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Harpin-elicited hypersensitive cell death and pathogen resistance require the *NDR*1 and *EDS*1 genes

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Abstract

Plants sprayed with harpin, a bacterial protein that induces hypersensitive cell death (HCD), develop systemic acquired resistance (SAR) without macroscopic necrosis. HCD sometimes accompanies the development of resistance conferred by resistance (*R*) genes. In *Arabidopsis*, some *R* genes require one or both of the signalling components NDR1 and EDS1 for function. This study addresses whether HCD, NDR1 and EDS1 are required for induction of SAR by harpin. When *Arabidopsis* and tobacco leaves were sprayed with harpin, microscopic hypersensitive response (micro-HR) lesions developed. Systemic expression of PR genes and the development of resistance were accompanied by micro-HR, except in the *ndr*1-1 mutant, in which harpin induced micro-HR without the development of resistance or expression of the *PR*-1 gene. Cell death and resistance did not occur following treatment with harpin in plants that could not accumulate salicylic acid. Harpin also failed to induce resistance in *Arabidopsis eds*1-1 mutants. Therefore, harpin-induced resistance seems to develop concomitantly with cell death and resistance requires *NDR*1 and *EDS*1.

Keywords: *Arabidopsis thaliana*; *Nicotiana tabacum*; *Pseudomonas syringae*; *Peronospora parasitica*; Tobacco mosaic virus; Harpin; HCD; SAR; NDR1; EDS1

1. Introduction

Harpins are glycine-rich, protease-sensitive, heat-stable, acidic proteins produced by Gram-negative plant pathogenic bacteria; they induce hypersensitive cell death (HCD) in non-host plants of bacteria [35]. Application of harpins to many plants enhances plant growth, and induces resistance to pathogens and insects [23,35,52,56]. These effects were found first in plants treated with harpin from *Erwinia amylovora* [56], the first reported bacterial cell-free HCD elicitor [57]; similar effects have been observed in plants treated with other harpins, such as $HrpZ_{Pss}$ from *Pseudomonas syringae* pv. *syringae* [52]. It has been suggested, but not yet proven, that harpins perform diverse functions by activating distinct signalling pathways [20,23,37,38,52].

The SAR pathway requires SA accumulation and regulation by the *NPR*1/*NIM*1 gene, and leads to expression of PR genes [6,14,15,24,44]. In addition, the pathway may involve many other components. HCD usually accompanies SAR mediated by plant resistance (*R*) genes [33,45,50]. However, SA-dependent pathogen defence develops in *Arabidopsis snc*1 (suppressor of *npr*1-1, constitutive 1) [39] and *dnd*1 (defence, no death) [59] mutants in the absence of cell death. Thus, HCD is not essential for resistance. The signal components EDS1 and NDR1, which are required for the function of some *R* genes [1,8,26,27,41,47], also are involved in *R*-mediated SAR. The *EDS*1 and *NDR*1 genes are required for lesion simulating disease resistance [21] and an *lsd*1-mediated runaway cell death pathway in *Arabidopsis* in responding to superoxide, SA and its structural analogs, or to infection with pathogens [32,41,43]. *EDS*1 also is required for constitutive SAR in the *Arabidopsis snc*1 [39] and *cpr*5 (constitutive expresser of

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PR genes) [5,11] mutants. Whether these signalling components are involved in resistance induced by elicitors has not been determined.

Harpin ($HrpN_{Ea}$) [23] and $HrpZ_{Pss}$ [52] have been shown to induce pathogen resistance by activating the SAR pathway. Harpin-induced SAR in *Arabidopsis* requires SA and *NPR*1/*NIM*1 [23]. PR gene expression and the resistance phenotype induced by harpin are compromised in NahG plants, which do not accumulate SA [17,28], and *nim*1 mutants [16,44], which contains a mutated and non-functional *NPR*1/*NIM*1 gene [6,44]. Harpins elicit an oxidative burst [18] and HCD [19,53] in both host and non-host plant cells and induces a macroscopic HR (macro-HR) in non-host plants following infiltration of leaf intercellular spaces. However, plants sprayed with harpins develop resistance in the absence of macroscopic necrosis [23,35,52,56]. Whether HCD is essential to, and whether NDR1 and EDS1 are required for harpin-induced SAR have been unclear.

We have attempted to identify components, in addition to SA and NPR1/NIM1 [23], which are involved in signalling harpin-induced resistance. Our data show that spraying plants with harpin coordinately induces expression of PR genes, systemic resistance to pathogens, and micro-HR, a form of HCD that occurs at low frequency without macroscopically visible signs [2]. Our results show that both *NDR*1 and *EDS*1 are required for the development of resistance in plants treated with harpin based on assays with *Arabidopsis eds*1-1 and *ndr*1-1 mutants.

2. Materials and methods

2.1. Plant growth and pathogen maintenance

Nicotiana tabacum variety Xanthi (nc) and its NahG transgenic plants were grown in a greenhouse for 6–7 weeks before use. *Arabidopsis thaliana* Columbia (Col-O) and Wassilewskija (Ws-O) ecotypes, NahG Col-O plants, *eds*1-1 and *ndr*1-1 mutants were grown in environmental control chambers at 18–20 °C and 14 h illumination for 3 weeks before treatment [36]. Tobacco mosaic virus (TMV) was maintained in an 18 mg ml^{-1} aqueous suspension at 4 °C. *Peronospora parasitica* strains Noco2 and Emwa were maintained by serial culture in ecotypes Col-O and Ws-O, respectively, of *Arabidopsis* at 18–20 °C [36]. *Pseudomonas syringae* pv. *tomato* DC3000 without any foreign avirulence genes and *Escherichia coli* DH5α strains were lyophilized and maintained at −80 °C.

