POTATO LEAFROLL VIRUS,

MOLECULAR ANALYSIS AND GENETICALLY ENGINEERED RESISTANCE

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POTATO LEAFROLL VIRUS,
MOLECULAR ANALYSIS AND GENETICALLY ENGINEERED RESISTANCE

Frank van der Wilk

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The research described in this thesis was part of the research programme of the DLO Research Institute for Plant Protection (IPO-DLO), Wageningen. The work was a concerted effort of IPO-DLO, Mogen International NV and the Department of Virology, WAU. It was supported in part by the Programme Committee on Agricultural Biotechnology (PcLB).

STELLINGEN

1. De aanwezigheid van het P1-eiwit van het aardappelbladrolvirus in de plantecel is voldoende om bladrolsymptomen in aardappelplanten te veroorzaken.
   Dit proefschrift.

2. Het opstellen van algemeen geldende theorieën betreffende mogelijke werkingsmechanismen van 'pathogen-derived resistance' op basis van resultaten verkregen in *Nicotiana* spp. alleen, dient vermeden te worden.

3. De door Smith *et al.* gedane suggestie dat resistentie tegen het aardappelvirus Y in transgene aardappel veroorzaakt wordt door een cellulair afbraakmechanisme van transgene en virale RNA sequenties wordt onvoldoende ondersteund door de gepresenteerde gegevens.
   Smith, H.A., *et al.*, (1995). Transgenic potato virus Y resistance in potato: evidence for an RNA-mediated cellular response. Phytopathology 85:864-870.

4. De naam 'symbionine' voor het 63 kDa eiwit, dat door de endosymbiotische bacteriën van bladluizen *in situ* gesynthetiseerd wordt, dient op grond van sequentiegegevens vervangen te worden door 'GroEL'.
   Fukatsu, H.E., & Ishikawa, H., (1992). Synthesis and localization of symbionin, an aphid endosymbiont protein. Insect Biochem. Mol. Biol. 22:167-17.

5. In het door Damsteegt *et al.* uitgevoerde onderzoek naar het voorkomen van luteovirussen in klaver wordt ten onrechte de sterke serologische kruisreactie tussen 'soybean dwarf virus' en het erwtetopvergelingsvirus (bean leafroll virus) genegeerd.
   Damsteegt, V.D., *et al.*, 1995. Soybean dwarf, bean leaf roll, and beet western yellows luteoviruses in southeastern U.S. white clover. Plant Dis. 79:48-50.

6. De door Yonaha *et al.* gepresenteerde gegevens ondersteunen onvoldoende de conclusie van de auteurs dat zij een nieuw luteovirus beschrijven.
   Yonaha, T., *et al.*, (1995). Pepper vein yellows virus, a novel luteovirus from bell pepper plants in Japan. Ann. Phytopathol. Soc. Jpn. 61:178-184.

7. Gezien de wisselvalligheid van de Nederlandse zomers dient er meer onderzoek verricht te worden naar de mogelijkheid om (picorna-achtige) bladluisvirussen als biologisch bestrijdingsmiddel te gebruiken.
   Laubscher, J.M., Von Wechmar, M.B., (1992). Influence of aphid lethal paralysis virus and *Rhopalosiphum padi* virus on aphid biology at different temperatures. J. Invert. Path. 60:134-140.

8. Bij de ontwikkeling van nieuwe taxonomische technieken die gebaseerd zijn op verschillen in nucleotidenvolgorden dient men 'lumping' boven 'splitting' te stellen.

9. Van het tellen van publikaties als maat voor de kwaliteit van een onderzoeksgroep worden alleen uitgevers wijzer.

10. Uit de naamgeving van het nieuwe computerbesturingsprogramma *Windows 95*™ blijkt dat de fabrikant zijn belofte dat de koper tot het jaar 2000 'up to date' is, niet serieus neemt.

Stellingen behorende bij het proefschrift van Frank van der Wilk: "PLRV, molecular analysis and genetically engineered resistance".

Wageningen, 13 december 1995.

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Scope of the investigation

The main objective of the investigations described in this thesis was to gain further insight in the genomic organization of the potato leafroll virus (PLRV) and to use this knowledge to develop new strategies for host plant resistance towards this virus. PLRV, a member of the genus *Luteovirus*, is still a problem in agriculture due to the lack of resistant potato varieties. The use of resistant potato plants would not only increase yields and reduce costs, but would also allow a considerable reduction in the use of insecticides, now being applied to control the aphid vector of the virus, thus reducing the spread of the virus. Knowledge about the molecular features of the virus is an absolute prerequisite for the development of new ways of virus resistance. Furthermore, information on the genomic organization of PLRV will lead to a correct classification of luteoviruses and provide new data about the relationship of this important virus group to other virus groups.

This chapter will present a general introduction to the luteoviruses, with special emphasis on PLRV, and to the transformation strategies aimed at increasing virus resistance levels of host plants by what is currently known as 'genetically engineered resistance'. Chapter 2 describes the unravelling of the complete nucleotide sequence of the genomic RNA of PLRV. Experiments aimed to obtain PLRV resistance by transforming potato with sense and anti-sense constructs of the viral coat protein gene are described in Chapters 3 and 4. In Chapter 3 the production of transgenic potato plants containing the wild-type coat protein gene of PLRV is reported. Chapter 4 describes the production of transgenic plants containing a mutated version of the PLRV coat protein gene. The coat protein gene was altered in order to increase its translational expression. Chapters 5 and 6 focus on the role of a non-structural (28 kDa) protein, encoded by the first open reading frame on the PLRV genome, during the infection process. The heterologous expression of this protein in both the *Escherichia coli* system

and the baculovirus/insect cell system, as well as the production of an antiserum against this protein is described in Chapter 5. Furthermore, transgenic potato plants containing the PLRV 28 kDa gene have been produced and tested for resistance to PLRV (Chapter 6 & 7). A general discussion of the experiments carried out and of the results obtained, are presented in Chapter 8.

Luteoviruses

In 1975, luteoviruses were recognized as a separate plant virus group, and in 1995 as a genus, by the International Committee on Taxonomy of Viruses. The name luteovirus is derived from the Latin word *luteus,* which means yellow. This refers to the yellowing symptoms that many of the viruses induce in their hosts. Barley yellow dwarf virus (BYDV) is the type species of the genus *Luteovirus*. Other, economical important species are PLRV and beet western yellows virus (BWYV).

Luteoviruses have small icosahedral particles, with reported sizes ranging from 24 to 30 nm in diameter. The viruses are not transmitted through seed or pollen, nor by mechanical inoculation, but exclusively by aphids in a persistent manner (Waterhouse *et al.*, 1988). The virus particles are confined to the phloem of the infected host plant. The viruses are believed to replicate in the companion cells of the sieve tubes. The genome is a single-stranded, messenger-sense, RNA molecule of M_r 2 x 10^6 (Brakke & Rochow, 1974; Hewings & D'Arcy, 1983; Rowhani & Stace-Smith, 1979; Takanami & Kubo, 1979). A small protein (VPg) is covalently attached to 5'-end of the RNA, which is not polyadenylated at the 3'-end (Mayo *et al.*, 1982; Murphy *et al.*, 1987). The coat protein consists of a single subunit of approximately 23 kDa (Miller *et al.*, 1988b). The nucleotide sequences of the genomic RNAs of BYDV (serotypes MAV, PAV and RPV) (Miller *et al.*, 1988a; Vincent *et al.*, 1991; Ueng *et al.*, 1992), BWYV (Veidt *et al.*, 1988), soybean dwarf virus (SDV) (Rathjen *et al.*, 1994) and cucurbit aphid-borne yellows virus (CABYV) (Guilley *et al.*, 1994) have been determined partly during the course of the investigation on PLRV described in this thesis.

Several luteoviruses cause severe damage to agricultural crops. The different serotypes of BYDV infect small-grain cereals and other grasses all over the world, resulting in high yield losses. BWYV infects a wide range of economical important crops, like sugarbeet and beans. PLRV is the causal agent of leafroll, one of the most

important viral diseases in potato. Although most luteoviruses have a narrow host range (PLRV, carrot red leaf virus (CRLV)), a few species have a very broad host range (BWYV, SDV). Control of luteoviruses has proven to be very difficult due to the widespread occurrence of their aphid vectors, infection of weeds which serve as inoculum sources, and the absence of resistance genes suitable for resistance breeding.

Classification of luteoviruses

A large number of viruses has been listed in the past as members, or possible members of the luteovirus group. Naming of luteoviruses and differentiation between luteovirus strains and separate viruses has been severely hampered in the past by the occurrence of low virus titers in the plant, the confinement of particles to the phloem and in consequence of this, difficulties in purification. At present, several improved methods for the purification of luteoviruses are available, for the most based on the use of enzymes to macerate cell tissue. While in the past, the biological properties of the viruses were the main criteria for differentiation between viruses, nowadays other data can be used for discrimination, including serology and nucleic acid analysis.

In spite of the availability of new techniques and increased knowledge about serology and physiology, classification of luteoviruses has remained difficult and subject to change. Most members of the genus *Luteovirus* are serologically interrelated. In the past host range and vector specificity of viruses have been major criteria for classification of luteoviruses. For BYDV five different serotypes (or isolates) have been described, namely PAV, MAV, SGV, RPV and RMV (Rochow, 1970a). The different serotypes are more or less specifically transmitted and named after their aphid vectors (MAV - *Macrosiphum avenae*; PAV - *Rhopalosiphum padi* and *M. avenae*; RMV - *R. maidis*; RPV - *R. padi*; SGV - *Schizaphis graminum*). The vector specifics of the various BYDV serotypes or isolates can be altered if these serotypes co-infect. One serotype can help another serotype to be transmitted by the vector aphid of the first serotype, which is a non-vector of the second serotype (Rochow, 1970b). This phenomenon, referred to as dependent transmission also occurs among other luteoviruses (Waterhouse *et al.*, 1988) and is probably caused by transcapsidation, occurring between the different BYDV serotypes (Wen & Lister, 1991).

The differentiation by vector specificity of the BYDV isolates approximately

corresponds to separation on basis of serotype differences. The BYDV isolates can be separated into two subgroups by serological relationships. The first group includes PAV, MAV and SGV, the second group includes RPV and RMV (Rochow, 1970a). The MAV and RPV isolates do not cross-react in immuno-diffusion tests with each others antisera. Also, plants infected by one of these two serotypes are not protected from infection by the other, while PAV-infected plants are protected against infection with the MAV serotype (Aapola & Rochow, 1971). However, BYDV-RPV is serologically very closely related to BWYV and it has also been suggested that RPV should be considered a BWYV isolate (Casper, 1988).

From the data mentioned above it follows that, classification of luteoviruses on the basis of biological properties and serology is difficult and not completely reliable. Nucleotide sequence analysis of the genomic RNAs of the different luteoviruses is an absolute prerequisite for a correct classification. Comparison of genomic nucleotide sequences viruses may help to clarify whether viruses should be considered to be isolates or distinct viruses. Sequence analysis also gives information about relationships between viruses belonging to different groups. The nucleotide sequences of the genomic RNAs of BWYV, SDV, CABYV and BYDV serotypes MAV, PAV and RPV have been determined (Veidt *et al.*, 1988; Miller *et al.*, 1988a; Vincent *et al.*, 1991; Ueng *et al.*, 1992; Guilley *et al.*, 1994; Rathjen *et al.*, 1994).

While the genomic organization of BYDV-RPV shows a high degree of similarity with that of BWYV, the genomic organization of the MAV and PAV isolates is quite different. From the deduced nucleotide sequences it is clear that the BYDV isolates PAV and RPV are actually two distinct viruses and that BYDV-MAV and PAV are closely related or even can be considered two strains of the same virus. Comparison of the amino acid sequences of the putative viral products revealed that the RNA-dependent RNA polymerase of BWYV shares a high homology with the putative RNA polymerases of southern bean mosaic virus (SBMV), SDV, CABYV and BYDV serotype RPV, while the RNA polymerase of BYDV serotypes MAV and PAV showed homology with the putative RNA polymerase of carnation mottle virus (CarMV) and not with the polymerases of BWYV or SBMV (Miller *et al.*, 1988a; Veidt *et al.*, 1988; see also Chapter 2).

As a consequence of the extended knowledge concerning both serology and molecular biology of luteoviruses, the number of viruses placed into this virus group

Table 1: Members of the genus *Luteovirus* (Randles & Rathjen, 1995).

Species in the genus *Luteovirus*:

BYDV subgroup I:
Barley yellow dwarf virus - MAV
Barley yellow dwarf virus - PAV
Barley yellow dwarf virus - SGV

BYDV subgroup II:
Barley yellow dwarf virus - RGV
Barley yellow dwarf virus - RMV
Barley yellow dwarf virus - RPV
Bean leafroll virus
Beet western yellows virus
Carrot red leaf virus
Groundnut rosette assistor virus
Indonesian soybean dwarf virus
Potato leafroll virus
Solanum yellows virus
Soybean dwarf virus
Tobacco necrotic dwarf virus
Tomato yellow top virus

Possible species in the genus *Luteovirus*:

Beet yellow net virus
Celery yellow spot virus
Chickpea stunt virus
Cotton anthocyanosis virus
Filaree red leaf virus
Grapevine ajinashika virus
Milk vetch dwarf virus
Millet red leaf virus
Physalis mild chlorosis virus
Physalis vein blotch virus
Raspberry leaf curl virus
Tobacco vein distorting virus
Tobacco yellow net virus
Tobacco yellow vein assistor virus

had been decreasing in the past years. In 1991 ten definitive and twelve possible members were recognized in the luteovirus group (Randles, 1991).

However, virus taxonomy has dramatically changed recently. In stead of the concept of virus groups, classification of viruses will be similar to the taxonomic system used for living organisms. Consequently, the luteovirus group has been recognized as a separate genus and the different viruses are being considered as species. This division in species appears to be mainly based upon the biological properties of viruses. Unfortunately, this has resulted in a sharp increase of the number of luteovirus species (Table 1) compared to the number of formerly recognized luteovirus members (Randles, 1991; Randles & Rathjen, 1995). Surprisingly, CABYV has not been recognized as a species, although

comparison of its genomic nucleotide sequence indicates that it is a distinct entity rather than a strain of a luteovirus. All the BYDV serotypes are now considered different species. Furthermore, tomato yellow top virus (TYTV) is distinguished as a species. This virus, a pathogen of tomato, causes no or few symptoms in potato and is readily transmitted by *Macrosiphum euphorbiae*. Since, no antigenic distinction could be detected between TYTV and PLRV isolates from Australia (Thomas, 1984) or between isolates from The Netherlands and Brazil (Van den Heuvel *et al.*, 1990), TYTV used to be considered a strain of PLRV (Casper, 1988). Undoubtedly the classification of luteoviruses has not been concluded and will certainly be subject to further changes in the future.

Potato leafroll virus

PLRV is of great economic importance all over the world. The virus is particularly damaging in tropical and subtropical areas where its vector, *Myzus persicae* (Sulzer), is present throughout the year. Yields of infected potato plants may be reduced by as much as 50%. The worldwide crop losses caused by PLRV is estimated at 10%, representing a yearly loss of 20 x 10^6 tons of potatoes (Kojima & Lapierre, 1988). Furthermore, PLRV causes damage to seed potato cultivation, a low incidence of PLRV infection in seed crops already leads to exclusion from certification schedules.

PLRV was first purified by Peters (1967) from the green peach aphid (*M. persicae*). Subsequently, Kojima *et al.* (1969) purified the virus from infected *Physalis floridana* plants. Virus particles are approximately 25 nm in diameter and contain a single protein subunit of approximately 23 kDa (Rowhani & Stace-Smith, 1979). The genome consists of a single-stranded messenger-sense RNA molecule of approximately 6000 nucleotides. The genomic RNA lacks a polyadenylate sequence at the 3'-end and contains a small protein (VPg), of approximately 7 kDa, covalently linked to the 5'-end (Mayo *et al.*, 1982). Like all other luteoviruses, the presence of virus particles in infected plants is limited to the phloem and the virus is transmitted by aphids in a persistent manner. *M. persicae* is considered to be the major vector of PLRV, but several other aphid species are also capable of transmitting the virus (Sylvester, 1980).

The host range of PLRV is mainly restricted to the plant family *Solanaceae*. PLRV is able to infect all *Solanum tuberosum* varieties. Although potato cultivars differ in

susceptibility to PLRV, there appears to be neither immunity nor major gene resistance to this virus within *S. tuberosum* species (Ross, 1986). *Solanum brevidens*, a wild non-tuber bearing species and sexually incompatible with *S. tuberosum*, has shown to be resistant to PLRV (Jones, 1979). Attempts to confer this resistance to *S. tuberosum* have failed until now, in spite of extensive research. Other PLRV-susceptible species include *Datura stramonium, Datura tatula, P. floridana, Physalis angualata, Lycopersicum esculentum* and *Nicotiana clevelandii.*

Infection of the plant by PLRV is followed by necrosis of the sieve tubes and the associated companion cells. Phloem parenchyma cells undergo hypertrophy and crush the necrotic cells (Esau, 1938). Abnormally large amounts of callous are deposited in the sieve tubes. As a consequence, the transport of assimilates through the sieve tubes becomes interrupted and the metabolism in the cells of the leaves disordered. External symptoms of PLRV infection are stunting of the plant, rolling or curling of the leaves, interveinal chlorosis (yellowing) and reddening of the leaves.

Genetically engineered resistance

Over the past years it has been shown that transgenic expression of viral genes induces host resistance to the homologous virus. This might be in accordance with the phenomenon of cross-protection whereby a virus is unable to infect plants which already have been infected by another strain of the same virus. This principle has been used, e.g. in the Netherlands to protect tomato plants against severe isolates of tomato mosaic virus (ToMV). Young tomato seedlings have been infected with a mild strain of the virus, which causes no yield losses, protecting the plant against severe strains of ToMV (Rast, 1972). A major disadvantage of this method is the continuous presence of ToMV in the crop. If the infected plants are infected with another virus too, serious yield losses can be encountered due to synergism between the two infections. There also is the risk that mild isolates may spontaneously mutate to more severe forms. Furthermore, a virus which causes mild symptoms in a certain crop may cause severe symptoms in another crop. For these reasons the use of cross-protection was abandoned after resistant tomato cultivars were obtained through breeding. Several different mechanisms for 'classical' cross-protection have been proposed. While, most reports indicate that viral coat protein is essential for protection, it has been shown that

infection of plants with the RNA-1 of tobacco rattle virus (TRV) is able to confer, in the absence of the RNA-2 encoded coat protein, protection to infection with a 'complete' TRV strain. (Cadman & Harrison, 1959). Furthermore, the occurrence of cross-protection between viroids has been demonstrated (Khoury *et al.*, 1988), clearly indicating that the presence of viral coat protein is not always necessary for protection.

The disadvantages of the classical method of cross-protection can be circumvented by expressing only a part of the viral genome in the plant to be protected. Initially, it has been shown that expression of viral coat protein genes in transgenic plants, conveyed protection from the virus of which the coat protein gene was derived (Beachy *et al.*, 1990). This was first shown for tobacco and tobacco mosaic virus (TMV) (Powell *et al.*, 1986). In these experiments tobacco plants were transformed with the coat protein gene of TMV, using an *Agrobacterium tumefaciens* Ti plasmid transformation system. The obtained transgenic plants, showed a delay or even complete inhibition of symptoms upon TMV infection. Similar results have since been obtained for many other plant-virus combinations (for a recent review, see Hackland *et al.*, 1994). Usually this principle is referred to as coat protein-mediated protection (Beachy, 1988) or genetically engineered cross-protection (Nelson *et al.*, 1987). Afterwards, other forms of genetically engineered resistance have been described. The expression of modified or 'wild-type' viral replicase genes in transgenic plants has been shown to confer resistance (Baulcombe, 1994). It also has been reported that the expression of defective mutants of movement protein genes in transgenic plants incited resistance (Beck *et al.*, 1994; Cooper *et al.*, 1995).

For most cases, the mechanisms involved in genetically engineered resistance have remained unknown sofar. In some cases it has been shown that the presence of transgenic viral protein is required for the expression of resistance, whereas in other cases the transgenic transcript induced resistance. In the initial experiments of Powell *et al.* (1986) it was shown that with increasing concentrations of the virus inoculum, both the delay in symptom development and the proportion of plants that escapes infection decreases. Also, it has been reported that the level of protection in transgenic plants expressing viral coat proteins was dependent on the presence and amount of transgenic protein expressed and not on the amount of RNA present (Van Dun *et al.*, 1988; Powell *et al.*, 1990; Taschner *et al.*, 1994). In experiments with transgenic tobacco plants expressing modified versions of the replicase gene P2 of alfalfa mosaic virus (AlMV)

resistance was observed in plants with relative high expression levels (Brederode *et al.*, 1995). Plants expressing altered versions of the P2 gene in which the catalytic GDD motif was modified into GGD, GVD or DDD exhibited resistance. Plants expressing a truncated gene or a gene in which the GDD motif was changed into VDD did not exhibit resistance, indicating that the (modified) P2 protein itself and not transcript sequences was involved in the conveyance of resistance. On the other hand, for transgenic potato plants expressing the coat protein gene of potato virus Y (PVY), the level of protection was independent of the level of transgenic coat protein produced (Lawson *et al.*, 1990) and plants in which accumulation of transgenic PVY coat protein could not be observed were shown to be highly resistant (Van der Vlugt *et al.*, 1992). Transgenic plants expressing modified and untranslatable coat protein gene sequences of tobacco etch virus (TEV) and PVY have been shown to be highly resistant (Lindbo & Dougherty, 1992a & 1992b). Similar results have been obtained for the replicase gene of PVX.

It appears that both the transgenic protein or the transgenic viral RNA sequences can be involved in the induction of resistance. The mechanisms underlying both the protein- or RNA-mediated resistance are still obscure. In the case of protein-mediated resistance several different mechanisms have been proposed. It has been reported for TMV and AlMV, that inoculation with viral RNA largely overcomes coat protein-mediated protection (Nelson *et al.*, 1987; Tumer *et al.*, 1987; Loesch-Fries *et al.*, 1987; Van Dun *et al.*, 1987). This seems to support the theory that coat protein-mediated protection results from interference with an early event (possibly uncoating) in infection (Register & Beachy, 1988). However, inoculation with potato virus X (PVX) RNA did not overcome protection in transgenic plants expressing the PVX coat protein gene (Hemenway *et al.*, 1988). Bertioli *et al.* (1992) suggested that for coat protein-mediated protection the production of empty viral particles is a prerequisite to mediate resistance. Another proposed mechanism for coat protein-mediated protection involves interference with long-distance movement (Wisniewski *et al.*, 1990). For plants expressing transgenic TMV coat protein it has been shown that spread of TMV to distant tissues was significantly reduced (Wisniewski *et al.*, 1990).

There is some evidence that protein-mediated genetically engineered resistance offers a type of broad spectrum resistance. Transgenic tobacco plants expressing the coat protein of the potyvirus soybean mosaic virus (SMV) have been shown to be

resistant against infection by two serologically unrelated potyviruses, tobacco etch virus (TEV) and PVY (Stark & Beachy, 1989). From amino acid sequence comparisons of the coat proteins of 40 strains of 18 different potyviruses it is known that, the N-terminal parts of the various proteins show major differences, but that the C-terminal parts share high sequence homologies (Ward & Shukla, 1991). This conserved region of the potyvirus coat protein or 'core' is probably involved in particle assembly. Sequence comparison of the coat protein core regions of TEV, SMV and PVY reveals considerable homologies (Ward & Shukla, 1991). Transgenic plants expressing the coat protein of TRV strain TCM, showed a considerable resistance to infection with another tobravirus, pea early browning virus (PEBV) (Van Dun & Bol, 1988). But, strikingly, the transgenic plants were not protected from infection with strain PLB of TRV. There is a 39% homology between the amino acid sequences of the coat proteins of TRV-TCM and TRV-PLB. The coat proteins of PEBV and TRV-TCM are supposed to be highly homologous in their amino acid sequences (Van Dun & Bol, 1988). These results suggest that at least a distinct part of the coat protein genes should be highly homologous or identical to offer protection from infection. This view is further supported by the observation that the second amino acid of the AlMV coat protein is critical for coat protein-mediated protection (Turner *et al.*, 1991).

It has been reported that transgenic tobacco plants expressing a defective mutant of the TMV movement protein showed a delay in symptom expression and reduced systemic accumulation of virus in the upper leaves of plants infected with a whole range of different viruses, unrelated to tobamoviruses (Cooper *et al.*, 1995). However, plants expressing the wild-type movement protein were more susceptible to viral infections, resulting in elevated virus titers, accelerated symptom development and enhanced symptom severity. All the above mentioned data suggest that for protein-mediated resistance, the transgenic protein based upon its intrinsic property, directly interferes with the viral infection cycle.

Recently, a possible mechanism for RNA-mediated resistance has been proposed. (Dougherty *et al.*, 1994; Smith *et al.*, 1994). In several reports it has been shown that the expression of untranslatable viral gene sequences in transgenic plants rendered resistance, but that the degree of resistance did not correlate with the steady state levels of transgenic transcripts (De Haan *et al.*, 1992; Lindbo & Dougherty, 1992a & 1992b). Strikingly, an inverse correlation has been observed between transgenic steady state

levels and acquired resistance (Lindbo *et al.*, 1993; Dougherty *et al.*, 1994; Smith *et al.*, 1994). Also, it was shown that some transgenic plants were susceptible for infection but able to recover from systemic viral infection. Tissues from recovered plants contained steady state levels of transgenic transcripts five to eightfold lower than those of unchallenged transgenic tissues (Dougherty *et al.*, 1994). Possibly, high levels of accumulation of transgenic viral transcripts lead to post-transcriptional breakdown of the transcripts and, ergo, to the breakdown of the genomic RNA sequences of the homologous infecting virus (Dougherty *et al.*, 1994; Smith *et al.*, 1994). Such an event would be in accordance with the well-known, but poorly understood, phenomenon of co-suppression, in which expression of transgenes in plants leads to the silencing of both the transgene and the homologous endogenous gene (for review, Finnegan & McElroy, 1994). Many different mechanisms have been suggested to play a role in co-suppression including mRNA breakdown (Van Blokland *et al.*, 1994). In support of the theory of specific post-transcriptional breakdown of RNA sequences is the observation that RNA-mediated resistance is highly virus-specific, indicating that only highly homologous RNA molecules are degraded.

