Molecular characterization of potato virus X: Development of detection probes and identification of the resistance-breaking capacity of strain HB

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Propositions

1. **The conclusion of Meshi *et al.* (1989) that the resistance provided by the *Tm-2* gene may be based on blockage of virus cell-to-cell movement, is premature.**

   Meshi, T., Motoyoshi, F., Maeda, T., Yoshiwoka, S., Watanabe, H., and Okada, Y. (1989). Mutations in the tobacco mosaic virus 30-kD protein gene overcome *Tm-2* resistance in tomato. *The Plant Cell* 1:515-522.

2. **The experiments reported by Truve *et al.* (1993) using potato plants transformed with either a mammalian 2'-5' oligoadenylate synthase gene or the PVX coat protein gene do not allow a comparison on protection efficiencies.**

   Truve, E., Aaspõllu, J., Puska, R., Mehto, M., Hassi, A., Teeri, T.H., Kelve, M., Seppänen, P., and Saarma, M. (1993). Transgenic potato plants expressing mammalian 2'-5' oligoadenylate synthetase are protected from potato virus X infection under field conditions. *Bio/Technology* **11**: 1048-1051.

3. **Studies of the mechanisms of natural resistance genes should be an integral component of plant breeding programs.**

4. **Research on genetically engineered plant resistance involving viral open reading frames, generally underestimates the role of transcribed viral RNA in resistance mechanisms.**

   Braun, C.J., and Hemenway, C.L. (1992). Expression of amino-terminal portions of full-length viral replicase genes in transgenic plants confers resistance to potato virus X infection. *The Plant Cell* 4:735-744.

   Maiti, I.B., Murphy, J.F., Shaw, J.G., and Hunt, A.G. (1993). Plants that express a potyvirus proteinase gene are resistant to virus infection. *Proc. Natl. Acad. Sci. USA* **90**: 6110-6114.

5. **Heteroencapsidation in transgenic crops expressing a viral coat protein is not a real ecological risk.**

Lecoq, H., Ravelonandro, M., Wipf-Scheibel, C., Monsion, M., Raccah, B., and Dunez, J. (1993). Aphid transmission of a non-aphid-transmissible strain of zucchini yellow mosaic potyvirus from transgenic plants expressing the capsid protein of plum pox potyvirus. *Molecular Plant-Microbe Interactions* 6:403-406.

Candelier-Harvey, P., and Hull, R. (1993). Cucumber mosaic virus genome is encapsidated in alfalfa mosaic virus coat protein expressed in transgenic tobacco plants. *Transgenic Research* 2:277-285.

6. **The use of the term "immunity" in connection with potato genes conferring resistance to PVX can be misleading and should be used with more caution.**

7. **As plant virology is a very useful but expensive science, virologists should seek ways to make this science cost wise to scientists in developing countries.**

8. **It is not sensible to use genomic material derived from heterokaryotic gill tissue for karyotyping of the cultivated mushroom *Agaricus bisporus*.**

Lodder, S., Gull, K., and Wood, D. (1993). An electophoresis karyotype of the cultivated mushroom - *Agaricus bisporus*. *Curr. Genet.* 24:496-499.

9. **Serious consideration should be given to the development and reinforcement of women's roles and perspectives in agricultural research.**

a mio padre

Preface

The help and support of many people have been essential for the successful completion of this work. First I would like to express my sincere gratitude to the International Potato Center, to all my colleagues of the Virology Laboratory, Dr. Enrique Fernandez-Northcote, Dr. Upali Jayasinghe and particularly to Dr. Luis Salazar for his constant guidance, help and support. Special thanks also to Ida Bartolini, Vidal Lazarte and Carlos Gutierrez for their friendly and dedicated assistance, to the photography and art sections, and the greenhouse staff. My thanks also to Dr. Roger Cortbaoui for stimulating me in the achievement of my Ph.D. degree.

I want to thank also, Dr. Bob Owens, Dr. Rose Hammond, Dr. John Hammond, and Dr. David Baulcombe for their help and stimulating discussions. I anticipate that our collaboration in the next future will be as fruitful and productive as it has been during my Ph.D. research.

Particular thanks also to The Wageningen Agricultural University and to everybody at the Virology Department for the help during my stay in Wageningen and especially to Dr. René van der Vlugt for the help during the preparation of the final version of the thesis. Particular thanks also to Prof. Rob Goldbach for his guidance, fruitful discussions and criticisms.

My thanks also to Tineke and Liesbeth for their hospitality and friendship.

Finally, I want to express my love and sincere thanks to Giori, my family, and particularly to my father, who supported me unconditionally and whose memory has always been heartening incentive in my work.

Contents

Chapter 1

Introduction

The potato (*Solanum* sp.) is the most important root crop grown in the world; its center of origin as a cultivated crop was probably in the Peruvian and Bolivian Andes (Burton, 1966), and particularly, it is in the area of the Lake Titicaca Basin that the greatest diversity of cultivated forms is found (Ford-Lloyd and Jackson, 1986).

Who first introduced the potato into Europe, and when, is not precisely known, but the most likely country to receive and first cultivate potato was Spain (Burton, 1966). Since then, the potato became a major European and North American foodstuff, and now it occupies the fourth position in the world among the principal food crops, only after wheat, rice and maize (FAO, 1992). The high content of fiber, vitamins, minerals, carbohydrates and proteins (Woolfe, 1987) as well as the high yield of edible energy and protein per hectare per day (Horton and Sawyer, 1985) make potato one of the major world food crops, especially in developing countries.

Over the years, considerable effort has been devoted to the collection and preservation of the genetic resources of the potato (Huamán, 1984) and to their utilization to increase potato productivity. However, one of the principal threats to potato cultivation is its susceptibility to pests and diseases. For that reason, sophisticated tools and modern plant breeding techniques have been applied to develop new varieties adapted to specific environments and, particularly, to introduce resistance to diseases.

These and other strategies have greatly increased agricultural productivity but, despite some successes, losses caused by pests and diseases are still significant. In particular, diseases caused by viruses invest a great importance in developing countries. At present, close to 30 different viruses are known to infect potato worldwide, causing qualitative and

quantitative losses (Salazar, 1990). In addition to viruses, the potato spindle tuber viroid (PSTVd) has been recognized for many years as an important pathogenic agent; crop losses caused by this viroid may reach 64% (Singh *et al.*, 1971).

The aim of my work, as presented in this thesis, was to develop sensitive and reliable probes for plant virus detection, and to gain insight in the genetic make up of viruses, which would help to understand the interactions between viral pathogens and plants in general, and the molecular aspects of plant virus resistance mechanisms in particular. Potato virus X, considered as the most common virus infecting potato (Schultz and Bonde, 1944), was chosen as the major subject in the studies described. Potato virus X (PVX), the type member of the genus *Potexvirus*, is a flexuous rod-shaped virus, known also as potato latent virus, potato mild mosaic virus, and *Solanum* virus 1 (Bercks, 1970). It is readily transmitted by mechanical means. Each particle contains a single molecule of positive sense, single-stranded RNA of about $2x10^6$ M_r.

The viral RNA, about 5% of the total particle weight (Knight, 1963) contains a m^7GpppG cap structure at the 5' end (Sonenberg *et al.*, 1978) and a poly(A) tail at the 3' end (Morozov *et al.*, 1981). The potexviral genome contains five open reading frames (ORF) coding for five proteins of 166 kDa, 25 kDa, 12 kDa, 8 kDa, and 25 kDa (coat protein), respectively (Huisman *et al.*, 1988) (Fig. 1.1)

Strains of PVX have been classified into four groups (Table 1.1) on the basis of their interactions with the dominant resistance genes Nx and Nb, which control a hypersensitive

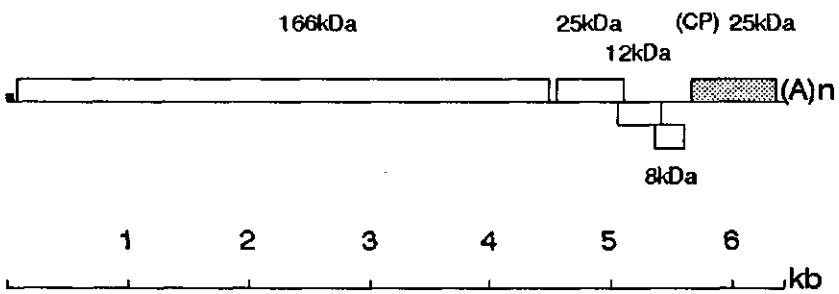

Fig. 1.1. Schematic representation of the organization of the potato virus X RNA genome (Huisman *et al.*, 1988, modified). CP: coat protein. ■: m^7GpppG cap structure. (A)n: poly(A) tail.

Table 1.1. Relation of known strains of potato virus X (PVX) and resistance genes in *Solanum* sp. (Cockerham, 1955, modified). R = resistant; s = susceptible.

Solanum sp. genotype	Strain group 1	2	3	4	HB
nx nb	s	s	s	s	s
Nx nb	R	s	R	s	s
nx Nb	R	R	s	s	s
Nx Nb	R	R	R	s	s
Rx	R	R	R	R	s

response, and the immunity gene Rx (Cockerham, 1955). A single "group 5" PVX strain, called PVX_{HB}, found in Bolivia, has been reported to be able to overcome all known resistance genes (Moreira *et al.*, 1980). This strain is unique in that it causes typical PVX symptoms in most common indicator species but it does not produce local lesions in inoculated leaves of *Gomphrena globosa* L. which is the main indicator host for PVX.

The research done in this thesis deals with both the development of virus detection techniques and the analysis of the molecular aspects related to virus resistance, in particular to the properties of PVX_{HB} and its ability to break immunity. An overview about some available techniques for virus and viroid detection using molecular hybridization, in both radioactive and nonradioactive formats, is presented in Chapters 2 and 3. In Chapter 4 a number of PVX isolates and strains are tested in both radioactive and nonradioactive detection assays using two selected cDNA probes derived from PVX strain cp (serotype PVX^A) and from an European strain-group 3 isolate (serotype PVX^O), respectively.

