Isolation and characterization of signal transduction mutants of *Arabidopsis thaliana* that constitutively activate the octadecanoid pathway and form necrotic microlesions

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Summary

Thionins are a group of antimicrobial polypeptides that form part of the plant's defense mechanism against pathogens. The *Thi* 2.1 thionin gene of *Arabidopsis thaliana* has been shown to be inducible by jasmonic acid (JA), an oxylipin-like hormone derived from oxygenated linolenic acid and synthesized via the octadecanoid pathway. The JA-dependent regulation of the *Thi* 2.1 gene has been exploited for setting up a genetic screen for the isolation of signal transduction mutants that constitutively express the *Thi* 2.1 gene. Ten *cet*-mutants have been isolated which showed a *c*onstitutive *e*xpression of the *t*hionin gene. Allelism tests revealed that they represent at least five different loci. Some mutants are dominant, others recessive, but all *cet* mutations behaved as monogenic traits when backcrossed with *Thi* 2.1-*GUS* plants. Some of the mutants overproduce JA and its bioactive precursor 12-oxophytodienoic acid (OPDA) up to 40-fold while others have the same low levels as the control wildtype plants. Two of the mutants showed a strong induction of both the salicylic acid (SA)- and the JA-dependent signaling pathways, while the majority seems to be affected only in the octadecanoid pathway. The *Thi* 2.1 thionin gene and the *Pdf* 1.2 defensin gene are activated independently, though both are regulated by JA. The *cet*-mutants, except for one, also show a spontaneous leaf cell necrosis, a reaction often associated with the systemic acquired resistance (SAR) pathway.

Keywords: Thionins, signal transduction mutants, plant defense, octadecanoid pathway

Introduction

Plants respond in a variety of ways to the presence of pathogens. Whereas some defense mechanisms are constitutive, others are induced during a pathogen attack (Hammond-Kosack and Jones, 1996). One of the best studied induced resistance responses is the systemic acquired resistance (SAR) that is triggered by pathogen-induced localized necrosis (Kuc, 1982). The inducing necrosis can result either from an endogenous plant cell death response following recognition of the pathogen by the host in an incompatible interaction, the so called hypersensitive reaction (HR), or from cell death caused by the action of the pathogen in a compatible interaction. The SAR leads to a long-lasting, systemic resistance to a broad range of virulent pathogens (Hunt and Ryals, 1996). Shortly after the onset of the localized necrosis the infected plant begins to express a subset of pathogenesis-related (*PR*) genes both locally, at the point of infection, and systemically, throughout the rest of the plant (Uknes *et al.*, 1992).

Considerable efforts have been directed towards identifying signaling molecules that are responsible for activating the systemic defense response throughout the infected plant. Salicylic acid (SA) has been shown to be involved in the signaling of the SAR response (Hammond-Kosack and Jones, 1996). During SAR formation, SA concentration increases throughout the plant. Exogenous application of

SA can promote the SAR and the removal of endogenous SA results in plants that are unable to establish an SAR response and are supersusceptible to pathogen infection (Gaffney *et al.*, 1993).

However, SA is not the only signaling compound produced by plants in response to pathogen attack that establishes a systemic resistance response. Also jasmonic acid (JA), an oxylipin-like hormone derived from oxygenated linolenic acid has been shown to increase strongly upon pathogen infection both in inoculated and in untreated leaves of inoculated plants and to induce the systemic accumulation of defense factors. Exogenous application of methyl jasmonate (MeJA) results in the activation of a specific subset of genes encoding antifungal proteins such as defensins and thionins that are not inducible by SA. Conversely, the synthesis of SA-induced PR proteins is not activated by MeJA (Epple *et al.*, 1995; Penninckx *et al.*, 1996; Vignutelli *et al.*, 1998). Thus, at least two different defense response pathways can be distinguished, an SA- and a JA-dependent one, that are turned on in the plant in response to pathogen attack (Epple *et al.*, 1995; Thomma *et al.*, 1998).

Various genetic approaches have been used to elucidate the SAR pathway. A large number of mutants with an altered resistance response in this pathway have been identified. These mutants can be broadly classified into two groups. The first group contains mutants that exhibit a constitutive SAR. They accumulate high levels of SA and express *PR* genes in the absence of pathogens. The second group consists of mutants that exhibit a compromised resistance to pathogens. These mutants are incapable of expressing the *PR* genes or developing SAR in response to SA. Based on the detailed characterization of these mutants various models have been proposed in which the SAR depends on the activation of a complex network of numerous genes, some of which appear to be required also for SA-independent defense responses (Dong, 1998; Farmer *et al.*, 1998; Maleck and Dietrich, 1999; Pieterse and van Loon, 1999).

Much less is known about the pathogen-induced defense response pathway that is activated by JA. Thus far only one mutant has been found that is unable to synthesize JA (McConn and Browse, 1996) and three mutant types have been described that are no longer able to respond to elevated JA-levels (Berger *et al.*, 1996; Feys *et al.*, 1994; Staswick *et al.*, 1992). The isolation and characterization of other mutants that are affected differently in this pathway would allow a more detailed analysis of this pathway and its interaction with other defense reactions during pathogen attack. In the present work we describe a mutant screen strategy that has enabled us to identify a novel class of signal transduction mutants of *Arabidopsis thaliana*, the so called *c*onstitutive *e*xpression of *t*hionin (*cet*) mutants that constitutively express the JA-inducible *Thi* 2.1 thionin gene.

Results

Isolation of mutants

The aim of this work was to isolate signal transduction mutants of *A. thaliana* that constitutively activate the octadecanoid pathway. The *Thi* 2.1 thionin gene is an ideal marker for this pathway. It is induced by MeJA and wounding, both locally and systemically (Epple *et al.*, 1995; Vignutelli *et al.*, 1998) but not by SA. Since there was no known phenotype associated with the constitutive expression of the *Thi* 2.1 gene, an alternative genetic approach that exploits its strict JA-dependent regulation was developed. The promoter of the *Thi*. 2.1 gene was fused to the coding region of the dominant marker gene *bar* (De Block *et al.*, 1987). This gene codes for phosphinotricin acetyl transferase that detoxifies the herbicide *Basta*. *Arabidopsis thaliana* ecotype Columbia was transformed with a gene construct consisting of a 1.3 Kb fragment of the *Thi* 2.1-promoter and the *bar* coding region (Figure 1). In addition, two control lines were used in which the *bar* gene was cloned behind the CAMV-35S promoter and behind the weaker *nos*-promoter, respectively (Figure 1). The rationale behind our screening strategy for the isolation of constitutively active signal transduction mutants was based on the assumption that the transgene-mediated herbicide resistance in a putative signal transduction mutant should also reflect the constitutive expression of the endogenous *Thi* 2.1 gene. Among the mutations that lead to an enhanced herbicide resistance it should be possible to identify transacting genes that do not only upregulate the *Thi* 2.1 promoter-*bar* reporter gene but at the same time also the endogenous *Thi* 2.1 gene.

