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Expression of Ribonuclease Gene in Mechanically Injured or Virus-Inoculated *Nicotiana tabacum* Leaves

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Summary

We characterized cDNAs encoding putative S-like RNases, RNase Nk1, Nk2 and Nk3, in *Nicotiana tabacum* L. cv. ky 57 leaf and examined the relation between these genes and *Cucumber mosaic virus* (CMV) infection. Of these genes, only RNase Nk1 was induced by wounding or CMV inoculation. The deduced amino acid sequence of RNase Nk1 is 97% identical to the *N. alata* RNase NE that is induced in roots in response to phosphate limitation. RNase Nk1 mRNA was highly induced in CMV-inoculated leaves within 3-6 hr after inoculation whereas a smaller increase was observed in mock- or CMV RNA-inoculated leaves. RNase Nk1 mRNA was also highly induced in leaves inoculated with CMV coat protein (CP) or BSA, suggesting that not only a mechanical damage but CP of CMV is the causal agent in the higher induction of RNase Nk1 gene immediately after virus infection in host plant cells.

Introduction

In plants, RNase is involved in RNA metabolic processes such as RNA processing, turnover and degradation. Recently, many plant RNases have been grouped into S-RNase and S-like RNase families by sequence similarity (6). S-RNase has been shown to be involved in control of self-incompatibility in certain plant species (6). In self-compatible plants, a number of S-like RNases have also been reported to be induced in response to diverse stresses. In

Arabidopsis thaliana, three RNase genes, RNS1, RNS2 and RNS3, have been identified. Further investigations suggested that these three RNases are induced by senescence, and that RNS1 and RNS2 are also induced in response to phosphate starvation (1, 19, 20). In *Zinnia elegans*, it was reported that ZRNase I is induced in response to xylogenesis, but ZRNase II is induced by wounding (24).

Many reports have shown that RNase activity is also increased in plants infected with pathogens (6, 22) including *Tobacco mosaic virus* or *Cucumber mosaic virus* (CMV) (12, 13, 14, 23). In many cases, higher RNase activity was observed in the leaves infected with virus than that with buffer (12, 13, 14). Thus, it is likely that S-like RNase may also be induced in response to the stress caused by plant virus infection.

Because encapsidated CMV RNA is easily degraded by ribonuclease *in vitro* (4, 7), it is likely that plant RNase could affect virus infection in host cells. However, the role of plant RNase in virus infection has not been clarified. In particular, it is not clear whether the increase of RNase activity of virus-infected plants is due to the continued stress by virus infection or to mechanical wounding.

In this paper, we have identified three putative S-like RNase genes, RNase Nk1, Nk2 and Nk3 in the self-compatible plant *Nicotiana tabacum* L. cv. ky 57. Of these genes, RNase Nk1 was most highly expressed in the tobacco leaves within 3 hr after CMV inoculation. We describe the RNase gene expression at the early stage of the infection process in CMV-inoculated tobacco leaves.

Materials and Methods

Plants and Virus

Tobacco (*Nicotiana tabacum* L. cv. ky 57) plants were grown in a growth chamber at 25°C under 1.6×10^4 lux illumination using 16 hr photoperiods. The ordinary strain of CMV was purified essentially by the method of Scott (18), followed by sucrose density-gradient ultracentrifugation. CMV-RNA was extracted from purified virus with phenol and SDS. CMV CP was prepared by the method of Kobayashi and Ehara (9). The fully developed 10th leaf at the 15-leaf stage was dusted with carborundum (600 mesh) and mechanically inoculated with a cotton swab moistened with 10 mM K-phosphate buffer, pH 8.0 (mock-inoculation) or CMV, CMV-CP, CMV-RNA in the same buffer.