Since the results obtained with these two probes revealed a great variability in detecting PVX isolates, a chimaeric recombinant probe was prepared, consisting of sequences from both original probes. It is shown that this recombinant probe gives a strong reaction (and therefore reliable detection) with all isolates tested so far (Chapter 5).

In order to localize the viral determinant(s) responsible for the ability of the PVX strain HB to overcome the immunity provided by the Rx gene, and to understand the mechanism involved, the genomic RNA of the strain HB was cloned, entirely sequenced, and analyzed

(Chapter 6). Comparisons between HB and three non resistance-breaking strains of PVX, i.e. strains cp (Orman *et al.*, 1990), X3 (Huisman *et al.*, 1988), and S (Skryabin *et al.*, 1988) are presented that indicate the presence of eight amino acid residues unique for PVX_{HB}. Computer-directed mutational analysis and secondary structure predictions of the viral coat proteins indicated that only two out of the eight amino acid changes may be considered as being involved in the differences between HB and the other strains of PVX.

Infectious clones of PVX, in which the residues possibly involved in Rx resistance-breaking had been modified, allowed to confirm *in vivo* the involvement of one of these two amino acid changes in symptom expression and in the resistance-breaking mechanism (Chapter 7).

REFERENCES

Bercks, R. (1970). Potato Virus X. *C.M.I./A.A.B. Descriptions of Plant Viruses*. No. 4.

Burton, W.G. (1966). *The potato: A survey of its history and of factors influencing its yield, nutritive value, quality and storage*. 2nd edn. H. Veenman and N.V. Zonen, Wageningen, 382 pp.

Cockerham, G. (1955). Strains of potato virus X. *Proceedings of the Second Conference on Potato Virus Diseases*, pp. 89-92. Lisse-Wageningen, 1954.

Food and Agriculture Organization (1992). *FAO Production Yearbook 1991*. Vol. 45. Rome, 265 pp.

Ford-Lloyd., B., and Jackson, M. (1986). *Plant genetic resources: An introduction to their conservation and use*. E. Arnold, London, 146 pp.

Horton, D., and Sawyer, R.L. (1985). The potato as a world food crop, with a special reference to developing areas. In Li P.H. (ed) *Potato physiology*, pp. 2-34. Academic Press, Orlando, Florida.

Huamán, Z. (1984). The evaluation of the potato germplasm at the International Potato Center. In Holden J.H.W. and Williams J.T. (eds) *Crop genetic resources: Conservation and evaluation*, pp. 199-201. George Allen and Unwin Publications, London.

Huisman, M.J., Linthorst, H.J.M., Bol, J.F., and Cornelissen, B.J.C. (1988). The complete nucleotide sequence of potato virus X and its homologies at the amino acid level with various plus-stranded RNA viruses. *Journal of General Virology* **69**: 1789-1798.

Knight, C.A. (1963). Chemistry of viruses. *Protoplasmatologia* 4 Wien: Springer-Verlag, 177 pp.

Moreira, A., Jones, R.A.C., and Fribourg, C.E. (1980). Properties of a resistance-breaking strain of potato virus X. *Annals of Applied Biology* **95**: 93-103.

Morozov, S.Yu., Gorbulev, V.G., Novikov, V.K., Agranovsky, A.A., Kozlov, Yu.V., Atabekov, J.G., and Baev, A.A. (1981). The primary structure of the 5 - and 3 -terminal regions of the RNA of potato virus X. *Doklady Akademii Nauk SSSR* **259**: 723-725.

Orman, B.E., Celnik, R.M., Mandel, A.M., Torres, H.N., and Mentaberry, A.N. (1990). Complete cDNA sequence of a South American isolate of potato virus X. *Virus Research* **16**: 293-306.

Salazar, L.F. (1990). Main virus diseases of potato. In *Control of virus and virus-like diseases of potato and sweet potato. Report of the III planning conference*, pp. 9-12. International Potato Center, Lima, 1989.

Schultz, E.S., and Bonde, R. (1944). The effect of latent mosaic (virus X) on yield of potato in Maine. *American Potato Journal* **21**: 278-283.

Singh, R.P, Finnie, R.F., and Bagnal, R.E. (1971). Losses due to PSTVd. *American Potato Journal* **48**: 262-267.

Skryabin, K.G., Kraev, A.S., Morozov, S.Yu., Rozanov, M.N., Chernov, B.K., Lukasheva, L.I., and Atabekov, J.G. (1988). The nucleotide sequence of potato virus X RNA. *Nucleic Acids Research* **16**: 10929-10930.

Sonenberg, M.N., Shatkin, A.J., Ricciardi, R.P., Rubin, M., and Goodman, R.M. (1978). Analysis of terminal structures of RNA from potato virus X. *Nucleic Acids Research* **5**: 2501-2512.

Woolfe, J.A. (1987). *The potato in the human diet*. Cambridge University Press, Cambridge, 231 pp.

Chapter 2

Detection of viroids and viruses by nucleic acid probes

INTRODUCTION

Molecular hybridization techniques for routine testing of viroids and viruses have gained worldwide acceptance in recent years. The technique involves the use of labeled complementary DNA or RNA (cDNA, cRNA) prepared from purified viroid or viral nucleic acid (or a recombinant clone of such nucleic acid) as a probe. On incubation with plant extracts these probes will detect the presence of viral or viroid nucleic acids by forming hybrids with them.

The degree of specificity of detection is determined by the degree of sequence complementarity between the RNA or DNA of the probe and the nucleic acid of the viroid or virus. Whereas the nucleotide sequence of a single isolate of a given virus or viroid is essentially constant, the sequences of different isolates of the same virus or viroid, or of unrelated viruses or viroids, can differ by as little as a few nucleotides up to a large portion of their nucleic acids. Nucleic acid probes prepared to one isolate of a viroid or virus will therefore form highly specific hybrids with the nucleic acid of that isolate, less specific ones with other isolates that differ in a few sequences and none at all with unrelated ones.

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Originally nucleic acid hybridization between DNA and RNA was done in liquid (Spiegelman, 1964), but nowadays it is commonly done by blotting the test samples onto a nitrocellulose or nylon-based membrane and then incubating the membrane in a solution of the probe. This technique is called the nucleic acid spot hybridization (NASH) test, although other names such as dot-blot or sap-blot hybridization are also used (Boulton *et al.*, 1984). In the NASH test, RNA or DNA can be hybridized with a RNA or DNA probe.

The NASH test was first used to detect viroids by Owens and Diener (1981). Thereafter it became the method of choice for viroids (Salazar *et al.*, 1983), because viroids, unlike viruses, do not have an antigenic protein coat and thus cannot be detected by immunological methods (Diener, 1979). Previous methods for detecting viroids were either not sensitive enough or were inadequate for large-scale testing. For example, detection of avocado sunblotch viroid (ASBVd) by symptom development in suitable indicator hosts may take up to 2 years. Even where symptom development takes only a few weeks, the requirement for large amounts of bench space in a heated glasshouse makes bioassays unsuitable for routine indexing.

Polyacrylamide gel electrophoresis (PAGE) of naturally infected plant samples on its own is not sensitive enough to detect low levels of viroid. However, the technique can be very sensitive for potato spindle tuber viroid (PSTVd) detection if the viroid is first inoculated from potato tissue onto tomato (in which PSTVd reaches much higher concentrations) prior to using PAGE (Harris *et al.*, 1984), but this is a cumbersome technique. Singh and Boucher (1987) have recently reported a "return" gel electrophoresis method with a sensitivity for the detection of PSTVd matching that of NASH, but we have been unable to confirm this in tests at the International Potato Center (CIP). A possible advantage of their technique is that it might distinguish between mild and severe strains of PSTVd. NASH, on the other hand, can detect as little as 0.33 pg of PSTVd in potato plants grown under high temperature regimes, but it cannot detect the viroid in plants or tubers which have been maintained below 10°C (Salazar *et al.*, 1988).

The NASH test has also been applied to the detection of viruses (Maule *et al.*, 1983; Baulcombe *et al.*, 1984 a, b). It has several advantages over serology. The latter is based on the detection of epitopes of the virus coat protein, the cistron for which represents only a small portion of the genetic information of the virus: for tobacco mosaic virus (TMV) less than 2% of the viral genome is involved in the antigenicity of the coat protein (Hull, 1986). By contrast, probes for hybridization analysis can represent the whole genome of the virus

or parts thereof, which opens up new possibilities for the study of relationships between viruses. Specific probes can be used for specific purposes: cDNA clones of one virus strain have been differentially hybridized with the RNA from other strains for a number of viruses (Baulcombe *et al.*, 1984a; Rosner and Bar-Joseph, 1984; Gallitelli *et al.*, 1985; Linthorst and Bol, 1986). Several probes can be combined simply by mixing them during hybridization, and can thus be used in a polyvalent manner to detect several virus strains simultaneously (Hopp *et al.*, 1988). Single probes can be constructed which contain short, specific sequences for each of several strains of a virus or even different viruses (M. Querci and L. F. Salazar, unpublished results).

Despite the wide application of enzyme-linked immunosorbent assay (ELISA; Clark and Adams, 1977) to the detection of many viruses there are instances where it is inapplicable. An example is tobacco rattle virus (TRV), where infection with TRV-RNA 1 will result in replication of RNA 1 but not in the production of virions, the coat protein for which is encoded by RNA 2 (Harrison and Robinson, 1982). Under such conditions serology cannot be applied and the detection of the TRV-RNA 1 is only possible by infectivity assays of extracted nucleic acids on suitable host plants, or by NASH. Linthorst and Bol (1986) were able to develop a number of probes that could be used to detect either a wide spectrum or specific groups of TRV isolates. Moreover, NASH was reported to be more sensitive than ELISA for detection of potato leafroll virus (PLRV) or potato virus X (PVX) in symptomless plants (Boulton *et al.*, 1984).

One drawback to the use of probes as commonly applied is that they are radioactive. This places limitations on their use in many countries and situations. The development of nonradioactive labels for probes makes possible wider application of the technology (Leary *et al.*, 1983; Vivian, 1992).

