

Genetic Elucidation of Nitric Oxide Signaling in Incompatible Plant-Pathogen Interactions^[w]

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Recent experiments indicate that nitric oxide (NO) plays a pivotal role in disease resistance and several other physiological processes in plants. However, most of the current information about the function of NO in plants is based on pharmacological studies, and additional approaches are therefore required to ascertain the role of NO as an important signaling molecule in plants. We have expressed a bacterial nitric oxide dioxygenase (NOD) in Arabidopsis plants and/or avirulent *Pseudomonas syringae* pv *tomato* to study incompatible plant-pathogen interactions impaired in NO signaling. NOD expression in transgenic Arabidopsis resulted in decreased NO levels in planta and attenuated a pathogen-induced NO burst. Moreover, NOD expression in plant cells had very similar effects on plant defenses compared to NOD expression in avirulent *Pseudomonas*. The defense responses most affected by NO reduction during the incompatible interaction were decreased H₂O₂ levels during the oxidative burst and a blockage of Phe ammonia lyase expression, the key enzyme in the general phenylpropanoid pathway. Expression of the NOD furthermore blocked UV light-induced Phe ammonia lyase and chalcone synthase gene expression, indicating a general signaling function of NO in the activation of the phenylpropanoid pathway. NO possibly functions in incompatible plant-pathogen interactions by inhibiting the plant antioxidative machinery, and thereby ensuring locally prolonged H₂O₂ levels. Additionally, albeit to a lesser extent, we observed decreases in salicylic acid production, a diminished development of hypersensitive cell death, and a delay in pathogenesis-related protein 1 expression during these NO-deficient plant-pathogen interactions. Therefore, this genetic approach confirms that NO is an important regulatory component in the signaling network of plant defense responses.

Plants have evolved several mechanisms to defend themselves from bacterial or fungal invasion. The rapid recognition of pathogenic microbes is based on the interaction of products from a pathogen-derived avirulence gene and a plant-derived resistance gene and represents a prerequisite to specific resistance in incompatible plant-pathogen interactions (Flor, 1956). The multicomponent defense responses associated with specific resistance include a burst of reactive oxygen intermediates (ROI; Lamb and Dixon, 1997), transcriptional activation of defense genes encoding phenylpropanoid pathway enzymes, lytic and antimicrobial pathogenesis-related (PR) proteins (Lamb et al., 1989), increase of intracellular levels of salicylic acid (SA; Malamy et al., 1990; Métraux et al., 1990), and development of the hypersensitive response (HR). The HR results in the rapid appearance of a dry, necrotic lesion at the infection site that is clearly delimited from surrounding healthy tissue and is thought to contribute to the limitation of pathogen spread (Keen, 1990).

One of the earliest events following pathogen recognition is a burst of oxidative metabolism leading

to the generation of superoxide (O₂⁻) and subsequent accumulation of H₂O₂ (Lamb and Dixon, 1997). These ROI are directly protective and drive the oxidative cross-linking of cell wall structural proteins (Brisson et al., 1994). The H₂O₂ originating from the oxidative burst induces some plant genes involved in cellular protection and defense such as glutathione S-transferase (GST) and is necessary for the initiation of host cell death following the HR (Levine et al., 1994).

Recent pharmacological experiments indicate that nitric oxide (NO), which acts as a signal in the immune, nervous, and vascular system in vertebrates (Schmidt and Walter, 1994), also plays an important role in plant disease resistance. Generation of NO by chemical NO donors augments the induction of hypersensitive cell death by H₂O₂ in soybean (*Glycine max*) suspension cultures (Delledonne et al., 1998, 2001). Likewise, inhibitors of NO synthesis as well as NO scavengers are able to block the HR induced by avirulent *Pseudomonas syringae* in soybean cell cultures and in Arabidopsis plants. Compared to ROI, NO induces a complementary set of plant defense genes, including two key enzymes of the phenylpropanoid pathway, namely Phe ammonia lyase (PAL) and chalcone synthase (CHS). Furthermore, NO-treated tobacco (*Nicotiana tabacum*) cells were shown to induce the pathogenesis-related protein 1 (PR-1) together

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with an accumulation of SA (Durner et al., 1998), a key molecule for the expression of systemic acquired resistance (Gaffney et al., 1993). Moreover, the molecular components of NO signaling in plants appear to be similar to those in animals, regarding the involvement of NO producing NO synthases (NOS; Chandok et al., 2003; Guo et al., 2003) and cGMP as a second messenger (Clark et al., 2000).

As an increasing number of recent reports suggests, a regulatory function of NO in plants seems to be essential in other physiological processes, including guard cell abscisic acid signaling (Desikan et al., 2002; Garcia-Mata and Lamattina, 2003), regulation of iron homeostasis (Graziano et al., 2002; Murgia et al., 2002), execution of programmed cell death in barley (*Hordeum vulgare*) aleurone layers (Beligni et al., 2002), root organogenesis (Pagnussat et al., 2002), and wound signaling (Orozco-Cardenas and Ryan, 2002). However, despite the recent identification of a pathogen-inducible NOS (Chandok et al., 2003), assumptions of NO function in plants emerging from all these studies are almost exclusively based on pharmacological studies, i.e. either exogenous application of NO donors, NO scavengers, and inhibitors of mammalian NOS or detection of NO by essentially indirect methods using fluorescent dyes or photometric indicator molecules (Delledonne et al., 1998; Foissner et al., 2000). If indeed the application of pharmacological compounds reflects a physiological NO situation without exerting nonspecific side effects is far from clear, and additional experimental approaches are therefore desirable.

We report here a novel genetic approach to manipulate NO levels in planta, which has been used to gain a better understanding of the function of NO in the signaling network underlying incompatible plant-pathogen interactions. We first generated transgenic *Arabidopsis* plants overexpressing the *Escherichia coli* *hmp* gene encoding NO dioxygenase (NOD), a flavo-hemoglobin capable of converting NO to nitrate by use of NAD(P)H and O₂ (Vasudevan et al., 1991; Gardner et al., 1998; Poole and Hughes, 2000). In this way, we attempted to directly reduce the levels of NO in plant cells. We then compared the defense responses of NO-deficient plants and wild-type *Arabidopsis* following challenge with avirulent *P. syringae* pv *tomato* (*Pst*) bacteria. Additionally, we employed avirulent *Pst* expressing the *hmpX* gene from *Erwinia chrysanthemi* [*Pst(avr-hmpX)*] (Favey et al., 1995) that encodes a highly similar NOD to lower NO levels specifically at the site of pathogen infection. We then challenged wild-type *Arabidopsis* and *hmp*-expressing *Arabidopsis* plants with *Pst(avr-hmpX)* to study the effect of NO removal at both the plant and the pathogen side.

RESULTS

Arabidopsis Plants Expressing a Bacterial NOD

For the production of *Arabidopsis* ecotype Col-0 plants expressing a functional NOD, the *hmp* coding

sequence from *E. coli* (Vasudevan et al., 1991) was cloned into the dexamethasone (DEX)-based pTA7001-inducible vector system (Aoyama and Chua, 1997). After *Agrobacterium*-mediated transformation of wild-type Col-0 plants with the vector construct, homozygous T3 plants segregating for a single T-DNA insertion were used for further experiments. Upon treatment with 3 μM DEX, the various lines expressed the *hmp* transgene at different levels (data not shown). We selected one of the lines that expressed *hmp* to a higher degree, designated *hmp8*, for more detailed analysis. In *hmp8* plants, *hmp* transcripts accumulated at 5 h after spraying rosette leaves with DEX (Fig. 1A). Transcriptional levels increased 1 d after DEX treatment and remained nearly constant for at least 1 week. Western-blot analysis with antibodies raised against *E. coli* Hmp revealed the production of a full-size Hmp (43 kD) protein in planta (Fig. 1B). The kinetics of Hmp protein expression was similar to transcriptional *hmp* induction.

