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New Antisense RNA Systems Targeted Against Plant Pathogens

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ABSTRACT tRNA and 7SL RNA based antisense vehicles were prepared by inserting conserved anti-viral and anti-viroid domains. Anti-PVS coat protein leader sequence (ACPL) and antistructural antihairpin domain of PSTVd (AHII) were inserted in tRNA cassette; anti- zing finger domain of PVS, AHII and anti hop latent viroid ribozyme were inserted in 7SL RNA gene isolated from *A. thaliana*. These constructs were shown to be transcribed both, in *in vitro* and in *in vivo* conditions. However, it followed from our work that closely linked position of PolII reference genes and PolIII antisense genes within T-DNA lead to the impairment of RNA expression in transgenic plants. To assay *in vivo* transcription of antisense genes, hairy root potato cultures were established using *A. tumefaciens* A4-24 bearing both, Ri plasmid and PolII-promoterless plant expression vectors with antisense RNA genes. Expression of antisense RNA in transgenic potato tissues was proven by specific RT-PCR reactions.

Key words: Antisense RNA, DNA-dependent RNA polymerase III transcription, hairy root tissue, PSTVd, PVS, RT-PCR, *Solanum tuberosum* L.

Abbreviations: GUS: ß-Glucuronidase, NPTII: Neomycin phosphotransferase II, PSTVd: Potato spindle tuber viroid, PVS: Potato virus S, RT-PCR: Reverse transcription polymerase chain reaction

Introduction

Antisense RNA-mediated inhibition of gene expression appears to be a common phenomenon observed in different experimental organisms. In plants this technology is used for various purposes, for instance, with the aim of modifying or improving plant performance, to determine function of cryptic genes or to analyze control steps in physiological processes (for review see e.g. van Blokland et al. 1993). The high level of antisense RNA expression in the cell is considered to be a prerequisite to be the antisense RNA system efficient. This requirement is important especially for blocking of viruses and virus-like pathogens, which represent extrachromosomally replicating units, reaching usually high copy number during the infections. In most cases 35S promoter of CaMV or other promoters specific for DNA-dependent RNA polymerase II were used for construction of plant antisense expression vectors (e.g. van Blokland et al. 1993; Wilson 1993). It is known that 35S promoter is strongly influenced by physiological and growth conditions and is not constitutively expressed in all tissue (e.g. Bourque and Folk 1992). Some antisense RNAs being transcribed as mRNAs are probably not stable enough and/or are not transported to the cytoplasm. These two factors contribute probably in general to low

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efficiency of blocking plant RNA viruses, which replicate in the cytoplasm (see Wilson 1993). Another factors like co-suppression and gene silencing (for recent review see e.g. Stam et al. 1997) in transgenic plants complicate efficient gene targeting by antisense RNAs. Two main pathways, transcriptional and posttranscriptional gene silencing are considered. Various factors seem to play role in these phenomena including DNA methylation, transgene copy number and the repetitiveness of the transgene insert, transgene expression level, possible production of aberrant RNAs, and ectopic DNA-DNA interactions (Stam et al. 1997). It seems likely that so-called RNA-directed *de novo* DNA methylation (Wassenegger et al. 1994) inactivated antisense genes targeted against potato spindle tuber viroid (Matoušek et al. 1994). In these experiments we used constructs driven by 35S promoter. Inhibitory effect of antisense RNA was stable in all transgenic lines agroinfected as leaf discs in tissue culture in the presence of methylase inhibitor, azacytidin, while quenching inhibition was observed for intact plants (Matoušek et al. 1994).