Field experiments and agronomic importance

Most of the data considering genetically engineered resistance, resulted from experiments carried out in greenhouses under controlled conditions, making an assessment of the value of the transgenic plants in agricultural practice difficult. However, there are some data available regarding field tests. Transgenic potato plants expressing both the coat protein of PVX and PVY have been tested for resistance under field conditions (Kaniewski *et al.*, 1990). In the case of the transgenic potato plants, four different transgenic plant lines were tested, with untransformed potato plants as a control. All four lines had been previously tested under greenhouse conditions and proved to show a high resistance against viral infection. In field tests, two transgenic plant lines demonstrated a significant resistance to PVX infection and another transgenic plant line proved to be highly resistant against infection with both PVY and PVX.

Also, transgenic tomato plants expressing the coat protein of TMV or ToMV, have been tested for resistance under field conditions (Nelson *et al.*, 1988; Sanders *et al.*,

1992). It was shown that plant lines expressing the TMV coat protein gene were protected from TMV infection, but were susceptible to ToMV infection, while plants expressing the ToMV coat protein gene were resistant to ToMV and susceptible to TMV. Tomato fruit yields of non-transgenic control plants decreased 20-69% due to virus infection, whereas the yields of the resistant transgenic tomato plants were unaffected (Sanders *et al.,* 1992).

Probably, the most elaborate studies have been carried out with potato cultivars Bintje and Escort, expressing the PVX coat protein gene (Jongedijk *et al.,* 1992 & 1993). Both the occurrence of resistance and cultivar properties were monitored under field conditions over a period of three years. It was shown that approximately 82% of the 'Escort' and 18% of the 'Bintje' derived transgenic plant lines were true to type and exhibited resistance to PVX. These results clearly demonstrate the potential value of genetically engineered resistance in agriculture.

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CHAPTER 2

Nucleotide sequence and organization of potato leafroll virus genomic RNA

ABSTRACT

The nucleotide sequence of the genomic RNA of potato leafroll virus (PLRV) was determined and its genetic organization deduced. The RNA is 5882 nucleotides long and contains 6 open reading frames (ORFs). In the 5'-terminal coding region of the RNA the ORFs 1 and 2, encoding 28 kDa and 70 kDa proteins respectively, are overlapping in different reading frames. The ORF3, encoding for another protein of 70 kDa, overlaps in a different reading frame the ORF2 and lacks a start codon. Circumstantial evidence suggests that this ORF is expressed via a -1 ribosomal frameshift. The C-terminal part of this protein contains the putative consensus sequence for RNA-dependent RNA-polymerases. The three ORFs in the 3' half of the PLRV RNA are preceded by a non-coding region of 197 nucleotides. The 23 kDa protein encoded by ORF4 shows a high homology with the putative coat proteins of barley yellow dwarf virus (BYDV) and beet western yellows virus (BWYV). The ORF6 encodes a 55 kDa protein and is contiguous with ORF4 being only separated by an amber stop codon. Since ORF6 lacks its own start codon, it is suggested that this ORF is expressed via translational readthrough from the ORF4 by suppression of the stop codon. The ORF5 of PLRV underlies ORF4 in a different reading frame and encodes a protein of 17 kDa.

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INTRODUCTION

Potato leafroll luteovirus (PLRV) is of great economic importance and infects potato plants worldwide (Kojima & Lapierre, 1988). Luteoviruses are transmitted by aphids in a persistent manner, their particles being confined to the phloem of the infected plants (Harrison, 1984). In spite of its economic importance, little is known about the genomic organization and expression of PLRV. PLRV has isometric capsids with a diameter of circa 28 nm, consisting of subunits of approximately 26 kDa. The capsid contains a 2 x 10^6 Da (6 kb) single-stranded messenger-sense RNA genome (Rowhani & Stace-Smith, 1979; Takanami & Kubo, 1979). The PLRV RNA is provided with a genome-linked protein (VPg) of approximately 7 kDa, but lacks a polyadenylate sequence at the 3'-end (Mayo *et al.*, 1982). The presence of a sub-genomic messenger RNA in infected plant cells has been reported (Mayo *et al.*, 1984).

PLRV is serologically related to several other luteoviruses, including beet western yellows virus (BWYV) and barley yellow dwarf virus (BYDV), the type species of the luteoviruses. The complete nucleotide sequences of the genomic RNAs of BYDV (serotype PAV) and BWYV have been determined (Miller *et al.*, 1988a; Veidt *et al.*, 1988). So far for PLRV cDNA physical maps and the sequence of the 3'-terminal 141 nucleotides have been published (Prill *et al.*, 1988; Smith *et al.*, 1988). Here the complete nucleotide sequence of the PLRV genomic RNA is reported with comparisons of its deduced amino acid sequences with those of other plant viral proteins.

MATERIALS AND METHODS

Materials

Enzymes were purchased from Bethesda Research Laboratories (BRL) and Amersham. Chemicals were obtained from Sigma and radiochemicals from Amersham. Computer programs used were developed by the Genetics Computer Group of the University of Wisconsin (UWGCG) (Devereux *et al.*, 1984).

Virus purification

PLRV isolate 'Wageningen' was transmitted using the aphid *Myzus persicae* (Sulzer) and propagated in *Physalis floridana* Rydb. plants. Virus was purified by a slightly modified method as described by Van den Heuvel *et al.* (1990). Infected leaf material (300 g) was thoroughly homogenized in 600 ml 0.1 M sodium citrate buffer pH 6.0, containing 0.5% macerozyme R-10 (Yakult Honsha Co. Ltd, Tokyo), 0.5% cellulase 'Onuzuka' R-10 (Yakult Honsha Co. Ltd, Tokyo), and 0.5% ethanol in a blender. The homogenate was stirred at 26 ^{0}C for 3-4 h and emulsified with a mixture of 150 ml chloroform and 150 ml 1-butanol. The emulsion was vigorously stirred for 5 min and broken by centrifugation at 10,000 rpm for 15 min in a Sorvall GSA rotor. The aqueous phase was collected and respectively 1% Triton X-100, 8% polyethylene glycol 6000 and 0.4 M sodium chloride were added to precipitate the virus. After stirring at room temperature for 1 h, the virus was collected by centrifugation at 10,000 rpm for 15 min in a Sorvall GSA rotor. The pellet was resuspended in 90 ml 0.1 M sodium citrate buffer pH 6.0 containing 5% ethanol. The supernatant was clarified by centrifugation at 7,000 rpm for 15 min in a Sorvall SS34 rotor. The partly purified virus suspension was layered on 30% sucrose (in citrate buffer) cushions and centrifuged at 30,000 rpm for 4 h in a Beckmann R45 rotor. Each pellet was resuspended 1 ml 0.1 M sodium citrate buffer pH 6.0. and loaded on a 5-45% linear sucrose gradient. After centrifugation at 30,000 rpm for 3 h in a Beckman SW41 Rotor, the purified virus was collected using an ISCO density gradient fractionator and resuspended in 0.1 M sodium citrate buffer pH 6.0.

RNA isolation and denaturing agarose gel electrophoresis

RNA was isolated from purified virus by phenol extraction. Purified virus was extracted with one volume phenol at 65 ^{0}C in the presence of 0.5% SDS, followed by two phenol extractions at room temperature. The PLRV RNA was ethanol precipitated and dissolved in sterile, diethyl pyrocarbonate (DEPC) treated, water. Denaturing agarose gel electrophoresis was performed as described (Maniatis *et al.*, 1982). 1 µg RNA was mixed with an equal volume 2x loading buffer (2.5% mercury methyl hydroxide, 20% glycerol, 1x electrophoresis buffer, 0.20% bromo phenol blue) and loaded onto a 1%

agarose gel. As electrophoresis buffer 50 mM boric acid, 5 mM sodium borate and 10 mM sodium sulphate was used. After electrophoresis the gel was stained with 0.5 M ammonium acetate containing 1 μg/ml ethidium bromide and examined under UV light.

In vitro translation of genomic PLRV RNA

A commercial kit (BRL) was used for the *in vitro* translation of purified PLRV RNA. 0.5 μg RNA was added to 30 μl reaction mixture containing 10 μl nuclease treated (3x) rabbit reticulocyte lysate or wheat germ lysate, 1 μl ribonuclease inhibitor, 50 μM amino acid mixture (minus methionine), 25 mM HEPES pH 7.2, 40 mM KCl, 10 mM creatine phosphate, 1 mM Mg^{2+} and 5 μCi [^{35}S]-methionine (1000-1500 Ci/mmol). After incubation at 30 ^{0}C for 1 h, the reaction mixture was placed on ice to stop the reaction. It was then incubated with 1 mg/ml pancreatic Rnase at 30 ^{0}C for 1 h to hydrolyse radioactive aminoacyl-tRNAs. The translation products were analyzed by polyacrylamide SDS gel electrophoresis, followed by fluorography. 10 μl of the translation reaction mixture was loaded onto a 15% SDS-polyacrylamide gel. After electrophoresis the gel was soaked in 10% HAc, 40% methanol (v:v) for 1 h to fix the proteins in the gel, followed by a 15 min incubation in amplify solution (Amersham). The gel was dried for 2 h at 80 ^{0}C on a geldryer. Fluorography was carried out by placing a XAR-5 (Kodak) film against the dried gel for 48 h.

cDNA synthesis and cloning

Two different strategies were followed to obtain cDNA clones. For first strand cDNA synthesis reverse transcription on the viral RNA was initiated by random priming with the use of calf thymus DNA fragments or by priming with specific synthetic oligonucleotides. Double-stranded cDNA fragments were synthesized using the RnaseH method (Gubler & Hoffman, 1983) with the use of (and according to the instructions of the manufacturer) a commercially available kit (Amersham). The first strand cDNA was synthesized using 20 units reverse transcriptase and 1 μg purified viral RNA. Second strand synthesis was performed using the RNA/cDNA hybrid as substrate. 1 u/μl RNaseH was utilized to produce nicks and gaps in the RNA. DNA polymerase (23 units) replaced the RNA strand utilizing the nicked RNA as primer. The double-

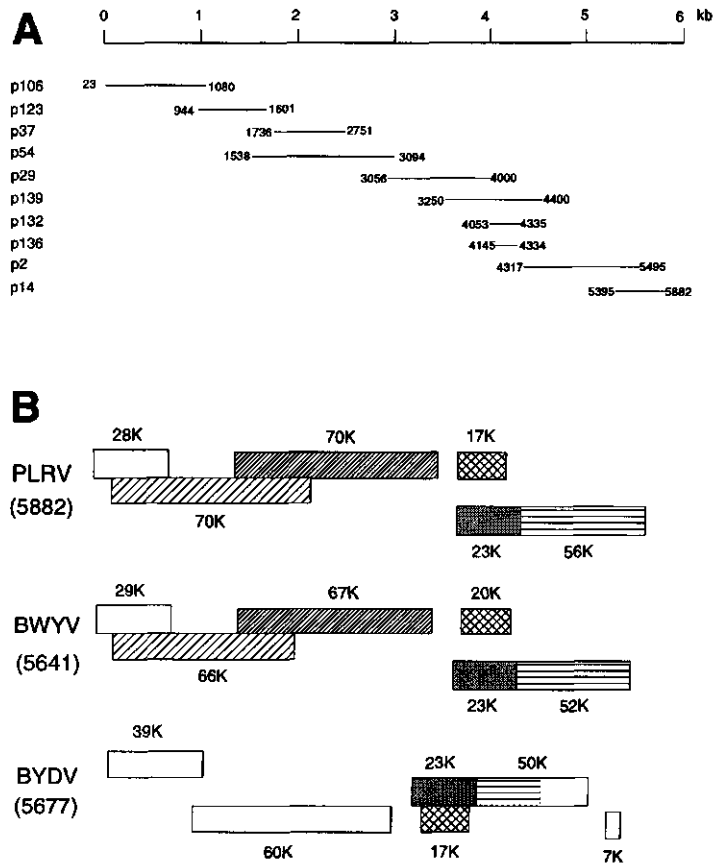


Figure 1: **(A)** Map of cDNA clones used to elucidate the nucleotide sequence of the PLRV RNA (p2, p14, p29, p37, p54 represent pUC19 clones; p106, p123, p132, p136, p139 represent lambda gt10 clones).
(B) Schematic representation of the organization of the PLRV genome and comparison of the open reading frames (ORFs) on the genomic RNAs of PLRV, BWYV and BYDV-PAV. The size in kDa (K) of the proteins encoded by each ORF is indicated. Similar shading indicates regions of high amino acid sequence homology amongst the ORFs of different viruses.

stranded cDNA was treated with 2 units T4 DNA polymerase to remove possible 3' overhangs from the first strand cDNA. The reaction was stopped by adding 1% SDS. The synthesized cDNA was purified by phenol extraction and ethanol precipitation.

In the first case the synthesized double-stranded cDNA fragments were blunt-end ligated into SmaI digested pUC19 followed by transformation of *Escherichia coli* strain DH5α. Recombinant plasmids were purified by a modified 'boiling preparation' procedure (Maniatis *et al.*, 1982). Transgenic bacteria were grown for 8 h in a rotary shaker at 37 ^{0}C. 1.5 ml of the bacterial culture was centrifuged for 1 min in an eppendorf centrifuge at full speed. The supernatant was carefully removed and the pellet was resuspended in 100 µl of STET (8% sucrose, 0.5% Triton X-100, 50 mM EDTA, 50 mM Tris-HCl pH 8.0), 5 µl lysozyme (10 mg/ml dissolved in STET) was added and the suspension was boiled for 1 min. Immediately afterwards the suspension was centrifuged for 10 min in an eppendorf centrifuge at full speed. The pellet was removed with a sterile tooth pick, and 105 µl of cold isopropanol was added to the supernatant followed by centrifugation for 5 min to precipitate the nucleic acids. The pellet was washed with 70% ethanol, dried and resuspended in 30 µl of sterile water. Recombinant plasmids were analyzed by restriction endonuclease digestion followed by agarose gel electrophoresis.

In the second case a commercially available kit (Amersham) was used to clone the cDNA fragments into lambda gt10 phages. According to the instructions of the supplier EcoRI adapters were added onto the cDNA fragments, followed by ligation into DNA lambda gt10 arms. The recombinant lambda DNA was *in vitro* packaged and *E. coli* strain NM514 was used for amplification of the recombinant phage. Recombinant lambda DNA was isolated by a modified method as described (Davis *et al.*, 1980). A single recombinant phage plaque was isolated and resuspended in 0.5 ml TM buffer (0.01 M Tris-HCl pH 7.5, 0.01 M $MgCl_2$). Bacterial cells (0.3 ml) at OD_{600}=0.6 were mixed and incubated with 0.3 ml resuspended phage particles. The cells were mixed with top-agarose and incubated for 8 h, until all cells were lysed. The plate was overlayed with 5 ml TM buffer and incubated for 8 h. The buffer was poured off and 500 µl was mixed with 1 µl DEPC, 12 µl 10% SDS and 60 µl 2 M Tris-HCl pH 8.5, 0.2 M EDTA to lyse the phage particles. Cell debris, proteins and the SDS were precipitated by adding 0.5 M KAc pH 6.0, followed by centrifugation. The supernatant was extracted with an equal volume phenol (phenol equilibrated with 10 mM Tris-HCl pH 7.6, 1 mM EDTA and 0.5 M KAc). After chloroform extraction the DNA was precipitated with ethanol. The DNA was analyzed by restriction endonuclease digestion. The inserted cDNA fragments were subcloned into EcoRI-digested pUC19 plasmids.

DNA sequence analysis

Sequence analysis was performed as previously described (Sanger *et al.*, 1977). Overlapping restriction fragments from cDNA clones were subcloned into M13 tg130/131 replicative form DNA (Kieny *et al.*, 1983) and sequenced using the dideoxy method with [α-^{35}S]thio-dATP as radioactive nucleotide. Both DNA polymerase I (Klenow fragment) and Sequenase (USB) were used as enzymes in sequencing reactions.

RNA sequence analysis

Direct dideoxy sequence determination on genomic RNA was performed as described (Huisman *et al.*, 1988). Synthetic oligonucleotides were used as primers and reverse transcriptase as enzyme. PLRV RNA was annealed to a suitable primer (in a 100-fold molar excess) in a buffer containing 50 mM Tris-HCl pH 8.3, 10 mM $MgCl_2$, 40 mM KCl by heating the mixture at 95 ^{0}C for 5 min and a subsequent incubation at 40 ^{0}C for 30 min. Dithiothreitol to a concentration of 10 mM, 2.5 units of reverse transcriptase and 10 µCi [α-^{35}S]dATP were added. This mixture was added to the termination mixtures. The final concentrations for dCTP, dGTP and dTTP were 62.5 µM and for dATP 12.5 µM. Each termination mixture contained one of the dideoxy NTPs at a final concentration of 18.75 µM; in the case of ddATP the final concentration was 6.25 µM. The samples were incubated for 30 min at 40 ^{0}C and were chased with 0.3 volumes of 0.5 mM of all dNTPs.

RESULTS

Viral RNA isolation

Prior to cDNA synthesis, the purified genomic viral RNA was analyzed on a denaturing agarose gel (Fig. 2). The molecular size of the PLRV RNA as estimated from the electrophoretic mobility was approximately 6 kilobases (kb), which is consistent with previously reported values (Mayo *et al.*, 1982). As shown in Figure 2, the purified RNA

was of high integrity and therefore suitable for cDNA synthesis.

Molecular cloning and sequence analysis

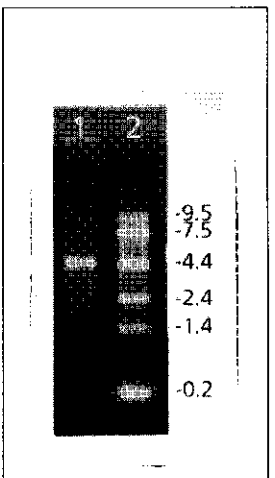


Figure 2: Denaturing agarose gel electrophoresis of PLRV RNA. Lane 1: viral RNA; Lane 2: RNA molecular weight marker RNA (BRL).

Two different procedures were used to obtain cDNA clones. The first procedure, in which reverse transcription on the viral RNA was initiated by random priming, followed by blunt-end cloning of the synthesized cDNA molecules in SmaI-digested pUC19 was less effective than the second procedure. The second procedure, initiation of the reverse transcription reaction by specific synthetic oligonucleotides and cloning of the double stranded cDNA in lambda gt10, produced 4 x 10^5 recombinant clones, while the first procedure yielded only 60 recombinant clones. Moreover cDNA clones obtained by the first procedure proved to be 'scrambled'. Nearly all clones contained several small cDNA fragments (approximately 80-100 nucleotides) not in accordance with the sequence of the other parts of the cDNA clones and actually corresponding to a different position on the PLRV RNA. A few recombinant clones were composed of 2 or 3 different cDNA fragments, each larger than 500 nucleotides. All clones, derived from the second procedure and used for sequence determination, were free from such aberrant sequences. The clones selected from both the libraries for sequence determination spanned approximately 99% of the viral genome (Fig. 1A). The 5'-proximal nucleotides were elucidated by direct dideoxy sequencing on PLRV genomic RNA by extension of an oligonucleotide complementary to nucleotides 98-115 (5'-ATCTTTGGTCAAAAAG C-3'). The sequence could be determined unequivocally up to the first nucleotide shown in Figure 3. The VPg covalently attached to the 5'-terminal nucleotide probably obscured one or two additional nucleotides from our scrutiny: they were observed as strong stops in all lanes of the sequence ladder. The total length of the sequence elucidated is 5882 nucleotides. This length corresponds well to the 6 Kb estimated by electrophoretic mobility in denaturing agarose gels, (Rowhani & Stace-Smith, 1979), and is close to the value of 6.1 Kb as estimated by

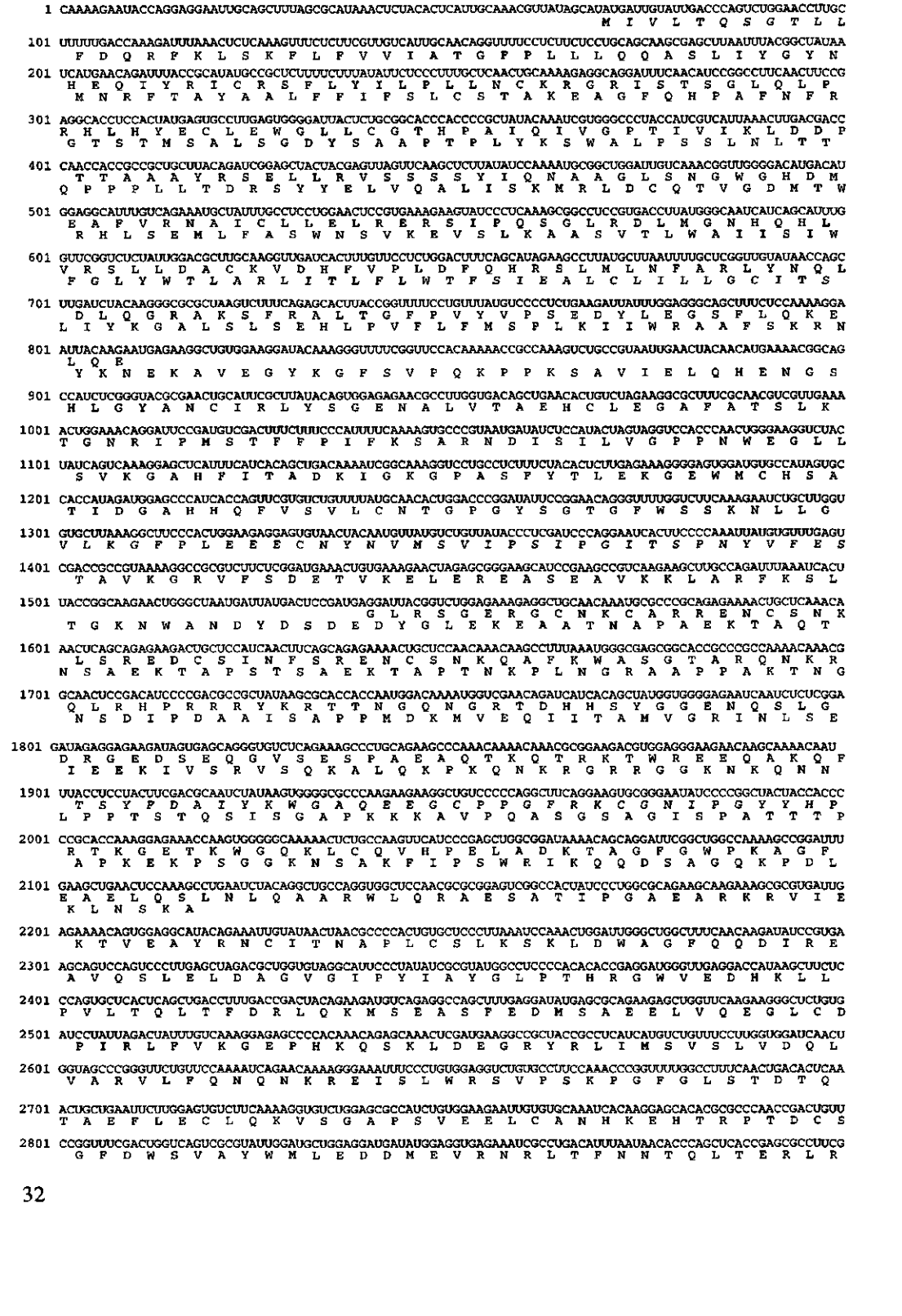
Prill *et al.* (1988), from endonuclease restriction mapping of cDNA.

Comparison of the deduced nucleotide sequence with the restriction endonuclease maps published by Prill *et al.* (1988) and Smith *et al.* (1988) shows no major differences. The XhoI site nearest the 3'-end reported by Prill *et al.* and Smith *et al.*, is absent in our sequence and five restriction sites present in our sequence are not shown by Prill *et al.*. All but three restriction sites present in our sequence are present in the restriction map as published by Smith *et al.* (1988).

Open reading frames

In Figure 3 the complete nucleotide sequence of the PLRV genomic RNA is shown. The first AUG start codon on the PLRV genome appears at position 70, thus the genome has a non-coding leader sequence of 69 nucleotides. Furthermore there are non-coding sequences in the middle (197 nucleotides) and at the 3'-terminal of the genome (140 nucleotides). In total the non-coding sequences of PLRV consist of 406 nucleotides, being 6.9% of the sequence. The minus strand does not contain ORFs of significant length.

