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HAHB4, a sunflower HD-Zip protein, integrates signals from the jasmonic acid and ethylene pathways during wounding and biotic stress responses

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Summary

The *Helianthus annuus* (sunflower) HAHB4 transcription factor belongs to the HD-Zip family and its transcript levels are strongly induced when sunflower plants are attacked by herbivores, mechanically damaged or treated with methyl-jasmonic acid (MeJA) or ethylene (ET). Promoter fusion analysis, in Arabidopsis and in sunflower, demonstrated that induction of *HAHB4* expression by these treatments is regulated at the transcriptional level. In transiently transformed sunflower plants *HAHB4* expression upregulates the transcript levels of several genes involved in JA biosynthesis and defense-related processes such as the production of green leaf volatiles and trypsin protease inhibitors (TPI). In *HAHB4* sunflower overexpressing tissue, increased activities of lipoxygenase, hydroperoxide lyase and TPI are detected whereas in *HAHB4*-silenced tissue these activities are reduced. Transgenic *Arabidopsis thaliana* and *Zea mays* plants ectopically expressing *HAHB4* also exhibit higher transcript levels of defense-related genes and when *Spodoptera littoralis* or *Spodoptera frugiperda* larvae are placed on each species, respectively, larvae consumed less and gain less mass compared with larvae feeding on control plants. Arabidopsis plants ectopically expressing *HAHB4* had higher amounts of JA, JA-isoleucine and ET compared with control plants both before and after wounding, but reduced levels of salicylic acid (SA) after wounding and bacterial infection. We conclude that *HAHB4* coordinates the production of phytohormones during biotic stress responses and mechanical damage, specifically by positively regulating JA and ET production and negatively regulating ET sensitivity and SA accumulation.

Keywords: HAHB4, jasmonic acid, ethylene, wounding, plant defense mechanisms, HD-Zip.

Introduction

When plants are wounded or attacked by herbivores and pathogens they can induce specific healing and defense responses. A set of signal molecules are synthesized in order to coordinate and specify these responses. A very delicate balance between jasmonic acid (JA), ethylene (ET), salicylic acid (SA) and abscisic acid (ABA) plays a critical role in this regulation and in the signaling pathways for these hormones. An extensive crosstalk during defense and wound responses take place between these hormones, acting either synergistically, additively or antagonistically to activate the expression of defense genes (Anderson *et al.*, 2004; Lorenzo *et al.*, 2003; O'Donnell *et al.*, 1996; Penninckx *et al.*, 1998; Xu *et al.*, 1994). Several transcription factors involved in the crosstalk of JA, ET, SA and ABA signaling pathways during wounding and biotic stress responses have been characterized thus far in different plant species, and most of them belong to the AP2, WRKY and MYB families of transcription factors (Denekamp and Smeekens, 2003; Li *et al.*, 2004; Miao and Zentgraf, 2007; Xu *et al.*, 2006).

HAHB4 is a member of the *Helianthus annuus* (sunflower) subfamily I of HD-Zip proteins that has been previously shown to be transcriptionally regulated by the availability of water and by ABA (Gago *et al.*, 2002). Members of the HD-Zip family exhibit the association of a leucine zipper (LZ) and a

homeodomain (HD), a feature unique to plants (Schena and Davis, 1992). The HD binds DNA through its helix III while the LZ acts as a dimerization motif (Palena *et al.*, 1999).

Previous studies have shown that *HAHB4* transcript levels are positively regulated by ET; however, once induced, HAHB4 negatively regulates the transcription of ET biosynthesis and signaling genes (Manavella *et al.*, 2006). This repression leads to strong inhibition of ET perception (Manavella *et al.*, 2006). Transgenic *Arabidopsis thaliana* plants ectopically expressing HAHB4 exhibit a characteristic phenotype that includes a strong tolerance to water stress and a reduction in growth rate and development (Dezar *et al.*, 2005a). Microarray studies of these transgenic lines also indicated that several JA-biosynthetic and JA-responsive genes are upregulated compared with control genotypes. These observations led us to hypothesize that HAHB4 could be involved in the regulation of defense-related responses in sunflower.

In this study, we show that HAHB4 participates in the regulation of the sunflower wound and biotic stress responses. HAHB4 expression is enhanced locally and systemically when sunflower plants are injured. This induction depends on elements present in the promoter region of *HAHB4* and also on methyl jasmonic acid (MeJA) and ET. Overexpression of *HAHB4* in sunflower results in increased transcript levels and protein activity of several genes associated with wounding and biotic stress responses. In contrast, specific silencing of *HAHB4* curtails their expression. In agreement with these observations, similar results were observed in Arabidopsis and maize plants ectopically expressing *HAHB4*. Quantitative phytohormone analysis showed that levels of jasmonates, ET and SA were affected during the wound and biotic stress responses in transgenic Arabidopsis, suggesting that HAHB4 plays a role in the integration and crosstalk of these phytohormones during these responses.

Results

HAHB4 is upregulated by mechanical damage, caterpillar feeding and jasmonic acid

To determine if the sunflower transcription factor HAHB4 is involved in the wound response, a series of kinetic assays testing its expression were performed. Sunflower leaves were mechanically damaged and RNA samples were prepared from both injured leaves and non-injured distal leaves. The results in Figure 1(a) showed that *HAHB4* transcript levels were induced ~12-fold locally and approximately sixfold systemically 1 h after sunflower plants were mechanically damaged. The peak of *HAHB4* mRNA induction was detected 1 h after wounding and transcript levels slowly decayed afterwards (Figure 1a). Because tissue damage and induction of JA biosynthesis are known to occur during insect attack, the effects of herbivory and exogenous application of MeJA on *HAHB4* expression were also tested. The results indicated that both caterpillar feeding and MeJA induced expression of *HAHB4* (Figure 1a). Differences in *HAHB4* mRNA levels between treatments indicated that transcript levels were maximal when leaves

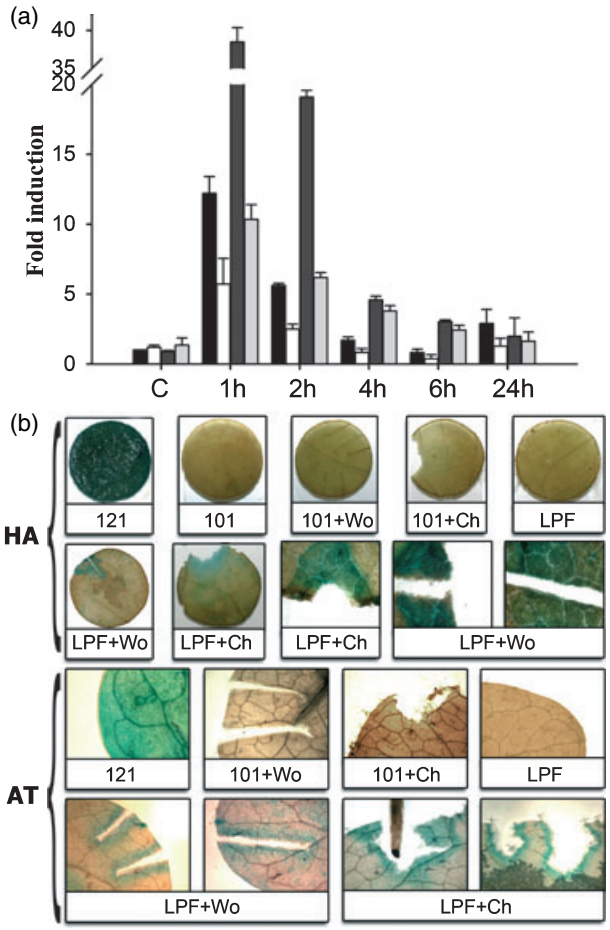

Figure 1. *HAHB4* transcript levels are upregulated by jasmonic acid (JA), wounding and insect attack.
(a) Kinetics of induction of *HAHB4* transcripts in sunflower leaves in control conditions (C) or when the plants were injured by mechanical damage (black bars), insect (*Spilosoma virginica*) chewing (dark grey bars) or treatment with 200 μM methyl-JA (MeJA) (light grey bars). White bars represent transcript levels in uninjured distal leaves when the applied treatment was a mechanical damage. Transcript levels were measured by quantitative RT-PCR and standard deviations calculated from three independent experiments in which actin transcripts (*ACTIN2* plus *ACTIN8*) were used as internal controls. Controls (C) for each condition were calculated as an average of the controls done simultaneously at each time in which the samples were taken. Differences were considered significant when the *P*-values were <0.05 (Student's *t*-test).
(b): Sunflower (HA) or Arabidopsis (AT) plants transiently or stably transformed with the indicated constructs and subjected to mechanical wounding (Wo) or damage caused by chewing insects (*S. virginica* for sunflower and *Bradysia impatiens* for AT; Ch). As negative and positive controls plants transformed with pBI101 (GUS without promoter, 101) or pBI121 (*35S:*GUS, 121) were used, respectively. LPF (1221 bp) represents the *HAHB4* promoter fused to the GUS cDNA.

