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STUDIES ON SPECIFIC AND BROAD SPECTRUM VIRUS RESISTANCE IN TRANSGENIC PLANTS

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PRESS

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To Liis and Katrin

To my parents

CONTENTS

1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers which will be referred to by their Roman numerals:

I. **Truve, E., Järvekülg, L., Bouscaren, M.-L., Aaspõllu, A., Priimägi, A., and Saarma, M.** (1995). Different propagation levels of potato virus X (PVX) isolates in PVX coat protein expressing tobacco plants and protoplasts do not correlate with coat protein sequence similarities. *Archiv für Phytopathologie und Pflanzenschutz* **30**: 15–30.

II. **Truve, E., Nigul, L., Teeri, T. H., and Kelve, M.** (1996). The effects of 2-5A on protein synthesis in wheat germ extracts and tobacco protoplasts. *Nucleosides and Nucleotides* **15**: in press.

III. **Truve, E, Kelve, M., Aaspõllu, A., Schröder, H. C., and Müller, W. E. G.** (1994). Homologies between different forms of 2-5A synthetases. *Progress in Molecular and Subcellular Biology* **14**: 139–149.

IV. **Truve, E., Aaspõllu, A., Honkanen, J., Puska, R., Mehto, M., Hassi, A., Teeri, T. H., Kelve, M., Seppänen, P., and Saarma, M.** (1993). Transgenic potato plants expressing mammalian 2'-5' oligoadenylate synthetase are protected from potato virus X infection under field conditions. *Bio/Technology* **11**: 1048–1052.

V. **Truve, E., Kelve, M., Aaspõllu, A., Kuusksalu, A., Seppänen, P., and Saarma, M.** (1994). Principles and background for the construction of transgenic plants displaying multiple virus resistance. *Archives of Virology* **S9**: 41–50.

IV. **Truve, E., Aaspõllu, A., Tamm, T., Järvekülg, L., Sareneva, T., Julkunen, I., Nigul, L., Saichenko, T. A, Skryabin, K. G., Kelve, M., and Saarma, M.** Transgenic tobacco plants expressing mammalian 2-5A synthetase show protection against independent infections of three unrelated viruses. Manuscript.

2. LIST OF ABBREVIATIONS

AA	—	amino acid
Ala	—	alanine
AlMV	—	alfalfa mosaic virus
Arg	—	arginine
ATP	—	adenosine triphosphate
AVF	—	antiviral factor
BMV	—	brome mosaic virus
bp	—	basepairs
cDNA	—	complementary DNA
CMV	—	cucumber mosaic virus
CP	—	coat protein
CP(+)	—	coat protein gene expressing
CPMP	—	coat protein-mediated protection
cv.	—	cultivar
D	—	dalton
DI	—	defective interfering
DNA	—	deoxyribonucleic acid
dsRNA	—	double-stranded RNA
eIF-2	—	eukaryotic initiation factor-2
ELISA	—	enzyme-linked immunosorbent assay
gp	—	glycoprotein
GTP	—	guanosine triphosphate
HIV	—	human immunodeficiency virus
HPLC	—	high performance liquid chromatography
HR	—	hypersensitive response
IFN	—	interferon
IgG	—	immunoglobin G
IκB	—	inhibitor of nuclear factor-κB
IL-1R	—	interleukin-1 receptor
kan^R	—	kanamycin-resistant
kb	—	kilobasepairs
Leu	—	leucine
LRR	—	leucine-rich repeat
Lys	—	lysine
MP	—	movement protein
mRNA	—	messenger RNA
NADPH	—	nicotinamide adenine dinucleotide phosphate
NF-κB	—	nuclear factor-κB
NIa	—	nuclear inclusion protein 'a'
PAP	—	pokeweed antiviral protein

pCp	—	cytidine 3',5'-bisphosphate
PCR	—	polymerase chain reaction
PKR	—	double-stranded RNA-dependent protein kinase
PLRV	—	potato leafroll virus
PR	—	pathogenesis-related
Pro	—	proline
pv.	—	pathovar
PVS	—	potato virus S
PVX	—	potato virus X
PVY	—	potato virus Y
RNA	—	ribonucleic acid
RNase	—	ribonuclease
S. E.	—	standard error
SAR	—	systemic acquired resistance
Ser	—	serine
T-DNA	—	transferred DNA
TEV	—	tobacco etch virus
TMV	—	tobacco mosaic virus
TSWV	—	tomato spotted wilt virus
TVMV	—	tobacco vein mottling virus
UV	—	ultraviolet
VPg	—	genome linked protein
WClMV	—	white clover mosaic virus
2-5A(+)	—	2-5A synthetase cDNA expressing
2-5A	—	2'-5' oligoadenylates
2' PDE	—	2' phosphodiesterase

3. INTRODUCTION

Plants, as well as animals, bacteria and other organisms are infected by viruses. Viruses are among plant pathogens which cause diseases of great economic importance. Virus infections, on average, account for about one third of crop losses to all diseases (Fraser, 1992). Despite active research since the 1950s no effective antiviral chemicals have been found for plants (Matthews, 1987). Therefore, besides long-lasting breeding programs, there is a need for the exploitation of naturally existing virus resistance mechanisms as well as for novel genetic engineering approaches to protect crops from viral diseases. As viruses are pathogens completely dependent on the host organism in their life cycle, studies on virus resistance mechanisms and virus-host interactions also provide better understanding of the functioning of the host organism. The last decade has provided us with many novel approaches to enhance virus tolerance in higher plants. However, most of these approaches do not confirm protection against multiple viruses, whereas crops are often infected simultaneously by several different plant viruses. During the very recent years information has also started to accumulate on the molecular mechanisms of natural virus resistance in plants. In the literature review of this thesis a short overview about some of the most significant natural virus resistance mechanisms in plants as well as about the approaches successfully taken to create virus resistance in transgenic plants is given. The experimental part of the work attempts to give some new information about the possibilities to generate virus tolerant transgenic plants, using genes derived from plant virus genome as well as from mammalian antiviral machinery. We first show that the expression of the virus coat protein gene in transgenic plants leads to protection against this virus. However, the level of protection against different isolates of the same virus remains unpredictable. As our final goal has been to introduce a broad-scale virus resistance in transgenic plants, we have chosen a mammalian 2'-5' oligoadenylate (2-5A) antiviral pathway as a possible means to achieve this aim. I report here that it is possible to construct transgenic plants displaying multiple virus protection by introducing the cDNA for the key enzyme of 2-5A pathway into the plants.

4. REVIEW OF LITERATURE

4.1. Natural plant virus resistance

Plants can defy some potential pathogen invaders but not others. Genetic studies have led to the "gene-for-gene" theory of plant pathogen interactions, which postulates that pathogens bear avirulence (*avr*) genes that elicits the production of molecules that are recognised by plant resistance (*R*) gene products (Keen, 1990). If a plant has an *R* product, it "detects" the pathogen bearing the corresponding *avr* gene and initiates a resistance response, such as rapid cell death at the site of infection, termed "hypersensitive" response (HR), thus preventing the establishment of infection (Chasan, 1993).

4.1.1. Plant resistance genes

The first plant resistance gene isolated was maize *HM1* gene conferring resistance to fungus *Cochliobolus carbonum* (Johal and Briggs, 1992). Two years later the cloning of the first plant virus resistance gene — *N* gene that mediates resistance to tobacco mosaic tobamovirus (TMV), was reported (Whitham *et al.,* 1994). N^+ tobaccos react with a typical HR response to infection by *N*-sensitive TMV serotypes. Surprisingly, the *N* gene showed considerable similarity to some other previously characterised plant resistance genes — to *RPS2* from *Arabidopsis* conferring resistance to bacterium *Pseudomonas syringae* (Bent *et al.*, 1994; Mindrinos *et al.*, 1994), to *Cf-9* from tomato (resistance to fungus *Cladosporium fulvum*, Jones *et al.*, 1994), and to L^6 from flax (resistance to fungal leaf rust, Lawrence *et al.*, 1995). As the lifecycles of these pathogens are completely different, the related structural features of their resistance genes are striking. All four gene products contain a number of leucine-rich repeats (LRRs), and three gene products (incl. *N*) have a nucleotide binding site. The significance of these similarities is unknown, although it has been proposed that all identified resistance genes are involved in the signal transduction pathways which lead to HR. N-terminal third of the N protein shares considerable homology with cytoplasmic domains of *Drosophila* protein Toll and interleukin-1 receptor (IL–1R) from mammals. Both these cell membrane associated proteins are involved in the rapid induction of gene expression leading to the dorsoventral polarity in *Drosophila* (St. Johnston and Nüsslein-Volhard, 1992) and immune response in mammals (Sims *et al.*, 1989), correspondingly. The N protein from tobacco is thought to be a cytoplasmic protein, but as its activator is the intracellular pathogen TMV, this difference between Toll and the IL–1R seem appropriate. The functioning of all proteins as direct activators of corresponding transcription factors leading to acute responses is highly probable. The currently cloned plant pathogen resistance genes are listed in Table 1.

Table 1. Plant pathogen resistance genes.

Gene	Plant species	Pathogen	Characteristic features of the resistance protein	First report
Hm1	maize	*C. carboneum*	NADPH-dependent HC-toxin reductase	Johal and Briggs, 1992
PTO	tomato	*P. syringae* pv. *tomato*	serine/threonine protein kinase	Martin *et al.*, 1993
Cf-9	tomato	*C. fulvum*	transmembrane protein with LRRs	Jones *et al.*, 1994
N	tobacco	TMV	cytoplasmic nucleotide-binding protein with LRRs	Whitham *et al.*, 1994
RPS2	*Arabidopsis*	*P. syringae* pvs. *tomato* and *maculicola*	cytoplasmic nucleotide-binding protein with LRRs	Bent *et al.*, 1994 Mindrinos *et al.*, 1994
RPS1	*Arabidopsis*	*P. syringae*	cytoplasmic nucleotide-binding protein with LRRs	Grant *et al.*, 1995
L^6	flax	*Melampsora lini*	cytoplasmic nucleotide-binding protein with LRRs	Lawrence *et al.*, 1995
Xa21	rice	*Xanthomonas oryzae* pv. *Oryzae*	serine/threonine protein kinase with LRRs	Song *et al.*, 1995
Cf-2	tomato	*C. fulvum*	transmembrane protein with LRRs	Dixon *et al.*, 1996

For several resistance genes providing protection against bacterial and fungal pathogens, the corresponding elicitors are known. Plant viruses do not possess dispensable avirulence functions, but some integral components of the virus are recognised by resistant host plant cultivars. For instance, the coat protein (CP) TMV has been identified as the elicitor of *N'* resistance gene-induced HR (Saito *et al.*, 1987) and the movement protein (MP) of TMV affects virulence in tomato carrying the *Tm-2* resistance gene (Meshi *et al.*, 1989, see Table 2). In the case of potato X potexvirus (PVX), it is the viral CP that elicits resistance in potato carrying the *Rx* and *Nx* resistance locuses, and the viral replicase that elicits resistance on *Nb* potatoes (Baulcombe *et al.*, 1995). However, for most identified and hypothetical plant virus resistance genes the viral proteins (and their domains) which function as elicitors remain to be identified.

Table 2. Examples of the viral elicitors of plant virus resistance genes.

Virus	Viral elicitor	Resistance gene	Plant	First report
TMV	CP	*N'*	*N. sylvestris*	Saito *et al.*, 1987
TMV	183 kD replicase	*Tm-1*	tomato	Meshi *et al.*, 1988
TMV	MP	*Tm-2*	tomato	Meshi *et al.*, 1989
PVX	CP	*Nx*, *Rx*	potato	Kavanagh *et al.*, 1992
TMV	183 kD replicase	*N*	tobacco	Padgett and Beachy, 1993
tomato mosaic tobamovirus	MP	*Tm-2²*	tomato	Weber *et al.*, 1993
PVX	replicase	*Nb*	potato	Baulcombe *et al.*, 1995

4.1.2. Hypersensitive response and nonhost resistance

HR is the only known active virus-resistance mechanism that is induced in plants after infection (Dawson and Hilf, 1992). It confines the virus to a small area surrounding the infection site. After HR induction, rapid accumulation of reactive oxygen intermediates occurs, which play a central role in the resulting hypersensitive cell death (Tenhaken *et al.*, 1995). To prevent the rapid breakdown of these active oxygen species, salicylic acid is believed to bind and thus inhibit the enzyme catalysing the degradation of H_2O_2 (Neuenschwander *et al.*, 1995). HR is specific and is induced only with certain plant-pathogen combinations, whereas very closely related plant cultivars or pathogen strains are unable to cause HR. At the same time resistance to viruses is the normal state since most plant species are resistant to most viruses. Complete resistance is probably due to the inability of virus to replicate in a given plant cell. In another case, the plant can be susceptible to the virus, but replication and/or movement along the plant is severely inhibited, resulting in mild or absent symptoms of the virus infection and no remarkable influence on normal plant life cycle. Actually, most viruses in the wild infect plants symptomlessly (Keese and Gibbs, 1993). Whether such general effects are also caused by specific plant resistance genes or, on the contrary, by the lack of plant genes essential for virus propagation, is unknown. However, it has been proposed that such "nonhost resistance" is also an active defense where several common resistance genes act against certain pathogens (Hadwiger and Culley, 1993). As such "passive" responses are dependent on not only different plant viruses attacking the same host, but also on the strain of the virus (sometimes being very

closely linked on the level of their genomic nucleotide sequence), it seems highly probable that some very specific regions of a plant virus genome can be responsible for very different reactions of the host plant to attack by a particular virus. However, if a symptom-inducing function was the primary role of one particular viral encoded protein or its domain, one might expect to find mutations in that gene that alter the symptoms without affecting virus titre (Daubert, 1988). Quite often, symptoms and titre are found to vary in parallel, suggesting that the symptomatology is not determined by a single viral gene. Virus host range appears to be determined by interactions between viral gene products and corresponding plant components, unlike bacteria and fungi, whose host range is determined by single virulence genes for every potent host (Dawson and Hilf, 1992). Interestingly, little correlation appears between the taxonomic relatedness of viruses and their host range. The systematic analysis of plant virus genes and genomes together with their host range and symptomatology in various host plants should shed some light on these questions.

4.1.3. Systemic acquired resistance

Ross (1962) was the first to describe a phenomenon of systemic acquired resistance (SAR), when noninoculated leaves were resistant to a secondary virus infection after the pre-inoculation of one leaf. SAR is unspecific, conferring resistance not only to other viruses but even to other types of pathogens (McIntyre *et al.*, 1981). SAR is expressed only against pathogens with localised infection, but not against pathogens able to infect the host sytemically. For example, potato Y potyvirus (PVY) necrotic strain does not induce SAR in tobacco in which it spreads systemically but yet causes necrosis in systemic leaves (Pennazio and Roggero, 1988). However, in some wild potatoes where the same strain causes necrotic local lesions but does not spread systemically, it causes also SAR (J. P. T. Valkonen, personal communication). This indicates that although SAR and HR are usually caused in parallel in local lesion hosts, HR is not inducing SAR *per se*. However, both types of host reaction to virus attack bear several similarities in their biochemistry. For instance, both responses cause the production of a set of new extracellular "pathogenesis-related" or PR proteins. It has been determined that the systemic signal for this induction is salicylic acid (Gaffney *et al.*, 1993). The resulting HR and subsequent cell death is at least in one step associated with proton influx. The expression of a bacterial proton pump in transgenic tobaccos activated a cell death pathway resembling HR and heightened resistance against TMV (Mittler *et al.*, 1995).

PR proteins are a heterogeneous group of proteins, which are not present in healthy leaves, but are induced by virus infections as well as by other pathogens and several chemicals. They were discovered in 1970 (Gianinazzi *et al.*, 1970; van Loon and van Kammen, 1970). Their production was shown to be

related to HR. PR-1a protein is expressed most actively in a ring of tissue around the necrotic lesion formed as a HR to virus infection (Antoniw and White, 1986). Transgenic plants expressing PR proteins constitutively do not exhibit enhanced protection against virus infection (Linthorst *et al.*, 1989; Cutt *et al.*, 1989). Constitutive PR proteins levels, however, were lower than found in the zone of cells around the local lesion (White and Antoniw, 1991). Interestingly, in transgenic plants where the ubiquitin system (reviewed by Hershko and Ciechanover, 1992) was perturbated, PR-1 expression was induced and TMV replication was inhibited (Becker *et al.*, 1993). This finding might lead to yet another approach to control viral diseases in transgenic plants.

4.2. Pathogen-derived resistance in transgenic plants

4.2.1. Coat protein-mediated resistance

The concept of pathogen-derived virus resistance was formulated in 1985 (Sanford and Johnston, 1985), and first realised a year later, when it was shown that transgenic plants expressing TMV CP gene exhibited either delayed symptom development or failed to develop any symptoms following challenge with TMV (Powell Abel *et al.*, 1986). Subsequently, it has been shown by many independent research groups that the expression of plant virus-derived nucleic acid sequences *in planta* confers resistance to the parental virus or, in some cases, also to closely related strains and viruses (reviewed by Wilson, 1993). The exploitation of virus CP gene for creating virus-resistant crops through genetic engineering has been the most widely used method of pathogen-derived resistance. As early as 1992, Howell and Zaitlin counted reports on virus coat protein-mediated protection (CPMP) against 14 different virus groups. Since then the list has expanded. This approach mimics the natural phenomenon of "cross-protection", first described over sixty years ago (McKinney, 1929). Cross-protection is based on the infection of the host plant with a mild strain of the virus, preventing subsequent superinfection by more severe strains of the same virus. The mechanisms responsible for cross-protection are still poorly understood, but generally the CP of the mild virus strain is thought to play a major role in it (Sherwood, 1987). Free CP may prevent the initial uncoating of the few particles of incoming virus, thus inhibiting their translation (Sherwood and Fulton, 1982; Wilson and Watkins, 1986). However, there are some reports suggesting that CP is not involved in conventional cross protection (Sarkar and Smitamana, 1981; Gerber and Sarkar, 1989).

Despite numerous reports of CPMP, the underlying mechanism that confers a resistant phenotype has not yet been elucidated. Moreover, recent reports suggest that the mechanism of CPMP possesses many details unique to nearly

every virus-plant system investigated to date. The first reports seemed to clearly show that functional CP was needed for the CPMP (Powell Abel *et al.*, 1990), where correlation existed between the levels of *in planta* expressed CP and the achieved protection. This rule has later, however, been broken. Examples obtained from CPMP against poty- (van der Vlugt *et al.*, 1992; Farinelli and Malnoë, 1993; Silva-Rosales *et al.*, 1994), luteo- (Kawchuk *et al.*, 1991), and tospoviruses (Pang *et al.*, 1992) have indicated that truncated or untranslatable CP genes can provide protection or even immunity against the parent virus. Moreover, in several cases plants accumulating low amounts of transgenic mRNA or protein were best protected against virus challenge (de Haan *et al.*, 1992; Pang *et al.*, 1992). A most intriguing and independent mechanism has been described for potyviruses, where low-level expression of CP was capable of inducing immunity in young, growing top leaves of the inoculated plant. This immunity, which could not be overcome by further virus challenge, was referred to as recovery from infection (Lindbo *et al.*, 1993). This phenomena resembles naturally occurring SAR in several aspects. A specific resistance state could also be achieved by expressing untranslatable potyviral mRNAs in transgenic plants (Lindbo and Dougherty, 1992a; 1992b). As transgenic mRNA levels (but not transcription rates) in these plants were reduced during virus infection, the protection mechanism is believed to be related with gene silencing or sense gene suppression phenomena. In both cases, attempts to overexpress transgenes resulted in a reduction of expression of both the transgene and a chromosomal homologue, when one existed. Gene silencing, first described for plants several years ago (Matzke *et al.*, 1989), is apparently a nuclear-based process during which transcription is down-regulated. Sense suppression (de Carvalho *et al.*, 1992) possibly results from post-transcriptional specific RNA degradation processes. In the case of RNA virus suppression, RNA should be the mediator of the silencing effect. RNA can dictate gene expression levels for example by RNA-directed gene methylation (Wassenegger *et al.*, 1994) or by small complementary RNAs, generated by host RNA-dependent RNA polymerase (Lindbo *et al.*, 1993). Recent findings indicate that both high-level resistance and recovery in plants expressing untranslatable tobacco etch potyvirus (TEV) CP RNA are mediated by the same cytoplasmic system in a dosage-dependent fashion (Goodwin *et al.*, 1996). Evidence is provided that cytoplasmic transgene RNA (and viral RNA) degradation is responsible for the achieved protection. The above research by W. Dougherty's group indicate that CPMP against potyviruses interferes with virus spread. Other reports have shown that early events in virus disassembly or later processes in virus replication cycle were inhibited by CPMP. Perhaps the step in the virus life cycle which is affected by CPMP varies from virus-host plant system to system (similarly to the mediator molecule of the protection). In cases where early steps of virus infection are altered, it is unlikely that recoating of virions by free CP synthesised from transgene is responsible for protection, as was first believed. For example, when a small amount of CP was removed from the 5' end of TMV RNA, CPMP was overcome (Register and Beachy, 1988). It is

probable that the uncoating of virions in CP(+) plants is blocked by occupation of CP-specific uncoating sites by endogenous CP (Register and Nelson, 1992). These sites, are, however, only hypothetical at present. But the fact that uncoating is a step which is inhibited in CP(+) plants has been proven experimentally (Osbourn *et al.*, 1989, 1990). For some virus groups, i.e. potexviruses (Hemenway *et al.*, 1988), carlaviruses (MacKenzie and Tremaine, 1990), and alfalfa mosaic virus (AlMV) (Tumer *et al.*, 1991), CPMP is not acting through uncoating since naked viral RNA cannot overcome the protection. It should also be noted that several different mechanisms might be responsible for protection in a single virus-host system. In TMV CP(+) plants, in addition to the interference with an early stage in infection, reduction of systemic movement of TMV in transgenic plants occurs (Wisniewski *et al.*, 1990).

CPMP is efficient with inoculum concentrations as high as 50 μg/ml (Cuozzo et al., 1988; Stark and Beachy, 1989; Tumer et al., 1987). Usually the protection level and homology between the transgene and the CP gene of challenging virus are in good correlation. However, again some contradictory results to this rule have been reported. Tobacco plants expressing tobacco vein mottling potyvirus (TVMV) CP showed resistance to TEV but not to TVMV (Shaw *et al.*, 1990).

4.2.2. Replicase-mediated resistance

Homology-dependent virus resistance has been reported in CP expressing plants as well as in transgenic crops expressing virus replicase constructs. The replicase was the first non-structural viral protein reported to mediate the pathogen-derived plant virus protection. The first successful replicase-mediated protection was reported using the TMV 54 kD gene (Golemboski *et al.*, 1990). The open reading frame encoding for the TMV 54 kD protein is located in-frame within the gene for the TMV 183 kD replicase. 54 kD protein is a putative component of the TMV replicase complex. Resistance mediated by the 54 kD protein is based on a different mechanism than that of CP-mediated resistance. The 54 kD protein is believed to compete with the viral replication complex and to, therefore, interfere with TMV replication. Resistance achieved using this approach is very effective, even with inoculum concentrations of 1 mg/ml (Carr and Zaitlin, 1991). It is also very specific, as 54K(+) protoplasts were resistant to challenge by the parent strain of TMV, but not by a closely related strain (Carr and Zaitlin, 1991). It seems that 54K protein was needed for the protection, although at very low levels (Carr *et al.*, 1992). More recent data strongly suggest that mutated or wild-type variants of replicase genes are capable of functioning as dominant negative mutants in transgenic plants and confer strong and specific virus immunity there. Such plants have been reported for tobra-, cucumo-, potex-, poty-, tombus-, and tobamoviruses (reviewed by Baulcombe, 1994). The very recent paper by D. Baulcombe's group (Mueller *et al.*, 1995) indicate that replicase RNA rather than the protein might be the active

mediator of the protection against PVX and that replicase-mediated protection against potexviruses might be caused by the gene sense suppression discussed above in relation to virus CP mRNA-mediated protection. A similar mechanism has been proposed for replicase-mediated resistance against tombusviruses (Rubino and Russo, 1995). Virus replicase mRNA-mediated protection was also shown by Sijen *et al.* (1995), who demonstrated that cowpea mosaic comovirus replicase gene RNA is responsible for strand-specific inhibition of comovirus replication. Support for the hypothesis that replicase RNA is inhibiting virus replication comes from the work on brome mosaic bromovirus (BMV) and tobacco protoplasts, where the expression of full-length genomic RNAs of BMV suppressed virus replication, whereas BMV RNA1 and RNA2 in which 3' nucleotides required for BMV RNA replication were deleted did not suppressed BMV infection (Kaido *et al.*, 1995). To make the picture even more complicated, Tenllado *et al.* (1995) reported a recovery phenomenon for *N. benthamiana* plants expressing the 54 kD protein of pepper mild mottle tobamovirus, which closely resembles the phenotype described by Lindbo *et al.* (1993) for TEV CP expressing plants. However, in contrast to previous "recovery" reports, this time the resistance seemed to be protein mediated. Resistance mediated by a truncated replicase protein which is not related to cosuppression has also gained support from the work on AlMV replicase expressing plants (Brederode *et al.*, 1995). Hellwald and Palukaitis (1995) have suggested that in the case of truncated cucumber mosaic cucumovirus (CMV) replicase expressing tobaccos, two different mechanisms are responsible for the achieved protection. One mechanism is believed to suppress viral replication. In addition, a newly proposed mechanism against viral movement was described. It's target is also viral RNA, but it does not cause RNA degradation (as occurs in the gene suppression pathway).

4.2.3. Other pathogen-derived resistance approaches

Plant virus MP is also capable of inducing resistance against the parent virus. White clover mosaic potexvirus (WClMV) 13 kD MP conferred protection against systemic infection by WClMV in *N. benthamiana* plants at inoculum concentrations of 250 μg/ml (Beck *et al.*, 1994). The characteristic features of MP-mediated protection seem also to vary from virus group to group. TMV dysfunctional MP has been reported to retard the local and systemic spread of tobamoviruses (Lapidot *et al.*, 1993; Malyshenko *et al.*, 1993), whereas a wild-type MP increased the susceptibility of plants to TMV infection (Cooper *et al.*, 1995). For comoviruses, wild-type MP RNA (but not the protein) has been demonstrated to mediate the protection (Sijen *et al.*, 1994; 1995).

Protection against viral infection is achieved by expressing *in planta* potato leafroll luteovirus (PLRV) 17 kD protein, which is the putative precursor of viral VPg (W. Rohde, cited by Wilson, 1993). Potyvirus protection has been

established in transgenic plants after transformation with two different potyviral protease genes. NIa protease has been shown to confer virus resistance in tobaccos (Maiti *et al.*, 1993; Vardi *et al.*, 1993) and P1 protein in potatoes (Pehu *et al.*, 1995). Whether these types of protection are protein- or RNA-mediated, is not yet known.

Synthesis of antisense RNA for viral transcripts in plants could inhibit virus propagation by annealing with viral RNA and thus preventing its expression, similar to the widely used antisense inhibition strategy for endogenous genes. The first transgenic plants expressing antisense RNA of virus CP genes (Cuozzo et al., 1988; Hemenway et al., 1988; Powell Abel et al., 1989) or part of the replicase gene with its leader sequence (Rezaian et al., 1988) have shown protection against virus infection, but only at low inoculum concentrations [max. 5 μg/ml (Rezaian et al., 1988)]. Here the exceptionally good protections originate from the same plant virus groups where RNA seems to also mediate virus protection triggered by CP sense constructs. For example, sense and antisense RNA constructs of the CP gene of PLRV worked equally well in protecting potato plants against PLRV (Kawchuk *et al.*, 1991). Antisense TEV CP construct conferred weak but broad-scale potyviral protection similar to translatable TEV CP gene (Lindbo and Dougherty, 1992a; 1992b). Recently, very strong protection to infection by 100 μg/ml inoculum has been reported for antisense bean yellow mosaic potyvirus CP RNA expressing plants (Hammond and Kamo, 1995).

Generally, it seems that any virus-derived sequence is potentially capable of inducing a virus tolerant state in transgenic plants. This is also true for virus noncoding sequences, both in sense (Zaccomer *et al.*, 1993) and antisense (Nelson *et al.*, 1993; Langenberg *et al.*, 1994) orientations. The only exception seems to be functional viral MP. As one of its most significant functions in cells is to increase the size exclusion limits of plasmodesmata (Lucas and Gilbertson, 1994), the expression of functional MPs from a transgene tends in some cases to enlarge plasmodesmata constitutively (Poirson *et al.*, 1993; Vaquero *et al.*, 1994) and thus increase the susceptibility of plants to virus infection (Cooper *et al.*, 1995). However, as discussed above, several examples exist where wild-type MPs cause virus protection in transgenic plants.

4.2.4. Virus satellites and defective interfering RNAs

Virus satellite RNAs are small RNA molecules that require a helper virus to replicate in the host plant and are encapsidated in the coat protein of the helper virus (Francki, 1985). The presence of some of satellite RNAs attenuates the production of helper virus infection symptoms (Baulcombe, 1989). Resistance to the helper virus [and to a related virus (Harrison et al., 1987)] has been induced by the introduction of a cDNA copy of satellite RNA into the host plant (Gerlach et al., 1987; Harrison et al., 1987; Jacquemond *et al.*, 1988). Toler-

ance in transgenic plants expressing satellite RNA was independent both of inoculum concentration and of the level of satellite RNA gene transcription, because the production of satellite RNA is switched on by the presence of the attacking virus. This feature overcomes a problem with the CP strategy, which in most cases requires constitutive expression of CP at high levels for effective protection. But this approach takes the risk that the symptom-reducing strain is converted into a hypervirulent satellite RNA, for which only a few base changes in the satellite RNA are required (Jacquemond and Lauquin, 1988).

Defective interfering (DI) RNA molecules are not very common among plant RNA viruses, occurring normally only in tombus- and carmovirus groups (Roux *et al.*, 1991). It as been shown that similar to satRNA protection, DI RNA can be used for transgenic protection in plants (Kollàr *et al.*, 1993). The resistance achieved is specific to the virus from which the DI RNA was derived. This circumstance limits the wider use of this approach since only few plant viruses possess naturally occurring DI RNAs.

4.3. Plant antibodies

Hiatt and colleagues (1989) demonstrated that plant cells are able to chaperone and assemble functional antibodies. Since then attempts have been made to provide protection against viral diseases by expressing appropriate IgGs or single-chain antibodies in plants. Tavladoraki and co-authors (1993) showed that the expression of single-chain antibody reduced the infection of artichoke mottled crinkle tombusvirus. Fecker *et al.* (1995) gave support to the possible successful exploitation of this approach in virus control with an example on beet necrotic yellow vein furovirus. Voss *et al.* (1995) managed to express *in planta* a TMV-specific full-size antibody, which protected plants against TMV. Antibody-mediated resistance is an attractive alternative to pathogen-derived resistance as it circumvents biosafety problems arising from phenomena such as transencapsidation or recombination of transgene transcripts with viruses (Tepfer, 1993). Antibodies could be used successfully only against closely related plant viruses, sharing common epitopes for the antibodies used.

4.4. Broad-spectrum virus tolerant transgenic plants

4.4.1. Broad-spectrum pathogen-derived virus resistance

Pathogen-derived resistance in transgenic plants has in many cases proved to be very efficient. Still, the expression of wild-type or mutated viral cDNAs *in planta* typically confers protection only against the particular virus itself or to closely related viruses of the same group. Clearly, the same occurs with antibody-mediated protection. However, there are some examples of pathogen-

derived protection in transgenic plants against heterologous infections of viruses from different systematic groups. Tobaccos expressing BMV 32 kD MP were reported to significantly reduce the accumulation of unrelated tobamovirus TMV strain U1 in addition to BMV itself (Malyshenko *et al.*, 1993). Mutated WClMV MP expression protected plants also against potato S carlavirus (PVS). However, transport proteins of BMV and TMV can functionally substitute for each other (De Jong and Ahlquist, 1992) and movement functions of potex- and carlaviruses are encoded by structurally closely related triple gene blocks (Rupasov *et al.*, 1989). It remains to be seen how general the protection mechanism reported for BMV and WClMV MPs is. Recently, very broad range virus protection was reported in tobaccos expressing a dysfunctional MP of TMV (Cooper *et al.*, 1995). These plants were, besides tobamoviruses, also protected against tobra-, nepo-, ilar-, and cucumoviruses and even against peanut chlorotic streak caulimovirus, which is a plant retrovirus with a DNA genome. These virus groups bear very different MPs, suggesting that protection was not achieved due to the similarity of MPs of challenge viruses. Infection by the same viruses was enhanced in plants expressing the wild-type MP of TMV (Cooper *et al.*, 1995), which contradicts earlier data with wild-type BMV MP (Malyshenko *et al.*, 1993). Support for the protection data with TMV MP comes from results with PLRV modified MP, which when expressed in potato protected also against PVX and PVY (Rohde, 1996). Anderson *et al.* (1989) have reported a broad-spectrum coat protein-mediated protection against low concentrations of systematically distinct viruses. Namely, TMV CP+ tobaccos exhibited a delay in symptom development when inoculated with PVX, PVY, CMV, and AlMV. Similarly, AlMV CP+ tobaccos showed a delay in the development of symptoms of PVX and CMV infections (Anderson *et al.*, 1989). The reason for this delay is not known, especially as several other authors have not been able to observe coat-protein-mediated protection against viruses from different groups (Hanley-Bowdoin and Hemenway, 1992). In general, MP-mediated resistance seems currently to be the only known pathogen-derived plant virus resistance approach, which can be promising also for the construction of broad-spectrum virus tolerant plants.

4.4.2. Other approaches for the broad-spectrum virus resistance

Broad spectrum virus resistance in plants expressing pokeweed (*Phytolacca americana*) antiviral protein (PAP) was reported (Lodge *et al.*, 1993). Remarkable protection against PVX, PVY and CMV (viruses belonging to three different virus groups) was achieved in transgenic *N. tabacum* plants, whereas PVX resistance was also detected in PAP expressing potatoes and PVY resistance in potatoes and *N. benthamiana* plants. PAP is a plant ribosome-inhibiting protein, which is capable of inhibiting the infection of both plant and animal viruses (Tomlinson *et al.*, 1974). The mechanism of PAP-mediated resistance in transgenic plants is presently not understood (Lodge *et al.*, 1993), Resistance to

viruses belonging to four taxonomic groups in tobaccos and potatoes has been reported for plants expressing another ribosome-inactivating protein from the related *Phytolacca* species (Moon *et al.*, 1994). However, the potential of this approach for crop improvement is restricted by the fact that PAP is capable of inhibiting the functions of ribosomes in higher mammals, *i.e.* to act as a mammalian protein synthesis inhibitor. It should be noted that phytotoxic proteins have been used also as specific suicide genes. When antisense RNA for such a protein is expressed constitutively in plants as a 3'-terminal fusion with a minus-sense plant viral subgenomic RNA promoter, infection by the cognate virus will lead to the transcription of nonsense RNA into mRNA, thus killing the cell. It has been reported for PVX and diphtheria toxin mRNA (J. G. Atabekov, referred from Wilson, 1993).

A promising approach for engineered broad spectrum virus tolerance has recently been reported by Masuta *et al.* (1995). They expressed antisense RNA of *S*-adenosylhomocysteine hydrolase cDNA in tobacco, which is an important enzyme in 5' capping of mRNA during viral replication. Indeed, transgenic tobaccos showed decreased levels of virus replication and symptom development when plants were challenged with CMV, TMV, or PVX. Interestingly, these plants displayed protection also against PVY, which does not contain a cap structure. As these plants showed also increased levels of cytokinins, which are known to trigger the induction of SAR to viruses, the protection effect could be indirect, not mediated by the undermethylation of the viral cap structure. The fact that elevated levels of cytokinins in transgenic plants can increase resistance to viruses was also demonstrated by Sano *et al.* (1994). They expressed a small Ras-related GTP-binding protein Rgp-1 in tobacco, which induced cytokinin levels in plants and increased protection against TMV.