Most of viruses and viroids are extremely variable entities, often forming quasispecies. The "silence" mutations, which accumulate in their genomes, could prevent antisense RNA systems to action efficiently. In order to develop more efficient antisense RNA systems against potato and hop viruses and viroids we analyze variable and conserved domains of these pathogens. For instance, using TGGE system and methods of pre-formed DNA heteroduplexes, we analyzed virus and viroid variability under control and stress conditions. It was shown that stress conditions induce HLVd mutagenesis (Matoušek et al. in press a). Also significant variations we revealed in PVS and PVY viruses within 3' part of genome and P1 gene, respectively (Matoušek et al. 2000a, b). Several conserved domains were selected to prepare pathogen-derived antisense RNAs, such as anti-HLVd ribozyme (Matoušek et al. 1995), anti-PSTVd anti hairpin II (Matoušek et al. 1999a), zinc finger domain of PVS (Matoušek et al. 2000a), anti P1 domain (Matoušek et al. 2000b) and anti HC-Pro domain of PVY. In addition, to overcome problems with RNA silencing we constructed DNA-dependent RNA polymerase III transcribed genes based on tRNA and 7SL RNA genes. In this study we investigate expression of newly modified antisense vehicles based on $tRNA^{Tyr}$ from tobacco and 7SL RNA from arabidopsis. We analyzed these vehicles by *in vitro* transcription as well as by RT-PCR in potato hairy root tissues transformed by *A. tumefaciens*.

Materials and Methods

Antisense chimeras and preparation of antisense RNA genes

A complete 7SL RNA gene containing both, promoter and terminator elements we isolated from *Arabidopsis thaliana* var. Columbia. This 305 nucleotide long 7SL RNA differs by several base changes from other arabidopsis 7SL sequences published so far [EMBL AC X72228, X72229] (Marques et al. 1993). 7SL RNA gene was modified by inserting a 31 bp MCS upstream the conserved motif ACCA at the 3' end of the gene as described by Matoušek et al. (in press b). This modified gene was designated A63M. *Srf*I restriction site within MCS was used to clone various antisense RNA in this 7SL RNA cassette. Flanking restriction sites (*Hind*III) were created to facilitate cloning these chimerical genes in plant expression vectors. Either 27-base AHII sequence (CCTACTCGCTCCCTTTGCGCTGTC GCT) or 87 base anti zinc finger domain of PVS (ACTAATGCCTG-GACGGCATGTCCTATTATCACACTTAGAATTG-CAAATAGGTGGATGACACGATAACATCGCCAGC ATCGCCCAAT) (Matoušek et al. in press b) were integrated into cassette. We used also 57 base anti HLVd ribozyme (Matoušek et al. 1995) to assay 7SL RNA expression vehicle.

tRNA-based expression cassette was constructed by modification of a $tRNA^{Tyr}$ from tobacco (Fuchs et al. 1992) *Eco*RI and *Sal*I cloning sites were created in the intron position of the modified tRNA gene. In this gene either 27-base AHII sequence or 84-base anti PVS coat protein leader (ACPL) (GATCTGGTTAGGCGGCATCTTC-TCTGTGGGACAGTTT CTTCGAACTCTTACCTGTGAAC-CTAAAGGTGTTTCAACGG) were cloned as described by Matoušek et al. (in press b). AHII was extended for ten C residues on its 5' and 3' ends respectively to prevent intramolecular hybridization. *Eco*RI and *Sal*I restriction sites respectively were attached to 5' and 3' cloning sites of AHII and ACPL sequences. Secondary structures for antisense vehicles were predicted using modified Zucker algorithm (Riesner and Steger 1990) and by DNASIS for Windows, version 2.5 (Hitachi).

Antisense RNA genes were ligated either in the *Hind*III site of plant expression vector pGPTV, bearing GUS and NPTII as marker genes or in pGPTVL vector having deleted both resistance and GUS marker genes. pGPTVL vector was prepared by *Eco*RI and *Bam*HI cleavage of pGPTV and by inserting new polylinker containing *Kpn*I and *Sac*I restriction sites. In these restriction sites the antisense tRNA or 7SL RNA genes were ligated. All plant expression vectors were electroporated into *A. tumefaciens* strain EHA 105.

