ENGINEERING RESISTANCE AGAINST POTATO VIRUS Y

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Stellingen

1. De conclusies van Pang *et al.* (1992), betreffende transgene resistentie tegen heterologe TSWV-isolaten uit verschillende serogroepen, worden niet ondersteund door de gepresenteerde resultaten.

   Pang S-Z, Nagpala P, Wang P, Slightom JL and Gonsalves D (1992). Resistance to heterologous isolates of tomato spotted wilt virus in transgenic tobacco expressing its nucleocapsid protein gene. *Phytopathology* **82**: 1223-1229.

2. De conclusie van Price, dat op grond van het voorkomen van diverse klassen van kortere virusdeeltjes van het aardappelvirus X, dit virus beschouwd dient te worden als een 'multipartite' virus, is onjuist.

   Price M (1993). Multipartite nature of potato virus X. *J Virology* **67**: 596-600.

3. Herschlag (1991) gaat er ten onrechte van uit dat binding van een 'hammerhead ribozyme' aan een substraat altijd resulteert in klieving van dat substraat.

   Herschlag D (1991). Implications of ribozyme kinetics for targeting the cleavage of specific RNA molecules *in vivo*: More isn't always better. *Proc Natl Acad Sci USA* **88**: 6921-6925.

4. De naam 'symbionine' voor het eiwit dat in grote hoeveelheid door de endosymbiont van *Acyrthosiphon pisum, in situ* wordt geproduceerd, dient wellicht te worden herzien nu is aangetoond dat dit eiwit enkel ten voordele van de endosymbiont lijkt te komen.

   Fukatsu T and Ishikawa H (1992). Synthesis and localization of symbionin, an aphid endosymbiont protein. *Insect Biochem Molec Biol* **22**: 167-174.

5. De virusfamilie *Filoviridae* dient als genus *Filovirus* binnen de familie *Paramyxoviridae* te worden geclassificeerd.

   Feldman H, Mühlberger E, Randolf A, Will C, Kiley MP, Sanchez A and Klenk H-D (1992). Marburg virus, a filovirus: messenger RNAs, gene order, and regulatory elements of the replication cycle. *Virus Research* **24**: 1-19.

   McCormick JB (1991). *Filoviridae*. In: *Classification and Nomenclature of Viruses, Fifth report of the International Committee on Taxonomy of Viruses, Arch Virology supplementum* **2**, pp. 247-249. Francki RIB, Fauquet CM, Knudson DL and Brown F (eds.). Springer-Verlag, Wien.

6. De aanduiding 'niet-coderende gebieden' voor niet-vertaalde gebieden in virale genomen, doet onrecht aan de grote biologische betekenis van deze regio's.

7. De architectuur van 'het Hoofdgebouw' suggereert ten onrechte een doorzichtige bestuursstructuur aan de Landbouwuniversiteit.

8. Het verschil tussen een systemische en een lokale infectie met 'het motorrijdersvirus' wordt slechts zichtbaar na een langere incubatietijd bij temperaturen beneden 5°C.

9. Emancipatie wordt niet door een wet maar door een samenleving geregeld.

Stellingen behorend bij het proefschrift:

Engineering resistance against potato virus Y

Wageningen, 26 januari 1993. René van der Vlugt

Voorwoord

Hoewel op de omslag van dit proefschrift slechts één naam vermeld staat, moge het duidelijk zijn dat dit 'boekje' nooit geschreven had kunnen worden zonder de hulp, inzet en het enthousiasme van vele mensen. Mijn dank gaat uit naar Rob Goldbach, als promotor heeft hij niet alleen de start tot dit onderzoek gegeven, maar heeft hij het ook, tot aan de laatste komma, steeds kritisch gevolgd. Harm Huttinga voor de vele nuttige adviezen, Peter de Haan, Hans van den Heuvel en alle andere 'plantevirologen' voor de vele (heftige) gedachtenwisselingen (over het werk en nog belangrijker zaken) en de gezellige samenwerking. De studenten Sjefke Allefs, Dianne van der Kop, Ambro van Hoof, Femke Blokker, René Ruiter, Marcel Prins, Karin Horsman en Doriet Willemen, die elk op hun eigen manier wezenlijk hebben bijgedragen aan het onderzoek. Jeroen Charité voor de hulp bij het begin van het ribozyme werk en Bert Essenstam voor de excellente verzorging van de transgene planten. Zonder iemand te willen vergeten, dank ook aan alle (ex-)medewerkers en (ex-)studenten van de vakgroep voor de prettige samenwerking en de goede werksfeer.
Rest mij nog slechts jou, Cécile, te bedanken voor je niet aflatende steun en je gewillig oor, misschien wel de meest essentiële bijdragen aan dit proefschrift.

René

P.S. Dat een infectie met een virus ook mooie gevolgen kan hebben, is mij nu wel duidelijk!

Suavis laborum est praeteritorum memoria

Cicero

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Chapter 1

General Introduction

1.1 The Potyviridae

Potato virus Y (PVY) is the type member of the *Potyviridae*, probably the largest and most wide-spread plant virus family in the world. This group of plant pathogens contains over 200 definitive and possible members (Barnett, 1991a), many of which occur in a wide range of crops in, especially, tropical and sub-tropical areas. Most viruses have narrow and often extremely restricted host ranges though some can infect plant species in up to 30 families (Hollings and Brunt, 1981; Barnett, 1991a). Members of the *Potyviridae* are generally classified on the basis of the following criteria:

- Flexuous filamentous particles, 680-900 nm long and 11 to 15 nm wide, with helical symmetry, containing one type of coat protein.
- A single-stranded positive sense RNA genome of 8.5-10 kb in length with at its 5'-end a protein denoted VPg (Viral Protein genome-linked) and at its 3'-end a poly(A)-tail. Functional proteins are generated by processing of polyprotein precursors.
- Virus infections induce the formation of characteristic cytoplasmic inclusion bodies ('pinwheels').
- Serologically clearly distinguishable from morphologically similar viruses.

Most potyviruses have been described as being transmitted by aphid vectors in a non-persistent, stylet-borne manner (Hollings and Brunt, 1981). Over the years however, a number of filamentous viruses have been described, which also induce the typical cytoplasmic inclusion bodies or pinwheels, but were shown to be transmitted by vectors other than aphids. Reported vectors were eriophydic mites, the fungus *Polymyxa graminis* and whiteflies. Though it has been suggested that, based on their different mode of transmission, these viruses should be excluded from the potyvirus group (Hollings and Brunt, 1981), they are nowadays included as possible members. Recently, a new system for the classification of this virus group was proposed with the new *Potyviridae* plant virus family that is to include, based on their mode of virus transmission, three definitive genera and one possible genus (Barnett, 1991b, 1992). The genus *Potyvirus* consists of the largest group, the aphid-transmitted viruses, with potato virus Y as the type species. Viruses belonging to the genus *Bymovirus* are fungal-borne with barley yellow mosaic virus (BaYMV) as the

type species, while the mite-transmitted viruses, with ryegrass mosaic virus (RGMV) as the type species, are included in the genus *Rymovirus*. The only whitefly transmitted virus reported sofar, sweet potato mild mottle virus (SPMMV), is included in the possible genus *Ipomovirus*.

Viruses belonging to the proposed genus *Potyvirus* possess one non-segmented RNA genome of approximately 10 kb, encoding one large polyprotein (Allison *et al.*, 1986; Domier *et al.*, 1986; Maiss *et al.*, 1989; Robaglia *et al.*, 1989; Johansen *et al.*, 1991). In contrast BaYMV, the type species of the baymoviruses, contains two genomic RNAs (Kashiwazaki *et al.*, 1990; 1991; Davidson *et al.*, 1991; Peerenboom *et al.*, 1992). This supported the previous observation of a bimodal particle distribution (Huth *et al.*, 1984). Wheat streak mosaic virus, a member of the ryemoviruses, contains a monopartite single-stranded RNA genome of approximately 8.5 kb and preliminary sequence data show a similar genome organization as observed for the genus *Potyvirus* (Niblett *et al.*, 1991). No data on the genome of the ipomovirus sweet potato mottle virus are available yet.

Genomic sequence comparisons have revealed that potyviruses resemble the plant bipartite como- and nepoviruses and the animal picornaviruses, both in general genome organization and expression. All viruses from these groups contain a set of genes, conserved in order, that encode non-structural proteins which, on the basis of a number of characteristic amino acid motifs, are thought to be involved in RNA-replication. This resemblance in genome structure and expression has prompted the suggestion that these plant virus groups can all be arranged in the supergroup of 'picorna-like' viruses (Goldbach, 1986, 1987, 1992; Goldbach *et al.*, 1990).

1.2 Potyviral RNA genome organization and expression

Viruses belonging to the genus *Potyvirus* share the same characteristics with respect to particle morphology and the organization and expression strategy of their RNA genomes. The flexuous and rod-shaped virus particles of 680 to 900 nm long and 11 to 15 nm wide consist of approximately 2000 units of one type of structural protein that encapsidate a non-segmented, single-stranded RNA molecule of positive polarity and with a length of approximately 9700 nucleotides. This RNA is at its 5'-end covalently coupled to a viral encoded protein (VPg) (Siaw *et al.*, 1985; Murphy *et al.*, 1990) and at its 3'-terminus it contains a poly(A)-tail (Hari *et al.*, 1979). The potyviral genome contains one large open reading frame (ORF) which is translated into a large polyprotein of 340 to 368 kDa, depending on the virus. Cleavage of the polyprotein, by three virally encoded proteases, results in maturation of 7 to 10 functional proteins (Dougherty and Carrington, 1988; Goldbach, 1990; Riechmann *et al.*, 1992, see Fig 1.1) for most of which their (likely) biological functions are now known. Starting with the N-terminal protein these are:

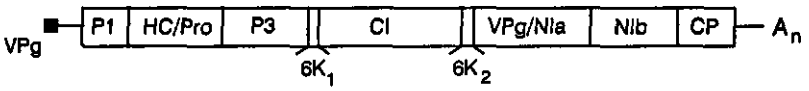

Fig. 1.1 Schematic representation of the organization of the potato virus Y RNA genome. See text for abbreviations of encoded proteins.

P1 with a sofar unknown function, however the C-terminal part of the protein is involved in autoproteolytic cleavage at its C-terminus between P1 and the second protein, the HelperComponent/Protease (HC-Pro) (Verchot *et al.*, 1991). This latter protein constitutes a factor necessary for aphid-transmission of the virus (Pirone and Thornbury, 1983; Thornbury *et al.*, 1985), while the C-terminal part is involved in autoproteolytic cleavage at its C-terminal cleavage site (Carrington and Herndon, 1992). The third protein (P3) also has an unknown function though the N-terminal part contains a motif that resembles a motif present in the 32 kDa protein of cowpea mosaic virus (CPMV), a protein involved in regulation of proteolytic processing of the polyprotein (Vos *et al.*, 1988). The fourth, putative, protein is a 6 kDa protein ($6K_1$), which is only partially released from the N-terminus of the cytoplasmic inclusion (CI) protein (García *et al.*, 1992). This CI-protein aggregates to form the typical cytoplasmic pinwheel inclusion bodies, found in potyvirus infected cells (Edwardson, 1974; Dougherty and Hiebert, 1980). It contains a nucleotide binding motif (NTBM) (Domier *et al.*, 1987; Laín *et al.*, 1989; Robaglia *et al.*, 1989) and was shown to have a nucleic acid stimulated ATPase and a RNA helicase activity and is therefore believed to be involved in potyviral RNA-replication (Laín *et al.*, 1990, 1991). At the C-terminus of the CI-protein a second, putative, 6 kDa protein ($6K_2$) is found. Both the $6K_2$ and $6K_1$ proteins resemble the picornaviral 2B and 3A peptides, as well as in relative genome location as in amino acid composition (Laín *et al.*, 1989), and these proteins are therefore also thought to be involved in potyviral replication (Riechmann *et al.*, 1992). The small nuclear inclusion protein (NIa), together with the large nuclear inclusion protein (NIb), forms the nuclear inclusion bodies found upon infection with some (e.g. TEV) but not all potyviruses. The most prominent function of the NIa protein is that of the main protease responsible for all but two cleavage events (those at the C-termini of both P1 and HC-Pro) in the potyviral polyprotein. The protease activity is located in the C-terminal part of the protein (García and Laín, 1991; Dougherty and Parks, 1991), while the complete NIa protein, or its N-terminal half, functions as the VPg (Shahabuddin *et al.*, 1988; Murphy *et al.*, 1990). A number of conserved motifs, characteristic of RNA-dependent RNA-polymerases, identified the

NIb protein as the potyviral polymerase (Domier *et al.*, 1987; Robaglia *et al.*, 1989; Poch *et al.*, 1989). Finally the last protein of the polyprotein is the viral coat protein (CP) of which the central part is highly conserved while in contrast, the N-terminus is extremely variable, both in length and in sequence, among the different potyvirus species. Comparisons of potyviral coat protein sequences therefore form the, nowadays widely accepted, molecular basis of potyvirus taxonomy (Ward and Shukla, 1991).

Potyviruses, unlike viruses that have segmented genomes or produce subgenomic RNAs, are unable to regulate their genome expression at either the transcriptional or translational level. It has been shown that for the majority of the potyviral polyprotein cleavage sites a conserved hepta-peptide sequence, characteristic of a particular potyvirus, determines the specificity and efficiency of the cleavage reaction (Dougherty *et al.*, 1989; Dougherty and Parks, 1989; García *et al.*, 1992). Potyviruses might therefore use post-translational, sequential polyprotein cleavage events to regulate the expression and function of their gene products. The possible role of partially processed intermediates (like e.g. the VPg/NIa precursor protein) however remains to be elucidated.

1.3 Potato virus Y

The type species of the genus *Potyvirus* potato virus Y (PVY) was first identified as a separate virus from a composite group of viruses causing mosaic diseases on potatoes (Smith, 1931). Virus particle dimensions are 730 x 11 nm, the apparent M_r of the coat protein is 34,000 (Huttinga and Mosch, 1974). Numerous studies on the host range have shown that the virus is mainly restricted to plants belonging to the *Solanaceae* but some plants from other families are also infected (Horváth, 1967). The virus is readily transmitted mechanically, however in the field it is mainly transmitted by aphids in a non-persistent manner, with *Myzus persicae* being the most efficient vector. Three major strains of the virus were identified, mainly on the basis of characteristic symptoms on different potato cultivars and indicator plants like *Nicotiana tabacum* 'White Burley' and 'Samsun NN' and *Physalis floridana* (De Bokx, 1961; De Bokx and Piron, 1977; De Bokx and Huttinga, 1981; Beemster and De Bokx, 1987):

- The PVY^0 strain (or 'common' strain), occurring worldwide and causing severe systemic crinkle symptoms, rugosity or leaf-drop streak in potato, systemic necrosis on *P. floridana* and systemic mottling in tobacco.
- The PVY^N strain (or 'tobacco veinal necrosis' strain) occurring in Europe, Africa and South-America. Isolates of this strain induce very mild mottling in almost all

potato cultivars, systemic mottling in *P. floridana* and severe systemic veinal necrosis in tobacco.

- The PVY^{C} strain (or 'stipple streak' strain) occurs in Europe, India and Australia. Most potato cultivars are hypersensitive to isolates belonging to this strain while susceptible cultivars show systemic mosaic or stipple streak. Infection with isolates from this group causes systemic necrosis on *P. floridana* and systemic mottling in tobacco.
- Several other isolates or strains, that appear to belong to none of the above strains, have been identified like an anomalous strain (PVY^{an}, Horváth, 1967), and recently, PVY^{NN}, a group of virus isolates highly resembling PVY^{N} however causing very characteristic necrotic ringspots on potato tubers (Potato Tuber Necrotic Ringspot Disease or PTNRD), (Van den Bovenkamp, 1992).

Though PVY can cause serious damage in pepper, tobacco and tomato, especially in warmer climates (Edwardson, 1974; De Bokx and Huttinga, 1981), its major impact is in potato crops all over the world. Infections generally result in yield losses up to 80%, depending on the virus strain, potato cultivar and time of infection (De Bokx and Huttinga, 1981). The virus translocates to the tubers, resulting in secondary infections upon replanting of infected tubers. Symptom expression varies greatly with the virus strain, potato cultivar and climate conditions (De Bokx and Piron, 1977) with secondary symptoms usually being more severe. In most potato cultivars PVY^{O} and PVY^{C} induce necrosis, mottling and other symptoms that are readily detectable (De Bokx and Huttinga, 1981). In contrast, PVY^{N} induces only mild mottling in most potato cultivars, making detection of infected plants in (seed) potato crops difficult. This facilitated the accumulation of the virus in potato stocks and its subsequent spread over Europe, Africa, Asia and South-America since it was first detected in a collection of wild potatoes in England (Munro, 1955; Weidemann, 1988).

Indirect control of PVY by spraying with insecticides and/or mineral oil to control natural aphid vectors is difficult because most compounds act too slow to prevent brief probing by aphids and in fact often cause more active probing behaviour of the aphids, resulting in increased virus acquisition and inoculation (Schepers *et al.*, 1984). In addition, the use of chemicals to control pests is more and more questioned as a result of the large impact such compounds can have on the environment. Breeding for resistance would therefore be the most acceptable and most efficient means of controlling virus diseases in potato. Several different types of resistance to PVY have sofar been reported and will be discussed below.

The effect that virus replication and translocation is slowed down in older potato plants thus preventing tubers from becoming infected is known as mature-plant resistance (Beemster, 1987). Large differences in the onset of this type of resistance however can occur depending on the potato cultivar, virus strain and environmental

conditions (Weidemann, 1988). More important than mature plant resistance is field resistance, also known as quantitative resistance. This polygenic resistance is effective against all PVY strains and occurs in a large number of cultivars. However, depending on the degree of resistance, a number of plants still becomes infected. Affording complete protection to PVY, are both 'hypersensitivity resistance' and 'extreme resistance' (Beemster, 1987; Colon, 1987). The first type of resistance leads to necrosis of infected cells, restricting the spread of the virus. This type of (dominant) monogenic resistance is available against PVY^N (N_y-gene) and PVY^C (N_c and N_y-gene). A number of N-genes appear to interact with virulence genes in a gene-for-gene relationship, and therefore potato cultivars hypersensitive to a particular strain may be normally susceptible to another strain (Beekman, 1987). Extreme resistance, or immunity, is the most important type of PVY-resistance in potato breeding, and depends on the presence of dominant genes ('R-genes'). These genes have been found in a number of wild *Solanum* species and *S. tuberosum* ssp. *andigena*. *S. stoloniferum* was shown to possess at least six resistance genes to PVY and potato virus A (PVA) (Cockerham, 1970). $R_{y(sto)}$ and $R_{y(and)}$ confer resistance to all known strains of PVY (Beekman, 1987; Colon, 1987; Weidemann, 1988). Introduction of these genes by breeding programs has resulted in a growing number of new resistant potato cultivars. Nowadays most potato cultivars grown in the Netherlands (and Western Europe) possess average to good levels of (field) resistance to PVY^N and other PVY strains (66* Beschrijvende Rassenlijst, 1991). However there are still a number of older cultivars of significant economic importance grown, in which only low levels of PVY-resistance are present, with as best known example 'Bintje'. Originally obtained in the beginning of this century, this cultivar is still a major potato crop worldwide, however its complex genetic background has sofar hampered the introduction of any of the available PVY-resistance genes into its genome. As in most cultivars PVY^N symptoms are not easy to detect and infected plants are therefore very hard to identify visually (Weidemann, 1988). Extensive screening for PVY^N is therefore required to guarantee virus-free potato stocks.

1.4 Outline of the thesis

Potato virus Y^N, as outlined above, represents an important pathogen of potato cultivars and other solanaceous crops like tomato and pepper. Though certain poly- and monogenic resistances to the virus are available, introduction in breeding programs or in registered cultivars is not always possible or very time consuming. Novel techniques, like genetic engineering, are expected to speed up the introduction of these, and other, traits in new crops.

At the start of this investigation a number of reports on genetically engineered virus resistance were published, to which generally is referred to as 'coat protein-

mediated resistance'. This coat protein-mediated resistance, is based on the *A. tumefaciens* mediated introduction of a viral coat protein gene into the genome of susceptible host plants. Expression of the coat protein gene may result in resistance to the homologous virus, and a number of closely related viruses (Powell *et al.*, 1986). Following these initial results on, mostly, well studied model viruses like tobacco mosaic virus (TMV), the aim of the research reported in this thesis was to establish some form of 'genetically engineered' resistance against PVY^N.

As a first step, part (4412 nt) of the nucleotide sequence of the PVY^N RNA genome (approx. 9500 nt) has been determined, i.e. the 3'-terminal half which contains the gene coding for the viral coat protein (Chapter 2). The cloning and sequence determination of the PVY^N RNA genome, necessary to enable engineered resistance involving viral genes, also enabled the investigation of the precise taxonomic position of this virus among a large number of (geographically) distinct PVY isolates. Sequence data, that became available during the course of this PhD research, allowed detailed comparisons of the amino acid and nucleotide sequences of their coat protein and 3'-non-translated regions (Chapter 3). Upon addition of the necessary transcriptional and translational start signals the PVY^N CP gene was transferred to the genome of potato cv. Bintje. The resulting transgenic plants were analyzed for the presence and expression of the viral transgene and possible protection against mechanical inoculation with the virus (Chapter 4). Constructs employing the PVY^N CP cistron, were also used for the transformation of tobacco cv. SR1. High levels of protection to PVY^N are described that are most likely based on the presence of viral RNA transcripts rather than on the presence of the coat protein itself (Chapter 5). This form of 'RNA-mediated resistance' was further analyzed for its potential to protect transgenic plants against different PVY isolates belonging to the major strains PVY^N, PVY^O and PVY^C, and challenging with viruliferous *Myzus persicae*, a natural aphid vector of PVY (Chapter 6). Finally the design and construction of a 'ribozyme' (a RNA sequence able to specifically cleave another RNA sequence), directed against a conserved region in the RNA-dependent RNA-polymerase gene of PVY^N, is described (Chapter 7). Some factors that influence the efficiency of cleavage of the substrate RNA by the ribozyme, and implications for the possible use of ribozymes as anti-viral agents, are discussed.

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Chapter 2

Nucleotide sequence of the 3'-terminal half of the potato virus Y^N RNA genome.

Summary

The nucleotide sequence of the 3'-terminal 4412 nucleotides (nt) of the RNA genome of the tobacco veinal necrosis strain of potato virus Y (PVY^N) was determined. The sequence revealed an open reading frame (ORF) of 4082 nt, of which the start was not identified, followed by an 3'-non-translated region (3'-NTR) of 329 nt upstream of a poly(A)-tract. The ORF corresponded with that part of the polyprotein that encompassed the C-terminal sequence of the cytoplasmic inclusion (CI) protein and the complete sequences of the putative $6K_2$ protein, the nuclear inclusion proteins NIa and NIb and the viral coat protein (CP). Cleavage sites in the polyprotein, releasing the functional viral proteins, were identified by comparisons with polyprotein sequences of a French isolate of PVY^N and several other potyviruses. In addition, the sequence of the amino terminal part of the CP was determined chemically, allowing precise mapping of its coding sequence.

The NIa, NIb and CP of PVY^N displayed significant sequence homology to the corresponding proteins of several other potyviruses like tobacco etch virus (TEV), tobacco vein mottling virus (TVMV), plum pox virus (PPV) and bean yellow mosaic virus (BYMV). Highest sequence homologies were observed to the NIa, NIb and CP of a French isolate of PVY^N and to the CP of another potyvirus, pepper mottle virus (PepMoV). Based on this sequence homology, and on the homology observed in the 3'-NTR, it should be concluded that PepMoV is to be regarded as a strain of PVY.

Parts of this chapter have been published as: Van der Vlugt *et al.* (1989). *J Gen Virology* **70**: 229-233; and R. van der Vlugt (1992). *Arch Virology, supplementum* 5: 327-335.

2.1 Introduction

The *Potyviridae* represent the largest plant virus family containing more than 70 established and 140 possible members (Barnett, 1991). Most of these viruses have been taxonomically included on the basis of their particle morphology and biological properties, such as distinct reactions on indicator plants and serological reactions. Viruses belonging to the genus *Potyvirus*, the largest genus within this family, have a single-stranded RNA genome of approximately 9700 nucleotides, provided with a small protein (VPg) at the 5'-end and a poly(A)-tail at the 3'-end. For several potyviruses e.g. tobacco etch virus (TEV), tobacco vein mottling virus (TVMV), plum pox virus (PPV) and pea seedborne mosaic virus (PSbMV) nucleotide sequence analysis of their genomes has revealed the presence of one large open reading frame (Allison *et al.*, 1986; Domier *et al.*, 1986; Maiss *et al.*, 1988; Johansen *et al.*, 1991). This open reading frame (ORF) encodes a high molecular weight (≈340 kDa) polyprotein which is processed into functional proteins by virally encoded proteinases (Dougherty & Hiebert, 1985; Riechmann *et al.*, 1992; Goldbach, 1990).

For a growing number of economically important plant viruses it has been shown that transformation of hosts with a translationally active form of the coat protein gene of a given virus, may confer resistance to infections and/or disease development by the corresponding virus. This form of engineered resistance is now generally referred to as 'coat protein-mediated resistance' (for reviews see Beachy *et al.*, 1990; Hemenway *et al.*, 1990; Nejidat *et al.*, 1990).

As a first step towards genetically engineered protection against PVY^N and to gain a better insight in the complex serological relationships existing among the potyviruses, the nucleotide sequence of the 3'-terminal region of the PVY^N RNA genome, containing the complete CP cistron, was determined (Van der Vlugt *et al.*, 1989). During the course of this investigation additional cDNA clones from the 3'-terminal half of the PVY^N genome were obtained and their sequences determined. This chapter reports the cloning and sequence determination of the 4412 3'-terminal nt of the PVY^N RNA genome, encompassing the coding regions for both NIa and NIb nuclear inclusion proteins, the viral CP and the 3'-NTR.

2.2 Materials and Methods

2.2.1 Virus and viral RNA purification

A Dutch isolate (PVY-NI1) of PVY^N (Hollings and Brunt, 1981), was obtained from Ing. D.Z. Maat (DLO Research Institute for Plant Protection; IPO-DLO, Wageningen) and purified from *Nicotiana benthamiana* 2-3 weeks after inoculation as described below. Purification steps were performed at 4°C.

Leaf material was homogenized in 2 volumes of citric acid-phosphate buffer (CAP-buffer: 0.14 M Na_2HPO_4/0.036 M citric acid, pH 7.0), containing 0.01 M sodium thioglycolate and 0.02 M sodium diethyldithiocarbamate. Triton X-100 was added to a final concentration of 1% (v/v) and the mixture was incubated for 18 h under continuous stirring. The homogenate was filtered through cheesecloth, centrifuged for 10 min at 6,500*g* and the resulting supernatant centrifuged for 2 h at 50,000*g*. The pellet was resuspended in 0.1 vol of CAP-buffer and the centrifugation steps repeated again. The final pellet was resuspended in 0.1 vol CAP-buffer and clarified by centrifugation for 10 min at 6,500*g*. The supernatant was layered on top of a 10-40% (w/v) sucrose gradient in the same buffer and centrifuged at 75,000*g* for 2 h. Pooled virus fractions were diluted 1:1 with CAP-buffer and centrifuged for 2.5 h at 52,500*g*.

The remaining virus pellet was resuspended in 21.6 ml of CAP-buffer, mixed with 38.4 ml of a 0.623 g/ml CsCl solution in distilled water and centrifuged for 22 h at 110,000*g* and 20°C. Virus fractions were collected and dialyzed against CAP-buffer. The CsCl centrifugation step was repeated and after dialysis against CAP-buffer the virus concentration determined. Virus yield was about 1-5 mg per 100 g of fresh leaves. Virus was stored in small aliquots at either -196°C or -20°C.

2.2.2 PVY^N RNA isolation

RNA was purified from virus preparations by a 15 min incubation at room temperature in 10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% SDS (final concentrations) followed by repeated phenol/chloroform extractions and ethanol precipitation of the RNA (Sambrook *et al.*, 1989). Purified RNA was resuspended in double distilled water and stored in small aliquots at -20°C.

2.2.3 Synthesis and cloning of complementary DNA

Synthesis of double-stranded cDNA of PVY^N RNA was done essentially by the method as described by Gübler and Hoffmann (1983) involving oligo-dT_{12-18} priming and AMV Reverse Transcriptase for first strand synthesis followed by second strand synthesis using RNaseH, *E. coli* DNA polymerase I and T_4 DNA ligase. cDNA was either made blunt-end by incubation with T_4 DNA polymerase and cloned directly into a *Sma*I-digested pUC19 plasmid (Yarrish-Perron *et al.*, 1985) or digested with an appropriate restriction-enzyme and cloned in the polylinker region of Bluescript (Stratagene) KS or SK vectors.

2.2.4 Identification of recombinant clones

Recombinant plasmids were transformed to *E. coli* strain MH1 using the $CaCl_2$ transformation method (Sambrook *et al.*, 1989) or to *E. coli* strain DH5αF' by electroporation. Ampicillin-resistant colonies, harbouring recombinant plasmids containing PVY^N-specific inserts, were identified by plating on LB-agar plates containing 20 μg/ml IPTG and X-gal and colony filter hybridization (Grunstein and Hogness, 1975) using oligo-dT_{12-18} primed first strand α-^{32}P-dATP labelled PVY^N cDNA. Plasmid DNA containing viral cDNA-inserts was analyzed by restriction enzyme mapping and Southern analysis (Southern, 1975) using first strand α-^{32}P-dATP-labelled cDNA as a probe. To definitely confirm their genetic origin the cDNA-inserts were isolated from Low Melting Point agarose gels, labelled with α-^{32}P-dATP in a nick-translation reaction (Rigby *et al.*, 1977) and hybridized with PVY^N RNA on Northern blots (Sambrook *et al.*, 1989).

2.2.5 Nucleotide sequence determination

Restriction fragments of cloned cDNA were inserted into the polylinker region of either M13mp18 or M13mp19 (Norrander *et al.*, 1983) or Bluescript KS and SK plasmids (Stratagene). Single stranded template DNA was isolated and sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using the universal M13 17-mer primer. Double stranded sequencing on plasmid DNA was performed as described in the "Sequenase" manual (United States Biochemical Corporation, 3rd edition). Chain elongation reactions were carried out using either the large fragment of DNA polymerase I ("Klenow fragment") or modified T_7 DNA polymerase ("Sequenase", USB) in the presence of α-^{32}P-dATP or α-^{35}S-dATP. Nucleotide sequences and protein data were analyzed using the Staden program (Staden, 1986) and University of Wisconsin GCG program package (Devereux *et al.*, 1984).