Amplification of RNase fragments by RT-PCR

Total RNA was prepared from plant tissues by the acid guanidium thiocyanate-phenol-chloroform extraction method (2). RT-PCR analysis was carried out on RNase-free DNase (DNase I, Takara, Tokyo)-treated RNA samples. For synthesis of first strand cDNA, 0.5 μg of oligo nucleotide $(dT)_{15-18}$ was annealed to 1 μg of total RNA by cooling from 70°C to 42°C. The annealing

mixture was incubated with 200 U of M-MLV reverse transcriptase (Promega, Tokyo) in reaction buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT and 2 mM dNTP, pH 8.3) for 60 min at 42°C. After incubation, 1 μl of the reaction mixture was used as template for PCR. The initial amplification was performed by 30 cycles of 95°C for 1 min, 42°C for 2 min and 72°C for 3 min. The amplification reaction contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM of each dNTP, 10 pmole of each degenerate primer (P1 and P2) and 2.5 U of Taq DNA polymerase. The major reaction products of about 150-250 bps were extracted from agarose gel and used for a second round of PCR with 40 cycles of 95°C for 1 min, 50°C for 2 min and 72°C for 3 min. The major PCR product was excised and cloned in a Bluescript SK+vector.

3′ and 5′ RACE

The downstream (3′) and upstream (5′) regions of RNase Nk1 were amplified by 3′ and 5′ RACE methods [16]. For 3′ RACE, cDNA was synthesized with the oligonucleotide GATCTAGAGGTACCGGATCC$(dT)_{18}$ (RACE primer) and used as template for PCR amplification with the oligonucleotide GATCTAGAGGTACCGGATCC (amplification primer) and a gene-specific primer (P3, AGAATGCAACAAAATTGGCC). The amplification was performed by 40 cycles of 95°C for 1min, 50°C for 2 min and 72°C for 3 min. For 5′ RACE, cDNA was synthesized with the gene-specific primer and oligo (dA) tails were added to the 3′ ends of the cDNA strand using 5 units of terminal deoxynucleotidyl transferase (Takara) with 100 μM dATP at 37°C for 30 min. The initial PCR amplification was performed with RACE primer and a gene-specific primer (P4, TAAGGACTGTTAGAATCAC) by 30 cycles of 95 °C for 1min, 42°C for 2 min and 72°C for 3 min. Then, the second round of PCR was performed with the oligonucleotide amplification primer and a second gene-specific primer (P5, GATGGCTCTTACCCATCA) by 30 cycles of 95°C for 1 min, 50°C for 2 min and 72°C for 3 min.

Northern blotting

Total RNA was extracted from tobacco leaves as described above and treated with sample buffer containing glyoxal and DMSO [17]. RNA samples separated on 1% agarose gel were transferred onto nylon membranes (Hybond-N^+, Amersham, UK) and hybridized with a digoxigenin (DIG)-labeled DNA probe prepared from cDNA clones by PCR. Prehybridization and hybridization of the membranes were performed at 65°C in 50% formamide, 5×SSC (0.75 M NaCl, 0.075 M $C_6H_5O_7Na_3$), 0.5% SDS, 10×Denhardt's solution (0.2% BSA, 0.2% Ficoll and 0.2% polyvinylpyrollidone) and 0.1 mg/ml denatured herring sperm DNA. Washing was in 2×SSC, 0.1% SDS at room temperature followed by final washing in 0.2×SSC, 0.1% SDS at 65°C. Chemiluminescent detection was performed using Phototope™ Star Detection Kit (New England Biolabs).

Results

Amplification of putative RNase cDNA fragments

A pair of PCR primers (P1 and P2) for amplification of RNase sequences was designed to code for the most conserved amino acid sequences of plant RNases (IHGLWP and KHGTC, respectively). The sequences of the primers were GAATNCAYGGNYTTTGGCC (P1) and AGCRCANGTNCCRTGTTT (P2). When cDNA from an intact *N. tabacum* leaf was used for PCR template, about 200 bp of PCR products were amplified. The PCR products were cloned into a Bluescript SK vector, and DNA sequences were determined. The sequencing of 24 individual independent clones identified three possible RNase fragments, designated RNase Nk1, Nk2, and Nk3 (Fig. 1). The deduced amino acid sequences of these cDNA fragments showed considerable similarity with other S-like RNases. Particularly, RNase Nk1 had 98% identical residues with *N. alata* RNase NE (3) or *N. glutinosa* RNase NW (8), Nk2 shared 68% identicality with *A. thaliana* RNS2 (19) and Nk3 shared 88% identicality with *L. esculentum* RNase LX (10) throughout the cloned region (data not shown). The deduced amino acid sequences of these clones included amino acids that were completely conserved among the S- and S-like RNases.