Among the primary transformants, *Thi* 2.1-*bar* lines were selected that contained only a single DNA insertion and that showed a MeJA-inducible herbicide resistance. Transgenic plants that showed a segregation ratio of approximately 3 : 1 for Kanamycin resistance were chosen for Southern blot analysis. Lines with only a single copy of the selectable marker gene were used for the following trials.

Initial experiments on agar plates had indicated that repeated MeJA-treatments of the seedlings significantly boosted the plants' herbicide resistance. In order to enhance this response further, plants grown on soil were not only exposed to MeJA but alternatively also to the culture filtrate of *Fusarium oxysporum*. This filtrate had been shown previously to be a much stronger inducer of the thionin gene expression than MeJA (Vignutelli *et al.*, 1998). Culture filtrate-treated seedlings grown on soil survived a herbicide concentration of 120 $\mu g\ ml^{-1}$, whereas

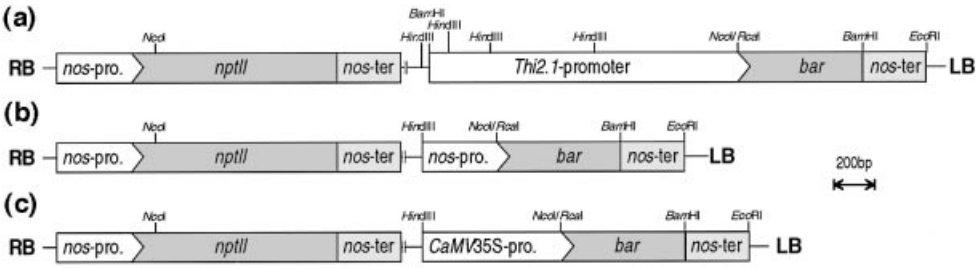

Figure 1. The promoter-*bar* constructs in pBIN 19 that were used for transformation of *Arabidopsis thaliana*.
(a) *Thi* 2.1-*bar*
(b) *nos-bar*
(c) CAMV 35S-*bar*.

the non-induced plants and the control line *Thi* 2.1-*GUS* died at a concentration of about 40 μg ml^{-1}. The LD_{50} of the *nos-bar* line was about 500 μg ml^{-1} and the 35 S-*bar* line survived *Basta* concentrations higher than 2500 μg ml^{-1}. Under optimized conditions seedlings were grown on soil for 9 days before they were sprayed on 4 consecutive days with water, 100 μM MeJA or the culture filtrate of *F. oxysporum*, respectively. On the fifth day, the seedlings were sprayed with the inducers in the morning followed by a treatment with 120 μg ml^{-1} *Basta* solution in the afternoon. The *Thi* 2.1-*GUS* line was used as a control. It is evident that only those seedlings that were induced with MeJA or the culture filtrate survived the herbicide treatment (Figure 2). Treatment with the culture filtrate resulted in a much higher *Basta* resistance than the treatment with MeJA. The conditions defined by this experiment should be sufficient to identify mutants that constitutively overexpress the selectable marker gene to the same high level as the original *Thi* 2.1-*bar* line after exposure to the culture filtrate.

400 mg of seeds from the homozygous *Thi* 2.1-*bar* line were mutagenized with ethylmethylsulfonate (EMS), allowed to germinate and grown in 40 pools of approximately 500 plants each. M2 seeds were harvested separately from each pool. Approximately 5% of the seeds did not germinate on agar and 2.5% of the seedlings showed a chlorophyll-deficient *albino*- or *xantha* phenotype when grown on MS agar plates.

A total of 200 000 M2 seeds from all 40 pools were screened. About 5000 seeds were sown on soil in a 1300-cm^2 tray. Unlike on agar, on soil only approximately 50% of the seeds germinated. Twelve days later the seedlings were sprayed with the herbicide *Basta*. 398 putative mutants were isolated in this first screen. The plants were allowed to self-fertilize and to set seeds. The M3 progeny of 366 of these putative mutants was re-screened with *Basta*. The seeds of each plant were sown in a separate pot and 12 days later the seedlings were treated with the herbicide. 59 lines showed *Basta* resistance again. These putative mutants were finally tested by Northern blot analysis for constitutive expression of the endogenous *Thi* 2.1 gene (Figure 3). The *Thi* 2.1-*bar* and *Thi* 2.1-*GUS* lines served as controls and they expressed large amounts of the thionin mRNA only after inoculation with *Fusarium oxysporum* or treatment with the culture filtrate (Figure 3). Ten lines that constitutively accumulated thionin mRNA were finally selected for a more detailed analysis. The mutants were called *cet* for their *c*onstitutive *e*xpression of *t*hionin. Each of them was backcrossed either once or twice to *Thi* 2.1-*GUS* plants. All mutants used for the subsequent studies were homozygous for the *Thi* 2.1-*GUS* reporter gene and, except for the *cet* 8 and *cet* 9 lines, were also homozygous for the *cet* mutation.

Genetic characterization of the cet mutants

For each of the backcrossed mutants test crosses were performed with the homozygous *Thi* 2.1-*GUS* line. The *cet* 7 mutant could not be included in this analysis because most of the seedlings died already prior to the formation of the primary leaves. The segregation of the activated *GUS* reporter gene in the F2 generation of the other crosses was consistent with single genes being responsible for the *cet*-phenotypes. Four of the mutations (*cet* 1, *cet* 2, *cet* 8 and *cet* 9) were dominant, while the remaining mutations were recessive (Table 1). With the recessive mutations a complementation analysis was performed. Plants homozygous for the *GUS* marker gene and their respective *cet* mutation were crossed. Seedlings of the F 1 generations were tested for their GUS activity. In most cases blue staining and white phenotypes could be clearly distinguished while in some F1 plants that were whitish a slight staining in the apex region could be detected. Since a similar staining was also occasionally seen in wildtype plants, these were considered as F1 plants of nonallelic parents (results not shown). The recessive mutants were assigned to four complementation groups. Only the *cet* mutants 4–1 and 4–2 were allelic.