were attacked by *Spilosoma virginica*. In order to determine if *HAHB4* upregulation by mechanical damage and herbivory was controlled by its promoter, sunflower leaves were transiently transformed with a construct bearing 1221 base pairs (bp) upstream of the *HAHB4* transcription initiation site fused to the reporter gene β-glucuronidase (GUS). After a stabilization period of 4 days, leaves were either subjected to mechanical damage or challenged with one *S. virginica* caterpillar. Histochemical analysis indicated that mechanical damage and larval feeding induced GUS expression directed by the *HAHB4* promoter in the affected area (Figure 1b). Leaves transformed with a promoter-less GUS construct (pBI101.3) did not show any activity in the affected area while leaves transformed with GUS under a constitutive promoter (pBI121) demonstrated high transformation efficiency.

Jasmonic acid and ET independently and additively exert the upregulation of HAHB4

It was previously demonstrated that *HAHB4* expression is induced by ET and that this induction results in a reduced sensitivity to ET in sunflower (Manavella *et al.*, 2006). A possible interaction between MeJA and ET in *HAHB4* regulation was evaluated by applying these two hormones independently or simultaneously to sunflower leaves. Figure 2(a) shows that *HAHB4* transcripts accumulate to higher levels when MeJA and ET are applied together than when they are applied independently. Several putative mechanisms schematized in Figure 2(b) may explain the regulation of *HAHB4* by these hormones. Two of them, I and II, are unlikely since they cannot explain the higher induction of *HAHB4* after a co-treatment with MeJA and ET. The remaining models, III (cooperative) and IV (additive), are therefore more plausible. To determine the mechanism operating to regulate *HAHB4*, either ET perception or its biosynthesis were first inhibited by $AgNO_3$ or α-aminoisobutyric acid (AIB), specific inhibitors of the perception and biosynthesis [1-aminocyclopropane-1-carboxylic acid (ACC) oxidase] of ET, respectively. The same approach was used to determine the influence of MeJA but, this time, by using SA and acetyl-salycylic acid (ASA) as indirect antagonists of the JA signaling pathway. After treatment with the different inhibitors, the seedlings were treated with ACC and MeJA and transcript levels were quantified by quantitative (q)RT-PCR. The rationale of the experiment is as follows: if both hormones are needed to exert the induction, the treatments with any of the inhibitors would suppress it. Otherwise, treatment with one of the hormones would activate *HAHB4* transcription (model IV) even in plants previously treated with the inhibitor of the second hormone. The results are shown in Figure 2(c) and indicated that MeJA treatment could upregulate *HAHB4*, even if the action of ET was suppressed, and vice

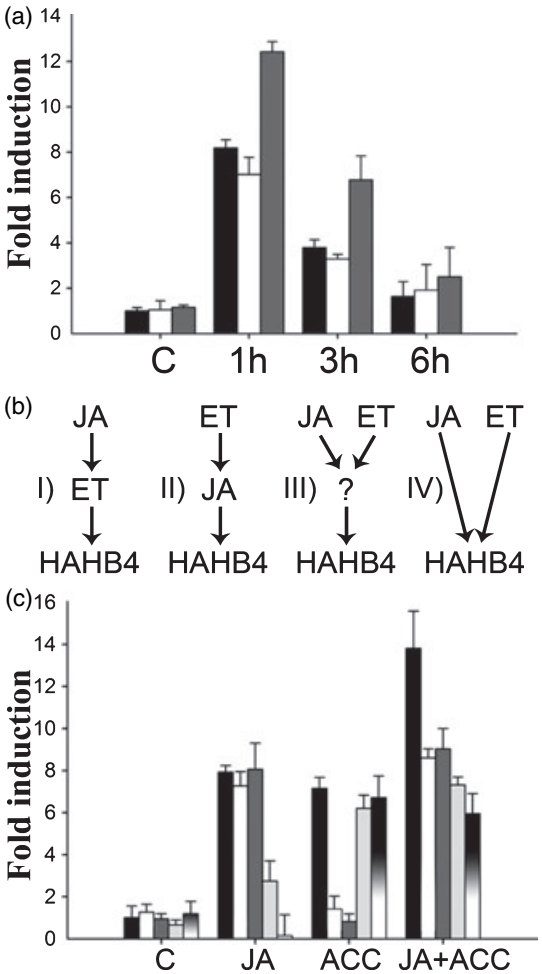

Figure 2. Methyl-jasmonic acid (MeJA) and ethylene (ET) act cooperatively to induce HAHB4 expression.

(a) *HAHB4* transcript levels in sunflower leaves treated with 200 μM MeJA (black bars), 30 μM 1-aminocyclopropane-1-carboxylic acid (ACC; white bars) or with both hormones together (grey bars) measured at different times after the treatment as indicated. Controls (C) represent the levels immediately after applying the treatment (time 0). Transcript levels were determined by quantitative RT-PCR and standard deviations calculated from three independent experiments in which actin transcripts (*ACTIN2* plus *ACTIN8*) were used as internal controls. Differences were considered significant when the *P*-values were <0.05 (Student's *t*-test).

(b) Four putative models explaining the action of these hormones. I, JA activates ET biosynthesis and ET induces *HAHB4* expression. II, ET activates JA biosynthesis and JA induces *HAHB4* expression. III, both hormones are necessary to activate *HAHB4* expression directly or through an unknown intermediate. IV, both hormones independently induce *HAHB4* expression.

(c) *HAHB4* transcript levels in sunflower leaves previously treated with 100 μM $AgNO_3$ (an inhibitor of ET perception, white bars), α-aminoisobutyric acid (AIB; an ET biosynthesis inhibitor, dark grey bars), salicylic acid and acetyl salicylic acid (SA and ASA – as indirect JA perception inhibitors, light grey and white/black bars, respectively), or untreated (black bars) measured after a treatment with 200 μM MeJA (JA), 30 μM ACC or with both hormones together.

Transcript levels were measured by quantitative RT-PCR and standard deviations calculated from at least three independent experiments in which actin transcripts (*ACTIN2* plus *ACTIN8*) were used as internal controls. Differences were considered significant when the *P*-values were <0.05 (Student's *t*-test).

versa. These observations led us to conclude that it is most likely that both hormones act independently in the regulation of *HAHB4* expression and that their effects are additive. This regulatory mechanism is therefore better represented by model IV (Figure 2b).

HAHB4 upregulates defense-related genes in sunflower leaves

We previously reported the microarray analysis of transgenic Arabidopsis plants carrying a *35S:HAHB4* construct (Manavella *et al.*, 2006). The comparison of this microarray data with a GenBank collection of sunflower expressed sequence tags (ESTs) and genomic clones served as a guide to look for possible wound-related genes regulated by *HAHB4* in sunflower. Although sunflower genomic sequence information is rather limited, we were able to identify several clones encoding for proteins with high homology to known proteins involved in the wound response and previously identified in the Arabidopsis microarray (Manavella *et al.*, 2006). Transcript levels of these genes were measured in control plants (either agroinfiltrated with *35S:*GUS or with the empty vector, EV) and in plants transformed with *35S:HAHB4* (H4) or with a construct bearing a RNAi cassette able to target *HAHB4* RNA (H4i). In all cases, leaves were also untreated or treated with JA. The aim of this treatment was to increase *HAHB4* levels and to establish the effect of *HAHB4* silencing, since basal mRNA levels of this transcription factor are normally very low. Transformation efficiency was checked by measuring transcript levels of the kanamycin resistance gene. Control assays to test RNAi efficiency were carried out and presented in the supplementary data as Figure S1. Transcripts levels of *VSP*, *LOX2*, *LOX3*, *TPI1*, *TPI2*, *DEF-L*, *SPI1*, *AOS*, *HPL1* and *NAC3* were clearly induced concomitantly with *HAHB4* (Figure 3). In contrast, plants unable to express *HAHB4* (RNAi) exhibited reduced levels of MeJA-mediated induction of these genes compared with control plants, supporting the hypothesis that *HAHB4* is an upstream regulator of their expression. *OPR2*, a gene not regulated by *HAHB4* (according to the microarray data), was used as a control in all cases to rule out artifacts caused by the methodology. *In silico* analysis revealed that among the ten tested genes, eight exhibited the core nucleotides of the pseudopalindro-

