Biosci. Biotechnol. Biochem., **67** (12), 2574–2583, 2003

Genomic Cloning of Ribonucleases in *Nicotiana glutinosa* Leaves, as Induced in Response to Wounding or to TMV-Infection, and Characterization of Their Promoters

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Received June 18, 2003; Accepted September 3, 2003

We previously cloned two distinct cDNA clones, *NGR1* and *NGR3*, encoding S-like ribonucleases (RNases) induced by wounding and tobacco mosaic virus (TMV) infection, respectively, in *Nicotiana glutinosa* leaves. To gain insight into the regulatory mechanism of the RNase genes, we analyzed nucleotide sequences of the genes *ngr1* (4.1 kbp) and *ngr3* (5.3 kbp), containing their structural genes as well as 5′-flanking regions. The *ngr1* gene is organized in three exons with two intervening introns, and *ngr3* has four exons interrupted by three introns. Primer extension analyses localized single transcription initiation sites at −32 and −99 upstream of the translation initiation codons ATG in the genes *ngr1* and *ngr3*, respectively. The β-glucuronidase (GUS) reporter gene analysis with serial 5′-deletion mutants as well as a gel shift assay defined the wound-responsive region at residues −509 to −288 in gene *ngr1* and a TMV-responsive region at the residues −401 to −174 in *ngr3*, respectively. Sequence search using PLACE and PlantCARE data bases showed that a wound-responsive element: the WUN-motif, occurs within the wound-responsive region in *ngr1*, while *ngr3* contains several potential *cis*-regulating elements, such as the elicitor responsiveness element: the W-box, a TMV responsive element: GT1, and the WUN-motif at positions between −401 and −174. These findings suggested that some of these *cis*-elements may be involved in inducible expressions of *ngr1* and *ngr3*. Furthermore, the gel shift assay suggested that the dissociation of protein factor(s) upon TMV-infection from the regulatory region may cause an inducible expression of *ngr3*.

Key words: *Nicotiana glutinosa*; promoter; ribonuclease; tobacco mosaic virus (TMV)-infection; wound-inducible

A variety of defense-specific events in higher plants are induced in response to various environmental stresses and developmental stimuli. The stress responses of plants are shown by altering expression of many specific genes encoding pathogenesis-related proteins, such as chitinases and glucanases,[1] and enzymes involved in production of phytoalexins and salicylic acid.[2] A vast amount of sequence information about *cis*-acting elements have been accumulated for the promoters of defense-related genes, and some trans-acting factors have also identified, including ethylene-responsive-element-binding factors, and the basic-domain leucine-zipper and WRKY protein families.[3] These studies are likely to lead to new ways to increase plant tolerance to disease and environmental stresses.

It was reported that RNase activity was induced in leaves of *Nicotiana glutinosa* by mechanical injury or by tobacco mosaic virus (TMV)-infection.[4,5] Matsushita and Shimokawa found two distinct RNase activities in TMV-infected *N. glutinosa* leaves, and showed that one RNase activity was induced by wounding and another RNase activity was induced 24 h after TMV-infection.[6,7] Lusso and Kuc reported that the increase of RNase activities in the *N. tabacum* leaves induced a systemic acquired resistance with TMV after challenge inoculation with viral and fungal pathogens,[8] and RNase NE was found to be induced in *N. tabacum* leaves upon infection by *Phytophthora cryptogea* and contribute to the control of fungal invasion in plants.[9] Besides *Nicotiana* species, the three distinct RNases RNS1, RNS2, and RNS3 were identified in *Arabidopsis thaliana*,[10] and RNS1 and RNS2 were found to be induced in response to phosphate starvation, whereas RNS3 was not.[11] Moreover, a mechanical wounding rapidly induced two S-like RNases, Z RNaseII in

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Abbreviations: bp, base pairs; GUS, β-glucuronidase; IPCR, inverse polymerase chain reaction; kbp, kilo bp; LA-PCR, long and accurate PCR; 4-MU, 4-methyl umbelliferone; 4-MUG, 4-methyl umbelliferyl-β-D-glucuronide; *NGR*, cDNAs encoding ribonuclease from *N. glutinosa*; *ngr*, genes encoding ribonuclease from *N. glutinosa*; TMV, tobacco mosaic virus

Zinnia elegans[12] and RNase LE in tomato.[13] In addition, the RNase gene expression was found to be induced in the common bean (*Phaseolus vulgaris* L.) leaves during senescence and in diseased states.[14] Although these findings strongly suggested the involvement of RNases in defense events in plants, little is known about the regulatory mechanisms of the gene expression of plant RNases.

