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Suppression of Cdc27B expression induces plant defence responses

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Title: Suppression of *Cdc27B* expression induces plant defense responses

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Abbreviations: APC/C, anaphase-promoting complex or cyclosome; BY, Bright Yellow; HCD, hypersensitive cell death; HR, hypersensitive response; PCR, polymerase chain reaction; PR, pathogenesis-related; SCF, Skp1/Cul1/F-box protein; TMV, tobacco mosaic virus; TRV, tobacco rattle virus.

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Summary (word count: 215)

Nonhost resistance is the most general form of disease resistance in plants because it is effective against most phytopathogens. The importance of hypersensitive responses (HRs) in nonhost resistance of *Nicotiana* species to the oomycete *Phytophthora* is clear. INF1 elicitin, an elicitor obtained from the late-blight pathogen *Phytophthora infestans*, is sufficient to induce a typical HR in *Nicotiana* species. The molecular mechanisms that underlie the nonhost resistance component of plant defense responses have been investigated using differential-display polymerase chain reaction (PCR) in a model HR system between INF1 elicitin and tobacco BY-2 cells. Differential-display PCR has revealed that *Cdc27B* is downregulated in tobacco BY-2 cells after treatment with INF1 elicitin. Cdc27B is one of thirteen essential components of the anaphase-promoting complex or cyclosome (APC/C) -type E3 ubiquitin ligase complex in yeast. This APC/C -type E3 ubiquitin ligase complex regulates G2-to-M phase transition of the cell cycle by proteolytic degradation. In this study, we investigated the roles of this gene, *NbCdc27B*, in plant defense responses using virus-induced gene silencing. Suppression of *NbCdc27B* in *Nicotiana benthamiana* plants induced defense responses and a gain of resistance to *Colletotrichum lagenarium* fungus. Elicitin-induced hypersensitive cell death (HCD) was inhibited mildly in plants silenced with TRV::Cdc27B. Cdc27B could manage the signaling pathways of plant defense responses as a negative regulator without HCD.

Introduction

Nonhost resistance is the most general form of disease resistance in plants because it is effective against the vast majority of phytopathogens. Most agriculturists advocate the use of nonhost resistance that is durable and not restricted to a specific species of pathogen (i.e., parasite-nonspecific; Heath, 2000). Although nonhost resistance can be generated in transgenic tobacco by introducing an elicitin gene coupled to a pathogen-inducible promoter, it is difficult to engineer nonhost resistance to a broad range of phytopathogens such as fungi, bacteria, and viruses, using known signal cascades (Keller *et al*., 1999). Although the mechanisms that underlie nonhost resistance are crucial to our understanding of host specificity and the pathogenesis of phytopathogens, these mechanisms are poorly understood.

Hypersensitive responses (HRs) are an important component of the nonhost resistance of *Nicotiana* species to the oomycete *Phytophthora* (Heath, 2000; Kamoun *et al*., 1998). *Phytophthora infestans*, which was responsible for the Irish potato famine, is a destructive pathogen that is still responsible for multibillion-dollar losses in potato and tomato production (Fry and Goodwin, 1997a, 1997b; Kamoun, 2000). Representative nonhost plants such as eggplant, parsley, tobacco, *Nicotiana benthamiana*, and *Arabidopsis thaliana* exhibit typical localized HRs at sites of infection. The HR is manifested by expression of defense genes, hypersensitive cell death (HCD), ion fluxes, phytoalexin production, and pathogenesis-related (PR) protein production (Colon *et al*., 1992; Kamoun *et al*., 1998, 1999; Naton *et al*., 1996; Schmelzer *et al*., 1995; Vleeshouwers *et al*., 2000). INF1 elicitin, an elicitor derived from the late-blight pathogen *P. infestans*, is sufficient to induce a typical HR in *Nicotiana* species (Kamoun *et al*., 1993, 1997). It was reported that the proteinaceous elicitor cryptogein induced cell cycle arrest at the G1 or G2 phase before the induction of HCD (Kadota *et al*., 2004).

The molecular mechanisms that underlie plant defense responses within nonhost resistance have been investigated previously using the differential-display polymerase chain reaction (PCR) in a model HR system in which INF1 elicitin was applied to tobacco Bright Yellow (BY)-2 cells. This

system has been used to identify and clone several INF1 elicitin-induced genes that are involved in tobacco HRs (Sasabe *et al*., 2000, 2002; Schenke *et al*., 2003). Differential-display PCR revealed that *Cdc27B* is downregulated in cultured tobacco cells in response to treatment with INF1 elicitin.

