

Overexpression of tobacco ethylene response factor *NtERF3* gene and its homologues from tobacco and rice induces hypersensitive response-like cell death in tobacco

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Abstract Among plant-specific transcription factors, ethylene response factors (ERFs) comprise one of the largest families. *ERFs* are unique to the plant kingdom and are considered to have crucial roles in plant response to various biotic and abiotic environmental stresses. Here, we report on the functional analysis of a transcriptional repressor, *NtERF3*, with regard to cell death associated with a hypersensitive response (HR), a plant-specific resistance reaction against pathogens. Expression of *NtERF3* was upregulated during HR induction by *Tobacco mosaic virus* (TMV) infection in tobacco plants harboring the resistance *N* gene to TMV. Transient overexpression of *NtERF3* by *Agrobacterium*-mediated gene delivery induced HR-like cell death in tobacco, associated with the production of reactive oxygen species and ion leakage. Deletion of the ERF-associated amphiphilic repression (EAR) motif from *NtERF3* resulted in no induction of cell death, while the deletion had no effect on nuclear localization of the proteins. After virus-mediated gene delivery, similar results also were observed in tobacco without the *N* gene. In addition to *NtERF3*, other EAR motif-containing ERFs from tobacco, *Nicotiana benthamiana* and rice also induced cell death when overproduced in tobacco

plants. The results suggested that many *ERF* genes encoding EAR motif-containing proteins might have the ability to induce cell death when overexpressed.

Keywords TMV · *N* gene · Hypersensitive response · HR cell death · *NtERF3* · EAR motif

Introduction

Because plants are continuously exposed to environmental changes and stresses and unable to escape the stress, it is important for them to respond to external stimuli in a timely fashion. Plants have evolved many plant-specific transcription factors to precisely regulate the expression of their genes. Among them, the AP2/ERF superfamily is one of the largest families of transcription factors in plants (Nakano et al. 2006; Zhang et al. 2008; Zhuang et al. 2011). Ethylene response factors (ERFs) bind with high affinity to the conserved promoter sequence, called the GCC-box through the highly conserved AP2/ERF domain (Hao et al. 1998; Ohme-Takagi and Shinshi 1995). The GCC-box is a short conserved *cis*-element found in the promoter regions of several ethylene-responsive genes, such as the gene for basic PR protein (Ohme-Takagi et al. 2000; Shinshi et al. 1995). There have been many reports of the involvement of ERFs in responses to biotic and abiotic stresses as well as in plant hormone signaling. ERFs are also reported to be involved in resistance against pathogens. Examples include an increase or a decrease in disease resistance in *ERF*-expressing transgenic plants (Berrocal-Lobo et al. 2002; Bethke et al. 2009; Century et al. 2008; Fischer and Dröge-Laser 2004; Gu et al. 2002; McGrath et al. 2005; Park et al. 2001; Zhang et al. 2009). ERFs seem to have important functions in transcriptional

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regulation of various responses to biotic or abiotic stresses (Century et al. 2008; Nakano et al. 2006).

Plants have evolved resistance (*R*) genes in response to biotic stresses such as infection by pathogens. *R* gene products recognize elicitors derived from pathogens and induce host resistance processes such as hypersensitive response (HR) (Jones and Dangl 2006; Soosaar et al. 2005). The *N* gene of *Nicotiana tabacum* (tobacco) is one of the TIR-NBS-LRR class of *R* genes (Whitham et al. 1994). When tobacco harboring the *N* gene (*NN* tobacco) is infected with *Tobacco mosaic virus* (TMV), the helicase domain (p50) of TMV replicase is recognized as an elicitor capable of inducing HR cell death, and under permissive temperatures, the viruses are confined to restricted necrotic areas. Some genes involved in the signal transduction pathway of HR cell death have the ability to induce HR-like cell death when ectopically overexpressed (Menke et al. 2005; Yang et al. 2001; Zhang and Liu 2001). Among ERF family genes, *NbCD1* gene from *Nicotiana benthamiana* was reported to induce HR-like cell death when overexpressed in *N. benthamiana* and *Arabidopsis thaliana* (Nasir et al. 2005). *NbCD1* is a transcriptional repressor that has a short, conserved ERF-associated amphiphilic repression (EAR) motif at its C-terminal region and is in the same class as tobacco *NtERF3* and *Arabidopsis AtERF3* and *AtERF4* (Nasir et al. 2005). *NtERF3* is one of four tobacco genes initially identified as *ERFs* (Ohme-Takagi and Shinshi 1995) and encodes an EAR motif-containing repressor protein (Ohta et al. 2000, 2001). The expression of *NtERF3* is upregulated rapidly and transiently by ethylene or salicylic acid treatment or by wounding at the transcriptional level (Nishiuchi et al. 2002, 2004; Suzuki et al. 1998). Because it is important to suppress the expression of genes when their product is not required, transcriptional repression of expression of other genes by repressor proteins such as *NtERF3* might play a key role in modulating plant stress responses (Kazan 2006). Although there have been some reports on the involvement of *NtERF3* in stress responses, whether *NtERF3* is involved in the signal transduction pathway to HR cell death is unknown. Though *NbCD1* is in the same class as *NtERF3*, regions outside the conserved domains and motifs are quite different from each other, and the amino acid identity between entire proteins is not very high (45%) (Nasir et al. 2005). This suggests that *NtERF3* is not an orthologue of *NbCD1*. Here, we report on the analysis of *NtERF3* function in HR-mediated cell death. We first characterized the expression of *NtERF3* during HR induction by TMV infection or ectopic expression of TMV p50 in *NN* tobacco. Then, we examined whether overexpression of *NtERF3* or its homologues from tobacco, *N. benthamiana* and rice would induce HR-like cell death in tobacco plants.

