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Original Paper

Overexpression of a tobacco Dof transcription factor BBF1 stimulates the transcription of the tobacco mosaic virus resistance gene *N* and defense-related responses including ROS production

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Abstract Dof proteins are known as plant-specific transcription factors. We previously reported that Dof proteins might be involved in expression of the tobacco resistance gene *N* for *Tobacco mosaic virus* (TMV). In *N*-carrying tobacco cultivars such as Samsun NN, a rapid upregulation of *N* transcription is induced by TMV infection, which is followed by the defense response called the hypersensitive response (HR). In this study, the role of a tobacco Dof protein BBF1 in *N* transcription was investigated. We cloned a *BBF1* ORF cDNA of Samsun NN and confirmed that a full-length recombinant BBF1 could bind *in vitro* to DNA with a Dof binding core motif. The transient overexpression of BBF1 alone did not induce any HR but activated the endogenous *N* gene expression in Samsun NN. The *N* promoter activation by BBF1 overexpression was also confirmed in the *N*-lacking Samsun nn plant by using the exogenously introduced *N* regulatory sequence connected to a reporter gene. Additional experiments suggested that BBF1 overexpression enhanced not only ROS production but also the transcription activity of certain defense signaling and HR marker genes even without HR induction. Regarding subcellular localization, BBF1 fused with a fluorescent protein was predominantly localized in the nucleus. Based on these data, we discuss potential roles of BBF1 and other Dof proteins as transcription factors for defense responses.

Key words: Dof transcription factor, HR, *N* gene, ROS, TMV.

Plants have evolved a variety of resistance (*R*) genes to defend themselves from virus infection (Maule et al. 2007). *R*-mediated defense responses often include the hypersensitive response (HR) that leads to cell death at the virus infection sites. The rapid induction of HR prevents the local and systemic virus spread. HR is triggered by a specific interaction between an *R* gene product and a virus-derived elicitor. The *N* gene of tobacco (*Nicotiana tabacum*) is known as a classical *R* gene for tobamoviruses including *Tobacco mosaic virus* (TMV) (Holmes 1938). The N protein consists of a Toll/interleukin-1 receptor (TIR) domain, a nucleotide binding site (NBS), and a leucine-rich repeat (LRR) domain (Whitham et al. 1994) and recognizes the 50-kDa helicase domain (p50) of TMV replicase as an elicitor (Abbink et al. 2001; Erickson et al. 1999). *Agrobacterium*-mediated transient expression of p50 can induce HR-like cell death (Abbink et al. 2001; Erickson et al. 1999). The N protein is present at a low level in the normal cells for the surveillance of TMV and ready for prompt amplification after the virus infection. The infection of TMV and the transient expression of p50 upregulate the transcription of the *N* gene prior to the appearance of cell death (Dinesh-Kumar and Baker 2000; Levy et al. 2004; Takabatake et al. 2006). The mRNA level of the *N* gene is not influenced by any biotic and abiotic stresses other than TMV infection (Levy et al. 2004; Takabatake et al. 2006). These reports suggest that the upregulation of *N* transcription may play an important role in *N*-mediated defense responses. However, molecular mechanisms of transcriptional regulation of the *N* gene are poorly understood.

Dof proteins are known as plant-specific transcription factors (Yanagisawa 2004). They contain a DNA binding domain with a single C_2–C_2 type zinc finger, which binds to an (A/T)AAAG or its inverse CTTT(A/T) sequence

Abbreviations: BBF, *rolB* domain B factor; DAPI, 4′,6-diamidino-2-phenylindole; Dof, DNA binding with one finger; GFP, green fluorescent protein; GST, glutathion S-transferase; hpi, hours post-inoculation; HR, hypersensitive response; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; TMV, *Tobacco mosaic virus*; YFP, yellow fluorescent protein

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as the recognition core. Functional analyses have indicated that Dof proteins play crucial roles in diverse biological phenomena including seed development, germination, plant growth, flowering, and responses to phytohormones, chemicals and light (Baumann et al. 1999; Gualberti et al. 2002; Guo et al. 2009; Imaizumi et al. 2005; Kang and Singh 2000; Mena et al. 2002; Papi et al. 2000; Yanagisawa and Sheen 1998). In addition, there are a few reports suggesting the involvement of Dof proteins in defense responses to biotic stresses. In Arabidopsis, OBPs expression is induced by salicylic acid, a plant defense signal molecule (Kang and Singh 2000). Recent microarray-based gene expression analysis using stable transformants expressing OBP2 has indicated that the Dof protein plays a role in biotic stress responses like herbivore feeding (Skirycz et al. 2006). In the case of pea, the expression of *PsDof1* is upregulated slightly by the treatment of the elicitor of a fungal pathogen (Seki et al. 2002).

In tobacco, there are at least three different classes of the *Dof* genes (*BBF1*, *BBF2*, and *BBF3*) (De Paolis et al. 1996). BBF1 has been characterized as a protein that can bind to the regulatory domain B of the promoter of the *Agrobacterium rolB* oncogene and may be involved in regulation of *rolB* expression (Baumann et al. 1999; De Paolis et al. 1996). The BBF1 target sequence ACTTTA in the domain B has been shown to play a pivotal role in tissue-specific and auxin-inducible expression of *rolB* (Baumann et al. 1999). As for BBF2 and BBF3, only partial cDNA sequences have been reported and little is known about their roles and functions.

Recently, we have cloned a 2.3-kb upstream region (NP2.3) of the *N* gene from *N*-containing tobacco (*N. tabacum* cv. Samsun NN). Reporter assays using the NP2.3-derived sequences with various deletions identified a 20-bp sequence essential for p50-triggered upregulation of the reporter gene expression (Haque et al. 2009). The presence of tandemly repeated Dof target AAAG motifs in the 20-bp regulatory sequence suggested the possibility that tobacco Dof proteins might be involved in the transcriptional upregulation of the *N* gene during HR induction. In this study, we cloned *BBF1* cDNA from Samsun NN tobacco and showed the ability of a recombinant BBF1 to bind preferentially *in vitro* to an *N* sequence with the TAAAG motif. The transient overexpression of BBF1 without HR induction activated *N* transcription and stimulated the production of reactive oxygen species (ROS). BBF1 also enhanced defense-related gene expression, when overexpressed transiently in the absence of HR induction. The subcellular localization of BBF1 in the nucleus was also observed. From these results, we discuss potential roles of BBF1 and other Dof proteins as transcription factors for the regulation of defense responses against pathogens.