2.3 Results

2.3.1 The sequence of the 3'-terminal half of PVY^N RNA genome

Double-stranded cDNA, transcribed from PVY^N RNA, was ligated into the *Sma*I-site of pUC19 or, upon digestion with restriction enzymes, in the polylinker region of Bluescript vectors and recombinant plasmids were transformed to *E. coli*. Using oligo-dT_{12-18} primed, α-^{32}P-dATP labelled PVY^N cDNA as a probe, recombinant plasmids containing PVY^N-specific cDNA inserts were identified, the inserts ranging in size from 300 to 2600 nucleotides. Sequence analysis of the 3'-end of the cDNA inserts demonstrated that three clones, with inserts of 1600, 1250 and 1200 base-

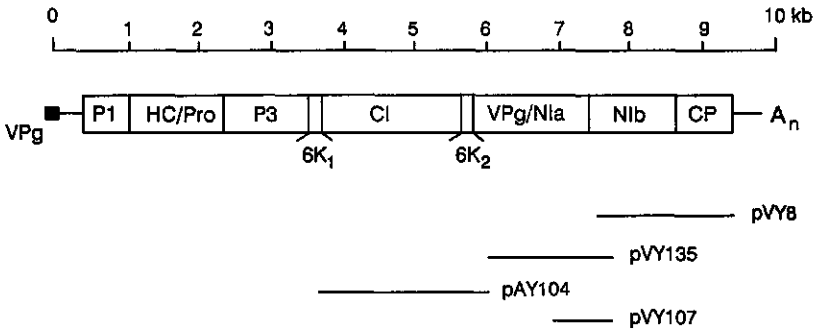
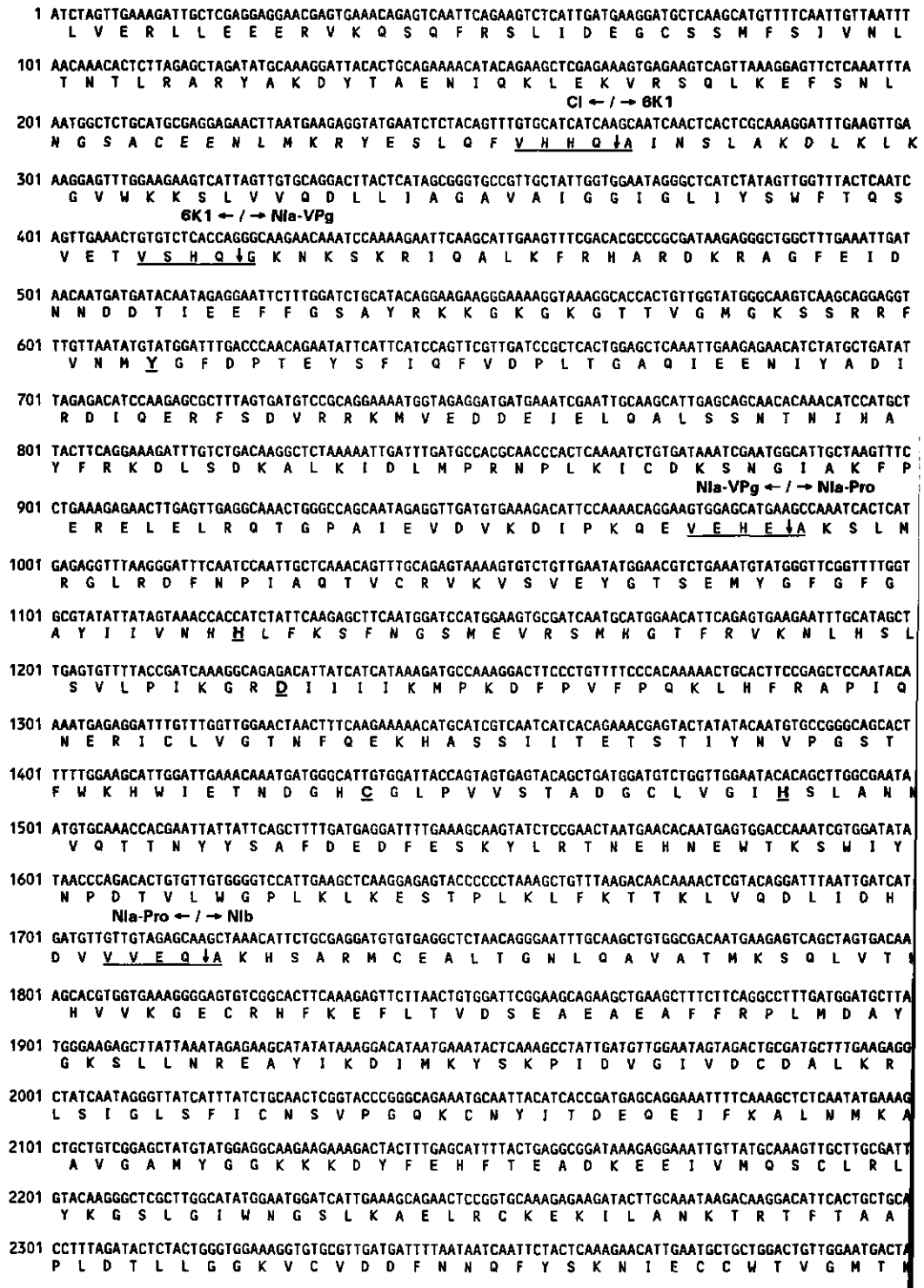


Fig. 2.1 Schematic representation of the organization of the PVY^N RNA genome and the localization of the different cDNA clones.

pairs (bp), contained a poly(A)-tract and therefore comprised the 3'-terminal sequence of PVY^N RNA. Sequence determination of clone pVY8, containing a 1600 bp cDNA insert that encodes the complete PVY^N CP cistron, has been described previously (Van der Vlugt *et al.*, 1989). The positions of additional clones on the viral genome were determined by restriction enzyme mapping and are shown in Figure 2.1. The cDNA insert contained in clone pAY104 had previously been prepared by Dr. H. Huttinga (IPO-DLO, Wageningen).

The sequence of the 4412 3'-terminal nt of PVY^N RNA, excluding the poly-(A) tail, is presented in Fig. 2.2. Computer analysis revealed a large ORF of 4080 nt in reading frame three of the (+) strand (virion polarity). The other reading frames of the (+) strand and all three reading frames in the (-) strand contained numerous stopcodons and few extended ORF's. The AUG start codon of the single ORF was not identified and is, in analogy with the other potyviral RNA genomes sequenced sofar, located far upstream of the sequence analyzed. The large ORF was terminated by a single opal stopcodon located 326 residues upstream of the 3'-poly(A)-tail. A second, amber stopcodon was found in the same reading frame two codons downstream of this first stopcodon (Fig. 2.2).

Cleavage sites in the polyprotein sequence were identified by comparison with the sequence of a French isolate (PVY-Fr) of PVY^N, published during the course of this work (Robaglia *et al.*, 1989). Different polyprotein cleavage sites have been identified for different potyviruses, however all cleavage sites located in the C-terminal half of the polyprotein of one potyvirus display a conserved heptapeptide sequence that is recognized by the NIa protease (Dougherty *et al.*, 1989; Riechmann *et al.*, 1992). The PVY^N-specific cleavage site sequence was identified as Vx(H/E)Q↓(G/S/A) (Robaglia *et al.*, 1989) and this sequence was found at positions 88/89, 140/141, 532/533 and 1093/1094 in the polyprotein (underlined in Fig. 2.2). Cleavage at these sites


1 ATCTAGTTGAAAGATTGCTCGAGGAGGAACGAGTGAAACAGAGTCAATTCAGAAGTCTCATTGATGAAGGATGCTCAAGCATGTTTTCAATTGTTAATTT
L V E R L L E E E R V K Q S Q F R S L I D E G C S S M F S I V N L
101 AACAAACACTCTTAGAGCTAGATATGCAAAGGATTACACTGCAGAAAACATACAGAAGCTCGAGAAAGTGAGAAGTCAGTTAAAGGAGTTCTCAAATTTA
T N T L R A R Y A K D Y T A E N I Q K L E K V R S Q L K E F S N L
CI ← / → 6K1
201 AATGGCTCTGCATGCGAGGAGAACTTAATGAAGAGGTATGAATCTCTACAGTTTGTGCATCATCAAGCAATCAACTCACTCGCAAAGGATTTGAAGTTGA
N G S A C E E N L M K R Y E S L Q F V H H Q ↓A I N S L A K D L K L K
301 AAGGAGTTTGGAAGAAGTCATTAGTTGTGCAGGACTTACTCATAGCGGGTGCCGTTGCTATTGGTGGAATAGGGCTCATCTATAGTTGGTTTACTCAATC
G V W K K S L V V Q D L L I A G A V A I G G I G L I Y S W F T Q S
6K1 ← / → Nla-VPg
401 AGTTGAAACTGTGTCTCACCAGGGCAAGAACAAATCCAAAAGAATTCAAGCATTGAAGTTTCGACACGCCCGCGATAAGAGGGCTGGCTTTGAAATTGAT
V E T V S H Q ↓G K N K S K R I Q A L K F R H A R D K R A G F E I D
501 AACAATGATGATACAATAGAGGAATTCTTTGGATCTGCATACAGGAAGAAGGGAAAAGGTAAAGGCACCACTGTTGGTATGGGCAAGTCAAGCAGGAGGT
N N D D T I E E F F G S A Y R K K G K G K G T T V G M G K S S R R F
601 TTGTTAATATGTATGGATTTGACCCAACAGAATATTCATTCATCCAGTTCGTTGATCCGCTCACTGGAGCTCAAATTGAAGAGAACATCTATGCTGATAT
V N M Y G F D P T E Y S F I Q F V D P L T G A Q I E E N I Y A D I
701 TAGAGACATCCAAGAGCGCTTTAGTGATGTCCGCAGGAAAATGGTAGAGGATGATGAAATCGAATTGCAAGCATTGAGCAGCAACACAAACATCCATGCT
R D I Q E R F S D V R R K M V E D D E I E L Q A L S S N T N I H A
801 TACTTCAGGAAAGATTTGTCTGACAAGGCTCTAAAAATTGATTTGATGCCACGCAACCCACTCAAAATCTGTGATAAATCGAATGGCATTGCTAAGTTTC
Y F R K D L S D K A L K I D L M P R N P L K I C D K S N G I A K F P
Nla-VPg ← / → Nla-Pro
901 CTGAAAGAGAACTTGAGTTGAGGCAAACTGGGCCAGCAATAGAGGTTGATGTGAAAGACATTCCAAAACAGGAAGTGGAGCATGAAGCCAAATCACTCAT
E R E L E L R Q T G P A I E V D V K D I P K Q E V E H E ↓A K S L M
1001 GAGAGGTTTAAGGGATTTCAATCCAATTGCTCAAACAGTTTGCAGAGTAAAAGTGTCTGTTGAATATGGAACGTCTGAAATGTATGGGTTCGGTTTTGGT
R G L R D F N P I A Q T V C R V K V S V E Y G T S E M Y G F G F G
1101 GCGTATATTATAGTAAACCACCATCTATTCAAGAGCTTCAATGGATCCATGGAAGTGCGATCAATGCATGGAACATTCAGAGTGAAGAATTTGCATAGCT
A Y I I V N H H L F K S F N G S M E V R S M H G T F R V K N L H S L
1201 TGAGTGTTTTACCGATCAAAGGCAGAGACATTATCATCATAAAGATGCCAAAGGACTTCCCTGTTTTCCCACAAAAACTGCACTTCCGAGCTCCAATACA
S V L P I K G R D I I I I K M P K D F P V F P Q K L H F R A P I Q
1301 AAATGAGAGGATTTGTTTGGTTGGAACTAACTTTCAAGAAAAACATGCATCGTCAATCATCACAGAAACGAGTACTATATACAATGTGCCGGGCAGCACT
N E R I C L V G T N F Q E K H A S S I I T E T S T I Y N V P G S T
1401 TTTTGGAAGCATTGGATTGAAACAAATGATGGGCATTGTGGATTACCAGTAGTGAGTACAGCTGATGGATGTCTGGTTGGAATACACAGCTTGGCGAATA
F W K H W I E T N D G H C G L P V V S T A D G C L V G I H S L A N
1501 ATGTGCAAACCACGAATTATTATTCAGCTTTTGATGAGGATTTTGAAAGCAAGTATCTCCGAACTAATGAACACAATGAGTGGACCAAATCGTGGATATA
V Q T T N Y Y S A F D E D F E S K Y L R T N E H N E W T K S W I Y
1601 TAACCCAGACACTGTGTTGTGGGGTCCATTGAAGCTCAAGGAGAGTACCCCCCTAAAGCTGTTTAAGACAACAAAACTCGTACAGGATTTAATTGATCAT
N P D T V L W G P L K L K E S T P L K L F K T T K L V Q D L I D H
Nla-Pro ← / → Nlb
1701 GATGTTGTTGTAGAGCAAGCTAAACATTCTGCGAGGATGTGTGAGGCTCTAACAGGGAATTTGCAAGCTGTGGCGACAATGAAGAGTCAGCTAGTGACAA
D V V V E Q ↓A K H S A R M C E A L T G N L Q A V A T M K S Q L V T
1801 AGCACGTGGTGAAAGGGGAGTGTCGGCACTTCAAAGAGTTCTTAACTGTGGATTCGGAAGCAGAAGCTGAAGCTTTCTTCAGGCCTTTGATGGATGCTTA
H V V K G E C R H F K E F L T V D S E A E A E A F F R P L M D A Y
1901 TGGGAAGAGCTTATTAAATAGAGAAGCATATATAAAGGACATAATGAAATACTCAAAGCCTATTGATGTTGGAATAGTAGACTGCGATGCTTTGAAGAGG
G K S L L N R E A Y I K D I M K Y S K P I D V G I V D C D A L K R
2001 CTATCAATAGGGTTATCATTTATCTGCAACTCGGTACCCGGGCAGAAATGCAATTACATCACCGATGAGCAGGAAATTTTCAAAGCTCTCAATATGAAAG
L S I G L S F I C N S V P G Q K C N Y I T D E Q E I F K A L N M K A
2101 CTGCTGTCGGAGCTATGTATGGAGGCAAGAAGAAAGACTACTTTGAGCATTTTACTGAGGCGGATAAAGAGGAAATTGTTATGCAAAGTTGCTTGCGATT
A V G A M Y G G K K K D Y F E H F T E A D K E E I V M Q S C L R L
2201 GTACAAGGGCTCGCTTGGCATATGGAATGGATCATTGAAAGCAGAACTCCGGTGCAAAGAGAAGATACTTGCAAATAAGACAAGGACATTCACTGCTGCA
Y K G S L G I W N G S L K A E L R C K E K I L A N K T R T F T A A
2301 CCTTTAGATACTCTACTGGGTGGAAAGGTGTGCGTTGATGATTTTAATAATCAATTCTACTCAAAGAACATTGAATGCTGCTGGACTGTTGGAATGACTA
P L D T L L G G K V C V D D F N N Q F Y S K N I E C C W T V G M T

2401 AGTTTTATGGAGGTTGGGACAAATTGCTTCGCGCTCTACCTGAAAATTGGGTGTACTGCGATGCTGATGGTTCACAATTCGATAGTTCACTCACCCCATA
F Y G G W D K L L R A L P E N W V Y C D A **D G S Q F D** S S L T P Y

2501 CCTAATCAATGCTGTTCTCATCATCAGAAGCACATACATGGAAGATTGGGAATTGGGGTTGCAAATGTTGCGCAATTTGTACACAGAAATAATTTACACA
L I N A V L I I R S T Y M E D W E L G L Q M L R N L Y T E I I Y T

2601 CCAATCTCAACTCCAGATGGAACAATTGTCAAGAAGTTTAGAGGTAATAATAGCGGTCAACCTTCTACCGTTGTGGATAATTCTCTCATGGTTGTACTTG
P I S T P D G T I V K K F R G N N **S G Q P S T V V D N S** L M V V L A

2701 CTATGCATTACGCTCTCATTAAGGAGGGCGTTGAGTTTGAAGAAATCGACAGCACGTGTGTATTCTTTGTTAATGGTGATGATTTATTGATTGCTGTAAA
M H Y A L I K E G V E F E E I D S T C V F F V N **G D D** L L I A V N

2801 TCCGGAGAAAGAGAGCATTCTCGATAGAATCTCACAACATTTCTCAGATCTTGGTTTGAACTATGATTTTTCATCGAGAACAAGAAGGAAGGAGGAATTG
P E K E S I L D R I S Q H F S D L G L N Y D F S S R T R R **K** E E L

2901 TGGTTCATGTCCCACAGAGGCCTGCTAATCGAGGGCATGTACGTGCCAAAGCTTGAAGAAGAGAGAATTGTATCCATTCTGCAATGGGATAGAGCTGATC
W F M S H R G L L I E G M Y V P K L E E E R I V S I L Q W D R A D L

3001 TGCCAGAGCACAGATTAGAAGCGATTTGTGCAGCAATGATAGAATCCTGGGGTTATTTTAAGTTAACGCACCAAATCAGGAGATTCTACTCATGGTTGTT
P E H R L E A I C A A M I E S W G Y F K L T H Q I R R F Y S W L L

3101 GCAACAGCAACCTTTTTCAACGATAGCGCAGGAAGGAAAAGCTCCATACATAGCGAGCATGGCATTGAAGAAGCTGTACATGGACAGGACAGTAGATGAG
Q Q Q P F S T I A Q E G K A P Y I A S M A L K K L Y M D R T V D E

NIb ← / → CP

3201 GAGGAACTGAAAGCTTTCACTGAAATGATGGTTGCCTTGGATGATGAATTTGAGTGCGATACTTATGAAGTGCACCATCAAGGAAATGACACAATCGATG
E E L K A F T E M M V A L D D E F E C D T Y E **V H H Q ↓G** N D T I **D A**

3301 CAGGAGGAAGCACTAAGAAAGATGCAAAACAAGAGCAAGGTAGCATTCAACCAAATCTCAACAAGGAAAAGGTAAAGGACGTGAATGTTGGAACATCTGG
G G S T K K D A K Q E Q G S I Q P N L N K E K V K D V N V G T S G

3401 AACTCACACTGTGCCACGAATTAAAGCTATCACGTCCAAAATGAGAATGCCCAAGAGTAAGGGTGCAACTGTACTAAATTTGGAACACCTACTCGAGTAT
T H T V P R I K A I T S K M R M P K S K G A T V L N L E H L L E Y

3501 GCTCCACAGCAAATTGAAATCTCAAATACTCGAGCAACTCAATCACAGTTTGATACATGGTATGAAGCAGTACAACTTGCATACGACATAGGAGAAACTG
A P Q Q I E I S N T R A T Q S Q F D T W Y E A V Q L A Y D I G E T E

3601 AAATGCCAACTGTGATGAATGGGCTTATGGTTTGGTGCATTGAAAATGGAACCTCGCCAAATATCAATGGAGTTTGGGTTATGATGGATGGAGATGAACA
M P T V M N G L M V W C I E N G T S P N I N G V W V M M D G D E Q

3701 AGTCGAATACCCACTGAAACCAATCGTTGAGAATGCAAAACCAACACTTAGGCAAATCATGGCACATTTCTCAGATGTTGCAGAAGCGTATATAGAAATG
V E Y P L K P I V E N A K P T L R Q I M A H F S D V A E A Y I E M

3801 CGCAACAAAAAGGAACCATATATGCCACGATATGGTTTAGTTCGTAATCTGCGCGATGGAAGTTTGGCTCGCTATGCTTTTGACTTTTATGAAGTTACAT
R N K K E P Y M P R Y G L V R N L R D G S L A R Y A F D F Y E V T S

3901 CACGTACACCAGTGAGGGCTAGAGAGGCACACATTCAAATGAAGGCCGCAGCTTTAAAATCAGCTCAATCTCGACTTTTCGGATTGGATGGTGGCATTAG
R T P V R A R E A H I Q M K A A A L K S A Q S R L F G L D G G I S

4001 TACACAAGAGGAAAACACAGAGAGGCACACCACCGAGGATGTTTCTCCAAGTATGCATACTCTACTTGGAGTGAAGAACATGTGATTGTAGTGTCTTTCC
T Q E E N T E R H T T E D V S P S M H T L L G V K N M * *

4101 GGACGATATATAGATATTTATGTTTGCAGTAAGTATTTTGGCTTTTCCTGTACTACTTTTATCGTAATTAATAATCGTTTGAATATTACTGGCAGATAGG

4201 GGTGGTGTAGCGATTCCGTCGTTGCAGTGACCTTAGCTGTCGTTTCTGTATTATTATGTTCGTATAAAAGTGCCGGGTTGTTGTTGTTGTGGCTGATCTA

4301 TCGATTAGGTGATGTTGCGATTCGTCGTAGCAGTGACTATGTCTGGATTTAGTTACTTGGGTGATGCTGTGATTCTGTCATAGCAGTGACTGTAAACTTC

4401 AATCAGGAGACAAAAAAAAAAAAAAAAAAAAAA

Fig. 2.2 Nucleotide sequence of the 3'-terminal half of the PVY^N RNA genome. The predicted amino acid sequence is shown under the nucleotide sequence. Asteriks (*) indicate stop codons. Hepta-peptide cleavage sites processed by the Nla protease are underlined, actual cleavge sites are indicated with an arrow (↓) and cistrons are indicated. Amino acid residues or motifs involved in specific protein functions (see text) are shown in bold and underlined lettering. Eight and eleven nucleotide direct repeats in the 3'-NTR are underlined.

would result in proteins of 88, 52, 432, 521 and 267 amino acids long, corresponding respectively with the C-terminal 88 amino acids of the cytoplasmic inclusion (CI) protein, the putative $6K_2$ protein, the NIa VPg/protease, the NIb RNA-dependent RNA-polymerase and the viral coat protein (Riechmann *et al.*, 1992).

The base composition of the 329 nt 3'-NTR showed a high percentage of uridine (39.3%) and a low percentage of cytosine (14.1%). Amounts of guanosine and adenosine were average to below average (resp. 25.4% and 21.2%). Analysis of this region revealed three regions, 35 to 40 nt in length, which were strongly homologous. In addition, 8-nucleotide and 11-nucleotide direct repeats were also present in the 3'-untranslated region of PVY^N-NI1 (Fig 2.1 and Van der Vlugt *et al.*, 1989). Considerable homology in nucleotide sequence and length could only be detected to the 3'-NTRs of other PVY isolates and PepMoV, while, in contrast, no homology could be detected with the 3'-region of other unrelated potyviruses or RNA plant viruses (data not shown).

2.3.2 Comparisons of PVY^N-specific proteins

The NIa protein has been identified as the protease involved in seven cleavage events in the central and C-terminal part of the potyviral polyprotein (Carrington and Dougherty, 1987; Hellman *et al.*, 1988; Chang *et al.*, 1988; García *et al.*, 1989; Ghabrial *et al.*, 1990; García *et al.*, 1992). The N-terminal region of the protein is proposed to contain the VPg protein while the proteolytic activity is located in its C-terminal part (Murphy *et al.*, 1990; Dougherty and Parks, 1991). Alignment of the NIa VPg/protease protein with the homologous proteins from the French PVY^N isolate (PVY-Fr; Robaglia *et al.*, 1989) and four other potyviruses revealed significant levels of overall sequence homology, ranging from 64 to 72% for the unrelated potyviruses to 97% for PVY-Fr (see Table 2.1A). No extended regions of strict sequence conservation are present but certain conserved amino acid residues and sequence motifs can be identified. A tyrosine residue, present at pos. 64 (Y^{64}), is thought to function as the VPg-RNA attachment site (Murphy *et al.*, 1991) and is strictly conserved in all potyviral NIa sequences. Two basic domains containing a high concentration of Arg and Lys residues were recently identified in the N-terminus of the TEV NIa protein as nuclear translocation signals (Carrington *et al.*, 1991). Similar regions are present in the NIa protein N-termini of the other potyviruses, though strict sequence conservation is not apparent. The suboptimal cleavage site QEVEHE↓A within NIa, at which the VPg domain is proposed to be released from the protease domain (García *et al.*, 1992), is located at pos. 188/189 of the PVY NIa protein (see Fig. 2.2) and conservation of residues between potyviruses appears to be limited to the dipeptide sequence at which cleavage is thought to occur (i.e. E↓(G/S/A). The residues of the catalytic triad [His, Asp, Cys], conserved in viral cysteine proteases

(Bazan and Fletterick, 1988; Gorbalenya *et al.*, 1989; Goldbach, 1990) are found at pos. 234, 269 and 339 respectively. Several other amino acid residues were identified as possibly involved in substrate recognition and binding of the NIa protease of TEV (Parks and Dougherty, 1991) however, only three amino aids (i.e. T^{334}, H^{355} and F^{373}) are strictly conserved among the different potyviruses.

The NIb protein has been identified as the RNA-polymerase protein on the basis of conserved sequence similarities with picorna- and como-viral polymerases (Allison *et al.*, 1986; Domier *et al.*, 1987). Alignment of the NIb polymerase with the homologous proteins from the French PVY^N isolate and four other potyviruses revealed even higher levels of overall sequence homology than previously observed for the NIa protein, ranging from 74 to 78% for the unrelated potyviruses to 95% for PVY-Fr (see Table 2.1B). The N- and C-termini are more variable in sequence while the central region of the polymerase contains a number of highly conserved sequence blocks (data not shown). Several conserved amino acid motifs, typical of plant viral proteins involved in replication (Kamer and Argos, 1984; Goldbach, 1986; Domier *et al.*, 1987; Candresse *et al.*, 1990; Koonin, 1991), could be identified (see Fig. 2.2) and were fully conserved in all potyviral NIb sequences under investigation. The first motif, DxxxxD, was present as a DGSQFD block, starting at pos 250 of the PVY^N NIb protein. The second motif, (T/S)GxxxTxxxN(T/S), started at pos. 312 and was present as a SGQPSTVVDNS block and the third motif, GDD, started at pos. 353. A fourth motif, a Lys residue typical of RNA-dependent RNA-polymerases (Poch *et al.*,1989) is located at pos. 391 (K^{393}).

The start of the PVY^N CP was identified both by multiple sequence alignment with other PVY isolates and by chemical determination of the N-terminal amino acids of the CP (Van der Vlugt *et al.*, 1989). This located the cleavage site in the polyprotein at pos. -267 corresponding to a CP M_r of 29,833. This is in reasonable agreement with the apparent M_r 33,000 to 34,000 as estimated from PAGE studies (Huttinga and Mosch, 1974). The DAG-motif, proposed to be involved in potyviral aphid-transmission (Harrison and Robinson, 1988; Atreya *et al.*, 1990) is located at pos. 6 of the PVY^N CP sequence. Multiple potyviral CP sequence alignments, including the CP sequence of pepper mottle virus (PepMoV; Dougherty *et al.*, 1985b) showed considerable homology throughout the CPs except for the N-terminal parts which were highly variable for the different viruses, both in length and in amino acid composition (Van der Vlugt *et al.*, 1989; Ward and Shukla, 1991). The highest degree of homology, of around 95%, was found between the capsid proteins of PVY-NI, PVY-Fr and PepMoV, with these viruses showing a CP N-terminus equal in length and sequence. CP sequence homologies for the other, non-related, potyviruses ranged from 69 to 79% (see Table 2.1C), with most differences located in the N-terminal regions.

Table 2.1 Percentages of homology between NIa proteins (A), NIb proteins (B) and coat proteins (C) of different potyviruses. Sources of sequence data are: PVY-NI1; Van der Vlugt *et al.*, 1989 and this Chapter, PVY-Fr; Robaglia *et al.*, 1989, TEV; Allison *et al.*, 1986, TVMV; Domier *et al.*, 1986, PPV; Maiss *et al.*, 1988, BYMV; Boye *et al.*, 1992, PepMoV; Dougherty *et al.*, 1985b.

A:

	NI1	Fr	TEV	TVMV	PPV	BYMV
PVY-NI1	100					
PVY-Fr	96.3	100				
TEV	62.3	63.0	100			
TVMV	61.1	61.1	64.9	100		
PPV	68.3	69.2	67.0	68.6	100	
BYMV	61.3	61.6	63.5	65.3	66.8	100

B:

	NI1	Fr	TEV	TVMV	PPV	BYMV
PVY-NI1	100					
PVY-Fr	94.8	100				
TEV	72.7	70.9	100			
TVMV	73.6	73.3	75.4	100		
PPV	74.5	73.6	75.0	74.2	100	
BYMV	72.8	72.3	76.2	73.1	75.5	100

C:

	NI1	Fr	PepMoV	TEV	TVMV	PPV	BYMV
PVY-NI1	100						
PVY-Fr	96.3	100					
PepMoV	94.4	95.1	100				
TEV	77.6	77.6	77.2	100			
TVMV	67.6	66.8	66.4	69.6	100		
PPV	75.7	75.3	75.7	75.3	67.9	100	
BYMV	73.8	74.2	73.8	77.2	67.6	71.4	100

2.4 Discussion

We have determined the nucleic acid sequence of the 3'-terminal half of the RNA genome of a Dutch isolate of PVY^N. The sequence obtained encodes respectively the C-terminal part of the cytoplasmic inclusion (CI) protein, the putative $6K_2$ protein, both NIa and NIb nuclear inclusion proteins and the viral coat protein.

Extensive sequence comparisons between the CPs of many potyvirus species has previously revealed a bimodal distribution of sequence homologies, with distinct species having 38 to 71% (average 54%) homology and isolates of one species showing 90 to 99% sequence homology (Shukla and Ward, 1989; Ward and Shukla, 1991). The 3'-NTRs of different potyviruses also display a high level of variability, both in length and in primary sequence (39 to 53%), whereas the homology in this region between strains of one virus species generally ranges from 83 to 99% (Frenkel *et al.*, 1989). This has prompted the suggestion that both the sequences of the CP and the 3'-NTR of the potyvirus genome can serve as accurate markers of genetic relatedness and should form the basis of potyvirus taxonomy (Ward and Shukla, 1991; Shukla *et al.*, 1991). Comparisons between the non-structural proteins of both a Dutch and a French isolate of PVY^N and a number of other distinct potyvirus species, show a similar bimodal distribution of homologies as observed for the CP and 3'-NTR sequences (see Table 2.1). Therefore, similar as the CP and 3'-NTR sequences, these sequences also appear suitable genetic markers for the taxonomic position of potyviruses as the CP and 3'-NTR sequences. It should be noted however, that large sequence divergences, possibly arising from genomic recombination events between different viruses, can not be ruled out as a cause for biological differences between isolates of distinct potyvirus species and that therefore preferably sequence data of complete potyviral genomes should serve to differentiate between distinct potyvirus species.