Materials and methods

Plant growth conditions and TMV treatment

Tobacco plants (*N. tabacum* cv. Samsun NN and nn) and *N. benthamiana* plants were grown on Rockfiber blocks (Nittobo, Tokyo, Japan) at 25°C with a 16 h light/8 h dark photoperiod for 2 weeks. Seedlings were then transferred to pots filled with vermiculite and grown in the same conditions with 0.1% (v/v) Hyponex fertilizer solution (Hyponex Japan, Osaka, Japan) added once a week. Plants at 8- to 10-weeks-old after sowing were used for all experiments. *Oryza sativa* cv. Nipponbare plants were grown as described previously (Hirabayashi et al. 2004).

For the induction of HR from the *N* gene, 25 µl of 100 ng/µl virion of the TMV-OM strain was inoculated onto leaves of *NN* tobacco mechanically with carborundum. The inoculated plants were incubated at 30°C for 40 h then all plants were shifted to 20°C to synchronously induce HR. Nonresistant *nn* tobacco plants were used as non-HR controls. As a control, the same volume of 10 mM sodium phosphate buffer (pH 7.0) was used as a mock inoculation solution.

Cloning of *NtERF3b*, *NbERF3*, *NtERF6a* and *NtERF6b*

NtERF3b, *NtERF6a* and *NtERF6b* were cloned from *N. tabacum* cv. Samsun NN while *NbERF3* was cloned from *N. benthamiana*. Details of the cloning process are described in supplementary materials. Nucleotide sequences of *NtERF3b*, *NbERF3*, *NtERF6a* and *NtERF6b* were submitted to DDBJ under the accession numbers AB573716, AB573717, AB573718 and AB573719, respectively.

Construction of plasmids

The details of construction of plasmids used in this study are described in the supplementary materials.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated using the RNAiso reagent (Takara BIO, Shiga, Japan) according to the manufacturer's instructions. cDNA was synthesized from total RNA with Oligo dT Primer and Random 6-mers using PrimeScript RT Reagent Kit (Takara BIO). Semi-quantitative expression analysis was performed by RT-PCR with GoTaq Green Master Mix (Promega, Madison, WI, USA). The cDNA for *Actin* was amplified as an internal control. Specific oligonucleotides for each gene were as follows: *Actin* (5'-GGGTTTGCTGGAGATGATGCT-3' and 5'-GCCTTTGCCAATCCACATCTGTTG-3'), *Hsr203J* (5'-CACCCCTATCGG

AGCAAATCGGAG-3' and 5'-GATGAACTCTGCAACG GCTTC-3'), *Hin-1* (5'-CACCGACCGATGCTACGTTG ACTC-3' and 5'-CTACCAATCAAGATGGCATCTG-3'), *acidic PR-1* (5'-TAGTCATGGGATTTGTTCTC-3' and 5'-TCAGATCATAACATCAAGCTG-3'), and *basic PR-1* (5'-GGGATACTCCACAACATTAG-3' and 5'-CACA TACATATACACACCTC-3'). Real-time quantitative PCR analysis was performed with the Smart Cycler II system (Takara BIO) using SYBR Premix Ex Taq (Takara BIO). The cDNA for *Actin* was amplified as an internal control to normalize the expression of each gene. The following oligonucleotides were used to detect the expression of each gene: *Actin* (5'-GGGTTTGCTGGAGATGATGCT-3' and 5'-GCTTCGTCACCAACATATGCAT-3'), *NtERF3* (5'-AGGAATTGATCTTGATCTTAAC-3' and 5'-ACAAAAT TCAACCATTAGTCTC-3'), *NtWRKY1* (5'-GATGTTACA GAGCTCTGGAAATTC-3' and 5'-TCGGCTTGATAT TATTCATGGGC-3'), and *PR-1b* (5'-CAATAGGTTAGC GGCCTTTG-3' and 5'-TTGCTTCTCATCAACCCACA-3').

Agrobacterium infiltration

Agrobacterium tumefaciens strain GV3101 (pMP90) was transformed with expression plasmids and tobacco leaves were infiltrated with bacteria essentially as described previously (Sasaki et al. 2009). The final concentrations of the bacteria were adjusted to an OD₆₀₀ of 0.4 with infiltration buffer containing 10 mM MES, pH 5.7, 10 mM MgCl₂ and 150 μM acetosyringone. The plants were kept at 20°C after *Agrobacterium* infiltration.

Determination of ion leakage

Leaf materials were excised from the infiltrated plants and soaked in H₂O at a ratio of 1 ml H₂O per 1 cm² of leaf materials. After incubation at 20°C for the indicated duration, conductivity of the water was measured using a conductivity meter (model B-173, Horiba, Kyoto, Japan) and was expressed as μS cm⁻¹ cm⁻² of leaf materials.

Measurement of reactive oxygen species (ROS) generation

ROS were detected using 8-amino-5-chloro-7-phenylpyrido [3,4-*d*] pyridazine-1,4-(2*H*, 3*H*) dione sodium salt (L-012) (Wako Chemical, Kanagawa, Japan). Tobacco leaves were infiltrated with L-012 solution (0.5 mM L-012, 10 mM MOPS-KOH, pH 7.4) using a needleless syringe to fill the *Agrobacterium*-infiltrated areas. After 5 min, chemiluminescence of the L-012-infiltrated leaves was measured for 5 min using LAS-3000 system (FujiFilm, Tokyo, Japan). The intensity of the chemiluminescence was converted into numerical values using MultiGauge ver.

2.2 analysis software (FujiFilm). The relative intensity per unit area of the *Agrobacterium*-infiltrated region was calculated after the subtraction of the background fluorescence value of the non-*Agrobacterium*-infiltrated regions from the treated leaves.

Recombinant virus inoculation

Template plasmids pTogJ-NtERF3bΔEAR-DHA, pTogJ-NtERF3b-DHA and the control plasmid pTocJ-GFP (Hori and Watanabe 2003) were digested with *Mlu*I and purified with UltraClean15 DNA Purification Kit (MO BIO Laboratories, Carlsbad, CA, USA). One microgram of the purified DNAs was subjected to in vitro transcription in a volume of 25 μl with T7 RNA polymerase as described (Hori and Watanabe 2003). Leaves of *nn* tobacco and *N. benthamiana* were mechanically inoculated with 8 μl of the transcripts using carborundum.