The functional effects of Hmp in transgenic plants were first investigated by studying the capability of isolated leaf protein extracts to degrade NO. Whereas wild-type plants and noninduced *hmp8* plants showed highly similar degradation kinetics for NO (Fig. 1C), plant extracts from the DEX-induced *hmp8* line significantly accelerated the degradation of NO (Fig. 1D).

In leaves, NO can be produced from nitrite by nitrate reductase, and this NO production is measurable as emission by chemiluminescence (Rockel et al., 2002). A temporary rise of NO emission resulting from increased nitrate reductase activity was detected when dark-adapted plants were transferred to light. When the light source was switched off again, a light-off peak caused by transient nitrite accumulation resulted (Kaiser et al., 2002). Both the light-induced NO emission and the NO light-off peak were significantly lower in DEX-treated *hmp8* plants compared to the wild type (Fig. 1E).

P. syringae Expressing a Bacterial NOD

To expand our genetic approach to study NO deficiency in incompatible plant-pathogen interactions, we transformed *Pst* (*avrB*) with the *hmpX* gene from *E. chrysanthemi*. Like Hmp from *E. coli*, *E. chrysanthemi* HmpX represents a NOD (M. Delledonne and R. Poole, unpublished data). The expression of HmpX in the bacterium should decrease the concentration of the diffusible molecule NO specifically at the site of pathogen infection. In this way, we were able to study plant-pathogen interactions in which NO is simultaneously removed at the infection site from both the plant and the pathogen side.

Plant Defense Responses under NO-Deficient Conditions

Pst carrying the *avrB* avirulence gene is recognized by *Arabidopsis* ecotype Col-0 carrying the Rpm1

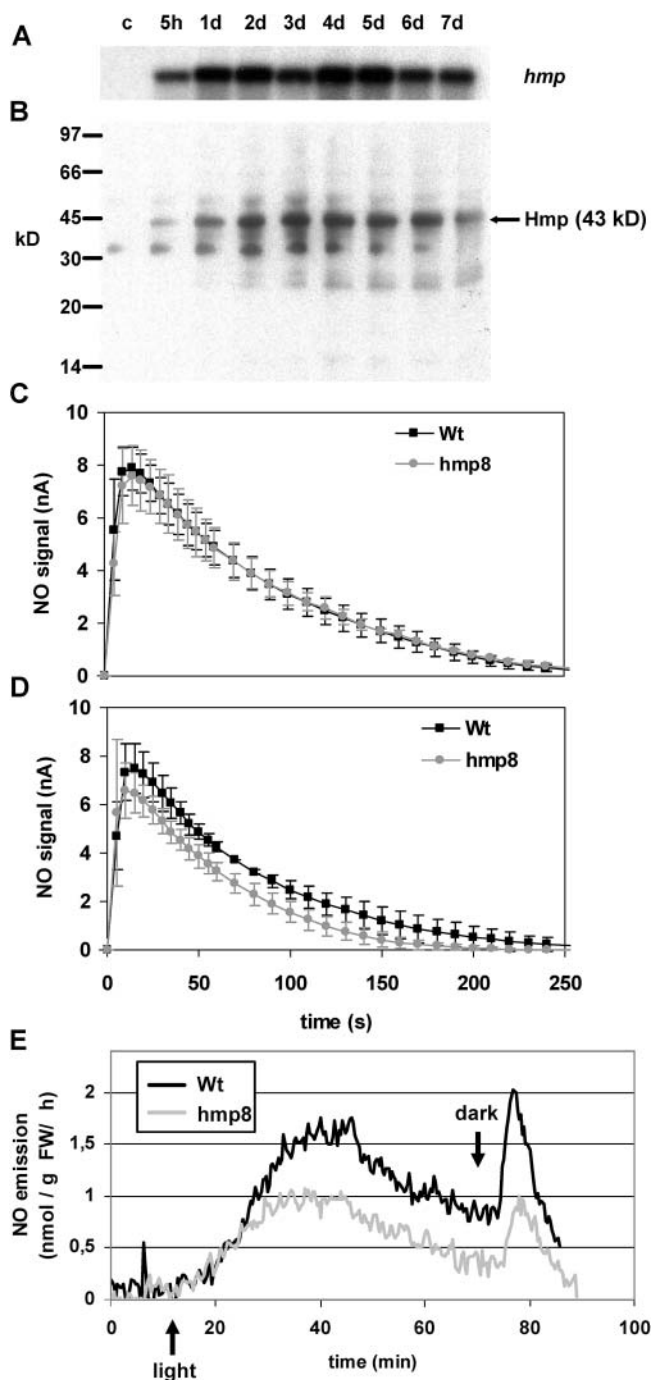


Figure 1. Expression of *E. coli hmp* in transgenic Arabidopsis as a functional NOD. A, Northern-blot analysis illustrating time-dependent accumulation of *hmp* transcripts after treatment of *hmp8* plants with 3 μM DEX (c, no DEX). B, Western blot demonstrating the appearance of correctly sized Hmp protein in leaf extracts; time course as in A. C and D, Electrochemically measured degradation kinetics of 10 μM NO in leaf extracts of wild-type and *hmp8* transgenic Arabidopsis. Error bars represent the sds of five independent measurements. C, *Hmp8* plants without DEX-induced transgene expression. D, Wild-type and *hmp8* plants 1 d after DEX treatment. NO concentrations of less than 1 μM were reached for wild-type, noninduced *hmp8*, and DEX-induced *hmp8* plants at 197 s, 202 s, and 131 s, respectively. E, NO emission from intact wild-type and *hmp8* plants 1 d after DEX treatment. Plants were first incubated in the dark for 10 min and then

resistance gene (Bisgrove et al., 1994). The recognition results in the oxidative burst, a production of ROI like H_2O_2 and O_2^- at the site of infection, induction of defense and cellular protectant genes, development of the HR, and a limitation of pathogen growth in comparison to isogenic virulent *Pst*.

NO Production

To investigate whether NO is produced during the infection of Col-0 plants with avirulent bacteria, we infiltrated DEX-treated wild-type leaves with the NO-sensitive, cell-permeable fluorescent dye 4,5-diaminofluorescein diacetate (DAF2-DA; Foissner et al., 2000) 3 h after challenge with 2×10^6 colony-forming units (cfu) mL^{-1} *Pst* (*avrB*). Inside the area of pathogen infiltration, various brightly green fluorescing cell groups were discernible 1 h after DAF2-DA treatment (Fig. 2C, white stars), whereas MgCl_2 -infiltrated control leaves only showed a weak fluorescence when treated with the fluorophore for the same period of time (Fig. 2B). Pathogen infiltration without fluorophore treatment did not cause any fluorescence (Fig. 2A). As compared to wild-type leaves, DEX-induced *hmp8* plants challenged with *Pst* (*avrB*) and subsequently treated with DAF2-DA showed a markedly reduced fluorescence at the site of pathogen infiltration (Fig. 2D). A similar reduction of DAF2-DA fluorescence was observed when wild-type leaves were challenged in the presence of 100 μM CPTIO, a NO scavenging compound (data not shown). These results suggest that NO is produced during the earlier stages of the Arabidopsis-*Pst* (*avrB*) interaction and that this NO burst is attenuated in NOD-expressing *hmp8* plants.