Materials and methods

Plants and growth conditions

Two tobacco cultivars (*N. tabacum* L. cv. Samsun NN and Samsun nn) were used. Seedlings were grown on Rock Fiber blocks (Nittobo, Tokyo, Japan) for 2 weeks at 25°C with a 16 h light/8 h dark photoperiod. They were transferred to pots filled with vermiculite and grown under the same conditions with weekly treatment of 0.1% Hyponex solution (N : P : K = 5 : 10 : 5) (Hyponex Japan, Osaka, Japan). For all the experiments, 50 to 60-day old plants were used.

cDNA synthesis of the BBF1 *gene*

Total RNA was isolated from Samsun NN tobacco leaves. Primers were designed to amplify the full-length *BBF1* cDNA according to the sequence of the *BBF1* genome published online (GenBank accession number AJ009594) (Baumann et al. 1999). The specific primers were as follows: BBF1/Xa/F01 (5′-GTCGAC*ATCGAAGGTCGT*ATGGATACTTCTCAC TGGCC-3′; a *Sal*I recognition site is underlined and a coding sequence of a Factor Xa Protease cleavage site is shown in italic), BBF1/Xa/R01 (5′-GCGGCCGCTTACCAAGATGATCC TCCGC-3′; a *Not*I recognition site is underlined).

Plasmid construction

pGEX-BBF1 was constructed as an expression plasmid for GST-BBF1. A *BBF1* cDNA fragment digested by *Sal*I and *Not*I was ligated into the multicloning site of pGEX-6P-3 (GE Healthcare, Little Chalfont, Buckinghamshire, UK). pART27-35S-BBF1 and pART7-BBF1-YFP were constructed by Gateway cloning techniques (Invitrogen, Carlsbad, CA, USA) as follows. For construction of pART27-35S-BBF1, the first PCR was performed with the *BBF1* cDNA as a template and a set of primers, BBF1/GW-pART27/F01 (5′-AAAAAGCAG GCTACCATGGATACTTCTCACTGGCC-3′) and BBF1/GW-pART27/R01 (5′-AGAAAGCTGGGTTTACCAAGATGATCC TCCGC-3′). After purification of PCR products, the second PCR was carried out with the attB1 adaptor primer (5′-GGG GACAAGTTTGTACAAAAAAGCAGGCT-3′) and the attB2 adaptor primer (5′-GGGGACCACTTTGTACAAGAAAGC TGGGT-3′). Second PCR products were cloned into the entry vector pDONR221 to obtain an entry plasmid for BBF1 (pDONR221-BBF1). pDONR221-BBF1 was recombined with the destination vector pART27-35Sa-GWB (Gao et al. 2007) to obtain pART27-35S-BBF1. In the case of construction of pART7-BBF1-YFP, the entry plasmid pDONR221-BBF1 was recombined with the destination vector pART7-GWC-YFP (Sasaki et al. 2009).

Gel shift assay

Double-stranded oligonucleotides used as 50-bp biotin-labeled probes and 30-bp competitors were obtained by annealing each set of single-stranded oligonucleotides at room temperature after denaturation for 5 min at 80°C. Regarding the labeled probes, biotinylation of the 3′-end of

single-stranded oligonucleotides was performed with the Biotin 3′End DNA Labeling kit (PIERCE, Rockford, IL, USA) according to the manufacturer's instructions. For the gel shift assay, GST and GST-BBF1 was expressed in *Escherichia coli* (Rosetta-gami B). The recombinant proteins were produced with 0.2 mM isopropyl-β-thiogalactopyranoside for 1 h at 37°C and purified by a batch method with Glutathione Sepharose 4B (GE Healthcare). Labeled probes (50 fmol) and recombinant proteins (200 ng) were mixed in 1×binding buffer [10 mM HEPES-KOH (pH7.8), 50 mM KCl, 5% glycerol, 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$] and incubated for 10 min at 4°C. In competition assays, a 500-fold excess of competitors (25 pmol) was added to the reaction. The reaction mixture was then resolved on a 4–20% Mini-PROTEAN TGX precast gel (Bio-Rad Laboratories, Hercules, CA, USA) and transferred onto a positively charged nylon membrane (Roche Applied Science, Mannheim, Germany). The labeled probes were detected chemiluminescently by using horseradish peroxidase-conjugated streptavidin of the Chemiluminescent Nucleic Acid Detection Module kit (PIERCE) according to the manufacturer's instructions.

Particle bombardment

Particle bombardment on Samsun NN tobacco leaves was carried out as described previously (Sasaki et al. 2009) except that gold particles were prepared in the presence of 1.1 M $CaCl_2$ and 87 mM spermidine.

Agroinfiltration

Transformation, growth and infiltration of *Agrobacterium tumefaciens* strain GV3101 (pMP90) were performed as described previously (Haque et al. 2009). Agrobacteria transformed with pART27-35S-BBF1 (see above), pART27 (Gleave 1992), pART27-35S-TomH1 (Gao et al. 2007) and pGWB4-NP2.3 (Haque et al. 2009) were grown in LB medium containing rifampicin (50 mg l^{-1}) and kanamycin (50 mg l^{-1}) in addition to streptomycin (50 mg l^{-1}) for pART27-based plasmids or hygromycin (50 mg l^{-1}) for pGWB4-NP2.3. The agrobacteria were collected by centrifugation for 10 min at 5000 rpm at 4°C, resuspended in an incubation buffer (10 mM $MgCl_2$, 10 mM MES, and 150 μM acetosyringone) to adjust the OD600 value to 0.5, and kept at room temperature for 1 h prior to infiltration. Infiltrated plants were kept at 25°C.

DAPI staining

For the staining of the nuclei, excised leaf tissues at 24 h after bombardment were placed on the surface of a 4′,6-diamidino-2-phenylindole (DAPI) solution (10 mg l^{-1}, Sigma, St Louis, MO, USA) for 1 h, keeping the abaxial side downward.

Fluorescence observation

In promoter assays, GFP fluorescence was observed at 36 h after infiltration under the epifluorescence microscope BX50 (Olympus, Tokyo, Japan) using the UMWIBA/GFP filter unit. In subcellular localization analysis, the fluorescence of DAPI, BBF1-YFP, and YFP was observed at 24 h after bombardment with the confocal laser scanning microscope LSM710NLO (Carlzeiss, Jene, Germany). DAPI and YFP were excited with the 405 nm and 514 nm line rays of the argon laser and detected in the range between 410 to 503 nm and 519 to 579 nm, respectively. Fluorescence images of DAPI and YFP were acquired in the sequential mode and processed by using the software ZEN 2009.