Potato transformation

Potato plants cv. Kamýk were transformed using *A. tumefaciens* transformation method of Rogers et al. (1986) and maintained on Murashige and Skoog (1962) medium containing initially 200 mg/l kanamycin (pGPTV antisense RNA vectors) as described previously (Matoušek et al. 1994). *In vitro* grown plants were supplied with light (16 h) of intensity 35 mmol m^{-2} s^{-1}. Day/night temperature were 25/18 C. Hairy root lines were established after injection of the suspension of *A. tumefaciens* A4-24 bearing Ri plasmid and pGPTVL derived antisense RNA vectors into *in vitro* grown potato plants. Hairy roots, which developed usually within two weeks, were excised and grown further on MS medium.

In vitro transcription and RT-PCR analyses

Transcription assays were carried out in a 10 µl reaction mixture containing 8 Mbq ml^{-1} ^{32}P [α-GTP] (400 µCi $mmol^{-1}$) as described by Stange and Beier (1987). In the standard reaction, we applied either 300 ng of plasmid DNA or 15 ng of PCR fragments purified using Qiagen protocols.

For RT-PCR analysis RNA was isolated using RNeasy Plant Total RNA Extraction Kit (Qiagen). If not otherwise stated this RNA was supplementary treated with DNAse I (RNAse-free, Roche Molecular Biochemicals) to avoid possible contamination with agrobacterial expression vectors. RT-PCR reactions were performed using Titan™ One Tube RT-PCR System (Roche Molecular Biochemicals). Primers Zn1 and Zn2 (above) were used to detect antisense RNA inserted in 7SL RNA cassette. Primers designated A63M start (GTCGAGCTAAGTAACAGTAG) and SHII (AGCGACAGCGCAAAGGGAGCGAGT AGG) were combined for detection of AHII-A63M (7SL RNA) chimera. The following primers designated 5tRNA (CCGACCTTAGCTCAG) and CPL-Eco (AAGAATTCCGTT GAAACACCTTTAGG) were used to detect part of ACPL-tRNA chimera. 5tRNA primer was combined with 3tRNA-AHIIC (CTAAGGATGTCGACG) primer to detect part of AHII-tRNA chimera. The additional control RT-PCR reactions were performed which included detection of potato 7SL RNA using primers α and ßanti described earlier (Matoušek et al. 1999b) and detection of expression of Rol A gene in potato hairy roots using primers RolA5 (TCGGAGTATTATCGCTCGTC) and RolA3' (CCCTAATTATGCGTAAAG CT). RT-PCR cycle included 30 min 53°C; 30 × (94°C - 30 sec; 50°C - 1 min; 68°C - 1 min); 68°C - 10 min.

Results and Discussion

To overcome gene silencing problems, RNA polymerase III driven genes were used for the construction of antisense RNA genes. For instance, 7SL RNA genes are driven by promoter elements for DNA-dependent RNA polymerase III similarly to 5S ribosomal RNA, U3, U6 snRNA and tRNA genes. Some of polymerase III driven genes has been already advantageously used for antisense RNA expression. These genes are suitable for expression of short RNA fragments or gene domains. For instance, efficient suppression of chloramphenicol acetyltransferase (CAT) using antisense tRNA chimeras was observed by Borque and Folk (1992). The most effective constructs contained only 184 and 164 base antisense RNAs and were complementary to 3' regions of CAT. A five-fold greater inhibitory effect was achieved using these constructs than an antisense construct driven by CaMV promoter (Borque and Folk, 1992). Even shorter construct containing hammerhead ribozyme was used by Perriman et al. (1995). In this work very effective ribozyme expression was achieved by the use of tRNA cassette in combination with geminivirus based vector. Efficient antisense ribozyme expression and inhibition of peroxidase gene was achieved by McIntyre et al. (1996) using tRNA promoter. U6 snRNA was used as expression vehicle by Fan and Sugiura (1995). 5S RNA and tRNA form very abundant RNA species. In our work we aimed to modify 7SL RNA and tRNA genes. 7SL RNA genes belong to the same category of RNAs associated with the protein synthesis machinery. We suppose that

antisense RNA systems based on these genes should not be sensitive to overproduction of homologous RNA gene products and should not be silenced by co-suppression or by RNA-directed *de novo* DNA methylation at least in such an extent as polymerase II-driven antisense genes.

