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Protection of transgenic tobacco plants expressing bovine pancreatic ribonuclease against tobacco mosaic virus

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Abstract Transgenic tobacco plants (*Nicotiana tabacum* cv. SR1) expressing extracellular pancreatic ribonuclease from *Bos taurus* and characterized by an increased level of ribonuclease activity in leaf extracts were challenged with tobacco mosaic virus. The transgenic plants exhibited a significantly higher level of protection against the virus infection than the control non-transformed plants. The protection was evidenced by the absence (or significant delay) of the appearance of typical mosaic symptoms and the retarded accumulation of infectious virus and viral antigen. These results demonstrate that modulation of extracellular nuclease expression can be efficiently used in promoting protection against viral diseases.

Keywords Pancreatic ribonuclease · Transgenic tobacco plants · Tobacco mosaic virus (TMV) · Protection

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Abbreviations

RNase   Ribonuclease
TMV   Tobacco mosaic virus
DAI   Days after inoculation
nptII   Neomycin phosphotransferase II

Introduction

Viruses are responsible for considerable losses of crops. Recently, a variety of gene engineering approaches have been employed to generate virus-resistant plants (for review, see Solomon-Blackburn and Barker 2001; Wassenegger 2002; Goldbach et al. 2003). Pathogen-derived resistance is the most common approach (Abel et al. 1986). Plants have been transformed to express certain viral proteins (e.g., coat protein, replicase) or antisense RNA (Goldbach et al. 2003). However, this strategy has one potential limitation: pathogen-derived resistance is efficient only against the parental virus or a few closely related viruses. In addition, some viruses encode proteins, suppressing the RNA-mediated resistance based on the posttranscriptional gene silencing (Wassenegger 2002; Goldbach et al. 2003; Voinnet 2005).

It has been shown that plants possess many various ribonuclease (RNase) activities (for review, see Green 1994). It was reported that RNase activity was higher in diseased plants (Barna et al. 1989; Green 1994; Lusso and Kuc 1995; Galiana et al. 1997; Hugot et al. 2002; Sindelarova et al. 2002; Malinovsky 2002). Wounding also causes induction of RNase expression in *Arabidopsis thaliana* (LeBrasseur et al. 2002), *Zinnia elegans* (Ye and Droste 1996), tobacco (Kurata

et al. 2002), and tomato (Lers et al. 1998). Some plant extracellular RNases participate in defense against various pathogens. *Nicotiana tabacum* extracellular RNase NE was induced by elicitors and pathogen invasion and provided resistance against TMV and *Phytophthora parasitica* (Galiana et al. 1997; Hugot et al. 2002). *Arabidopsis thaliana* extracellular RNase RNS1 was assumed to be a defense-related enzyme, because its expression was induced by wounding, both locally and systemically (LeBrasseur et al. 2002).

It was suggested that the introduction of RNase-encoding genes could be used to protect plants against various pathogens. Transgenic plants expressing bacterial or yeast intracellular RNases specific for double-stranded RNAs have recently been characterized. It was shown that the expression of the heterologous dsRNA-specific intracellular RNases increased plant resistance to a wide range of viruses, although the protective effect varied considerably (Watanabe et al. 1995; Langenberg et al. 1997; Zhang et al. 2001). It is possible that the transgenes encoding extracellular ribonucleases could also protect plants against RNA viruses, but this hypothesis has not been tested yet.

We have transformed tobacco (*Nicotiana tabacum* SR1) with the *Bos taurus* pancreatic ribonuclease gene and selected transformants with elevated levels of RNase activity in leaf extracts. Bovine RNase A is a non-toxic enzyme, efficiently degrading single-stranded RNA (Kim et al. 1995). The enzyme is activated only after maturation (the deletion of *N*-end leader peptide during secretion), while the unmaturated form of the protein is inactive. In this paper, we present data that demonstrate the protection of pancreatic ribonuclease-producing tobacco plants against tobacco mosaic virus (TMV). The protection was assessed using (1) the time of formation and the levels of expression of the disease symptoms, and (2) the levels of accumulation of the infectious virus and the virus antigen in leaves.