The coding regions comprise six large open reading frames (ORFs) (Fig. 1B) of which the amino acid sequences are noted below the PLRV genomic RNA sequence in Figure 3. For brevity, ORFs will be referred to by their position on the genome, starting with the 5' proximal ORF as ORF1. ORF1 starts at the first AUG codon (position 70), terminates with a UGA stop codon (position 811) and could encode a product of 28,127 Da. The second ORF overlaps ORF1 by a start at position 203, in a different phase from ORF1, and stops at position 2120, corresponding with a putative translation product of 69,674 Da. Although lacking an AUG start codon ORF3 is proposed to start at position 1540, overlapping the second ORF, and to terminate at the UGA stop codon at position 3388. ORF3 encodes a putative protein of 69,622 Da. ORF4 is separated from ORF3 by a non-coding sequence of 197 nucleotides, starts at position 3588 and extends to a UAG stop codon at position 4212, hence coding for a protein of 23,233 Da. The ORF5 underlies ORF4 from position 3613 to the UGA stop codon at position 4081 and encodes for a protein of 17,344 Da. PLRV ORF6 is contiguous with ORF4, separated only by the amber stop codon of ORF4 and lacks its own AUG start codon. ORF6 extends to a stop codon (UGA) located at position 5739, thus corresponding



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2901 GGCUGCCUGGUUGAAGUGCAUAGGAAACUCCGUCCUAUGCCUGUCCGAUGGCACUUUACUUGCCCAAACUGUUCCCGGUGUGCAAAAGAGCGGAAGUUAC
       A  A  W  L  K  C  I  G  N  S  V  L  C  L  S  D  G  T  L  L  A  Q  T  V  P  G  V  Q  K  S  G  S  Y

3001 AAUACAAGUUCUUCCAACUCUAGAAUCCGGGUUAUGGCUGCCUAUCACUGUGGCGCCGACUGGGCAAUGGCCAUGGGGGACGAUGCUCUCGAAGCCCCCA
       N  T  S  S  S  N  S  R  I  R  V  M  A  A  Y  H  C  G  A  D  W  A  M  A  M  G  D  D  A  L  E  A  P  N

3101 ACUCCGAUCUAGAGGAGUAUAAAACACUAGGUUUCAAAGUCGAGGUAGGUCGAGAACUCGAAUUCUGUUCACACAUCUUCAGAAAUCCGACCCUCGCCGU
       S  D  L  E  E  Y  K  T  L  G  F  K  V  E  V  G  R  E  L  E  F  C  S  H  I  F  R  N  P  T  L  A  V

3201 UCCGGUCAACACCAACAAAAUGCUUUACAAGUUGAUCCAUGGUUAUAAUCCGGAAUGUGGCAAUCCAGAAGUGAUUCAAAACUAUCUGGCUGCAGUUUUC
       P  V  N  T  N  K  M  L  Y  K  L  I  H  G  Y  N  P  E  C  G  N  P  E  V  I  Q  N  Y  L  A  A  V  P

3301  UCUGUGCUGCAGGAACUCCGACACGAUCGUGAGCUCGUUGCCAAGCUCCACCAGUGGUUGGUUCCGAGUGCCACCACAAAAGAACACUGAAGGAGCUCAC
      S  V  L  Q  E  L  R  H  D  R  E  L  V  A  K  L  H  Q  W  L  V  P  S  A  T  T  K  E  H

3401 UAAAACUAGCCAAGCAUACGCGAGUUGCAAGCAUUGGAAGUUCAAGCCUCGUUACAUCAACCGGAUAAAAAUAGAUUUUAAAUUCUUAGCGGGAUUUGCUU

3501 UAGGAUUCUCAUCCGCAAUCCCAUUUUCAGUAGCCGGUUUAUAUUUAGUUUACCUAAAGAUUUCCUCCCACGUGCGAUCAAUUGUUAAUGAGUACGGUCG
                                                                                                  M  S  T  V  V

3601 UGGUUAAAGGAAAUGUCAAUGGCGGUGUACAACAACCAAGAAGGCGAAGAAGGCAAUCCCUUCGCAGGCGCGCUAACAGAGUUCAGCCAGUGGUUAUGGU
                    M  S  M  A  V  Y  N  N  Q  E  G  E  E  G  N  P  F  A  G  A  L  T  E  F  S  Q  W  L  W  S
       V  K  G  N  V  N  G  G  V  Q  Q  P  R  R  R  R  R  Q  S  L  R  R  A  N  R  V  Q  P  V  V  M  V

3701 CACGGCCCCUGGGCAACCCAGGCGCCGAAGACGCAGAAGAGGAGGCAAUCGCCGCUCAAGAAGAACUGGAGUUCCCCGAGGACGAGGCUCAAGCGAGACA
       R  P  L  G  N  P  G  A  E  D  A  E  E  E  A  I  A  A  Q  E  E  L  E  F  P  E  D  E  A  Q  A  R  H
       T  A  P  G  Q  P  R  R  R  R  R  R  G  G  N  R  R  S  R  R  T  G  V  P  R  G  R  G  S  S  E  T

3801 UUCGUGUUUACAAAGGACAACCUCAUGGGCAACUCCCAAGGAAGUUUCACCUUCGGGCCGAGUCUAUCAGACUGUCCGGCAUUCAAGGAUGGAAUACUCA
       S  C  L  Q  R  T  T  S  W  A  T  P  K  E  V  S  P  S  G  R  V  Y  Q  T  V  R  H  S  R  M  E  Y  S
       F  V  F  T  K  D  N  L  M  G  N  S  Q  G  S  F  T  F  G  P  S  L  S  D  C  P  A  F  K  D  G  I  L  K

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

4001 UUGCAAAGUAUCAUCCCUCCAGUCCUACGUCAACCAGUUCCAAAUUCCUCAGGGCGGCGCCAAAACUUAUCAAGCGCGGAUGAUAAACGGGGUAGAAUGG
       A  K  Y  H  P  S  S  P  T  S  T  S  S  K  F  L  R  A  A  P  K  L  I  K  R  G
       C  K  V  S  S  L  Q  S  Y  V  N  Q  F  Q  I  P  Q  G  G  A  K  T  Y  Q  A  R  M  I  N  G  V  E  W

4101 CACGAUUCUUCUGAGGAUCAGUGCCGGAUACUGUGGGAAAGGAAAUGGAAAAUCUUCAGAUACCGCAGGAUCCUUCAGAGUCACCAUCAGGGUGGCUUUGC
       H  D  S  S  E  D  Q  C  R  I  L  W  K  G  N  G  K  S  S  D  T  A  G  S  F  R  V  T  I  R  V  A  L  Q

4201 AAAACCCCAAAUAGGUAGACUCCGGACCAGAGCCUGGUCCAAGCCCACAACCAACACCCACUCCAACUCCCCAGAAGCACGAGCGAUUUAUUGCUUAUGU
       N  P  K  *  V  D  S  G  P  E  P  G  P  S  P  Q  P  T  P  T  P  T  P  Q  K  H  E  R  F  I  A  Y  V

4301 UGGCAUACCUAUGCUAACCAUUCAGGCCAGGGAGAGCGACGACCAAAUCAUAUUGGGUUCCUUAGGGAGCCAAAGGAUGAAAUAUAUAGAGGACGAGAAC
       G  I  P  M  L  T  I  Q  A  R  E  S  D  D  Q  I  I  L  G  S  L  G  S  Q  R  M  K  Y  I  E  D  E  N

4401 CAGAACUAUACAAAUGUUAGUUCUGAGUAUUACUCUCAAUCGAGCAUGCAAGCCGUCCCUAUGUAUUACUUCAAUGUCCCGAAAGGGCAAUGGUCAGUCG
       Q  N  Y  T  N  V  S  S  E  Y  Y  S  Q  S  S  M  Q  A  V  P  M  Y  Y  F  N  V  P  K  G  Q  W  S  V  D

4501 ACAUCAGCUGCGAAGGGUAUCAACCCACUAGCAGCACCUCGGAUCCAAACCGGGGUAGGAGUGACGGGAUGAUCGCGUAUUCCAAACGCGGAUUCCGAUUA
       I  S  C  E  G  Y  Q  P  T  S  S  T  S  D  P  N  R  G  R  S  D  G  M  I  A  Y  S  N  A  D  S  D  Y

4601 UUGGAAUGUUGGUGAAGCGGAUGGUGUAAAAAUUUCGAAGCUACGCAACGAUAACACCUACCGCCAAGGUCACCCAGAACUUGAAAUUAACUCGUGUCAU
       W  N  V  G  E  A  D  G  V  K  I  S  K  L  R  N  D  N  T  Y  R  Q  G  H  P  E  L  E  I  N  S  C  H

4701 UUUCGCGAGGGCCAACUCCUUGAACGGGACGCUACAAUUAGCUUCCACGUUGAAGCGCCUACUGAUGGGGCGAUUCUUUCUCGUUGGUCCCGCUAUCCAGA
       F  R  E  G  Q  L  L  E  R  D  A  T  I  S  F  H  V  E  A  P  T  D  G  R  F  F  L  V  G  P  A  I  Q  K

4801 AAACCGCAAAGUAUAACUAUACUAUCUCAUACGGUGACUGGACGGACCGAGACAUGGAACUGGGGCUGAUCACCGUGGUGCUUGAUGAACAUUUAGAAGG
       T  A  K  Y  N  Y  T  I  S  Y  G  D  W  T  D  R  D  M  E  L  G  L  I  T  V  V  L  D  E  H  L  E  G

4901 CACUGGUUCGGCUAACAGAGUGCGGCGGCCCCCACGGGAGGGCCACACCUAUAUGGCCUCGCCGCGCGAACCGGAAGGAAAACCGGUUGGAAAUAAACCA
       T  G  S  A  N  R  V  R  R  P  P  R  E  G  H  T  Y  M  A  S  P  R  E  P  E  G  K  P  V  G  N  K  P

5001 AGGGACGAAACCCCGAUACAAACGCAGGAAAGACAACCUGAUCAAACUCCGUCUGACGACGUAUCCGAUGCUGGUUCGGUAAACAGCGGCGGCUCAACUG
       R  D  E  T  P  I  Q  T  Q  E  R  Q  P  D  Q  T  P  S  D  D  V  S  D  A  G  S  V  N  S  G  G  S  T  E

5101 AGUCGCUGCAAUUGGAGUUCGGGGCAAAUUCAGAUAGUACCUACGAUGCUACAGUCGAUGGUACAGACUGGCCCAGAAUUCCUCCACCAAGGCACCCAGC
       S  L  Q  L  E  F  G  A  N  S  D  S  T  Y  D  A  T  V  D  G  T  D  W  P  R  I  P  P  P  R  H  P  P

5201 UGAACCUAGAGUUUCCGGCAAUUCAAGAACUGUUACUGACUUUUCUCCGAAAGCCGAUCUAUUGGAGAAUUGGGAUGCCAAACACUUCGACCCUGGUUAU
       E  P  R  V  S  G  N  S  R  T  V  T  D  F  S  P  K  A  D  L  L  E  N  W  D  A  K  H  F  D  P  G  Y

5301 UCCAAAGAAGAUGUCGCUGCUGCUACUAUUAUAGCGCACGGCAGUAUUCAAGAUGGGCGAAGUAUGUUAGAGAAGAGAGAGGAAAAUGUCAAGAACAAAA
       S  K  E  D  V  A  A  A  T  I  I  A  H  G  S  I  Q  D  G  R  S  M  L  E  K  R  E  E  N  V  K  N  K  T

5401 CCUCCUCCUGGAAGCCCCCGUUACCUAAAGCGGUGAGCCCAGCCAUAGCCAAAUUGCGCUCGAUUCGCAAAUCCCAACCCCUCGAGGGAGGGACCCUUAA
       S  S  W  K  P  P  L  P  K  A  V  S  P  A  I  A  K  L  R  S  I  R  K  S  Q  P  L  E  G  G  T  L  K

5501 GAAAGACGCCACUGAUGGUGUCUCAUCUAUUGGCAGUGGUUCUCUAACAGGUGGCACGCUUAAGAGGAAGGAAACUAUCGAAGAGCGUUUACUGCAGACC
       K  D  A  T  D  G  V  S  S  I  G  S  G  S  L  T  G  G  T  L  K  R  K  E  T  I  E  E  R  L  L  Q  T

5601 UUAACAACUGAACAAAGGCUGUGGUACGAGAAUUUGAAGAAAACUAACCCUUUAGCCUGCUACCCAAUGGCUGUUUGAAUAUCAGCCACCUCCCCAAGUGG
       L  T  T  E  Q  R  L  W  Y  E  N  L  K  K  T  N  P  L  A  A  T  Q  W  L  F  E  Y  Q  P  P  P  Q  V  D

5701 AUAGAAACUUAGCUGAAAAGCCAUUCCAAGGGAGGAAAUGAGUCGACUCACGACUUAAAACUGAGUGUCCGCCGGACAUUAAGCGGAACGAAAGCCGAAA
       R  N  L  A  E  K  P  F  Q  G  R  K

5801 GGUGAUUAGGCUCUCAACGCCUGCUAGAGACCGUCGAAAGACGCGACUGUGUACCCAGGAUCCUCUUACAGGGUUGUGUAGU 5882
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Figure 3: The nucleotide sequence of PLRV RNA. The deduced amino acid sequences of long open reading frames are depicted below the nucleotide sequence.

with a coding capacity for a protein of 56,476 Da.

In Table 1, the main characteristics of the proteins encoded by the various ORFs of the three sequenced luteoviruses are summarized. Most proteins have high isoelectric points, except for the ORF5 product of PLRV, BWYV and the corresponding ORF4 product of BYDV.

Expression of the PLRV ORFs

As shown in Figure 4, *in vitro* translation of purified PLRV RNA, using rabbit reticulocyte lysate and wheat germ lysate, resulted in two major products synthesized in both reactions. The largest protein is approximately 70 KDa and probably corresponds to the PLRV ORF2 product. The smaller protein is approximately 28 kDa, which is similar in size as the ORF1 protein. Besides these two proteins present in both reactions, several other translation products are present in one translation only. Possibly, these products resulted from proteolytic degradation or reflect the different preferences for translational sequences of both translation lysates employed. A proteins of approximately the size (23 kDa) of the PLRV coat protein could be detected in the translation products of the reticulocyte lysate, however, such a product was absent in the translation products from the wheat germ lysate. Possibly, the coat protein is expressed via a sub-genomic messenger. Mayo *et al.* (1984) reported the presence of sub-genomic RNA in infected cells. In the non-coding regions of the three sequenced luteoviruses similar nucleotide sequences can be distinguished. These sequences might function as promoter sequences for the sub-genomic RNAs.

Table 1: Molecular weights (MW) and isoelectric points (pI) of the proteins encoded by the ORFs of PLRV, BWYV and BYDV RNA.

	PLRV		BWYV		BYDV-PAV	
	MW	pI	MW	pI	MW	pI
ORF1	28.1	8.2	29.1	8.2	-	-
ORF2	69.7	10.1	66.2	9.9	38.7	5.9
ORF3	69.6	7.8	66.8	8.3	60.4	9.2
ORF4	23.3	11.9	22.5	11.9	22	12
ORF5	17.3	6.8	19.6	8.5	17.1	9.3
ORF6	56.5	5.2	51.4	4.8	49.7	4.8

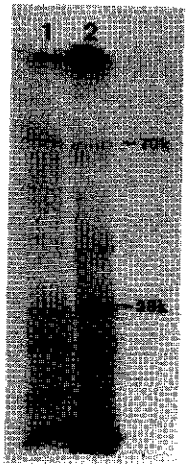

Figure 4: Fluorogram showing the products of an *in vitro* translation of PLRV genomic RNA, using rabbit reticulocyte lysate or wheat germ lysate. Lane 1: PLRV RNA translation products employing reticulocyte lysate; lane 2: PLRV RNA translation products employing wheat germ lysate.

The ORF1 and ORF2 are probably directly expressed from the genomic RNA. Usually, viral RNAs are expressed in a mono-cistronic fashion, though exceptions on this rule are known (Kozak, 1986). The start codon of the ORF1 has to be considered as suboptimal compared to the start codon of ORF2 (Kozak, 1989). Possibly, ribosomes scanning the RNA slip past the first, suboptimal and therefore leaky, start codon and initiate translation at the, more optimal, ORF2 start codon located 133 nucleotides downstream of the first one.

The first AUG codon in ORF3 is located at position 2440, more than 900 nucleotides downstream from the preceding stop codon. Initiation of translation from this start codon would result in the synthesis of a product of 35,607 Da, while the complete coding capacity of the ORF2 corresponds to a protein of 69,674 Da. The putative PLRV ORF3 product shows a high homology with the BWYV ORF3 product. For BWYV ORF3, evidence has been obtained that it is expressed via a -1 translational frameshift (Brault & Miller, 1992). Moreover, in the ORF 2 and 3 overlapping region of the RNA a pseudoknot structure can be formed (Ten Dam *et al.*, 1990). Pseudoknots are proposed to play an important role in the -1 ribosomal frameshift in the F_1 and F_2gene overlap region of the coronaviral avian infectious bronchitis virus (IBV) (Brierley *et al.*, 1989). On the basis of both the presence of a pseudoknot structure and the strong sequence homology between the ORFs of PLRV and BWYV, we propose that PLRV ORF3 is expressed via translational frameshift, like the analogous ORFs in BYDV and BWYV RNA.

As mentioned above, PLRV ORF6 lacks its own AUG start codon and is contiguous with ORF4, separated only by the amber stopcodon of ORF4. The same situation is observed for ORF5 of BYDV and ORF6 of BWYV, which show homologies to PLRV ORF6 of 45 and 59%, respectively. The sequences flanking the amber stop codons are identical in all three viruses (CCAAA<u>UAG</u>GUAGAC). Amber stop codons are

renowned for their leakiness (Van Tol *et al.*, 1980). Furthermore, for BWYV evidence was presented that readthrough of ORF4/6 indeed occurs (Veidt *et al.*, 1988). Therefore it seems likely that ORF6 is expressed via translational readthrough, by suppression of the amber stopcodon, which would result in a protein of 79,709 Da.

Homologies with other plant viruses

The genomic organization of PLRV seems to be very similar to the genomic organization of BWYV and, to a lesser extent, BYDV-PAV. Figure 1B compiles the genomic organization of all three luteoviruses. Comparison of the deduced amino acid sequences of the PLRV ORFs with the sequences of BWYV and BYDV reveals homology with nearly all of the BWYV ORFs and with several of the BYDV ORFs (Fig. 2B). The total genome lengths of the three viral genomes are comparable, the PLRV genome being the longest (5882 nucleotides). Also the base composition of the PLRV RNA (28%A; 25%C; 24%G; 23%U) is similar to those of BYDV RNA (29.5%A; 24%C; 24.5%G; 22%U) and BWYV RNA (28%A; 25%C; 24%G; 23%U).

The PLRV ORF1 product shows no amino acid homology with any of the different ORFs of BWYV or BYDV, in spite of the fact that the BWYV ORF1 is similar in size and position on the genome. The PLRV ORF1 peptide also shows no homology with any other published plant viral proteins. In a database search, the protein showed a low degree of similarity with various membrane-associated proteins, like cytochrome d, terminal oxidase and NADH dehydrogenase. All similarities were located in the, strongly hydrophobic, N-terminal part of the ORF1 protein. These data suggest that the PLRV ORF1 protein is membrane-linked.

The putative product of PLRV ORF2 shows homology (54%, calculated with the UWGCG programme GAP) with the BWYV ORF2 product. The ORF2 product contains amino acid sequence motifs found in plant viral proteases. The putative protease belongs to the type of the trypsin/3C-like proteases, with a serine protease catalytic activity (Gorbalenya *et al.*, 1989; Bazan & Fletterick, 1989). Using sequence analysis, four conserved regions in these proteases can be distinguished (Fig. 5). These boxes encase the conserved putative catalytic triad of His, Asp, and Ser residues as well as residues that contribute to substrate binding. The function of this protease in the expression of the PLRV-encoded proteins is unclear.

BOX I *BOX II*

ENALVTAE**H**^{255}CLEGAFATSLK-aa$_{17}$-RN**D**^{268}ISILVGPP-

BOX III *BOX IV*

-aa$_{51}$-LCNT**G**PGY**S**^{354}**G**TGFWSSK NLL**GV**LKGFPL

Figure 5: Trypsin Ser-proteases motifs as found in the ORF2 product of PLRV. The putative catalytic residues are represented in bold and underlined. Positions in the ORF2 protein of the amino acids constituting the putative catalytic triad are indicated.

The putative PLRV ORF3 peptide contains the amino acid sequence motifs (---$^{S}/_{T}$G--T---N$^{S}/_{T}$18-37aaGDD---) which are found in all RNA-dependent RNA polymerases of eukaryotic RNA viruses sequenced to date (Koonin, 1991) and therefore this product most likely represents the PLRV-encoded RNA-dependent RNA polymerase. The predicted amino acid sequence of ORF3 shows considerable homology with BWYV ORF3 (70%) and the putative RNA-dependent RNA polymerase (43%) of southern bean mosaic virus (SBMV) (Wu *et al.*, 1987). Strikingly ORF2 of BYDV showed no homology with the PLRV ORF3 but, instead, showed homology with the putative RNA polymerase of carnation mottle virus (CarMV) (Miller *et al.*, 1988a).

Two nucleic acid helicase consensus motifs (IV and VI) can be distinguished in the ORF2 and ORF3-encoded peptides (Habili & Symons, 1989). Helicase motif VI (QSKLDE**GRY**RLIMSVS), believed to provide the nucleic acid binding site, is located in the ORF3-encoded part. The helicase motif IV (N**YFV**ESTAV) is located in the ORF2-encoded part, no putative function has yet been stipulated for this sequence motif. No NTP-binding site consensus sequence could be detected in any of the PLRV ORFs (Higgins *et al.*, 1986).

As shown in Figure 6 PLRV ORF4 shows considerable homology with BYDV ORF3 (57%) and BWYV ORF4 (72%). Since BYDV ORF3 has been shown to correspond to the BYDV coat protein (Miller *et al.*, 1988b), the conclusion seems justified that ORF4 of both BWYV and PLRV encodes the respective viral coat proteins. The strong homology in amino acid sequences can explain the serological

cross-reactivity between the different luteoviruses. The putative coat protein of PLRV has a high isoelectric point, resulting in a positive charge. This high positive charge of the ORF4 product is mainly caused by the arginine-rich sequence near the N-terminus. Several other viruses, including SBMV have coat proteins positively charged at the N-terminal end. For SBMV it has been suggested that the positively charged part of the coat protein reacts with negatively charged groups of the RNA (Hermodson *et al.*, 1982). While the ORF4 protein has a positive charge, the ORF6 protein has a very low isoelectric point, resulting in a negative charge. Thus the complete readthrough product (ORF4+6) has a negatively charged C-terminal end and a positively charged N-terminal end, the net charge of the overall protein being positive. It has been shown that the so-called readthrough proteins of luteoviruses are associated with virus particles and are suggested to play a role in aphid transmission (Bahner *et al.*, 1990; Reutenauer *et al.*, 1993).

For BYDV and BWYV it has been proposed that the ORFs, underlying the coat protein gene, code for the respective VPg's. However, the VPg of PLRV has been estimated to have a molecular mass of 7 kDa only (Mayo *et al.*, 1982), whereas the

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          1                                                        50
cpBWYV    MNTVVgRrii NG....rRRp RRQT..RRAq RpQPVVVVQT sRAtqRRPRR
cpPLRV    MsTVVvkGnv NGgvqqpRRR RRQslrRRAn RVQPVVmV.T aPgqPRRRRR
cpBYDV    MNsVgrRGpr ranqngtRRR RRrT...... .VrPVVVVQp nrAgPRRRng

          51                                                      100
cpBWYV    RRrGNnRtGR TvptRGaGSS ETFVFSKDNL aGsSSGAITF GPSLSDCPAF
cpPLRV    RRgGNrRsrR TgVpRGrGSS ETFVFtKDNL mGNSqGAfTF GPSLSDCPAF
cpBYDV    RRkG..RgGa nfVfRptGgt EvFVFSvDNL kaNSSGAIkF GPSLSDCPAl

          101                                                     150
cpBWYV    SnGmLKAYHE YKIsmvILEF VSEASSqnSG SIAYELDPHC KlnSLsStIN
cpPLRV    kDGILKAYHE YKITSILLqF VSEASStsSG SIAYELDPHC KvSSLqSYvN
cpBYDV    SDGILKsYHr YKITSIrvEF kShASantaG aIfiELDtaC KqSaLgSYIN

          151                                                     200
cpBWYV    kFgItKpGkr aFtAsyINGt EWHDvaEDQF RILYKGNG.S SsiAGSFRIT
cpPLRV    qFqIpqgGaK TyqArmINGv EWHDSsEDQc RILwKGNGkS SDTAGSFRvT
cpBYDV    sFtIsKtasK TFrseaINGk EfqeStiDQF wmLYKaNGtt tDTAGqFiIT