Total RNA extraction and real-time PCR analysis

Total RNA was isolated by using the NucleoSpin RNA Plant kit (MACHEREY-NAGEL, Duren, Germany). cDNA was synthesized from 350 ng of the total RNA by using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa BIO, Shiga, Japan). To quantify the gene expression, real-time PCR analysis was performed on the Thermal Cycler Dice Real Time System TP800 (TaKaRa BIO) by using of cDNA corresponding to 35 ng of the total RNA and SYBR Premix Dimer Eraser (TaKaRa BIO). PCR cycles were as follows: 1 cycle for 30 s at 95°C, 40 cycles each for 5 s at 95°C, 30 s at 55°C and 30 s at 72°C (for all cDNAs except *p50*); 1 cycle for 30 s at 95°C, 45 cycles each for 5 s at 95°C, 30 s at 60°C (for *p50*). The dissociation curves were generated by incubation for 15 s at 95°C, 30 s at 60°C and 15 s at 95°C. The specific oligonucleotides were as follows: Actin/real/F1 (5′-CTATTCTCCGCTTTGGACTTGGCA-3′) and Actin/real/R1 (5′-AGGACCTCAGGACAACGGAAACG-3′) for the *actin9* cDNA (GenBank accession number X69885); N/real/F1 (5′-TTCTTTGTACCTTTTGCTGGCTTAT-3′) and N/real/R1 (5′-CTCTGGTCCTTCTTTATACAACAAAC-3′) for the *N* cDNA; BBF1/real/F1 (5′-GGAGAAACTGCTGGAGCAAA-3′) and BBF1/real/R1 (5′-TCAGCAGCTTGCCCTCTATT-3′) for the *BBF1* cDNA; GFP/real/F1 (5′-AGGAGCGCACCATCTTCTTC-3′) and GFP/real/R1 (5′-TCCTTGAAGTCGATGAAATT-3′) for the *GFP* cDNA; p50/real/F2 (5′-GACATGCGATGATTGGAGAAGAG-3′) and p50/real/R2 (5′-GGCTGGACAACGGAGAGTAG-3′) for the *p50* cDNA; PR1a/real/F1 (5′-TAGTCATGGGATTTGTTCTC-3′) and PR1a/real/R2 (5′-TTTTGGGCACGGCAAGAGTG-3′) for the acidic *PR1a* cDNA (GenBank accession number D90196); PR1b/real/F2 (5′-AGCAAGGGGTGGATTTACTG-3′) and PR1b/real/R2 (5′-CGTTGACTCATCACCTTGTT-3′) for the basic *PR1b* cDNA (GenBank accession number X66942); Hsr203/real/F2 (5′-CAATAATGGAGTGGGACATAGC-3′) and Hsr203/real/R1 (5′-GATGAACTCTGCAACGGCTTC-3′) for the *Hsr203j* cDNA (GenBank accession number AB091430); Hin1/real/F2 (5′-GCGTATTAGGCTTAAGGTTGG-3′) and Hin1/real/R1 (5′-CTACCAATCAAGATGGCATCTG-3′) for the *Hin1* cDNA (GenBank accession number Y07563).

ROS measurement

The relative amount of ROS was determined by infiltration of 10 mM MOPS-KOH (pH 7.4) buffer containing 0.5 mM L-012 (Wako, Osaka, Japan) to tobacco leaves. Chemiluminescence at 24 h after infiltration was monitored with the LAS3000 image analyzer (FUJIFILM, Tokyo, Japan) and quantified using the

Multi Gauge software (FUJIFILM).

Results

BBF1 binds specifically to DNA with the Dof binding motif

A cDNA clone of *BBF1* from Samsun NN tobacco was obtained by RT-PCR based on the genomic sequence of *BBF1* clone from SR1 tobacco (NCBI accession no. AJ009594) (Baumann et al. 1999). There were no differences in the nucleotide sequence between our *BBF1* cDNA clone and the *BBF1* ORF predicted from the SR1 genome (data not shown). Another *BBF1* cDNA encodes a homologous protein (hereafter referred to as BBF1*) (De Paolis et al. 1996) that has an almost identical Dof domain, but is varied in both the N- and C-terminal regions (Figure S1). The variations in BBF1* may have suggested the existence of BBF1 homologues or possibly arisen through the assembly process of different genomic and cDNA sequences (NCBI accession nos. X97942, X97943, and X97944).

For BBF1*, the N-terminally located Dof domain with a C_2–C_2 zinc finger was used to demonstrate the ability for DNA binding to the ACTTTA sequence (De Paolis et al. 1996). To examine the DNA binding ability of BBF1, we performed gel shift assays with recombinant GST-fused BBF1 (GST-BBF1) protein and 50-bp DNA probes consisting of a 20-bp sequence (N20A) and its flanking regions (Figure 1). N20A, a possible elicitor responsive element of the *N* gene, contains two AAAG motifs (Haque et al. 2009). The migration of the labeled DNA was retarded by incubation with GST-BBF1 but not GST (Figure 1). In addition, the labeled DNA at the shift position was reduced drastically by the competition with excess of 30-bp unlabeled probes (W) consisting of N20A and its downstream sequence (Figure 1). These data indicated that the full-length BBF1 was able to bind specifically to the AAAG-containing DNA. For other Dof proteins including BBF1*, the 5′ proximal nucleotides adjacent to the binding motif AAAG were preferably A or T in efficient binding (De Paolis et al. 1996; Yanagisawa and Schmidt 1999). For BBF1, the upstream TAAAG rather than the downstream CAAAG might be the recognition core in N20A. To test the possibility, we also carried out competition assays with unlabeled mutant probes, M1, M2, and M3 (Figure 1). M1 had mutations in the both AAAG motifs, whereas M2 and M3 had ones in the upstream and the downstream motif, respectively. A remarkable reduction of the shift DNA indicated the binding of excess competitor M3, but not M1 or M2 (Figure 1). This result suggested that BBF1 might bind to the target DNA through preferential recognition of the TAAAG sequence. We found multiple (A/T)AAAG and CTTT(T/A) sequences in the upstream region of the *N* gene, including N20A (Figure S2),