Materials and methods

Transgene construct

The intronless gene of pancreatic ribonuclease was isolated from bovine genomic DNA by means of polymerase chain reaction (PCR). Primer sequences were chosen from the known nucleotide sequence of the gene (Carsana et al. 1988; GenBank Acc. number X07283): P1 (5′-ATCATGGCTCTGAAGTCCC-3′) and P2 (5′-CCTACACTGAAGCATCAAAG-3′). The amplified DNA fragment was cloned in the pBlue-

Script KS (Stratagene) polylinker at the *Eco*RV site. Then the gene was transferred to the PC27 vector (Alliotte et al. 1988), cut with *Cla*I and *Eco*RI under the control of the mannopin synthase 2′ promoter. The resulting plasmid PC27bov was transferred to *Agrobacterium tumefaciens* (pGV2260) cells by triparental mating with pRK2013 helper plasmid (Deblaere et al. 1987).

Plant material

Leaf discs from 3-week tobacco plants (*Nicotiana tabacum* cv. SR1) were transformed as described by Deblaere et al. (1987). Ten R0 transformants were regenerated by selection on the MS medium containing kanamycin (100 μg/ml). The genomic DNA from each regenerated tobacco line was assayed for the presence of the transgene by PCR analysis with the primers described above. Selected transformants were selfed to generate R1 and R2 plants. The number of transgene insertions was evaluated by *nptII* segregation analysis. R0 plants were clonally multiplied on the MS medium containing kanamycin (100 μg/ml).

Ribonuclease assay

The level of RNase activity in crude leaf extracts of transgenic plants was evaluated by the change in the amount of acid-soluble matter in total yeast RNA (Blank and McKeon 1989). Leaf tissue 1 g, was ground in liquid nitrogen, suspended in 1 ml of 50 mM Tris–HCl (pH 7.0) and centrifuged for 10 min (12,000*g*; 4°C). The total protein was assayed in the supernatant according to Bradford (1976). The extracts, containing 25 μg of total protein, were added to the reaction mixture containing 0.4% total yeast RNA, 0.1% bovine serum albumin, and 0.1 M Tris–HCl (pH 7.0). The total volume of the reaction mixture was 300 μl. The reaction was stopped by adding 1 ml of 3.4% $HClO_4$. The test tubes were cooled at 4°C for 10 min and centrifuged at 12,000*g* and 4°C for 5 min. The optical densities of the supernatants were measured at 260 nm relative to the control (reaction mixture without leaf extract).

Ribonulease activity was also tested in the apoplast fraction. For this purpose, apoplastic fluid was extracted from the leaves using a procedure described by Huang et al. (2002). The leaves were cut into 1 cm length, inserted into a 5 ml syringe, and placed in a 1.5 ml centrifuge tube. After centrifugation for 20 min at 830*g*, the apoplastic extract was recovered from the bottom of the tube, and the remaining debris from the syringe.

Correspondence of the increased RNase activity in the leaf extracts to the transgene-encoded enzyme was demonstrated using the activity gel according to Yen and Green (1991), with minor modifications. Separating gels included 0.1 mg/ml bovine fibrinogen and 0.2 mg/ml total RNA from baker's yeast (Sigma). After electrophoresis at room temperature, SDS was washed from gels with two 15-min treatments in 25% (v/v) isopropanol in 0.01 M Tris–HCl buffer, pH 7.0. The isopropanol was then removed with two 10-min preincubations in 0.01 M Tris–HCl, pH 7.0, and the gels were incubated in 0.1 M Tris–HCl, pH 7.0 for 3 h to permit in situ degradation of RNA by resolved and renatured RNases. Following incubation, the gels were rinsed in 0.01 M Tris–HCl, pH 7.0 for 10 min and stained in 0.2% toluidine blue O (Sigma) in 0.01 M Tris–HCl, pH 7.0. The gels were destained once for 10 min and twice for 15 min in 0.01 M Tris–HCl, pH 7.0.

Virus material and plant inoculation

The Japanese strain OM TMV (Nozu and Okada 1968) was used in the experiments. Purified TMV preparations were obtained from the sap of diseased tobacco plants (cv. Samsun) with well-expressed mosaic symptoms, 2 weeks after inoculation, using polyethylene glycol-6000 precipitation, followed by three cycles of differential centrifugation (Boedtker and Simmons 1958). The average yield of purified TMV was ca. 1 g per 1 kg of fresh leaf tissue.