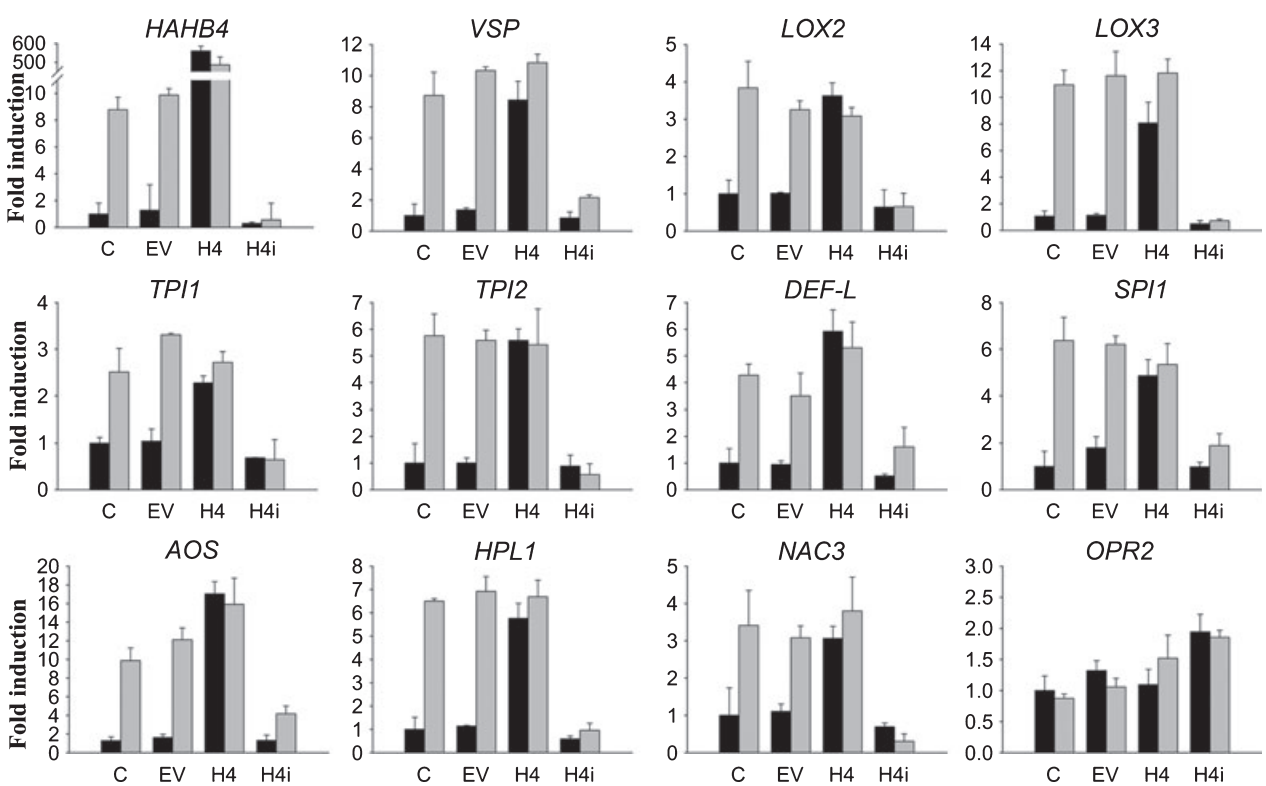

Figure 3. *HAHB4* expression influences transcript levels of wound-responsive genes in sunflower leaves.
Sunflower leaves were transformed with a $MgCl_2$ solution (C), empty vector (EV), *35S:HAHB4* (H4) or an RNAi construct silencing endogenous *HAHB4* (H4i). Transcript levels of different wound-responsive genes were measured by qRT-PCR after the plants were treated with 200 μM methyl jasmonic acid (MeJA; grey bars) or in control conditions (black bars) 1 h after the treatment was applied. *ACTIN* genes (*ACTIN2* plus *ACTIN8*) were used as an internal control and standard deviations were calculated from at least three independent experiments. Differences were considered significant when the *P*-values were <0.05 (Student's *t*-test). *VSP*, vegetative storage protein; *LOX2*, lipoxygenase 2; *LOX3*, lipoxygenase 3; *TPI1*, trypsin inhibitor 1; *TPI2*, trypsin inhibitor 2; *DEF-L*, defensin-like protein; *SPI1*, serin protease inhibitor 1; *AOS*, allene oxide synthase; *HPL1*, hydroperoxide lyase 1; *NAC3*, NAC-type transcription factor 3; *OPR2*, 12-oxophytodienoate reductase.

mic sequence AAT(A/T)ATT within the 1000-bp region upstream of their transcription start site (Figure S2). This element is the core sequence bound by HAHB4 (Palena *et al.*, 1999) and its presence in the promoter regions of the tested genes suggests that they may be direct targets of HAHB4.

HAHB4 induces the activity of trypsin protease inhibitors and enzymes involved in JA and green leaf volatile biosynthesis

Since the expression of several genes involved in JA biosynthesis and defense responses seems to be regulated by HAHB4, we quantified the activities of lipoxygenase (LOX), the first committed enzyme in JA and green leaf volatile (GLV) biosynthesis, and also of hydroperoxide lyase (HPL), the second enzyme in GLV biosynthesis. Activities of these enzymes were measured in sunflower control plants and in plants agro-infiltrated with the control construct (EV) or with the constructs overexpressing (H4) or suppressing (H4i) *HAHB4*. The results are presented in Figure 4(a,b) and showed that sunflower leaves overexpressing *HAHB4* contained increased specific activities of both LOX and HPL. In contrast, activities of the same enzymes were lower in leaves suppressed in *HAHB4* expression (either in the presence or absence of JA). Similar to LOX and HPL, the activity of trypsin protease inhibitors (TPI) increased concomitantly with *HAHB4* expression, reaching almost the same levels as in controls plants pre-treated with JA. Consistently, the activity remained almost unaffected when *HAHB4* expression was suppressed (either in the presence or absence of JA; Figure 4c). These results are consistent with the increased transcript levels of some JA and GLV biosynthesis enzymes, and provide further evidence for the participation of this transcription factor in the activation of JA and GLV biosynthesis genes together with direct defense mechanisms such as TPI expression.

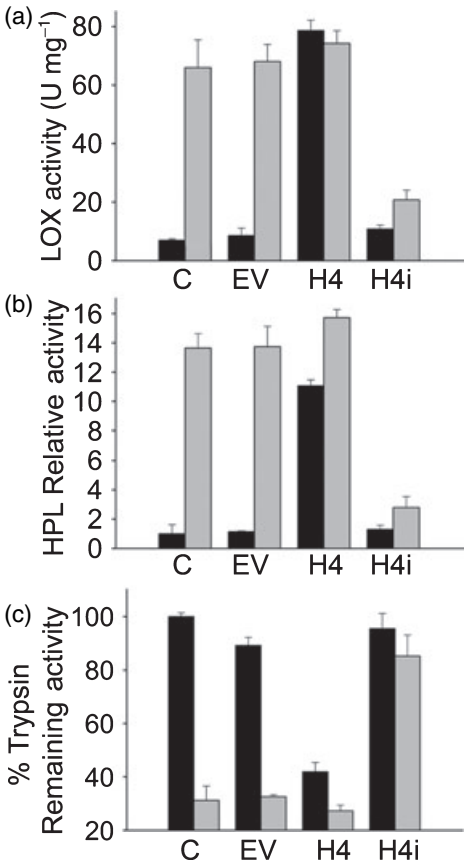

Figure 4. Transcript levels of defense genes regulated by *HAHB4* correlate with the encoded proteins activities.
Sunflower leaves were transformed with a $MgCl_2$ solution (C), empty vector (EV), *35S:HAHB4* (H4) or a RNAi construct silencing endogenous *HAHB4* (H4i), and protein extracts prepared from these samples. Lipoxygenase (LOX), hydroperoxide lyase (HPL) and trypsin protease inhibitor (TPI) activities were measured in proteins extracts from transformed leaves untreated (black bars) or treated with 200 μM methyl jasmonic acid (MeJA; grey bars). The standard deviations were calculated from four independent assays and differences were considered significant when the *P*-values were <0.05 (Student's *t*-test).

