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High-throughput *in planta* expression screening identifies a class II ethylene-responsive element binding factor-like protein that regulates plant cell death and non-host resistance

Khairun Hisam Bin Nasir[1,2], Yoshihiro Takahashi[1], Akiko Ito[1], Hiromasa Saitoh[1,3], Hideo Matsumura[1], Hiroyuki Kanzaki[1], Takeshi Shimizu[1], Minako Ito[1], Shizuko Fujisawa[1], Prakash C. Sharma[1,4], Masaru Ohme-Takagi[5], Sophien Kamoun[6] and Ryohei Terauchi[1,2,*]

[1]*Iwate Biotechnology Research Center, 22-174-4, Narita, Kitakami, Iwate 024-0003, Japan,*
[2]*The United Graduate School of Agricultural Sciences, Iwate University, 3-18-8, Ueda, Morioka, Iwate 020-8550, Japan,*
[3]*Max-Planck-Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, 50829 Cologne, Germany,*
[4]*School of Biotechnology, Guru Gobind Singh Indraprastha University, 110006 New Delhi, India,*
[5]*Gene Function Research Center, National Institute of Advanced Industrial Science and Technology (AIST), Central 4, Tsukuba 305-8562, Japan, and*
[6]*Department of Plant Pathology, Ohio Agricultural Research and Development Center, Ohio State University, Wooster, OH 44691, USA*

Received 11 April 2005; revised 20 May 2005; accepted 23 May 2005.
*For correspondence (fax +81 197 68 3881; e-mail terauchi@ibrc.or.jp).

Summary

We performed high-throughput screening using the potato virus X (PVX) system to overexpress *Nicotiana benthamiana* genes *in planta* and identify positive regulators of cell death. This screening identified *NbCD1*, a novel class II ethylene-responsive element binding factor (ERF), as a potent inducer of the hypersensitive response (HR)-like cell death. *NbCD1* expression was induced by treatments with INF1 elicitor and a non-host pathogen *Pseudomonas cichorii*. NbCD1 exhibited transcriptional repressor activity through its EAR motif, and this motif was necessary for NbCD1 to cause cell death. We identified 58 genes that displayed altered transcription following *NbCD1* overexpression. *NbCD1* overexpression downregulated the expression of *HSR203*, a negative regulator of hypersensitive death. Conditional expression of *NbCD1* in Arabidopsis also caused cell death, indicating that *NbCD1* downstream cascades are conserved in dicot plants. To further confirm the role of *NbCD1* in defense, we used virus-induced gene silencing to demonstrate that *NbCD1* is required for non-host resistance of *N. benthamiana* to the bacterial pathogen *P. cichorii*. Our data point to a model of transcriptional regulatory cascades. *NbCD1* positively regulates cell death and contributes to non-host resistance, possibly by downregulating the expression of other defense response genes.

Keywords: cell death, class II ERF, repressor, SuperSAGE, VIGS, non-host resistance.

Introduction

Programmed cell death (pcd) plays key roles in plant development, senescence and defense response against pathogens (reviewed in Kuriyama and Fukuda, 2002; Lam, 2004). Substantial information on plant pcd came from studies on the hypersensitive response (HR), a rapid death of plant cells that is associated with restriction of pathogen growth (Heath, 2000). Signaling cascades leading to the HR and disease resistance, particularly following R-gene-mediated pathogen recognition, have been the focus of intensive cellular and genetic studies (for recent reviews, see Greenberg and Yao, 2004; Martin *et al.*, 2003; Shirasu and Schulze-Lefert, 2003). Upon R-gene-mediated perception of pathogens, early cellular responses are triggered including changes in ion flux, generation of reactive oxygen species (ROS), and in most cases, rapid development of cell death (HR). The HR seems to require ROS generation mediated by NADPH oxidase activation (Torres *et al.*, 2002). Balance in cellular concentration of H_2O_2 and NO (Delledonne *et al.*, 2001) as well as

cellular levels of salicylic acid (Gaffney *et al.*, 1993) may define the rate of the HR. Several positive and negative regulators of the HR have been identified using genetic approaches (see Shirasu and Schulze-Lefert, 2000). Genes required for some, but not all, *R*-gene-mediated HR have been identified and include *Rar1* (Shirasu *et al.*, 1999), *Sgt1* (Azevedo *et al.*, 2002), *Hsp90* (Hubert *et al.*, 2003; Takahashi *et al.*, 2003), *Ndr1* (Century *et al.*, 1995), and *Eds1* (Parker *et al.*, 1996). Protein phosphorylation cascades involving the kinases CDPK and MAPKs also appear to be important for transducing signals from pathogen recognition to the HR and disease resistance (Romeis, 2001). Nonetheless, despite the significant knowledge accumulated in the last decade, our understanding of the HR and cell death, particularly of downstream signaling events, remains fragmentary. Many important players involved in positive and negative regulation of cell death remain to be discovered. The recent finding of a plant vacuolar protease VPE, necessary for virus-mediated HR, and similar in activity to the animal cell death executioners caspases, suggests that much remains to be discovered about plant cell death (Hatsugai *et al.*, 2004).

To date, genetic approaches to explore regulators of plant defense and cell death have emphasized loss-of-function mutagenesis (Glazebrook, 2001). However, many genes cannot be uncovered using this approach because of lethal effects or functional redundancy, especially in complex reiterative signal networks. Therefore, Xia *et al.* (2004) employed a gain-of-function mutation approach using T-DNA activation tagging in *Arabidopsis thaliana*. Screening of approximately 5000 lines with random insertion of cauliflower mosaic virus (CaMV) 35S enhancer identified an aspartic protease, CDR1, as a positive regulator of defense. An alternative to activation tagging is functional expression screening of cDNAs. Karrer *et al.* (1998) constructed a tobacco cDNA library in a tobacco mosaic virus (TMV)-based expression vector. Infectious transcripts were generated and used to inoculate tobacco plants resulting in the identification of 12 unique cDNAs that cause HR-like cell death. The identified cDNA sequences showed similarity to ubiquitin, a tumor-related protein, and various unknown proteins. However, there was no clear evidence linking the observed cell death and overexpression of the cloned cDNA, and no detailed characterization of the cDNAs was carried out. The observed cell death may have been caused by co-suppression (ubiquitin gene) or overexpression (tumor-related protein gene) (Karrer *et al.*, 1998). In addition, virus vectors have tremendously improved since the study of Karrer *et al.* (1998) and current virus expression systems are particularly adapted to high-throughput screens (see Huitema *et al.*, 2004). For example, Takken *et al.* (2000) developed a functional cloning strategy based on a binary potato virus X (PVX)-expression vector and applied it to expressing pathogen genes *in planta*. In that study, cDNAs of the tomato fungal pathogen, *Cladosprorium fulvum*, were cloned into a PVX vector, and transferred to *Agrobacterium tumefaciens*. A total of 9600 clones were tooth pick-inoculated onto tobacco leaves, among which four cDNAs were identified to cause necrotic lesion around the inoculation site. One of the identified cDNAs encoded AVR4, a race-specific elicitor. Similar high-throughput approaches have been used to identify cDNAs from the oomycete plant pathogen *Phytophthora* that elicit defense responses in plants (Qutob *et al.*, 2002; Torto *et al.*, 2003).

In this study, we performed high-throughput expression of plant cDNAs to identify novel factors that positively regulate cell death. We carried out functional screening of plant cell death-causing genes by transiently overexpressing a 40 000 *Nicotiana benthamiana* cDNA library *in planta* using the binary PVX-vector pSfinx (Takken *et al.*, 2000). This high-throughput functional screening resulted in the identification of several genes that cause cell death upon overexpression. Here, we present and characterize one of these genes, a novel ethylene response factor (ERF) that functions as a strong cell death-inducing factor.