Imaging by fluorescence microscopy

GFP fluorescence in *Agrobacterium*-infiltrated leaf tissues was observed with a BX50 fluorescence microscope equipped with a UMWIBA/GFP filter unit (OLYMPUS, Tokyo, Japan). For staining the nucleus, leaves that were sampled at 32 h after *Agrobacterium* infiltration were infiltrated with 10 μg/ml of 4',6-diamidino-2-phenylindole (DAPI). After incubation for 30 min to 1 h, the DAPI-stained leaves were observed with the fluorescence microscope and filter unit U-MWU (OLYMPUS). Images were taken with a DP71 digital camera (with DP Controller ver. 3.1.1 and DP Manager ver. 3.1.1, OLYMPUS).

Results

Expression of *NtERF3* is upregulated during HR cell death induction by *N* gene

Nicotiana tabacum is thought to be an amphiploid derived from *Nicotiana sylvestris* and *Nicotiana tomentosiformis* (Murad et al. 2002). Therefore, it was thought that the tobacco genome contained two types of *NtERF3* genes, one from each ancestor. To characterize the expression of *NtERF3* during HR induction, we first isolated each type of cDNA for *NtERF3* from *N. tabacum* by utilizing DNA sequence information for *NtERF3* (DDBJ accession D38124) from *N. tabacum* and *NsERF3* (DDBJ accession AB016265) from *N. sylvestris*. One type of cDNA was 100% identical to *NtERF3* (DDBJ accession D38124) at the nucleotide level, while the other type was almost identical to *NsERF3* with a single-base difference in the protein-coding region. To distinguish the two types of

NtERF3, a novel *N. sylvestris*-type of *NtERF3* was designated as *NtERF3b* (deposited in DDBJ as accession AB573716), and the other type of *NtERF3* (DDBJ accession D38124) called *NtERF3a* in this work. The deduced amino acid sequences had a single-amino acid difference between *NtERF3b* and *NsERF3*, and 16 amino acids differing (94% identity) between *NtERF3a* and *NtERF3b*, including the insertion of two amino acids in *NtERF3b*. On the basis of the nucleotide sequence data, a set of oligonucleotides was prepared to amplify both *NtERF3a* and *NtERF3b*.

For HR induction, we used a combination of TMV and its specific resistance gene *N* as a model system. The HR controlled by the *N* gene is temperature-dependent and does not occur under nonpermissive temperatures. This feature is often exploited to uniformly and synchronously induce HR in several samples (Takabatake et al. 2007). Using a synchronous HR-induction system based on a temperature shift from 30°C, a nonpermissive temperature for the *N* gene, to 20°C, a permissive temperature, we investigated the expression level of *NtERF3* during HR induction. In the *N* gene-containing *NN* tobacco infected with TMV, cell death lesions became visible from 5 to 7.5 h after the temperature shift (data not shown), and ion leakage, an indicator of cell death, also increased from 5 to 7.5 h after the temperature shift (Fig. 1a). In contrast, no cell death was observed, and ion leakage did not significantly change in either TMV-infected *nn* tobacco or mock-inoculated *NN* tobacco (Fig. 1a). Gene expression of *NtERF3* was upregulated significantly from 2.5 to 5 h after the temperature shift, and this increased expression state lasted during the cell death progression only in TMV-infected *NN* tobacco (Fig. 1b).

The expression levels of *NtWRKY1* and *PR-1b* genes were also examined because it was known that both genes were upregulated during HR and that the promoter region of *NsERF3* contains a *cis*-element, the W-box, to which *NtWRKY1* binds in vitro (Nishiuchi et al. 2004). Both genes were upregulated during the progression of cell death only in the TMV-infected *NN* tobacco, as in the case of *NtERF3* (Fig. 1c, d). Upregulation of *NtWRKY1* was comparable to that of *NtERF3*, while *PR-1b* was upregulated later than *NtERF3* (Fig. 1b–d). *Agrobacterium*-mediated ectopic expression of the TMV *p50*, an elicitor to the *N* gene, is also known to induce HR cell death in *NN* tobacco (Erickson et al. 1999). We used this *Agrobacterium*-infiltration method for the induction of HR. In this system, cell death lesions became visible from 36 to 48 h after the infiltration of *p50* (data not shown). The mRNA level of *NtERF3* in the *p50*-expressing *NN* tobacco was higher at 32 h after