HAHB4 induces defense-related responses in Arabidopsis and maize

Thus far we have demonstrated that *HAHB4* is upregulated locally and systemically when sunflower plants are mechanically injured. This local upregulation is also observable when the plants are attacked by *S. virginica* or when treated with MeJA and ET. Moreover, HAHB4 is able to induce the transcription of several genes involved in direct and indirect defense responses in sunflower. To test whether this sunflower transcription factor has a conserved function and could therefore also mediate defense responses in other plant species, transgenic Arabidopsis and maize plants ectopically expressing *HAHB4* were also evaluated for changes in defense-related gene expression and caterpillar performance. Three independent homozygous transgenic lines for each species were used for all experiments. Plants ectopically expressing GUS were used as controls and the results are shown in Figure 5. Transcript levels of *LOX2*, *AOS* and *VSP* were highly induced in transgenic Arabidopsis (about seven, four and eightfold, respectively) while *LOX2*, *AOS* and *HPL* were induced in transgenic maize (about five, four and sixfold, respectively).

Transgenic Arabidopsis and maize plants were challenged with *Bradysia impatiens* and *Spodoptera frugiperda* larvae, respectively. Several parameters were chosen to compare the performance of the different larvae on both plant species. First, the feeding activity of the larvae was measured by quantifying the leaf area consumed (Table 1, Figure 6a and Figure S3a). In both Arabidopsis and maize this area was smaller when the larvae fed on plants expressing *HAHB4*, even if these plants were their only food source. Similar results were obtained with *S. frugiperda* and *Diatraea saccharalis* (not shown). Accordingly, when *B. impatiens* and *Spodoptera litoralis* larvae fed only on

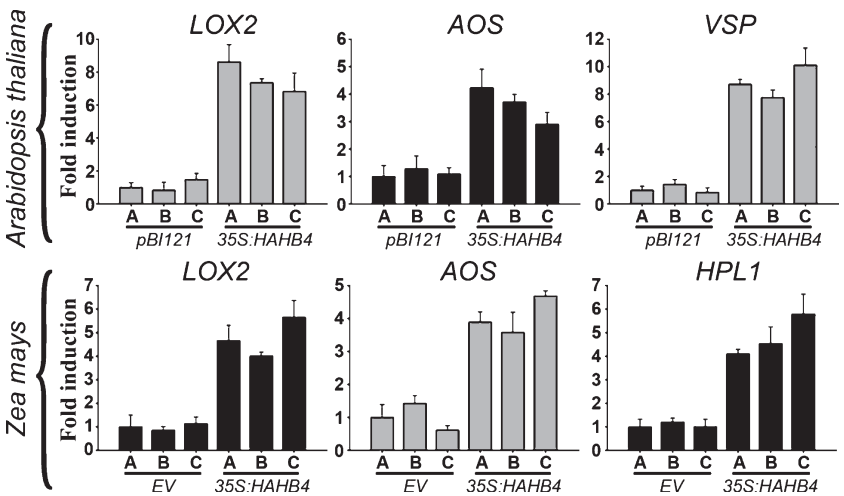

Figure 5. Ectopic expression of *HAHB4* in Arabidopsis and maize upregulates defense-related gene expression.
Transcript levels of several genes associated with defense responses were analyzed by qRT-PCR in three independent Arabidopsis and maize lines transformed with a CaMV *35S:HAHB4* construct (*35S:HAHB4*). Control plants were transformed with pBI121 or with the empty vector pTF101 (EV). Quantifications were repeated at least three times and the differences were considered significant when the *P*-value was <0.05 (Student's *t*-test).

Table 1 Performance of *Bradysia impatiens* and *Spodoptera frugiperda* when fed with Arabidopsis and maize transgenic plants

	Daily eaten surface (mm^2)	Survival rate (%)
Line AT		
pBI121 A	83.3 ± 8.7	94 ± 5
pBI121 B	95.1 ± 7.3	96 ± 4
pBI121 C	92.9 ± 6.8	98 ± 2
35S:HAHB4 A	3.5 ± 1.6	26 ± 5
35S:HAHB4 B	4.7 ± 2.1	37 ± 7
35S:HAHB4 C	5.6 ± 2.8	41 ± 3
Line ZM		
Empty vector A	186.5 ± 9.2	95 ± 2
Empty vector B	189.8 ± 11.4	85 ± 4
Empty vector C	167.9 ± 14.7	90 ± 3
35S:HAHB4 A	61.1 ± 7.2	10 ± 5
35S:HAHB4 B	52.0 ± 5.6	35 ± 2
35S:HAHB4 C	39.1 ± 8.7	30 ± 3

Transgenic *Zea mays* (ZM) and *Arabidopsis thaliana* (AT) plants constitutively expressing *HAHB4* were subjected to herbivory attack as described in Experimental procedures. The eaten area, the time spent to proceed from larva to pupa and the percentage of survivors were quantified in five independent experiments with 15 insects per genotype and the average values expressed as well as standard deviations. Controls were carried out with an empty vector construct.

HAHB4-expressing plants, they show a significant reduction in body mass compared with larvae fed on control plants (Figure 6d,e and Figure 3b). The activity of TPI was also measured in transgenic plants and compared with their controls (Figure 6b,c). Like in sunflower, TPI activity was enhanced in transgenic plants ectopically expressing *HAHB4*. These results were in agreement with the reduced larval performance on these plants.

To indirectly evaluate if the larvae exhibit a differential preference between Arabidopsis and maize *HAHB4* transgenic plants compared with control plants, we performed choice assays. The larvae were allowed to choose between transgenic and wild-type plants. The results shown in Figure 6(f,g) indicated a significant tendency of the larvae to avoid the *HAHB4*-expressing lines, choosing the control plants even when they had not been exposed to them before.

HAHB4 positively regulates JA and ET levels but negatively regulates SA after mechanical damage

We previously demonstrated that the ectopic expression of *HAHB4* in Arabidopsis reduces sensitivity to ET (Manavella *et al.*, 2006). However, it remained unclear whether ET biosynthesis was also affected in these transgenic lines. To answer this question, we quantified ET levels in both Arabidopsis transgenic plants ectopically expressing *HAHB4* and in plants transformed with pBI121 used as controls. *HAHB4*-expressing plants presented levels of ET approximately 50% higher than in control plants after mechanical damage (Figure 7a, *t*-test, *P*-value < 0.05). This observation is in agreement with our previous study showing that Arabidopsis plants expressing *HAHB4* present a strong reduction of *EIN3*/*EIL1* transcript levels and lower ET sensitivity (Manavella *et al.*, 2006). Arabidopsis plants mutated in *EIN3*/*EIL1* also accumulate higher levels of ET due to a reduced capacity to perceive this hormone (Chen *et al.*, 2005).

We also tested if the upregulation of JA biosynthesis genes, observed when *HAHB4* is expressed, is reflected in increased production of JA and its conjugates. Jasmonic acid and JA-isoleucine (Ile) were quantified in leaves before and after mechanical damage. The results presented in Figure 7(b,c) showed that *HAHB4* transgenic Arabidopsis plants accumulated levels of JA in leaf tissue ~40% higher, and on average about twofold more after mechanical damage. Consistently, they also accumulated levels of JA-Ile about twofold higher. The kinetics of JA and JA-Ile accumulation were similar between the genotypes. In con-