In conclusion, much more work is needed to evaluate the potency of the above referred approaches for the construction of transgenic crop plants with broad spectrum virus tolerance. It seems to me that, although they provide protection against different viruses under the greenhouse conditions, the wider usage of toxins and ribosome-inactivating proteins for the contruction of virus-tolerant crop plants is improbable because of the harmful properties of these proteins to human and cattle.

As mentioned above, most viruses are not pathogens for most plant species. This might indicate that powerful, yet unidentified universal natural virus resistance genes are expressed in plants. Undoubtedly, the overexpression of these genes would be the most "natural" way to protect crop plants from virus diseases. From mammals such universal virus resistance genes have been identified as the components of the 2-5A pathway. As controversial reports for the presence of the plant homologs of the same pathway were available from the literature, we hypothesised that these putative homologs could function as plant universal natural virus resistance pathway components.

A summary of different approaches successfully taken to generate virus tolerant transgenic plants is given in Table 3.

Table 3. Successfully taken approaches to induce virus tolerant state in transgenic plants.

Nature of the cDNA conferring virus tolerant state in transgenic plants	First report
1. Pathogen-derived	
Virus coat protein	Powell Abel *et al.*, 1986
Virus antisense RNA	Cuozzo *et al.*, 1988 Hemenway *et al.*, 1988
Virus replicase	Golemboski *et al.*, 1990
Virus movement protein	Lapidot *et al.*, 1993
Virus protease	Maiti *et al.*, 1993
Virus noncoding sequences	Zaccomer *et al.*, 1993
Virus satellite RNA	Gerlach *et al.*, 1987 Harrison *et al.*, 1987
Virus defective interfering RNA	Kollàr *et al.*, 1993
2. Antibodies against the virus	Tavladoraki *et al.*, 1993
3. Ribosome-inactivating proteins	Lodge *et al.*, 1993
4. Mammalian 2-5A system components	Truve *et al*, 1993
5. Antisense RNA to ubiquitin system	Becker *et al.*, 1993
6. Ras-related GTP-binding proteins	Sano *et al.*, 1994
7. Enzyme involved in RNA capping	Masuta *et al.*, 1995
8. Bacterial proton pump	Mittler *et al.*, 1995

4.5. 2-5A system

4.5.1. 2-5A system in mammals

Three types of enzymes are involved in this animal antiviral pathway (Fig. 1):
a) the 2-5A synthetases which synthesize 2-5A;
b) the 2' phosphodiesterase which degrades 2-5A;
c) the 2-5A-dependent ribonuclease or RNase L.

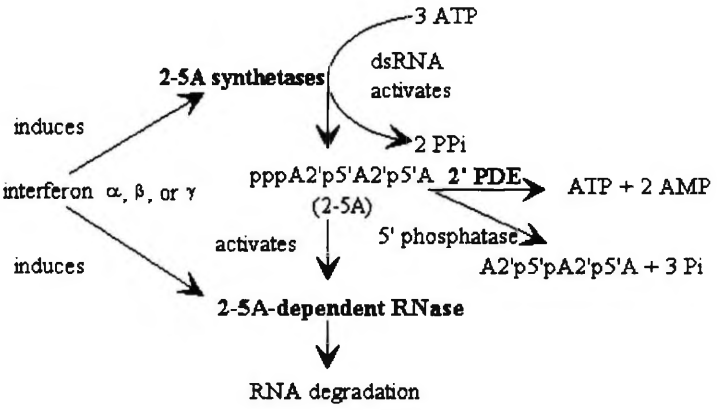

Fig. 1. Schematic representation of the 2-5A pathway.

Constitutive levels of all of these enzymes are present in most mammalian cells and tissues. The level of 2-5A synthetase activity, however, increases substantially in response to interferons (IFNs). 2-5A synthetase is activated by the double-stranded replicative intermediates of viral RNA (dsRNAs) (Hovanessian, 1991). This activation leads to the synthesis of a family of oligoadenylates with unusual 2'–5' phosphodiester bonds, abbreviated 2-5A (Fig. 2). Virus replication is inhibited due to the rapid degradation of viral RNA by the specific 2-5A-activated ribonuclease — RNase L (Zhou *et al.*, 1993). RNase L is a constitutively present enzyme in most mammalian tissues (although at extremely low levels). But usually it is latent, and only the binding of 2-5A is capable to activate RNase L. As activated RNase L degrades both viral and cytoplasmic mRNA, the consequence of 2-5A action is an inhibition of protein synthesis (Kerr and Brown, 1978). Activation of the 2-5A system is transient. This is due to the degradation of 2-5A by 2' phosphodiesterase (Schmidt *et al.*, 1979). Although in mammalian cells the 2-5A pathway inhibits picornavirus infections preferentially (Samuel, 1991), theoretically the multiplication of all RNA viruses could be inhibited *via* this pathway. As a majority of plant viruses possess genomes based on RNA, such a possibility might have great impact on genetic engineering for virus resistant transgenic plants.

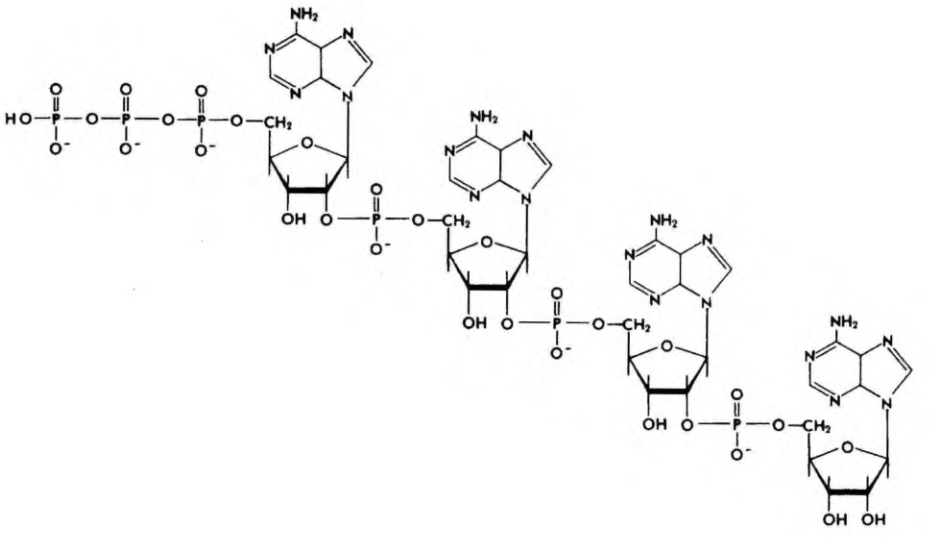

Fig. 2. Structure of the triphosphorylated form of 2'-5' oligoadenyate tetramer.

2-5A pathway is not the only IFN-activated dsRNA-dependent antiviral response system in mammals. IFN is also able to induce a dsRNA-dependent protein kinase, designated PKR, in most animal cells. Human 68 kD PKR , in the presence of dsRNA, autophosphorylates and phosphorylates the eukaryotic translation initiation factor eIF-2, thereby throttling protein synthesis (Hovanessian, 1989). In addition, PKR is capable to phosphorylate IκB, thus activating NF-κB, which leads to the activation of transcription of several genes, including IFN-β (Kumar *et al.*, 1994). PKR has also been cloned (Meurs *et al.*, 1990).

At least three major forms of 2-5A synthetase have been reported in mammalian cells: 40–46 kD, 69 kD, and 100 kD (Hovanessian, 1991). The cDNA sequences encoding the small form of the 2-5A synthetase have been identified from human (Benech *et al.*, 1985; Saunders *et al.*, 1985; Shiojiri *et al.*, 1986; Wathelet *et al.*, 1986) and mouse (Ghosh *et al.*, 1991; Ichii *et al.*, 1986; Rutherford *et al.*, 1991) libraries. In humans, from a single gene mapped to chromosome 12 (Williams *et al.*, 1986), two mRNA species with approximate sizes of 1.6 and 1.8 kb are derived by differential splicing (Benech *et al.*, 1985; Saunders *et al.*, 1985). These mRNAs encode the 40 kD and 46 kD forms of the 2-5A synthetase, respectively. Similar mRNAs also have been described from mouse (Ghosh *et al.*, 1991). Corresponding human and mouse 2-5A synthetase cDNAs are very highly conserved except that the larger mRNAs are spliced in a different manner (Ghosh *et al.*, 1991). In addition, a separate genetic locus in mouse containing two small-type 2-5A synthetase genes has been reported (Cohen *et al.*, 1988). cDNA for the 69 kD 2-5A synthetase has also been cloned

and reported to possess two similar domains both sharing strong homology to the small 2-5A synthetase isoform (Marié and Hovanessian, 1992). The authors speculate that the functioning of 2-5A synthetase activity might require the presence of four catalytic domains which can be provided by the tetramer of 40–46 kD synthetase, dimer of 69 kD synthetase, or the monomer of 100 kD. Indeed, it has been demonstrated that these isoforms of 2-5A synthetase tend to exist in cells as tetramers, dimers, and monomers, respectively (Marié *et al.*, 1990). The final proof of this speculation would come from the cloning of the cDNA for 100 kD 2-5A synthetase. A recent report on the cloning of the partial cDNA for 100 kD 2-5A synthetase (Rebouillat and Hovanessian, 1995) seems to support this idea. Despite of their strong homology, different isoforms of 2-5A synthetase might be involved in different cellular functions. This is proposed because different forms of 2-5A synthetase have different subcellular locations, different IFN dose-responses, and different post-translational modifications (Hovanessian, 1991).

4.5.2. 2-5A system in other animals and lower organisms

The presence of the 2-5A system in birds was confirmed long ago (Stark *et al.*, 1979). At least some components of the pathway have been detected in reptilia and amphibia (Cayley *et al.*, 1982), in fish (Sokawa *et al.*, 1990), insects (Laurence *et al.*, 1984), sponges (Kuusksalu *et al.*, 1995), yeast and even bacteria (Laurence *et al.*, 1984; Trujillo *et al.*, 1987).

4.5.3. 2-5A system components in plants

The existence of 2-5A pathway components in higher plants has remained unclear. dsRNA-dependent ATP polymerising activity in *Nicotiana glutinosa* and *N. tabacum* leaves after treatment with a so-called "antiviral factor" (AVF, Sela, 1981) or after TMV infection (Devash *et al.*, 1981; Reichman *et al.*, 1983) has been reported. Later, the ability of plant extracts to synthesise *in vitro* oligoadenylate-like compounds and their antiviral activity was shown (Devash *et al.*, 1985, 1986a; Sher *et al.*, 1990; Babosha *et al.*, 1990). Also, inhibition of TMV multiplication by chemically synthesised dephosphorylated 2-5A has been demonstrated (Devash *et al.*, 1982, 1984, 1986b). A probe of human 2-5A synthetase gene was reported to hybridise with tobacco genomic DNA and to mRNA of TMV-infected tobacco (Sela *et al.*, 1987). However, the cloning and sequencing of this hypothetical tobacco gene has never been reported. Kulaeva *et al.* (1992) demonstrated that 2-5A (as well as human IFN) has the ability to increase the cytokinin content and induce the synthesis of various proteins in plant cells.

Data obtained mainly by I. Sela's group do not answer the question how putative plant oligoadenylates affect the cell response to virus infection. Al-

though nuclease activity in AVF treated cells was first reported (Devash *et al.*, 1981), later it was declared that plant 2-5A do not activate a 2-5A-dependent endoribonuclease. It was proposed that plant oligoadenylates themselves, and the plant 2-5A pathway probably substantially differ from their mammalian counterparts (Devash *et al.*, 1985). Independently, another group failed to detect any 2-5A-binding protein in tobacco (Cayley *et al.*, 1982).

The possible inducers of putative plant oligoadenylate synthetase are also unidentified. Similarities between plant AVF and chick IFN were noted more than 30 years ago (Fantes and O'Neill, 1964). But as AVF has not been cloned and molecularly characterised, these similarities are only hypothetical. Two glycoproteins (gp22 and gp35), which production is stimulated by virus infection, were purified from plants and found to cross-react with human β-IFN polyclonal antibodies (Edelbaum *et al.*, 1990). Later, however, they were identified as an isoform of the PR protein 5 and β-1,3-glucanase (Edelbaum *et al.*, 1991). Contradictory data is available about the influence of exogenous IFN on plant virus propagation. Treatment of tobacco leaf discs (Orchansky *et al.*, 1982; Reichman *et al.*, 1983; Kaplan *et al.*, 1988), protoplasts (Rosenberg *et al.*, 1985, Sela, 1986), callus culture (Reichman *et al.*, 1983) or intact plants (Quanyi *et al.*, 1989) with human IFN-α was shown to inhibit TMV replication. Replication of potato viruses X, Y, and F in tobacco plants and potato virus M in potato leaf discs was also inhibited by human IFN-α (Kaplan *et al.*, 1988). Vicente *et al.* (1987) reported the inhibitory effect of human γ-IFN on TMV in *Datura stramonium* and on PVX in *Gomphrena globosa.* Later the same group showed also that human amniotic IFN had an inhibitory effect on TMV in *D. stramonium* and *N. glutinosa*, on PVX in *G. globosa*, and on tomato spotted wilt tospovirus (TSWV) in tobacco (Vicente *et al.*, 1988). However, Antoniw *et al.* (1984) could not detect any effect of human IFN-α on the multiplication of TMV in tobacco. Human IFN-α and IFN-β did not protect cowpea protoplasts against AlMV (Huisman *et al.*, 1985) or human IFN-α tobacco leaf discs and alfalfa protoplasts against the same virus (Loesch-Fries *et al.*, 1985). *In planta* produced IFN-α was according to De Zoeten *et al.* (1989) unable to hamper the infection of turnip yellow mosaic virus. Again, in contradiction, Smirnov *et al.* (1991) reported that transgenic tobacco plants expressing IFN-α were protected against TMV. IFN-β, expressed in tobaccos, could not establish protection against TMV (Edelbaum *et al.*, 1992).

In conclusion, the presence of antiviral compounds and enzymes in plants resembling those of the mammalian 2-5A pathway has remained unclear. The information which was available from the literature provided contradictory data for the presence and/or activity of 2-5A synthetase and 2'-5' oligoadenylates in plants. The attempts to detect 2-5A-binding proteins from plants, however, failed in two independent experiments. As far as I am aware, the third key enzymatic activity for the entire 2-5A system — 2' phosphodiesterase, has not been analysed from plants.

4.5.4. Possible functions of the 2-5A pathway in different organisms

In mammals, it has been clearly shown that the very important and maybe dominant function of the 2-5A pathway is to mediate resistance to virus infection (Hovanessian, 1991). However, accumulating data indicate that the same pathway is involved in several other cellular processes. There has been much speculation about its role in the control of cell growth (Stark *et al.*, 1979), proliferation (Wells and Mallucci, 1985), and differentiation (Krause *et al.*, 1985). For instance, 2-5A synthetase activity is remarkably increased in the end of the S phase of the cell cycle (Wells and Mallucci, 1985) and the cells where 2-5A synthetase is blocked by the corresponding antisense RNA are growing much more rapidly than the parental cell line (De Benedetti *et al.*, 1985). The reason for the ability of 2-5A to arrest cell growth can be at least partially due to its inhibitory effect on DNA topoisomerase I (Castora *et al.*, 1991), known to be important for normal cell growth. 2-5A synthetase activity is increased on induction of neuronal (Saarma *et al.*, 1986) and hematopoietic differentiation (Ferbus *et al.*, 1985). Furthermore, 2-5A synthetase has been reported to be an immunomodulator (Mayumi *et al.*, 1989) and to be involved in pre-mRNA splicing (Sperling *et al.*, 1991). These findings together with the fact that 2-5A synthetase exists in multiple forms with different intracellular locations (Hovanessian *et al.*, 1987) could indicate diverse functions for the 2-5A system in mammals. What the functions of the 2-5A pathway are in lower vertebrates and other organisms and whether these functions are similar to those in mammals, is largely unknown. Keeping in mind that some components of the pathway have been reported from bacteria and from such distinct eukaryotes as yeasts, higher plants, and mammals, we have a working hypothesis that 2-5A pathway is an ancient system that responds to stress factors. Support for this idea came from the recent finding of the very active 2-5A production in lowest multicellular animals — sponges (Kuusksalu *et al.*, 1995). According to this hypothesis the original pathway evolved later to several independent ones, which are now characterised for mammals. Strong evidence that the response to viral attack was perhaps one of the most ancient functions of 2-5A pathway was given by Trujillo *et al.* (1985). They were able to demonstrate that infection with bacteriophages M13 and λ brought about significant increase in levels of 2'-5' oligoadenylates in *E. coli*. In relation to these data one could assume that in all organisms possessing entire or partial 2-5A pathway, one of its main properties should be the inhibition of virus infection. Previous data on the 2-5A inhibitory effect on plant virus propagation fit well with this idea. Thus, 2-5A system could be the first example of a common virus resistance mechanism for plant and animal kingdoms (and maybe even for prokaryotes).

Interestingly, some data that support the presence in plants of an homolog to another dsRNA-dependent mammalian antiviral enzyme — PKR, have also been obtained. Namely, a 68 kD protein has been identified that is phosphorylated in extracts from TMV-infected (Jessen Crum *et al.*, 1988) or potato spindle tuber viroid-infected (Hiddinga *et al.*, 1988) tissues. It contains an ATP

binding site and is immunologically related to human PKR. The phosphorylation of this putative plant kinase was enhanced by the addition of dsRNA (Jessen Crum *et al.*, 1988). The more complete molecular characterisation of this protein is not available, but it might indicate that higher plants and animals possess universal antiviral pathways with similar properties.

5. AIMS OF THE PRESENT STUDY

The main purpose of the current study was to construct transgenic plants tolerating infection by several different plant RNA viruses. As shown already in the literature review, several different approaches to achieve virus resistant state in transgenic plants are currently available. Unfortunately, most of them provide very specific resistance to one virus (or some closely related viruses). Several years ago, when this project was initiated, it was not yet so definite. Therefore, we first wanted to obtain plants expressing the coat protein gene of PVX, and test the capabilty of these plants to suppress infections of different plant viruses. Soon it became evident that this approach could only lead to the specific virus resistant state. Therefore, our second goal was to construct plants bearing gene(s) of a more general antiviral pathway. As no such pathways has been found from plant kingdom, and no reports on whatever type of broad spectrum virus resistance in transgenic plants were available, we decided to take advantage of the reasonably well-characterised universal antiviral 2-5A pathway from mammals. Furthermore, as several groups have since reported components similar to the essential parts of this pathway were also present in organisms besides mammals, incl. higher plants. The more specific aims of this part of the study were:

a) to check the reproducibility of the previously reported *in vitro* data on the presence of some 2-5A pathway components in plants;
b) to clone into plants the components missing from this type of organisms to reconstruct a more complete 2-5A pathway *in planta*;
c) to test the susceptibility of these plant to different RNA viruses.

Finally we wanted to test the susceptibility of the obtained transgenic plants under field conditions. The reasons for that were the following:

a) we wanted to analyse virus protection levels with virus challenge closer to natural infection pressure (*versus* very high inoculation pressure used in greenhouse studies);
b) we wanted to evaluate the phenotype of commercial 2-5A(+) transgenic potato cultivar, tuber yield and general properties in the field;
c) we wanted to compare the efficiency of coat protein-mediated protection *versus* 2-5A synthetase-mediated protection in a commercial crop cultivar under field conditions.

Taken together, the aims of the present study can be figured out as the following:

1. **Transform tobacco plants with the coat protein cDNA of potato virus X. Analyse the replication of different potato virus X isolates in the potato virus X coat protein expressing plants and protoplasts.**
2. **Analyse the effects of 2-5A on *in vitro* and *in vivo* protein synthesis in plant systems. Identify the presence of 2-5A-binding proteins in plant extracts.**
3. **Isolate a cDNA for rat 40 kD 2-5A synthetase and determine its primary structure.**
4. **Transform tobacco and potato plants with rat 2-5A synthetase cDNA. Analyse the plants expressing 2-5A synthetase with respect to multiplication of different plant RNA viruses.**
5. **Carry out field tests with 2-5A synthetase expressing potato plants. Analyse the potato virus X content in field grown transgenic potato leaves and tubers.**

6. MATERIALS AND METHODS

Nicotiana tabacum cv. Petite Havana SR1 seeds were originally kindly provided by Dr. Teemu H. Teeri and later propagated by ourselves. *Solanum tuberosum* cv. Pito was obtained from the Seed Potato Centre, Tyrnävä, Finland. Sources of viruses, cDNA clones and library, and antibodies are indicated in the original publications.

All molecular biology procedures were performed according to standard practices (Sambrook *et al.*, 1989). Most of the methods used are described in detail in the publications of this thesis. The methods include:

Agrobacterium-mediated plant transformation (I, IV, V, VI).
Analysis of amino acid incorporation into protein (II, V).
Analysis of potato tuber yields, tuber sprouting (IV).
Chemical and UV-crosslinking of proteins to oligonucleotides (II, V).
Computer analysis, statistics (I, III, IV, V).
Dephosphorylation, labelling, HPLC-purification of oligonucleotides (II, V).
DNA recombination between *E. coli* and *Agrobacterium* (I, IV, V, VI).
DNA sequencing (I, III, IV, V).
ELISA and time-resolved fluoroimmunoassay for virus detection (I, IV, V, VI).
Field tests with transgenic potato plants (IV).
Immunoprecipitation (VI).
In vitro translation, coupled transcription/translation (II, V, VI).
Isolation of cDNA clones by library screening with DNA probes (III, IV, V).
Laser densitometer scanning (II, V).
Plasmid transformation, isolation, restriction analysis, subcloning (I, III, IV, V, VI).
Polyacrylamide gel electrophoresis, Western analysis (I, II, VI).
Polymerase chain reaction (I, VI).
Preparation of extracts from murine cell cultures and organs (II, V).
Preparation and virus infection of tobacco protoplasts (I, II, V).
Protein expression and purification in *E. coli*, production of antibodies in rabbits (VI).
Regeneration of plants from leaf discs or stem cuttings (I, IV, V, VI).
Southern analysis, Northern analysis, autoradiography (I, II, IV, V, VI).
Thin layer chromatography on silica gel plates (IV).
Total bacterial DNA isolation (I, IV, V, VI).
Total plant DNA, RNA, and protein extraction (I, IV, V, VI).
Virus inoculation of intact plants and leaf discs (I, IV, V, VI).

All nucleic acid sequences determined by us and reported in this thesis are available from EMBL databank (under acc. nos. Z18877, Z29333, Z29334, and Z29335).

7. RESULTS AND DISCUSSION

7.1. Construction of PVX tolerant tobacco plants expressing PVX coat protein (I)

As a first step towards engineering plants to tolerate virus infections, we constructed several transgenic potato lines expressing PVX CP gene in the sense or antisense orientation. These plants, constructed by Dr. Andres Priimägi and me six-seven years ago, were the first transgenic plants in Estonia and perhaps also in the Baltics. The plants obtained from regenerated tobacco leaf discs after cocultivation with recombinant *Agrobacteria* were kanamycin-resistant, indicating that the hybrid nopalin synthetase-neomycin phosphotransferase II gene (included into the T-DNA segment of our plant transformation vector pHTT202) was integrated into the plant genome. Southern and Northern analysis of total DNA and RNA, respectively, isolated from the leaves of transgenic lines revealed that PVX CP cDNA was present in the genomic DNA of transformed tobaccos and was transcribed at levels detectable in Northern blottings (I, Fig. 1). Transgenic lines expressing the PVX CP sense construct, which were later subjected to infection tests, also synthesised detectable amounts of virus coat protein (I, Fig. 2). From Western blottings, we have calculated that the amount of the coat protein in transgenic plants is around 0.02–0.1% of total soluble protein of leaf mesophyll. Thus, we believe that resistance we were able to demonstrate in subsequent infection tests was protein-mediated. However, we did not include in the experiments tobaccos expressing the untranslatable sense RNA of PVX CP. As an RNA-mediated protection control only plant lines expressing the antisense RNA of PVX CP were used.

Tobacco lines 2S4 and 2S5 were used for challenge inoculation with seven different PVX isolates. Virus amplification of all seven isolates was inhibited at least one month postinoculation (I, Fig. 3 and 4). However, there were significant differences in the levels of protection. Propagation of PVX_{KD} and PVX_{KD4} was completely inhibited throughout the experiment. Later we have shown that detectable amounts of PVX_{KD} and PVX_{KD4} appear in the end of the second month postinoculation, which is accompanied by the appearance of disease symptoms. However, even then the concentration of these isolates remains much lower than other PVX isolates (Raudsepp, Järvekülg, and Truve, unpublished). The most surprising fact was that the propagation of PVX_S, from which the transgenic CP originated, was much more efficient in CP(+) lines than PVX_{KD} and PVX_{KD4}. PVX_S concentrations in transgenic lines were comparable to those of isolates HB and cp2. The weakest protection in CP(+) lines was observed against PVX_{S6111} and PVX_{P551}. Detectable propagation of these isolates in transgenic lines started appr. a week earlier than that of other isolates tested.

Transgenic lines expressing the antisense RNA of PVX CP did not exhibit protection against any of the isolates tested at inoculum concentrations of 10 µg of virions per millilitre.

The fact that CPMP was not the most efficient against the virus strain which provided the transgene is contrary to most data reported in the literature. I am not aware of similar results except the even more striking data reported by Shaw and co-authors, who showed that TVMV CP(+) tobaccos were not protected against TVMV, but were resistant to another potyvirus — TEV (Shaw *et al.*, 1990).

To be able to evaluate, whether the protection against other PVX isolates in the study somehow correlated with their CP sequence similarity to that of PVX_S CP, we first had to sequence the CP cDNAs of three PVX isolates [CP gene sequences for PVX_S, PVX_{HB}, PVX_{KD}, and PVX_{KD4} were available from other studies (Skryabin *et al.*, 1988; Kavanagh *et al.*, 1992; Santa Cruz and Baulcombe, 1993)]. When the CP sequences for all seven isolates were aligned (I, Fig. 5 and 6), we were unable, despite some amino acid mutations (discussed in I) find any remarkable differences. We were unable to correlate these changes to the different protection levels in transgenic PVX_S CP(+) plants. This result, again, contradicts most reports from similar experiments on other plant viruses. In a classical paper by Nejidat and Beachy (1990), the authors conclude that tobamoviruses sharing ≥60% homology in the CP amino acid sequence with TMV, are inhibited in TMV CP(+) plants. The protection efficiency correlated with the increasing levels of CP homology. However, examples obtained from CP(+) plants of CMV (Namba *et al.*, 1992) and AlMV (Taschner *et al.*, 1994) are lacking reciprocity in CPMP between virus strains.

It has been demonstrated that CPMP can function *via* interfering either with virus replication or cell-to-cell transport. To evaluate which type of protection we were studying in PVX_S CP(+) tobaccos, we carried out inoculation experiments with protoplasts isolated from these transgenic plants. Protoplasts derived from PVX_S CP(+) tobaccos exhibited protection against challenge with 1 µg of PVX_S, PVX_{S6111}, or PVX_{P551} virions per 500,000 protoplasts. When inoculum concentration was increased five-fold, the susceptibility of transgenic protoplasts to PVX isolates did not differ significantly from that of SR1 control cells (I, Table 1). To our surprise, the different propagation levels of PVX isolates detected in intact transgenic tobacco plants were not reproduced in protoplasts. Here, the protection effect at low inoculum pressure was the same for all three isolates tested. I am not aware of any previous studies on PVX CPMP at the single cell level. The data reported so far for CPMP in protoplasts using other virus-host systems are relatively controversial. AlMV CP(+) protoplasts did not support AlMV replication (Hill *et al.*, 1991), whereas protoplasts expressing the CP of tobacco rattle tobravirus were not protected against the virus challenge (Angenent *et al.*, 1990). The expression of TEV CP in protoplasts did not protect cells from TEV (Lindbo and Dougherty, 1992a; 1992b), whereas protoplasts derived from young virus-free leaf tissue of TEV CP(+) plants, "recovered" from the virus infection, were resistant to TEV (Lindbo *et al.*,

1993). Truncated CP (Silva-Rosales *et al.*, 1994) or untranslatable CP mRNA (Lindbo and Dougherty, 1992b) of TEV conferred resistance to TEV in protoplasts. Transgenic TMV CP(+) protoplasts were protected against TMV, when TMV virions were used as inoculum (Register and Beachy, 1988). However, in a few CP(+) cells, where TMV infection was initiated, virus replication rates did not differ from nontransgenic protoplasts (Register and Beachy, 1988). As the comparative multiplication of different PVX isolates in our constructed PVX_S CP(+) plants and protoplasts were different, our working hypothesis is that two different mechanisms are responsible for the protection against PVX in CPMP: one inhibiting virus replication at low inoculum pressure in strain-independent fashion and another being a strain-dependent virus transport reducing mechanism.

Our results as well as these obtained by others meanwhile clearly indicated that CPMP can in most cases be efficient only against the virus which provided the CP gene [and even then some strains are more potent to multiply in CP(+) plants than others]. Therefore, we decided to study the possibilities for exploiting some general antiviral pathways in plants using genetic engineering techniques. Our CP(+) plants were later successfully used as a reference for comparing the efficiency of 2-5A synthetase-mediated protection. The same construct which was used for the construction of PVX CP(+) tobacco, was exploited for the creation of PVX-tolerant commercial Finnish potato varieties (Mehto, 1991).

7.2. Effects of exogenous 2'-5' oligoadenylates on protein synthesis in plant systems (II, V)

Previous reports have indicated that 2-5A can have an effect on protein synthesis in plant cells, similar to the well-established influence on translation in mammalian systems (Devash *et al.*, 1982; 1984; 1986b). In mammals 2-5A inhibit protein synthesis through their ability to induce the degradation of the cytoplasmic RNA pool, incl. mRNA and disrupting, therefore, the polysomes. Our first aim was to check whether the data obtained by Devash and co-authors are reproducible and whether 2-5A induce plant RNA degradation. We analysed the influence of chemically synthesised 2-5A di-, tri-, tetra-, and pentamers with different degree of phosphorylation on *in vitro* translation of TMV or BMV RNA in wheat germ extracts. Nonphosphorylated 2-5A tri-, tetra- (II, Table 1), and pentamers (data not shown) were potent inhibitors of virus RNA translation in wheat germ extract. 2-5A dimers, independent of their phosphorylation state, had no effect on protein synthesis in the same system, indicating that the chain length of these oligonucleotides was perhaps too short to cause any effect (data not shown). Phosphorylated 2-5A tri-, tetra, and pentamers were less potent inhibitors of *in vitro* translation in wheat germ extract or did not inhibit translation at all. Addition of phosphorus groups to the 5' end of 2-

5A reduced the potential of the oligomers to inhibit plant protein synthesis. This is opposite to the situation in mammals, where di- and triphosphorylated forms of 2-5A are capable of inhibiting protein synthesis *via* the induction of RNA degradation. We propose that the requirement for 2-5A with different structures to inhibit plant protein synthesis indicate also that the putative 2-5A-binding plant protein(s) differ structurally from their mammalian counterparts. Around 1 μM of nonphosphorylated 2-5A was needed to reduce protein synthesis 4–6 times in wheat germ extract (II, Fig. 2). This concentration is two to three orders of magnitude higher than needed for the same effect with phosphorylated 2-5A in mammalian cell-free extracts (Floyd-Smith *et al.*, 1981; Wreschner *et al.*, 1981). This might be an indication that the putative plant 2-5A-binding protein has lower affinity for 2-5A oligomers analysed by us. The fact that nonphosphorylated forms of 2-5A were inhibiting plant protein synthesis fit well with results reported by others (Devash *et al.*, 1982; 1984).

In mammalian systems 2-5A inhibit protein synthesis by inducing RNA degradation. We decided to follow the TMV RNA degradation rates in wheat germ extracts. We detected rapid TMV RNA degradation in wheat germ extract in the presence of nonphosphorylated 2-5A (II, Fig. 5). 2-5A trimer triphosphate had no effect on RNA degradation in wheat germ extract, but led to the rapid destruction of TMV RNA in rabbit reticulocyte lysates. These data gave indirect evidence that 2-5A activated a ribonuclease in plants.

In mammals, 2-5A bind to and activate a latent ribonuclease — RNase L. The presence of the 2-5A-binding protein(s) in cell extracts can be detected by chemical crosslinking (Wreschner *et al.*, 1982), UV crosslinking (Nolan-Sorden *et al.*, 1990) or affinity blotting (Bayard and Zhou, 1992) methods. We have used all three methods for the detection of putative plant 2-5A-binding proteins. The best results were obtained using chemical crosslinking with periodate oxidation. We were able to repeatedly show the presence of a 70 kD protein in potato leaf extracts, which bound [^{32}P]-labelled 2-5A specifically (II, Fig. 4). A 2-5A-binding protein was also detected in wheat germ extract (data not shown). [^{32}P]-labelled 3'–5' oligoadenylates did not bind the protein (II, Fig. 4). These findings contradict reports by Cayley *et al.* (1982) and Devash *et al.* (1985), who could not detect plant 2-5A-binding proteins. We do not know the reason for their results, but a possible explanation is that the labelled triphosphorylated 2-5A forms they were using for the crosslinking experiments are unable to bind plant 2-5A-binding proteins.

It has been demonstrated previously that nonphosphorylated 2-5A are capable of penetrating through the mammalian cell membrane (Kimchi *et al.*, 1981; Suhadolnik *et al.*, 1983). We repeated this with tobacco mesophyll protoplasts. This enabled us to show that 2-5A inhibit protein synthesis, not only in wheat germ cell-free system, but in plant cells (II, Fig. 6).

In mammals the action of 2-5A is transient due to its degradation by rather unspecific phosphodiesterase. We have followed whether plant cell extracts contain enzymatic activities capable of degrading these oligonucleotides. As revealed from thin layer chromatography analysis, tobacco leaves contain en-

zymes which degrade 2-5A at 25° C even more efficiently than it occurs in mouse cell extracts (V, Fig. 1).

Four lines of evidence support the presence of components analogous to the 2-5A system in plants: 1) nonphosphorylated 2-5A inhibit protein synthesis in wheat germ extract; 2) 2-5A induce RNA degradation in wheat germ extract; 3) 70 kD plant protein is specifically crosslinked to 2-5A; 4) 2-5A are rapidly degraded in plant cell extracts. These facts indicate that enzymatic activities resembling 2-5A-dependent ribonuclease and 2' phosphodiesterase might be present in higher plants. As all our attempts to identify 2-5A synthesising activity or DNA and/or RNA sequences hybridising to 2-5A synthetase cDNA in plants have been unsuccessful, we assumed that a functional 2-5A pathway in plants could be reconstituted only after the expression of functional 2-5A synthetase cDNA in transgenic plants.

7.3. Cloning of a rat 2-5A synthetase cDNA (III)

2-5A synthetases in mammals form a multienzyme family with three principle isoenzymes (with molecular weight of 40–46, 69, and 100 kD; Chebath *et al.*, 1987). We used a mouse L3 2-5A synthetase cDNA probe (Ichii *et al.*, 1986) to isolate the corresponding cDNA from rat (*Rattus norvegicus)* hippocampus cDNA library. The isolated cDNA was 1421 bp long (III, Fig. 1), and it contained a single open reading frame consisting of 1077 nucleotides. It encodes a protein with calculated molecular weight of 41,582 kD. Rat 2-5A synthetase cDNA showed high homology to previously characterised human and mouse 2-5A synthetases. Multiple alignment of six amino acid (AA) sequences revealed 70.2% similarity between primary structures of these proteins. CLUSTAL computer program revealed that rat 2-5A synthetase is most closely related to both L2 and L3 2-5A synthetases from mouse (III, Fig. 9). L2 cDNA has 600 untranslated nucleotides at its 3' end not present in L3 cDNA (Rutherford *et al.*, 1991). As the length of the 3' untranslated region of our isolated cDNA is nearly identical to mouse L3 cDNA, we believe that we have cloned the rat analogue of mouse L3 2-5A synthetase.