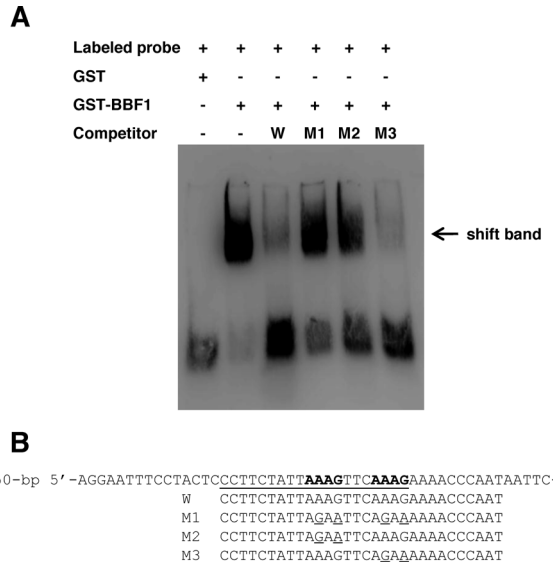

Figure 1. DNA binding property of BBF1. (A) The binding of recombinant GST and GST-BBF1 to a 50-bp biotinylated probe (50-bp) consisting of N20A (underlined) and its 5′- and 3′-proximal sequences in the *N* gene was analyzed by the gel shift assay. Biotinylated probes were detected chemiluminescently by using HRP-conjugated streptavidin. Competition was examined with 500-fold molar excess of unlabeled 30-bp probes consisting of the wild-type (W) or mutated (M1, M2, and M3) N20A and the 3′-proximal sequence. (B) Nucleotide sequences of 50-bp, W, M1, M2, and M3 are shown. Two AAAG motifs in N20A are indicated in bold. Mutated nucleotides in M1, M2, and M3 are underlined.

implying that some of these sequences might be the functional binding targets for BBF1 *in vivo*.

Transient overexpression of BBF1 can enhance the activity of an exogenous N *promoter*

To investigate the role of BBF1 on the activation of the *N* promoter, we carried out *in vivo* promoter assays by agroinfiltration of the binary plasmid pGWB4-NP2.3, in which the 2.3-kb region upstream of the transcriptional initiation codon of the *N* gene is linked to the reporter *GFP* cDNA (Haque et al. 2009). Agrobacteria carrying pGWB4-NP2.3 were mixed with those carrying pART27-35S-BBF1 for BBF1 expression or the empty vector pART27 and infiltrated to leaves of Samsun nn tobacco lacking the *N* gene. Samsun nn tobacco was used to avoid possible effects of the endogenous *N* gene or its gene products on the promoter activity. At 36 and 42 h post-infiltration (hpi), tissues coexpressing BBF1 showed stronger GFP fluorescence than the control leaves (Figure 2A). To quantify the relative levels of *GFP* mRNA, we carried out real-time RT-PCR with these tissues at 24, 36, and 42 hpi. The levels of *GFP* mRNA in BBF1-overexpressing tissues were always higher and approximately doubled when compared with those in the control (Figure 2B, C). These results suggested that the transient overexpression of BBF1 could enhance the transcription from the exogenous *N* promoter.

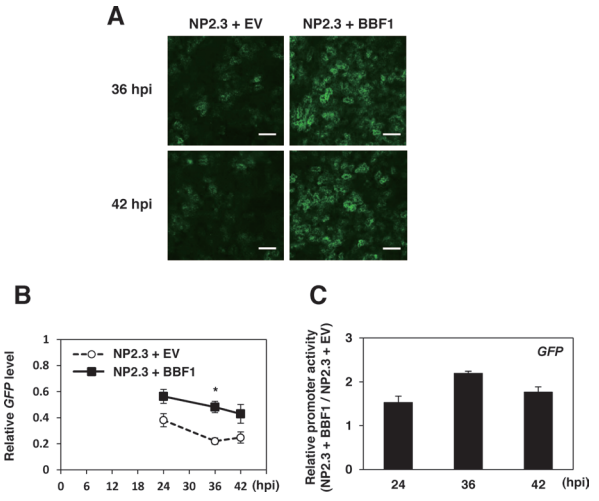

Figure 2. The effect of the transient overexpression of BBF1 on the *N* promoter activity. Agrobacteria with the plasmid encoding the GFP reporter under the regulation of the NP2.3 sequence, together with those with the expression plasmid for the BBF1 protein (+BBF1) or with the empty vector (+EV), were infiltrated to Samsun nn tobacco. (A) Images of GFP fluorescence in epidermis were captured at 36 and 42 hpi with an epifluorescence microscope. Scale bar, 200 μm. (B) mRNA levels of *GFP* in NP2.3+BBF1 (■) or NP2.3+EV (○) infiltrated leaves were determined by real-time RT-PCR. The levels of *GFP* mRNA were normalized with those of *actin9* mRNA. Error bars represent the standard errors of the means (n=3). Statistical significance was analyzed using Student's *t*-test: * $p<0.05$. (C) Relative NP2.3 promoter activity in the presence and absence of BBF1 overexpression was calculated based on *GFP* mRNA levels at each time point indicated in B.

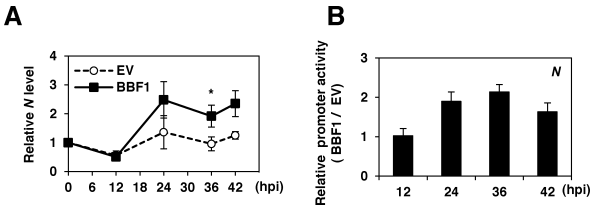

Figure 3. Transcription of the endogenous *N* gene under the transient overexpression of BBF1. (A) Expression of the endogenous *N* gene of Samsun NN tobacco was measured by real-time RT-PCR. Agrobacteria for the expression of BBF1 or those carrying the empty vector (EV) were infiltrated to Samsun NN tobacco. The mRNA level at 0 hpi was fixed as 1.0 to determine the relative *N* mRNA levels. The relative mRNA levels were normalized with *actin9* mRNA levels. Error bars represent the standard errors of the means (n=3). Statistical significance was analyzed using Student's *t*-test: * $p<0.05$. (B) Relative *N* promoter activity in the presence and absence of BBF1 overexpression was calculated based on mRNA levels at each time point indicated in A.