2.2. Harpin preparation, plant treatment and cell death assays

Harpin and an appropriate control were prepared as cell-free elicitor preparation (CFEP) and cell-free vector preparation (CFVP), respectively. CFVP and CFEP were produced by *E. coli* DH5α strains harboring pCPP50 or the $HrpN_{Ea}$ gene cloned in pCPP50, known as pCPP2139 [23,35]. Based on assays by established methods [23,56], CFVP contains inactive proteins that do not induce the HR, expression of PR genes, resistance to pathogens or to insects, and do not enhance plant growth. The concentration of total proteins in both preparations and harpin in CFEP was determined by the BCA Protein Assay Reagent Kit (Epicenter Biotechnologies, Rockford, IL) and high performance chromatography, respectively. CFEP and CFVP were used at the same concentration of total proteins.

Plants were sprayed with high-purity water, CFVP containing 17 μg ml^{-1} total protein, or CFEP containing 15 μg ml^{-1} harpin and 2 μg ml^{-1} inactive protein, except when otherwise specified. Leaves were detached and examined for micro-HR at appropriate intervals following treatment. Dose effects of harpin were determined by using several concentrations and evaluating leaves 16 h after treatment. Micro-HR development was determined at three-hour-intervals over 24 h following spraying with harpin. Leaves of control plants, treated similarly with water or CFVP, were observed at the same times as the harpin-treated plants.

Micro-HR was monitored based on observing dead cells in leaves after staining with lactophenol trypan blue [23,55]. Briefly, 1–1.5 cm squares of tobacco leaves or whole leaves of *Arabidopsis* were placed in multi-well tissue culture plates (Falcon 3027, Becton Dickinson, Lincoln Park, NJ). Lactophenol trypan blue solution (10 ml 85% lactic acid aqueous, 10 ml water-saturated phenol, 10 ml 98% glycerol, 10 ml distilled water, 15 mg trypan blue) was added and infiltrated into leaf intercellular spaces with the aid of a vacuum pump and a bell jar. Leaves were then heated in a boiling water bath for 5–8 min and incubated at room temperature for 6–8 h. Stained leaves were cleared in chloral hydrate solution (2.5 g ml^{-1}) and observed using an Olympus BX60 microscope.

2.3. Determination of transcript levels

Northern blot hybridization was done with replicate blots of plant RNA hybridized to ^{32}P[dCTP]-labeled probes of *PR*-1 and *PR*-2 from *Arabidopsis*, or *PR*-1*a* and *PR*-3 from tobacco [9,10,23]. Loading had been standardized based on the amount of rRNA. Semi-quantitative RT-PCR for *Arabidopsis PR*-1 and *NDR*1 genes was performed using RT-PCR Beads (Amersham Pharmacia Biotech. Inc., Piscataway, NJ) in US and Promega M-MLV kits in China. *PR*-1 cDNA sequence (M90508 in GenBank) was used as the basis for synthesizing primers (5′-CACAACCAGGCACGAGGAGC-3′; 5′-GGCTTCTCGTTCACATAATTCCCACG-3′) that specifically yielded a 346-bp amplification product. Primers for *NDR*1 (5′-ATAATCAAAATGAAGACACAGAAGGTGGTC-3′; 5′-AACGAATAGCAAAGAATACGAGTAAATTCA-3′) were synthesized based on its DNA sequence (AF021346.1

in GenBank), which amplifies a 654-bp fragment. The translation elongation factor gene *EF*1α, which is constitutively expressed and highly conserved in eukaryotes [4,29], was used as a standard. Specific primers (5′-AGACCACCAAGTACTACTGCAC-3′; 5′-CCAC-CAATCTTGTACACATCC-3′), producing a 495-bp sequence, were synthesized based on the highly conserved region of *EF*1α cDNA sequences (for example, AJ223969, X97131, AF181492 and AF120093 in GenBank). Prior to running RT-PCR, RNA samples were treated with DNAse (Promega). Reaction conditions were optimized by testing five template concentrations (0, 10, 20, 30 and 60 ng μl^{-1}) for the first-strand cDNA synthesis and different numbers of PCR cycles (25, 30 or 35) for cDNA amplification. Accordingly, 20 ng μl^{-1} template and 25 PCR cycles were used. Equal amounts of RT products, based on cDNA quantification by spectrophotometry, were amplified subsequently by PCR. RT-PCR products were cloned into the pGEM®-T Easy Vector (Promega), sequenced (Takara Biotech. Co., Ltd. Dalian, China), and compared with published gene sequences using Blast searches. Gene expression levels were estimated based on intensities of hybridization signals on films and band intensities on gels of PCR products following staining with ethidium bromide, as determined by digital gel documentation.

2.4. Pathogen inoculation, infection evaluation, resistance scoring, and data analyses

Inoculation was done 5 days after plant treatment; infection was evaluated 7 days later in all experiments. Tobacco plants were inoculated with 1 $\mu g\ ml^{-1}$ TMV by rubbing leaves using a finger together with abrasive diatomaceous earth; inoculated plants were maintained in a glasshouse. Inoculation of *Arabidopsis* with *P. parasitica* (4×10^5 spores ml^{-1}) and *P. syringae* pv. *tomato* DC3000 (10^6 cfu ml^{-1}) followed previous methods [23,30,36]. Inoculated plants were incubated at room temperature (*P. syringae* pv. *tomato*) or 18 °C (*P. parasitica*). Lesions on tobacco leaves caused by TMV were counted and the diameters of 10–20 lesions per leaf were measured [22]. Conidiospores of *P. parasitica*, produced on inoculated *Arabidopsis* leaves, were counted in leaf washes by haemocytometry; oomycete growth in leaf tissues was examined after staining with trypan blue [23]. The bacterial populations in *Arabidopsis* leaves were determined [23] and chlorotic and necrotic symptoms on plants were observed.