2-5A synthetase must contain two functionally important domains, one binding the activator of the enzyme — dsRNA, and another being the catalytic site responsible for the synthesis of 2'-5' oligoadenylates. Ghosh *et al.* (1991) have mapped the dsRNA-binding site for 2-5A synthetase small isoform. According to the sequence alignments the dsRNA-binding site for rat 2-5A synthetase is located at the AA residues 105–159. This region is characterised by several Arg and Lys residues known to be crucial for RNA binding. According to the analysis *in silico*, dsRNA-binding motif should contain the longest helical stretch in the 2-5A synthetase protein (III, Fig. 7). Perhaps the binding properties are largely confirmed by the conserved secondary structure elements, as primary structure comparisons of this region did not reveal very high identity among six 2-5A synthetases of human and murine origin (III, Fig. 2).

Ghosh *et al.* (1991) have found by the analysis of deletion mutants that the catalytic site of the mouse 2-5A synthetase lies between AA residues 321 and 345. This region corresponds to AA 322–346 in rat 2-5A synthetase. The region is well conserved when six sequenced 2-5A synthetase cDNAs were compared (60% identical). Very recently the same group has reported that first nine N-terminal AA residues are essential for the 2-5A synthetase activity (but not for dsRNA binding) (Ramaraj *et al.*, 1995). They have established that conserved AA residues Leu_3, Pro_7, and Ala_8 are functionally important for 2-5A synthetase. Leu_3 and Pro_7 are present also in rat 2-5A synthetase, but at position 9 a change $Ala_8 \rightarrow Ser$ has occurred. Therefore, at least for the rat 2-5A synthetase, Ala_8 is not needed for the functional enzyme. AA sequence analysis of rat 2-5A synthetase revealed an ATP/GTP binding motif A (P-loop) from AA residues 68 to 75. A similar sequence is also present in mouse 2-5A synthetase. It is the most polar neutral part of the entire rat 2-5A synthetase AA sequence (III, Fig. 3). 2-5A synthetase polymerises ATP, but its ATP-binding domain has not been located. Usually it is believed that it is structurally related to the catalytic centre in the C-terminus of the protein, but this idea lacks experimental proof. Our finding of the putative ATP/GTP-binding motif in the N-terminus of rat (and also mouse) 2-5A synthetase serves as an alternative to the previous model for the ATP-binding domain location in 2-5A synthetase protein.

7.4. Construction of transgenic tobacco and potato plants expressing rat 2-5A synthetase, which display a broad spectrum virus protection (IV–VI)

For the transformation of rat 2-5A synthetase cDNA into tobacco and potato plants we utilised the same plant transformation vector which was used for the transfer of PVX_S CP gene into tobaccos (IV, Fig. 1). Transformation vector pHTT2-5A+ was obtained after subcloning the entire rat 2-5A synthetase cDNA into *Bam*HI site of the vector pHTT202. For control transformations, the same cDNA was inserted into the plasmid pHTT202 in an antisense orientation, resulting in plasmid pHTT2-5A-. The initial screening for successfully transformed plantlets was carried out on kanamycin-containing selective plates. With both species used, tens of putative transformants were selected as kan^R plants. According to our knowledge, it was the first published report of successful gene transfer to the Finnish commercial potato cv. Pito. Later, an independent detailed protocol for the transformation of the same cultivar has been published (Koivu *et al.*, 1994). Several kan^R plantlets were subjected to further molecular analysis. Southern analysis of transformed 2-5A(+) tobacco lines revealed that from six lines analysed, two contained a single copy of 2-5A synthetase cDNA and two others had incorporated at least two copies of the same cDNA. Two clones out of six were apparently picked up as false positives. According to Southern analysis these lines did not contain 2-5A syn-

thetase cDNA-specific genomic sequence(s). From analysed potato lines, six plants were shown to contain a single copy of 2-5A synthetase (IV, Fig. 2). All tobacco and potato lines containing genomic sequences for 2-5A synthetase expressed detectable amounts of 2-5A synthetase mRNA and protein, as revealed by Northern and Western analyses (IV, Fig. 3; VI, Fig. 1 and 2). Therefore, they were suitable for the analysis of the properties of transgenic plants expressing the mammalian antiviral enzyme — 2-5 synthetase. The only exception was tobacco line T6, where mRNA and protein of that enzyme were not detected (data not shown). The Southern analysis of the genomic DNA of this line revealed that some rearrangements could have been occurred since the *Pvu*II site present in the original sequence of the cDNA was absent.

Plant lines expressing 2-5A synthetase protein were used for independent inoculation tests with multiple plant viruses. The results with tobacco plants are summarised in Fig. 3–5 (VI). Briefly, 2-5A(+) tobaccos showed protection against three independent plant viruses PVX, PVS, and TMV, belonging to three different virus groups. In each case, at least one 2-5A(+) line showed significantly lower amounts of virus than nontransgenic control plants up to one month post inoculation. With PVS, all three transgenic 2-5A(+) lines showed strong protection against this carlavirus. With TMV, correlation between the inhibition of symptom development and the suppression of virus titre was observed. The infection of the other two viruses in SR1 tobacco plants was nearly symptomless.

However, in addition to these three viruses we have also tested the ability of 2-5A(+) tobaccos to suppress the multiplication of PVY infection. We were unable to identify any tobacco lines that were protected against infection by this potyvirus (V, Table 2). Hence, we cannot yet conclude that the protection achieved by expressing mammalian 2-5A synthetase *in planta* confers an absolutely universal protection against all plant RNA viruses.

Greenhouse tests with 2-5A(+) potato plants revealed that at least some lines were protected against both PVX and PVY. When inoculated with PVX, several lines were protected against the virus. Fig. 3 represents the differences between the concentrations of PVX in control plants and one transformant during the first month post inoculation. With PVY, most plant 2-5A(+) lines were as susceptible as control plants [similar to 2-5A(+) tobaccos]. However, potato lines P5 and P6 showed protection to a certain extent also against this virus (Fig. 4).

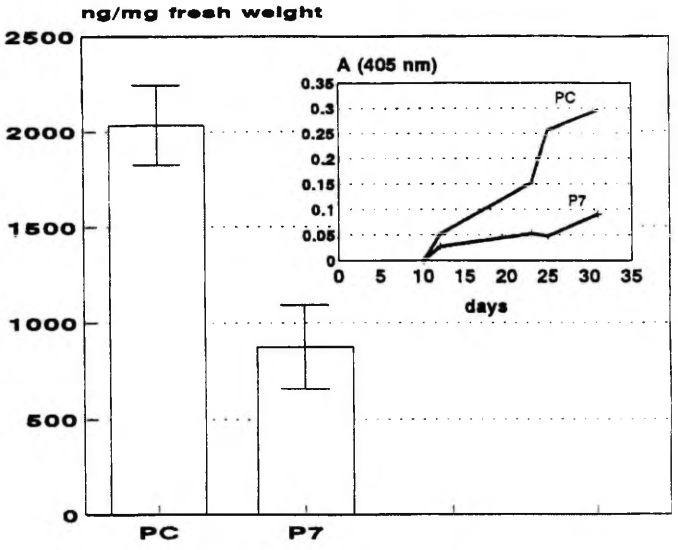

Fig. 3. Rates of PVX infection in potato (PC = control, P7 = transgenic 2-5A(+) line), greenhouse test. ELISA absorbances followed 10–30 days post inoculation, n = 2 (insert figure). PVX concentrations 20 days post inoculation, n = 9 and n = 10, ± S. E. (main figure).

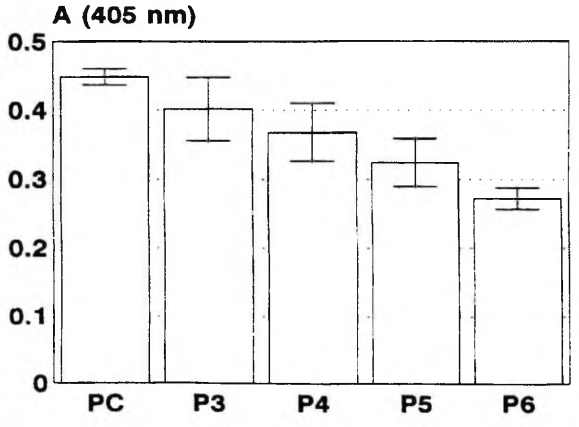

Fig. 4. ELISA absorbances of PVY in potato (PC = control, P3 – P6 = transgenic 2-5A(+) lines), greenhouse test. n = 2 and n = 3, ± S. E.

In conclusion, we have been able to show that transgenic tobacco and potato plants expressing rat 2-5A synthetase are protected against challenge inoculation of plant viruses belonging to different taxonomic groups. This resembles the situation in mammals, where it has been demonstrated that the constitutive expression of 2-5A synthetase in human (Rysiecki *et al.*, 1989) and mouse (Chebath *et al.*, 1987; Coccia *et al.*, 1990) cells confers resistance to picornavirus infection. Constitutive expression of 2-5A synthetase also enhances cellular protection against HIV infection (Schröder *et al.*, 1992). Furthermore, in mammals the importance of 2-5A synthetase in antiviral action has been demonstrated by the reverse experiment, where blocking the enzyme with antisense RNA resulted in enhanced susceptibility to picorna-, rhabdo-, and togaviruses (De Benedetti *et al.*, 1987).

Protection against viruses belonging to three (or even four) different systematic groups is one of the most broad spectrum virus resistances ever achieved in transgenic plants. To my knowledge it was the first report about the successful utilisation of an animal antiviral gene that protects transgenic plants. In subsequent years others have successfully repeated our experiments with different virus-host systems. It has been independently demonstrated that tobacco plants expressing human 2-5A synthetase exhibit resistance to TMV and to several CMV strains (Ehara *et al.*, 1994; Nakamura *et al.*, 1994). Furthermore, very recently it has been reported that plants expressing both 2-5A synthetase and RNase L have been constructed (Silverman *et al.*, 1995). The expression of both enzymes together *in planta* caused the formation of local necrotic lesions on leaves after infection with three different viruses instead of typical systemic infection. This reaction resembles HR, which is a natural protection mechanism against virus infections in plants. However, Ishida *et al.* (1995) were unable to detect protection against TMV in tobaccos expressing human 2-5A synthetase. More detailed information about this work is not yet available, but as the authors themselves conclude, more extensive infection tests are needed to understand the background of these discrepancies.

7.5. Field testing of 2-5A synthetase expressing potato plants (IV)

Field tests at Kotkaniemi Research Station, Finland were carried out after mechanical inoculation of field growing potato plants with sap from PVX-infected *N. glutinosa* plants. We were able to show that three lines of six 2-5A(+) lines used in the study remained virus-free 35 days post inoculation (IV, Table 1). This indicates that 2-5A synthetase-mediated virus protection is effective also under field conditions. It should be noted that virus resistance effects determined in the greenhouse are not always functional in the field (Jongedijk *et al.*, 1993). Lower or absent concentrations of PVX in transgenic potato leaves correlated with reduced numbers of infected tubers harvested from the field-grown transgenic plants. However, all transgenic lines contained at least some PVX-

infected tubers, so the expression of 2-5A synthetase had not led to absolute immunity against PVX (IV, Fig. 4).

The phenotype of transgenic plants in the field as well as the yield and morphology of transgenic tubers were indistinguishable from nontransgenic control plants (data not shown).

To compare the efficiency of CP-mediated protection with protection conferred by 2-5A synthetase, we used our transgenic potato plants, expressing PVX_S CP (Mehto, 1991). PVX CP(+) potatoes showed strong protection against PVX under field conditions. Still, PVX CP(+) leaves contained detectable levels of the virus 35 days post inoculation [differently from three 2-5A(+) potato lines; IV, Table 1]. PVX CP(+) plants also showed a higher percentage of PVX-infected tubers than one 2-5A(+) line (IV, Fig. 4). This is a strong indication that in field the expression of this mammalian antiviral enzyme in plants can produce virus protection levels comparable to that of conventional CP-mediated protection in transgenic plants.

8. CONCLUSIONS AND FURTHER PERSPECTIVES

1. **Tobacco plants expressing PVX_S CP have been shown to be protected against PVX. Resistance levels did not correlate with the homology between the challenging virus CP and the transgene. From experiments with whole plants and protoplasts we proposed two different mechanisms for CP-mediated protection — one functioning on the level of replication and another on virus movement.**

Our results served as an additional proof to the view that CPMP is virus- and even strain-specific, and cannot be used for the construction of broad-spectrum virus tolerant transgenic plants.

Currently, we are analysing two Estonian PVX isolates, which, despite their close evolutionary relationship, produce very different disease symptoms on indicator plants. We plan to use these isolates for closer characterisation of PVX CP role in natural and transgenic virus protection. CP cDNAs of these isolates have recently been sequenced (Bouscaren, 1996).

2. **Using transgenic tobacco and potato plants, we have clearly shown that mammalian 2-5A synthetase cDNA can be utilised as a plant antiviral gene for broad spectrum virus resistance.** This approach has now also been patented (Saarma *et al.*, 1992; 1993).

However, several questions remain to be answered. The first important issue concerns the stability of the 2-5A synthetase cDNA in the plant genome and the heritability of the achieved protection. We are currently actively examining these subjects. We have obtained three subsequent generations of tobacco lines expressing 2-5A synthetase. The analysis of R_1 and R_2 generations have revealed that 2-5A synthetase cDNA is stable and the plants have remained protected against PVX and TMV (Saichenko, unpublished). Furthermore, we have extended our studies also to TSWV, a plant virus with negative-sense RNA genome. R_1 tobacco plants expressing 2-5A synthetase were protected also against challenge by this virus. According to my information it has not been previously reported that transgenic plants are simultaneously protected against both (+) – and (–) – strand RNA viruses.

Another set of experiments have been started to focus on understanding the actual mechanism by which 2-5A synthetase confers resistance in transgenic plants. We have shown that 2-5A induces RNA degrading activity in plant extracts and that plants contain protein(s) that specifically bind 2-5A. Thus, we have proposed that the expression of 2-5A synthetase leads to the activation of mammalian-like 2-5A system in transgenic plants (V, Fig. 2). To prove whether this is true or not, our current aim is to clone plant 2-5A-binding protein cDNA. We have taken three different approaches to fullfill this goal: a) screening plant cDNA libraries with mammalian RNase L cDNA probe. For that purpose we have PCR-cloned a partial cDNA for rat RNase L (Aaspõllu

and Truve, unpublished); b) screening plant expression libraries with [^{32}P]-labelled 2-5A; c) purification of plant 2-5A-binding protein(s) on 2-5A-agarose columns. As a result of these experiments we should also be able to determine the actual role(s) of the putative plant 2-5A-binding protein. Currently, we cannot rule out the possibility that this protein is bearing functions not related at all to the functions of mammalian 2-5A-binding proteins. If it is the case, it could explain also the relatively low binding efficiency of this plant protein to 2-5A.

We have been able to show that universal virus protection mechanisms identified from one kingdom can be exploited for virus tolerance in organisms from another kingdom by means of genetic engineering. The rat 2-5A synthetase expressing plants were protected against at least three distinct viruses from three different systematic groups. It is one of the most broadest virus protections ever reported for transgenic plants. It seems to be unlimited by too narrow specificity, which is the main drawback for most pathogen-derived resistance approaches. 2-5A synthetase *per se* is also not toxic for human and mammals, which is the case for some proteins used for broad scale virus resistance approaches in plants. Mammalian IFN-induced antiviral pathways are at present the best studied universal antiviral mechanisms. However, other mechanisms from other organisms are also found. For example, yeast *Saccharomyces cerevisiae* has a purely intracellular antiviral system — SKI, which consists of at least six genes. *SKI3* product is a 163 kD nuclear protein of unknown function (Rhee *et al.*, 1989), *SKI8* protein has been identified as β-transducin (Matsumoto *et al.*, 1993). *SKI2* gene encodes a 145 kD protein with motifs characteristic of helicases and nucleolar proteins. It blocks specifically translation of viral mRNAs (Widner and Wickner, 1993). It is proposed that *SKI2* protein can recognise mRNAs with absent cap and/or poly(A) tail (Widner and Wickner, 1993). Although no similar pathways to *SKI* from other organisms are known, it can have a potential for engineered virus protection in transgenic organisms when all essential genes of the pathway are cloned and characterised. The immunity of most plants to natural infections of most plant viruses suggests that still undiscovered universal virus protection mechanisms can function also in higher plants. The use of these, still hypothetical pathways for protecting crops from virus diseases might be the preferred approach for tomorrow. It keeps the door open for many interesting experiments and successful thesis in the future.

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10. SPETSIIFILISE VÕI LAIA SPEKTRIGA VIIRUSRESISTENTSIGA TRANSGEENSED TAIMED

Kokkuvõte

On konstrueeritud kahte tüüpi transgeensed taimed, mis ekspresseerivad vastavalt kartuliviiruse X (PVX) kattevalku ning roti 2-5A süntetaasi.

PVX-i kattevalku ekspresseerivad taimed on resistentsed PVX-i eri isolaatide suhtes, kusjuures kaitse tugevus ei korreleeru isolaatide kattevalkude homoloogiaga transgeeni suhtes. Tervete taimede ja protoplastide nakatamistest saadud tulemuste põhjal oleme püstitanud hüpoteesi, mille kohaselt viiruse kattevalgu vahendatud resistents transgeensetes taimedes toimib kahe erineva mehhanismi kaudu. Üheks mehhanismiks on viiruse replikatsiooni inhibeerimine ja teiseks viiruse rakust rakku liikumise takistamine.

Tubaka- ja kartulitaimedel, mis ekspresseerivad meie kloneeritud roti 2-5A süntetaasi, on laia spektriga viiruskaitse erinevatesse süstemaatilistesse gruppidesse kuuluvate taimeviiruste suhtes. *In vitro* katsetega oleme tõestanud, et 2'-5' oligoadenülaadid (2-5A süntetaasi produktid) suudavad nagu imetajatelgi indutseerida RNA lagunemist ja inhibeerida valgusünteesi ka taimedes. Kuna oleme suutnud detekteerida ka spetsiifiliselt 2-5A-d siduvat taimevalku, siis usume, et meie konstrueeritud taimedes funktsioneerib indutseeritav viiruskaitse mehhanism, millel on suurt sarnasus imetajate 2-5A süsteemiga. Põllukatsetega oleme ka demonstreerinud, et saavutatud viiruskaitse toimib ka põllul ja et roti antiviraalse geeni ekspressioon taimes ei muuda viimase sordiomadusi, fenotüüpi ega saagikust. Sellel uuel põhimõttel laia spekriga viiruskindlate taimede saamiseks on perspektiivi laiemaks kasutamiseks põllukultuuride puhul, mida nakatavad samaaegselt erinevad taimsed RNA viirused. Laia spektriga viiruskindlate taimede konstrueerimise printsiip on nüüdseks ka patenteeritud.

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DIFFERENT PROPAGATION LEVELS OF POTATO VIRUS X (PVX) ISOLATES IN PVX COAT PROTEIN EXPRESSING TOBACCO PLANTS AND PROTOPLASTS DO NOT CORRELATE WITH COAT PROTEIN SEQUENCE SIMILARITIES

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We have obtained transgenic tobaccos expressing the coat protein (CP) (CP+plants) or coat protein antisense RNA [CP(−) plants] of the Russian isolate of potato virus X (PVX_S). When these plants were challenged with seven different PVX isolates, two CP+ lines showed delay in virus propagation, whereas CP(−) lines were not protected at inoculum concentrations of 10 μg/ml. The level of protection in CP(+) lines was different for different PVX isolates. Sequence comparisons revealed that similarity between CPs of different PVX isolates to PVX_S CP do not correlate with the protection achieved against these isolates in tobaccos expressing PVX_S CP. We analyzed the replication of PVX isolates S, S6111 and P551 in protoplasts derived from PVX CP+ transgenic tobaccos. In contrast to studies with intact plants, we were not able to detect any differences in propagation of these isolates in individual CP+ tobacco cells. With 1 μg PVX inoculum, in CP+ protoplasts the replication of all three isolates was inhibited, whereas at higher inoculum concentrations no difference existed between CP+ and control cells. These data indicate that two mechanisms could be involved in PVX CP-mediated virus protection: at low virus concentrations PVX replication is inhibited, and at higher virus concentrations PVX spread is perturbed in transgenic plants.

KEYWORDS: potato virus X, coat protein, transgenic tobacco, virus isolates, virus protection, protoplasts

INTRODUCTION

The concept of pathogen-derived virus resistance was formulated in 1985 (Sanford and Johnston, 1985), and first realized a year later, when it was shown that transgenic plants expressing the tobacco mosaic virus (TMV) coat protein (CP) gene exhibited either delayed symptom development on failed to develop any symptoms following challenge with TMV (Powell Abel *et al.*, 1986). Subsequently, it has been shown by many independent research groups that the expression of plant virus-derived nucleic

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acid sequences *in planta* confers resistance to the parental virus or, in some cases, also to closely related strains and viruses (reviewed by Wilson, 1993). The exploitation of virus CP gene for creating virus-resistant crops through genetic engineering has been the most widely used method of pathogen-derived resistance. Already in 1992, Hull and Davies cited reports on virus coat protein-mediated protection (CPMP) against 12 different virus groups and since then the list has expanded. Despite numerous reports of CPMP, the underlying mechanism that confers a resistant phenotype has not yet been elucidated. Moreover, recent reports suggest that the mechanism of CPMP possesses many details unique to nearly every virus-plant system investigated to date.

Potato virus X (PVX), a filamentous virus with single messenger-sense RNA, is the type member of the potexvirus group. PVX was one of the first examples for which CPMP was demonstrated. *Nicotiana tabacum* cv. Samsun plants were transformed with a construct containing the full-length PVX CP coding sequence between 18 bp and 72 bp of 5′ and 3′ non-coding regions, respectively, under the control of cauliflower mosaic virus (CaMV) 35S promoter (Hemenway *et al.*, 1988). The obtained transgenic lines expressed detectable amounts of PVX CP and generated significantly lower concentrations of PVX than the control plants at inoculum concentrations of up to 5 μg/ml. The protection in CP+ plants was accompanied by a reduced number of lesions on inoculated leaves and delayed or absent development of systematic infections, as compared to control plants. The protection effects were dependent upon PVX CP expression levels in different transgenic lines. Similar protection was observed when the same plant lines were inoculated with up to 5 μg/ml of naked PVX RNA (Hemenway *et al.*, 1988).

Later on, CPMP to PVX was also demonstrated in transgenic potatoes (*Solanum tuberosum*). Hoekema *et al.* (1989) transformed two commerical potato cultivars using a plant expression vector, where the full-length PVX CP gene with 8 bp 5′ and 10 bp 3′ non-coding flanking sequences, respectively, was cloned downstream from CaMV 35S promoter. Transgenic CP+ plants of both cultivars showed a considerable delay in the accumulation of PVX (at inoculum concentration of 1 μg/ml), whereas higher endogenous levels of PVX CP yielded greater protection (Hoekema *et al.*, 1989). The potato lines maintained their virus protection abilities during four years of field trials (van den Elzen *et al.*, 1993). Fehér *et al.* (1992) cloned PVX CP gene together with 65 bp 5′ upstream and 75 bp 3′ downstream regions under the control of the carrot ethylene-inducible extensin promoter. Transformed potatoes expressing PVX CP accumulated PVX RNA to a lesser extent than control plants, when inoculated with 0.5 μg/ml PVX inoculums (Fehér *et al.*, 1992). Basically the same construct was earlier transfered to tobacco under the extensin promoter, which resulted in strong protection against PVX at inoculum concentrations of 10 μg/ml (Zakhar'ev *et al.*, 1989).

PVX strains of different geographical origins are well characterized. They have been classified into four groups according to their reactions with the potato dominant hypersensitive resistance genes *Nb* and *Nx* (Cockerham, 1970). At present CP amino acid sequences of at least 8 PVX isolates are available: South-American strain cp2 (Orman *et al.*, 1990), belonging to resistance group 2; group 3 strains X3 (Huisman *et al.*, 1988) and S (Skryabin *et al.*, 1988), both from continental Europe, and British strains UK3 (Kavanagh *et al.*, 1992) and DX (Santa Cruz and Baulcombe, 1993); group 4 South-American strains HB (Kavanagh *et al.*, 1992) and cp4 (Goulden *et al.*, 1993); plus a Chinese isolate with unknown grouping (Wang *et al.*, 1991). However, resistance

studies with PVX CP expressing transgenic plants have been carried out using a single PVX isolate, usually the one from which the CP was cloned.

In this study we describe the construction of transgenic *N. tabacum* SR1 plants expressing the CP of the PVX Russian strain – PVX_S (Skryabin *et al.*, 1988). Transgenic plants and protoplasts were challenged with seven and three different PVX isolates, respectively. Additionally, the CP gene sequence of the isolates S6111, P551 and cp2 were analyzed. The results indicate that expression level of different PVX isolates in PVX_S CP+ transgenic plants and protoplasts do not correlate with corresponding CP sequence similarities.

MATERIALS AND METHODS

Materials

N. tabacum L. cv. Petite Havana SR1 (Maliga *et al.*, 1973) plants were propagated on MS medium (Murashige and Skoog, 1962) without hormones at 25 °C with a photoperiod of 16 h. PVX strain S was obtained from Dr. Y. Varitsev, strains HB and cp2 were obtained from Prof. B. D. Harrison, recombinant strains KD and KD4 derived from British strains UK3 and Dx or Dx4, respectively (Santa Cruz and Baulcombe, 1993), were kindly provided by Dr. D. Baulcombe, and isolates S6111 and P551 (Kurppa, 1983) by Dr. A. Kurppa. Radiolabeled nucleotides were purchased from Radiopreparat (Uzbekistan) or Amersham (England), restriction endonucleases from Fermentas (Lithuania), avian myeloblastosis virus (AMV) reverse transcriptase and RNasin ribonuclease inhibitor from Promega (USA), Klenow fragment of DNA polymerase I from New England Biolabs (USA) and *Taq* DNA polymerase from Perkin Elmer (USA). Plant growth hormones and antibiotics were from Sigma (USA) and macerating enzymes from Yakult Honsha Co. (Japan).

Primers for polymerase chain reaction (PCR), recognizing the conserved regions along PVX genome were as follows: "upstream" primer complementary to the N-terminal half of PVX ORF4: 5′ TGCTTGTGGT/CAACAATCATAGCA; "downstream" primer complementary to the very beginning of PVX 3′ untranslated region: 5′ CGTCGGTTATGTG/AGACGTAGTT.

Construction of plasmids and tobacco transformation

All cloning steps were carried out using standard protocols (Maniatis *et al.*, 1982). PVX_S CP cDNA originated from plasmid pX72 (Zakhar'ev *et al.*, 1989), kindly provided by Prof. K.G. Skryabin. PVX_S CP cDNA contained 53 bp of 5′ noncoding region, 711 bp of the structural gene, 3′ untranslated region of 75 bp plus 15 bp of poly (A) tail. cDNA was excised from pX72 with *Bam*HI and *Hin*dIII. The insert was subcloned into the plant expression vector pHTT202 (Truve *et al.*, 1993) *Bam*HI site after DNAs were blunted using the Klenow enzyme. The resulting constructs were named pHTTCP+ (sense orientation) and pHTTCP(–) (antisense orientation), respectively. PVX CP cDNA in both orientations was integrated into *Agrobacterium tumefaciens* Ti-plasmid pGV2260 (Deblaere *et al.*, 1985) via homologus recombination as described by Van Haute *et al.* (1983). Recombinant *Agrobacteria* were verified by isolating total

bacterial DNA (Dhaese *et al.*, 1979) and carrying out Southern analysis. Leaf discs from *N. tabaccum* SR1 plants were cocultivated with *Agrobacterium* according to Horsh *et al.* (1985). Shoots were induced from the transformed discs on selective LS medium (Linsmaier and Skoog, 1965) with 50 μg/ml kanamycin, 1 μg/ml benzylaminopurine (BAP) and 500 μg/ml Claforan (Hoechst, Germany). Roots were induced on the same medium except that BAP was omitted. Fully rooted explants were grown on MS medium without hormones and plants were transferred to fresh medium once a month. To select for the transformants, Southern, Northern, and Western analysis were carried out.

Analysis of transgenic plants

For Southern analysis, DNA was extracted from transformed and control plants according to Dellaporta *et al.* (1983). 2 μg of total DNA was digested with *Pst*I and *Xho*I, both having a unique clevage site in PVX_S CP cDNA. The cleavage products were separated on 0.8% agarose gel. DNA was blotted to Hybond-N nylon membranes (Amersham) and hybridized at 42 °C in the presence of 50% formamide according to Amersham membrane transfer and detection protocols. Full-length PVX_S CP cDNA, labeled with [α-^{32}P]dCTP according to Feinberg and Vogelstein (1983) was used as a probe. The hybridized filters were washed for 2 × 15 min with 2 × SSC (300 mM NaCl, 30 mM Na-citrate, pH 7.0)+0.1% sodium dodecyl sulfate (SDS) at 65 °C, 30 min with 1 × SSC+0.1% SDS at 65 °C and 10 min with 0.1 × SSC+0.1% SDS at room temperature. The filters were air-dried and exposed to Hyperfilm-MP (Amersham) at −40 °C using intensifying screens. Total RNA was extracted from plants for Northern as described by Verwoerd *et al.* (1989). After the electrophoresis in formaldehyde/agarose gel, RNA was Northern blotted to Hybond-N filters using Amersham protocols. The [^{32}P]-labeled probe, hybridization, washing of the filters and autoradiography were as in Southern analysis.

For Western analysis, total soluble proteins were extracted from transgenic and control leaves by homogenizing frozen samples with a pestle and a mortar in 50 mM K/Na-phosphate buffer, pH 7.2 (10 ml buffer/1 g leaf material) and low-speed centrifugation in a table-top centrifuge. Supernatant was collected and proteins from 15 μl aliquots were separated on 12% SDS-polyacrylamide gel electrophoresis (Laemmli 1970). Proteins were electroblotted onto Hybond-C (Amersham) membranes for 2 h at 200 mA, using 40 mM NaH_2PO4, pH 6.5 as a blotting buffer. Blotted filters were washed in Tris-buffered saline (TBS) and blocked with 3% bovine serum albumin in TBS containing 0.05% Tween 20 (TBS/Tw) at room temperature. After washing the filters five times with TBS/Tw, the filters were reacted overnight at 4 °C with rabbit polyclonal antibodies to PVX_S (Sõber *et al.*, 1988) diluted 1:500 in TBS/Tw. After five washes with TBS/Tw the filters were incubated for 2 h at room temperature with anti-rabbit horseradish peroxidase conjugate (Sigma, dilution 1:200). The filters were washed three times with TBS/Tw and the color reaction was developed as previously described (Sõber *et al.*, 1988).

Virus infection of tobacco plants

Primary transformants of each tobacco clone were multiplied by rooting stem pieces with two leaves on fresh MS medium. Plants were transferred from agar to soil two

weeks before the inoculation and were kept at room temperature with a 16 h photoperiod. For the inoculation, 10 µg/ml of purified virus in 5 mM K/Na-phosphate buffer, pH 7.2 was manually inoculated onto 1–2 carborundum-dusted lower leaves of plants bearing 6–8 leaves. Carborundum was washed away after 10 min with sterile water. Plants mock-inoculated with phosphate buffer were used as negative controls throughout all experiments. After inoculation, plants were kept for 24 h in darkness and thereafter as before inoculation. Three plants of each clone were inoculated in each experiment. Samples were collected by taking a top leaf from each inoculated plant and freezing it in liquid nitrogen. Inoculated leaves were not tested.

Protoplast preparation and inoculation

Tobacco mesophyll protoplasts were prepared from leaves of *in vitro* grown axenic shoot cultures, or alternatively of glasshouse-grown plants. In the latter case, leaves were sterilized with 3% sodium hypochlorite. Leaves were cut on a Petri dish in Man-pp medium [B5 salts (Gamborg *et al.*, 1968) supplemented with 500 mM mannitol, 2% sucrose and 0.5% MES; pH 5.7], plasmolysed for 30 min in 20 ml Man-pp and incubated overnight in sterile enzyme solution containing 0.5% cellulase and 0.2% macerase in Man-pp. Protoplast suspension was centrifuged for 10 min at 500 rpm in Jouan CR4.22 centrifuge (France). The pellet was resuspended in 35% Percoll (Sigma) and overlaid with 20% Percoll and Man-pp. After centrifugation for 10 min at 1000 rpm protoplasts on top of the 20% Percoll were collected and resuspended in Man-pp. The protoplasts were centrifuged for 10 min at 500 rpm, resuspended in virus inoculation solution (15 mM $MgCl_2$ in 500 mM mannitol), and were counted.

Protoplasts were infected with PVX essentially according to Valkonen *et al.* (1991) using polyethylene glycol (PEG). Desired amounts of virus in 5 mM K/Na-phosphate buffer (pH 7.0) were mixed with 0.5 ml of virus inoculation solution containing 5×10^5 protoplasts, and the mixture was incubated for 10 min before 0.5 ml of 40% PEG 4000 (Ferak, Germany) and 100 mM $Ca(NO_3)_2$ (pH 8.0) in 500 mM mannitol was added. Protoplasts were incubated with virus for 20 min, spun down for 5 min at 7000 rpm in table-top centrifuge (Eppendorf 5415C, Germany) and washed three times with Man-pp. Thereafter protoplasts were plated with 3 ml Man-pp and cultured for 2 days in darkness. For further analyses, cells were frozen in liquid nitrogen.

Determination of PVX

For the determination of virus concentrations in infected tobacco leaves, frozen leaf samples were separately homogenized with a pestle and a mortar in immunoassay buffer at 1 g/ml. Frozen protoplasts were disrupted by three freeze-thaw cycles. The debris was removed by centrifugation (5 min in table-top centrifuge) in both cases. For the PVX CP detection, monoclonal antibodies 21XD2 and time-resolved fluoroimmunoassay (TRFIA) or double-antibody sandwich ELISA were used (Sinijärv *et al.*, 1988). Immunoassays were carried out individually for each plant and protoplasts culture. For quantification, TRFIA or ELISA values of serially diluted purified PVX preparations were measured. Values obtained from mock-inoculated leaves or protoplasts were taken as nonspecific background of TRFIA or ELISA, respectively.

PVX RNA was extracted from tobacco protoplasts and analyzed with Northern blottings according to Baulcombe *et al.* (1984).

Cloning and sequencing of CP genes

For PVX CP cDNA synthesis, total RNA was isolated from PVX-infected leaf samples according to Verwoerd *et al.* (1989). First strand CP cDNA synthesis was carried out in a mixture containing 1 μg of total plant RNA, 1 mM of each dNTPs, 10 mM $MgCl_2$, 20 pmol PVX "downstream" primer, 1 mM DTT, 20 u of RNasin and 8 u of AMV reverse transcriptase in 1 × PCR buffer (50 mM KCl, 10 mM TrisHCl pH 9.0, 0.1% Triton X-100). The synthesis was carried out for 60 min at 42 °C, followed by the single-stranded cDNA denaturation at 95 °C for 5 min, and chilling on ice. PCR was performed using the total first-strand cDNA synthesis mix (50 μl), 20 pmol of PVX "upstream" primer, 1.25 u of *Taq* DNA polymerase and the appropriate volume of PCR buffer. The mixture was heated at 95 °C for 2 min before adding the enzyme. PCR was performed in Perkin Elemer DNA Thermal Cycler as follows: 30 cycles consisting of 1 min at 94 °C, 1 min at 51 °C and 1.5 min at 72 °C, followed by the incubation for a final 3 min at 72 °C.

PVX_{cp2} CP PCR product was cloned to the vector pBluescript SK(+) (Stratagene, USA) as described by Liu and Schwartz (1993). PVX_{S6111} CP cDNA fragment was cloned to the above named plasmid vector after cleaving it with *Sma*I and tailing the linearized plasmid with a 3′ deoxythymidylate extension in a reaction mixture containing 1 × PCR buffer, supplemented with 1 mM dTTP, 20 μM $MgCl_2$ and 5 u of *Taq* DNA polymerase, for 4 h at 70 °C. PVX_{P551} PCR product was also cloned to the pBluescript SK(+) *Sma*I site, after the single 3′ deoxythymidylate extensions of the PCR fragment were trimmed at 22 °C for 30 min with 3 u of Klenow enzyme. Sequencing of the cloned PCR fragments was performed by the dideoxynucleotide chain termination method using the Sequenase Version 2.0 kit (United States Biochemicals, USA).