Expression of RNase Nk1, Nk2, and Nk3 mRNAs by wounding or CMV inoculation

Early studies showed that RNase activities in virus-inoculated plants increased within 4–6 hr after inoculation (12, 13). The expression of RNase genes in the early period after CMV inoculation was analyzed by Northern blotting of total RNA extracted from tobacco leaves at 6 hr after mock or CMV inoculation. When RNase Nk1, Nk2, and Nk3 cDNA clones were used to probe Northern blots, no cross-hybridization was observed between these genes. Neither RNase Nk2 nor Nk3 mRNA increased in response to wounding or CMV (100 μg/ml) inoculation. However RNase Nk1 mRNA increased more in CMV-inoculated leaves than mock-inoculated leaves (Fig. 2), suggesting that RNase Nk1 is CMV-reproducible.

```
Nk1 -HGLWPNNNDGSYPSNCDSNSPYDQSQVSDLISRMQQNWPTLACPSDTG-----SAFWSHEWEKHGTC

Nk2 -HGLWPEYNDGTWPACCSGKA-FDEREISTLLEPLRKYWPSLSCGSPRSCHHRKGSFWAHEWEKHGTC

Nk3 -HGLWPNYENGKWPQNCDRESSLDESKISDLISTMEKNWPSLACPSSDG-----VRFWSHEWLKHGTC
     *****    * *  *      *    * *       ** * * *          ** *** *****
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FIG. 1. Deduced amino acid sequences of RNase Nk1, Nk2 and Nk3. The cDNA fragments encoding RNase were amplified by RT-PCR using oligonucleotide primers P1 and P2. Asterisks under the sequences denote residues that are conserved among RNase Nk1, Nk2 and Nk3.

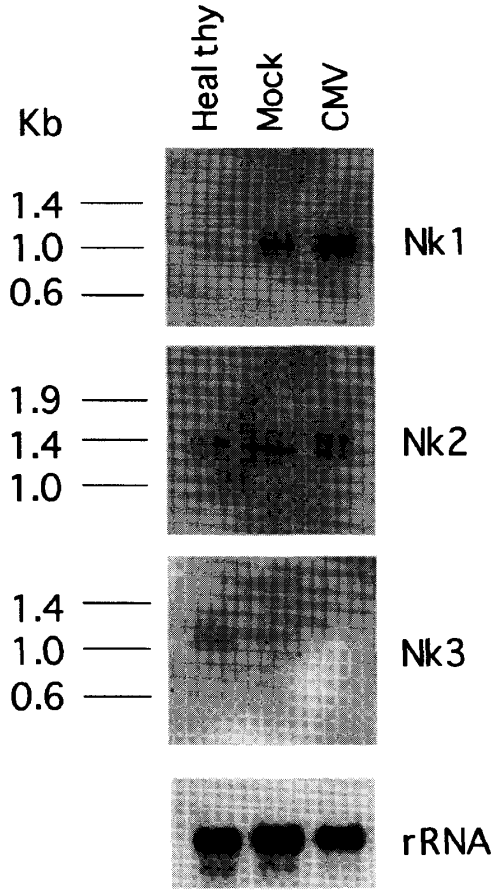

FIG. 2. Northern blot analysis of RNase Nk1, Nk2 and Nk3 mRNAs in tobacco leaves inoculated with buffer (mock) or CMV (100 μg/ml). Two μg of total RNA extracted from the leaves at 6 hr after inoculation were separated on agarose gels and blotted onto nylon membranes. The blots were hybridized with the RNase Nk1, Nk2 and Nk3 probes. As a control, the blot was hybridized with a probe corresponding to the 18S ribosomal RNA (rRNA).

The time course of RNase Nk1 mRNA expression in mock-, CMV- or CMV RNA-inoculated leaves was examined by northern blot hybridization using a cDNA fragment amplified by 3′RACE as a probe (Fig. 3). RNase Nk1 mRNA increased markedly within 3-6 hr after CMV (100 μg/ml) inoculation and then gradually decreased during the 24 hr after inoculation. RNase Nk1 mRNA also increased in mock- or CMV RNA (100 μg/ml)-inoculated leaves within 3-6 hr after inoculation, but the mRNA levels were less than that in CMV-inoculated leaves.

RNase Nk1 mRNA expression by CMV CP- or BSA- inoculation