The NASH test, with several methods of probe preparation, has been used at CIP since 1983 for the routine detection of PSTVd (Salazar *et al.*, 1988), and since 1987 its use has been expanded to the detection of several viruses and viroids also on crops other than potato (Fig. 2.1).

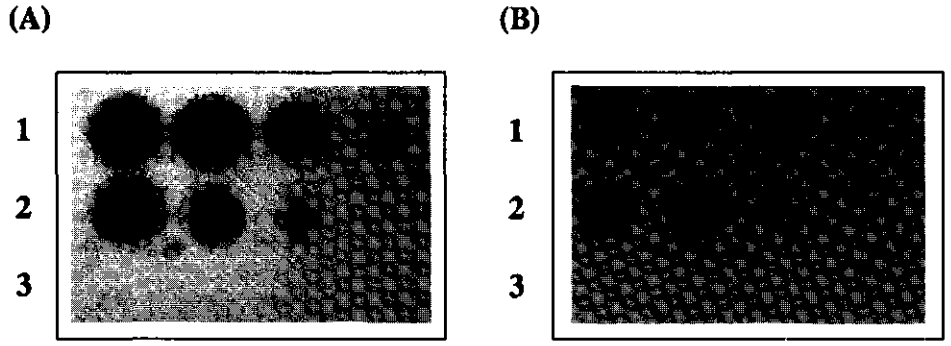

Fig. 2.1. Detection of potato virus X (PVX) by nucleic acid spot hybridization. Comparison of (A) a $\alpha(^{32}P)$RNA probe and (B) a biotin-labeled DNA probe. 1 = tenfold dilutions (1/10-1/10,000) of PVX infective potato sap in 2x SSC; 2 = purified PVX: 100 ng, 10 ng, 1 ng, 0.1 ng; 3 = tenfold dilutions (1/10-1/10,000) of non-infective potato sap in 2x SSC.

PREPARATION OF NUCLEIC ACID PROBES

Nucleic acid probes can be of two types: cDNA or cRNA probes. The probe consists of a strand of DNA or RNA, complementary to the target nucleic acid, which is conjugated to a label. The label can be a radioisotope such as ^{32}P or ^{35}S, or nonradioactive such as biotin.

Preparation of recombinant DNA clones

Despite the relative ease by which cDNA probes can be obtained from purified viroid or virus nucleic acid most are prepared using some form of recombinant DNA technology. There are several ways that this can be done and we give details of the methods used by Owens and Cress (1980) and Cress *et al.* (1983) to produce probes to PSTVd.

Purified PSTVd is treated with alkaline phosphatase from *Escherichia coli* to remove 3'-terminal phosphate residues from linear molecules. A polyadenylate tail is added by incubation with poly(A) polymerase, and the polyadenylated form of the PSTVd is

recovered by phenol:chloroform extraction and ethanol precipitation. Single-stranded cDNA of PSTVd is synthesized by incubation with reverse transcriptase and the four deoxynucleoside triphosphates (dNTPs). For synthesis of the second strand, the single-stranded cDNA is denatured at 100°C, quenched at 0°C, and added to a reaction mixture containing DNA polymerase I and the four dNTPs. The non-base-paired regions of the double-stranded DNA are removed by incubation with S1 nuclease.

Hybrids are constructed from plasmid pBR322 and S1-digested double-stranded PSTVd cDNA, and annealed before transformation of *E. coli* C600 ($rk^- mk^+$). Tetracycline-resistant and ampicillin-sensitive transformants are screened, the cloned DNAs are isolated and their size and reactivity assessed. Two clones obtained in this way, pDC-29 and pDC-22, which contained overlapping partial sequences of PSTVd cDNA, were used by Cress *et al.* (1983) to ligate specific fragments and thereby reconstruct full-length double-stranded PSTVd cDNAs.

Preparation of cDNA probes

Radioactive and nonradioactive labels can be added to double-stranded cDNA by 'nick-translation' (Rigby *et al.*, 1977; Vivian, 1992). The method outlined in Appendix 1 (Method A) is used for preparing probes with ^{32}P-labeled nucleotides. It can also be used with some modifications to label cDNA with biotinylated nucleotides (Appendix 1, Method B).

Single-stranded cDNA probes

cDNA for a number of viroids has been prepared successfully by direct synthesis on purified viroid molecules (Palukaitis and Symons, 1979; Randles and Palukaitis, 1979; Imperial *et al.*, 1981; Palukaitis *et al.*, 1981). A prerequisite is several micrograms of highly purified viroid nucleic acid, free of contaminating host RNA (Symons, 1984). After incubating the viroid RNA with S1 nuclease which cleaves only a few internucleotide bonds in each molecule, a short polyadenylate tail is synthesized on the 3'-OH end of each fragment (Sippel, 1973). To form cDNA probes labeled with ^{32}P, the fragments are incubated with labeled and unlabeled dNTPs in the presence of reverse transcriptase.

Single-stranded cDNA in two orientations ('plus' and 'minus') have been produced for ASBVd (Barker *et al.*, 1985) and PSTVd (D.E. Cress, personal communication) using full-length double-stranded cDNA inserted into the M13mp9 phage according to the procedures

described by Messing (1983). Two methods of preparing radioactive probes for ASBVd (Barker *et al.*, 1985) and PSTVd (Salazar *et al.*, 1988) have been compared. Method A uses a downstream primer on the M13 'plus' cDNA clone as a template to synthesize a single-stranded cDNA radioactive probe specific to the viroids. Method B uses an upstream primer on a M13 'minus' clone to produce a probe which is single-stranded in the region of the insert and double stranded in a portion of the phage vector. The double-stranded region contains the radioactive label.

Comparison of both methods indicates that the probe prepared by Method A is highly specific and less susceptible to background reactions.

RNA probes

Melton *et al.* (1984) constructed RNA probes by transcribing plasmid DNA templates containing a promoter for bacteriophage SP6 polymerase. The insertion of the required cDNA sequence into vectors flanked by both SP6 and T7 polymerase promoters allows the construction of probes specific for 'plus' or 'minus' sequences. Salazar *et al.* (1988) inserted a full-length double-stranded PSTVd cDNA into plasmid pSP65, thereby creating a template for synthesis of RNA probes for PSTVd. A method for ^{32}P-labeling of RNA transcripts is given in Appendix 2.

Comparison of probes

Four radioactive-labeled probes were compared for their sensitivity of detection of PSTVd. RNA probes were found to be the most sensitive, detecting as little as 0.33 pg of PSTVd, followed by probes prepared by Method A (M13 primer extension plus-sense insert), and nick-translation of double-stranded cDNA. Probes prepared by Method B (M13 primer extension minus-sense insert) were the least sensitive (Salazar *et al.*, 1988). RNA probes are currently used at CIP for both viroid and virus detection.

DETECTION OF VIRUSES AND VIROIDS WITH cDNA PROBES

Sample preparation

Viroids: The protocols of Owens and Diener (1981) and Salazar *et al.* (1988) are adequate for the detection of PSTVd and other viroids where these are found in high

concentration in plant tissue. The tissue is triturated in a ratio of 1 g tissue to 2 ml of a 1:1 (v/v) mixture of formaldehyde (37%) and 10x SSC (1x SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The homogenate is then mixed with an equal volume of chloroform and water-saturated phenol (1:1, v/v) before centrifugation, or standing overnight at 4°C, to separate the aqueous phase.

It is not necessary to deproteinize (Sambrook *et al.*, 1989) leaf samples by addition of phenol and chloroform to detect PSTVd with radioactive probes. However, deproteinization is necessary when testing botanical or true potato seed (TPS) and potato tuber flesh or sprouts. Also, leaf samples must be thoroughly deproteinized if biotin-labeled probes are to be used, otherwise non-specific reactions may occur.

At CIP, leaf or sprout samples are routinely collected and crushed with buffer in plastic bags. Samples are transferred to test tubes, mixed with an equal volume of phenol:chloroform (1:1, v/v) and left until the aqueous phase separates. The mixing can also be done in the bags if they are resistant to solvents.

Samples of TPS are soaked for at least 2 h (usually overnight) in distilled water before crushing in a mortar as for leaf or sprout samples. A single sample can contain from one to 100 seeds, depending on the size of the stock to be tested (Salazar *et al.*, 1988).

To detect viroids which occur in low concentration in plant tissue such as ASBVd, nucleic acid must be extracted carefully from large amounts of tissue. The best method, which was described by Palukaitis and Symons (1980) and modified subsequently by Allen and Dale (1981) and Barker *et al.* (1985), is described in detail in Appendix 3.

Viruses: Boulton *et al.* (1986) successfully detected virus RNA in extracts of undiluted plant sap, and at CIP 0.1-0.5 g leaf samples extracted in two volumes of 2x SSC have given consistent results (L.F. Salazar and M. Querci, unpublished results). By simply touching a recently cut section of stem or rolled leaves onto a membrane, enough sap was obtained to detect sweet potato feathery mottle virus and some other potato viruses (J. Abad and J. Moyer, personal communication).

Spotting of samples

Nitrocellulose membranes are rinsed thoroughly in distilled water, avoiding the formation of air bubbles, and then washed immediately with two or three changes of 5x-20x

SSC. After drying on filter paper at room temperature they are stored until required in a dessicator over silica gel (note that the membranes must never be handled with bare hands).

Each spot on the membrane, located by prestamped vertical and horizontal numbers, receives 3-5 μl of the sample supernatant, usually delivered by micropipettes fitted with disposable tips, although other devices such as Pasteur pipettes or capillary tubes can be used. Once spotted the membranes are usually baked for 1 hour at 80°C but good results have been obtained with only a few minutes baking (L.F. Salazar and M. Querci, unpublished results).

Membranes can be spotted with samples and stored either until more samples are processed and added or until the probe is ready, thereby making the best use of the ^{32}P-labeled probes which have a relatively short half-life. Membranes spotted with samples can also be sent through the post, a procedure employed at CIP to support testing for viroids or viruses by NASH in developing countries. The same procedure is also used for samples to be tested by nitrocellulose membrane-ELISA (Lizárraga and Fernandez-Northcote, 1989) or nonradioactive NASH.