Results

Functional screening of plant cell death factors in N. benthamiana *identifies NbCD1*

To search for plant genes that cause cell death upon overexpression, we constructed a *N. benthamiana* cDNA library in the binary PVX-expression vector pSfinx developed by Takken *et al.* (2000). Complementary DNA was synthesized from mRNA extracted from *N. benthamiana* leaves infiltrated with the HR elicitor INF1 of *Phytophthora infestans* (Kamoun *et al.*, 1998) to activate defense-related genes, and directionally cloned into pSfinx in the sense orientation. We screened 40 000 cDNAs by toothpick inoculation of *N. benthamiana* leaves with *A. tumefaciens* carrying recombinant pSfinx. Four to 14 days post-inoculation, cell death became visible around the inoculated site if the vector insert contained a cDNA of a gene for cell death-inducing protein. Such candidate *A. tumefaciens* clones were liquid-cultured, and infiltrated into *N. benthamiana* leaf blades by a needleless syringe to test whether cell death can be reproducibly observed. This functional screening identified a total of 30 cDNA clones that consistently caused cell death. The cDNA inserts were sequenced, and similarity searches were carried out with the BLAST program (Altschul *et al.*, 1990) to annotate the genes. The sequenced cDNAs showed significant similarity to ubiquitin-like protein (eight cDNA clones), RNA recognition motif protein (five), ethylene-responsive element binding factor (ERF)-like protein (one), and MAPKK-like protein (one) among others. We elected to focus on the ERF-like protein cDNA (henceforth named *NbCD1*) because it: (i) exhibited the strongest cell death-inducing phenotypes, causing cell death on *N. benthamiana* leaves 7 days

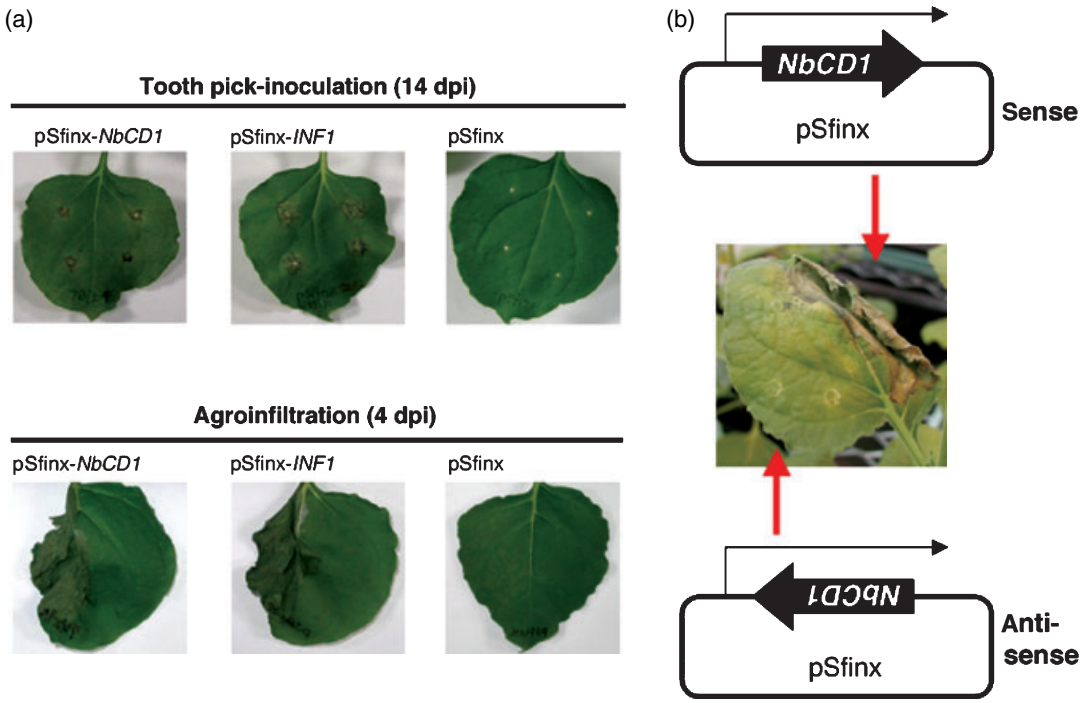

Figure 1. Overexpression of *NbCD1* causes cell death.
(a) *Agrobacterium tumefaciens* clones transformed with the pSfinx vector harboring *NbCD1* cDNA (left), *INF1* cDNA (center), or no insert (right) were toothpick-inoculated (top) or infiltrated (bottom) to *Nicotiana benthamiana* leaves. Photographs were taken 14 and 4 days after toothpick inoculation and infiltration to the half leaf, respectively. Necrotic cell death after toothpick inoculation of *A. tumefaciens* with pSfinx-NbCD1 became visible as early as 7 days post-inoculation.
(b) *Agrobacterium tumefaciens* clone harboring the pSfinx vector with *NbCD1* in the sense orientation caused cell death, whereas that with *NbCD1* in the antisense orientation did not.

after toothpick inoculation and 4 days after infiltration (Figure 1a); and (ii) encoded a novel putative transcription factor of the class II ERF family, a family of proteins that have been previously implicated in defense. Detailed description of the other identified cDNAs will be reported elsewhere. Cell death was only induced when the *NbCD1* cDNA was inserted in the vector in the sense, but not in the anti-sense, orientation (Figure 1b), indicating that overexpression, not virus-induced gene silencing (VIGS), of *NbCD1* induced cell death. This was further confirmed by agroinfiltration and by correlating the presence of NbCD1 protein with cell death (see below).

NbCD1 belongs to class II ERF

The *NbCD1* cDNA insert in the pSfinx vector contained 965 nucleotides (GenBank accession no. AB196362). This DNA sequence had no perfect similarity to known sequences deposited in public databases, indicating that *NbCD1* is a novel plant gene. This *NbCD1* cDNA harbored a single ORF corresponding to a protein of 231 amino acids in size (Figure 2a) with a high sequence similarity to the class II ethylene-responsive element binding factor (ERF, Figure 2b). Class II ERF proteins are characterized by the presence of the EAR (ERF-associated amphiphilic repression) motif (L/FDLNL/F(x)P) near their C-terminus in addition to the AP2/ERF DNA-binding domain and acidic domain common to all classes of ERFs (Fujimoto *et al.*, 2000; Ohta *et al.*, 2001). The NbCD1 predicted protein also contains a sequence with high similarity to the EAR motif (Figure 2a). NtERF3, a member of class II ERF, is known to function as a strong transcriptional repressor through its EAR motif (Ohta *et al.*, 2001).

NbCD1 functions as an active repressor

To test whether, similar to other class II ERFs, NbCD1 displays transcriptional repressor activity, we carried out a transient expression assay (Figure 3a) as described by Fujimoto *et al.* (2000). The reporter plasmid contained a firefly luciferase gene located downstream of 5× yeast GAL4 sequence and 4× GCC box, a target *cis*-element of ERFs (Ohme-Takagi and Shinshi, 1995). This reporter plasmid, together with an effector plasmid(s), was delivered to *A. thaliana* leaves by particle bombardment. Effector plasmids contained GAL4 DNA-binding domain (GAL4DB) only, GAL4DB fused to the transcriptional activation domain of viral protein 16 (VP16: GAL4DB-VP16), NbCD1, or NbCD1 lacking the EAR motif (NbCD1ΔEAR), all under the control of the CaMV35S promoter. Expression of GAL4DB did not induce the transcription of luciferase, whereas GAL4DB-VP16 expression