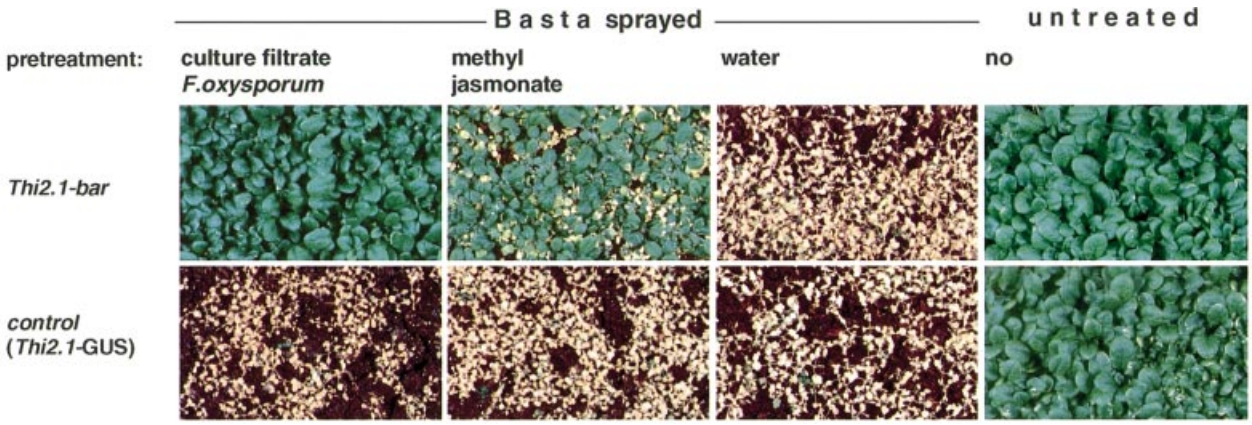

Figure 2. Functional analysis of the line *Thi* 2.1-*bar* 17.5 that was chosen for mutagenesis.
The transgenic lines *Thi* 2.1-*bar* and, as a control, *Thi* 2.1-*GUS* were pretreated with the culture filtrate of *F. oxysporum*, methyl jasmonate or water, respectively, before spraying them with *Basta*.

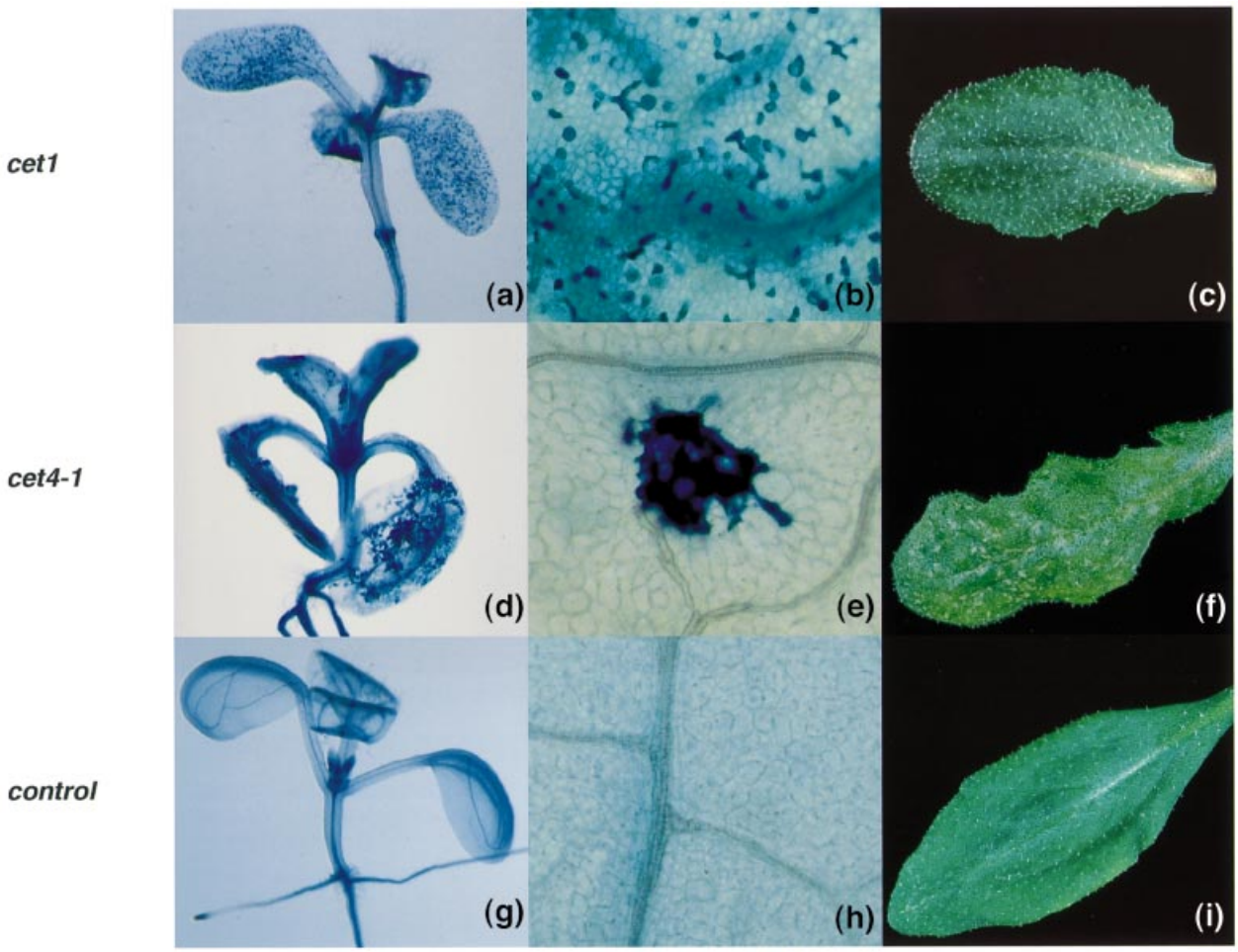

Figure 6. Necrotic lesion formation in the dominant *cet* 1 (a–c) and the recessive *cet* 4–1 (d–f) mutants. The *Thi* 2.1-*GUS* line was used as a control that does not show any lesions (g–i). Lesion formation was detected by trypan blue staining. Plants were grown for 9 d on MS agar and 0.5% sucrose under a 16-h light/8-h dark regime (a, b, d, e, g, h) or on soil for 70 d under short day conditions (8 h light/16 h dark) (c, f, i). Pictures were taken from whole seedlings (a, d, g), primary leaves (b, e, h) or rosette leaves (c, f, i). Note that necrotic lesions were macroscopically visible only on rosette leaves of the *cet* 4–1 mutant (f).

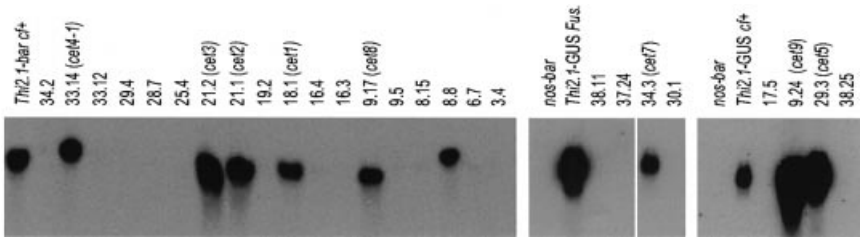

Figure 3. Identification of putative signal transduction mutants that were selected after spraying M2-seedlings of the mutagenized *Thi* 2.1-*bar* line with 120 μg ml^{-1} *Basta*.
Putative mutants were subjected to two rounds of screening. Afterwards RNA was extracted from each of the selected plant lines and analyzed on Northern blots, using a *Thi* 2.1-DNA probe for hybridization. In several of the selected mutants the constitutive expression of the endogenous *Thi* 2.1 gene reached a similar high level as in the control plants (*Thi* 2.1-*bar*, *Thi* 2.1-*GUS*) that were exposed to the culture filtrate (cf +) or to *F. oxysporum* (Fus.). *nos-bar*: Control plants that were treated with water. Numbers on top of the gel refer to the batch number from which a putative mutant had been isolated (first number) and the number of that particular mutant plant (second number).