Interestingly the first PepMoV isolate whose sequence was determined (Dougherty *et al.*, 1985), shares a much higher homology in its CP sequence to PVY^N than the other potyviruses. This homology is extended to the 3'-non-coding region of the RNA where it shares about 84% nucleotide homology to both PVY^N isolates and up to 94% with other PVY isolates (see Chapter 3 and Van der Vlugt *et al.*, 1993). No extensive sequence homology to the 3'-NTR of other potyviral genomes was detected. This and other comparisons of the sequence of the PepMoV CP with those of other PVY isolates and unrelated potyviruses (Van der Vlugt *et al.*, 1989, Van der Vlugt, 1992) show that PepMoV is much more related to PVY than was previously believed from serological studies (Bartels, 1964; Purcifull & Gooding, 1970; Purcifull *et al.*, 1975; Nelson & Wheeler, 1978). The high levels of homology in both the polymerase coding region and the 3'-NTR sequence confirm this close relationship. On the basis of host range studies and serological tests PepMoV was first described as an atypical strain of PVY (PVY^S or PVY-speckling, Zitter, 1972). On the basis of

serological distinction and differences in symptoms on indicator plants, PVY^S has later been tentatively renamed PepMoV and proposed to be a distinct potyvirus (Zitter, 1972; Zitter and Cook, 1973; Nelson and Wheeler, 1972; 1978). On the basis of the published sequence information and the sequence comparisons made here, it should be concluded that PepMoV can be regarded as a strain of PVY.

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Chapter 3

Taxonomic relationships between distinct potato virus Y isolates based on detailed comparisons of the viral coat proteins and 3'-nontranslated regions.

Summary

Detailed comparisons were made of the sequences of the coat protein (CP) cistrons and 3'-nontranslated regions (3'-NTR) of 21 (geographically) distinct isolates of potato virus Y (PVY) and a virus isolate initially described as pepper mottle virus (PepMoV). Multiple sequence alignments and phylogenetic relationships based on these alignments resulted into a subgrouping of virus isolates which largely corresponded with the historical strain differentiation based on biological criteria as host range, symptomatology and serology. Virus isolates belonging to the same subgroup shared a number of characteristic CP amino acid and 3'-NTR nucleotide residues indicating that, by using sequences from the 3'-terminal region of the potyvirus genome, a distinction could be made between different isolates of one virus species as well as between different virus species. RNA secondary structure analysis of the 3'-NTR of twelve PVY isolates revealed four major stem-loop structures of which, surprisingly, the loop sequences gave a similar clustering of isolates as resulting from the overall comparisons of CP and 3'-NTR sequences. This implies a biological significance of these structural elements.

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3.1 Introduction

The *Potyviridae* represent the largest and economically most important plant virus family and for that reason also one of the best characterized. For this virus family, in which members are defined on the basis of particle morphology, a positive sense ssRNA genome, and intercellular formation of characteristic cylindrical inclusion bodies, the Family - Genus - species concept has recently been proposed as the main system of virus classification (Barnett, 1991, 1992). Within the *Potyviridae*, four genera are distinguished on the basis of their mode of transmission i.e. the genus *Potyvirus* (aphid-borne), the genus *Bymovirus* (fungal-borne), the genus *Rymovirus* (mite-borne) and the possible genus *Ipomovirus* (whitefly-borne)(Barnett, 1991, 1992). Of these four, the genus *Potyvirus* is the best characterized, though classical biological approaches to identify separate potyviruses and their strains, like host range, symptomatology and serology, have sofar failed to supply reliable criteria for the distinction between all viruses within this genus. With a growing number of protein and genome sequence data of potyviruses becoming available, it has become clear that these data can be used for a reliable identification and classification of these viruses. Coat protein (CP) and 3'-nontranslated region (3'-NTR) sequence comparisons nowadays form the widely accepted, molecular basis of distinction between the different potyvirus species (Shukla and Ward, 1988; Shukla and Ward, 1989a, 1989b; Ward and Shukla, 1991; Rybicki and Shukla, 1992; Ward *et al*, 1992). Extensive comparisons between the CP sequences of many potyvirus species have revealed a bimodal distribution of sequence homologies with distinct virus species having 38 to 71% (average 54%) homology and isolates belonging to one species showing higher than 90% homology (Shukla and Ward, 1988). The central and C-terminal parts of the potyviral CPs are highly homologous while major differences are found in sequence and length of the CP N-termini of different species. In contrast, different strains of a distinct virus all show highly homologous N-terminal sequences. The 3'-NTR of different potyviruses also displays a high degree of sequence variability (39 to 53%) whereas the homology between strains of the same virus generally ranges from 83 to 99% (Frenkel *et al.*, 1989).

For potato virus Y (PVY), the type species of the genus *Potyvirus*, genomic and CP sequence data on as many as 21 different isolates have become available. This large number of sequences of a single potyvirus species led us to the comparison on both the CP and 3'-NTR sequence level to investigate whether a similar, molecular distinction of strains within a given virus species is possible as it is for distinct species. This paper describes these detailed comparisons and phylogenetic relationships derived from multiple sequence alignments. Evidence is presented that it is indeed possible to distinguish between different strains of one potyvirus species on the basis of both CP cistron and 3'-NTR sequences.

3.2 Materials and Methods

3.2.1 Virus strains and isolates

Coat protein and 3'-NTR sequences from the following virus isolates were used for comparisons: PVY-Go16 (Wefels *et al.*, 1989), PVY-NI1 (Van der Vlugt *et al.*, 1989), PVY-NI2 (MOGEN NV., The Netherlands, unpublished), PVY-NI3 (KeyGene NV. The Netherlands, unpublished), PVY-Jp (Oshima *et al.*, 1991), PVY-T (Hataya *et al.*, 1990), PVY-Hu (Dalmay and Balázs, 1990), PVY-Nz (Hay *et al.*, 1989), PVY-Ru (Puurand and Saarma, 1990), PepMoV (PeMV; Dougherty *et al.*, 1985), PVY-Us (Griffin *et al.*, 1992, unpublished, EMBL accession no. M81435), PVY-Fr (Robaglia *et al.*, 1989), PVY-Ch (Zhou *et al.*, 1990), PVY-O1 (Bravo-Almonacid and Mentaberry, 1989), PVY-O2 (Lawson *et al.*, 1990), PVY-O3 (Oshima *et al.*, 1991), PVY-O4 (Hikada *et al.*, 1992), PVY-Is (Rosner *et al.*, 1988), PVY-D, PVY-10, PVY-18 and PVY-43 (Shukla *et al.*, 1988).

3.2.2. Data analysis

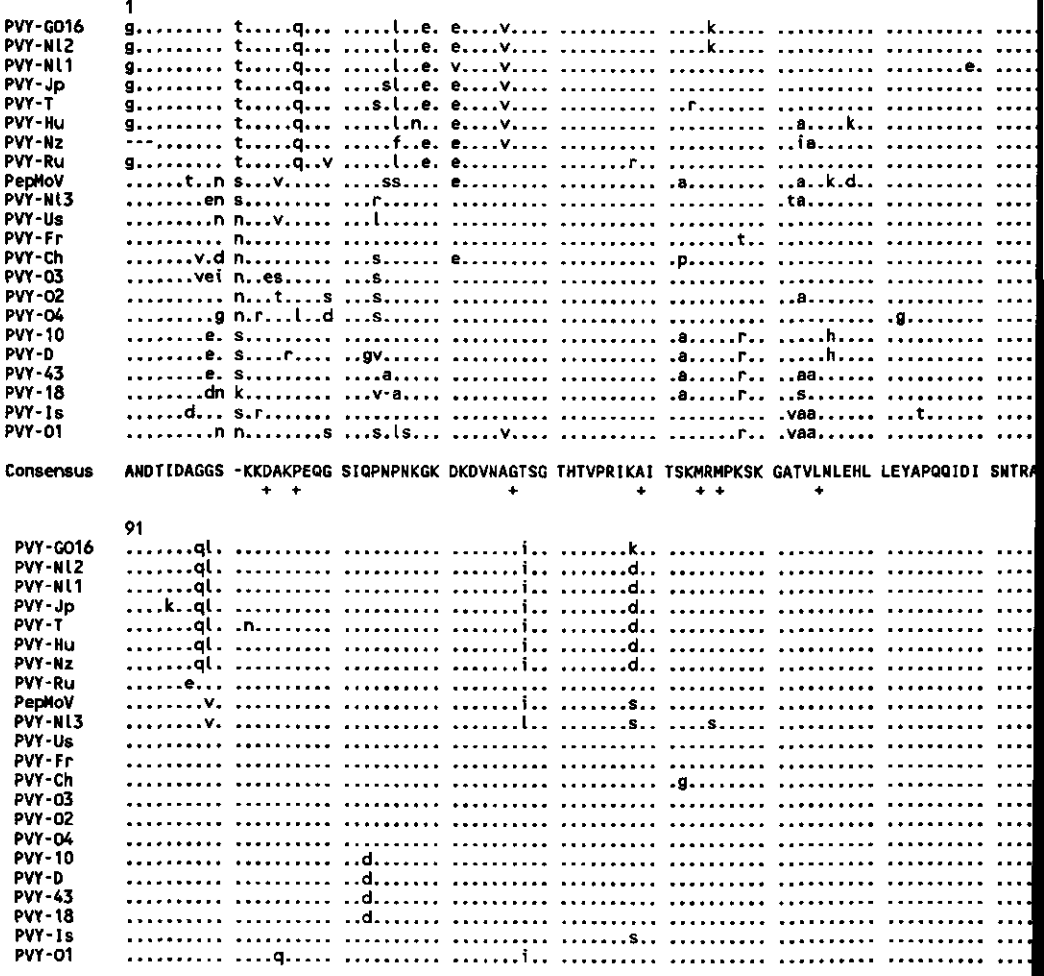
Sequence data, multiple alignments and phylogenetic relationships were analyzed using the University of Wisconsin GCG program package version 7.1 (Devereux *et al.*, 1984), the PHYLIP (Phylogeny Inference Package, version 3.4) program package (Felsenstein, 1991) and STructure Analysis of RNA ('STAR') program (Abrahams *et al.*, 1990).

3.3 Results

3.3.1 PVY CP sequence comparisons

Since the first reports on the nucleotide sequences of the 3'-terminal regions of strains of PVY (Rosner *et al.*, 1988; Van der Vlugt *et al.*, 1989), numerous reports on the sequence analysis of this region of the genome of PVY and a great number of other potyviruses have been published. Over the last three years this has resulted in a large number of CP sequences of isolates of both PVY^N ('the tobacco veinal necrosis') and PVY^O ('the common') strains (De Bokx and Huttinga, 1980). In an attempt to establish more precisely the exact relationships between all these isolates, detailed sequence comparisons were made, employing all available CP amino acid and 3'-NTR nucleotide sequence data. Sequence data of a virus isolate described as pepper mottle virus (PeMV or PepMoV; Dougherty *et al.*, 1985) were included since on the basis of CP and 3'-NTR sequence comparisons this particular isolate was proposed to be an isolate of PVY (Van der Vlugt *et al.*, 1989; Van der Vlugt, 1992).

It should be noted, however, that recent sequence analysis of a Californian isolate of pepper mottle virus (PepMoV-C; Bowman-Vance *et al*, 1992a,b) and comparisons between a Florida isolate of PepMoV and PVY (Hiebert and Purcifull, 1992) suggest that PepMoV, though more closely related to PVY than to any other potyvirus, represents a distinct potyvirus. If this is true, then the PepMoV sequence, as published by Dougherty *et al.* (1985), and included in our comparisons, may represent a PVY-isolate contaminating the PepMoV preparation analyzed. In spite of the uncertainties with respect to the identity of the various proposed PepMoV isolates, the sequence published by Dougherty *et al.* (1985) is, for reasons of simplicity, still referred to as PepMoV throughout this chapter.



```
                181                                                                                                  267
PVY-GO16  ......v... .......... .......... .......... .........s .......... .......... .......... .......
PVY-NL2   ......v... .......... .......... .......... .........s .......... .......... .......... .......
PVY-NL1   ......v... .......... .......... .......... .........s .......... .......... .......... .......
PVY-Jp    ......v... .......... .......... .......... .........s .......... .......... .......... .......
PVY-T     ......v... .......... .......... ..t....... .........s .......... .......... .......... .......
PVY-Hu    ......v... .......... .......... .......... .........s .......... .......... .......... .......
PVY-Nz    ......v... .......... .......... .......... .........s .......... .......... .......... .......
PVY-Ru    .......... ..vg...... .......... .......... .......... .......... .......... .......... h......
PepMoV    ......v... ..a....... .......... .......... .........s .........v. .......... .......... .......
PVY-NL3   .......... .......... .......... .......... .........s .........v. .......... .......... .......
PVY-Us    .......... ..vg...... .......... .......... .......... .......... .......... .......... .......
PVY-Fr    .......... ..mg...... .......... .......... .......... .......... .......... .......... .......
PVY-Ch    .......... ..vg...... .......... .......... .......... .......... .......... .......... .......
PVY-O3    .......... ..vg...... .......... .......... .......... .......... .......... .......... .......
PVY-O2    ......v... ..vg...... .......... .......... .......... .......... .........r .......... .......
PVY-O4    .......... ..vg...... .......... .......... .......... .......... .......... .......... .......
PVY-10    .......... ..vg...... .......... .......... .......... .......... ........g. .......... .......
PVY-D     .......... ..vg...... .......... .......... .......... .......... ........g. .......... .......
PVY-43    .......... .......... .......... .......... .......... .......... ........g. .......... .......
PVY-18    .......... ...g...... .......... .......... .......... .........v. ........g. .......... .......
PVY-Is    .......... ..i....... .......... e......... .......... .......... .......... .......... .......
PVY-O1    .......... ..i....... .......... .......... .......... .......... .......... .......... ...g...

Consensus MPRYGLIRNL RDGSLARYAF DFYEVTSRTP VRAREAHIQM KAAALKSAQP RLFGLDGGIS TQEENTERHT TEDVSPSMHT LLGVKNM
            +                                                                 +                    +
```

Fig. 3.1 Multiple alignment of the CP sequences of 21 PVY isolates and PepMoV (sources; see Materials and Methods). A derived consensus sequence is shown under the alignment with CP amino acids identical to the consensus indicated by dots (.). Residues differing from the consensus are shown in small lettering, gaps are indicated by dashes (-). The + indicates conserved amino acid changes within the following groups (Kamer and Argos, 1984): acidic and polar (D,E,N,Q), basic (K,R), hydrophobic (A,C,F,H,I,L,M,V,W,Y), polar (S,T) or strong turn formers (D,G,N,P).

Using the PILEUP, PRETTY and DISTANCES programs of the GCG program package (Devereux *et al.*, 1984) the CP amino acid sequences of 21 PVY isolates and this PepMoV isolate were aligned, a CP consensus sequence deduced (see Fig. 3.1) and the levels of sequence similarities calculated (see Table 3.1). All CP sequences contain 267 amino acids, except for PVY-18 (266 aa) and PVY-Nz whose three most N-terminal residues were not determined. As found for different potyviral species, the N-terminus appears to be the most heterogeneous region among the various isolates of PVY, showing most amino acid substitutions. The one amino acid gap introduced to align the PVY-18 CP sequence is also located in this region. The CP core regions are highly homologous with most differences between isolates restricted to only a few amino acid positions, indicating 'hot-spots' of mutation (see Fig. 3.1). This CP sequence alignment indicates a division of the different PVY isolates into two major groups. The first eight isolates (PVY-GO16 to PVY-Ru) can be placed into a first subgroup since they all share a glycine residue as the first CP amino acid (with the exception of PVY-Nz whose N-terminus was not determined), as well as a number of non-conserved amino acid mutations in their N-termini and core-regions. These differences clearly separate them from the other PVY strains (see Fig. 3.1). Most

viruses in this subgroup were described as typical PVY^N ('necrosis') isolates, indicating that the observed amino acid differences are (PVY^N)-strain specific.

The other isolates, characterized by the presence of an alanine residue as the first CP amino acid, appear to be more diverse in CP sequence, with more variable N-termini and only a few shared non-conserved amino acid substitutions separating them from the PVY^N group. There is a less clear grouping between different isolates in this group with the exception of the four Australian PVY isolates (Shukla *et al.*, 1988), which share a number of identical differences from the consensus sequence throughout their CPs. Most viruses in this group, with the exception of PepMoV, were classified as typical PVY^O ('common') strains suggesting that the second subgroup in the CP alignment overlaps with this strain.

Interestingly, PepMoV and PVY-NI3 share a number of amino acid substitutions suggesting a more close relationship between these two viruses than with the other members of the PVY^O subgroup. In this respect it should be noted that both viruses were originally isolated from pepper ('*Capsicum annuum*' L.) (Zitter, 1972; J. Pot, KeyGene NV., personal communication) suggesting a possible host adaptation. From figure 3.1, it can also be noted that PVY-Fr, a French PVY strain originally described as a typical PVY^N strain (Robaglia *et al.*, 1989), clearly falls into the PVY^O subgroup since it does not share any of the amino acid mutations typical of the isolates in the PVY^N subgroup.

On the basis of the multiple CP sequence alignment, a matrix of pairwise sequence dissimilarities was calculated using the program HOMOLOGIES (J. Leunissen., unpublished results). This matrix was used as input in the NEIGHBOR program from the PHYLIP programs package (Felsenstein, 1991) to construct a phenogram (phylogenetic tree) of the 22 virus isolates. The NEIGHBOR program employs a Neighbor-joining method (Saitou and Nei, 1987) and was run several times with the Global option and randomized order of input of the sequences, to generate a optimal tree topology. Furthermore, several unrelated potyviral CP sequences were incorporated as an outgroup (one in every run) to simulate a rooted tree. A typical tree, showing the generally obtained grouping of sequences, is presented in Figure 3.2. This tree shows the same clustering of isolates as observed with the multiple alignment (Fig. 3.1) and this same clustering was confirmed in trees generated with other PHYLIP programs like FITCH, KITSCH and PROTPARS (results not shown). The isolates belonging to the Y^N subgroup (PVY-Go16 - PVY-Ru) again form a subcluster, clearly separated from the ones in the Y^O subgroup, which again contains PVY-Fr. Remarkably, both PepMoV and PVY-NI3 are placed in an additional subgroup separate from both the PVY^N and PVY^O subgroups.

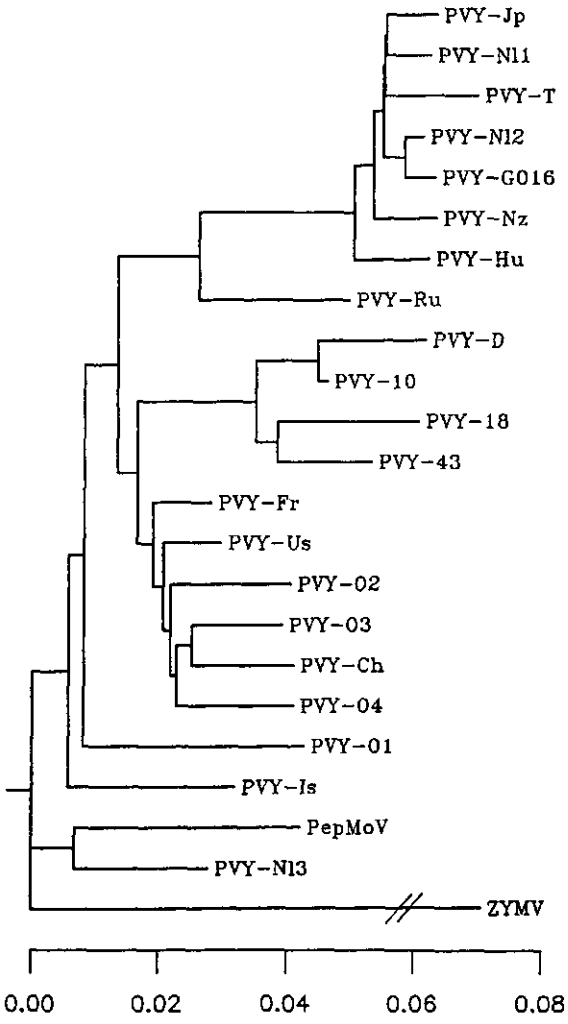


Fig. 3.2 Unrooted NEIGHBOR phylogenetic tree based on the multiple alignments of the CP amino acid sequences of 21 PVY isolates and PepMoV. The CP sequence of zucchini yellow mosaic virus (ZYMV) (Grumet and Fang, 1990) is included as an outgroup to enable the simulation of a root to the tree. The scale of the branch lengths is in minimum mutational distances /amino acid residue.

3.3.2 PVY 3'-NTR sequence comparisons

Like for the PVY CP sequences, similar multiple alignments were made for all PVY 3'-NTR sequences known sofar. The alignment obtained by PILEUP was further refined by eye and using the PRETTY program a consensus nucleotide sequence was obtained (see Fig. 3.3). All PVY 3'-NTRs contain between 329 and 335 nt and are highly homologous in sequence with, in analogy with the CP sequences, most nucleotide substitutions clustered in certain regions. Although only twelve 3'-NTR

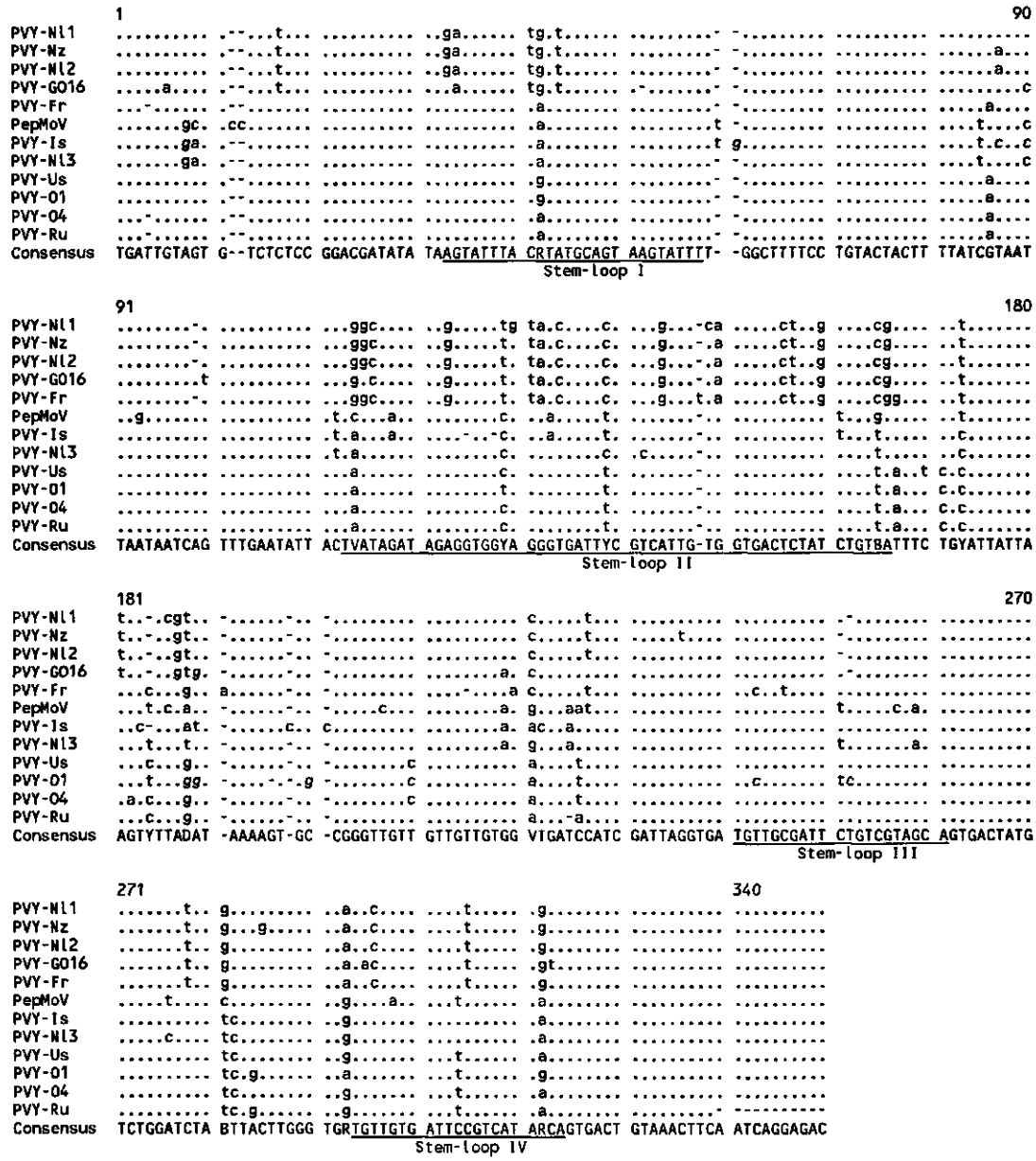


Fig. 3.3 Multiple alignment of the 3'-NTR sequences of 11 PVY isolates and PepMoV. A derived consensus sequence is shown under the alignment with R = A/G, V = A/C/G and Y = C/T. Nucleotides identical to the consensus are indicated by dots (.), nucleotides differing from the consensus are shown in small lettering and gaps are indicated by dashes (-). Nucleotide sequences involved in formation of stem loop structures are underlined.

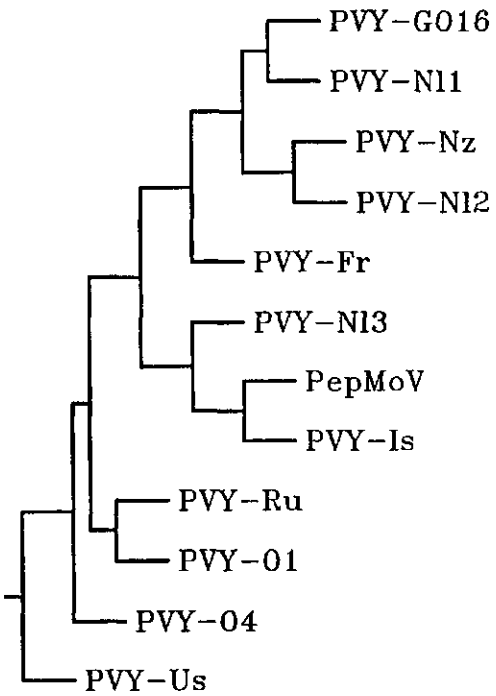


Fig. 3.4 Unrooted DNAPENNY phylogenetic consensus tree of 11 PVY isolates and PepMoV based on the alignment of their 3'-NTR nucleotide sequences.

sequences were available for this comparison a similar clustering of sequences can be observed as for the CPs. Again PVY-NI1, -Nz, -NI2 and -Go16 form a PVY^N sub-group and, in contrast to the clustering based on the CP amino acid sequences, PVY-Fr can now clearly be included in this subgroup, while PVY-Ru appears to group into the PVY^O subgroup. This latter group is again somewhat more diverse, with PepMoV, PVY-NI3, and PVY-Is, appearing to be more closely related to each other than to the other isolates. All these relationships were confirmed by the levels of sequence similarities, as calculated using the GCG DISTANCES program (Devereux *et al.*, 1984), ranging from 99-91% for related and below 89% for more distantly related isolates (see Table 3.1).

The multiple alignment of the 3'-NTRs was also used for the construction of a phylogenetic tree using the DNAPENNY program from the PHYLIP program package (Felsenstein, 1991). This program finds all most parsimonious trees by the branch and bound method and from these, highly similar, trees a unrooted consensus tree was derived. This tree is presented with an arbitrarily chosen root in Fig. 3.4. The PVY^N strains all cluster into one sub-group together with PVY-Fr. PepMoV, PVY-Is and PVY-NI3 again form a closely related sub-group, while the remaining PVY^O strains appear to form a more diverse group with isolates less well related.

Table 3.1 Percentages sequence similarity between the coat protein sequences (below diagonal) and the 3'-nontranslated regions (above diagonal) of 21 geographically distinct potato virus Y isolates and PepMoV. Sources of sequence data: see Materials and Methods.