We earlier cloned three distinct cDNAs (*NGR1*, *NGR2*, and *NGR3*) encoding RNases in *N. glutinosa* leaves and showed that the *ngr1* transcript was induced within 4 h after wounding and then gradually decreased during 24 h, while the expression of the *ngr3* gene was significantly induced upon TMV-infection after 48 h and this increase was observed through 72 h. In contrast, the gene *ngr2* was found to be constitutively expressed in the *N. glutinosa* leaves.[15,16] Subsequently, *NGR1* and *NGR3* were expressed in *E. coli* cells and their translational products, RNase NW and RNase NGR3, respectively, were characterized in terms of their enzymatic properties.[17] The result indicated that the two enzymes have a slightly distinct substrate specificity and specificity constants, suggesting that RNase NW and RNase NGR3 may have different roles in *N. glutinosa* leaves. Moreover, the three-dimensional structure of RNase NW was recently determined at 1.5 Å resolution, and a structural basis for its guanosine preference was solved at an atomic level.[18] To extend the study of the *N. glutinosa* RNases, we cloned genes *ngr1* and *ngr3* encoding wounding and TMV-infection inducible RNases, RNase NW and RNase NGR3, respectively, and characterized their transcription initiation sites and their promoters in the expectation that this would be of help in elucidation of the regulatory mechanism of plant RNases. In this study, we describe the genomic organizations of *ngr1* and *ngr3* and identify their exon-intron boundaries. We have also characterized the 5′-flanking regions of *ngr1* and *ngr3* and showed the presence of potential *cis*-acting regulatory elements that may be involved in inducible expression of *ngr1* and *ngr3*.

Materials and Methods

Materials. Oligonucleotides used in this study were purchased from Hokkaido System Science Co. The *ExTaq* DNA polymerase and DNA ligation kit were purchased from Takara Shuzo, and used as recommended by the supplier. Restriction endonucleases and DNA modifying enzymes were purchased from MBI Fermentas. The plasmid vector, pGEM-T EASY vector, was obtained from Promega and the *E. coli* strain JM109 was used as a host cell for cloning. The binary vector plasmid pBI101 was from Clontech and *E. coli* strain DH10B was used as a host cell for the cloning. Lambda EMBL4/*Eco* RI vector and Gigapack Gold packing extract were purchased from Stratagene. Nitrocellulose filters were obtained from Schleicher and Schuell. *Agrobacterium tumefaciens* strain LBA4404 was kindly given by Prof. Y. Takanami (Kyushu University). 4-Methyl umbelliferone (4-MU) and 4-methyl umbelliferyl-β-D-glucuronide (4-MUG) were purchased from Wako Pure Chemicals. All other chemicals were of analytical grade for biochemical use.

Plant materials. N. glutinosa plants were grown as previously described.[7] Mature leaves of 8-week-old *N. glutinosa* were dusted with carborundum (600 mesh) and rubbed with cotton pads moistened with 0.1 M Na-phosphate buffer (pH 7.0) or with TMV (10 μg/ml) in the same buffer. Leaves were harvested after appropriate intervals, immediately frozen in liquid nitrogen, and stored until use for RNA extraction. *N. tabacum* cv. Sumsun NN, which was given to us by Prof. Y. Takanami (Kyushu University), was used for transformation. Sterilized seeds were grown on solid MS medium.[19]

Cloning procedures. General genetic procedures were essentially done as described in ref. 20. Cloning of the *ngr1* gene was done by screening a partial genomic library using the cDNA *NGR1* as a probe. A partial genomic library was made as follows. The *N. glutinosa* chromosomal DNA was digested with *Eco* RI and size-fractionated on 0.6% agarose. The DNA fragments of 15 kb, which gave a clear hybridized band with *NGR1*, were cloned in the phage vector EMBL4. The plaque was screened by plaque hybridization and positive clones were amplified and analyzed by Southern hybridization to define the gene *ngr1* in the 4.1-kb fragment.

Cloning of the *ngr3* gene was done as follows. The structural gene of *ngr3* was cloned by using the long and accurate PCR (LA-PCR) with the following three sets of oligonucleotide primer pairs: S1-forward primer, 5′-TCATTATTGGTCAAGATTTTC-CTTGTAC-3′; S1-reverse primer, 5′-TGGCCAT-AGACCATGGATGCTAAAATCTTC-3′; S2-forward primer, 5′-GAAGATTTTAGCATCCATGGT-CTATGGCCA-3′; S2-reverse primer, 5′-TTCGCC-AAGAGCAGAACAGGTCCCATGTTT-3′; S3-forward primer, 5′-AAACATGGGACCTGTTCTGCT-CTTGGCGAA-3′; S3-reverse primer, 5′-AAATTC-ATCATGGTCAGACTCAGAGGAGAA-3′; which were chosen from the *NGR3* cDNA sequence. The samples were first denatured by heating at 98°C for 1 min and then incubated for 35 cycles at 98°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The samples were finally incubated for 10 min at 72°C. The amplified products were purified on a 1% agarose gel, ligated into the pGEM-T EASY vector, and sequenced by a DNA sequencer D-100 (Shimadzu) using a cycle sequencing kit (Amersham). The 5′-regulatory region of *ngr3* was cloned as

follows. The *N. glutinosa* genomic DNA (1 μg) was digested with *Bam* HI overnight and self-ligated by using T4 DNA ligase (Takara Shuzo). The circularized DNA was ethanol-precipitated, dried, and resuspended in 5 μl of distilled water. The inverse PCR (IPCR) was made up with 100 ng of circularized DNA and used LA-taq DNA polymerase (Takara Shuzo). The forward primer, 5′-TCTGAGTCTGACCATGATGAATTTTAATTC-3′; and reverse the primer, 5′-CCTCCTCCAATAAACTTTCGATGTTGCTTC-3′, were designed on the basis of the nucleotide sequences of *NGR3*.[16] The samples were first denatured at 94°C for 10 min, which was followed by 35 cycles for 30 sec of denaturation at 94°C, primer annealing for 2 min at 55°C, and extension for 1 min at 72°C, with a final extension at 72°C for 10 min. The amplified products were analyzed in a manner identical with the procedures described for LA-PCR.