In the most common host resistances and occasional nonhost resistances, the various HRs (which include HCD and activation of defense genes at the site of infection) are associated with pathogen limitation. Activation of defense genes may be more important than HCD in inhibiting pathogen growth, and it has been proposed that HCD and activation of defense genes are independent processes (Bendahmane *et al*., 1999; Clough *et al*., 2000; Jakobek and Lindgren, 1993). Several of the elements that are involved in HCD and apoptosis (either directly or indirectly) have been identified, which include the following: *Acd11*, lipid transfer protein; *Cpr5*, type IIIa transmembrane protein; *Dnd1*, cyclic-nucleotide-gated channel; *Lsd1*, zinc-finger protein; *Paf* and *Rpn9*, alpha6 of 20S proteasome subunits and 19S regulatory complex; and *Ssi4*, TIR-NB-LRR protein (Brodersen *et al*., 2002; Clough *et al*., 2000; Dietrich *et al*., 1997; Kim *et al*., 2003; Kirik *et al*., 2001; Lorrain *et al*., 2003; Shirano *et al*., 2002).

HCD and apoptosis are linked to the regulation of the cell cycle and proteolysis. Multiple rounds of ubiquitination of the initial ubiquitin conjugate lead to the formation of polyubiquitin chains that are recognized by the proteasome, which degrades the ubiquitinated protein. Some ubiquitination reactions, such as those that involve the SCF-mediated pathway, require that the substrate is phosphorylated to be recognized by E3. In other reactions, such as those that involve the anaphase-promoting complex or cyclosome (APC/C), E3 is the target of regulation by phosphorylation, and substrate recognition does not require that the substrate be phosphorylated. With the APC/C, phosphorylation can either positively or negatively regulate activity, depending on the context.

The APC/C- type E3 ubiquitin ligase complex comprises thirteen subunits with RING finger, scaffold, adapter, and receptor functions in yeast (Harper *et al*., 2002). These complexes mediate the proteolytic degradation of short-lived regulatory proteins, including cyclins and other regulators

of the cell cycle. Cdc27B is an adapter subunit of APC/C-type E3 ubiquitin ligase that regulates the G2-to-M phase transition via proteolytic degradation. This protein is essential for the correct functioning of the cell cycle and for the normal development of plants. A RING-finger component, namely Rbx1 protein, was identified subsequently (Blilou *et al*., 2002; Harper *et al*., 2002; Liu *et al*., 2002; Nakayama *et al*., 2001). There is recent evidence that *N. benthamiana Skp1* (*NbSkp1*) is an essential component of *N*-gene-mediated resistance responses to TMV. For example, suppression of *NbSkp1* expression compromises *N*-mediated resistance to TMV (Liu *et al*., 2002).

Here we report on the roles of Cdc27B in plant defense responses. Suppression of *Cdc27B* induces plant defense responses and does not greatly compromise HCD induced by elicitin. It is likely that Cdc27B is not greatly involved in HCD signaling pathway.

Results

Identification of *Cdc27B*

Differential-display PCR has been used previously to reveal INF1 elicitin-induced gene expression, including the expression of *NtHSR203J*, *NtPAL*, and *NtPDR1* (Matsamura *et al*., 2003; Sasabe *et al*., 2000, 2002). Expression of genes that are involved in plant defense was upregulated in tobacco BY-2 cells and *N. benthamiana* leaves in response to treatment with INF1 elicitin (Fig. 1A, B). However, *Cdc27B* gene expression was greatly reduced 3–6 h after treatment (Fig. 1A, B). We used a 450-bp-long Cdc27B sequence isolated by differential-display PCR to identify three distinct *Cdc27B*-like genes in tobacco BY-2 cells and *N. benthamiana* leaf cDNA, namely *NtCdc27B1*, *NtCdc27B2*, and *NbCdc27B*. Only one kind of *Cdc27B* cDNA was got from *N. benthamiana*. Although there is only one copy of *Cdc27* in the genome of humans, *Caenorhabditis elegans*, yeast, and drosophila, there are two copies of this gene in the *Arabidopsis* genome, which are designed *Cdc27A* and *Cdc27B* (Fig. 1C). *NbCdc27B* encodes a protein that comprises 750 amino acids and *NbCdc27B* is substantially homologous to *Arabidopsis* Cdc27A (59% identity), Cdc27B (71% identity), and NtCdc27B1 and NtCdc27B2 (>98% identity for both). The

aforementioned proteins contain nine typical tetratrico peptide repeat domains within their C-termini (Fig. 1D).