Hybridization

Full details of the hybridization procedures used for ^{32}P-labeled DNA probes, for biotinylated DNA probes and for ^{32}P RNA probes are given in Appendix 4, Methods A-C. The longest procedure takes less than a week and the others 1-2 days.

CONCLUSIONS: FUTURE PROSPECTS

Virus detection technology has changed greatly in the last 10 years with the development of ELISA and the use of recombinant DNA. Manipulation of hybridization conditions has improved the detection, and the sensitivity of detection, of viroids and viruses. For example, selection of appropriate stringency conditions during hybridization can help to control the specificity of the probe to a large extent. NASH and ELISA are generally complementary with similar sensitivities. NASH can be used in situations where ELISA cannot, but it has the disadvantage of employing radioactive labels. This shortens the useful life of the probes and restricts their use to countries where radioactive labels can be obtained and handled. Nonradioactive labels are being increasingly used but non-specific

reactions with sap extracts can interfere with the results. Samples therefore require further purification which increases the labour and time involved. New labels will undoubtedly be developed and new procedures for more expeditious handling of samples are being investigated in several laboratories around the world. Furthermore, with NASH, unlike ELISA, it is possible to select and prepare probes which detect specific parts of the virus genome.

REFERENCES

Allen, R.N., and Dale, J.L. (1981). Application of rapid biochemical methods for detecting avocado sunblotch disease. *Annals of Applied Biology* **98**: 451-461.

Barker, J.M., McInnes, J.L., Murphy, P.J., and Symons R.H. (1985). Dot-blot procedure with (^{32}P) DNA probes for the sensitive detection of avocado sunblotch and other viroids in plants. *Journal of Virological Methods* **10**: 87-98.

Baulcombe, D.C., Boulton, R.E., Flavell, R.B., and Jellis, G.J. (1984a). Recombinant DNA probes for detection of viruses in plants. *Proceedings of the 1984 British Crop Protection Conference*, pp. 207-213. British Crop Protection Council, Thornton Heath, Surrey.

Baulcombe, D.C., Flavell, R.B., Boulton, R.E., and Jellis, G.J. (1984b). The sensitivity and specificity of a rapid nucleic acid hybridization method for the detection of potato virus X in crude sap samples. *Plant Pathology* **33**: 361-370.

Boulton, R.E., Jellis, G.J., Baulcombe, D.C., and Squire, A.M. (1984). The practical application of complementary DNA probes to virus detection in a potato breeding programme. *Proceedings of the 1984 British Crop Protection Conference*, pp. 177-180. British Crop Protection Council, Thornton Heath, Surrey.

Boulton, R.E., Jellis, G.J., Baulcombe, D.C., and Squire, A.M. (1986). The application of complementary DNA probes to routine virus detection with particular reference to potato viruses. In Jones R.A.C. and Torrance L. (eds) *Developments and Applications in Virus Testing*, pp. 41-53. Association of Applied Biologists, Wellesbourne.

Clark, M.F., and Adams, A.N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* **34**: 475-483.

Cress, D.E., Kiefer, M.C., and Owens, R.A. (1983). Construction of infectious potato spindle tuber viroid cDNA clones. *Nucleic Acids Research* **11**: 6821-6835.

Diener, T.O. (1979). Viroids: Structure and function. *Science* **205**: 859-866.

Gallitelli, D., Hull, R., and Koenig, R. (1985). Relationships among viruses in the tombusvirus group: nucleic acid hybridization studies. *Journal of General Virology* **66**: 1523-1531.

Harris, P.S., James, C.M., Liddell, A.D., and Okeley, E. (1984). Viroid detection in potato quarantine: The cDNA probe and other methods. *Proceedings of the 1984 British Crop Protection Conference*, pp. 187-191. British Crop Protection Council, Thornton Heath, Surrey.

Harrison, B.D., and Robinson, D.J. (1982). Genome reconstitutions and nucleic acid hybridization as methods of identifying particle-deficient isolates of tobacco rattle virus in potato plants with stem-mottle disease. *Journal of Virological Methods* **5**: 255-266.

Hopp, H.E., Giavedoni, L., Mandel, M.A., Arese, A., Orman, B., Bravo Almonacid, F., Torres, H.N., and Mentaberry, A.N. (1988). Biotinylated nucleic acid hybridization probes for potato virus detection. *Archives of Virology* **103**: 231-241.

Hull, R. (1986). The potential for using dot-blot hybridisation in the detection of plant viruses. In Jones R.A.C. and Torrance L. (eds) *Developments and Applications in Virus Testing*, pp. 3-12. Association of Applied Biologists, Wellesbourne.

Imperial, J.S., Rodriguez, J.B., and Randles, J.W. (1981). Variation in the viroid-like RNA associated with cadang-cadang disease: Evidence for an increase in molecular weight with disease progress. *Journal of General Virology* **56**: 77-85.

Leary, J.J., Brigati, D.J., and Ward, D.C. (1983). Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: Bio-blots. *Proceedings of the National Academy of Science USA* **80**: 4045-4049.

Linthorst, H.J.M., and Bol, J.F. (1986). cDNA hybridisation as a means of detection of tobacco rattle virus in potato and tulip. In Jones R.A.C. and Torrance L. (eds) *Developments and Applications in Virus Testing*, pp. 25-39. Association of Applied Biologists, Wellesbourne.

Lizárraga, C., and Fernandez-Northcote, E.N. (1989). Detection of potato viruses X and Y in sap extracts by a modified indirect enzyme-linked immunosorbent assay on nitrocellulose membranes (NCM-ELISA). *Plant Disease* **73**: 11-14.

Maule, A.J., Hull, R., and Donson, J. (1983). The application of spot hybridization to the detection of DNA and RNA viruses in plant tissues. *Journal of Virological Methods* **6**: 215-224.

Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K., and Green, M.R. (1984). Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Research* **12**: 7035-7056.

Messing, J. (1983). New M13 vectors for cloning. *Methods in Enzymology* **101**: 20-78.

Owens, R.A., and Cress, D.E. (1980). Molecular cloning and characterization of potato spindle tuber viroid cDNA sequences. *Proceedings of the National Academy of Science USA* **77**: 5302-5306.

Owens, R.A., and Diener, T.O. (1981). Sensitive and rapid diagnosis of potato spindle tuber viroid disease by nucleic acid hybridization. *Science* **213**: 670-672.

Palukaitis, P., and Symons, R.H. (1979). Hybridization analysis of chrysanthemum stunt viroid with complementary DNA and the quantitation of viroid RNA sequences in extracts of infected plants. *Virology* **98**: 238-245.

Palukaitis, P., and Symons, R.H. (1980). Purification and characterization of the circular and linear forms of chrysanthemum stunt viroid. *Journal of General Virology* **46**: 477-489.

Palukaitis, P., Rakowski, A.G., Alexander, D.M., and Symons, R.H. (1981). Rapid indexing of the sunblotch disease of avocados using a complementary DNA probe to avocado sunblotch viroid. *Annals of Applied Biology* **98**: 439-449.

Randles, J.W., and Palukaitis, P. (1979). *In vitro* synthesis and characterization of DNA complementary to cadang-cadang-associated RNA. *Journal of General Virology* **43**: 649-662.

Rigby, P.W.J., Dieckmann, M., Rhodes, C., and Berg, P. (1977). Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA Polymerase I. *Journal of Molecular Biology* **113**: 237-251.

Rosner, A., and Bar-Joseph, M. (1984). Diversity of citrus tristeza virus strains indicated by hybridization with cloned cDNA sequences. *Virology* **139**: 189-193.

Salazar, L.F., Balbo, I., and Owens, R.A. (1988). Comparison of four radioactive probes for the diagnosis of potato spindle tuber viroid by nucleic acid spot hybridization. *Potato Research* **31**: 431-442.

Salazar, L.F., Owens, R.A., Smith, D.R., and Diener, T.O. (1983). Detection of potato spindle tuber viroid by nucleic acid spot hybridization: evaluation with tuber sprouts and true potato seed. *American Potato Journal* 60: 587-597.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Singh, R.P., and Boucher, A. (1987). Electrophoretic separation of a severe from mild strains of potato spindle tuber viroid. *Phytopathology* 77: 1588-1591.

Sippel, A.E. (1973). Purification and characterization of adenosine triphosphate: ribonucleic acid adenyl transferase from *Escherichia coli*. *European Journal of Biochemistry* 37: 31-40.

Spiegelman, S. (1964). Hybrid nucleic acids. *Scientific American* 228: 34-42.

Symons, R.H. (1984). Diagnostic approaches for the rapid and specific detection of plant viruses and viroids. In Kosuge T. and Nester E.W. (eds) *Plant Microbe Interactions: Molecular and Genetic Perspectives*, vol. I, pp. 93-124. Macmillan Publishing Company, New York.

Vivian, A. (1992). Identification of plant pathogenic bacteria using nucleic acid technology. In Duncan J.M. and Torrance L. (eds) *Techniques for the rapid detection of plant pathogens*, pp. 145-161. Blackwell Scientific Publications, Cambridge.

Appendix 1. Labeling of double-stranded cDNA probes

The Laboratory Manual *Molecular Cloning* by Sambrook *et al.* (1989) is an invaluable source of information about the reagents and techniques described here.

Method A: Radioactive labeling of cDNA probes by nick-translation.

Materials

- 0.2 mM deoxyribonucleoside triphosphates (dNTPs): deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP) and deoxythymidine triphosphate (dTTP).
- Nick-translation buffer (10x)
  - 0.5 M Tris-HCl, pH 7.8
  - 50 mM $MgCl_2$
  - 100 mM 2-mercaptoethanol
  - 100 μg ml^{-1} nuclease-free bovine serum albumin (BSA).
- DNA polymerase I/DNase I mixture
  - 0.4 μg μl^{-1} DNA polymerase I/40 pg μl^{-1} DNase I in 50 mM Tris-HCl, pH 7.5
  - 5 mM magnesium acetate
  - 1 mM 2-mercaptoethanol
  - 50% (v/v) glycerol
  - 100 μg ml^{-1} BSA.
- Substrate DNA: 1 μg at a concentration of 0.1 - 0.5 μg μl^{-1}.
- Stop buffer: 300 mM EDTA, pH 8.0.
- Sterile distilled water (SDW).