Primer extension analysis. The *N. glutinosa* leaves were wounded or infected with TMV, as described above and detached 4 h after wounding or 72 h after TMV-infection. Total RNA was purified from the treated *N. glutinosa* leaves using a plant RNA isolation kit (Qiagen). The transcription initiation site was found by primer extension using a primer extension kit (Promega). The oligonucleotides complimentary to nucleotides 37 to 66 and 51 to 80 downstream from the translation initiation codons ATG in *NGR1*[15] and *NGR3*,[16] respectively, were synthesized and end-labeled with [α-^{32}P] ATP and T4 polynucleotide kinase. For the primer extension reaction, end-labeled oligonucleotides and 3 μg total RNA were annealed at 58°C for 20 min and then cooled to room temperature. The extension reaction was done using 1 unit of AMV reverse transcriptase at 42°C for 30 min, as recommended by the supplier. The reaction products were heated at 90°C for 10 min and analyzed on a 8% polyacrylamide-7 M urea denaturing gel. The gels were autoradiographied using Fuji Film FLA 5000.

Database searching. Putative transcription factor binding sites in *ngr1* and *ngr3* genes were analyzed using the online facilities at the PLACE[21] and PlantCARE.[22]

Construction of GUS-fusion genes and Ti-mediated gene transfer. GUS-fusion genes were constructed by inserting 5′-flanking regions of *ngr1* and *ngr3* into the multi-cloning site of the binary vector plasmid pBI101 (Clontech). Plasmids were used to transform *E. coli* DH10B and mobilized into *Agrobacterium tumefaciens* strain LBA4404 before leaf disc transformation of sterile *N. tabacum* cv. Sumsun NN shoots.

Fluorometric quantification of GUS activity. Wounding or TMV-infection of the transformant leaves were done in a manner identical to those described for the *N. glutinosa* leaves. Leaves treated for 4 h after wounding or for 72 h after TMV-infection were homogenized in GUS extraction buffer (50 mM sodium phosphate pH 7.0, 10 mM EDTA, 0.1% triton X-100, 0.1% sarkosyl, 10 mM 2-mercaptoethanol) on ice followed by centrifugation. Fluorometric GUS enzyme assay was done by the method of Jefferson.[23] Protein concentrations were measured using Bradford methods.[24]

Gel mobility shift assay. Preparation of nuclear extracts was done as described.[25] Briefly, mature leaves of 8-week-old *N. glutinosa*, which were detached 72 h after TMV-infection as described above, were homogenized in liquid nitrogen with extraction buffer (25 mM MES, pH 6.0, 250 mM sucrose, 5 mM EDTA, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.5 mM spermidine, 0.3% Triton X-100), and the homogenate was filtered through five layers of cheesecloth. Nuclei were pelleted by centrifugation (1,000 *g*, 4°C, 10 min), washed three times with the extraction buffer, and resuspended in a small volume of the dialysis buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 12.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, 1 mM PMSF, 2 mM DTT). After sonication for 5 sec with 5 cycles, they were shaken on ice for 45 min, and then high-speed centrifugation (10,000 *g*, 4°C, 50 min) was done to obtain the supernatant. The yields of nuclear proteins were about 1.5 mg from 100 g of leaves. The DNA fragments used for the gel mobility shift assay were amplified by PCR and labeled by the Klenow fragment using [α-^{32}P]dTTP. For the gel mobility shift assay, the nuclear extracts (0.1 μg) were first incubated at 25°C for 15 min, and DNA fragments and 1 μg of poly (dI-dC) were added. The mixtures were analyzed by electrophoresis through non-denatured 5% polyacrylamide gels containing 1× TBE buffer at 1,000 V for 1.5 h.

Results and Discussion

Cloning and nucleotide sequences of ngr1 and ngr3

The gene *ngr1* was cloned by screening a partial genomic library, as described under Materials and Methods. Screening the partial genomic library followed by Southern blotting analysis localized the *ngr1* gene within a 4.1-kbp *Bam* HI/*Eco* RI DNA fragment (data not shown). The nucleotide sequence of the entire 4.1-kbp fragment was identified, as shown in Fig. 1A. The nucleotide sequence consists of 4,104 bp including the structural gene as well as 2,364 bp and 350 bp in the 5′- and 3′-flanking regions, respectively. The structural gene of *ngr3* was amplified by LA-PCR with a genomic DNA, using

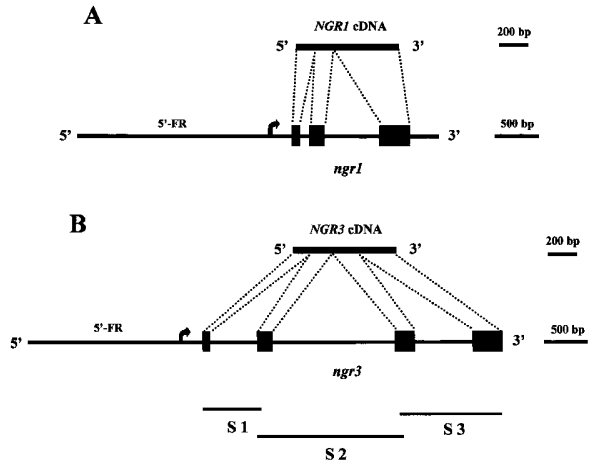

Fig. 1. Structures of the *N. glutinosa ngr1* and *ngr3* Genes.