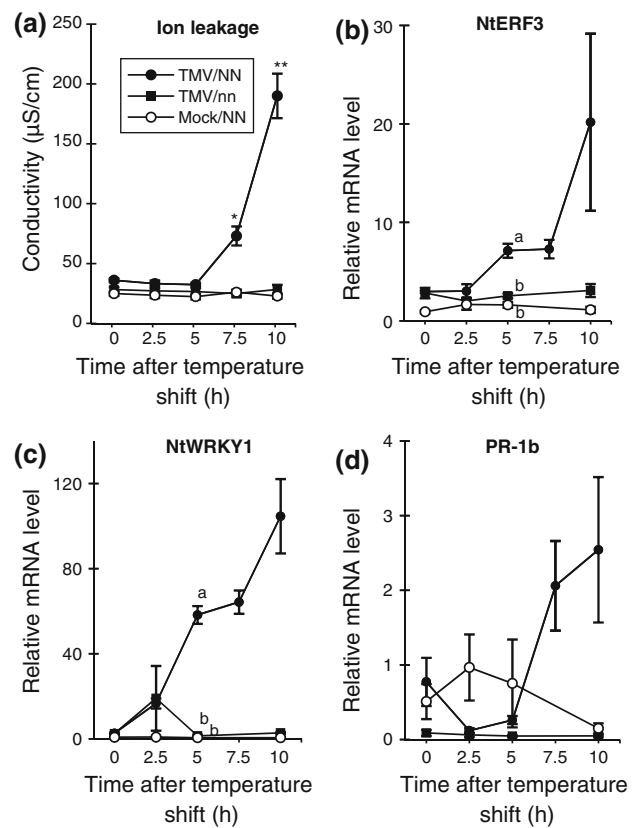


Fig. 1 Upregulation of *NtERF3* during hypersensitive response induction by the *N* gene. **a** Ion leakage from tobacco leaves inoculated with *Tobacco mosaic virus* (TMV) or buffer (TMV/NN, TMV/nn and Mock/NN) after the temperature shift from 30 to 20°C. Leaf materials were excised at the indicated time points after the temperature shift, soaked in water for 30 min, and conductivity of the water was measured. Values are presented as mean \pm SE ($n = 6$), and significant differences between data at each time point with respect to 0 h were assessed with Dunnett's test; * $P < 0.05$, ** $P < 0.01$. Black and white circles in **a–d** indicate TMV/NN and Mock/NN, and black squares indicate TMV/nn, respectively. Real-time PCR analysis of *NtERF3* (**b**), *NtWRKY1* (**c**) and *PR-1b* (**d**) expression at the indicated time points after the temperature shift. Values are mean \pm SE ($n = 6$) relative to mRNA levels in untreated *NN* tobacco (untreated *NN* tobacco; set arbitrarily at 1). Relative mRNA levels were normalized to the expression of *Actin* gene measured in the same samples. Different letters (a, b) at 5 h indicate significant differences among the three plants as determined by Tukey's test ($P < 0.01$)

infiltration than that in the control *sGFP*-expressing or mock-infiltrated *NN* tobacco, in which HR had not been induced (Fig. S1a). *NtERF3* was known to be one of the wound-induced genes whose expression was induced rapidly and suppressed to the normal level soon afterward (Nishiuchi et al. 2002, 2004; Suzuki et al. 1998). The expression levels of *NtERF3* in the wound-treated *NN* tobacco increased about 30-fold by 30 min following treatment relative to the untreated control tobacco (Fig. S1b).

Overexpression of *NtERF3* induces HR-like cell death in tobacco

NtERF3a and *NtERF3b* were transiently overexpressed in tobacco treated by the *Agrobacterium*-infiltration method. Overexpression of *NtERF3a* and *NtERF3b* induced cell death, just as did the TMV elicitor (*p50*) in *NN* tobacco (Fig. 2a, *NtERF3a*, *NtERF3b* and *p50*). Addition of an HA-tag at the C-terminus did not affect cell death induction (*NtERF3a::HA* and *NtERF3b::HA*). In contrast, deletion of the EAR motif from *NtERF3b* (*NtERF3b-ΔEAR::HA*) resulted in no induction of cell death, just as with inoculation with *sGFP*, empty vector (Empty vector) or mock infiltration (Mock). Similar results were obtained in *nn* tobacco, in which, due to the absence of *N* gene, *p50* did not induce cell death (Fig. S2a). Overexpression of *NtERF3a* and *NtERF3b* in *N. benthamiana* also induced cell death, although the manifestation of cell death was not as clear as that in tobacco (data not shown). HA-tagged proteins (*NtERF3a::HA*, *NtERF3b::HA* and *NtERF3b-*

ΔEAR::HA) became detectable at 24 h following infiltration into *NN* tobacco, and their level continued to increase (Fig. S2b), while cell death lesions became visible from 36 to 48 h after the infiltration of *NtERF3a::HA*- or *NtERF3b::HA*-expressing *Agrobacterium* (data not shown). It is known that HR cell death is associated with the production of reactive oxygen species (ROS) and an increase in ion leakage (Jones and Dangl 2006; Soosaar et al. 2005; Takabatake et al. 2007). Cell death induced by overexpression of *NtERF3s* was associated with an increase of ion leakage (Fig. 2b) and ROS production (Fig. 2c), as in the case of HR cell death induced by the TMV *p50* in *NN* tobacco. In addition, expression of marker genes for the HR was upregulated in the *Agrobacterium*-infiltrated regions of *NtERF3a*- and *p50*-treated leaves, but not in those of *sGFP*-treated leaves (Fig. 2d).

To further examine the ability of *NtERF3* to induce cell death, we used a virus vector, pTogJ (Hori and Watanabe 2003), as another system for ectopic gene expression. The recombinant virus RNA encoding *NtERF3b* (Fig. 3a, b)