Analyses of the nucleotide and corresponding amino acid sequences were performed with PCGENE (University of Geneva, Switzerland) and DNAsis (Hitachi Software Engineering Co., Japan) computer programs.

RESULTS

In this study, we constructed two plant expression vectors pHTTCP+ and pHTTCP(−) containing PVX_S CP cDNA in messenger-sense and -antisense orientation, respectively. The inserts were cloned between CaMV 35S promoter and the T-DNA gene 7 polyadenylation signal. The T-DNA segment of the vectors also contained the hybrid nopaline synthetase-neomycin phosphotransferase II (*nos-npt*II) gene conferring kanamycin resistance (Horsch *et al.*, 1985), which was used to select for transgenic shoots from *Agrobacterium*-transformed tobacco cells. Morphologically normal kanamycin-resistant regenerants were selected for further analyses.

Southern hybridization of *Pst*I-*Xho*I digested total plant DNA with a PVX CP specific cDNA probe revealed three CP DNA-specific bands in four CP+ clones and in five CP(−) clones (data not shown). Restriction analysis with *Pst*I and *Xho*I, both having a unique cleavage site in PVX_S CP cDNA (Skryabin *et al.*, 1988), indicated that a single copy of PVX CP cDNA was incorporated into the genome of at least 9 transformed plants. The expression of PVX CP mRNA was analyzed on Northern blots (Fig. 1). All CP+lines expressed detectable amounts of PVX CP mRNA of

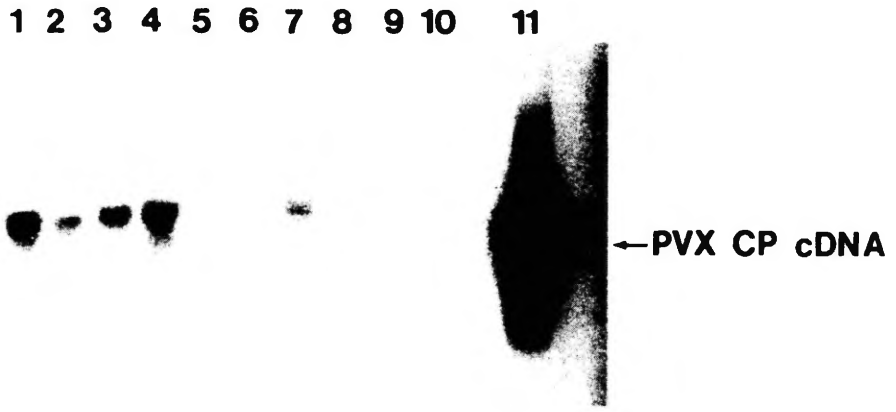

Fig. 1 Northern blot analysis of tobacco lines transformed with PVX_S CP cDNA either in sense (lanes 1–4) or in antisense (lanes 5–9) orientation. 5 μg of total tobacco RNA was separated on 1% agarose/formaldehyde gel, blotted to a nylon filter and hybridized with [^{32}P]-labeled PVX_S CP cDNA. Lane 1–line 2S1; 2–2S2; 3–2S4; 4–2S5; 5–3S1; 6–3S2; 7–3N5; 8–3N14; 9–3N16; 10–untransformed SR1 tobacco; 11–100 ng of linear PVX_S CP cDNA

identical size – about 850 bp. The expression levels were the highest in lines 2S1 and 2S5, whereas expression was weakest in the line 2S2. CP(–) clones expressed CP-specific RNA at much lower levels than CP+ lines (Fig. 1). Lines 3N14 and 3N16 did not express detectable amounts of PVX CP antisense RNA, whereas lines 3S1, 3S2 and 3N5 weakly expressed CP-specific RNA of the expected size (Fig. 1). No signal was observed in the nontransgenic control plants using the PVX CP gene as a probe in Southern and Northern analysis (Fig. 1).

Rabbit PAbs against PVX_S CP were used to study PVX_S CP expression in transgenic tobacco lines (Fig. 2). All four CP mRNA-expressing tobacco lines produced a 25 kD protein recognized by anti-PVX PAbs. The size of the protein was identical to PVX CP (Fig. 2). Surprisingly, the levels of protein expression did not correlate to large extent with CP mRNA expression levels. Lines 2S2, 2S4, and 2S5 expressed PVX CP nearly at the same level, although 2S2 contained the least amount of CP mRNA (Fig. 1). Line 2S1, which expressed high levels of PVX_S CP mRNA, expressed less CP than the other CP+ lines. The reason for such discrepancies is not known. CP(–) lines (data not shown) as well as nontransgenic control plants (Fig. 2) did not express PVX CP.

Lines 2S4 and 2S5 expressed PVX CP efficiently and were inoculated with seven different PVX isolates from four different geographical regions. Virus multiplication was inhibited in CP+ lines 2S4 and 2S5 (Fig. 3 and 4). For the first 15 days post inoculation (p. i.) multiplication of five tested isolates was greatly reduced. It did not exceed 20% of the corresponding virus concentrations in SR1 controls [and also in CP(–) plants, see below]. The main exceptions were the Finnish isolates P551 and S6111. Both CP+ lines 2S4 and 2S5 supported PVX_{P551} and PVX_{S6111} multiplication 10 days p.i. P551 concentrations were 1.2 to 2 times higher than those of S6111 (Fig. 3 and 4). Furthermore, in line 2S4 PVX_{cp2} virus concentrations were 10–20% of those in nontransgenic tobaccos 10 and 15 days p.i. (Fig. 3). The replication of PVX strains S, HB, cp2, P551 and S6111 in CP+ lines was 20–50% of that in nontransgenic

Fig. 2 Western blot analysis of tobacco lines expressing PVX_S CP mRNA. Proteins from 15 µl of plant leaf extract were separated on 12% SDS-PAAG, blotted to a nitrocellulose filter and probed with anti-PVX_S polyclonal antibodies. Lane 1–line 2S5; 2–2S4; 3–2S2; 4–2S1; 5–untransformed SR1 tobacco; 6–2 µg of purified PVX_S virions

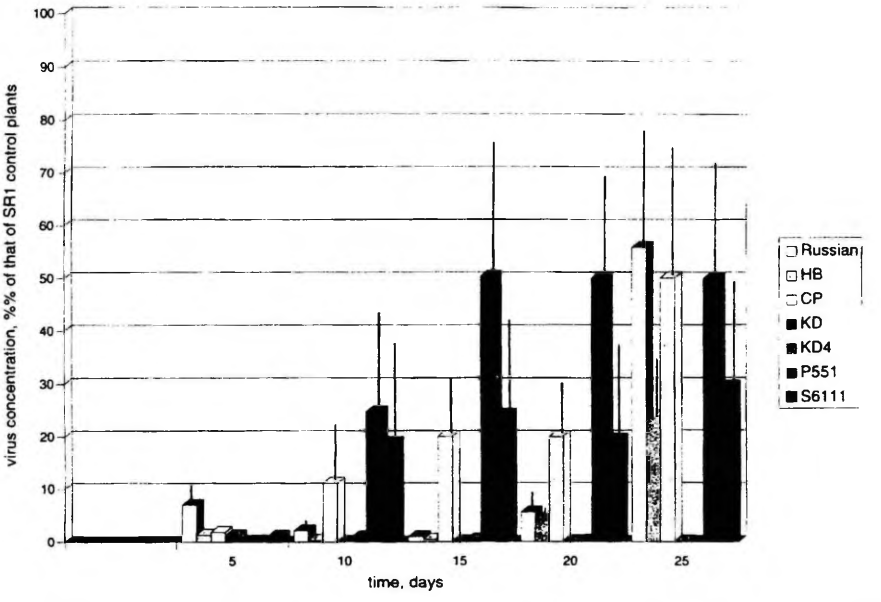

Fig. 3 Propagation levels of different PVX isolates in PVX_S CP+ tobacco line 2S4. Virus concentrations were estimated by TRFIA or ELISA analysis of leaf samples. One leaf sample from three independently inoculated plants challenged with one of the isolates used were analyzed separately for each timepoint, and the obtained results were pooled afterwards to calculate the average virus concentration. PVX isolates' concentrations in transgenic tobacco lines are expressed as a percentage of the virus concentration in nontransgenic SR1 tobaccos at the same timepoint. PVX concentrations in tobaccos expressing PVX CP antisense RNA did not differ from SR1 plants and are not shown on the figure

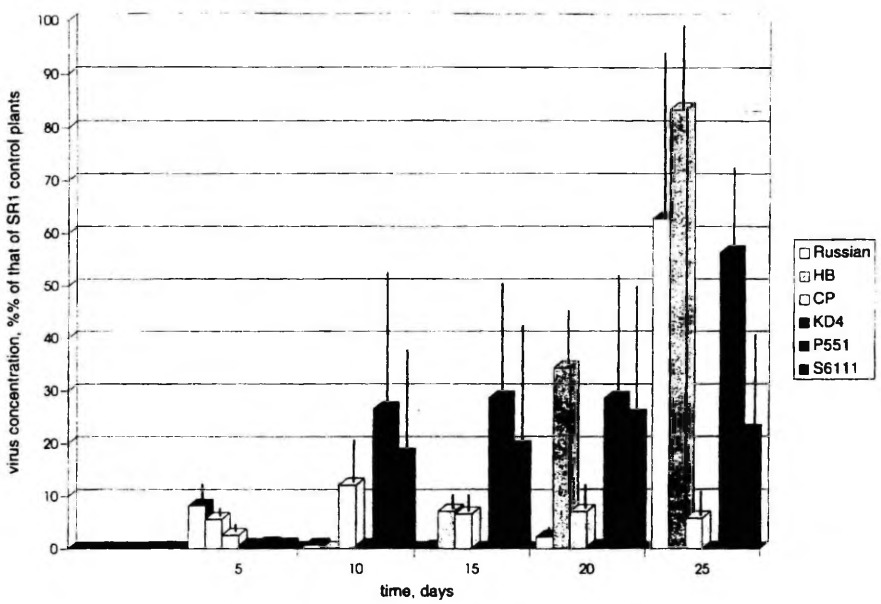

Fig. 4 Propagation levels of different PVX isolates in PVX_S CP+ tobacco line 2S5. The analyses of virus concentration, the number of analyzed samples per line and the controls were the same as in Fig. 3

plants 20 to 25 days p.i. The multiplication of strains KD and KD4, however, was completely inhibited until the end of the experiment (25 days p.i.) in both CP+ lines (Fig. 3 and 4). However, despite detectable levels of five PVX strains in CP+tobaccos, none of them were able to multiplicate in these lines as efficiently as in the nontransgenic SR1 tobaccos and transgenic plants expressing PVX_S CP antisense RNA. These plants supported rapid multiplication of all seven PVX isolates. Virus concentrations reached a plateau of more than 10 μg/ml in plant sap 5–10 days p.i. (data not shown). We were not able to detect any inhibition of virus propagation in PVX CP(−) plants with any of the isolates at inoculum concentrations of 10 μg/ml. All strains, except PVX_S, that was nearly symptomless on SR1 tobaccos, produced typical mild yellow mottling on systemic leaves. The development or absence of systemic symptoms correlated with high and low concentration of PVX CP antigen in analyzed leaf samples.

In addition to the intact tobacco plants, we determined the replication of three PVX strains in protoplasts derived from both transgenic CP+ and nontransgenic control tobacco leaves. Virus infection was observed when 500 ng of PVX virions were inoculated per 500,000 protoplasts (data not shown). At low inoculum concentrations (1 μg/500,000 cells) protoplasts expressing endogenous PVX CP were protected against different PVX isolates (S, S6111 and P551) (Table 1). At inoculum concentrations of 1 μg of PVX_S virions, replication in CP+ cells was inhibited 5.1 fold compared to replication in nontransgenic control cells. For PVX_{S6111} and PVX_{p551} the average inhibition rates were 9.8 and 5.2 folds, respectively. At 5 μg or more virions per 500,000 protoplasts, no significant differences between PVX multiplication in transgenic CP+ and nontransgenic cells were found (Table 1). Again, all three isolates tested behaved similarly. The virus concentrations measured by ELISA in protoplasts were later confirmed by the quantification of viral RNA in Northern blotting (data not shown).

Table 1 Replication of PVX isolates in tobacco protoplasts

PVX isolate	*Inoculum*	*Amount of virus (ng/100.000 cells)*[1]	
		SR1 controls	*CP+*[2]
PVX_S	1 µg	355 ± 131	69 ± 13
	5 µg	521 ± 286	421 ± 99
PVX_{P551}	1 µg	301 ± 37	58 ± 3
	5 µg	577 ± 320	397 ± 138
PVX_{S6111}	1 µg	598 ± 10	61 ± 1
	5 µg	925 ± 170	645 ± 211

[1] Protoplasts were lysed two days after inoculation and the virus content was measured by DAS-ELISA

[2] "CP+" indicates the values pooled together from data obtained with protoplasts derived from both transgenic tobacco clones 2S4 and 2S5. The clones did not display significant differences in PVX isolates replication at both concentrations tested

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S6111       TTAGTGCTTCCTTAGTGAGGACTGAACCTTGTGTCATCAAGATTAC  50
P551    GTCATTAGTGCTTCCTTAGTGAGGACTGAACCTTGTGTCATCAAGATTAC
cp2     GTCATTAGCGTGGGCTTGACTCGGACAGAGCCATGCACCATCAGAATTAC
          **** *     ***       **** ** ** **   *****  *****

S6111   TGGAGAATCAATCACAGTGTTGGCCTGCAAATTAGACGCAGAAACCATTA 100
P551    TGGAGAATCAATCACAGTGTTGGCCTGCAAATTAGACGCAGAAACCATTA
cp2     CGGTGAGTCGATCACGGTACACGCTTGCCACATAGACAGCGAGACGATCA
         ** ** ** ***** **     ** *** *  *****    ** ** ** *

                                                    ORF4→|
S6111   GAGCCATTGCCGATCTCAAGCCACTCTCCGTTGAACGGTTAAGTTTCCAT 150
P551    GAGCCATTGCCGATCTCAAGCCACTCTCCGTTGAACGGTTAAGTTTCCAT
cp2     AGGCTCTGGCTAATCTCAAACCACTCTCACTTGAGCGGTTAAGTTTCCAG
          **  * **  ******** ********  **** ***************

                  |→ORF5
S6111   TGATACTCGAAAGATGTCAGCACCAGCTAGCACAACACAGGCCACAGGGT 200
P551    TGATACTCGAAAGATGTCAGCACCAGCTAGCACAACACAGGCCACAGGGT
cp2     TGATAATTGAAAGATGACTACACCAGCCAACACCACTCAATCTGTAGGAT
        ***** * ******** *  ******** * *** ** **   *   *** *

S6111   CAACTACCTCAACTACCACAAAAACTGCAGGCGCAACTCCTGCCACAGCT 250
P551    CAACTACCTCAACTACCACAAAAACTGCAGGCGCAACTCCTGCCACAGCT
cp2     CCACCACATCAACTACCACCACTACTGCAGGCGCAACTCCTGCCA---AT
        * ** ** ************ *  ************************   *

S6111   TCAGAACTGTTCACCATCCCGGATGGGGATTTCTTTAGCACTGCCCGTGC 300
P551    TCAGGACTGTTCACCATCCCGGATGGGGATTTCTTTAGCACTGCCCGAGC
cp2     TCAGGGCTATTCACTATACCAGATGGGGACTTCTTTAGCACCGCGAAGGC
        ****  ** ***** ** ** ******** ************ **    **

S6111   CATAGTAGCCAGCAATGCCGTTGCAACAAATGAGGACCTCAACAAGATTG 350
P551    CATAGTAGCCAGCAATGCCGTTGCAACAAATCAGGACCTCAACAAGATTG
cp2     TGTGGTCGCTAGCAATGCCGTGGCCACCAACGAGGATCTCGCCAAAATAC
          * ** ** ************ ** ** **   **** ***  *** **

S6111   AGGCTATTTGGAAGGACATGAAGGTGCCCACAGACACTATGGCACAGGCT 400
P551    AGGCTATTTGGAAGGACATGAAGGTGCCCACAGACACTATGGCACAGGCT
cp2     AGGAGATCTGGAAGGACATGAAAATTCCTTCAGACACTATGGCTCAGGCA
        ***  ** ***************  * **  ************** *****

S6111   GCTTGGGACTTAGTCAGACACTGTGTTGATGTGGGCTCATCTGCTCAAAC 450
P551    GCTTGGGACTTAGTCAGACACTGTGTTGATGTGGGCTCATCTGCTCAAAC
cp2     GCTTGGGACTTAGTGAGACACTGTGCCGACGTCGGGTCCTCTGCCCAAAC
        *************** ***********  ** ** ** ** ***** *****

S6111   AGAAATGATAGATACAGGTCCCTATTCAAACGGCATCAGCAGAGCTAGAC 500
P551    AGAAATGATAGATACAGGTCCCTATTCAAACGGCATCAGCAGAGCTAGAC
cp2     TGAAATGATAGGCACCGGTCCATACTCCAATGGGGTCAGCCGGGCTAGAC
         **********  ** ***** ** ** ** **  ***** * ********

S6111   TGGCAGCAGCGATTAAAGAGGTGTGTACACTTAGGCAATTCTGCATGAAG 550
P551    TGGCAGCAGCGATTAAAGAGGTGTGCACACTTAGGCAATTCTGCATGAAG
cp2     TGGCAGCTGCCATTAAGGAGGTGTGCACACTGAGGCAGTTCTGCAAAAAG
        ******** ** *****  ******* ***** ***** ********  ***
```

Fig. 5a

5b

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S6111  TACGCCCCAGTGGTATGGAACTGGATGTTGACTAACAACAGTCCACCTGC 600
P551   TATGCCCCAGTGGTATGGAACTGGATGTTGACTAACAACAGTCCACCTGC
cp2    TATGCCCCCGTGGTCTGGAACTGGATGCTCACAAACAACAGCCCACCGGC
       ** ***** ***** ************ * ** ******** ***** **

S6111  TAACTGGCAAGCACAAGGTTTCAAGCCTGAGCACAAATTCGCTGCATTCG 650
P551   TAACTGGCAAGCACAAGGTTTCAAGCCTGAGCACAAATTCGCTGCATTCG
cp2    CAACTGGCAGGCACAAGGCTTCAAGCCGGAGCACAAATTCGCAGCCTTTG
        ******** ******** ******** ************ ** ** *

S6111  ACTTCTTTAATGGAGTCACTAACCCAGCTGCCATCATGCCCAAAGAGGGG 700
P551   ACTTCTTTAATGGAGTCACTAACCCAGCTGCCATCATGCCCAAAGAGGGG
cp2    ACTTCTTTGATGGAGTCACCAATCCTGCAGCTATCACTCCAAAAGAAGGG
       ******** ********** ** ** ** ** ****  ** ***** ***

S6111  CTCATCCGGCCACCGTCTGAAGCTGAAATGAATGCTGCCCAAACTGCTGC 750
P551   CTCATCCGGCCACCGTCTGAAGCTGAAATGAATGCTGCCCAAACTGCTGC
cp2    CTCATGAGACCTCCGTCTGAAGCAGAAATGAATGCCGCCCAAACTGCTGC
       *****  * ** *********** *********** **************

S6111  CTTTGTGAAGATTACGAAGGCCAGGGCACAATCCAACGACTTTGCCAGCC 800
P551   CTTTGTGAAGATTACGAAGGCCAGGGCACAATCCAACGACTTTGCCAGCC
cp2    CTTTGTGAAGATCACCAAGGCGAGGGCGCAATCCAACGACTTTGCCAGTC
       ************ ** ***** ***** ********************* *

S6111  TAGATGCAGCTGTCACTCGAGGTCGCATCACTGGAACAACAACCGCTGAG 850
P551   TAGATGCAGCTGTCACTCGAGGTCGCATCACTGGAACAACAACCGCTGAG
cp2    TGGATGCCGCGGTCACTAGGGGCCGCATCACAGGAACGACTGTTGCAGAA
       * ***** ** ****** * ** ******** ***** **     ** **

                          ORF5→|
S6111  GCTGTTGTCACTCTCCCACCACCATAA
P551   GCTGTTGTCACTCTCCCACCACCATAA
cp2    GCAGTTGTTTCACTACCCCCACCATAA
       ** *****  * ** ** *********
```

Fig. 5a–b Multiple nucleotide sequence alignment of the 3'-terminal part (except 3' noncoding region) of PVX isolates S6111, P551 and cp2. Conserved nucleotides among all three isolates are marked with "*". The start-point for open reading frame 5 (ORF 5, coding for the CP) and end-points for ORFs 4 and 5 are indicated

Of the seven PVX strains used in this study, CP amino acid sequences for four strains have been reported previously (S: Skryabin *et al.*, 1988; HB: Kavanagh *et al.*, 1992; KD and KD4: Santa Cruz and Baulcombe, 1993). We have PCR-cloned and sequenced the cDNAs encoding the CP of three remaining strains: P551, cp2, and S6111 (EMBL database accession nos. Z29333, Z29333, and Z29335, respectively) (Fig. 5). Overall nucleotide identity among the three strains was 67.1% in the central and C-terminal parts of PVX ORF4 (coding for 7K protein) and 79.7% in the CP gene. Amino acid sequence comparisons with PVX_S, PVX_{HB}, PVX_{KD}, and PVX_{KD4} revealed that CPs of Finnish isolates S6111 and P551 were remarkably homologus to PVX_S (Fig. 6). Overall PVX_{S6111} and PVX_{P551} amino acid sequence identities to PVX_S were 97.9% in both cases. Four out of five amino acid substitutions in PVX_{S6111} and PVX_{P551} were identical, whereas changes $D_{51}\rightarrow N$, $S_{60}\rightarrow N$ and $E_{61}\rightarrow K$ were located at positions where PVX_S CP generally differes from most other PVX isolates analyzed up to now (Fig. 6). The CP amino acids typical for only the Finnish isolates were the following (when compared with PVX_S CP): E_{31} in PVX_{S6111}, Q_{57} in PVX_{P551} and V_{88} in both isolates. Central and C-terminal parts of the CPs of these isolates were completely identical to the corresponding areas of PVX_S. The South-American isolate cp2 was almost similar to another PVX strain of the same geographic origin, namely HB (CP amino acid identity between the two strains was 96.6%, Fig. 6). The homology of cp2 CP with the PVX_S CP was much less significant – 89.5%. The amino acid sequence of PVX_{cp2} CP determined by us was, however, not identical with the sequence reported by Orman *et al.* (1990). The two PVX_{cp2} CP sequences differed at the amino acid level in 14 positions, the overall CP identity being only 94.1% (data not shown). Meanwhile, the PVX_{cp2} CP sequence has been determined also at the Sainsbury Laboratory, Norwich, and it has turned out to be 100% identical with ours (D. Baulcombe, personal communication).

```
S       MSAPASTTQATGSTTSTTTKTAGATPATASGLFTIPDGDFFSTARAIVAS
HB      -TT--N----V--------T--------N*---------------K-V---
cp2     -TT--N---SV--------T--------N*---------------K-V---
KD      --------------------------------------------------
KD4     --------------------------------------------------
S6111   ----------------------------E---------------------
P551    --------------------------------------------------

S       DAVATNEDLSEIEAIWKDMKVPTDTMAQAAWDLVRHCADVGSSAQTEMID
HB      N---------TK-QK------I-S--------------------------G
cp2     N---------AK-QE------I-S--------------------------G
KD      N---------K-------------------------------------
KD4     N---------K----------------P----------------------
S6111   N--------NK--------------------------V------------
P551    N-----Q--NK--------------------------V------------

S       TGPYSNGISRARLAAAIKEVCTLRQFCMKYAPVVWNWMLTNNSPPANWQA
HB      -------V-------------K-----R----------------------
cp2     -------V-------------------K----------------------
KD      --------------------------------------------------
KD4     --------------------------------------------------
S6111   --------------------------------------------------
P551    --------------------------------------------------

S       QGFKPEEKFAAFDFFNGVTNPAAIMPKEGLIRPPSEAEMNAAQTAAFVKI
HB      ---------------D--------T----------------------
cp2     ---------------D--------T-----M------------------
KD      --------------------------------------------------
KD4     --------------------------------------------------
S6111   --------------------------------------------------
P551    --------------------------------------------------

S       TKARAQSNDFASLDAAVTRGRITGTTTAEAVVTLPPP
HB      ----------------------A----IS----
cp2     ----------------------V-----S----
KD      ------------------------------------
KD4     ------------------------------------
S6111   ------------------------------------
P551    ------------------------------------
```

Fig. 6 Amino acid sequence alignment of CPs of seven PVX isolates. "-" marks the amino acid residue identical to PVX_S. "*" marks the deletion of the amino acid residue. CP sequences of isolates cp2, P551 and S6111 were determined in this study, sequence data for other isolates are taken from literature: S–Skryabin *et al.*, 1988; HB–Kavanagh *et al.*, 1992; KD and KD4–Santa Cruz and Baulcombe, 1993

DISCUSSION

The data that have accumulated on CPMP during the last years have revealed that nearly all transgenic virus CP-plant systems possess many unique features (Wilson, 1993). The underlying mechanism responsible for the resistant phenotype has not yet been elucidated. We have analyzed the ability of seven different PVX isolates to multiply in transgenic tobacco plants and protoplasts expressing the CP of PVX_S strain. Since several PVX isolates from different geographical regions have been characterized, PVX seems to be a good model for studying the propagation of different strains of the same virus in CP+ transgenic host. In transgenic, intact CP+ plants the multiplication levels of all seven PVX isolates were reduced when compared to the corresponding levels in the nontransgenic plants. Furthermore, the transgenic CP+ tobaccos were almost completely resistant to PVX strains KD and KD4. Surprisingly, PVX_S from which the CP transgene was derived, was among the five strains to which the resistance was less effective. This is in contrast to most previous reports because the strongest CPMP is usually achieved against the strain from which the transgenic CP is derived. However, tobacco plants expressing the coat protein of tobacco vein mottle potyvirus (TVMV) were protected against tobacco etch potyvirus but not against TVMV itself (Hull and Davies, 1992). Usually, it is believed that the efficiency of CPMP is correlated with the extent of homology between the transgenic CP and the CP of the challenging virus (see for example Stark and Beachy, 1989; Nejidat and

Beachy, 1990). This was not so with PVX strains in PVX_S CP+ tobaccos in our study. The best replicating strains S6111 and P551 shared considerable CP homology with PVX_S. S6111 and P551 possessed only one unique mutation in CP amino acid sequence $-A_{88} \rightarrow V$, when compared to other PVX strains used in this study. In addition, PVX_{S6111} CP had an amino acid substitution in position 31, where glycine was replaced by glutamic acid and PVX_{P551} CP substitution $E_{57} \rightarrow Q$. The substitution $A_{88} \rightarrow V$ may change the packing of the secondary structure elements, as valine has a larger volume than alanine. The substitutions $G \rightarrow E$ and $E \rightarrow Q$, as well as other CP mutations in strains S6111 and P551 compared to PVX_S are pseudohomologous ones (A. Efimov, personal communication). Strains HB and cp2, which differ greatly from PVX_S, replicated in CP+ lines at levels similar to or less than PVX_S (PVX_{cp2} in line 2S5, Fig. 4). PVX_S CP+ lines 2S4 and 2S5 were nearly completely resistant to hybrid PVX strains KD and KD4. The primary structures of CPs of these two strains, however, are remarkably similar to PVX_S CP. Besides two amino acid changes in KD and KD4 (which are characteristic of all PVX strains studied except PVX_S), strain KD4 has a unique mutation at position 78 ($Q \rightarrow P$). This amino acid change enables PVX_{KD4} to overcome *Nx* gene induced hypersensitivity (Santa Cruz and Baulcombe, 1993). However, since both strains were similarly unable to multiply in CP+ clones 2S4 and 2S5, this amino acid substitution has no major role in the resistance phenomenon in the CP+ transgenic plants.

In conclusion, our data indicate that the similarity of the infecting virus CP to the transgenic CP does not determine the virus replication rates in the transgenic host. This observation is not consistent with most data reported on analogous virus-host plant interactions. However, the CPMP to potexviruses seems to possess some unique details in comparison to other plant RNA virus groups. PVX RNA is not able to overcome CPMP, whereas CPMP to naked viral RNA was not observed with most other viruses studied (other exceptions besides potexviruses to this rule are alfalfa mosaic virus and nepo- and carlaviruses; Fitchen and Beachy, 1993). We are currently studying the multiplication of different PVX strains RNAs in PVX_S CP+plants and protoplasts.

The lack of correlation between protection levels and homology between challenging viruses and PVX_S CP indicates that, in addition to the CP gene, other as yet undetermined portions of the PVX genome could be responsible for the interactions resulting in reduced PVX replication and movement in PVX CP+ plants.

Finnish PVX isolates S6111 and P551, which multiplied in intact PVX_S CP+ tobacco plants faster than PVX_S and other strains, did not, however, differ from PVX_S in their ability to replicate in CP+ tobacco protoplasts. At low inoculum concentrations the replication of all three isolates was suppressed in CP+ cells. By increasing the inoculum concentrations protection effect was abolished. We are not aware of any earlier studies on PVX replication in PVX CP+ protoplasts. Data obtained on other model systems are rather controversial. Alfalfa mosaic virus (AIMV) CP+ protoplasts did not support AIMV replication (Hill *et al.*, 1991). The expression of TEV CP in protoplasts did not protect cells from TEV (Lindbo and Dougherty; 1992a, b), whereas protoplasts derived from young, virus-free leaf tissue of TEV CP+ plants, "recovered" from the virus infection, were resistant to TEV (Lindbo *et al.*, 1993). Transgenic TMV CP+ protoplasts were protected against TMV, when TMV virions were used as inoculum (Register and Beachy, 1988; Clark *et al.*, 1990). However, in a few CP+

cells, where TMV infection was initiated, virus replication rates did not differ from nontransgenic protoplasts (Register and Beachy, 1988). As PVX_{S6111} and PVX_{P551} started to replicate in intact CP+ plants faster and more efficiently than PVX_S, our hypothesis is that two different mechanisms are involved in CPMP against PVX – one functioning on the replication level at low virus concentrations (as shown in protoplast tests) and the other affecting virus spread in intact plants.

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II

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THE EFFECTS OF 2-5A ON PROTEIN SYNTHESIS IN WHEAT GERM EXTRACTS AND TOBACCO PROTOPLASTS

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Abstract. Nonphosphorylated 2-5A inhibited translation and caused RNA degradation in wheat germ extract, whereas 3-5A had no effect. Protein synthesis inhibition by 2-5A was observed in tobacco protoplasts. 70 kD 2-5A-binding protein was found in potato leaf extracts by chemical crosslinking.

Interferons (IFNs) are antiviral proteins secreted by animal cells in response to not only viral infection, but also cell proliferation and various immunological processes.[1] The 2'-5' oligoadenylate pathway is also affected by IFN. IFN binding to cell surface receptors induces the synthesis of 2'-5' oligoadenylate synthetase, which in the presence of double-stranded RNA polymerizes ATP to form a series of unique 2'-5' oligoadenylates (2-5A). 2-5A trimers and longer oligomers bind to and activate a latent endoribonuclease (RNase L), which leads to the inhibition of protein synthesis by degrading mRNA. Some components of the 2-5A pathway have been detected in plants. Devash *et al.* have demonstrated that 2-5A can inhibit tobacco mosaic virus (TMV) replication in tobacco protoplasts, leaf discs, and intact plants. 2-5A trimer "cores" are the most potent inhibitors.[2,3] On the basis of these data Devash *et al.* postulated that a system analogous to the mammalian 2-5A pathway may exist in higher plants. Two glycoproteins (gp22 and gp35), which production is stimulated by virus infection, were purified from plants and found to cross-react with human β-interferon polyclonal antibodies.[4] Later, they were identified as an isoform of the pathogenesis-related protein 5 and β-1,3-glucanase.[5] Reichman *et al.* reported ATP-polymerizing activity in antiviral factor-treated leaves of *Nicotiana glutinosa* giving rise to plant

oligonucleotides with antiviral activity.[6] Later Devash *et al.* demonstrated the poly(rI)· poly(rC)-dependent synthesis of oligoadenylates in tobacco leaves and cell cultures.[7] They claimed, however, that these oligoadenylates differed substantially from the mammalian 2-5A.[7] Recently Kulaeva *et al.* have shown that human IFN and 2-5A increase the cytokinin content and induce the synthesis of various proteins in plant cells.[8] However, IFN did not appear to inhibit the replication of turnip yellow mosaic virus and alfalfa mosaic virus.[9-11] DNA sequences homologous to human 2-5A synthetase were found in tobacco genomic DNA and a 2-5A synthetase was purified from tobacco and found to cross-react with human 2-5A synthetase antibodies.[12] We have not detected the sequences hybridizing to rat 2-5A synthetase cDNA in tobacco and potato mRNA or genomic DNA.[13,14] But we have found 2-5A-degrading activity in tobacco leaf extracts.[14] Little is known about other 2-5A-binding proteins of the plant 2-5A pathway. Devash *et al.* could not detect (2'-5')p_3A_4[^{32}P]pCp-binding proteins in *N. glutinosa* leaves.[7] As plant oligoadenylates could not activate RNase L from rabbit reticulocyte lysates (RRLs) and did not compete with (2'-5')p_3A_4[^{32}P]pCp for binding to mammalian RNase L, Devash *et al.* concluded that the oligoadenylate-dependent protein synthesis inhibition in plants occurs without activating a 2-5A-dependent RNase.[7] Cayley *et al.* could not detect 2-5A-binding proteins in tobacco either.[15]

Mammalian 2-5A-synthetase has recentiy been expressed in transgenic plants and provides protection against several plant RNA viruses.[13,14,16,17] These data indicate that 2-5A-binding protein(s) may exist in plant cells. In this study we report for the first time the presence of a 2-5A-binding protein in potato leaf extracts. We show that the 2-5A trimer "core" is the most potent inhibitor of translation in a plant cell-free system and *in vivo* in tobacco protoplasts.

MATERIALS AND METHODS

Purification of 2-5A oligomers

2'-5' and 3'-5' oligoadenylates, chemically synthesized by Prof. I. A. Mikhailopulo, Institute of Bioorganic Chemistry, Minsk, Belarus, as nonphosphorylated "core" forms, were finally purified by HPLC.[18] Synthesis of the mono-, di- and triphosphate forms was performed by Prof. Mikhailopulo's group by sequential addition of 5' phosphate groups to the "core" molecules.[19] 2-5A trimers and tetramers with differing degrees of phosphorylation were purified with HPLC on amino-Sil-X-1 columns (Tessek, Czechia) using a DuPont 8810 chromatograph with UV detector. A concentration gradient of buffer A (20% acetonitrile in 6.25 mM K-phosphate buffer, pH 6.45) and buffer B (13% acetonitrile with 0.6 M $LiClO_4$ in 6.25 mM K-phosphate buffer, pH 6.45) was used with a gradient of 50% to 100% of buffer B in 15 min. Samples were desalted by lyophilization and stored at -20°. Prior to use the samples were dissolved in sterile RNase-free double-distilled water.

In addition, we used independently synthesized 2-5A trimers (a mixture of oligomers with varying degrees of phoshorylation) provided by Prof. W. E. G. Müller, University of Mainz, Germany. These oligomers were treated with calf intestine alkaline phosphatase (Boehringer Mannheim) according to the manufacturer's instructions. The resulting nonphosphorylated trimers were purified on HPLC using Supelcosil LC-18 column (Supelco) in a concentration gradient of buffer A (0.1 M triethylammoniumacetate, pH 7) and buffer B (0.1 M triethylammoniumacetate in 40% acetonitrile) with a gradient of 10% to 30% of buffer B in 30 min. Samples were lyophilized, stored, and dissolved as described above.

ATP, AMP and adenosine were purchased from Sigma.