Table 1. Segregation of the *cet* mutations in the F2 generation of crosses between the *cet*-mutants and *Thi* 2.1-*GUS* plants. GUS staining was used to discriminate between mutant and wildtype phenotypes. All parent plants used for these crosses were homozygous for the *GUS* marker gene

line	blue/white	segr.	χ^2
cet1 × *Thi2.1*-GUS *	059 : 028	2.1 : 1.0	2.39464
Thi2.1-GUS × *cet1* *	062 : 020	3.1 : 1.0	0.01626
cet2 × *Thi2.1*-GUS	129 : 044	2.9 : 1.0	0.01734
cet3 × *Thi2.1*-GUS	016 : 053	1.0 : 3.3	0.12077
Thi2.1-GUS × *cet3*	023 : 058	1.0 : 2.5	0.49794
cet4.1 × *Thi2.1*-GUS	024 : 059	1.0 : 2.5	0.67871
Thi2.1-GUS × *cet4.1*	022 : 070	1.0 : 3.2	0.05797
cet4.2 × *Thi2.1*-GUS	043 : 112	1.0 : 2.6	0.62151
cet5 × *Thi2.1*-GUS	012 : 050	1.0 : 4.2	1.05376
Thi2.1-GUS × *cet5*	012 : 046	1.0 : 3.8	0.57471
cet6 × *Thi2.1*-GUS	026 : 072	1.0 : 2.8	0.12245
Thi2.1-GUS × *cet6*	014 : 052	1.0 : 3.7	0.50505
cet8 × *Thi2.1*-GUS **	119 : 045	2.6 : 1.0	0.52032
cet9 × *Thi2.1*-GUS **	120 : 048	2.5 : 1.0	1.14286

*Adult homozygous plants were smaller than heterozygous plants.
**About 1/3 of the GUS stainable seedlings were extremely small. Their leaves were bent downwards (*cet8:* 40 of 110; *cet9*: 39 of 120). Homozygous plants do not survive standard growth conditions in the green house.

Levels of JA and 12-oxophytodienoic acid (OPDA) in cet mutants

The constitutive expression of the *Thi* 2.1 gene could be due to an upregulation of JA biosynthesis in the *cet* mutants or to an enhanced sensitivity to JA. Alternatively, it might also be caused by an alteration of a downstream component of JA-dependent signaling pathways. The levels of JA were measured in *cet* 1, *cet* 3 and *cet* 4–1 that could easily be propogated as homozygous mutants. In addition to JA the concentration of one of its precursors, 12-oxophytodienoic acid (OPDA), was also determined (Figure 4a,b). OPDA has previously been shown to be even more active than JA in triggering one of the most sensitive, octadecanoid-dependent responses, the tendril coiling of *Bryonia dioica* (Blechert *et al.*, 1999). The concentrations of JA and OPDA were remarkably different in the three *cet* mutants. *cet* 1 was grown in the presence of 0.5% sucrose while the two other mutants were kept on 2% sucrose. The JA content of *cet* 1 was almost 40-fold higher than in wildtype control plants (Figure 4b). In *cet* 3 the OPDA and JA levels were also increased by a factor of 4 and 8, respectively. In both mutants the molar concentrations of OPDA were approximately 10-fold higher than those of JA (Figure 4). In marked contrast to *cet* 1 and *cet* 3, JA and OPDA levels in *cet* 4–1 were very low and not different from those of the control plants (Figure 4). These differences in JA and OPDA concentrations had no apparent effect on the concentrations of free SA in the three mutants. In *cet* 1 and *cet* 4–1 the levels were only twice the amount present in control plants, while in *cet* 3 the level of free SA was similar to that in the control (Figure 5a).

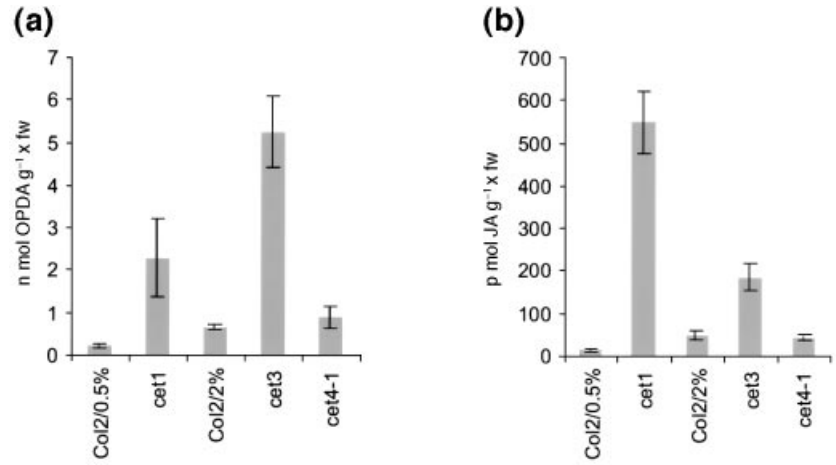

Figure 4. Levels of OPDA (a) and JA (b). *cet* mutants and wildtype seedlings were grown for 12 d under long day conditions on MS agar in the presence of 0.5% (*cet* 1) and 2% (*cet* 3, *cet* 4–1) sucrose, respectively.

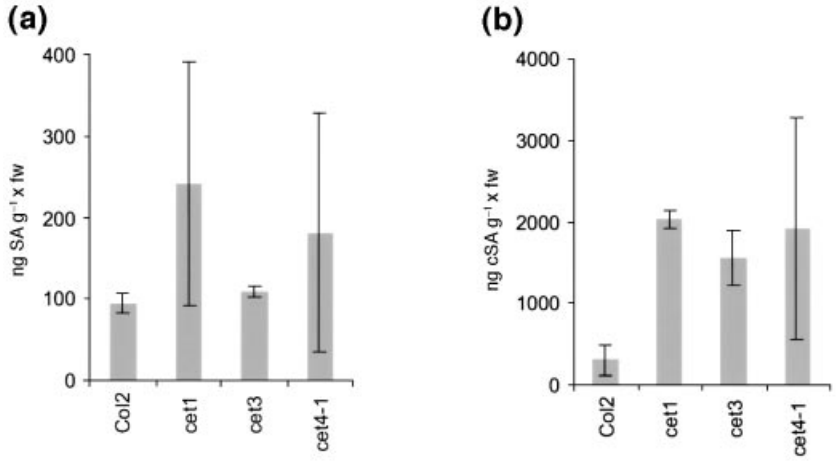

Figure 5. Levels of (a) free (SA) and (b) sugar-conjugated (cSA) salicylic acids. Plants were grown on 2% sucrose for 12 d under long day conditions.