All plants were grown in pots under natural light in a greenhouse. Leaves of 14- to 16-day-old plants were dusted with carborundum and inoculated by rubbing with TMV suspensions in redistilled water at the concentrations of 0.01–10 μg/ml.

Virus assay

One to 3 weeks after inoculation, we determined the sample infectivity as described earlier (Malinovsky et al. 2001). Infectivity tests were done using tobacco plants cv. Xanthi nc (the hypersensitive host of TMV). Each sample was homogenized in 0.1 M phosphate buffer, pH 7.0 (ratio 1:50 w/v) containing carborundum, and rubbed into the Xanthi nc leaves. Local lesions were counted 3 days after inoculation (DAI). To carry out the statistical treatment, the number of local lesions was expressed in arbitrary units (decimal logarithms of local lesion number) (Kleczkowski 1968).

The content of TMV antigen in the samples was determined by the quantitative microplate method of enzyme-linked immunosorbent assay (ELISA) according to Clark and Adams (1977) with rabbit anti-TMV antibodies and peroxidase labeled antibodies prepared against our TMV strain. The virus content was estimated on the basis of a calibration curve of the purified TMV.

Statistical treatment

Each experiment with TMV inoculations was performed in five replicates (a single plant represented one replicate). The results in the tables are presented as mean values (±SE) for the combined data of three independent experiments. The t-test was employed to evaluate the statistical significance of differences.

Results

Transgene construction and characteristics of transgenic plants

Intron-less gene coding for pancreatic ribonuclease (Carsana et al. 1988) was isolated from bovine genomic DNA by PCR and cloned in the PC27 vector under the control of mannopin synthase 2′ promoter (Alliotte et al. 1988). This promoter is active in roots and leaves, and its activity is strongly induced locally by wounding (Langridge et al. 1989). Ten kanamycin-resistant transformants were selected. These R0 plants were additionally checked for the presence of transgene by PCR (not shown). Analysis of crude leaf extracts identified three transformants with a high level of ribonuclease activity (10–14 times greater than non-transgenic control), six transformants with a low level (two to four times greater than control), and one transformant with a medium level (seven times greater than control; Table 1). Leaf extracts of the transgenic

Table 1 RNase activity in leaves of non-transgenic (SR1) and transgenic (transformants 1–10) tobacco plants

Plants	Crude leaf extract, average	Standard error	Apoplastic fraction	Debris fraction
SR1	0.8	0.07	0.5	0.3
1	2.7	0.35	2.3	1.3
2	7.6	0.15	8.0	1.4
3	2.4	0.13	2.4	1.1
4	2.2	0.11	1.9	1.1
5	1.7	0.11	nt	nt
6	1.2	0.07	nt	nt
7	8.1	0.07	7.1	1.4
8	9.7	0.44	12	1.4
9	1.8	0.11	nt	nt
10	5.1	0.51	6.0	1.4

Optical density at 260 nm

nt Not tested

plants contained additional ribonuclease activities that were electrophoretically indistinguishable from that of commercial bovine ribonuclease (Fig. 1; several additional bands on the activity gel might possibly be explained by posttranslational modification of bovine ribonuclease). Notably, the apoplast fraction of transgenic leaf extracts was characterized by a higher level of RNase activity in comparison with the apoplast fraction of non-transgenic leaf extracts (24 times greater than control for line 8): thus, bovine pancreatic ribonuclease was secreted from plant cells (Table 1).

According to a segregation analysis of *nptII* marker, line 7 contained single T-DNA insertion [it was also verified by Southern blot hybridization with bovine pancreatic RNase gene used as a probe (not shown)]. This line was selected for further investigations and selfed to obtain R1 and R2 offsprings. Seeds from R1 plants 7-2, 7-3, 7-8 were found to be homozygous for kanamycin resistance. These R2 plants were checked for RNase activity levels (7.6 ± 0.07 for 7-2 plants, 8.0 ± 0.05 for 7-3 plants, 8.2 ± 0.07 for 7-8 plants), and were used to analyze the transgene influence on plant resistance to TMV. Under greenhouse conditions without any nutritional limitation, the expression of the transgene had no visible effect on plant growth and development.