A, The 4.1-kbp *Bam* HI/*Eco* RI fragment containing the structural gene as well as the 5′- and 3′-flanking regions of *ngr1* was cloned by a partial genomic DNA library screening. B, The *ngr3* gene was amplified by LA-PCR followed by IPCR, as described under Materials and Methods. S1, S2, and S3 indicate DNA fragments amplified by LA-PCR. The exons and introns are indicated by boxes and solid lines, respectively. Arrows show the transcription start sites in both genes.

three sets of the oligonucleotide primers designed on the basis of the nucleotide sequence of *NGR3*. The LA-PCR using primers S1, S2, and S3 produced three DNA fragments, 0.65 kbp, 2.0 kbp, and 0.95 kbp, and the DNA fragments thus obtained were sequenced. The complete sequencing of the three DNA fragments overlapped them, thereby providing an entire nucleotide sequence of the structural gene of *ngr3*, as shown in Fig. 1B. The 5′-flanking region of *ngr3* was amplified with IPCR, as described under Materials and Methods. The IPCR yielded a 2.4-kbp DNA fragment and its nucleotide sequencing provided 1,949 bp in the 5′-flanking region of *ngr3* (Fig. 1B). The nucleotide sequence data for *ngr1* and *ngr3* appear in DDBJ, EMBL, and GeneBank nucleotide sequence data bases under the accession numbers AB112028 and AB112026, respectively.

On the basis of comparison of the nucleotide sequences of *ngr1* and *ngr3* with those of the cDNAs *NGR1* and *NGR3*, respectively, the genomic structures of *ngr1* and *ngr3* were established, as shown in Fig. 1. The gene *ngr1* consists of three exons, which were interrupted by two introns, while *ngr3* is composed of four exons and three introns. All introns except for the first intron in *ngr3* contain the conserved GT and AG dinucleotides at the 5′ and 3′ splice sites, respectively.[25,26] The first intron in *ngr3* has GC in stead of GT at 5′ splice site. The first intron (106 bp) in *ngr1* occurs between codons for amino acids Gln12 and Trp13 in RNase NW, and the second intron (594 bp) locates between those for amino acids Gln64 and Val65 in RNase NW. Hence, the two introns in *ngr1* are in phase 0. Similarly, the first and second introns in *ngr3* occur between codons for Gln12 and Trp13 and for Glu64 and Ile65, respectively; the two introns 1 and 2 in *ngr3*, like those in *ngr1*, are in phase 0. Hence, the exon-intron splice junctions in *ngr1* and *ngr3* occur at the same positions for the first two introns with respect to the amino acid sequences of the two RNases. The additional intron (third intron) found in *ngr3* is located within the codon (GAG) for Glu129 in RNase NGR3; the third intron in *ngr3* is in phase 1.

Recent phylogenetic analyses of plant RNases, together with information on the intron number and position grouped them into three classes: the class I contains S-like RNases the genes of which consist of three or four exons interrupted by two or three introns, while the class II includes RNases the genes of which are split into a number of exons and introns, more than eight, and the class III contains S-RNases whose genes have one intron.[27] In the phylogenetic classification, cDNAs *NGR1* and *NGR3* belong to the class I on the basis of their sequence similarity to those of S-like RNases.[27] The intron number and position found by the present analysis of *ngr1* and *ngr3* are in good agreement with those of genes for S-like RNases classified into class I. It is however clear that RNase NW and RNase NGR3 form distinct groups in the class I RNases: RNase NW is homologous to RNase NE from *N. alata*[28] and RNase LE from tomato,[13] while RNase NGR3 is to RNase LX from tomato[29] and Z RNaseII from *Zinnia elegans*.[12] This finding suggested occurrence of distinct evolutionary events in RNase genes belonging to class I, and that S-like RNases forming distantly related groups in class I may have distinct roles in plants.

Location of transcriptional start site

The transcriptional start sites of *ngr1* and *ngr3* were located by primer extension analysis, as described under Materials and Methods. As shown in Fig. 2, 98 and 179 nucleotide primer extension products were identified in total RNA from wounded and TMV-infected leaves, respectively. Based on comparison with the sequence ladder, transcription initiation sites could be localized at adenosine −32 and at adenosine −99 upstreams from the translation initiation sites, respectively, of the *ngr1* and *ngr3* genes.

