

Genetic evidence that expression of NahG modifies defence pathways independent of salicylic acid biosynthesis in the *Arabidopsis*–*Pseudomonas syringae* pv. *tomato* interaction

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Summary

The salicylic acid (SA)-induction deficient (*sid*) mutants of *Arabidopsis*, *eds5* and *sid2* accumulate normal amounts of camalexin after inoculation with *Pseudomonas syringae* pv. *tomato* (*Pst*), while transgenic NahG plants expressing an SA hydroxylase that degrades SA have reduced levels of camalexin and exhibit a higher susceptibility to different pathogens compared to the *sid* mutants. SID2 encodes an isochorismate synthase necessary for the synthesis of SA. NahG was shown to act epistatically to the *sid* mutant phenotype regarding accumulation of camalexin after inoculation with *Pst* in *eds5*NahG and *sid2*NahG plants. The effect of the *pad4* mutation on the *sid* mutant phenotype was furthermore tested in *eds5pad4* and *sid2pad4* double mutants, and it was demonstrated that PAD4 acts epistatically to EDS5 and SID2 regarding the production of camalexin after inoculation with *Pst*. NahG plants and *pad4* mutants were also found to produce less ethylene (ET) after infection with *Pst* in comparison to the wild type (WT) and *sid* mutants. Both PAD4 and NahG acted epistatically to SID regarding the *Pst*-dependent production of ET that was found to be necessary for the accumulation of camalexin. Early production of jasmonic acid (JA) 12 h after inoculation with *Pst/avrRpt2* was absent in all plants expressing NahG compared to the other mutants tested here. These genetic studies unravel pleiotropic changes in defence signalling of NahG plants that are unlikely to result from their low SA content. This adds unexpected difficulties in the interpretation of earlier findings based solely on NahG plants.

Keywords: NahG, salicylic acid, jasmonic acid, ethylene, defence signalling.

Introduction

Plants have evolved an array of inducible defence responses to defend themselves against different types of pathogens. If efficient responses can be mounted rapidly, e.g. when a product resulting from the expression of an avirulence gene is recognized specifically by the plant carrying a resistance gene, the plant is able to stop the growth of the invading pathogen and establishes a resistance response that is often accompanied by a hypersensitive response (HR). Disease ensues when defence responses are mounted too slowly and/or they are not effective, leading to the spread of the attacking pathogen (Dangl and Jones, 2001; Tao *et al.*, 2003). Interestingly, HR can be uncoupled from resistance as well as pathogen growth from disease symptoms (Bent *et al.*, 1992; Clough *et al.*, 2000; Lund *et al.*, 1998; Yu *et al.*, 1998).

The best-characterized signal molecules acting in plant defence against pathogens in *Arabidopsis* are salicylic acid (SA), ethylene (ET) and jasmonic acid (JA; Dong, 1998; Reymond and Farmer, 1998). These defence signals do not work independently; positive and negative cross-talks have been observed between the pathways leading to an intricate plant defence network (Feys and Parker, 2000; Genoud and Métraux, 1999; Pieterse and van Loon, 1999). For example, JA and ET act synergistically to induce certain defence genes such as PDF1.2, PR-3 and PR-4 (Penninckx *et al.*, 1998). SA and JA can act synergistically or antagonistically during the activation of gene expression (Chen and Chen, 2002; Gupta *et al.*, 2000; Schenk *et al.*, 2000). After inoculation of *Arabidopsis* with strains of *Pseudomonas syringae* pv. *tomato* (*Pst*), effective defence

reactions depend strongly on SA, but not on ET and JA, while symptom development is influenced by ET (Bent *et al.*, 1992; Thomma *et al.*, 2001; Uknes *et al.*, 1992). However, defence reactions are induced in *Arabidopsis* by *P. syringae* pv. *maculicola* (*Psm*) and *Pst* that are not essential for the defence against these bacteria, e.g. the production of the phytoalexin camalexin (Glazebrook and Ausubel, 1994; Nawrath and Métraux, 1999).

The SA-dependent pathway has been analysed in detail in tobacco and *Arabidopsis* using transgenic NahG plants expressing an SA hydroxylase that degrades SA to catechol (Delaney *et al.*, 1994; Gaffney *et al.*, 1993). In tobacco, NahG plants show an increased susceptibility to viral and bacterial pathogens that is correlated to a block in expression of PR-1 (Gaffney *et al.*, 1993; Mur *et al.*, 1997). NahG plants of tobacco also exhibit a delayed development of an HR after inoculation with TMV (Mur *et al.*, 1997) as well as an attenuation of the oxidative burst after inoculation with avirulent bacteria (Mur *et al.*, 2000). In *Arabidopsis*, NahG plants are blocked in the expression of PR-1, PR-2 and PR-5 that contribute to the resistance pathways against virulent *Pst* and *Peronospora parasitica* (Delaney *et al.*, 1994). In addition, they show an increased susceptibility to a variety of other fungal, bacterial, oomycetes and viral pathogens (Delaney *et al.*, 1994; Kachroo *et al.*, 2000), including the non-host pathogen *P. syringae* pv. *phaseolicola* (*Psp*; Lu *et al.*, 2001; van Wees and Glazebrook, 2003). Furthermore, a strong decrease in the accumulation of camalexin has been observed in *Arabidopsis* NahG plants (Nawrath and Métraux, 1999; Zhou *et al.*, 1998).

During the past years, a number of other phytoalexin- and SA-deficient *Arabidopsis* mutants have been characterized. The camalexin-deficient *pad4* mutant was shown to have reduced accumulation of SA after inoculation with *Psm*. However, the block in production of camalexin as well as the deficiency in accumulation of SA was only seen after infection with certain pathogens, indicating that PAD4, a protein carrying a lipase domain, may have a pleiotropic regulatory function (Glazebrook *et al.*, 1997; Jirage *et al.*, 1999; Zhou *et al.*, 1998). This hypothesis is supported by the transcriptional regulation of *PAD3*, a gene encoding for a cytochrome P450 necessary for the biosynthesis of camalexin, and of *EDS5*, a gene necessary for the accumulation of SA by PAD4 (see below; Nawrath *et al.*, 2002; Zhou *et al.*, 1999).