- Labeled dNTP: e.g. $\alpha(^{32}P)$dCTP, ~24TBq mmol^{-1} in aqueous solution (370 MBq ml^{-1}).
- Yeast transfer RNA: 20 mg ml^{-1} stock solution.
- 20% SDS.
- 7.5 M ammonium acetate.
- 3 M sodium acetate, pH 5.2.
- Buffered phenol (0.1 M Tris-HCl, pH 7.6).
- Chloroform.
- $T_{10}E_1$ buffer: 10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA.

Method

- Pipette the following into a 1.5 ml Eppendorf tube sitting on ice:
  5 μl 10x nick-translation buffer
  5 μl each of 0.2 mM dGTP, dATP, dTTP (or all nucleotides except the labeled one)
  1 μg substrate DNA
  156 pmol labeled dNTP (2.4 MBq).
- Make up to 45 μl with SDW.
- Mix and add 5 μl of the DNA polymerase I/DNase I mixture.
- Mix again, centrifuge and then incubate at 16°C for 1 h.
- Add in order:
  3 μl H_2O
  5 μl stop buffer
  1 μl 20% SDS
  1 μl yeast transfer RNA
  30 μl 7.5 M ammonium acetate.
- Extract with an equal volume of phenol:chloroform (1:1, v/v), and transfer the aqueous phase into a clean Eppendorf tube.
- Add 225 μl ethanol and precipitate at -70°C for 1 h or at -20°C overnight.
- Centrifuge for 15 min, remove the supernatant with a pipette and resuspend the pellet in 90 μl $T_{10}E_1$. Add 10 μl of 3 M sodium acetate and 225 μl ethanol and precipitate at -70°C for 1 h or at -20°C overnight.
- Recover the pellet by centrifugation, dry it under vacuum and resuspend in 100 μl $T_{10}E_1$.
- Determine the incorporation of label into the probe by trichloroacetic acid (TCA) precipitation as follows:

Materials

- 5% TCA in 0.02 M sodium pyrophosphate.
- 70% ethanol.
- 95% ethanol.
- Whatman 3MM filter paper cut into 2 x 0.5 cm pieces.

- Scintillation counter.

Method

- Spot 1 μl of the sample to be assayed onto the center of a 2 x 0.5 cm piece of Whatman 3MM filter paper and let it dry completely.
- Wash the paper strip three times in ice cold 5% TCA, once in 70% ethanol and once in 95% ethanol (5 min per wash).
- Dry under a lamp and put the paper strip into a scintilliation vial containing toluene-based scintillation fluid. Measure the radioactivity in a liquid scintillation counter and calculate the μl of probe to be used according to the required c.p.m. ml^{-1}.

Method B: Biotin labeling of cDNA probes by nick-translation.

The procedure is the same as for radioactive labeling (Method A) except for the following:

- 2.5 μl of 0.4 mM labeled nucleotide, either biotin-7-dATP (Gibco-BRL) or another biotinylated nucleotide, is used in a 50 μl reaction mixture with 1 μg of substrate DNA.
- The mixture is incubated at 15°C for 90 min.
- The biotinylated DNA must not be extracted with phenol:chloroform. Separate it from the unincorporated nucleotides by two ethanol precipitations in the presence of 2.5 M ammonium acetate. Resuspend it in 100 μl of 1x SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The labeled probe can be stored at 4°C for a few weeks or at -20°C for several months.

Appendix 2. Radioactive labeling of RNA probes.

Materials

- 5x transcription buffer
  200 mM Tris-HCl, pH 7.5
  30 mM $MgCl_2$
  10 mM spermidine
  50 mM NaCl.
- 10 mM stock solutions of rNTPs: ATP, CTP, UTP, GTP, pH 7.0.
- 100 mM dithiothreitol (DTT).
- Ribonuclease inhibitor, 25 units μl^{-1} (e.g. RNasin from PROMEGA).
- Labeled NTP, e.g. $\alpha(^{32}P)$UTP in aqueous solution (370 MBq ml^{-1}; 24 TBq $mmol^{-1}$).
- SDW treated with 0.1% diethylpyrocarbonate (DEPC) before autoclaving (Sambrook *et al.*, 1989).
- SP6 RNA polymerase or T7 RNA polymerase, 20 units μl^{-1} (PROMEGA). (Use appropriate polymerase according to RNA polymerase promoter contained in the plasmid used).

- Template plasmid with insert, previously linearized with the appropriate restriction enzyme downstream from the insert and resuspended in $T_{10}E_1$, at a final concentration of 0.5 $\mu g\ \mu l^{-1}$.
- DNase I, 1 unit μl^{-1} (PROMEGA).

Method

- Pipette the following into a sterile Eppendorf tube at room temperature:
  - 4 μl 5x transcription buffer
  - 2 μl of 100 mM DTT
  - 0.8 μl ribonuclease inhibitor (final concentration 1 unit μl^{-1})
  - 1 μl each of 10 mM ATP, CTP and GTP solutions
  - 0.3 μl of 10 mM UTP
  - 2 μl linearized plasmid template DNA (1μg)
  - 5 μl $\alpha(^{32}P)$UTP (1.85 MBq)
  - 1 μl RNA polymerase.
- Add DEPC-treated SDW to give a final volume of 20 μl.
- Mix carefully, centrifuge briefly and incubate at 38°C for 90 min.
- Add 1 μl of DNase I (final concentration 1 unit μg^{-1} DNA) and incubate at 37°C for 20 min.
- Add 179 μl of DEPC-treated SDW.
- Extract with an equal volume of phenol:chloroform (1/1, v/v).
- Recover the aqueous phase and extract again with an equal volume of chloroform. Recover aqueous phase again and precipitate RNA by adding 0.1 volume 3 M sodium acetate, pH 5.2 and 2.5 volumes of cold ethanol. Incubate at -70°C for at least 30 min.
- Centrifuge for 15 min in a microcentrifuge and resuspend the pellet in 50 μl of $T_{10}E_1$ containing 1% 2-mercaptoethanol. Store at -70°C until required.

Before hybridization, measure the incorporation of the labeled nucleotide as described in Appendix 1, Method A, and calculate the amount of probe to be used.

Appendix 3. Extraction of viroids present at low concentration in host tissue.

Materials

- TSS buffer
  - 0.1 M Tris-HCl, pH 8.5
  - 0.5 M NaCl
  - 0.5% SDS
  - 2.5% PVP-40
  - 0.1 mM magnesium acetate

Add 1% DIECA immediately before use.

- Tris-HCl buffer
  75 mM Tris-HCl, pH 7.0
  0.15 M NaCl
  1.5 mM EDTA.
- Sodium acetate buffer
  0.5 M sodium acetate, pH 5.0
  0.2 M NaCl
  1 mM EDTA.
- Cold ethanol.
- Phenol:chloroform (1:1, v/v).
- 12 mM LiCl.
- 1.0 M magnesium acetate.

Methods

-25-30 g of tissue are triturated for 1 min at room temperature in 90 ml of TSS buffer.
- NaCl (7.9 g) is added and the mixture is triturated for a further minute.
- Incubate at -15°C for 1 h.
- Centrifuge at 10,000 *g* for 20 min at 2°C.
- Add 2 volumes of cold ethanol to 50 ml of supernatant.
- Incubate at -15°C for 1 h.
- Centrifuge to recover the nucleic acids and resuspend the pellet in 12.5 ml of Tris-HCl buffer.
- Add an equal volume of phenol:chloroform and centrifuge to separate the aqueous phase.
- Nucleic acids are precipitated from the aqueous phase with an equal volume of 12 mM LiCl and 0.01 volume of 1.0 M magnesium acetate.
- Centrifuge at 10,000 *g* for 20 min.
- Resuspend the pellet in 10 ml sodium acetate buffer.
- Precipitate the nucleic acids once more by adding 2.5 volumes of cold ethanol.
- Centrifuge, dry and resuspend the pellets in 0.5 ml of 0.1 mM EDTA.
- Store at -20°C.

Appendix 4. Hybridization protocols.

Method A: Hybridization with ^{32}P-DNA probes.

Materials

- Hybridization solution
  50% deionized formamide

5x SSC
50 mM sodium phosphate, pH 6.5
2.5 mM EDTA
0.6% SDS
5x Denhardt's solution (0.1% Ficoll, 0.1% PVP, 0.1% BSA).

- 50% dextran sulphate.
- ^{32}P-labeled DNA probe (specific activity should be about 108 c.p.m. μg^{-1}).
- 10 mg ml^{-1} herring sperm DNA.
- SSC/SDS wash buffers
  I: 2x SSC/0.1% SDS
  II: 0.2x SSC/0.2% SDS
  III: 0.1x SSC/0.2% SDS

Method

- Put the membrane in a suitable heat-sealable polythene bag.
- For a 12 x 16 cm membrane add 9 ml of hybridization solution (adjust the volume according to the membrane size - a suitable ratio is at least 1 ml of solution per 20 cm^2).
- Denature the herring sperm DNA by heating it at 100°C for 10 min.
- Chill on ice and add 120 μl to the bag (final concentration 120 $\mu g\ ml^{-1}$).
- Add 1 ml of 50% dextran sulphate (final concentration 5%) and mix. Incubate for 2 h at 55°C for viroids (45°C for viruses).
- Denature the correct amount of probe (about 1-2.5 x 10^6 c.p.m. ml^{-1}) by heating at 100°C for 7 min. Chill quickly on ice and add to the hybridization bag. Seal and avoid trapping air bubbles.
- Hybridize for 18-24 h at 55°C for viroids and 45°C for viruses.
- Recover the membrane from the bag. Dispose of the buffer safely and remember that it is highly radioactive.
- Wash the membrane in a tray on a rotary shaker:
  twice for 15 min at room temperature in SSC/SDS I;
  twice for 15 min at 37°C in SSC/SDS II;
  twice for 15 min at 55°C in SSC/SDS III;
  final rinse in 0.1x SSC.
- Allow the membrane to dry on tissue paper at room temperature and then autoradiograph for 24-48 h at -70°C with Kodak X-Omat AR film (or similar) using an intensifying screen such as Dupont Cronex Lightning Plus.

