Isolation and Characterization of a Wound-inducible Ribonuclease from *Nicotiana glutinosa* Leaves

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A wound-inducible ribonuclease (RNase NW) was purified from leaves of *Nicotiana glutinosa*. The purified RNase NW has an optimum pH around 5 and 7, and its base specificity is suggested based on the relative rates of hydrolysis of homopolyrribonucleotides to be a preference for guanine base. The complete amino acid sequence of RNase NW was deduced by a combination of protein and cDNA sequencings. The cDNA sequence includes the entire coding sequence for a polypeptide with 229 amino acids including a putative secretion signal peptide at the N-terminal composed of 25 amino acids. The amino acids identified by the protein chemical methods are unambiguously localized within the deduced amino acid sequence from the cDNA sequence. Comparison of the sequence of RNase NW with those of other known plant RNases showed that it was identical except for eight residues to that of *N. alata* RNase NE, which is present in the style and pollen under normal conditions and is induced in roots in response to phosphate starvation [Dodds et al., Plant. Mol. Biol., 31, 227-238 (1996)]. RNase NW shows considerable sequence similarity to other known RNases, sharing 57% to 84% identical residues. Northern blot analysis using an RNase NW cDNA fragment as a probe showed that the RNase NW transcript was not detected in leaves without wounding, but it was induced within 4 h after wounding and then gradually decreased during 20 h.

Key words: amino acid sequence; cDNA sequence; *Nicotiana glutinosa*; ribonuclease; wound-inducible RNase

Introduction

Although the induction of ribonuclease (RNase) activity by mechanical injury, osmotic shock, or TMV infection is known to be a factor in the defence response of plants, very few studies on plant RNases had been done for many years. However, since a recent discovery that S-glycoproteins associated with gametophytic self-incompatibility in the Solanaceae had ribonuclease activity, 1, 2 a large amount of physiological information has been accumulated on plant RNases. 3 That is, Taylor and Green identified three RNase genes, *RNS1*, *RNS2*, and *RNS3*, in the self-compatible species *Arabidopsis thaliana* by PCR and found that these three RNases were senescence-induced RNases, and *RNS1* and *RNS2* were also induced in response to phosphate starvation, but *RNS3* was not. 4 It was further reported that several RNases were induced in cultured tomato cells extracellularly 5 and intracellularly 6 upon phosphate starvation of tomato cell culture, suggesting that RNases function as scavengers of exogenous phosphate. Recently, it was reported that two RNases, ZRNase I and II, were present in *Zinnia elegans*, and ZRNase I was induced in response to xylogenesis and phosphate starvation, but ZRNase II was induced by wounding. 7 Furthermore, a few RNases were found in the seeds of the Cucurbitaceae, such as *Momordica charantia* 8, 9 and *Cucumis sativus* 10 and characterized in terms of their amino acid sequences and subunit specificities. However, despite the vast amount of physiological information about the plant RNases that has been accumulated for both self-incompatible and self-compatible species, the mechanism by which the plant RNases are induced in response to diverse stimuli is still not known and studies on their structure-function relationships have not thus far been pursued.

It was previously described that RNase activity was induced in leaves of *Nicotiana glutinosa* by tobacco mosaic virus (TMV) infection. 11 Meanwhile, Matsushita and Shimokawa found two distinct RNase activities in TMV-infected *N. glutinosa* leaves, and subsequently showed that one RNase designated RNase M was induced by wounding and its induction was inhibited by cycloheximide, while another RNase designated RNase F was induced by TMV infection and its induction was not inhibited by cycloheximide. 12 In this study, as an initial step toward understanding structure-function relationships of RNases in the *N. glutinosa* leaves, we have purified the wound-inducible RNase to homogeneity by column chromatography, and examined its enzymatic properties. Furthermore, we have analyzed tryptic and cyanogen bromide peptides, amplified the full-length of cDNA by PCR methods, and sequenced its nucleotides.