The SA-induction deficient (*sid*) mutants contain very little SA and display a decreased resistance to *Pst* and *P. parasitica*, broadly confirming the results obtained with other SA-deficient plants (Nawrath and Métraux, 1999). Surprisingly, the *sid* mutants are not as susceptible to *Pst* and *P. parasitica* as NahG plants (Nawrath and Métraux, 1999). Furthermore, *sid* mutants display only a decreased expression of PR-1, whereas the expression of PR-2 and PR-5 after inoculation with *P. syringae* is normal, as observed

in the *pad4* mutant (Glazebrook *et al.*, 1996; Nawrath and Métraux, 1999). In addition, camalexin accumulates to normal levels in infected *sid* mutants, whereas infected NahG plants show low levels (Nawrath and Métraux, 1999). *SID2* encodes a plastidic isochorismate synthase that is necessary for the biosynthesis of SA (Wildermuth *et al.*, 2001), while *EDS5*, identified to be *SID1*, encodes a multidrug and toxin extrusion (MATE) protein (Nawrath *et al.*, 2002). *EDS5* is expressed normally in *sid2* mutants after inoculation with *Pst*, indicating that the transcription of *EDS5* does not depend on SA. However, the expression of *EDS5* is clearly delayed in NahG plants (Nawrath *et al.*, 2002). A number of differences between NahG and other SA-deficient plants indicate that NahG plants may have other changes in addition to their SA deficiency that are still to be elucidated.

Little is known about the biosynthesis of ET and JA after pathogen infection of SA-deficient plants. Recently, a deficiency in the accumulation of ET after infection with *Xanthomonas campestris* has been reported in NahG plants (O'Donnell *et al.*, 2003). The dependence on functional ET- and JA-signalling pathways for a certain defence response is often investigated by the analysis of mutants having a block in the pathways (Dong, 1998), e.g. *etr1* and *ein2* for the ET pathway (Alonso *et al.*, 1999; Guzman and Ecker, 1990; Schaller and Bleeker, 1995) and *jar1* and *coi1* for the JA pathway (Feys *et al.*, 1994; Staswick *et al.*, 1992, 2002; Xie *et al.*, 1998).

In the present study, the production of camalexin, ET and JA was investigated in various SA-deficient plants after inoculation with virulent and avirulent strains of *Pst*. In addition, *sid* mutants expressing NahG, i.e. *eds5NahG* and *sid2NahG*, as well as *eds5pad4* and *sid2pad4* double mutants were generated and integrated in the analysis. Several alterations in the production of the different defence molecules have been discovered in SA-deficient plants. Interestingly, the expression of NahG or the *pad4* mutation acted in all cases epistatically to accumulation of SA, providing genetic evidence that NahG plants not only have a strongly reduced accumulation of SA, but also potentially additional changes leading to alterations in plant defence.

Results

Accumulation of SA and camalexin in SA-deficient plants

The influence of the NahG transgene on the accumulation of SA and camalexin was investigated in the *sid* mutant background after inoculation with a virulent strain of *Pst* and the isogenic avirulent strain carrying the *avrRpt2* gene (*Pst/avrRpt2*). *eds5NahG* and *sid2NahG* plants were analysed in comparison to their progenitor plants as well as

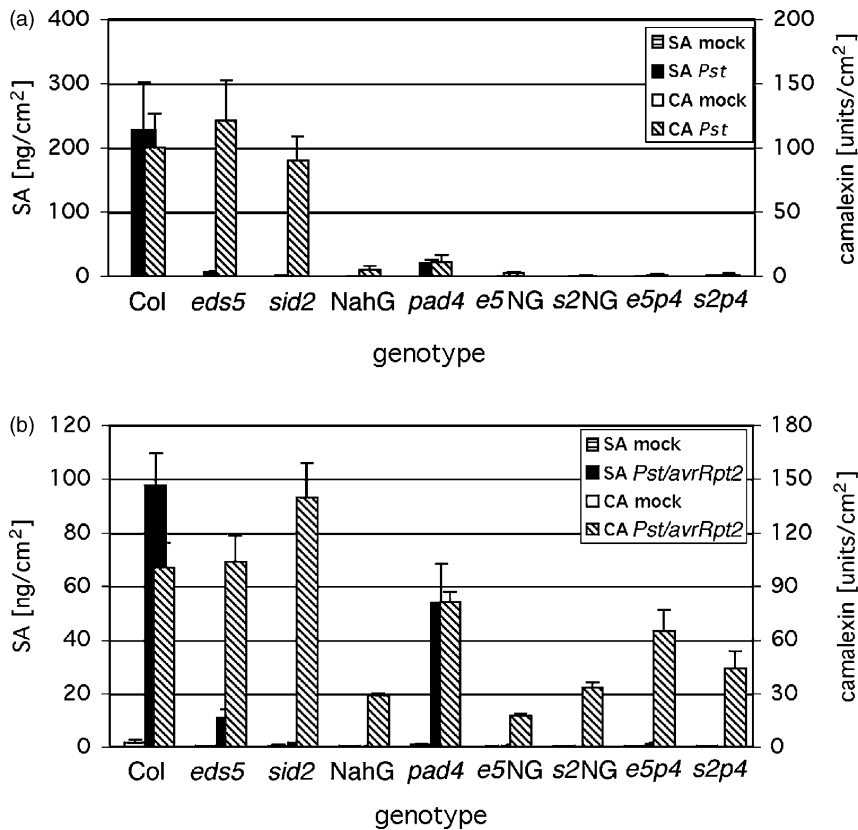


Figure 1. Accumulation of SA and camalexin after inoculation with different *Pst* strains. The accumulation of SA and camalexin was measured 48 h after hand-inoculation with (a) *Pst* or (b) *Pst/avrRpt2* at a concentration of 2×10^6 cfu ml⁻¹. 10 mM MgCl₂ was injected as mock control. The amounts of SA and camalexin represent the means of five to six measurements, including their SE. The experiment was repeated once with similar results.

pad4 and *eds5pad4* and *sid2pad4* double mutants. Measurements were made at 48 hours post inoculation (hpi), to ensure the accumulation of large quantities of SA and camalexin (Glazebrook *et al.*, 1997; Nawrath and Métraux, 1999; Summermatter *et al.*, 1995).