In our experiments, two tRNA and three 7SL RNA antisense vehicles were constructed. The secondary structures of these RNAs were calculated for physiological temperature conditions using modified Zucker algorithm (Riesner and Steger 1990) (Figure 1 and Figure 2).

Anti-PVS coat protein leader sequence (ACPL) and antistructural anti-hairpin II domain of PSTVd (AHII) were inserted in tRNA cassette. The secondary structures predicted are depicted in (Figure 1). The CPL domain is relatively stable and it contains conserved nucleotide motif CCTTTAGGTT within the translational enhancer (Turner and Foster 1997), which is probably essential for virus coat protein synthesis. Targeting this domain could inhibit coat protein synthesis. In our experiments this domain was derived from 3' terminal region of ordinary PVS strain Kobra (EMBL AC Y15625), which we characterized recently (Matoušek et al. 2000a). Hairpin II structure is believed to be either part of the promoter element or polymerase recognition site (Loss et al. 1991; Qu Feng et al. 1993). Therefore targeting this structure could inhibit viroid replication. According to our unpublished results 27 base AHII oligo form specific complexes with viroid target. In order to prevent intramolecular pairing with tRNA specific sequence, AHII was extended for ten C residues on its 5' and 3' ends respectively. It was predicted that both antisense RNAs form small 9-22 base internal loops which could facilitate interaction with the target sequences (Figure 1).

Anti-zing finger domain of PVS, AHII and anti hop latent viroid ribozyme HR57 were selected to construct chimerical 7SL RNA genes. The structures predicted for these chimerical antisense RNAs are depicted in figure 2. "Zinc finger" domain [Zn1Zn2] is localized within 11K protein (position 3325-3411 in 3' part of PVS Kobra genome). This domain is also relatively conserved having general consensus C-$X_{(2-4)}$-C-$X_{(2-15)}$-C(or H)$_{(2-4)}$-C(or H)

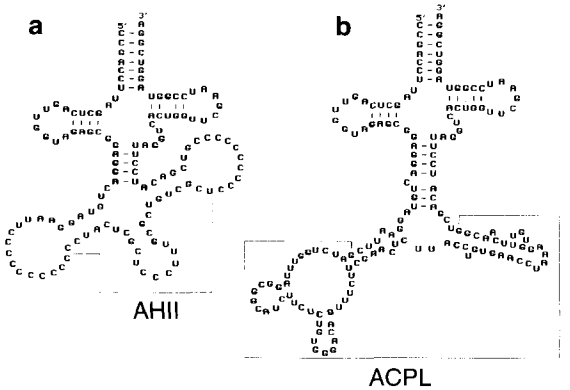

Figure 1. The secondary structures of tRNA antisense RNA chimeras AHII and ACPL. The structure was predicted using program DNASIS and is based on well known and stable tRNA folding.