For determining systemic development of resistance, three lower leaves of tobacco and *Arabidopsis* were sprayed or infiltrated with harpin or water, respectively. Untreated upper tobacco leaves were inoculated with TMV and whole plants of *Arabidopsis* were inoculated with *P. syringae* or *P. parasitica*. The inoculated leaves of both plants were evaluated for extent of infection. To determine possible correlation of cell death levels with resistance levels, three lower leaves of 30-day-old *Arabidopsis* and 60-day-old tobacco plants were sprayed and infiltrated with water or harpin over a range of concentrations (0–90 $\mu g\ ml^{-1}$), respectively. Treated leaves were stained 16 h post treatment and dead epidermal cells were counted under the microscope, as described above. Two untreated upper leaves of tobacco and whole of *Arabidopsis* plants were inoculated with TMV and *P. parasitica*, respectively. Infection of untreated upper leaves of inoculated plants was evaluated. Resistance of tobacco to TMV [22], resistance of *Arabidopsis* to *P. parasitica* and *P. syringae* [23] were scored as percent reduction in lesion numbers or conidiospores on leaves, or bacterial cells recovered from leaves, of plants treated with harpin, relative to numbers in controls.

In every experiment appropriate numbers of replicates were treated to ensure adequate data for statistical analyses. Cell death assays were based on examining 3000–4500 epidermal cells in three replicates for each treatment. Resistance was evaluated based on assays of three to five replicates, each containing 30–50 *Arabidopsis* seedlings or 25–50 TMV lesions on tobacco leaves. Data were subjected to F tests using the Data Analyses Tool of Microsoft Excel.

3. Results

3.1. Harpin induces micro-HR

We first investigated whether micro-HR occurs in tissues of harpin-treated plants that develop resistance but do not show macro-HR. *Arabidopsis* and tobacco were sprayed with water, CFVP containing inactive proteins, or CFEP containing harpin at 15 $\mu g\ ml^{-1}$, a concentration that is sufficient to cause macro-HR when infiltrated into tobacco leaves. Only a few dead cells were observed in plants treated with CFVP (Fig. 1a) or water. In contrast, many dead cells (micro-HR) were seen in the CFEP-treated plants 8 h after treatment (Fig. 1b and c). Because CFVP caused only minimal response in plants (see also Ref. [23]), we used water as a control in further studies.

Secondly, we counted dead cells in the epidermis of treated leaves (Fig. 1c, left panel), estimated the extent of harpin-induced micro-HR and related it to phenotypic resistance. Fig. 2a indicates that the extent of micro-HR that occurred in *Arabidopsis* and tobacco was related to the concentration of harpin used to treat plants. Micro-HR developed at a similar threshold dose of harpin, 3–5 $\mu g\ ml^{-1}$. In both plants, the spontaneous cell death (SCD) rate was approx. 1% in water-treated leaves. Leaves treated with harpin at 3–5 $\mu g\ ml^{-1}$ sustained approx. 8% cell death. Three to five microgram per milliliter was the lowest harpin concentration tested that caused cell death significantly different from the SCD rate. As the harpin concentration was increased from 5 to 30 $\mu g\ ml^{-1}$, the cell death rate increased to 15 and 20% on *Arabidopsis* and tobacco, respectively. Little change in the percentage of

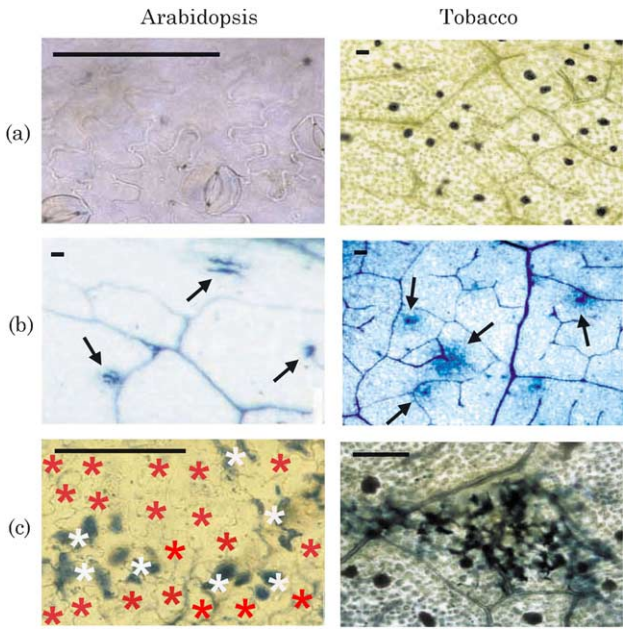

Fig. 1. Induction of micro-HR by harpin in *Arabidopsis* and tobacco. Leaves of *Arabidopsis* and leaf squares of tobacco were sampled following treatment, stained with trypan blue, and observed under the microscope. Dead cells stain blue or dark blue; healthy cells, except veins, appear hyaline. Projecting lipoglands in tobacco leaves appear black in (b) and (c) are not related to any particular treatment. Scale bars = 1 mm. Determinations were repeated three times with similar results. (a) Absence of cell death in plant leaves sprayed with a preparation containing inactive proteins, determined 16 h post treatment. (b) Cell death in leaves sprayed with harpin at 15 μg ml^{-1}. Samples were observed 8 h post treatment. Arrows indicate areas of cell death. (c) Close-up views of dead individual cells (left, 16 h) and cell death areas (right, 12 h). Dead and live cells are indicated by white and red asterisks, respectively (left).

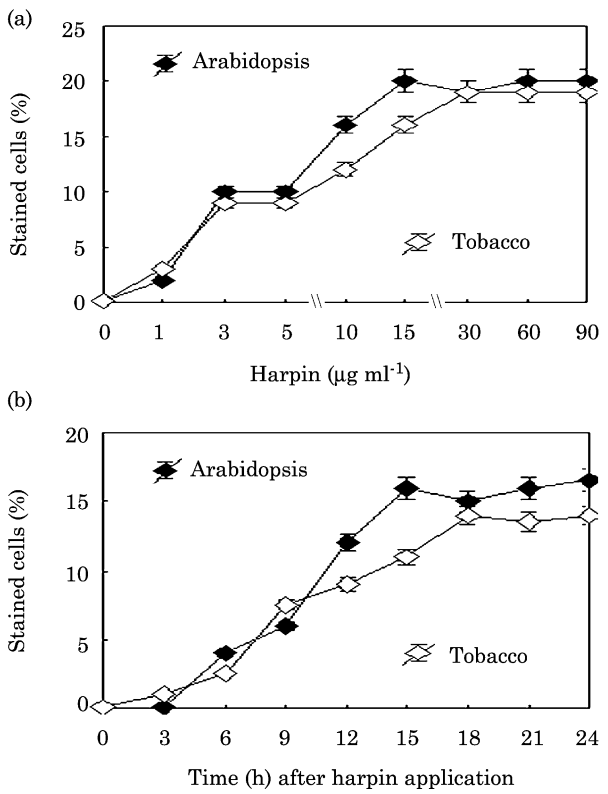

Fig. 2. Effects of harpin concentrationon on extent of cell death (a); time course of cell death development (b) in *Arabidopsis* and tobacco. Plants were sprayed with harpin at indicated concentrations and examined 16 h after treatment in (a), or sprayed with harpin at 15 μg ml^{-1} and observed at indicated times in (b). In total, 1000–1500 epidermal cells were observed and the stained cells counted for each treatment. Data are means ± SD for three replicates.