The total amounts of SA after infection with the virulent *Pst* strain were drastically lower in all mutants than in wild-type (WT) plants (Figure 1a). The amounts of SA in *sid2*, *eds5* and *NahG* plants were close to the detection limit, while *pad4* had a low amount of SA that was, however, significantly more than that measured in the other SA-deficient plants investigated here. The *sid* mutants expressing *NahG* had amounts of SA as low as those in *NahG* plants, while the *sidpad4* double mutants contained as little SA as that in the *sid* mutants. The presence of some SA in all these plants was clearly established by GC-MS analysis (results not shown).

The accumulation of camalexin was comparable in WT and *sid* mutants, while drastically reduced in *NahG* and *pad4* mutants, in agreement with earlier results (Nawrath and Métraux, 1999; Zhou *et al.*, 1998). The accumulation of camalexin in *sid* mutants carrying the *NahG* transgene was at least as low as that in *NahG* plants, showing that *NahG* acts epistatically to the *SID* genes regarding this phenotype. Similarly, the accumulation of camalexin was very low in *sidpad4* double mutants as expected because *PAD4* is

thought to act as a regulatory protein in both the SA and the camalexin biosynthetic pathways (Jirage *et al.*, 1999; Zhou *et al.*, 1998).

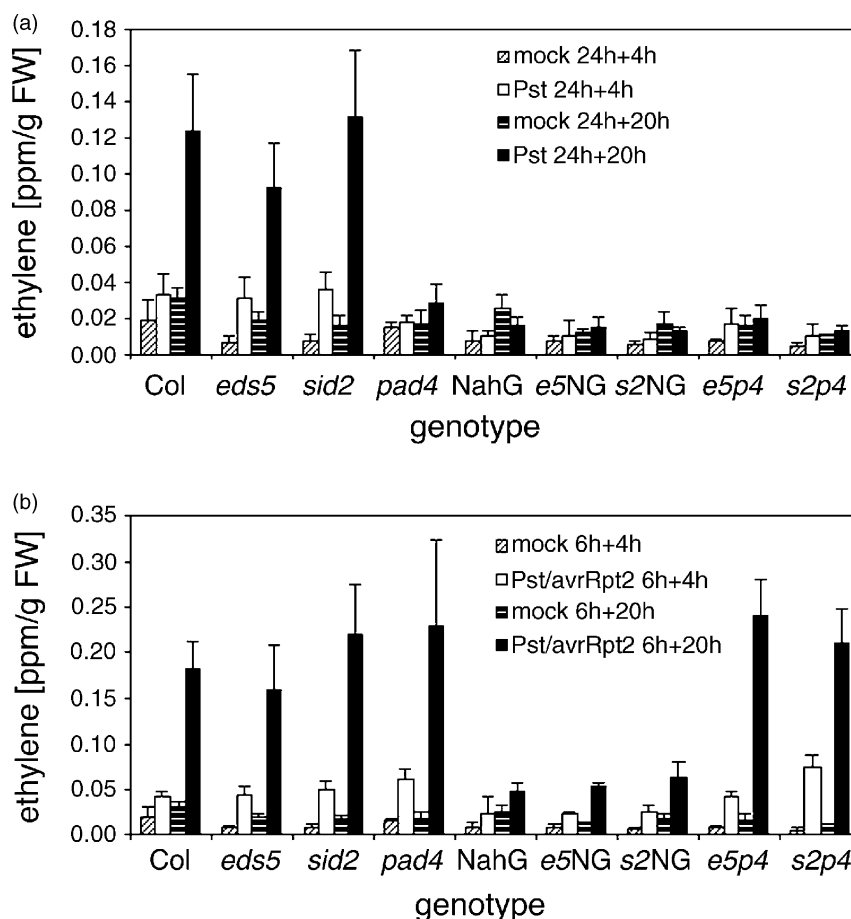
After inoculation with *Pst/avrRpt2*, the accumulation of SA in *pad4* mutants was similar to that in WT plants, in accordance with the observation that *pad4* does not display an enhanced susceptibility to this pathogen strain (see Figure 1b; Zhou *et al.*, 1998). The levels of SA in all other mutants and double mutants were very low in comparison to those in WT plants. The reduced production of camalexin after inoculation with *Pst/avrRpt2* in *NahG* plants and in *sid* mutants expressing *NahG* again demonstrates that *NahG* acts epistatically to the *SID* genes with respect to accumulation of camalexin.

Production of ET in SA-deficient plants

As cross-talk has been observed between different signaling pathways activated during plant defence, all SA-deficient mutants and double mutants were investigated for their production of ET after infection with virulent and avirulent *Pst* strains. A significant production of ET began in WT plants approximately at 24 hpi with the virulent *Pst* strain (data not shown). The amount of ET produced was therefore determined in leaves detached from plants at 24 hpi and enclosed in gas-tight vials. A low, but significant,

Figure 2. Production of ET after inoculation with different *Pst* strains.

The production of ET was measured after inoculation with (a) *Pst* and (b) *Pst/avrRpt2* at a concentration of 2×10^6 cfu ml⁻¹, as well as after mock-inoculation with 10 mM MgCl₂ as controls. Inoculated leaves were enclosed in gas-tight vials at 24 and 6 hpi, respectively. Measurements of the composition of head space were taken after exactly 4 and 20 h. Bars represent the means of five to six measurements with their SE. The experiment was repeated two times with similar results.



increase in ET production could be reproducibly detected after 4 h in the wild type that increased further over the next 16 h. Thus, samples were taken after 4 and 20 h. The amounts of ET produced by both *sid* mutants were similar to those in the case of WT plants, while the levels of ET in *pad4* and NahG plants were approximately 50% of those in WT plants during the 4-h-measurement period and not significantly different from the production in mock-inoculated plants (Figure 2a). Measuring the accumulation of ET at 24 hpi showed more pronounced differences (Figure 2a). Thus, the production of ET in NahG plants as well as in *pad4* mutants is reduced compared to that in *sid* mutants. Interestingly, NahG and PAD4 also acted epistatically to the SID genes, regarding this decrease in production of ET, as seen in both *sidpad4* double mutants and *sid* mutants expressing NahG.