Dwarf phenotypes induced by suppression of *Cdc27B*

Approximately 300-bp-long cDNA fragment of *NtCdc27B1* was cloned into the multicloning site of the tobacco rattle virus (TRV)-silencing vector, pTV00, in an inverse orientation relative to the cauliflower mosaic virus 35S promoter to obtain virus-induced gene silencing of *NbCdc27B* (Fig. 2A) (Ratcliff *et al*., 2001). As the aforementioned fragments of *NtCdc27B1* and *NbCdc27B* were highly homologous (>97% homology; only 7 of 296 nucleotides are mismatched), we used the 300-bp fragment of *NtCdc27B1* alone to silence *NbCdc27B* (Fig. 2A).

The plants silenced with TRV::Cdc27B were stunted (Fig. 2B). The morphology of cells from the leaves of these plants was investigated by staining them with toluidine blue; this revealed shrunken or expanded phenotypes (Fig. 2C). A toluidine blue-stained section of a leaf from a plant silenced with TRV::Cdc27B revealed slightly enlarger vacuoles (Fig. 2C).

Staining with toluidine blue revealed the presence of cells within the mesophyll that accumulated crystal-like structures and exhibited unusual phenotypes in the plants silenced with TRV::Cdc27B (Fig. 2C, Fig. 3A–C). Intensely stained cells (Fig. 3B and C) were distributed uniformly and at similar positions in the mesophyll layer of leaves from the various gene-silenced plants (Fig. 3A). The sections presented in Fig. 3B, D, and E, illustrate the features of these failed cells by vacuolation. Those in Fig. 3C, F, and G, also illustrate the features of these failed cells by crystalization. Degradation of the vacuoles and organelles is visible in Fig. 3B, D, and E. In Fig. 3C, F, and G, accumulated crystal-like structures are visible despite the absence of obvious fragmentation of nucleus. In the sections presented in Fig. 3D to G, features of apoptosis are visible, including vacuolation, expansion of the vacuole, and degradation of the chloroplast. However, we were unable to determine whether a general apoptosis signaling pathway caused the aforementioned two phenotypes. In addition, it is difficult to obtain direct evidence of apoptosis,

because these phenotypes were relatively rare in gene-silenced plants. The appearance of the failed cells is a rare phenomenon, only observed in less than 1% of the cells (Fig. 3A). In the sections that exhibited unique failed cells, vacuolation and accumulation of crystal-like structures within the vacuole (Fig. 3D and F) and degradation of the chloroplast (Fig. 3D, F, and G) were observed, but there was no degradation of the nucleus (Fig. 3F). Plastglobules were visible in the chloroplasts (arrowheads in Fig. 3G). A previous report suggested that the vacuole within the leaf mesophyll cells of *N. tabacum* accumulates crystal-like structures during development that might be a SiO_2 gel (Neumann and De Figueiredo, 2002).

Although we expected constitutive HCD without INF1-elicitin stimulation in plants silenced with TRV::Cdc27B, the plants silenced with TRV::Cdc27B had no constitutive HCD phenotype. To investigate whether *Cdc27B* is involved in IFN1-elicitin-induced HCD, crude INF1 elicitin protein was applied to each of the plants silenced with TRV::Cdc27B as well as a negative control (empty TRV). Figure 4A illustrates that suppression of *Cdc27B* had a partial effect on IFN1-elicitin-induced HCD. Specifically, in plants silenced with the empty TRV vector, 72.7% and 27.3% of cells exhibited intense and no staining, respectively; in plants silenced with TRV::Cdc27B, 39.7%, 46.6 %, and 13.8% of cells exhibited intense, moderate, and no staining, respectively. These findings indicate that while typical elicitin-induced HCD occurred in the control plants, elicitin-induced HCD was inhibited mildly by the silencing of Cdc27B. Therefore, it is likely that Cdc27B is not so much a negative regulator as a weak positive regulator to HCD signaling pathway.

Defense responses induced by suppression of *Cdc27B*

Although *Cdc27B* did not appear to be involved essentially in the signaling pathway that leads to HCD (Fig. 4A), there is evidence that defense responses were activated constitutively in plants silenced with TRV::Cdc27B (Fig. 4B, C, and D). Partial, drastic, and strong callose deposition

were observed in the plants silenced with TRV::Cdc27B (data not shown). There was strong induction of defense genes in plants silenced with TRV::Cdc27B (Fig. 4B); semiquantitative reverse transcriptase (RT)-PCR revealed that *PR1a*, *1c*, *2*, *Hin1*, *SAR8.2*, and *HSR203J* were activated (Fig. 4B). Knockout of *Cdc27B* in *Arabidopsis* is lethal, while the mutant *Hobbit* (*Hbt*), which has a dwarf phenotype, exhibits weak *Cdc27B* activity due to a point mutation (Blilou *et al*., 2002). Similarly, we found that *Cdc27B* was not silenced completely in the plants silenced with TRV::Cdc27B (Fig. 4B).