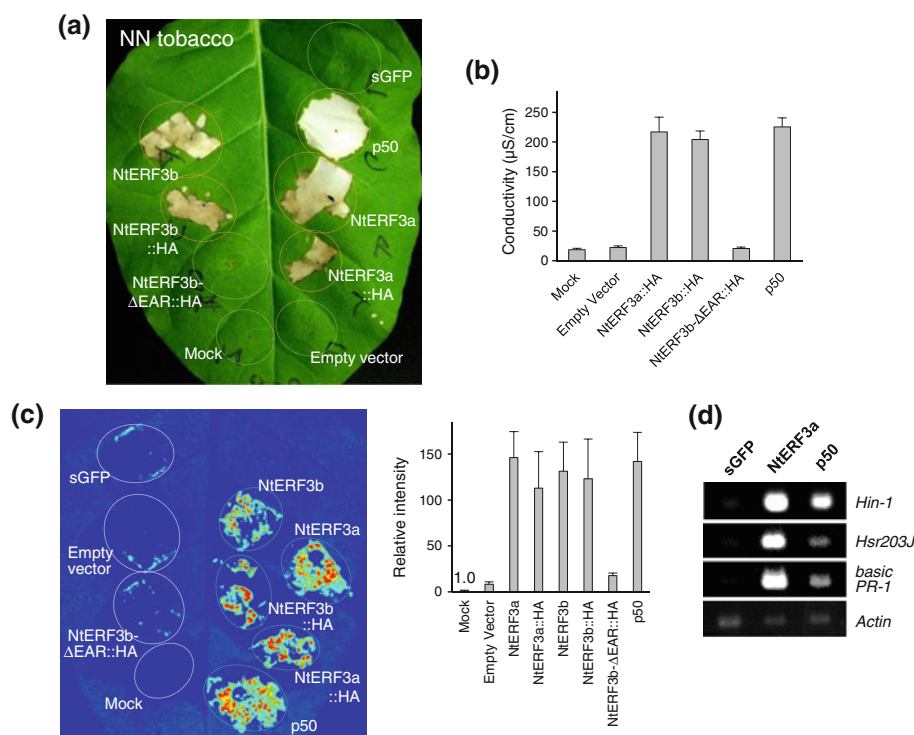


Fig. 2 Hypersensitive response (HR)-like cell death induction by *Agrobacterium*-mediated transient overexpression of *NtERF3* in tobacco. **a** *NtERF3a*, *NtERF3b* and their HA-tag-fused derivatives were transiently expressed in *NN* tobacco by *Agrobacterium* infiltration. Empty vector, *sGFP* and the infiltration buffer (Mock) were used as negative controls. The helicase domain of *Tobacco mosaic virus* (TMV) replicase (*p50*), the elicitor from the *N* gene, was used as a positive control for induction of HR cell death. The photograph was taken at 7 days after infiltration. Circles generally indicate each infiltrated area. **b** Ion leakage from tobacco leaves at 48 h after *Agrobacterium* infiltration. Leaf materials were excised and soaked in

water for 4 h, and conductivity of the water was measured. Values are mean \pm SE ($n = 5$). **c** Production of reactive oxygen species (ROS) in *NN* tobacco leaves at 32 h after *Agrobacterium* infiltration. The image of chemiluminescence due to ROS production is shown on the left panel. Circles generally indicate each infiltrated area. Relative intensities of chemiluminescence at each infiltrated area are shown on the right graph. Values are mean \pm SE ($n = 5$) relative to the value of mock-infiltrated region (Mock; set arbitrary at 1). **d** RT-PCR analysis of the expression of *Hin-1*, *Hsr203J* and *basic PR-1* in *NN* tobacco at 42 h after *Agrobacterium* infiltration. Expression of *Actin* was investigated as a control

induced cell death lesions in the leaves of *nn* tobacco and *N. benthamiana*, while those encoding NtERF3b without the EAR motif (Fig. 3d, e) and GFP (Fig. 3g, h) did not induce cell death. In *nn* tobacco and *N. benthamiana* inoculated with the NtERF3b-encoding recombinant virus RNA, cell death lesions became visible at 3–4 days after inoculation, while in those inoculated with GFP-encoding recombinant virus RNA, GFP fluorescence was confirmed at 3–4 days after inoculation (data not shown). Because pTogJ virus vector encodes TMV replicase, the recombinant viruses are able to induce *N* gene-mediated HR cell death in *NN* tobacco. This was utilized to confirm the infection of each recombinant virus in *nn* tobacco. Crude extracts prepared from the leaves of *nn* tobacco inoculated with each recombinant virus RNA (Fig. 3a, d, g) were secondarily inoculated to *NN* tobacco. Such inoculation resulted in the appearance of cell death lesions (Fig. 3c, f, i). Expression of the marker genes for HR was upregulated in the leaves inoculated with the NtERF3b-encoding recombinant virus RNA, but was not in those inoculated with the GFP-encoding recombinant virus RNA (Fig. 3j).

EAR motif of *NtERF3* is essential for cell death induction but not for NtERF3 subcellular site

The EAR motif has been shown to function as a repression motif in some ERF proteins and C2H2 zinc-finger proteins (Ohta et al. 2001). In Figs. 2 and 3, the deletion of the EAR motif from NtERF3b was shown to affect the induction of cell death. Although it was previously reported that NtERF3 localized to nuclei in tobacco BY-2 cell (Ohta et al. 2000), the influence of its EAR-motif on subcellular localization has not been comprehended. To examine whether deletion of the EAR motif would affect the subcellular localization of the NtERF3 protein, expression plasmids for the sGFP-fused proteins NtERF3b::sGFP and NtERF3b-ΔEAR::sGFP were constructed and transiently expressed in *NN* tobacco by the *Agrobacterium*-infiltration method. At 32 h after *Agrobacterium* infiltration, GFP fluorescence was observed exclusively in the nuclei of the NtERF3b::sGFP- and NtERF3b-ΔEAR::sGFP-producing cells, but not in those of the in sGFP-producing cells (Fig. 4a). In addition, attachment of the EAR motif to the C-terminus of sGFP had no effect on the subcellular localization of the protein (Fig. 4b). Cell death lesions became visible in the *Agrobacterium*-infiltrated area of NtERF3b::sGFP leaves, but not in those of NtERF3b-ΔEAR::sGFP nor sGFP leaves at 36 h after the infiltration (data not shown), and the cell death lesions induced by NtERF3b::sGFP became more distinct (Fig. 4c). Reattachment of the EAR motif to the C-terminus of the EAR motif-deleted mutant restored the ability of the protein to induce cell death (Fig. 4d, NtERF3b-ΔEAR::EAR::HA). When the EAR motif was fused to the wild type NtERF3b, the cell