Oxidative Burst

To assess the accumulation of ROI in response to infection with an avirulent pathogen, DEX-treated leaves from wild-type and *hmp8* plants were infiltrated with 2×10^6 cfu mL^{-1} *Pst* (*avrB*) and stained with diaminobenzidine (DAB), a histochemical reagent that forms a reddish-brown precipitate upon contact with H_2O_2 (Thordal-Christensen et al., 1997). Wild-type leaves showed a strong production of H_2O_2 at the infection site 4 h after pathogen challenge (Fig. 3). By contrast, leaves from *hmp8* plants showed significantly lower levels of H_2O_2 compared to the wild type when challenged with *Pst* (*avrB*). Moreover, when DEX-treated leaves from *hmp8* plants were challenged with *Pst* (*avrB/hmpX*) bacteria, the suppression of H_2O_2 levels during the oxidative burst was even more pronounced.

illuminated for 60 min to cause nitrate reductase-dependent NO emission. The light was switched off again, which gave rise to a characteristic light-off peak (see "Results"). Experiments were repeated three times with similar results.

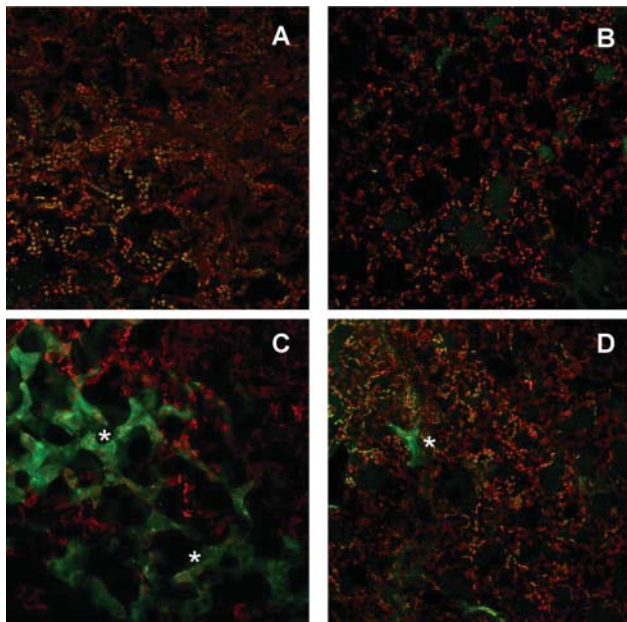


Figure 2. Pathogen-induced DAF2-DA fluorescence as a measure for NO production in DEX-treated wild-type and *hmp8* plants. Leaves were pretreated with *Pst* (*avrB*) or $MgCl_2$ for 3 h and subsequently infiltrated with $10 \mu M$ DAF2-DA or control buffer (10 mM Tris/KCl, pH 7.2). Infiltrated leaf areas were analyzed 1 h later by confocal laser scanning microscopy. DAF2-DA fluorescence (green) was recorded using a channel with a 505- to 530-nm band-pass filter, and autofluorescence of chloroplast (red) was captured with a channel equipped with a 560-nm long-pass filter. A, Treatment of a wild-type *Arabidopsis* leaf with *Pst* (*avrB*) and control buffer. B, Wild-type *Arabidopsis*- $MgCl_2$ and DAF2-DA. C, Wild-type *Arabidopsis*-*Pst* (*avrB*) and DAF2-DA. D, *Hmp8*-*Pst* (*avrB*) and DAF2-DA. Seven independent samples were recorded for each condition, and representative leaf areas are shown.

This observation prompted us to test whether the reduced H_2O_2 levels were a consequence of less H_2O_2 production or, once produced, an effect of increased H_2O_2 degradation. We infiltrated equal amounts of the H_2O_2 -generating system Glc/Glc oxidase into leaves of wild-type and *hmp8* plants and performed DAB staining 1 h after infiltration. Again, wild-type leaves showed stronger staining patterns with respect to induced *hmp8* leaves (Fig. 4), suggesting that the action of *Hmp* increased the ability of the plants to degrade H_2O_2 .

Defense Gene Expression

We next examined whether the expression of three typical defense-related genes, GST, PAL, and PR-1, was affected in the *hmp8* line (Fig. 5). GST functions in cellular protection, and *gst* transcripts are induced during the oxidative burst (Levine et al., 1994; Delledonne et al., 2001). *Gst* transcripts accumulated 4 to 10 h after DEX-treated wild-type plants were challenged with *Pst* (*avrB*). Similar induction kinetics were observed when DEX-induced *hmp8* plants were challenged with *Pst* (*avrB*) or *Pst* (*avrB/hmpX*). Only in

the latter case was the amount of gene induction slightly diminished, presumably reflecting the extremely low H_2O_2 levels during the oxidative burst.

PAL catalyzes the first step in phenylpropanoid biosynthesis and possibly initiates the synthesis of lignin, antibiotics, and SA. A strong induction of *pal* transcripts occurred 4 h after pathogen infection in wild-type plants challenged with *Pst* (*avrB*) (Fig. 5). This strong induction of *pal* was highly suppressed in NO-deficient interactions, i.e. when DEX-induced

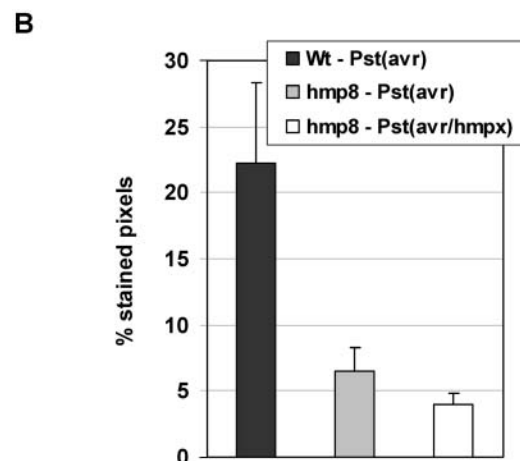
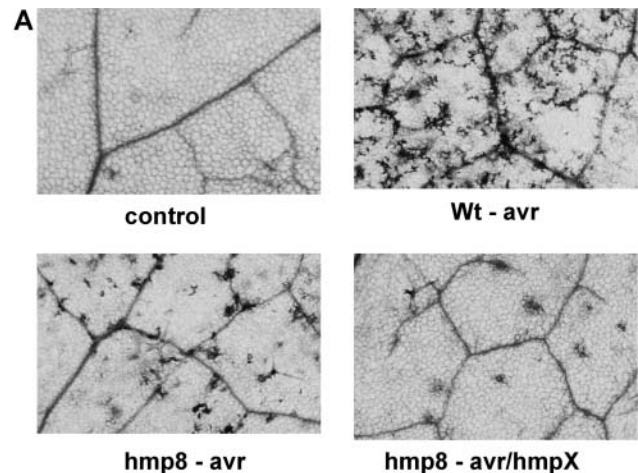


Figure 3. DAB staining of *Arabidopsis* leaves to assess H_2O_2 accumulation during the oxidative burst in DEX-treated wild-type and *hmp8* transgenic plants. Solutions of *Pst* (*avrB*) were infiltrated into *Arabidopsis* leaves, and DAB staining was initiated 4 h after infection. A, Staining patterns of representative, $MgCl_2$ -infiltrated wild-type or *hmp8* leaves (control), *Pst* (*avrB*)-infected wild-type *Arabidopsis* leaves (Wt-*avr*), *Pst* (*avrB*)-infected *hmp8* plants (*hmp8*-*avr*), and *Pst* (*avrB/hmpX*)-infected *hmp8* plants (*hmp8*-*avr/hmpX*) 4 h after the respective treatment (100-fold magnification). B, Quantification of DAB staining in *Pst* (*avrB*)-infected wild-type and *hmp8* leaves. The percentage of stained pixels inside the infiltration area was assessed as described in "Materials and Methods." Values are shown as the mean \pm SD of at least five leaves from different plants. Experiments were repeated three times with similar results.