A significant increase in the production of ET in WT plants could be detected at approximately 6 hpi with *Pst/avrRpt2* (data not shown). This time was thus chosen to determine the production of ET. Leaves were detached from plants at 6 hpi with *Pst/avrRpt2* and enclosed in gas-tight vials for 4 or 20 h. The level of ET was found to be comparable to WT in

both *sid* and *pad4* mutants as well as in the *sidpad4* double mutants (Figure 2b). In contrast, *sid* mutants expressing NahG and NahG plants produced only about 50% of the ET found in WT in a 4-h-measurement period, and the values were even lower in a 20-h-measurement period. However, there was still a significant increase in production of ET in comparison to mock-inoculated plants. Thus, NahG plants have deficiencies in the production of ET after inoculation either with a virulent or an avirulent strain of *Pst*, while in *pad4* mutants, the deficiency is strain dependent.

Accumulation of JA in SA-deficient plants

The accumulation of JA was observed in WT plants at different time points over a period of 48 hpi with *Pst*. A significant accumulation of JA could only be observed at 48 hpi with bacteria when tissue damage became obvious (Figure 3a). The production of JA in all SA-deficient plants occurred also at 48 hpi and was approximately three to four times as much as that of WT controls with no significant difference between the different classes of mutants (Figure 3a).

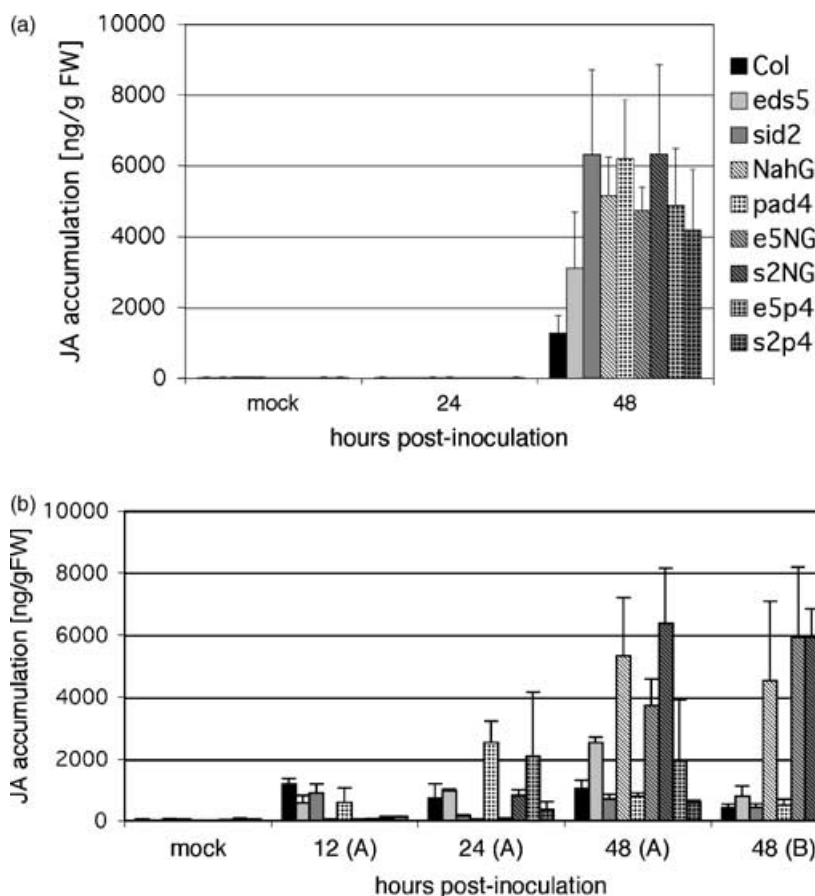


Figure 3. Accumulation of JA after inoculation with different *Pst* strains.

The accumulation of JA was measured 12, 24 and 48 hpi with (a) *Pst* at a concentration of 2×10^5 cfu ml⁻¹ or with (b) *Pst/avrRpt2* at a concentration of 2×10^6 cfu ml⁻¹. Control samples were mock-inoculated with 10 mM MgCl₂. Bars represent the means of four measurements including their SE. The experiment was repeated two times with similar results. The legend shown in (a) applies for both graphs. Data of two independent experiments are indicated as (A) and (B).

The production of JA was also determined after inoculation with *Pst/avrRpt2*. In the WT, a significant amount of JA had already accumulated at 12 hpi and approximately the same amount was maintained up to 48 hpi (Figure 3b). *eds5*, *sid2* and *pad4* mutants also produced JA at all time points investigated (Figure 3b). In contrast, no JA had accumulated in NahG plants and *sid* mutants expressing NahG at 12 hpi. The accumulation of JA in plants expressing NahG began between 24 and 36 hpi (data not shown). At 48 hpi, high amounts of JA had accumulated in NahG plants as well as in *sid* mutants expressing NahG. NahG-expressing plants showed an altered pattern of JA accumulation even in situations when SA is not synthesized. Interestingly, the early production of JA was also strongly reduced in *sidpad4* double mutants in several experiments, indicating that PAD4 might have a role in the accumulation of JA after inoculation with this pathogen.

Although the observations that are highlighted above were reproducible, a greater overall variability in the accumulation of JA was observed in comparison to production of SA and ET. From these observations, it is possible to deduce that each mutant produces JA with specific kinetics that might overlap only in part with those of the disease development.

Accumulation of SA and camalexin in mutants blocked in ET and JA signalling

As accumulation of camalexin in the different mutants paralleled the production of ET, we hypothesized that the synthesis of camalexin might be under the control of ET. However, it is also possible that the production of both camalexin and ET is regulated in the same way, independently of each other. In order to distinguish these possibilities, measurements of the amount of camalexin in different mutants blocked in ET and JA signalling were undertaken after inoculation with both *Pst* and *Pst/avrRpt2*. Production of camalexin after inoculation with *Pst* clearly depended on the perception of ET, as evidenced by the lower production of camalexin in the *etr1* and *ein2* mutant (Figure 4). In contrast, the accumulation of camalexin is independent of JAR1, indicating that the synthesis of camalexin is not regulated by JA. The production of camalexin after inoculation with *Pst/avrRpt2* showed the same trends, but was not always significantly different from WT (data not shown).

The accumulation of SA was determined in parallel in the three different mutants (*jar1*, *etr1* and *ein2*) and was not significantly different from the WT (data not shown).