          201
cpBWYV    IkcqfhNPK*
cpPLRV    IrVaLqNPK*
cpBYDV    msVsLmtaK*
```

Figure 6: Alignment of the coat protein sequences of BYDV, PLRV and BWYV. Amino acid residues differing from the consensus are shown in small lettering, gaps are indicated by dots.

coding capacity of ORF5 predicts a protein of 17 kDa. Possibly, this ORF encodes a VPg-precursor molecule from which, at the onset of RNA synthesis, the VPg molecule is released, as has been suggested for CPMV (Goldbach & Van Kammen, 1985).

DISCUSSION

In this chapter the nucleotide sequence of the PLRV genomic RNA has been determined and described. The genomical RNA is 5882 nucleotides long and contains six different ORFs. Comparison of the deduced PLRV sequence with the recently determined nucleotide sequences of BWYV and BYDV-PAV reveals both similarities and intriguing differences between the luteovirus genomes. The PLRV genome as determined is 5882 nucleotides in length, whereas both BYDV (5677 nucleotides) and BWYV (5641 nucleotides) are smaller. The genomic organization of PLRV appears to be similar to that of BWYV and to a lesser extent to that of BYDV-PAV. The putative products of the ORFs of PLRV and BWYV show high homologies in amino acid sequence except for the ORF1 products. Although both ORF1s are similar in size, they are completely different in amino acid sequence. An equivalent of this ORF is even completely absent in BYDV-PAV RNA. The function of the ORF1 is unclear, for PLRV there is circumstantial evidence that the ORF1 protein is membrane-associated. For BWYV, however, the ORF1 amino acid sequence does not suggest an association to membranes. In their ecological aspects, PLRV and BWYV mainly differ in their potential host range: PLRV is only able to infect a restricted number of plant species, while BWYV infects a wide range of plant species (Rochow & Duffus, 1981). Therefore, it is tempting to suggest that the ORF1 proteins play a role in host determination, but more data will be needed to verify this hypothesis.

Figure 7: Putative functions of the PLRV ORFs. GDD = putative polymerase amino acid sequence motif; IV = helicase motif IV; VI = helicase motif; PRO = Ser protease (3-C like); CP = coat protein.

Remarkably the putative RNA-dependent RNA polymerases of PLRV and BWYV (ORF3 products) show a high homology to the (putative) RNA polymerase of SBMV, while the RNA polymerase of BYDV-MAV shows homology with the RNA polymerase of CarMV and no homology with the other luteoviral or SBMV polymerases. Until now this is the only example of viruses of the same taxonomic group having RNA polymerases corresponding to different gene families.

In contrast to the genes located in 5' halves of the genomes, comparison of the 3' terminal gene clusters of all three luteoviruses reveals a similar gene organization and high amino acid sequence homologies (Fig. 1B). The 3'-terminal gene clusters are obviously evolutionary related, while the RNA-polymerases of PLRV (and BWYV) and BYDV seem to have different ancestors. This observation strongly supports the hypothesis that RNA viruses evolve by interviral gene exchange.

Considering the homology between the different luteoviruses it seems that PLRV is more closely related to BWYV than to BYDV. It is suggested that BYDV and BWYV might be considered as distinct subtypes of the luteovirus group. In view of the homologies observed between the ORFs of PLRV and BWYV, PLRV appears to be part of the BWYV subgroup. However, differences between PLRV and BWYV, e.g. the non-homologous ORF1s, make PLRV and BWYV distinctly different members.

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CHAPTER 3

Expression of the potato leafroll luteovirus coat protein gene in transgenic potato plants inhibits viral infection

ABSTRACT

Transgenic potato plants, cultivar Désirée, were produced that contained the coat protein gene of potato leafroll luteovirus (PLRV). The transformed potato plants expressed the PLRV coat protein (CP) RNA sequences but accumulation of coat protein in transgenic tissues could not be detected. Upon inoculation with PLRV, the PLRV-CP RNA expressing potato plants showed a reduced rate of virus multiplication.

This chapter is a slightly modified version of Van der Wilk *et al.*, (1991). Plant Mol. Biol. 17:431-439.

INTRODUCTION

Potato leafroll luteovirus (PLRV), the causal agent of one of the major viral diseases of potato, is responsible for large crop losses. Yields of PLRV-infected plants may be reduced by as much as 50% while the worldwide damage is estimated at approximately 10% yield loss or a yearly loss of 20 x 10^6 tons of potatoes (Kojima & Lapierre, 1988).

All commercial potato varieties are, to a greater or lesser extent, susceptible to PLRV infection. Resistance breeding to control the virus has sofar been hampered by the lack of readily available resistance genes. All resistance traits available in potato breeding lines are thought to be multigenic and monogenic dominant resistance genes conferring a high level resistance against PLRV are absent (Gibson *et al.*, 1988). Genetically engineered coat protein-mediated protection has been shown to be a promising approach for the control of a number of plant viral diseases. In some aspects coat protein-mediated protection is similar to 'cross-protection', i.e. the phenomenon whereby infection of a plant with a mild strain of a virus is used to reduce susceptibility to infection with a virulent strain of the same virus (Sherwood, 1987).

By expression of the coat protein of a virus in transgenic plants, a delay or even complete inhibition of infection with the corresponding virus has been observed. This has been shown for viruses belonging to different taxonomic groups, like tobamo- (Nelson *et al.*, 1987), tobra- (Van Dun & Bol, 1988), potex- (Hemenway *et al.*, 1988; Hoekema *et al.*, 1989), poty- (Lawson *et al.*, 1990), ilar- (Loesch-Fries *et al.*, 1987; Tumer *et al.*, 1987) and cucumoviruses (Cuozzo *et al.*, 1988). All these viruses differ substantially in morphology, genome organization and replication strategy, but they are all mechanically transmissible and the presence of their viral particles in infected plants is not confined to specific plant tissues.

PLRV is, like all other luteoviruses, dependent on aphids for its transmission and is not mechanically transmissible (Harrison, 1984). In infected plants the isometric virus particles are found in the phloem only. Aphids acquire the virus by feeding on the phloem of infected plants and transmit the virus, in a persistent manner, directly to the phloem of healthy plants.

The genome of PLRV is a single-stranded, messenger-sense RNA which is characterized by a small protein (VPg) covalently attached to the 5' terminus (Mayo *et al.*, 1982). The nucleotide sequence of the genomic RNA of PLRV has been determined

(Van der Wilk *et al.*, 1989). The sequence revealed six open reading frames. The open reading frame corresponding with the coat protein gene was identified by amino acid sequence homology with the coat protein of barley yellow dwarf virus, the type species of the luteovirus genus (Miller *et al.*, 1988). In this study, the potato cultivar Désirée was transformed with the PLRV-CP gene. The resulting transgenic plants were tested for expression of this viral gene and for protection against aphid inoculation with PLRV.

Materials and methods

Cloning of the PLRV coat protein constructs

Several cDNA clones collectively spanning the complete PLRV genome were isolated (Van der Wilk *et al.*, 1989). For construction of the PLRV-CP expression vectors, a fragment encoding the CP sequence was excised from a pUC18 plasmid and cloned into M13mp18. By site-directed mutagenesis the DpnI site, located eight nucleotides upstream of the AUG start codon, was modified into a BglII site and the fragment was recloned into pUC18. The AccI site immediately downstream of the UAG stop codon was filled in using DNA polymerase I (Klenow fragment) and the blunt end supplied with a BglII linker. The resulting fragment was excised with BglII and cloned in the BamHI site of pMOG181 (Fig. 1). The construct was checked for deletions or mutations by sequence analysis and *in vitro* translation. Two constructs were selected, one with the CP gene in the sense orientation and the other with the CP gene in the antisense orientation. These constructs were digested with EcoRI and HindIII and the fragments containing the chimaeric CP gene were placed into the binary vector pMOG23 (Sijmons *et al.*, 1990), giving rise to plasmids pLRV-S and pLRV-A.

In vitro translation of the coat protein construct

The coat protein was subcloned into pBluescript SK^+. The construct DNA was digested with EcoRI, RNase A treated and subsequently incubated with proteinase K (50 µg/ml), in the presence of 0.5% SDS. After phenol extraction and ethanol precipitation,

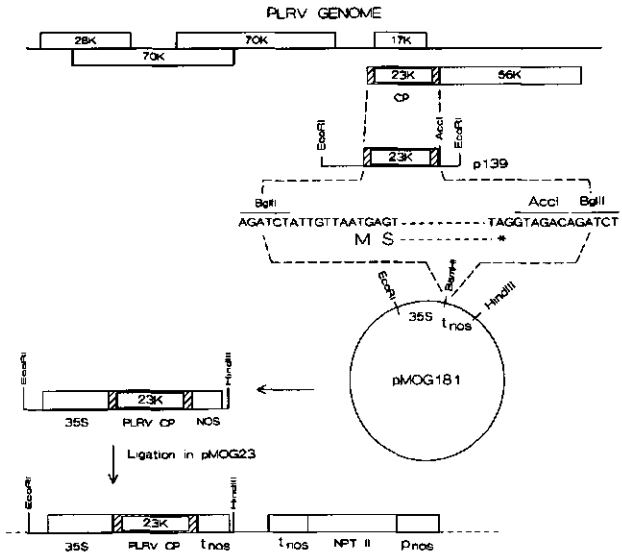

Figure 1: Schematic representation of the construction of the plant expression vectors pLRV-S and pLRV-A used to express the PLRV-CP gene in transgenic potato plants. 35S = cauliflower mosaic virus (CaMV) 35S promoter; p_{nos}/t_{nos} = nopaline synthase promoter/ terminator sequence

the DNA was resuspended in sterile water and used for synthesizing T3 RNA transcripts. RNA was synthesized using a commercially available kit (Stratagene), following the manufacturers instructions. The transcripts were checked for size on a denaturing agarose gel and translated *in vitro*, using a commercial kit (Bethesda Research Laboratories). 0.5 µg RNA was added to 30 µl reaction mixture containing 10 µl nuclease treated (3x) rabbit reticulocyte lysate, translation buffer, 1 µl ribonuclease inhibitor, 50 µM amino acid mixture (minus methionine), 5 µCi [^{35}S] methionine (1000-1500 Ci/mmol). After incubation at 30 ^{0}C for 1 h, the mixture was placed on ice to stop the reaction. Subsequently, it was incubated with 1 mg/ml pancreatic RNase at 30 ^{0}C for 1 h to hydrolyse radioactive aminoacyl-tRNAs. The translation products were analyzed by polyacrylamide SDS gelelectrophoresis, followed by fluorography. 10 µl of the

translation reaction mixture was loaded onto a 15% SDS polyacrylamide gel. After electrophoresis the proteins were fixed in the gel, followed by a 15 min incubation in amplify solution (Amersham). Fluorography was carried out by placing a XAR-5 (Kodak) film against the dried gel for 48 h.

Potato transformation

Transformation of potato plants was done as previously described (Hoekema *et al.*, 1989). Potato tuber discs, cultivar Désirée, were cocultivated with *Agrobacterium tumefaciens*, strain LBA 4404, harbouring plasmid pLRV-S or pLRV-A. For each cocultivation 500 tuber discs were used. Selection for transformation was done on medium containing kanamycin (100 µg/ml). Kanamycin resistant shoots were cut off and placed onto rooting medium, propagated axenically and transferred to soil.

Analysis of transgenic potatoes on Northern and Southern blots

For Northern analysis, total RNA was isolated from transgenic leaf material as previously described (Verwoerd *et al.*, 1989) and 20 µg aliquots were loaded onto a denaturing 1% agarose gel containing 2.2 M formaldehyde. After electrophoresis the RNA was transferred to a Hybond-N membrane (Amersham) and hybridized with a radiolabelled PLRV-CP probe.

For Southern analysis high molecular weight DNA was isolated as described (Mettler, 1987). DNA was digested with EcoRI and HindIII and 10 µg of DNA was loaded on a 1% agarose gel. After gel electrophoresis the DNA was transferred to GeneScreen-Plus (NEN-DuPONT) and probed with a radiolabelled CP fragment.

Immunoblot analysis and enzyme-linked immunosorbent assay (ELISA)

For immunoblot analysis leaf discs of one centimeter diameter were ground in 200 µl 3x Laemmli buffer (Laemmli, 1970). Protein concentrations were determined using BCA protein assay reagent (Pierce Chemical Co.). Different amounts, ranging from 10 to 200 µg, of soluble leaf proteins were boiled for 5 minutes, loaded onto a 12.5% polyacrylamide SDS gel and run at 200 mA (Bio-Rad Mini-PROTEAN II

electrophoresis system). The gels were blotted onto nitrocellulose using a semi-dry transfer system. The blots were preincubated with a blocking reagent (Boehringer Mannheim) and incubated with anti-PLRV IgG. Several different antisera were used, including polyclonal IgG's from rabbit, mouse and rat, Mabs and IgG-conjugates. The antisera were kind gifts of J.F.J.M. van den Heuvel (Department of Virology, Agricultural University Wageningen), D.Z. Maat (Research Institute for Plant Protection), T. Kühne (Institut für Phytopathologie Aschersleben, GDR) or purchased from Boehringer Mannheim. All antisera were raised against intact, non-denatured virions.

ELISA was performed as described, including amplification of the enzyme reaction (Van den Heuvel & Peters, 1989). Leaf discs of one centimeter diameter were ground in 0.5 ml extraction buffer (0.2% ovalbumin, 2% polyvinylpyrrolidone, 0.05% Tween 20 in PBS), 200 µl of the suspension was used per well in the test. The samples were simultaneously incubated with the conjugate for 16 h at 4 ^{0}C. For amplification of the enzyme reaction 100 µl of 0.2 mM NADP in 0.05 M diethanolamine (pH 9.5) was added to each well. After incubation at 20 ^{0}C for 30 min. alkaline phosphatase activity was blocked by adding 15 µl of 0.5 M 4-nitrophenyl disodium ortho-phosphate in 0.025 M phosphate buffer pH 7.4 to each well. Subsequently 150 µl of amplification reaction mixture was added per well. The stock amplification reaction mixture consisted of 700 units of alcohol dehydrogenase, 100 units of lipoamide dehydrogenase (type VI), 3% ethanol and 1 mM p-iodonitrotetrazolium violet in 15 ml of 0.025 M phosphate buffer pH 7.0. Colour development was allowed to proceed at room temperature and absorbency values were read at 492 nm.

Transmission of PLRV to transgenic plants

Myzus persicae (Sulzer), Wmp1, was maintained on *Brassica napus* L. susp. *oleifera* (oilseed rape) in a greenhouse under controlled conditions (Reinink *et al.*, 1989). Mature apterae, confined to leaf cages, were transferred daily to leaves of oilseed rape plants. Nymphs were used for the protection experiments. The aphids acquired the virus by feeding for 4 days on virus-infected young *Physalis floridana* Rydb. plants. Inoculation of the transgenic potato plants was carried out by confining 10 viruliferous nymphs per plant for a 9 day access period. Since the virus titer is not uniform within

the infected plant, virus infection was monitored for each individual plant by taking samples of the lower, middle and top leaves at each time-point. For each time-point, 3 leaf discs of each plant were pooled, ground in extraction buffer, and screened for the presence of virus using the 'cocktail' ELISA procedure without enzyme amplification.

Tubers from infected control and transgenic plants were harvested and stored at 4 ^{0}C. After 4 to 6 months of storage at 4 ^{0}C, the tubers were planted and subsequently virus titers were determined in the emerging shoots by ELISA.

Statistical analysis

Statistical analysis of the A_{405} ELISA-values was done with the help of the computer programme Statistical Application Systems of the SAS Institute Inc. The general linear models procedure was used to calculate the standard error of the mean (MSE). Statistical significance was determined with the procedure of the least significant difference (LSD). $LSD=t_{0.025[r]}[\sqrt{(2/n)}].MSE$

RESULTS

Construction of the PLRV expression vector and transformation of potato plants

As shown in Figure 1, the coat protein gene of PLRV starts on the viral genomic RNA at position 3588 and extends to an UAG stop codon at position 4212. Contiguous with the coat protein gene another open reading frame, separated only by the amber stop codon, is present. This open reading frame is probably expressed by translational readthrough from the coat protein gene, to yield a protein of approximately 79 kDa (Veidt *et al.*, 1988). In spite of the putative leakiness of the UAG stopcodon and sub-optimal start codon (Kozak, 1989), the coat protein gene was cloned in its unaltered, natural appearance.

For this purpose a BglII site was created by site directed mutagenesis, at a position seven nucleotides upstream of the AUG start codon. The AccI site directly downstream of the UAG stop codon was used to create a BglII site (Fig. 1). The CP gene was cloned in both the sense and antisense orientation in the BamHI cloning site of the plasmid

pMOG181 between the cauliflower mosaic virus (CaMV) 35S promoter, with a double enhancer, and the transcription termination signal of the nopaline synthase *(nos)* gene. These constructs were placed in a binary vector (pMOG23) (Sijmons *et al.*, 1990) (Fig. 1). Transformation of tuber discs of potato cultivar Désirée was mediated by *A. tumefaciens* (Hoekema *et al.*, 1989). Using this procedure, we analyzed twenty-five independent transformants containing the CP gene in the sense orientation (CP-s) and forty-one transformants containing the CP gene in the antisense orientation (CP-a).

Expression of the PLRV-CP gene constructs in transgenic potatoes

All transgenic potato plants were analyzed on Northern blots for expression of PLRV-CP gene transcripts. Using a CP coding probe, a single transcript of the expected size of approximately 800 nucleotides was detected in all transformed plants tested. Remarkably, the transgenic plants containing the CP gene in the antisense orientation showed a relatively lower expression of CP gene RNA sequences than the CP-s transgenic plants. The levels of expression for two plants containing the sense and for two plants containing the antisense CP gene construct are shown in Figure 2. When signal intensities of the transcripts were compared to known standards, it was estimated that the transgenic tissues contained between 30 and 100 pg of transcript RNA per µg of total plant RNA, depending on the transgenic plant line tested (data not shown).

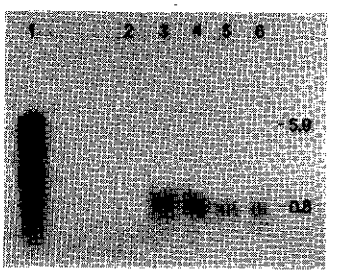

Figure 2: Northern analysis of PLRV-CP transgenic potato plants. Twenty µg of total plant RNA purified from transgenic leaf material were loaded onto a 1% agarose gel containing formaldehyde. Lane 1: 25 ng PLRV RNA; lane 2: RNA from control Désirée plants; lane 3-6: RNA from transgenic plant lines, CP^S19, CP^S31, CP^A2 and CP^A26, respectively.

Southern analysis was carried out on four transformed plants (Fig. 3). It was estimated that the tested transformants contained one to four copies of the CP gene per tetraploid genome. In the case of one of the transformants analyzed, the size of the detected fragment in the Southern analysis was larger than expected (Fig. 3). This might indicate the occurrence of a mutation during the integration

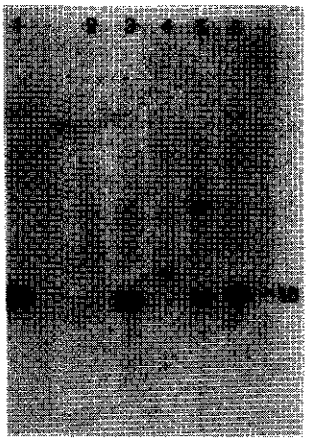

Figure 3: Southern analysis of PLRV-CP transgenic potato plants. Ten µg of DNA, purified from transgenic leaf material, and digested with EcoRI and HindIII were loaded onto a 1% agarose gel. Lane 1 represents two copies of EcoRI-HindIII double-digested pLRV-S per tetraploid genome; Lane 2: DNA from control Désirée plants; lane 3-6: DNA from transgenic plant lines, CP^S19, CP^S31, CP^A2 and CP^A26, respectively.

of the construct in the plant genome. Since the CP transcript detected in this transformant was of the expected size (Fig. 2), it is assumed that mutational events occurred in sequences flanking the actual PLRV-CP gene.

All the transgenic plants containing the sense CP gene construct were analyzed on Western blots and screened for possible accumulation of PLRV coat protein. None of the twenty-five CP-s transgenic plants analyzed contained detectable levels of coat protein. However, the antisera used in the Western blot procedure showed only low affinity to SDS-denatured purified virus. This might be due to the fact that all antisera tested, were raised against intact, non-denatured virions. The antiserum showing the highest affinity for purified virus, still did not allow detection of less then 10 ng denatured virus. For this reason all transgenic CP-s plants were also tested in a 'cocktail' ELISA procedure (Van den Heuvel & Peters, 1989). Using this technique, together with amplification of the enzyme reaction, it is possible to detect as little as 60 pg of purified virus or 20 pg of viral coat protein per 100 µg soluble leaf protein. However, even in this more sensitive procedure, none of the transgenic potato plants accumulated detectable levels of coat protein. Hence, it was concluded that the CP-s transgenic plants produced either extremely low amounts of coat protein (less than 2×10^{-5}% of soluble leaf protein, as calculated on basis of ELISA measurements), produced no coat protein at all, or produced protein which was rapidly degraded.

Inoculation of transgenic potato plants with PLRV

On the basis of highest level of expression of the PLRV-CP RNA four transgenic plant lines (two sense lines: CP^S19, CP^S31 and two antisense lines: CP^A2 and CP^A26) were

Table 1: Development of viral multiplication in transgenic and control potato plants after inoculation with PLRV, as determined by 'cocktail' ELISA. Ten plants per plant line were used. Plants were inoculated by ten viruliferous aphids. The ELISA plate reader was not blanked. Values within a column not followed by the same letter are significantly different. Dpi = days post infection; Dreg = non transformed, regenerated potato plants; LSD = least significant difference.

	A_{405}(ELISA values) (x ± SE)			
Dpi	21	29	36	45
Dreg	0.288±0.060 (a)	0.636±0.188 (a)	0.966±0.258 (a)	0.695±0.209 (a)
CP^S19	0.124±0.028 (b)	0.173±0.051 (b)	0.168±0.044 (b)	0.178±0.032 (b)
CP^S31	0.184±0.026 (a)	0.138±0.025 (b)	0.244±0.055 (b)	0.253±0.055 (b)
CP^A2	0.216±0.044 (a)	0.543±0.155 (a)	0.404±0.128 (b)	0.411±0.102 (a)
CP^A26	0.167±0.030 (b)	0.209±0.056 (b)	0.179±0.053 (b)	0.263±0.060 (b)
healthy	0.037±0.002	0.052±0.002	0.048±0.003	0.048±0.002