Method B: Hybridization with biotinylated DNA probes

Materials

- Prehybridization solution

50% deionized formamide
5x SSC
5x Denhardt's solution (0.1% Ficoll, 0.1% PVP, 0.1% BSA)
25 mM sodium phosphate, pH 6.5
0.5 mg ml^{-1} freshly denatured, sheared herring sperm DNA.

- Hybridization solution
  45% formamide
  5x SSC
  1x Denhardt's solution
  20 mM sodium phosphate, pH 6.5
  5% dextran sulphate
  0.2 mg ml^{-1} freshly denatured, sheared herring sperm DNA
  0.1-0.5 μg ml^{-1} freshly denatured, biotinylated DNA probe.
- Buffer 1
  0.1 M Tris-HCl, pH 7.5
  0.15 M NaCl.
- Buffer 2
  0.1 M Tris-HCl, pH 9.5
  0.1 M NaCl
  0.05 M $MgCl_2$.
- Streptavidin-alkaline phosphatase conjugate (SA-AP).
- Nitro blue tetrazolium chloride, grade III (NBT).
- 5-bromo-4-chloro-3-indolyl phosphate (BCIP).
- Blocking solution: 3% (w/v) BSA (fraction V) in buffer 1.
- SSC/SDS wash buffers:
  I: 2x SSC/0.1% SDS
  II: 0.2x SSC/0.1% SDS
  III: 0.16x SSC/0.1% SDS

Method

- The prehybridization and hybridization stages are again performed in heat-sealable polythene bags with approximately 1 ml of solution per 20 cm^2 of membrane.
- Denature sheared herring sperm DNA by heating at 100°C for 10 min, chill immediately on ice, add the prehybridization solution and mix well.
- Pour the appropriate amount of prehybridization solution into the bag, seal without trapping air bubbles and incubate in a water bath at 42°C for 2 h.
- Replace the prehybridization solution with the same amount of hybridization solution containing denatured herring sperm DNA and an adequate amount of biotinylated probe, which is denatured and chilled on ice just before use.
- Seal the bag, mix well and allow to hybridize at 42-45°C (the hybridization solution containing the probe can be reused if stored at -20°C, and denatured again before use).

- The membrane is placed in a plastic tray and washed with 250 ml of solution at each step on a rotary shaker. Washing is carried out at room temperature (if not otherwise indicated) as follows:
  twice for 5 min in SSC/SDS I;
  twice for 5 min in SSC/SDS II;
  twice for 15 min at 50°C in SSC/SDS III;
  final rinse in 2x SSC buffer.
- Incubate the membrane at 60°C for 1 h with 50 ml of preheated blocking solution in a covered plastic tray.
- Incubate on a rotary shaker for 20 min at room temperature with 20 ml buffer 1 containing SA-AP conjugate, diluted 1:1000.
- Wash twice for 15 min in buffer 1 containing 0.05% Tween 20, and then for 10 min in buffer 2.
- Transfer to a clean tray with a lid containing the color development solution made up as follows: 6 mg NBT and 3 mg BCIP in 30 ml of buffer 2 (both can be kept as stock solutions in dimethyl formamide). *Wear gloves throughout this procedure.*
- Incubate on a shaker in the dark until color develops and stop the reaction by rinsing in distilled water.

Method C: Hybridization with RNA probes

Materials

- Hybridization solution
  40% deionized formamide
  0.18 M NaCl
  10 mM sodium cacodylate
  1 mM EDTA
  0.1% SDS.
- 50% dextran sulphate.
- Calf thymus DNA 4 mg ml^{-1}.
- Wash buffer 1
  0.36 M NaCl
  10 mM Tris-HCl, pH 7.5
  0.1% SDS.
- Wash buffer 2
  0.1x SSC
  0.1% SDS.
- Wash buffer 3
  2x SSC.
- RNase A stock solution 10 mg ml^{-1}.

Method

- Place the membrane in a heat-sealable polythene bag and add 9.6 ml of hybridization solution (enough for a 12 x 16 cm membrane).
- Incubate at 55°C for 10 min.
- Denature the calf thymus DNA by heating at 100°C for 5 min, chill on ice and add 1 ml to the bag to give a final concentration of 300 μg ml^{-1}.
- Incubate again at 55°C for 10 min.
- Add 2.4 ml of 50% dextran sulphate and incubate at 55°C for 10 min.
- Add enough probe to give approximately 400,000 c.p.m. ml^{-1} of hybridization solution.
- Immerse the bag overnight in a water bath at 55°C for viroids and 45°C for viruses.
- Remove the membranes and wash as follows:
  twice for 20 min with wash buffer 1 at room temperature;
  once for 30 min with wash buffer 2 at 65°C;
  twice for 10 min with wash buffer 3 at room temperature;
  once with wash buffer 3 containing RNase A at a final concentration of 2 μg ml^{-1} at room temperature.
- Dry the membrane over tissue paper at room temperature or under an incandescent lamp.
- Autoradiograph overnight at -70°C with Kodak X-Omat AR film (or similar) using an intensifying screen such as Dupont Cronex Lightning Plus.

Chapter 3

Nonradioactive approaches for the detection of viroids and viruses

SUMMARY

In recent years several new rapid techniques for virus detection via nucleic acid hybridization have been developed. Most of these hybridization techniques have traditionally relied on radioactively labeled hybridization probes. But the hazard, expense and instability of radioactive labels led to the search for alternative, nonradioactive DNA or RNA labeling and detection methods. Some of the currently available approaches for nonradioactive detection of viruses and viroids are summarized.

INTRODUCTION

There is an increasing need for the improvement and further development of procedures for the rapid and specific detection of plant viruses and viroids of agricultural importance. Ideally, these procedures should allow the assay of a large number of samples with high sensitivity to reliably detect low levels of the disease agent as well as of its various strains that may occur in the field.

The more specific diagnostic methods are: 1) immunological approaches which are based on the use of antibodies prepared against the viral coat protein and, 2) the use of nucleic acid hybridization methods. Viruses have been efficiently detected by using both approaches while viroids, which lack a protein coat, can only be detected by nucleic acid hybridization methods (Owens and Diener, 1981; Palukaitis *et al.*, 1981). Previous methods used for viroid detection, such as inoculation onto tomato plants, which show clear symptoms after 2-3 weeks, or electrophoretic separation of the viroid RNA on acrylamide gels, were indeed demonstrated to be inadequate when large number of samples had to be analyzed and when high sensitivity of detection was required (Salazar, 1989).

The concept of nucleic acid hybridization is based on the reassociation between two homologous complementary strands of nucleic acids thereby forming either double-stranded DNA or RNA, or hybrid molecules that consist of strands of RNA and DNA. In all cases a single-stranded nucleic acid probe that has been labeled in some fashion is allowed to form a hybrid with homologous sequences. The double-stranded labeled material is then either quantitated, visualized, or further analyzed. The principal uses of nucleic acid hybridization techniques are detection of specific genomes or portions of them, determination of homology between genomes, characterization of DNA structure, and detection and analysis of RNA transcripts.

Most of the important advances in molecular biology, and among them pathogen detection, have relied on the use of radioisotopes to label the probes. The use of radiation, however, had always drawbacks, safety being the most important one.

Beyond this most basic concern, the use of radioactivity is associated with problems of regulatory restrictions, high costs and, in some places such as in South America and in many developing countries, limited availability. In addition, difficulty in obtaining short-lived isotopes of certain biomolecules, the physical and chemical instability of radioisotope preparations, and difficulties in transportation and custom clearance have to be considered

as potential factors for the lost of shipments and extensive delay which result in losses of activity.

Alternative technologies, based on the use of nonradioactive labels, as well as detection methods that employ colored and luminescent reaction products, have become available and are competitive with radioisotopic techniques.

Nonradioactive detection of target nucleic acids bound to membranes ideally should approach the sensitivity and simplicity of methods using probes containing radioactive isotopes. Traditionally, probes have been substituted with affinity tags such as biotin (Langer *et al.*, 1981) or a hapten and, after hybridization and washes, the tags have been detected with enzyme conjugates such as alkaline phosphatase linked to streptavidin or an antibody. Recently several additional techniques have been developed allowing easier interpretation of the results, increased sensitivity and simplification of the procedure.

The purpose of this chapter is to provide a compendium of some of the most useful techniques of molecular hybridization based on the use of nonradioactive alternatives to radioisotopes use. By avoiding radioactivity, these methods have opened the field of application for nucleic acid hybridization also to laboratories which are not equipped for working with isotopes.

Nonradioactive labels

Several approaches have been taken in the development of nonradioactive (or "cold") probes. "Cold" probes include the use of reporter groups ("labels" or "tags") that can be detected directly or indirectly following the hybridization. The label or tag can be introduced into the nucleic acid chemically (Renz and Kurz, 1984; Tchen *et al.*, 1984; Forster *et al.*, 1985) or enzymatically (Langer *et al.*, 1981; Leary *et al.*, 1983). One of the first nonradioactive tags investigated is biotin (vitamin H). The interaction between biotin and avidin (or avidin-like proteins) is one of the strongest noncovalent interactions known: the dissociation constant for the complex is 10^{-15} M. Avidin, from egg white (a 68,000 dalton glycoprotein) and streptavidin, from *Streptomyces avidinii* are tetrameric proteins containing four affinity binding sites for biotin. Streptavidin is preferred to avidin because being slightly acidic (whereas avidin is basic), is less likely to bind nonspecifically to

cellular glycoproteins and to acidically charged cell components such as nucleic acids, and therefore causes lower backgrounds (Wilchek and Bayer, 1988).