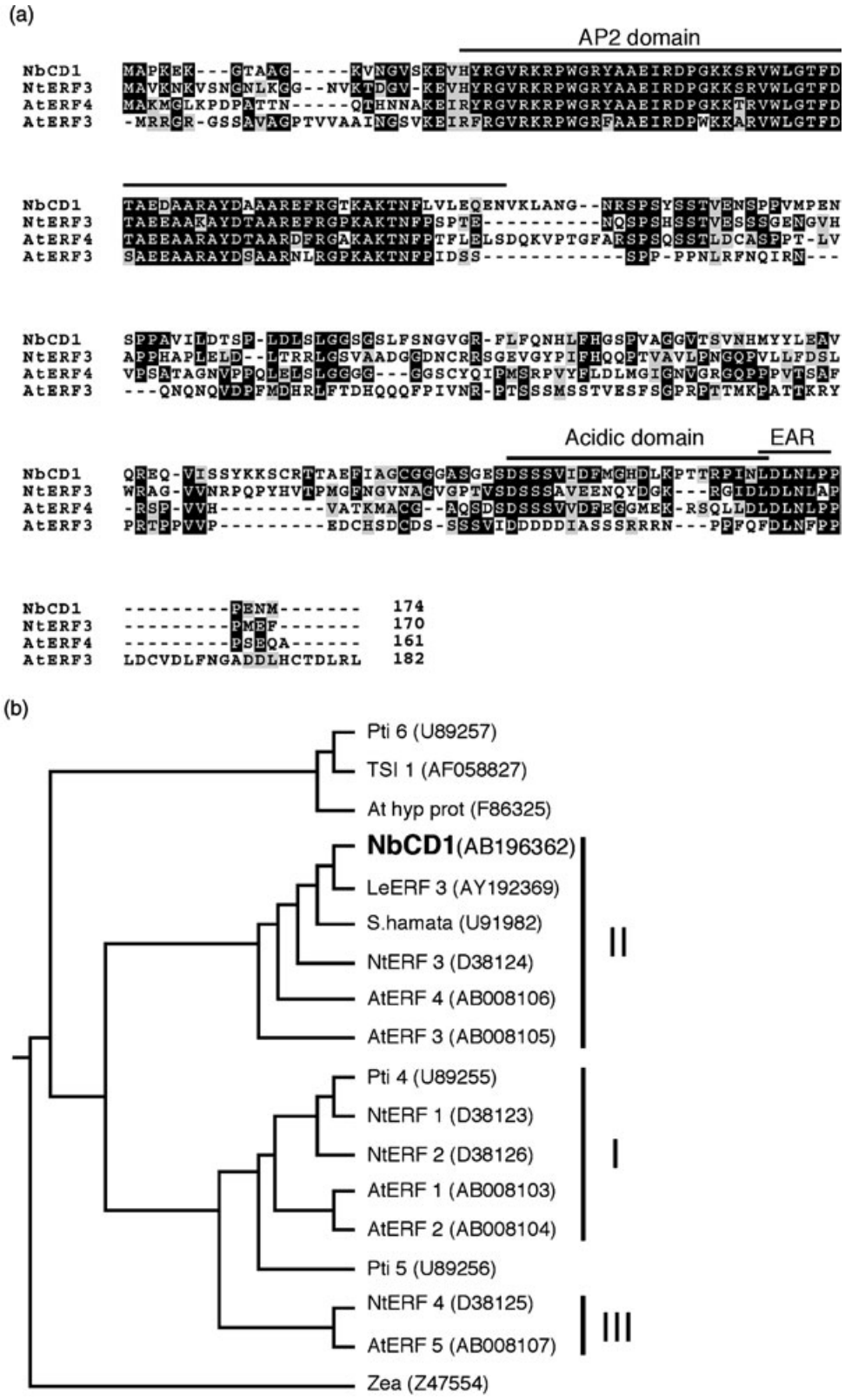

Figure 2. *NbCD1* encoded a protein with similarity to class II ethylene-responsive element binding factor (ERF).
(a) Amino acid sequence alignment of NbCD1, NtERF3 (Ohme-Takagi and Shinshi, 1995), AtERF3, and AtERF4 (Fujimoto *et al.*, 2000).
(b) A phylogenetic tree of ERF proteins. Gene names or species names followed by GenBank accession numbers in parenthesis.

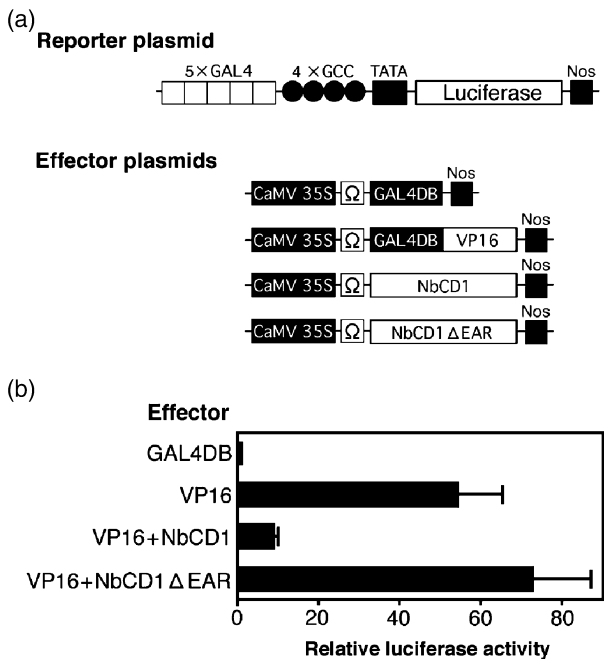

Figure 3. NbCD1 is an active repressor.
(a) Schematic diagram of the reporter and effector plasmids used. The GAL4 binding site (open boxes) and GCC box sequence (filled circles) were fused to a minimal TATA box and the luciferase gene. The effector plasmid contains either the GAL4 DNA binding domain (GAL4DB), GAL4DB fused with the VP16 activation domain (GAL4DB-VP16), NbCD1, or NbCD1 lacking the EAR motif (NbCD1ΔEAR), all under the control of the CaMV 35S promoter. Nos denotes the terminator signal of the gene for nopaline synthase. Ω indicates translational enhancer of tobacco mosaic virus.
(b) NbCD1 represses VP16-mediated transactivation. Values shown are averages of results from three independent experiments. Error bars indicate standard deviation. All luciferase activities are expressed relative to the reporter construct alone (value set to 1).

significantly activated luciferase transcription (Figure 3b). This VP16-mediated transactivation was abrogated by co-expression of NbCD1, suggesting that NbCD1 has an intermolecular repressor activity. This repressor activity was not observed with the construct expressing NbCD1 with a deleted EAR motif. These results demonstrate that NbCD1, similar to other class II ERFs, functions as a transcriptional repressor through its EAR motif.

NbCD1 is a single-copy gene, and its expression is induced by treatments with INF1 elicitor and Pseudomonas cichorii*, a non-host pathogen*

Southern blot analysis indicated that *NbCD1* is a single-copy gene (Figure 4a). To see the expression changes in *NbCD1* during defense responses of *N. benthamiana*, Northern blot analysis was carried out using RNA extracted from leaves collected at defined time intervals after infiltration with INF1 HR elicitor (Kamoun *et al.*, 1998), *P. cichorii*, a non-host bacterial pathogen (Hikichi *et al.*, 1998), and water as control (Figure 4b). Hybridization probe used for Northern blot analysis (Figure 4b) was derived from *NbCD1* cDNA region showing low sequence similarity to other members of the ERF gene family, and is the same as the one used for Southern blot analysis (Figure 4a), suggesting that transcripts detected here are *NbCD1* gene specific. *NbCD1* expression is absent in intact leaves. One hour after all treatments, strong transient induction of *NbCD1* was observed, presumably caused by hypo-osmotic stimulus following infiltration. While *NbCD1* expression was shut off 4 h after water treatment, its expression continued in INF1- and *P. cichorii*-treated leaves up to 12 h after the treatments, indicating that *NbCD1* expression is induced by INF1 and *P. cichorii* treatments.

Inducible expression of NbCD1 protein triggers H_2O_2 generation and cell death

To correlate NbCD1 protein production and leaf cell death, *NbCD1* cDNA was cloned into the glucocorticoid-inducible plant expression vector GVG as developed by Aoyama and

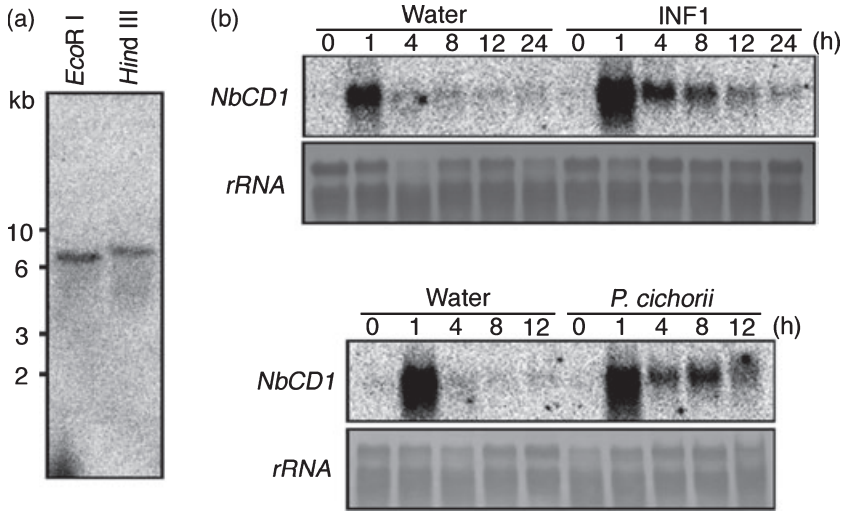

Figure 4. *NbCD1* is a single-copy gene, and its expression is induced by elicitor and a pathogen.
(a) Southern blot analysis of *NbCD1*. Genomic DNA of *Nicotiana benthamiana* was digested with *Eco*RI or *Hin*dIII, transferred to a membrane, and hybridized with a partial fragment of *NbCD1* cDNA.
(b) Northern blot analysis of *NbCD1* expression after treatments with *Phytophthora infestans* INF1 elicitor protein and a non-host pathogen, *Pseudomonas cichorii*. The DNA fragment used for the probe was the same as the one used in (a). Numbers indicate hours after infiltration.