Nomenclature. A wound-inducible RNase from the *N. glutinosa* leaves was initially designated RNase M on the basis of the mobility on the gel electrophoresis. 13 Since a fungal RNase isolated from *Aspergillus saitoi* is known to be referred to as RNase M, 14 this study, to
avoid confusion, we renamed the *N. glutinosa* wound-inducible RNase “RNase NW”.

**Materials and Methods**

*Plant materials. N. glutinosa* was grown as previously described. The leaves of *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 in the same buffer. After harvest, leaves were immediately frozen with liquid nitrogen and stored at −80°C until use. Seeds of bitter gourd (*Momordica charantia*) were purchased from Takii Seeding Co. (Kyoto) and RNase MC1 was isolated from them as described previously.

**Purification of RNase NW.** Extraction of RNases from the *N. glutinosa* leaves infected by TMV and the separation of RNase isozymes by hydroxylapatite column chromatography were done as described previously. Purification of RNase NW was done by ion-exchange chromatography on a Q-Sepharose column (1 × 14 cm) equilibrated with 10 mM Na-phosphate buffer, pH 7.2. The proteins were eluted with a linear gradient of 0–0.4 M NaCl in the buffer. Further purification of RNase NW was done by reverse-phase HPLC (RP-HPLC) on a YMC gel C18 column (4.6 × 250 mm) with a linear gradient of 0–56% acetonitrile in 0.1% trifluoroacetic acid (TFA).

**Protein chemical methods.** RNase NW was hydrolyzed in the vapor of 5.7 N HCl containing 0.02% 2-mercaptoethanol at 110°C for 24 h in an evacuated sealed tube. The amino acids were analyzed on a Shimadzu LC6A system amino acid analyzer after derivatization of amino acids with phenylisothiocyanate, as described by Heinrikson and Meredith. (19) The amino acids were sequenced by a gas-phase protein sequencer, PSQ-1 (Shimadzu). RNase NW was reduced and pyridylethylated as described previously. (19) One hundred micrograms of the pyridylethylated RNase NW (PE-RNase NW) was digested with 2 µg of trypsin in 0.2 M N-methylmorpholine acetate buffer, pH 8.2, at 37°C for 6 h. Cyanogen bromide (CNBr) cleavage of PE-RNase NW (100 µg) was done with 100 µg of CNBr in 70% formic acid overnight. The resulting peptides derived from tryptic digestion and CNBr cleavage were resuspended in 0.1% TFA and isolated by RP-HPLC on a C18 column, as described above. SDS-PAGE was done by the Laemmli method using 15% acrylamide, (20) and the proteins were stained by silver. (21)

**Assay for RNase activity.** The enzymatic activity of RNase NW on yeast RNA or homopolyribonucleotides was assessed at 37°C by measuring the uranyl acetatesoluble material absorption at 260 nm, principally as described in reference 22. To find the pH optimum, the buffers were as follows: Na-acetate for pH 3–5; Na-phosphate for pH 6 and 7; Tris-HCl for pH 8 and 9; Tris-borate for pH 10; and Gly-NaOH for pH 11 and 12.

**Nucleotide sequence of cDNA encoding RNase NW.** The *N. glutinosa* leaves were wounded by Carborundum as described above, and after 4 h, total RNA was isolated by the method of Chirgwin. (23) Poly(A)+ RNA was purified with Oligotex-dT30 Super (Takara) as recommended by the manufacturer. The double stranded cDNA was synthesized with a cDNA rapid cloning module (Amersham), and ligated to the phage vector λgt11 (Promega). The cDNA fragments encoding RNase NW were amplified by consecutive PCRs including RT-PCR, 3′ RACE, (24) and RAGE (25) methods, using the oligonucleotide primers listed in Table 1. The amplified DNA was purified by agarose gels and recovered by adhesion to a silica matrix according to the manufacturer’s instructions (GENECLEAN II, BİO 101 Inc.) and ligated to the plasmid pGEM-T Vector (Promega). The double-stranded DNA sequencing was performed with a DNA sequencer D-1000 (Shimadzu) using a cycle sequencing kit (Amersham).