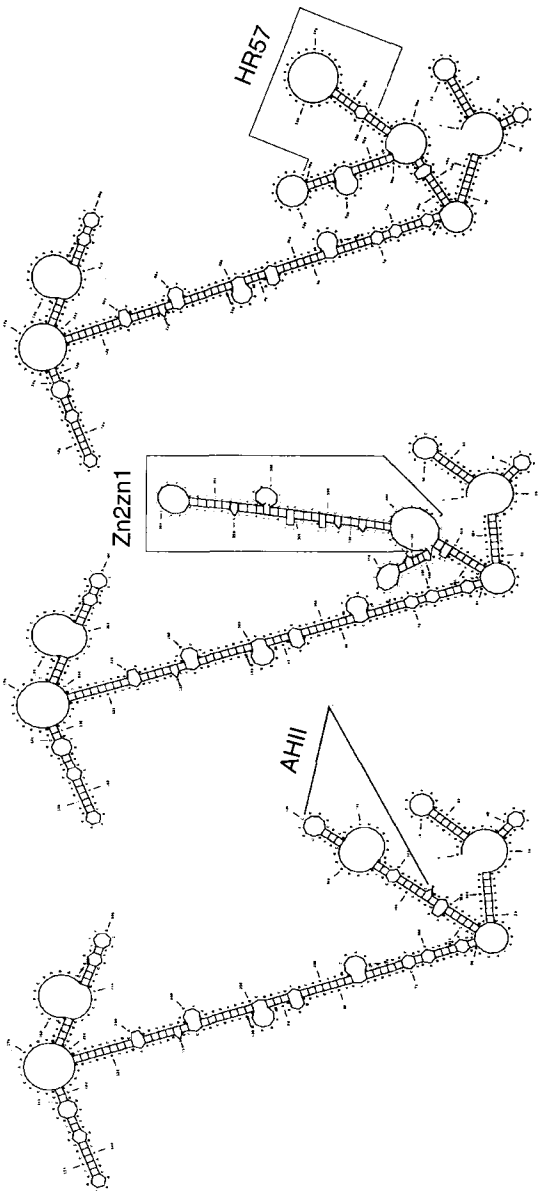

Figure 2. The secondary structures of 7SL RNA A63M antisense RNA chimeras AHII, HR57 and Zn2Zn1. The calculation of RNA folding simulates low salt and temperature conditions.

(Klug and Rhodes 1987). Anti viroid ribozyme was designed against minus viroid replication intermediates. HR57 is targeted against 50 base intramolecular loop and scissile GUC at position 111 in HLVd genome (Matoušek et al. 1995). It is shown in figure 2 that all antisense sequences form separate hairpins within chimerical structures. The presence of internal loops within these sequences could facilitate interaction with corresponding targets.

All these construct were shown to transcribe *in vitro* using PolIII extract from HeLa cells (Figure 3) and recently also using tobacco PolIII extract (unpublished), suggesting that designed modifications do not impair promoter activity of tRNA and 7SL RNA genes and that chimerical antisense RNAs are, in principle, stable in plant extracts.

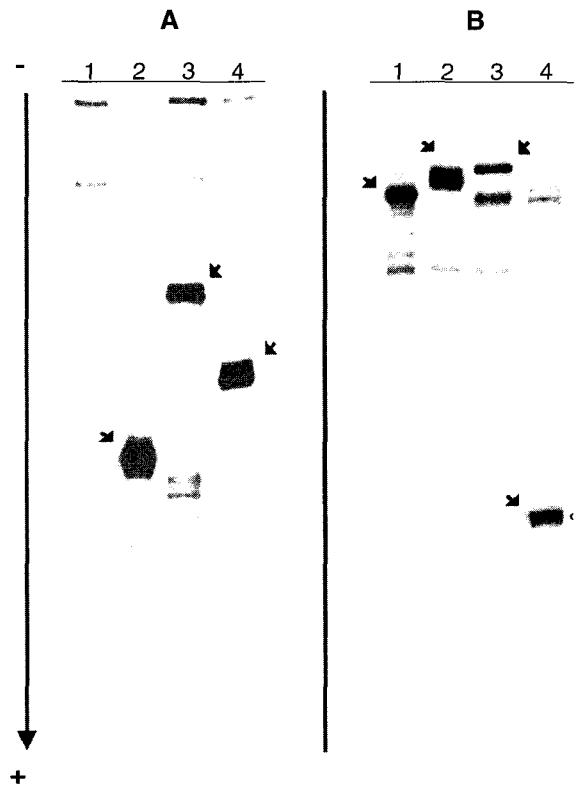

Figure 3. *In vitro* transcription of antisense tRNA and 7SL RNA chimeras. Transcription was performed from pBlueScript KS+ plasmids containing chimerical sequences using protein extracts from HeLa cells and radioactively labeled GTP. RNA transcripts were then electrophoresed in 8% sequencing gels. Panel A: 1 - pBlueScript control; 2 - wild type tRNA clone AtY3II; 3- ACPL-tRNA chimera; 4 - AHII-tRNA chimera. Panel B: 1 - AHII-A63M chimera ; 2 - ribozyme HR57-A63M chimera; 3 - Zn2Zn1-A63M chimera (modified chimerical molecule containing anti "zinc fingers" antisense RNA); 4 - wild type $tRNA^{Tyr}$-control.