However, in all three *cet* mutants the levels of sugar-conjugated SA were higher compared with the wildtype controls (Figure 5b).

Phenotypic characterization of the cet mutants

Seedlings were grown on MS agar plates supplemented with either 0.5% or 2% sucrose. Plants grown on 2% sucrose have been shown to have a higher inducibility of the *Thi* 2.1 gene than plants grown on 0.5% sucrose (Vignutelli *et al.*, 1998). However, some of the mutant seedlings were severely retarded or developed no roots when grown on 2% sucrose. The four dominant mutants were all significantly smaller than wildtype plants. At seedling stage their leaves bent downwards and their youngest leaves often had a light green color. On soil the homozygous *cet* 1 mutant grew normally, while the homozygous *cet* 2 mutant often died and produced only very few seeds. Homozygous *cet* 8 and *cet* 9 mutants did not survive on soil and could be propagated only as heterozygous plants. All homozygous recessive mutants were viable. In some cases (*cet* 3, *cet* 4) seedlings developed callus- and blister-like structures on their leaves.

During an attempted infection by an avirulent pathogen the induced SAR reaction is often preceded by the collapse of the challenged plant cell. Such hypersensitive response-like lesions also develop spontaneously in several mutants that constitutively activate the SAR reaction in the absence of pathogens (Dietrich *et al.*, 1994; Greenberg and Ausubel, 1993). On the other hand, exposure of wildtype plants to the culture filtrate of *F. oxysporum* or MeJA activates the octadecanoid-dependent pathway and induces several defense-related genes but does not trigger any cell death events. Most of the *cet* mutants, however, that constitutively activate the expression of the MeJA-inducible *Thi* 2.1 gene also formed spontaneous lesions. The cotyledons of all dominant *cet* mutants (*cet* 1, *cet* 2, *cet* 8, *cet* 9) formed lesions at a high frequency. As an example of this group the *cet* 1 mutant is shown in Figure 6(a)–(c). The true

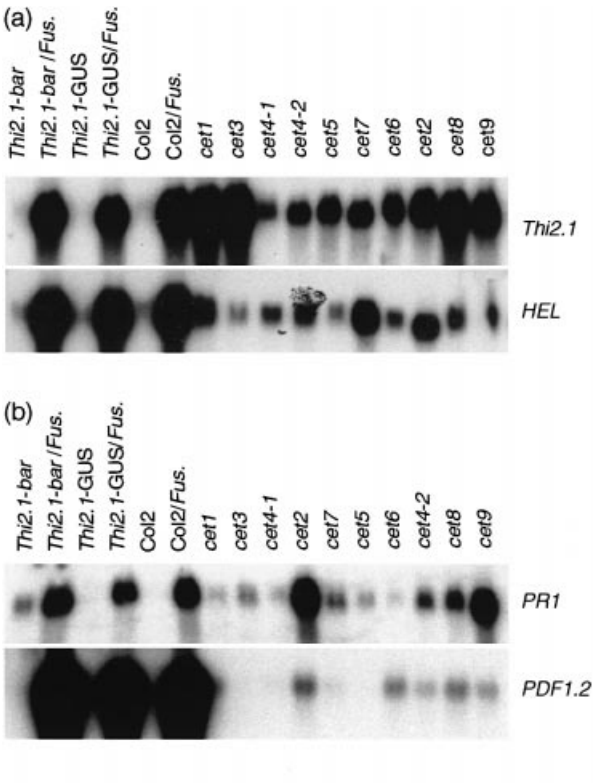

Figure 7. Constitutive expression of (a) the *Thi* 2.1 thionin gene (*Thi* 2.1) and the hevein-like protein gene (*HEL*) and (b) the pathogenesis-related protein 1 gene (*PR1*) and the Pdf 1.2-defensin gene (*Pdf* 1.2) in the *cet* mutants of *Arabidopsis thaliana*. As controls the *Thi* 2.1-*bar* and the *Thi* 2.1-*GUS* lines and the non-transgenic wildtype plants (Col 2) that were inoculated with *F. oxysporum* (Fus.) or sprayed with water were used. Transcript levels in the inoculated control plants were used as an internal reference to estimate the level of constitutive gene expression in the various *cet* mutants. RNA was isolated and analyzed on Northern blots. Seedlings were grown for 12 d on MS agar and 2% sucrose.

leaves were scattered with single cell corpses or small clusters of a few dead cells. These lesions were not visible macroscopically and they did not spread further; 18-d-old plants showed the same pattern as 9-d-old plants. In *cet* 1 mutants and, with a lower frequency, also in *cet* 2 mutants these microlesions were even found on stems, cauline leaves and young siliques. As mentioned above it was very difficult to grow homozygous *cet* 2, *cet* 8 and *cet* 9 mutants under standard conditions in the greenhouse. The rosettes of the majority of these plants turned whitish and died. It is not clear, whether this collapse of the plants was due to an environmentally triggered spreading of the microscopic lesions. *cet* 2 plants that had been transferred from agar to soil very late generally started to flower and form siliques. However, shoot tips of these plants frequently died before the seeds matured. The recessive line *cet* 5 also formed microlesions on cotyledons and rosette and cauline leaves, but at an extremely low frequency. A second group of mutants, *cet* 3 and *cet* 4–1 and 4–2, formed larger lesions. Numerous necrotic spots were found on cotyledons, rosette and cauline leaves. As an example *cet* 4–1 is shown in Figure 6(d)–(f). In the cases of the lines *cet* 4–1 and 4–2 areas of dead cells were even detectable on stems and siliques. The lesions did not spread, but were detectable before staining (Figure 6f). In the *cet* 6 mutant as well as in wildtype, the *Thi* 2.1 *GUS* and the *Thi* 2.1-*bar* lines no spontaneous appearance of dead cells has been observed (Figure 6g–i).