	Virus isolates																					
	GO16	NI1	NI2	Jp	T	Hu	Nz	Ru	Pep	NI3	Us	Fr	Ch	O3	O2	O4	10	D	43	18	Is	O1
GO16	-	95.7	95.4				95.4	85.0	83.6	86.9	85.7	92.1				85.7					84.8	85.4
NI1	99.3	-	97.9				97.9	85.3	83.9	86.0	86.0	93.9				86.0					83.6	85.1
NI2	99.6	99.6	-				98.8	85.9	84.2	86.9	86.6	93.9				86.6					84.5	85.7
Jp	98.9	98.9	99.3	-																		
T	98.9	98.9	99.3	98.5	-																	
Hu	98.1	98.1	98.5	97.8	97.8	-																
Nz	98.9	98.9	99.3	98.5	98.5	98.1	-	85.3	83.6	86.3	86.0	93.9				86.0					83.9	85.1
Ru	97.0	97.0	97.4	96.6	96.6	95.9	96.6	-	90.6	93.4	98.8	88.4				98.8					92.8	96.6
Pep	94.8	94.4	94.8	94.8	94.0	94.8	94.3	93.6	-	93.4	90.0	84.0				90.0					92.5	88.8
NI3	95.1	94.8	95.1	94.4	94.8	95.1	94.7	94.0	96.3	-	93.1	86.4				93.1					94.6	91.8
Us	95.5	95.5	95.9	95.1	95.5	95.1	95.1	96.3	95.9	96.3	-	88.5				99.4					92.5	97.0
Fr	96.3	96.3	96.6	95.9	95.9	95.9	95.8	97.0	95.1	95.9	98.5	-				88.5					84.3	87.3
Ch	94.8	94.8	95.1	94.4	95.1	94.4	94.3	95.5	94.8	95.5	98.1	97.8	-									
O3	94.8	94.8	95.1	94.4	95.1	94.4	94.3	95.5	94.4	95.9	98.1	97.8	98.1	-								
O2	95.1	95.1	95.5	94.8	95.5	95.5	95.1	95.9	95.1	95.9	98.1	98.1	97.4	97.8	-							
O4	95.9	95.9	96.3	95.5	96.3	95.5	95.5	96.6	94.4	95.5	98.1	98.5	98.1	97.8	97.8	-					92.5	97.0
10	95.1	95.1	95.5	94.8	94.8	94.8	94.7	95.9	95.5	95.9	97.0	97.8	96.6	97.0	96.6	97.0	-					
D	94.4	94.4	94.8	94.0	94.4	94.0	93.9	95.1	94.8	95.5	97.0	97.0	96.3	96.6	96.3	96.6	99.3	-				
43	94.8	94.8	95.1	94.8	94.4	95.1	95.5	94.8	95.9	96.3	95.9	96.6	95.5	95.9	96.3	95.9	98.1	97.4	-			
18	95.1	95.1	95.5	95.1	95.1	95.1	94.7	95.1	96.2	96.2	97.4	96.6	96.6	96.2	96.2	96.2	97.4	97.4	98.1	-		
Is	94.4	94.0	94.4	93.6	93.6	94.4	94.7	94.8	95.1	95.9	95.9	96.6	95.1	95.1	96.3	95.9	95.9	95.1	96.3	94.7	-	90.6
O1	94.8	94.8	95.1	94.4	95.1	95.1	95.5	94.8	94.0	95.1	96.6	96.3	96.3	95.9	96.6	95.9	94.8	94.4	95.1	95.5	95.9	-

3.3.3 PVY 3'-NTR structural comparisons

Of all available 3'-NTR sequences the secondary RNA structures were determined using the STructure Analysis of RNA ('STAR') program of Abrahams *et al.* (1990). Several potential stem-loop structures could be found in all 3'-NTRs with four major stem-loop structures present in all PVY sequences. These stem-loops were denoted no. I to IV and their general structures and positions are shown in Fig. 3.4. Stem-loops I and II are clearly distinct while stem-loops III and IV are nearly identical. Surprisingly, all loop regions are highly homologous with a strong preference for a UUCG or UUCCG sequence in loops II, III and IV (see Table 3.2). A similar loop sequence (UUCG) has been described as an unusually stable hairpin loop, abundantly present in prokaryotic 16S ribosomal RNAs (Cheong *et al.*, 1990; Varani *et al.*, 1991) and intercistronic regions of the bacteriophage T4 (Tuerk *et al.*, 1988). In these RNAs, UNCG sequences have been shown to be the dominant tetra-loop sequences, sometimes being replaced by penta-loops containing a closely related sequence (Woese *et al.*, 1990). Stable hairpin structures are thought to provide potential nucleation sites for RNA folding and interaction with other nucleic acids and proteins (Carey *et al.*, 1983; Feng and Holland, 1988; Witherell and Uhlenbeck, 1989). The stem-loop structures with the conserved loop sequences in the PVY 3'-NTRs could have similar biological functions. Additional evidence for this assumption comes from the observation that loop sequences from PVY isolates which, on the basis of direct CP and 3'-NTR sequence comparisons, belong to the same subgroup or strain (see Fig. 3.1 to 3.3), usually contain identical sequences which differ distinctly from the sequences in the loop regions of the isolates belonging to the other subgroup (see Table 3.2). In this respect again the position of PVY-Fr should be noted since the sequences of loop I and III of this isolate are specific for PVY^{O}-type loops, while loops II and IV are identical to PVY^{N}-type loops. Also the observation that the stems of stem-loops II contain two mismatched basepairs (shown for PVY-NI1, see Fig. 3.4), that are closely coupled and separated by two G-U and U-G non-Watson and Crick basepairs, supports the possible biological function of the PVY 3'-NTR secondary structure elements. These G-U base pairs have been shown to be slightly less stable when in the middle of a helix region (Westhof *et al.*, 1985) and this region could potentially loop out to form a symmetrical internal loop. In all PVY stem-loop II structures the right bulge thus formed, consists of a GNRA sequence (i.e. GUGA), a sequence also dominantly present in hairpin loops of 16S ribosomal and T4 RNAs (Woese *et al.*, 1990).

Generally, support for the existence of stem-loop structures is thought to come from comparative sequence analysis and the preservation of secondary helical regions by complementary changes in the primary sequence ('co-variation'). Although the sequences under investigation are highly homologous with small conserved regions, and identical secondary RNA structures are therefore likely to occur, all observed

stems contain at least two covariant basepairs. In addition, essentially all loop sequences present in the structures I to IV contain UNCG or GNRA sequences that occur exceptionally often in hairpin loops in ribosomal and other RNAs and are thought to be of biological importance (Woese *et al.*, 1990). Further support for the existence of the PVY 3'-NTR stem-loop structures comes from the observation that grouping of isolates, based on loop sequences (see Table 3.2), coincides with the strain differentiation previously established on the basis of biological data and on comparisons of the CP and 3'-NTR sequences. This may imply a function of these sequences in symptom expression, possibly through specific interactions with the viral replication complex. A 58 nt insertion in a stem-loop structure in the 3'-NTR of TVMV, comparable to stem-loop I of PVY, attenuated symptom expression, thus locating a determinant of symptom severity to this region of the potyviral genome (Rodríguez-Cerezo *et al.*, 1991). Preliminary secondary structure analyses of the 3'-NTRs of other potyviruses for which the sequences of several isolates have been determined, showed comparable structural elements as in the 3'-NTR of PVY, i.e. a small stem-loop on the 5'-side of this region, a larger stem-loop more or less situated in the middle and one or two stem-loops at the 3'-side of the 3'-NTR (results not

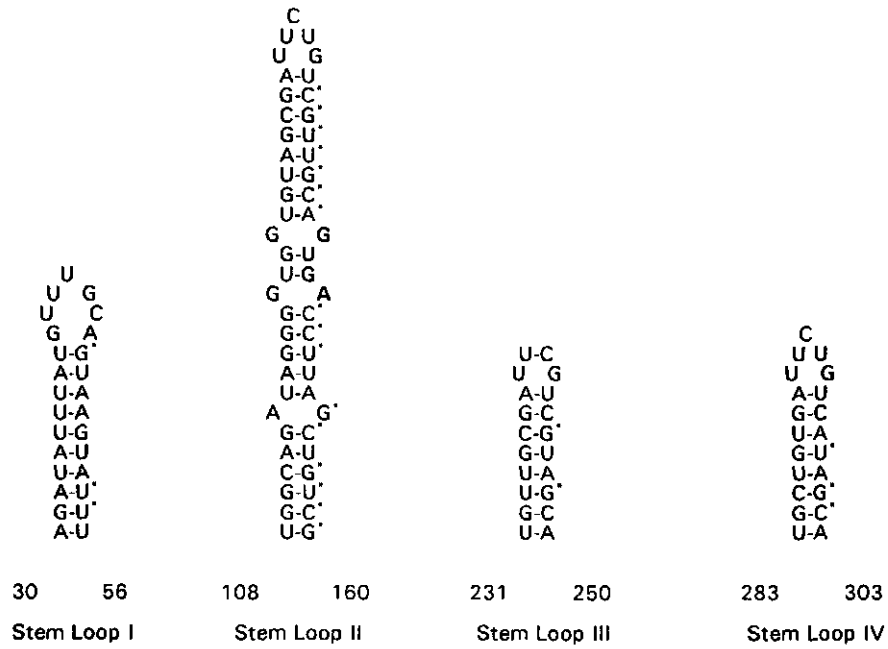


Fig. 3.4 Secondary stem-loop structures and their positions in the 3'-NTR of PVY-NI1 RNA. An asterisk (*) indicates basepairs in which covariants occur, the GUGA tetra-loop in stem-loop II is shown in bold lettering.

Table 3.2 Conserved nucleotide sequences of the loop regions in the stem-loop structures I - IV present in the 3'-NTR of eleven differerent PVY isolates and PepMoV. For sources of sequence data see Materials and Methods.

	Loop sequences			
Isolate	*Loop I*	*Loop II*	*Loop III*	*Loop IV*
NI1	GUUUGCA	UUCCG	UUCG	UUCUG
Nz	GUUUGCA	UUCCG	UUCG	UUCUG
NI2	GUUUGCG	GUCCG	UUUG	UUCUG
GO16	GUUUGCA	UUCCG	UUCG	UUCUG
Fr	AUAUGCA	UUCCG	UUCUG	UUCUG
PepMoV	AUAUGCA	UUUCG	UUUUG	UUUCG
Is	AUAUGCA	UUUCG	UUCUG	UUCCG
NI3	AUAUGCA	UUCCG	UUUUG	UUCCG
Us	AUAUGCA	UUUCG	UUCUG	UUUCG
O1	GUAUGCA	UUUCG	UUUCG	UUUCG
O4	AUAUGCA	UUUCG	UUCUG	UUUCG
Ru	AUAUGCA	UUUCG	UUCUG	UUUCG

shown). A similar structure was suggested for the 3'-NTR of TVMV (Rodríguez-Cerezo *et al.*, 1991), however, since no sequence conservations between different viruses could be observed and considering the small number of sequences per virus species (three to four), this prevents definitive conclusions on their existence.

3.4 Discussion

Detailed comparisons of both CP and 3'-NTR sequences of a large number of (geographically) distinct isolates of one potyvirus species, potato virus Y, have shown that these data can be used to discriminate between different strains of one virus species. The results obtained are in major agreement with the historical classification of these viruses into strains, based on biological criteria like host range and symptomatology, and on serology. Based on CP amino acid and 3'-NTR sequences a clear distinction between PVY^N- and PVY^O-specific isolates can be made, in which the differences are apparently limited to a small number of amino acid and nucleotide sequence residues, respectively. It is tempting to assume that one or more of the observed sequence differences, typical for each strain, are responsible for their

biological differences, as was shown for amino acid substitutions in the CPs of several viruses (Culver and Dawson, 1989; Petty and Jackson, 1990; Neeleman *et al.*, 1991) and mutational analysis of the 3'-NTR of TMV (Ishikawa *et al.*, 1988; Takamatsu *et al.*, 1990) or cowpea mosaic virus (CPMV) (Eggen *et al.*, 1989). Comparison of CP sequences of seven isolates of sugarcane mosaic virus (SCMV) also suggested a grouping of isolates, which correlated well with the biological data e.g. the host plant origin and the reactivities on differential sorghum cultivars (Shukla *et al.*, 1991). These results suggest that sequences in the potyviral CP and 3'-NTR can influence virulence and symptom expression. However, as demonstrated for other plant viruses and their satellites, mutations in regions other than the CP or the 3'-NTR can also be responsible for dramatic changes in virulence (Meshi *et al.*, 1989; Holt *et al.*, 1990; Collmer *et al.*, 1992). The generation of full-length cDNA clones from which infectious transcripts can be obtained, should enable mutational analysis to study the involvement of these and other sequences in symptom expression and potyvirus strain specificity. These studies will also enable the elucidation of the functions of the highly conserved regions in the potyviral CP. Their high sequence conservation throughout the potyvirus genus suggests common biological functions like protein-RNA or protein-protein interactions involved in RNA encapsidation. Expression of the CP of Johnson grass mosaic virus (JGMV) in microbial expression systems has shown, that only the core domain of the potyviral CP is required for formation of virus-like particles (Jagadish *et al.*, 1991) and has indicated the possible involvement of specific amino acid residues in the viral assembly process. In addition, expression of the CP and other potyviral proteins in microbial systems and the use of *in vitro* RNA transcription systems to generate potyviral 3'-NTR specific RNAs, will enable the study of the possible binding of viral proteins to the PVY 3'-NTR secondary structure elements and their involvement in virus replication and particle assembling.

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Chapter 4

Transformation of potato cultivar Bintje with the PVY^N coat protein cistron

Summary

The tobacco veinal necrosis strain of potato virus Y (PVY^N) is one of the important viruses in potato seed cultures mainly because of its lack of primary symptoms. Introduction of the single dominant R_y resistance genes in important potato cultivars like 'Bintje' by conventional breeding programs has sofar proven extremely difficult or even impossible because of the tetraploid nature of the potato genome. 'Coat protein-mediated resistance' has been described to confer resistance to several important plant viruses by transformation and expression of their coat proteins (CP) genes in transgenic plants. To study the possible virus protection upon the expression of the PVY^N CP in transgenic potato plants, the CP cistron, supplemented with additional start signals, was cloned in both orientations in an *Agrobacterium tumefaciens* Ti-plasmid based transformation vector. Transformation of large numbers of potato cv. Bintje tuber discs resulted in 20 transgenic lines with the CP in the correct orientation (CP-sense) and 16 transgenic lines with the CP in the reverse orientation (CP-antisense). All transgenic lines were analyzed for the presence and expression of the CP gene at both the transcriptional and translational level. In none of the transgenic lines, all expressing CP specific RNA transcripts, however, viral CP could be detected. Some lines expressing CP specific RNA were subsequently tested for their protection against mechanical inoculation with PVY^N. No significant levels of virus protection were found in these transgenic lines, containing the CP gene in either orientation.

4.1 Introduction

Potato virus Y, the typemember of the potyvirus group (De Bokx and Huttinga, 1981; Barnett, 1991), the world largest plant virus family, forms a serious problem in potato cultures worldwide. The virus, mechanically transmissible but in the field mainly transmitted by aphid vectors, can cause considerable damages with yield losses up to 80% depending on virus strain, potato cultivar and time of infection. Of the three PVY strains known (Y^O, Y^N, Y^C), Y^O is the most widespread while PVY^N ('the tobacco veinal necrosis strain') is the most problematic in West-European seed potato cultures as primary PVY^N symptoms are rare in most cultivars grown and infected plants are visually almost indistinguishable from healthy plants (Weidemann, 1988). To guarantee virus free stocks extensive screening for PVY^N is therefore required.

Among the criteria that determine the final commercial value of seed potato stocks are the levels of several important potato viruses, and virus resistance in seed potato cultivars is therefore considered to be an important trait. Two types of resistance to PVY^N are known among potato cultivars; firstly, partial resistance or field resistance which is polygenic and secondly, extreme resistance, conferring complete protection, based on two single dominant R_y genes derived from *Solanum stoloniferum* or *S. tuberosum* ssp. andigene (Colon, 1987). Introduction of these R_y genes in important potato cultivars like 'Bintje' by conventional breeding programs is hampered by the tetraploid nature of the potato genome and has sofar proven to be extremely difficult or even impossible.

In 1986 a new method to introduce induced resistance to the plant virus TMV was reported (Powell-Abel *et al.*, 1986). This method, based on genetic modification of the host plant, employed the long known principle of 'cross-protection' by which a plant can be protected from a severe strain of a virus by pre-inoculation with a mild strain of the same virus (McKinney, 1929). *Agrobacterium tumefaciens* mediated transformation of tobacco plants with the coat protein (CP) gene of tobacco mosaic virus (TMV) resulted in plants expressing the TMV CP. These plants were proven to be protected from virus infection and the level of virus resistance in these plants appeared to be linked to the level of expression of the TMV CP. This type of induced resistance is now generally referred to as 'coat protein-mediated resistance' and has sofar been described for viruses from at least seven different plant virus groups (for reviews see Beachy *et al.*, 1990; Hemenway *et al.*, 1990; Nejidat *et al.*, 1990).

In contrast to the other viruses for which CP-mediated resistance has been reported the potyviral CP is not expressed from a distinct, separate CP gene. All potyviral proteins are released from a large polyprotein (the primary translation product) by proteolytic cleavages performed by virally encoded proteinases (Dougherty and Carrington; 1988, Goldbach, 1990; Riechmann *et al.*, 1992). To enable PVY^N CP expression in transgenic plants a translational initiation codon (AUG) was provided by introduction of a linker sequence containing such a codon directly

upstream of the cloned CP encoding sequence (see Chapter 2). The resulting construct was cloned in both orientations in an *A. tumefaciens* Ti-plasmid based plant transformation vector and by tuber disc transformation introduced in the genome of potato cv. Bintje. The resulting transgenic CP-sense and -antisense plants were subsequently analyzed for the presence and expression of the PVY^N CP gene and their possible resistance to PVY^N infection.

4.2 Materials and Methods

4.2.1 Virus and plants

The tobacco veinal necrosis strain of potato virus Y (PVY^N), obtained from the Institute for Plant Disease Research (IPO-DLO), was maintained in tobacco (*Nicotiana benthamiana* or *Nicotiana tabacum* var. SR1) by mechanical inoculation. For the transformation experiments, potato virus Y free potato (*Solanum tuberosum*) tubers of the tetraploid cultivar Bintje were obtained from the Center of Plant Breeding and Reproduction Research (CPRO-DLO) in Wageningen. Transgenic potato plants were kept under restricted greenhouse conditions (PK II level), according to the legislation imposed by the Dutch authorities (Voorlopige Commissie Genetische Modificatie: VCOGEM)

4.2.2 Construction of PVY^N CP cistron transformation vectors

The 1006 basepairs (bp) long *Cla*I fragment from pVY8, a cDNA clone containing the complete PVY^N CP encoding cistron (Van der Vlugt *et al.*, 1989 and Chapter 2) was supplemented at its 5'-end with an oligonucleotide linker sequence encompassing a translational start signal in an optimal context (Kozak, 1986), directly preceding the first 16 nt of PVY^N CP encoding sequence (see Fig. 4.1). The linker was first cloned into a *Bam*HI linearized pUC18 vector (Yanisch-Perron *et al.*, 1985).

```
                M  G  N  D  T  I
5'  GATCCACCATGGGAAATGACACAAT   3'
3'      GTGGTACCCTTTACTGTGTTAGC 5'
```

Fig 4.1 The oligonucleotide linker used to introduce a translational start signal in front of the PVY^N CP cistron. The linker contains the Kozak translational start consensus sequence such that the **ATG** start codon is positioned directly upstream of the first PVY^N coat protein amino acid (G) encoding triplet. At its 5'- and 3'-end the linker contains *Bam*HI and *Cla*I sticky cloning sites. The encoded amino acid sequence is indicated on top of the nucleotide sequence.

The resulting plasmid, containing two inverse copies of the linker sequence, was linearized with *Cla*I and ligated with the 1006 bp *Cla*I fragment from pVY8. This *Cla*I fragment contains the complete PVY^N CP cistron, except for the first 14 nt, followed by 219 nt of the 3'-NTR.

Insertion of the fragment in the correct orientation resulted in plasmid pCP5. Downstream of the CP encoding insert in pCP5 a *Bam*HI compatible cloning site (*Bgl*II) was introduced to facilitate cloning of the CP sequence into a plant transformation vector. For this an 2988 bp SspI fragment was isolated from pCP5 and provided with *Bgl*II-linkers (8-mer, Boehringer Mannheim). Upon digestion with *Eco*RI the DNA fragment was ligated in plasmid pUC19 (Yanisch-Perron, 1985), previously linearized with *Hin*cII and *Eco*RI, resulting in plasmid pDK1 (fig 4.2). Presence and orientation of the PVY^N CP insert were confirmed by restriction enzyme digest analysis and Southern blot hybridization using a ^{32}P-α-dATP labelled PVY^N CP specific cDNA probe (results not shown). Nucleotide sequence analysis confirmed the sequence surrounding the translational start codon.

The PVY^N CP cistron, at its 5'-end supplemented with translational start signals, was subcloned into pGH2, a plant transformation vector derived from pBI121 (Jefferson *et al.*, 1987) by deletion of a *Bam*HI/*Sst*I fragment containing the ß-glucuronidase (ß-GUS) encoding region. A 915 bp *Bam*HI/*Bgl*II fragment from plasmid pDK1 was inserted in both orientations in vector pGH2 using an unique *Bam*HI site positioned between the cauliflower mosaic virus (CaMV) 35S promoter and the nopalin synthase (nos) transcription termination sequence of the *A. tumefaciens* Ti-plasmid. This resulted in plasmids pCPY1 and pCPY2 (Fig. 4.3).

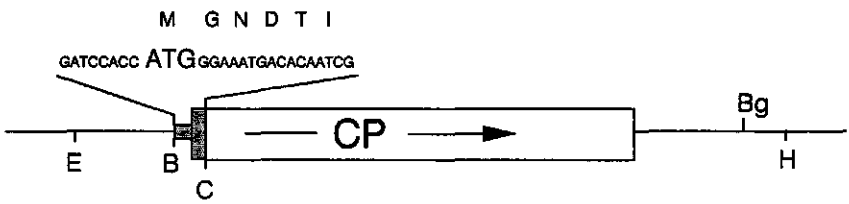

Fig. 4.2 The region of plasmid pDK1 containing a uan unique copy of the PVY^N CP gene at its 5'-end supplemented with a ATG start codon and followed by 102 nt of the viral 3'-NTR. The shaded box indicates the linker sequence containing the translational start signal (ATG) and black marker indicated the *Bgl*II linker. The amino acid sequence of the N-terminus of the CP is indicated on top of the linker sequence. B = *Bam*HI, Bg = *Bgl*II, C = *Cla*I, E = *Eco*RI and H = *Hin*dIII.

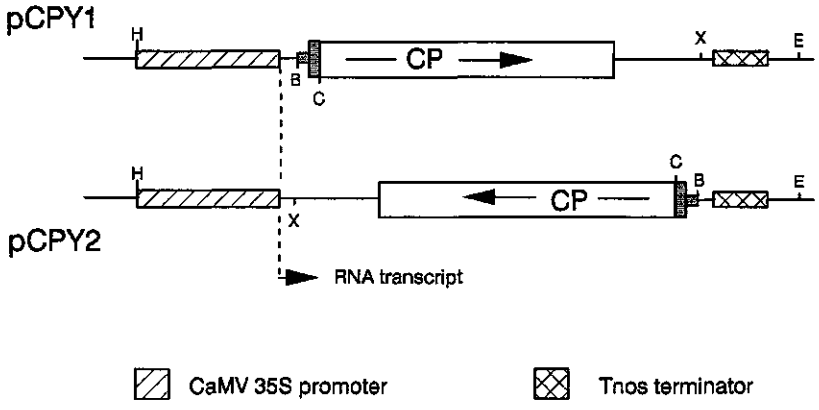

Fig 4.3 Schematic representation of plant transformation vectors pCPY1 (CP-sense) and pCPY2 (CP-antisense) with the PVY^N CP gene inserted in both orientations between the CaMV 35S promoter and the nos transcription terminator region (Tnos). The shaded box indicates the linker sequence containing the translational start codon. B = *Bam*HI, C = *Cla*I, E = *Eco*RI, H = *Hin*dIII and X = *Xho*II.

4.2.3 Transformation of potato plants

Plant transformation vectors pGH2, pCPY1 and pCPY2 were mated into the disarmed *A. tumefaciens* C58 strain LBA4404 (Hoekema *et al.*, 1983) by a triparental mating procedure using pRK2013 as a helper plasmid (Ditta *et al.*, 1980). The resulting recombinant *A. tumefaciens* strains were checked for the presence and orientation of the PVY^N CP gene by restriction enzyme digestions and Southern blot analysis. Transformation and regeneration of potato cultivar 'Bintje' tuber discs was done as described by Stiekema *et al.* (1988). Potato transformants were selected for resistance to kanamycin (100 μg/ml), rooted and after potting in soil transferred to the greenhouse. Transgenic plants were grown in the greenhouse under PKII conditions, according to the legislation imposed by the Dutch authorities (Voorlopige Commissie Genetische Modificatie; VCOGEM).

4.2.4 In vitro transcription and cell-free translation

For *in vitro* transcription purposes a 915 bp *Bam*HI/*Bgl*II cDNA fragment from pDK1, harbouring the CP encoding sequence, was subcloned in the transcription vector Bluescript SK$^+$ (Stratagene). One μg of plasmid DNA was linearized downstream of the PVY^N CP insert using *Xba*I and treated with 20 μg proteinase K. Proteins were removed by phenol:chloroform (1:1) extraction and the DNA ethanol

precipitated. Messenger sense 'run-off' transcripts were synthesized using T_7 RNA-polymerase (Promega) according to manufacturer's conditions for 1 hour at 37°C. One μg of transcript was translated *in vitro* using a rabbit reticulocyte lysate system (Boehringer, Mannheim) in the presence of 50μCi ^{35}S-Methionine (37 TBeq/nmole) for 1 hour at 37°C. Translation products were analyzed directly by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), or immunoprecipitated first using polyclonal antibodies directed against purified PVY^N particles, as described by Bernstein and Hruska (1981), prior to electrophoresis. Following electrophoresis gels were dried and autoradiographed.

4.2.5 Southern and Northern blot analysis

Total DNA was extracted from plants as described (Murray and Thompson, 1980). Five μg of DNA was digested with the appropriate restriction enzymes, separated on 1.2% agarose gels and blotted onto Genescreen Plus membrane (NEN). Total RNA was isolated from plants (Hooft van Huijsduijnen *et al.*, 1985), separated by electrophoresis and blotted onto Hybond-N membrane (Amersham). Southern and Northern blots were hybridized with a random-primed α-^{32}P-dATP labelled probe spanning the complete CP coding region. Hybridization and washing of the blots was done according to the manufacturers recommended method at 65°C.

4.2.6 Protein analysis in transgenic plants

The production of the PVY^N CP in transgenic plants was monitored by cocktail double antibody sandwich (DAS) ELISA (Van den Heuvel and Peters, 1989) and Western blot analysis of immunoprecipitated total leaf protein (Bernstein and Hruska, 1981) using either a polyclonal rabbit antiserum raised against purified virus or a monoclonal antibody (MAb) reacting with the core region of the potyviral CP. Both antisera were obtained from Dr van den Heuvel from the Institute for Plant Disease Research (IPO-DLO) in Wageningen. Total amounts of leaf protein were quantified using the BioRad protein assay.

4.2.7 Analysis of protection against PVY^N infection

Original transformants were reproduced by cuttings and plants were inoculated mechanically. One leaf was dusted with carborundum powder and inoculated with 50 μl inoculum containing 500 ng of purified PVY^N. Plants were monitored for systemical virus infection by subjecting samples of leafs younger than the inoculated leaf to cocktail DAS-ELISA using a polyclonal rabbit antiserum against purified virus. Virus titers were calculated from control amounts of purified virus included in the DAS-ELISA reaction.

4.3 Results

4.3.1 Construction of PVY^N CP cistron potato transformation vectors

The nucleotide sequence of the 3'-terminal part of the potyviral PVY^N genome, containing the complete CP encoding region, has been reported previously (Van der Vlugt *et al.*, 1989; see also Chapter 2). To enable expression of this CP in transgenic plants, a start signal in an optimal translational initiation context (Kozak, 1986) was introduced directly upstream of the viral CP encoding sequence by insertion of a synthetic linker sequence. Translation from this startcodon should result in a CP with an extra N-terminal methionine (M) residue. A second start codon present in the linker sequence and located 7 nt downstream the newly introduced ATG codon is out of frame with respect to the CP encoding sequence. Translation from this weak start codon would result in a short 29 amino acid polypeptide sharing no sequence homology with the PVY^N CP.

Insertion of a *Bgl*II linker 102 nt downstream of the CP stop codon finally yielded plasmid pDK1 (see Fig. 4.2) harbouring a 915 bp *Bam*HI/*Bgl*II fragment, containing the complete PVY^N CP encoding region, supplemented at its 5'end with an ATG start codon and at its 3'-end with 102 nt of the viral 3'-NTR.

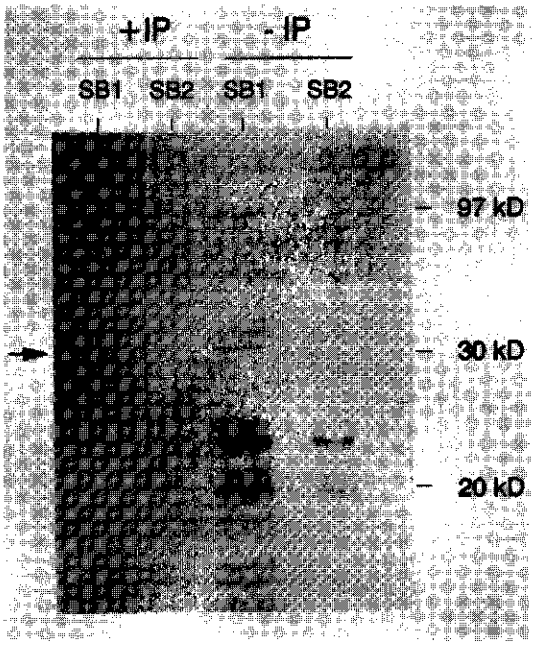

Fig 4.4 SDS-PAGE of cell free translation (-IP) and immunoprecipitation products (+IP) of *in vitro* T_7 RNA transcripts of plasmids pSB1 (CP-sense) and pSB2 (CP-antisense). The arrow indicates the PVY^N CP specific translation product of the expected size of 30 kDa. The extra band observed at the top is presumed to be a CP-dimer. Protein size markers are indicated on the right.

To confirm the translational integrity of the sequence surrounding the start of the CP cistron the 915 bp *Bam*HI/*Bgl*II fragment from pDK1 was also subcloned into the *Bam*HI site of RNA-transcription vector Bluescript KS$^+$ (Stratagene). Insertion of the fragment in the correct orientation with respect to the T_7 RNA-polymerase promoter yielded plasmid pSB1, insertion in the reverse orientation plasmid pSB2. Both plasmids were linearized just downstream of the CP insert using *Xba*I and run-off RNA was transcribed from the T_7 RNA-polymerase promoter. One μg of transcript was used in a rabbit reticulocyte *in vitro* translation reaction in the presence of ^{35}S-Methionine and the resulting translation products were subsequently immunoprecipitated with PVY^N CP specific polyclonal antiserum. SDS-PAGE of translation and immunoprecipitation products of RNA from pSB1 revealed the presence of a 30 kDa PVY^N specific translation product which is consistent with the expected size of 29.8 kDa. This product could not be detected in the translation and immunoprecipitation products from plasmid pSB2 (see Fig 4.4). Additional bands present in the non-immunoprecipitated lanes (-IP) most likely represent non-PVY specific translation products since they are absent from the immunoprecipitated translation products.

To construct PVY^N CP plant transformation vectors the 915 bp long, CP coding *Bam*HI/*Bgl*II fragment from pDK1 was subcloned in a unique *Bam*HI site positioned directly downstream of the CaMV 35S promoter of plant transformation vector pGH2. The orientation of the insert in recombinant plasmids was determined by restriction mapping and Southern blot analysis of resulting fragments (results not shown). Two plasmids, pCPY1, with the PVY^N CP sequence in the correct orientation with respect to the CaMV 35S promoter, and pCPY2, with the PVY^N CP sequence in the reverse orientation (see Fig. 4.3) were subsequently used for potato tuber discs transformations.

Plasmids pCPY1, pCPY2 and transformation vector pGH2, the latter containing no inserted sequences, were introduced into *A. tumefaciens* and recombinant strains were selected for kanamycin and rifampicin resistance. Colony filter hybridization with a PVY^N CP specific probe confirmed the presence of the CP gene in pCPY1 and pCPY2 harbouring *A. tumefaciens* strains (results not shown). Transformation of three hundred and fifty tuber discs (Stiekema *et al.*, 1988) of virus free potatoes cv. Bintje with each recombinant *A. tumefaciens* strain, resulted in large amounts of tuber discs forming shoots. Upon transfer to root inducing medium however only a fraction of the shoots showed formation of roots. Finally, 20 pCPY1 transformants, denoted C to X, and 16 pCPY2 transformant, denoted E to U, were obtained and transferred to soil. Most transgenic plants exhibited normal phenotypic appearances and produced normal tubers. Upon replanting of tubers however several transgenic pCPY1 and pCPY2 lines failed to grow normally and could not be maintained. Only those plants showing no apparent phenotypic deviation were selected for further analysis.