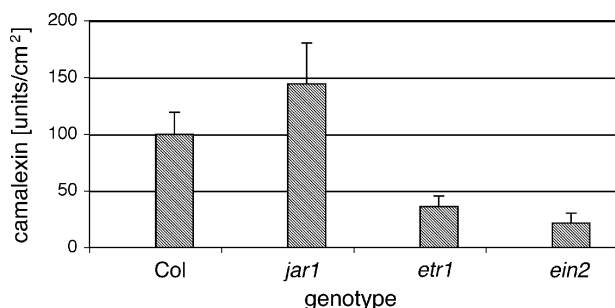


Figure 4. Accumulation of camalexin after inoculation with *Pst* in mutants blocked in JA and ET signalling.

The accumulation of camalexin was measured at 48 hpi with *Pst* at a concentration of 2×10^6 cfu ml⁻¹ (hatched). Controls were mock-inoculated with 10 mM MgCl₂ (black). Bars represent the means of five to six measurements, including their SE. The experiment was repeated once with similar results. The control values and their associated SE are too small to be visible.

Digital representation of the effects of the different genetic backgrounds leading to low SA levels on the signalling network of plant defence

The effect of NahG expression or of various mutations on the accumulation of SA, ET, JA and camalexin is first represented using a convention model (Figure 5a,b). This intuitive model is based on arrows and inhibitor elements and represents the flow of information in the signalling network from the initial signal generated by the pathogen

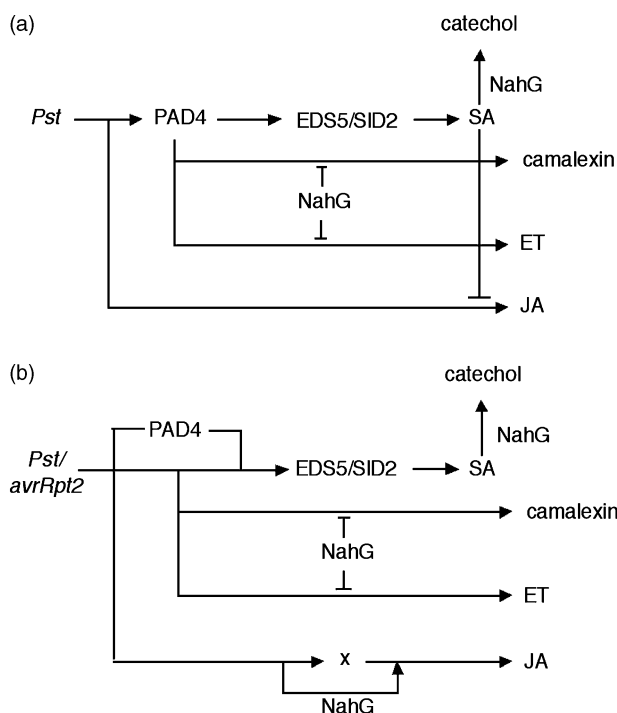


Figure 5. Conventional intuitive model of the signalling network after inoculation with *Pst* (a) or *Pst/avrRpt2* (b).

to the various responses. This type of representation invariably presents a difficulty in interpretation, particularly when several elements are modified. For example, the outcome of the combination of an infection with *Pst* with mutations in EDS5/SID2 and expression of NahG is not easy to read from the model. One way to improve the representation is to use a Boolean formalism as shown in Figure 6(a,b) (Genoud and Métraux, 1999; Genoud *et al.*, 2001). The models show outputs (levels of SA, ET, camalexin and JA) that were observed after various single or combined inputs (represented in the input table by columns A–G and A–H, respectively). Inputs are represented by switch elements that can be set ‘on’ or ‘off’. In contrast to the conventional model, existing knowledge from various experiments is incorporated to model exactly the results obtained. Thus, it is possible to combine inputs and observe the effects on the output. The models presented in Figure 6(a,b) provide a description of the network to which qualitative features have been added in the form of several output options for one molecule. In this way, the high levels of JA or the low levels of ET observed in certain situations could be taken into account. Low levels are found when only one output symbol is ‘on’, while high levels are found when both outputs are ‘on’. The effect of pathogen inoculation on WT plants is represented in column B.

Figure 6(a) represents the situation for *pad4* infected by *Pst*. The input switches for *Pst* and *pad4* are both in position ‘on’ (switch ‘on’ for *pad4* means PAD4 is inactivated by a mutation; column F). *Pad4* mutants accumulate no SA, ET or camalexin, but high levels of JA after inoculation with this pathogen strain, as given from the model. As can easily be seen in the output table, the *pad4* mutation (column F) or the presence of the NahG transgene (column D) leads to the same outputs independent of the presence of a mutation in EDS5 and SID2 (columns E and G).

Figure 6(b) provides an overview of the variety of changes caused by the expression of the NahG gene after inoculation with the avirulent *Pst* strain. As the Boolean symbolism is a static representation of a certain situation, the results obtained at different time points after inoculation are represented as different switches (e.g. NahG 12 hpi, column E; and NahG 48 hpi, column G) that should not be switched on in combination. In the core part of Figure 6(b), the switches of *Pst/avrRpt2* and NahG 12 hpi are ‘on’. At 12 hpi, the production of a small amount of ET is the only defence reaction that was observed in NahG plants during this study (column E), while *eds5* and *sid2* plants produce more ET as well as camalexin and JA (column D). Again, the tables easily give an overview of the effects of several changes in the genetic background. For example, the introduction of the *eds5/sid2* mutations in the NahG background does not change the outputs of the NahG plant alone, as seen in columns F and H. Furthermore, it can be seen from the network that the interference of NahG expression with

