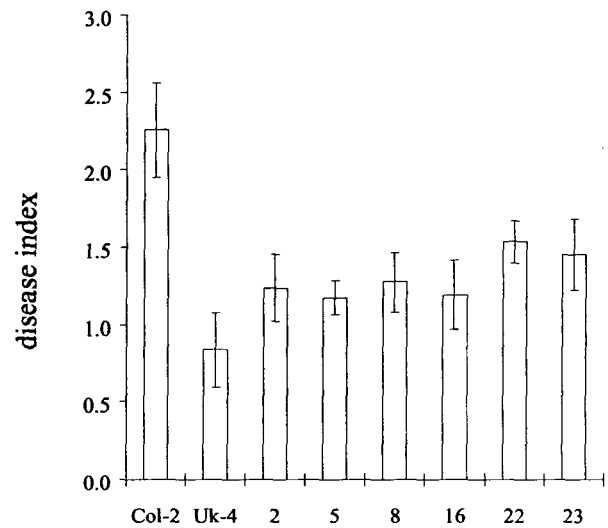

Figure 6. The Disease Index of THI2.1 Overexpressing Lines Is Significantly Lower than That of the Parental Line.

For each line, the mean disease index calculated from three independent experiments with a total of five time points is shown together with the standard deviation.

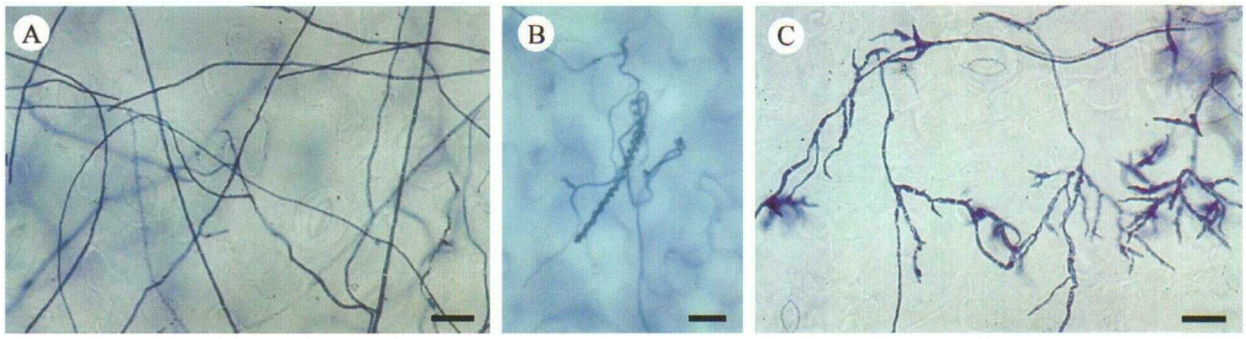

Figure 7. Fungal Hyphae Show Growth Anomalies on Transgenic Lines.

(A) Normal appearance of *F. o. matthiolae* hyphae on the susceptible ecotype Col-2.
(B) and **(C)** Abnormal growth of hyphae on cotyledons constitutively expressing THI2.1. **(B)** shows the coiling of hyphae, and **(C)** shows hyperbranching.
Bars = 20 μm.

tubers on *F. culmorum*, has been demonstrated (Terras et al., 1996).

One might expect to find such hyperbranching growth on the resistant ecotype Uk-4 as well, perhaps to an even greater extent. But fungal growth on the cotyledons of this ecotype was even more suppressed (disease index of ~1) than on the transgenic overexpressing lines. Suppression was so strong that these fungal structures could not develop. However, in one control experiment with a high disease index, hyperbranching was also found on Uk-4 cotyledons (data not shown). Hyperbranching has never been observed on the susceptible ecotype Col-2.

At this point, it should be stressed that Arabidopsis plants induce several other defense genes in addition to the *Thi2.1* gene. These include *PR-1* and *PR-5* as well as the plant defensin gene *Pdf1.2* (Figure 8; Epple et al., 1997). The products of these and perhaps other still unknown defense genes might act together with THI2.1. Plant defensins have in vitro antimicrobial activity (e.g., Terras et al., 1995), and we predict that coexpression of the induced plant defensin with THI2.1 will result in higher protection against *F. o. matthiolae.* Transcripts for *Pdf1.2* are highly induced by *F. o. matthiolae* as are *Thi2.1* transcripts, and both genes are probably regulated by the same signal transduction pathway (Epple et al., 1997). The toxic mechanisms by which thionins and plant defensins exert their antimicrobial activity are different (Thevissen et al., 1996), and both THI2.1 and PDF1.2 might display a synergistic effect as has been found for hordothionin and other cysteine-rich proteins in vitro (Molina et al., 1993; Terras et al., 1993).