**Northern blotting.** Total RNA was extracted, as described above, from the wounded *N. glutinosa* leaves after the indicated times. About 30 µg/lane was separated on 1.0% formaldehyde agarose gel, stained with ethidium bromide, and transferred to a nylon membrane. (25) Prehybridization, hybridization, and washing were done by the method described in reference 27. For detection of RNase NW transcripts, the cDNA fragment amplified by 3′ RACE (650-bp fragment) was used as a hybridization probe. Probe labeling and detection were done using DIG-High Prime Labeling and DIG Detection kits (Boehringer Mannheim), respectively.

**Results**

**Purification and characterization of RNase NW**

In the foregoing work, a wound-inducible RNase was separated from the isozyme (RNase F) by hydroxylapatite column chromatography, (26) but it was still con-

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence</th>
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<tr>
<td>P1: TTCAGAAATTCAG/TGGNC/TTNTGGCC</td>
<td></td>
</tr>
<tr>
<td>P2: TCGGGGATCCAGTCAC/ACCAGT</td>
<td></td>
</tr>
<tr>
<td>P3: AATAAATGATGTCCTTACCCATCA</td>
<td></td>
</tr>
<tr>
<td>P4: CCAAAAGATTAAATTGATTTTGAG</td>
<td></td>
</tr>
<tr>
<td>P5: CGTCAGGGTTTAAATCCAGCAGCATG</td>
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</tr>
<tr>
<td>P6: GCAGGCTTTTCAGGTACATTTT</td>
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<td>P7: CGCCAGCGTTCCTCCAGTCGAC</td>
<td></td>
</tr>
<tr>
<td>P8: GTGGGAGCAGGATCCTGAGCCTC</td>
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</tbody>
</table>

Table 1. Oligonucleotide Primers Used in This Study
Fig. 1. Purification of RNase NW from N. glutinosa Leaves on a Column of YMC gel C₄ and SDS-PAGE of the Purified RNase NW.

The protein fractions obtained from Q-Sepharose column chromatography was put onto an RP-HPLC column (4.6 × 250 mm) and eluted with increasing acetonitrile concentration in 0.1% aqueous TFA at the flow rate of 1.0 ml/min. Solvent B: 80% acetonitrile in 0.1% TFA. The insert shows the SDS-PAGE pattern of the purified RNase NW. Lane 1, marker proteins; lane 2, the purified RNase NW. The marker proteins used were BSA (67 kDa), ovalbumin (45 kDa), chymotrypsinogen (29 kDa), myoglobin (17 kDa), and lysozyme (14 kDa).

Fig. 2. The Effects of pH and Temperature on the Activity of RNase NW from N. glutinosa Leaves.

Enzymatic activity of RNase NW was measured in 50 mM sodium phosphate buffer, pH 6.0, by the method of Uchida and Egami, using yeast RNA as a substrate. A, Optimal pH of the activity of RNase NW. RNase activities were measured at 37°C at various buffer as described under Materials and Methods. The amount of RNase NW used for assay was about 0.2 μg. B, Effects of pH on the stability of RNase NW. RNase NW (about 0.2 μg) was preincubated in 100 μl of various buffer for 10 min at 37°C and then, the enzymatic activity was measured at pH 6.0 at 37°C. C, Heat stability of RNase NW. RNase NW (about 0.2 μg) were preincubated in 100 μl of 50 mM sodium phosphate buffer, pH 6.0, for 10 min at various temperatures. The enzymatic activity of each sample was measured at pH 6.0 and 37°C. (---), RNase NW; (-----), RNase MC1 from bitter gourd seeds.