We investigated whether gene-silenced plants developed increased resistance to virulent pathogens by infecting the leaves of these plants with *Colletotrichum lagenarium*, which is a virulent pathogen of *N. benthamiana* (Takano *et al*., 2006). Assay of lesion formation (Fig. 4C and Table 1) and the microscope photographs of infection (Fig. 4D) show that plants silenced with TRV::Cdc27B was significantly more resistant to *C. lagenarium* than the control plants. We stained for dead cells and *C. lagenarium* using trypan blue. A few spores of *C. lagenarium* was found on the leaves of plants silenced with TRV::Cdc27B (Fig. 4D). It is likely that most unfixed spores were washed away. We are able to assume that any subsequent development of lesions reflected susceptibility but not HRs.

Discussion

APC/C controls the G2-to-M phase transition during mitosis (Harper *et al*., 2002; Zachariae and Nasmyth, 1999). A mutation in the *Arabidopsis Hbt* gene that encodes a *Cdc27B* homologue interferes with postembryonic cell division and the differentiation of the distally located quiescent center, columella root cap, and lateral root cap cells. *Hbt* is essential for normal plant development (Blilou *et al*., 2002; Willemsen *et al*., 1998). *Cdc27B* encodes an adapter subunit of the APC/C, a multisubunit E3 ubiquitin ligase that triggers proteolytic destruction of mitotic cell cycle. We found in the present study that expression of *Cdc27B* was reduced in response to treatment with INF1 elicitin. Cdc27B binds Cdc16 and Cdc23 via tetratrico peptide repeat domains to form the acceptor

subunit of the APC/C-type E3 ubiquitin ligase. It would appear that Cdc16 and Cdc23 have functions that are similar to those of Cdc27B (Harper *et al*., 2002). In future, we will investigate the effects of silencing the aforementioned genes.

Several types of cell death have been identified in living plants, including necrotic, apoptotic, and HCD. HCD induces the same morphological features as apoptosis and these two types of cell death may have signaling pathways in common. However, unlike apoptosis, HCD is not initiated by endogenous signaling but rather requires external elicitors. Although HCD is triggered by balanced production of nitric oxide and reactive oxygen species and involves a mitogen-activated protein kinase cascade, crosstalk between HCD signaling pathways and concrete defense responses (e.g., PR gene expression, callose deposition) is highly complicated and poorly understood. Disruption of cellular homeostasis caused by the loss of function of core cell-cycle proteins can induce defense responses. For example, silencing of 26S ubiquitin ligase (proteasome) subunits results in cell death and the induction of plant defenses (Kim *et al.*, 2003). In the present study, we investigated the functions of a protein that regulate the G2-to-M phase transition of the cell cycle, namely Cdc27B. We found that suppression of *Cdc27B* leads to the activation of defense responses and the development of failed cells. In Fig. 4, silencing of Cdc27B induced expression of defense-related genes and resistance to virulent *Colletotrichum lagenarium* was increased, whereas it inhibited mildly elicitin-induced HCD. In a relationship between INF1 elicitin and *Nicotiana benthamiana*, we believed that the resistance must depend on HRs including HCD. However, a phenotype of resistance to virulent pathogen does not depend on HCD, but appears as a sum of defense responses without HCD. Our observation that (1) there were failed cells within the mesophyll, and this phenotype was restricted to individual cells and did not spread to other cells in the immediate vicinity of the affected cell and (2) elicitin-induced HCD was inhibited mildly in plants silenced with TRV::Cdc27B allows us to conclude that Cdc27B could not manage negatively the HCD signaling pathways.

The first identified target of APC/C is cyclin B, which regulates the cell cycle. However, many other targets of APC/C-dependent ubiquitination have been identified in yeast and animals. Jasmonic acid plays a crucial role in plant defense responses. Jasmonic acid induces G2 arrest, prevents the accumulation of cyclin-B-dependent kinases, and inhibits the expression of cyclin B1-1; the latter two of the aforementioned processes are essential for the initiation of mitosis (Swiatek *et al*., 2004). Therefore, it is possible that cyclin B degradation is prevented by the suppression of *Cdc27B*, while the subsequent accumulation of cyclin B can induce excess activation of the defense genes (Harper *et al*., 2002; Nakayama *et al*., 2001). However, it is not yet clear whether defense responses are induced via an injury-induced signaling pathway due to damage to failed cells or a genuine signal derived from defense responses.