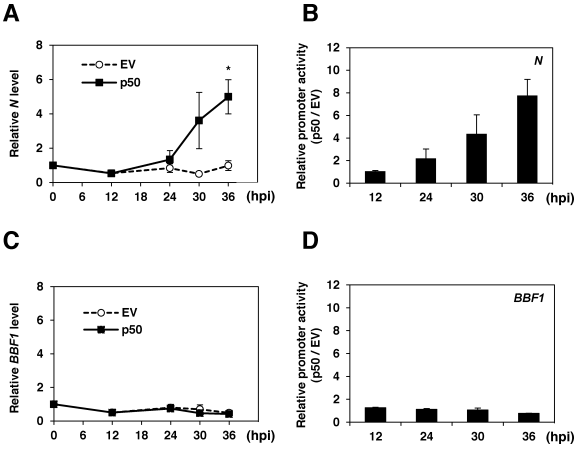

Figure 4. Expression of the endogenous *N* and *BBF1* genes during the HR induction by the expression of p50. Agrobacteria for the expression of p50 or those carrying the empty vector (EV) were infiltrated to Samsun NN tobacco. mRNA levels of the endogenous *N* (A) and the *BBF1* (C) genes in p50 (■) or EV (○) infiltrated leaves were determined by real-time RT-PCR. The mRNA level at 0 hpi was fixed as 1.0 to determine the relative mRNA levels. The levels of *N* and *BBF1* mRNA were normalized with those of *actin9* mRNA. Error bars represent the standard errors of the means (n=3). Statistical significance was analyzed using Student's *t*-test: * $p<0.05$. Relative promoter activity of the *N* gene (B) and the *BBF1* gene (D) was calculated based on mRNA levels at each time point indicated in A and C.

Effects of transient overexpression of BBF1 on the transcriptional activation of the endogenous N *gene*

We investigated the effects of the overexpression of BBF1 on the endogenous *N* gene expression. For infiltration, we used agrobacteria carrying pART27-35S-BBF1 for the expression of BBF1. Agrobacteria carrying the empty vector pART27 were used as a negative control. Gene expression analysis by real-time RT-PCR at 0, 12, 24, 36, and 42 hpi demonstrated that the levels of *N* mRNA in BBF1-overexpressing tissues were approximately twice the control at 24 hpi and thereafter (Figure 3). This result indicated that the overexpression of BBF1 alone led to transcriptional activation of the endogenous *N* gene. We did not observe any symptoms for HR in the BBF1 overexpressing leaves after infiltration, suggesting that the *N* gene activation may be prerequisite for but separable from the onset of HR, which requires yet other factors specifically induced by the elicitor p50.

p50 expression has no influence on the transcription levels of BBF1

To investigate the transcription of the endogenous *N* and the *BBF1* gene during p50-mediated HR induction, leaves of Samsun NN tobacco were infiltrated with agrobacteria carrying pART27-35S-TomH1 for p50 expression and the levels of these mRNAs were determined by real-time RT-PCR at various times (0, 12, 24, 30, and 36 hpi). The mRNA levels of the *N* gene increased remarkably under the expression of p50 (Figure 4A, B), as reported previously (Takabatake et al. 2006). In contrast, mRNA levels of the *BBF1* gene did not change even at 36 hpi under the expression of p50 (Figure 4C, D). Thus, the p50-mediated endogenous *N* gene activation did not require the concomitant activation of the *BBF1* gene.

Transient overexpression of BBF1 enhances ROS production and upregulates defense-related gene expression

Production of ROS is associated with *N*-mediated HR upon TMV infection or p50 expression in tobacco (Allan et al. 2001; Király et al. 2008; Ogata et al. 2012). We examined the effect of BBF1 overexpression on ROS production in both Samsun nn and Samsun NN tobacco. At 24 hpi of agroinfiltration to tobacco leaves, ROS production was determined with chemiluminescence from L-012, which is a chemiluminescence probe reacting with superoxide, hydrogen peroxide or their metabolites (Imada et al. 2002). In a control experiment, infiltration of agrobacteria carrying the empty vector pART27 led to weak chemiluminescence indicating a low level of ROS production due to some pathogenic effect of agrobacterium infection. The level of the background chemiluminescence caused by the bacterial infection was set to one as the basal level. As expected, ROS production was not induced by the expression of p50 in Samsun nn but elevated to four times as high as the basal level in Samsun NN (Figure 5). Interestingly enough, the expression of BBF1 tripled and doubled the basal levels of ROS production in Samsun nn and Samsun NN, respectively. These results indicated that p50-mediated ROS production was *N*-dependent and thus linked to the defense mechanisms. In contrast, BBF1-mediated ROS production was not *N*-dependent: it occurred in Samsun nn as well as Samsun NN.

Since the overexpression of BBF1 stimulated the ROS production that is related to the defense responses in Samsun NN tobacco, we also investigated effects of the overexpression of BBF1 on defense-related gene expression. It was reported that gene expression of *PR1a*, *PR1b*, *Hsr203j*, and *Hin1* in Samsun NN tobacco was upregulated during HR induced by p50 expression (Ogata et al. 2012) as well as infection of avirulent *Ralstonia* species (Kiba et al. 2003). Acidic *PR1a* and basic *PR1b* are defense marker genes in salicylic acid and jasmonic acid/ethylene signaling, respectively, and *Hsr203j* and *Hin1* are HR marker genes. Time-course expression analysis by quantitative real-time RT-PCR showed that the expression of all genes examined was increased after agrobacterium infiltration for the overexpression of BBF1. The upregulated promoter activity was prominent especially at 24 hpi for *Hsr203j* and *Hin1* while at 36 hpi for *PR1a* and *PR1b* (Figure 6). The upregulation of these genes, however, was also observed considerably in the control experiment with the empty vector, which may be caused by some defense responses to the agrobacterium infection itself. We could show statistical differences only for *Hin1* expression. The differences for the other genes were not statistically significant due to rather wide variations of the gene expression levels.

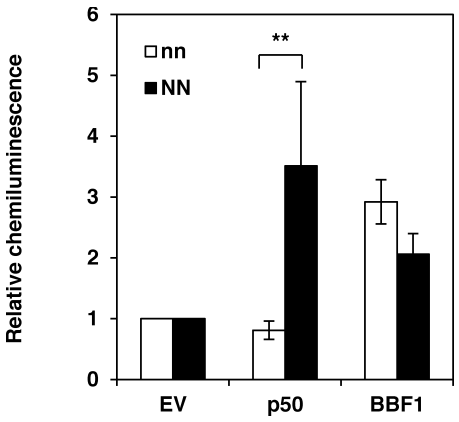

Figure 5. The effect of BBF1 expression on ROS production. Agrobacteria for the expression of p50 or BBF1 or those carrying the empty vector (EV) were infiltrated to Samsun NN and Samsun nn tobacco. The relative levels of chemiluminescence in infiltrated areas are shown. The fluorescence level of EV was fixed as 1.0. Error bars represent the standard errors of the means ($n=6$). Statistical significance was analyzed using Student's *t*-test: ** $p<0.01$.