Biotinylated bases are incorporated into the probe which, after hybridization, is detected with avidin (or streptavidin) or anti-biotin antibodies conjugated to either alkaline phosphatase or peroxidase enzymes (Forster *et al.*, 1985; Van Brunt and Klausner, 1987) by addition of a colored precipitating substrate (Fig. 3.1). During the test, biotinylated probes are hybridized to target nucleic acid in the same manner as are ^{32}P-labeled probes. After hybridization, the filter is washed to remove excess probe and incubated with the conjugate, which binds to the biotin. The filters are washed and the precipitating substrate is added. The colored precipitate indicates hybridization between the probe and the target nucleic acid. The biotin can be incorporated into DNA by nick-translation as a biotin analogue of thymidine triphosphate (dTTP), in which the biotin is separated from the nucleotide by a seven to sixteen-carbon spacer arm (Bio-n-dUTP), making the biotin more accessible to the avidin (or streptavidin) (Langer *et al.*, 1981). Kits for preparing nick-translated probes with biotinylated nucleotides are now available commercially. Biotinylated-n-UTP can also be incorporated into RNA in *in vitro* transcription reactions (Theissen *et al.*, 1989). RNA or DNA probes prepared with biotinylated nucleotides have a sensitivity similar to radioactive probes, they can detect as little as 1 pg of membrane-bound target sequence.

The results obtained in sensitivity, the advantage of using non-toxic materials and the possibility of long-term storage of biotinylated probes, greatly increased their application. The detection of viruses and viroids using biotin-labeled probes have been reported for potato virus S (Eweida *et al.*, 1989), papaya mosaic virus (Roy *et al.*, 1988), potato virus X (Eweida *et al.*, 1990) and for the potato spindle tuber viroid (Roy *et al.*, 1989) and others. In addition, Hopp *et al.* (1988; 1991) reported the use of specific biotinylated probes for the simultaneous detection of potato virus X, potato virus Y, potato leafroll virus and the potato spindle tuber viroid (PSTVd).

Nucleic acids have also been biotinylated chemically with photobiotin, which is biotin coupled with a photoactivable group that reacts with any organic material under intense light (Forster *et al.*, 1985). The additional advantage of this method is that no expensive enzymes are required. Habili *et al.* (1987) used a specific photobiotin-labeled DNA probe for the routine detection of barley yellow dwarf virus (BYDV) in infected plant sap with the same level of sensitivity as ^{32}P-labeled probes.

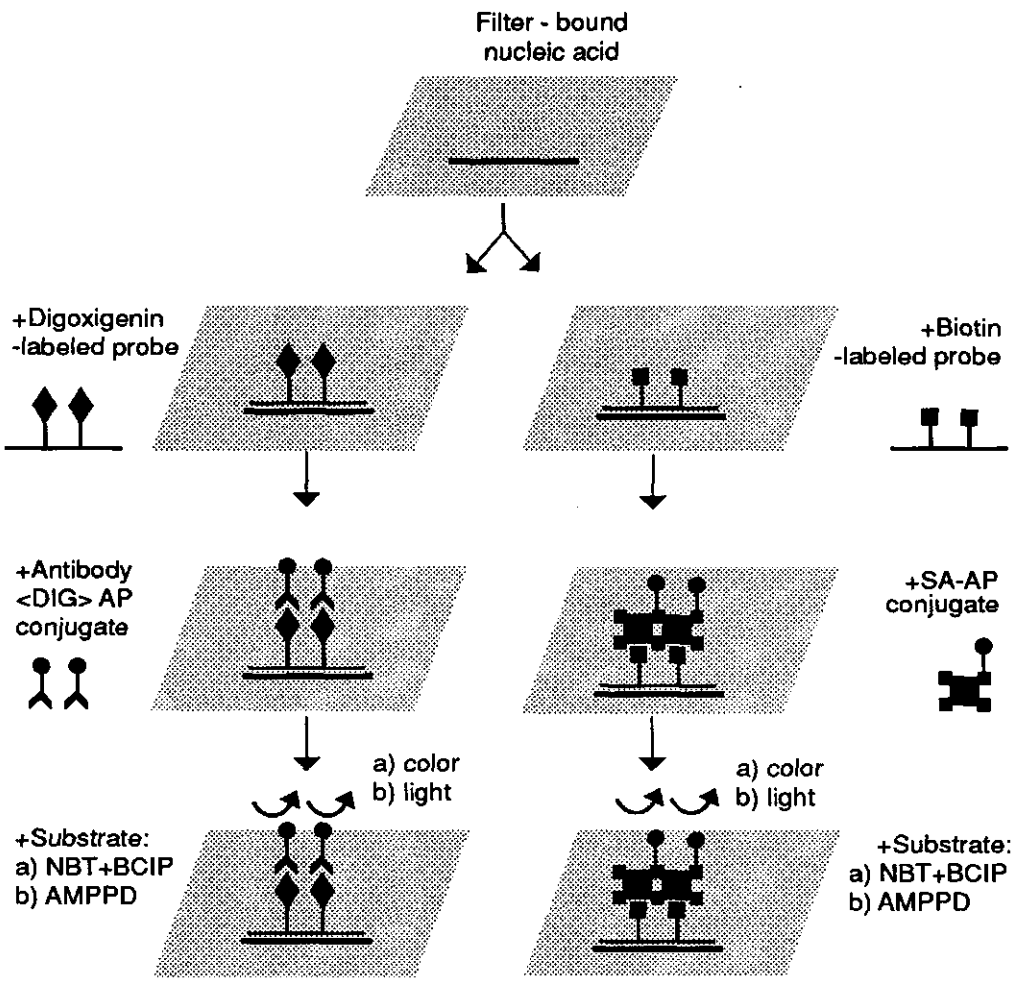

Fig. 3.1. Schematic diagram of nonradioactive detection of target nucleic acids using biotin or digoxigenin-labeled probes.

Another technique investigated is the chemical modification of bases (haptens) which can be detected after hybridization with anti-hapten antibodies. One example is the sulphonation of cytosine residues, marketed as Chemiprobe™ (Bioproducts). A kit is used to insert antigenic sulphone groups into cytosine residues of the probe. Following hybridization, the probe is detected using a sandwich immuno-enzymatic reaction. Monoclonal antibodies bind to the sulphone residues and then to an alkaline phosphatase-anti-immunoglobulin conjugate. Addition of the NBT/BCIP chromogenic alkaline phophatase substrate (NBT: nitroblue tetrazolium salt + BCIP: 5-bromo-4-chloro-3-indolyl phosphate) produces a blue color in the presence of the hybridized probe.

Other alternative haptens such as dinitrophenol (Shoyer and Nakane, 1983), 2-acetylaminofluorene (Landegent *et al.*, 1984) and digoxigenin (Boehringer-Mannheim, GmbH., Mannheim, Germany) have been recently used as substitutes for biotin.

The hapten digoxigenin (DIG), extracted from *Digitalis purpurea* plants, is bound via a spacer arm to uridine-nucleotides (Fig. 3.2) and incorporated enzymatically (Höltke and Kessler, 1990) or chemically (Mühlegger *et al.*, 1990) into nucleic acid probes. After hybridization and blocking, DIG-labeled probes are detected by high affinity anti-digoxigenin-antibody Fab-fragments conjugated to alkaline phosphatase (AP) (Fig. 3.1) with a system similar to the one used for detection of biotinylated probes.

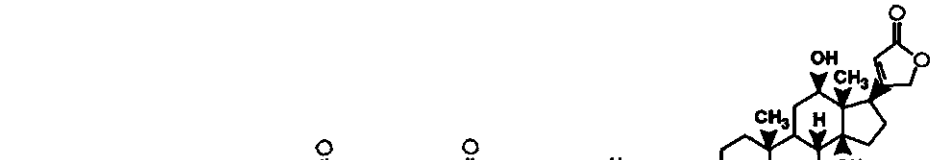

Fig. 3.2. Structure of digoxigenin-UTP (DIG-UTP). The digoxigenin hapten is connected to UTP via a 9 atoms spacer arm.

Chemiluminescence

Improvements in nonradioactive detection techniques, such as chemiluminescent detection, have made it possible to decrease the use of radioisotopes without forgoing advantages and versatility of radioactivity.

Recently an alternative substrate for alkaline phosphatase (AP)-mediated detection of biotin or digoxigenin-labeled probes has been introduced. Specifically bound to the UV-immobilized target DNA or RNA via a biotin (or digoxigenin)-streptavidin complex, alkaline phosphatase catalyzes a color or light reaction depending on the substrate added.

Using a chemiluminescent substrate (the compound is called AMPPD= 3-(2'-spiroadamantane)-4-methoxy-4-(3''-phosphoryloxy)phenil-1,2-dioxetane, disodium salt) (Bronstein *et al.*, 1990), the cleavage of the phophate group by alkaline phosphatase in the presence of basic buffer, destabilizes the 1,2 dioxetane ring structure and the molecule decomposes with emission of visible yellow-green light at 477 nm. Signal detection is accomplished by a short exposure of blots to ordinary X-ray or Polaroid films. For the chemiluminescent detection, positively charged nylon membranes are required as solid support. The light emission is stable so that several exposures can be made on different films. The intensity can also be further enhanced in the presence of an appropriate enhancer. An additional advantage is that normal stripping procedures for removing the probe allow the blot to be used several times and the probing of the target sequence with different labels (Allefs *et al.*, 1990; Kreike *et al.*, 1990).

Until recently cold probes have not been as sensitive as radio-labeled probes. However, several of the recent introductions, including both AMPPD and digoxigenin, offer extended shelf-life of up to 1 year or more, together with a high degree of sensitivity. Chemiluminescent detection has already found application in DNA sequencing (Beck *et al.*, 1989) and in virus detection (Choi and Sano, 1990).

Polymerase Chain Reaction

The most recent development in DNA technology, and probably one of the most promising techniques, is the Polymerase Chain Reaction (PCR), which in few years, since its introduction, has already become a widespread research tool. The PCR is an *in vitro*

method for the enzymatic synthesis of specific nucleic acid sequences (Saiki *et al.*, 1985; Mullis and Faloona, 1987).

To apply the technique, the nucleotide sequence of the DNA of interest, or sequences near it, must be at least partially known. In fact the PCR is a mechanism for amplifying a DNA sequence comprised between two oligonucleotide primers. Design of the primers is a very important aspect of the process. The two primers are synthesized to be complementary to known sequences flanking the target sequence to be amplified (<100 to 10,000 bases) on opposite strands and are oriented so that DNA synthesis by the polymerase proceeds across the region between the primers.