LSD 5%	LSD=0.114	LSD=0.331	LSD=0.389	LSD=0.322

selected for analysis of their sensitivity to PLRV infection. All four transgenic plant lines were phenotypically identical to control plants (Désirée) and were able to produce tubers. Ten plants from each line were inoculated by viruliferous aphids and ten non-transformed, regenerated potato plants (cultivar Désirée) were used as a control. All plants were propagated on rockwool microplugs and infected one week after their transfer to soil. For inoculation, ten aphids (*Myzus persicae*) were used per plant. Previous experiments had shown that ten viruliferous aphids per plant never failed to initiate a full blown infection in control plants, whereas with lower aphid numbers some plants did not become infected. For tissue culture propagated potato plants, development of primary infection symptoms upon PLRV infection was absent. Therefore virus replication and spread were monitored by ELISA on leaf discs sampled at various times for each plant. The results obtained are shown in Table 1. For statistical analysis the general linear models procedure was used to calculate the standard error of the mean.

Significant differences between the A_{405} values were determined by calculating the least significant difference (LSD) with a 95% probability. The statistical analysis of the A_{405} values indicated that the infected transgenic plant lines CP^S19, CP^S31 and CP^A26 contained a significantly lower amount of viral antigen during the experiment than the control plants (Table 1). The different A_{405} values in the experiment reflect differences,

Table 2: Development of viral multiplication in transgenic and control shoots emerging from infected tubers, as determined by 'cocktail' ELISA. The ELISA plate reader was not blanked. Eight to ten tubers per plant line were used. Values within a column not followed by the same letter are significantly different. Dreg = non transformed, regenerated potato plants. **T** = Days following the emerging of the first shoots.

	A_{405} (ELISA values) (x ± SE)			
	experiment 1		*experiment 2*	
T	42	56	21	35
Dreg	0.951±0.127 (a)	1.369±0.180 (a)	1.706±0.140 (a)	2.178±0.155 (a)
CP^S19	0.236±0.019 (b)	0.281±0.040 (b)	0.696±0.162 (b)	0.754±0.132 (b)
CP^S31	0.729±0.152 (a)	0.781±0.163 (b)	0.769±0.219 (b)	1.460±0.128 (b)
CP^A2	0.560±0.092 (b)	1.097±0.126 (a)	1.267±0.129 (a)	1.856±0.375 (a)
CP^A26	0.572±0.121 (b)	0.546±0.103 (b)	1.205±0.101 (a)	1.237±0.183 (b)
healthy	0.070±0.010	0.080±0.020	0.090±0.015	0.085±0.010

ranging from 4 to 10 times, in virus titers between control and transgenic plants. This indicates a lower rate of multiplication of the virus in the transgenic plants compared to the control plants and thus a significant interference of the expressed CP gene sequences with the viral replication cycle. By repetition of the inoculation experiment as described in Table 1 the conclusion, that lines CP^S19, CP^S31 and CP^A26 show a significant level of PLRV resistance, was confirmed.

Potato tubers, harvested from transgenic and control plants used in the inoculation experiments, were planted and the emerging shoots were tested in an ELISA procedure. The results obtained are shown in Table 2. Shoots from infected tubers of the transgenic plant lines CP^S19, CP^S31 and CP^A26 contained a significantly lower amount of viral antigen compared to the shoots of the control line. These results are in accordance with the results obtained from the initial inoculation experiments.

DISCUSSION

The potato cultivar Désirée was transformed with the PLRV-CP gene by use of the binary vector system of *A. tumefaciens*. Two transgenic plants, that expressed the CP RNA sequences in sense orientation (CP^S19, CP^S31) and one of the two tested

transgenic plants expressing the CP RNA sequences in antisense orientation (CP^A26), showed inhibition of viral multiplication upon infection with PLRV by aphids or after secondary infection. The transgenic plant line CP^A2 did not show a statistical significant reduction of virus titer upon infection compared to the control plants, although it contained similar levels of (antisense) transcript RNA as CP^A26. In all four transgenic plant lines coat protein could not be detected by immunoblot or by ELISA procedures. Although the presence of coat protein below detection levels could not be ruled out, it seems likely that the coat protein was absent in these plants, taking into account the low detection level of the ELISA procedure used, i.e. 20 pg coat protein per 100 µg of soluble leaf protein.

The mechanism of coat protein-mediated protection has remained unknown sofar. The current hypothesis is that the coat protein present in the transgenic plants interferes with an early event (possible uncoating) during viral infection (Register & Beachy, 1988; Powell *et al.*, 1990). Data supporting this hypothesis include the observation that the level of protection is dependent on the presence and the level of coat protein in the transgenic plant and not on the amount of mRNA produced (Van Dun *et al.*, 1988; Powell *et al.*, 1990). Moreover, inoculation with viral RNA has been shown to overcome the protection (Van Dun *et al.*, 1987; Loesch-Fries *et al.*, 1987; Nelson *et al.*, 1987). However, plants expressing the coat protein of potato virus X (PVX) have been reported to be protected against inoculation with viral RNA (Hemenway *et al.*, 1988). Additionally, it was reported that the level of protection in transgenic potato plants, expressing the CP gene of potato virus Y (PVY), against infection with this virus, was independent of the level of accumulation of expressed coat protein (Tumer *et al.*, 1987).

The results reported here indicate that protection against PLRV may be achieved solely by transcriptional expression of the PLRV-CP gene. The virtual absence of accumulated coat protein in CP-s transgenic plants and the protection conferred by the expression of antisense CP RNA sequences in the CP-a transgenic plants might support this view. Likewise, several distinct mechanisms for cross-protection, based on interference on either protein or RNA level, have been proposed. The majority of reports indicate that viral coat protein is responsible for the mechanism of cross-protection. However, infection of plants with the RNA-1 of tobacco rattle virus (TRV) is able to confer, in the absence of the RNA-2-encoded coat protein, protection towards infection with a complete TRV strain (Cadman & Harrison, 1959). Additionally, the

occurrence of cross-protection between viroids indicates that proteins are not always involved (Khoury *et al.*, 1988).

Several explanations for the observed, engineered protection against PLRV can be envisaged. Firstly, even an extremely low level of presence of coat protein in transgenic plants is possibly enough to confer protection against this phloem-restricted virus. Secondly, coat protein is possibly not involved at all and the observed protection is conferred by the CP RNA sequences. If the first model is true, then we are studying a similar phenomenon as observed in all previous studies on coat protein-mediated protection, though strikingly low levels of coat protein are involved in this case. However, the second explanation seems more plausible, since it is supported by the protection conferred by the expression of antisense CP RNA sequences in the $CP^{A}26$ transgenic plants. Therefore we favour the hypothesis, that the protection observed for the transformed plant lines, expressing the PLRV-CP gene in either sense or antisense orientation, is based on interference of viral multiplication on the RNA level. This protection could be a phenomenon comparable to the cross-protection, observed for viroids or TRV RNA-1, in which RNA molecules have also been proposed to be involved.

Further experiments are needed to clarify the mechanism of the protection described. However, from the data presented in this paper, it is clear that irrespective of the mechanism underlying the protection observed, transformation of potato plants with genomic sequences of PLRV leads to significant levels of protection against the virus.

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CHAPTER 4

Expression of a modified coat protein gene of potato leafroll virus in transgenic potato plants

ABSTRACT

The PLRV coat protein gene was cloned and subsequently altered with the aim to optimize its translational expression upon transformation into potato plants and to study its potential as virus resistance trait. To this end, the sequences flanking the ATG start codon were modified into a potentially optimal context and the start codon of the open reading frame (ORF5) underlying the coat protein gene was deleted. Transgenic potato plants, expressing the modified PLRV coat protein gene on transcriptional level, did not accumulate detectable levels of coat protein. Upon inoculation with PLRV the transgenic potato plants showed a reduced rate of virus multiplication, as compared to non-transgenic control plants. These results are similar to previous results obtained with the wild-type PLRV coat protein gene.

INTRODUCTION

The production of transgenic potato plants expressing the coat protein gene of potato leafroll virus (PLRV), in both sense as anti-sense orientation, has been described (Kawchuk *et al.*, 1990 & 1991; Van der Wilk *et al.*, 1991). The transgenic plants showed relatively high levels of expression of RNA sequences, but accumulation of transgenic coat protein was extremely low (Kawchuk *et al.*, 1990) or even below detection limits (Kawchuk *et al.*, 1991; Van der Wilk *et al.*, 1991). Upon inoculation with PLRV, both the transgenic potato plants expressing the sense construct as the plants expressing the anti-sense construct, showed a reduced rate of virus multiplication, as compared to non-transgenic control plants. Although, considerable reductions in virus titers have been observed, the decrease was less extreme as reported for transgenic plants expressing coat protein genes of other viruses. In most instances the presence of coat protein in the transgenic plants has been suggested to be a prerequisite for protection. For transgenic tobacco plants expressing the coat protein of tobacco mosaic virus (TMV) has been reported that the presence of coat protein, and not coat protein RNA sequences was required for protection (Powell *et al.*, 1990).

The reason for the lack of a significant accumulation of transgenic PLRV coat protein has remained unknown, but the translational initiation site of the coat protein gene can be considered as sub-optimal (Kozak, 1989) and may therefore have caused an inadequate synthesis of transgenic coat protein. Transient expression studies of a PLRV coat protein construct in protoplasts have revealed that the open reading frame 5 (ORF5), underlying the coat protein gene, was 3 to 7 times higher expressed than the coat protein gene itself (Tacke *et al.*, 1990). The coat protein gene translational initiation site sequence (CAAUUGUUAAUGA) differs strongly from the consensus sequence (GCCGCCA/GCCAUGG) reported to be present in more efficiently translated eukaryotic mRNA's (Kozak, 1989; Cavener & Stuart, 1991). However, both potential translational initiation sites (UUAAAGGAAAUGUCAAUGG) of PLRV ORF5 differ at most positions from this consensus sequence, possibly the tandem array of the two AUG codons favour translation. On the other hand, it has been reported that dicotelydonous plant mRNA's exhibit a strong preference for a G at position +4 and a weak preference for A's at all positions upstream (-9 to -1) of the start codon (Cavener & Stuart, 1991). Possibly, preference for nucleotides also depends on the type of tissue

where translation takes place.

In this paper, it was investigated wether the translational expression of the PLRV coat protein gene would be amplified in transgenic plants by mutations that lead to a translationary optimized context of the start codon. The modifications in the sequences were selected on basis of consensus sequences of dicot plant mRNA's and the initiation sites sequences of the other PLRV ORFs.

Additionally, to rule out the possibility that the earlier observed inhibition of viral multiplication in transgenic plants (Van der Wilk *et al.*, 1991) was caused by expression of the ORF5 underlying the coat protein gene, the ORF5 start codon was deleted.

MATERIALS AND METHODS

Mutagenesis of the PLRV coat protein gene

The cloning of the PLRV coat protein gene has been described previously (Van der Wilk *et al.*, 1989). A modified coat protein construct was produced by synthesizing an oligonucleotide nearly identical to the first 41 nucleotides of the coat protein gene. The synthetic oligonucleotide differed from the PLRV coat protein gene at the sequences flanking the start codon, changing the translational initiation site from sub-optimal to potentially optimal (Fig. 1). Furthermore, the translational initiation site of the open reading frame (ORF5) underlying the coat protein gene, was deleted. Additionally, restriction sites were placed at the 3'- and 5'-end of the oligonucleotide to facilitate cloning. The linker was cloned in plasmid pUC19 and subsequently, checked for mutations or deletions by nucleotide sequence analysis. The cloned linker (pMH1) was digested with AccI and subsequently treated with nuclease S1. A plasmid containing the cloned PLRV coat protein gene (pFW139) (Van der Wilk *et al.*, 1989) was digested with StyI and SstI. The resulting 445 base pairs (bp) fragment was isolated and treated with RsaI. The generated RsaI-StyI fragment was ligated into StyI/AccI/nuclease treated pMH1, giving rise to the plasmid pMH2. This plasmid was digested with SstI and StyI and the resulting fragment was ligated into StyI/SstI digested pFW139. The AccI site immediately downstream of the altered PLRV coat protein gene was filled in using DNA polymerase I (Klenow fragment) and the blunt end supplied with a BglII linker.

The generated fragment was digested with BglII and cloned in BamHI digested pMOG181. The created construct, with the coat protein gene in the sense orientation, was digested with EcoRI and HindIII and the fragment containing the chimaeric coat protein gene placed into the binary vector pMOG23 (Sijmons *et al.*, 1990), giving rise to the plasmid pLRV-T.

In vitro translation experiments

To investigate whether the mutations introduced in the coat protein gene construct influenced the translation efficiency, *in vitro* translation experiments were performed. To this end, the wild-type and modified coat protein gene was placed under the control of the SP6 promoter. *In vitro* translations were performed using the TnT system (Promega) according to the manufacturers instructions. The synthesized proteins were separated on SDS-polyacrylamide gel and visualized by autoradiography.

Potato transformation

Transformation of potato plants was done as described by Hoekema *et al.* (1989). Potato tuber discs, cultivar Désirée, were cocultivated with *Agrobacterium tumefaciens*, strain LBA 4404, harbouring plasmid pLRV-T. For cocultivation 500 tuber discs were used. Selection for transformation was done on medium containing kanamycin (100 µg/ml). Kanamycin resistant shoots were cut off and placed onto rooting medium, propagated axenically and transferred to soil.

Northern and Southern blot analysis of transgenic potatoes

For Northern analysis, total RNA was isolated from transgenic leaf material as described by Verwoerd *et al.* (1989). 20 µg aliquots were loaded onto a denaturing 1% agarose gel containing 2.2 M formaldehyde. After electrophoresis the RNA was transferred to a Hybond-N membrane (Amersham) and hybridized with a radiolabelled PLRV-CP probe.

For Southern analysis high molecular weight DNA was isolated as described (Mettler, 1987). DNA was digested with the restriction endonucleases EcoRI and

HindIII and 10 µg of DNA was loaded on a 1% agarose gel. After gel electrophoresis the DNA was transferred to Hybond-N and probed with a radiolabelled CP fragment.

Immunoblot analysis and enzyme-linked immunosorbent assay (ELISA)

For immunoblot analysis three different methods of sample preparation were tested. In the first method leaf discs of one centimeter diameter were ground in 200 µl 3x Laemmli buffer (Laemmli, 1970). The second method was performed essentially as described (Van Etten *et al.*, 1979; Kawchuk *et al.*, 1991). Leaf material was ground in a mixture of 20% buffer (80 mM Tris-HCl pH 6.8, 1 mM dithiothreitol, 10 mM EDTA, 2% SDS, 50 µg/ml phenylmethylsulfonyl fluoride (PMSF)) and 80% phenol (containing 0.1 M ammonium acetate and 0.1 M dithiothreitol). The phenol and aqueous layers were separated by centrifugation. The phenol layer was extracted three successive times with equal volumes of buffer (80 mM Tris-HCl pH 6.8, 1 mM dithiothreitol, 10 mM EDTA, 0.1 M ammonium acetate). The protein was precipitated from the phenol phase by adding five volumes of methanol, containing 0.1 M ammonium acetate, and stored at -20 ^{0}C for 2 h. The protein was collected by centrifugation, washed three times with methanol and one time with acetone. The pellet was dried and resuspended in Laemmli buffer.

The third method has been developed by Dr M.A. Mayo of the Scottish Crop Research Institute. Leaf material was powdered in liquid nitrogen, suspended in buffer consisting of 75 mM Tris-HCl pH 6.8, 9 M urea, 4.5% SDS and 7.5% ß-mercaptoethanol and incubated for 30 min at 65 ^{0}C. Proteins were precipitated from the suspension by adding nine volumes of 10% TCA, dissolved in acetone, incubation overnight at -20 ^{0}C, followed by centrifugation. The pellet was washed three times with acetone (of -20 ^{0}C), two times with ether and subsequently resuspended in Laemmli buffer.

Protein concentrations were determined using BCA protein assay reagent (Pierce Chemical Co.). Different amounts, ranging from 10 to 200 µg, of soluble leaf proteins were boiled for 5 minutes, loaded onto a 12.5% polyacrylamide SDS gel and run at 200 mA (Bio-Rad Mini-PROTEAN II electrophoresis system). The gels were blotted onto nitrocellulose using a semi-dry or buffered transfer system. The blots were preincubated with a blocking reagent (Boehringer Mannheim) and incubated with anti-PLRV IgG.

The antiserum used, was purchased from Boehringer Mannheim and raised against intact, non-denatured virions.

ELISA was performed as described, including amplification of the enzyme reaction (Van den Heuvel & Peters, 1989). Leaf material was ground in extraction buffer (0.2% ovalbumin, 2% polyvinyl-pyrrolidone, 0.05% Tween 20 in PBS). The samples were simultaneously incubated with the conjugate for 16 h at 4 ^{0}C. For amplification of the enzyme reaction 100 µl of 0.2 mM NADP in 0.05 M diethanolamine (pH 9.5) was added to each well, followed by an incubation at 20 ^{0}C for 30 min. Alkaline phosphatase activity was blocked by adding 15 µl of 0.5 M 4-nitrophenyl disodium ortho-phosphate in 0.025 M phosphate buffer pH 7.4 to each well. Subsequently, 150 µl of amplification reaction mixture was added per well. This reaction mixture consisted of 700 units of alcohol dehydrogenase, 100 units of lipoamide dehydrogenase (type VI), 3% ethanol and 1 mM p-iodonitrotetrazolium violet in 15 ml of 0.025 M phosphate buffer pH 7.0. Colour development was allowed to proceed at room temperature and absorbency values were read at 492 nm.

Transmission of PLRV to transgenic plants

Aphids, *Myzus persicae* (Sulzer) Wmp1, were maintained on oilseed rape, *Brassica napus* L. susp. *oleifera* in a greenhouse under controlled conditions (Reinink *et al.*, 1989). Mature apterae, confined to leaf cages, were transferred daily to leaves of oilseed rape plants. The aphids acquired the virus by feeding for 4 days on virus-infected young *Physalis floridana* Rydb. plants. Inoculation of the transgenic potato plants was carried out by confining 10 viruliferous nymphs per plant for a 9 day access period. Since the virus titer is not uniform within the infected plant, virus infection was monitored for each individual plant by taking samples of the lower, middle and top leaves at each time-point. For each time-point, 3 leaf discs of each plant were pooled, ground in extraction buffer, and screened for the presence of virus using the 'cocktail' ELISA procedure without enzyme amplification.

Statistical analysis

Statistical analysis of the A_{405} ELISA values was done employing the Statistical

Application Systems programme of the SAS Institute Inc. The general linear models procedure was used to calculate the standard error of the mean (MSE). Statistical significance was determined with the procedure of the least significant difference (LSD). $LSD=t_{0.025[r]}[\sqrt{(2/n)}].MSE$

RESULTS

Modification of the PLRV coat protein gene and construction of a plant transformation vector

Potato plants (cultivar Désirée) have been transformed with the coat protein gene of PLRV in which mutations were introduced, affecting the translational initiation site. Using a synthetic oligonucleotide, the translational initiation site of the coat protein gene was altered from sub-optimal to a theoretically more optimal context, and the putative start codon of the open reading frame, underlying the coat protein gene (ORF5), was deleted. Since it was not clear which of the two AUG codons present at the 5' proximal end of the ORF5 acts as the translational start codon, both were deleted by replacement of the thymidines (located on the viral genome at nucleotide positions 3614 and 3620 (van der Wilk *et al.*, 1989)), with cytidines. As shown in Figure 1, the modifications in the sequences flanking the start codon of the gene, resulted in the substitution of the serine, being the second amino acid of the coat protein, into a glycine. The deletion of the translational initiation site of the ORF5 did not affect the amino acid sequence of the coat protein. The modified gene was ligated in the plasmid