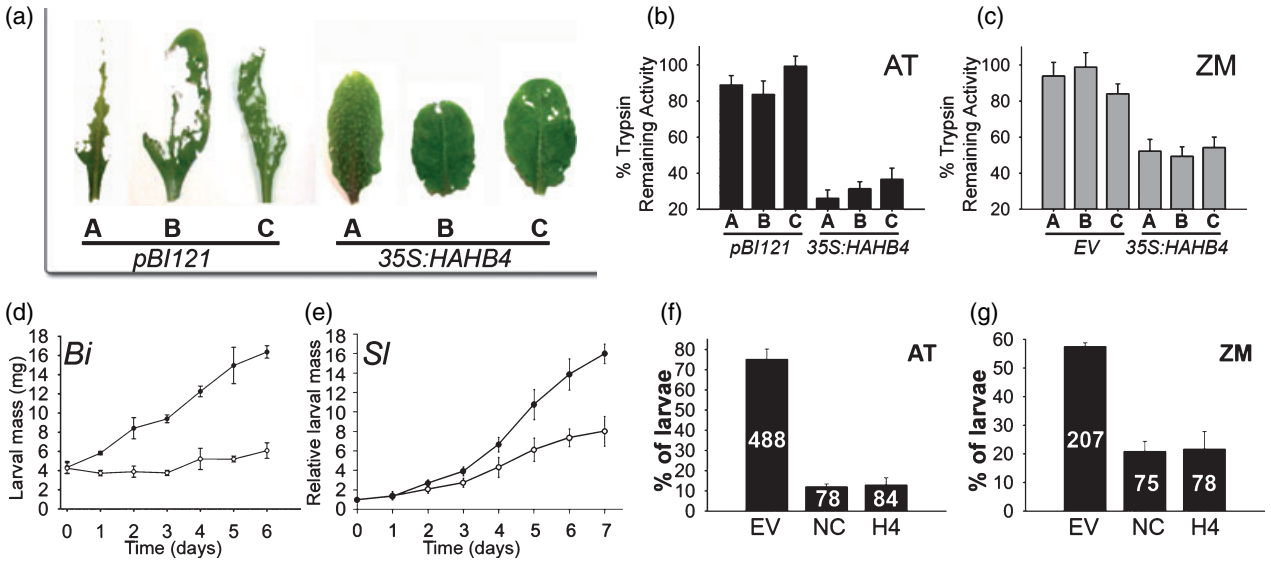

Figure 6. *HAHB4* alters insect resistance in transgenic Arabidopsis and maize plants.
(a) Transgenic Arabidopsis plants ectopically expressing *HAHB4* (right panel, in three independently transformed lines A, B and C) and plants transformed with pBI121, which contains GUS cDNA (left panel) challenged with *Bradysia impatiens* larvae. Photographs were taken 1 day after the initiation of feeding.
(b, c) Trypsin residual activity was measured in extracts of transformed plants as described in the Experimental procedures. AT, Arabidopsis; ZM, maize; *35S:HAHB4* A, B and C, pBI121/EV plants.
(d, e) Mass gain of *Bradysia impatiens* and *Spodoptera litoralis* larvae on *HAHB4*-expressing transgenic plants (○) or Arabidopsis plants transformed with pBI121 (•).
(f, g) Differential preference of the larvae between transgenic AT or ZM and control plants. Bars represent the overall percentages of larvae choosing either of the odor sources [plants transformed with empty vector (EV) or with *35S:HAHB4* (H4)] or larvae that did not make any choice (NC) (remained in the original position or near it). Numbers inside the bars represent the total number of larvae choosing each odor source. Error bars represent the standard deviation calculated from 10 repetitions of each assay.
For all the assays described the differences were considered significant when the *P*-values were <0.05 (Student's *t*-test).

trast to JA, levels of SA in the *35S:HAHB4* plants were about twofold lower both before and after mechanical damage compared with control plants (Figure 7d).

Arabidopsis plants ectopically expressing HAHB4 *are more sensitive to bacterial infection*

To test if the reduced SA accumulation observed in the *HAHB4*-expressing Arabidopsis transgenic plants could affect the normal response to *Pseudomonas syringae*, we quantified levels of SA during infection with virulent (DC3000) and avirulent (DC3000/*avrRpt2*) strains of this bacterium. Similar to the wound response, Arabidopsis *HAHB4*-expressing plants produced lower SA levels at 24 and 48 h post-infection with both virulent and avirulent strains of this pathogen compared with controls (Figure 8a). The levels of JA and JA-Ile remained low and similar to control levels (before treatment) during infection in both control and *HAHB4*-expressing plants (Figure S4). In agreement with the reduced SA levels, evident signs of *P. syringae* infection were observed in the transgenic plants (Figure 8b). To quantify these differences in sensitivity between genotypes we determined the number of bacteria in leaf tissue after 24 and 48 h post-infection. The results indicated increased bacterial growth (assessed as colony-forming units, CFU) in *HAHB4*-expressing lines compared with control lines, for both virulent and avirulent strains (Figure 8c,d).

Discussion

Signaling pathways involved in plant defense responses against herbivores are complex and specific. It is known that certain transcription factors orchestrate anti-herbivore defense responses, but only a few have thus far been characterized (Denekamp and Smeekens, 2003; Li *et al.*, 2004; Miao and Zentgraf, 2007). The HD-Zip proteins are transcription factors unique to plants, and previous studies have associated them with abiotic stress responses (Ariel *et al.*, 2007). In this study we present evidence indicating that the sunflower HD-Zip I transcription factor HAHB4 is involved in the regulation of defense mechanisms against biotic stresses and wounding.

After a mechanical injury or insect attack, several plant species elicit a defense response that includes a quick burst of ET and jasmonates. The signaling events mediated by these two hormones occur rapidly, and they can be enhanced if the damage is caused by insect herbivory (von Dahl *et al.*, 2007; McCloud and Baldwin, 1997). This stronger response is a direct consequence of the presence of compounds in the insect's oral secretions which are recognized by the plant. Consistent with this previous

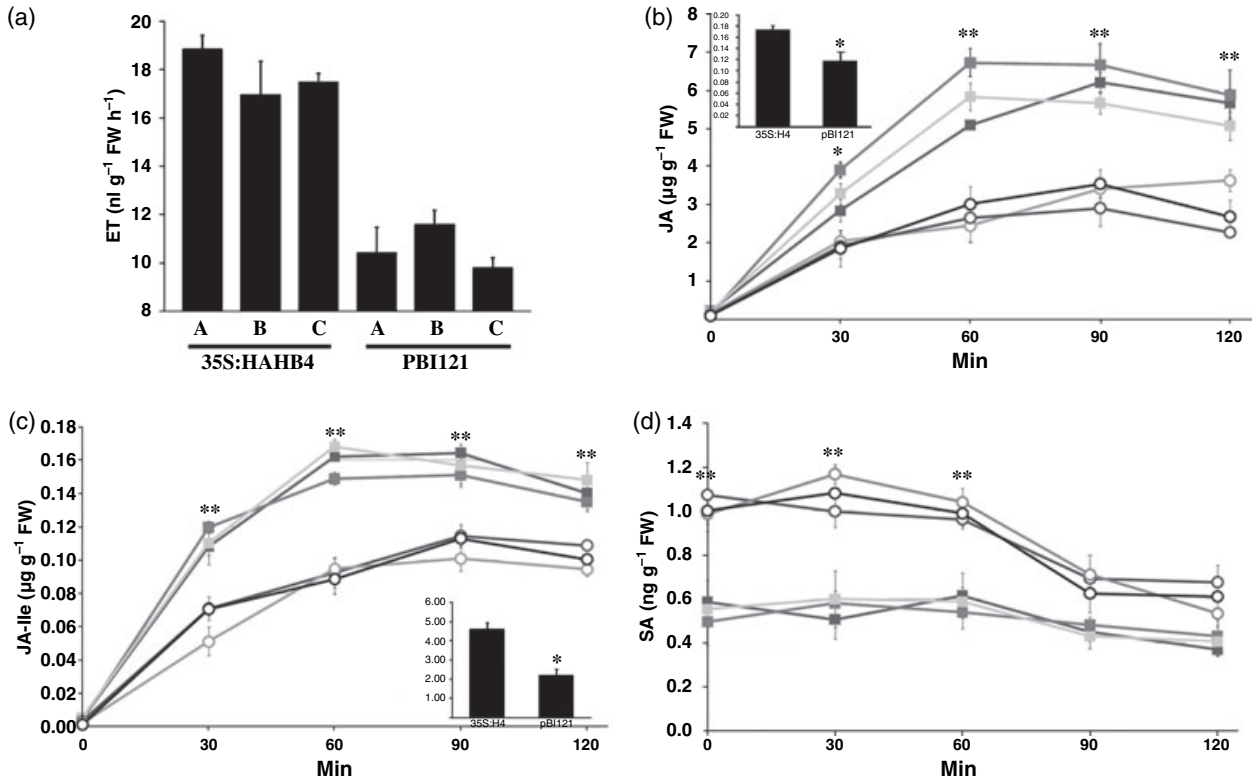

Figure 7. Ectopic *HAHB4* expression modulates the biosynthesis of jasmonic acid (JA), ethylene (ET) and salicylic acid (SA).
(a) Ethylene production in three independent lines of Arabidopsis plants transformed with CaMV *35S:HAHB4* compared with those present in plants transformed with pBI121, which contains the 35S CaMV promoter and the GUS cDNA . Quantifications were repeated at least three times and the differences between genotypes present a statistical significance with a *P*-value <0.05 (Student's *t*-test).
(b–d) Jasmonic acid, JA-isoleucine (Ile) and SA measurements performed in three independent lines of Arabidopsis plants transformed with CaMV *35S:HAHB4* (■) or with pBI121 (○). All plants were mechanically wounded and the phytohormone levels determined after 0, 30, 60, 90 and 120 min of the stimulus. The inserts in panels (b) and (c) represent hormone levels in resting tissue (before wounding) in a smaller scale to distinguish the differences. For each line, measurements were performed in triplicate and analyzed by ANOVA $*P < 0.05$; $**P < 0.01$.