The SCF-type E3 ubiquitin ligase complex is also involved in regulating the cell cycle via protein degradation. *NbSkp1* appears to be an essential component of *N*-gene-mediated resistance response to TMV, because suppression of *NbSkp1* compromises *N*-mediated resistance to TMV (Liu *et al*., 2002). Skp1 is an adapter protein of the SCF-type E3 ubiquitin ligase complex (Nakayama *et al*., 2001). Future investigations should indicate if other components of this complex are also negative regulators of plant defenses.

Experimental procedures

N. benthamiana plants, tobacco BY-2 suspension cultures, and the elicitor

N. benthamiana was grown in a greenhouse at 26˚C under standard light:dark (16:8 h) conditions. Suspension-cultured tobacco BY-2 cells were maintained as described previously (Nagata *et al*., 1982; Sasabe *et al*., 2000, 2002). Donor material obtained from *N. benthamiana* plants for the elicitin-sensitivity test was restricted to the fourth to eighth leaves from the top of 8-week-old plants. INF1 elicitin protein (the *inf1* gene product of *P. infestans*) was prepared as a crude recombinant protein using a method that has been described previously (Kamoun *et al*., 1997; Sasabe *et al*., 2000). INF1 was concentrated using ammonium sulfate and the final concentration

quantified using the Bradford assay. Concentrated crude INF1 elicitin protein (10 μg ml^{-1} as a bovine serum albumin equivalent) was injected into *N. benthamiana* leaves. Crude INF1 elicitin protein used to treat the tobacco BY-2 cells was prepared as unconcentrated crude recombinant protein (Sasabe *et al.*, 2000, 2002). Samples of the *N. benthamiana* plant and tobacco BY-2 cells were obtained at various times after inoculation.

Differential-display PCR

Differential-display PCR was performed according to the method described by Yoshida *et al.* (1994). The nucleotide sequence of B61 (an arbitrary primer) was 5-AGACCTGCTTCT-3 (Wako, Osaka, Japan). The underlined nucleotides of the arbitrary primer (i.e., CTGCTTCT) correspond to both terminals of the identified aforementioned DNA fragment (*NtCdc27B*; positions 1131–1580 in AB207046). We obtained a 450-bp-long DNA fragment that was revealed by a DNA Data Bank of Japan (DDBJ) blast search to be homologous to *AtCdc27B* (Sasabe *et al.*, 2002).

Isolation of *NtCdc27B1*, *NtCde27B2*, and *NbCdc27B* cDNAs

To isolate and sequence *NtCdc27B1*, *NtCde27B2*, and *NbCdc27B* cDNAs, a tobacco cDNA library was constructed from BY-2 cells treated with INF1 elicitin protein (10 μg ml^{-1} for 6 h) (Sasabe *et al.*, 2002). Another cDNA library was constructed from the mRNA obtained from the leaves of *N. benthamiana* plants that were collected 0, 3, 6, 9, and 12 h after the injection of INF1 elicitin protein. The double-strand cDNA was inserted into the *Eco*RI–*Xho*I site of pINA177Hyg-vector-derived pGreen0179 (Hyg^r) for making the library (a gift from Prof. D. Baulcombe, The Sainsbury Laboratory, Norwich, UK). pINA177Hyg is a vector that is expressed strongly in plants, and expression of this vector is driven by the 35S promoter of the cauliflower mosaic virus with an intron from the *CAT1* gene (a gift from Dr. K Nakamura, Nagoya University, Japan). If transformed into *Agrobacterium*, which harbors the transformation helper plasmid pSoup,

the pINA177Hyg plasmid is available for plant transformation as a binary vector (Hellens *et al.*, 2000).

We cloned numerous segments of three *Cdc27B* genes (*NtCdc27B1*, *NtCdc27B2*, and *NbCdc27B*) from the tobacco BY-2 cell and *N. benthamiana* leaf cDNA libraries using PCR amplification of the 450-bp-long nucleotide sequence that was identified using differential-display PCR (see above). Thereafter, we arranged these segments to identify the *Cdc27B* genes. The nucleotide sequences reported here have been deposited in the DDBJ under accession numbers AB207046 (*NtCdc27B1*), AB207047 (*NtCdc27B2*), and AB207048 (*NbCdc27B*).