```
wild-type      3579CAATTGTTAATGAGTACGGTCGTGGTTAAAGGAAATGTCAATGGCG3624
sequence                 M  S  T  V  V  V  K  A  N  V  N  G  G
                                                  M  S  M  A

modified         CTATAAACAATGGGTACGGTCGTGGTTAAAGGAAACGTCAACGGCG
sequence                 M  G  T  V  V  V  K  A  N  V  N  G  G
                                                  T  S  T  A
```

Figure 1: Mutations introduced in the PLRV CP gene. Mutated nucleotides and amino acids residues are underlined. Nucleotide positions are indicated according to Van der Wilk *et al.* (1989).

pMOG181 between the cauliflower mosaic virus (CaMV) 35S promoter, with a double enhancer sequence, and the transcription termination signal of the nopaline synthase *(nos)* gene. The created chimaeric gene was placed in the binary vector pMOG23 (Sijmons *et al.*, 1990). Potato tuber discs, cultivar Désirée, were transformed using an *Agrobacterium tumefaciens* mediated transformation system (Hoekema *et al.*, 1989). Employing this procedure, 63 independent transformants were obtained.

Expression of the altered PLRV-CP gene construct in transgenic potato plants

All transgenic potato plants obtained were analyzed for transcriptional expression. In all transgenic plants transcripts of the expected size of 800 nucleotides, encoding the PLRV coat protein, could be detected. The levels of accumulation of transgenic transcripts for three plants lines are shown in Figure 2. Generally, the levels of expression of the altered PLRV coat protein gene were approximately equal to those of the transgenic wild-type coat protein gene.

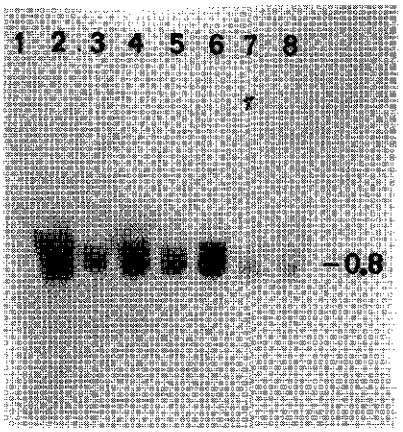

Figure 2: Detection of PLRV CP transcripts in transgenic potato plants. Twenty µg of total plant RNA purified from leaf material was loaded onto a 1% agarose gel, containing formaldehyde. After transfer of the RNA, the blot was probed with a cDNA fragment comprising the PLRV coat protein sequences. Lane 1: RNA from control Désirée plants; lane 2-7: RNA from transgenic plant lines, CP^S19, CP^S31, CP^T13, CP^T14, CP^T20, CP^A12, CP^A26, respectively.

The copy number(s) of the integrated coat protein gene in the transgenic plant lines CP^T13, CP^T14, CP^T20, as determined by Southern analysis, was between one and three (Fig. 3). A similar copy number has been determined for transgenic potato plants containing the wild-type PLRV coat protein gene (Chapter 3, this thesis).

All the transgenic potato plants were analyzed on Western blot for accumulation of PLRV coat protein. For sample preparation transgenic leaf

material was ground in Laemmli buffer (Laemmli, 1970). None of the analyzed plant lines contained detectable levels of coat protein.

The applied method of sample preparation is commonly used in immunoblot analysis procedures for the detection of viral coat proteins in transgenic plants. However, two other methods of sample preparation have been developed to enable the detection of PLRV coat protein in transgenic potato plants (Kawchuk *et al.*, 1991; Mayo, personal communication). Both methods are based on the purification of total protein from the transgenic leaf material. In the first method phenol-soluble proteins are extracted from the leaf material and precipitated with methanol (Van Etten *et al.*, 1979). The second method involves the incubation of powdered leaf material in a buffer containing urea, SDS and ß-mercaptoethanol at 60 ^{0}C, followed by precipitation of the proteins with the use of trichloric acid (Mayo, unpublished). On the basis of highest levels of expression of PLRV coat protein transcripts five transgenic plant lines were selected and tested on Western blot for the presence of coat protein, using all three different methods of sample preparation. Regardless of the method used, in none of the plant lines analyzed, coat protein could be detected. Twenty of the transgenic plant lines were analyzed in a 'cocktail' ELISA procedure, including amplification of the enzyme reaction. This procedure has shown to be extremely sensitive for the detection of PLRV in infected plants and viruliferous aphids, allowing the detection of 60 ng of virus (Van den Heuvel & Peters, 1989). However, despite this high sensitivity in none of the ELISA analyzed plant lines, coat protein could be detected.

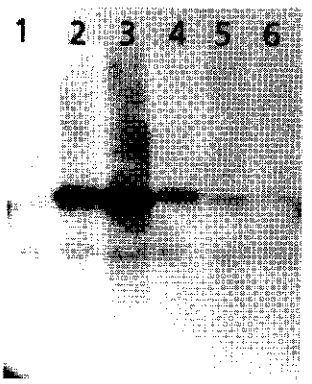

Figure 3: Southern blot analysis of PLRV CP transgenic potato plants. Ten µg of DNA, purified from transgenic leaf material, and digested with EcoRI and HindIII was loaded onto a 0.8% agarose gel. After transfer of the DNA, the membrane was probed with a cDNA fragment comprising the PLRV coat protein sequence. Lane 1: DNA derived from control Désirée plants; lane 2-6: DNA from transgenic plant lines, $CP^{S}19$, $CP^{T}13$, $CP^{T}14$, $CP^{T}20$ and $CP^{A}26$, respectively.

It is therefore concluded that accumulation of PLRV coat protein in the transgenic potato plants did not occur or only at extremely low amounts. Hence, the introduced

modifications in the PLRV coat protein gene did not positively influence its accumulation in transgenic potato plants. Comparison of the products of *in vitro* translation experiments with the modified and non-modified coat protein gene revealed that expression of the 23 kDa coat protein was markedly higher in case of employing the modified construct (Fig. 4). This indicates that the observed low accumulation of transgenic coat protein in plants (Chapter 5) is possibly due to proteolytic breakdown rather than faulty translation of the transgenic transcripts.

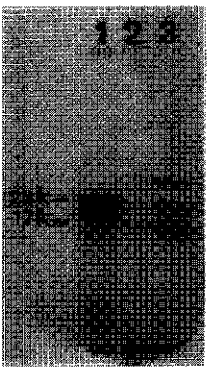

Figure 4: *In vitro* translation of the PLRV-CP constructs used in potato transformation experiments.
Lane 1: translation products of the modified coat protein transcripts; lane 2 :control translation products employing an empty vector construct; lane 3: translation products of 'wild-type' coat protein transcripts. The size in kDa (K) of the translation products is indicated

Inoculation of transgenic potato plants with PLRV

Three independent obtained transgenic potato lines (CP^T13, CP^T14, CP^T20), containing the modified PLRV coat protein gene and expressing the highest levels of coat protein mRNA, were selected for further experiments. All three plant lines were phenotypically indistinguishable from non-transformed control plants (cultivar Désirée) and were able to produce viable tubers. The selected transgenic plants were analyzed for their possibly altered susceptibility to infection with PLRV. In the experiments non-transformed, regenerated potato plants (cultivar Désirée) and two transgenic plant lines, containing the unaltered PLRV coat protein gene in the sense (CP^S19) and antisense orientation (CP^A26), respectively (Van der Wilk *et al.*, 1991) were used as controls. All plants were propagated in tissue culture and infected two weeks after their transfer to soil. Ten plants from each line were inoculated by viruliferous aphids. For inoculation, ten aphids (*M. persicae)* were used per plant. Accumulation of virus in the infected plants was monitored by ELISA. Leaf discs were sampled from the plants at different time points after inoculation. The results obtained in two of these experiments are shown in Table 1. For statistical analysis the general linear models procedure was used to calculate the

standard error of the mean. Significant differences between the A_{405} values were determined by calculating the least significant difference (LSD) with a 95% probability.

All analyzed transgenic plant lines showed to be susceptible to PLRV infection. Statistical analysis of the A_{405} values indicated that the infected transgenic plant lines contained significant lower amounts of viral antigen during the experiments than the control plants. The actual differences in virus titers between the transgenic and control plants ranged from 4 to 10 times, as calculated from the ELISA values. No statistical significant differences were observed between the mutual A_{405} values of the different transgenic plant lines.

These results indicate a lower rate of multiplication of the virus in the transgenic plants as compared to the control plants. Furthermore, the modification of the second amino acid of the expressed coat protein did not influence the reduction of viral multiplication. From the results obtained with the transgenic plant lines, containing the modified PLRV coat protein gene, it can be concluded that the putative product of the ORF5, underlying the coat protein gene, did not play a role in the observed reduction of viral multiplication.

Table 1: Development of viral multiplication in transgenic and control plants after inoculation with PLRV, as determined by ELISA. The ELISA plate reader was not blanked. Values within a column not followed by the same letter are significantly different. Dreg = non-transformed, regenerated potato plants (Désirée).

	A_{405} (ELISA values) (x ± SE)	
	experiment 1	*experiment 2*
Dreg	0.801±0.181 (a)	1.084±0.142 (a)
$CP^{S}19$	0.273±0.132 (b)	0.556±0.399 (b)
$CP^{T}13$	0.431±0.178 (b)	0.822±0.527 (a)
$CP^{T}14$	0.331±0.145 (b)	0.620±0.142 (b)
$CP^{T}20$	0.551±0.131 (b)	0.743±0.188 (b)
$CP^{A}26$	0.296±0.091 (b)	0.596±0.200 (b)
healthy	0.040±0.010	0.065±0.030

DISCUSSION

Translational expression of the PLRV coat protein gene in transgenic plants has been shown to be extremely low or possibly absent (Van der Wilk *et al.*, 1991; Kawchuk *et al.*, 1991). In an attempt to enhance the synthesis of the coat protein in transgenic plants, the viral gene was modified. The sequences flanking the start codon of the coat protein gene, which can be considered as sub-optimal, were altered and the start codon of the ORF5 deleted. The mutations induced in the translational initiation site of the coat protein gene could theoretically lead to a higher level of protein expression (Kozak, 1989; Cavener & Stuart, 1991). *In vitro* translation experiments employing both the modified and wild-type coat protein gene showed that synthesis of the 23 kDa protein was indeed enhanced by the mutations introduced. However, attempts to detect accumulated coat protein in transgenic potato plants, containing the modified gene, failed. Although the presence of coat protein below detection levels can not be ruled out, it seems likely that the mutations, introduced in the translational initiation site of the coat protein gene, did not lead to a considerable increase in protein accumulation as compared to the wild-type coat protein accumulation. Possibly, the extremely low amounts or even complete absence of coat protein is caused by rapid degradation of the protein, thereby masking any difference in rate of synthesis.

Both the modified and the wild-type coat protein gene, induced in transgenic plant lines reduction of PLRV multiplication, but no immunity. Statistical analysis revealed that no discrimination could be made between the viral titers in infected transgenic plants expressing the wild-type and those of transgenic plants expressing the modified coat protein gene. For the coat protein of alfalfa mosaic virus it has been reported that the second amino acid was critical for coat protein-mediated protection (Tumer *et al.*, 1991). However, the substitution of the serine with an glycine as the second amino acid of the PLRV coat protein did not lead to an altered sensitivity for PLRV infection. This observation does not contradict the hypothesis that the observed inhibition of viral multiplication in transgenic potato plants expressing the PLRV coat protein gene, is caused by RNA sequences rather than coat protein (Van der Wilk *et al.*, 1991).

In the modified PLRV coat protein expression vector, the start codon of ORF5 was deleted, to prevent interference of the expression of the coat protein by the expression of the ORF5 17 kDa product. This deletion clearly demonstrates that the earlier reported

inhibition of viral multiplication in transgenic plants, expressing the wild-type PLRV coat protein gene, was not caused by translational expression of this viral gene.

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CHAPTER 5

SYNTHESIS OF PLRV P1 IN *ESCHERICHIA COLI* AND INSECT CELLS

ABSTRACT

To study the role of the hypothetical translation product (P1) of the ORF1 of PLRV in the viral infection cycle, several antisera directed against this protein were produced. Neither antisera raised against synthetic peptides nor antisera raised against an in *E. coli* produced P1-GST fusion protein were able to detect the presence of P1 in PLRV-infected plant material, although these antisera allowed detection of 1 ng of protein on Western blot. The data presented suggest that P1 accumulates to extreme low levels in infected plant cells and is possibly rapidly degraded in the cell. As an alternative approach to study the function of P1, this protein was produced in Sf21 insect cells using the baculovirus expression system. Despite of using the strong polyhedrin promotor, P1 accumulated only to low levels in the insect cells, underlining the instable character of this protein and making purification infeasible. In immunolabelling experiments the synthesized protein was shown to accumulate in the cytoplasm of the Sf21 cells.

INTRODUCTION

The genome of potato leafroll virus (PLRV), a species of the genus *Luteovirus*, consists of a single-stranded RNA molecule of 5882 nucleotides long containing six open reading frames (ORFs) (Van der Wilk *et al.*, 1989; Mayo *et al.*, 1989; Keese *et al.*, 1990). An intergenic region located in the middle of the RNA separates two clusters of three genes each. The genes at the 3'-end (ORF4-6) are highly conserved among all luteoviruses and are translated from a subgenomic messenger RNA. The largest ORF (ORF6) is contiguous with ORF4, only separated by an amber stop codon, and is expressed via translational readthrough from ORF4. ORF4 encodes for the major coat protein subunit. ORF6 is to believed to play a role in the transmission of the virus (Brault *et al.*, 1995). The function of ORF5, underlying ORF4, has not been clarified yet, although some evidence has been presented that it encodes a movement protein (Tacke *et al.*, 1991). The ORFs located at the 5'-terminus are less conserved among luteoviruses. The equivalent of the first ORF of PLRV is even absent in the MAV and PAV strains of barley yellow dwarf virus (BYDV) (Miller *et al.*, 1988; Ueng *et al.*, 1992). ORF3 overlaps with ORF2 and is expressed via a -1 translational frameshift (Prüfer *et al.*, 1992). The translational fusion protein contains motifs characteristic for proteases (Gorbalenya *et al.*, 1989; Bazan & Fletterick, 1989) and the viral replicase (Van der Wilk *et al.*, 1989; Mayo *et al.*, 1989; Habili & Symons, 1989).

The genomic organization of PLRV is very similar to the genomic organization of other luteoviruses, especially beet western yellows virus (BWYV). Comparison of the products of the analogous ORFs of both luteoviruses shows a high amino acid sequence homology for all the ORFs except the ORF1 product. The function of the ORF1 encoded protein remains unclear. Since the most notable difference between PLRV and BWYV is in their host range, it has been suggested that the function of the ORF1 products is associated with host range specificity (Veidt *et al.*, 1992). The PLRV ORF1 encodes a 28 kDa protein (P1) which contains a putative membrane binding site (Mayo *et al.*, 1989) and shows low sequence homology with several membrane-associated proteins (Chapter 2, this thesis).

To gain insight in the function of the PLRV P1 the protein was expressed in *E. coli* and *Spodoptera frugiperda* cells as to study its intracellular location and to produce specific antisera to allow *in situ* studies on PLRV-infected potato leaf tissues. Both

these heterologously expressed P1 sequences as well as synthetic peptides were used to raise antisera against P1. The obtained antisera were used in experiments to detect P1 in infected plants and ORF1-recombinant *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV)-infected *S. frugiperda* cells.

MATERIALS AND METHODS

Production of P1 in *E. coli*

A PLRV ORF1 construct was synthesized from an existing cDNA clone (Van der Wilk *et al.*, 1989) using specific primers and polymerase chain reaction (PCR). The construct was supplied with unique BamHI restriction sites directly in front of the start and after the stop codon. The synthesized PCR fragment was cloned with the help of the TA cloning system (Invitrogen) according to the instructions of the manufacturer. Upon *in vitro* translation to verify whether the cloned sequence encoded a protein of the expected size, a P1-related protein was disclosed on the fluorogram. *In vitro* translation was performed using the TnT system (Promega) according to the manufacturers instructions, synthesized proteins were separated on SDS-polyacrylamide gel (SDS-PAGE). After sequencing to exclude undesired mutations, the ORF1 fragment was excised from the pCR1000 vector using BamHI and the fragments were separated on a 1% agarose gel. After isolation of the ORF1 fragment from the gel it was ligated in pGEX-2T and the obtained construct (pGEXP1) was used to transform JM101 cells. Cells harbouring the plasmid were grown to mid-log phase (OD_{600} = 0.6-1.0) at 37 ^{0}C after which expression was induced by adding isopropyl-ß-D-thiogalactoside (IPTG) to a final concentration of 1.0 mM to the culture. The cells were allowed to grow further for 5 h and consequently harvested by centrifugation. To monitor expression of P1, the cells were resuspended in Laemmli buffer (Laemmli, 1970) and boiled for ten minutes. The samples were loaded onto a 12.5% SDS-polyacrylamide gel and subjected to electrophoresis. After electrophoresis the proteins were either visualized with Coomassie Brilliant Blue or by silver staining.

Non-denatured P1 was purified by disruption of the bacterial cells using sonification followed by high speed centrifugation. The pellet containing P1 was dissolved in 5 M

urea. After incubation for 1 h the suspension was centrifuged at 14000 rpm for 30 min. The pellet was dissolved in 9 M urea/5% ß-mercaptoethanol at 65 ^{0}C. After centrifugation, the supernatant was dialyzed against 5 M urea at room temperature. P1 was collected from the suspension by centrifugation at 10000 rpm for 30 min. The pellet was sonified to dissipate the aggregate. Two rabbits were four times injected subcutaneously at three week intervals with 100 µg purified protein samples emulsified in Freund's incomplete adjuvant (Difco Laboratories). One week after each injection the rabbits were bled. Gamma-globulin fractions were isolated from the blood by ammonium sulphate precipitation (Clark & Adams, 1977).

Denatured P1 was isolated from SDS-polyacrylamide gel using a Prep-Cell electrophoresis apparatus (BioRad). Purified denatured protein (5 µg) was emulsified in Freund's incomplete adjuvant (Difco Laboratories) and injected four times into two mice at three week intervals. Gamma-globulin fractions were isolated according to Clark and Adams (1977).

Production of P1 in insect cells using the baculovirus expression system

A transfer vector (pFWAc03) containing the PLRV ORF1 was constructed by ligation of the ORF1 DNA into BamHI digested plasmid vector pJR1. Insect cells (*S. frugiperda*, Sf21) were cotransfected (Smith *et al.*, 1983) with pFWAC01 and NheI-linearized AcPAK6 baculoviral DNA. After three days at 27 ^{0}C, virus was collected from the medium and titrated in dilutions to render single plaques (Brown & Faulkner, 1977). Recombinant viruses (AcMU19) were further plaque purified.

S. frugiperda 21 or *Trichoplusia ni* (High Five) cells were either inoculated with recombinant (AcMU19) or wild-type AcNPV and incubated at 27 ^{0}C. The infected cells were harvested 24 to 48 h post infection and monitored for P1 production by SDS-PAGE and Western blotting. The cells were collected by centrifugation, resuspended in Laemmli buffer (Laemmli, 1970) and boiled for ten minutes. After electrophoresis the separated proteins were transferred to nitrocellulose using a buffered transfer system. The blots were pre-incubated with 5% skimmed milk powder in phosphate buffered saline (PBS)/0.1% Tween. Subsequently, the proteins were probed for P1 expression by incubation with anti-P1 IgG's for 16 h at 4 ^{0}C. After washing with 0.05% Tween 20 in PBS the blot was incubated with alkaline phosphatase conjugated anti-mouse IgG

(Sigma) for 3 h at room temperature. Bound conjugate was visualized using nitro blue tetrazolium (NBT) and bromo-chloro-indolyl-phosphate (BCIP) as substrate.

Raising of antisera against P1 using synthetic peptides

The amino acid sequence of P1 was analyzed with the help of the GCG computer programme Peptidestructure. Two domains with a high antigenic index were identified at amino acid positions 64-83 and 205-224, respectively. Two synthetic peptides identical to the identified domains were synthesized, with amino acid sequences CKRGRISTSGLQLPRHLHYE (SYN1) and ARLYNQLDLQGRAKSFRALT (SYN2), respectively. The synthetic peptides were covalently conjugated to the carrier protein keyhole limpet haemocyanin (KLH; MW 4.5 x 10^4 to 1.3 x 10^7) using 1-ethyl-3-3-dimethylaminopropyl carbodiimide hydrochloride (EDC) as a coupling reagent. Coupling reactions and subsequent purification of the conjugate was carried out using a commercially available kit (Imject Immunogen EDC Conjugation Kit) and according to the manufacturers instructions (Pierce). Antisera were raised by a two times, at a three week interval, repeated subcutaneous injection of two rabbits with approximately 100 µg of conjugate emulsified with equal volumes of Freund's incomplete adjuvant, followed three weeks later with a booster injection with approximately 200 µg conjugated protein. One week after the third injection blood was taken from the rabbits and tested for immunogenic response in enzyme-linked immunosorbent assay (ELISA). Antibodies were isolated from the blood according to Clark and Adams (1977).

Immunofluorescence microscopy

Sf21 insect cells were infected with AcMU19 or AcPAK6 baculovirus. After washing the cells to remove inoculum, the cell suspension (10^5 cells in 100 µl) was placed on a sterile coverslip for 1 h. Subsequently, 1 ml of medium was added and the cells were allowed to grow for 24 to 72 h. To fix the cells the coverslips were placed in acetone for 15 min at -70 ^{0}C. After fixing of the cells, the coverslips were incubated in PBS for 15 min followed by an incubation in PBS containing 1% BSA. Immunodetection was tested by incubation of the coverslips in (1:100-1000) diluted antiserum. The coverslips were washed three times in PBS to remove unbound antibodies, followed by incubation

with 1:1000 diluted FITC conjugated anti-rabbit or anti-mouse IgG's (Sigma) for 1 h. The coverslips were washed three times in PBS, covered with glycerine/PBS/cityfluor and placed on microscope slides. The slides were examined under UV light using a light microscope.

Immunogold detection

Insect cells infected with AcPAK6 or AcMU19 baculovirus were harvested by centrifugation, washed twice with PBS to remove medium and fixed in 2% paraformaldehyde/3% glutaraldehyde in PBS for 3 h. Subsequently, the cells were washed and resuspended in 2% gelatine. The gelatine encapsidated cells were fixed with 2% paraformaldehyde/3% glutaraldehyde in PBS for 16 h at 4 ^{0}C. Afterwards, the encapsidated cells were dehydrated with ethanol and embedded in LRGold (London Resin Co) (Van Lent *et al.*, 1990). For immunolabelling, unstained ultra-thin sections were pre-incubated with 1% BSA/PBS for 1 h followed by incubation with anti-P1 antibodies for 3 h. After rinsing with BSA/PBS three times, the grids were incubated with colloidal gold-antibody complexes (Jansens Chimica) for 1 h.

RESULTS

Expression of P1 in *E. coli* and production of antisera

Several bacterial expression vectors were tested to express P1 in *E. coli* cells. With the exception of the glutathione S-transferase (GST) gene fusion vector pGEX-2T, none of the tested vectors expressed detectable amounts of P1, indicating a possible cytotoxic character of P1. The recombinant P1-GST fusion vector was synthesized by extending the PLRV ORF1 with unique BamHI restriction sites and ligation in the BamHI site of pGEX-2T, located directly after the GST gene. Upon induction of expression with IPTG, the fusion protein was readily detectable in bacterial cell proteins separated on SDS-polyacrylamide gel (Fig. 1). However, the estimated molecular weight of the fusion protein was approximately 45 kDa in stead of the expected 54 kDa. Sequence

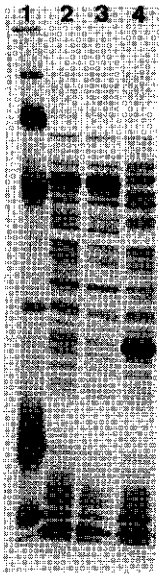

Figure 1: Expression of PLRV P1 in *E. coli* cells, employing the GST fusion vector pGEX-2T. Proteins from total bacterial cell extracts were separated on a 12.5% polyacrylamide gel and subsequently stained with Coomassis Brilliant Blue. Lane 1; molecular weight markers, 200, 97.4, 68, 43, 29, 18.4 and 14.3 kDa, respectively; lanes 2 & 3: extracts from cells harbouring pGEXP1 and expressing the GST-P1 fusion protein; lane 4: extracts from control cells harbouring the plasmid pGEX-2T.

analysis of the P1-GST expression vector revealed no mutations in both reading frames. Thus, part of the fusion protein was cleaved off and probably degraded, since no extra proteins were observed on gel as compared to the lanes loaded with proteins from cells containing the 'empty' GST vector (Fig. 1).

Upon lysis of the bacterial cells, the fusion protein showed to be nonsoluble, making it impossible to purify the fusion protein from bacterial lysate by affinity chromatography employing glutathione-coupled Sepharose. Solubilization of the protein proved to be only possible under highly chaotropic conditions e.g. in 9 M urea at 65 ^{0}C. However, after solubilization under these conditions the protein precipitated again if temperature and molarity urea were lowered. Exploiting these conditions it was possible to refine the fusion protein from the bacterial proteins to a 90% purity. The purified protein was used to immunize rabbits, but the antisera obtained proved to be ineffective in both ELISA and Western blot procedures, probably because the antigenicity of the protein was strongly affected due to the harsh conditions used during purification. Therefore, the fusion protein was purified under denaturing conditions using preparative SDS-polyacrylamide electrophoresis. Two mice (Balb-c) were injected with the denatured fusion protein to raise specific antisera. Strikingly, the serum of one mouse reacted strongly on Western blot with the fusion protein and only weakly to GST while the serum obtained from the second mouse reacted exactly the opposite, although both mice were immunized with the same batch of protein. Using the antiserum derived from the first mouse it was possible to detect 1 ng of purified fusion protein on Western blot.

However, the antiserum was unable to recognize the non-denatured protein in ELISA procedures, indicating that the antibodies in the serum were exclusively directed at linear epitopes which are not exposed in the native protein.

Production of antisera raised against synthetic peptides

To obtain antisera able to detect the non-denatured P1, synthetic proteins mimicking putative epitopes present on the surface of the protein, were used to raise antisera. To this end, the amino acid sequence of P1 was analyzed for surface probability and antigenic index. Predictions were made according the Emini-method (surface probability) and the Jameson-Wolf method (antigenic index) employing the computer programme Peptidestructure (Jameson & Wolf, 1988; Devereux *et al.*, 1984). Two areas with a high antigenic index and surface probability were identified located approximately in the middle and at the C-terminal part of the protein. Two synthetic peptides of twenty amino acids identical to these areas were synthesized (SYN1 & SYN2). To obtain a high immunoresponse the synthetic peptides were covalently coupled to a carrier protein (KLH), making them more immunogenic. Two rabbits were immunized with the different conjugated peptides. Both obtained antisera displayed a similar, slightly lower affinity for P1 on Western blot as compared to the antiserum raised against the *E. coli* fusion protein (Fig. 2).

Analysis of PLRV-infected plant tissues for the presence of P1

PLRV-infected *Physalis floridana* and *Solanum tuberosum* plants were analyzed on Western blot and in ELISA for the presence of P1. Leaves, roots and stems from infected plants were examined separately for the presence of P1, since it can not be excluded that P1 accumulation is tissue specific. For Western blot analysis plant tissues were ground in Laemmli buffer (Laemmli, 1970), boiled and subsequently separated in SDS-PAGE. After transfer to nitrocellulose the proteins were probed with all the obtained antisera for the presence of P1. However, no detectable levels of P1 were observed.

In experiments to detect P1 in infected plant material employing ELISA, leaf tissues were ground in SEB (0.2% ovalbumin, 2% polyvinylpyrrolidone, 0.05% Tween 20 in

PBS). For detection, the separate antisera raised against the synthetic peptides as well as a mixture of both, were used. In all instances the obtained ELISA readings were statistically indifferent from those derived of control samples.

Possibly, the detection of P1 in infected plant tissues was hampered due to low accumulation of P1. Since, it is conceivable that P1 only accumulates in distinct parts of infected tissues or cells, several procedures were tested to obtain cell fractions enriched for the presence of P1. Computer analysis of the amino acid sequence suggests that P1 is membrane-linked, therefore, membrane fractions were isolated from infected plant tissues and analyzed for the presence of P1. Two procedures were tested to isolate membrane fractions. The first method used has been shown to enable the detection of the movement proteins MP of tobacco mosaic virus (TMV) and BL1 of squash leaf curl virus (SqLCV) in plasma membrane and crude cell wall fractions of infected and transgenic tobacco plants (Deom *et al.*, 1990; Pascal *et al.*, 1993). The second method has been shown to facilitate the isolation of crude membrane fractions containing viral replicase activity from plum pox virus-infected plants (Martin & Garcia, 1991). However, in none of the fractions obtained from PLRV-infected plant tissues, the presence of P1 could be detected. In addition, a method was tested which among others has been used to detect transgenic PLRV coat protein (P4) in potato plants (Van Etten *et al.*, 1979; Kawchuk *et al.*, 1990). But again, accumulation of P1 employing this procedure, which involves the isolation of phenol-soluble protein fractions, was not detectable on Western blot.

The aforesaid results indicate, that either extreme low levels of P1 are present in infected plant material or that extraction of P1 from plant material is hampered, possibly due to presence of P1 in insoluble fractions.

Expression of P1 in insect cells

Since, P1 approved to be non-detectable in PLRV-infected plant material, an alternative approach was followed to gain insight in the intracellular behaviour of P1. It was decided to express this protein in the eukaryotic baculovirus/insect cell system. Insect cells (Sf21) were infected with the ORF1-recombinant baculovirus AcMU19 and expression of P1 was monitored by SDS-PAGE and Western blotting, using AcPAK6 baculovirus-infected Sf21 cell extracts as a control. No extra protein band was observed

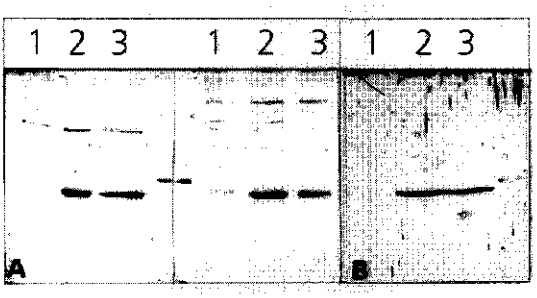