Chua (1999). Insert cDNA was modified so that the C-terminus of the protein was tagged with the triple HA (influenza haemagglutinin) epitope. This vector GVG-NbCD1-HA was transferred to *A. tumefaciens* by electroporation. Liquid culture of the transformed *A. tumefaciens* was infiltrated into *N. benthamiana* leaves by a needleless syringe to establish transient transformation of the leaves. Two days later, a glucocorticoid inducer, dexamethasone (DEX), was infiltrated to induce NbCD1-HA expression. At a given time after DEX treatment, the leaf sample was collected and kept for further analysis. NbCD1-HA protein was detected by Western blot analysis using an anti-HA antibody. As shown in Figure 5(a), the NbCD1-HA protein became detectable 4 h after DEX treatment and abundantly produced at 24 h after DEX treatment. Leaf cell death became visible at 12 h after DEX treatment, and complete desiccation of the leaf was observed 48 h after the treatment. By contrast, in leaves that were treated with 0.1% ethanol instead of DEX, no production of NbCD1-HA protein was detected and no cell death was observed. This result demonstrates that overproduction of NbCD1 protein caused leaf cell death. It is noteworthy that multiple fragments with different molecular sizes were detected in the Western blot analysis of NbCD1-HA, suggesting possible protein modification (Figure 5a).

We studied in detail the early cellular events leading to leaf cell death triggered by induction of NbCD1. In *NbCD1-HA*-transformed leaves that were treated with DEX, massive H_2O_2 generation started at 4 h and continued until 12 h after the treatment (Figure 5b), exactly corresponding to the timing of the accumulation of NbCD1-HA protein in the leaf

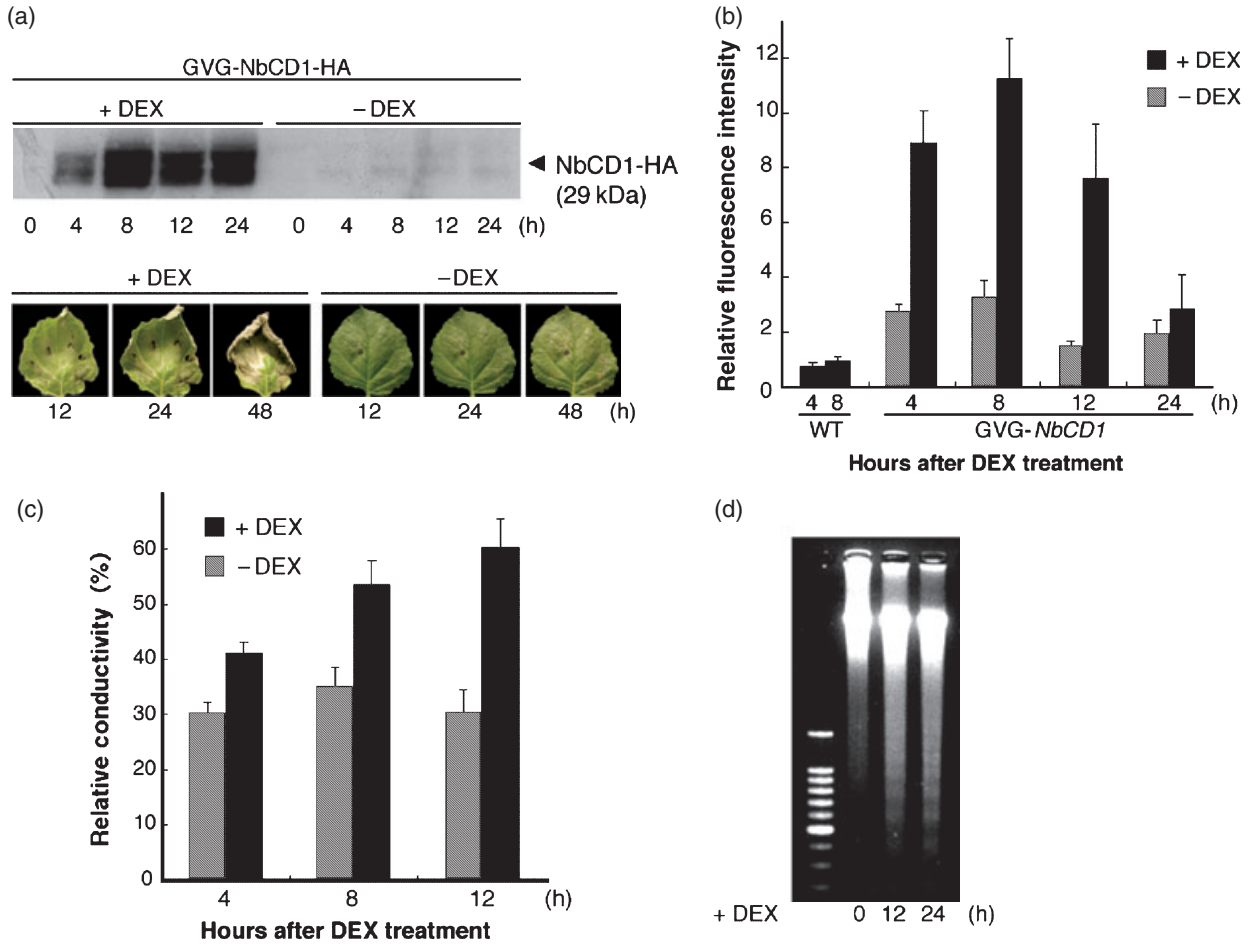

Figure 5. NbCD1 overexpression induces HR-like cell death.
(a) Western blot analysis of NbCD1-triple HA protein after transient transformation of *Nicotiana benthamiana* leaves with *Agrobacterium tumefaciens* containing GVG-NbCD1-triple HA followed by treatment with DEX (+DEX) or 0.1% ethanol (–DEX) (top). Leaf phenotypes of DEX-treated and 0.1% ethanol-treated leaves (bottom).
(b) Hydrogen peroxide generation in non-transformed leaves treated with DEX (WT), GVG-NbCD1-transformed leaves treated with DEX (+DEX; black bar), or with 0.1% ethanol (–DEX; dark bar).
(c) Ion leakage from NbCD1-transformed leaves treated with DEX (+DEX; black bar) or with 0.1% ethanol (–DEX; dark bar).
(d) Agarose gel (1.0%) electrophoresis of genomic DNAs extracted from *N. benthamiana* leaves transformed with NbCD1 and treated with DEX.

(Figure 5a). This H_2O_2 production is specifically induced by NbCD1-HA protein, as no H_2O_2 burst was observed in *NbCD1-HA*-transformed leaves without DEX treatment nor in untransformed leaves treated with DEX (Figure 5b). Ion leakage from *NbCD1-HA*-transformed cells was detected as early as 4 h after DEX treatment, indicating that the cellular membrane became permeable (Figure 5c). Genomic DNA extracted from leaf tissue 12 h after DEX treatment exhibited a ladder-like pattern after agarose gel electrophoresis, indicating that DNA fragmentation, a hallmark of apoptotic cell death, is taking place in NbCD1-overexpressing cells (Figure 5d). Taken together, these results suggest that *NbCD1* causes HR-like cell death.

NbCD1 is localized in the plant nucleus

Subcellular localization of NbCD1 was studied by transiently transforming *N. benthamiana* leaves with *A. tumefaciens* harboring the GVG vector containing *NbCD1* fused to *GFP* at its N-terminus (GVG-GFP-NbCD1), followed by DEX induction (Figure 6). GFP fluorescence became visible in nuclei 6 h after DEX treatment. Twenty-four hours after DEX treatment, GFP fluorescence accumulated in the nuclei of GVG-GFP-NbCD1-transformed leaves. After this time, nuclei expressing GFP became deformed and irregular (data not shown). Leaf cell death became evident between 48 and 72 h after DEX treatment. Thus, NbCD1 localized to the nucleus prior to the onset of the HR, and likely functions in the nucleus to trigger cell death.

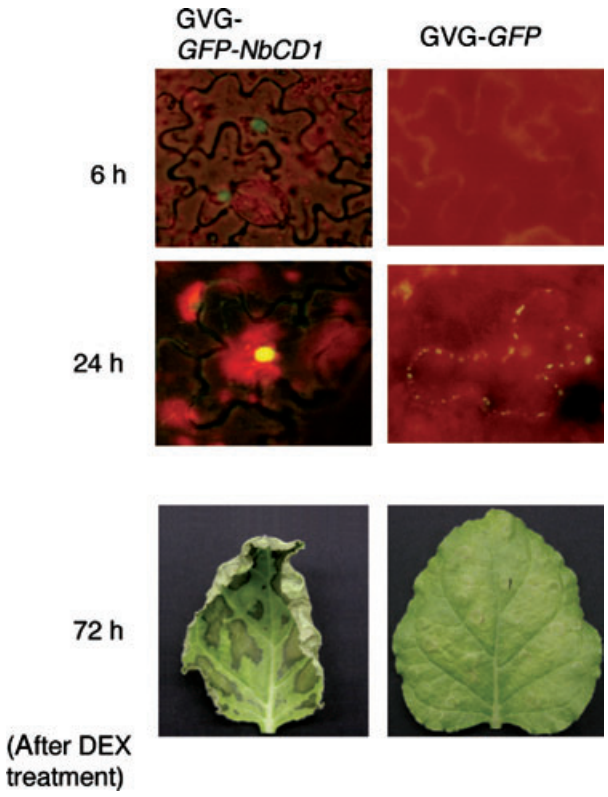

Figure 6. NbCD1 is localized in nuclei prior to triggering cell death. GVG-GFP-NbCD1 (left) and GVG-GFP (right) were transiently transformed to *Nicotiana benthamiana* leaves. Two days later, leaves were treated with DEX to induce transgene expression. Leaf tissue was observed under a UV microscope 6 h (top) and 24 h (middle) after DEX treatment. Leaf phenotypes 72 h after DEX treatment are also shown (bottom).