All chimerical cassettes were initially integrated in plant expression vector pGPTV in-between GUS and NPTII genes, regulated from 35S and P_{nos} promoters, respectively. Both these promoters are driven by DNA-dependent RNA polymerase II. PolIII cassettes were in tight neighborhood to these promoters. Using *Agrobacterium tumefaciens* strains harboring these vectors, 1686 potato explants were transformed; 1893 potato regenerants were transferred to selection media and 146 potato regenerants rooting on media containing kanamycin were selected. Except for one plant regenerant transformed with Zn2Zn1-A63M, non of them contained chimerical antisense gene. In addition, non of these regenerants expressed GUS activity. These results led to the assumption that there is strong selection against our particular T-DNA expression cassette due to some impairment of simultaneous transcription from closely linked PolII and PolIII genes. This possibility is supported by the finding that 7SL RNA genes are often surrounded by AT-rich regions, which could be important for their activity (Matoušek et al. 1999b). Some tRNA genes are also positioned in AT-rich regions (e.g. Arnold et al. 1986). In addition, it can be assumed that some recombination process eliminated PolIII and GUS genes at least in some kanamycin resistant transgenotes.

In order to overcome a potential problem of PolII and PolIII interaction, we constructed PolII-promoterless pGPTVL vector by deleting both reference genes from T-DNA. AHII and ACPL tRNA chimeras, as well as AHII and anti Zn2Zn1 7SL RNA chimeras were ligated in pGPTVL vector and used for transformation of potato tissues mediated by *A. tumefaciens* strain A4-24. Induced hairy roots were then used to assay antisense RNA expression. Although significant chimerism of hairy roots can be expected in the absence of any selection marker, the expression of antisense tRNA and 7SL RNA chimeras was proven in our experiments by RT-PCR (Figure 4 and Figure 5 A, B). In all cases specific PCR products were observed corresponding to antisense RNAs. RT-PCR signals were comparable or slightly weaker that signals for internal potato 7SL RNA genes (Figure 4) or Rol genes introduced by A4-24 strain of *Agrobacterium tumefaciens*.

It can be concluded from our experiments that new PolIII-driven antisense RNA chimeras we developed, can be successfully expressed in plant tissues. However, to