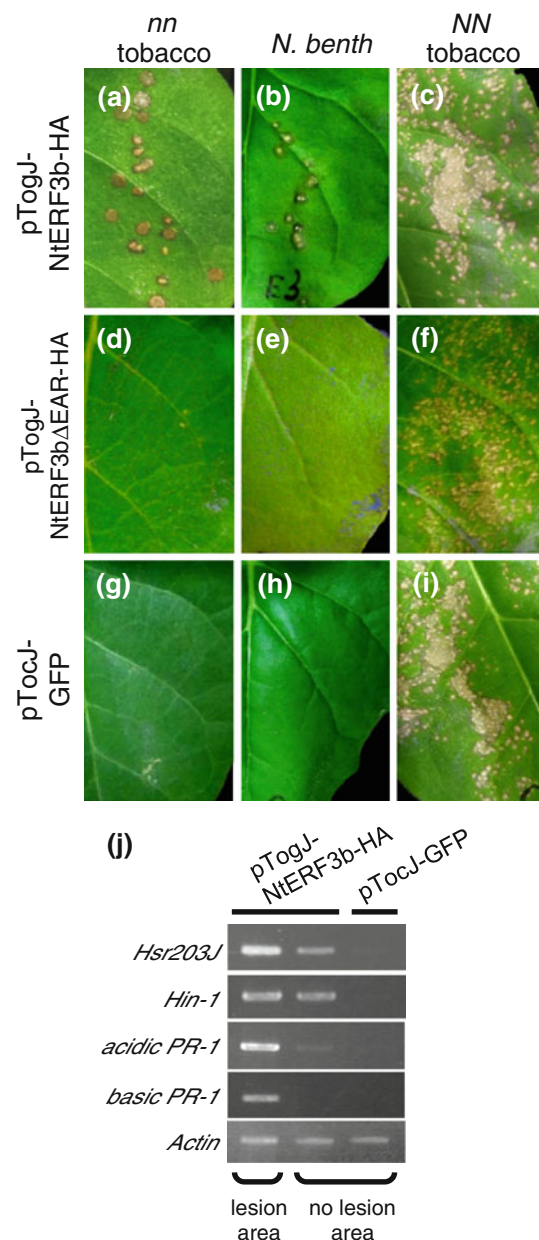


Fig. 3 Hypersensitive response-like cell death induction by virus vector-mediated overexpression of *NtERF3*. Leaves of *nn* tobacco (a, d, g) and *Nicotiana benthamiana* (*N. benthamiana*) (b, e, h) were inoculated with in vitro transcripts derived from pTogJ-NtERF3b-DHA (a, b), pTogJ-NtERF3bΔEAR-HA (d, e) and pTocJ-GFP (g, h). Crude extracts prepared from inoculated leaves of *nn* tobacco were secondarily inoculated to *NN* tobacco (c, f, i). Photographs were taken at 7 or 6 days after the inoculation of tobacco or *N. benthamiana*, respectively. **j** Expression analysis of *Hsr203J*, *Hin-1*, *acidic PR-1* and *basic PR-1* by RT-PCR in *nn* tobacco at 7 days after the inoculation of in vitro transcripts from TogJ plasmids. For pTogJ-NtERF3b-HA, leaf materials were prepared from areas with (lesion area) or without (no lesion area) cell death lesions. Expression of *Actin* was examined as a control

death-inducing ability of the resultant protein NtERF3b::EAR::HA was comparable to that of NtERF3b::HA (Fig. 4d). In contrast, the ectopic expression of the EAR