AP2/ERF domain, acidic domain and EAR motif of NbCD1 are necessary and sufficient to cause cell death

To identify the domains of NbCD1 responsible for induction of cell death, a series of deletion mutants of *NbCD1* were cloned into the pSfinx vector and tested for their potency in causing cell death upon transient overexpression by agroinfiltration (Figure 7). Results show that the N-terminal region upstream of AP2/ERF domain is dispensable for cell death (Figure 7a,b). Lack of either the intact AP2/ERF domain (Figure 7c,d), acidic domain (Figure 7l) or EAR motif (Figure 7h) abrogates the function of NbCD1 as a cell death inducer. The intervening region between AP2/ERF domain and acidic domain was dispensable for causing cell death (Figure 7k). Taken together, these results suggest that the AP2/ERF domain, acidic domain and EAR motif of NbCD1 are necessary and sufficient to cause HR-like cell death in *N. benthamiana* leaves.

SuperSAGE reveals NbCD1 downstream targets

To gain an insight into the genes regulated by *NbCD1*, SuperSAGE (Matsumura *et al.*, 2003) was applied to *N. benthamiana* to compare gene expression profiles of *NbCD1-HA*-overexpressing and *GFP*-overexpressing (control) leaves. GVG-NbCD1-HA and GVG-GFP plasmids were separately transferred to *A. tumefaciens*, and used for transient transformation of *N. benthamiana* leaves. Forty-eight hours later, DEX was infiltrated to induce gene expression from the GVG vectors, and leaf samples were collected 4 h after DEX treatment. Messenger RNA was extracted from the leaf samples, and subjected to SuperSAGE. For each of GVG-NbCD1-HA- and GVG-GFP-transformed leaves, a total of 10 269 tags corresponding to 6659 genes and 10 884 tags corresponding to 6796 genes were isolated, respectively. After comparison of frequencies of each tag in the two samples, 138 tags were identified to be differentially represented between GVG-NbCD1-HA- and GVG-GFP-transformed leaves by criteria that either the tag frequencies are statistically significantly different or the tag frequencies are different more than fourfold between the two samples. Of these, 68 tags were overrepresented, whereas 70 tags were underrepresented in *NbCD1*-overexpressing leaves. To further confirm the SuperSAGE results, RT-PCR was carried out to compare the expression

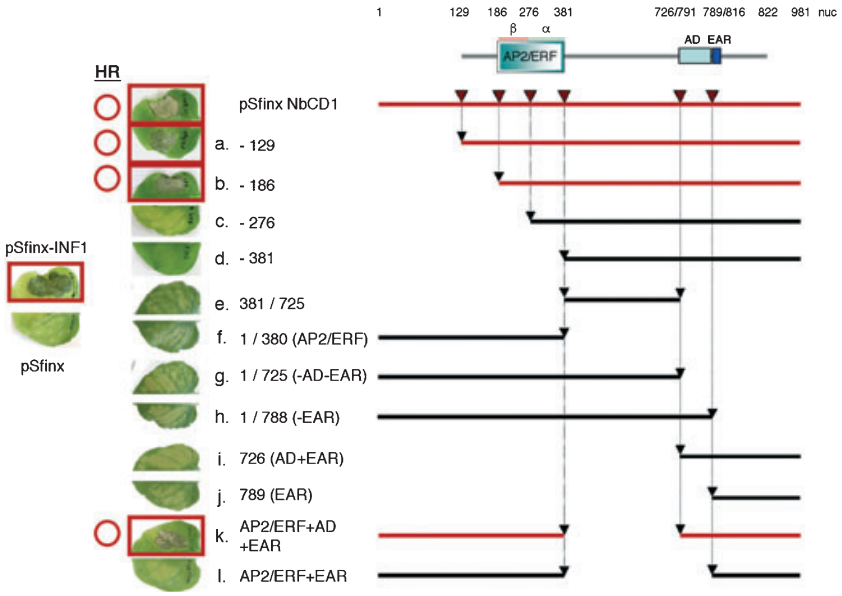

Figure 7. AP2/ERF domain, acidic domain and EAR motif of NbCD1 are necessary and sufficient to cause HR-like cell death in *Nicotiana benthamiana*. Deletion series of NbCD1 cDNA (right) were cloned into the pSfinx vector, and used for transient transformation of *N. benthamiana* leaves by agroinfiltration. One week after infiltration, occurrence of HR-like cell death (HR) was recorded (left). Phenotypes caused by the positive control pSfinx-INF1 and negative control pSfinx empty vector are shown.

of identified genes. For this purpose, the 26-bp tag sequences obtained by SuperSAGE were directly used as 3′-RACE PCR primers to amplify the regions between the tag and poly-A tail (Table 1; Figure 8). The expression changes observed by SuperSAGE were confirmed by 3′-RACE-PCR for 58 genes. For the rest of the tags, specific PCR products were not obtained. We performed BLASTX searches of the DNA sequence of the 3′-RACE product of each tag against GenBank non-redundant database, resulting in the successful annotation of about half of the sequences. The negative regulator of the HR *HSR203* (ERSAGE24), and the pathogenesis-related *PR-1b* (ERSAGE23), were among the repressed genes, whereas the genes for caffeoyl-CoA-O-methyltransferase (ERSAGE1), hin1-like protein (ERSAGE6), ERF1 (ERSAGE8), 5-epi-aristolochene synthase (ERSAGE14), and PR-1a (ERSAGE16) were among the NbCD1-induced genes.

NbCD1 expression induces cell death in A. thaliana

To test whether NbCD1 overexpression causes cell death in plants other than *Nicotiana*, we constructed *A. thaliana* lines harboring a GVG-NbCD1-HA transgene by the floral-dip transformation method (Clough and Bent, 1998). Thirty-three independent transgenic T_0 individuals were obtained. These plants were selfed, and their T_1 progeny were grown. Application of DEX to the plants identified 24 families with T_1 progeny segregating in normal versus dying plants. A typical result is shown in Figure 9. In family #2, the progeny with and without *NbCD1* transgene as checked by PCR segregated in an approximately 3:1 ratio (data not shown), suggesting incorporation of the transgene in a single locus. Treatment with DEX caused no change in *NbCD1*$^-$ progeny (#2-30) whereas it caused rapid cell death in most of the *NbCD1*$^+$ individuals (#2-28, #2-29). In the latter, the plants were completely desiccated within 4 days after DEX treatment. This result demonstrates that NbCD1 is functional in *A. thaliana*, and cell death pathways downstream of NbCD1 are conserved between *Nicotiana* and Arabidopsis.

VIGS of NbCD1

We carried out VIGS to knock down expression of *NbCD1*. A partial fragment of the *NbCD1* 3′-UTR region was cloned into a PVX vector (pPC2S, Baulcombe *et al.*, 1995) resulting in PVX–*NbCD1*, and its transcript was inoculated onto *N. benthamiana* leaves. Three weeks after inoculation, silencing of *NbCD1* gene expression was confirmed in the leaves located three to four leaves above the inoculated leaf (Figure 10a). To see the effect of *NbCD1* silencing on disease resistance, *P. cichorii*, a non-host bacterial pathogen, was inoculated onto *N. benthamiana* leaves silenced for *NbCD1* (Figure 10b). At 36 h post-inoculation, *P. cichorii* growth was significantly enhanced in *NbCD1*-silenced leaves. The same result was obtained in three independent experiments. We challenged *NbCD1*-silenced plants with HR or cell death-inducing factors including *P. infestans* INF1 protein, *P. infestans* PiNPP1.1 protein (T.-D. Kanneganti, E. Huitema, C. Cakir and S. Kamoun, unpublished data), paraquat, and *P. cichorii*. Only in the treatment with PiNPP1.1, *NbCD1*-silenced plants showed a delay in cell death development, but time difference of the onset of cell death between *NbCD1*-silenced plants and control was subtle and was only observed in two of five experiments (data not shown).