Mapping of putative cis-acting elements in the 5′-flanking regions

Potential transcriptional factor binding sites and regulatory elements in the 5′-flanking regions of *ngr1* and *ngr3* were searched for by computational analysis using the databases PLACE[21] and PlantCARE.[22] Analysis of the nucleotide sequences of 2.3 kb and 1.9 kb of the 5′-flanking regions in *ngr1* and *ngr3* showed canonical TATA and CAAT boxes at 32 bp

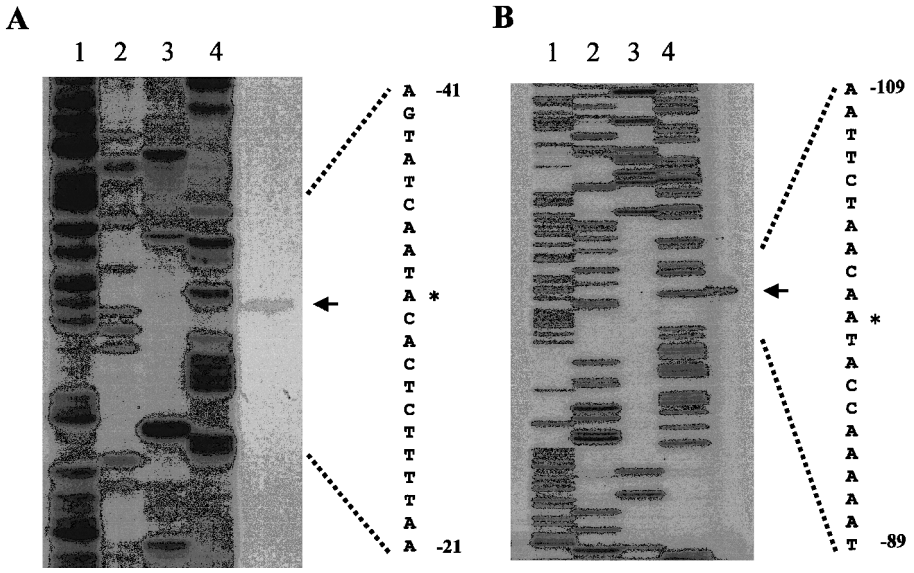

Fig. 2. Mapping of Transcription Initiation Sites in *ngr1* and *ngr3* by Primer Extension Analysis.

Oligonucleotides complementary to the 5′-regions of *NGR1* and *NGR3* cDNAs were annealed to 3 μg total RNA from *N. glutinosa* leaves treated by wounding and by wounding followed by TMV-infection and extended, as described under Materials and Methods. Sequence ladders of 5′-flanking regions of *ngr1* and *ngr3* primed with the same primers as in primer extension reactions are shown in lanes 1–4 (representing A, C, G, and T ladders, respectively). The arrows indicate the primer extension products. Asterisks indicate transcription initiation sites in *ngr1* and *ngr3*.

and 150 bp upstream from the transcription initiation site for *ngr1*, respectively, (Fig. 3A) and at 33 bp and 109 bp upstream of the transcription initiation site for *ngr3* (Fig. 3B). The sequence search in the 5′ flanking region of *ngr1* found several regulatory elements, such as WUN-motif, SARE, ERE, TCA-element, EIRE, and AT-rich sequence, as shown in Fig. 3A. Similarly, WUN-motif, TC-rich repeats, and EIRE for defense and stress-responsiveness element were located upstream from the transcription initiation site of *ngr3*. ELI-box 3 and W-box for elicitor responsiveness elements were also detected. Furthermore, we detected many TMV and salicylic acid responsiveness elements for GT1 binding site and EIR element upstream the transcription initiation site of *ngr3*, as shown in Fig. 3B.

Promoter analysis of ngr1 and ngr3

To define the wound-responsive region within the 5′-flanking region of *ngr1* and TMV-responsive region within that of *ngr3*, we analyzed the individual expressions of the GUS reporter gene, which was fused to several unidirectional 5′-deleted *ngr1* and *ngr3* promoters. For this purpose, the individual DNA fragments were amplified by PCR and ligated to the multi-cloning site of plasmid pBI101, as described under Materials and Methods. The transformation of *N. tabacum* cv. Sumsun NN by individual constructs was confirmed by PCR using specific primers for the GUS reporter gene. As results, three to ten transformants for each construct were obtained and used for expression of the GUS activity in response to wounding or TMV-infection. About 8-week-old transformants were treated with either wounding or TMV infection, and the leaves were detached 4 h after wounding and 72 h after TMV infection. The result indicated that the full-length constructs ($-891/+27$ and $-1841/+201$) demonstrated approximately 2-fold inductions of GUS activity at 4 h after wounding and at 72 h after TMV-infection, respectively, in the leaves of seven and ten independent transformants (Fig. 4), being consistent with expression patterns of *ngr1* and *ngr3* as seen by Northern blot analysis.[16] These findings indicated that the 5′flanking regions contain individual responsive elements. Next, we analyzed the wound-responsive or TMV-responsive expression of GUS activity of a series of 5′-deletion mutants using leaves wounded for 4 h or infected with TMV for 72 h. In cases for leaves infected with TMV, they displayed discrete lesions within approximately 48 h. The transformants ($-509/+27$) containing the promoter sequence deleted up to -509 of *ngr1* had a significant promoter activity, which was more than twofold of that obtained without wounding (Fig. 4A). However, a further deletion up to -287 in the promoter led to a failure in the induction of GUS activity by wounding (Fig. 4A). These results indicated that the promoter region from -509 to -288 contributes to the wound-responsive expression of *ngr1* in leaves. As described above, a wound-responsive element: the WUN-motif (TAATTACTC), occurs at positions -457 to -449 in *ngr1*, suggesting that it may participate in the inducible expression of *ngr1* by wounding (Fig. 3A).