Additionally, we analyzed the profile of the expression of *NtrbohD*, a NADPH oxidase gene that may be involved in ROS production during TMV-induced HR (Király et al. 2008). Although the gene expression of *NtrbohD* in BBF1-overexperssing and control leaves increased after agrobacterium infiltration, there were no significant differences in mRNA levels between the two leaves. These results indicated that the overexpressed BBF1 might upregulate the expression of some specific defense-related genes.

BBF1 is localized in the nucleus

Dof proteins of several plant species are reported to localize in the nucleus (Diaz et al. 2005; Yanagisawa and Sheen 1998). To determine the subcellular localization of BBF1, the plasmid pART7-BBF1-YFP encoding BBF1 fused with YFP (BBF1-YFP) was introduced to epidermal cells of Samsun NN tobacco by a bombardment method. The plasmid pART7-YFP encoding YFP was used as a control (Sasaki et al. 2009). The fluorescence of YFP was observed with a confocal laser scanning microscope at 24 h after bombardment. As shown in Figure 7, BBF1-YFP was detected predominantly in the nucleus. In contrast, YFP was detected in both the cytoplasm and the nucleus. In the nucleus, BBF1-YFP was localized in the nucleoplasm as distinct aggregates. BBF1-YFP and YFP were not localized in the nucleolus. This result was consistent with the assumption that BBF1 was a nuclear protein serving as a transcription factor for defense-related genes.

Discussion

To analyze a possible involvement of Dof transcription factors in regulation of the *N* gene, we have cloned

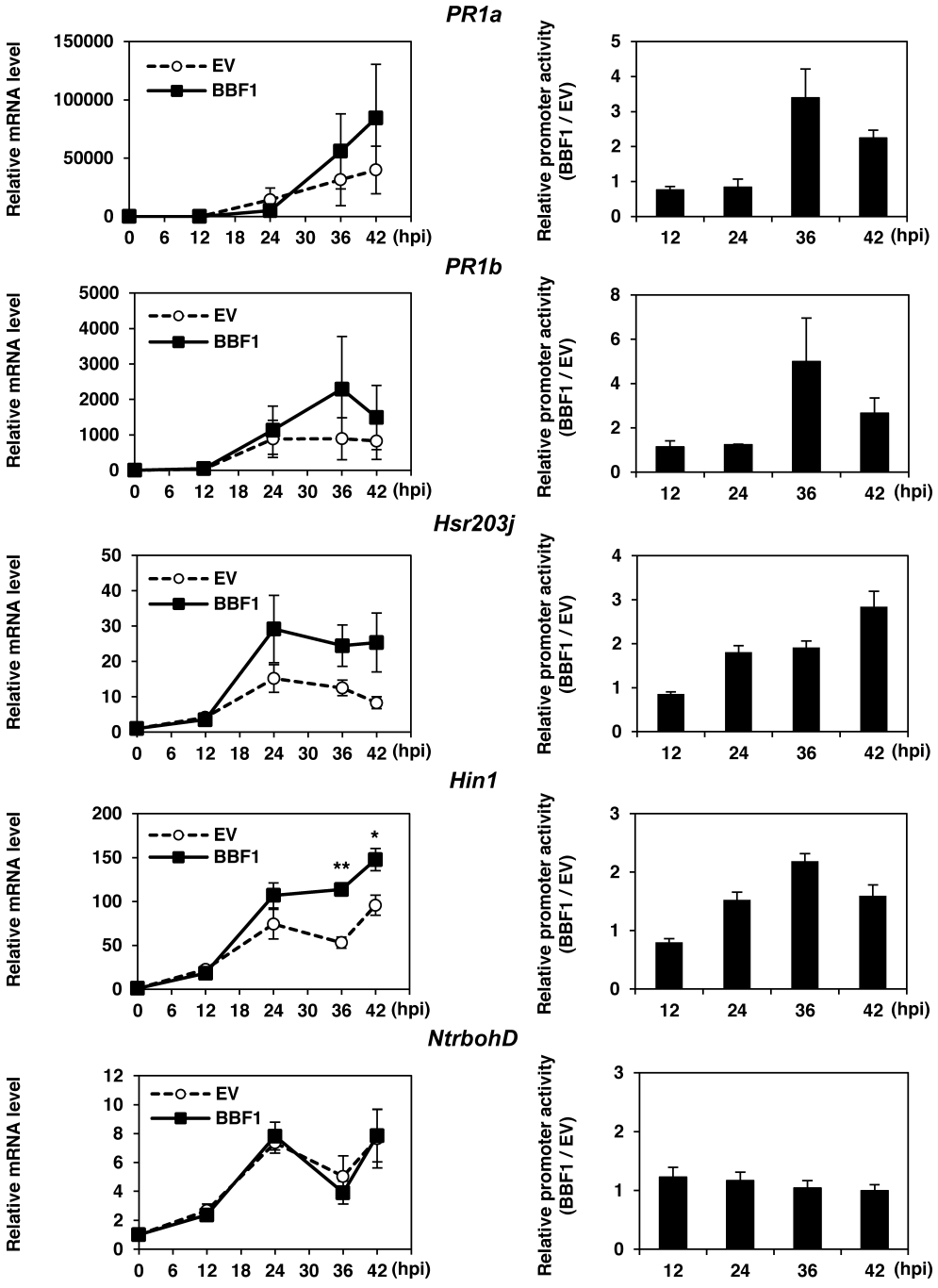

Figure 6. Expression of defense-related genes under the transient overexpression of BBF1. Agrobacteria for the expression of BBF1 were infiltrated to Samsun NN tobacco. Agrobacteria carrying the empty vector (EV) were used as a negative control. Left panels: mRNA levels of the *PR1a*, *PR1b*, *Hsr203j*, *Hin1*, and *NtrbohD* genes in BBF1 (■) or EV (○) infiltrated leaves were determined by real-time RT-PCR. The mRNA level at 0 hpi was fixed as 1.0 to determine the relative mRNA levels. The mRNA levels of the defense-related genes were normalized with those of *actin9* mRNA. Right panels: Relative promoter activity of each gene was calculated based on mRNA levels at each indicated time point. Error bars represent the standard errors of the means ($n=3$). Statistical significance was analyzed using Student's *t*-test: * $p<0.05$, ** $p<0.01$.