Table 1 Differentially expressed genes in NbCD1- versus GFP-overexpressing *Nicotiana benthamiana* leaves (4 h after DEX treatment for the induction of transgene expression)

Tag sequence[a]	Count of tags NbCD1	GFP	Putative encoded protein[b]	ID no.
ACTCAAATACTTGTGCACGAGG	8	33	PR-1b	ERSAGE23
TGCCGTCTTGATTGTCACGTTC	8	22	HSR203	ERSAGE24
ACAGCAGCAGCAGCGACAGCGA	7	22	No homology	ERSAGE25
ATAAGCTTTAAGGGATTAGTCG	1	20	Cytosolic aldolase	ERSAGE26
CGCCCCCCGTCCGCTTGCCGAC	3	16	Senescence-associated protein	ERSAGE27
GCCGACTTGCTGCACGTCAACC	1	13	60S ribosomal protein	ERSAGE28
ACAATATGCTCTGTCTTGTCTG	3	11	No homology	ERSAGE29
GCTAATGCTGGACCTGGAACCA	2	10	Cyclophilin	ERSAGE30
CGCCGTTTTGGCTGTAGAATGG	0	7	Hypothetical protein similar to NtPR27	ERSAGE31
ACCGTGGAGCCTTGATCATTTT	0	7	No homology	ERSAGE32
GATAGTCCTTCACATTGGCACG	0	7	Chaperonin 21	ERSAGE33
AGCAGCTAAGTGAAGAAACTTG	1	6	Glutamate-ammonia ligase	ERSAGE34
ATCAAAATAGATTTCAGTTGGG	1	6	Phospho glycerate kinase	ERSAGE35
TAATTTCCCAAATCGAACTGTA	1	6	Carbonic anhydrase	ERSAGE36
GACGCTTCCAGACTACACAGGA	1	6	No homology	ERSAGE37
CCAGCTGGGAGAGCTAATCCGC	0	6	Copper chaperone	ERSAGE38
GGGGTATACCACACTGTCTTTG	0	6	Aspartic proteinase	ERSAGE39
TGCTGCAGGCAGTGCTTCCGCA	0	6	40S ribosomal protein	ERSAGE40
GATGAGCTTTTAAGGGGACTAGT	0	6	No homology	ERSAGE41
ATGCAGCTGGGTTGTGATGGCG	0	5	No homology	ERSAGE42
TAATTTGGCGGGGAGTAATGTA	0	5	No homology	ERSAGE43
TGAAAGAACAGACTGAGCTTGT	0	5	No homology	ERSAGE44
GATGGTATGTGCCTGCTCCAGT	0	5	Ribosomal protein	ERSAGE45
CAAAACACTCTCATCCCCCCTA	0	4	Tonoplast intrinsic protein	ERSAGE46
GAGGCATTCTCCCGTACGTCAT	0	4	Cytosolic acotase	ERSAGE47
TCTACGGAGGCTGTAACTTTTT	0	4	Calmodulin	ERSAGE48
GGTAGAGCCAAAGAGTGTGAAC	0	4	Ribosomal protein	ERSAGE49
TTCTGCTACTCGACTATGAGAC	0	4	No homology	ERSAGE50
TGCTTCAAGACGTATCACTTGT	0	4	SAR8.2c protein	ERSAGE51
TACACTTCAAGAATCCTACTCC	0	4	No homology	ERSAGE52
GGTAGATGGATGGTTTGCTTAG	0	4	Hypothetical protein (*A. thaliana*)	ERSAGE53
GCACAGTTAAAGGATTCTCTCT	0	4	40S ribosomal protein	ERSAGE54
GATGAAGAAGCTGCTGGGTTTT	0	4	RNA binding glycine-rich protein	ERSAGE55
CAAAACACTCTCATCCCCCCTA	0	4	ADP-glucose pyrophosphorylase	ERSAGE56
ACACGGTCAAGCAAAGATCTGT	0	4	No homology	ERSAGE57
GAATGCATTGTAGAATACTGTG	0	4	No homology	ERSAGE58
GATCATATGATTTCATATTTGT	22	3	Caffeoyl-CoA O-methyltransferase	ERSAGE1
GGCAGATCAATGGGATCCAGCC	16	2	Hypothetical protein (*A. thaliana*)	ERSAGE2
ACGTATTACAAGTACCAAAAGC	15	2	Hypothetical protein similar to NtSA1	ERSAGE3
GAAGAAGCAACTTTAGTGTGGT	14	3	Calmodulin	ERSAGE4
TACATTGAAAGATGGAGGCGGA	13	0	No homology	ERSAGE5
GTACCATCTTGTTATATTTGGA	10	1	hin1-like protein	ERSAGE6
GTGGTGGGTACATCGTTAGAAG	9	1	Epoxide hydrolase	ERSAGE7
TTGATTATATGACCGGAGGGTA	7	0	ERF1	ERSAGE8
GGGTGTTGACCAAGACGCACTT	7	1	Unknown protein	ERSAGE9
AGTGCAAGCGTTCGAGGTTCCT	7	1	No homology	ERSAGE10
GGCAGTGAAACTGGGAAGAAGA	6	0	No homology	ERSAGE11
ATTACTATTCTATCAAGGGACT	6	1	60S ribosomal protein	ERSAGE12
GATCGGCAAACAAAGAGATAAT	6	0	No homology	ERSAGE13
TCGTATAAAGTTGTAACGGAGT	6	0	5-epi-aristolochene synthase	ERSAGE14
AGTCTCAACATTAGGTGGATTA	6	0	No homology	ERSAGE15
CACTAATAATGCTACTTCAAGT	5	0	PR-1	ERSAGE16
TGGAGTTAGATCCAAATTTTCC	4	0	No homology	ERSAGE17
GTACTACTCCTGGAAGATCATT	4	0	No homology	ERSAGE18
GATTCCAAAAAAGAGCAAAAGC	4	0	Phenylpropanoid:glucosyltransferase	ERSAGE19
GATATTGATGATCAGAATAATG	4	0	No homology	ERSAGE20
CTAATAAGGAAATTGATGCTGC	4	0	No homology	ERSAGE21
ACTTCTTGGGACTGATGTACAT	4	0	Cysteine proteinase	ERSAGE22

[a]Tags represented as a 22-bp sequence excluding the *Nla*III site (CATG).
[b]Encoded proteins were deduced by BLAST search with 3′RACE fragment recovered by using a 26-bp-tag primer. No homology indicates that no homologous proteins were identified by BLAST search.

Figure 8. NbCD1 target genes of *Nicotiana benthamiana* as revealed by SuperSAGE and 3′-RACE PCR. The 26-bp SuperSAGE tag sequence listed in Table 1 was used as 3′-RACE PCR primers to amplify the regions between tags and poly-A tail for NbCD1-overexpressed and GFP-overexpressed leaves. The 3′-RACE PCR products were sequenced and annotated by BLAST searches.

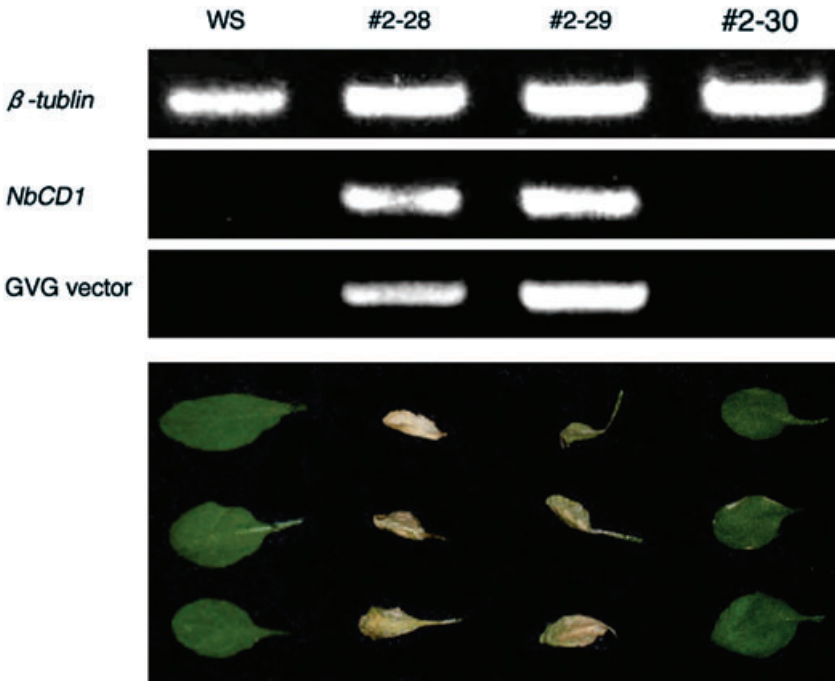

Figure 9. *NbCD1* overexpression causes cell death in *Arabidopsis thaliana*. Genomic DNAs of a wild type (WS) and three T_1 progeny (#2-28, #2-29, and #2-30) of the GVG-NbCD1-HA transgenic line were used as templates for PCR amplification of β-tublin, NbCD1 transgene, and GVG vector arm (top). These plants were treated with DEX, and phenotypes observed 4 days after treatment (bottom).