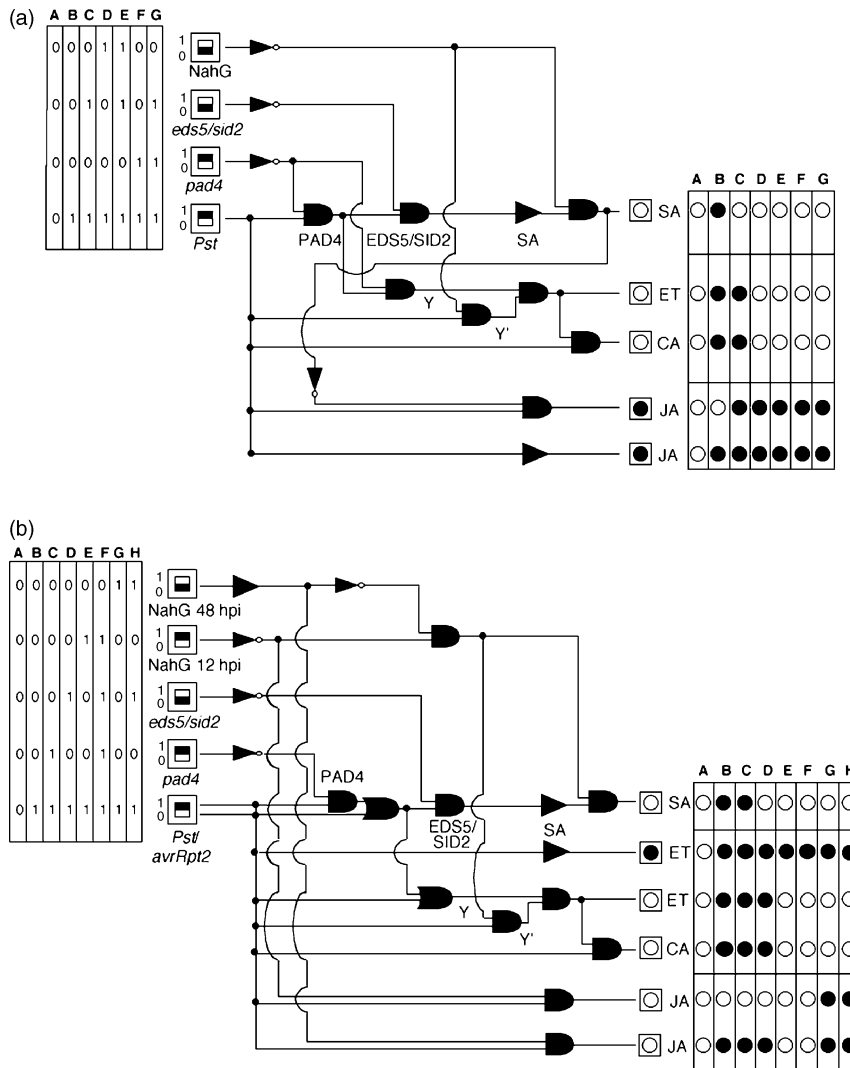


Figure 6. Models of the signalling pathways after inoculation with the virulent strain (a) *Pst* or (b) *Pst/avrRpt2* using Boolean formalism. The inputs are represented at the left (stimulus by inoculation with *Pst* or *Pst/avrRpt2*; effect of a mutation in *eds5/sid2* or *pad4*; effect of NahG expression at different time points or combinations). As *eds5* and *sid2* led to similar responses, they were treated together as a single input for the sake of simplicity. On the right, the outputs are represented by the expression of SA, ET, camalexin (CA) or JA levels. The table on the left of the model represents the different combinations of inputs (A–G and A–H, respectively; 1: input ‘on’; 0: input ‘off’). On the right, the table represents the outputs for the same combinations (A–G and A–H, respectively; full circles: output ‘on’; open circles: output ‘off’). The Boolean symbols have been described elsewhere (Genoud and Métraux, 1999; Genoud *et al.*, 2001). It is possible that the logic elements Y and Y’ are identical. In (a), the model depicts column F, where the *pad4* mutant is infected with *Pst*. The switches for *Pst* and *pad4* are ‘on’ (position 1 of the switch symbol). Both outputs for JA are ‘on’ (black) meaning the plants had high JA levels. In (b), the model depicts column E, when NahG plants were analysed at 12 hpi with *Pst/avrRpt2*. The switches *Pst/avrRpt2* and NahG 12 hpi are ‘on’. Only one of the outputs for ET is ‘on’, meaning that the plants produced only little ET.

ET, camalexin and JA production is independent of SA, as the interfering signal starts upstream of SA.

Discussion

The analysis of the accumulation of camalexin in different SA-deficient plants after inoculation with *Pst* and *Pst/avrRpt2* confirmed the existence of two classes of SA-deficient mutants: one class accumulates camalexin normally, i.e. *eds5* and *sid2*; and another class shows decreased accumulation of camalexin, i.e. *pad4* and NahG. These results are in good accordance with published data (Glazebrook and Ausubel, 1994; Nawrath and Métraux, 1999; Zhou *et al.*, 1998).

In order to understand the epistatic relationship of the accumulation of camalexin and SA in the different SA-deficient plants, the accumulation of SA and camalexin was determined in *eds5pad4* and *sid2pad4* double mutants, as well as in *eds5NahG* and *sid2NahG* plants. PAD4 is

known to be a regulator of defence reactions, and accordingly was found to act epistatically to both the accumulation of SA and the production of camalexin (Jirage *et al.*, 1999; Zhou *et al.*, 1998).

The expression of NahG acts epistatically in the *sid* mutants with regard to the production of camalexin. This phenotype is surprising, as the degradation of SA to catechol has been widely assumed, so far, to be the only metabolic contribution of NahG expression to plants. This has led, for example, to the conclusion that camalexin synthesis depends on SA or on its degradation products (Zhou *et al.*, 1998). Interestingly, this epistasis is unlikely to function via the reduction of SA content in the *sid* mutants because the *pad4* mutant does not have a lower amount of SA than have the *sid* mutants, while having a very similar phenotype as that of NahG plants, regarding camalexin accumulation after inoculation with *Pst* (Figure 1a). Therefore, the epistasis of NahG expression to SID2 with respect to the production of camalexin provides genetic evidence

that in NahG plants, pathways other than the SA pathway might be affected. This observation prompted the present detailed study of the production of ET and JA in the different classes of SA-deficient plants.

The production of ET after infection was unaffected in *sid* mutants but strongly reduced in NahG and *pad4* plants. Again, mutations in the *PAD4* locus as well as expression of NahG act epistatically to SID2 and EDS5, indicating that the lower amount of ET may not be related to biosynthesis/degradation of SA, but that production of ET may be independently influenced by *pad4* and NahG. A reduced production of ET in NahG plants has also recently been reported after infection with *X. campestris* (O'Donnell *et al.*, 2003). In the absence of a more detailed analysis, these authors concluded that the production of ET depends on SA (O'Donnell *et al.*, 2003). This example shows why the SA dependency of various phenotypes in NahG plants should be re-evaluated.