Table 2. Fungal Hyphae Show Growth Anomalies on Cotyledons of THI2.1 Overexpressing Lines[a]

Lines	Σ[b]	Altered Morphology	%[c]	Hyperbranching	%[c]
Uk-4	60	4	6.6	0	0
Col-2	60	4	6.6	0	0
2	60	11	18.3	2	3.3
5	60	18	30.0	7	11.6
8	60	14	23.3	7	11.6
16	60	6	10.0	2	3.3
22	60	15	25.0	5	8.3
23	60	9	15.0	4	6.6

[a] Evaluation of *F.o. matthiolae* growth anomalies on cotyledons of the susceptible wild-type Col-2, several transgenic lines overexpressing THI2.1, and the resistant ecotype Uk-4 at 8 dpi. Given are the number of cotyledons with hyphae showing an altered morphology, especially coilings (Figure 7B), and the number of cotyledons with hyphae showing intensive hyperbranching (Figure 7C).
[b] Σ, total number of cotyledons.
[c] %, percentage of the cotyledons with hyphae showing the effect.

PR proteins are induced by a variety of pathogens in a salicylate-dependent pathway and are thought to mediate a protective effect during SAR (Alexander et al., 1993). Chemicals that can induce SAR also induce PR proteins and have been shown to protect the plant against pathogen attack. Such protection has been achieved, for instance, by the application of benzothiadiazole against several biotrophic pathogens but not against necrotrophic pathogens such as *A. alternata* and *B. cinerea* (Friedrich et al., 1996). SAR in radish and Arabidopsis can also be induced by biocontrol bacteria in the soil. This resistance is not associated with an accumulation of PR proteins (Hoffland et al., 1995; Pieterse et al., 1996). In the case of Arabidopsis, it has been shown that the resistance is also independent of salicylate by using NahG plants that are disturbed in the accumulation of salicylate (Pieterse et al., 1996).

The *Thi2.1* gene is highly induced by *F. oxysporum* and other necrotrophic fungi but is only weakly induced by *Peronospora parasitica* and probably also other biotrophic fungi and bacteria (P. Epple, K. Apel, and H. Bohlmann, unpublished results). In addition, the signal transduction pathway

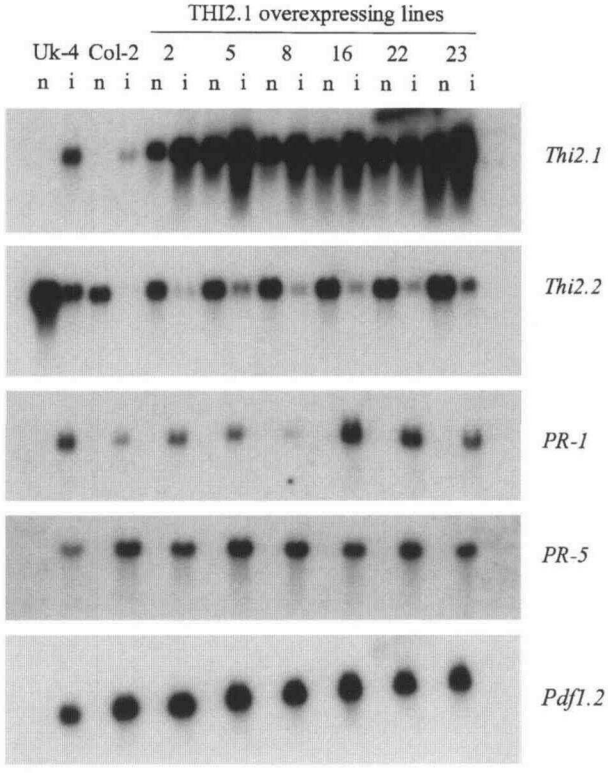

Figure 8. THI2.1 Overexpression Does Not Induce Other Defense-Related Genes.