knowledge, the results presented here indicate that *HAHB4* expression is quickly induced when sunflower leaves suffer an injury and that this induction is stronger when the injury is caused by an insect. Accordingly, *HAHB4* is also upregulated by MeJA and ET (Figure 1) as has been observed for other transcription factors associated with the response to wounding and insect attack (Boter *et al.*, 2004; Lorenzo *et al.*, 2003). Since JA biosynthesis is induced locally and systemically after wounding and insect attack, it was not surprising to find that *HAHB4* transcript levels are also induced locally and systemically after an injury (Figure 1).

The signaling pathways for JA, ET and SA are known to extensively crosstalk during biotic stresses. Jasmonic acid and ET frequently act synergistically to induce defense-related gene expression, whereas they frequently act antagonistically with the SA signal transduction pathway. For example, protease inhibitors (PI), defensins and certain pathogenesis-related (PR) proteins are induced by both JA and ET signaling cascades (O'Donnell *et al.*, 1996; Penninckx *et al.*, 1998; Thomma *et al.*, 1998). However, in some cases ET- and JA-mediated responses are antagonistic. An example of this antagonism is the effect produced in tobacco plants by *Manduca sexta*: ET accumulates and negatively interferes with the JA-mediated induction of nicotine biosynthesis (Winz and Baldwin, 2001). Regarding the SA/JA antagonism, several transcription factors, particularly some belonging to the WRKY family, have been reported as mediators of the antagonistic effect between these two hormones (Li *et al.*, 2004; Miao and Zentgraf, 2007). *HAHB4* expression is regulated by both ET and MeJA (Manavella *et al.*, 2006 and the present work). The fact that ET biosynthesis and perception inhibitors disrupt only the ET-mediated but not the JA-mediated *HAHB4* induction supports the conclusion that these two hormones act additively. Similar conclusions can be drawn from the fact that the indirect antagonism of SA over JA signaling doesn't affect ET-mediated *HAHB4* induction (Figure 2). The transcript levels of several wound-related genes – *LOX*2, *LOX*3 and *AOS* (encoding enzymes participating in JA biosynthesis), *VSP* (a gene induced after wounding), *NAC3* (a NAC-type transcription factor regulated by MeJA and wounding), genes encoding protease inhibitors such as *TPI1*, *TPI2*, *DEF-L* and

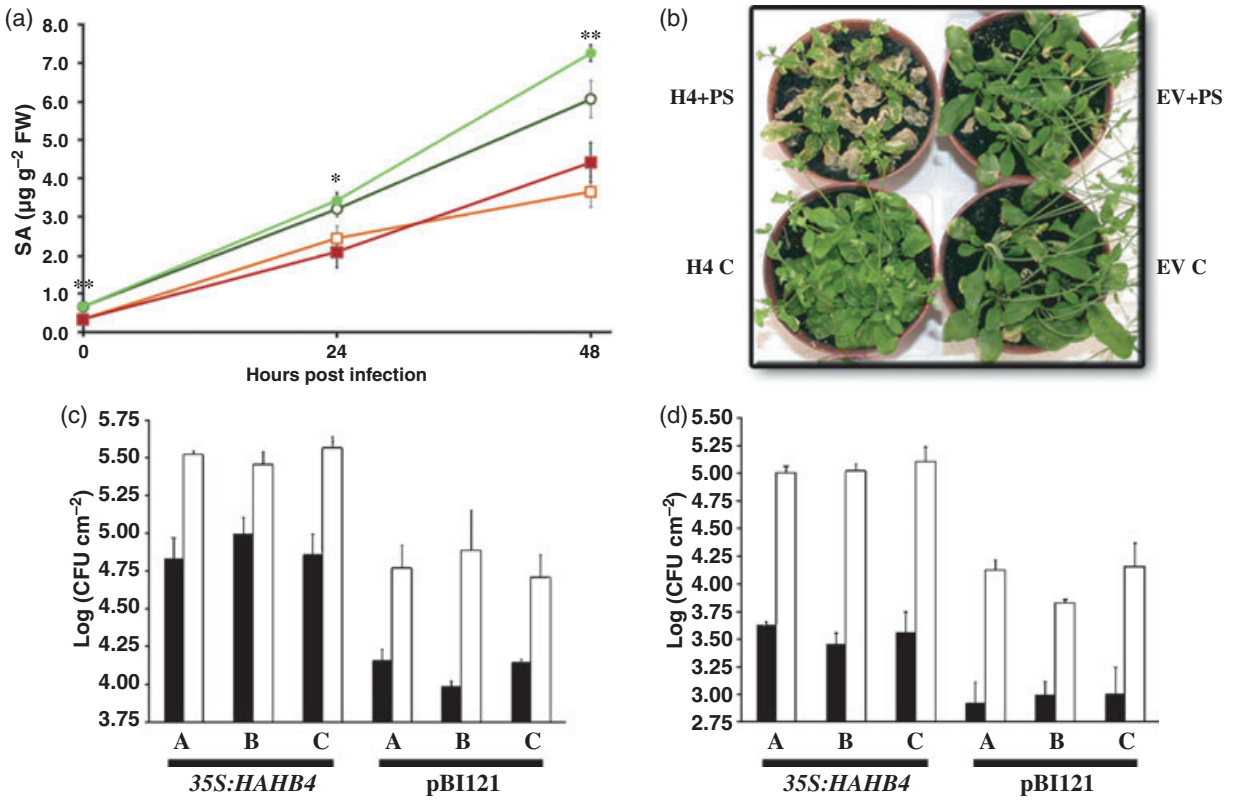

Figure 8. The reduction of salicylic acid (SA) in Arabidopsis transgenic plants expressing *HAHB4* affects their susceptibility to pathogen infections.
(a) Levels of SA were quantified after infection with a virulent [*35S:HAHB4* (■) and pBI121 (•)] and avirulent [*35S:HAHB4* (□) and pBI121 (○)] strain of *Pseudomonas syringae* DC3000. Phytohormone levels were determined in leaf tissue 0, 24 and 48 h post-infection in triplicate in three independent transgenic lines for each genotype. Results are presented as the average value between the different lines. **P*-value < 0.05; ***P*-value < 0.01 (ANOVA).
(b) Transgenic Arabidopsis plants ectopically expressing *HAHB4* (H4) and plants transformed with pBI121 (EV) were inoculated with the *P. syringae* virulent strain DC3000 and with the avirulent strain *Pst* DC3000/avrRpt2 [infected (+PS), un-infected (C)]. Photographs were taken 2 days after the initiation of the infection.
(c), (d) Quantification of colony forming units (CFU) in three independent lines of Arabidopsis transformed with CaMV 35S:HAHB4 (*35S:HAHB4*-A, -B and -C) or pBI121 (pBI121-A, -B and -C). Bacterial density was quantified 24 h (black bars) and 48 h (white bars) post-infection with a virulent (C) and an avirulent (D) strain of *P. syringae* (DC 3000). For each time point the quantification was repeated three times for each line. The statistical analysis of the data indicated a significant difference between genotypes with a *P*-value < 0.05 (*t*-test).