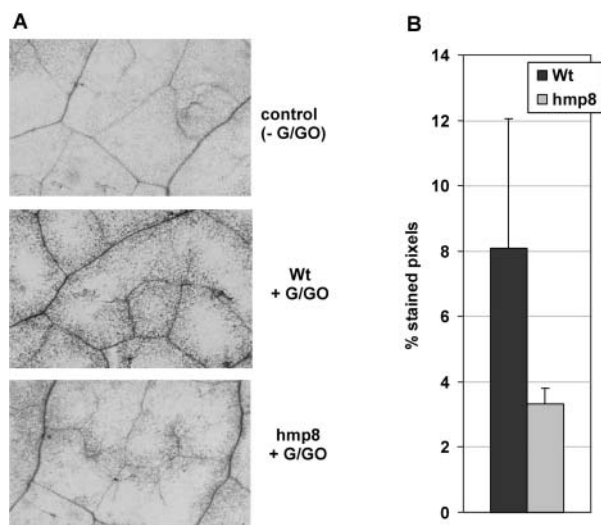


Figure 4. DAB staining of *Arabidopsis* leaves to assess their capability to degrade H_2O_2 in DEX-treated wild-type and *hmp* transgenic plants. Solutions of 2.5 mM Glc/2.5 units mL^{-1} Glc oxidase were infiltrated into leaves and DAB staining was performed 1 h after infiltration. A, Staining patterns of representative leaves inside the infiltration zone (100-fold magnification). B, Percentage of stained pixels inside the infiltration zone. Values are shown as the mean \pm SD of at least five leaves from different plants. Experiments were repeated three times with similar results.

hmp8 plants were challenged either with *Pst (avrB)* or *Pst (avrB/hmpX)*.

Transcriptional induction of the antimicrobial PR-1 protein occurred 10 h after challenge of both wild-type and *hmp8* plants with *Pst (avrB)* (Fig. 5). PR-1 induction was delayed, albeit not fully suppressed, when *hmp8* plants were challenged with *Pst (avrB/hmpX)*.

The finding that pathogen-induced *pal* expression is blocked under NO-deficient conditions prompted us to test whether NO could act as a general signal for the activation of the phenylpropanoid pathway. Another stimulus activating this pathway is UV light (Chappell and Hahlbrock, 1984), and we examined the UV-induced expression of PAL and CHS, the key enzyme for flavonoid biosynthesis, in DEX-treated wild-type and *hmp8* plants (Fig. 6). Whereas wild-type plants strongly expressed *chs* and *pal* after 24 h of UV

treatment, this UV-induced gene expression was markedly attenuated in *hmp8* plants, indicating that NO is required for phenylpropanoid pathway activation by different environmental stimuli.

Development of Hypersensitive Cell Death

When leaves from wild-type plants were challenged with *Pst (avrB)* at concentrations of 5×10^6 cfu mL^{-1} , a dry, colorless lesion limited to the site of pathogen infiltration developed within 2 d in at least 5 out of 7 leaves (Fig. 7A). These macroscopic symptoms characteristic of hypersensitive cell death developed to the same extent when noninduced *hmp8* plants were used (data not shown). However, when infected with *Pst (avrB)*, DEX-treated *hmp8* plants showed a more chlorotic lesion that was reminiscent of the symptoms caused by isogenic virulent *Pst* in about 50% of leaves (Fig. 7B). Infection of induced *hmp8* plants with *Pst (avrB/hmpX)* further increased this percentage. In this case, virtually every challenged leaf developed chlorotic symptoms (Fig. 7C).

To characterize the HR development in more detail, we performed Trypan blue staining of infected leaves 24 h after bacterial inoculation (Fig. 8). Blue-stained dead cells or patches of dead cells appeared to a similar extent at the sites of *Pst (avrB)* infiltration in DEX-treated wild-type leaves and nontreated *hmp8* leaves. In DEX-treated *hmp8* leaves, the density of dead cells was reduced when challenged with *Pst (avrB)*, and *Pst (avrB/hmpX)* challenge led to a dramatic reduction of HR lesions.

SA Levels and Bacterial Growth

SA plays a central role in the activation of plant defense responses, and a relationship between NO and SA signaling pathways has been discussed (Klessig et al., 2000). To determine the effects of NO on SA levels in the *Pst (avrB)*-Col-0 interaction, we determined the levels of SA and SA glucoside (SAG) in our system 8 h after pathogen infection (Fig. 9). DEX-treated wild-type and *hmp8* plants infiltrated with 10 mM $MgCl_2$ had SA levels of about 120 ng g^{-1} leaf fresh weight and SAG contents of about 600 ng g^{-1} . Upon *Pst (avrB)* challenge, SA levels increased in wild-

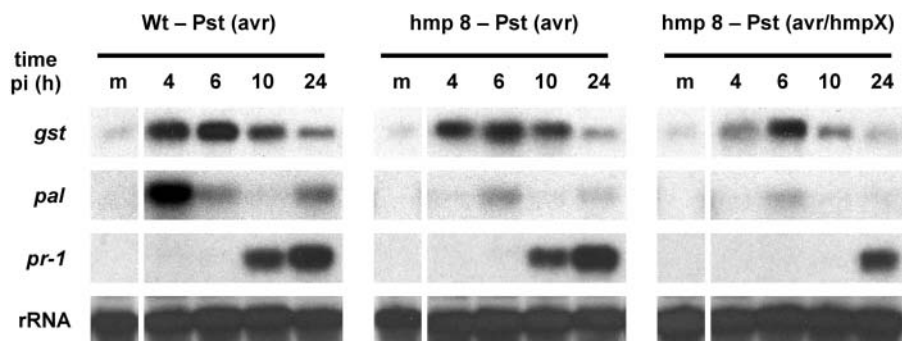


Figure 5. Expression of defense and cellular protectant genes in wild-type and *hmp8* transgenic plants after *Pst (avrB)* challenge. Three parallel leaf samples were collected at the indicated times after infection for RNA extraction and northern-blot analysis. $MgCl_2$ (m)-infiltrated leaves were collected 4 h after infection. Experiments were repeated three times with similar results.

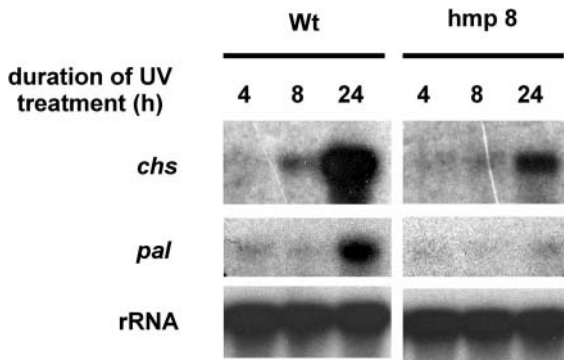
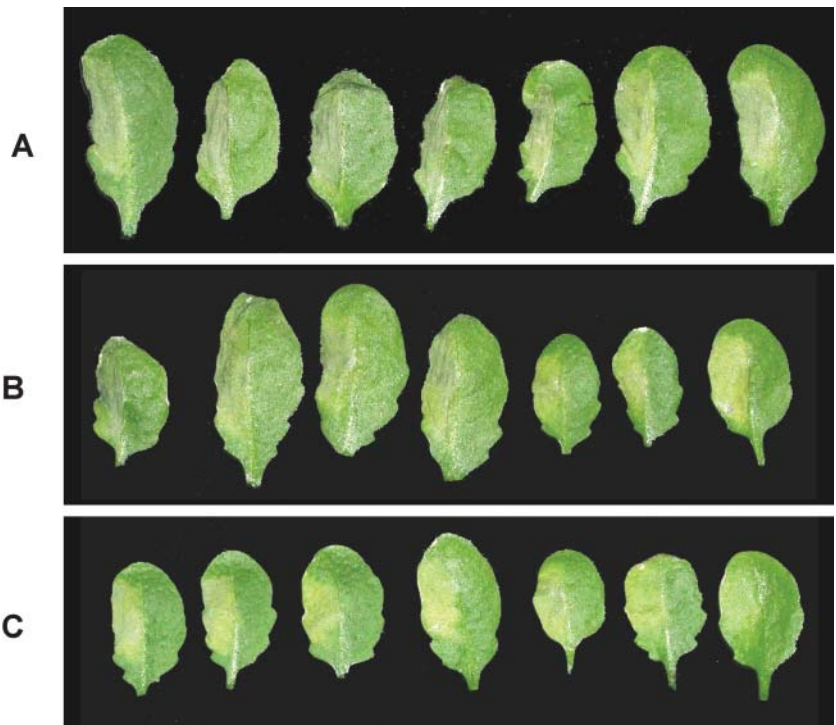