In vitro *translation assays*

Assays in wheat germ extract (WGE, Amersham or Promega) and rabbit reticulocyte lysate (RRL, Amersham) were carried out according to

manufacturers' instructions, using 0.8 μg of TMV RNA (prepared from TMV particles according to[20]) or 0.5 μg of brome mosaic virus (BMV) RNAs (Promega) and 5 μCi (WGE) or 3.5 μCi (RRL) of DL-[4,5-^{3}H]Leu (34 Ci/mmol, Amersham) or 10 μCi of L-[^{35}S]Met (>1000 Ci/mmol, Amersham) per reaction (30 μl). Samples (5 μl) were collected on Whatman GF/C filters, incubated on ice 5 min with cold 5% trichloroacetic acid (TCA), heated 10 min at 90°C, incubated again on ice for 5 min with cold TCA, washed sequentially with 30% H_2O_2 (omitted for WGE), water, ethanol, and acetone and then dried with a warm air blower. The dried filters were counted using a toluene-based scintillant on LKB Wallac Rackbeta 1215 scintillation counter. BMV RNA translation products were separated by 12% PAGE in the Laemmli system[21], gels dried and autoradiographed. Autoradiographs were scanned with an LKB 2202 UltroScan laser densitometer to quantify the amount of synthesized proteins.

Covalent binding of [^{32}P]pCp-labeled 2-5A to proteins in cell extracts

Nonphosphorylated HPLC-purified 2-5A and 3-5A tetramers were ligated to cytidine-3',5'-[5'-^{32}P]-bisphosphate (pCp, 3000 Ci/mmol, Amersham) following the method described by Knight *et al.*[22] and A_4[^{32}P]pCp purified in an HPLC system using a reverse phase ODC C18 column (DuPont) according to.[23] The 3'-phosphate was removed by *Escherichia coli* alkaline phosphatase type III (Sigma) according to[24] and the dephosphorylated products purified by HPLC on an ODC C18 column. The chemical crosslinking procedure was carried out essentially as described by Wreschner *et al.*[24] UV crosslinking was done essentially as described by Nolan-Sorden *et al.*,[25] performing the irradiation in Stratalinker (Stratagene) twice at maximum energy at the distance of 6 cm from the bulbs. Extracts of potato (*Solanum tuberosum*) leaves, wheat germ, mouse L-cells, and mouse spleen were used as the sources of oligoadenylate-binding proteins. The binding specificity of labeled 2-5A to proteins was controlled in competition assays, by adding excess (100 μM) unlabeled 2-5A and 3-5A to the reaction mixtures. The

reaction mixtures were separated on 12% SDS-PAGE as described above.

RNA degradation analysis

Samples were taken from *in vitro* translation mixtures after 0, 15, and 30 min incubation and frozen in liquid nitrogen. RNA was isolated from the samples as described by us earlier,[26] analyzed by electrophoresis in 1% agarose/formamide gels and Northern blotted to Hybond™ nylon membranes according to Amersham protocols. ^{32}P-labeled cDNA was synthesized on TMV RNA templates according to[27] using hexanucleotide random primers and [^{32}P]dNTP (4000 Ci/mmol, Radiopreparat, Uzbekistan). To quantify the RNA degradation, autoradiographs were scanned by laser densitometer as described above.

Protein synthesis in tobacco protoplasts

Protoplasts from *N. tabacum* SR1 leaves (grown on MS[28] agar medium at 24°C during a 16 h photoperiod) were obtained according to[29], except that after enzyme treatment protoplasts were washed and maintained in K3 medium[30] containing 400 mM sucrose. 1 μCi of ^{14}C-labeled protein hydrolysate (0.5 Ci/milliatom C, Chemapol, Czechia) per 10^6 protoplasts was added. Samples were collected after 0, 30, 60, 90, and 120 min incubation and frozen in liquid nitrogen. After homogenization with half a volume of 100 mM Tris-HCl pH 7.8, proteins were precipitated with three volumes of ice-cold 10% TCA for 30 min and collected on Schleicher & Schuell No. 6 filters. The filters were dried and counted in toluene-based scintillant. To ensure that the 2-5A "core" does not inhibit the uptake of amino acids by tobacco protoplasts, a control experiment was carried out. In the control, besides the ^{14}C-labeled protein hydrolysate, cycloheximide at a final concentration of 1 mM was added to prevent *de novo* protein synthesis. After a 30 min incubation, the cells were washed three times with growth medium and total cellular radioactivity was counted in a dioxane-based scintillant.

RESULTS

Purification of 2-5A oligomers

The separation of 2-5A oligomers having the same chain length but different degrees of phosphorylation was achieved with HPLC using a weak anionite amino-Sil-X-1 columns and a lithium perchlorate gradient in volatile buffer (FIG. 1). The purity of individual 2-5A oligomers was greater than 99%. The purity of "core" forms, obtained from the Institute of Bioorganic Chemistry, Minsk was checked on HPLC in methanol gradient according to Brown *et al.*[23] It was always higher than 99%. 2-5A trimers from the University of Mainz, after the enzymatic dephosphorylation and HPLC purification, had the same degree of purity.

The effect of 2-5A, 3-5A, ATP, AMP, and adenosine on TMV RNA translation in WGE and RRL

The effects of various 2-5A derivatives, 3-5A tetramers, ATP, AMP, and adenosine on the cell-free translation of TMV RNA in WGE and RRL are presented in TABLE 1. The 2-5A trimer "core" (A_3) and tetramer "core" (A_4) were the most potent inhibitors among the 2-5A derivatives. The inhibitory effects of the phosphorylated 2-5A trimers pA_3, ppA_3, and $pppA_3$ were significantly lower than that of the 2-5A trimer "core". The same is true for 2-5A tetramer derivatives (TABLE 1). Unlike the triphosphorylated trimers, 200 nM $pppA_4$ activated TMV RNA translation in WGE. Micromolar concentrations of 3-5A tetramer "core", ATP, AMP, and adenosine had no effect on TMV RNA translation in WGE. No differences were observed in translation inhibition rates when 2-5A trimer "cores" provided either by Prof. Mikhailopulo or Prof. Müller were used (data not shown).

In RRL, the most potent inhibitors of protein synthesis, as expected, were triphosphorylated oligoadenylates. The tetramer triphosphate showed a greater inhibitory effect than corresponding trimer (TABLE 1). The concentration dependence of 2-5A trimers and tetramer "cores" on the

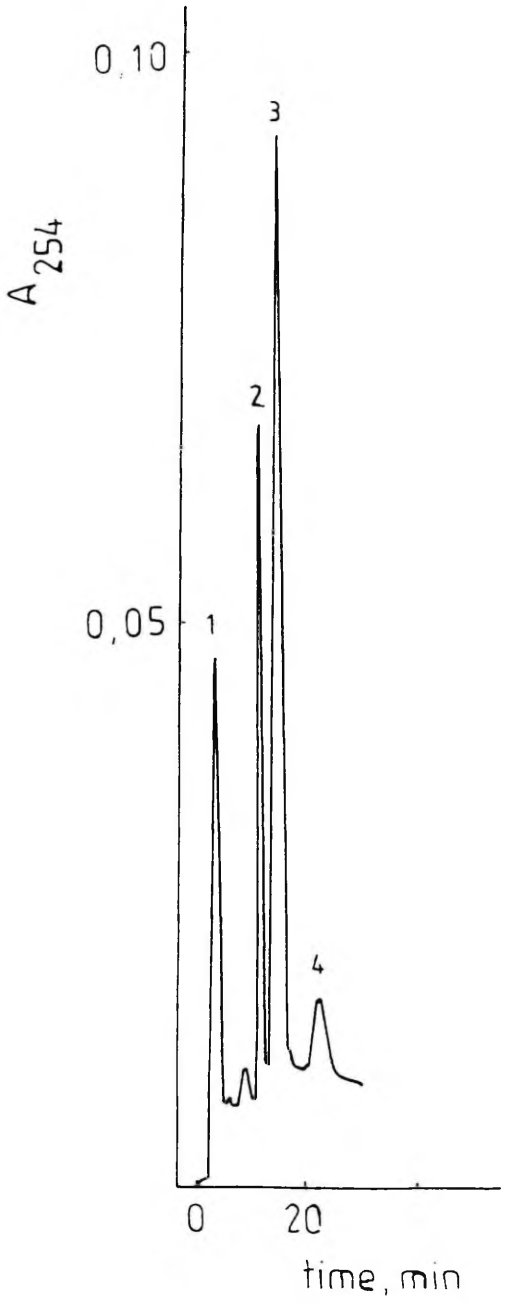

FIG. 1. Purification of 2-5A trimers with HPLC. Column (3.5 x 140 mm) with amino-Sil-X-1, sample volume was 20 µl. 1 - p_3A_3, 2 - p_2A_3, 3 - pA_3, 4 - A_3.

TABLE 1. The inhibition of TMV RNA translation in WGE and RRL by 2-5A, 3-5A, ATP, AMP, and adenosine. The translation rate in WGE and RRL in the presence of 2-5A tri- and tetramers with the different phosphorylation, 3-5A tetramers, ATP, AMP, and adenosine is shown as % of that without 2-5A (positive control).

Added nucleotide	WGE[a]	RRL[a]
positive control	100	100
3 μM adenosine	102.5 ± 11.9	nd[b]
3 μM AMP	108.2 ± 16.9	nd
3 μM ATP	104.6 ± 15.0	nd
1 μM 3'-5' A_4	100.3 ± 9.2	nd
1 μM A_3	27.4 ± 7.4	93.6 ± 4.6
1 μM pA_3	92.3 ± 7.3	95.9 ± 2.8
1 μM ppA_3	66.9 ± 4.5	78.4 ± 5.3
1 μM $pppA_3$	87.9 ± 6.6	63.1 ± 5.4
200 nM A_4	62.5 ± 3.6	81.0 ± 7.0
200 nM pA_4	82.0 ± 4.3	63.0 ± 8.1
200 nM ppA_4	84.5 ± 9.9	55.0 ± 12.1
200 nM $pppA_4$	121.6 ± 2.3	44.8 ± 11.3

[a]The samples were taken from the *in vitro* translation mix and the incorporation of [^{3}H]-leucine into protein was measured as described in Materials and Methods.
[b]Not determined.

inhibition of WGE protein synthesis was studied separately. Micromolar concentrations of the 2-5A trimer and tetramer "cores" were necessary for the inhibitory effect (FIG. 2 A and B).

2-5A tetramer "core" also inhibits BMV RNA *in vitro* translation in WGE.In the presence of 1 μM A_4 "core" the amount of four major BMV translation products is reduced at least five-fold when compared to the control (Fig. 3). Laser densitometer scanning revealed an equal reduction in translation of all four major proteins.

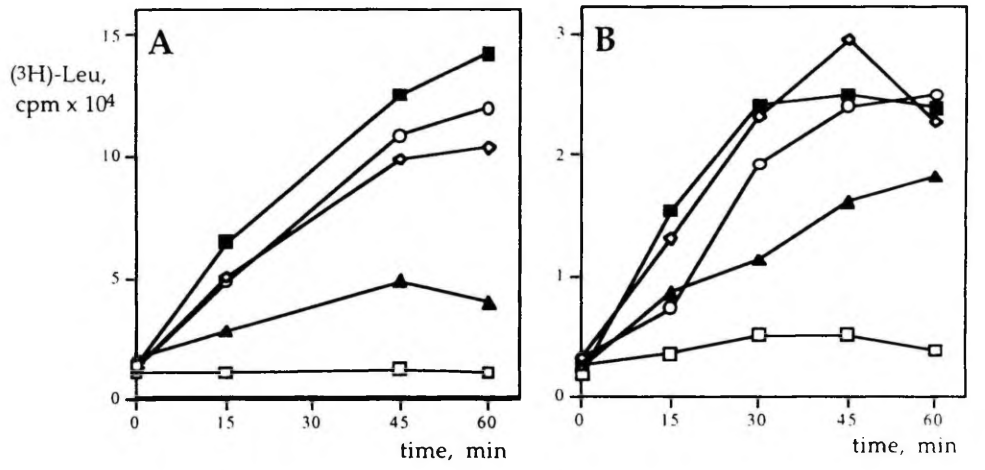

FIG. 2. The inhibition of TMV RNA translation in WGE by 2-5A. The dependence of inhibition on 2-5A trimer (A) and tetramer (B) "core" concentrations. —■— pos.control (no 2-5A added) , —◇— 1 nM 2-5A, —○— 100 nM 2-5A, —▲— 1000 nM 2-5A, —□— neg. control (no TMV RNA added).

Covalent cross-linking of [^{32}P]pCp-labeled 2-5A to proteins in cell extracts

The chemical crosslinking of [^{32}P]pCp-labeled 2-5A "cores" to proteins in cell extracts and the subsequent separation of labeled products by electrophoresis in SDS gels revealed an approximately 70 kD 2-5A-binding protein in potato leaf extracts (FIG. 4, line 5) and an 80 kD protein in mouse L-cell extracts (FIG. 4, line 3). A weak 70 kD band was also observed in WGE (data not shown). In mouse L-cell extracts, an additional minor crosslinked protein of approximately 40 kD was also found. The specificity of the binding reaction in potato leaf extracts (FIG. 4, line 4) and mouse L-cell extract (FIG. 4, line 2) was shown in competition assays where labeled 2-5A was completely displaced by unlabeled 2-5A. When the chemical crosslinking experiment was repeated with potato leaf extracts using A_3[^{32}P]pCp, a more potent protein synthesis inhibitor in plant cells, essentially the same results were obtained (data not shown).

FIG. 3. The inhibition of BMV RNA translation in WGE by 2-5A tetramer "core". Translation products were labeled with [^{35}S]Met, separated by electrophoresis in 12% SDS-PAGE, dried, and autoradiographed. 1 - BMV RNA translation with 1 μM of 2-5A$_4$ "core", 2 - BMV RNA translation without 2-5A, 3 - no added RNA.

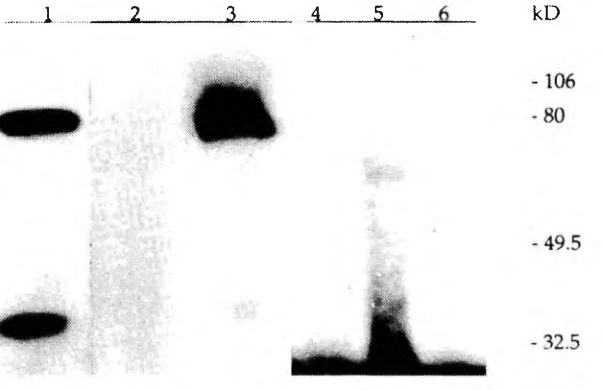

FIG. 4. Covalent binding of [^{32} P]pCp-labeled 2-5A (lines 1--5) and 3-5A (line 6) tetramer "cores" to proteins in cell extracts, using the periodate oxidation method and UV-crosslinking. 100 nM of [^{32}P]pCp-labeled 2-5A was used per reaction. Electrophoresis in 12% SDS-polyacrylamide gel. 1 - UV-crosslinking of mouse spleen extract, 2 - chemical crosslinking of mouse L-cell extract in the presence of unlabeled 2-5A, 3 - chemical crosslinking of mouse L-cell extract, 4 - chemical crosslinking of potato leaf extract in the presence of unlabeled 2-5A, 5 - chemical crosslinking of potato leaf extract, 6 - chemical crosslinking of potato leaf extract with 3-5A[^{32}P]pCp . The apparent molecular masses of protein standards are indicated in kD.

When ^{32}P-labeled 3-5A tetramer "core" was chemically crosslinked to potato leaf extract, no proteins were detected (FIG. 4, line 6), even after the very long exposure times.

Surprisingly, when mouse spleen extract was used for chemical crosslinking under the same conditions, no 2-5A-labeled proteins were detected (data not shown). Using an independent method of UV crosslinking, we found 80 kD and 40 kD 2-5A tetramer-binding proteins in mouse spleen extract (FIG. 4, line 1), which were efficiently displaced by nonlabeled 2-5A tetramer (data not shown). In potato leaf extracts, UV crosslinking did not produce any labeled proteins. The results of the UV crosslinkings of ^{32}P-labeled 3-5A tetramer "cores" with mouse spleen and potato leaf extracts were also negative (data not shown).

TMV RNA degradation in cell-free systems

The time dependence of RNA degradation in the presence or absence of 2-5A trimer triphosphate or trimer "core" in WGE was studied. Addition of trimer "core" led to a rapid degradation of TMV RNA in WGE, which was not observed with control RNA (FIG. 5). As expected, in RRL the addition of 2-5A trimer triphosphate caused rapid degradation of TMV RNA. $pppA_3$ treated TMV RNA in WGE was almost as stable as the control sample without 2-5A (FIG. 5). 2-5A "core"-dependent TMV RNA degradation indicates the presence of a 2-5A "core" activated ribonuclease in plant extracts. The opposite effects of different 2-5A forms on RNA degradation rates in WGE and RRL indicate also that the protein synthesis inhibition was not caused by ribonuclease contamination in 2-5A samples.

Inhibition of protein synthesis in protoplasts

Nonphosphorylated derivatives of 2-5A, in contrast to phosphorylated, are able to penetrate through the mammalian cell membrane.[31,32] Our results show that this may also occur in plant protoplasts. The addition of 2-5A trimer "cores" to *N. tabacum* SR1 protoplasts (final concentration 1 μm)

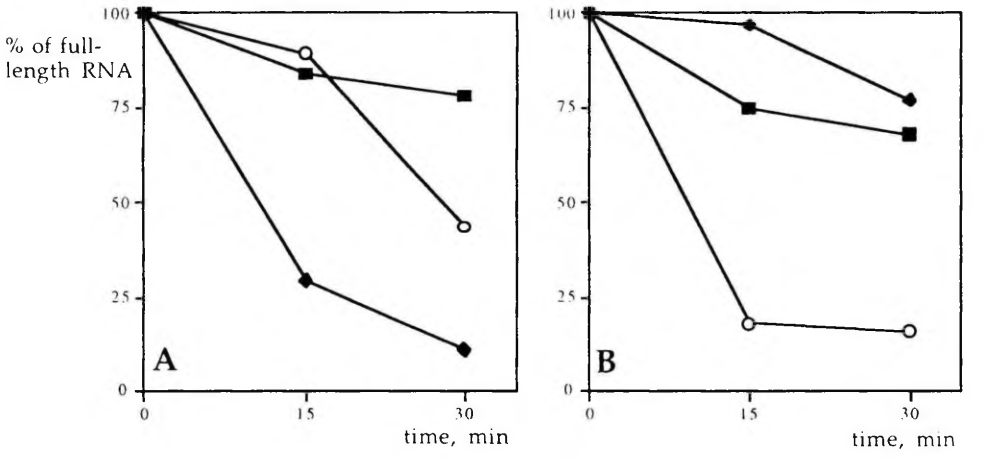

FIG. 5. The effect of 1 μm concentration of 2-5A trimer triphosphate and trimer "core" on TMV RNA degradation in WGE (A) and in RRL (B). RNA was electrophoresed on 1% agarose/formamide gels, blotted to nylon filter and hybridized with ^{32}P-labeled TMV cDNA. The autoradiographs were scanned with a laser densitometer. —■— pos. control (no 2-5A added), —◆— A_3, —○— $pppA_3$

reduced the rate of protein synthesis by up to two-fold (FIG. 6). The control experiment demonstrated that total cellular radioactivity in the presence or absence of 2-5A remained the same when *de novo* protein synthesis was blocked by cycloheximide and the protoplasts were exposed to radioactive amino acids (data not shown). This indicates that lower incorporation of labeled amino acids in the presence of 2-5A was not due to the inhibition of cellular amino acid uptake but rather to protein synthesis inhibition in both *in vitro* and *in vivo* in plant systems.

DISCUSSION

In contrast to mammalian cell-free systems, 2-5A trimer and tetramer "cores" were the most potent inhibitors of the *in vitro* translation in the

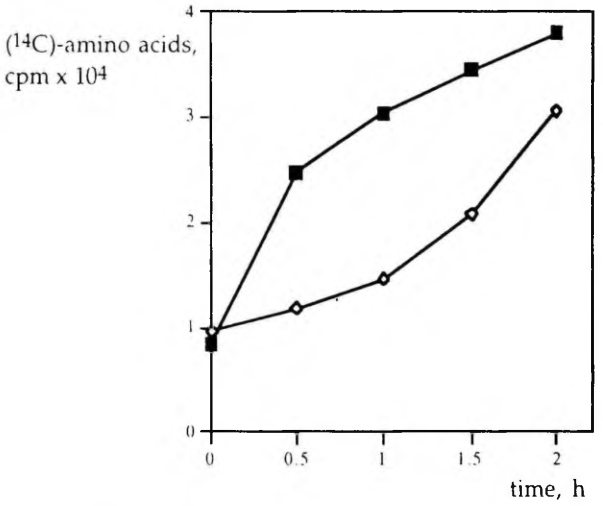

FIG. 6. The effect of 2-5A trimer "cores" on *in vivo* protein synthesis in *N.tabacum* SR1 protoplasts. —■— without 2-5A, —◇— with 1μM 2-5A_3

plant cell-free system. The inhibitory effect of phosphorylated 2-5A derivatives was significantly lower. Therefore, the inhibitory effect of "cores" due contamination by phosphorylated 2-5A forms is ruled out. The purity of "cores" was shown also by HPLC analysis. The contamination of "core" preparations with enzymes such as phosphatases is also very unlike, as these were prepared using direct chemical synthesis. The concentration of 2-5A needed for efficient inhibition of viral RNA translation in WGE was about 1 μM (FIG. 2 A and B), which is about two to three orders of magnitude higher than that needed for the inhibitory effect of di- or triphosphorylated 2-5A trimers and tetramers in mammalian systems.[33,34] Although relatively high concentrations of nonphosphorylated 2-5A are needed for the *in vitro* inhibition of TMV and BMV RNA translation, this inhibition is 2-5A-specific since 3-5A, ATP, AMP, and adenosine nucleotides have no effect on translation in WGE (TABLE 1). The inhibition of protein synthesis by contaminating salts or ribonucleases is ruled out since the inhibition was

specific to certain 2-5A forms. "Core" forms inhibited protein synthesis in WGE, but were inactive in the mammalian cell-free system. Phosphorylated forms had an opposite effects in these two systems. In addition, we have shown that the traces of salts present in the HPLC-purified 2-5A samples do not inhibit *in vitro* protein synthesis.[35] Micromolar concentrations of 2-5A trimer "cores" also inhibited protein synthesis *in vivo* in protoplasts of *N. tabacum* (FIG. 6). Our finding that dephosphorylated 2-5A molecules were the best inhibitors of protein synthesis in plant systems agree with the results of Devash *et al.*[2,3]

TMV RNA translation inhibition by 2-5A trimer and tetramer "cores" is accompanied by TMV RNA degradation. An activation of a putative plant 2-5A-dependent ribonuclease may occur. This degradation is not TMV-specific, since the levels of translation products of all four BMV RNAs were diminished in the presence of 2-5A tetramer "core".

We demonstrated the existence of an approximately 70 kD 2-5A-binding protein in potato leaf extracts (FIG. 4). In plant cells, the 2-5A-binding protein had a lower molecular weight than in mammalian cells - about 70 kD versus 80 kD in mouse L-cell and mouse spleen extracts. In mammalian extracts we observed a smaller 40 kD 2-5A-binding protein, which was reported by Bisbal *et al.*[36] We were able to detect plant 2-5A-binding protein by the periodate oxydation method, but not by UV crosslinking.

Three lines of evidence support the existance of a 2-5A-activated ribonuclease in plants: i) micromolar concentrations of 2-5A trimer and tetramer "cores" inhibit protein synthesis in wheat germ extract and *N.tabacum* protoplasts; ii) the addition of 2-5A "cores" induces the degradation of TMV RNA in wheat germ extract; iii) 70 kD protein is specifically crosslinked to [^{32}P]2-5A in potato leaf extracts.

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III

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Homologies Between Different Forms of 2-5A Synthetases

E. Truve[1], M. Kelve[1], A. Aaspollu[1], H.C. Schröder[2], and W.E.G. Müller[2]

1 Introduction

(2'-5') Oligoadenylate synthetases (2-5A synthetases; EC 2.7.7.19) are present in mammalian cells and tissues and synthesize from ATP a series of oligomers termed 2-5A [general formula: $ppp(A2'p)_nA$; with $1 \leq n < 18$ and usually $1 \leq n < 6$] (Hovanessian 1991). For full enzymic activity of the 2-5A synthetases, binding of double-stranded RNA is required (Sen 1982). Three principal 2-5A synthetase isoenzymes have been described with M_r's of 40–46, 69, and 100 kDa (Chebath et al. 1987; Hovanessian et al. 1987, 1988). In the following they are classified as 2-5A synthetase I [M_r 40–46 000], II [M_r 69 000] and III [M_r 100 000]. All three isoforms are induced in cells by interferon (Cohen et al. 1988; Rutherford et al. 1988). 2-5A synthetases I and II are present in both the nucleus and the mitochondria as well as in the rough/smooth microsomal fraction, while 2-5A synthetase III is associated with the rough microsomal fraction only (Hovanessian et al. 1987). The enzymic product, 2-5A, functions as an activator of the endoribonuclease L. 2-5A is rapidly degraded either by the relatively unspecific phosphodiesterase (Schmidt et al. 1979; Johnston and Hearl 1987) or the specific 2',3'-exoribonuclease (Müller et al. 1980; Schröder et al. 1980, 1984).

Until now the 2-5A synthetase I has been cloned from humans (1.6 kb mRNA; accession numbers: X04371 and M25352; Wathelet et al. 1986) and mice (X58077: Rutherford et al. 1991; P11928: Ichii et al. 1986; M63849: Ghosh et al. 1991; M63860: Ghosh et al. 1991). Here, we present the nucleotide sequence of rat 2-5A synthetase I cDNA (Z18877). The human 2-5A synthetase II (M87284) has recently been cloned by Marié and Hovanessian (1992). No species of 2-5A synthetase III has so far been cloned.

2 Primary Structure of the Rat 2-5A Synthetase cDNA

The cDNA insert coding for 2-5A synthetase I was isolated from *Rattus norvegicus* (hippocampus cDNA library) and is 1421 nt long; it is termed

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RN25ASYN; the base composition (in %) was A = 25.6, T = 20.1, G = 26.8 and C = 27.0. The open reading frame with the ATG codon for methionine is 1077 bp long and follows downstream the sequence CCGGAGGTC, a consensus sequence for the translational start (Kozak 1984). An inverted repeat GAAAGCTTTC is present close to the initiation codon (nt −13 to nt −4). The stop codon is TGA (nt +1074). The typical signal polyadenylation site AATAAA (Zarkower et al. 1986) is found at nt + 1333 to 1338 (Fig. 1). One inverted repeat each is present in the 5'- and 3'-untranslated region of RN25ASYN. No relevant homologies were found on the nt level for invertebrates in the EMBL Data Bank (CDEM33IN).

3 Amino Acid Sequence of Rat 2-5A Synthetase

The aa sequence of rat 2-5A synthetase was deduced from the nucleotide sequence of RN25ASYN cDNA (PC/Gene: TRANSL) (Fig. 2). The cDNA encodes a 41 582 dalton primary translation product, indicating that the cloned rat 2-5A synthetase belongs to class I. It consists of 12% aromatic, 35% hydrophobic and 25% charged aa, resulting in an aliphatic index of 84.6. The

```
                                                5'-end      - 69   AGCTCCACC
-   60 GCGGTGGCGCCGCGCGAGACACAGGACCTGCAGGCTGCAGAGGCAAAAGCTCCGGAGGTC
+    1 ATGGAGCAGGAACTCAGGAGCACCCCGTCCTGGAAGCTGGACAAGTTCATAGAGGTTTAC
+   61 CTCCTTCCAAACACCAGCTTCCGTGATGATGTCAAATCAGCTATCAATGTCCTGTGTGAT
+  121 TTCCTGAAGGAGAGATGCTTCCGAGATACTGTCCACCCAGTGAGGGTCTCCAAGGTGGTG
+  181 AAGGGTGGCTCCTCAGGCAAAGGCACCACACTCAAGGGCAAGTCAGACGCTGACCTGGTG
+  241 GTGTTCCTTAACAATTTCACCAGCTTTGAGGATCAGTTAAACAGACGGGGAGAGTTCATC
+  301 AAGGAAATTAAGAAACAGCTGTATGAGGTTCAGCGTGAAAAACATTTTAGAGTGAAGTTT
+  361 GAGGTCCAGAGTTCATGGTGGCCCAACCCCCGGGCTCTGAGCTTCAAGCTGAGTGCACCA
+  421 CACCTCCAACAGGAGGTGGAGTTTGATGTGCTTCCAGCCTATGATGTCCTAGGTCATGTT
+  481 AGCCTCTACAGCAATCCTGATCCCAAGATCTACACCATCCTCATCTCCGAATGTATCTCC
+  541 CTGGGGAAGGATGGCGAGTTCTCTACCTGCTTCACGGAGCTCCAGAGGAACTTCCTGAAG
+  601 CAGCGCCCAACCAAGCTGAAGAGTCTCATCCGCCTGGTCAAGCACTGGTACCAACTGTGT
+  661 AAGGAGAAGCTGGGGAAGCCGCTGCCCCCACAGTACGCCCTGGAGCTGCTCACGGTCTAT
+  721 GCCTGGGAACGTGGAAATGGAATTACTGAGTTCAACACAGCTCAGGGCTTCCGGACAATC
+  781 TTGGAACTGGTCACAAAGTACCAGCAGCTTCGAATCTACTGGACAAAGTATTATGACTTT
+  841 CAACACCCAGATGTCTCCAAATACCTACACAGACAGCTCAGAAAATCCAGGCCTGTGATC
+  901 CTGGACCCTGCTGACCCAACAGGGAACGTGGCTGGTGGGAACCAAGAAGGCTGGCGGCGG
+  961 TTGGCCTCAGAGGCGAAGCTGTGGCTGCAGTACCCATGTTTTATGAACACCGGTGGTTCC
+1021 CCAGTGAGTTCCTGGGAAGTGCCGGTGGATGAGGCCTGGTCATGCATCCTGCTGTGA #
+1078 ACCCAGCAGCACCAGCCCAGGAGGCTCCGGAGTCAGGGGCACGTGCTGCTCTGCTGCAGG
+1138 ACCTTGACACAGTGAGGGAGGCCCCACTCGGGATCACAGTCCATGCTTCTGATGCCCGCC
+1198 CGCCATGGTTGAATACTGTCCAATCACAGACAGCCTTCCTCAACAGATTCAGAAGGGGCG
+1258 GAAAGAACTCAAGCTTGACTTCCATCTGACCGTCCACCTGTTGGGAGGTTCTGTCCAACC
+1318 ATGTCTGTCAACAACAATAAAGTACAGCAGGTGCC(A)n      3'-end
```