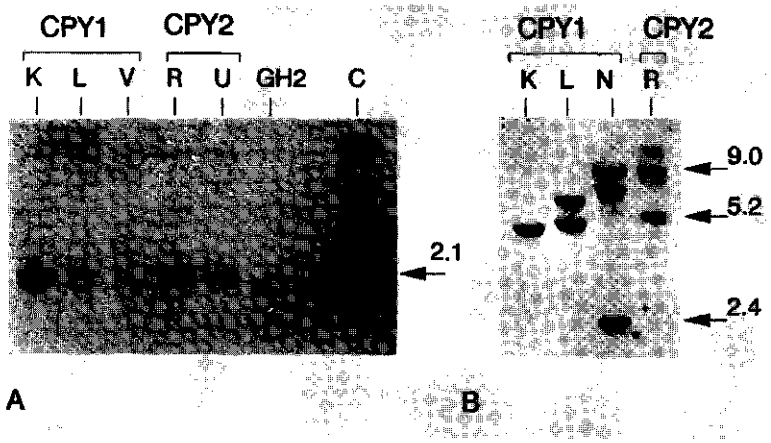

Fig 4.5 Southern blot analysis of total genomic DNA isolated from some sense (pCPY1: K, L, N and V) and antisense (pCPY2: R and U) PVY^N CP transgenic potato lines. Panel A: DNA was digested with *Hin*dIII and *Eco*RI, separated on a 1.2% agarose gel, blotted to GeneScreen Plus membrane and hybridized to a ^{32}P-labelled DNA fragment containing the PVY^N CP gene to reveal the complete 1600 bp CaMV 35S promoter - PVY^N CP - Tnos cassette. Panel B: DNA digested only with *Eco*RI to reveal the number of copies of the PVY^N CP gene cassette inserted in different sites in the potato genomic DNA. GH2; pGH2 transformed control plant, C; plasmid pCPY1 digested with *Hin*dIII and *Eco*RI. Sizes of DNA-fragments (in Kb) are indicated.

4.3.2 Analysis of transgenic potato plants

Transgenic pCPY1 potato plants were analyzed for the presence of PVY^N CP by cocktail DAS-ELISA. Using a PVY^N polyclonal antiserum or a MAb specifically reacting with the core region of the CP, a region highly conserved between different potyviruses (Ward and Shukla, 1991), no PVY^N CP could be detected in any of the transgenic plants although 1 ng of purified virus could reliably be detected in healthy plant sap by the DAS-ELISA format used. Alternative protein extraction methods under which a method employing SDS by which researchers from Monsanto Co. were able to detect PVY^N CP in transgenic potato plants (W. Kaniewski, personal communication) all failed to detect viral CP in the transgenic plants. Western blot analysis of immunoprecipitated total transgenic plant sap also failed to detect PVY^N CP. It was concluded that transgenic pCPY1 potato plants contained no CP or less then 0.0001% of total soluble leaf protein.

Southern blot analysis of total transgenic plant DNA probed with an PVY^N CP specific probe revealed that all CP-sense and -antisense transgenic plants contained CP specific DNA. Analysis of the copy number of the CP gene in different transgenics showed that in most plant lines 1-3 copies were inserted (Fig. 4.5). RNA spot blots probed with a CP specific ^{32}P-labelled DNA fragment demonstrated that the PVY^N CP was transcribed in all transgenic potato plants (results not shown).

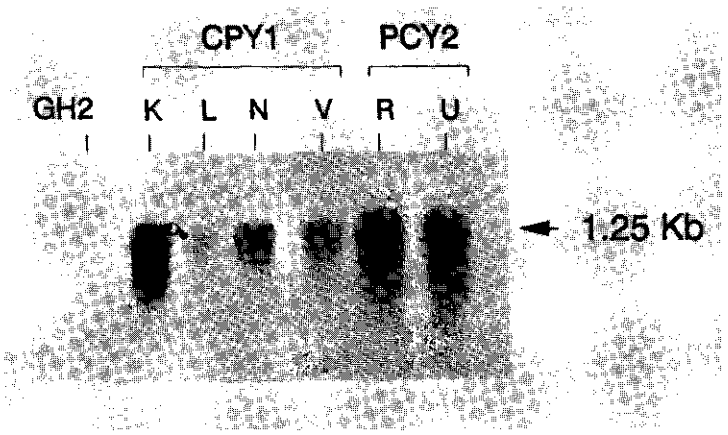

Fig 4.6 Northern blot analysis of total RNA isolated from some CPY1 (CP-sense: K, L, N, and V), CPY2 (CP-antisense: R and U) and empty vector (GH2) transformed potato plants and probed with a ^{32}P-labelled DNA fragment containing the PVY^N CP gene. The expected size of 1,25 Kb of the transcript is indicated on the side.

Northern blot analysis of total RNA showed the presence of a RNA transcript of the expected size of ± 1.25 Kb, composed of 950 PVY^N specific nt, 150 nt derived from the Tnos region and a poly-(A$^+$) tail with an assumed length of 150 nt, in the transgenic plants (see Fig. 4.6).

4.3.3 Analysis of virus protection in transgenic potato plants

To assess possible reduced susceptibility to PVY^N infection, transgenic potato plants were challenged with PVY^N by mechanical inoculation. Transgenic lines were propagated by cuttings as was a pGH2 transformed control plant. Eight progeny plants of each line were inoculated with 500 ng of purified virus, two plants of each line were left untreated as controls. Since distinct primary symptoms of PVY^N infection in potato cv. Bintje are absent, viral infection of inoculated plants was monitored by determining virus titers in a DAS-ELISA on leaf samples taken from inoculated leafs and younger leafs at different days post inoculation (dpi). Polyclonal antiserum against purified PVY^N could be used in the DAS-ELISA since CP production in the transgenic plants was at undetectable low levels (see above) while virus infection was always readily detected within 30 minutes in the ELISA format used.

Transgenic plants and virus challenge experiments were restricted to a special greenhouse modified to meet the safety standards (PK-II level) set by the Dutch authorities (Voorlopige Commissie Genetische Modificatie: VCOGEM). As a consequence of these technical modifications however correct temperature and humidity regimes in the greenhouse, necessary for successful PVY^N inoculation experiments of potato plants, could not reliably be maintained. Of all transgenic potato lines tested

only five CPY1 (sense) lines (K, L, N, V, and W), two CPY2 (antisense) lines (R and U) and one GH2 control line gave reliably results in the tests for possible resistance to PVY^N infection. In all other protection studies high temperature conditions (> 30°C) prevented successful PVY^N infections in control and transgenic lines using several different PVY^N inoculum concentrations. The results of successful inoculation experiments are listed in Table 4.1.

Table 4.1 shows the average virus titers observed in the inoculated transgenic potato plant lines. At 11 dpi all plants were systemically infected and contained virus but line CPY2-U contained a significant lower amount of virus when compared to the GH2 control line. At 17 and 23 dpi however all plants lines were heavily infected and contained similar amounts of virus. Therefore none of the transgenic plant lines appeared to be significantly protected against PVY^N infection. During this experiment two out of eight inoculated plants from line CPY1-K did not become infected. This finding however was not observed in other experiments in which none of the plants of line CPY1-K challenged with similar amounts of PVY^N showed protection to the virus (results not shown).

Table 4.1 Development of virus titer (ng virus/ mg leaf) in PVY^N CP-sense (CPY1; K-W), CP-antisense (CPY2; R-U) and empty vector (GH2) transformed transgenic potato plants. Eight plants of each line were mechanically inoculated with 500 ng of PVY^N and the amount of PVY^N in systemically infected leafs determined in duplo by DAS-ELISA. Virus titers were calculated for each inoculated plant using standard amounts of purified virus in healthy plant sap included in each ELISA experiment. For each transgenic plant line the average virus titer and the standard deviation (SD) were calculated from the ELISA values obtained from the individual systemically infected plants after correction for background values obtained for healthy plant sap. n = number of systemically infected plants 23 dpi. nd = not determined.

	Average virus titers in ng virus / mg leaf (± SD)			
Plant line	*11 dpi*	*17 dpi*	*23 dpi*	*n*
GH2	71.3 ± 12.0	106.3 ± 17.6	96.2 ± 14.6	8
CPY 1 K	58.0 ± 32.0	112.2 ± 14.3	121.4 ± 16.3	6*
L	88.0 ± 10.6	88.0 ± 11.0	86.8 ± 20.8	8
N	93.0 ± 20.3	nd	nd	8
V	95.0 ± 22.3	nd	nd	8
W	100.0 ± 16.3	nd	nd	8
CPY 2 R	54.6 ± 14.4	108.5 ± 25.0	97.7 ± 1.3	8
U	31.0 ± 11.3	101.9 ± 14.0	105.5 ± 13.4	8

* : Two out of eight inoculated plants did not become infected.

4.4 Discussion

In this chapter the transformation of tuber discs of potato cv. Bintje with the coat protein cistron of PVY^N has been described as well as the subsequent analysis of possible protection to PVY^N virus infection of the resulting transgenic plants.

Transformation of potato tuber discs resulted in a large number of shoots (on average one per disc), however only 10 percent of these shoots were able to form roots on root-inducing medium. Transgenic plants were propagated by stem cuttings and after rooting transferred to soil. Most transgenic plants showed normal phenotypes with the exception of one plant exhibiting a very stunted morphology with small, very hairy leafs. This plant and several others could not be maintained since either no tubers were formed or tubers proved not to be viable. The overall efficiency of transformation and regeneration of transgenic plants was rather low for potato cv. Bintje. Stiekema *et al.* (1988) observed that about 1% of shoots formed on tuber discs developed into kanamycin resistant transgenic plants. Bintje also appeared to be more sensitive for kanamycin selection during regeneration than potato cv. Désirée. The use of alternative selection markers in plant transformation vectors might therefore aid in lifting transformation and regeneration efficiencies for potato cv. Bintje.

Twenty CP-sense (CPY1) and 16 CP antisense (CPY2) transformed potato plants were finally obtained. These plants were all tested for the production of PVY^N CP by DAS-ELISA and Western blot analysis of immunoprecipitated total leaf sap. Although all plants produced PVY^N CP RNA, in none of the plants accumulation of PVY^N CP could be detected. It is not known whether this is caused by relatively inefficient protein extractions from transgenic leaf material, low expression levels or reduced stability of the viral CP. The expression of the CP from a 'sub-genomic' mRNA might result in a CP lacking essential folding structures and thereby stability since potyviral CPs are normally released from polyproteins by proteolytic processing mediated by the viral encoded Nla proteinase (Carrington and Dougherty, 1987). Protein structures appear to be involved in this cleavage as the requirement of specific domains in the carboxyl-terminal region of the Nla proteinase seem to indicate (Parks and Dougherty, 1991). Whether transformation of plants employing constructs in which a biologically active cis-acting Nla proteinase releases the PVY^N CP from a precursor protein, will result in higher expression levels of this CP remains to be tested. Introduction of the tobacco mosaic virus (TMV) 5'-untranslated leader sequence which is known to act as a translational enhancer (Gallie *et al.*, 1987) upstream of the PVY^N CP encoding sequence might also result in higher expression levels of CP. The use of alternative transformation vectors employing more optimized CaMV 35S promoters could also help in raising expression levels of the PVY^N CP. Reiteration of cis-acting subdomains of the CaMV 35S promoter region has been shown to greatly enhance expression of specific genes in transgenic plants (Kay *et al.*, 1987; Fang *et al.*, 1989) as had a

chimeric promoter combining elements from the 35S promoter and the mannopine synthetase (mas) promoter from the *A. tumefaciens* Ti-plasmid (Comai *et al.*, 1990).

Despite the absence of detectable levels of PVY^N CP in the transgenic plants several CP-sense and -antisense potato lines expressing CP specific RNA were challenged with PVY^N. In none of the plant lines tested a significant protection to PVY^N infection could be observed since 17 dpi all plants contained similar, high amounts of virus in systemically infected leafs. At 11 dpi plant lines CPY1-K (CP-sense) and CPY2-U (CP-antisense) accumulated relative low amounts of virus when compared to control line GH2. Line CPY1-K also showed a wide spread in virus titers between different infected plants. At 17 dpi however all plants from both lines were heavily infected with PVY^N. Interestingly these two lines also displayed the fittest behavior under tissue culture conditions with explants rooting very well and developing relatively large and dark green leafs. This could indicate that the reduced virus levels observed at 11 dpi are based on general physiological properties of these two plant lines.

Protection to PVY infection has previously been described for potato plants transformed with the CPs of both PVY and potato X potexvirus (PVX) (Lawson *et al.*, 1990) and tobacco plants transformed with the CP of soybean mosaic potyvirus (SMV) (Stark and Beachy, 1989). The level of virus protection in these plants however was not directly correlated with the level of CP accumulation. Potato plants transformed with the CP of potato leafroll luteovirus (PLRV), another major virus disease in potato crops, also didn't show any clear correlation between production of CP and level of virus protection (Van der Wilk *et al.*, 1991; Kawchuk *et al.*, 1991). Despite the absence of detectable levels of PLRV CP, transgenic plants were found to be partially protected to PLRV infection i.e. they accumulated significantly lower virus titers as compared to untransformed control plants.

Successful protection of PVY^N CP transgenic potato cv. Bintje to PVY^N infection remains to be established. Better controllable greenhouse temperature regimes will allow for more reliable virus protection studies and might reveal PVY^N protection of the transgenic potato lines already obtained. The apparent lack of correlation between the level of potyvirus, and luteovirus, CP production and the level of virus protection (Lawson *et al.*, 1990; Stark and Beachy, 1989; Van der Wilk *et al.*, 1991; Kawchuk *et al.*, 1991) however implies that large numbers of transgenic potato plant lines should be tested for presence and level of virus resistance.

To circumvent the problems of transformation efficiency, plant regeneration and maintenance and greenhouse climate conditions, associated with transformation and virus protection studies of potato plants, it was decided to also investigate the possible introduction of CP-mediated resistance to PVY^N in transgenic tobacco plants. Tobacco, being a very efficient model plant system for *A. tumefaciens* based plant transformation, is a very susceptible host for PVY^N showing a strong necrotic reaction to infection. Transformation of tobacco leaf discs should therefore result in large

numbers of transgenic plants that can subsequently be tested for the production of PVY^N CP and upon self-pollination, their progeny can visually be screened for possible virus resistance. The results of these experiments will be discussed in the next chapter.

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Chapter 5

Evidence for sense RNA-mediated protection to PVY^N in tobacco plants transformed with the viral coat protein cistron

Summary

The coat protein (CP) cistron of the tobacco veinal necrosis strain of potato virus Y (PVY^N), supplemented with translational start signals, was cloned into an *Agrobacterium tumefaciens* Ti-transformation vector. Transformation of tobacco leaf discs resulted in 99 transgenic lines which were subsequently analyses for the presence and expression, at both the transcriptional and translational level, of the CP gene. Although CP specific RNA transcripts were produced in all plants no CP could be detected by several sensitive immunological techniques. Upon mechanical inoculation of progeny lines of self-pollinated original transformants (S1) with PVY^N, protection levels of 20 and 95% respectively could be observed in two out of ten lines tested. This level of protection increased to 100% in the S2 progeny obtained from self-pollination of virus-protected S1 plants.

Transformation of tobacco leaf discs with a PVY^N CP construct from which the ATG start codon had been removed by site-directed mutagenesis resulted in 57 transgenic lines that all produced CP specific transcripts. Mechanical inoculation with PVY^N of S1 progeny plants of several of these lines resulted in resistance to a similar level and extent as in the S1 progeny of plants transformed with the intact CP cistron. The results obtained strongly suggest that the resistance observed in the transgenic plants is principally based on the presence of PVY^N CP RNA sequences rather than on the accumulation of viral coat protein.

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5.1 Introduction

'Coat protein-mediated resistance', generally defined as the resistance to a given virus caused by the expression of its coat protein (CP) gene in transgenic plants, is now considered to be a general method to achieve virus protection in plants. For a growing number of economically important plant viruses it has been shown that accumulation of the CP in transgenic plants confers resistance to infections and/or disease development by the virus from which the CP gene is derived and related viruses (for reviews see Beachy *et al.*, 1990; Hemenway *et al.*, 1990; Nejidat *et al.*, 1990). Although the mechanism underlying the resistance is not fully understood and seems to differ with the virus involved, it has been shown for several viruses that the presence of the CP and not only the mRNA is essential for protection (Van Dun *et al.*, 1988; Powell *et al.*, 1990).

Potyviruses form the largest and economically most important group of plant viruses and the establishment of CP mediated resistance to these viruses would have an enormous economical impact. In the past few years the sequence of the CP cistron has been determined for a considerable number of potyviruses, often with the intention to achieve CP mediated resistance. Despite all efforts this type of engineered resistance has sofar been reported for only two potyviruses. Transformation of the non-host plant tobacco with the soybean mosaic virus (SMV) CP gene resulted in reduced virus accumulation in transgenic plants upon inoculation with two non-related potyviruses, tobacco etch virus (TEV) and potato virus Y (PVY) (Stark and Beachy, 1989). Both viruses only share approximately 60% CP amino acid sequence homology to each other and to SMV. Transformation of potato cv. Russet Burbank with the CP genes of both PVY and the potexvirus potato virus X (PVX) resulted in plants that were resistant to both mechanical inoculation with PVY (and PVX) and inoculation using viruliferous aphids (Lawson *et al.*, 1990). Surprisingly the transgenic potato line that showed the highest level of resistance to both PVX and PVY, contained the lowest amounts of PVX and PVY CP. Both reports seem to indicate a less prominent involvement of the potyviral CP in the engineered resistance than reported for other virus groups.

One complication with respect to the expression of potyviral CPs in transgenic plants is, that in contrast to the other viruses for which CP-mediated resistance has been described sofar, they are not expressed from a distinct, separate CP gene. All potyviral proteins are generated from a large, primary translation product (polyprotein) by proteolytic cleavages performed by virally encoded proteinases (Dougherty and Carrington, 1988; Goldbach, 1990). As a consequence the CP coding region does not start with a translational initiation codon (AUG) which, therefore, should be provided artificially to enable protein expression in transgenic plants.

The previous chapter describes the transformation of potato cv. Bintje tuber discs with the CP of PVY^N and subsequent analysis of the resulting transgenic potato plants

for the production of PVY^N CP and possible protection against PVY^N virus infection. In none of the transgenic potato plants obtained however, detectable levels of viral CP were present and screening for possible PVY^N protection also revealed no significant virus resistance. Under given greenhouse conditions however (i.e. high temperatures and humidity) only seven out of 36 transgenic potato lines gave reliable results in virus protection studies in that control plant lines became infected under given conditions in the same experiment. Tobacco (*Nicotiana tabacum*) is a very efficient model plant system for *A. tumefaciens* based plant transformation and a very susceptible host for PVY^N showing a strong necrotic systemic reaction to infection. Tobacco plants are also less susceptible for the higher greenhouse temperatures impeding the virus protection studies of the transgenic potato lines. To circumvent further problems of transformation efficiency, plant regeneration and maintenance and greenhouse climate conditions, associated with transformation and virus protection studies of potato plants, it was therefore decided to also investigate the possible introduction of CP-mediated resistance to PVY^N in transgenic tobacco plants.

This chapter reports the transformation of tobacco plants with the PVY^N CP encoding sequence, supplemented with an AUG start codon in a translational favorable context, and the high levels of resistance to mechanical inoculation with PVY^N present in the transgenic tobacco lines obtained. As a control, and to determine whether the observed resistance is caused by expression of the viral CP gene on the RNA or on the protein level, plants were also transformed with the CP cistron devoid of a translational start signal. Surprisingly these transgenic plants show similar levels of resistance to PVY^N as observed in plants containing a translational functional CP gene.

5.2 Materials and Methods

5.2.1 Virus and plants

The tobacco veinal necrosis strain of potato virus Y (PVY^N) was maintained in tobacco (*Nicotiana benthamiana* or *Nicotiana tabacum* var. SR1) by mechanical inoculation. For the transformation experiments, *in vitro* grown *N. tabacum* var. SR1 was used as an recipient. Transgenic tobacco plants were kept under safe greenhouse conditions (PK II level), according to the legislation imposed by the Dutch authorities (Voorlopige Commissie Genetische Modificatie: VCOGEM)

5.2.2 *Construction of PVY^N CP cistron transformation vectors and transformation of plants*

The 1006 basepairs (bp) long *Cla*I fragment from pVY8, a cDNA clone encompassing the complete PVY^N CP cistron (Van der Vlugt *et al.*, 1989), was supplemented at its 5'-end with a translational start signal. To this end the first 14 nucleotides (nts) of the CP encoding sequence were replaced by a *Bam*HI/*Cla*I linker sequence comprised of two complementary oligonucleotides. This linker contained a 5' compatible *Bam*HI site followed by an ATG start codon in a favourable translation initiation context (Kozak, 1981) directly upstream the first 14 nts of the CP encoding sequence giving an *Cla*I compatible 3'-end. Using a unique *Ssp*I site a *Bam*HI linker was inserted 102 nts downstream of the CP stop codon. From the resulting plasmid pCPYS1 a 933 bp *Bam*HI fragment, containing the complete CP cistron of PVY^N with an additional 102 nts of 3'-non-translated region (3'-NTR), was subcloned into pGH2, a plant transformation vector, derived from pBI121 (Jefferson *et al.*, 1987) by deletion of a *Bam*HI/*Sst*I GUS encoding fragment. Insertion of the CP cistron in the correct orientation with respect to the CaMV 35S promoter resulted in plasmid pTCPYS1 (Fig. 5.1). The ATG start codon was deleted from pCPYS1 by *Nco*I digestion followed by mung bean exonuclease treatment of the protruding 5'-ends. Insertion of the resulting 927 bp *Bam*HI fragment in the correct orientation in pGH2 resulted in plasmid pTCPY2 (Fig. 5.1). Both recombinant transformation vectors and pGH2 were mobilized into *Agrobacterium tumefaciens* C58 strain LBA4404 (Hoekema *et al.*, 1983) by a triparental mating procedure using pRK2013 as a helper plasmid (Ditta *et al.*, 1980). Transformation of tobacco leaf discs was done essentially as described (Horsch *et al.*, 1984).

5.2.3 *Southern and Northern blot analysis*

Total DNA was extracted from plants as described (Murray and Thompson, 1980). Five μg of DNA was digested with *Eco*RI, separated on 1.2% agarose gels and blotted onto Genescreen Plus membrane (NEN). Total RNA was isolated from plants (Verwoerd *et al.*, 1989), separated by electrophoresis and blotted onto Hybond-N membrane (Amersham). Southern and Northern blots were hybridized with a random-primed α-^{32}P-dATP labelled probe spanning the complete CP coding region. Hybridization and washing of the blots was done according to the manufacturers recommended method at 65°C.

5.2.4 *Protein analysis*

The production of the PVY^N CP in transgenic plants was monitored by cocktail double antibody sandwich (DAS) ELISA both with and without an additional ampli-

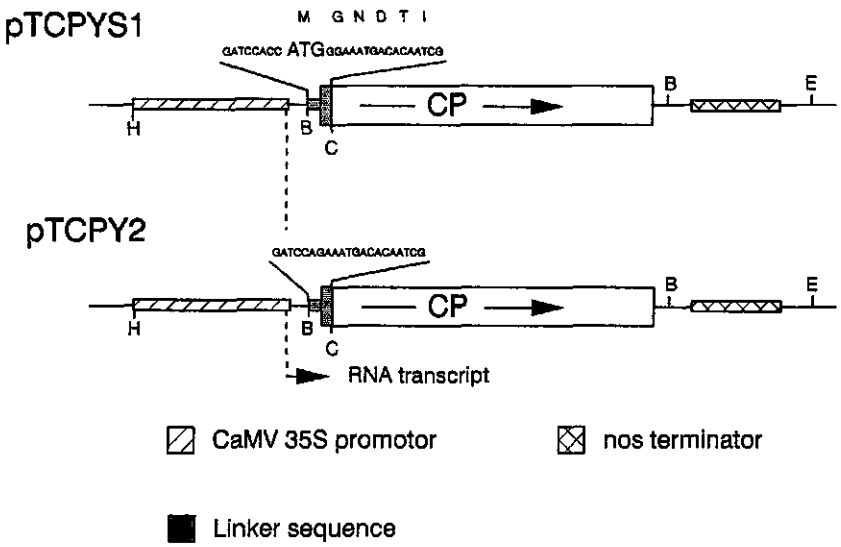

Fig. 5.1 Schematic representation of plant transformation vectors pTCPYS1 and pTCPY2, containing the PVY^N CP gene inserted between the CaMV 35S promoter and the nos terminator region. In pTCPYS1 the amino acid sequence of the N-terminus of the PVY^N CP is indicated on top of the linker sequence. B = *Bam*HI, C = *Cla*I, E = *Eco*RI, H = *Hin*dIII.

fication of the enzyme reaction (Van den Heuvel and Peters, 1989) and Western blot analysis of immunoprecipitated total leaf protein (Bernstein and Hruska, 1981) using either a polyclonal rabbit antiserum raised against purified virus or a monoclonal antibody reacting with the core region of the potyviral CP. Total amounts of leaf protein were quantified using the BioRad protein assay.

5.2.5 Analysis of PVY^N protection

Progeny plants of self-pollinated original transformants were mechanically inoculated approximately 6 weeks after sowing (four leaf stage). The largest leaf was dusted with carborundum powder and inoculated with 50 μl inoculum containing 500 ng of purified PVY^N. Plants were monitored daily for the development of systemical symptoms. Approximately 7-8 days after inoculation of *N. tabacum* var SR1 with PVY^N, plants developed vein chlorosis followed by vein necrosis on systemically infected leafs. All inoculated plants were checked for the presence of virus 10-14 days post inoculation (d.p.i.) by cocktail DAS-ELISA using a polyclonal rabbit antiserum against purified virus.

5.3 Results

5.3.1 Construction of PVY^N CP cistron transformation vectors

The nucleotide sequence of the 3'-terminal part of the potyviral PVY^N genome, containing the viral CP encoding region, has been reported previously (Van der Vlugt *et al.*, 1989; see also Chapter 2). *In vivo* the PVY^N CP is cleaved from a polyprotein precursor at a glu/gly (Q/G) dipeptide sequence by the viral encoded NIa proteinase (Carrington and Dougherty, 1987). To enable specific expression of the CP cistron in transgenic plants a translationally favourable ATG start codon (i.e. ***A**ATGG****, see Fig. 5.1) was introduced directly upstream of the CP cistron. In principle, translation from this newly introduced start codon results in a CP with an extra methionine (M) residue at its N-terminus. The sequence of the modified start region was confirmed by sequence analysis and the integrity of the CP reading frame by *in vitro* translation (data not shown). Addition of a second BamHI cloning site 102 nt downstream of the CP stop codon yielded plasmid pCPYS1. The resulting 933 bp BamHI fragment from pCPYS1 was subcloned into a unique *Bam*HI site positioned between the CaMV 35S promoter sequence and the transcription terminator sequence of the nopaline synthase (nos) gene of plant transformation vector pGH2. The correct orientation of the CP insert in the resulting plasmid pTCPYS1 (see Fig. 5.1) was confirmed by restriction analysis and Southern blot hybridizations (data not shown).

To verify whether the viral CP or CP mRNA is responsible for protection, an additional vector (pCPY2) was derived from pCPYS1 in which the ATG start codon was removed by Mungbean exonuclease treatment as described in materials and methods. Sequence analysis of the relevant region in plasmid pCPY2 revealed the deletion of the hexanucleotide ACCATG. The next ATG start codon in the CP-encoding frame in pCPY2 is located 162 nts downstream of the deleted ATG codon and in theory translation from this start codon would result in a truncated CP lacking the 54 N-terminal amino acids. A second weak ATG start codon, present in the linker sequence (see Fig. 5.1), is out of frame with the CP and would result in a short polypeptide of 29 amino acids without sequence homology to the PVY^N CP. *In vitro* translation of T_7-derived RNA transcripts of the CP encoding regions of plasmid pCPYS1 and plasmid pCPY2, followed by immunoprecipitation of the translation products with a monoclonal antibody specifically reacting with the potyviral CP core region, clearly showed the presence of the PVY^N CP of the expected size of 30 kDa for the CP^{+ATG} transcript of pCPYS1, while no truncated PVY^N CP products for the CP^{-ATG} transcript of pCPY2 were detected (data not shown). This result indicated that removal of the ATG start codon was effective and silenced the cistron completely. Subcloning of the 927 bp BamHI fragment from pCPY2 in pGH2 resulted in transformation vector pTCPY2 (see Fig. 5.1).

5.3.2 *Analysis of transgenic tobacco lines*

Plant transformation vectors pGH2, pTCPYS1 and pTCPY2 were mated into *A. tumefaciens*. Colonies of *A. tumefaciens* containing recombinants were selected for kanamycin and rifampicin resistance and by subsequent colony filter hybridization using a PVY^N CP specific cDNA probe. Transformation of one hundred leaf discs of tobacco var. SR1 (Horsch *et al.*, 1984) using recombinant *A. tumefaciens* strains, resulted in numerous shoots. Of each leaf disc only one shoot was transferred to root inducing medium and most shoots developed roots. Finally 99 pTCPYS1 (CP^{+ATG}) and 57 pTCPY2 (CP^{-ATG}) transformed tobacco plants were maintained and together with 10 pGH2-transformed control plants transferred to the greenhouse. All transgenic tobacco plants showed normal phenotypes and set seed after self-pollination.

All originally obtained CP^{+ATG} transformants were analyzed for the presence of PVY^N CP by cocktail DAS-ELISA using polyclonal PVY^N antiserum, whereas upon amplification of the enzyme reaction (Van den Heuvel and Peters, 1989), 150 pg of purified virus in healthy plant sap could reliably be detected, production of viral CP could not be detected in the transgenic plants. Western blot analysis of total transgenic plant sap immunoprecipitated with a Mab against the potyviral CP core, also failed to detect PVY^N CP. It was concluded that the transgenic CP^{+ATG} plants either contained no CP or less than 0.0001% of total soluble leaf protein. As expected, similar ELISA analyses of the CP^{-ATG} transformants were also negative. RNA spot blot analysis however revealed that the viral CP gene was transcribed in virtually all plants (data not shown).

5.3.3 *Virus protection in CP^{+ATG} transgenic plants*

To determine whether the transgenic tobacco plants are protected to PVY^N infection, ten randomly selected S1 CP^{+ATG} progeny lines, obtained after self-pollination of the original transformants, were mechanically inoculated with 500 ng of PVY^N. As controls, pGH2 transformed SR1 progeny plants were also challenged with PVY^N.