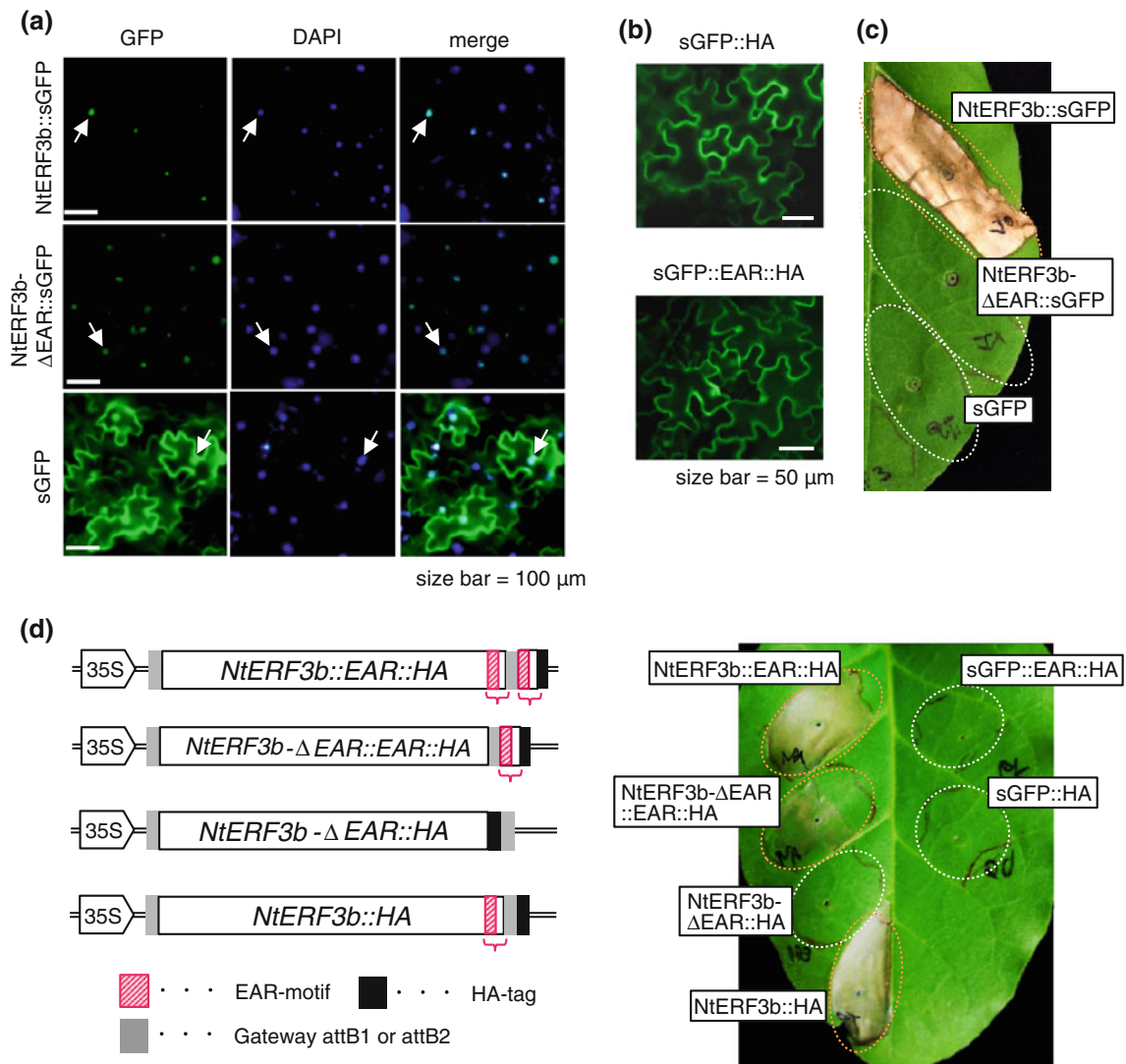


Fig. 4 Nuclear localization and cell death-inducing ability of the EAR motif-deleted NtERF3. **a** *NtERF3b::sGFP*, *NtERF3b-ΔEAR::sGFP* or *sGFP* were transiently overexpressed in tobacco leaves by *Agrobacterium* infiltration. GFP and DAPI fluorescence were observed by microscopy at 32 h after the infiltration. Both fluorescent images are merged in the *third row* (merge). *White arrows* indicate one of the images for nuclei showing the fluorescence of GFP or DAPI. **b** *sGFP::HA* and *sGFP::EAR::HA* were expressed in tobacco leaves, and GFP fluorescence was observed at 3 days after

Agrobacterium infiltration. **c** Photograph of leaf in **a** was taken at 7 days after the infiltration. **d** *NtERF3b::EAR::HA*, *NtERF3b-ΔEAR::EAR::HA*, *NtERF3b-ΔEAR::HA*, *NtERF3b::HA*, *sGFP::EAR::HA* or *sGFP::HA*, were overexpressed by *Agrobacterium* infiltration. Photograph was taken at 3 days after the infiltration. Schematic drawing of plasmid constructs is on the *left*. *Boxes with slanted lines* depict the EAR motifs, while *black and gray boxes* depict HA-tags and the additional amino acids derived from the attB Gateway recombination sequences, respectively

motif-fused (*sGFP::EAR::HA*) or non-fused *sGFP* (*sGFP::HA*), as well as the EAR motif-deleted NtERF3b (*NtERF3b-ΔEAR::HA*) induced no visible change at the *Agrobacterium*-infiltrated area at 3 days after the infiltration (Fig. 4d).

NtERF3 homologues from tobacco, *N. benthamiana* and rice induce cell death in tobacco

The deduced amino acid sequences of NtERF3a and NbCD1 were highly conserved in the AP2/ERF domain

and EAR motif, but not in other regions (Fig. S3). Therefore, genes with higher identity to *NbCD1* than *NtERF3a* and *NtERF3b* were expected in the genome of *N. tabacum*, and vice versa for *N. benthamiana*. Such genes were isolated by RT-PCR using the oligonucleotides designed based on the nucleotide sequence information of *NbCD1*, *NtERF3a* and *NtERF3b*. Two kinds of *NbCD1* homologues were isolated from *N. tabacum* and named *NtERF6a* and *NtERF6b*, respectively, while one type of *NtERF3* homologue was isolated from *N. benthamiana* and named *NbERF3*. In contrast to the amino acid identity of NtERF3a

and NtERF3b to NbCD1 (45 and 40%, respectively), NtERF6a and NtERF6b were 89 and 89% identical to NbCD1, respectively, while NbERF3 was 93 and 92% identical to NtERF3a and NtERF3b, respectively (Fig. S3). When these newly isolated genes were transiently overexpressed in tobacco after *Agrobacterium* infiltration, HR-like cell death was induced, as in the case of *NtERF3a* and *NbCD1* (Fig. 5a, b). The results raised the question whether other *ERF* genes encoding EAR motif-containing proteins would have the ability to induce cell death when overexpressed, because many *ERF* genes encoding the EAR motif-containing proteins have been reported for various plant species (Nakano et al. 2006; Ohta et al. 2001; Zhang et al. 2008). Two genes, *OsERF#074* (DDBJ accession AK111755) and *OsERF#075* (DDBJ accession AK069262, reported as *OsERF3*) were isolated from a monocotyledonous plant, *O. sativa*, based on nucleotide sequence information (Nakano et al. 2006). Transient overexpression of *OsERF#074* and *OsERF#075* by the *Agrobacterium*-infiltration method also induced HR-like cell death in tobacco (Fig. 5c).

Discussion

In this study, a tobacco transcriptional repressor NtERF3 was analyzed for its putative function in HR cell death. *NtERF3* was cloned from two types of tobacco *NtERF3* (*NtERF3a* and *NtERF3b*), one from each ancestor of amphiploid tobacco, *N. sylvestris* or *N. tomentosiformis*.

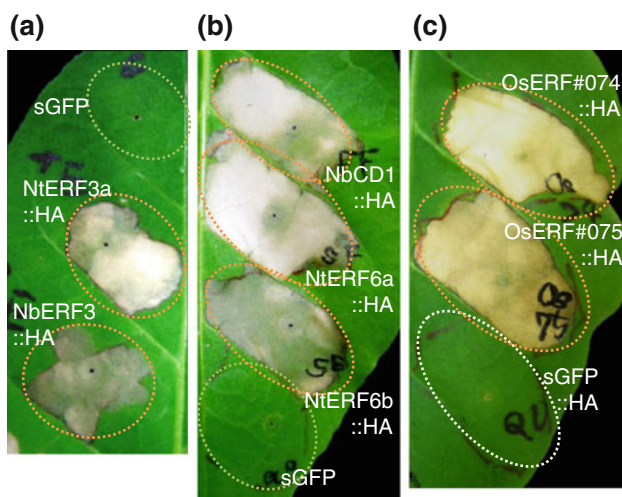


Fig. 5 Cell death induction by *NtERF3* homologues. HA-tag-fused derivatives of *Nicotiana benthamiana* *NbERF3* (a), tobacco *NtERF6a* and *NtERF6b* (b) and *Oryza sativa* *OsERF#074* and *OsERF#075* (c) were transiently expressed in *NN* tobacco leaves by *Agrobacterium* infiltration. *sGFP* and *sGFP::HA* were used as negative controls. Photographs were taken at 5 (b) or 7 days (a, c) after the infiltration, respectively