Figure 2: Detection of P1 in total cell extracts from AcMU19-infected insect cells by Western blot analysis. **A.** Western blot probed with antibodies raised against synthetic peptides. Lanes 1: lysates from AcPAK6-infected insect cells; Lane 2 & 3: lysates from AcMU19-infected cells. The western blot membrane was cut in two halves. The left hand lanes were probed with antibodies raised against synthetic peptide SYN1, while the right hand lanes were probed with antibodies raised against SYN2.
B: Western blot probed with antibodies raised against GST-P1 fusion protein. Lane 1: lysate from wild-type AcNPV-infected insect cells; Lane 2 & 3: lysates from AcMU19-infected insect cells.

in Coomassie Brilliant Blue or silver stained protein patterns of AcMU19-infected cells on SDS-PAGE as compared to the control patterns. However, Western blots of extracts of AcMU19-infected cells probed with the antisera raised against the *E. coli* P1-GST fusion protein or the synthetic peptides disclosed a P1-related protein band of 28 kDa (Fig. 2). To determine at which time point the accumulation of P1 reached its maximum, cell extracts were analyzed on Western blot at different time points after infection (p.i.). AcMU19-infected cells displayed the highest levels of P1 accumulation at the end of the infection cycle (72 h p.i.), shortly before lysis, but also breakdown products were observed. However, the amount of P1 produced was insufficient to allow purification.

Upon lysis of the infected cells small amounts of P1 were detectable in the medium, indicating that P1 itself is stable and that the observed breakdown in the cells was probably caused by proteolytic activity of the cells. To determine whether other insect cell lines would allow higher levels of P1 expression *T. ni* ('High Five') and Sf9 cells were infected with AcMU19. However, both cell types produced similar levels of P1 and further experiments were performed using Sf21 cells. To see whether the low accumulation of P1 was caused by impeded transcription rather than by breakdown of the protein or poor translation, mRNA was isolated from infected Sf21 cells and

analyzed for expression of ORF1 mRNA on Northern blot. Using radiolabelled ORF1 DNA as a probe it was disclosed that ORF1 mRNA was abundantly present in AcMU19-infected cells, ruling out the possibility of faulted transcription of the PLRV ORF1.

Localization of P1 in Sf21 cells

To determine the intracellular location of P1 and ergo to gain insight in the nature of the protein, AcMU19-inoculated Sf21 cells were prepared for immunofluorescence and immunogold studies. All available P1 antisera were tested in the experiments. The *E. coli* P1-GST fusion protein raised antiserum displayed no affinity for P1 in immunolabelling experiments, probably since it was raised against SDS-denatured protein. The antisera raised against the synthetic peptides were tested in immunolabelling experiments both separate and in a mixture. Using a mixture of both antisera, in approximately 10% of the AcMU19-infected Sf21 cells high levels of immuno-fluorescence were observed in the cytoplasm of the cells (Fig. 3). In labelling experiments using the separate antisera immunofluorescence was markedly reduced.

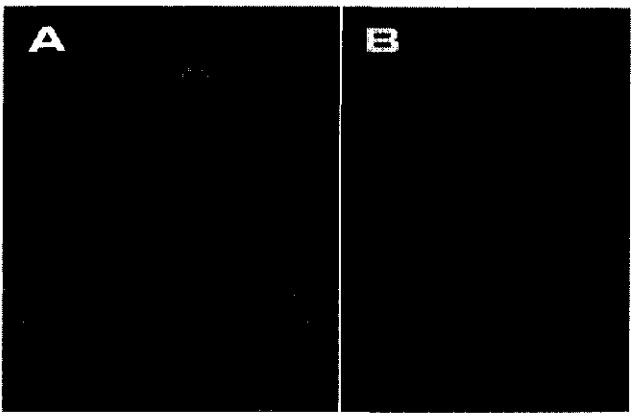

Figure 3: Detection of P1 in AcMU19-infected insect cells by immunofluorescence. **A:** AcMu19-infected insect cells; **B:** AcPAK6-infected cells.

Immuno gold labelling experiments of ultrathin sections of AcMU19-infected insect cells revealed that P1 was present at low levels in the cytoplasm. P1 seemed not to be membrane-associated, but rather to occur randomly dispersed throughout the cytoplasm of the insect cells (data not shown).

DISCUSSION

In this study several antisera were raised against the putative ORF1-product of PLRV with the aim to determine its intracellular localization and consequently to gain insight in the role of this non-structural protein in the viral infection cycle. Antisera were raised against synthetic peptides as well as against in *E. coli* expressed P1. Remarkably, production of P1 in *E. coli* cells employing non-fusion expression vectors failed while expression employing the pGEX-2T GST-fusion vector rendered a product approximately 10 kDa smaller in size as expected. Possibly, P1 is rapidly degraded in the bacterial cell except in case it is fused to another protein conveying stability to at least part of the protein.

Although, the antiserum obtained from the immunization experiments with the GST-P1 fusion protein allowed detection of less then 1 ng P1 in Western blot procedures, the presence of P1 could not be detected in PLRV-infected plant material. Also the antisera raised against the synthetic peptides were unable to detect P1 in Western blot or ELISA procedures.

From these data it was concluded that the detection of P1 was infeasible in infected plant material and an alternative strategy had to be examined. The baculo virus expression system has been shown to enable a high production of a wide range of different proteins in eukaryotic cells, ergo presenting an opportunity to study the intracellular localization of P1 in a heterologous system. Hence, P1 was expressed in insect cells. Surprisingly, accumulation of P1 in AcMU19-infected insect cells was exceptionally low, although ORF1 mRNA was abundantly present in the cells. Besides, considerable

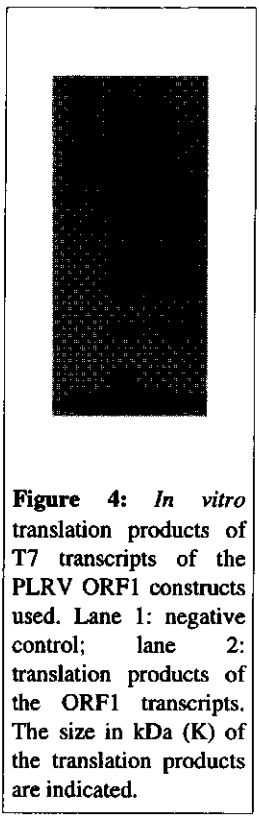

Figure 4: *In vitro* translation products of T7 transcripts of the PLRV ORF1 constructs used. Lane 1: negative control; lane 2: translation products of the ORF1 transcripts. The size in kDa (K) of the translation products are indicated.

breakdown of the protein was observed at the peak of accumulation of P1 in the cells, shortly before lysis of the AcMU19-infected cells while the use of different types of host cells (Sf9, High Five) generated similar results. P1 released in the medium upon lysis of the cells showed to be stable and *in vitro* translation experiments of the ORF1 construct used, showed that the sequence was efficiently translated to high levels and breakdown of the synthesized product was not observed (Fig. 4). These data suggest that P1 is readily degraded upon synthesis before it accumulates to substantial amounts in the cell. Conceivably, P1 is highly susceptible to breakdown by proteases present in the cell, but this seems to be contradictory with the fact that P1 released from the cells appears to be stable and unaffected by proteases present in the medium. Therefore, a more likely explanation is that P1 conveys a toxic effect to both bacterial and insect cells. Consequently, only cells which display a high proteolytic activity resulting in low accumulation of P1 would manage to persist. Supporting the latter hypothesis are the results obtained regarding the expression of P1 in *E. coli* and the observation that expression of P1 in transgenic potato plants leads to phenotypic alterations resembling viral infection, possibly caused by metabolic changes in the plant cells (Chapter 6, this thesis).

The immunofluorescence and immunogold labelling experiments showed that P1 appeared not to accumulate in distinct regions of the insect cells but to occur randomly dispersed throughout the cytoplasm of the insect cells. Based upon amino acid sequence homology and predictions made over the amino acid sequences present at the N-terminal part of the protein it has been suggested that P1 is membrane-associated (Mayo *et al.*, 1989). Strikingly, the BWYV P1 sequence does not suggest membrane-linking. The immunolabelling experiments showed that transgenic PLRV P1 present in AcMU19-infected Sf21 cells was not membrane-associated, however it can not be ruled out that the low level of accumulation hampered detection of membrane-linked P1 or that binding of P1 to cellular membranes is a specific process only occurring in plant cells.

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CHAPTER 6

Expression of the potato leafroll virus ORF1 induces viral disease-like symptoms in transgenic potato plants

ABSTRACT

The open reading frame 1 (ORF1) of potato leafroll virus (PLRV) was transformed into potato cv Désirée. From a total of 44 transgenic potato plant lines none contained detectable levels of P1 protein and only 14 produced detectable levels of ORF1 mRNA. The transgenic potato plants accumulating detectable levels of ORF1 transcripts displayed an altered phenotype resembling virus-infected plants. Potato plants transformed with a modified, untranslatable ORF1 sequence were phenotypically indistinguishable from wild-type control plants. These data indicate that P1 is involved in viral symptom expression.

Manuscript in preparation

INTRODUCTION

The genome of potato leafroll virus (PLRV) consists of a single-stranded messenger-sense RNA molecule containing six open reading frames (ORFs) (Van der Wilk *et al.*, 1989; Mayo *et al.*, 1989; Keese *et al.*, 1990). The genomic RNA contains a VPg at the 5'-terminus and is encapsidated in an icosahedral particle. The major coat protein subunit is encoded by the open reading frame 4 (ORF4) present in the 3'-half of the genomic RNA. The ORF6 which is expressed by translational readthrough of the ORF4, is believed to be involved in the transmission of the virus (Brault *et al., 1995).*

Presently, little is known about the role of most of the products of the non-structural genes of PLRV. The ORF3 represents the putative viral polymerase gene, since its product contains the GxxxTxxxN(x_{25-40})GDD amino acid sequence motif, which is conserved in all known RNA-dependent RNA polymerases (Koonin, 1991). The ORF2 product contains a protease motif (Bazan & Fletterick, 1989; Gorbalenya *et al.*, 1989), but the role of this putative protease in the viral infection cycle is still concealed. The ORF5 product has been shown to bind single-stranded nucleic acids and therefore has been suggested to play a role in the cellular transport of the virus (Tacke *et al.*, 1991 & 1993). The function of the ORF1 product is still a mystery. ORF1 encodes a 28 kDa protein which is highly hydrophobic and shows a very weak homology with several membrane-linked proteins. Analysis of its amino acid sequence has revealed a putative membrane binding site between residues 21 and 32 (Mayo *et al.*, 1989).

The genomic organization of PLRV is very similar to those of other luteoviruses especially beet western yellows virus (BWYV) (Veidt *et al.*, 1988). Comparisons made between the PLRV and BWYV-encoded proteins revealed that all the viral proteins shared a high homology in amino acid sequence except for the ORF1s. Although, the ORF1s of both viruses are similar in size and position on the genome, their respective products (P1) share no homology whatsoever. Furthermore, there are no indications that the BWYV ORF1 product is membrane-linked. In ecological view the main difference between both viruses lies in their host range, PLRV being only capable of infecting a limited number of plant species (mostly *Solanaceae*) and BWYV able to infect many different plant species. Since the ORF1 products seem to compose the main genetical difference between the viruses, it has been suggested that ORF1 plays a role in host recognition (Veidt *et al.*, 1992).

The transformation of plants with viral non-structural genes has been shown to be most beneficial both for gaining more insight in the function of these genes and for obtaining resistance. For example, transgenic plants expressing functional or dysfunctional viral polymerases (Golemboski *et al.*, 1990; Longstaff *et al.*, 1993; Brederode *et al.*, 1995) have been shown to convey resistance, while the expression of the two movement proteins of squash leaf curl virus (SqLCV) in transgenic plants induced viral disease-like symptoms and provided insight in the cellular localization of the proteins (Pascal *et al.*, 1993). To investigate the function of PLRV P1, potato plants were constructed containing the ORF1. Plants expressing the ORF1 displayed a viral diseased-like phenotype, indicating that P1 interferes with the metabolism of the plant.

MATERIALS AND METHODS

Production of transgenic plants

The sequence encoding the PLRV ORF1 was excised from an existing cDNA clone (Van der Wilk *et al.*, 1989) employing the polymerase chain reaction (PCR). Using different oligonucleotides, two ORF1 constructs were synthesized. The first construct (pORF1) was analogous to the wild type PLRV ORF1 sequence, while the second one (pORFM) contained a stop codon 6 nucleotides downstream of the ORF1 start codon, ergo inhibiting translation of the sequence (Fig. 1). To facilitate further sub-cloning the ORF1 sequences were supplied with BamHI restriction sites located immediately in front and after the start and stop codons. The obtained fragments were translated *in vitro* and sequenced to exclude the occurrence of possible mutations. Subsequently, the fragments were placed between the cauliflower mosaic virus (CaMV) 35S-promoter and the nopaline synthase terminator (nos) by ligation in BamHI digested pMOG181 (Chapter 3, this thesis). The resulting plasmids (pMOGORF1 & pMOGORFM) were digested with EcoRI and HindIII and the fragments containing the ORF1 cassette inserted in the binary vector pMOG402, giving rise to the plasmids pBWTO and pBMO. Transformation of potato plants was done as previously described (Hoekema *et al.*, 1989) Potato tuber discs, cultivar Désirée) were cocultivated with *Agrobacterium tumefaciens* LBA4404 harbouring the plasmid pBWTO or pBMO. For cocultivation

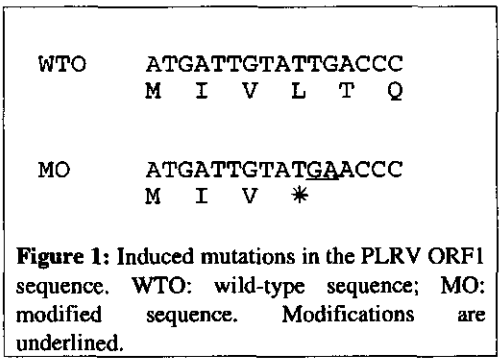

Figure 1: Induced mutations in the PLRV ORF1 sequence. WTO: wild-type sequence; MO: modified sequence. Modifications are underlined.

1000 tuber discs in the initial experiments were used and 500 discs in the other experiments. Selection for transformation was done on medium containing kanamycin (100 µg/ml). Kanamycin resistant shoots were cut off and placed onto rooting medium, propagated axenically and transferred to soil.

Analysis of transgenic potato plants

The transgenic plants were analyzed on Northern blot for expression of ORF1 encoding mRNA. Total RNA was isolated from plant material as described (Verwoerd *et al.*, 1989). Aliquots of approximately 20 µg of total RNA were loaded onto a denaturing 1% agarose gel containing 2.2 M formaldehyde. After electrophoresis the RNA was transferred to a blotting membrane (Hybond-N) and probed with a radiolabelled ORF1 fragment.

For Southern analysis genomic DNA was isolated from the plant material as previously described (Dellaporte *et al.*, 1983). The genomic DNA was digested with restriction enzymes, separated on agarose gel and subsequently transferred to a blotting membrane (Hybond-N). The blots were hybridized with a radiolabelled ORF1 probe.

All the transgenic plants containing the wild-type ORF1 sequence were analyzed for transgenic protein expression using enzyme linked immunosorbent assay (ELISA) and Western blot procedures. To analyze the presence of accumulated P1, antisera were used raised against two synthetic peptides (ELISA) and raised against a P1-glutathione-S-transferase (GST) fusion protein produced in *Escherichia coli* (Western blot) (Chapter 5, this thesis). ELISA was performed as described (Van den Heuvel & Peters, 1989), transgenic plant tissues were ground in extraction buffer (0.2% ovalbumin, 2% polyvinylpyrrolidone, 0.05% Tween-20 in phosphate buffered saline (PBS)). Two hundred microliter of the suspension was used per well. ELISA plates were coated with (0.5 µg/ml) IgG's raised against synthetic peptides. IgG's conjugated to alkaline phosphatase were used to detect bound P1. Western blot was carried out using a

buffered transfer system. Leaf material was ground in Laemmli buffer (Laemmli, 1970), boiled for 10 minutes and loaded onto a 12.5% polyacrylamide SDS gel. After electrophoresis, the proteins were transferred to nitrocellulose and probed with antiserum.

RESULTS

Analysis of transgenic ORF1 potato plants

Transgenic potato plants, cultivar Désirée, were produced containing the PLRV ORF1. To this end, the ORF1 sequence was supplied with BamHI restriction sites and cloned into a binary transformation vector. Employing the *A. tumefaciens* transformation system, forty four independent transformants were obtained.

Strikingly, approximately one third of the produced plant lines was phenotypically dissimilar from the other obtained transformants or non-transgenic potato plants. As shown in Figure 2, the phenotypic aberrations included yellowing and rolling of the leaves, anthocyan formation in the leaf, slow and (severe) stunted growth of the plant. The leaves of these transgenic plants were thickened and showed a loss in flexibility making the leaves brittle. Moreover, the plants displaying the more extreme phenotypic aberrations exhibited slow or even impeded root formation both in soil and on medium containing or devoid of kanamycin. Several of the obtained transgenic plants were not able to produce viable tubers. In general, the observed phenotypic aberrations resembled strongly viral disease-like symptoms. The changes in the phenotypic appearance of the plants were not due to the possibly unfavourable conditions in tissue culture, since plants grown from tubers displayed exactly the same phenotype.

The manifestation and extension of the phenotypic aberrations was strongly influenced by the light intensity used to grow the plants. High light intensities induced strong phenotypic aberrations, while at low light intensities phenotypic changes were milder. This is analogous to the situation with PLRV-infected plants, in which symptoms are enhanced by elevated light intensities.

All of the obtained transgenic plants were tested on Northern blot (Fig. 2) to detect the presence of ORF1 specific mRNA. Fourteen of the transgenic plants accumulated

detectable levels of ORF1 mRNA. The presence of detectable amounts of ORF1 mRNA in the transgenic potato plants coincided with the observed phenotypic aberrations, with the exception of one plant line (WTO34). This plant line was phenotypically indifferent of non-transgenic potato plants but contained detectable levels of ORF1 mRNA. In all other cases, severity of the phenotypic aberrations was directly correlated with the levels of accumulation of ORF1 transcripts, suggesting that expression of PLRV ORF1 sequences caused the appearance of viral diseased-like symptoms.

To determine if the observed correlation between ORF1 mRNA accumulation and severity of phenotypic aberrations was not generated by an overall impeded mRNA accumulation due to a disturbed metabolism in the plant tissues, the obtained transgenic plants were tested for accumulation of mRNA encoding actin and the small sub-unit of ribulose-biphosphate-carboxylase (rubisco). Whereas all the plants contained comparable amounts of mRNA coding for actin, the accumulation of rubisco mRNA was strongly inhibited in the plants displaying a changed phenotype, suggesting that ORF1 expression interfered with the process of photosynthesis but did not have an overall effect on mRNA synthesis.

To examine wether both transgenic plant lines displaying the altered phenotype and plant line WTO34 contained complete, non-mutated ORF1 sequences, PCR was carried out using isolated genomic DNA as a template. The 5'-terminal primer used in the PCR experiments was identical to a sequence internally located in the CaMV 35S promoter (92 nucleotides upstream of the start codon of ORF1 in the binary construct), while the downstream primer was complementary to the 3'-end of ORF1. PCR performed with genomic DNA, isolated from both transgenic plants expressing and plants not expressing ORF1 mRNA, rendered in all cases products of the expected size. Sequence analysis of these PCR products, including WTO34 derived products, showed that all plants tested contained unaltered copies of the PLRV ORF1 sequence.

Expression of P1

All the obtained transgenic plants were tested on Western blot or in ELISA for the accumulation of transgenic P1. To detect P1 on Western blot an antiserum was used raised against an *E. coli* expressed P1/GST fusion protein, for ELISA antisera were used raised against synthetic peptides (Chapter 5, this thesis). Since detection of P1 in

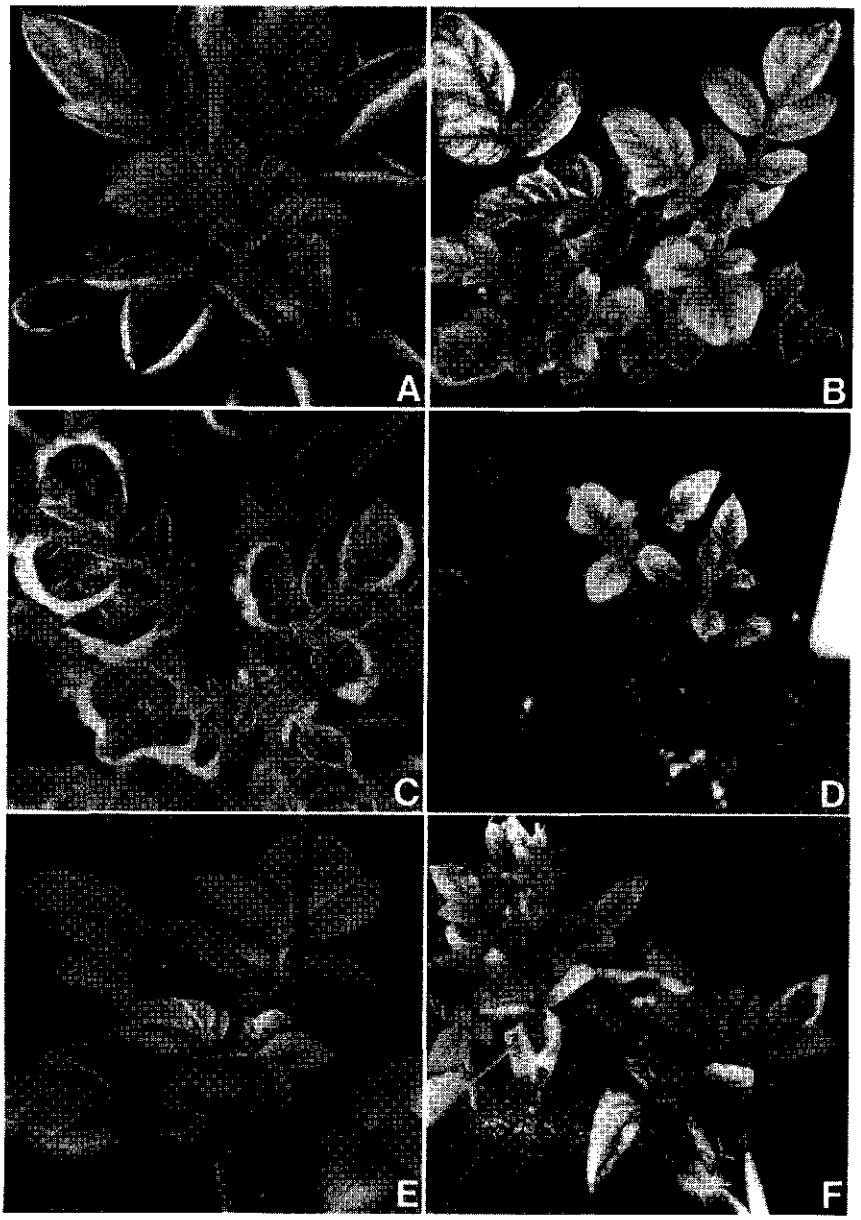

Figure 2: Phenotypes of PLRV ORF1 transgenic potato plants. **A, B, C, D:** transgenic WTO plants, expressing levels of transgenic transcripts detectable on Northern blot, WTO24, WTO26, WTO4, WTO18, respectively; **E**: wild-type potato plant cv Désirée; **F:** potato plant cv Désirée infected with PLRV.

PLRV-infected plants has been shown to be infeasible, as a control P1 produced in the baculovirus expression system was used (Chapter 5). Although several different procedures were examined to isolate P1 from transgenic plant tissue, including methods specifically designed to isolate membrane-linked proteins (Martin & Garcia, 1991; Deom *et al.*, 1990, Chapter 5) transgenic P1 protein could neither be detected on Western blot nor by ELISA.

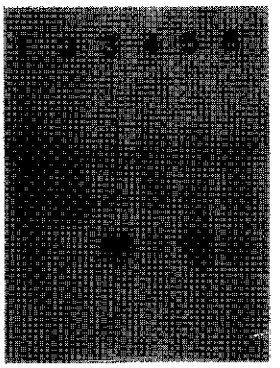

Figure 3: Northern analysis of PLRV ORF1 transgenic potato plants. Twenty µg of total plant RNA purified from transgenic leaf material were loaded onto a 1% agarose gel containing formaldehyde. Lane 1: RNA from control Désirée plants; lane 2-6: RNA from transgenic plant lines, WTO15, WTO24, WTO25, WTO26, WTO34, respectively

Analysis of transgenic plants containing a modified ORF1 sequence

To investigate whether translational expression of PLRV ORF1 is responsible for the observed viral disease-like symptoms, potato plants (cultivar Désirée) were transformed with a modified ORF1 sequence (MO). This altered sequence contained a stop codon (UGA) 6 nucleotides downstream of the start codon, thus blocking the translation of the ORF1 reading frame (Fig. 1). Remarkably, a significant higher number (more than 80) of transgenic MO plant lines than WTO plant lines (approximately 20) was obtained in the transformation procedure. Moreover, compared to the MO transgenic plants WTO plants were slow in shooting and rooting (3 to 9 weeks longer).

Nearly all of the MO plants were phenotypically similar to wild-type potato plants and none of the obtained transgenic MO plant lines displayed phenotypic aberrations similar to those of the WTO plants. Five to six MO plant lines displayed growth and morphological aberrations. These aberrations were believed to be caused by the occurrence of somaclonal variation and included stunting and disformation of the leaves. Rolling of the leaves, yellowing of the leaves and anthocyan formation, however, were never observed. Northern blot analysis of forty transgenic MO plant lines disclosed that 90% of the examined plants contained detectable levels of ORF1

transcripts.

The results from the transformation experiments with the wild-type ORF1 sequence were similar to the results from the initial transformation experiment. Transgenic WTO plant lines, expressing levels of ORF1 transcripts detectable on Northern blot, displayed viral disease-like symptoms similar to the earlier observed phenotype. These results indicate that translational expression of PLRV ORF1 product in transgenic potato plants is responsible for the induction of the disease-like symptoms.

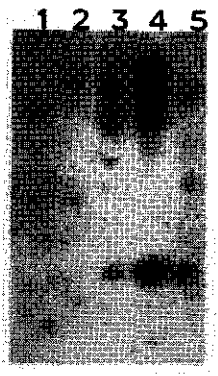

Figure 4: Southern blot analysis of PLRV ORF1 transgenic potato plants. Ten µg of DNA, purified from transgenic leaf material, and digested with EcoRI and HindIII were loaded onto a 1% agarose gel. Lane 1-5: DNA from transgenic plant lines, WTO1, WTO15, WTO25, WTO26, WTO34, respectively.

DISCUSSION

Presently, surprisingly little is known about the functions of the putative products of the open reading frames present on the genome of PLRV in particular and of luteoviruses in general. While, the genes encoding the coat protein subunits are known, it is still obscure which ORF codes for the VPg and concerning the non-structural genes only the putative RNA-dependent RNA polymerase has been identified by sequence comparison. To gain insight in the function of the non-structural ORF1 of PLRV, potato plants have been transformed with this sequence. Surprisingly, the resulting transgenic (WTO) plants which accumulated detectable levels of ORF1 transcripts were phenotypically different from wild-type potato plants and from transgenic plants which did not accumulate transgenic mRNA. Since, transgenic plants expressing a modified, untranslatable ORF1 sequence were phenotypically identical to wild-type potato plants, it is concluded that translational expression of PLRV ORF1 provokes the observed viral disease-like symptoms. The altered phenotype included yellowing, rolling and thickening of the leaves, anthocyan formation and stunted growth, thus resembling plants displaying viral disease symptoms.

Northern blot analysis of the transgenic WTO plants revealed that accumulation of mRNA encoding rubisco was also impeded. Both the change in phenotype and the decrease in accumulation of rubisco mRNA indicate that expression of ORF1 sequences interfered with the metabolism in the plant cells. Remarkably, only approximately one third of the obtained transgenic WTO plants accumulated levels of ORF1 mRNA detectable in Northern blot procedures. These findings strongly deviate with the results of the transformation experiments in which a modified, untranslatable ORF1 sequence was transformed into potato plants. Ninety percent of transgenic MO plants analyzed were shown to accumulate detectable levels of transgenic mRNA. Moreover, the level of accumulation of transgenic mRNA in plants expressing the wild-type ORF1 was notably lower than in transgenic plants expressing the modified ORF1 sequence. In all experiments the same binary transformation vector and thus promoter was used to express the different ORF1 sequences in plants. Differences in accumulation of mRNA have been shown to be caused by variations in the level of transcription due to methylation of promoter sequences (Razin & Cedar, 1991) and by post-transcriptional degradation of the mRNA (Van Blokland *et al.*, 1994). Possibly, expression of ORF1 sequences is down-regulated in the plant cell to minimize the amount of P1 present in the plant cell (Chapter 5).

Attempts to detect the ORF1 product in transgenic tissues proved to be unsuccessful. P1 has been suggested to be membrane-linked (Mayo *et al.*, 1989). However, employing procedures which have been shown to be successful for the isolation of viral membrane-bound proteins, no P1 could be detected in both ELISA and Western blot procedures. These findings coincide with the results of the experiments described in Chapter 5, in which the detection of P1 showed to be infeasible in PLRV-infected plants. Conceivably, P1 is rapidly degraded in plant cells. It has been shown that expression of P1 in both *E. coli* cells and the baculovirus/insect cell expression system was burdensome, due to the fact that accumulation of high levels of transgenic P1 was obstructed by proteolytic degradation (Chapter 5). Conceivably, breakdown of the protein circumvents a toxic effect imparted by the accumulation of P1 in the cell.

Previously, it has been reported that expression of the movement protein BL1 gene of the geminivirus squash leaf curl virus (SqLCV) in *Nicotiana benthamiana* (Pascal *et al.*, 1993) and the gene VI of the pararetrovirus cauliflower mosaic virus (CaMV) in non-host tobacco species (Baughman *et al.*, 1988) induced viral disease-like

phenotypes. While expression of gene VI in susceptible host plants has been shown not to convey virus disease-like symptoms (Goldberg *et al.*, 1989), expression of BL1 in its permissive host induced viral disease-like symptoms. Interestingly, SqLCV, a bipartite geminivirus, is phloem-limited like PLRV and its movement proteins BL1 and BR1 have been shown to be important determinants of viral host range properties (Lazarowitz, 1991; Ingham & Lazarowitz, 1993). Both P1 and BL1 are moderately hydrophobic (isoelectric points 8.4 and 8.6, respectively) and are approximately similar in size, 28 and 33 kDa respectively). However, SqLCV is a circular single-stranded DNA virus and the BL1 and BR1 proteins of the different bipartite geminiviruses share an extensive amino acid sequence homology (Smith & Maxwell, 1994) while the ORF1 products of the luteoviruses display no amino acid sequence homology whatsoever. Nonetheless, based upon the available data on BL1 and the results of the experiments described in this chapter it is tempting to suggest a BL1 analogous function for PLRV P1.

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CHAPTER 7

Resistance in transgenic potato plants expressing the PLRV ORF1

ABSTRACT

Transgenic potato plants containing the open reading frame 1 (ORF1) of potato leafroll virus (PLRV) were tested for acquired resistance. From a total of 34 transgenic potato plant lines one showed to be highly resistant upon inoculation with PLRV. The acquired resistance was expressed as near immunity, under high inoculation pressure only a low percentage of the plants became infected.

Manuscript in preparation

INTRODUCTION

Pathogen-derived resistance (PDR) (Sanford & Johnston, 1985), the phenomenon that a host is protected from infection by expressing pathogen-derived sequences, is currently widely applied to obtain resistance to plant viruses. Both the expression of wild-type and modified versions of viral genes encoding replicases, coat proteins, or movement proteins have been shown to convey resistance. PDR has now been shown to be effective against viruses in 14 different taxonomic groups and in a growing number of crop plants (for review, see Wilson, 1993; Hackland *et al.*, 1994).

In most cases the mechanisms underlying PDR have remained unknown. Initially it was believed that expression of transgenic protein was indispensable to incite resistance, later it has been shown that expression of untranslatable mRNA sequences also induced resistance (Smith *et al.*, 1994). Conceivably, different mechanisms are involved, in some cases the presence of transgenic protein is required while in other cases the transgenic transcript itself mediates resistance (for review, see Lindbo *et al.*, 1993; Wilson, 1993; Hackland *et al.*, 1994). However, the molecular mechanisms underlying both the 'protein-mediated' and 'RNA-mediated' resistance are still obscure and subject to further investigations.

Recently, evidence has been presented that high expression of transgenic viral sequences in plants leads to specific degradation of the transgenic mRNA, resulting in low accumulation of the transgenic RNA. As a consequence the genomic RNA of the analogous challenging virus would be degraded by the same cellular pathway, resulting in immunity (Smith *et al.*, 1994; Dougherty *et al.*, 1994). Thus, plant lines accumulating low levels of transgenic mRNA should exhibit high levels of resistance. Acquired resistance by specific degradation of RNA sequences corresponds to the phenomenon of 'co-suppression', in which transgenes silence homologous endogenous plant genes. Co-suppression has been reported for several different genes in various plant species (for review, see Finnegan & McElroy, 1994). The mechanism underlying the event of co-suppression is poorly understood. Both methylation of promoter sequences (Hobbs *et al.*, 1990 & 1993), the spontaneous occurrence of anti-sense mRNA (Mol *et al.*, 1991), and post-transcriptional degradation of mRNA (Van Blokland *et al.*, 1994) have been reported to play a role in gene silencing.

Potato leafroll virus (PLRV) is the causal agent of one of the major diseases of