In several independent experiments, eight out of the ten CP^{+ATG} lines tested, showed no protection against PVY^N infection just as the pGH2-transformed SR1 control plants. Two transgenic lines (A80 and A30) however, showed clear levels of protection (see Figure 5.2A) despite the absence of any detectable levels of viral CP in the progeny plants. Twenty percent of the progeny from line A80 showed no systemic symptoms 14 d.p.i. Progeny of line A30 even showed 95% protected plants. The absence of virus in symptomless plants was confirmed by DAS-ELISA. Similar to the control SR1 plants no delay in symptom development or decrease in virus titre (as estimated by DAS-ELISA) could be observed in the systemically infected plants of both protected lines.

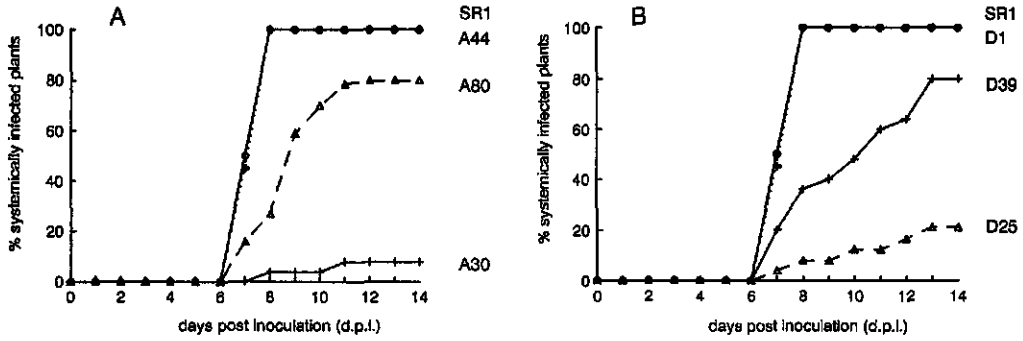

Fig. 5.2 Levels of virus resistance of tobacco lines transformed with the CP cistron of PVY^N. Twenty five S1 progeny plants obtained after self-pollination of original transformants (CP^{+ATG}, CP^{-ATG} and pGH2) were mechanically inoculated with 500 ng of purified virus and assessed daily for symptom development. Presence of virus was monitored 14 d.p.i. by DAS-ELISA. Panel A: three CP^{+ATG} lines; A80 and A30 (protected), A44 (unprotected) and pGH2 control (denoted as SR1). Panel B: three CP^{-ATG} lines; D39 and D25 (protected), D1 (unprotected) and pGH2 control (SR1).

Both CP^{+ATG} lines which were protected to mechanical inoculation with PVY^N (lines A30 and A80) were further analysed in terms of copy number and RNA expression of the introduced PVY^N CP cistron. Southern hybridizations of *Eco*RI digested total plant DNA with a PVY^N CP specific cDNA probe revealed the presence of two independently inserted copies of the CP gene in both lines A30 and A80 (see Fig. 5.3).

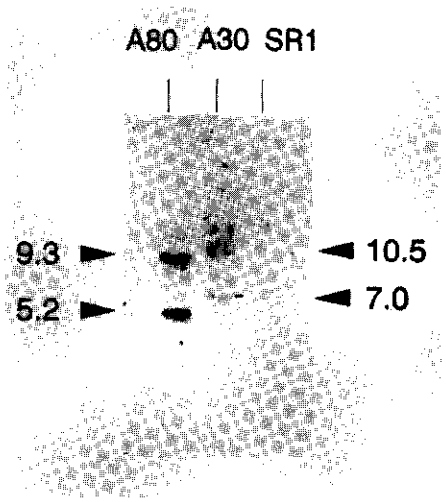

Fig. 5.3 Southern blot analysis of *Eco*RI digested total DNA isolated from CP^{+ATG} lines A80 and A30 and a pGH2 transformed control line (SR1), probed with a ^{32}P labelled DNA fragment containing the PVY^N CP encoding sequence. Sizes of DNA fragments (in Kb) are indicated.

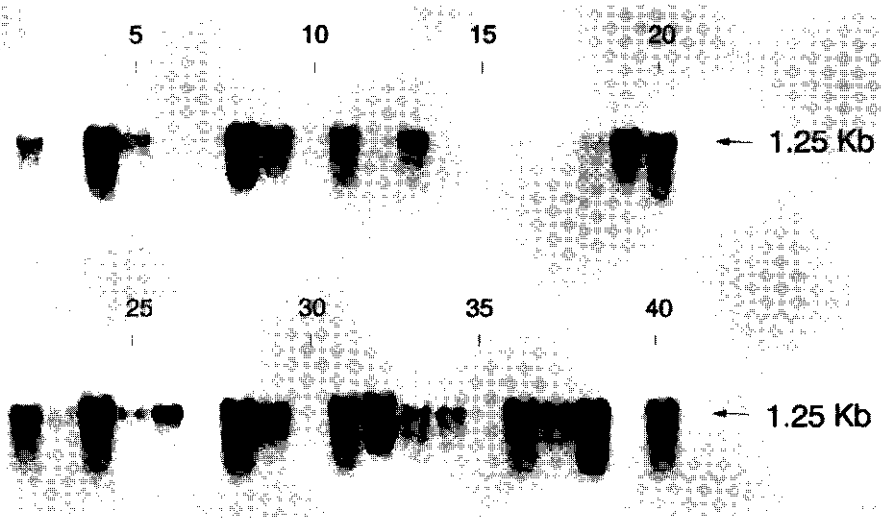

Fig. 5.4 Northern blot analysis of total RNA isolated from 40 S1 progeny plants from original CP^{+ATG} transformant line A30 hybridized to a ^{32}P labelled DNA fragment containing the PVY^N CP cistron. Plant numbers are indicated.

This finding was confirmed by the segregation ratio of 15:1 (expressors : non-expressors) observed on Northern blots of 5 μg of total RNA isolated from self-pollinated progeny plants from both lines (Fig. 5.4 shows the results for line A30). Transcripts were of the expected size of ± 1.25 Kb. In both lines both copies are expressed, but apparently at different levels. The observed differences in levels of the PVY^N CP mRNAs among the various plants of line A30 reflected true differences in transcriptional activity of the PVY^N CP gene, since a control hybridization of the same blots using a 5S ribosomal RNA probe revealed equal amounts of corresponding RNA for all plants (data not shown). From the Northern blot analysis it was concluded that all progeny plants lacking a transcriptionally active CP gene were not protected to PVY^N-infection.

Co-segregation of virus resistance and transcriptionally active CP genes was further confirmed by challenging S2 progeny plants obtained after self-pollination of virus-protected S1 plants from line A80. The level of protection increased from 20% in the original S1 plants to 100% in a number of S2 lines indicating that the obtained protection is genetically stable.

5.3.4 Virus protection in CP^{ATG} transgenic plants

From the experiments above it is not clear whether the observed resistance is mediated by undetectable trace amounts of the CP or by accumulation of the CP

mRNA. In order to determine whether the CP or CP gene transcripts are involved in PVY^N protection, tobacco plants were transformed with a CP DNA construct devoid of a translational start signal. To this end a deletion of 6 nts, including the ATG start codon, was introduced in transformation vector pTCPYS1, resulting in vector pTCPY2 (Fig. 5.1). Transformation of tobacco leaf discs with this vector yielded 57 transgenic (CP^{-ATG}) plants. As expected none of these plants showed the production of detectable levels of (a truncated form of) viral CP in sensitive enzyme-amplified ELISA experiments using a monoclonal antibody directed against the core region of the potyviral CP. Mechanical inoculation of progeny plants of ten randomly selected, self-pollinated CP^{-ATG} plants revealed that two lines (D25 and D39) were protected to PVY^N infection at a similar level and extent as found for the two protected CP^{+ATG} lines. Lines D39 and D25 showed protection levels of 20% and 80% respectively (see Fig. 5.2B). Symptomless plants were completely virus free. Similar to the control SR1 plants no delay in symptom development or decrease in virus titer (as estimated by DAS-ELISA) could be observed in the systemically infected plants of both protected lines while infected plants showed no decrease in virus titer (as determined by ELISA) or a delay in symptom development. Southern and Northern analysis of the two protected CP^{-ATG} S1 progeny lines D25 and D39 showed that both lines each contained two transcriptionally active copies of the PVY^N CP gene (data not shown).

5.4 Discussion

In this chapter the engineered protection against PVY^N infection by transformation of tobacco plants with the CP coding domain of this potyvirus is described. In contrast to "coat protein-mediated resistance", as reported for several viruses belonging to other taxonomic groups (Beachy *et al.*, 1990; Hemenway *et al.*, 1990; Nejidat *et al.*, 1990), the protection reported here is apparently not based on the translational expression of the viral gene. Both the very low titers (or even complete absence) of viral CP in two protected CP^{+ATG} transgenic lines (A30 and A80) and the similar levels of protection observed in two lines transformed with a CP gene construct lacking a translational start codon (lines D25 and D39) strongly suggest that the observed resistance is primarily based on the presence of the CP sense RNA rather than on the CP itself. Although one could argue that even in the CP^{-ATG} transgenic lines the resistance is based on a non-detectable level (i.e. less than 0.0001% of total soluble protein) of translation into a truncated viral polypeptide (from a internal AUG start codon), the evidence collected points towards a role of the viral transcripts (of sense polarity) in the resistance obtained. Involvement of CP RNA transcripts in virus resistance has also been found for transgenic potato plants expressing either sense or anti-sense constructs of the CP gene of potato leafroll luteovirus (PLRV) (Van der Wilk *et al.*, 1991; Kawchuk *et al.*, 1991). Despite the

presence of PLRV CP specific transcripts no viral CP could be detected. The transgenic potato plants however, were found to be not completely resistant to PLRV infection but accumulated significantly lower virus titers as compared to untransformed control plants. On the contrary the protection to PVY^N reported here appears to be absolute i.e. plants are either susceptible (and contain high amounts of virus) or resistant (protected plants being completely free of systemically spread virus). These dissimilar results may reflect different mechanisms involved in the protection to both virus groups. Previous reports show that the level of protection to PVY infection in different CP transgenic tobacco lines (Stark and Beachy, 1989) and potato plants (Lawson *et al.*, 1990) is not directly correlated with the level of CP accumulation. However from these studies it has remained unclear whether the CP encoding RNA or the protein itself is responsible for the virus resistance observed, since no control plants were tested that only produced viral CP sense transcripts.

In this study all transgenic tobacco lines challenged for virus protection showed clear production of CP specific transcripts, yet only four out of twenty lines tested showed significant levels of protection to PVY^N. Obviously there is no direct correlation between the amounts of CP RNA expressed and the level of resistance among different transgenic lines. A similar lack of correlation between expression level and protection level has also been reported for several proposed cases of "protein-mediated" virus resistances (TSWV: Gielen *et al.*, 1991; PVY: Lawson *et al.*, 1990; TEV/PVY: Stark and Beachy, 1989; CMV: P. Palukaitis, personal communication). In this context it is worthwhile to note that the expression pattern of the CaMV 35S promoter, which consists of several elements that control developmentally regulated and tissue-specific expression in plants, is thought to depend on its integration site in the chromosome (Benfey *et al.*, 1990a, 1990b). Transcriptional expression of integrated PVY^N CP genes may therefore differ in time and tissue among the various transgenic lines during their development, thus explaining the variable levels of resistance found. This implies that, irrespective of the level of transcription, each transgenic plant line should be tested for presence and level of virus resistance.

At least two possible mechanisms can be proposed for the involvement of the PVY^N CP transcripts in virus-protection. The positive sense RNA may hybridize to viral negative sense RNA replication intermediates, thereby blocking further virus replication. Alternatively, the transcripts may compete with viral RNA molecules for host or viral factors involved in RNA replication. Expression of partial CP or other PVY^N genomic sequences in transgenic plants might aid in identifying the protection mechanism(s) involved and show whether other regions than the CP encoding domain can be equally effective in conferring virus resistance.

The question why only trace amounts of CP are produced in transgenic plants, though optimal translational start signals have been provided, remains to be answered. Normally potyviral CPs are released by proteolytic processing from

polyproteins. This cleavage might be a protein structure related event as the involvement of specific subdomains in the carboxyl-terminal region of the NIa proteinase seem to indicate (Parks and Dougherty, 1991). Thus expression of the CP from a 'sub-genomic' mRNA might result in a CP lacking essential folding structures and thereby stability. Furthermore relatively inefficient extraction methods of PVY^N CP from transgenic tissue may also contribute to the undetectability of CP in spite of the high sensitivity of the detection methods used.

In contrast to the high levels of virus protection in transgenic tobacco plants described in this chapter no protection could be observed in the transgenic potato plants (see previous chapter) though basically the same transformation vectors were employed in both experiments. The protection is apparently not based on translational expression of the PVY^N CP (this chapter) and the absence of protection in the transgenic potato lines is therefore not likely to be based on the absence or low level of expression of the viral CP in these plants. The overall low number of protected tobacco lines (i.e. four out of 20 lines tested) and the low number of potato lines that could reliably be tested for virus resistance, implies that testing of more transgenic potato lines, under well defined climatological conditions, is likely to reveal significant levels of protection to PVY^N infection.

This chapter describes high levels of genetically stable resistance to a plant virus that appears to be principally RNA-mediated. The next chapter describes experiments to determine whether the obtained PVY^N resistance in transgenic plants is maintained during inoculation by aphids and to what level this resistance is capable of protecting plants against other related and unrelated potyviruses.

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Chapter 6

Tobacco plants transformed with the PVY^N coat protein gene are protected against different PVY isolates and aphid-mediated infection.

Summary

Tobacco plant lines transformed with the coat protein (CP) gene of the tobacco veinal necrosis strain of potato virus Y (PVY^N), and previously shown to be protected against mechanical inoculation with the virus, have now been tested for specificity and protection against virus infection mediated by viruliferous aphids. To determine the specificity of virus protection, two transgenic tobacco lines, A30 and A80, were challenged with several isolates of distinct PVY strains (PVY^N, PVY^O and PVY^C) by mechanical inoculation. Clear levels of protection against the PVY^O-isolates tested were maintained in the transgenic plants although these levels were slightly lower than the protection against the homologous PVY^N strain from which the CP gene was derived. Interestingly, no protection against mechanical virus inoculation with the 'Gladblaadje' isolate of PVY^C could be observed. To assess the levels of protection against aphid-mediated virus infection, two transgenic plant lines, A30 and D25, showing levels of protection of respectively 95 and 80% against mechanical virus inoculation, were challenged using PVY^N viruliferous *Myzus persicae*. Virus inoculation using six aphids per plant, resulted in similar levels of protection in both transgenic lines as found previously for mechanical inoculation. Protection was maintained in both lines even when as many as 60 viruliferous aphids were used per plant in the inoculation experiments.

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6.1 Introduction

The *Potyviridae* represent the largest and economically most important plant virus family with over 150 members (Barnett, 1991). Potyviruses are mechanically transmissible but in the field they are mainly transmitted by aphid vectors in a non-persistent manner. The type species, potato virus Y (PVY), forms a serious problem in potato crops worldwide and may cause considerable yield losses depending upon virus strain, potato cultivar and time of infection. Of the three known PVY strains (PVY^N, PVY^O and PVY^C), PVY^N ('the tobacco veinal necrosis strain') is the most problematic in West-European seed potato production. In most commercial cultivars primary PVY^N symptoms are rare and infected plants are therefore very hard to identify visually (Weidemann, 1988). Extensive screening for PVY^N is therefore required to guarantee virus-free potato stocks. Introduction of known R_y resistance genes in economically important potato cultivars by conventional breeding has so far proven to be extremely difficult or even impossible (Colon, 1987).

'Coat protein-mediated protection' whereby transformation of a susceptible plant with an expressionally active viral coat protein (CP) gene confers protection against the corresponding virus, has now been described for viruses from at least seven different plant virus groups (for reviews see Beachy *et al.*, 1990; Hemenway *et al.*, 1990 and Nejidat *et al.*, 1990). The achievement of this type of resistance against potyviruses would be of great economical importance. However, despite the numerous potyviral CP sequences that have been determined over the last years, often with the intention to achieve CP-mediated resistance, the number of successful reports describing this type of engineered resistance against potyviruses has been disappointingly low. Stark and Beachy (1989) reported reduced virus accumulation of PVY and tobacco etch virus (TEV) in tobacco plants transformed with the CP cistron of soybean mosaic virus (SMV), for which tobacco is in fact not a host. Both the CP's of PVY and TEV share approximately 60% homology to each other and with the CP of SMV. Lawson *et al.* (1990) described the protection to PVY, both against mechanically inoculated and aphid-transmitted virus, in 'Russett Burbank' potato plants transformed with the CP genes of PVY and the potato potexvirus X (PVX). Ling *et al.* (1991) transformed tobacco plants with the CP cistron of papaya ringspot virus (PRSV), a potyvirus, like SMV, also not infectious on tobacco. These transgenic plants, mechanically challenged with TEV, PVY and pepper mottle virus (PepMoV), a potyvirus closely related to PVY (Van der Vlugt *et al.*, 1989; Van der Vlugt, 1992), all showed a delay and attenuation in symptom development. Recently Lindbo and Dougherty (1992) described protection ranging from near wild type to immunity in tobacco plants transformed with several altered forms of the TEV CP sequence. In all these reports a less prominent correlation between the level of potyviral CP expression and the level of virus protection than reported for other virus groups seems to exist and moreover, in only a very small number of the transgenic plants

complete immunity to virus infection could be observed (Lawson *et al.*, 1990; Lindbo and Dougherty, 1992).

Recently we described the transformation of tobacco with the CP encoding region of PVY^N and the subsequent analysis of virus protection in the progeny lines of self-pollinated transgenic plants (Van der Vlugt *et al.*, 1992, see also Chapter 5). Despite high levels of viral CP specific mRNA in all transgenic plants obtained, CP production was undetectable. However levels of over 80% immunity to mechanical inoculation with PVY^N were observed in plant lines transformed with either a CP cistron supplemented with a translational ATG start signal (CP^{+ATG}) or a CP cistron devoid of such a signal (CP^{-ATG}). These results suggest that the resistance observed is predominantly based on the presence of the viral CP gene transcripts and not on the accumulation of the viral CP as such.

This chapter describes the further investigations of this form of "RNA-mediated protection". To test the specificity of the observed protection transgenic plant lines were mechanically inoculated with various isolates belonging to the three major strains of PVY. Furthermore it was investigated whether the resistance is also maintained when transgenic plant lines are challenged with viruliferous aphid vectors.

6.2 Materials and Methods

6.2.1 Viruses, aphids and plants

An tobacco veinal necrosis strain of potato virus Y (PVY^N) obtained from IPO-DLO Wageningen, a Wageningen isolate of PVY^O (PVY^O-Wag) and a pepper isolate of PVY^O (PVY^O-pep) were maintained in tobacco (*Nicotiana benthamiana* or *Nicotiana tabacum* var. SR1) by aphid-inoculation. PVY^C 'Gladblaadje' was obtained from plants grown from replanted potato tubers collected from plants systemically infected by an aphid-transmissible isolate of the virus. *Myzus persicae* biotype WMp2 (Reinink *et al.*, 1989) was cultured on *Brassica napus* L. subsp. *oleifera* (oilseed rape) in a greenhouse compartment at 20 ± 3°C. Cohorts of nymphs differing in age less than 24 hrs were produced by transferring daily mature apterae, confined to leaf cages, to fresh plants. Generation of PVY^N CP transgenic plants has been described previously (Van der Vlugt *et al.*, 1992). Transgenic tobacco plants were kept at 20-26°C with a 16 h light period under safe greenhouse conditions (PK II level), according to the legislation imposed by the Dutch authorities (Voorlopige Commissie Genetische Modificatie: VCOGEM)

6.2.2 ELISA

The presence of PVY^N in transgenic plants was monitored using either a polyclonal rabbit antiserum, raised against purified virus, in a cocktail double antibody sandwich (DAS) ELISA (Van den Heuvel and Peters, 1989) or a monoclonal antibody reacting with the core region of the potyviral CP, in a triple antibody sandwich (TAS) ELISA, done essentially as described by de Avila *et al.* (1990).

6.2.3 Analysis of virus protection in transgenic plants

S1 progeny plants of self-pollinated original transformants were mechanically inoculated approximately six weeks after sowing (four leaf stage). The largest leaf was dusted with carborundum powder and inoculated with 50 μl inoculum containing 500 ng of purified virus. For aphid inoculations, *Myzus persicae* L2 nymphs were placed on plants infected with aphid-transmissible isolates of virus strains and allowed an acquisition access period (AAP) of 20-30 minutes. Subsequently, nymphs were transferred to 3-4 weeks old tobacco seedlings for an inoculation access period (IAP) of 16 hours before being killed by an insecticide. All plants were monitored daily for the development of systemical symptoms. Approximately 7-8 days after inoculation of *N. tabacum* var SR1 with PVY^N, plants developed vein chlorosis followed by vein necrosis on systemically infected leafs. Plants inoculated with PVY^O of PVY^C developed only a very mild systemic mottling. All inoculated plants were checked for the presence or absence of virus 10-14 days post inoculation (dpi) by cocktail DAS- or TAS-ELISA.

6.3 Results

6.3.1 Protection of PVY^N CP gene transgenic plants against different PVY isolates

To investigate whether the resistance of tobacco plants, transcribing the PVY^N CP gene (Van der Vlugt *et al*, 1992), also extends to other PVY strains, plants from both a highly protected and a moderately protected transgenic line were challenged with isolates from three different PVY strains.

Two PVY^N CP^{+ATG} transgenic lines, denoted A30 and A80 and showing protection levels of respectively 95% and 20% upon mechanical inoculation with PVY^N (Van der Vlugt *et al.*, 1992), and a pGH2 transformed control line (from here on denoted SR1), were mechanically inoculated with 500 ng of purified virus of PVY^N, a Wageningen isolate of PVY^O (PVY^O-Wag), a pepper isolate of PVY^O (PVY^O-pep) and the 'Gladblaadje' isolate of PVY^C (PVY^C-Gb, Rozendaal *et al.*, 1971). Fourteen days after inoculation the presence or absence of virus in each plant was determined by DAS-

ELISA. Antisera reacting with the PVY CP region could be used in ELISA experiments since CP production in the transgenic plants was at undetectable low levels ($\leq$ 0.0001% of total soluble leaf protein, Van der Vlugt *et al.*, 1992) while virus infection was always readily detected within 30 minutes in the ELISA format used. Individual plants were scored as positively infected when ELISA readings were more than twice the average of healthy uninfected SR1 controls. Table 6.1 shows the number of virus-free plants 14 dpi from a total of 20 inoculated plants per line. Inoculation of lines A30 and A80 with PVY^N resulted in respectively 95% and 25% protected plants i.e. similar levels of protection as observed previously (Van der Vlugt *et al.*, 1992; Chapter 5). Inoculation with the two PVY^O isolates (PVY^O-Wag and PVY^O-pep) consistently resulted in lower levels of virus protection in both lines (i.e. up to 80% protection in line A30 and 5% protection in line A80, see Table 6.1 and Fig. 6.1A and 6.1B), indicating a slightly lower level of protection to PVY^O than to PVY^N. Most infected plants displayed normal virus titers and symptom development but interestingly a small number of plants in both transgenic lines displayed delayed and attenuated symptoms. These plants developed small numbers of necrotic spots on systemically infected leafs 3-4 weeks after inoculation with all PVY strains while development of virus titers in these plants was comparable to normally infected plants. Therefore these disease-less plants were scored as unprotected. Interestingly, mechanical inoculation of both transgenic lines with 500 ng of purified PVY^C-Gb, did not result in clear levels of protection, neither in the moderately protected line A80 nor the highly protected line A30 (Table 6.1). Also no protection could be observed against a unrelated virus (tomato spotted wilt virus) in any the transgenic plant lines or against any of the different PVY strains in the pGH2 transformed control line SR1. These results seem to indicate that the virus protection mechanism in PVY^N CP gene transgenic plants responsible for resistance against the homologous virus can also provide significant levels of protection towards isolates of strain PVY^O but not to the 'Gladblaadje'-isolate of PVY^C.

Table 6.1 Number of virus infected plants of PVY^N CP^{+ATG} transgenic lines A30 and A80 and transformation vector pGH2 transformed control line SR1, challenged with different PVY strains.

	Virus strain			
	No. of infected plants / No. of test plants			
Transgenic line	*PVY^N*	*PVY^O-Wag*	*PVY^O-pep*	*PVY^C-Gb*
SR1	20/20	20/20	20/20	18/20
A30	1/20	4/20	5/20	18/20
A80	15/20	19/20	20/20	19/20

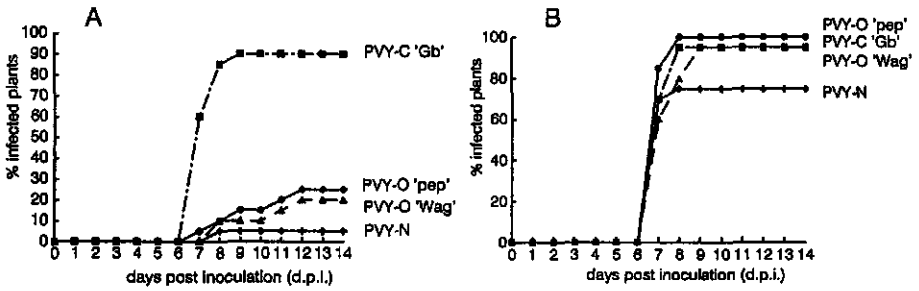

Fig. 6.1 Levels of virus protection in CP^{+ATG} tobacco lines A30 (panel A) and A80 (panel B) transformed with the CP gene of PVY^N. Twenty S1 progeny plants, obtained after self-pollination of original transformants, were mechanically inoculated with 500 ng of purified virus of the three PVY strains (PVY^N, PVY^O-Wag, PVY^O-pep and PVY^C-Gb) and monitored daily for the development of systemical symptoms. Presence or absence of virus was determined 14 dpi by DAS-ELISA.

6.3.2 Protection of transgenic plants to aphid-mediated inoculation

To determine whether the resistance of transgenic tobacco lines to PVY^N (Van der Vlugt *et al.*, 1992) also holds upon virus inoculation using aphids, plants from two highly protected transgenic lines were challenged with viruliferous *Myzus persicae*. Twenty five plants of transgenic lines A30 (CP^{+ATG}) showing 95% protection against mechanical inoculation with PVY^N, and D25, (CP^{-ATG}) showing 80% protection, were challenged with six viruliferous aphids per plant. Starting five dpi, plants were scored daily for the development of typical systemical PVY^N symptoms. Fourteen dpi presence or absence of virus in each plant was confirmed by DAS-ELISA. Individual plants were scored as infected when ELISA readings were at least twice the average of healthy uninfected SR1 controls. Both lines A30 (CP^{+ATG}) and D25 (CP^{-ATG}) showed high levels of protection (84% and 76% respectively) against aphid-transmitted PVY^N, while SR1 control plants showed no significant protection with 96% of the plants becoming infected (Fig. 6.2). The observed levels of protection against aphid-mediated PVY^N infection are in close agreement with the levels previously obtained in mechanical inoculation experiments for both lines A30 (95%) and D25 (80%) (Van der Vlugt *et al.*, 1992; Chapter 5).

Previously the high levels of protection against PVY^N in lines A30 (CP^{+ATG}) and D25 (CP^{-ATG}) have been shown to be maintained upon mechanical inoculation with as much as 10 μg purified virus per plant (data not shown). To test whether these levels of resistance would also hold under high viruliferous vector pressure, four groups of six

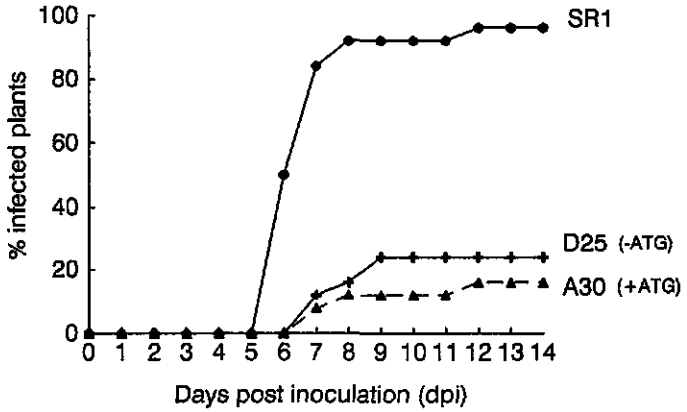

Fig. 6.2 Levels of virus protection in tobacco lines transformed with the PVY^N CP encoding sequence (A30 and D25) and empty transformation vector pGH2 (SR1). Twenty five S1 progeny plants were each challenged with six PVY^N viruliferous *Myzus persicae*. Plants were monitored daily for the development of systemical symptoms. Presence or absence of virus was determined 14 dpi by DAS-ELISA.

plants of each of the transgenic lines A30, D25 and SR1 were inoculated with different numbers of aphids. *M. persicae* L2 nymphs were allowed an AAP of 30 minutes on tobacco infected with PVY^N and subsequently numbers of 7, 15, 30 or 60 aphids were transferred to the plants in each group. After an IAP of 16 hours the aphids were killed and the plants were scored daily for the development of systemical symptoms. Fourteen dpi the absence or presence of PVY^N in each plant was confirmed by ELISA. Table 2 shows the numbers of infected plants of lines A30, D25 and SR1 for the increasing numbers of aphids. No apparent differences can be observed in the number of infected plants of each CP transgenic line with increasing numbers of aphids. Apart from confirming the high levels of protection against aphid-

Table 6.2 Number of infected plants of PVY^N CP transgenic A30 (CP^{+ATG}) and D25 (CP^{-ATG}) and pGH2 transformed control line SR1, challenged with PVY^N by inoculation with an increasing number of viruliferous *Myzus persicae*.

	No. of aphids			
	No. of infected plants / No. of test plants			
Transgenic line	*7*	*15*	*30*	*60*
SR1	6/6	6/6	6/6	6/6
A30	1/6	2/6	1/6	0/6
D25	2/6	3/6	2/6	1/6

transmitted PVY^N in both CP transgenic lines A30 and D25, this experiment shows that this resistance is still effective against as many as 60 viruliferous aphids per plant.

Preliminary results of aphid-mediated inoculation experiments in which plants from lines A30, D25 and the control line SR1, were inoculated with PVY^C-Gb demonstrate that both transgenic lines display no significant protection to this virus, in agreement with what was concluded from the mechanical inoculation experiments reported above.