Transient overexpression of *NtERF3a* and *NtERF3b* induced cell death in tobacco following *Agrobacterium* infiltration (Figs. 2a, S2a), as well as by the direct inoculation of an *NtERF3*-containing recombinant virus RNA (Fig. 3a). The *NtERF3*-induced cell death could be described as HR-like cell death because it was associated with an increase in ion leakage (Fig. 2b), ROS production (Fig. 2c), and upregulation of marker genes for HR (Figs. 2d, 3j), as in the case of HR cell death induced by the TMV elicitor p50 in *NN* tobacco. The EAR motif of the NtERF3 protein had some crucial role on its cell death-inducing ability though it had no effect on nuclear localization of NtERF3 (Figs. 2, 3, 4). In addition to demonstrating cell death-inducing ability of NtERF3, we showed that other EAR motif-containing ERFs from tobacco, *N. benthamiana* and rice also induced cell death when overproduced in tobacco plants (Fig. 5). These results suggest that many *ERF* genes encoding EAR motif-containing proteins have the ability to induce cell death when overexpressed and that the cell death-inducing processes downstream of NtERF3 are conserved among monocot and dicot plant species.

The alignment of amino acid sequences of the EAR motif-containing ERF proteins used in this work showed that the AP2/ERF domain in the middle region and the EAR motif (DLNxxP) in the C-terminal region were highly conserved (Fig. S3). The consensus sequence of the EAR motif was at first reported L/FDLNL/FxP in tobacco NtERF3, and in *Arabidopsis* AtERF3 and AtERF4 (Ohta et al. 2001). Recently, Kagale et al. (2010) reported two distinct conservation patterns of EAR motif (DLNxxP and LxLxL) and identified 219 EAR motif-containing proteins belonging to 21 different families in *Arabidopsis*. The LxLxL type of EAR motif was first reported in the SUPERMAN protein of *Arabidopsis* (Hiratsu et al. 2002). The ERF proteins shown in Fig. S4 also have the LxLxL type of EAR motif in their middle region, as well as in their C-terminal region with overlapping the DLNxxP type of EAR motif. Two serine-rich regions exist between the AP2/ERF domain and the acidic region (Fig. S3). Nasir et al. (2005) reported that the AP2/ERF domain, acidic region and EAR motif were essential for the cell death-inducing ability of NbCD1, but that the region between the AP2/ERF domain and the acidic region was not required. The LxLxL type of EAR motif and the serine-rich region in the middle area might have other functions than cell death induction.

There are many possible downstream target genes for NtERF3, which contain the GCC box in their promoter regions. Non-GCC box-containing promoters could also be the targets of NtERF3, as Chakravarthy et al. (2003) reported that tomato transcriptional activator Pti4, a homologue of tobacco NtERF2, regulated defense-related

gene expression via non-GCC box *cis* element. Elucidation of the downstream target genes of *NtERF3* required for the cell death induction will facilitate an understanding of the HR cell death-inducing pathway in tobacco.

It was reported that *NtERF1* and *NtERF2* were upregulated during the HR cell death induction from *N* gene (Kim et al. 2003). In this study, *NtERF3* was revealed to be upregulated during HR cell death induction by the *N* gene (Figs. 1b, S1a). Before the cell death lesions became visible, *NtERF3* had started to be upregulated in *NN* tobacco at 0–5 h after the temperature shift to 20°C, and the state of upregulation lasted during the cell death progression (Fig. 1b). However, the extent of the *NtERF3* upregulation during HR cell death induction (Figs. 1b, S1a) was much lower than that induced by the wounding treatment (Fig. S1b). It was reported that the mRNA level of *NtERF3* by wounding became maximal about 1 h after the wounding treatment and decreased rapidly afterward (Nishiuchi et al. 2002, 2004; Suzuki et al. 1998). However, the upregulation of *NtERF3* by wounding did not induce cell death. The long-lasting upregulation of *NtERF3* might be important for HR cell death induction. From this point of view, it would be interesting to note that a salicylic acid-induced protein kinase, SIPK and a wounding-induced protein kinase, WIPK, were regulated in different manners depending on the stress. For example, the SIPK protein kinase activity was transient in the wound response, but lasted long in HR cell death induction (Zhang and Klessig 1998; Zhang and Liu 2001).

In addition to the upregulation of *NtERF3* during HR cell death induction by the *N* gene, we tried to show the effect of loss of function of *NtERF3* on TMV-*N* gene-mediated HR cell death. In transgenic *NN* tobacco plants expressing antisense or inverted-repeat transcripts of *NtERF3*, cell death by TMV infection still occurred although there was a tendency that the size of the cell death lesions was larger than that in wild-type plants (Fig. S4). Because the AP2/ERF superfamily is one of the largest families of transcription factors in plants (Nakano et al. 2006), there may be functional redundancy in the cell death induction between *NtERF3* and its homologous genes. Silencing such genes altogether may be effective to clearly demonstrate the involvement of *NtERF3* in TMV-*N* gene-mediated HR.

McGrath et al. (2005) reported that a transcriptional repressor *AtERF4*, an *Arabidopsis* homologue of *NtERF3*, functioned as a negative regulator in the expression of a jasmonate (JA)-responsive gene *PDF1.2*, one of the defense-related genes containing GCC-box in its promoter region. In contrast, a truncated-type of *AtERF4* without the EAR motif acted as a positive regulator of *PDF1.2* in a T-DNA insertion mutant of *AtERF4* (*erf4-1*). *AtERF4*-overexpressing transgenic plants had decreased resistance

to a necrotrophic fungal pathogen *Fusarium oxysporum*, while *erf4-1* mutant plants had increased resistance in compared to the wild-type plants (McGrath et al. 2005). The involvement of *AtERF4* in the basal disease resistance to pathogens may suggest that *NtERF3* could be concerned with multiple processes such as the HR and basal disease resistance. Analysis of *NtERF3* using pathogens other than TMV will provide new insight into the roles of *NtERF3* in many biological processes, including the defense responses in tobacco. Further studies of *NtERF3* and its homologues in other plant species, such as *Arabidopsis* and rice, will contribute to a better understanding of the defense responses in plants.

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