Figure 6. Expression of PAL and CHS in DEX pretreated wild-type and hmp8 plants after UV exposure. Three parallel leaf samples were collected at the indicated times after the beginning of UV treatment. Experiments were repeated three times with similar results.

type plants about 4-fold to 500 ng g⁻¹, and SAG levels reached values of 1,700 ng g⁻¹, representing a 3-fold increase. In DEX-treated hmp8 plants, the SA content in *Pst* (*avrB*)-challenged leaves showed a small, albeit statistically not significant, decrease to about 80% of the wild-type value (Fig. 9A). A similar trend was observed for the glucoside (Fig. 9B). Again, the most striking effect was detected when induced hmp8 plants were infiltrated with *Pst* (*avrB/hmpX*). Here, SA and SAG were reduced to about 50% of the wild type-*Pst* (*avrB*) values, reflecting a pathogen-induced increase of only about 2-fold (SA) and 1.5-fold (SAG), respectively.

Figure 7. Macroscopic HR symptoms 2 d after infiltration of DEX-pretreated wild-type and hmp8 plants with avirulent *Pseudomonas*. Bacteria were infiltrated into the left side of leaves. Seven parallels are shown for each condition. A, Wild-type plants-*Pst* (*avrB*). B, hmp8 plants-*Pst* (*avrB*). C, hmp8 plants-*Pst* (*avrB/hmpX*).



To test whether these changes of defense responses in our genetically different pathosystems affected bacterial growth in planta, we determined the number of colony-forming bacteria in the apoplast 2 d after leaf inoculation (Table I). Compared to wild-type plants, bacterial growth was slightly, but statistically insignificantly, enhanced in DEX-treated hmp8 plants when challenged with *Pst* (*avrB*). A more pronounced growth enhancement was detected when DEX-treated hmp8 plants were challenged with *Pst* (*avrB/hmpX*). However, this enhancement did not reach the extent of growth found in the compatible interaction of wild-type plants and the isogenic virulent *Pst* strain (Table I).

DISCUSSION

Pharmacological methodologies in different laboratories using mainly mammalian NOS inhibitors, NO scavengers, and NO-releasing systems have implicated a pivotal role for NO in plant disease resistance (Delledonne et al., 1998; Durner et al., 1998). Moreover, the recent report that activity suppression of a pathogen-inducible NOS in tomato (*Lycopersicon esculentum*) increases susceptibility to *P. syringae* demonstrates the involvement of a NO-generating enzyme in plant defense (Chandok et al., 2004). This plant pathogen-inducible NOS represents a variant form of the P protein of the Gly decarboxylase complex that shares some biochemical characteristics with animal NOS, such as sensitivity to inhibitors (Chandok et al.,

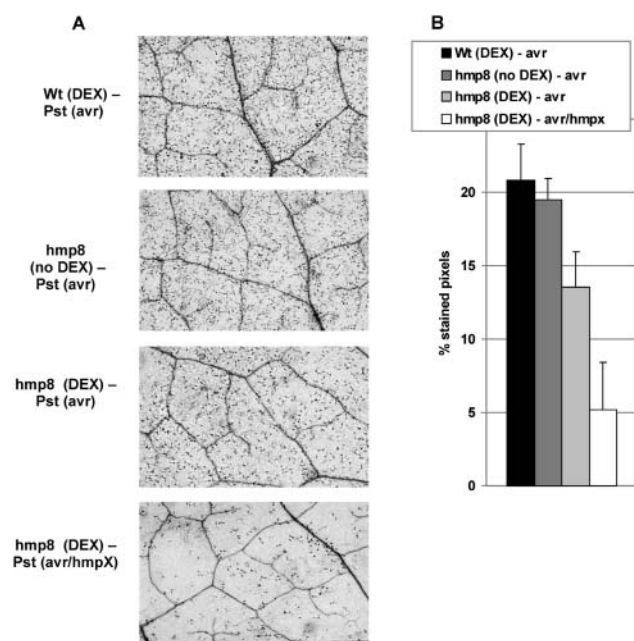


Figure 8. Microscopic cell death after pathogen challenge of DEX-pretreated wild-type and *hmp8* plants. Trypan blue staining was performed 24 h after infection. A, Staining patterns of representative leaves inside the infiltrated area (100-fold magnification). B, Percentage of stained pixels inside the infiltration area. Values are shown as the mean \pm SD of at least six leaves from different plants. Experiments were repeated three times with similar results.

2003), indicating the validity of the data obtained with the use of pharmacological approaches. However, it cannot be fully excluded that pharmacological compounds not only interfere with the metabolic pathway of interest but also have nonspecific effects. The widely used NADPH oxidase inhibitor diphenylene iodonium, for instance, has been shown to be a potent inhibitor of mammalian NOS and other flavoproteins (Stuehr et al., 1991; Bolwell, 1999). To generalize and broaden the knowledge of NO function in plant defense, we employed a genetic approach to interfere with NO signaling by expressing a NOD in transgenic *Arabidopsis* plants as well as in avirulent *P. syringae*.

Transgenic *Arabidopsis* plants were produced that express the Hmp protein from *E. coli*, and whole-plant NO emission was evaluated. The emission was shown to be markedly reduced in Hmp-expressing plants, and leaf extracts from transgenic plants degraded NO significantly faster than extracts from control plants. These findings demonstrate that Hmp is a functional NOD in planta (Fig. 1). Using the NO-sensitive fluorescence indicator DAF2-DA, we furthermore showed that NO is produced during the incompatible interaction of *Arabidopsis* and *Pst (avrB)* and that this NO burst is attenuated in NOD-expressing plants (Fig. 2). We also produced avirulent *Pseudomonas* expressing HmpX, a similar NOD from *E. chrysanthemi*. Biochemical experiments suggest that HmpX is located in the periplasm and represents a functional NOD in trans-

formed *Pseudomonas* (R. Poole and M. Delledonne, unpublished data).

With these genetic tools, we examined the hypersensitive disease resistance response when NO accumulation was attenuated by the action of NODs in two different surroundings. We could generally state that the removal of NO from the plant resulted in strikingly similar tendencies compared to NO removal from the pathogen side. When comparing the interaction of *hmp8* plants with *Pst (avrB)* on the one hand and the interaction of wild-type plants with *Pst (avrB/hmpX)* on the other hand, we found very similar tendencies (data not shown). Moreover, when combining the two genetically modified systems, i.e. the interaction of *hmp8* plants with *Pst (avrB/hmpX)*, we observed additive effects in all examined defense responses.