potato. Since, all commercial potato cultivars are, to a greater or lesser extent, susceptible to the virus, resistance is of great economic value. It has been shown that the expression of the open reading frame 1 (ORF1) of potato leafroll virus (PLRV) induced viral disease-like symptoms in transgenic potato plants (Chapter 6). Furthermore, it has been shown that most of the transgenic plants are capable of suppressing the accumulation of ORF1 transcripts to circumvent the toxic effect imparted by the expression of P1. Therefore, in concordance with the above mentioned concept of co-suppression-like RNA-mediated resistance, it would be expected that transgenic ORF1 plants expressing low levels of transgenic transcripts display high levels of resistance. To investigate this supposition, transgenic ORF1 plants both non-expressing and expressing extreme low or high levels of transgenic transcripts have been inoculated with PLRV and monitored for infection.

MATERIAL AND METHODS

Inoculation of plants and assessment of PLRV resistance

Transgenic and wild-type potato plants (cv Désirée) were inoculated with PLRV as previously described (Van der Wilk *et al.*, 1991) using 10-15 viruliferous aphids (*Myzus persicae* (Sulzer) Wmp1) per plant. The aphids were allowed 3-7 days to feed on the plants and thereby to transmit the virus. Infection was monitored for each individual plant by taking samples of the lower, middle and top leaves at each time point. The three samples of each plant obtained were pooled, ground in extraction buffer and tested in an enzyme-linked immuno sorbent assay (ELISA) procedure for the presence of PLRV. In the first preliminary tests 3 to 5 plants per transgenic plant line were screened; lines which showed a reduction in viral incidence as compared to the controls were examined in more detail. In the additional screenings 10 to 15 plants of each line were inoculated and monitored for acquired resistance. Tubers of plants showing up negative in the screenings were harvested and stored for 4 to 6 months at 4 ^{0}C. After replanting the shoots emerging from the tubers were tested in ELISA for the presence of PLRV.

Enzyme-linked immuno sorbent assay (ELISA)

ELISA was performed as described (Van den Heuvel & Peters, 1989). Leaf discs of one centimeter diameter were ground in 0.5 ml extraction buffer (0.2% ovalbumin, 2% polyvinylpyrrolidone, 0.05% Tween 20 in phosphate buffered saline (PBS)), 200 µl of the suspension was used per well in the test. ELISA plates were coated with (1 µg/ml) IgG's raised against intact purified virus. The samples were incubated for 16 h at 4 ^{0}C. Subsequently, the wells of the plates were washed three times with PBS/0.05% Tween 20. IgG's conjugated to alkaline phosphatase (1 µg/ml) were used to detect bound virus.

Reverse transcription (RT) and polymerase chain reaction (PCR)

Transgenic plants were analyzed for the presence of ORF1 transcripts by reverse transcription (RT) of purified mRNA followed by amplification of the transgenic cDNA using the polymerase chain reaction (PCR) and employing specific primers. Total RNA was isolated as described (Verwoerd *et al.*, 1989). cDNA was synthesized using moloney leukemia virus (M-MLV) reverse transcriptase (Gibco-BRL) and an oligo(dT)$_{12\text{-}18}$ as primer. PCR was performed using oligonucleotides identical to 5'-end sequence of the ORF1 and complementary to a sequence located internally in the ORF1. PCR-products were visualized by agarose gel electrophoresis.

RESULTS

Inoculation of transgenic potato plants with PLRV

Thirty-four transgenic plants containing the PLRV ORF1 sequence were tested for possible acquired resistance to PLRV. All transgenic plants were derived from one transformation experiment (Chapter 6).

Inoculation experiments employing viruliferous aphids disclosed that one plant line (WTO25) exhibited resistance, while all other plant lines were susceptible to infection. In five separate experiments, using 10 to 15 plants per tested plant line, line WTO25 was found to be highly resistant. Upon inoculation of WTO25, using ten viruliferous

aphids per plant, none of the tested plants became infected, while all the other inoculated transgenic and non-transgenic control plants showed a 90-100% infection incidence. Experiments on WTO25 with an increased inoculation pressure employing higher numbers (15-30) of viruliferous aphids resulted in an infection rate of approximately 10%. The infected WTO25 plants contained similar amounts of viral antigen as compared to infected wild-type potato plants. To determine if possible infections could have been obscured by low virus titers resulting in low ELISA readings, tubers of plants used in the inoculation experiments were harvested and the emerging sprouts of the replanted tubers were monitored for viral infection. None of the tested sprouts derived from plants assessed in earlier experiments as non-infected contained detectable levels of PLRV.

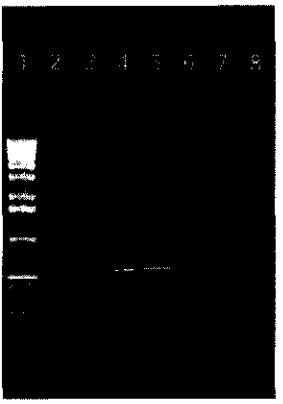

Figure 1: Analysis of transgenic potato plants for the presence of ORF1 transcripts by RT-PCR. All plants tested had previously been shown to contain levels of ORF1 transcripts undetectable on Northern blot. Lane 1: DNA marker; lanes 2-6: RT-PCR products from WTO2, WTO5, WTO25, WTO32, WTO15, respectively; lane 7: empty; lane 8: non-transgenic control plants.

WTO25 was phenotypically indifferent from wild-type potato plants. Previously, plant line WTO25 had been shown to accumulate levels of ORF1 mRNA undetectable on Northern blot. RT-PCR, using total RNA extracted from WTO25 as a template, showed that extreme low amounts of ORF1 mRNA were present in the transgenic tissue (Fig. 1). From southern blot analysis it was estimated that the genome of WTO25 contained one copy of the ORF1 sequence. PCR and subsequent sequence analysis disclosed that the inserted ORF1 sequence did not contain any mutations or deletions, indicating that the observed resistance was not due to expression of a modified ORF1-product.

DISCUSSION

Transgenic plants containing the PLRV ORF1 were tested for possible acquired

resistance. All the transgenic plants were derived from one transformation experiment. In Chapter 6 of this thesis it was shown that only approximately one third of transgenic ORF1 plant lines tested accumulated notable amounts of transgenic transcripts. Most likely the accumulation of transgenic transcripts in the cell is impeded to suppress the toxic effect caused by expression of the P1 protein (Chapters 5 & 6). This appears to make these plant lines a possible model system to examine the recently proposed mechanism of cosuppression-like RNA-mediated resistance (Smith *et al.*, 1994; Dougherty *et al.*, 1994).

Upon inoculation using viruliferous aphids, one plant line (WTO25) showed to be resistant. All other thirty three analyzed transgenic plant lines were similar susceptible to viral infection as wild-type potato plants. Remarkably, the acquired resistance in WTO25 expressed itself as nearly absolute immunity rather than a decrease in viral titers as observed for transgenic potato plants expressing the PLRV ORF4 (Van der Wilk *et al.*, 1991; Kawchuk *et al.*, 1991). Only if high inoculation pressure was applied a small percentage of the WTO25 plants became infected. Infected WTO25 plants contained levels of viral antigen similar to infected control plants. Shoots emerging of tubers derived of plants inoculated with PLRV and assessed as non-infected, did not contain detectable levels of virus. This indicates that possible infection of the plants was not obscured by extreme low viral titers, not detectable in ELISA.

The mechanism of the observed resistance is unclear. Previously it has been shown that none of the transgenic plants accumulated detectable amounts of P1 protein, secluding a possible role for the ORF1 product. Since only one plant line showed to be resistant it is highly unlikely that expression of ORF1 sequences itself conveyed resistance. Besides, WTO25 contained extreme low amounts of ORF1 transcripts, solely detectable by RT-PCR. These findings seem to support the hypothesis that a co-suppression-like event is involved in the observed resistance in WTO25. However, 20 out of 34 transgenic plant lines tested did not contain significant amounts of transgenic ORF1 mRNA, probably due to specific degradation of the transgenic transcripts or silencing of the transgene. If the observed resistance was imparted by post-transcriptional breakdown of the viral RNA or another regulatory mechanism it would be expected that all plants, accumulating low levels of ORF1 transcripts showed to be resistant instead of only plant line WTO25. Ergo, it appears that the observed resistance in plant line WTO25 can not be attributed to a co-suppression-like phenomenon.

Another possible explanation for the acquired resistance is that it is induced by somaclonal variation. WTO25 appeared to be phenotypically indistinguishable from wild-type potato plants cv Désirée. Nevertheless, it can not be excluded that somaclonal variation occurred. In field experiments with transgenic potato plants, cultivars Bintje and Escort, expressing the potato virus X (PVX) coat protein gene, it was found that approximately 82% and 18%, respectively, of the transgenic plant lines were not true to type in all 50 morphological traits examined (Jongedijk *et al.*, 1992). In both the experiments described here and the experiments described by Jongedijk *et al.* essentially the same protocol for transformation was used. Interestingly, recently it has been described that transformation of potato with binary vector sequences alone induced resistance against PLRV (Presting *et al.*, 1995). Although this resistance expressed itself as a reduction in viral titer rather than immunity as described here, it appears from these data that the procedure of transformation itself can introduce unexpected variation among the transformants, including the manifestation of resistance.

Also, it can not be excluded that by insertion of the transgene in the potato genome a gene conferring susceptibility was inactivated. However, the existence of such a gene has never been reported and, ergo, the occurrence of insertional inactivation seems to be only a remote possibility.

Irrespectively from the fact that further experiments will be needed to clarify the mechanism of resistance, a potato line resistant to PLRV has been generated. Since, PLRV is the causal agent of leafroll, one of the most important diseases in potato, extreme resistance is an economical highly important trait.

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CHAPTER 8

GENERAL DISCUSSION

Potato leafroll virus causes a destructive disease of potato plants and is consequently responsible for significant economic losses world-wide. Beside causing severe yield reductions, PLRV incidence above certain thresholds leads to rejection of seed potato lots from certification schedules. Although potato cultivars differ in resistance to PLRV, there appears to be neither immunity nor major gene resistance to the virus in *Solanum tuberosum* cultivars (Ross, 1986). Resistance to PLRV presently available in cultivated forms is believed to be polygenically controlled and can be overcome by high inoculation pressure. The aim of this study was to gain more insight in the molecular biology of this virus and to utilize this knowledge to obtain resistance, according to the principal of pathogen-derived resistance (Sanford & Johnston, 1985).

To this end the nucleotide sequence of the PLRV genomic RNA was determined and its organization deduced. The PLRV genomic organization was shown to contain six open reading frames (ORFs). To express its genes the virus appears to employ all presently known strategies in viral gene expression: initiation at downstream AUG start codons by leaky scanning, shifting of ribosomes during translation from one reading frame to another, suppression of stop codons resulting in translational readthrough, translation of sub-genomic messenger RNAs and maturation of proteins by proteolytic cleavage.

By interviral sequence comparison the genes encoding for the coat protein subunit and the putative RNA-dependent RNA polymerase have been identified. The amino acid sequences of the coat proteins of different luteoviruses share a substantial homology, which complies with the fact that many luteoviruses are immunogenic related. Remarkably, the putative replicase of PLRV shares sequence homology with both the replicases of BWYV and barley yellow dwarf virus serotype RPV (BYDV-RPV) and the replicase of southern bean mosaic virus (SBMV), but not with those of BYDV-MAV and PAV, which resemble the replicase of carnation mottle virus (CarMV) (Miller *et al.*, 1988; Ueng *et al.*, 1992). This makes the luteovirus group the

only virus group harbouring members with different types of RNA-dependent RNA polymerases. Therefore, based on sequence data two different subgroups have been recognized. For subgroup I (BYDV-MAV & PAV) the term "alloluteoviruses" has been proposed while for subgroup II (PLRV, BWYV, BYDV-RPV) the original name "luteovirus" could then be reserved (Koonin & Dolja, 1993).

The role of most of the putative viral proteins in the infection cycle remains unclear. The ORF2 product contains sequence motifs believed to be typical for chymotrypsin-like serine proteases (Bazan & Fletterick, 1989; Gorbalenya *et al.*, 1989). However, the role of such a protease in the expression strategy of the virus is unknown. Possibly, this protease cleaves autocatalytical and conceivably plays a role in the maturation of the VPg. The ORF coding for the VPg has not been identified yet, but the most likely candidates are the ORF5 or the 5'-terminal part of the ORF2. Irrespective of which ORF encodes the VPg, proteolytic cleavage has to play a role in the expression of the mature protein since its reported size of 7 kDa (Mayo *et al.*, 1982) is much smaller than the coding capacity of the different ORFs present on the PLRV genome. All the putative PLRV-encoded proteins exhibit a certain degree of homology with other putative luteoviral products except for the PLRV ORF1 product (P1). Not only PLRV P1 exhibits no amino acid sequence homology with the analogous ORF1 products of other luteoviruses but an equivalent ORF is even absent on the genome of the BYDV-MAV and PAV isolates. The genomic organization of the PLRV and BWYV genomes are very similar (Van der Wilk *et al.*, 1989; Veidt *et al.*, 1988) and the respectively encoded putative viral proteins share extensive amino acid sequence homologies, with the exception of the ORF1 products. The most notable difference between PLRV and BWYV is in their host range. While BWYV is able to infect a wide range of different plant species, the host range of PLRV is restricted to a few plant species. It has been speculated that the luteoviral ORF1s determine host range, since they appear to represent the primary difference between the various luteoviruses genomes (Veidt *et al.*, 1992). In experiments described in this thesis it was attempted to elucidate the role of the PLRV ORF1 product (P1) in the viral infection cycle. Unexpectedly, expression of this protein in both prokaryotic (*Escherichia coli*) and eukaryotic cells (baculovirus/ insect cell expression system) demonstrated to be burdensome, probably due to high sensitivity to post-translational proteolytic activity. PLRV-infected plants analyzed for the presence of P1, appeared not to contain detectable levels of the protein, although the

antiserum used was able to detect as little as 1 ng of recombinant protein. It seems therefore justified to conclude that only extreme low levels of P1 are accumulated during PLRV infection. Remarkably, only approximately one-third of transgenic potato plants, containing the PLRV ORF1 sequence, accumulated detectable levels of ORF1 transcripts and none of the plants contained detectable levels of transgenic protein. The transgenic potato plants expressing ORF1 transcripts to detectable levels were phenotypically different from wild-type plants, strongly resembling viral-diseased plants. These data indicate that expression of PLRV ORF1 leads to distortion of the metabolism in the plant cell, possibly mimicking the events associated with viral infection. Such a putative toxic effect of P1 could possibly also offer an explanation for the difficulties encountered in both the experiments aimed at the expression of the protein in *E. coli* or the baculovirus/insect cell system and the detection of P1 in infected and transgenic plants. In order to avert the toxic effect generated by P1 expression, cells are possibly capable of limiting the accumulation of the protein by down-regulation of the translation or by proteolytic breakdown.

To incite resistance, potato plants have been transformed with PLRV genes. Pathogen-derived resistance, the phenomenon that expression of viral sequences in plants confers resistance, has been shown to be potentially extremely useful. Initially, attention has been focused on the expression of viral coat protein genes, later it has also been shown that the expression of other viral (nonstructural) genes could induce resistance (for reviews, see Wilson, 1993; Hackland *et al.*, 1994). Since the initial experiments of Powell *et al.* (1986), coat protein-mediated resistance has been shown to be an effective strategy to acquire resistance against a whole range of different viruses (for recent review, see Hackland *et al.*, 1994). The PLRV ORF4 encodes the major coat protein subunit present in the viral particle. In experiments described in this thesis, transgenic potato plants, were produced that contained the PLRV ORF4 sequence in sense or antisense orientation. Although, the obtained transgenic potato lines expressed ORF4 transcripts, none of the plants accumulated detectable levels of transgenic protein. Upon inoculation with PLRV the obtained transgenic plants showed a reduced rate of virus accumulation as compared to wild-type control plants. However, complete immunity was not obtained. Initially, it has been reported for other plant-virus systems that a direct correlation existed between the amount of coat protein expressed *in planta*

and the efficacy of coat protein-mediated protection (for recent review, see Hackland *et al.*, 1994). Therefore, to determine whether it was feasible to optimize the translational expression of the PLRV ORF4 in transgenic plants and thereby to enhance the induced resistance, the ORF4 sequences were modified. The sequences flanking the sub-optimal start codon were modified into a potentially translationally optimized context and the start codon of the ORF5, underlying the ORF4, was deleted. The results obtained from experiments with the transgenic plants harbouring this modified ORF4 sequence were identical to the results obtained with the wild-type ORF4 transgenic plants. All the obtained transgenic potato plants were shown to express the modified ORF4 transcripts but still were not able to accumulate detectable levels of transgenic protein and upon inoculation with PLRV the transgenic potato plants showed a reduced rate of virus accumulation similar to the transgenic lines containing the wild-type ORF4.

Since upon inoculation both plants expressing sense and those expressing antisense transgenic ORF4-transcripts were shown to inhibit viral accumulation, and accumulation of transgenic coat protein could not be detected in the plants expressing messenger-sense (wild-type or modified ORF4) transcripts, it is most likely that expression of RNA sequences alone effected the observed inhibition rather than the expression of transgenic protein.

Besides transgenic plant lines containing the PLRV coat protein gene, potato lines containing the PLRV ORF1 have been produced. As aforesaid, only approximately one-third of the obtained transgenic plants accumulated detectable levels of ORF1-transcripts and none of the plants contained detectable levels of transgenic protein. All transgenic ORF1 plant lines obtained have been tested for possibly acquired resistance against PLRV. Out of thirty-four plant lines tested one showed to be resistant upon inoculation with viruliferous aphids. This resistant plant line was phenotypically similar to wild-type potato plants and was shown to accumulate extreme low levels of ORF1-transcripts. The acquired resistance in this plant line expressed itself as immunity instead of a decrease in virus accumulation as observed for the transgenic plants expressing the ORF4. Only under high inoculation pressure a small percentage of the inoculated plants became infected.

Presently, it is generally accepted that several different mechanisms are involved in specific resistance resulting from the expression of particular viral genes in plants. Both the expression of transgenic RNA alone as well as the expression of transgenic protein

has been shown to confer resistance. Recently, a mechanism underlying the phenomenon of genetically engineered RNA-mediated resistance has been proposed (Dougherty *et al.*, 1994; Smith *et al.*, 1994) which becomes more and more accepted. This mechanism would closely resemble the processes involved in co-suppression, i.e. the phenomenon that expression of transgenes in plants can provoke silencing of both transgenes and homologous endogenous genes (for review, see Flavell, 1994). The mechanism involved has not yet been elucidated, but it has been suggested that high accumulation of transgenic products would trigger cellular processes directed to suppress the transgene expression. The virus resistance generated in transgenic plants would possibly be incited by a similar mechanism, cellular processes involved in breakdown of transgenic sequences leading to suppression of the homologous sequences of the infecting virus. This theory is supported by the observation that RNA-mediated resistance is prevalently observed in plant lines accumulating low levels of transgenic transcripts.

However, it is questionable wether the observed, partial inhibition of viral accumulation in the PLRV ORF4 transgenic plants or the nearly complete immunity in the transgenic ORF1 lines can be explained by such a mechanism. The transgenic ORF4 plants tested for resistance accumulated high levels of ORF4 transcripts, ergo, excluding the occurrence of silencing of transgenic and viral sequences. The results from the inoculation experiments with the resistant ORF1 plant line appear to conform to the findings reported concerning the above mentioned genetically-engineered RNA-mediated protection or co-suppression (Dougherty *et al.*, 1994; Smith *et al.*, 1994). However, it is puzzling that only one plant line showed to be resistant. Altogether, twenty transgenic plant lines were shown to contain non-detectable or extreme low levels of ORF1-transcripts. If the observed low accumulation of transgenic ORF1 transcripts in these plants was due to specific cellular processes, like post-transcriptional breakdown, it would be expected that all these plant lines exhibited a resistant phenotype. Conceivably, also other and still unknown factors are involved in the expression of genetically engineered resistance.

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SUMMARY

The nucleotide sequence of the genomic RNA of potato leafroll virus (PLRV) was elucidated and its genetic organization deduced (Chapter 2). Six open reading frames (ORFs) were shown to be present on the genome. Both the PLRV coat protein gene and the RNA-dependent RNA polymerase gene were identified by interviral sequence comparison. The PLRV genomic organization was shown to be highly similar to that of beet western yellows virus (BWYV) and except for the ORF1 products all PLRV and BWYV coded proteins displayed an extensive amino acid sequence homology.

In order to obtain resistance following the principle of pathogen-derived resistance, the PLRV coat protein gene was placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter and used to transform potato (Chapter 3). Upon analysis of the transgenic plants obtained it was shown that, although transgenic transcripts were abundantly present in the plant tissues, the presence of transgenic coat protein could not be detected. The transgenic potato plants were shown to be susceptible to PLRV infection but contained significant lower virus titers as compared to infected wild-type potato plants. To enhance the translational expression of the coat protein gene the sequences flanking the start codon were modified to a theoretically optimized context (Chapter 4). Potato plants were transformed with the altered coat protein gene and analyzed for the presence of transgenic coat protein. Despite of the induced mutations transgenic protein could not be detected. The results from inoculation experiments with PLRV were identical to those obtained with the transgenic plants containing the unaltered coat protein gene, the transgenic plants containing less viral antigen than infected wild-type plants.

To investigate the role of the PLRV ORF1 product (P1) in the viral infection process and to define its intracellular location in infected plant cells, the protein was expressed in *Escherichia coli* and in the baculovirus expression system and used to raise an antiserum (Chapter 5). Expression of P1 proved to be difficult, possibly due to a toxic effect imparted by the protein. Using an antiserum raised against a recombinant P1 fusion protein, it was determined that P1 did not accumulate in infected plant tissues to

detectable levels.

To further investigate the function of the ORF1, its sequence was transformed into potato (Chapter 6). Surprisingly, the transgenic plants expressing detectable levels of ORF1 transcripts displayed an altered phenotype closely resembling that of virus-diseased plants. Plants expressing a modified and therefore untranslatable, version of the ORF1 sequence were phenotypically indistinguishable from wild-type control plants, indicating that the expression of the P1 protein induced virus disease-like symptoms. The transgenic potato plants containing the ORF1 sequence were analyzed for possibly acquired resistance (Chapter 7). Upon infection one plant line showed to be highly resistant while all other plant lines were susceptible to PLRV-infection similar to wild-type plants. The resistance obtained expressed itself as near immunity, only under high inoculation pressure a low percentage of the plants became infected.

SAMENVATTING

Het aardappelbladrolvirus (potato leafroll virus, PLRV) is de veroorzaker van een van de belangrijkste ziekten (bladrol) in de aardappelteelt. Mondiaal wordt het jaarlijkse oogstverlies ten gevolge van PLRV-infecties geschat op 20 x 10^6 ton aardappelknollen. Het virus is in Nederland vooral schadelijk voor de pootaardappelindustrie. Jaarlijks worden vele partijen pootgoed afgekeurd of verlaagd in kwaliteitsklasse ten gevolge van infectie met PLRV. Alle aardappelrassen zijn vatbaar voor dit virus en bladrol wordt op dit moment voornamelijk bestreden door bespuitingen uit te voeren tegen de overbrenger van het virus, de bladluis *Myzus persicae*. De symptomen die het virus in geïnfecteerde aardappelplanten veroorzaakt zijn het karakteristieke oprollen van de bladrand, vergeling van het blad, de vorming van anthocyaan in blad en stengel, achterblijven in groei van de plant en het achterblijven in groei van de knollen. PLRV behoort tot het genus *Luteovirus*. Virussen behorende tot dit genus infecteren alleen het floeem van hun gastheer, waardoor de virusconcentratie in de totale plant laag blijft en dientengevolge de zuivering van virusdeeltjes uit de plant moeizaam is. Hierdoor is het onderzoek naar de economisch zeer belangrijke luteovirussen relatief laat op gang gekomen. Het onderzoek beschreven in dit proefschrift was erop gericht om meer kennis over de moleculaire biologie van het virus te verkrijgen en deze kennis te gebruiken om resistentie op te wekken door middel van genetische modificatie van aardappelplanten.

Bij de aanvang van het onderzoek was de samenstelling van het RNA-genoom van PLRV niet bekend. In hoofdstuk 2 van dit proefschrift is beschreven hoe de nucleotidenvolgorde van het PLRV-genoom is bepaald. Het genomische RNA bleek 5882 basen lang te zijn en op het genoom waren 6 mogelijke genen te onderscheiden. De genen coderend voor het manteleiwit en het RNA-afhankelijke RNA-polymerase konden geïdentificeerd worden door de aminozuurvolgorden van de respectievelijke produkten te vergelijken met die van andere virale eiwitten. Verder bleek dat de door PLRV-gecodeerde eiwitten een grote homologie vertonen met de eiwitten gecodeerd door een ander luteovirus, het slavergelingsvirus (beet western yellows virus, BWYV),

met uitzondering van het produkt (P1) van het gen gesitueerd aan het uiterste 5'-einde van het genoom. Uit vergelijkingen met de beschikbare sequenties van andere luteovirussen bleek in dit gen een grote variatie te bestaan en bij het gerstevergelingsvirus (barley yellow dwarf virus, BYDV-MAV) zelfs geheel te ontbreken.

Het PLRV-manteleiwitgen is onder de controle van de 35S promoter van het bloemkoolmozaïekvirus (cauliflower mosaic virus, CaMV) geplaatst en m.b.v. een *Agrobacterium tumefaciens* transformatiesysteem in het genoom van aardappelplanten ingebouwd. De op deze wijze verkregen transgene planten bleken wel transgeen mRNA, maar geen aantoonbare hoeveelheden transgeen manteleiwit te produceren (Hoofdstuk 3). De transgene aardappelplanten werden in inoculatie-experimenten met virusdragende bladluizen getoetst op resistentie. Hoewel de planten vatbaar waren voor PLRV, was de concentratie van het virus in de geïnfecteerde transgene planten aanzienlijk lager dan in de niet transgene controle-planten. Een gemuteerde vorm van het PLRV-manteleiwitgen werd in aardappelplanten ingebouwd om te onderzoeken of door de aangebrachte modificaties de synthese van transgeen manteleiwit in de plant verhoogd zou kunnen worden en daarmee wellicht het niveau van resistentie. Na analyse bleek dat ook in deze transgene planten wel transgeen mRNA maar geen eiwit aangetoond kon worden. De resultaten van inoculatie-experimenten waren identiek aan die van de eerdere experimenten met de transgene planten waarin het wildtype manteleiwitgen was ingebracht (Hoofdstuk 4).

Het PLRV-P1-eiwit is onderzocht om de rol van dit eiwit in het virale infectieproces en de lokalisering ervan in de plant te doorgronden. Het eiwit is zowel in *Escherichia coli* als in het baculovirusexpressiesysteem tot expressie gebracht, met de bedoeling een antiserum te maken. Hierbij bleek het eiwit zeer moeilijk te produceren en mogelijk toxisch van aard te zijn (Hoofdstuk 5). Opvallenderwijs was P1 niet in aantoonbare hoeveelheden aanwezig in PLRV-geïnfecteerd plantmateriaal. Teneinde het gen verder te onderzoeken is het onder de controle van de CaMV 35S-promoter in aardappelplanten ingebouwd (Hoofdstuk 6). Transgene planten die aantoonbare hoeveelheden transgeen mRNA bevatten, vertoonden een zeer grote gelijkenis met virus-geïnfecteerde planten. Transgene planten waarin een gemodificeerd, voor

translatie geblokkeerd, P1-gen ingebouwd was, waren uiterlijk eender aan niet-transgene planten. Hieruit kon geconcludeerd worden dat de aanwezigheid van het P1-eiwit op zichzelf al voldoende is om symptomen te veroorzaken. De transgene P1-planten zijn getoetst op resistentie tegen PLRV. Eén transgene plantelijn bleek resistent te zijn terwijl alle andere transgene planten normaal vatbaar waren. De verkregen resistentie bleek absoluut te zijn en was alleen te doorbreken bij zeer hoge infectiedruk.

CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 6 september 1961 te Rotterdam. Na het behalen van het V.W.O. examen aan de Christelijke Scholengemeenschap "Comenius" te Capelle a/d IJssel in 1980, begon hij met de studie Planteziektenkunde aan de Landbouwuniversiteit Wageningen. In 1987 studeerde hij af met als hoofdvakken Fytopathologie, Virologie en Moleculaire Biologie. Sinds zijn afstuderen is hij werkzaam bij het Instituut voor Planteziektenkundig Onderzoek (IPO-DLO), aanvankelijk in dienst van de Landbouwuniversiteit en later in tijdelijke en sedert 1992 in vaste dienst van het IPO-DLO.

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