TMV accumulation and the development of disease symptoms

Two R0 transgenic lines (8 and 10), homozygous R2 plants of line 7, and control plants were inoculated with
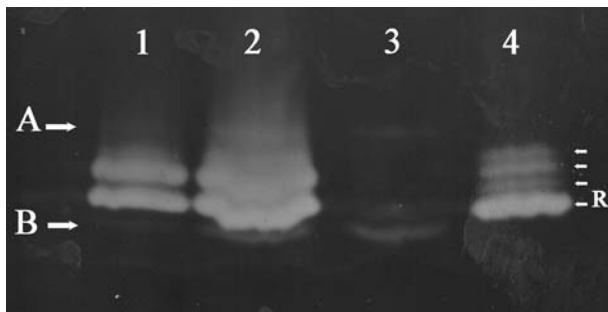

Fig. 1 RNase activity in leaf extracts of transgenic tobacco plants corresponding to *Bos taurus* pancreatic RNase. The extracts were electrophoresed and activity stained in Toluidine blue O. Activity gel contains leaf extract of transgenic tobacco (plant 7-3) corresponding to 10 μg of total soluble protein (*lane 1*), leaf extract of transgenic tobacco (plant 7-3) corresponding to 50 μg of total soluble protein (*lane 2*), leaf extracts of non-transgenic tobacco corresponding to 50 μg of total soluble protein (*lane 3*), and 0.25 μg of purified bovine pancreatic ribonuclease (Sigma) (*lane 4*). *A*, *B* correspond to tobacco proteins possessing ribonuclease activity; *R* corresponds to pancreatic ribonuclease

different TMV concentrations. The occurrence of disease symptoms and the accumulation of the viral antigen (coat protein) were tested. Transgenic plants demonstrated either absence of disease symptoms or a significant delay in their appearance, depending on the virus concentration in the inocula and level of ribonuclease activity (Table 2). In the control and the transgenic tobacco plants, TMV caused a similar sequence of disease symptoms: rugosity and deformation of leaf blades, chlorotic spots, lightening of leaf veins, and islands of dark green tissue. Rugosity and deformation of leaf blades were predominantly expressed in the very young leaves of both plant groups.

We found that the accumulation of viral antigen was significantly retarded in the inoculated leaves of the transgenic plants in comparison with the control plants ($P < 0.05$). Moreover, quantitative viral antigen accumulation negatively correlated with the level of ribonuclease activity (Table 2). Similar results were obtained on comparison of infectious virus contents in the leaves of transgenic and control plants (Table 3). In general, if the virus concentration in the inoculum was low or medium (0.01–0.1 μg/ml), transgenic plants

Table 2 Symptomic responses and TMV antigen content in the leaves of the control (SR1) and the transgenic tobacco plants (μg/cm^2 of leaf surface)

Plants	Concentration of TMV in the inocula (μg/ml)			
	0.01	0.1	1	10
7 DAI				
SR1	0	19 ± 1.2	33 ± 2.1	93 ± 2.6
7-2	0	0	0	0
7-3	0	0	9 ± 1.6	20 ± 2.1
7-8	0	0	0	0
8	0	0	6 ± 1.8	11 ± 1.2
10	0	0	30 ± 2.2	31 ± 1.4
14 DAI				
SR1	0[a]	60 ± 4.4[b]	100 ± 4.2[b]	162 ± 2.5[b]
7-2	0[a]	0[a]	70 ± 3.3[a]	127 ± 6.8[b]
7-3	0[a]	0[a]	32 ± 2.2[a]	86 ± 4.4[a]
7-8	0[a]	0[a]	41 ± 2.0[a]	76 ± 3.1[b]
8	0[a]	0[a]	13 ± 1.4[a]	29 ± 2.4[b]
10	0[a]	45 ± 2.4[b]	86 ± 2.1[b]	141 ± 3.1[b]
21 DAI				
SR1	29 ± 3.6[b]	147 ± 6.4[b]	126 ± 8.4[b]	191 ± 5.3[b]
7-2	0[c]	0[c]	104 ± 6.4[d]	137 ± 6.0[b]
7-3	0[c]	0[c]	88 ± 5.9[d]	150 ± 4.8[b]
7-8	0[c]	0[c]	75 ± 3.4[d]	134 ± 2.7[b]
8	0[c]	0[c]	32 ± 4.4[d]	50 ± 2.4[b]
10	0[c]	145 ± 2.4[b]	116 ± 2.4[b]	215 ± 3.4[b]