To clarify the reason RNase Nk1 mRNA expression strongly increased in the leaves inoculated with CMV but not with CMV RNA, the effect of CMV CP on RNase Nk1 mRNA expression was examined. Tobacco leaves were inoculated with CMV CP (10 or 100 μg/ml). Northern blot analysis showed that RNase Nk1

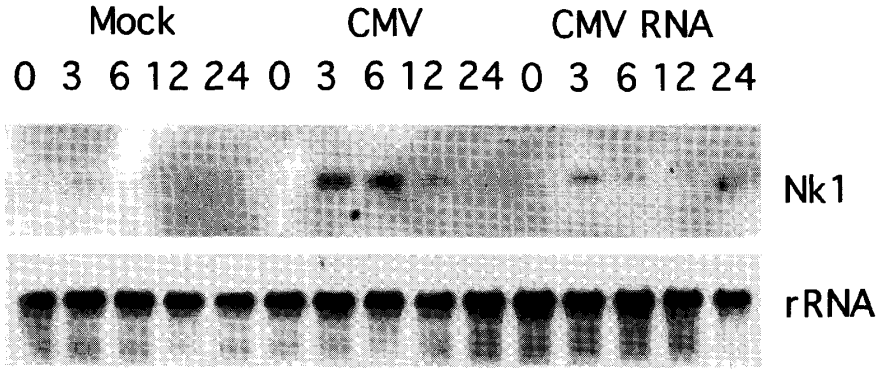

FIG. 3. Northern blot analysis of RNase Nk1 mRNA in tobacco leaves inoculated with buffer, CMV or CMV RNA (100 μg/ml each). Two μg of total RNA extracted from the leaves at the indicated times (hr) after inoculation were separated on agarose gels and blotted onto nylon membranes. The blots were hybridized with the RNase Nk1 probe. As a control, the blot was hybridized with a probe corresponding to the 18 S ribosomal RNA (rRNA).

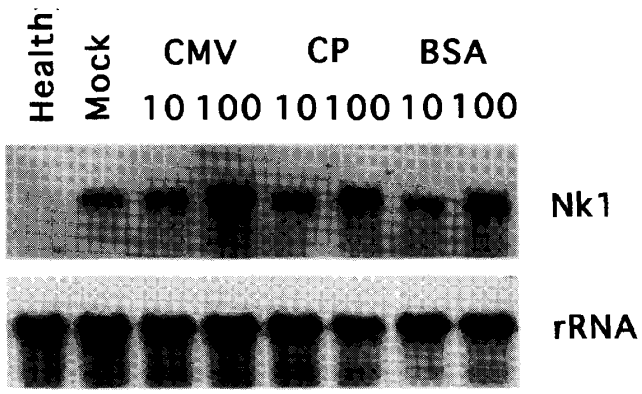

FIG. 4. Northern blot analysis of RNase Nk1 mRNA in tobacco leaves inoculated with buffer, CMV, CMV coat protein or BSA (10 or 100 μg/ml each). Two μg of total RNA extracted from the leaves 6 hr after inoculation were separated on agarose gels and blotted onto nylon membranes. The blots were hybridized with the RNase Nk1 probe. As a control, the blot was hybridized with a probe corresponding to the 18S ribosomal RNA (rRNA).

mRNA was induced by CMV CP inoculation to almost the same levels as by CMV virion 6 hr after inoculation (Fig. 4). RNase Nk1 mRNA was also induced by BSA inoculation. The expression of RNase Nk1 mRNA was higher with higher CMV, CP or BSA concentrations.

Nucleotide sequence of RNase Nk1 cDNA

The nucleotide sequence at the 3′ and 5′ terminal end of RNase Nk1 was obtained using 3′- and 5′-RACE procedures, respectively (Fig. 5). Oligonucleotide P3 derived from the Nk1 sequence was used for the 3′-RACE method. Similarly, oligonucleotides P4 and P5 were used for 5′-RACE method. The

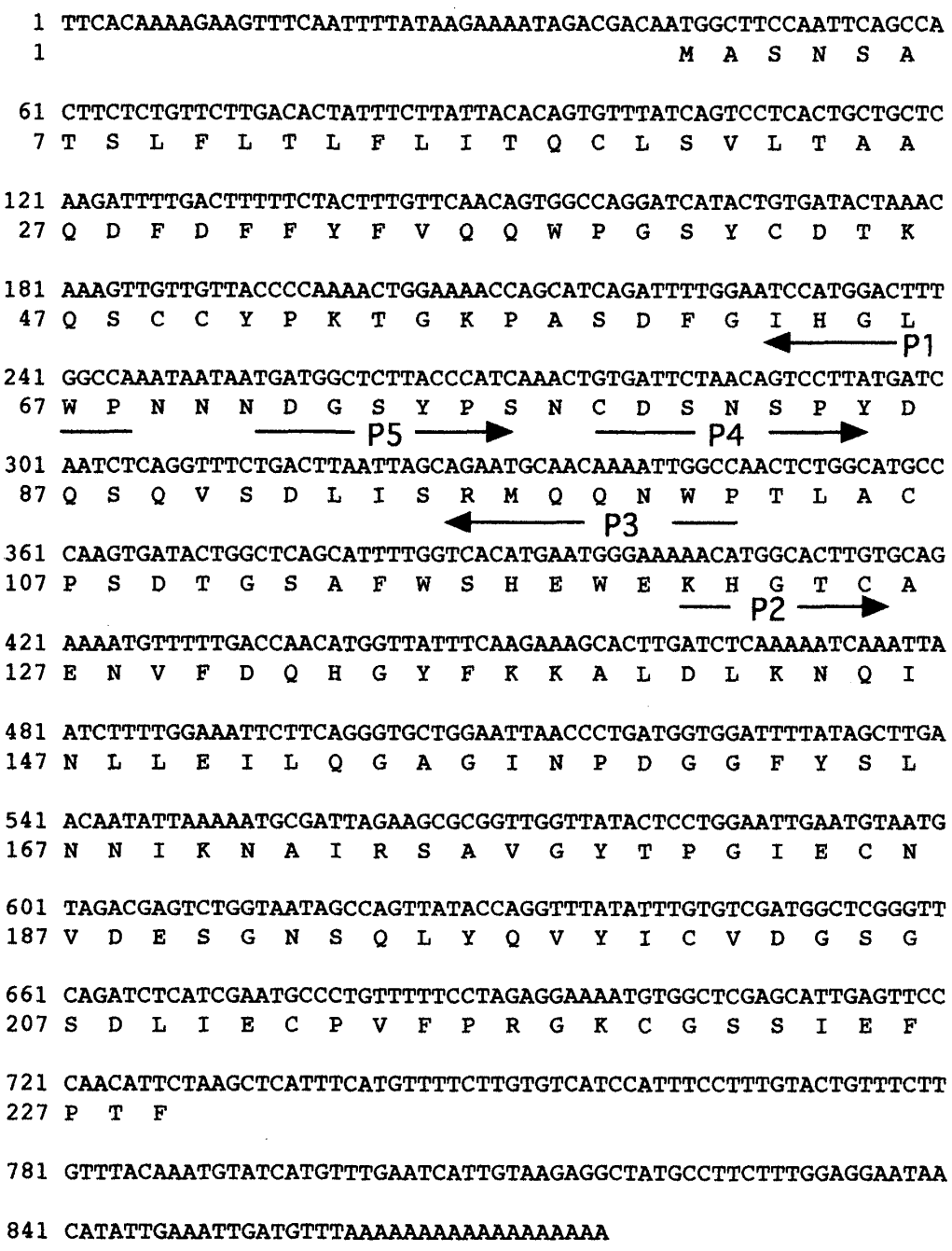

FIG. 5. Nucleotide and deduced amino acid sequences of RNase Nk1 cDNA. The positions of gene specific primers used for amplification of cDNA fragments are shown by arrows. P1 and P2 were primers used in initial amplification. Primer P3 was used for 3′-RACE and primers P4 and P5 were used for 5′-RACE.

complete nucleotide sequence of RNase Nk1 cDNA consists of 877 nucleotides and has an open reading frame of 687 nucleotides with a poly(A) tail on the 3′ end. The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number