We first detected significantly lower H_2O_2 levels during the oxidative burst in NO-deficient interactions, and removal of NO from both the plant and the pathogen side had an additive effect (Fig. 3). Less H_2O_2 staining in the presence of NOD was also obvious

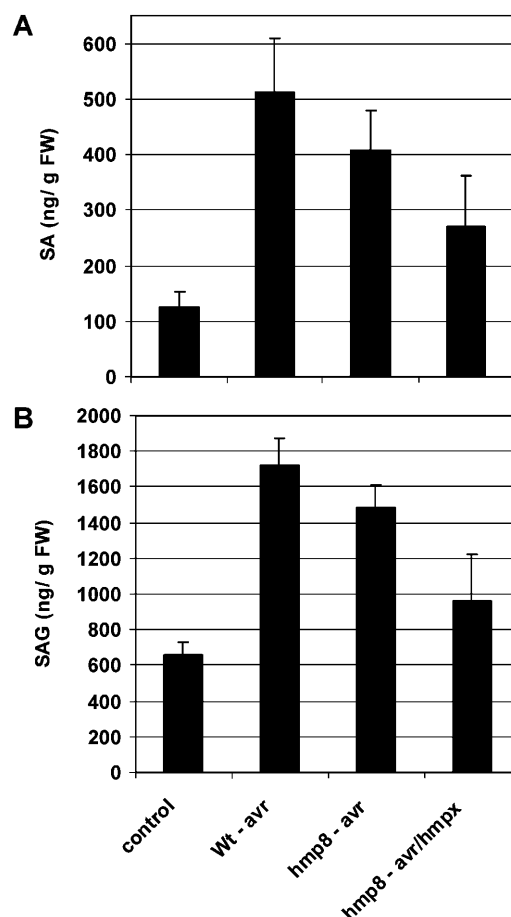


Figure 9. SA contents of wild-type and *hmp8* transgenic plants after challenge with avirulent *Pst* (\pm *hmpX*). Leaf samples were collected 8 h postinfection. Leaves were pretreated with DEX for 16 h. Bars indicated mean values of three independent measurements. Control, $MgCl_2$ -infiltrated plants. A, Free SA. B, SAG.

Table 1. Bacterial growth of different *Pst* in *Arabidopsis* leaves of wild-type *Arabidopsis* and *hmp8* transgenic plants

Leaves were pretreated with DEX 16 h before pathogen inoculation, infiltrated with 10^6 cfu mL⁻¹ *Pst*, and harvested 1 h and 2 d after inoculation. Values $\times 10^4$ represent means of cfu per cm² (\pm SD), each from five sets of three leaf discs. Experiments were repeated twice with similar results. *vir*, Virulent; *avrB*, avirulent; *avrB/hmpX*, avirulent expressing *hmpX*.

Time	Wild Type <i>vir</i>	Wild Type <i>avrB</i>	<i>hmp8</i> <i>avrB</i>	<i>hmp8</i> <i>avrB/hmpX</i>
1 h	2.33 (\pm 1.14)	2.2 (\pm 0.67)	2.00 (\pm 0.76)	1.57 (\pm 0.77)
2 d	2,700 (\pm 361)	152 (\pm 47)	170 (\pm 53)	267 (\pm 76)

when plants were infiltrated with Glc/Glc oxidase, a H₂O₂-generating system (Fig. 4), indicating that the in planta capability to degrade H₂O₂ was increased by the action of the NOD. This effect might be due to the NO-degrading function of the NOD or possibly by direct degradation of H₂O₂ by NOD. Because the rate of H₂O₂ degradation was identical in leaf extracts from wild-type and *hmp8* plants (data not shown), we conclude that direct H₂O₂ degradation through NOD does not take place. Rather, a factor differing in *hmp8* and wild-type plants but not in the corresponding extracts might account for the different observation in intact plants and extracts, respectively. This factor might be the concentration of NO, which is delivered by the intact plant continuously but not necessarily by extracts. Following this interpretation, the higher in planta H₂O₂ degradation capability of NO-deficient *Hmp* plants suggests an inhibitory effect of NO toward H₂O₂-degrading enzymes. In fact, the predominant H₂O₂ scavenging enzymes are catalase and ascorbate peroxidase. Mammalian catalase is reversibly inhibited by NO (Brown, 1995), and it has been shown in vitro that NO inhibits both tobacco catalase and ascorbate peroxidase (Clark et al., 2000). In accordance to previous reports (Delledonne et al., 1998; Foissner et al., 2000), our data reveal that a local burst of NO coincides with the oxidative burst at the site of pathogen infection. Therefore, it is conceivable that this NO burst locally contributes to maintain more sustained and higher H₂O₂ levels that can then either act directly as an antimicrobial oxidant or indirectly by triggering various defense responses (Lamb and Dixon, 1997).

The most striking differences in defense gene expression concerned the induction of *pal* transcripts, which was significantly attenuated in *hmp* plants and in the presence of avirulent *Pseudomonas* expressing *HmpX* (Fig. 5). This observation confirms pharmacology-based findings demonstrating a reduction of *pal* expression by NOS inhibitors in soybean cells and *pal* induction by NO donors and recombinant NOS in soybean and tobacco, respectively (Delledonne et al., 1998; Durner et al., 1998). In addition, the UV-induced expression of *pal* and *chs*, the first committed enzyme in anthocyanin biosynthesis, was strongly

repressed in *hmp* plants (Fig. 6). Based on this experimental evidence, NO appears to play a pivotal regulatory role in the signaling processes leading to expression of phenylpropanoid pathway genes.

PAL is the key enzyme for the general phenylpropanoid pathway, and possible outcomes are lignin, anthocyanin, and/or SA biosynthesis. However, despite the strong repression of *pal*, SA levels only showed a 20% reduction in the presence of *Hmp* in plants or *HmpX* in bacteria (Fig. 9). This supports the findings that in *Arabidopsis*, SA is produced by alternative routes, e.g. by the isochorismate pathway (Wildermuth et al., 2001). The observed induction of *pal* in the *Pseudomonas*-*Arabidopsis* pathosystem might then feed alternative processes like lignification or the production of other phenylpropanoid compounds to a significant extent. If SA is the causative agent of *PR-1* induction (Yalpani et al., 1991; Uknes et al., 1992), the rather weak attenuation of SA induction might explain the fact that in these cases the expression of *PR-1* is not affected (Fig. 5). The levels of SA might still be above a threshold value necessary for full pathogen-induced *PR-1* expression. In the double experiment in which NO is reduced from both the pathogen and the plant, however, SA was reduced to 50% of the usual value and *PR-1* expression was clearly delayed, although not fully suppressed. It is worth noting that the expression of *PR-1* is also up-regulated in plants challenged with virulent pathogens but slower than in the corresponding incompatible interaction (de Torres et al., 2003). Compared to pharmacological experiments with tobacco and soybean cells (Delledonne et al., 1998; Durner et al., 1998), our results demonstrate a similar tendency of NO involvement in SA and *PR-1* production and a strong regulatory role of NO toward the synthesis of phenylpropanoid pathway enzymes like PAL.

When NO is scavenged by *Hmp* or *HmpX* alone, dry lesion development is delayed but not eliminated (Fig. 7), and the appearance of microscopic HR lesions is only moderately suppressed (Fig. 8). However, when NO is scavenged by the simultaneous action of *Hmp* and *HmpX*, the macroscopic dry HR lesions are yellowish with less pronounced symptoms, and the microscopic HR lesions are significantly reduced (Fig. 8). Therefore, HR lesion development is clearly affected by a reduced NO content in *Arabidopsis*, and the correlation with SA levels suggests a mediatory role of SA in these processes. This is in accordance with findings that SA is required for induction of the HR in response to bacterial pathogens in soybean (Tenhaken and Rubel, 1997) and that SA is needed for cell death initiation in *Arabidopsis lsd* mutants (Weymann et al., 1995). The execution of hypersensitive cell death in soybean cells challenged with avirulent *P. syringae* is strongly diminished by NO scavenging compounds and NOS inhibitors (Delledonne et al., 1998). Furthermore, a poised production of ROI and NO is necessary to trigger the HR, and NO together with H₂O₂, but not O₂, are

indicated as the essential players in this process (Delledonne et al., 2001). One possible mechanism for this cooperation could be that NO ensures maintenance of high, persistent H₂O₂ levels that are necessary to trigger the HR. The threshold H₂O₂ levels for HR development in soybean cells is about 6 μ M (Levine et al., 1994), and in our experiments, H₂O₂ levels may not have significantly fallen below a comparable threshold value in the cases of Hmp or HmpX action alone. In the double experiment, however, when H₂O₂ levels showed the strongest decrease (Fig. 3), the H₂O₂ threshold might not have been exceeded and, consequently, a significant reduction in HR development could be observed. A similar reasoning could explain why a significant induction of *gst* transcription still took place under NO deficiency, even in the double experiment (Fig. 5). For *gst* expression, H₂O₂ threshold levels have been shown to be below the HR value, which are around 2 μ M in soybean cells (Levine et al., 1994).