Fig. 1. Nucleotide sequence analysis of RN25ASYN, a 1421-nt-long cDNA fragment (accession number: Z18877) which encodes rat 2-5A synthetase I. The putative stop codon is indicated (#). The putative polyadenylation signal is *double underlined*. The inverted repeats in the 5'- and 3'-untranslated region are in *boldface* and *underlined*

```
RN25ASYN     MEQELRSTPSWKLDKFIEVYLLPNTSFRDDVKSAINVLCDFLKERCFRDT  50
MM25ASYN     -------------DKFIEDYLLPDTTFGADVKSAVNVVCDFLKERCFQGA  37
O25S-MOUSE   MEHGLRSIPAWTLDKFIEDYLLPDTTFGADVKSAVNVVCDFLKERCFQGA  50
HS25ASYN     M-MDLRNTPAKSLDKFIEDYLLPDTCFRMQINHAIDIICGFLKERCFRGS  49
MMOLISAA     M-MDLRNTPAKSLDKFIEEYLLPDTCFRMQINHAIDIICGFLKERCFRGS  49
MMOLISAB     M-MDLRNTPAKSLDKFIEDYLLPDTCFRMQINHAIDIICGFLKERCFRGS  49
                      ***** ****.* *  ... *....*.*******...

RN25ASYN     VHPVRVSKVVKGGSSGKGTTLKGKSDADLVVFLNNFTSFEDQLNRRGEFI 100
MM25ASYN     AHPVRVSKVVKGGSSGKGTTLKGKSDADLVVFLNNLTSFEDQLNRRGEFI  87
O25S-MOUSE   AHPVRVSKVVKGGSSGKGTTLKGRSDADLVVFLNNLTSFEDQLNRRGEFI 100
HS25ASYN     SYPVCVSKVVKGGSSGKGTTLRGRSDADLVVFLSPLTTFQDQLNRRGEFI  99
MMOLISAA     SYPVCVSKVVKGGSSGKGTTLRGRSDADLVVFLSPLTTFQDQLNRRGEFI  99
MMOLISAB     SYPVCVSKVVKGGSSGKGTTLRGRSDADLVVFLSPLTTFQDQLNRRGEFI  99
              .** ****************.*.*********. .*.*.**********

RN25ASYN     KEIKKQLYEVQREKHFRVKFEVQ-SSWWPNPRALSFKLSAPHLQQEVEFD 149
MM25ASYN     KEIKKQLYEVQHERRFRVKFEVQ-SSWWPNARSLSFKLSAPHLHQEVEFD 136
O25S-MOUSE   KEIKKQLYEVQHERRFRVKFEVQ-SSWWPNARSLSFKLSAPHLHQEVEFD 149
HS25ASYN     QEIRRQLEACQRERAFSVKFEVQ-APRWGNPRALSFVLSSLQLGEGVEFD 148
MMOLISAA     QEIRRQLEACQRERAFSVKFEVQ-APRWGNPRALSFVLSSLQLGEGVEFD 148
MMOLISAB     QEIRRQLEACQRERAFSVKFEVQEAPRWGNPRALSFVLSSLQLGEGVEFD 149
              .**..** . *.*. *.****** ...* *.*.*** **. .* ..****

RN25ASYN     VLPAYDVLGHVSLYSNPDPKIYTILISECISLGKDGEFSTCFTELQRNFL 199
MM25ASYN     VLPAFDVLGHGSINKKPNPLIYTILIWECTSLGKDGEFSTCFTELQRNFL 186
O25S-MOUSE   VLPAFDVLGHVNTSSKPDPRIYAILIEECTSLGKDGEFSTCFTELQRNFL 199
HS25ASYN     VLPAFDALGQLTGSYKPNPQIYVKLIEECTDLQKEGEFSTCFTELQRDFL 198
MMOLISAA     VLPAFDALDQLTGSYKPNPQIYVKLIEECTDLQKEGEFSTCFTELQRDFL 198
MMOLISAB     VLPAFDALGQLTGSYKPNPQIYVKLIEDCTDLQKEGEFSTCFTELQRDFL 199
             ****.*.*.. .   .*.* **. ** .*..* *.************.**

RN25ASYN     KQRPTKLKSLIRLVKHWYQLCKEKLGKPLPPQYALELLTVYAWERGNGIT 249
MM25ASYN     KQRPTKLKSLIRLVKHWYQLCKEKLGKPLPPQYALELLTVYAWEQGNGCN 236
O25S-MOUSE   KQRPTKLKSLIRLVKHWYQLCKEKLGKPLPPQYALELLTVFAWEQGNGCY 249
HS25ASYN     KQRPTKLKSLIRLVKHWYQNCKKKLGK-LPPQYALELLTVYAWERGSMKT 247
MMOLISAA     KQRPTKLKSLIRLVKHWYQNCKKKLGK-LPPQYALELLTVYAWERGSMKT 247
MMOLISAB     KQRPTKLKSLIRLVKHWYQNCKKKLGK-LPPQYALELLTVYAWERGSMKT 248
             ******************** **.**** ************.***.*.

RN25ASYN     EFNTAQGFRTILELVTKYQQLRIYWTKYYDFQHPDVSKYLHRQLRKSRPV 299
MM25ASYN     EFNTAQGFRTVLELVINYQHLRIYWTKYYDFQHKEVSKYLHRQLRKARPV 286
O25S-MOUSE   EFNTAQGFRTVLELVINYQHLRIYWTKYYDFQHQEVSKYLHRQLRKARPV 299
HS25ASYN     HFNTAQGFRTVLELVINYQQLCIYWTKYYDFKNPIIEKYLRRQLTKPTPV 297
MMOLISAA     HFNTAQGFRTVLELVINYQQLCIYWTKYYDFKNPIIEKYLRRQLTKPRPV 297
MMOLISAB     HFNTAQGFRTVLELVINYQQLCIYWTKYYDFKNPIIEKYLRRQLTKPRPV 298
             .*********.****..**.* *********..  ..***.*** *. **

RN25ASYN     ILDPADPTGNVAGGNQEGWRRLASEAKLWLQYPCFMNTGGSPVSSWEV-- 347
MM25ASYN     ILDPADPTGNVAGGNPEGWRRLAEEADVWLWYPCFMKNDGSRVSSWDV-- 334
O25S-MOUSE   ILDPADPTGNVAGGNPEGWRRLAEEADVWLWYPCFIKKDGSRVSSWDV-- 347
HS25ASYN     ILDPADPTGNLGGGDPKRWRQLAQEAEAWLNYPCFKNWDGSPVSSWILLV 347
MMOLISAA     ILDPADPTGNLGGGDPKGWRQLAQEAEAWLNYPCFKNWDGSPVSSWILLV 347
MMOLISAB     ILDPADPTGNLGGGDPKGWRQLAQEAEAWLNYPCFKNWDGSPVSSWILLT 348
             **********..**... **.** **. ** **** . .**.**** .

RN25ASYN     ----P---------------------------------------------- 348
MM25ASYN     ----PTVVP-----------------------------VPFE--------- 343
O25S-MOUSE   ----PTVVP-----------------------------VPFE--------- 356
HS25ASYN     RPPASS-------------------------------LPF---------- 356
MMOLISAA     RPPASS-------------------------------LPF---------- 356
MMOLISAB     QHTPGSIHPTGRRGLDLHHPLNASASWGKGLQCYLDQFLHFQVGLLIQRR 398

RN25ASYN     -VDEAWSCILL------ 358
MM25ASYN     QVEENWTCILL------ 354
O25S-MOUSE   QVEENWTCILL------ 367
HS25ASYN     ----------IPAPLHEA 364
MMOLISAA     ----------IPAPLHEA 364
MMOLISAB     QSSSVSWCIIQDRTQVS 415
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Fig. 2. Homologies between the deduced aa sequence of RN25ASYN with the following 2-5A synthetases I: *MM25ASYN* (mouse mRNA for 2-5A synthetase L2; accession number: X58077; Rutherford et al. 1991); *O25S-MOUSE* (mouse 2-5A synthetase 1; P11928; Ichii et al. 1986); *HS25ASYN* (human 1.6-kb mRNA for 2-5A synthetase; X04371, M25352; Wathelet et al. 1986); *MMOLISAA* (mouse 2-5A synthetase gene; M63849; Ghosh et al. 1991); *MMOLISAB* (mouse 2-5A synthetase gene; M63860; Ghosh et al. 1991). *Stars* indicate identical aa; *dots* well-conserved aa; *dashes* arbitrary gaps. The putative ATP/GTP-binding motif in RN25ASYN and MM25ASYN is *double underlined*

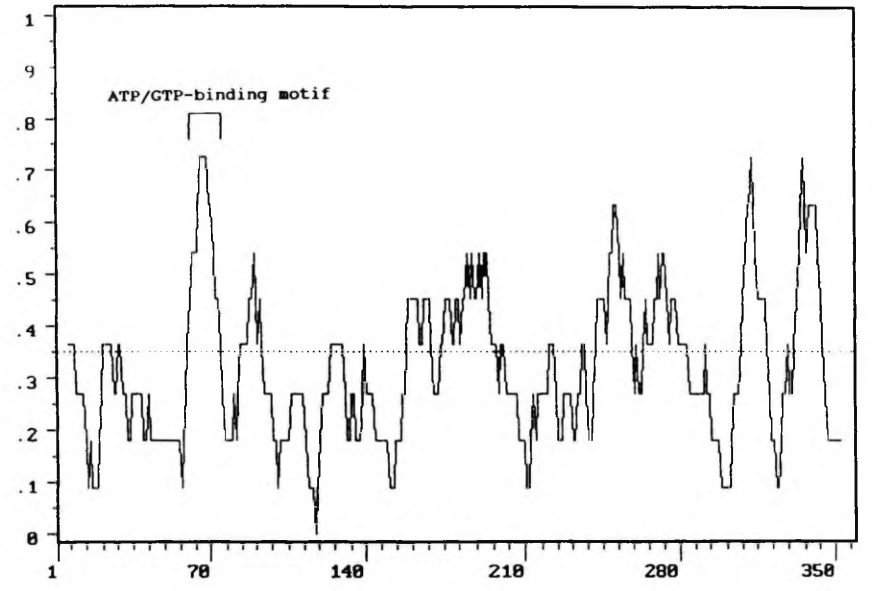

Fig. 3. Plot of C, G, N, Q, S, T, Y (polar/neutral aa) proportion for the aa sequence of RN25ASYN. The ATP/GTP-binding motif is indicated

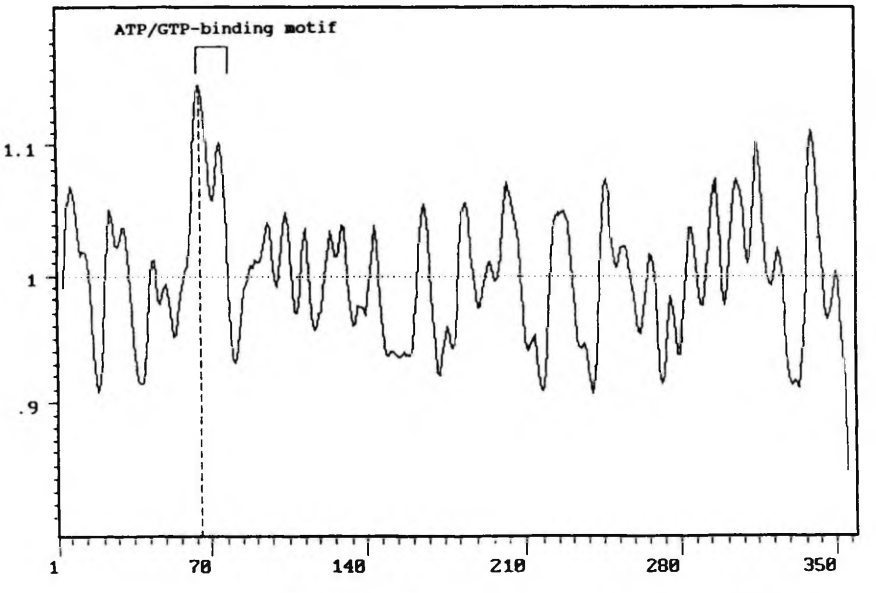

Fig. 4. Plot of the prediction of chain flexibility for the aa sequence of RN25ASYN. The ATP GTP-binding motif is indicated

```
          60         70         80         90         100
VHPVRVSKVVKGGSSGKGTTLKGKSDADLVVFLNNFTSFEDQLNRRGEFI
------------>**>------>**XX------*****XXXXXXXXXXXX
------------>**>------>**XX------*****XXXXXXXXXXXX
```

Fig. 5. Semigraphic plot of protein secondary structure prediction around the ATP/GTP-binding motif (*double underlined*) of the aa sequence of RN25ASYN. Symbols: ×, helical; −, extended; >, turn; *, coil conformation

isoelectric point (pI) is 9.22. The protein can be considered according to the instability index as a stable protein with a half-life of 36.6 h (PC/Gene: PHYSCHEM); no "good" PEST region (rich in the aa Pro, Glu, Ser and Thr) is present (PC/Gene: PESTFIND). Experimental evidence obtained from Chang and Wu (1991) from human cells indicated that the half-life of the 2-5A synthetase I is >2 h. No strong secretory signal sequence was found. The deduced aa sequence of RN25ASYN displays, like other class I 2-5A synthetases, potential protein kinase C phosphorylation sites, casein kinase II phosphorylation sites and N-myristoylation sites (PC/Gene: PROSITE).

In contrast to human 1.6 kb mRNA for 2-5A synthetase (HS25ASYN; Wathelet et al. 1986) and mouse 2-5A synthetase gene (MMOLISAB; Ghosh et al. 1991), the rat RN25ASYN is provided both with additional N-glycosylation sites and one farnesyl group binding site of the sequence CILL (nt + 355 to + 358). These two types of sites are also present in the mouse 2-5A synthetase L2 mRNA (MM25ASYN; Rutherford et al. 1991) and mouse 2-5A synthetase 1 (O25S-MOUSE; Ichii et al. 1986). The existence of a binding site for a farnesyl group may indicate that rat 2-5A synthetase is membrane-associated (Sinensky and Lutz 1992).

The ATP/GTP-binding motif A (P-loop) (Moller and Amons 1985), GTTLKGKS, is found in RN25ASYN from nt + 68 to + 75 and is present also in the sequence MM25ASYN. Analyses of the aa around the putative ATP/GTP-binding motif displayed the following characteristics. (1) Determination of the clustering of aa groups according to their physico-chemical properties showed that the site of the motif is the most polar neutral part of the total sequence (PC/Gene: PRESIDUE) (Fig. 3). (2) The motif is located in the groove of the protein segment with the highest flexibility and flanked by the segments aa 62 to 68 (GGSSGKG) and aa 71 to 77 (LKGKSDA) (PC/Gene: FLEXPRO) (Fig. 4). (3) In the predicted secondary structure of the rat 2-5A synthetase , the motif which displays extended character is surrounded by turns (Fig. 5) (PC/Gene: GARNIER; Garnier et al. 1978).

Ghosh et al. (1991) have analyzed the region within two mouse 2-5A synthetases I (MMOLISAA-MMOLISAB) to which dsRNA binds. They delimited it to the aa residue 104 to 158, corresponding to the segment aa 105 to 159 in rat 2-5A synthetase RN25ASYN. Analysis of this region by PC/Gene: PRESIDUE revealed a clustering of the aa Arg-Lys, known to be crucial for RNA binding (Fig. 6). The deduced aa sequence of RN25ASYN was further analyzed by the

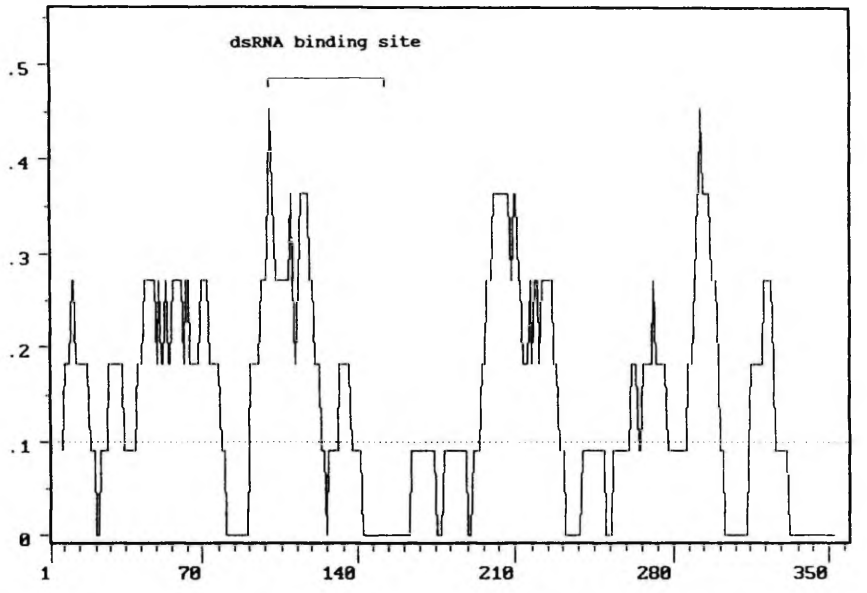

Fig. 6. Putative dsRNA binding site in the Arg-Lys-rich region of the deduced aa sequence of RN25ASYN according to data published by Ghosh et al. (1991)

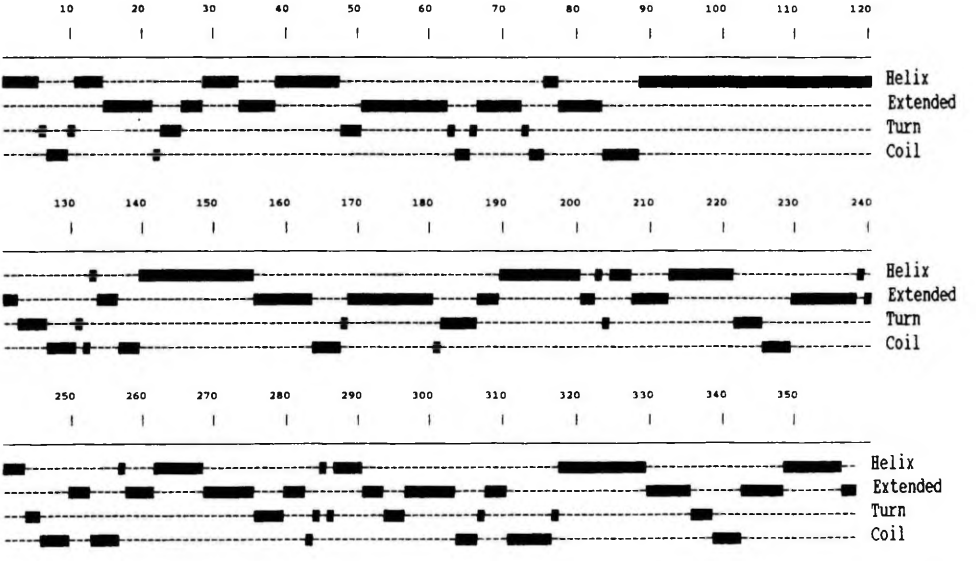

Fig. 7. Secondary structure of rat 2-5A synthetase by Garnier analysis. The helical, extended, turn and coil conformations along the deduced aa sequence are depicted

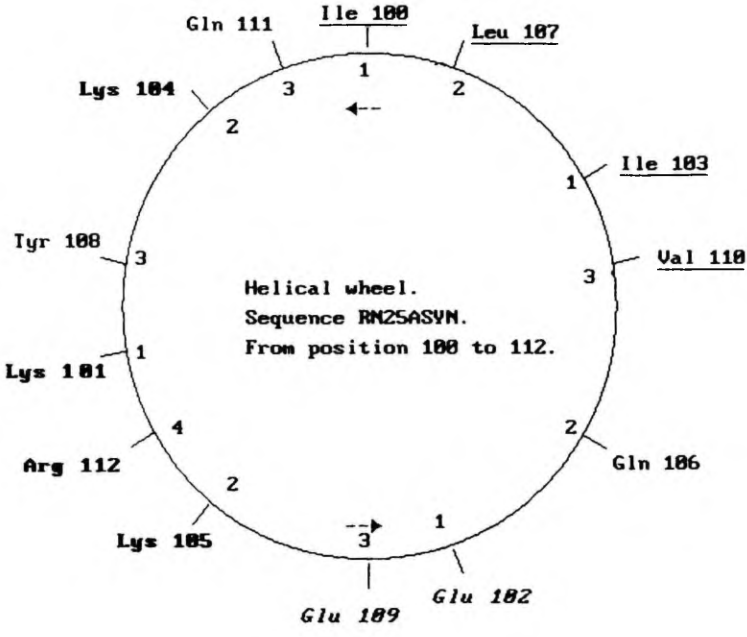

Fig. 8. Helical wheel analysis of the putative dsRNA binding site of the deduced aa sequence of RN25ASYN (aa 100–112). The basic aa Lys and Arg are in *bold face*; the acidic aa Glu in *italics*; the hydrophobic aa are *underlined*; the others are polar aa. The figures within the wheel denote the number of turns; a periodicity of four residues is assumed

procedure of Garnier (PC/Gene: GARNIER). The putative dsRNA binding site was found to be the longest helical stretch, spanning from aa 89 to 120 (Fig. 7). This part of the sequence is interesting because "helical wheel" analysis (PC/Gene: HELWEEL) revealed that the Arg-Lys residues are clustered on one side of the helix, while the hydrophobic aa are located on the opposite side (Fig. 8).

4 Comparison with Other Sequences of 2-5A Synthetases

The rat 2-5A synthetase I, RN25ASYN, was compared with five human and mouse sequences on the deduced aa level (Fig. 2). The comparisons revealed a high homology. The consensus length is 417 aa; among them 220 aa (52.8%) show identity and 75 aa (18%) similarity. If the sequence aa 14 to 347 (using the rat clone RN25ASYN) is selected, the value for identity among the six sequences increases to 66% and similarity to 23%. If the rat RN25ASYN sequence is compared with mouse MM25ASYN, the identity on both the nt and aa level is almost 90%.

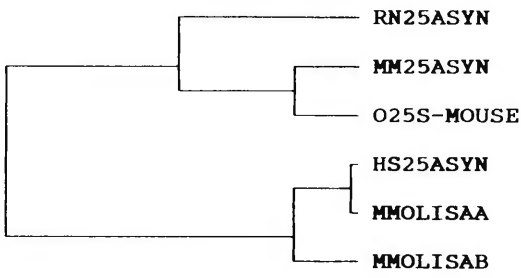

Fig. 9. Dendrogram based on the comparisons of the deduced aa sequences of 2-5A synthetases I shown in Fig. 2

```
RN25ASYN    M---EQELRSTPSWKL----------------------------------   13
HSSYN69KD   MGNGESQLSSVPAQKLGWFIQEYLKPYEECQTLIDEMVNTICDVCRNPEQ   50
            *   *  **  *  **

RN25ASYN    ----------------------------------------------------   13
HSSYN69KD   FPLVQGVAIGGSYGRKTVLRGNSDGTLVLFFSDLKQFQDQKRSQRDILDK   100

RN25ASYN    ----------------------------------------------------   13
HSSYN69KD   TGDKLKFCLFTKWLKNNFEIQKSLDGSTIQVFTKNQRISFEVLAAFNALS   150

RN25ASYN    ----------------------------------------------------   13
HSSYN69KD   LNDNPSPWIYRELKRSLDKTNASPGEFAVCFTELQQKFFDNRPGKLKDLI   200

RN25ASYN    ----------------------------------------------------   13
HSSYN69KD   LLIKHWHQQCQKKIKDLPSLSPYALELLTVYAWEQGCRKDNFDIAEGVRT   250

RN25ASYN    ----------------------------------------------------   13
HSSYN69KD   VLELIKCQEKLCIYWMVNYNFEDETIRNILLHQLQSARPVILDPVDPTNN   300

RN25ASYN    ----------------------------------------------DK   15
HSSYN69KD   VSGDKICWQWLKKEAQTWLTSPNLDNELPAPSWNVLPAPLFTTPGHLLDK   350
                                                          **

RN25ASYN    FIEVYLLPNTSFRDDVKSAINVLCDFLKERCFRDTVHPVRVSKVVKGGSS    65
HSSYN69KD   FIKEFLQPNKCFLEQIDSAVNIIRTFLKENCFRQSTAKI---QIVRGGST   397
            **   * **  *     ** *    **** ***          * ***

RN25ASYN    GKGTTLKGKSDADLVVFLNNFTSFEDQLNRRGEFIKEIKKQLYEVQREKH   115
HSSYN69KD   AKGTALKTGSDADLVVFHNSLKSYTSQKNERHKIVKEIHEQLKAFWREKE   447
             *** **  ******** *   *   * * *    ***  **    **

RN25ASYN    FRVKFEVQSSWWPNPRALSFKLSAPHLQQEVEFDVLPAYDVLGHVSLYSN   165
HSSYN69KD   EELEVSFEPPKWKAPRVLSFSLKSKVLNESVSFDVLPAFNALGQLSSGST   497
                  *  ** *** *    *   * ******  **  *  *

RN25ASYN    PDPKIYTILISECISLG-KDGEFSTCFTELQRNFLKQRPTKLKSLIRLVK   214
HSSYN69KD   PSPEVYAGLIDLYKSSDLPGGEFSTCFTVLQRNFIRSRPTKLKDLIRLVK   547
            * *  *  **    *     ******** *****   ****** *******

RN25ASYN    HWYQLCKEKLGKP---LPPQYALELLTVYAWERGNGITEFNTAQGFRTIL   261
HSSYN69KD   HWYKECERKL-KPKGSLPPKYALELLTIYAWEQGSGVPDFDTAEGFRTVL   596
            ***  *  ** **   *** ******* **** * *   * ** **** *

RN25ASYN    ELVTKYQQLRIYWTKYYDFQHPDVSKYLHRQLRKSRPVILDPADPTGNVA   311
HSSYN69KD   ELVTQYQQLGIFWKVNYNFEDETVRKFLLSQLQKTRPVILDPGEPTGDVG   646
            **** **** * *   * *    * * *  ** * *******  *** *