The presence of phenotypic disease symptoms was evaluated 14, 21, and 28 DAI

[a] Symptoms were absent

[b] Symptoms were present

[c] Symptoms were absent even 28 DAI

[d] Symptoms were detected only 28 DAI

Table 3 Infectivity of the discs from the infected leaves of the control (SR1) and the transgenic tobacco plants (14 DAI), arbitrary units

Plants	Concentration of TMV in the inocula (µg/ml)			
	0.01	0.1	1	10
SR1	1.03 ± 0.03	2.01 ± 0.05	2.20 ± 0.08	2.07 ± 0.06
7-2	1.06 ± 0.06	1.29 ± 0.06	1.91 ± 0.03	2.15 ± 0.08
7-3	1.00 ± 0.00	1.04 ± 0.04	1.81 ± 0.05	1.97 ± 0.09
7-8	1.00 ± 0.00	1.23 ± 0.09	1.57 ± 0.06	1.98 ± 0.05
8	1.00 ± 0.00	1.00 ± 0.00	1.24 ± 0.04	1.32 ± 0.05
10	1.00 ± 0.00	1.87 ± 0.04	2.16 ± 0.05	2.21 ± 0.03

were characterized by the absence or considerable delay of virus accumulation and appearance of disease symptoms in comparison with the control plants. At higher TMV concentrations in inoculum (10 µg/ml), the differences between the control and transgenic plants were negligible in the later stages of infection (14–28 DAI) (Tables 2, 3).

Discussion

In this work, we have demonstrated that tobacco plants expressing bovine pancreatic ribonuclease are protected against TMV infection. This protection was evidenced by the absence or significant delay of the appearance of typical mosaic symptoms and the retarded accumulation of viral antigen (Table 2) and infectious virus (Table 3). The level of the protection observed in transgenic plants depended on the virus concentration in the inocula and the level of ribonuclease activity (Tables 2, 3).

Currently, the mechanisms of extracellular RNase antiviral effects are poorly known. It was recently proposed that these enzymes might regulate yeast membrane permeability or stability (MacIntosh et al. 2001), so it may be suggested that extracellular RNases inhibit fungal pathogens by this mechanism (Hugot et al. 2002). It may be hypothesized that apoplastic RNases can degrade viral genomic RNAs during some stage of the infection process. The alternative hypothesis might be that ribonuclease molecules enter into plant cells together with viral RNA during the inoculation process. In this case, ribonucleases could kill the cells before viral replication occurs. If the initial quantity of transfected virus particles were large enough, TMV could overcome the nuclease "barrier" and penetrate into plant cells. Note that at the low virus concentrations in the inocula, the transgenic plants were characterized by the absence of both infection symptoms and viral antigen. To the best of our knowledge, this is the first example of the antiviral activity of the transgene-encoded extracellular RNase in plants. This work also supports earlier reports of the antiviral effect of exogeneous ribonuclease application in extracellular space of plant leaves (Galiana et al. 1997). Expression of the heterologous secretory RNase is likely to enhance the intrinsic mechanisms of plant antiviral defense based on plant extracellular RNases [e.g., *Nicotiana tabacum* RNase NE (Galiana et al. 1997; Hugot et al. 2002) and *Arabidopsis thaliana* RNS1 (LeBrasseur et al. 2002)].