Substrate specificity, comparing them with those of RNase MC1 from the seeds of bitter gourd10, 11, and Phnaris, unpublished results). The optimum pH for its action on yeast RNA was found to lie between 5 and 7 (Fig. 2A). Upon incubations at different pHs, RNase NW almost lost its activity under acidic and basic conditions, and was found to be stable only around pH 6 (Fig. 2B). Incuba-
tion at temperatures higher than 50°C led to its inactivation, which was completed above 60°C as shown in Fig. 2C. To analyze the base specificity of RNase NW, the rates of hydrolysis of four homopolynucleotides were measured and expressed as that of yeast RNA as 100. RNase NW cleaved polyinosinic acid most rapidly and the rates of hydrolysis of the other three polynucleotides were much lower than that of yeast RNA (Table 2).

The direct N-terminal sequencing with a gas-phase sequencer was unsuccessful, suggesting that the N-terminal amino acid of RNase NW is probably modified. To obtain an internal amino acid sequence, RNase NW was first reduced and pyridylethylated, and then cleaved with trypsin and cyanogen bromide as described under Materials and Methods. Figures 3A and 3B show the elution patterns of tryptic and cyanogen bromide peptides, respectively, separated by RP-HPLC on a column of YMC gel C4. The amino acids of these peptides were sequenced by a gas phase sequencer, as summarized in Fig. 4B. By sequencing tryptic peptides, four peptides provided distinct amino acid sequences. Cyanogen bromide cleavage, as predicted from the presence of one methionine residue, yielded two peptides, and the sequence analysis of the peptide CB2 gave a single amino acid sequence, which agreed well with that of the methionine-containing tryptic peptide T-2, while that of CB1 yielded no amino acid, suggesting that CB1 was probably the N-terminal CB peptide of RNase NW. Eighty-eight amino acids were identified in this analysis, accounting for about 40% of total residues in RNase NW.

**Nucleotide sequence of cDNA encoding RNase NW**

The total RNA was isolated from 10 g of the wounded *N. glutinosa* leaves, as described under Materials and Methods, and 28 µg poly(A)+ RNA was purified using Oligotex-dT30. The double-stranded cDNA was synthesized and inserted into the phage vector λgt11 at Eco RI site. The strategy used for sequence analysis of cDNA encoding RNase NW is given in Fig. 4A. First, a pair of PCR primers, P1 and P2 (Table 1), were designed on the basis of the two consensus amino acid sequences IHGLWP and KHGT, respectively, found in plant RNases.3 Using these primers and poly(A)+ RNA as a RT-PCR template, specific amplified products of 200 bp were obtained. The DNA fragments recovered from an agarose gel were cloned into the plasmid vector pGEM-T-vector, and then the nucleotides were sequenced. This analysis identified four distinct clones, TR1, TR2, TR3, and TR4, encoding possible RNase fragments (data not shown). By comparison of the amino acid sequences deduced from four nucleotide sequences with those of peptides of RNase NW, it was found that the amino acid sequence of the CB peptide (CB2) agreed well with that of the TR4 clone. Thus, it was assumed that the

### Table 2. Hydrolysis of Homopolynucleotides and RNA with RNase NW

<table>
<thead>
<tr>
<th>RNase NW</th>
<th>Substrate</th>
<th>RNA</th>
<th>Poly A</th>
<th>Poly C</th>
<th>Poly I</th>
<th>Poly U</th>
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<td></td>
<td></td>
<td>100</td>
<td>8.8</td>
<td>0</td>
<td>96.5</td>
<td>0</td>
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</table>

One hundred µg of the substrate was incubated with RNase NW (10 nM) in 400 µl of 0.2 M sodium acetate buffer, pH 6.0, at 37°C for 10 min. The reaction was stopped by the addition of 0.2 ml of 0.75% uranyl acetate in 25% perchloric acid. The reaction mixture was centrifuged at 5000 rpm for 10 min. The supernatant was diluted with 10-fold of water. The absorbancy of the diluted solution was measured at 260 nm. The rates of hydrolysis were expressed as that of yeast RNA as 100.