6.4 Discussion

Transformation of tobacco plants with the CP encoding domain of PVY^N resulted in high levels of protection against mechanical inoculation with this potyvirus (Van der Vlugt *et al.*, 1992; Chapter 5). Inoculation of two CP^{+ATG} transgenic lines A30 and A80, which are highly (95%) and moderately (20%) protected against mechanical inoculation to PVY^N, with two PVY^O isolates (PVY^O-Wag and PVY^O-pep) revealed a significant though somewhat reduced protection when compared to inoculation with the homologous virus PVY^N (see table 6.1 and Fig 6.1A,B). This result agrees with the results reported for CP-mediated protection which was effective against closely related strains or viruses sharing a high level of CP sequence homology (Nelson *et al.*, 1987; 1988; Van Dun en Bol, 1988; Van Dun *et al.*, 1988; Nejidat and Beachy, 1990). All four PVY^O isolates for which the CP gene sequence has been determined so far (Bravo-Almonacid and Mentaberry, 1989; Lawson *et al.*, 1990; Ohshima *et al.*, 1991; J.E. Pot, unpublished results) show a high degree of CP nucleotide sequence homology to PVY^N (88.4% - 89.0%). Interestingly both transgenic lines A30 and A80 showed no protection against the 'Gladblaadje' isolate of PVY^C. Unfortunately, no sequence data on PVY^C-isolates, needed to establish their taxonomic relation to PVY^N, are currently available. It would be especially worthwhile to determine the CP sequence of the PVY^C-Gb isolate, certainly since this virus has now been shown to break the engineered resistance based on the CP gene sequence of PVY^N, but also in view of earlier reports (Fribourg and Nakashima, 1984) in which, on the basis of biological properties, the taxonomic position of the Gladblaadje isolate within the PVY^C strain has been questioned. Failure of hybridization of purified 'Gladblaadje' RNA with a PVY-specific cDNA probe from the 3'-nontranslated region (3'-NTR) of PVY^N and the absence of a cross-reaction with a PVY^N polyclonal antiserum in ELISA (results not shown) seem to support the proposition (Fribourg and Nakashima, 1984), that 'Gladblaadje' represents a potyvirus distinct from PVY.

The inoculation experiments with PVY^N viruliferous *M. persicae* show that the RNA-mediated protection in CP transgenic tobacco extends to virus transmitted by its natural aphid vector. Furthermore they confirm the effectiveness of resistance in

the transgenic plants under high inoculum pressure as was previously observed in mechanical inoculation experiments (data not shown). Despite the considerable number of reports on CP-mediated protection against viruses from different families, reports on engineered resistance against viruses when inoculated by their natural vectors have been very limited. Lawson *et al.* (1990) described protection in only one PVY/PVX CP transgenic potato line (line 303), also completely immune against mechanical inoculation, while two additional lines that demonstrated high levels of protection to mechanical PVY infection were found to be not protected against aphid-mediated infection. Quemada *et al.* (1991) inoculated plants from one protected CMV-C CP transgenic tobacco line with three CMV strains by aphid inoculation. Although plants were claimed to be protected against all three strains the poor transmissibility of the strains tested do not allow a direct comparison to mechanical inoculations.

The "RNA-mediated" resistance studied here is primarily based on the presence of the CP sense RNA rather than on the CP itself. For practical purposes this novel mechanism of virus protection might be preferred over CP-mediated resistance since no foreign protein accumulates in the plant (except of course for the selectable marker protein). Furthermore there is no risk of transcapsidation with other viral RNAs.

Our results demonstrate that tobacco plants transformed with the PVY^N CP encoding sequence can acquire high levels of resistance against PVY infections irrespective whether the virus is provided mechanically or by viruliferous aphid vectors. Analysis of S2 progeny lines obtained from self-pollinated protected S1 plants from line A80 revealed that the level of resistance can be raised to 100% within one generation (Van der Vlugt *et al.*, 1992; Chapter 5). Also our results show that the protection obtained is operational against isolates of the two economically most important strains of PVY, i.e. PVY^N and PVY^O. Therefore introduction of PVY CP specific sequences may be used to achieve a highly efficient resistance against this important pathogen under natural field conditions.

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Chapter 7

Complex formation determines the activity of ribozymes directed against potato virus Y^N genomic RNA sequences

Summary

A ribozyme was synthesized against a conserved region in the RNA-dependent RNA-polymerase encoding cistron of the important plant pathogen potato virus Y (PVY). This ribozyme was shown to cleave PVY specific RNA transcripts efficiently *in vitro*, with up to 95% of the substrate RNA being cleaved within 2 hrs incubation at 37°C. A second ribozyme, designed with much shorter viral complementary arms in an attempt to optimize the efficiency of the cleavage reaction, surprisingly failed to cleave the substrates previously cleaved by the longer ribozyme. A much shorter PVY specific RNA transcript of only 37 nucleotides however, was cleaved by this short ribozyme proving its ribozymic activity and indicating that the cleavage activity of the ribozyme is, in part, determined by the substrate involved. Analysis of cleavage reactions on non-denaturing polyacrylamide (PA)-gels indicated that incorrect basepairing, interferring with correct formation of the hammerhead structure, was likely to be responsible for the absence of detectable cleavage of the larger substrates by the short ribozyme.

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7.1 Introduction

Since the discovery that RNA molecules can act as enzymes and catalyze (self)-cleavage reactions in the absence of proteins (Cech *et al.*, 1981), several types of these RNA enzymes or "ribozymes" have been described, each differing in primary, and proposed secondary structures, and progress of the cleavage reaction. Group I and II introns and the RNA component of RNase P contain large catalytic structures of several hundreds of nucleotides involved in the cleavage reaction (Cech *et al.*, 1981; Van der Veen *et al.*, 1986; Guerrier-Takada *et al.*, 1983). In contrast to these large domains, much smaller catalytic regions have been identified in a satellite RNA transcript of the newt (*Notophthalmus viridescens*; Epstein and Gall, 1987) and several plant viroids and satellite RNAs (Prody *et al.*, 1986; Hutchins *et al.*, 1986; Buzayan *et al.*,1986; Forster and Symons, 1987). Typical of these smaller catalytic regions is a secondary structure composed of two single-stranded regions, containing 13 highly conserved nucleotides, and three non-conserved stem-loop structures. Because of its typical basepairing, this secondary structure motif was originally named 'hammerhead' (Forster and Symons, 1987). This motif is essential in the rolling circle replication mechanism of some plant viroids, virusoids and satellite RNAs where it catalyzes cis-cleavage at specific sites of the multimeric replication intermediates to generate monomers of the pathogenic RNA (Symons, 1989). Cleavage catalyzed by the hammerhead structure requires divalent cations, usually Mg^{2+}, and occurs directly at the 3'-side of the trinucleotide GUC. *In vitro* muta-genesis of the GUC target site has shown that cleavage can also occur at a NUX trinucleotide, though usually at markedly slower rates. In these new target sites N can be any nucleotide and X preferably is a cytidine but can not be a guanine (Haseloff and Gerlach, 1988; Koizumi *et al.*, 1989; Sheldon and Symons, 1989; Ruffner *et al.*, 1990), while recently it was shown that even this general NUX ruling may not hold since an A or C in the central position also allowed cleavage provided certain basepairings in the first position were present (Perriman *et al.*, 1992). In the presence of Mg^{2+} the hammerhead is thought to form a complex of, as yet, unknown tertiary structure in which the 2'-OH group on the ribose of the C-residue is enabled to attack the 3' adjacent phosphodiester bond which is then cleaved, forming free 5'-hydroxyl and cyclic 2'3'-cyclic phosphate termini (Buzayan *et al.*, 1986; Prody *et al.*, 1986).

While naturally occurring hammerhead structures, containing both the substrate and the enzyme as part of a single RNA molecule, normally cleave in an intramolecular reaction, *in vitro* catalytic intermolecular (trans) cleavage was shown by separation of the enzyme and substrate domains of the hammerhead structure on two RNA molecules (Uhlenbeck, 1987; Haseloff and Gerlach, 1988). This separation of the catalytic domain from the substrate domain has allowed for the design and production of ribozymes possessing high endonuclease activities against specific

target RNA molecules (Koizumi *et al.*, 1988a, 1988b; Cotten and Birnstiel, 1989; Lamb and Hay, 1990; Goodchild and Kohli, 1991).

The catalytic nature of ribozymes and the possibility to target them to unique RNA sequences seem to make them ideal for the inhibition of expression of specific genes. Indeed ribozymes have been utilized in the inactivation of a growing number of cellular and viral RNA molecules both *in vitro* and *in vivo* (Scanlon *et al.*, 1991; Steinecke *et al.*, 1992; Xing and Whitton, 1992; Dropulic *et al.*,1992 and references therein). Their potential as antiviral agent *in vivo* was shown by the reduction in expression of HIV-1 specific RNAs and the reduction of virus replication in cells stably expressing anti-HIV ribozymes (Sarver *et al.*, 1990; Weerasinghe *et al.*, 1991; Dropulic *et al.*, 1992; Chen *et al.*, 1992). Effective therapeutic use of these ribozymes however will ultimately rely on their delivery into mammalian target cells either by the administration of synthetic RNA ribozymes or the introduction of ribozyme-encoding genes into cells by gene-therapy.

In contrast to these problems associated with animal virus-specific ribozymes, the intercellular delivery of ribozymes directed against plant RNA viruses appears to pose no major problems due to the development of efficient transformation procedures. Ribozyme technology therefore appears to offer an attractive expansion to the antisense strategies employed against plant viruses. Sofar however, only two examples of ribozymes directed against plant viral RNAs have been described. Lamb and Hay (1990) demonstrated specific cleavage of potato leafroll luteovirus (PLRV) RNA *in vitro* by ribozymes designed against regions encoding the viral coat protein (CP) and RNA polymerase. Ribozymes directed against tobacco mosaic virus (TMV) RNA were also reported to cleave the viral RNA *in vitro* and in addition appeared to inhibit virus replication both in protoplasts and transgenic plants (Edington and Nelson, 1992).

Here we report the design and synthesis of ribozymes directed against a highly conserved region in the RNA genome of potato virus Y, the type species of the *Potyviridae* family. Analysis of *in vitro* cleavage reactions revealed the efficient cleavage of virus specific RNA transcripts at the predicted site. A ribozyme with much shorter complementary arms, designed to enhance cleavage efficiency, however failed to cleave the substrates efficiently cleaved by a ribozyme with longer antisense arms. Cleavage studies using a short PVY specific RNA transcript revealed that lack of cleavage is most likely caused by incorrect hammerhead complex formation between the longer substrates and the short arms of the ribozyme.

7.2 Materials and Methods

7.2.1 Construction of ribozyme plasmids

A 103 bp *Bgl*II/*Hin*dIII fragment from pVY8, a cDNA clone comprising the 1611 3'-terminal nucleotides (nt) of the tobacco veinal necrosis strain of potato virus Y (PVY^N, Van der Vlugt *et al.*, 1989) was cloned into a *Bam*HI/*Hin*dIII digested M13mp18 vector (Norrander *et al.*, 1983). Through site-directed mutagenesis (Kunkel, 1985) a GA-dinucleotide was inserted at position 65/66 of the *Bgl*II/*Hin*dIII fragment to create a unique *Sal*I-site. After *Sal*I-digestion and subsequent removal of the 5'-protruding ends by Mungbean Exonuclease digestion, a 23 nt linker sequence, composed of two complementary oligonucleotides comprising the 23 nt hammerhead sequence of the satellite of Tobacco Ringspot Virus (sTobRV, Haseloff and Gerlach, 1988), was inserted in both orientations. The resulting 124 nt *Xho*II/*Hin*dIII fragment were cloned into a *Hin*dIII/*Bam*HI Bluescript SK$^+$ vector (Stratagene) resulting in plasmids pRIBO184 and pOBIR184 (Fig. 7.1). Nucleotide sequences and hammerhead orientations were confirmed by sequence analysis (Sanger *et al*, 1977). *In vitro* transcription, using T_7 RNA polymerase, of both *Xba*I linearized plasmids pRIBO184 and pOBIR184 resulted in transcripts R_{184} and O_{184} of 184 nt, with respec-tively 47 nt and 8 nt vector-derived sequences at their 5'- and 3'-ends, and 63 and 43 nts PVY-complementary sequences on the 5'- and 3'-side of the hammerhead insert.

Short ribozyme plasmid pRIBO59 was constructed using two partially complementary oligonucleotides (A: 5'-CGGAGCTCGCCTCTGTGGCTGATGAGTCCGTGAGG-3', and B: 5'-GCACTAGTGGTTCATGTTTCGTCCTCACGGACT-3'). Both oligo's were annealed at their 3' 11 nt overlap and the protruding ends were filled in using Klenow polymerase. The resulting 57 bp fragment was digested at both a 5' unique *Sst*I-site and a unique 3' *Spe*I-site. The 43 bp fragment thus obtained was subsequently cloned in a *Sst*I/*Spe*I digested KS$^+$ Bluescript vector resulting in plasmid pRIBO59. *In vitro* transcription, using T_7 RNA polymerase, of pRIBO59 linearized with *Spe*I, resulted in transcript R_{59} of 59 nt, composed of 17 nt vector-derived sequence followed by the 23 nt hammerhead sequence flanked by respectively 10 and 9 nt PVY-complementary sequences on the 5'- and 3'-side.

7.2.2 Construction of substrate plasmids

Substrate plasmid pS202/S321 (Fig. 7.1) was constructed by subcloning the 268 bp *Bam*HI/*Hpa*I fragment from pVY8 (Van der Vlugt *et al.*, 1989) in a *Bam*HI/*Hin*cII digested Bluescript KS$^+$ vector. Upon linearization with *Hin*dIII, transcription using T_7 RNA polymerase resulted in transcript S_{202} of 202 nt in length with at its 5'-end 54 nt of vector-derived sequence followed by 148 nt of PVY RNA sequence. Linearization with *Xho*I, followed by T_7 RNA transcription, generated transcript S_{321} of 321

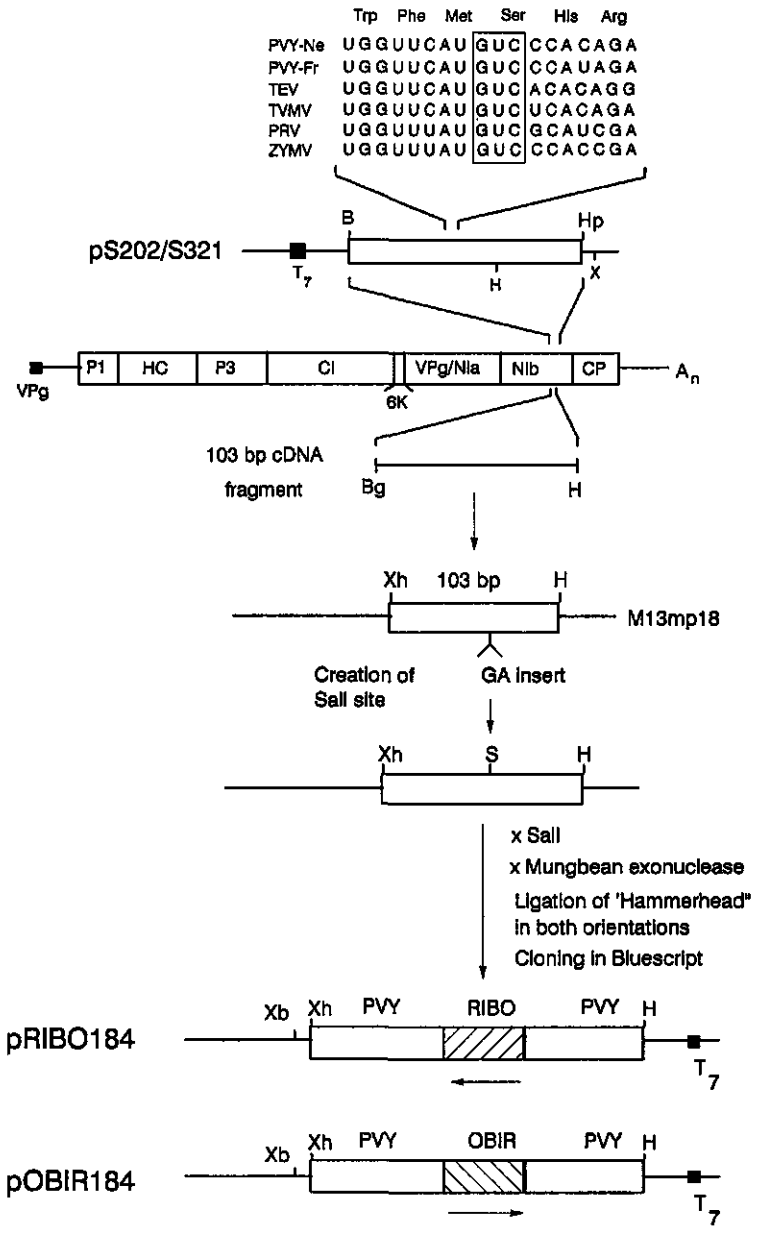


Fig. 7.1 Cloning of substrate plasmid pS202/S321 (top) and ribozyme plasmids pRIBO184 and pOBIR184 (bottom). The relative positions of the cloned fragments from the RNA-dependent RNA-polymerase (NIb) cistron are indicated on the PVY^N RNA genome. The nucleotide- and amino acid-sequences surrounding the conserved GUC ribozyme cleavage site (boxed) in the NIb cistron of several potyviral RNA genomes is shown above plasmid pS202/S321. B = *Bam*HI, Bg = *Bgl*II, E = *Eco*RI, H = *Hin*dIII, Hp = *Hpa*I, S = *Sal*I, Xb = *Xba*I, Xh = *Xho*I, T_7 = T_7-promotor. Sources of viral sequences are: PVY-Ne: Van der Vlugt *et al.*, 1989; PVY-Fr: Robaglia *et al.*, 1989; tobacco etch virus (TEV): Allison *et al.*, 1985; tobacco vein mottling virus (TVMV): Domier *et al.*, 1986; papaya ringspot virus (PRV): Quemada *et al.*, 1990; zucchini yellow mosaic virus (ZYMV): Grumet and Fang, 1990.

nt with at its 5'-end 54 nt of vector-derived sequence followed by 263 nt of PVY RNA sequence and 4 nt vector-derived sequence. Both T_7 RNA-transcripts S_{202} and S_{321} contain the same GUC ribozyme cleavage site, located in the viral genome at position 8122-8124 (Robaglia et al., 1989). This triplet is part of a highly conserved amino acid motif (i.e. W-F-M-S-H-R) in the RNA-dependent RNA-polymerase (NIb-protein) of different potyviruses (Fig. 1) in which the G-residue is the last nucleotide of a methionine encoding triplet and the UC residues are the first two of a serine encoding triplet (UCN) which makes any conserved base substitutions in the GUC triplet unlikely.

The second ribozyme substrate plasmid pS37 was constructed by annealing two complementary oligonucleotides (C: 5'-CGTGGTTCATGTCCCACAGAGG-3' and D: 5'-GATCCTCTGTGGGACATGAACCACGAGCT-3'). Cloning of the resulting double stranded fragment, with a 5' *Sst*I compatible overhang and a 3' *Bam*HI compatible overhang, in a *Sst*I/*Bam*HI digested Bluescript KS$^-$ vector yielded plasmid pS37. Linearization with *Bam*HI followed by T_7 RNA transcription resulted in the 37 nt transcript S_{37} composed of 15 nt vector derived sequence followed by 22 nt PVY specific RNA.

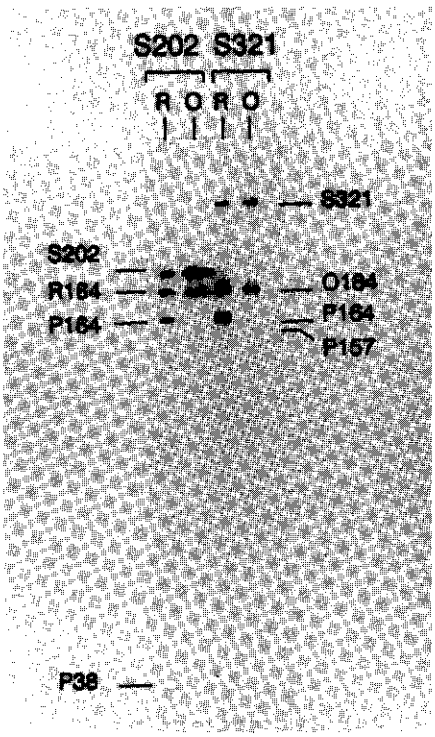


Fig. 7.2 Cleavage of substrate RNAs S_{202} and S_{321} by ribozyme transcript R_{184} (lanes denoted R) and reverse hammerhead transcript O_{184} (lanes denoted O). Positions of transcripts and cleavage products are indicated.

7.2.3 *In vitro RNA transcription reactions*

Ten μg of plasmid DNA was linearized with the appropriate restriction enzyme and treated with 20 μg proteinase K for 30 min at 37°C. After protein extraction with phenol and phenol/chloroform (1:1) the DNA was ethanol precipitated and dissolved in H_2O to a concentration of 1 μg/μl.

Ribozyme and substrate RNAs were transcribed using 1 μg of linearized template DNA in a reaction containing 40 mM Tris pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 40 μM NTPs (A, C, and G), 4 μM UTP, 15 μCi α-^{32}P-UTP, 1U/μl T_7 RNA polymerase (Promega) and 2U/μl RNAse inhibitor (Boehringer), for 1.5 hours at 37°C. Non-radioactive transcripts were synthesized by substituting the 4 μM UTP and α-^{32}P-UTP for 40 μM UTP. Transcription mixtures were treated with 1U RNAse-free DNAse for 30 min at 37°C to remove DNA templates.

7.2.4 *Analysis of ribozyme cleavage reactions*

A typical ribozyme reaction was carried out by mixing 3 μl volumes of total ribozyme and substrate transcription reaction mixtures with 3 μl of 30 mM $MgCl_2$ followed by incubation at the appropriate temperature. Two μl samples were taken at desired time intervals and 1 μl of 0.1 M EDTA pH 8.0 was added to terminate the cleavage reaction. Reaction samples were mixed with 3 μl of sequencing stopbuffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), denatured for 3 min at 95°C, snap-cooled on ice and analyzed by electrophoresis on a 8% polyacrylamide (PA)-gel in 1X TBE containing 8 M urea. Upon drying gels were subjected to autoradiography. For analysis of ribozyme cleavage reactions under non-denaturing conditions, samples were mixed with 3 μl of DNA-loading buffer (30% glycerol, 0.05% bromophenol blue, 0.05% xylene cyanol) and loaded on 12.5% non-denaturing PA-gels in 1x TBE. Gels were run at 5 W constant current to avoid heat-denaturation, vacuum dried and subjected to autoradiography. Initial amounts of ribozymes and substrates and the resulting products were quantified from autoradiographs using a laser densitometer (LKB Ultroscan XL) and the LKB 2400 GelScan XL software package.

7.3 Results

7.3.1 *Ribozyme cleavage of PVY specific substrate RNAs*

Initial analysis of α-^{32}P-UTP labelled transcripts on a 8% denaturing PA-gel showed the intactness and expected sizes of all transcripts. Equal amounts of α-^{32}P-UTP labelled transcription reaction mixtures of both substrates S_{202} and S_{321}, ribozymes

R_{184} and O_{184} and 30 mM $MgCl_2$ were mixed and incubated for 1 hr at 37°C. PA-gel electrophoresis (PAGE) of cleavage reactions (Fig. 7.2) showed that R_{184} cuts both substrates S_{202} and S_{321} into the expected product fragments of 164 and 38 nt (P_{164} and P_{38}) and 164 and 157 nt (P_{164} and P_{157}) respectively. Reverse hammerhead transcript O_{184} showed no cleavage activity on either of the substrates indicating that indeed the hammerhead sequence, inserted in the reverse orientation, lacks detectable ribozyme activity. PAGE of ribozyme reactions in which either the ribozyme or the substrate transcripts were α-^{32}P-UTP labelled showed that the appearance of cleavage products is ribozyme (R_{184}) mediated and not caused by non-specific breakdown of any of the transcript RNAs (Fig. 7.3).

To determine the efficiency of the ribozyme-mediated cleavage, a time course experiment was performed in which both PVY specific transcripts S_{202} or S_{321} were incubated with equal volumes of either R_{184} or O_{184} at 37°C. Reaction samples were taken at 0, 5, 15, 60 and 120 min after addition of the $MgCl_2$, quenched by the addition of 0.1 M EDTA and analyzed by denaturing PAGE (see Fig. 7.4A). Quantification of substrate RNAs and cleavage products in time samples on the resulting autoradiographs using a LKB densitometer confirmed the efficient cleavage

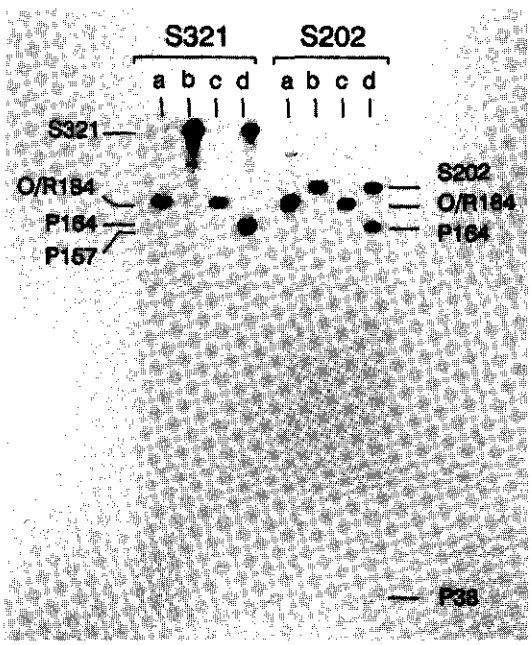
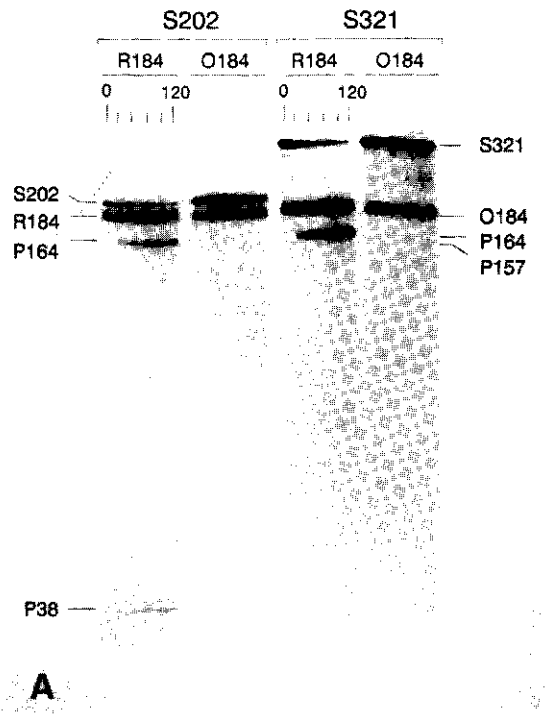


Fig. 7.3 Cleavage of substrate RNAs S_{321} and S_{202} by reverse hammerhead transcript O_{184} and ribozyme transcript R_{184}. Cleavage reactions were preformed with unlabelled substrate RNAs and ^{32}P-UTP labelled O_{184} (lanes a) or R_{184} (lanes c) and with ^{32}P-UTP labelled substrates and unlabelled O_{184} (lanes b) or unlabelled R_{184} (lanes d). Positions of transcripts and cleavage products are indicated.



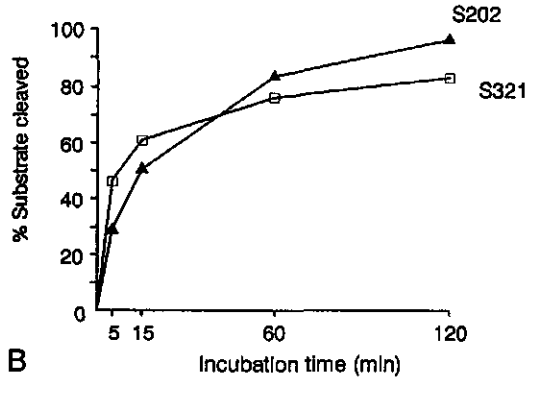


Fig. 7.4 **A:** time course experiment of cleavage of PVY^N specific substrate RNAs S_{202} and S_{321} by ribozyme R_{184} and reverse hammerhead transcript O_{184}. Reaction samples taken before (t=0) and 5, 15, 60 and 120 min. after the addition of the $MgCl_2$, were analyzed on a 8% PA gel under denaturing conditions.

Fig. 7.4 **B:** efficiency of cleavage of PVY^N specific substrate RNA transcripts S_{202} and S_{321} by ribozyme R_{184}.

of both substrate RNAs by R_{184}. After 5 min incubation 30% of the S_{202} transcript had been converted to the cleavage products while as much as 47% of the S_{321} transcripts had been cleaved (Fig. 7.4B). The cleavage levels were raised to 96% for S_{202} and 83% for S_{321} after 120 min incubation. This analysis again confirmed the absence of any detectable cleavage activity by the O_{184} transcript. These results show that a ribozyme construct with rather long antisense arms each with a length of approximately 50-55 nt, recognizing PVY specific RNA transcripts, can cleave more then 95% of the substrate in two hours at 37°C.

In an attempt to further improve the efficiency of the cleavage reaction a plasmid yielding a ribozyme transcript with much shorter antisense arms was constructed. The rational behind this was to obtain a ribozyme which was able to detach from substrate sequences and thus would be really catalytic in cleavage activity. Transcription of this plasmid pRIBO59 upon linearization with *Spe*I would result in a short ribozyme (R_{59}) of 59 nt. Cleavage reactions performed under the same conditions used previously for the large ribozyme (i.e. 120 min at 37°C), however failed to show specific cleavage of either the S_{202} or S_{321} substrate RNAs. Prolonged cleavage reactions with up to 8-fold excess of R_{59} over the substrate RNAs or at reactions temperatures up to 55°C also failed to detect specific cleavage products (results not shown).