RNA gel blots demonstrate the expression of several defense-related genes (indicated at right) in THI2.1 overexpressing lines, in the parental line Col-2, and in the resistant ecotype Uk-4 before and 4 days after infection with *F. o. matthiolae*. i, induced; n, not induced.

for this gene is independent of salicylate and therefore different from that of the PR proteins (Epple et al., 1995). The same results have been obtained for the *Pdf1.2* gene (Epple et al., 1997). It is therefore possible that the systemic protection of Arabidopsis and also of radish by biocontrol bacteria uses the salicylate-independent pathway that induces the *Thi2.1* and *Pdf1.2* genes, leading to the accumulation of THI2.1 and PDF1.2 antimicrobial proteins.

METHODS

Cloning

The coding sequence of THI2.1 was amplified by polymerase chain reaction (PCR) with the following primers: PE.1, 5′-AAATCATGAAAGGAAGAATTTTG-3′; and PE.2, 5′-TATGGATCCATTACAACAGTTTAGGC-3′. Primer PE.1 introduced a RcaI site at the start codon without altering the nucleotide sequence of the coding region, and primer PE.2 introduced a BamHI site just behind the stop codon. The PCR product was digested with RcaI and BamHI and was gel purified. A cauliflower mosaic virus (CaMV) promoter with double enhancer plus Ω element was made by inserting the CaMV enhancer from pBI121.1 (Clontech, Palo Alto, CA) into the EcoRV site of the CaMV promoter from pSH9 (Holtorf et al., 1995b) to give pHB1802. The insert from pHB1802 was cut out with HindIII and cloned into pUK19 (pUC19 with kanamycin instead of ampicillin resistance) in the reverse orientation to give pHB2531. This vector was cut with EcoRI and NcoI, and the promoter fragment was gel purified. The final construct (pPE13) resulted from a triple ligation of the coding fragment, with the promoter fragment containing the CaMV double enhancer plus Ω element into pBIN19Ter digested with EcoRI and BamHI. pBIN19Ter is pBIN19 (Bevan, 1984) containing the CaMV terminator from pRT101 (Töpfer et al., 1987). The correct sequence of the coding region was verified by sequencing.

Generation of Transgenic *Arabidopsis thaliana* Lines

Agrobacterium tumefaciens C58 was transformed as described by Holsters et al. (1978). Integrity of the transformed plasmid was confirmed by DNA gel blotting. Transformation of Arabidopsis by vacuum infiltration was performed as described by Bechtold et al. (1993) with the following modifications. Ecotype Columbia (Col-2) plants were first grown under short-day conditions for ~2 months. Bolting was induced by long-day treatment, and the plants were used when the shoots were 5 to 10 cm long. Only the shoots were infiltrated in the Agrobacterium suspension. Transformants were selected on Murashige and Skoog (MS) agar (Murashige and Skoog, 1962) containing 1% sucrose, 250 μg/mL timenten, and 50 μg/mL kanamycin. Kanamycin-resistant seedlings were planted in the soil and grown to maturation.

Growth and Treatment of Plants

We used the Arabidopsis ecotypes Col-2 and Umkirch (Uk-4). For seed production, plants were grown in soil in a greenhouse. For treatment with pathogens, seeds were sterilized, sown on MS plates with vitamins (glycine [2 mg/L], nicotinic acid [0.5 mg/L], pyridoxine-HCl [0.5 mg/L], thiamine-HCl [0.1 mg/L]), 0.5% sucrose, and 0.8% agar, stored at 4°C for 2 days, and grown in a growth chamber (16 hr of light at 20°C and 8 hr of dark at 18°C) for 12 days.

Fusarium 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 Miracloth (Calbiochem-Novabiochem, San Diego, CA), and counted with a Fuchs/Rosenthal chamber (Merck ABS, Dietikon, Switzerland). Spore suspensions were diluted to 10^5 spores mL^{-1}. Seedlings were grown as described above, and they were sprayed with a spore suspension (1 mL per 5-cm Petri dish). Petri dishes were closed and incubated again in the growth chamber (the first 24 hr in the dark) until harvest of the infected plants.

For chlorophyll measurements, plant material was ground with liquid nitrogen, and 100 mg was extracted with 2 mL of 95% ethanol overnight. The chlorophyll content of the samples was determined spectrophotometrically, according to the formula $C_{a+b} = 5.24\ A_{664} + 22.24\ A_{648}$, where C is the chlorophyll concentration in micrograms per milliliter and A is absorption (Lichtenthaler, 1987).