A few approaches were found to protect plants against a broad range of pathogens including the expression of pokeweed antiviral proteins (Zoubenko et al. 2000) and 2′,5′-oligoadenylate synthetase (Honda et al. 2003). Plants have also been transformed to express heterologous intracellular RNases specific to double-stranded RNA. It was found that the expression of heterologous dsRNA-specific RNases, as well as their mutant forms (lacking hydrolase activity, but capable of binding to the dsRNA molecules), protected plants against a wide range of pathogenic viruses, although the level of resistance varied considerably (Watanabe et al. 1995; Langenberg et al. 1997; Zhang et al. 2001). As we have mentioned above, bovine pancreatic ribonuclease specifically degrades single-stranded RNAs. We suggest that the approach presented here can be efficiently used in combination with the expression of RNases of other types [e.g., intracellular PR-10-related ribonucleases (Park et al. 2004) or dsRNA-specific ribonucleases (Watanabe et al. 1995; Sano et al. 1997; Langenberg et al. 1997; Zhang et al. 2001)], as well as with a pathogen-derived resistance strategy to maximize protection efficiency and durability. One may also suggest that extracellular ribonuclease-producing plants can be resistant to some pathogenic fungi (e.g., Galiana et al. 1997; Hugot et al. 2002; Park et al. 2004), but this assumption should be verified in further experiments.

References

Abel PP, Nelson RS, De B, Hoffmann N, Rogers SG, Fraley RT, Beachy RN (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232:738–743

Alliotte T, Zhu LH, Van Montagu M, Inze D (1988) Plant expression vectors with the origin of replication of the W-type plasmid Sa. Plasmid 19:251–254

Barna B, Ibenthal WD, Heitefuss R (1989) Extracellular RNase activity in healthy and rust infected wheat leaves. Physiol Mol Plant Pathol 35:151–160

Blank A, McKeon TA (1989) Single-stranded preferring nuclease activity in wheat leaves is increased in senscense and is negatively photoregulated. Proc Natl Acad Sci USA 86:3169–3173

Boedtker H, Simmons NS (1958) The preparation and characterization of essentially uniform tobacco mosaic virus particles. J Am Chem Soc 80:2550–2556

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248–254

Carsana A, Confalone E, Palmieri M, Libonati M, Furia A (1988) Structure of the bovine pancreatic ribonuclease gene: the unique intervening sequence in the 5′ untranslated region contains a promoter-like element. Nucleic Acids Res 16:5491–5502

Clark MF, Adams AN (1977) Characteristic of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J Gen Virol 34:475–483

Deblaere R, Reynaerts A, Hofte H, Hernalsteens J-P, Leemans J, Van Montagu M (1987) Vectors for cloning in plant cells. Methods Enzymol 153:277–292

Galiana E, Bonnet P, Conrod S, Keller H, Panabieres F, Ponchet M, Poupet A, Ricci P (1997) RNase activity prevents the growth of a fungal pathogen in tobacco leaves and increases upon induction of systemic acquired resistance with elicitin. Plant Physiol 115:1557–1567

Goldbach R, Bucher E, Prins M (2003) Resistance mechanisms to plant viruses: an overview. Virus Res 92:207–212

Green PJ (1994) The ribonucleases of higher plants. Annu Rev Plant Physiol Plant Mol Biol 45:421–445

Honda A, Takahashi H, Toguri T, Ogawa T, Hase S, Ikegami M, Ehara Y (2003) Activation of defense-related gene expression and systemic acquired resistance in cucumber mosaic virus-infected tobacco plants expressing the mammalian 2′5′oligoadenylate system. Arch Virol 148:1017–1026

Huang T, Nicodemus J, Zarka DG, Thomashov MF, Wisniewski M, Duman JG (2002) Expression of an insect (Dendroides canadensis) antifreeze protein in Arabidopsis thaliana results in a decrease in plant freezing temperature. Plant Mol Biol 50:333–344

Hugot K, Ponchet M, Marais A, Ricci P, Galiana E (2002) A tobacco S-like RNase inhibits hyphal elongation of plant pathogens. Mol Plant Microbe Interact 15:243–250

Kim J-S, Soucek J, Matousek J, Raines RT (1995) Mechanism of ribonuclease cytotoxicity. J Biol Chem 270: 31097–31102

Kleczkowski A (1968) Experimental design and statistical methods of assay. Meth Virol 4:615–730