SPI1 (components of the direct defense against insects attack) and *HPL1* (a gene encoding an enzyme that participates in GLVs biosynthesis) – are upregulated together with *HAHB4* in transiently transformed sunflower leaf tissue. Furthermore, silencing *HAHB4* expression produces a partial or complete reduction of the normal induction of these genes when the plants are treated with MeJA, indicating that *HAHB4* acts upstream of them. This conclusion is also supported by the presence of the core target sequence recognized by HAHB4 *in vitro* (Palena *et al.*, 1999) in most of the promoters of these genes (Figure S2). Increased transcript levels of two enzymes participating in biosynthesis of JA and GLV were correlated with their increased activities in *HAHB4* overexpressing tissue (Figure 4). This is the case for LOX and HPL. In addition, increased activity of TPI was also detected in *HAHB4* overexpressing tissue. Additionally, reduced activities of these three proteins were observed in *HAHB4*-silenced tissue. Together, these observations led us to conclude that *HAHB4* regulation of wound-related genes is reflected in the encoded expressed proteins.

When *HAHB4* was heterologously expressed in Arabidopsis and maize, a similar induction of defense-related responses to those observed in sunflower was observed, including the upregulation of defense-related transcripts. Moreover, these transgenic plants presented enhanced resistance to insect herbivory. Given that *B. impatiens* (fungus gnat) uses fungi as its primary food source the presence of such organisms in the culture chamber cannot be ruled out; the results obtained with *S. littoralis* were thus crucial to confirm the effect. Choice tests also suggested that these transgenic genotypes have altered indirect defense responses (Figure 6). Quantification of jasmonates in Arabidopsis transgenic lines showed that basal levels of JA in leaves were higher than in control lines. Furthermore, after mechanical damage, accumulated levels of JA and JA-Ile were about twofold higher throughout the time

course of the experiment (Figure 7b,c). These results are consistent with the increased LOX activity and transcript levels for JA biosynthetic enzymes in Arabidopsis plants ectopically expressing *HAHB4*. The morphological phenotype of these transgenic plants expressing *HAHB4* (Dezar *et al.*, 2005a) is characteristic of Arabidopsis mutant plants slightly overproducing JA in resting tissue. The hallmarks of this phenotype include arrested growth, epinastic leaves and accumulation of anthocyanins in the petioles and base of the stems (Bonaventure *et al.*, 2007; Ellis and Turner, 2001; Hilpert *et al.*, 2001; Jensen *et al.*, 2002). The levels of SA were reduced in leaves both before and after mechanical damage (Figure 7d). This effect is most likely indirect and related to the increased levels of jasmonates in these lines. However, we cannot rule out at this point a more direct control of SA levels by *HAHB4*. Consistent with the curtailed SA production, Arabidopsis *HAHB4*-expressing lines showed enhanced susceptibility to both virulent and avirulent strains of *P. syringae*. Finally, ET levels were also increased after wounding in Arabidopsis *HAHB4*-expressing lines (Figure 7a). It has been shown that *HAHB4* expression in Arabidopsis downregulates the transcript levels of some ET biosynthesis enzymes and signal transduction components such as EIN3/EIL1 (Manavella *et al.*, 2006). The increased levels of ET produced after wounding in these plants are probably a result of a decreased sensitivity to ET, as has been previously proposed (Manavella *et al.*, 2006). Enhanced biosynthesis of ET due to a reduced perception, by for example downregulation or mutation of receptors, or signaling components, has been reported in different plant species (Chen *et al.*, 2005; von Dahl *et al.*, 2007). Thus, expression of *HAHB4* appears to desensitize plants' ET perception and it may thereby regulate ET-dependent gene expression during the wound and biotic stress responses.

In Arabidopsis, two members of the HD-Zip I subfamily, namely ATHB7 and ATHB12, were identified as being most closely related to HAHB4. They present a high homology in the HD-Zip domain (but not outside it) and their expression is upregulated by ABA and drought as in the case of *HAHB4* (Lee and Chun, 1998; Söderman *et al.*, 1996). However, in contrast to *HAHB4*, when *ATHB7* and *ATHB12* were overexpressed, the transgenic plants did not exhibit an enhanced drought tolerance (Olsson *et al.*, 2004). Thus far, no reports on maize members of HD-Zip subfamily I are available.

Based on previous and present results, we propose a model for the regulation of the crosstalk between ET and JA mediated by *HAHB4*. The levels of *HAHB4* are normally very low (Gago *et al.*, 2002). Upon mechanical damage or insect attack, the expression of this transcription factor is induced via production of JA and ET. Induction of *HAHB4* leads to a positive feedback over JA and volatile biosynthesis via the transcriptional activation of several enzymes involved in these processes such as LOX, AOS and HPL. This accumulation of JA is associated with a decrease in levels of SA (Figure 7) which may explain the plants' sensitivity to infection by phytopathogens. Simultaneously, induction of *HAHB4* mediates a negative feedback mechanism over the ET signaling pathway that results in a decreased ET sensitivity. Additionally, *HAHB4* induction may directly promote the transcriptional activation of several other defense-related genes, such as *TPI*, via a direct interaction with their promoters (Figure 9). Many stress responses require the coordinated interaction of several hormone-mediated signaling pathways. Given that *HAHB4* expression is also upregulated by drought and ABA (Dezar *et al.*, 2005a; Gago *et al.*, 2002) and that these two factors participate in the defense response (Lorenzo and Solano, 2005), crosstalk between these signal transduction pathways in which this transcription factor plays a role may be occurring.

The present work demonstrates the participation of the HD-Zip transcription factor HAHB4 in the response to wounding and biotic stress in sunflower and a conserved response when expressed heterologously in Arabidopsis and maize. Their effects are most likely brought about by the direct regulation of the expression of defense-associated genes including those involved in the LOX biosynthesis pathway and ET sensitivity and production. These changes result in an enhanced defense response against insect herbivores but at the same time in an increased susceptibility to bacterial infection.

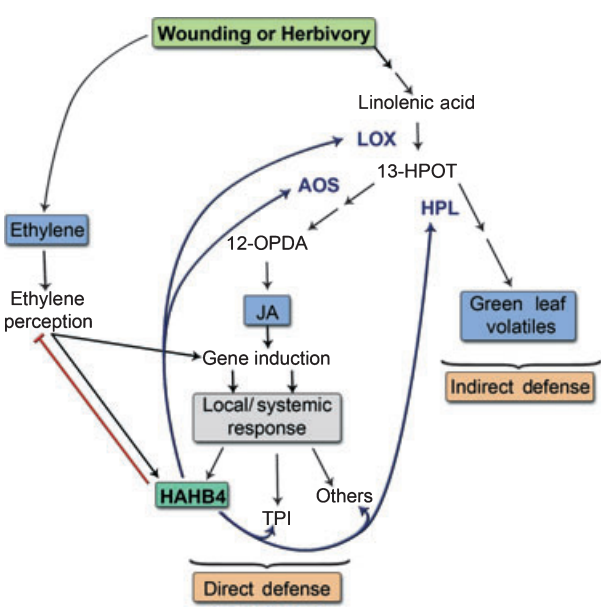

Figure 9. Putative model of the role of *HAHB4* in biotic stress responses.
Blue lines indicate activation and red lines repression. LOX, lipoxygenase; TPI, trypsin inhibitors; AOS, allene oxide synthase; HPL, hydroperoxide lyase; 13-HPOT, 13-hydroperoxylinolenic acid; 12-OPDA, 12-oxo-phytodienoic acid.

Experimental procedures

Plant material and growth conditions

Arabidopsis thaliana Heyhn. ecotype Columbia (Col-0), Landsberg *erecta* (L*er*-0), *Zea mays* (Hi II hybrid genotype) and *Helianthus annuus* L. (sunflower cv. contiflor 15, from Zeneca) were grown directly on MS medium or soil as previously described (Dezar *et al.*, 2005a,b; Manavella *et al.*, 2006).

Treatments

Twenty-one-day-old Arabidopsis plants grown in MS Petri dishes were transferred to a fresh MS medium dish supplemented with either 30 μM ACC or 200 μM MeJA. In order to inhibit the signaling pathways of these hormones, 100 μM $AgNO_3$, 2 mM α-aminoisobutyric acid (AIB), 1 mM SA or 1 mM acetyl-SA were applied 1 h before the treatment with the respective hormone. Seedlings were harvested and immediately frozen in liquid nitrogen before RNA isolation. When the treatments were applied to sunflower transiently transformed leaves, 11 mm diameter disks were used and the reagents applied during 15 min under vacuum.

For wounding of sunflower leaves, one half of the leaves were damaged by thoroughly crushing them with fine tweezers (approximately 50% of the surface was damaged). For systemic analysis, samples were taken from undamaged leaves of the same wounded plants. Feeding effects were measured in leaves challenged with 5–10 larvae during 1 h. For wounding of Arabidopsis plants, one-third of the leaf (tip) was crushed with fine tweezers and the entire leaf was used for analysis.