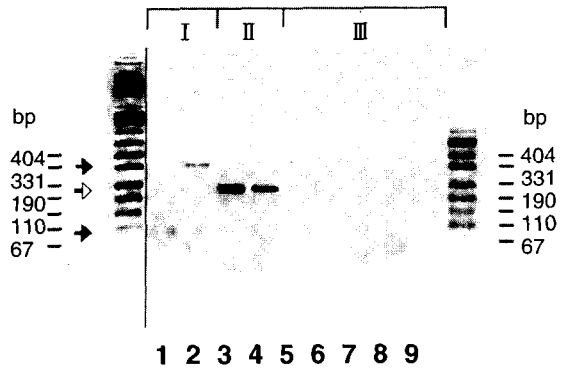

Figure 4. Analysis of anti-PSTVd antisense RNA chimeras in transgenic potato tissues by RT-PCR. RNA was extracted from hairy roots as described in Material and methods, treated with DNAse I and subjected to analysis. Part I: detection of AHII-chimeras; Part II: detection of cellular potato 7SL RNA. Part III: control PCR reactions with RNA templates. 1 - AHII-tRNA chimera (specific band indicated by the filled arrow); 2 - AHII-A63M chimera (specific band indicated by the filled arrow); 3 - potato 7SL RNA signal in hairy roots transformed with tRNA chimera (specific bands indicated by the empty arrow); 4 - potato 7SL RNA signal in hairy roots transformed with 7SL RNA chimera (specific band indicated by the empty arrow); 5 - PCR with primers for modified tRNA with RNA template from hairy roots transformed with tRNA chimera; 6 - PCR with primers for modified tRNA no template added; 7- PCR with primers for modified 7SL RNA with RNA template from hairy roots transformed with 7SL RNA chimera; 8- PCR with primers for modified 7SL RNA no RNA template added; 9 control PCR with 7SL RNA primers no RNA template added. Molecular weight markers are shown on the sides.

produce transgenic plants, some selection marker genes are desirable to be included. In this case, some additional modification of T-DNA has to be performed to stabilize the expression. It is supposed that the splitting of PolII and PolIII genes with MAR element, which represents AT-rich scaffold attachment region (e.g. Nap et al. 1997) could stabilize T-DNA with PolIII cassettes in potato transgenotes. These vectors were recently prepared and plant transformation is in progress.

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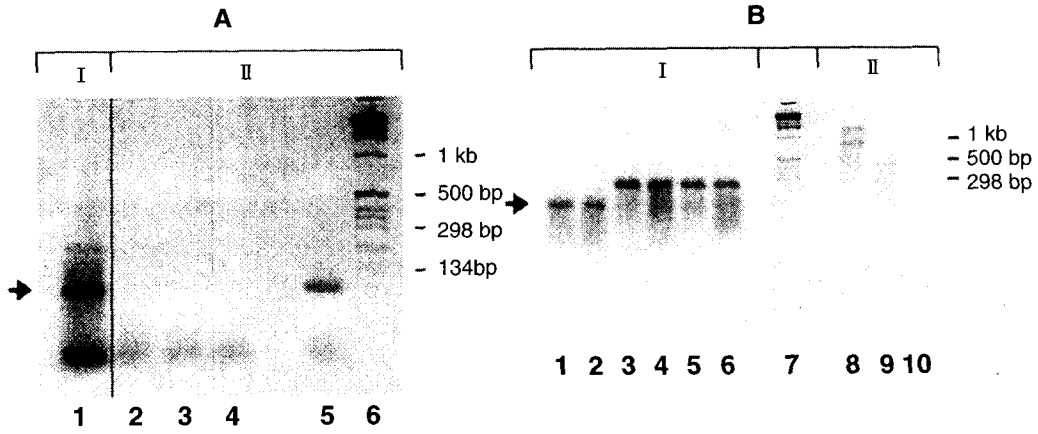

Figure 5. Analysis of anti-PVS antisense RNA chimeras in transgenic potato tissues by RT-PCR. Gel A: detection of Zn2Zn1-A63M RNA; Gel B: detection of ACPL-tRNA. The products of RT-PCR reactions are analyzed in parts I of the corresponding gels, while products from control PCR reactions were applied on the parts II of the gels. Gel A: 1 - RT-PCR of Zn2Zn1-A63M after RNA treatment with DNAse I; 2 - control PCR reaction with Zn1xZn2 primers using RNA treated with DNAse I; 3 - the same reaction as in lane 3 using untreated RNA, 4 - PCR reaction without template; 5 - PCR reaction with Zn1xZn2 primers from DNA clone of Zn2Zn1-A63M; 6 - 1 kb ladder. Gel B: 1 - detection of ACPL-tRNA (untreated RNA); 2 - ACPL-tRNA (DNAse I); 3 - detection of RolA (untreated RNA); 4 - RolA (DNAse I); 5 - detection of 7SL RNA (untreated RNA); 6 - 7SL RNA (DNAse I); 7 - 1 kb ladder; 8 and 9 control PCR reactions using DNAse I treated and untreated RNA templates, respectively; 10 - PCR reaction from ACPL-tRNA cloned in plasmid.

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