Kurata N, Kariu T, Kawano S, Kimura M (2002) Molecular cloning of cDNAs encoding ribonuclease-related proteins in Nicotiana glutinosa leaves, as induced in response to wounding or to TMV-infection. Biosci Biotechnol Biochem 66:391–397

Langenberg WG, Zhang L, Court DL, Giunchedi L, Mitra A (1997) Transgenic tobacco plants expressing the bacterial rnc gene resist virus infection. Mol Breed 3:391–399

Langridge WHR, Fitzgerald KJ, Koncz C, Shell J, Szalay AA (1989) Dual promoter of Agrobacterium tumefaciens mannopine synthase genes is regulated by plant growth hormones. Proc Natl Acad Sci USA 86:3219–3223

LeBrasseur ND, MacIntosh GC, Perez-Amador MA, Saitoh M, Green PJ (2002) Local and systemic wound-induction of RNase and nuclease activities in Arabidopsis: RNS1 as a marker for a JA-independent systemic signalling pathway. Plant J 29:393–403

Lers A, Khalchitski A, Lomaniec E, Burd S, Green PJ (1998) Senescence-induced RNase in tomato. Plant Mol Biol 36:439–449

Lusso M, Kuc J (1995) Increased activities of ribonuclease and protease after challenge in tobacco plants with induced systemic resistance. Physiol Mol Plant Pathol 47:419–428

MacIntosh GS, Bariola PA, Newbigin E, Green PJ (2001) Characterization of Rny1, the Saccharomyces cerevisiae member of the T2 RNase family of RNases: unexpected functions for ancient enzymes? Proc Natl Acad Sci USA 98:1019–1023

Malinovsky VI (2002) The study of physiology of diseased plant in Russian Far East. In: Malinovski VI (ed) The conception of phytovirology and its development in the Russian Far East. Dalnauka, Vladivostok (In Russian), pp 59–79

Malinovsky VI, Pisetskaya NF, Gnutova RV, Sibiryakova II, Tolkach VF (2001) Accumulation and transport of two strains of TMV (OM and Far-Eastern, detected on pepper) in tobacco plants. J Russ Phytopathol Soc 2:31–34

Nozu Y, Okada Y (1968) Amino acid sequence of a common japanese strain of tobacco mosaic virus. J Mol Biol 35:643–646

Park C-J, Kim K-J, Shin R, Park JM, Shin Y-C, Paek K-H (2004) Pathogenesis-related protein 10 isolated from hot pepper functions as a ribonuclease in an antiviral pathway. Plant J 37:186–198

Sano T, Nagayama A, Ogawa T, Ishida I, Okada Y (1997) Transgenic potato expressing a double-stranded RNA-specific ribonuclease is resistant to potato spindle tuber viroid. Nat Biotechnol 15:1290–1294

Sindelarova M, Sindelar L, Burketova L (2002) Glucose-6-phosphate dehyfrogenase, ribonucleases and esterases upon tobacco mosaic virus infection and benzothiodiazole treatment in tobacco. Biol Plant 45:423–432

Solomon-Blackburn RM, Barker H (2001) Breeding virus resistant potatoes (Solanum tuberisum): a review of traditional and molecular approaches. Heredity 86:17–35

Voinnet O (2005) Induction and suppression of RNA silencing: insights from viral infections. Nat Rev Genet 6:206–220

Wassenegger M (2002) Gene silencing-based disease resistance. Transgenic Res 11:639–653

Watanabe Y, Ogawa T, Takahashi H, Ishida I, Takeuchi Y, Yamamoto M, Okada Y (1995) Resistance against multiple plant viruses in plants mediated by a double stranded-RNA specific ribonuclease. FEBS Lett 372:165–168

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

Yen Y, Green PJ (1991) Identification and properties of the major ribonucleases of Arabidopsis thaliana. Plant Physiol 97:1487–1493

Zhang L, French R, Langenberg WG, Mitra A (2001) Accumulation of barley stripe mosaic virus is significantly reduced in transgenic plants expressing a bacterial ribonuclease. Transgenic Res 10:13–19

Zoubenko O, Hudak K, Tuer NE (2000) A non-toxic pokeweed antiviral protein mutant inhibits pathogen infection via a novel salicylic acid-independent pathway. Plant Mol Biol 44:219–229