Pseudomonas syringae infections were made out by spray inoculation using the virulent strain *Pst* DC3000 and the avirulent strain *Pst* DC3000/avrRpt2 as previously described (Katagiri *et al.*, 2002).

Constructs

Constructs containing the *HAHB4* cDNA fused to the 35S cauliflower mosaic virus promoter (35S CaMV) and GUS cDNA fused to the *HAHB4* promoter were previously described (Dezar *et al.*, 2005a,b). Constructs for RNAi assays were carried out as follows: a PCR-amplified 300-bp fragment (primers RNAi H4S F: 5′-GGGCTCGAGCTACTCAATCAGTTGGAGG-3′; RNAi H4S R: 5′-CCCGGTACCAGTGTAAACCAACTATCTGG-3′; RNAi H4AS F: 5′-GGGTCTAGACTACTCAATCAGTTGGAGG-3′; RNAi H4AS R: 5′-GGGAAGCTTAGTGTAAACCAACTATCTGG-3′) bearing a segment of the *HAHB4* non-conserved coding region plus a segment of the 3′ non-coding region (between oligonucleotides 369 and 669 from the transcription initiation site) were cloned in the pHANNIBAL vector restricted with *Xho*I/*Kpn*I (sense) and *Hin*dIII/*Xba*I sites (antisense). The complete iRNA cassettes were subcloned in the *Not*I site of the pART27 plasmid and used to transiently transform sunflower leaves. Negative and positive controls were performed with pBI101.3, pBI121 and pART27 following the same transformation technique as described below.

Transformation and identification of transformed plants

Transformation of Arabidopsis plants was carried out by the floral dip procedure (Clough and Bent, 1998) as previously described (Dezar *et al.*, 2005a,b; Manavella *et al.*, 2006). At least three independent lines of each transgenic genotype, previously characterized (Dezar *et al.*, 2005a), were used for all the assays described in this manuscript. Transgenic maize plants were obtained by *Agrobacterium tumefaciens*-mediated transformation of embryos in the Iowa State University Plant Transformation Facility following the method described by Frame *et al.* (2002). Sunflower leaf disks (11 mm diameter) were transiently transformed as previously described (Manavella *et al.*, 2006). For each construct, at least six disks originating from three different plants were analyzed. All the experiments with transformed plants were repeated at least four times. To test the infiltration and transformation efficiencies the expression of the simultaneously introduced kanamycin resistance gene was measured by real time RT-PCR as described bellow.

RNA isolation and analysis by real time RT-PCR measurements

Total RNA for real time was prepared with TRIZOL® reagent (Invitrogen, http://www.invitrogen.com/) following commercial instructions (Invitrogen). Quantitative RT-PCRs were carried out using a MJ-Cromos 4-(Bio-Rad, Hercules, CA, USA) apparatus as previously described (Manavella *et al.*, 2006).

Enzymatic activity determination

In situ assays of GUS activity were performed as described by Jefferson *et al.* (1987). Briefly, whole plants were immersed in a 1 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid solution in 100 mM sodium phosphate, pH 7.0, 0.1% (vol/vol) Triton X-100 and, after applying a vacuum for 5 min, they were incubated overnight at 37°C. Chlorophyll was cleared from plant tissues by immersing them in 70% ethanol. Lipoxygenase and HPL activities were determined as previously described (Perez *et al.*, 1999). Trypsin inhibitor activity was determined using 250 μM *N*-α-benzoyl-L-arginine ethyl ester (BAEE) as a substrate for trypsin (1000 U/reactions). Protein extracts were incubated for 5 min with a trypsin suspension (1000 U; Sigma-Aldrich Co., http://www.sigmaaldrich.com/) before the addition of BAEE and the increase in absorbance (253 nm) caused by BAEE conversion was monitored for 5 min with a spectrophotometer DU530 (Beckman, http://www.beckmancoulter.com/).

Insect bioassays

Larval mass gain and feeding rate were determined as follows: 10 *B. impatiens* (isolated from soil in the second instar) and one *S. littoralis*, *D. saccharalis* or *S. frugiperda* larvae (fed in an adequate medium) were placed together with Arabidopsis or maize plants, respectively, either from transgenic or control plants. Experiments with *B. impatiens* were carried out in Petri dishes either with MS-agar or filter paper, whereas the experiments with caterpillars were performed directly on the respective plants as indicated in the figure legends. The leaves (for *B. impatiens*) or the whole plants (for caterpillars) were replaced daily with fresh ones and the consumed area of each removed leaf was measured to determine the feeding rate of the larvae. Larval mass was also determined daily for 6–7 days. Survival rates were calculated from adult populations fed with fresh leaves. These tests were repeated at least ten times using three independent transgenic lines for each genotype.

Choice assays were performed in two alternative forms. The first approach involved the design of special acrylic tubes (150 × 20 mm diameter) with sealing lids in both sides. A fine layer of 0.8% agar was applied on the bottom of the tubes and four to five leaves from each genotype were placed in the tips. The experiment was carried

out placing 10 *Bradysia* larvae in the middle of the tube and the number of larvae in each side was counted 2 h later. Alternatively, and with the aim of avoiding the possible effects of placing elicited leaves, the choice assays were performed as follows: three plants of each genotype were grown for 2 weeks in the borders of MS/agar-containing Petri dishes (as schematized in the Figure S5); 10 larvae were placed in the center of each dish and it was quickly closed. Counting of larvae was performed 2 h later.

Phytohormone extraction and quantification

Leaf tissue (~200 mg fresh weight) was homogenized in FastPrep tubes containing 1 g of FastPrep matrix (Bio 101, http://www.bio101.com/) and 1 ml of ethyl acetate spiked with 100 ng of D_2-JA, $^{13}C_6$-Ile-JA, and D_4-SA as internal standards. The supernatant was transferred to fresh 2-ml microcentrifuge tubes and the pellet was re-extracted with 0.5 ml of ethyl acetate. The solvent evaporated to dryness. The dry residue was reconstituted in 100 μl of 70% (v/v) methanol. One hundred microliters of the supernatant was transferred to HPLC vials and analyzed in a Varian 1200L Triple-Quadrupole-LC-MS system (Varian, http://www.varianinc.com/). Ten microliters of each sample was injected onto a ProntoSIL column (C18; 5 μm, 50 × 2 mm, Bischoff, http://www.bischoff-chrom.de/) attached to a pre-column (C18; 4 × 2 mm, Phenomenex, http://www.phenomenex.com/). The mobile phase comprised solvent A (0.05% formic acid) and solvent B (0.05% formic acid in acetonitrile) used in a gradient mode [time/concentration (min/%) for B: 0:00/15; 1:30/15; 4:30/98; 12:30/98; 13:30/15; 18:00/15] with a flow rate of [time/flow (min ml^{-1}): 0:00/0.4; 1:00/0.4; 1:30/0.2; 10:00/0.2; 10:30/0.4; 18:00/0.4]. The compounds were detected in the electro spray ionization (ESI) negative and multiple reaction (MRM) modes. Molecular ions (M–H) with *m/z* 209 (JA), 213 (D_2-dhJA), 322 (JA-Ile), 328 ($^{13}C_6$-Ile-JA), 137 (SA) and 141 (D_4-SA) were fragmented under a 12 V collision energy and their respective daughter ions at *m/z* 59, 61, 130, 138, 93 and 97 were used to quantify endogenous phytohormones.

For quantification of ET, rosette leaves from five 4-week-old plants were mechanically wounded by crushing their tips with sharp forceps, excision and placement in 100-ml air-sealed glass vessels for 5 h under white light (100 μE m^{-2} sec^{-1}) at 22°C. After this treatment, ethylene emissions were measured non-invasively with a stop-flow procedure with a photo-acoustic spectrometer according to von Dahl *et al.* (2007).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The *HAHB4* RNAi cassette is able to silence *HAHB4* expression.

Figure S2. The core sequence of the HAHB4 binding site is present in most of the promoters of the regulated genes.

Figure S3. *HAHB4* mediates insect resistance responses in transgenic Arabidopsis plants.

Figure S4. *Pseudomonas syringae* infection does not affect production of jasmonic acid (JA) in *HAHB4* transgenic Arabidopsis plants.

Figure S5. Schematic representation of choice assays performed in Petri dishes.

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