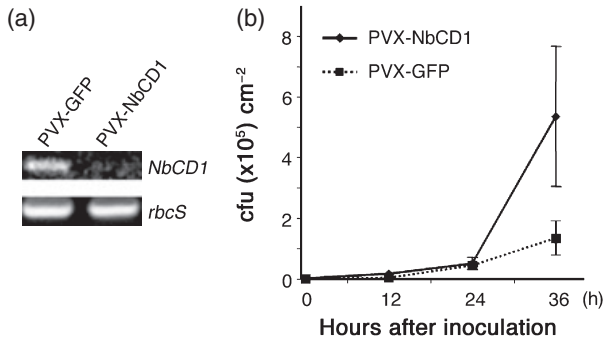

Figure 10. VIGS of *NbCD1* compromises non-host resistance of *Nicotiana benthamiana* to *Pseudomonas cichorii*.
(a) RT-PCR of *NbCD1* and *rbcS* genes in PVX-GFP-inoculated plant and PVX-NbCD1-inoculated plant.
(b) Leaves of *N. benthamiana* exhibiting *NbCD1* silencing were inoculated with *P. cichorii* and bacterial growth was monitored at 1, 12, 24, and 36 h after the inoculation. PVX-GFP-inoculated plants were used as control.

Discussion

Functional screening of plant genes identifies novel positive regulators of cell death

To search for plant genes that positively regulate cell death, we employed a high-throughput overexpression screening method similar to the one described by Takken *et al.* (2000). Overexpression of anonymous cDNAs *in vivo* followed by screening for cells showing phenotypic changes is a routine approach to functionally identify novel genes in prokaryotes, yeasts, and animal cells (Grimm, 2004; Rine, 1991). The approach we employed in this study proved useful in identifying new genes in plants solely based on their function and is an alternative strategy to activation-tagging screens (Xia *et al.*, 2004). Use of a binary-PVX vector system allows reliable transient overexpression and is therefore superior to the TMV expression system (Karrer *et al.*, 1998). The overexpression screens we performed in *N. benthamiana* are complementary to the VIGS approach taken by Lu *et al.* (2003). In that study, approximately 5000 normalized random cDNA fragments were used to silence their corresponding genes and identify genes involved in HR caused by the interaction between the *Pto* resistance gene and *Pseudomonas syringae* (Lu *et al.*, 2003). This VIGS screening identified *Hsp90* among 79 genes required for R-gene-mediated HR. In a similar VIGS screen in *N. benthamiana*, Del Pozo *et al.* (2004) identified MAPKKKα as a positive regulator of HR and disease resistance against *P. syringae*. The loss-of-function screens used by Lu *et al.* (2003) and Del Pozo *et al.* (2004), as well as the gain-of-function strategy described here and elsewhere (Kamoun *et al.*, 2003; Takken *et al.*, 2000), provide a useful set of high-throughput techniques for isolating novel factors involved in plant cell death in particular, and plant defense in general.

NbCD1 positively regulates cell death and contributes to non-host resistance

NbCD1 encoded a protein with a high sequence similarity to class II ERFs (Figure 2). ERFs form a distinct family of plant-specific transcription factors with similarity to AP2 transcription factors (Ohme-Takagi *et al.*, 2000). Although

several class I and class III ERFs have been linked to defense, this study reports a class II ERF that contributes to cell death and defense. *NtERF1*, *2*, *3* and *4* of tobacco were first isolated as genes coding for proteins that specifically bind to a *cis*-element, GCC-box, known to be necessary and sufficient for ethylene-induced response of promoters of a variety of genes (Ohme-Takagi and Shinshi, 1990, 1995). ERFs were subdivided into three classes according to their amino acid sequence similarity (Fujimoto *et al.*, 2000): class I ERFs include tobacco NtERF1 and NtERF2, as well as *A. thaliana* AtERF1 and AtERF2 and tomato Pti4 and Pti5 (Zhou *et al.*, 1997); class II ERFs consist of NtERF3, AtERF3, and AtERF4; and class III ERFs comprise NtERF4 and AtERF5. Members of all ERF classes were shown to bind to GCC-box sequences (Ohme-Takagi and Shinshi, 1995; Solano *et al.*, 1998; Zhou *et al.*, 1997), whereas binding of Pti4 to non-GCC-box *cis*-element was recently demonstrated (Chakravarthy *et al.*, 2003). ERFs belonging to class I and class III function as transcriptional activators, and plants overexpressing some of these factors showed enhanced disease resistance (Berrocal-Lobo *et al.*, 2002; Gu *et al.*, 2002). In contrast, class II ERFs function as active transcriptional repressors (Fujimoto *et al.*, 2000), based on their C-terminal EAR motifs (Ohta *et al.*, 2001).

Co-expression assay of NbCD1 and GAL4-VP19 showed that similar to other class II ERFs, NbCD1 functions as a transcriptional repressor through its EAR motif (Figure 3). The EAR motif, as well as the AP2/ERF and acidic domains, were necessary for NbCD1 to exert cell death (Figure 7). It is well known that overexpression of some cellular factors cause titration of interacting proteins (squelching) and this squelching effect is responsible for the alteration of phenotypes (Ptashne and Gann, 2002). In such circumstances, overexpression of isolated domains involved in protein–protein interaction or protein–DNA interaction alone would be able to cause the phenotype. However, this was not the case for NbCD1, which required all three functional domains to exert cell death (Figure 7). Altogether, these results suggest that transcriptional repressor activity is necessary for NbCD1 to cause cell death.

We observed that overexpressed NbCD1 was targeted to plant nuclei (Figure 6), where it is likely to bind to DNA regions so far unidentified. We hypothesize that NbCD1 represses the expression of unidentified target genes, some of which are presumably negative regulators of cell death (see below). Cell death caused by *NbCD1* overexpression displayed many similarities to pathogen-induced HR (Figure 5). It was associated with rapid generation of H_2O_2, ion leakage, and DNA fragmentation. We envisage that expression changes in NbCD1 target gene(s) triggered the generation of H_2O_2 and ultimately cell death. It is well established that accumulation of H_2O_2 to high concentration leads to the HR (Alvarez *et al.*, 1998; Dat *et al.*, 2003). Apparently, such a mechanism operates not only in *Nicotiana* but also in Arabidopsis, suggesting a conserved cell death-inducing process downstream of NbCD1 in dicot plants (Figure 9).

NbCD1 expression was induced by HR elicitor INF1 and a non-host pathogen, *P. cichorii* (Figure 4). VIGS of *NbCD1* partially compromised plant defense against *P. cichorii* (Figure 10). These results suggest that *NbCD1* positively regulates defense response, including non-host resistance. However, VIGS of *NbCD1* did not affect the timing of onset of cell death caused by INF1, paraquat, and *P. cichorii*. We hypothesize that either (i) *NbCD1* is not involved in the signaling pathways emanating from tested HR- or cell death-inducing agents but plays a role in other pathways of cell death signaling, or (ii) *NbCD1* actually is involved in tested cell death pathways, but because of redundancy of signaling pathways, VIGS of *NbCD1* alone did not affect the final output. Further studies with other pathogens and cell death-inducing agents are needed to obtain a clear picture of function of NbCD1.