As for the gene *ngr3*, all constructs conferred the

A

Fig. 3.

characteristic of TMV-induced expression, as shown in Fig. 4B. The result suggested that the region up to 500 bp upstream from the translation initiation codon may contain the TMV-response promoter element of *ngr3*.

To further define the TMV-response promoter element within the 500 bp upstream region in *ngr3*, a gel-shift assay was done using two DNA fragments (fragment I, +98/−173 and fragment II, −174/−401) amplified in the account of the 5′-deletion analysis constructs of *ngr3*. The nuclear extracts from non-treatment or TMV-infected leaves were incubated with double-strand ^{32}P-labeled DNA probes. To avoid non-specific binding, 1 μg of poly (dI-dC) was added to the reaction mixtures. The mobility shift assays revealed the formation of DNA-

B

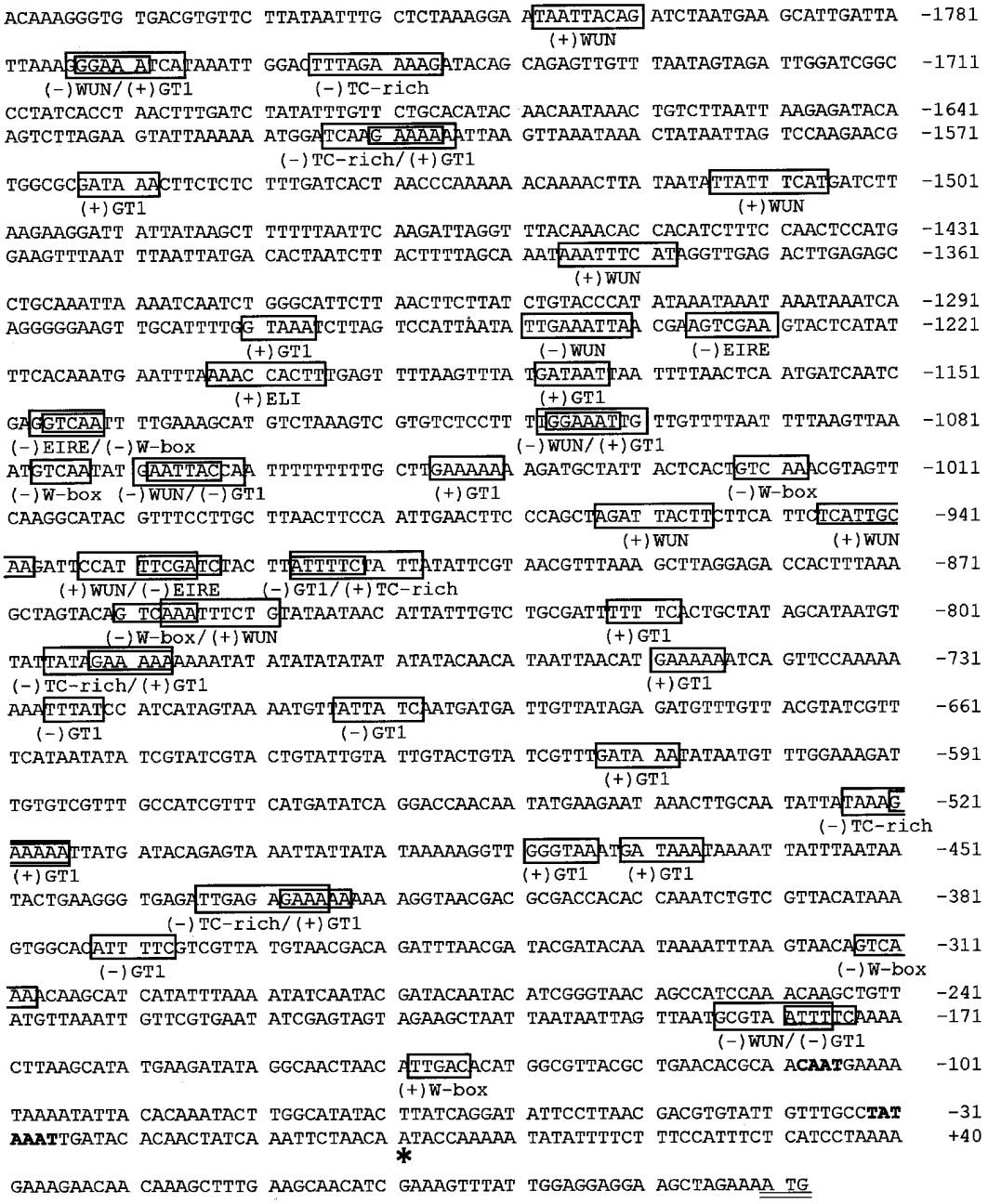

Fig. 3. 5′-Upstream Sequences of the *ngr1* and *ngr3* Genes.