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                                                                    40
Nk1       MASNSATSLFLTLFLITQCLSVLTAAQDFDFFYFVQQWPGSYCDTKQSCCYPKTGKPA
RNaseNE   :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
RNaseNW   :::D::::V:::::::::::::A:::::::::::::::::::::::::::::::::::
RNaseLE   ::::::F::::I:LI::::::::N::K:::::::::::::::::::::::::T:::::

                                        80
Nk1       SDFGIHGLWPNNNDGSYPSNCDSNSPYDQSQVSDLISRMQQNWPTLACPSDTGSAFWS
RNaseNE   ::::::::::::::::::::::::::::::::::::::::::::::::::G:::::::
RNaseNW   ::::::::::::::::::::::::::::::::::::::::::::::::::G:::::::
RNaseLE   A::::::::::::::T::::::P::::::::I:::::S::::::::::::GS::T:::

            120                                                 160
Nk1       HEWEKHGTCAENVF-DQHGYFKKALDLKNQINLLEILQGAGINPDGGFYSLNNIKNAI
RNaseNE   :::::::::S:SI:-:::::::::::::::::::::::::::::::::::::::S:::::
RNaseNW   ::::::::::::::-:::::::::::::::::::::::::::::::::::::::S:::::
RNaseLE   ::::::::::::S:LTN::A::::::::::::D::S:::::D:H:::ES:D:V::R:::

                                        200
Nk1       RSAVGYTPGIECNVDESGNSQLYQVYICVDGSGSDLIECPVFPRGKCGSSIEFPTF
RNaseNE   :::I::::::::::::D::::::::::::::::N::::::::::::::::::::::::
RNaseNW   :::I::A::::::::::::::I::::::::::::N:::::I::::::::::::::::
RNaseLE   K::I::::W:Q::::Q:::::::::::::::::::S:::::I::G::::T:::::::
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FIG. 6. Comparison of the amino acid sequence of RNase Nk1 with those of RNase NE (*N. alata*), RNase NW (*N. glutinosa*) and RNase LE (*L. esculentum*): indicates amino acids identical with RNase Nk1.

AB034638. Like the predicted amino acid sequences of RNase LE and NE, the predicted N-terminal sequence of RNase Nk1 has an extracellular secretion signal sequence (3, 8, 10). It is likely that the cleavage of the signal peptide occurs between Ala-25 and Ala-26. The deduced amino acid sequence of RNase Nk1 exhibits 58–97% similarity with other S-like RNases. In particular, the deduced amino acid sequence of RNase Nk1 showed higher similarity with that of RNase NE and RNase NW, sharing 97% and 96% identical residues, respectively (Fig. 6).

Discussion