RN25ASYN    GGNQEGWRRLASEAKLWLQYPCFMNTGGSPVSSWEVPVDEAWSCILL      358
HSSYN69KD   GGDRWCWHLLDKEAKVRLSSPCFKDGTGNPIPPWKVPVKVI          687
            **    *  *  **   *  ***    * *   * ***
```

Fig. 10. Homologies between the deduced aa sequence of RN25ASYN (2-5A synthetase I) and the aa sequence of the human 69-kDa (2'-5')oligoadenylate synthetase (p69 2-5A synthetase) (HSSYN69KD; M87284; Marié and Hovanessian 1992)

Multiple sequence alignments were performed to obtain a dendrogram and to estimate the relative relationships between the five 2-5A synthetase clones, applying the CLUSTAL program (Fig. 9). The closest relationship was found between RN25ASYN and mouse MM25ASYN and O25S-MOUSE; common to all three sequences is that they are provided with the N-glycosylation sites and the farnesyl group binding site, none of which is present in any of the other sequences.

The deduced rat 2-5A synthetase aa sequence, RN25ASYN, was compared with the only available sequence of 2-5A synthetase class II, the human 69-kDa 2-5A synthetase (HSSYN69KD; Marié and Hovanessian 1992). The comparison revealed that the first half of the human sequence does not show homology with the rat sequence, while the second half shows a remarkably high homology; on both the nt and aa level the identity was 60 and 51%, respectively (Fig. 10). The high identity of the second half to 2-5A synthetase I has been previously reported leading to the suggestion that 2-5A synthetase II might have two catalytic domains (Marié and Hovanessian 1992).

5 Summary

Sequence analyses of 2-5A synthetases of class I (M_r 40000–46000) revealed high homology among them. The cDNA coding for the M_r 69000 2-5A synthetase of class II displayed in the second half a likewise high homology to the complete sequences of class I enzymes. This high degree of conservation of the 2-5A synthetases supports the assumption that these enzymes play important roles during virus infection (Williams et al. 1979; Coccia et al. 1990) and in the control of growth and differentiation of mammalian cells (Williams and Silverman 1985).

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IV

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Transgenic Potato Plants Expressing Mammalian 2′-5′ Oligoadenylate Synthetase are Protected From Potato Virus X Infection Under Field Conditions

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We cloned and sequenced a rat cDNA encoding the 2′-5′ oligoadenylate synthetase, a component of the mammalian interferon-induced antiviral response, and used *Agrobacterium*-mediated transformation to generate transgenic potato clones expressing this mammalian enzyme. In transgenic plants infected with potato virus X and followed under field conditions, virus concentrations in leaves and in tubers were significantly lower than in nontransgenic controls. Additionally, virus concentration in the leaves of five transgenic clones and in tubers of one clone was also lower than in transgenic potatoes expressing potato virus X coat protein.

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The expression of viral coat protein (CP) genes and of viral nonstructural proteins, the use of artificial antisense genes, and the expression of nucleic acid sequences encoding viral satellite RNAs, have all been used to generate virus-resistant plants (reviewed in ref. 1). Of these, the most common strategy is "coat protein-mediated resistance"[2]. Although the mechanism of protection via the expression of free virus CP in transgenic plants is not fully understood, this technique is widely used, and resistance against viruses of at least 12 different groups has been reported[3]. None of these methods, however, confer absolute immunity and resistance is generally against only one specific virus. Although a broader CP-mediated protection has been reported against tobamo-[4] and potyviruses[5,6], it appears to be restricted to closely related viruses. Thus a technique that provides simultaneous protection against a wide range of viruses still remains a goal.

In mammals, the interferon system provides such a universal antiviral response. Interferons (IFNs), proteins secreted by animal cells after viral infection, during cell proliferation, and in several immunological processes[7], induce the synthesis of additional proteins that directly lead to the inhibition of virus multiplication[8]. One of these is the 2′-5′ oligoadenylate synthetase. It is activated by double-stranded RNA (dsRNA), the usual replicating intermediate of RNA viruses, and as a rule only poorly activated by cellular dsRNAs[9]. Once activated, the enzyme polymerizes ATP to a series of 2′-5′ oligoadenylates (2-5A), whose monomers are linked via 2′-5′ phosphodiester bonds rather than the usual 3′-5′ linkage. 2-5A activates a latent endoribonuclease (RNase L) which degrades viral and cellular RNAs and is itself degraded by a cellular 2′ phosphodiesterase[8].

The existence of 2-5A pathway components in higher plants has remained unclear. dsRNA-dependent ATP polymerizing activity in the leaves of *Nicotiana glutinosa* L. and *N. tabacum* L. after treatment with so-called "antiviral factor"[10] or after tobacco mosaic virus (TMV) infection[11,12] has been reported. Additionally, the ability of plant extracts to synthesize *in vitro* oligoadenylate-like compounds having antiviral activity[13,14] and inhibition of TMV multiplication by chemically synthesized dephosphorylated 2-5A have also been demonstrated[15,16].

A probe for the human 2-5A synthetase gene was reported to hybridize with tobacco genomic DNA and to the mRNA of TMV-infected tobacco[16], and the putative 2-5A synthetase was partially purified from tobacco and shown to cross-react with antibodies against the human enzyme[16]. During recent years only one short report[19] has been published to confirm these results, and no paper describing the cloning of a sequence homologous to the mammalian 2-5A synthetase gene from plants has yet appeared.

Here we report the construction of transgenic potato plants expressing the murine 2-5A synthetase gene and compare the virus resistance of these transgenic plants with those expressing the potato virus X (PVX) coat protein. Both types of transgenic plants were protected against PVX infection under field conditions, and in some clones expressing the 2-5A synthetase, the level of virus accumulation was lower than in potatoes expressing PVX CP.

Results

Characterization of rat cDNA encoding 2-5A synthetase. Rat 2-5A synthetase cDNA was isolated from a rat hippocampus cDNA library, cloned in the pBluescript SK(+) vector and sequenced (EMBL acc. no. Z18877). The cDNA was more than 1500 base pairs (bp) long, containing a 69 bp 5′ nontranslated leader, a 1074 bp open reading frame, a 3′ nontranslated sequence of 277 bp with the putative polyadenylation signal, and an approximately 100 bp poly(A) tail.

Nucleotide sequence analysis (data not shown) revealed that the isolated rat 2-5A synthetase cDNA encodes the small, 43 kD form of the enzyme. A comparison of the rat and corresponding mouse[20,21] cDNAs revealed 86% homology at the nucleic acid level. The predicted amino acid sequence (Fig. 1A) showed 82% identity with the mouse small 2-5A synthetase form, with most of the mismatches due to conservative[22] exchanges, probably having only small effects on protein structure (Fig. 1A). Only 16 mismatches (4%) out of the 367 residue mouse sequence were nonconservative. The main difference between the rat and mouse small 2-5A synthetases was found in the rat enzyme's C-terminal region where a nine amino acid-long sequence (TVVPVPFEQ) was deleted (corresponding to mouse amino acids 349–357).

Expression of a 2-5A synthetase gene in *Solanum tuberosum* L. For the transformation of potato plants, the 2-5A synthetase cDNA was cloned between the cauliflower mosaic virus 35S promoter and the T-DNA gene 7 polyadenylation signal of the plant expression vector pHTT202 (ref. 23, Fig. 1B). The transferred segment of the vector also contained the hybrid nopaline synthetase-neomycin phosphotransferase II (*nos-nptII*) gene determining kanamycin resistance[24], which was used to select shoots induced from *Agrobacterium*-transformed potato

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                    10         20         30         40         50         60
Rat       MEQELRSTPSWKLDKFIEVYLLPNTSFRDDVKSAINVLCDFLKERCFRDTVHPVRVSKVV
Mouse     ..HG...I.A.T......D....D.T.GA.....V..V.........QGAA.........

                    70         80         90        100        110        120
Rat       KGGSSGKGTTLKGKSDADLVVFLNNFTSFEDQLNRRGEFIKEIKKQLYEVQREKHFRVKF
Mouse     .............R...........L.........................H.RR.....

                   130        140        150        160        170        180
Rat       EVQSSWWPNPRALSFKLSAPHLQQEVEFDVLPAYDVLGHVSLYSNPDPKIYTILISECIS
Mouse     .........A.S.........H..........F......NTS.K...R..A...E..T.

                   190        200        210        220        230        240
Rat       LGKDGEFSTCFTELQRNFLKQRPTKLKSLIRLVKHWYQLCKEKLGKPLPPQYALELLTVY
Mouse     ..............................................................F

                   250        260        270        280        290        300
Rat       AWERGNGITEFNTAQGFRTILELVTKYQQLRIYWTKYYDFQHPDVSKYLHRQLRKSRPVI
Mouse     ...Q...CY..........V....IN..H............QE...........A....

                   310        320        330        340        350        360
Rat       LDPADPTGNVAGGNQEGWRRLASEARLWLQYPCFMNRGGSPVSSWEVP---------VDE
Mouse     .............P.......E..DV..W....IKKD..R....D..TVVPVPFEQ.E.

                   367
Rat       AWSCILL
Mouse     N.T....
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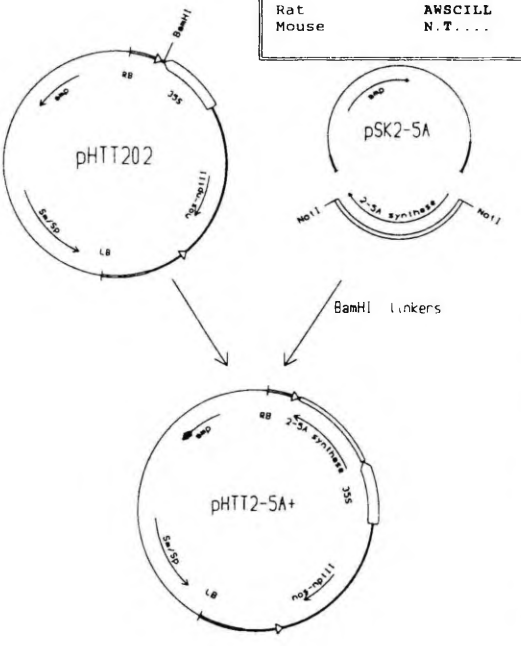

FIGURE 1. (A) Amino acid sequence comparison of the mouse and rat small 2-5A synthetases. Identical amino acids are marked with ".", conservative[22] changes of amino acid residues with bold letters and nonconserve replacements in Roman. (B) Construction of the plasmid pHTT2-5A+. The NotI fragment containing the rat 2-5A synthetase cDNA was cleaved from plasmid pSK2-5A, the cohesive termini filled in, and the insert ligated to pHTT202 vector BamHI site using BamHI linkers.

TABLE 1. Evaluation of PVX infection in transgenic potato clones P1–P6 (expressing 2-5A synthetase) and P7 (expressing PVX CP) under field conditions. Virus concentrations were calculated from ELISA values of pooled samples of each clone after 21, 28 and 35 days after infection.

Clone	PVX concentration (μg/g fresh weight) after 21	28	35 days	Percent of infected plants (n = 20)
Control	0	42.0	3020.4	25
P1	0	0	0	0
P2	0	0	841.0	10
P3	0	242.2	2943.4	20
P4	0	0	0	0
P5	0	0	0	0
P6	0	55.4	1456.4	5
P7	0	0	1764.0	15

cells. From putative potato transformants, which were resistant to kanamycin, 6 morphologically normal plants were selected for further tests. Southern hybridizations of HindIII-digested total plant DNA (HindIII has a unique restriction site near the *nos-npt*II gene, but does not cut 2-5A synthetase cDNA) with a 2-5A synthetase specific cDNA probe revealed the incorporation of a single copy of 2-5A synthetase cDNA into unique sites of the genome of all six transformed plants (Fig. 2). Sequences homologous to rat 2-5A synthetase were not found in nontransformed potato plants. The weaker hybridization signal in clone P3 DNA reflects the smaller amount of DNA loaded onto the gel. The expression of 2-5A synthetase mRNA was further analyzed with Northern blots (Fig. 3). All transgenic clones expressed detectable amounts of 2-5A synthetase mRNA of identical size. The expression level of 2-5A synthetase mRNA in clones P1, P3, P4 and P5 correlates well with the virus resistance data (Table 1, Figs. 3, 4). However, there is almost no correlation between the high level of 2-5A synthetase mRNA and the virus resistance of clones P6 and P2. In mammals the antiviral state depends on the coordinated expression of three enzymes of the 2-5A pathway—2-5A synthetase, RNase L and 2′ phosphodiesterase[7]. It is possible that the lack of correlation between the level of 2-5A synthetase mRNA and virus resistance in potato clones P6 and P2 reflects somaclonal variation in the two other components of the system. We are presently investigating this possibility directly. Control potato plants (Fig. 3) did not contain any sequences which hybridized to the rat 2-5A synthetase gene under the conditions used to detect such sequences in the transgenic plants. The absence of hybridizing sequences in potato mRNA as well as in genomic DNA indicates that these plants most likely do not possess a close homologue of a mammalian 2-5A synthetase.

Propagation of PVX in 2-5A+ potatoes. The transgenic clones for the field studies were selected based on their performance in preliminary greenhouse experiments. Potatoes were nine weeks old at the time of inoculation and the height of the inoculated plants was about 35–40 cm. Untransformed potatoes propagated in tissue culture in the same way as transformed plants were used as controls. Field-growing plants were inoculated with the sap from PVX-infected *N. glutinosa L.* leaves

The virus concentration in the diluted sap was 1 μg/ml. The PVX concentrations in field grown plants (20 plants treated with PVX and 10 uninfected negative control plants from each clone) were analyzed 21, 28 and 35 days after infection. In the ELISA used (see Experimental Protocol), 0-5 individuals from each clone were virus positive (Table 1). Negative controls all remained uninfected. ANOVA statistical analyses showed that the probability of infection was highest for control plants and lowest for transgenic clones P1, P4, P5, P6, and P2 (data not shown). The first three of these remained uninfected (Table 1). The PVX concentration used in the infection was low in order to simulate natural infection. Compared to the control plants, PVX concentrations were lower in all but one of the transgenic clones (P3) (Table 1). A transgenic clone (P7) expressing PVX CP was also included in this experiment. Although virus concentration in this clone was lower than in controls (Table 1), it was still significant at 35 days post infection (1.8 μg/mg fresh weight). By contrast, in five clones (P1, P2, P4, P5, P6) expressing 2-5A synthetase, the amounts of virus remained lower or were undetectable (Table 1). No visible symptoms were observed in the infected potatoes. This was expected as PVX infection symptoms in *S. tuberosum* are often minimal[25] depending on virus strain and cultivar.

Tubers harvested from the field experiment were analyzed using a procedure which is used to test the vigor of seed potatoes. The percentages of infected tubers were lower than the controls in all transgenic clones, and in four clones (P5, P4, P1 and P2) the difference was more than two-fold. In clone P3 almost the same number of tubers were infected as in the controls. These data correlate well with virus concentrations of leaf samples. However, only in one 2-5A+ clone (P5) was the number of infected tubers lower than the PVX CP expressing clone P7 (4.8% versus 10.7%, Fig. 4). No differences in tuber yield were seen between PVX infected transgenic and control clones or between infected and uninfected negative control plants (data not shown). It should be noted that the morphology of the tubers from the transgenic plants did not differ from those of the controls.

Discussion

We have shown that protection against PVX infection in potato plants can be achieved by the expression of a mammalian 2-5A synthetase in *planta*. In theory, the presence of an active 2-5A pathway may lead to a generalized protection against RNA viruses, and allow the construction of plants with multiple virus resistance. We have in fact, preliminary evidence for such a broad spectrum of resistance in these 2-5A synthetase-expressing transgenic plants.

While the precise mechanism of this protection and the practicability of this approach to construct plants with a broad spectrum tolerance remain to be elucidated, we none-the-less offer the following speculations based on some of our recent unpublished observations.

We have evidence that dephosphorylated forms of 2-5A inhibit TMV RNA translation *in vitro* in wheat germ extract and *in vivo* in tobacco protoplasts. We have also detected the presence of a 70 kD and a 40 kD 2-5A binding protein in plant extracts. Since the inhibition of TMV RNA translation induced by 2-5A is accompanied by a rapid RNA degradation, we think that the 2-5A-binding protein(s) is (are) a ribonuclease analogous to the mammalian 2-5A-dependent RNase. We have also detected a 2-5A-degrading activity, similar to the mammalian 2′ phosphodiesterase, in tobacco leaf extracts. Our working hypothesis is therefore that the expression of the rat 2-5A synthetase leads to the elaboration of an active mammalian-like 2-5A system in these transgenic plants. Although we expressed the enzyme under the control of a constitutive promoter, the enzyme should normally remain quiescent, requiring the presence of a dsRNA for activation. We are currently testing these predictions directly as well as concluding experiments on the segregation and heritability of the observed protection.

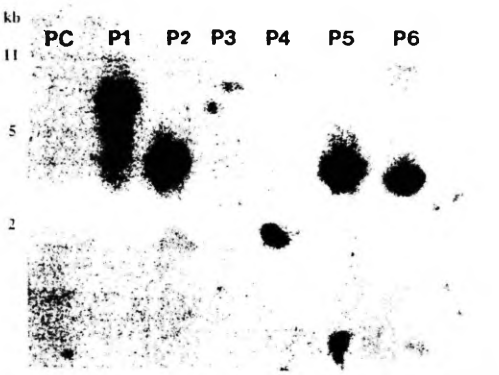

FIGURE 2. Southern blot analyses of potato DNA cut with HindIII (PC = control, P1-P6 = transgenes). ^{32}P-labeled 2-5A synthetase cDNA was used as a probe.

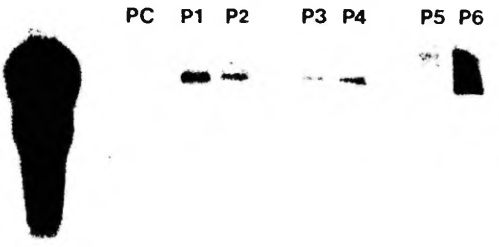

FIGURE 3. Northern blot analyses of potato (PC = control, P1-P6 = transgenes). The first lane from the left is circular DNA of the plasmid pSK2-5A containing the 2-5A synthetase cDNA insert. ^{32}P-labeled 2-5A synthetase cDNA was used as a probe.

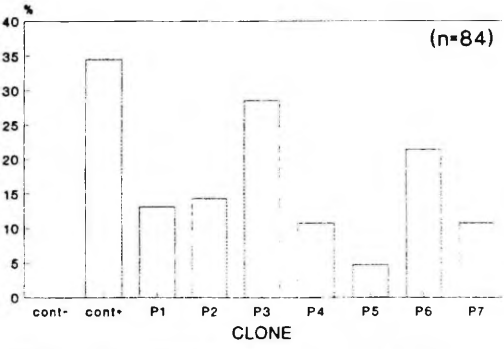

FIGURE 4. Percentages of PVX infected potato tubers in transgenic clones expressing 2-5A synthetase (P1–P6), PVX CP (P7) and in nontransgenic controls. The cut off limit in ELISA absorbances between healthy and infected tubers was 0.1.

Experimental Protocol

Materials. Virus-free *Solanum tuberosum L.* cv. Pito plants (obtained from The Seed Potato Center of Agricultural Research Centre of Finland, Tyrnävä) were propagated and rooted on MS medium[26] without hormones. The transgenic clone of cv. Pito expressing the PVX CP[27] was developed at Espoo Research Centre[28]. Isolates of PVX were obtained from the Institute of Plant Protection, Jokioinen, Finland. The rat hippocampus cDNA library in λgt10 was a gift from H. Persson, Karolinska Institute, Stockholm, Sweden. The mouse 2-5A synthetase cDNA was kindly provided by Y. Sokawa, Kyoto University, Japan. [^{35}S]dATP was purchased from Amersham (England), [α-^{32}P]deoxynucleotides from Amersham or Radiopreparat (Tashkent, Uzbekistan). BamHI linkers and DNA modifying enzymes were from New England Biolabs (USA) except that T4 DNA ligase was from Promega (USA), calf intestine alkaline phosphatase from Boehringer Mannheim (Germany) and some restriction endonucleases from Promega or Fermentas (Vilnius, Lithuania). Plant growth hormones and antibiotics were purchased from Sigma (USA). Claforan from Hoechst (Germany).

Cloning and sequencing of rat 2-5A synthetase cDNA. All nucleic acid manipulations throughout this study were performed according to standard methods[29] unless stated otherwise. An adult rat hippocampus cDNA library in λgt10 (ref. 30) was plated at 4×10^5 pfu per 22 × 22 cm plate using LE 392 (Promega) plating cells[31]. Replica filters were made on Amersham Hybond-N membranes, hybridized with radioactive probe and washed following the manufacturer's recommendations. Mouse 2-5A synthetase cDNA, labeled with [α-^{32}P]dATP by random priming[31] was used as a probe. Hybridized filters were exposed to Hyper-film-MP (Amersham) using intensifying screens. Positive phage plaques were plated on 9 cm Petri dishes and rescreened, using the same probe. Phage DNA was isolated and its NotI fragment was subcloned into the pBluescript SK(+) (Stratagene, USA) NotI site to create the plasmid pSK2-5A. Sequencing of the NotI fragment was performed by the dideoxynucleotide chain termination method[32] using the Sequenase Version 2.0 kit (United States Biochemicals, USA). Analyses of the nucleotide and corresponding amino acid sequences were performed with the Genetics Computer Group (University of Wisconsin, USA) Sequence Analysis Software Package and PCGENE computer programs (University of Geneva, Switzerland).

Construction of plasmid vectors. Rat 2-5A synthetase cDNA was excised from pSK2-5A by NotI, the cohesive termini filled in, and the cDNA ligated to the BamHI-linearized plant expression vector pHTT202 using synthetic BamHI linkers. The resulting plasmids were desginated pHTT2-5A+ and pHTT2-5A−, indicating the sense or antisense orientation of the 2-5A synthetase cDNA insert, respectively. 2-5A synthetase cDNA was integrated into *Agrobacterium tumefaciens* Ti-plasmid pGV2260 *via* homologous recombination as described[33]. Recombinant *Agrobacteria* were verified by isolating total bacterial DNA[34] and carrying out Southern analysis. The construction of the transgenic potato plants expressing the coat protein of potato virus X and the properties of these plants have been described earlier[28]. Briefly, PVX coat protein cDNA[27] was cloned into the BamHI site of pHTT202 vector with BamHI linkers. The recombinant plasmid pHTTPVXcp was used for *Agrobacterium* recombination. Altogether 29 potato clones, expressing the PVX CP were obtained and the resistance to PVX of 9 of these clones were tested in field tests[28]. Two of the clones showed clear protection to PVX infection[28] and one of these clones (P7) was used as the reference plant in this study.

Transformation and regeneration of potato. Stems of *in vitro* grown potato plantlets were cut into small pieces (5 mm) without axillary buds in 6 to 8 ml of liquid MS medium containing 2.25 μg/ml BAP and 0.03 μg/ml 1-naphthaleneacetic acid. Twenty μl of recombinant *Agrobacterium* culture grown overnight in Luria broth was added into medium. Stem pieces were cocultivated for two days with *Agrobacterium* using gentle shaking. After cocultivation explants were washed three times with sterile water and three times with sterile water containing Claforan (500 μg/ml). After washing explants were transferred to solid regeneration medium on Petri dishes. Shoots were induced using MS medium containing BAP (6-benzylaminopurine) 2.25 μg/ml, 1-naphthaleneacetic acid 0.03 μg/ml, Claforan (500 μg/ml) and 100 μg/ml kanamycin. After two weeks hormones were changed to BAP 0.5 μg/ml and gibberellic acid 0.5 μg/ml. Kanamycin was omitted from the control plates. Explants were transferred to fresh medium in every two weeks. Cultures were grown in light (1800 lx) with a photoperiod of 16 h light and 8 h dark. Two experiments, each with 500 explants were conducted. To select for true transformants, 20 shoots regenerated on selective medium were further rooted in the presence of kanamycin (100 μg/ml) and Claforan (500 μg/ml) on MS medium without hormones[35]. These 20 plantlets, which rooted well on kanamycin containing medium were transferred into greenhouse conditions in order to evaluate the plants further. Six true to type plants were selected as material for field tests. Total DNA and RNA from leaf tissue was isolated from these plants for Southern and Northern analyses.

Southern analysis. Total genomic DNA was isolated from greenhouse material of each transgenic clone and controls using CTAB method[36]. Ten μg of DNA was digested with HindIII, electrophoresed overnight on 0.8% agarose gel and transferred to Hybond-N membrane. Hybridization was done at 42°C in the presence of 50% formamide. 2-5A synthetase cDNA specific EcoRI-PstI fragment from pSK2-5A plasmid, labeled with ^{32}P-deoxynucleotides using the Boehringer Mannheim nick translation kit, was used as a probe. The blots were washed 3 × 10 min with 1 × SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0) and 0.1% sodium dodecyl sulfate (SDS) at room temperature and 2 × 15 min at 65°C and exposed to Kodak X-ray film at −80°C.

Northern analysis. Total RNA was isolated from greenhouse material (leaves) using the guanidinium isothiocyanate method and CsCl cushion[37,38]. Twenty μg of total RNA per lane was separated on denaturating formaldehyde gel and blotted to Hybond-N membrane. Northern blots were hybridized with ^{32}P-labeled plasmid pSK2-5A. Hybridization conditions were the same as in the Southern hybridization. The blots were washed 2 × 30 min at 60°C with 2 × SSC, 0.1% SDS and 2 × 15 min at 60°C with 0.2 × SSC, 0.1% SDS.

Infection of potato with PVX in the field. Field experiment was done in the summer of 1992 at Kotkaniemi Research Station, Finland (location 60°22'N, 24°22'E). The field was divided into three separate plots. Each plot contained 10 plants from each clone planted in a row. Two of the plots were infected with PVX and one plot was left as a negative control. Micropropagated plantlets from controls and from each transgenic clone were acclimatizated for one week at 23°C in 85% relative humidity after which they were transferred to the greenhouse. After 2 weeks, plants were transferred into field and grown 7 weeks before infection with PVX. Fully opened leaflets were selected in the middle part of the plants for inoculation. Juice from PVX-infected *N. glutinosa* leaves was used for infection. The juice was diluted in 50 mM K,Na-phosphate buffer (pH 7.0), so that the final PVX concentration was 1 μg/ml. After treating three randomly selected potato leaflets of plants with carborundum, the diluted sap was spread over them using cotton wool swaps. As samples, three leaflets from fully opened (with 5–7 leaflets) topmost leaves were collected 21, 28, and 35 days after infection from each individual. Leaf samples were stored in ice during transportation to the laboratory where they were analyzed immediately. Tubers were harvested individually from each plant in the autumn and yield was measured. Tubers were put into cold storage for four months. After the cold storage 4–5 tubers from each individual in each clone were pooled and followed to sprout. The sprouts were analyzed by ELISA for PVX.

Detection of virus concentration in infected plant leaves and sprouts of collected tubers. For PVX detection in potato, leaflets were homogenized in 2 ml cold sample buffer (Boehringer Mannheim) per 1g tissue in plastic bags. Extract was diluted into cold sample buffer (1:5000) and analyzed using the Boehringer Mannheim PVX enzyme immunoassay kit. For quantification a purified PVX virus preparation was used. The dormancy of the tubers was broken with Rindle treatment[39]. Three or four weeks after treatment sprouts were analyzed using an ELISA[40,41]. Extract from sprouts was obtained by a special pressing machine (Meku, E. Pollähne, Germany). The extract was diluted into cold sample buffer (1:4). Antisera for PVX analyses was produced in rabbits at the Finnish Agricultural Research Centre and IgG was conjugated with alkaline phosphatase.

Statistical analysis. Statistical analyses of the infection data was carried out using ANOVA nested design.

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Principles and background for the construction of transgenic plants displaying multiple virus resistance

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Summary. We investigated the possibility of reconstructing the 2′–5′ oligoadenylate (2–5A) pathway into the plant kingdom to achieve multiple virus resistance. Differently phosphorylated 2–5A trimers and tetramers inhibited TMV RNA translation in cell-free systems. In wheat germ extracts the most potent inhibitors were nonphosphorylated forms of 2–5A. Triphosphorylated forms of 2–5A were deposphorylated and hydrolysed in plant extracts. Since we could not detect homologous DNA to mammalian 2–5A synthetase cDNA in tobacco or potato, we cloned rat 2–5A synthetase cDNA and transformed it by the *Agrobacterium*-mediated mechanism into tobacco and potato. Transformed tobacco plants were resistant to PVS infection and propagation of PVX was reduced. In transgenic potatoes tolerance to PVX and, in one transgenic clone, also to PVY was observed.

Introduction

The 2′–5′ oligoadenylate (2–5A) pathway is part of the mammalian antiviral response system induced by interferons. The key enzyme of the pathway, the 2–5A synthetase, polymerizes ATP to a family of oligonucleotides, the 2–5A. Virus replication is inhibited due to the degradation of viral RNA by the specific 2–5A-activated ribonuclease, RNase L. Recently we have demonstrated that exogenously added 2–5A molecules can inhibit protein synthesis in plant cell-free extracts and protoplasts [21]. This inhibition was accompanied by the degradation of translated RNA. Thus, we concluded that 2–5A can activate a putative plant RNase. In this paper, the effects of exogenous 2–5A on protein synthesis in plant systems, the cloning of a novel mammalian 2–5A

synthetase cDNA, and the properties of transgenic tobacco and potato plants expressing 2–5A synthetase are discussed.

Approaches to engineered resistance against plant viruses

The following approaches have successfully been taken to make plants virus-resistant by means of genetic engineering: (1) expression of the viral coat protein (CP) gene [1]; (2) expression of viral nonstructural proteins [10]; (3) use of artificial antisense genes [2]; (4) expression of nucleic acid sequences encoding viral satellite RNAs [8, 11]. The main drawback of all approaches here mentioned for the construction of virus-resistant transgenic plants is that resulting plants are resistant to only one specific virus. There is some evidence for a broad-spectrum protection being raised by viral CP expression [15, 17] but nevertheless this is limited to a closely related group of viruses.

2–5A system in mammals

The 2–5A synthetase polymerizes ATP in the presence of double-stranded RNA (dsRNA, for example replicative intermediates of RNA viruses) to produce a family of oligonucleotides with the general structure ppp(A2′p5′)$_n$A with $n \geq 2$, abbreviated 2–5A. These oligonucleotides possess 2′–5′ phosphodiester bonds that are unusual in comparison with ordinary 3′–5′ links in the nucleic acids. Two other enzymes involved in the 2–5A system are: (i) 2′–5′phosphodiesterase which degrades 2–5A, and (ii) 2–5A-dependent ribonuclease (RNase L). The 2–5A synthetase is expressed as an inactive enzyme. For its activation, the presence of dsRNA is required. Only dsRNA molecules at least about 50 base pairs and with no more than one mismatch per 45 nucleotides can activate the synthesis of 2–5A [16]. Viral RNA has been shown to be a very potent activator of the 2–5A pathway [14]. Actually, 2–5A is not a single compound but a mixture of oligoadenylates with different chain lengths and states of phosphorylation. Oligomers with at least three residues are required to activate RNase L. Another requirement for 2–5A activation of RNase L in mammalian cells is a 5′ di- or triphosphate group. The existence of nonphosphorylated "core" 2–5A molecules in cells also has been reported, but they neither bind to nor activate the RNase L [12]. Activation of the RNase L in mammalian cell extracts is observed already at nanomolar concentrations of 2–5A [12]. Due to the activity of 2′–5′phosphodiesterase in cells, the activation of the 2–5A-dependent RNase is transient without the persistent de novo synthesis of 2–5A.

Components of the 2–5A pathway in plants

In plants ATP polymerizing activity was reported from tobacco mosaic virus (TMV)-infected or "antiviral factor" treated *Nicotiana glutinosa* leaves, which mediated the discharge of histidinyl-TMV-RNA [6]. The polymerization product was synthesized in vitro using a partially purified enzyme from *N. glutinosa* or *N. tabacum* immobilized on a poly(rI:rC) column [7]. An inhibitory effect on TMV replication was demonstrated as well with the exogenous 2–5A trimer "core" at a concentration of 100–200 nM in *N. glutinosa* leaf discs as at 10 nM in *N. tabacum* protoplasts [5]. It was suggested that plant oligoadenylates differ from the mammalian analogs because they did not compete with (2′–5′) $pppA_4[^{32}P]pCp$ for binding on the RNase L [7]. Furthermore, no 2–5A-binding proteins were detected in plant extracts [7], despite the fact that 2–5A was able to activate the discharging factor [6] referred above. The absence of (2′–5′)$pppA_nA$-binding proteins in plants has been independently demonstrated [4]. From metabolic stability assays, plants were thought to lack the 2′–5′ phosphodiesterase activity [5]. A probe of the human 2–5A synthetase was shown to hybridize to tobacco genomic DNA and mRNA from TMV-infected tobacco [19] although 2–5A synthetase activity had not been detected in tobacco earlier [4]. The partially purified ATP-polymerizing plant enzyme also reacted with antibodies to human 2–5A synthetase [19]. Recently, exogeneous nonphosphorylated 2–5A molecules longer than trimers were demonstrated to induce both increased cytokinin activity and the synthesis of pathogenesis-related and heat shock proteins in tobacco and wheat [13].

Detection of 2–5A-dependent ribonuclease activity and 2–5A degradation in plant system

We have followed the in vitro translation rate of TMV RNA in wheat germ extract and rabbit reticulocyte lysate in the presence and absence of different 2–5A forms [21]. In the case of rabbit reticulocyte lysate, di- and triphosphorylated forms of 2–5A trimers and tetramers were the most potent inhibitors of in vitro translations (Table 1). These results fit well with those obtained by several other groups [12]. We also showed a clear inhibition of TMV RNA translation by 2–5A in wheat germ extract. However, in contrast with a mammalian cell-free system, in wheat germ extract nonphosphorylated forms of 2–5A were the best inhibitors of protein synthesis, whereas 5′ phosphorylated compounds had much weaker inhibitory effects on the translation efficiency (Table 1). Addition of the 2–5A "core" resulted in the greatest reduction of the in vitro translation rate, consistent with the in vitro data obtained by Devash et al. [5].

Table 1. Effect of differently phosphorylated forms of 2–5A trimers and tetramers on TMV RNA in vitro translation in wheat germ extract and rabbit reticulocyte lysate[a]

Compound	Incorporation of the [^{3}H]-Leu (%)	
	wheat germ extract	rabbit reticulocyte lysate
Control without 2–5A	100	100
1 μM A_3 "core"	19.4	84.4
1 μM pA_3	84.3	90.2
1 μM ppA_3	42.6	73.8
1 μM $pppA_3$	70.4	54.9
200 nM A_4 "core"	61.5	69.7
200 nM pA_4	67.9	53.8
200 nM ppA_4	68.8	40.9
200 nM $pppA_4$	118.8	40.2

[a] 1 μg of TMV RNA was translated in the cell-free extract in the presence of [^{3}H]-labelled leucine; the rate of in vitro translation was measured by TCA precipitation of the translation products on Whatman GF/C filters and counting the radioactivity of the filters [21]

The ability of the 2–5A trimer "core" to inhibit protein synthesis was examined in tobacco mesophyll protoplasts. Addition of the 2–5A trimer "core" resulted in an at least two-fold reduction in protoplast protein synthesis compared to that of 2–5A-untreated control cells [21].

When TMV RNA was isolated from the wheat germ in vitro translation mix with and without the 2–5A "core", a three to seven-fold faster degradation of TMV RNA in the mix with the 2–5A "core" was observed [21]. This means that a rapid degradation of RNA is induced by 2–5A in the plant cell-free extracts. This is similar to the activity found in mammalian cells, in which 2–5A-dependent RNase L is responsible for the cleavage of cytoplasmic RNAs, thus inhibiting the protein synthesis. Using chemical crosslinking method, we detected a 70 kD plant protein from potato leaf extracts that specifically bind (2′–5′)A_4[^{32}P]pCp [21]. This observation is contrary to that of Cayley et al. [4] and Devash et al. [5] who could not detect 2–5A-binding protein in plants. The reason for such a difference may be the use of radioactively labeled triphosphorylated 2–5A forms instead of "core" molecules by the other groups. Because 2–5A inhibits protein synthesis and stimulates the hydrolysis of RNA in wheat germ extract, the 2–5A-binding 70 kD plant protein may be the plant 2–5A-dependent RNase, analogous to the mammalian RNase L. We emphasize that the putative plant 2–5A-dependent RNase differs from its mammalian analogs in that the plant

Fig. 1. Separation of 2–5A degradation products analyzed by silica gel thin layer chromatography. *1* $pppA_4$, incubated 1 h at 37°C with 0.5 units of calf intestine alkaline phosphatase; *2* A_3 "core", incubated 1 h at 37°C with 0.5 units of calf intestine alkaline phosphatase; *3* $pppA_4$, incubated overnight at 25°C with mouse L cell extract; *4* $pppA_4$, incubated overnight at 25°C with tobacco leaf extract; *5* $pppA_4$, untreated

enzyme seems to be activated preferentially by the 2–5A "core" whereas in mammals "cores" neither bind nor activate RNase L [12].

Thin layer chromatography on silica gel plates revealed that (2′–5′) $pppA_4[^{32}P]pCp$ was efficiently degraded in tobacco leaf extracts (Fig. 1). As triphosphorylated forms of 2–5A remain in the start on silica gel plates (Fig. 1, lane 5), our results show that enzymes that both dephosphorylate 2–5A and hydrolyze 2′–5′ phosphodiester bonds are present in plant extracts. These data contradict those reported by Devash et al. [5].

In plant extracts we have found enzyme activities resembling 2–5A-dependent RNase and 2′–5′ phosphodiesterase activities of mammalian cells. However, we have been unable to detect 2–5A synthetase activity in healthy or virus-infected plants. We did not detect neither tobacco or potato genomic DNA sequences nor mRNAs from virus-infected tobacco or potato hybridizing to the murine 2–5A synthetase cDNA probe. Therefore we assume that plants do not exhibit an enzyme activity similar to that of mammalian 2–5A synthetase.

As plants do contain a 2–5A-dependent ribonuclease, we assumed that expression of the mammalian 2–5A synthetase gene in transgenic plants might simultaneously protect against infection by many different RNA viruses. Mammalian 2–5A synthetase is normally expressed in an inactive form, so the pathway in transgenic plants, as we assume, would be switched on only after the appearance of dsRNA, i.e. after viral

infection. As the product of the 2–5A synthetase, 2–5A is degraded by plant enzymes, the effect would be transient and the reconstructed pathway is switched off after the disappearance of dsRNA, i.e. after the degradation of viral RNA.

Cloning of a novel 2–5A synthetase cDNA from rat

At least three major forms of 2–5A synthetase have been reported in mammalian cells: 40–46 kD, 69 kD, and 100 kD. The cDNA sequences encoding the small form of the 2–5A synthetase have been identified from human [3] and mouse [9, 18]. We have used the mouse L3 2–5A synthetase cDNA, which is derived from the unique mouse genetic locus not present in human and which encodes a 40 kD 2–5A synthetase [18]. Using L3 cDNA as a probe, we isolated 2–5A synthetase cDNA from a rat hippocampus cDNA library. We have cloned and sequenced this cDNA (EMBL Acc. No. Z18877), which is 1.5 kb long and contains a 1 074 bp open reading frame. The nucleotide sequence showed 86% homology to the mouse L3 2–5A synthetase cDNA. Homology to the mouse synthetase L2 cDNA was even higher (89%). The L2 cDNA is characterized by an additional 600 bp untranslated sequence at its 3′-end, which is not present in the L3 cDNA [18]. Since in our cloned rat 2–5A synthetase cDNA the starting point for the poly(A) tail was nearly at the same position as in the mouse L3 cDNA (−3 bp compared to L3) we believe that the cloned sequence is the rat analog of mouse 2–5A synthetase L3 cDNA. The amino acid sequence is 82% identical to the corresponding mouse 2–5A synthetase. The main difference between rat and mouse 40 kD 2–5A synthetases was the nine amino acid long deletion at the C-terminal region of the rat enzyme [20].