![Fig. 3. HPLC Patterns of Peptides Derived from Tryptic and Cyanogen Bromide Cleavages of RNase NW on a Column of YMC gel C4.](image)

RNase NW was cleaved with trypsin (A) and cyanogen bromide (B), and the resulting peptides were separated on a YMC gel C4 column (4.6 × 250 mm). The peptides were eluted as described in the legend to Fig. 1.
Fig. 4. The Strategy for Nucleotide Sequencing of RNase NW cDNA, and Its Nucleotide and Deduced Amino Acid Sequences. A. The strategy for nucleotide sequencing of RNase NW cDNA is shown. Three cDNA fragments, RT-PCR (pos. 201-387), 3’ RACE (pos. 223-879), and RAGE (pos. 1-462), are illustrated. B. The nucleotide sequence is presented with corresponding amino acid translations.
clone TR4 might be the cDNA fragment encoding RNase NW.

To obtain the nucleotide sequence of the 3' terminal end of the RNase NW cDNA, 3' RACE was done with the RNase NW specific primer P3 and 3' RACE primers (P6 and P7 primers) (Table 1). Sequence analysis of the product could give the nucleotide sequence of the 3' terminal end including the stop codon (TAG: nucleotides 703-705) and the poly A tract (Fig. 4B).

The sequence information at the 5' terminal end was obtained as follows. The RAGE method was used with the RNase NW specific primers (P4 and P5) and Lgt11 specific primer P8 (Table 1), using total cDNA extracted from N. glutinosa cDNA library as a template. Sequence analysis of the product could identify the nucleotide sequence of the 5' terminal end of RNase NW cDNA including the possible initiation codon ATG (nucleotides 16-18). In this way, the complete nucleotide sequence of the cDNA was established, as shown in Fig. 4B. The cDNA sequence consists of 879 nucleotides and has an open reading frame of 687 nucleotides with 174 nucleotides at the 3'-untranslated region containing the poly A tail. The amino acid sequence of RNase NW found by protein chemical methods could be unambiguously localized within the deduced amino acid sequence, as shown in Fig. 4B. This strongly demonstrated that the cDNA sequenced indeed encodes RNase NW.

Expression of the RNase NW gene in response to wounding

To further corroborate that the cDNA sequenced encodes the wound-inducible RNase NW, induction of the transcript for RNase NW in response to wounding was examined by Northern blot hybridization using the RNase NW cDNA fragment amplified by the 3' RACE method as a probe. Figure 5 shows the hybridization of the cDNA fragment to a gel blot of total RNA isolated from leaves without or with wounding. No transcript was detected in total RNA isolated from the healthy leaves. By contrast, a single transcript band of 0.9 kb was detected in each RNA isolated from the wounded leaves. This result indicated that the expression of the RNase NW gene was induced within 4 h and this transcript gradually decreased during 20 h. This is consistent with the earlier observation that the enzyme activity increased by injury started to increase within 4 h and reached a maximum 16 h after treatment.146