In this study, we have isolated cDNA fragments encoding three putative S-like RNases, RNase Nk1, Nk 2 and Nk 3, in *N. tabacum* L. cv. ky 57 leaves. Of these genes, only RNase Nk1 mRNA increased by mock- or virus-inoculation. RNase Nk1 was expressed more highly in CMV-inoculated leaves than in mock-inoculated leaves 6 hr after inoculation. In addition, we investigated wound- and virus infection-inducible RNase Nk1. The deduced amino acid sequence of RNase Nk1 has high sequence similarity with other S-like RNases (58–97%). Particularly, RNase Nk1 shared 97% identical amino acid residues with RNase NE in *N. alata* (3) and 96% identity with RNase NW in *N. glutinosa* (8).

RNase Nk1 cDNA was amplified by RT-PCR using DNase-treated total RNA

from intact *N. tabacum* leaves as sample. In fact, RNase Nk1 mRNA was detected in intact leaves by northern blotting when 2 μg of poly(A) RNA was used as sample (data not shown). Consistent with the production of wound-inducible RNase NW, RNase Nk1 was induced in response to the wounds formed on the leaves. These results show that a small amount of RNase Nk1 mRNA is expressed in the leaves under normal conditions. RNase Nk1 may play a role in RNA metabolism in *N. tabacum* leaves under natural conditions and increase in response to some stresses such as mechanical damage.

The expression of RNase Nk1 mRNA was higher in the leaves inoculated with CMV (100 μg/ml) than in those with buffer or CMV RNA (100 μg/ml). Western blot analysis showed that virus multiplication was higher in the leaves inoculated with CMV RNA than in those with CMV virion (data not shown). Although no virus multiplication was observed, a significant increase of RNase Nk1 expression was detectable in CMV-inoculated leaves 3-6 hr after inoculation and then gradually decreased. Thus, in CMV-inoculated leaves, higher RNase Nk1 induction may not a result of continued stress by virus multiplication. However, RNase Nk1 mRNA induction in CMV-inoculated leaves may not be exclusively due to mechanical damage during the inoculation. Because RNase Nk1 mRNA was highly induced in the leaves inoculated with CMV CP or BSA, protein may strengthen the mechanical damage. In the infection of CMV, CP part of the virus may produce a stronger wounding effect. It is also possible that CMV CP which has dissociated from CMV virion after entering the host cells may be recognized by host cells as a foreign protein.