The nucleotide sequences of the 5′-flanking regions of *ngr1* (A) and *ngr3* (B) are shown. Numbers at right are based on the transcription initiation sites (+1) as determined by primer extension analysis. Transcription initiation sites are indicated by asterisks. The translation start sites (ATG) are double underlined. Canonical TATA box and CAAT box are shown in bold letters. Nucleotide sequences homologous to consensus sequences for binding to the regulatory factors are boxed. (+) and (−) indicate nucleotide sequences homologous to consensus sequences and to complementary sequences of the consensus sequences, respectively. WUN (WUN-motif), wound-responsive element; ERE, ethylene-responsive element; SARE, salicylic acid responsive element; TCA (TCA-element), *cis*-acting element involved in salicylic acid responsiveness; EIRE, elicitor-responsive element; AT-rich (AT-rich sequence), elicitor-mediate activated element; GT1 (GT1 consensus), GT1 binding site; TC-rich (TC-rich repeats), *cis*-acting element involved in defense and stress responsiveness; ELI (ELI-box3), elicitor box3; W-box (TTTGACT), wounding and pathogen response; EIR (EIR-element), elicitor-responsive element. Nucleotide sequences in parenthesis indicate consensus sequences. R and W indicate A or G and A or T, respectively.

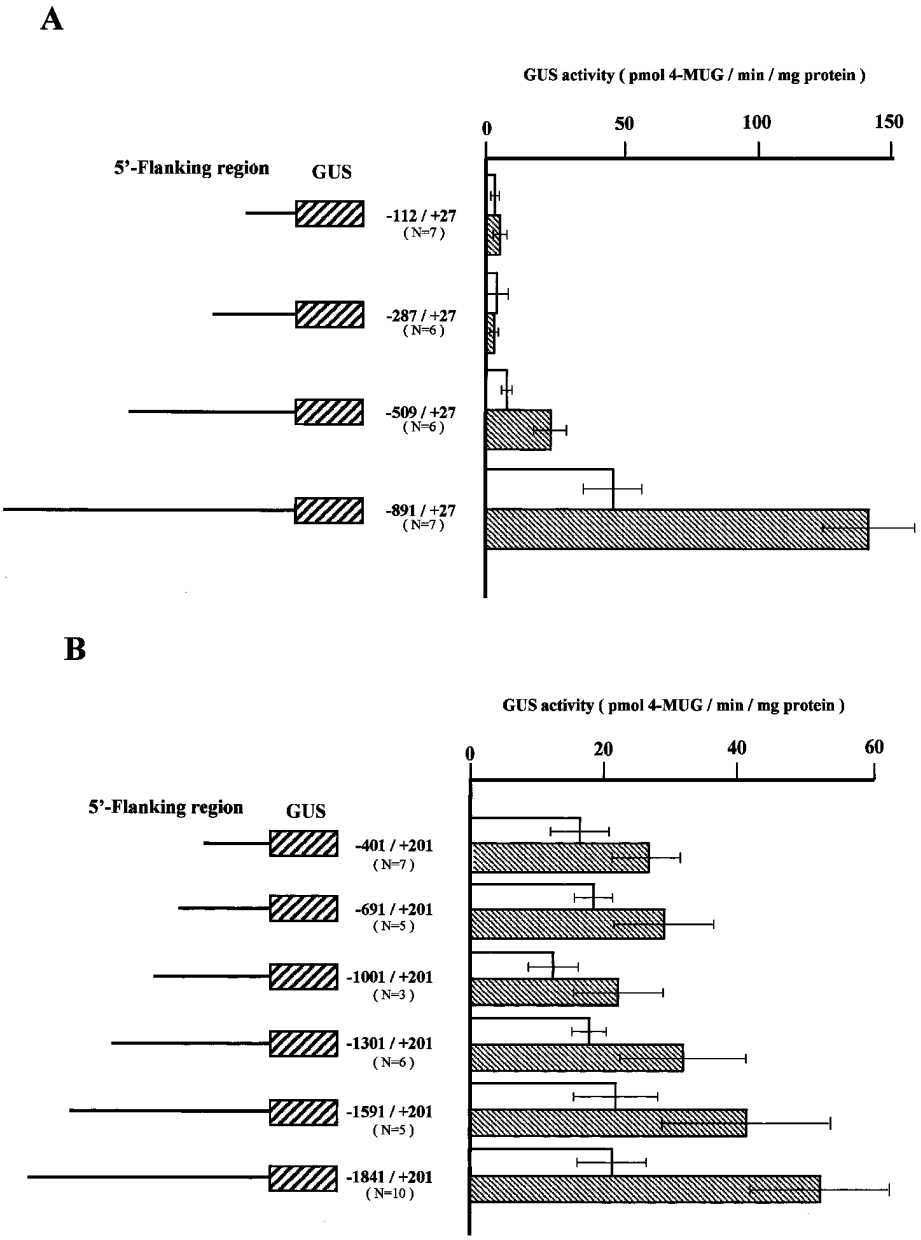

Fig. 4. Measurement of *ngr1* and *ngr3* Promoter Activities.