Trypan blue staining (Keogh et al., 1980) was performed as described by Mauch-Mani and Slusarenko (1996). Seedlings were harvested and boiled in the staining solution for 1 min and left in the solution for 4 hr at room temperature. They were destained in chloral hydrate for 4 and 24 hr. Seedlings were stored and viewed in 50% glycerol. Microscopy was done with a Zeiss Axiophot (Oberkochen, Germany). The fungal growth on a cotelydon 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: $DI = \Sigma\, i \times j/n$, where i is infection class, j is the number of cotyledons in each class, and n is the total number of cotyledons.

RNA Gel Blots

Plants were grown on MS agar plates and treated as described above. Seedlings were harvested by pouring liquid nitrogen 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 (Ausubel et al., 1994). Ethidium bromide was included to verify equal loading of RNA. After transfer to Gene Screen membranes (New England Nuclear, Beverly, MA), filters were hybridized with 10^6 cpm mL^{-1} ^{32}P-labeled probes (Feinberg and Vogelstein, 1983) in HYBSOL (Yang et al., 1993). *Thi2.1* and *Thi2.2* probes were prepared from the corresponding cDNAs (Epple et al., 1995). Pathogenesis-related protein gene *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 cut out from an expressed sequence tag clone (37F10T7) with SalI and NotI and was gel purified.

Filters were washed for 20 min at 60°C with 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS, and then 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 hr to 6 days. Probes were stripped from the membrane in boiling 0.2% SDS solution, according to the manufacturer's instructions (New England Nuclear).

DNA Gel Blots

Genomic DNA from 6-week-old plants was isolated according to the method of Tai and Tanksley (1990). Three micrograms of DNA was digested with the restriction enzyme HindIII (Boehringer Mannheim), according to the manufacturer's instructions, and separated on a 0.8% agarose gel. Afterward, the DNA was transferred to Pall Biodyne A membranes (PALL, Muttenz, Switzerland), and the blots were hybridized (Sambrook et al., 1989) with a ^{32}P-labeled neomycin phosphotransferase (*nptII*) probe. Filters were washed twice with 2 × SSC, 0.1% SDS at 65°C, once with 0.5 × SSC, 0.1% SDS at 62°C, and once with 0.1 × SSC, 0.1% SDS at 62°C, and exposed for 4 days.

Production of Antibodies

The coding sequence for the THI2.1 proprotein was PCR amplified with the following primers: PE.2, 5′-TATGGATCCATTACAACAGTTTAGGC-3′; and PE.7, 5′-CAAGTAGAATTTAAAATCTGCTG-3′. PE.2 introduced a BamHI site behind the stop codon, and PE.7 introduced a DraI site at the beginning of the thionin domain. The PCR product was digested, gel purified, and cloned into pExSec1 (Brünen-Nieweler et al., 1994) digested with SmaI and BamHI to give pPE17. The correct sequence was verified, and pPE17 was subsequently transformed into *Escherichia coli* BL21 (Studier and Moffatt, 1986). The protein A fusion protein was isolated from the periplasma, according to Ausubel et al. (1994), and purified with IgG–Sepharose columns (Pharmacia, Dübendorf, Switzerland). Polyclonal antiserum was raised against the fusion protein of the protein A–THI2.1 proprotein in rabbits (Eurogentech, Seraing, Belgium).

Protein Gel Blots

Plants were grown on MS agar plates with vitamins, as described in the section "Growth and Treatment of Plants." Plant material was ground with liquid nitrogen, and 1 mL of pulverized material was homogenized in 4 mL of Laemmli gel loading buffer (Laemmli, 1970) and incubated at 95°C for 10 min. Cell debris was pelleted, and the supernatant was precipitated with 4 volumes of acetone at −20°C for 30 min. Proteins were pelleted, dried, and dissolved in Laemmli gel loading buffer. The protein concentration was determined according to Esen (1978). Proteins (30 μg) were separated on Tricine–SDS-polyacrylamide gels, according to Schägger and von Jagow (1987), and electroblotted onto polyvinyl difluoride membranes (Bio-Rad, Glattbrugg, Switzerland). THI2.1 was detected with a polyclonal antibody raised against a THI2.1–protein A fusion protein. The antibody was diluted 1:1000 and detected by using the BM Chemiluminescence Western Blotting Kit (Boehringer Mannheim).

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