SuperSAGE revealed downstream targets of NbCD1

We exploited the SuperSAGE technique to identify downstream targets of NbCD1. SuperSAGE (Matsumura *et al.*, 2003) is an improved version of serial analysis of gene expression (SAGE, Velculescu *et al.*, 1995) that is particularly adapted to non-model organisms, such as *N. benthamiana*. SuperSAGE, along with RT-PCR validation, applied to NbCD1-overexpressing and control leaves identified 58 differentially expressed transcripts between the two samples (Table 1; Figure 8). Although NbCD1 was shown to function as an active repressor (Figure 3), its overexpression caused induction as well as repression of many genes. Several of the differentially expressed genes were previously tied to pathogen defense and hypersensitive death. *HSR203* (ERSAGE24) was among the NbCD1-repressed genes. This gene codes for a serine hydrolase and is known to be rapidly induced in incompatible host–pathogen interactions (Pontier *et al.*, 1994). Transgenic tobacco plants with antisense-mediated reduced *HSR203* transcript levels exhibited a remarkably accelerated HR response when inoculated with incompatible pathogens, leading the authors to suggest that HSR203 is a negative regulator of the HR (Tronchet *et al.*, 2001). It is possible that NbCD1 repression of *HSR203* expression contributes to the rapid development of the HR. The induced genes included a gene for ERF1 (ERSAGE8), a class I ERF suggesting that expression of repressor and activator ERF types is interlinked. Other induced genes included several HR marker genes (*hin1*; Gopalan *et al.*, 1996), genes for acidic PR protein (PR1a), lignin biosynthesis (caffeoyl-CoA-O-methyltransferase) and phytoalexin biosynthesis (5-epi-aristolochene synthase). Induction of these defense-related genes could be a secondary effect of NbCD1-mediated repression of key genes that negatively

regulate defense responses. About half of the genes listed in Table 1 had no similarities to sequences in public database or code for unknown proteins. In the future, transient overexpression of *NbCD1*-induced genes as well as VIGS of *NbCD1*-repressed genes will identify the downstream factors directly involved in *NbCD1*-mediated HR-like cell death.

In conclusion, gain-of-function screening of plant cDNAs implicates *NbCD1* in cell death signaling and regulation of non-host resistance. Our data point to a model of transcriptional regulatory cascades. NbCD1 may positively regulate cell death through its EAR motif-mediated repressor activity and contributes to non-host resistance by downregulating the expression of other defense response genes. Future studies will focus on the key target genes of NbCD1 responsible for cell death and non-host resistance.

Experimental procedures

Plant material and INF1 treatment

Nicotiana benthamiana plants were grown in a glasshouse at 20°C. INF1 elicitor (100 nM) was prepared according to Kamoun *et al.* (1998) and infiltrated to well-developed leaf blades. Leaves were collected 0, 15, 30, 60, 120 and 240 min after infiltration and used for isolation of RNA for cDNA library construction.

cDNA library construction in pSfinx vector

Messenger RNA (mRNA) was isolated from total RNA by the mRNA purification kit (Amersham Biosciences, Little Chalfont, UK). Double-stranded cDNAs were synthesized by a SuperScript Plasmid System kit (Gibco BRL, Gaithersburg, MD, USA). These cDNAs with the *Sal*I site in the 5′-ends and the *Not*I site in the 3′-ends were directionally cloned into a modified pSfinx vector (Takken *et al.*, 2000) whereby the original *Cla*I–*Sfi*A–*Sfi*B–*Asc*I–*Not*I–*Sal*I cloning cassette was replaced with the *Asc*I–*Sal*I–*Not*I cassette using the *Sal*I and *Not*I sites. Competent *Escherichia coli* (DH10α) cells were transformed with the cDNAs by electroporation. From more than 500 000 independent *E. coli* colonies, plasmids were isolated in bulk. Using this bulked cDNA, *A. tumefaciens* strain MOG101 cells were transformed by electroporation. From more than 100 000 transformed *A. tumefaciens* colonies, approximately 40 000 clones were transferred to 384-well microtiter plates filled with LB-agar medium, and kept for further use.

Toothpick inoculation of A. tumefaciens

From 384-well microtiter plates, *A. tumefaciens* clones were transferred to 96-well microtiter plates filled with LB liquid medium, and cultured for 48 h at 28°C. Liquid-cultured cells were lifted by a toothpick, and inoculated to *N. benthamiana* leaves basically following the method of Takken *et al.* (2000).

Transient assay of NbCD1 repressor activity

Reporter plasmid, construction of effector plasmids, and delivery of plasmids into *A. thaliana* leaves by particle bombardment are as described (Fujimoto *et al.*, 2000). For the reporter plasmid, firefly luciferase gene was used. To monitor cell viability, another plasmid containing *Renilla* luciferase under the control of the CaMV35S promoter was co-bombarded with reporter and effector plasmids. Firefly luciferase activity was normalized as its raw value divided by *Renilla* luciferase activity.

Northern blot analysis

The probe used was a PCR-amplified partial fragment of *NbCD1* cDNA corresponding to the amino acid residue nos 85–231, downstream of the AP2 domain of the NbCD1 protein. This region of the *NbCD1* cDNA exhibits low sequence similarity to other ERF genes. After hybridization, the membrane was washed in high-stringency condition (0.1x SSC, 0.1% SDS at 60°C).

Inducible expression of NbCD1-triple HA and NbCD1-GFP

To the 3′-end of the ORF of *NbCD1*, an extension of the DNA sequence (5′- GCTTCTAGATATCCATATGATGTTCCAGATTATGCTGGTTATCCATATGATGTTCCAGATTATGCTGGTTCTTATCCATATGATGTTCCAGATTATGCTTCTAGATGA-3′) corresponding to the triple-HA tag (ASRYPYDVPDYAGYPYDVPDYAGSYPYDVPDYASR) was added by PCR resulting in NbCD1-triple HA cDNA. This fragment was cloned into *Xho*I and *Spe*I sites of a GVG-vector, pTA7001 (Aoyama and Chua, 1999). Engineered green fluorescent protein (mGFP, Haseloff and Amos, 1995) gene was fused to the 3′-end of NbCD1 cDNA, and cloned into pTA7001. These binary vectors were used for transformation of *A. tumefaciens* MOG101 by electroporation. *Nicotiana benthamiana* leaves were infiltrated with *A. tumefaciens* cells to establish transient transformation. Two days after *A. tumefaciens* infiltration, DEX (30 μM in 0.1% ethanol) was further infiltrated to the same leaves to induce gene expression.

Measurement of H_2O_2 and ion leakage

H_2O_2 generation was measured by using DCFH-DA as described by Sanchez *et al.* (1990). Ion leakage was measured according to Kim *et al.* (2003).

SuperSAGE

Four hours after induction of *NbCD1* and *GFP* from the GVG vector, leaf samples (approximately 5 g) were collected. Total RNA was extracted from them according to the standard method, and mRNA isolated by Oligo-dT columns. SuperSAGE was carried out as described (Matsumura *et al.*, 2003) using 5 μg mRNA as the starting material. Agroinfiltration causes defense response in plants (Ditt *et al.*, 2001). In this experiment, we focused on the genes that are differentially transcribed between the *NbCD1* and control treatments. Using the same RNA, cDNA was synthesized separately using an anchored oligodT primer (5′-biotin-CTGATCTAGAGGTACCGGATCCCAGCAGTTTTTTTTTTTTTTTT-3′). For 3′-RACE PCR, 26-bp primers corresponding to the SuperSAGE tags were used in combination with the primer (polyT anc: 5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3′) complementary to the cDNA ends.

Arabidopsis transformation

Arabidopsis thaliana landrace *WS* was grown for 50 days under light. Floral dip transformation of *A. thaliana* plant with the GVG-NbCD1-HA construct was carried out according to Clough and Bent (1998). Transformed T_0 seeds were selected by 30 μg ml^{-1} hygromycin and 500 μg ml^{-1} cefotaxim. DEX (50 nM in 0.1%

ethanol) was used to induce the expression of NbCD1-HA from the GVG vector.

VIGS of NbCD1

The *NbCD1* cDNA fragment corresponding to positions 654–834, whereby the first nucleotide of the first codon was set to position 1, was cloned into a PVX vector pPC2S in the antisense orientation resulting in pTXS.NbCD1. pTXS.NbCD1 was linearized by a restriction endonuclease *Spe*I, and *in vitro* runoff transcripts were synthesized by T7 RNA polymerase. The transcripts were inoculated to *N. benthamiana* plants as described elsewhere (Saitoh *et al.*, 2001). Confirmation of gene silencing of *NbCD1* was made by using a primer pair, 5′-CCGTCGACTGGTTTTAGAGCAGGAGA-3′ and 5′-CCGATATCTGAAGAAATCACTTGCTC-3′ annealing to position 361 and position 656 of *NbCD1* cDNA.

Inoculation with P. cichorii *and determination of growth kinetics*

Pseudomonas cichorii SPC9001 (Hikichi *et al.*, 1998) was grown at 28°C in nutrient broth medium (Difco, Detroit, MI, USA) containing ampicillin (10 μg ml^{-1}) overnight. After centrifugation, bacterial cells were resuspended in 10 mM $MgCl_2$ ($OD_{600} = 0.01$). Bacterial suspensions were inoculated onto leaves using a needleless syringe. The increase in the numbers of bacteria was estimated in leaf disks. Further details are available in Sharma *et al.* (2003).

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