Some of the Arabidopsis mutants that either constitutively activate the SAR resistance response or are blocked in this pathway are also affected in SA-independent defense responses. For instance the *cpr* 5 and *ssi* 1 mutants constitutively activate the expression of the *PR*-genes and the MeJA-inducible *Pdf* 1.2 defensin gene (Bowling *et al.*, 1997; Shah *et al.*, 1999). Thus, these mutations define genetic loci that seem to control points at which different signal transduction pathways converge and which may form an important link between distinct defense pathways that allow the coordinated activation of several defense reactions. Crosses between the recessive *cpr* 5 mutant and the *cet* 3 and *cet* 4–1 mutants revealed that the mutated genes were not allelic. We were interested in identifying possible links that may exist between the MeJA-inducible defense reactions and other defense pathways. Since all *cet* mutants were identified based on their constitutive overaccumulation of thionin mRNAs that reached levels in the mutants similar or even higher than in wildtype plants exposed to the strongly inducing culture filtrate of *F. oxysporum,* we analyzed the expression level of other genes known to be activated through the SA- and the ethylene-dependent signal transduction pathways during a pathogen-induced defense reaction. The expression of the *Pdf* 1.2 defensin gene is induced by MeJA but not by SA, similar to the *Thi* 2.1 gene. However, in contrast to the thionin gene, the defensin gene expression also depends on ethylene (Penninckx *et al.*, 1998). The hevein-like protein HEL is strongly inducible by ethylene (Potter *et al.*, 1993). The accumulation of the pathogenesis-related protein PR 1 of Arabidopsis is strongly induced by SA but not by MeJA and is used as a molecular marker for the SA-dependent defense pathway (Hunt and Ryals, 1996; Malamy and Klessig, 1992).

The control plants were challenged with *F. oxysporum* in order to reach a maximum level of expression for all marker genes. These levels were used as reference points to evaluate the expression intensity of the marker genes in the various *cet* mutants. As shown in Figure 7(a), the expression of the *Thi* 2.1 gene is induced strongly in all *cet* mutants and in some cases it even exceeds the high transcript level of control wildtype plants that have been inoculated with *F. oxysporum*. Under these conditions the expression level of the *Thi* 2.1 gene in inoculated wildtype plants is even higher than after exposure to the culture

filtrate (Figure 3). When the same RNA samples were used to analyze the expression of the *PR* 1 gene a different expression profile was obtained for the *cet* mutants (Figure 7b). In the wildtype controls that had been inoculated with *F. oxysporum* a strong induction of the *PR* 1 gene expression was observed, indicating that under these conditions not only the MeJA-dependent pathway but also the SA-dependent defense response was activated. In most of the *cet* mutants that had not been exposed to pathogens only a very weak expression of *PR* 1 could be observed. Only in the *cet* 2, *cet* 8 and *cet* 9 mutants a strong constitutive expression of this gene occured. In *cet* 2 and *cet* 9 mutants the *PR* 1 mRNA level was even higher than in the control plants that had been challenged with the fungal pathogen (Figure 7b).

The expression of the *HEL* gene was induced in the *cet* mutants. The highest mRNA levels were found in *cet* 1, *cet* 2 and *cet* 7, but even in these mutant lines the mRNA levels were much lower than in wildtype plants after *F. oxysporum* infection (Figure 7a). The expression of the *HEL* gene can be stimulated more than 100-fold by ethylene, but also elevated SA-levels can result in a 5–10-fold increase in *HEL* mRNA concentration (Potter *et al.*, 1993). Therefore, the increase in *HEL* mRNA levels in most of the *cet* mutants may not be due to a constitutive activation of the ethylene-dependent pathogen-defense pathway but instead could reflect a secondary response to a slight increase in SA concentrations as seen for example in *cet* 1, *cet* 3 and *cet* 4–1 (Figure 5).

The expression of the *Pdf* 1.2 gene was induced in most of the *cet* mutants (Figure 7b). When the *Pdf* 1.2 gene expression in the wildtype control plants was used as an internal reference, it became evident that in the *cet* mutants the mRNA level for this gene reached only a very minor fraction of less that 2% of the level induced in the control plants after inoculation with *F. oxysporum*. Only an extended exposure of the X-ray films clearly revealed the enhanced levels of this transcript (Figure 7b). In *cet* mutants that had been inoculated with *F. oxysporum* the *PR* 1, *HEL* and *Pdf* 1.2 genes were all strongly upregulated and reached similar high transcript levels as in the pathogen-treated wildtype control plants (results not shown).

Discussion

Thionins are a group of small and cysteine-rich polypeptides, most of which exhibit antimicrobial activities (Bohlmann, 1994; Broekaert *et al.*, 1997). For a long time they have been thought to play an important role in many higher plant species during pathogen defense. In our previous work we have used Arabidopsis to study the function of these proteins in greater details. Two thionin genes (*Thi* 2.1 and *Thi* 2.2) were identified in Arabidopsis and were shown to be regulated differently (Epple *et al.*, 1995). Induction of the *Thi* 2.1 gene correlated with resistance against *Fusarium oxysporum* and overexpression of this gene in the susceptible ecotype Columbia resulted in an enhanced resistance of the transgenic lines against this fungal pathogen (Epple *et al.*, 1997, 1998). The *Thi* 2.1 gene was found to be inducible by MeJA, wounding and pathogenic fungi, leading to local and systemic expression. SA and ethephon, however, did not induce the *Thi* 2.1 gene, indicating that it is regulated exclusively via the octadecanoid pathway (Bohlmann *et al.*, 1998). The aim of the present work was to identify components that are involved in the regulation of the *Thi* 2.1 gene and to isolate signal transduction mutants that constitutively activate the octadecanoid pathway. Since there was no known phenotype associated with a constitutive expression of the *Thi* 2.1 gene, the MeJA-dependent regulation of this gene was exploited for setting up an efficient genetic selection scheme using the *bar* gene as a selectable marker. The *bar* gene confers resistance to the herbicide *Basta*. It codes for phosphinotricin acetyltransferase, which converts the toxic phosphinotricin, the active compound of *Basta*, into the non-toxic acetylated form (De Block *et al.*, 1987). The *Thi* 2.1-promoter fragment, which was chosen to control the *bar* gene expression, had previously been placed in front of the *GUS* marker gene and had been shown to be tightly regulated via the octadecanoid pathway (Vignutelli *et al.*, 1998). Using the *Thi* 2.1-*bar* construct it became possible to discriminate clearly between mutagenized seedlings that did not and those that did show a constitutive and enhanced activity of the *Thi* 2.1 promoter. Among the selected mutants 10 were isolated and showed not only an enhanced *Basta* resistance but at the same time constitutively expressed the endogenous *Thi* 2.1 gene and the *Thi* 2.1 promoter-controlled *GUS* reporter gene (results not shown). Thus, these mutants proved to be affected in transacting genes that control the promoter activity of the *Thi* 2.1 gene.

Most of the homozygous mutants grew very poorly on soil, their leaves often necrotized and in several cases plants died before flowering. Three of the mutants (*cet* 1, *cet* 3, *cet* 4) that could easily be propagated on soil were used for determining levels of JA and OPDA. Based on the results of this analysis two different types of *cet* mutants could be distinguished; those that drastically overproduce JA and OPDA, such as *cet* 1 and *cet* 3, and others, such as *cet* 4–1 that contain the same low levels as non-stressed wildtype control plants. The constitutive upregulation of the *Thi* 2.1 gene in members of the second group of *cet* mutants could be due to an enhanced sensitivity to JA or the constitutive activation of a downstream component of JA-dependent signaling pathways. Further genetic and biochemical work is required to define more precisely the

exact nature of the mutations that lead to the activation of the *Thi* 2.1 gene.