Northern hybridization analysis

For the Northern hybridization analysis, 15 μg of total RNA was separated on a 1% agarose gel that contained formaldehyde before being blotted onto a Hybond-N nylon membrane (Amersham Pharmacia Biotech, UK). Full-length RNA probes *NtPDR1* and *NtPAL* were generated using an RNA transcription kit (Stratagene, USA). These probes were labeled with digoxigenin (DIG)-UTP using a DIG RNA labeling mix (Boehringer, Mannheim, Germany) (Sasabe *et al.*, 2002). The full-length DNA probe *NtHSR203J* was generated by PCR with *NtHSR203J* cDNA as the template (a gift from Dr. Yasufumi Hikichi, Kochi University, Japan) and the primers 5’-GAAGACGGTTCAGTAGACCG-3’ and 5’-TCGTTGTTGATGAACTCTGC-3’ (DDBJ accession number X77136). The DNA probe used for the Northern blot analysis of *NtCdc27B1*, namely *Cdc27B(N)*, was generated by PCR with a cDNA subclone of *NtCdc27B1* as the template and the primers 5’-CAACACAGACTCTCTCTCTC-3’ and 5’-GGGCCTGAATGGTTGAATTG-3’ (positions 60–1001 of AB207046). Control data for the Northern blot analysis (treatment with Luria-Bertani medium) are not presented here but have been published elsewhere (see Sasabe *et al.*, 2002). The hybridization was carried out at 37°C overnight in low-stringency conditions [5× saline sodium citrate (SSC), 50% formamide, 0.02% sodium dodecyl sulfate (SDS), 2% blocking reagent (Boehringer), and 0.1 % lauroyl sarcosine]. The final

washing step was performed at 42˚C in 0.1× SSC and 0.1% SDS. Hybridization signals were detected using an anti-DIG antibody conjugated to alkaline phosphatase (Boehringer) and the corresponding chemiluminescent substrate, CDP-Star (Boehringer).

DNA constructs for virus-induced gene silencing

A 308-bp cDNA fragment of *NtCdc27B1* was generated by PCR using subclones of *NtCdc27B1* isolated from our cDNA libraries as templates and the following primers: 5'-CGCCCGTCGACCTGCTTCTGG-3' and 5'-TTCCCCATCGATCCTGAAAAGGG-3' (positions 1131–1426 of AB207046) for *Cdc27B* (restriction sites are underlined). This cDNA fragment was cloned into the *Cla*I–*Sal*I and *Sal*I sites of the TRV-silencing vector, pTV00, and the multicloning site at contrary directions to the cauliflower mosaic virus 35S promoter, respectively (gifts from Prof. D. Baulcombe) (Ratcliff *et al.*, 2001). Sequences were confirmed using primers located on either side of the multicloning site (TRV-FWClaI and TRV-RVSalI corresponding to 5'-CACCGCCTCCAGCGAGTGGAG-3' and 5'-ACTTCAGGCACGGATCTACTT-3', respectively). TRV that contained no inserts was used as a control. Virus infection of *N. benthamiana* was achieved by *Agrobacterium*-mediated transient gene expression of infectious constructs from the T-DNA of a binary plasmid (pTV00 and pBINTRA6). These binary plasmids were transformed into *Agrobacterium*, which harbors pSoup (Hellens *et al.*, 2000). The *Agrobacterium* was injected into the abaxial side of leaves of 5- to 6-week-old *N. benthamiana* plants using a syringe. Synthesis of viral RNA from the construct and inoculation of *N. benthamiana* leaves were carried out as described by Ratcliff *et al*. (2001). After approximately 20 days, one of the fourth to eighth leaves from top of each plant was checked for silenced levels of the target genes.

Preparation of specimens for optical and electron microscopy

Samples of leaves were cut into small pieces (~3 × 4 mm) with a razor blade. These samples were then prefixed with 2.5% glutaraldehyde in 60 mM sodium phosphate buffer (pH 7.4) at 4˚C overnight before being postfixed with 1% buffered OsO_4 at room temperature for 1 h. The fixed specimens were dehydrated in a graded series of ethanol (10–100%) before being embedded in Quetol 651 resin mixture (Nisshin EM, Tokyo, Japan). For optical microscopy, thin (700-nm-thick) sections that were cut from the resin-embedded material using a diamond or glass knife were stained with a solution of 0.6% toluidine blue. These sections were examined under an inverted optical microscope (IX70; Olympus, Tokyo, Japan). For electron microscopy, ultrathin (70-nm-thick) sections that were cut from the resin-embedded material using a diamond knife were stained with 4% uranyl acetate for 10 min before being counterstained with a solution of lead for 10 min. These sections were examined with a transmission electron microscope (H-7500; Hitachi, Tokyo, Japan).