The increased production of JA in all SA-deficient plants after the inoculation with the virulent bacterial strain might reflect the negative cross-talk between the SA and the JA pathways observed in certain plant-pathogen interactions (Gupta *et al.*, 2000). The production of JA only at 48 hpi indicates that JA may not be part of the signalling pathway-limiting disease but might rather be related to cellular damage caused by disease. Thus, elevated amounts of JA might reflect a higher degree of damage, although no differences in the production of JA in the different mutants investigated could be detected.

Measurements of JA after the inoculation of SA-deficient plants with the *Pst/avrRpt2* gene revealed an early accumulation of JA that occurs in WT, *sid* and *pad4* mutants, while in plants carrying the NahG gene, JA is not produced at this time. These results indicate that NahG affects the signalling that leads to the production of JA in the incompatible *Pst* interaction. Thus, NahG plants may have an altered HR, as has been hypothesized earlier by Delaney *et al.* (1994). In tobacco, an attenuation of the HR and a reduced production of reactive oxygen species were observed in NahG plants (Mur *et al.*, 1997, 2000). These phenotypes were correlated to early transient changes in SA accumulation, while other parameters were not measured. An early transient increase of SA has not been described in *Arabidopsis*, and mutants blocked in SA biosynthesis do not exist in tobacco. Thus, it is difficult to decide if the situation in tobacco resembles that in *Arabidopsis* or not. Interestingly, *eds5pad4* and *sid2pad4* double mutants also showed a reduced early production of JA, indicating that PAD4 might have an effect on the early production of JA. The very high accumulation of JA observed in the different NahG-expressing plants at 48 hpi may be caused by the stronger level of disease that ensues in such plants in comparison to other SA-deficient plants. Remarkably, the production of JA after inoculation

with *Pst/avrRpt2* does not show a significant down-regulation by SA because *sid* mutants produce approximately the same amounts of JA as in the case of WT plants.

The higher variability in the amounts of JA produced in *Arabidopsis* may be a result of the metabolic flux through JA that leads to other active signal molecules that have been recently discovered (Seo *et al.*, 2001; Staswick *et al.*, 2002). Thus, the accumulation of JA itself might only be an incomplete view of the flux through this/these pathway(s).

The increased susceptibility of NahG plants in comparison to *sid* mutants to virulent and avirulent strains of *Pst* and *P. parasitica* is therefore likely to be caused by the number of additional changes in the defence pathways (Nawrath and Métraux, 1999), of which the modifications in the ET and JA pathway have been identified in this study.

All the additional phenotypes of NahG plants observed here were epistatic to SID2, the protein essential for induced SA biosynthesis in *Arabidopsis* (Nawrath and Métraux, 1999; Wildermuth *et al.*, 2001) and seem therefore to be independent of an increased biosynthesis and degradation of SA. Even if widely used, the transgenic NahG plant remains an artificial system in which unexpected side reactions might possibly take place. A detailed study on the specificity of this SA hydroxylase expressed at high levels in plants has not been published so far.

Similarly, no information is available on the reactivity of catechol or some of its degradation products in plants. Recently, it was shown that catechol rather than low SA amount is responsible for the loss in non-host resistance of NahG plants to *Psp* (van Wees and Glazebrook, 2003), unlike assumed earlier (Lu *et al.*, 2001). Obviously, NahG plants need to be better studied to exclude phenotypes caused by something else than the low SA content.

In contrast to the results by van Wees and Glazebrook (2003), the NahG-specific changes in defence pathways described here are clearly not based on the production of catechol because *sid2NahG* plants have an NahG phenotype. We therefore hypothesize that the SA hydroxylase of *P. putida* expressed in NahG plants may well act on substrates other than SA, a property that has been overlooked so far.

Thus, NahG plants are likely to have pleiotropic changes in their metabolism altering the signalling in the defence network at different levels.

The studies reported here demonstrate that the production of camalexin depends at least partially on the ET pathway. A dependence of camalexin production on JA was proposed earlier by Glazebrook (2001). In our study, the production of camalexin was found to be independent of JA, but this discrepancy might be because of the type of mutant that was chosen for the analysis. For instance, *jar1* is likely to block only one branch of the JA pathway (Staswick *et al.*, 1992, 2002), while *coi1* seems to have a more pleiotropic block in JA signalling (Feys *et al.*, 1994; Xie *et al.*,

1998). It is therefore likely that the production of camalexin is co-regulated by ET and a specific branch of the JA pathway that is controlled by COI1, but not by JAR1. A positive cross-talk between the JA and ET pathway has already been found for the induction of PDF1.2, PR-3 and PR-4 (Penninckx *et al.*, 1998).

It is striking that the modifications in the production of defence molecules after infection with *Pst* in *pad4* and NahG are so similar despite their difference in the content of SA. A recent large-scale gene expression study of various mutants after infection with *Psm* gave similar results (Glazebrook *et al.*, 2003). NahG was found to cluster in the same group as *pad4* and not in the group of the *sid* mutants (Glazebrook *et al.*, 2003). This similarity between *pad4* and NahG plants further supports the idea that side effects caused by NahG are independent of the biosynthesis/degradation of SA, particularly as the overall gene expression pattern of NahG plants is not altered in *sid2*-NahG plants (Nawrath and Glazebrook, unpublished results). Whether a biochemical basis exists to explain this similarity, i.e. that NahG modifies a signal molecule formed by PAD4, remains to be discovered.

Our findings can be represented by digital simulation using Boolean operators. This language was found to be appropriate for a logical representation of signalling networks (Genoud and Métraux, 1999; Genoud *et al.*, 2001).

The results presented here on the production of SA, JA and ET as well as camalexin in the *Arabidopsis*-*Pst* interaction give new insights into the signalling network of plant defence. Furthermore, the alterations discovered in the defence network in NahG plants that are unrelated to accumulation of SA add unexpected difficulties to the analyses of NahG plants.

Experimental procedures

Plants, bacteria and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used throughout the study, and all other mutants and transgenic plants were in a Col background. Transgenic NahG plants have been described by Delaney *et al.* (1994) and Lawton *et al.* (1995). The *Arabidopsis* mutant *eds5* was *eds5-3* (Nawrath and Métraux, 1999), *sid2* was *sid2-1* (Nawrath and Métraux, 1999) and *pad4* was *pad4-1* (Glazebrook and Ausubel, 1994; Jirage *et al.*, 1999). The *etr1* mutant was *etr1-1* (Schaller and Bleecker, 1995; Nottingham Arabidopsis Stock Center), *ein2* was *ein2-1* (Alonso *et al.*, 1999; Guzman and Ecker, 1990) and *jar1* was *jar1-1* (Staswick *et al.*, 1992, 2002).