From these results it was unclear whether the failure of the short ribozyme R_{59} to cleave substrates S_{202} and S_{321} was due to the absence of detectable ribozyme activity or to other factors like e.g. improper complex formation. Therefore the ribozymic activity of R_{59} was tested on a third, much smaller RNA substrate, S_{37}, deficient in secondary structure formation. Ribozyme cleavage of this small substrate RNA of only 37 nt should result in products of 29 and 8 nt (P_{29} and P_8), the latter product containing no U-residues and therefore not visible in the gel analysis. Both α-^{32}P-UTP labelled S_{37} and R_{59} were incubated in a typical cleavage reaction for 120 min at 37°C which was analyzed by denaturing PAGE (Fig. 7.5). The presence of the 29 nt cleavage reaction product clearly showed that the short ribozyme R_{59} has ribozymic activity and is capable to cleave the short substrate S_{37}. In contrast, again no cleavage of the longer substrate S_{202} could be observed. This seemed to indicate that the specific cleavage activity of the short ribozyme can be determined by the length of the substrate involved. Computer analyses of possible RNA secondary structures of ribozyme-substrate complexes using the FOLD program of the University of Wisconsin GCG package (Devereux *et al.*, 1984) indeed gave indications that within the longer substrate molecules, alternative secondary structures might prevent the short ribozyme R_{59} from correct basepairing i.e. positioning the hammerhead sequence opposite the GUC target sequence (see Fig 7.6A). In contrast, basepairing between the longer substrates and the long ribozyme molecules can result, by means of their long antisense arms, in very stable, correctly positioned structures (Fig. 7.6B) that appear to be energetically much more favourable (-218 KJ) than the internal

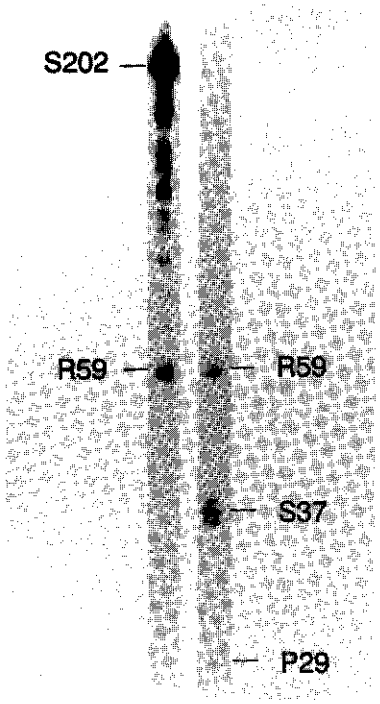


Fig. 7.5 Analysis of cleavage of substrate RNAs S_{202} and S_{37} by the short ribozyme transcript R_{59}. Position of transcripts and of cleavage product P_{29} are indicated.

structures of the separate molecules, i.e. -39 KJ for R_{184} and -50 KJ for S_{202}. Decreasing the length of the antisense arms of the PVY specific ribozymes therefore did not seem to increase the catalytic efficiency of the cleavage reaction but rather decreased cleavage by hampering correct complex formation between the ribozyme and its substrate.

To test the hypothesis that cleavage of longer PVY specific substrates by R_{59} is hampered by incorrect complex formation, time course samples taken from typical cleavage reactions of both S_{202} and S_{37} with the short ribozyme R_{59} were also analyzed on non-denaturing 12.5% PA-gels. Fig. 7.7A shows that already after 5 min most of the S_{202} substrate was converted into a larger complex that hardly migrated into the gel while in the sample taken before the addition of R_{59} ($t=0$), S_{202} is clearly visible. Since the analysis of reaction products under denaturing conditions had previously shown the absence of any detectable cleavage of S_{202} by R_{59} (Fig. 7.5), this result indicates that the secondary structure complex formed between S_{202} and R_{59} is most likely incorrect with respect to the position and formation of the hammerhead structure. The R_{59} band still present after incubation is thought to represent an excess fraction not involved in complex formation. In contrast, no stable complex can be observed between the S_{37} substrate and R_{59}, while already 30 min

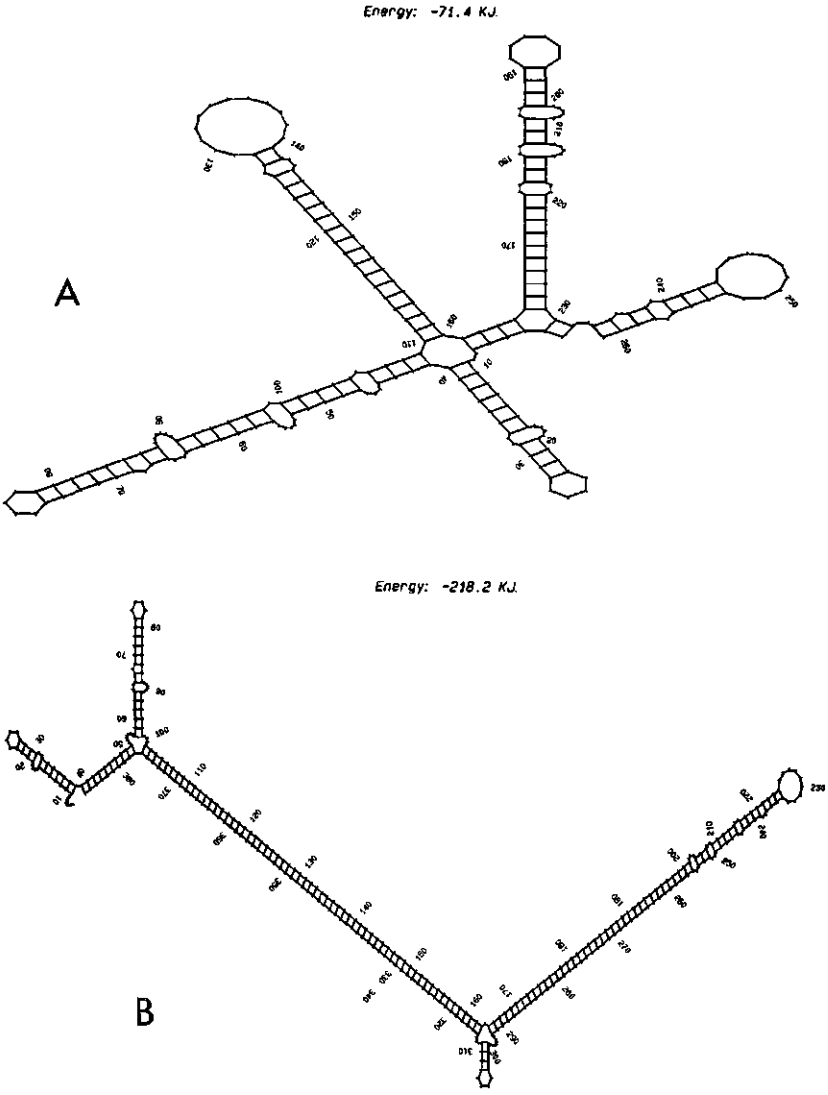


Fig. 7.6 Possible secondary structures of complexes between substrate RNA S_{202} and short ribozyme R_{59} (A) and S_{202} and ribozyme R_{184} (B). The GUC target sequence is found in both structures at pos. 162-164. Hammerhead sequences run from pos. 229-251 (A) and pos. 292-314 (B).

after the start of the reaction the specific cleavage reaction product P_{29} is clearly present with its amount increasing in time (Fig. 7.7B). This would fit with formation of a correct hammerhead structure opposite the GUC target site in S_{37} followed by cleavage and product dissociation. In this case R_{59} would have real catalytic activity, i.e. the potential to cleave more than one substrate molecule. These results show that the limited cleavage of the longer PVY specific substrates is caused by incorrect

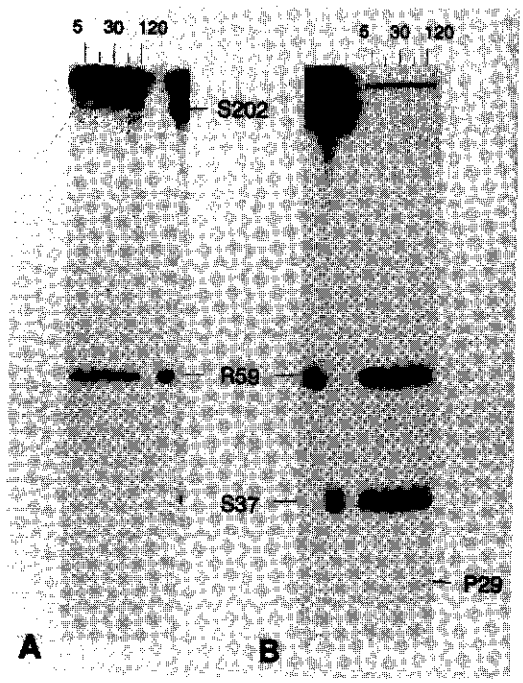


Fig. 7.7 Cleavage by short ribozyme R_{59} of substrates S_{202} (panel A) and S_{37} (panel B). Reaction samples were taken 5, 15, 30, 60 and 120 min after the addition of $MgCl_2$ and analyzed on a 12.5% non-denaturing PA gel. Individual transcripts were included on the gel as t=0 samples. Positions of transcripts and of cleavage product P_{29} are indicated.

basepairing between the substrate and the short ribozyme R_{59} preventing the formation of the hammerhead structure. It can thus be concluded that both the lengths of the complementary ("antisense") arms of the ribozymes and the lengths, and internal folding, of the substrate RNAs determine the occurrence and efficiency of the ribozyme cleavage reaction.

7.4 Discussion

Ribozymes have the potential to inhibit the expression of specific genes by cleavage of the corresponding mRNA and they offer a powerful tool in the functional studies of RNA molecules. This has prompted the design and production of ribozymes directed against a growing number of specific cellular and viral RNAs. In this paper we report the synthesis of a ribozyme (R_{184}) capable of efficient *in vitro* cleavage of genomic sequences of the important plant pathogen potato virus Y (PVY^N). Incubation at 37°C resulted in up to 45% cleavage of the substrate within 5 min after the start

of the reaction. This level of cleavage was even raised to 95% after 2 hours of incubation. In comparison with most reports, ribozyme R_{184} has relatively long (43 and 63 nt) complementary arms. Increasing complementary arm length will increase the difference in free energy between the complex and the unpaired substrate and ribozyme RNAs and may therefore enhance the rate of complex formation (Haseloff and Gerlach, 1988), however, longer stems are also likely to decrease product dissociation by increasing complex stability. This was illustrated by the analysis of time samples taken from a reaction between S_{202} and R_{184} on a non-denaturing PA-gel, clearly showing the formation of a complex between S_{202} and R_{184}, while no dissociated products could be detected (results not shown). Dissociation of the cleavage products from the complex appears to be an important rate-limiting factor in the ribozyme reaction (Goodchild and Kohli, 1991) and will obviously affect the turnover number of the ribozyme. Therefore, a second ribozyme was synthesized (R_{59}) with much shorter viral complementary arms (9 and 10 nt) in an attempt to further improve the efficient cleavage of the substrates. Surprisingly however, no cleavage products could be detected. This small ribozyme therefore either lacked cleavage activity or cleaved substrates S_{202} and S_{321} far less efficient than the longer ribozyme. The ribozymic ability of the short ribozyme R_{59} was demonstrated by cleavage of a much shorter PVY specific RNA transcript (S_{37}) of only 37 nt (Fig. 7.5) and this result provided an indication that the cleavage activity of this ribozyme is mainly determined by the secondary structure of the substrate involved. Analysis of cleavage reactions of the long (S_{202}) substrate RNA by the short ribozyme R_{59} on non-denaturing PA-gels showed the formation of a stable complex (Fig. 7.7A). This indicates that alternative, incorrect, basepairing between S_{202} and R_{59}, preventing the formation of the hammerhead structure, has been responsible for the absence of detectable cleavage. Incorrect basepairing has previously been reported to influence the cleavage activity of ribozymes (Forster and Symons, 1987; Epstein and Gall, 1987; Heus *et al.*, 1990) and computer assisted predictions of possible secondary structures between S_{202} and R_{59}, using the MFOLD program (Zuker, 1989), indeed indicated several alternative structures in none of which the correct hammerhead structure was formed (results not shown).

Unknown internal secondary structures in target RNAs will also influence the accessibility of the ribozyme cleavage site (Fedor and Uhlenbeck, 1990). Our results suggest that ribozymes with short (up to 20 nt) complementary arms will be relatively strongly influenced by these structures since the formation of the hammerhead is not likely to be energetically favoured over the pre-existing secondary structures in both the substrate and the ribozyme. Ribozymes with much longer antisense arms are more likely to form the correctly positioned hammerhead structures since the long perfect basepairing will decrease the free energy of the complex considerably compared to the energy values for the unpaired substrate and ribozyme RNAs.

For *in vivo* applications of ribozymes on larger substrate RNAs, like cellular mRNAs and full-length viral RNAs, that are likely to possess extensive secondary structures, the possible catalytic but low cleavage activity of short ribozymes will have to be compared with the highly efficient though non-catalytic cleavage of longer ribozymes. As antiviral agents ribozymes with long antisense arms could prove to be an efficient expansion of the antisense technology already successfully employed (Argawal, 1992; Hemenway *et al.*, 1990). In view of the recently reported sense RNA-mediated protection against three important plant viruses (De Haan *et al.*, 1992; Lindbo and Dougherty, 1992; Van der Vlugt *et al.*, 1992; Chapters 5 and 6), aiming of (multiple) ribozymes (Chen *et al.*, 1992) with long antisense arms against highly conserved and accessible regions in the less abundant viral-complementary of plant viral genomes could prove an efficient strategy.

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Chapter 8

Summary and concluding remarks

Potato virus Y is the type species of the potyvirus genus, the largest genus of the plant virus family *Potyviridae*. The virus causes serious problems in the cultivation of several Solanaceous crops and although certain poly- and monogenic resistances are available, these can not always be employed, e.g. R_y genes in potato cv. 'Bintje'. The aim of the research described in this thesis was to establish new forms of resistance against PVY by genetic modification of host plants. One such form of genetic engineered resistance is 'coat protein-mediated resistance', whereby expression of a viral coat protein (CP) in a transgenic plant may confer resistance against infection with the homologous virus, and some closely related viruses.

At the start of this investigation no sequence data on the RNA genome of PVY were available, therefore cDNA synthesis and subsequent sequence determination was performed to obtain the necessary PVY CP gene sequence as well as additional sequences from the 3'-terminal region of the viral genome (Chapter 2 and Van der Vlugt *et al.*, 1989). This enabled the determination of the exact taxonomic position of the PVY^N ('tobacco veinal necrosis strain') isolate used in these experiments, among other PVY isolates from at least two different strains. Detailed comparisons of the PVY^N CP and 3'-non translated (3'-NTR) sequences with those from a large number of geographically distinct PVY isolates that became available during the course of this investigation, showed that these sequences, in addition to distinguish between different potyvirus species (Ward and Shukla, 1991; Frenkel *et al.*, 1989), can also be used for the distinction between strains of one potyvirus (Chapter 3, Van der Vlugt *et al.*, 1992a). Several strain specific amino acid sequences in the CPs and nucleotide sequences in the 3'-NTRs could be discerned, that are possibly involved in virulence and/or symptom expression. Further experiments are required to elucidate the precise biological significance of these sequence motifs. Interestingly the sequence comparisons as complied in Chapter 3 also confirmed the high levels of CP and 3'-NTR sequence identity between the PVY isolates at one hand and one putative isolate of pepper mottle virus (PepMoV, Dougherty *et al.*, 1985) at the other, as described previously (Van der Vlugt *et al.*, 1989; Van der Vlugt, 1992). Initially described as an atypical strain of PVY (PVY-S, Zitter, 1972) PepMoV was later found to be serologically and biologically distinct from PVY (Purcifull *et al.*, 1973, 1975; Zitter and Cook, 1973). Recent determination of the complete genomic RNA sequence of a Californian isolate of pepper mottle virus (PepMoV-C; Bowman-Vance *et al*, 1992a,b) and comparisons between a Florida isolate of PepMoV and PVY (Hiebert and Purcifull, 1992) however, suggest that PepMoV represents a distinct

potyvirus though more closely related to PVY than to any other potyvirus. Additional sequence information of other, biologically well characterized, isolates of PepMoV, like a virus isolate apparently intermediate between PepMoV and PVY (Nelson and Wheeler, 1978), will hopefully aid in establishing the exact taxonomic position of this pepper infecting virus in the genus *Potyvirus*. Generally it is to be recommended that of all virus isolates whose (partial) sequences are under investigation, precise origin and other relevant biological characteristics are also accurately documented.

In contrast to all other viruses for which 'CP-mediated resistance' has been described sofar, potyviruses do not express their CPs from a distinct, separate gene but through proteolytic cleavage of a polyprotein precursor. This necessitated the addition of translational start signals, directly upstream of the CP encoding sequence, in order to enable expression of the PVY^N CP in transgenic potato and tobacco plants. Potato tuber disc and tobacco leaf disc transformations with these constructs resulted in large numbers of transgenic plants (Chapters 4 and 5). Despite the fact that a large number of transgenic plants was tested for CP expression, using a highly sensitive enzyme-amplification based ELISA format, in none of the plants significant amounts of viral CP could be detected. Whether this is caused by the extra N-terminal methionine residue, or improper folding of the CP, resulting in decreased stability of the protein, or by inefficient protein extractions, possibly resulting from protein insolubility, is not known. It remains to be tested whether transformation of plants with a construct in which a functional protease domain is coupled to a potyviral CP with an intact protein processing sequence, will result in high levels of expression of the CP. For more practical purposes however, PVY CP expression levels appear not to be of significant importance since the protection against PVY, observed in the transgenic tobacco plants (Chapter 5 and 6), is apparently RNA-mediated, i.e. primarily based on the presence of the CP encoding RNA rather than on the coat protein itself. Transgenic tobacco lines expressing PVY CP transcripts devoid of a translational start signal (CP^{-ATG}), possess equal levels of protection against both mechanically inoculated virus and virus transmitted by the natural aphid vector *Myzus persicae* (Chapter 5 and 6). It seems highly unlikely that the protection in these CP^{-ATG} plants is based on minute amounts (i.e. less then 0.0001% of the total soluble protein) of a truncated viral polypeptide since the presence of six translational stopcodons preceding the first in-frame AUG startcodon, 162 nucleotides downstream the 5'-end of the CP encoding sequence, will prevent expression of such a polypeptide.

Analysis of the transgenic potato lines (Chapter 4) showed that most lines, as the transgenic tobacco lines, expressed CP specific RNA transcripts. Under the given greenhouse conditions, however, in none of the transgenic plants protection to PVY could be determined. In view of the results obtained with the transgenic tobacco lines, it may be anticipated that virus challenging of additional transgenic potato lines, under more optimal greenhouse conditions, will reveal similar levels of RNA-mediated

virus resistance as observed in tobacco. For all practical purposes genetically engineered resistance based on the presence of RNA molecules is to be preferred over forms of resistance that are based on the expression of a (foreign) protein. Apart from being energetically more favourable for the plant, it is likely to aid in the acceptance of genetically modified crop plants by both politicians and the public, something which might, in the next few years, turn out to be the major obstacle in the successful application of plant transformation techniques.

At this stage one can only speculate on the mechanism(s) on which this RNA-mediated resistance is based. Transformation of plants with partial CP or other PVY^N genomic sequences will help in identifying the protection mechanism(s) involved and show whether regions other than the CP-encoding domain can be equally effective in conferring virus resistance. If the resistance is based on a 'sense-RNA' effect, i.e. hybridization of the positive sense transgenic RNA to negative-sense viral RNA replication intermediates, thereby blocking further virus replication, the ribozyme technology might prove an efficient expansion of this genetically engineered type of resistance. Ribozymes, RNA sequences capable of specific and catalytic cleavage of other RNA-sequences, are able to cleave target RNAs efficiently and catalytically *in vitro*. The antiviral application of ribozymes in transgenic plants however has sofar demonstrated not to be very successful and reported protection levels are not yet exceeding those obtained with antisense RNAs (Edington and Nelson, 1992). Chapter 7 describes the design and synthesis of hammerhead ribozymes capable to cleave a highly conserved region from the PVY RNA dependent RNA-polymerase cistron. It was shown that the correct formation of the hammerhead cleavage complex, determined at least in part by the lengths of the antisense arms of the ribozyme, forms an important factor in the efficiency of cleavage. Cellular and full-length viral RNA molecules generally posses extended, unknown secondary structures which are likely to hamper precise formation of hammerhead structures, which requires bimolecular basepairing. Correct hammerhead formation and efficient cleavage of these RNAs will therefore require ribozymes with rather long basepairing arms. These long antisense arms however will make catalytic cleavage rather unlikely since complex dissociation will probably become the rate limiting factor. For this reason one can assume that ribozymes will only be successful when introduced into specific anti-sense RNA molecules, directed against the less abundant viral complementary strands, rather than as highly efficient RNA cleaving "enzymes".

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Samenvatting

Aardappel virus Y, ofwel potato virus Y (PVY), is de naamgever van 's werelds grootste plantevirus familie de *Potyviridae*. Potyvirussen komen in een groot aantal gewassen voor en vormen vooral in tropische en sub-tropische gebieden een probleem. Dit wordt mede veroorzaakt door het feit dat de meeste erg gemakkelijk, op een niet-persistente manier, overgedragen worden door diverse soorten bladluizen. Potyvirussen bezitten de volgende algemene kenmerken:

- Het zijn flexibele, draadvormige deeltjes met een lengte van 600 tot 900 nm, opgbouwd uit ongeveer 2000 eenheden van één type manteleiwit.
- Ze bezitten een enkelstrengs RNA genoom, van positieve polariteit, met een lengte van 8500 tot 10.000 nucleotiden. Het RNA draagt aan het 5' uiteinde een eiwit, VPg genoemd (Viral Protein genome-linked), en aan het 3' uiteinde een poly-A staart. Het RNA codeert voor een polyproteïne dat door 2 à 3 virale proteases in functionele eiwitten geknipt wordt.
- In het cytoplasma van geïnfecteerde cellen worden karakteristieke insluitsels gevormd, de zgn 'pinwheels' of 'cytoplasmic inclusions'.

Op grond van het type vector dat voor de verspreiding van de diverse virussen zorgt, wordt de familie *Potyviridae* onderverdeeld in vier genera. Het genus *Potyvirus* vormt de grootste groep en wordt door bladluizen overgebracht. Potato virus Y is het voorbeeld-virus van dit genus. Virussen uit het genus *Bymovirus* worden door schimmels overgebracht, terwijl virussen uit het genus *Rymovirus* overgedragen worden door mijten. Het vierde genus *Ipomovirus*, met een nog voorlopige status, wordt gevormd door de virussen die overgebracht worden door witte vliegen.

PVY kan een groot probleem zijn in een aantal belangrijke gewassen uit de familie van de *Solanaceae* ("nachtschade-achtigen") waaronder aardappel en tomaat. De laatste jaren zijn een aantal poly- en monogene resistenties beschikbaar gekomen die een goede bescherming tegen dit virus kunnen geven. Het is echter niet altijd mogelijk om deze genen in bestaande, economisch belangrijke gewassen te introduceren. Dit geldt bijvoorbeeld voor de aardappelcultivar 'Bintje'. In dit belangrijke (poot)aardappelras vormt met name de 'N-stam' ('tobacco veinal necrosis') van PVY een probleem terwijl het, mede door de complexe genetische achtergrond van Bintje, nog niet gelukt is om resistentiegenen tegen het virus in te kruisen. Deze PVY 'N-stam' (PVY^N) is vooral problematisch omdat een infectie met deze stam in Bintje (en in de meeste andere aardappelcultivars) geen duidelijke symptomen teweeg brengt. Dit bemoeilijkt de certificering van pootaardappelpartijen in ernstige mate en maakt kostbare laboratorium-toetsingen op PVY^N noodzakelijk.

Dit proefschrift beschrijft het onderzoek dat gedaan is naar de ontwikkeling van nieuwe vormen van resistentie tegen PVY^N door middel van de genetische modificatie van waardplanten, daarbij gebruik makend van het virale manteleiwitgen.

Bij de aanvang van het onderzoek was nog niets bekend over de samenstelling van het RNA genoom van PVY^N. Allereerst werd de volgorde bepaald van 4412 nucleotiden, die gelegen zijn in het 3' gedeelte van het genoom. Op deze manier kwam de primaire structuur van de genen beschikbaar die coderen voor een gedeelte van het cytoplasmatisch insluitsel (CI) eiwit, één van de protease eiwitten, het RNA-afhankelijke RNA-polymerase en het manteleiwit (Hoofdstuk 2). Naast de mogelijkheid tot klonering en modificatie van het PVY^N manteleiwitgen bood deze sequentiebepaling ook de mogelijkheid om de preciese taxonomische positie van het virus tussen een groot aantal andere PVY isolaten te bepalen. Gedetailleerde vergelijkingen van deze isolaten op basis van de manteleiwit aminozuur-volgorden en de nucleotiden-volgorden van het 3'-niet vertaalde gebied (3'-NTR), lieten zien dat deze niet alleen kunnen dienen om onderscheid te maken tussen verschillende potyvirus species, maar ook tussen verschillende stammen behorend tot eenzelfde virus species (Hoofdstuk 3).

Om expressie van het virale manteleiwit in transgene planten mogelijk te maken, moest het gen voorzien worden van startsignalen voor zowel transcriptie als translatie. Na klonering van het manteleiwitgen in een *Agrobacterium tumefaciens* Ti-plasmide, werden de resulterende constructen, door middel van transformatie van aardappelknolschijfjes, overgebracht naar het genoom van aardappel cv. Bintje (Hoofdstuk 4). De verkregen transgene planten werden geanalyseerd op de aanwezigheid van het PVY^N manteleiwitgen en de expressie ervan op RNA- en eiwitniveau. Ondanks de aanwezigheid van voldoende manteleiwit-specifiek RNA kon echter in geen van de transgene planten het manteleiwit aangetoond worden. De verkregen transgene planten werden vervolgens getoetst op hun mogelijke resistentie tegen een mechanische inoculatie met PVY^N. Onder de gegeven niet-optimale klimatologische omstandigheden in de PKII kas, kon echter in geen van de planten bescherming tegen virusinfectie vastgesteld worden (Hoofdstuk 4).

Om de problemen met transformatie-efficiëntie, de regeneratie van planten en klimatologische kascondities, verbonden met het transformeren en toetsen van aardappelplanten te omzeilen, werd besloten om de PVY^N manteleiwitgen-constructen ook te transformeren naar tabak (*Nicotiana tabacum*). Naast een efficiënt modelsysteem voor op *A. tumefaciens*-gebaseerde transformatie is tabak ook een zeer gevoelige waardplant voor PVY^N. Infectie resulteert in sterke necrose hetgeen toetsing op resistentie vergemakkelijkt. Transformatie van tabaksbladschijfjes met manteleiwit-constructen resulteerde in grote hoeveelheden transgene planten. Ook in deze planten kon echter, ondanks de goede expressie van manteleiwit-specifiek RNA, geen viraal

manteleiwit aangetoond worden. Na zelfbevruchting van de originele transformanten werden de resulterende S1 planten getoetst op virusresistentie hetgeen bij twee van de tien getoetste lijnen resulteerde in resistentieniveaus van respectievelijk 20 en 95%. Dit niveau van bescherming steeg zelfs tot 100% in de S2 nakomelingen van zelfbevruchte beschermde S1 planten (Hoofdstuk 5). Transformatie van tabak met manteleiwitgen-constructen waaruit het ATG startcodon verwijderd was (CP^{-ATG}), resulteerde eveneens in grote aantallen planten die alle het manteleiwit-specifiek RNA produceerden. Mechanische virusinoculaties van de uit zelfbevruchting verkregen S1 planten resulteerden in resistentieniveaus gelijk aan die van planten getransformeerd met constructen met een functioneel ATG startcodon (CP^{+ATG})(Hoofdstuk 5). Deze resultaten wijzen erop dat de gevonden resistentie in de transgene tabaksplanten 'RNA-mediated' is d.w.z dat deze hoofdzakelijk gebaseerd is op de aanwezigheid van PVY^N specifieke manteleiwit RNA sequenties en niet op de aanwezigheid van het virale manteleiwit zelf.

Om de specificiteit van de gevonden virusresistentie te bepalen, werden de twee beschermde CP^{+ATG} transgene lijnen mechanisch geinoculeerd met isolaten van verschillende PVY stammen (PVY^N, PVY^O en PVY^C). Ook hier werden duidelijke niveaus van bescherming gevonden, al waren deze iets lager dan de bescherming tegen de homologe PVY^N stam. Ook bleek de resistentie in zowel de CP^{+ATG} als de CP^{-ATG} lijnen gehandhaafd te blijven tegen virus dat overgebracht werd door de natuurlijke vector, de bladluis *Myzus persicae* (Hoofdstuk 6). Deze resultaten zijn veelbelovend voor een toepassing van deze vorm van resistentie in de praktijk.

Hoofdstuk 7 tenslotte beschrijft het ontwerp en de constructie van zgn. 'hammerhead' ribozymen gericht tegen een geconserveerd gebied van het PVY^N viraal RNA. Ribozymen zijn RNA structuren die in staat zijn tot enzymatische klieving van andere, specifieke RNAs en daarom mogelijk als nieuw anti-viraal middel ingezet zouden kunnen worden. *In vitro* klieving van PVY specifieke substraat-RNAs laat zien dat correcte complexvorming tussen het ribozyme en het substraat-RNA een belangrijke factor is in de klievings-efficiëntie. Dit heeft belangrijke gevolgen voor de *in vivo* toepassing van ribozymen als mogelijke anti-virale middelen.

Curriculum vitae

Op 12 augustus 1959 werd ik, René Andries Antonius van der Vlugt, samen met mijn broer Rob in Eindhoven geboren. Na het behalen van het mavo-diploma in 1976 op Mavo Selsterhorst te Veldhoven en vervolgens het havo- (1978) en het Atheneum-B-diploma (1980) aan het Van Maerlantlyceum te Eindhoven, begon ik in september 1980 aan een studie Planteziektenkunde aan de toenmalige Landbouw Hogeschool te Wageningen. Na het behalen van het kandidaatsexamen in september 1984, volgde de doctoraalstudie met als hoofdvakken Celbiologie, Moleculaire Biologie en Moleculaire Virologie. Van november 1985 tot augustus 1986 werkte ik, in het kader van mijn praktijktijd, gedurende 9 maanden onder de bezielende leiding van Phil Mullineaux in het Virology Department van het John Innes Insitute te Norwich, Engeland. Het doctoraal examen Planteziektenkunde werd in september 1987 afgelegd.

Van augustus 1987 tot oktober 1991 was ik als Assistent in Opleiding, met financiële ondersteuning van het Innovatiefonds Planteveredeling (InPla), onder begeleiding van prof. Goldbach, werkzaam bij de Vakgroep Virologie van de Landbouwuniversiteit Wageningen (LUW). Het onderzoek tijdens deze periode, beschreven in dit proefschrift, was vooral gericht op de moleculair-genetisch analyse van het aardappelvirus Y en de mogelijkheden om, door middel van genetisch modificatie van planten met delen van het genoom van dit virus, resistentie te verkrijgen in aardappel en tabak tegen dit belangrijke pathogeen. Van oktober 1991 tot januari 1992 was ik als toegevoegd onderzoeker, en na die tijd als gastmedewerker werkzaam op de Vakgroep Virologie van de LUW.

Vanaf december 1992 ben ik als Post-doc werkzaam bij het Proefstation voor de Champignoncultuur te Horst-America (L) aan de genoomkartering van de cultuurchampignon *Agaricus bisporus*.