Blockage of the accumulation of NO by NO scavengers or mammalian NOS inhibitors has previously been shown to enhance bacterial growth of avirulent *Pseudomonas* in *Arabidopsis* leaves, although to a lower extent in comparison to the growth of virulent strains (Delledonne et al., 1998). In this work, NO scavenging by NOD led to a similar, albeit weaker, tendency of bacterial growth enhancement of avirulent *Pst* (Table 1). Obviously, the removal of NO in these and other recent experiments is not sufficient for a strong growth enhancement of avirulent pathogens, despite the apparent effects on progression of the described defense responses. This behavior is in some respects reminiscent of the *dnd* (defense, no death) class of *Arabidopsis* mutants that show the ability to limit pathogen growth in a gene-for-gene resistance without developing an HR (Yu et al., 1998). *Dnd1* encodes a cyclic nucleotide-gated ion channel (Clough et al., 2000). In mammals, cyclic nucleotides like cGMP or cADP-Rib are second messengers closely associated with NO signaling (Galione and White, 1994; Schmidt and Walter, 1994), and this has been suggested for plants as well (Pfeiffer et al., 1994; Durner et al., 1998; Clarke et al., 2000). It will be of interest to investigate if generation of NO leads to activation of *Dnd1*, which contributes to the development of symptoms characteristic of the HR.

The complete scavenging of a highly diffusible and reactive molecule like NO is difficult to achieve for a single protein, and the (physiologically active) reaction of NO with other cellular molecules might to a certain extent still compete with the NOD reaction. This is also reflected by the fact that Hmp-overexpressing plants still emitted about one-half the amount of gaseous NO than wild-type plants (Fig. 1E). Therefore, not all cellular NO-mediated effects might have been fully suppressed by this transgenic approach. Compared to the very similar salicylate hydroxylase (NahG) strategy applied by Gaffney et al. (1993), which addressed the role of the far less reactive signaling compound SA in disease resistance, these

data have some limitations due to the physicochemical properties of NO. In general, the possibility that protein overexpression in plants leads to side effects not associated with the physiological process under investigation cannot be fully excluded. For instance, it was demonstrated recently that effects on plant defense responses observed by overexpression of NahG might not necessarily result from a lack of SA accumulation but could partly be a consequence of the presence of the SA degradation product catechol (van Wees and Glazebrook, 2003). Because Hmp converts NO, O₂, and NAD(P)H to nitrate in equistoichiometric amounts, it is conceivable that physiological side effects resulting from oxygen and NAD(P)H consumption or nitrate accumulation exist in hmp-overexpressing plants. Such side effects, however, should be minimal in hmp plants considering the comparatively low NO levels produced in plants (Fig. 1E) and taking into account the use of an inducible vector system, which restricted the action of Hmp to a small experimental window. The parallel tendencies observed in two different genetic backgrounds, i.e. the action of Hmp in *Arabidopsis* and HmpX in *Pseudomonas*, further support that the described effects on plant defenses were a direct consequence of NO degradation rather than a result of indirect effects caused by NOD on plant metabolism. Besides its NOD activity, however, it was shown that Hmp-overexpressing *E. coli* strains are capable of generating ROI (Poole and Hughes, 2000). We can rule out that this metabolic activity took place in transgenic hmp plants during the course of our pathogen and UV experiments due to the findings that transcriptional up-regulation of *gst*, a sensitive marker for ROI production (Levine et al., 1994), and a positive DAB reaction could not be detected in uninfected but DEX-induced hmp plants (Figs. 3 and 5). The latter statement, however, could not be maintained when hmp plants were exposed to DEX for longer periods of time. About 4 d after transgene induction, we detected elevated levels of ROI in leaves of hmp plants by DAB staining and the expression of *gst* and *pr-1* in the absence of pathogens (data not shown). Obviously, a new physiological situation in hmp plants appeared when plants perpetually accumulated Hmp protein, either caused by the constant removal of NO or by an emerging ROI-generating activity. Interestingly, in a recent transgenic approach, the constitutive overexpression of an alfalfa (*Medicago sativa*) hemoglobin with putative NO scavenging properties in tobacco similarly resulted in increased basal ROI levels, besides elevated pathogen-induced SA levels and reduced disease symptoms after *P. syringae* infection (Seregélyes et al., 2003). These findings indicate that a temporally controlled expression rather than constitutive or prolonged overexpression of certain transgenes like (flavo) hemoglobins might be crucial in overexpression studies. The use of the inducible vector system permitted us to use a window of 3 to 4 d after transgene expression to perform pathogen and phys-

iological experiments without undesirable side effects like ROI production.

MATERIALS AND METHODS

Generation of Hmp-Overexpressing Arabidopsis

To generate transgenic Arabidopsis overexpressing the *Escherichia coli* hmp gene, pTA7001, a dexamethasone-inducible expression system, was used (Aoyama and Chua, 1997). The hmp coding sequence was generated by PCR with the primers 5'-CGGCTCGAGATGCTTGACGCTCAAACCATC-3' and 5'-GGACTAGTACGCGCAATTTAAACCGCGTC-3' using a full-length hmp clone as a template, which was kindly provided by A.M. Gardner (University of Cincinnati). The PCR product was subcloned into pGEMT-Easy (Promega, Madison, WI), sequenced, and introduced into pTA7001 by the use of 5'-*Xho*I and 3'-*Spe*I restriction sites. The construct was transformed into *Agrobacterium tumefaciens* (strain GV3101) and the latter used for plant transformation of Arabidopsis ecotype Col-0 by the floral dip method (Clough and Bent, 1998). After transformation, seeds were harvested from T0 plants and surface sterilized, and positive transformants were selected on phytagar plates supplemented with Murashige minimal organics medium (Life Technologies, Paisley, UK) containing 15 $\mu\text{g L}^{-1}$ hygromycin.

Homozygous T3 plants from single insert lines were used for all experiments, and plants were grown at 22°C under a 9-h-light/15-h-dark cycle. For transgene induction, hmp plants were sprayed with a solution of 3 μM DEX in 0.01% Tween 20. Control experiments were performed with wild-type Col-0 plants treated with 3 μM DEX in 0.01% Tween 20 and/or hmp transgenic plants solely sprayed with 0.01% Tween 20. Pathogen infiltrations followed 16 h after DEX/Tween 20 treatment.

Growth of Plant-Pathogens and Infection

Pseudomonas syringae pv *tomato* carrying the avirulence gene *avrB* were transformed with a pRK415 broad host vector (Keen et al., 1988) carrying the complete coding sequence of hmpX (Favey et al., 1995) under control of the Lac promoter, which is constitutively active in *Pseudomonas*. Detailed description of the construction of pRK415-hmpX and maintenance in *Pseudomonas* is not provided (M. Boccara, C. Mills, J. Zeier, C. Anzi, C. Lamb, R. Poole, and M. Delledonne, unpublished data).