The double mutants *eds5-3*NahG (*e5*NG), *sid2-1*NahG (*s2*NG), *eds5-3pad4-1* (*e5p4*) and *sid2-1pad4-1* (*s2p4*) were generated by genetic crosses between the respective plants. Homozygous double mutants were identified in the F₂ generation via PCR-based markers. For the identification of the *nahG* gene, the forward primer 5'-ACTGGAAGCTCTGCCGCTA-3' and the reverse primer 5'-TGAGTTACTAGGGCGTCG-3' were used in the PCR reaction

carried out with an annealing temperature of 54°C resulting in a fragment of 293 bp. For the identification of *pad4-1*, the forward primer 5'-GCGATGCATCAGAAGAG-3' and the reverse primer 5'-TTAGCCCCAAAGCAAGTATC-3' were used in a PCR reaction carried out with an annealing temperature of 55°C. The resulting 391-bp-long PCR product can be cleaved by *BsmFI* resulting in a 110-bp fragment when amplified from WT DNA, but not when amplified from *pad4-1* DNA (personal communication from Jean Greenberg, University of Chicago, IL, USA). For the identification of *eds5-3*, a cleaved amplified polymorphic sequence (dCAPS) marker was developed. The forward primer 5'-ACAGGTCCGGC-GATGGGGAGGTG-3' and the reverse primer 5'-CAGCTTAGC-TATTGGGTAG-3' resulted in a 185-bp-long PCR fragment when the reaction was carried out with an annealing temperature of 56°C. This PCR fragment was cleaved by *HphI* when amplified from *eds5-3* DNA resulting in two fragments of 160 and 25 bp long, but not when amplified from WT DNA. *sid2-1* was identified by a CAPS marker by using the forward primer 5'-AATCAAAAGCCTTCTTC-3' and the reverse primer 5'-CATTCTTGGATAATAGTTTGG-3' in a PCR reaction carried out with an annealing temperature of 56°C. The resulting 337-bp-long PCR fragment was cleaved by *MseI* once when WT DNA was amplified resulting in two fragments (101 and 236 bp), and two times when *sid2-1* DNA was amplified, resulting in three fragments (100, 101 and 136 bp).

Plants were grown at 20°C/18°C under a 12-h light/12-h dark cycle for inoculation with *P. syringae* and under an 18-h light/6-h dark cycle for genetics.

Pseudomonas syringae DC3000 pv. *tomato* was cultured in King's B or Luria-Bertani (LB) medium at 28°C containing 50 mg ml⁻¹ rifampicin, and the isogenic strain carrying the *avrRpt2* gene was grown in medium containing, in addition, 50 mg ml⁻¹ kanamycin (Bent *et al.*, 1993). Leaves were infiltrated with a suspension of bacteria in 10 mM MgCl₂ by using a needleless syringe at the concentrations given depending on the experiment performed.

Determination of SA and camalexin

Five leaves of two 5-week-old plants were inoculated with a suspension of 2×10^5 cfu ml⁻¹ of *P. syringae* DC3000 pv. *tomato* or 2×10^6 cfu ml⁻¹ of *P. syringae* DC3000 pv. *tomato* carrying the *avrRpt2* gene, respectively, on the entire spread of the leaf. Ten leaf discs of 0.5 cm² were harvested at 48 hpi and assayed for total SA (free SA and SA conjugated with glucose) and camalexin as described by Nawrath and Métraux (1999). The amounts of camalexin in the different mutants were normalized to the amount in WT (100%).

Determination of JA

All the leaves of 5-week-old plants were syringe-inoculated with a bacterial suspension of 2×10^5 cfu ml⁻¹ of *P. syringae* pv. *tomato* DC3000 or 2×10^6 cfu ml⁻¹ of *P. syringae* pv. *tomato* DC3000 carrying the *avrRpt2* gene, respectively, on the entire spread of the leaf. At indicated time points, the entire rosettes were harvested and two plants were pooled giving an FW of approximately 0.8–1.0 g. JA was extracted according to a modified protocol proposed by Gundlach *et al.* (1992) and quantified by GC-MS. Samples were separated on a capillary column (25 m × 0.2 mm) of BP-225 (SGE, Ringwood, Vic., Austria) fitted to a Hewlett Packard 6890 gas chromatograph (Palo Alto, CA, USA) coupled with a mass-specific detector. The methyl esters of JA and 9,10-dihydrojasmonate were detected by selective ion monitoring (electron

impact at 70 eV) at m/z 224 and 156, respectively. The detection limit for JA was 10 ng g⁻¹ FW. Two to four replicates were made per measurement.

Determination of ET

Ethylene measurements were performed, as described earlier, with some modifications (Penninckx *et al.*, 1998). Leaves of 5-week-old plants were syringe-inoculated over the entire leaf spread with a bacterial suspension of *P. syringae* pv. *tomato* DC3000 (titre: 2×10^5 cfu ml⁻¹), with the isogenic strain carrying the avirulent gene *avrRpt2* (titre: 2×10^6 cfu ml⁻¹) or with 10 mM MgCl₂ as control and kept under high humidity in continuous light. Three leaves were harvested at 24 hpi with the virulent strain or 6 hpi with the avirulent strain; their FW was determined (70–80 mg on average) and they were enclosed in gas-tight 8-ml glass vials fitted with a septum. The vials were placed back in the growth chamber for the indicated time periods, and 250 µl of the gas phase was analysed by gas chromatography on a Hewlett Packard gas chromatograph (HP 5890) with a Poraplot Q column (10 m × 0.5 mm; Chrompack, Antwerp, Belgium) and a flame ionization detector. For each measurement, six replicates were analysed and the amounts of ET were calculated by comparison with known amounts of ET.

Modelization using Boolean formalism

The signal transduction pathway in WT and infected plants was translated into Boolean formalism using the digital simulation program LOGICSIM created by Arnaud Masson (available at <http://www.planete.net/~amasson/logicsim.html>).

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