Semiquantitative RT-PCR

To detect the mRNAs of defense genes that were expressed in INF1 elicitin-inoculated *N. benthamiana* leaves, 2 μg of total RNA extracted from each leaf were used for semiquantitative RT-PCR (Chelly and Kahn, 1994; Inagaki *et al*., 1999). The first-strand cDNA was synthesized from total RNA (2 μg) and was then diluted 10-fold. After the synthesis of cDNA, PCR was performed with denaturing (1 min) at 95˚C, annealing (1 min) at 53˚C, and extension (45 s) at 72˚C. the PCR primers and the number of PCR cycles used to amplify the various genes were as follows: 5’-GGACGACATCAGTCCTAGGC-3’, 5’-GCAATTACCCATAGCACACC-3’, and 30 cycles for *NbCdc27B* (accession number AB207048); 5’-GTCGGGCGGTCCTGCTGACT-3’, 5’-ATTTGTAACACCCGCCACTG-3’, and 28 cycles for the *Coat Protein* of TRV (accession number Z36974); 5’-AATATCCCACTCTTGCCG-3’, 5’-CCTGGAGGATCATAGTTG-3’, and 28 cycles for *NbPR1a* (Kim *et al*., 2003); 5’-CTTGTCTCTACGCTTCTC-3’, 5’-AACACGAACCGAGTTACG-3’, and 28 cycles for *NbPR1c* (Kim *et al*., 2003);

5’-ACCATCAGACCAAGATGT-3’, 5’-TGGCTAAGAGTGGAAGGT-3’, and 25 or 28 cycles for *NbPR2* (Kim *et al*., 2003); 5’-GAGCCATGCCGGAATCCAAT-3’, 5’-GCTACCAATCAAGATGGCATCTGG-3’, and 25 or 30 cycles for *NbHIN1* (Kim *et al*., 2003); 5’-CTTTGCCTTTCTTTGGCT-3’, 5’-GACATTTAGGACATTTGCTGC-3’, and 25 or 28 cycles for *NbSAR8.2a* (Kim *et al*., 2003); 5’-TGTGTCAGCCATGCTGATTG-3’, 5’-CCGATAGGACCGCACGAAAC-3’, and 28 cycles for *NbHSR203J* (Yoshioka *et al*., 2003); and 5’-CTTCCTACCTCAAGAAGGTAGGATACAAC-3’, 5’-TGCCTCCTGAAGAGCTTCGTGGTGCAT-3’, and 25 cycles for *NbEF1alpha*; (Yoshioka *et al*., 2003). We confirmed that genomic DNA was not amplified by RT-PCR using the same PCR conditions in the absence RTase (negative control).

Assay of lesion formation by *C. lagenarium*

C. lagenarium strain 104-T (a stock culture from the Laboratory of Plant Pathology at Kyoto University, Japan) was used as the wild-type strain. *C. lagenarium* exhibits pathogenicity against *N. benthamiana* and a penetration frequency of ~70% (Takano *et al*., 2006). The *C. lagenarium* culture was maintained on potato dextrose agar medium (3.9% w/v; Difco, USA) at 24°C in the dark. Conidia were obtained by gentle scraping of cultures that had been incubated for 5–7 days. Inoculation on the first to fourth leaves from top of the silenced *N. benthamiana* plants was performed as described by Takano *et al*. (1997). A 3-μl aliquot of conidial suspension (10^5 conidia ml^{-1}) was spotted onto the surface of the leaves of the silenced *N. benthamiana*. After inoculation, the leaves were kept in humid Petri dishes at 24°C under a light:dark cycle of 16:8 h. The number of lesions was counted 9 days after inoculation.

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Table 1. ***Number of lesions formed on the leaves of control and gene-silenced*** **N. benthamiana** ***plants in response to inoculation with*** **C. lagenarium.**

	Total number of spots[b]	Total number of lesions[b]	Frequency ± SE (%)[c]
Control (buffer)	189	102	54.0 ± 9.0
Control (TRV)	178	79	44.4 ± 11.0
TRV::Cdc27B[a]	83	11	13.3 ± 4.8

[a] Plants in which gene was silenced with TRV::Cdc27B. For each treatment, [b] five repeats were analyzed at least, and [c] means ± standard errors are shown.