dead cells resulted when the concentration of harpin was increased above 30 μg ml^{-1}. Further, we observed the time course of development of micro-HR in *Arabidopsis* and tobacco sprayed with harpin at 15 μg ml^{-1}. As shown in Fig. 2b, significant cell death appeared 6 h after application of harpin. Cell death reached its maximum of approx. 15% in 16 h in both *Arabidopsis* and tobacco. Thus, micro-HR did not progress after 16 h.

3.2. Resistance develops coordinately with the micro-HR

The relationship between micro-HR and resistance was determined by observing the occurrence of micro-HR in lower leaves infiltrated with harpin, and assessing infection of untreated upper leaves of treated plants (tobacco) and whole treated plants (*Arabidopsis*) according to established criteria [22,23]. Fig. 3 shows the correlation between the level of micro-HR and the level of resistance as a function of harpin concentration. In both tobacco and *Arabidopsis*, the lowest concentration of harpin that resulted in resistance was 3–5 μg ml^{-1}, the same threshold concentration of harpin needed to induce micro-HR. A dramatic increase in resistance was achieved when the concentration of harpin was increased from 5 to 15 μg ml^{-1} in *Arabidopsis*. However, concentrations of harpin greater than 15 μg ml^{-1} did not result a significant increase in resistance for *Arabidopsis* or tobacco.

3.3. Expression of PR genes is coordinated with the occurrence of micro-HR

Harpin was shown previously to induce expression of PR genes [23], which are considered good molecular markers of SAR [45]. To determine whether gene activation depends on harpin concentration, we assayed *Arabidopsis* and tobacco for PR gene expression, in response to a range of harpin concentrations. Fig. 4 shows that PR genes were activated coincidentally with induction of micro-HR. Expression of *PR*-1 and *PR*-2 in *Arabidopsis* (Fig. 4a), and *PR*-1*a* in tobacco (Fig. 4b), was induced when plants were treated with ≥5 μg ml^{-1} harpin, the threshold concentration for induction of micro-HR in both plants. *PR*-3 of tobacco was expressed at a lower level under the same conditions (Fig. 4b). The pattern of the gene expression induced in *Arabidopsis* differed from that in tobacco. The levels of *PR*1 and *PR*2 gene expression in *Arabidopsis* were similar when harpin was applied at either 5 or 15 μg ml^{-1}. In tobacco, however, application of harpin

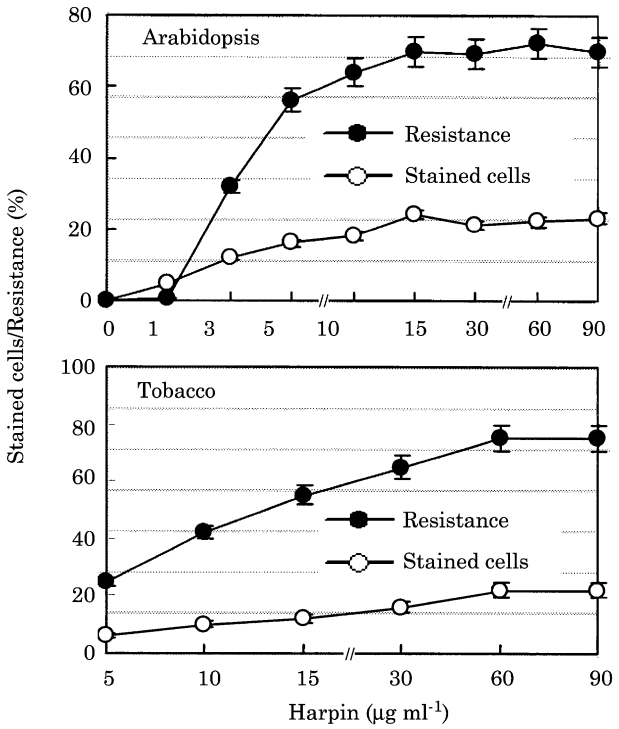

Fig. 3. Correlation of cell death with pathogen resistance in *Arabidopsis* and tobacco treated with harpin. The lower three leaves of plants were sprayed with harpin at the indicated concentrations and examined 16 h after treatment. In total, 1000–1500 epidermal cells were observed and the stained cells counted for each treatment. Untreated upper leaves of *Arabidopsis* and tobacco were inoculated with *P. parasitica* and TMV, respectively, 5 d after treating lower leaves with harpin or water. Resistance was determined in plants treated with harpin, based on percentage reduction in conidiospores per leaf and the number of viral lesions, relative to controls, 7 d after inoculation, respectively. Data are means ± SD for three replicates.

at 15 μg ml^{-1} resulted in a much higher level of induction than 5 μg ml^{-1}. These response concentrations correlate well with cell death response ranges.