DNA fragments containing different lengths of the 5′-flanking regions of *ngr1* (A) and *ngr3* (B) were cloned upstream from the GUS reporter gene in plasmid pBI101, and the promoter activity of the cloned DNA was established by expressing promoter-reporter constructs in *N. tabacum* Sumsun cv. NN. For each construct, three to ten independent transgenic lines were investigated. Leaves wounded for 4 h or infected for 72 h with TMV were detached from transgenic plants, and the GUS activity was assayed as described under Materials and Methods. The specific GUS activity is given as the conversion rate of 4-MUG to 4-MU (pmol 4-MUG/min/mg protein). Slash bar in (A), wounding; slash bar in (B), infection with TMV; open bars, no-treatment. Bars represent standard errors.

protein complexes by the fragment II (−174/−401) with nuclear extracts from non-treatment leaves, whereas the incubation of fragment I (+98/−173) with nuclear extracts showed no band shift (Fig. 5A). Furthermore, gel shift assays using the amplified DNA fragments derived from the 5′-flanking region between −1841 and −402 gave no specific complexes with nuclear extracts (data not shown). These experiments revealed that TMV-responsive element(s) may occur at positions −401 to −174 in the 5′-flanking region of *ngr3*. The result further indicated that nuclear extracts from non-treatment leaves may contain protein factor(s) which would suppress expression of *ngr3*, and that the dissociation of nuclear factor(s) upon TMV-infection would cause an inducible expression of *ngr3*.

To corroborate a specific interaction between nuclear proteins and fragment II, further gel shift assays were done. Unlabeled fragments representing a 500-times molecular excess of the labeled fragment

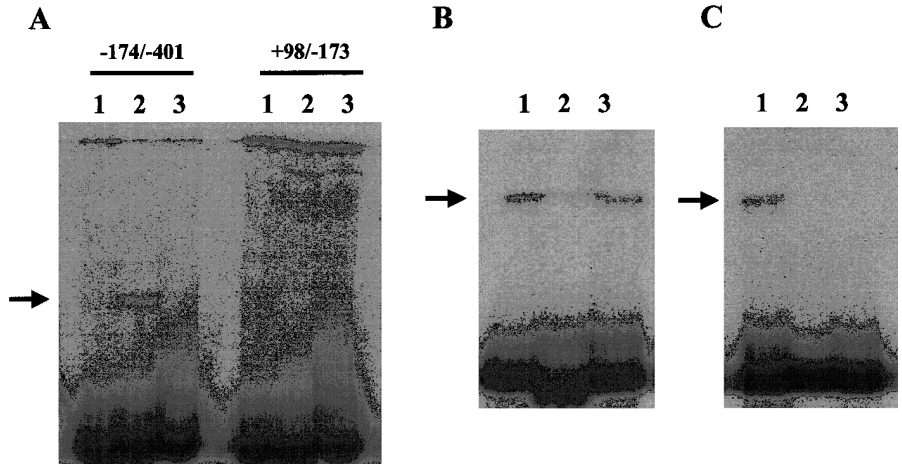

Fig. 5. Gel Shift Assay of the 5′-Flanking Region of *ngr3*.

A, Gel-shift assays of nuclear extracts to labeled +98/−173 and −174/−401 fragments of the *ngr3* promoter. Lane 1, DNA; lanes 2 and 3, DNA and the nuclear extracts from non-treated and TMV-treated leaves, respectively. B, Competition binding assays using the −174/−401 labeled probe of the *ngr3* promoter. Unlabelled fragments −174/−401 and −402/−691 were added to the binding reactions at 500-fold molar excesses before the addition of 0.1 μg of tobacco nuclear protein from non-treated leaves. Lane 1, no competition; lane 2, competition with −174/−401; and lane 3, competition with −402/−691. C, Heat and Protease K treatments of nuclear extracts. Lane 1, −174/−401 and the nuclear extract from non-treated leaves; lane 2, −174/−401 and the nuclear extract incubated at 90°C for 30 min, and lane 3, −174/−401 and the nuclear extract digested by Protease K at 37°C for 4 h.

II were added to the DNA-protein interaction reaction mixture. Addition of the unlabeled fragment II was sufficient to reduce the DNA-protein complex formation (lane 2 in Fig. 5B), whereas the DNA fragment derived from positions −402 to −691 in the 5′-flanking region had little effect on formation of the complex (lane 3 in Fig. 5B). Furthermore, nuclear extracts were subject to Protease K digestion or heat treatment before incubation with the fragment II. Both heat and Protease K treatments completely abolished the binding activity with the DNA fragment (lanes 2 and 3 in Fig. 5C). These results demonstrated that nuclear extracts from non-treated leaves contain protein factor(s) that specifically interact with the nucleotide sequence at positions −401 to −174 in the 5′-flanking region, while those from TMV-treated leaves do not. It could be thus speculated that an inducible expression of *ngr3* upon TMV-infection is caused by dissociation of protein factor(s) from the TMV-inducible element occurred at positions −401 to −174 in the 5′-flanking region. In other words, protein factor(s) may interact with the TMV-inducible element as a repressor, thereby suppressing the expression of *ngr3*. Further biochemical and molecular studies of the nuclear extracts will address this assumption.

Acknowledgments

We should like to thank Prof. Y. Takanami and Dr. M. Takeshita of Kyushu University for valuable comments and suggestions throughout this study, and also for free access to the greenhouses.

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