Plant viruses infect host plants mainly through wounds or vector transmission. Under natural conditions, RNase Nk1 may be induced in CMV-infected leaves. There is no report that plant RNase can inhibit virus multiplication after it has entered host cells. On the contrary, early reports have suggested that a higher RNase level may favor virus infection because virus RNA might be synthesized from nucleoside precursors released by degraded host RNA (16, 17). Thus, it is unlikely that induced RNase Nk1 inhibits CMV multiplication after it has entered host plant. Previously, we showed that RNase exuded from wounded tobacco epidermal cells easily attaches to the surface of the CMV virion and destroys its infectivity (15). Thus, it is conceivable that RNase Nk1 induced by wounds or virus infection may play a defensive role against virus re-infection at the infection sites on the surface of tobacco epidermis.

References

1) Bariola, P.A., Howard, C.J., Taylor, C.B., Verburg, M.T., Jaglan, V.D. and Green, P.J., The Arabidopsis ribonuclease gene RNS1 is tightly controlled in response to phosphate limitation. *Plant J.*, **6**, 673-685

(1994)
2) Chomczynski, P. and Sacchi, N., Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156-159 (1987)
3) Dodds, P.N., Clarke, A.E. and Newbigin, E., Molecular characterisation of an S-like RNase of Nicotiana alata that is induced by phosphate starvation. *Plant Mol. Biol.*, **31**, 227-238 (1996)
4) Francki, R.I.B., Inactivation of cucumber mosaic virus (Q strain) nucleoprotein by pancreatic ribonuclease. *Virology*, **34**, 694-700 (1968)
5) Frohman, M.A., Dush, M.K. and Martin, G.R., Rapid production of full length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. *Proc. Nat. Acad. Sci. USA*, **85**, 8998-9002 (1988)
6) Green, P.J., The ribonucleases of higher plants. *Annu. Rev. Plant. Physiol. Mol. Biol.*, **45**, 421-445 (1994)
7) Habili, N. and Francki, R.I.B., Comparative studies on tomato aspermy and cucumber mosaic viruses. *Virology*, **60**, 29-36 (1974)
8) Kariu, T., Sano, K., Shimokawa, H., Itoh, R., Yamasaki, N., and Kimura, M., Isolation and characterization of a wound-inducible ribonuclease from *Nicotiana glutinosa* leaves. *Biosci. Biotechnol. Biochem.*, **62**, 1144-1151 (1998)
9) Kobayashi, A. and Ehara, Y., *In vitro* encapsidation of cucumber mosaic virus RNA species. *Ann. Phytopathol. Soc. Jpn.*, **61**, 99-102 (1995)
10) Köck, M., Löffler, A., Abel, S., and Glund, K., cDNA structure and regulatory properties of a family of starvation-induced ribonucleases from tomato. *Plant Mol. Biol.*, **27**, 477-485 (1995)