3.4. NahG plants fail to develop cell death in response to harpin

NahG plants do not develop SAR following infection by pathogens or treatment with SA or its structural analogs because they do not accumulate SA due to the transgenic expression of an SA-degrading enzyme, salicylate hydroxylase [28]. To determine whether accumulation of SA is required for harpin-induced micro-HR, *Arabidopsis* and tobacco NahG transgenic plants were tested and compared to wild-type plants. No micro-HR was observed in NahG plants (Fig. 5a and b), even when a greater concentration of harpin was used than is needed to cause micro-HR in wild-type plants (Figs. 1, 5a and b). Macro-HR also was not observed in NahG tobacco following application of harpin at 15 μg ml^{-1} by spraying and infiltration, which is sufficient to induce micro-HR (Figs. 1 and 5b) and macro-HR (Fig. 5c, top panel) in wild-type

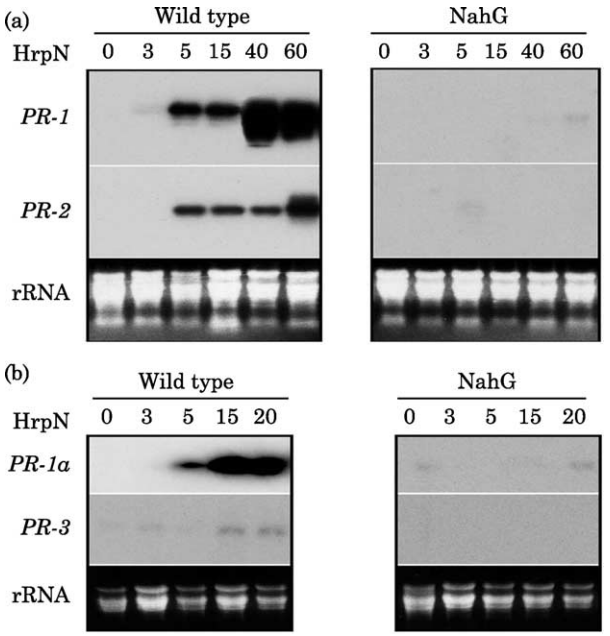

Fig. 4. Effects of harpin concentration on PR gene expression in wild-type *Arabidopsis* (a) and tobacco (b) plants, and NahG transgenic plants. RNA was isolated from untreated young leaves of plants whose lower leaves had been infiltrated with harpin (HrpN) at the indicated concentration (μg ml^{-1}) 5 d prior to sampling. RNA accumulation was detected by RNA gel blot analyses. Uniform loading was standardized based on the amount of rRNA, as visualized by staining with ethidium bromide. The assay was repeated three times with similar results.

plants. Thus, the inability to accumulate SA greatly affected the occurrence of macro-HR and micro-HR in plants treated with harpin. Finally, tobacco NahG plants failed to develop resistance to TMV, based on numbers of lesions caused by the virus (Fig. 5d), which is consistent with our previous results with *Arabidopsis* NahG plants [23].

3.5. The NDR1 gene is required for resistance but not cell death induced by harpin

Expression of the *NDR*1 gene in *Arabidopsis* was determined by semi-quantitative RT-PCR and confirmed by northern blot hybridization (Fig. 6a). Then we found that the optimized RT-PCR protocol was reliable in detecting the desired genes based on Blast comparison (data not shown), and more sensitive than northern in evaluating levels of gene expression (Fig. 6a and 7a and data not shown). Fig. 6a shows the accumulation of *NDR*1 mRNA in plants treated with water or harpin, or inoculated with *P. syringae* pv. *tomato* strain DC3000, which contains no foreign avirulence genes and is highly virulent to *Arabidopsis* accessions [1]. *NDR*1 expression was enhanced in plants treated with harpin, as compared to plants treated with water, suggesting that *NDR*1 might be involved in the development of harpin-induced resistance. To examine this possibility, we determined PR gene expression levels in the *ndr*1-1 mutant in response to treatment with harpin. Fig. 6b shows that *PR*-1 was expressed only in wild-type plants

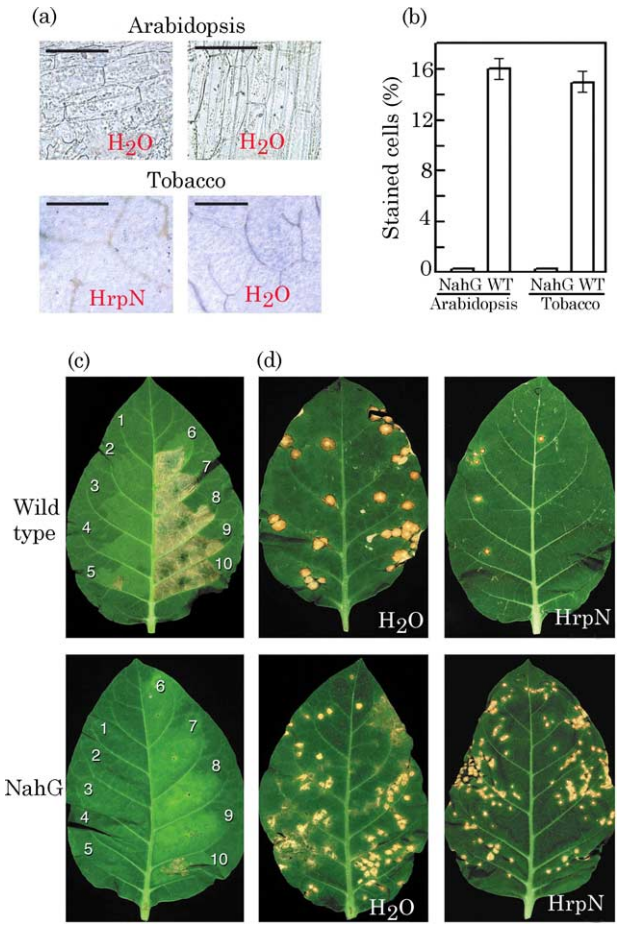

Fig. 5. Induction of cell death and resistance to TMV by harpin in wild-type but not in NahG transgenic plants. (a) Absence of cell death in NahG plant leaves. Plants were sprayed with water or harpin (HrpN) at 15 μg ml^{-1}. Trypan blue-stained leaves were observed 16 h later. Scale bars = 1 mm. (b) Quantification of dead cells in leaves of NahG and wild-type (WT) plants. In total, 500–1000 epidermal cells were observed and dead cells were counted for each treatment. Data represent the mean of three replicates ± SD. (c) The occurrence of macro-HR in wild-type but not NahG plants, photographed 24 h (wild-type) and 96 h (NahG) after infiltration of panels 1–10 with harpin at 0, 0.5, 1, 3, 5, 10, 20, 40, 60, and 90 μg ml^{-1}, respectively. (d) Symptoms caused by TMV on leaves of plants. The lower three leaves of the same plants had been infiltrated with water or harpin (HrpN) at 15 μg ml^{-1}, 5 d prior to inoculation. Leaves were photographed 7 d after inoculation. Five replicate determinations gave similar results.

following treatment with harpin. The gene was not expressed in the mutant *ndr*1-1 regardless of whether it was treated with water or harpin, or inoculated with *P. syringae* pv. *tomato*. Thus, *NDR*1 appears to be involved in the induction of *PR*-1 expression by harpin.