There were two main characteristics that were common to most of the *cet* mutants. Firstly, in addition to the *Thi* 2.1 gene, genes were upregulated that are known to be controlled by other signal transduction pathways and, secondly, in most of these *cet* mutants necrotic lesions formed spontaneously.

Plants are capable of activating different signaling pathways to induce the expression of specific subsets of defense-related genes in response to a particular pathogen. An SA-dependent pathway has been shown to activate the SAR response against bacterial and fungal pathogens (Bowling *et al.*, 1997). The JA-dependent octadecanoid pathway is also involved in controlling some of the antifungal defense reactions, but in addition plays a major role in wound responses (Farmer *et al.*, 1998). Besides the SA- and JA-dependent reactions ethylene-dependent steps in the signaling cascade have also been shown to be involved in the control of the expression of certain antimicrobial proteins (O'Donnell *et al.*, 1996; Penninckx *et al.*, 1998; Pieterse and van Loon, 1999). These three signaling pathways do not operate independently but have been shown to interact. Several mutations like *ssi* 1 (Shah *et al.*, 1999) and *cpr* 5 (Bowling *et al.*, 1997) that define branch points within the signaling cascade, at which these signaling pathways may converge or diverge, have been described.

In addition to the *Thi* 2.1 gene we have analyzed the expression of three other marker genes that are known to be activated specifically by one or two of the known pathogen-induced signaling pathways: The SA-dependent *PR* 1 gene (Uknes *et al.*, 1992), the ethylene-inducible *HEL* gene (Potter et al., 1993) and the *Pdf* 1.2 gene which requires a concomitant activation of the ethylene- and the octadecanoid pathways (Penninckx *et al.*, 1998). All these genes were upregulated in the *cet* mutants. However, the extent of upregulation varied extensively among the different mutants. In order to assess the degree of upregulation and to facilitate the interpretation of these transcript changes, we have used as an internal reference the transcript levels of all genes reached in wildtype and mutant plants after inoculation with *Fusarium oxysporum*. It became evident that the constitutive expression of the *Thi* 2.1 gene in the *cet* mutants led to very high transcript levels that were either close to or, in some mutants such as *cet* 1, *cet* 3, *cet* 8 and *cet* 9, even surpassed those of the pathogen-treated control plants. In most of the *cet* mutants these constitutive levels were higher than in wildtype plants treated with a culture filtrate of *Fusarium oxysporum* and drastically exceeded the amounts of *Thi* 2.1 transcripts that were recovered from MeJA-treated plants (results not shown). Thus, the enhanced *Thi* 2.1 transcript levels in the *cet* mutants seem to reflect a constitutive activation of components of the octadecanoid pathway that control the *Thi* 2.1 expression.

In contrast to the high expression level of the *Thi* 2.1 gene the constitutive transcript levels of the three other marker genes were much lower in most of the *cet* mutants, when compared with the levels of the corresponding transcripts in the pathogen-treated control plants. This became particularly obvious in the case of the *Pdf* 1.2 gene. In most of the *cet* mutants, the transcript levels of this gene were slightly enhanced when compared with the non-treated control plants. However, after infecting these control plants with *Fusarium oxysporum,* their transcript levels exceeded those of the non-treated *cet* mutants by more than 50 fold. Expression of the *Pdf* 1.2 gene depends on the concomitant activation of the JA-dependent and the ethylene-dependent pathways (Penninckx *et al.*, 1998). The very low expression level of this gene in the *cet* mutants indicates that levels of ethylene in the *cet* mutants seem to be too low to mimic the full activation of the defensin gene by a pathogen. It also strongly suggests that the *cet* mutations define genes that are selectively involved in the activation of the octadecanoid pathway and that the activation of the *Thi* 2.1 gene occurs independently of ethylene. This interpretation concurs with the levels of *HEL*-transcripts found in the *cet* mutants. The *HEL* gene is strongly induced by ethylene and can also be activated by elevated levels of SA. In all *cet* mutants the levels of this transcript are higher than in non-treated control plants. However, when compared with the pathogen-infected control plants, in none of the *cet* mutants similar high levels of *HEL* transcripts were present. Since in *cet* 1, *cet* 3 and *cet* 4–1, slightly higher concentrations of free and/or sugar-conjugated SA were found, it seems likely that the elevated levels of SA contributed to the increase in *HEL* transcripts in these mutants.

Of particular interest were the results of the *PR* 1 expression studies. *PR* 1 is considered to be a marker gene of the SA-dependent systemic acquired resistance response in Arabidopsis. In most of the *cet* mutants only a minor increase in the activity of this gene could be observed that could easily be attributed to the slightly elevated levels of SA and its derivatives in these mutants. However, in sharp contrast to most of the *cet* mutants, in *cet* 2 and *cet* 9 *PR* 1 transcript levels were reached that exceeded even those in the pathogen-treated control plants. It is not known whether the high *PR* 1 transcript levels in these two *cet* mutants correlate with drastically enhanced endogenous SA concentrations. It will be important to analyze genetically whether the expression of the *PR* 1 gene in the *cet* 2 and the *cet* 9 mutants is maintained at a similar high level after these mutations have been combined with the *NahG* gene. The *NahG* gene encodes a bacterial salicylic acid hydroxylase that leads to a drastic reduction in the SA content (Delaney *et al.*, 1994;

Gaffney *et al.*, 1993). Both *cet* mutants resemble other mutants such as *cpr* 5 and *ssi* 1 that define sites at which different signaling pathways may meet and interact (Bowling *et al.*, 1997; Shah *et al.*, 1999). Even though test crosses between these two mutants and the dominant *cet* 2 and *cet* 9 mutants have not been performed it seems likely that both *cet* 2 and *cet* 9 are not allelic to *cpr* 5 and *ssi* 1. *cpr* 5 and *ssi* 1 constitutively overexpress the SA-dependent *PR* 1 gene and the *Pdf* 1.2 gene that requires JA and ethylene (Penninckx *et al.*, 1998), while *cet* 2 and *cet* 9 overexpress the *PR* 1 but not the *Pdf* 1.2 gene.