Discussion

It was previously described that RNase activity was induced in N. glutinosa leaves by either injury or TMV-infection.13 However, very few studies on the RNases were done at the molecular level possibly because of their limited amounts when induced. Meanwhile, Matsushita and Shimokawa146 found two distinct RNase activities in the N. glutinosa leaves when infected with TMV, and showed on the basis of an experiment using cycloheximide that RNase NW was de novo synthesized, but RNase F was not de novo synthesized. In this study, RNase NW was purified to homogeneity, judging from SDS-PAGE and RP-HPLC, and characterized its enzymatic properties, comparing with those of RNase MC1 from the bitter gourd seeds. RNase NW actively digested yeast RNA around pH 5.0 and 7.0, and retained its activity until treatment at 60°C, which are slightly different from those of RNase MC1, as shown in Fig. 2. As for the pH stability, the two RNases showed considerably different properties. RNase NW was stable only around pH 6, while RNase MC1 retained a full activity at a wide range of pH. This result suggests that the two plant RNases are probably structurally divergent and may play different role(s) in plants. Further, we investigated the action of RNase NW on homopolynucleotides. RNase NW promoted an effective degradation of polyinosinic acid. By contrast, polyadenylic acid was only slightly digested, and neither polyuridylic nor poly-cytidylic acids were attacked at all. On the basis of substrate specificity, it was suggested that plant RNases are classified into two groups: the tomato type is G>A>U, C and the seed type is U>G>A>C.111 Although it is premature on the basis of only the hydrolysis rate of homopolynucleotides and further studies will be required to conclude its base specificity, this study suggests that RNase NW may be classified into the tomato type.

It is known that the extracellular and intracellular RNases in the cultured tomato cells are synthesized with signal peptides at either N- or C-termini. By comparison of the N-terminal sequence of the predicted amino acid sequence of RNase NW with that of the cultured tomato RNase LE (Fig. 6), it was found that the N-terminal 25 amino acids of the predicted sequence is very simi-
Fig. 6. Comparison of the Amino Acid Sequence of RNase NW from *N. glutinosa* Leaves with Those of Other Known Plant RNases. RNases NE, LE, LX, ZI, RNs 2, MC1, and S indicate RNases from tobacco, 
*tomato, Zinnia elegans, Arabidopsis,* bitter gourd seeds, and tobacco. + indicates residues identical with RNase NW. Amino acid residues are numbered according to RNase NW.

...
ta not shown), and its molecular mass on the basis of the amino acid sequence is calculated to be 22,551 Da. It was found that RNase NW, unlike the cases for S-RNases, contains no potential N-glycosylation sites.

The deduced amino acid sequence of RNase NW was compared with other known plant RNases (Fig. 6). Surprisingly, this analysis indicates that the sequence is in perfect agreement except for eight residues with that of the recent published ribonuclease RNase NE in *N. alata* 27 as shown in Fig. 6. It is reported that RNase NE is present in the style and pollen, but not in leaves under normal condition, and it is induced in root in response to phosphate starvation. The absence of RNase NE in leaves is consistent with our result obtained for RNase NW by Northern blotting of total RNA isolated from the *N. glutinosa* leaves. It is highly likely from this finding as well as from sequence similarity that RNase NE in *N. alata* is probably a homologue of RNase NW. If this is true, it is suggested that RNase NW may be present in the style and pollen in *N. glutinosa* under normal conditions and induced in different tissues, leaves and roots, in response to two different stimuli, wounding and phosphate starvation, respectively.

Sequence comparison showed that RNase NW shares 57–84% identical amino acid residues with the other S-like RNases. Particularly, RNase NW shows considerable similarity to the tomato extracellular RNase LE among S-like RNases, sharing 84% identical residues. Interestingly, this value is higher than that between two isoenzymes, RNase LE and RNase LX, in tomato (62%). Furthermore, in this study three putative RNase cDNA fragments TR1, TR2, and TR3, were amplified in addition to RNase NW. Although only partial amino acid sequences have been deduced, the percentages of identical amino acid residues between the RNase NW and other three RNase sequences lie between 45 and 65%. These observations demonstrate the recent finding that a divergencies of RNase isoenzymes in plants had occurred before speciation.

In this study, we obtained cDNA fragment encoding a wound-inducible RNase NW in *N. glutinosa* leaves. This will enable us to obtain its genomic clone and also to analyze its regulatory gene(s). Furthermore, it will make it possible to overproduce RNase NW by a genetic engineering. These studies will provide us more insight into the mechanism of wound induction and also structure-function relationships of RNase NW in detail.

References