We further determined whether the induction of HCD and resistance is affected by the loss of *NDR*1 function. We found that *ndr*1-1 plants developed micro-HR, but they did not develop resistance following treatment with harpin. Micro-HR was observed in both the *ndr*1-1 mutant and its wild-type parent by 6 h after treating with 15 μg ml^{-1} harpin (Fig. 6c and d). Whereas both wild-type and *ndr*1-1 mutant plants exhibited harpin-induced micro-HR, only wild-type plants exhibited harpin-induced resistance to *P. syringae* pv. *tomato*. In the wild-type, after 5 days, the bacterial population increased approx. 10^2-fold in harpin-treated plants, as compared with 10^6-fold increase in wild-type plants treated with water and in the harpin-treated *ndr*1-1 mutant plants (Fig. 6e). In contrast, the bacterial numbers increased equally by about 10^7-fold in harpin-treated and control plants of the *ndr*1-1 mutant during the same period (Fig. 6e). Symptoms developed only in the control plants of the wild-type and similar severity of symptoms was observed in the mutant plants regardless of treatment (Fig. 6f). These data suggest that *NDR*1 is required for the development of resistance, but it is not required for HCD in plants, in responding to harpin.

3.6. The EDS1 gene is required for harpin-induced resistance

The *Arabidopsis eds*1-1 mutant was identified by Parker et al. [42] based on compromised resistance to *P. parasitica*. To determine whether *EDS*1 is required for SAR elicited by harpin, we assayed *eds*1-1 and wild-type (Ws-O) plants for expression of *PR*-1 and development of resistance to the oomycete in response to treatment with harpin. We found that *PR*-1 expression (Fig. 7a) and resistance (Fig. 7b and c) were compromised in *eds*1-1 plants. *PR*-1 was highly expressed in the wild-type but not in *eds*1-1 plants within 3 d after treatment with harpin (Fig. 7a). Accordingly, the pathogen produced a large number of spores over most of the leaf surfaces of *eds*1-1 plants (Fig. 7b), and it grew extensively in leaf tissues regardless of treatment with harpin or water (Fig. 7c). The same effects were seen in wild-type plants treated with water. However, in harpin-treated wild-type plants, few conidiophores were evident on the leaf surfaces (Fig. 7b), and few oospores were produced and limited hyphal growth occurred within leaf tissues (Fig. 7c). Therefore, *EDS*1 is required for induction of resistance by harpin.

4. Discussion

We used harpin to characterize the relationship between cell death and induced resistance and effects of NDR1 and EDS1 on both responses with the following results: (i) micro-HR develops in *Arabidopsis* and tobacco following treatment with harpin; (ii) correlations were found between the presence of micro-HR, expression of PR genes and the development of phenotypic resistance in harpin-treated plants; (iii) *EDS*1 and *NDR*1, genes previously hypothesized to regulate the function of several *R* genes, are involved also in harpin-activated processes.