Construction of plants simultaneously tolerant to different plant viruses

We have used an *Agrobacterium*-mediated transfer to obtain transgenic tobacco and potato plants expressing the rat 2–5A synthetase. Plants expressing detectable amounts of the 2–5A synthetase mRNA were used for the virus infection experiments. In a series of independent experiments transgenic tobacco plants were infected with potato virus X (PVX), potato virus S (PVS), and potato virus Y (PVY), belonging to the potex-, carla- and potyvirus groups, respectively. The propagation of the viruses was followed for a month after infection. The results revealed that all transgenic tobacco clones were resistant to infection by PVS and in some clones the propagation rate of PVX was considerably reduced. However, protection to PVY was not observed (Table 2).

Leaf disc experiments using both PVX and TMV, a member of tobamovirus group, were done with transgenic tobacco. Again, some

Table 2. Propagation of four different plant viruses in leaf discs and intact transgenic tobacco plants expressing 2–5A synthetase[a]

Virus	Intact plants	Leaf discs
Potato virus X	+/−	+/−
Potato virus S	−	N.D.
Potato virus Y	+	N.D.
Tobacco mosaic virus	N.D.	+/−

[a] Virus concentration was measured a week (leaf discs) or a month (intact plants) after infection by homogenizing the leaf material and detecting the virus by ordinary ELISA or time-resolved fluoroimmunoassay (TRFIA) measurement [20]. Specific monoclonal antibodies against PVX, PVS and PVY were used, TMV was detected by polyclonal antibodies

−: virus propagation is inhibited in all tested transgenic lines

+/−: virus propagation is inhibited in some transgenic lines, but not in all

+: virus propagating as in nontransgenic control tobacco plants

N.D. Not determined

tobacco clones markedly reduced the infection rate of both viruses, as determined one week following infection (Table 2).

Transgenic potato plants were infected with PVX and PVY, using sap from infected plant leaves [20]. Some clones exhibited remarkable tolerance, not only to PVX infection as with tobacco, but one potato clone also showed slight tolerance to PVY infection, which was not the case in transgenic tobaccos (data not shown). Infection experiments with PVS in potato are now in progress.

We conclude that the expression of the mammalian 2–5A synthetase in transgenic plants leads to reconstruction of a pathway analogous to that of the mammalian 2–5A system. As plants contain endogenous 2–5A-dependent ribonuclease as well as the 2–5A degrading enzyme activity, we propose a model for the reconstituted 2–5A pathway (Fig. 2). As revealed in the infection experiments, transgenic plants expressing 2–5A synthetase exhibit the broadest spectrum of protection against virus infections ever reported. Recently a patent has been filled for this novel approach.

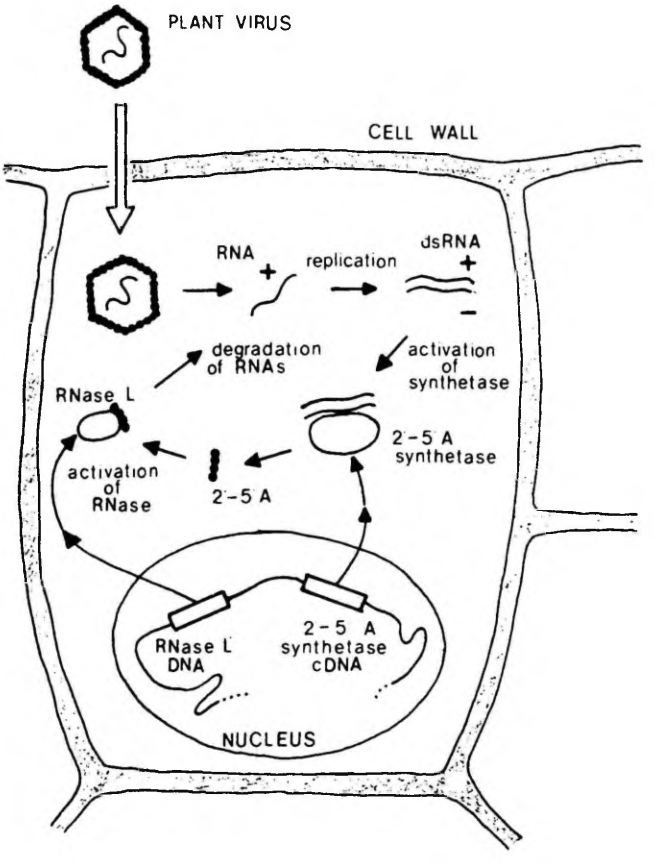

Fig. 2. A model for the reconstructed 2–5A pathway in transgenic plants expressing the mammalian 2–5A synthetase. The putative plant 2–5A-dependent RNase is marked as RNase L′

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VI

Manuscript

TRANSGENIC TOBACCO PLANTS EXPRESSING MAMMALIAN 2-5A SYNTHETASE SHOW PROTECTION AGAINST INDEPENDENT INFECTIONS OF THREE UNRELATED VIRUSES

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Key words: potato virus S, potato virus X, tobacco mosaic virus, transgenic tobacco, virus protection, 2'-5' oligoadenylate synthetase

Abstract

We have transformed and expressed in *Nicotiana tabacum* SR1 plants rat 2'-5' oligoadenylate (2-5A) synthetase, an enzyme involved in mammalian antiviral action. Three transgenic tobacco clones (T2, T3, and T5) were obtained and they expressed both 2-5A synthetase mRNA and protein. When they were challenged in separate experiments to infection by potato virus S (PVS) or X (PVX), or tobacco mosaic virus (TMV) all three tested clones exhibited resistance to PVS infection. In one transgenic clone (T3) the propagation of symptomless $PVX_{Russian}$ and PVX_{KD} strains was significantly inhibited. In PVX_{KD} infection no symptoms developed. TMV strain *vulgare* propagation was also inhibited in clone T3 and was again accompanied by the absence of symptoms. In clone T5, TMV reproduction was delayed, but symptom development did not differ significantly from that of control plants. Controls (nontransformed SR1 plants and 2-5A synthetase antisense constructs) were efficiently infected with $PVX_{Russian}$, PVX_{KD}, PVS, and $TMV_{vulgare}$, except that in plants expressing 2-5A synthetase antisense RNA massive TMV propagation was delayed. We conclude that tobacco clone T3 expressing mammalian 2-5A synthetase displays protection against three plant viruses (PVX, PVS and TMV) belonging to different systematic groups.

Introduction

Recently, we have reported that transgenic potato plants expressing rat 2'-5' oligoadenylate (2-5A) synthetase contained significantly lower concentrations of PVX under field conditions than similarly infected untransformed controls [24]. 2-5A synthetase is a mammalian enzyme involved in viral resistance. It is activated by the double-stranded replicative intermediates of viral RNA (dsRNAs) [8]. This activation leads to the synthesis of a family of oligoadenylates with 2'-5' phosphodiester bonds, abbreviated 2-5A. Virus replication is inhibited due to the rapid degradation of viral RNA by the specific 2-5A activated ribonuclease - RNase L. Although in mammalian cells the 2-5A pathway inhibits picornavirus infections preferentially [17], theoretically the multiplication of all RNA viruses could be inhibited *via* this pathway. As a majority of plant viruses possess genomes based on RNA, such a possibility might have a great impact on genetic engineering for virus resistant transgenic plants.

We have utilized the antiviral potential of the mammalian 2-5A system and constructed several tobacco clones expressing the rat 2-5A synthetase (2-5A+ plants). Three independent clones were chosen for infection experiments with three plant viruses belonging to the tobamo-, potex-, and carlavirus groups, respectively. In independent inoculation experiments at least one clone showed protection against the infection of all three viruses tested, thus exhibiting simultaneous defense mechanisms against plant viruses belonging to different systematic groups.

Materials and methods

Materials

Nicotiana tabacum L. cv. Petite Havana SR1 [12] plants were propagated and rooted on MS medium [15] without hormones at 25° C with a photoperiod of 16 h. Russian isolates of PVX and PVS were obtained from NPF Biotekhtsentr, Korenevo, Moscow Region, Russia. PVX strain KD was a gift from Dr. D. Baulcombe, Sainsbury Laboratory, Norwich, England. TMV strain *vulgare* and anti-TMV polyclonal antibodies were kindly provided by Dr. V. Novikov, Moscow University, Russia. [α-^{32}P] deoxynucleotides were purchased from Radiopreparat (Tashkent, Uzbekistan), L-[^{35}S]-methionine from Amersham (England), restriction endonucleases from Fermentas (Vilnius, Lithuania), plant growth hormones and antibiotics from Sigma (USA), and Claforan from Hoechst (Germany).

Transformation and regeneration of tobacco plants

The construction of a plant expression vector containing rat 2-5A synthetase cDNA under the control of 35S promoter and its integration to *Agrobacterium tumefaciens* Ti-plasmid pGV2260 was as described by us earlier [24]. Leaf discs from *N. tabacum* SR1 plants were cocultivated with *Agrobacterium* according to [7]. Shoots were induced from the transformed discs on selective LS medium [10] with 50 μg/ml kanamycin, 1 μg/ml benzylaminopurine (BAP), and 500 μg/ml Claforan. Roots were induced on the same medium by omitting BAP. Fully rooted explants were grown as before the transformation, transferring plants to fresh medium in every month. To verify that true transformants were selected, Southern, Northern, and Western analyses were carried out.

Southern analysis

DNA was extracted from transformed and control plants according to [3]. 10 μg of total DNA was digested with *Pvu*II (having a unique internal cleavage site in rat 2-5A synthetase cDNA) and electrophoresed on 0.8% agarose gel using standard protocol [13]. After the electrophoresis, DNA was blotted to Hybond-N nylon membrane (Amersham) and hybridized at 42° C in the presence of 50% formamide according to Amersham membrane transfer and detection protocols. Full-length 2-5A synthetase cDNA, labelled with [α-^{32}P]dCTP according to [4], was used as a probe. The hybridized filters were washed 2 x 15 min with 2 x SSC (300 mM NaCl, 30 mM Na-citrate, pH 7.0) + 0.1% sodium dodecyl sulfate (SDS) at 65° C, 30 min with 1 x SSC + 0.1% SDS at 65° C and 10 min with 0.1 x SSC + 0.1% SDS at room temperature. The filters were air dried and exposed to Hyperfilm-MP (Amersham) at -40° C using intensifying screens.

Northern analysis

Total RNA from transformed and control plant leaves was extracted as by [26]. Electrophoresis in formaldehyde/agarose gel and Northern blotting was carried out using Amersham protocols. The synthesis of the [^{32}P]-labelled cDNA probe, hybridization, washing of the filters and autoradiography was as in Southern analysis.

Production of polyclonal antibodies against 2-5A synthetase

The cDNA of rat 2-5A synthetase was modified by polymerase chain reaction (PCR) in order to insert *Bam*HI restriction sites just prior the ATG codon (upstream oligonucleotide 5'GCAAAAGCTCCGGGATCCATGGAGCAGGAA) and after the stop codon (downstream oligonucleotide 5'CCTGGGCAGGATCCGGAATTCTCACAGCAGGATACATGTC). The PCR product was ligated into the *Bam*HI site of the pGEM-3Zf(+) vector (Promega, USA), subcloned into the pGEX-2T (Pharmacia, Sweden) expression vector and transformed into the *Escherichia coli* B strain BL21(DE3) [22]. Glutathione S-transferase (GST) 2-5A synthetase fusion protein was produced as described [21] and it was purified using ProSieve Gel System (FMC BioProducts, USA) under denaturing conditions. The fusion protein recovered from gels was used to immunize the rabbits with three injections (20 µg/injection/animal) at 0, 2, and 6 weeks and the animals were bled one week after the last injection. To confirm the specificity of the antiserum, the rat 2-5A synthetase in the pGEM-3Zf(+) vector was expressed *in vitro* using a simultaneous transcription/translation TNT Coupled Reticulocyte Lysate System (Promega). The *in vitro*-produced 2-5A synthetase was immunoprecipitated with preimmune, immune and GST-2-5A synthetase protein absorbed antisera. The precipitated proteins were separated on 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [9] followed by autoradiography.

Western analysis

For the protein analysis, frozen leaf samples were homogenized to powder in a prechilled mortar and centrifuged for 5 min at 10,000 rpm in a microcentrifuge. Supernatant fluids were collected and proteins from 20 µl aliquots were separated on 10% SDS-polyacrylamide gel electrophoresis [9]. Subsequentially proteins were electroblotted onto Hybond-C membranes, using 40 mM NaH_2PO_4, pH 6.5 as a blotting buffer. Blotted filters were rinsed with phosphate buffered saline containing 0.1% of Tween 20 (PBS/Tw) and blocked for 1 h at 37° C with 5% skim-milk in PBS/Tw on shaker. Filters were incubated for 1 h at room temperature with anti-2-5A synthetase rabbit polyclonal antibodies (PAbs) diluted 1:800 in PBS/Tw as primary antibodies and for an additional 1 h at room temperature with anti-rabbit IgG horseradish peroxidase (HRP) conjugate (Sigma) (dilution 1:250). Both incubations were carried out on shaker in the presence of 5% skim-milk. Between every step, filters were washed 1 x 15 min and 2 x 5 min with PBS/Tw. HRP activity was enhanced using Amersham ECL Western blotting reagents; thereafter the filters were exposed to Hyperfilm-MP for up to 10 min.

Inoculation of tobacco with various plant viruses

In order to obtain enough clonal individuals for several independent inoculation experiments, primary transformants of each clone were multiplied by rooting stem pieces with two leaves on fresh MS medium. Tobacco plants were transferred from agar medium to soil two weeks before the inoculation and were kept at room temperature under a 16 h photoperiod. For the inoculation, 10 μg/ml of purified virus in 5 mM K/Na-phosphate buffer, pH 7.2, were manually inoculated onto 1-2 carborundum-dusted lower leaves of plants with 6-8 leaves. Carborundum was washed away after 10 min with sterile water. Plants "inoculated" with K/Na-phosphate buffer alone were used as negative controls throughout all experiments. Plants were kept for 24 h in darkness and thereafter as before the inoculation. Three independent plants of each clone were inoculated in all experiments. Samples were collected by taking one of the top leaves of each inoculated plant and freezing it in liquid nitrogen. Inoculated leaves were not analyzed.

Leaf disc inoculations

One leaf of three independent plants of each transgenic and control clone (grown on soil for two weeks) were inoculated as described above. 20 discs with a diameter of 8 mm were punched out of every leaf inoculated and kept upside down in sterile water for five days in the same conditions as intact plants. Then the discs were frozen in liquid nitrogen and analyzed as samples from intact plant inoculations.

Determination of virus concentration in infected tobacco leaves

Frozen tobacco leaf samples were separately homogenized in a mortar, adding 1 ml of the immunoassay buffer per gram of leaf material. For PVX detection, monoclonal antibodies (MAbs) 21XD2 and time-resolved fluoroimmunoassay (TRFIA) were used as described by us earlier [19]. PVS and TMV concentrations were evaluated with ordinary double antibody sandwich (DAS) ELISA, using MAbs S4A4 [19] and anti-TMV polyclonal antibodies, respectively. ELISA tests were carried out on individual samples of every plant inoculated and the results were pooled thereafter. For quantification, TRFIA or ELISA values of serially diluted purified virus preparations were measured. Values obtained from leaf samples of buffer-"inoculated" plants were taken as nonspecific background of TRFIA or ELISA, respectively.

Results

Construction of transgenic tobacco plants

The plant expression vector pHTT2-5A+ [24], containing full-length rat 2-5A synthetase cDNA under the control of the cauliflower mosaic virus 35S promoter, and nopaline synthetase-neomycin phosphotransferase II gene determining kanamycin resistance, was used to *Agrobacterium*-transform tobacco (*Nicotiana tabacum* L.) leaf

discs. From kanamycin-resistant shoots, 12 independent tobacco clones were selected for further analysis. Southern analysis of six transformed tobacco clones is shown on Fig. 1A. It revealed that four out of six clones analyzed were 2-5A synthetase cDNA positive. Multiple bands on Southern hybridization obtained with *Pvu*II-cut total DNA of clones T3 and T5 indicate that more than one copy of 2-5A synthetase cDNA was integrated into the plant genome (Fig. 1A, lanes 5 and 7). In case of clone T2, one copy of the transgene was integrated into plant chromosomal DNA, as *Pvu*II-cut DNA (having a unique cleavage site in rat 2-5A synthetase cDNA) gave two specific hybridzation bands on Southern analysis (Fig. 1A, lane 4). In clone T6 DNA rearrangements had perhaps occurred as only one 2-5A synthetase specific DNA band was detected in Southern analysis (Fig. 1A, lane 8). Later it was shown that this clone was able neither to synthesize 2-5A synthetase mRNA nor immunologically detectable enzyme (data not shown). Southern blots of uncut total plant DNA were probed with the same 2-5A synthetase sequence (data not shown). This experiment confirmed that clones T2, T3, T5 and T6 had integrated mammalian 2-5A synthetase cDNA into the chromosomal DNA. No hybridization signals were seen with nontransformed SR1 tobacco DNA (Fig. 1A, lane 2). Altogether five (T2, T3, T5, T7, T8) out of twelve kanamycin resistant clones were efficiently transformed with full-length 2-5A synthetase cDNA (data not shown for independently analyzed clones T7 and T8, where the pattern and intensity of 2-5A synthetase cDNA-specific bands were similar to clones T3 and T5).

We transformed the same SR1 tobacco cultivar also with the plasmid pHTT2-5A(-), where 2-5A synthetase cDNA was cloned under the control of the 35S promoter in the antisense orientation. Nine clones obtained contained 2-5A synthetase-specific DNA sequence [data not shown, abbreviated as 2-5A(-) plants].

Expression of 2-5A synthetase mRNA and protein in transgenic plants

Five tobacco clones containing 2-5A synthetase cDNA were analyzed with Northern blots (Fig. 1B). All transgenic plants expressed detectable amounts of 2-5A synthetase 1.5 kb mRNA although their expression levels were different. Clones T3, T5 and T8, having more than one 2-5A synthetase cDNA copy integrated, also expressed higher levels of 2-5A synthetase compared to the single transgene copy clone T2. Clone T7 expressed as a major product a truncated form of 2-5A synthetase mRNA (Fig. 1B, lane 2). The reason of this phenomenon is unknown, because clone T7 bears a full-length 2-5A synthetase cDNA fragment and produces a mRNA of the expected size, although at a very low level. Control tobacco plants lacked 2-5A synthetase homologous mRNA whereas all 2-5A(-) clones expressed RNA hybridizing with 2-5A synthetase cDNA (data not shown).

Rabbit PAbs against *E. coli*-produced rat 2-5A synthetase fusion protein were used to study 2-5A synthetase protein expression in transgenic plants (Fig. 2). The specificity of the antibodies was confirmed by immunoprecipitation (Fig. 2A). Tobacco clones T2, T3 and T5 produced a protein recognized by anti-2-5A synthetase PAbs with the expected size of 40 kD (Fig. 2B). Clones T7 and T8 produced 2-5A synthetase at extremely low levels (Fig. 2B) and only with very long exposure times faint 40 kD bands were seen. In the T5 clone, the expression level of 2-5A synthetase was higher as

compared to the ones of clones T2 and T3 (Fig. 2B). Control and 2-5A antisense plant cells did not express any 2-5A synthetase (Fig. 2B).

Replication of PVX in transgenic tobacco plants

Three transgenic tobacco clones T2, T3 and T5 were selected for virus resistance studies. Transgenic plants expressing 2-5A synthetase antisense RNA and untransformed SR1 tobaccos were used as controls. Tobacco plants were inoculated about two weeks after the transfer to the soil at a 6-8 leaf stage. One or two lower leaves of the plantlets were inoculated mechanically with 10 μg/ml of potexvirus PVX (Russian strain [20]). Morphologically the transgenic plantlets looked normal and were indistinguishable from the nontransformed tobaccos of the same age. The age and morphology of plants was also the same in the case of PVS and TMV inoculations.

One month after the inoculation all transgenic tobaccos contained detectable levels of PVX. In clone T2 the concentration of the virus and the infection curves did not differ significantly from that of nontransgenic control plants (Fig. 3A). In clones T3 and T5 inhibition of PVX replication during the first two weeks post infection was observed, whereas detectable levels of virus were observed about five days later than in control plants. Moreover, in clone T3 the virus concentration was much lower than in control plants throughout the experiment (Fig. 3A). Plants expressing 2-5A synthetase antisense RNA did not exhibit protection against PVX since the virus yields in these plants were similar to those of nontransformed SR1 plants (Fig. 3A).

Next we studied whether the inhibition of PVX propagation was accompanied with the delay or absence of symptoms development. Since under our experimental conditions we were not able to detect any visible symptoms in tobaccos infected with $PVX_{Russian}$, we inoculated sister plants of the same transgenic clones with hybrid PVX strain KD [18], which is capable of producing yellow mosaic on SR1 tobacco leaves during a systemic infection (our unpublished data). According to ELISA data the infection curves of PVX_{KD} and $PVX_{Russian}$ in 2-5A+ and 2-5A(-) tobaccos were similar (data not shown). The rate of PVX_{KD} replication in clone T2 did not differ from control plants, whereas clones T3 and T5 exhibited a low protection. However, in the case of PVX_{KD} the protection level was lower than in the case of infection by $PVX_{Russian}$. PVX_{KD} concentration in clone T3 was 65-70% of that in control plants one month after the infection. Surprisingly, as the virus concentration in PVX_{KD}-infected T3 clone was only slightly lower as compared to controls, all three inoculated plants remained completely symptomless even one month after the infection. All control plants, 2-5A synthetase antisense RNA-expressing plants and 2-5A+ clones T2 and T5 developed clear yellow mosaic on leaves with systemic infection (data not shown). The development of symptoms in three T5 plants was delayed about for one week, as compared to other plants developing symptoms.

To follow more precisely the local infection by $PVX_{Russian}$ in inoculated tobacco leaves, we carried out leaf disc inoculations where discs were punched out from PVX-inoculated leaves and analyzed after five days. The protection was again not seen in clone T2 where the PVX level was the same as in nontransformants. Both clones T3 and T5 exhibited protection against PVX in this assay, whereas in T3 the inhibition of

virus reproduction was more effective than in T5 (38% *versus* 59% compared to control plants, Fig. 4).

Replication of PVS in 2-5A+ plants

The T2, T3, and T5 tobacco clones were inoculated with 10 μg/ml of a Russian strain of PVS, member of carlavirus group [16]. Control and 2-5A(-) tobaccos were clearly infected with PVS after 25 days of inoculation (Fig. 3B). At the same time, all three transgenic 2-5A+ tobacco clones contained PVS at almost undetectable levels according to ELISA (Fig. 3B). Only clone T2 contained about 20 ng/ml of PVS in fresh leaf material 20 days post infection, but thereafter the virus concentration also dropped in this clone. In contrast to the data obtained with PVX strains, 2-5A+ clones behavied quite similarly to each other when infected with the same concentration of PVS, and displayed nearly complete immunity against this plant virus. No symptoms could be detected, neither in PVS-inoculated transgenic nor in control tobacco plants.

TMV infections in tobacco plants expressing 2-5A synthetase

TMV, the type member of tobamoviruses, was chosen as a third plant virus since unlike PVX and PVS, the natural host for TMV is *N. tabacum*. The inoculation conditions were the same as in previous experiments with PVX and PVS. One month after the inoculation with 10 μg/ml of TMV strain *vulgare* [5], the concentrations of TMV were in similarly high levels in control, 2-5A antisense and 2-5A+ T2 and T5 plants (Fig. 5). At 25 days post infection the TMV concentrations in the T3 clone plants were about 400 ng/ml whereas in other clones they were over 3 μg/ml. The analysis of infection kinetics revealed that in clone T2 and in nontransformed plants TMV concentrations were already beyond the upper plateau of ELISA sensitivity six days post infection (Fig. 5). In transgenic tobacco clone T3, the TMV concentration was significantly lower than in other plants throughout the experiment. In clone T5, the virus concentrations remained low for the first two weeks, after which they were similar to those of the controls (Fig. 5). Surprisingly, in the case of TMV infection, the 2-5A(-) plants also exhibited a certain level of protection against the virus during the first 12 days post infection (Fig. 5).

$TMV_{vulgare}$ causes typical pale green mottle and leaf distortions in SR1 tobaccos. Clones T2, T5 and 2-5A(-) developed the $TMV_{vulgare}$ infection symptoms similar to those of controls (data not shown). Only in the case of T5 was the development of symptoms delayed about five days. No differences could be detected among individual plants of the same clone. Plants of clone T3 remained symptomless throughout the experiment, even though the TMV concentrations were about 400 ng/ml at 25 days post infection. The virus levels in T3 were clearly lower as compared to the other clones.

Similarly to PVX, also with $TMV_{vulgare}$ leaf disc inoculations were carried out. Five days after the inoculation, the leaf discs from clones T3 and T5 contained significantly less virus than controls and clone T2 (Fig. 4). Surprisingly, in leaf disc inoculations clone T5 was even more resistant to TMV than T3.

Discussion

We have transformed rat cDNA encoding for the small, 40 kD form of 2-5A synthetase into tobacco cultivar SR1. Five independent transformants were shown to have integrated 2-5A synthetase cDNA. All five clones expressed 2-5A synthetase specific mRNA, whereas clones T2, T3, T5, and T8 synthesized as a major product 2-5A synthetase mRNA with the expected size of 1.5 kb (Fig. 1B). Western blots revealed that clones T2, T3, and T5 expressed a 40 kD protein readily detectable by antisera against 2-5A synthetase, whereas the expression levels of 40 kD protein in clones T7 and T8 were scarcely detectable (Fig. 2B). Tobacco clones T2, T3, and T5 were chosen for virus-resistance studies. As revealed from Northern blots, clone T3 expressed the highest amounts of 2-5A synthetase mRNA among these three clones, whereas the specific mRNA levels in clone T2 were much lower than in the two other clones (Fig. 1B). Surprisingly, at the protein level the clone T5 showed the highest levels of 2-5A synthetase, whereas the expression levels in clones T2 and T3, although different in sense of transgene copy number and levels of 2-5A synthetase mRNA expression, were quite similar to each other (Fig. 2B). Infection experiments showed that clone T3 exhibited complete resistance to PVS infection, whereas the propagation of $PVX_{Russian}$, PVX_{KD} and $TMV_{\textit{vulgare}}$ was reduced as compared to those of nontransformed and 2-5A synthetase antisense RNA expressing tobaccos. Clone T5 also displayed protection against PVX and TMV infections, but at lower levels than T3. The kinetics of virus infections in clone T2 did not significantly differ from those of controls, except in the case of PVS infection, in which all three 2-5A+ clones exhibited nearly complete virus resistance. Protection against $PVX_{Russian}$ and $TMV_{\textit{vulgare}}$ in clones T3 and T5 was further confirmed by leaf disc inoculation experiments. Although leaf disc experiments are often hard to interprete because of complications with senesence and would response processes, our data correlated well with results obtained from intact plant inoculations and gave results statistically differing in 2-5A+ clones T3 and T5 when compared to nontransgenic controls. In conclusion, at least tobacco clone T3 was protected aginst three different plant viruses - PVX, PVS and TMV, belonging to potex-, carla- and tobamovirus groups, respectively. The level of achieved protection was in a correlation with the copy number of and transcription levels from the transgene, as nonresistant clone T2 also contained the single copy of 2-5A synthetase and expressed 2-5A synthetase mRNA at lower levels as compared to clones T5 and T3. Correlation at steady state 2-5A synthetase protein levels was not so obvious, as the clone T3 expressed 2-5A synthetase in lower levels than the clone T5. At present we do not know the significance of this phenomenon, but our hypothesis is that the optimal levels of 2-5A synthetase are needed for most efficient virus protection in plants. As we are not aware about the cellular concentrations of double-stranded forms of RNA viruses tested in our experiments, and we do not know their ability to activate 2-5A synthetase, too high concentrations of expressed synthetase might not give the best protection against virus attack.

The exact mechanism, how 2-5A synthetase is activated in plants and how it mediates the antiviral resistance, is presently not known. We are currently studying the possibility that plants have an endogenous ribonuclease, which is activated by 2-5A. Preliminary results indicate that external 2-5A is capable of inducing ribonuclease

activity in plants [25]. As we have also been able to show that chemically synthesized 2-5A is efficiently degraded in plant extracts [25], we propose that higher plants might exhibit enzymatic activities resembling those of mammalian RNAse L and 2' phosphodiesterase, the latter being the enzyme degrading 2'-5' phosphodiester bonds. Despite many attempts we have not been able to detect either 2-5A synthetase-like enzymatic activity or DNA and/or mRNAs hybridizing to mammalian 2-5A synthetase cDNA in plants [24]. Therefore our hypothesis is that the expression of functional 2-5A synthetase *in planta* may complement a full 2-5A antiviral pathway in plants. Although the existance and original functions of a putative plant "RNase L" and "2' phosphodiesterase" remain to be elucidated, results reported in this paper refer to the possibility that this approach might be promising in order to construct plants with multiple virus resistance. Up to now such attempts have not been very successful.

The widely used approach of pathogen-derived resistance in transgenic plants (reviewed by [27]) has in many cases proved to be very efficient. Still, the expression of wild-type or mutated viral cDNAs *in planta* typically confers protection only against the particular virus itself or to closely related viruses of the same group. However, there do exist some examples of pathogen-derived protection in transgenic plants against heterologous infections of viruses from different systematic groups. Tobaccos expressing brome mosaic bromovirus (BMV) 32 kD transport protein were reported, besides of BMV itself, to significantly reduce the accumulation of unrelated tobamovirus TMV strain U1 [14]. As far as transport proteins of BMV and TMV can functionally substitute each other [2], it remains to be seen how general the protection mechanism reported for BMV transport protein is. Anderson *et al.* have reported a broad-spectrum coat protein-mediated protection against low concentrations of systematically distinct viruses [1]. Namely, TMV CP+ tobaccos exhibited delay in symptom development when inoculated with potexvirus PVX, potato virus Y (PVY, potyviruses), cucumber mosaic virus (CMV, cucumovirus), and alfalfa mosaic virus (AlMV). Similarly, AlMV CP+ tobaccos showed a delay in the symptoms' development of PVX and CMV infections [1]. The reason for this delay is not known, especially as several other authors have not been able to observe coat-protein-mediated protection against viruses from different groups [6].

Recently, a broad spectrum virus resistance in plants expressing pokeweed antiviral protein (PAP) was reported [11]. Remarkable protection against PVX, PVY and CMV (viruses belonging to three different virus groups) was achieved in transgenic *N. tabacum* plants, whereas PVX resistance was also detected in PAP expressing potatoes and PVY resistance in potatoes and *N. benthamiana* plants. PAP is a plant ribosome-inhibiting protein, which is capable of inhibiting the infection of both plant and animal viruses [23]. The mechanism of PAP-mediated resistance in transgenic plants is presently not understood [11]. However, the potential of this approach for crop improvement is restricted by the fact that PAP is capable of inhibiting the functions of ribosomes in higher mammals, *i.e.* to act as mammalian protein synthesis inhibitor.

We have also obtained transgenic tobacco plants displaying protection against plant viruses belonging to three different virus groups. Besides the reports by Anderson *et al.* [1] and by Lodge *et al.* [11], this is to our knowledge the most broad spectrum protection against viruses ever reported for transgenic plants. In tobacco clone T3, both the concentration of inoculated viruses was reduced and the symptom development of

PVX$_{KD}$ and TMV*vulgare* was absent while using relatively high concentrations of inoculum (10 μg/ml). We believe that expression of antiviral genes of animal or plant origin may offer a more broad spectrum and more general means to develop virus resistant plants.

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Legends to the figures

Fig. 1. (A) Southern blot analysis of tobacco DNA cut with *Pvu*II. DNA was separated on a 0.8% agarose gel, blotted to a nylon filter and hybridized with [^{32}P]-labeled rat full-length 2-5A synthetase cDNA. Lane 1 - pHTT2-5A+/*Pvu*II; 2 - nontransformed SR1 tobacco; 3 - 2-5A+ tobacco clone T1; 4 - 2-5A+ tobacco clone T2; 5 - 2-5A+ tobacco clone T3; 6 - 2-5A+ tobacco clone T4; 7 - 2-5A+ tobacco clone T5; 8 - 2-5A+ tobacco clone T6. (B) Northern blot analysis of tobacco total RNA. RNA was separated on 1% agarose/formaldehyde gel and blotted and hybridized as in Southern analysis. Lane 1 - tobacco clone T8; 2 - tobacco clone T7; 3 - tobacco clone T5; 4 - tobacco clone T3; 5 - tobacco clone T2; 6 - circular DNA of plasmid pSK2-5A (Truve *et al.*, 1993). The arrow indicates the position for 1.5 kb 2-5A synthetase mRNA.

Fig. 2. Specificity of 2-5A synthetase antibodies and expression of 2-5A synthetase in transgenic plants. (A) Rat 2-5A synthetase cDNA was *in vitro* translated in reticulocyte lysate in the presence of [^{35}S]-methionine. The labeled proteins were immunoprecipitated with rabbit antisera (except lane 1) and separated on 12% SDS-PAGE followed by autoradiography. Lane 1 - *in vitro* translation product; 2 - immunoprecipitates with immune serum; 3 - immunoprecipitates with preimmune serum; 4 - immunoprecipitates with *E. coli* expressed glutathione S-transferase (GST) protein absorbed immune sera; 5 - immunoprecipitates with GST-2-5A synthetase fusion protein absorbed immune serum. (B) Western blot of transgenic plant cell extracts. 20 µl normalized cell extract aliquots were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes and stained with rabbit anti-2-5A synthetase antibodies followed by HRP labeled goat anti-rabbit immunoglobulins. The bands were visualized by ECL reagent. Lane 1 - 2-5A synthetase antisense control plant; 2 - 2-5A+ tobacco clone T5; 3 - 2-5A+ tobacco clone T3; 4 - 2-5A+ tobacco clone T2; 5 - 2-5A+ tobacco clone T7; 6 - 2-5A+ tobacco clone T8; 7 - nontransformed SR1 tobacco. The arrow indicates the position for 40 kD 2-5A synthetase protein.

Fig. 3. (A) Evaluation of PVX infection in transgenic and control intact tobacco plants. Virus concentrations were estimated by TRFIA analysis of leaf samples. One leaf sample from three independently inoculated plants from each clone were analyzed separately for each timepoint, and the obtained results were pooled afterwards to calculate the average virus concentration. Since the initial leaf extract was not further diluted, the virus concentration of 0.5 µg/ml shows the upper limit of sensitivity of the immunoreaction rather than the real virus concentrations in leaves. TRFIA values of samples from buffer-"inoculated" SR1 plants (~1500 cps) were taken as background of the test, and were used as values for the virus concentration 0 µg/ml. Clones T2, T3 and T5 are 2-5A+ plants, 2-5A(-) refers to clones expressing 2-5A synthetase antisense RNA, SR1 tobaccos were used as controls. (B) Evaluation of PVS infection in transgenic and control intact tobacco plants. Standard DAS ELISA test was used. The number of analyzed samples per clone, estimation of virus concentrations and used clones were the same as in (A), the ELISA background from mock-inoculated samples being 0.096 at OD_{450}.

Fig. 4. Evaluation of PVX and TMV infections in tobacco leaf discs. Virus concentrations were estimated by TRFIA (PVX) or ELISA (PVS) analysis of homogenized discs, taking 20 of 8 mm discs per each inoculated leaf. One leaf from three independent plants of each analyzed clone was inoculated and analyzed. The immunoassay values of each sample analyzed were pooled and used to calculate the average virus concentration with standard error for each clone. Amount of virus in nontransgenic SR1 control tobaccos was taken as 100%. T2, T3 and T5 were 2-5A+ clones.

Fig. 5. TMV infection in transgenic and control intact tobacco plants. The number of analyzed samples per each clone, estimations of TMV concentrations in individual samples and used clones were the same as in PVX and PVS infections (Fig. 3). ELISA background for mock-inoculated plants was 0.127 at OD_{450}. TMV concentrations of 3 μg/ml reflect the upper limit of ELISA sensitivity and not the real final virus concentrations.

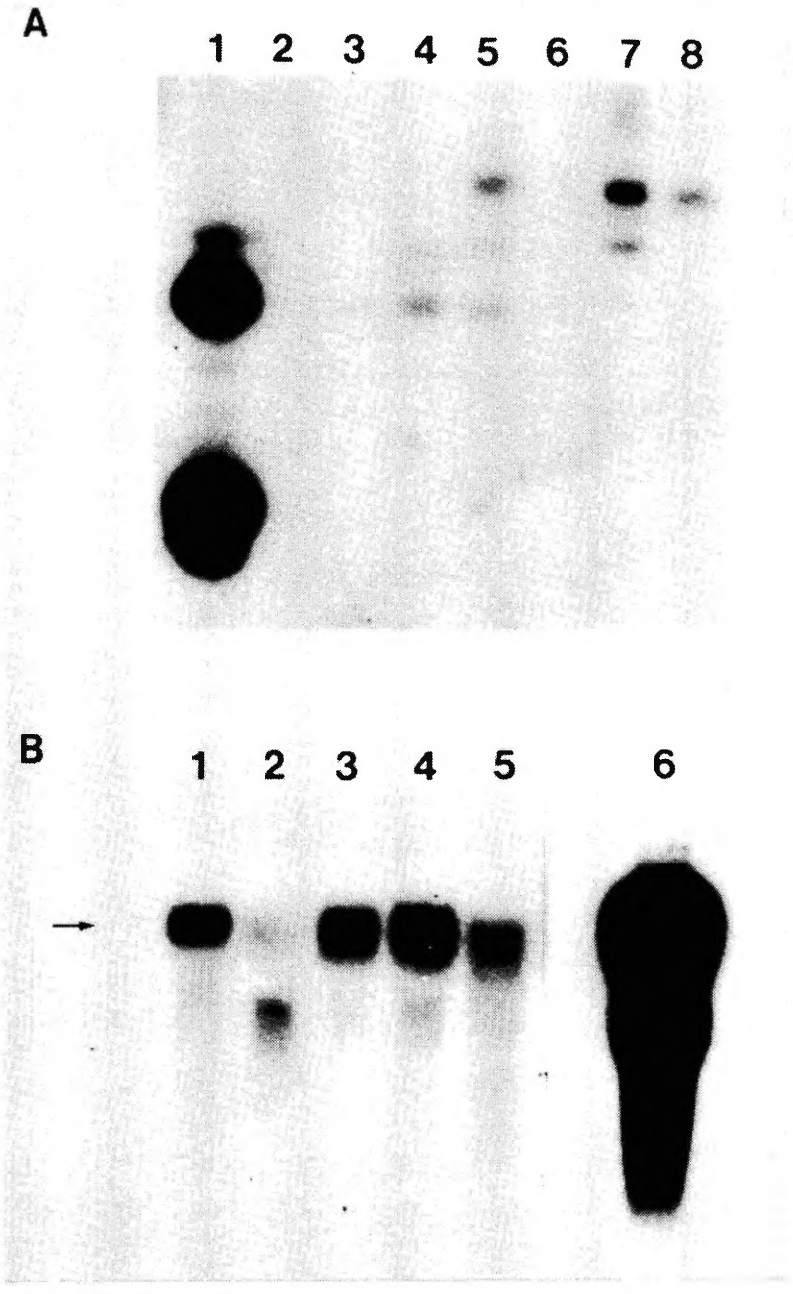


Fig. 1.

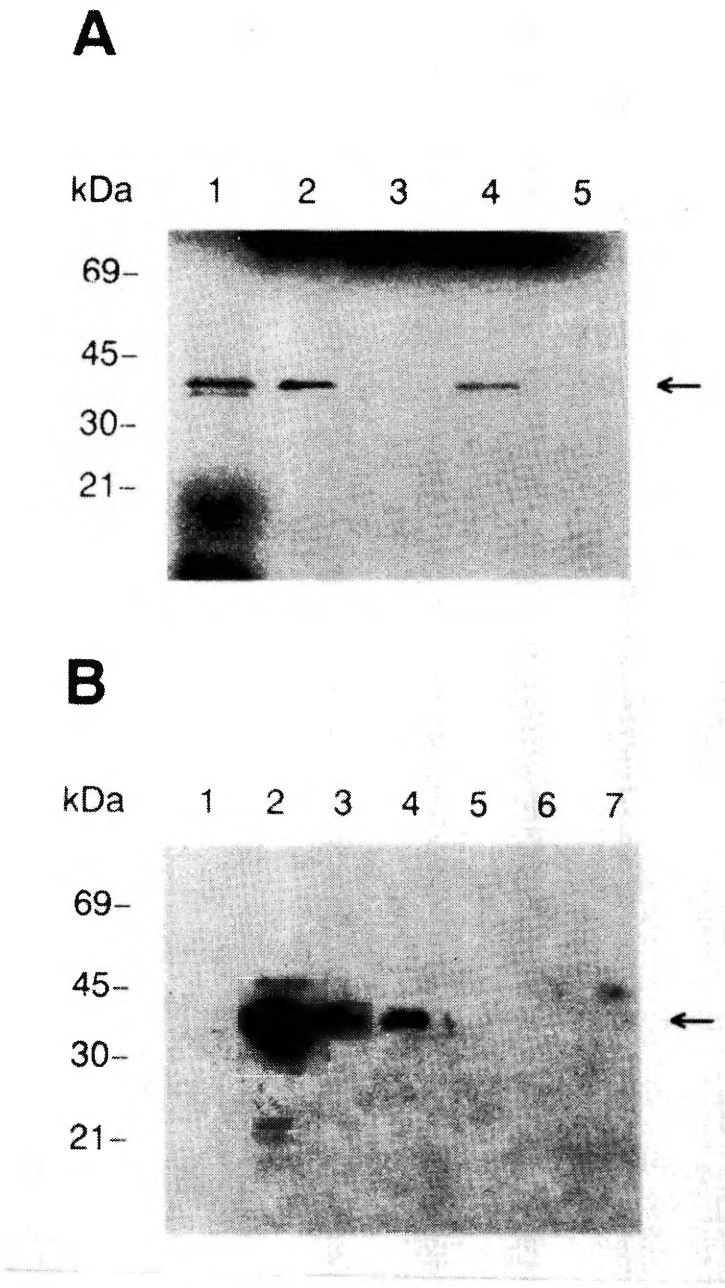


Fig. 2.

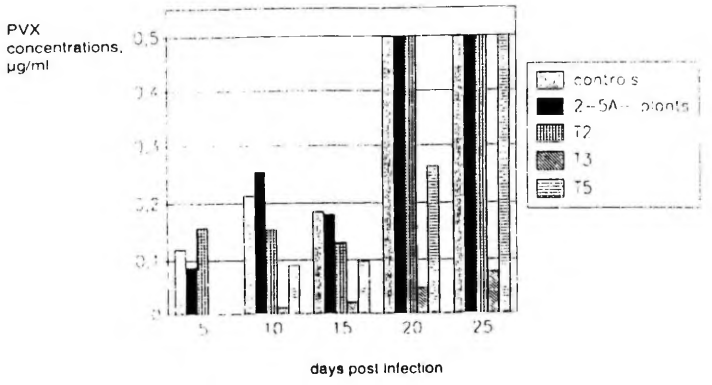
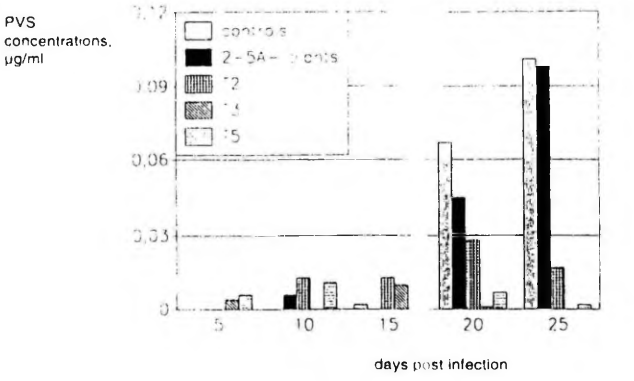


Fig. 3A.



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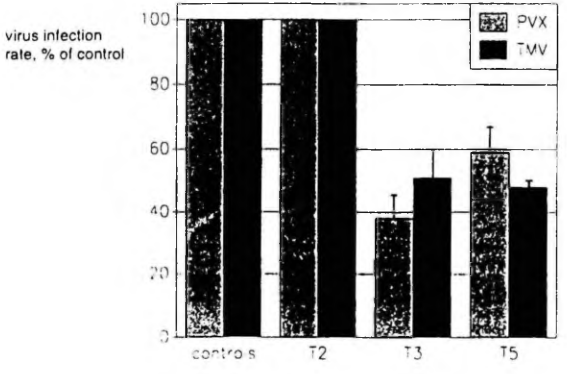

Fig. 4.

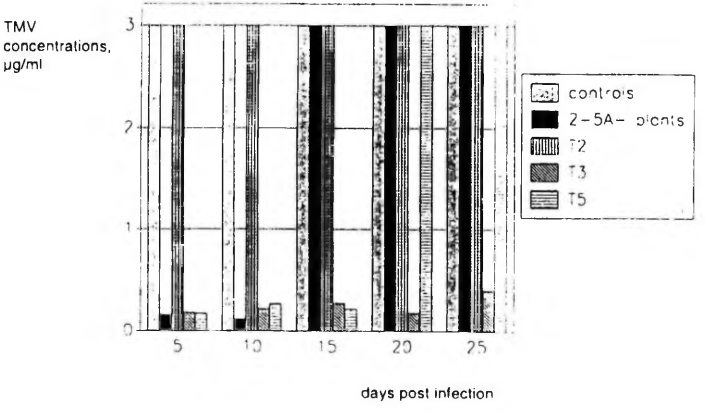

Fig. 5.

CURRICULUM VITAE

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Scientific work

Since 1989 I have studied the mechanisms of viral infection and viral resistance in plants. I have followed the effect of 2'-5' oligoadenylates on the protein synthesis and RNA stability in plants. I have constructed transgenic tobacco and potato plants expressing mammalian 2'-5' oligoadenylate synthetase which are resistant to infection of several different plant viruses. Presently I am characterizing the second enzyme of the 2-5A pathway — RNase L. I have studied also the functioning of 2-5A system in neuronal differentiation and apoptosis of rat cell lines.

I have also constructed transgenic tobaccos expressing PVX coat protein. Here I am at the moment studying the molecular mechanisms of plant virus coat protein-mediated resistance and natural resistance to plant viral diseases.

I am also involved in a project on characterization of the structure and biology of cocksfoot mottle sobemovirus. We have sequenced the virus and characterized the *in vitro* translation products, translational strategies of the virus and its subgenomic RNA.

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Nooremteadur	1991–1996

Teadustegevus

Alates 1989. aastast olen uurinud taimede viirusinfektsiooni ja viirusresistentsi mehhanisme. Olen analüüsinud 2'-5' oligoadenülaatide mõju valgusünteesile ja RNA stabiilsusele taimedes. Olen konstrueerinud transgeensed tubaka- ja kartulitaimed, mis ekspresseerivad imetajate 2-5A süntetaasi ja on resistentsed mitmete taimeviiruste suhtes. Praegu iseloomustan 2-5A süsteemi teist ensüümi: RNaas L-i. Olen uurinud ka 2-5A süsteemi funktsioneerimist roti rakuliinide neuraalses diferentseerumises ja apoptoosis.

Olen konstrueerinud ka transgeensed tubakad, mis ekspresseerivad kartuliviiruse X kattevalku. Praegu uurin molekulaarseid mehhanisme, mis tagavad taimeviiruste kattevalgu vahendatud resistentsi ning loodusliku kaitse taimede viirushaiguste vastu.

Olen seotud ka projektiga, mis tegeleb keraheina laiguviiruse struktuuri ja bioloogia iseloomustamisega. Oleme sekveneerinud selle viiruse genoomi, iseloomustanud *in vitro* translatsiooniprodukte ja -strateegiaid ning viiruse subgenoomset RNA-d.

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