11) Löffler, A., Abel, S., Jost, W., Beintema, J.J. and Glund, K., Phosphate-regulated induction of intracellular ribonucleases in cultured tomato (*Lycopersicon esculentum*) cells. *Plant Physiol.*, **98**, 1472-1478 (1992)
12) Lusso, M. and Kuc, J., Increased activities of ribonuclease and protease after challenge in tobacco plants with induced systemic resistance. *Physiol. Mol. Plant Pathol.*, **47**, 419-428 (1995)
13) Matsushita, K. and Shimokawa, H., Behaviors of ribonuclease in tobacco mosaic virus-infected *Nicotiana glutinosa* leaves. *Ann. Phytopathol. Soc. Jpn.*, **49**, 206-213 (1983)
14) Misawa, T., Kato, S. and Suzuki, T., Changes of ribonuclease activity in tobacco-leaf infected with CMV. *Jubilee Pub. in Commemoration of 60th Birthday of Prof. M. Sakamoto.*, 183-189 (1968)
15) Ohno, H., Hase, S. and Ehara, Y., Analysis of the pH effect on infectivity of cucumber mosaic virus: A possible role of ribonuclease. *Ann. Phytopathol. Soc. Jpn.*, **63**, 445-449 (1997)
16) Reddi, K.K., Studies on the formation of tobacco mosaic virus ribonucleic acid, II. Degradation of host ribonucleic acid following infection. *Proc. Natl. Acad. Sci. USA*, **50**, 75-81 (1963)
17) Santilli, V., Nepokroeff, C.M. and Gagliardi, N.C., Susceptibility of pinto bean leaves to tobacco mosaic virus and its relationship to leaf ribonuclease content. *Nature*, **193**, 656-658 (1962)
18) Scott, H., Purification of cucumber mosaic virus. *Virology*, **20**, 103-106 (1963)
19) Taylor, C.B., and Green, P.J., Genes with homology to fungal and S-gene

RNases are expressed in *Arabidopsis thaliana*. *Plant Physiol.*, **96**, 980-984 (1991)

20) Taylor, C.B., Bariola, P.A., delCardayre, S.B., Raines, R.T. and Green, P.J., RNS2 : A senescence-associated RNase of *Arabidopsis* that diverged from the S-RNases before speciation. *Proc. Natl. Acad. Sci. USA*, **90**, 5118-5122 (1993)

21) Thomas, P.S., Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA*, **77**, 5201-5205 (1980)

22) Wilson, C.M., Plant nucleases. *Ann. Rev. Plant Physiol.*, **26**, 187-208 (1975)

23) Wyen, N.V. Udvardy, J. Erdei, S. and Farkas, G.L., The level of a relatively purine-specific ribonuclease increases in virus-infected hypersensitive or mechanically injured tobacco leaves. *Virology*, **48**, 337-341 (1972)

24) Ye, Z-H. and Droste, D.L., Isolation and characterization of cDNAs encoding xylogenesis-associated and wounding-induced ribonucleases in *Zinnia elegans*. *Plant Mol. Biol.*, **30**, 697-709 (1996)