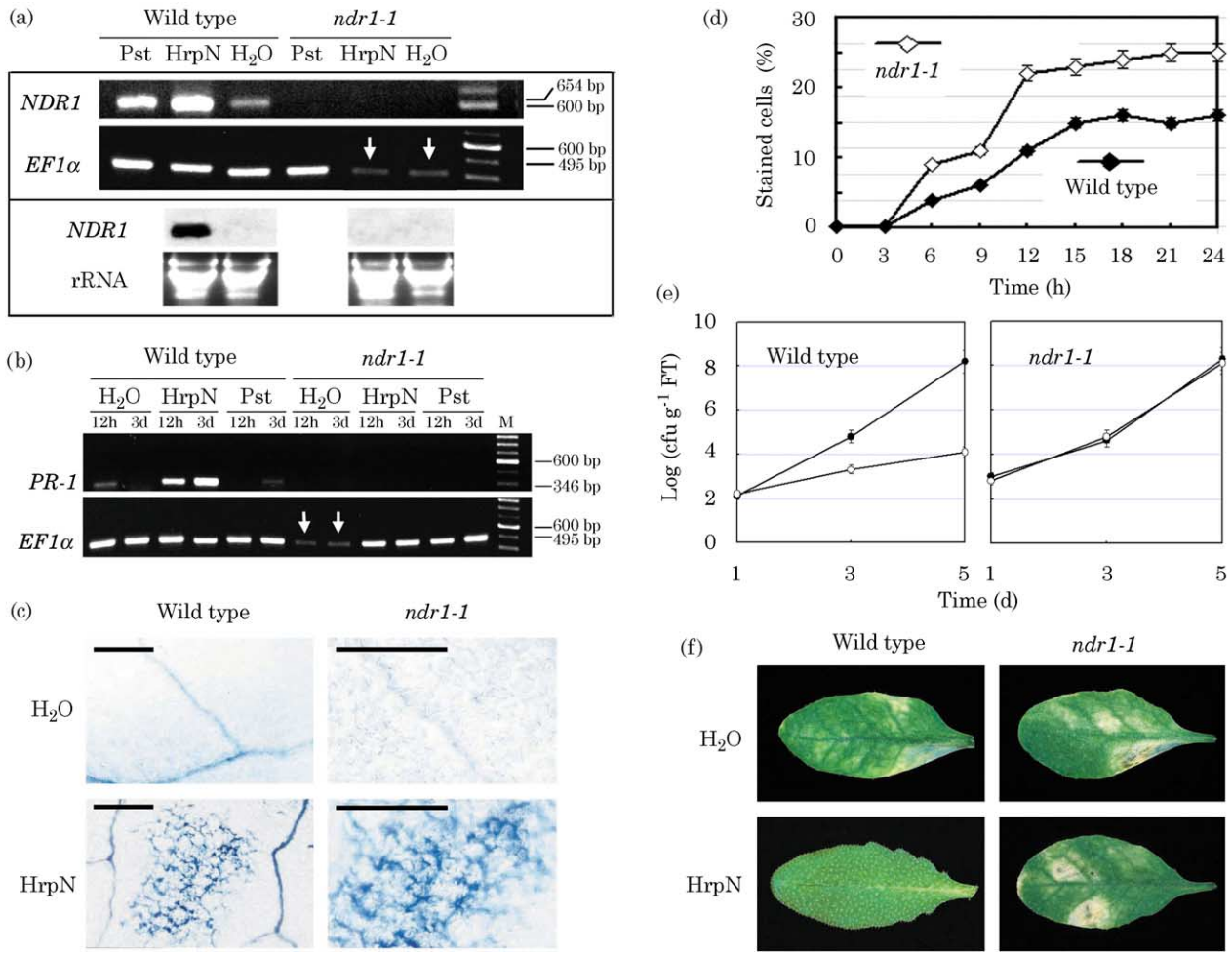

Fig. 6. Effects of *ndr*1 on *NDR*1 and *PR*-1 gene expression, cell death, and pathogen resistance in *Arabidopsis* treated with harpin. (a) Expression of *NDR*1 determined by semi-quantitative RT-PCR (top) and northern blot hybridization (bottom). (b) Expression of *PR*-1. RNAs used as templates were isolated at 3 days for (a) or intervals for (b) from plants treated with water or harpin (HrpN), or inoculated with *P. syringae* pv. *tomato* DC3000 (Pst). Primers specific to *NDR*1 and *PR*-1 yield a 654- and a 346-bp product, respectively. Size of the RT-PCR product of *EF*1α, the quantitative control gene, is 495 bp. To confirm reliable quantitative detection of gene expression levels, the RT-PCR product of *EF*1α was loaded in the two lanes that show weaker bands (arrows) at 0.2 the amount of the other preparations. Procedures used for northern blotting were as used for Fig. 4. Four and two replicate determinations for the RT-PCR and northern blot, respectively, gave similar results. (c) Microscopic view of cell death in leaf tissues. Cell death was examined 16 h after leaf infiltration with harpin or water and was observed with a microscope after staining with trypan blue and clearing as described in the text. Dead cells stain blue, while healthy tissues except veins remain hyaline. Scale bar = 1 mm. (d) Quantitative determination of dead cells in leaves of plants sprayed with harpin. Data were obtained from three replicates of the assay and error bars are shown on curves. (e) Multiplication of Pst in plants. Bacteria were recovered from fresh tissues (FT) of plants 1, 3 and 5 d after inoculation. Plants had been sprayed with harpin (open circles) or water (solid circles) 5 d prior to inoculation. Curves represent the mean ±SD for 3 replicates. (f) Symptom development affected by *ndr*1-1 and treatment with harpin, as observed 7 d after inoculation.

4.1. The correlation between HCD and resistance induced by harpin

Dangl and coauthors [13] asked in their review on HCD, 'Is the HR a requirement for successful activation of resistance at and immediately surrounding the site of infection? Is cell death required for the onset of SAR?'. These questions have not yet been satisfactorily answered. Relationships between HCD and resistance vary in different systems and for different elicitors. Cell death often accompanies, but may not be essential to, the induction of resistance by elicitors such as fungal glycoproteins [12,25] and elicitins, the HR-eliciting proteins produced by *Phytophthora* spp. and *Pythium* spp. [34]. How HCD is correlated with resistance in plants treated with harpin heretofore has not been clear.

In the present studies we found that HCD concomitantly developed with resistance in *Arabidopsis* and tobacco following treatment with harpin. Evidence supporting a linkage between these responses was found in the temporal coordination of micro-HR development, induction of PR gene expression and expression of resistance in harpin-treated plants. Furthermore, in wild-type *Arabidopsis* and tobacco, induction of micro-HR, PR gene expression, and resistance required the same threshold dose (3–5 μg ml^{-1}) of harpin. A similar harpin concentration (15–30 μg ml^{-1})