The second feature common to most of the *cet* mutants is the formation of spontaneous lesions. Thionins are phytotoxic (Reimann-Philipp *et al.*, 1989) and overexpression of some of these proteins in a heterologous plant system may induce various stress symptoms such as enhanced anthocyanin synthesis and spontaneous necrosis in older leaves (P. Malnoe, H. Bohlmann and K. Apel, unpublished results). Thus, it is conceivable that the constitutive activation of the *Thi* 2.1 gene leads to the accumulation of larger amounts of thionins in the *cet* mutants that may trigger the formation of necrotic lesions. We have used two different experimental approaches to test this possibility. First, control plants were treated with MeJA or with the culture filtrate of *F. oxysporum*. Both treatments activate the *Thi* 2.1 gene and lead to enhanced levels of thionins. Second, transgenic lines of *A. thaliana* that constitutively overexpress the *Thi* 2.1 gene and accumulate large amounts of thionins were analyzed. In contrast to non-transgenic controls, these lines show an enhanced resistance against *F. oxysporum* (Epple *et al.*, 1997). In both plant samples that contain elevated thionin levels no spontaneous necrotic lesions were observed. Collectively, these results indicate that formation of necrotic lesions in the *cet* mutants does not seem to be caused by an overaccumulation of thionins.

Necrotic lesion formation has been described as part of the hypersensitive response of plants to pathogens. The cell death response associated with the HR requires higher concentrations of SA and is normally restricted to a limited number of cells adjacent to the site of pathogen attack. The *cet* mutants resemble mutants that mimic this defense response (Dietrich *et al.*, 1994; Greenberg *et al.*, 1994; Hu *et al.*, 1998). Some of the lesions occur independently of SA, while others require elevated levels of SA (Hunt *et al.*, 1997; Weymann *et al.*, 1995). The wildtype copies of the mutated genes had been thought to be involved in the genetic control of the HR response (Dietrich *et al.*, 1994; Greenberg and Ausubel, 1993). However, identification of some of these genes has revealed that the perturbance of normal metabolic pathways that seem unrelated to defense reactions may also result in formation of necrotic lesions (Hu *et al.*, 1998; Mach *et al.*, 2001). If one accepts the SA-dependency of cell death execution as a criterion to identify cell death associated with the hypersensitive reaction, necrotic lesion formation in the *cet* mutants may not necessarily represent this pathogen defense reaction. It is not known whether the slightly increased levels of free SA in some of the *cet* mutants are sufficient to support the activation of an HR-like cell death pathway. An answer to this question may come from combining the *cet* mutations with genes that either prevent the accumulation of SA or that block the SA-dependent signaling, and analyzing the effects of these genetic changes on necrotic lesion formation in the *cet* mutants.

Experimental procedures

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* ecotype Columbia were surface-sterilized with 80% ethanol, 0.1% Tween 20 for 2 min. They were then treated with 6% sodium hypochlorite for 15 min, washed 4 times with sterile tap water and plated onto Petri dishes containing 0.8% agar, MS medium plus vitamins pH 5.7, and various concentrations of sucrose and antibiotics. The seedlings were grown at 22°C under a 16-h light/8-h dark cycle.

Tests with Fusarium oxysporum

Spores of *Fusarium oxysporum* f. sp. *mathiolae* strain 247.61 were produced by growing the fungus on PDA (potato dextrose broth, 1.5% agar; Difco, Detroit, MI, USA) at room temperature for 2–3 wk. Spores were suspended in sterile tap water, filtrated through Miracloth (Calbiochem-Novabiochem, San Diego, CA, USA) and counted with a Fuchs/Rosenthal chamber (Merck ABS, Dietikon, Switzerland). The culture filtrate was produced by growing the fungus in potato dextrose broth at room temperature without shaking for 6 wk. The nutrient solution was then sterile-filtrated and diluted.

For inoculation with *Fusarium oxysporum* the seedlings were grown on MS medium containing 0.5% sucrose for 10 d in a growth chamber. Each Petri dish was then sprayed with 2 ml of the spore suspension (10^7 spores ml^{-1}) of *F. oxysporum*. The plates were closed and kept again in the growth chamber. During the first 24 h after inoculation the plates were kept in the dark.

Determination of JA, OPDA and SA

For the measurement of JA and OPDA plant material frozen in liquid nitrogen (max. 1 g per sample) was homogenized with 10 ml methanol and, as internal standards, 100 ng ($^2H_6$) JA (Miersch, 1991) and ($^2H_5$) OPDA, prepared from (17–$^2H_2$, 18–$^2H_3$) linolenic acid according to Zimmerman and Feng (1978). Separations by HPLC and GC/MS-SIM were performed as described previously (Kramell *et al.*, 2000). The SA content of wildtype and mutant plants was determined according to Meuwly and Métraux (1993).

Cloning of the bar-constructs

The coding region of a synthetic *bar* gene (D'Halluin *et al.*, 1992) was amplified by PCR with the primers PPT 1 (5'-GTC ATG AGT

CCG GAG AGG AGA CCA) and PPT 5(5′-GCA GGA TCC ATC ATA TCT GGG TAA CTG) and cloned into the *Sma* 1 site of pUC 18. After sequence verification and determination of the correct orientation, the *bar* gene was isolated as a *Sac* I/*Hind* III fragment, gel-purified (Hansen *et al*., 1993) and ligated into the *Sac* I/*Hind* III site of the vector pHB 234 (*nos* terminator in pUC 18). The *bar*::*nos*-terminator cassette was excised with *Rca* 1 and *Eco* RI. The *Thi* 2.1-promoter was isolated as a *Bam* HI/*Nco* I fragment from a pUC BM 21 derivative (Vignutelli *et al*., 1998). Both fragments were gel-purified (Hansen *et al*., 1993) and coligated into the *Bam* H I/*Eco* RI site of the binary vector pBin 19 (Bevan, 1984) to give pHB 2209 harboring the *Thi* 2.1-promoter::*bar*::*nos*-terminator construct (*Thi* 2.1-bar). Correct ligation was verified by sequence analysis. In case of the two control constructs 35 S-*bar* and *nos-bar* a *Hind* III/*Nco* I fragment of the respective promoter was ligated together with the *Rca* I/*Eco* RI digested *bar*::*nos*-terminator casette into the *Hind* III/*Eco* RI site of pBin 19.

In planta-transformation of Arabidopsis thaliana

All binary vectors were transformed into *Agrobacterium tumefaciens* strain C 58 (Holsters *et al*., 1978) following a standard protocol. *A. thaliana* ecotype Columbia was transformed by an *in planta* transformation method (Bechtold *et al*., 1993) as described by Vignutelli *et al*. (1998).

Other methods

DNA was isolated according to Tai and Tanksley (1990). RNA was isolated according to Melzer *et al*. (1990) and analyzed on Gene Screen membranes (NEN, Boston, MA, USA) as described in Ausubel *et al*. (1994). To identify mutant plants in a segregating population, the primary mutants were backcrossed with the *Thi* 2.1-*GUS* line. Ten to 14 d after plating, the seedlings were numbered and a single leaf from each seedling was used for histochemical GUS staining (Jefferson *et al*., 1987). Dead plant cells were stained with an alcoholic lactophenol-trypan blue solution (Keogh *et al*., 1980). EMS mutagenesis of seeds of the *Thi* 2.1-*bar* line was done as described previously (Runge *et al*., 1995).

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