Pst strains were grown overnight at 28°C in King's B medium containing the appropriate antibiotics (concentrations: rifampicin 50 $\mu\text{g L}^{-1}$, kanamycin 50 $\mu\text{g L}^{-1}$, tetracycline 15 $\mu\text{g L}^{-1}$). Bacteria were pelleted, washed three times with 10 mM MgCl₂, resuspended, and diluted in 10 mM MgCl₂ to the desired concentration (generally 2×10^6 cfu mL⁻¹, for symptom development 5×10^6 cfu mL⁻¹, for bacterial growth 10^6 cfu mL⁻¹). The bacterial solutions were infiltrated from the abaxial side into one-half of a sample leaf using a 1-mL syringe without a needle. Control (mock) inoculations were performed with 10 mM MgCl₂. Macroscopic symptoms were documented 2 d after infection. Bacterial growth was assessed by homogenizing discs originating from infiltrated areas of three different leaves in 1 mL of 10 mM MgCl₂, plating appropriate dilutions on King's B medium containing Rifampicin, and quantifying colony numbers after 2 to 3 d.

UV Treatment of Arabidopsis Plants

Five-week-old Arabidopsis wild-type and hmp8 plants were pretreated with DEX for 16 h and placed into a growth chamber equipped with UV-A light-emitting black light tubes (Phillips TL 8 W/08; Eindhoven, The Netherlands).

Histochemical Staining and Quantification of H₂O₂ Levels and Microscopic HR Lesions

DAB and Trypan blue staining were performed as described by Thordal-Christensen et al. (1997) and Koch and Slusarenko (1990), respectively. Exogenous H₂O₂ was generated by infiltrating 10 μL of 2.5 mM D-Glc and 2.5 units mL⁻¹ *Aspergillus niger* Glc oxidase (Calbiochem, San Diego) in 20 mM Na phosphate buffer, pH 6.5, into Arabidopsis leaves.

For quantification of the number of stained pixels inside the infected leaf area, the histogram function of Adobe Photoshop 6.0 (Adobe Systems,

Mountain View, CA) was used. Microscopic photographs were reduced to grayscale mode, and all pixels inside the infiltration zone with a gray tone value <125 were quantified. To account for background staining, the corresponding value for an area of equal size inside the noninfected opposite side of the leaf was subtracted from the latter value and the result divided by the total amounts of considered pixels to yield the relative number of stained pixels in percentage.

RNA and Protein Analysis

Total RNA was isolated from Arabidopsis leaves using Trizol reagent (Life Technologies) following the manufacturer's instructions. RNA-blot hybridization (Levine et al., 1994) was performed with probes of the Arabidopsis *gst*, *pal*, and *PR-1* genes: ATGST (GenBank accession no. U70672), ATPAL1A (X62747), and ATHRPRP1A (M90508). Equal loading was verified by gel staining with ethidium bromide and by hybridization with an rDNA probe.

For protein extraction, three leaves were homogenized with 1 mL of extraction buffer (15 mM HEPES, 40 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.6). The mixture was centrifuged for 30 min at 19,000g and 4°C. The supernatant constituted the protein extract. Protein samples were subjected to SDS-PAGE on 10% (w/v) polyacrylamide (Sambrook et al., 1989) and electroblotted to a polyvinylidene difluoride membrane (Hybond-P; Amersham Pharmacia, Little Chalfont, UK). Probing and detection of western blots were performed as described in the ECL western-blotting detection kit (Amersham). The primary antibody raised against *E. coli* Hmp was kindly provided by Robert Poole (Sheffield, UK) and used at a dilution of 1:3,000. A goat anti-rabbit IgG horseradish peroxidase conjugate (Sigma, St. Louis) was used as the secondary antibody with a dilution of 1:10,000.

Determination of SA Levels

Measurements of SA and SAG essentially followed the protocol of Raskin et al. (1989). Briefly, 0.15 g of frozen leaf tissue was homogenized in 1 mL of 90% methanol and extracted for 10 min at 40°C. The mixture was centrifuged for 5 min at 14,000g, and the pellet was extracted for another 10 min at 40°C with 100% methanol. Supernatants from both extractions were combined and dried under a gentle stream of N₂ at 40°C. The residue was resuspended in 1.5 mL of 0.1 M HCl, and 100 ng of *o*-anisic acid was added as an internal standard. After centrifugation for 10 min at 14,000g, the aqueous solution was extracted three times with 2 mL of cyclopentane/ethylacetate (1:1). The extracts were combined and the solvent removed under N₂ at 40°C. The residue was dissolved in 50 μL of methanol and passed through a solid-phase extraction column filled with ODS-H optimal packing material (Capital HPLC, Broxburn, UK) using 6 mL of methanol. The eluate was dried under N₂ at 40°C, dissolved in 100 μL of methanol, and used for HPLC analysis. For detection of SAG, the aqueous, acidic phase from the first extraction step was heated to 100°C for 30 min to convert the glucoside to free SA. The above protocol was repeated starting with addition of the internal standard.

HPLC analysis was performed using an ODS-H optimal column (10 \times 2.1 mm, Capital HPLC) on a Shimadzu (Columbia, MD) LC-5A chromatograph. For separation, a linear gradient from 95% of H₂O/BuOH/HOAc (98.3/1.2/0.5) to 90% acetonitrile/BuOH/HOAc (98.3/1.2/0.5) in 20 min and flow rate of 0.7 mL min⁻¹ was applied. For detection, a Waters (Milford, MA) 474 scanning fluorescence detector with an excitation wavelength of 300 nm was used. The emission wavelength was switched at 7 min elution time from 365 nm to 405 nm to ensure highest sensitivities for *o*-anisic and SA, respectively.

NO Degradation Assay

The kinetics of NO degradation were measured electrochemically using an Iso-NO meter (World Precision Instruments, Sarasota, FL). A saturated, 2 mM NO solution was prepared by bubbling 10 mL of NO gas through 5 mL of HEPES buffer (see above). Protein extracts were prepared as described above using six fully grown Arabidopsis leaves and 1 mL of HEPES-extraction buffer (without dithiothreitol). For NO degradation measurements, 1 mL of plant extract was supplemented with 10 μL of 10 mM NADH and the temperature of the solution kept at 24°C in a water bath. An Iso-NO electrode was calibrated according to the manufacturer's instructions and submerged into the protein solution inside a gas-tight vial. Under stirring, 5 μL of NO solution was added, and the time-dependent changes of the NO signal were recorded.

NO Emission Measurements

Rosette leaves were cut from root parts of *Arabidopsis* plants and immediately floated on deionized water. The leaves from three different plants were placed in a transparent lid container with 2 L of air volume. A constant flow of measuring gas (NO-free air conducted through a custom-made charcoal column) of 1.5 L/min was pulled through the container and subsequently through the chemiluminescence detector (CLD 770 AL ppt; Eco-Physics, Dürnten, Switzerland) by a vacuum pump connected to an ozone destroyer. Light was provided by a 400 W HQI-lamp (Schreder, Winterbach, Germany) above the container. The quantum flux density could be adjusted at $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic active radiation by a polyester sieve (pore size is $210 \mu\text{m}$) on the lid of the container. Air temperature in the container was usually about 23°C in the dark and 23°C to 25°C under light conditions.

NO Detection using DAF2-DA Fluorescence

Arabidopsis leaves were treated with 2×10^6 cfu mL^{-1} *Pst* (*avrB*) or 10 mM MgCl_2 as described above, and 3 h later, $10 \mu\text{M}$ DAF2-DA (Sigma) dissolved in 10 mM Tris/KCl, pH 7.2, was infiltrated into the pretreated leaf areas. One hour after DAF2-DA infiltration, leaf areas were analyzed microscopically using a Zeiss Axioskop 2 fluorescence microscope equipped with a confocal laser scanner (LSM 5 PASCAL; Zeiss, Oberkochen, Germany). Leaves were excited with an argon laser (488 nm). DAF2-DA fluorescence was recorded using a channel with a 505- to 530-nm band-pass filter, and autofluorescence of chloroplast was captured with a channel equipped with a 560-nm long-pass filter.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers X58872, X75893, U70672, X62747, and M90508.

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