a cDNA coding for the tobacco Dof protein BBF1. Our *in vitro* binding assays have shown that the intact recombinant BBF1 is able to bind to a 50-bp DNA fragment derived from the *N* upstream region through the preferential recognition of the TAAAG sequence of N20A. This is consistent with the previous reports that Dof proteins including a BBF1 homolog were shown to bind to DNA with the (A/T)AAAG core motif (De Paolis et al. 1996; Yanagisawa 2004; Yanagisawa and Schmidt 1999). In addition to this DNA binding ability, our promoter assays revealed that the expression of BBF1 in Samsun nn tobacco increased the levels of *GFP* mRNA transcribed from the NP2.3 promoter that spans about 2.3-kbp upstream of the translational start codon of

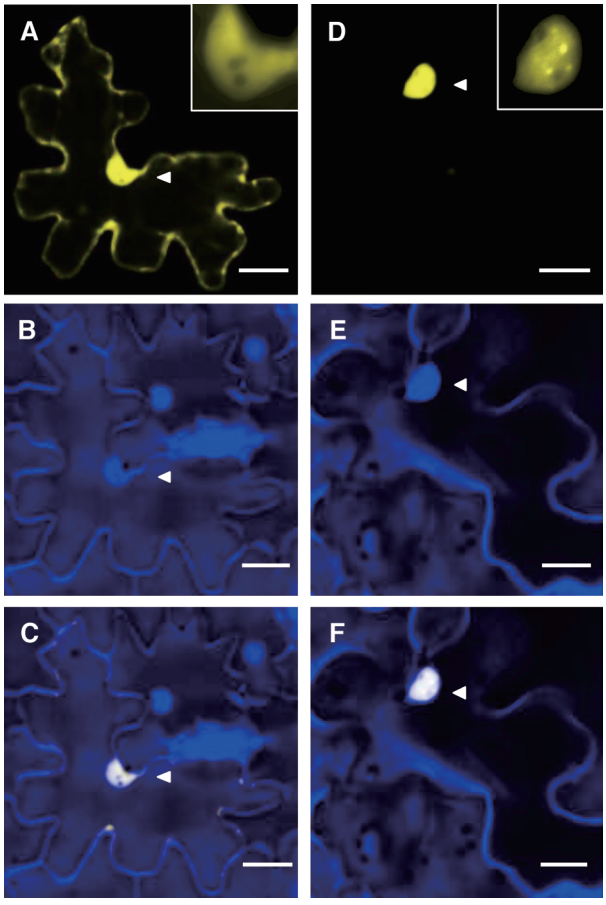

Figure 7. Nuclear localization of the BBF1 protein in Samsun NN tobacco. Confocal images of epidermal cells expressing YFP (A–C) and YFP-BBF1 (D–F) are shown. The epidermal cells were stained with DAPI at 24 h after bombardment. The fluorescence of YFP (A and D) and DAPI (B and E) are shown. Merged images are shown in C (A+B) and F (D+E). Small boxes in A and D are enlarged images of nuclei. White arrowheads indicate nuclei. Scale bar, 20 μm.

the *N* gene. In line with this result, the overexpression of BBF1 in Samsun NN tobacco could also upregulate the endogenous *N* gene expression in the absence of the elicitor p50. NP2.3 contains (A/T)AAAG and CTTT(T/A) sequences at multiple sites including N20A (from −269 to −250). Although N20A was shown in our previous report to function as a minimum enhancer element in response to p50 and the N protein (Haque et al. 2009), other transcription factors could recognize different *cis*-acting elements and cooperate with BBF1. In fact, Kobayashi et al. (2010) reported a distinct regulatory element located just upstream of the N20A sequence. More detailed studies on NP2.3 are necessary to determine the actual target sequence(s) for BBF1 and also other *trans*-acting factors.

The activation of the *N* promoter by the overexpression of BBF1 raised a possibility that BBF1 might be involved in the upregulation of the *N* transcription in response to the TMV elicitor. Time-course expression analysis on the endogenous *N* and *BBF1* genes (Figure 4) has demonstrated that the p50-triggered upregulation of the *N* transcription may not require a concomitant transcriptional activation of *BBF1*, indicating that BBF1 may not play a direct role in the upregulation of the *N* gene. Consequently, the elicitor-triggered upregulation of *N* transcription may require additional transcription factors including different Dof proteins such as BBF2 and/or BBF3. On the other hand, BBF1 may have some role in the basal level expression of the *N* gene in normal cells. Assuming that the endogenous BBF1 level is a limiting factor for the basal expression of the *N* gene, it is plausible that the overexpression of BBF1 expression should result in an enhancement of the *N* gene transcription in the absence of the elicitor. In any case, the effects of BBF1 overexpression in our experimental system may reflect a certain regulatory aspect of BBF1 as one of the transcription factors for the basal expression of the *N* gene. To prove our hypothesis, it will be necessary to test whether the deletion or mutation of N20A may affect the basal expression of the *N* gene.

The overexpression of BBF1 was shown to increase the level of ROS in not only Samsun NN but also Samsun nn tobacco, indicating that BBF1 has the ability to enhance the ROS production in an *N*-independent manner. Although the function of ROS is not fully understood, ROS was shown to act as secondary signals in the activation of the expression of defense-related genes (Torres 2010). Consistently with this, the transient overexpression of BBF1 in Samsun NN tobacco was shown in this study to enhance the transcription of defense-related genes such as *Hin1* and possibly *PR1a*, *PR1b*, and *Hsr203j*. These observations suggest that BBF1 may be involved in the transcriptional regulation of a variety of defense-related genes, including those for the activation of ROS production. Further studies are necessary to elucidate the molecular mechanisms in which the overexpression of BBF1 stimulates the production of ROS and the expression of the defense-related genes as well as the *N* gene.