Figure Legends

Fig. 1. Analysis of *Cdc27B* and defense-related gene expression and comparison with homologues. **A**, Expression patterns of *Cdc27B* and defense-related genes in BY-2 cells treated with crude INF1 elicitin (10 μg ml^{-1}). Samples were fixed 0, 3, 6, 9, or 12 h after treatment. Total RNA was isolated using TRIzol (Invitrogen, USA) and Northern blot hybridization was performed using *Cdc27B(N)* and defense-related genes as probes. Control RNA (sampled at time 0) was extracted from untreated BY-2 cells. **B**, *N. benthamiana* leaves were treated with crude INF1 elicitin (10 μg ml^{-1}) and samples were fixed 0, 3, 6, 9, or 12 h later. Total RNA was isolated using TRIzol method (Invitrogen) and was analyzed using semiquantitative RT-PCR. Control RNA (sampled at time 0) was extracted from *N. benthamiana* leaves as soon after the application of INF1 elicitin as possible. **C**, Alignment of the amino acid sequences of Cdc27B from several different organisms. The tree was constructed by the neighbor-joining method (Saitou and Nei, 1987) using GENETYX-MAC software (version 11.0.2, Software Development, Tokyo, Japan). The six Cdc27 homologues registered in the DNA database and the three newly characterized *Nicotiana* sequences were used to calculate the phylogenetic distances. The gene accession numbers are as follows: AtCdc27A, NM_112503; AtCdc27B, AJ487669; CeCdc27, AY081955; DmCdc27, U18298; HsCdc27, XM_067035; and ScCdc27, NC_001134. The scale bar at the bottom indicates 0.1 amino acid substitutions per site. **D**, Comparison of Cdc27 amino acid sequences. The open boxes indicate TPR domains. Abbreviations: Dm, *Drosophila melanogaster*; Sc, *Saccharomyces cerevisiae*; Ce, *C. elegans*; Hs, *Homo sapiens*; Nt, *N. tabacum*; Nb, *N. benthamiana*; At, *A. thaliana*.

Fig. 2. Plant and leaf-cell phenotypes of *N. benthamiana* after silencing with TRV::Cdc27B. **A**, Constructions of plasmids used for gene silencing. The structure of pTV00::Cdc27B. Abbreviations: P35S, cauliflower mosaic virus 35S promoter; CP, coat protein; Tnos, transcriptional terminator of nopaline synthase. The polylinker includes *Cla*I and *Sal*I sites (Ratcliff *et al*., 2001; see Experimental procedures). **B**, Phenotypes of *N. benthamiana* plants after silencing

with TRV::Cdc27B. **C**, Fixed, toluidine blue-stained samples of the leaves of each type of gene-silenced plant (upper and lower). Magnified view of representative cells from the leaves of each type of gene-silenced plant (lower). The scale bar represents 20 μm. The outline in the upper and right panel indicates typical failed cell.

Fig. 3. Failed cells in plants silenced with TRV::Cdc27B. A–C, Thin-layer sections of fixed specimens of stained with toluidine blue illustrating the presence of failed cells within the mesophyll of leaves from plants silenced with TRV::Cdc27B. The scale bars in A–C represent 20 μm. The electron micrographs in D–G revealed failed cells within the mesophyll of leaves from plants silenced with TRV::Cdc27B. The scale bars in D and F represent 10 μm. The scale bars in E and G represent 1 μm. Abbreviations: ch, chloroplast; c, crystal-like material; cw, cell wall; m, mitochondria; n, nucleus; v, vacuole. Micrographs E, and G are magnified views of the areas within the open boxes shown in D and F.

Fig. 4. Defense responses and resistance to the virulent fungus *C. lagenarium* in gene-silenced plants. **A**, Effect of suppression of *Cdc27B* on elicitin-induced HCD. Blue, red, and yellow indicate typical phenotypes of leaf disks in the following categories: blue indicates no staining, red indicates slight trypan blue-staining, and yellow indicates intense trypan blue-staining. Percentage of the y-axis indicates leaf disk number of each phenotype per total number of leaf disk. Leaves from gene-silenced plants were treated with crude INF1 elicitin protein (25 μg ml^{-1}) for 24 h, after which 8-mm-wide disks were stamped out of the leaves and stained with trypan blue. A total of 22 samples were obtained from 3 independent plants silenced with TRV (control), 58 samples were obtained from 6 independent plants silenced with TRV::Cdc27B. **B**, Defense-related gene expression in gene-silenced *N. benthamiana* plants. The third or fourth leaves from the top of gene-silenced *N. benthamiana* plants were fixed 20 days after TRV infection to check the status of

the gene silencing. Total leaf RNA was isolated using TRIzol (Invitrogen) and semiquantitative RT-PCR was performed with *Cdc27B*, *CP* of *TRV*, defense-related genes, and *EF1alpha* as a control. **C**, Lesion formation in response to infection on the leaves of the gene-silenced *N. benthamiana* plants with *C. lagenarium* strain 104-T. A 3-μl aliquot of conidial suspension (10^5 conidia ml^{-1}) was spotted onto the surface of the leaves, and the number of lesions that formed subsequently was counted 9 days later. **D**, Infection of *C. lagenarium* is observed under microscope. Hypha of *C. lagenarium* was stained with trypan blue. The microscope photographs of infection were taken 4 days after inoculation. Samples were stained with trypan blue.