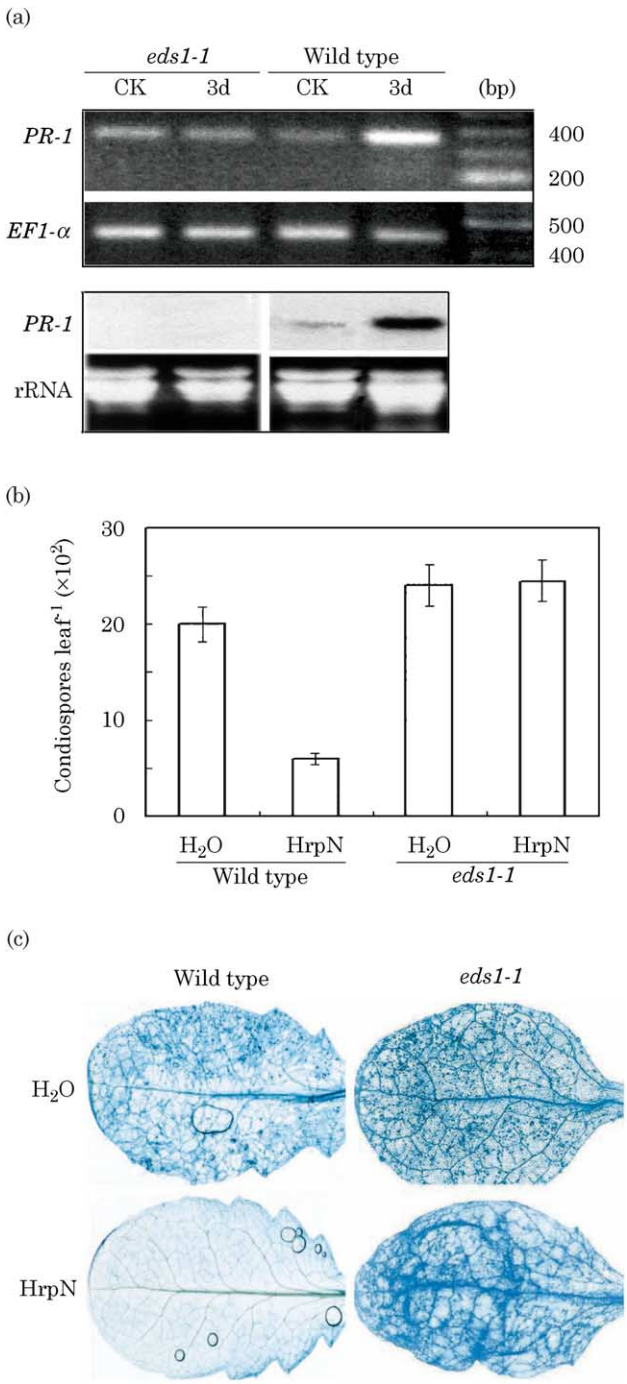

Fig. 7. Effects of *eds*1 on *PR*-1 gene expression and pathogen resistance in *Arabidopsis* treated with harpin. (a) *PR*-1 expression determined by semi-quantitative RT-PCR (top) and northern blot hybridization (bottom). Primers specific to *PR*-1 yield a 346-bp product. RNA used as templates was isolated from plants immediately (CK) and 3 d after treatment with harpin. Size of the RT-PCR product of *EF*1α, the quantitative control gene, is 495 bp. Procedures used for northern blotting were as used for Fig. 4. Four replicate determinations gave similar results. (b) Number of conidiospores on leaves. Plants were inoculated with *P. parasitica* 5 d after spraying with harpin or water. Conidiospores per leaf were determined 7 d after inoculation. Curves were drawn between means ± SD for three replicates. (c) Oomycete growth in leaf tissues. Growth of the oomycete in tissues was observed, seven days post inoculation, after staining the leaves with trypan blue, which stains mycelia and oospores blue.

saturated PR gene expression, micro-HR, and phenotypic resistance in *Arabidopsis*. Thus, PR gene expression and resistance occurred whenever micro-HR developed in plants treated with harpin.

4.2. Sequence of SA accumulation and cell death initiated by harpin

Previous reports regarding the sequence of cell death and SA accumulation in the SAR pathway have shown that the pathway is complex, and it may be under feedback control [45]. Cell death is believed to occur before SA accumulation, based on epistasis analyses of crosses between NahG plants with some lesion-mimic mutants of *Arabidopsis* [31]. However, experiments with other mutants indicate that feedback mechanisms may modify regulation of cell death by an SA-dependent pathway [45,58]. In our experiments with harpin-treated *Arabidopsis* and tobacco, micro-HR, or micro-HR and macro-HR, did not occur in NahG *Arabidopsis* and tobacco, respectively. This supports the notion that SA is required for harpin-induced HCD. However, whether SA accumulation precedes or follows cell death, and whether feedback regulation occurs, are unclear with respect to the mode-of-action of harpin.

4.3. The requirement for NDR1 and EDS1 by harpin-induced SAR

The requirement for *NDR*1 or *EDS*1 for the function of *R* genes is related to structural features of their products. Almost 70% of over 40 cloned *R* genes encode NBS-LRR ('nucleotide-binding site plus leucine-rich repeat') proteins [14]. The proteins are further classified based on their N-terminal structure as those possessing only NBS-LRR, those containing a coiled-coil (CC) or leucine zipper (LZ) motif, and those carrying a domain homologous to the intracellular signalling domains of the *Drosophila* Toll and mammalian interleukin (IL)-1 receptors (TIR) [3,14,49,51]. *EDS*1 usually is required for function of some TIR-NBS-LRR *R* genes, while *NDR*1 is required for function of several CC-NBS-LRR *R* genes [1,40]. Structural differences of R proteins affect the responses they initiate [14]. The requirement for *EDS*1 or *NDR*1 for harpin-induced resistance may indicate that harpin acts on plants as do R proteins with different structures.

This study demonstrates the involvement of both *EDS*1 and *NDR*1 in harpin-induced resistance. Both *ndr*1 and *eds*1 abolish the induction of PR gene expression and the development of resistance. Therefore, *NDR*1 and *EDS*1 are required not only for *R*-mediated resistance but also for resistance induced by harpin. Presumably, harpin has the potential to activate distinct pathways that are activated by different R proteins and elicitors [14,24], which can lead to multiple repertoires of plant defence.

The extent to which *NDR*1 and *EDS*1 operate coordinately in harpin-activated processes is not clear. Analyses of

RPS-4-specific responses in wild-type and *eds*1 plants revealed that *EDS*1 operates upstream of SA-dependent defence [26]. In the *cpr*5–*eds*1 double mutant, *EDS*1 acts downstream of cell death and before SA accumulation [11]. The gene also functions in the mutant *snc*1 before SA while no cell death occurs [39]. On the other hand, the *ndr*1-1 mutant exhibits cell death following inoculation with avirulent *P. syringae* [7], and INA induces resistance in the mutant [45]. This is consistent with our data that micro-HR develops in *ndr*1-1 plants, although harpin fails to induce either PR gene expression or resistance in the mutant. Thus, HCD may accompany, but may not be required for resistance induced by harpin.

In conclusion, we have demonstrated here that harpin requires both NDR1 and EDS1 for the induction of resistance that coordinately develops with HCD in plants. Harpin seems to signal plants to activate basal defence pathways, like the SAR pathway [14], in a manner analogous to R-Avr recognition [46,54]. Whether harpin acts directly on plant signalling pathways or through its own receptors [48] remains to be determined.

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