Subcellular localization analysis showed that BBF1-YFP was observed exclusively in the nucleus, similarly to the cases for Dof proteins of other species such as maize and barley (Diaz et al. 2005; Yanagisawa and Sheen 1998). The exclusive nuclear localization of BBF1 reinforces the idea that the protein functions as a transcription factor. Here, our functional analyses with a transient expression system have also demonstrated that BBF1 has the ability to activate the promoter of the virus resistance gene, ROS generation, and defense-related gene expression. To our knowledge, this is the first report to provide direct biological evidence for the implication of a Dof transcription factor in resistance gene regulation. It is important to investigate whether BBF1 and other Dof proteins have similar or distinct

functions in defense responses mediated by the *N* gene and also other *R* genes. Further analyses with gain-of-function and loss-of-function of tobacco Dof proteins including BBF1 will shed more light on their roles in the regulation of the target gene transcription in pathogen resistance.

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BBF1       ------------------------------------------------------------
BBF1*      MKFQNMLSRLLTDIWNRQRDHLKLSGIFLALGPEPHVLGVRCFHHLLCDSLPLFPHLLFR 60


BBF1       -----------MDTSHWPQGIGLVKAVEPSKPVP-TERKPRPQKEQAINCPRCNSTNTKF 48
BBF1*      LRFCLPLPFFNMDTSHWPQGIGLAKAVEPSKPVPVTERKPRPQKEQAINCPRCNSTNTKF 120
                     ++++++++++ +++++++++ ++++++++++++++++++++++


BBF1       CYYNNYSLSQPRYFCKTCRRYWTEGGSLRNVPVGGGSRKNKRSSSSSNNSSSSTSSSYKK 108
BBF1*      CYYNNYSLSQPRYFCKTCRRYWTDGGSLRNVPVGGGSRKNKRSNSSSNNSSSSTSSSYKK 180
           ++++++++++++++++++++++ ++++++++++++++++++ ++++++++++++++++


BBF1       IPDLTIPTSS--QNPKIINEPHDLNLAFNPSATSNFSNISEFMALPLMNPNSTTSFMSSI 166
BBF1*      IPDLTIPTSSSTQNPKIINEPHDLNLTFNPSTTSNFSNISEFMALPLMNPNSTTSFMSSI 240
           +++++++++  ++++++++++++++ ++++ ++++++++++++++++++++++++++++


BBF1       MPQLSDSNNIMYSSSSTGLPNLHDLKPTLNFSLDGFDNNNGYGSLQGETAGXKLFFPLDD 226
BBF1*      MPQISDSNNIMYSSSSTGLPNLHDLKPTLNFSLDGFDNNNGYGSLQGETAGAKLFFPLDD 300
           +++ ++++++++++++++++++++++++++++++++++++++++++++++++ ++++++++


BBF1       LKNVSTPNDDHEFDEQNRGQAAESHGFWNGMLGGGSSW 264
BBF1*      ------------FEECFNGK------------------ 308
```

Supplemental Figure S1. Alignment of amino acid sequences of BBF1 and BBF1*. The amino acid sequences of BBF1 (NCBI accession no. AJ009594) (Baumann et al. 1999) and BBF1* (NCBI accession nos. X97942, X97943, and X97944) (De Paolis et al. 1996) are aligned by using the multiple sequence alignment program ClustalW (http://www.ebi.ac.uk/clustalw). An underline indicates the Dof domain of BBF1*, which was used for the previous *in vitro* DNA binding assay (De Paolis et al. 1996). Plus symbols (+) indicate the identical residues. Hyphens (-) indicate deletions.

-2335 caagttgacagttacctctttctcctcccgtacaacCTTTTcaccaccacccctccatgtccatgatttgttggtcccTAAAGtt -2251

taaataataataaataaataaaaaattgtaattaaaatttagagatcaactttggtcgttaaatatatattattaaaatattatacc -2161

gaccgaagttggtcggtattttatttatcctaaatatttggttCTTTTaacttagtgaccaacgttggtcgctaaattAAAAGgaccacc -2071

aatatagcgaccaatccattttggacgcgttttggtcggtatattgtgataagcgaccaactttggtcgctatttgtggtctCTTTTtgc -1981

cggatttctagcagtgtgtacacgcaaatcgAAAAGgataaaatgagatttttaaggctaacgagtgcagaattaaatttttaaaacgtaa -1891

gtttaggtcatcacatattatgtgatttttaaaaaaatgatcttcatatagaatacacacgtaacacgcttgcccaaaaactattagaac -1801

aaaataagtaacggctatttttaaaccttcaatccgtagcagcccactaatccctggctccaattttcttcaataataagttgtatgcag -1711

aaggaAAAAGattgttcctagaagttgtatgcgatactaaacaccttccccctgttatttttctgtctgttttCTTTAAAGcaacgaatc -1621

ctgtgccttgattctttcttgtttcctgtgttagttataagtttcaataatgaaaaataatatattatattgggcgtaggatcacaaggg -1531

attcaagaagcaacactagtcgggaatagaTAAAGgaacataatcaataatcagcatggAAAAGgaagaagtagcgaaaattcggcaaga -1441

ataatcaatttaattaattacagtagctaattcttatatattaagtttctgagAAAAGtaacatttcttcacatttatggacctacattt -1351

gttgtcactttctatctgcgcaaagaaaaataagaccatagtactgCTTTTggttagtacaactgttgacaaagaaaattactgggatat -1261

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ttttagAAAAGaaaaaataacaataagattgtcataggaAAAAGgattgaaaggtcgaccagataatttttttttttttttttttaccag -541

aatgatatgttccacaatatattgtacaattttgtcgaaaCTTTAtaataactttcttaacgttaataaattgggaacaagtttacgatt -451

aaatttcacatgtgatcattcaactttgtgtttattatccaacaaaaatgaaaaatattttgctagatgaagactttgtcatcctcggta -361

gaaaactaaaatagaAAAAGaattcaatcaatggagacCTTTTtctctttggagcaataattcaattcaattgggaaggaatttcctact -271

cccttctatTAAAGttcaaagaaaacccaataattcCTTTTattgcattaagaagaattttcctactagtgtatatcagttgactaggac -181

accaataattctatggagtagagcccatctcacacaaaCTTTTtccaatagcaatataactcttatctcttctaatatatataaaaattt -91

gttgaaaatatcatctattattctcttaccacaatcacaatttttcacatacagtttcttactCTTTTcagagaattaacgttgagtcc -1

Supplemental Figure S2. Location of the putative Dof binding motifs in the NP2.3 sequence of the *N* gene. (A/T)AAAG and CTTT(T/A) sequences are underlined. A double line indicates the position of the TAAAG motif in N20A (from −269 to −250). The nucleotide C at position −1 is followed by the initiation codon of the *N* gene.