The double-stranded DNA is first heated at a temperature of around 95°C to separate the strands in the presence of the primers. When the temperature is reduced to around 30°C, the primers anneal. At a selected temperature a DNA polymerase extends the primers to copy the DNA. Successive series of cycles involving template denaturation, primer annealing, and the extension of the annealed primers by a DNA polymerase, result in the exponential accumulation of a specific region whose termini are defined by the 5' ends of the primers.

In principle, because each extension product, synthesized in a cycle, along with the original target, can serve as a template in the following one, each new cycle doubles the amounts of product DNA, and after n cycles of denaturation, annealing and extension, the target DNA sequence is amplified 2^n-fold. The recent utilization of a thermostable DNA polymerase purified from the thermophilic bacterium *Thermus aquaticus* (*Taq*) instead of the thermolable Klenow fragment of *Escherichia coli* DNA polymerase I, greatly simplified the procedure (Saiki *et al.*, 1988). Since this heat-resistant polymerase is not affected by the denaturation step, it does not need to be replenished at each cycle. This modification not only made the procedure amenable to automation, it also substantially improved the overall performance of the reaction by increasing the specificity, yield, sensitivity and length of targets that can be amplified.

In vitro nucleic acid amplification by the polymerase chain reaction has become one of the most powerful and versatile molecular biological techniques available; PCR products may be used for a number of applications which have already been described in recently published handbooks (Ehrlich, 1989; Innis *et al.*, 1990).

Reverse transcription (RT) combined with PCR amplification (RT-PCR) makes it possible to generate cDNA from rare RNAs: the polymerase chain reaction allows

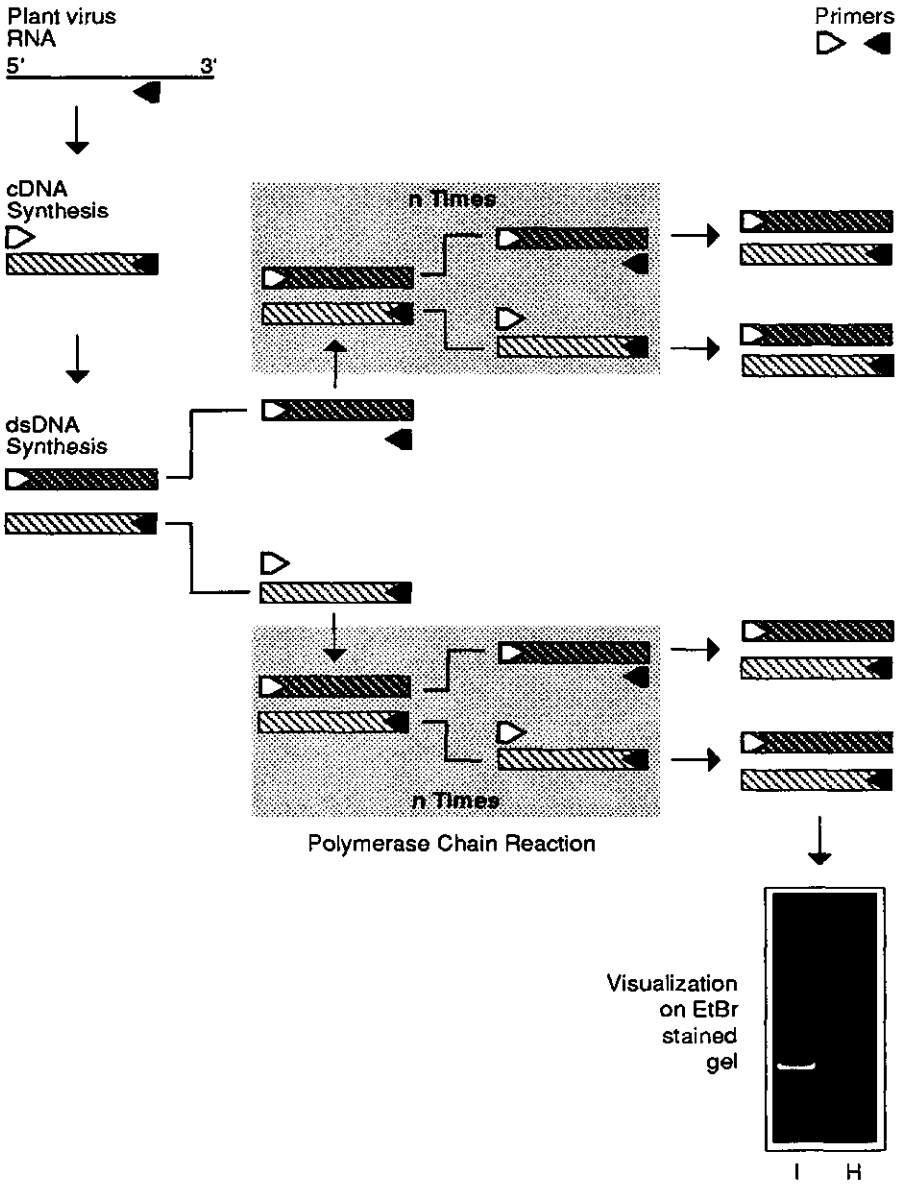

Fig. 3.3. Schematic representation of the steps involved in the detection of a plant virus single-stranded RNA by Polymerase Chain Reaction. (I) infected and (H) healthy sample.

overcoming the difficulty in detecting DNA/RNA sequences occurring as a very low proportion of the nucleic acid sample, for example, single-copy genes or nucleic acids associated with low levels of virus infection, in fact the target DNA of interest, being amplified several million times, can be visualized on a gel (Fig. 3.3). An additional application of RT-PCR, now largely used in virology, is the identification and characterization of viral strains or types within the family (Rybicki and Hughes, 1990; Langeveld *et al.*, 1991).

REFERENCES

Allefs, J.J.H.M., Salentijn, E.M.J., Krens, F.A., and Rouwendal, G.J.A. (1990). Optimization of non-radioactive Southern blot hybridization: single copy detection and reuse of blots. *Nucleic Acids Research* **18**: 3099-3100.

Beck, S., O'Keeffe, T., Coull, J.M., and Köster, H. (1989). Chemiluminescent detection of DNA: application for DNA sequencing and hybridization. *Nucleic Acids Research* **17**: 5115-5123.

Bronstein, I., Voyta, J.C., and Edwards, B. (1990). A comparison of chemiluminescent and calorimetric substrates in a Hepatitis B virus DNA hybridization assay. *Analytical Biochemistry* **180**: 95-98.

Choi, J.K., and Sano, T. (1990). Hybridization detection of CMV satellite RNA using enhanced chemiluminescence. *Korean Journal of Plant Pathology* **6(4)**: 507-510.

Erlich, H.A. (ed) (1989). *PCR Technology. Principles and Applications for DNA Amplification*. M. Stockton Press, New York. 246 pp.

Eweida, M., Sit, T.L., and AbouHaidar, M.G. (1989). Molecular cloning of the genome of the carlavirus potato virus S: biotinylated RNA transcripts for virus detection in crude potato extracts. *Annals of Applied Biology* **115**: 253-261.

Eweida, M., Xu, H., Singh, R.P., and AbouHaidar, M.G. (1990). Comparison between ELISA and biotin-labelled probes from cloned cDNA of potato virus X for the detection of virus in crude tuber extracts. *Plant Pathology* **39**: 623-628.

Forster, A.C., McInnes, J.L., Skingle, D.C., and Symons, R.H. (1985). Nonradioactive hybridization probes prepared by the chemical labeling of DNA and RNA with a novel reagent, photobiotin. *Nucleic Acids Research* **13**: 745-761.

Habili, N., McInnes, J.L., and Symons, R.H. (1987). Nonradioactive, photobiotin-labelled DNA probes for the routine diagnosis of barley yellow dwarf virus. *Journal of Virological Methods* **16**: 225-237.

Höltke, H.-J., and Kessler, C. (1990). Non-radioactive labeling of RNA transcripts *in vitro* with the hapten digoxigenin (DIG); hybridization and ELISA-based detection. *Nucleic Acids Research* **18**: 5843-5851.

Hopp, H.E., Giavedoni, L., Mandel, M.A., Arese, A., Orman, B., Bravo Almonacid, F., Torres, H.N., and Mentaberry, A.N. (1988). Biotinylated nucleic acid hybridization probes for potato virus detection. *Archives of Virology* **103**: 231-241.

Hopp. H.E., Hain, L., Bravo Almonacid, F., Tozzini, A.C., Orman, B., Arese, A.I., Ceriani, M.F., Saladrigas, M.V., Celnik, R., del Vas, M., and Mentaberry, A.N. (1991). Development and application of a nonradioactive nucleic acid hybridization system for simultaneous detection of four potato pathogens. *Journal of Virological Methods* **31**: 11-30.

Innis, M.A., Gilfand, M.A., Sninsky, J.J., and White, T.J. (eds) (1990). *A guide to methods and applications. PCR protocols*. Academic Press, New York. 482pp.

Kreike, C.M., de Koning, J.R.A., and Krens, F.A. (1990). Non-radioactive detection of single-copy DNA-DNA hybrids. *Plant Molecular Biology Reporter* **8(3)**: 172-179.

Langer, P.R., Waldrop, A.A., and Ward, D.C. (1981). Enzymatic synthesis of biotin-labelled polynucleotides: Novel nucleic acid affinity probes. *Proceedings of the National Academy of Science USA* **78**: 6633-6637.

Langeveld, S.A., Dore, J.-M., Memelink, J., Derks, A.F.L.M., van der Vlugt, C.I.M., Asjes, C.J., and Bol, J.F. (1991). Identification of potyviruses using the polymerase chain reaction with degenerate primers. *Journal of General Virology* **72**: 1531-1541.

Leary, J.J., Brigati, D.J., and Ward, D.C. (1983). Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: Bio-blots. *Proceedings of the National Academy of Science USA* **80**: 4045-4049.

Mullis, K.B., and Faloona, F.A. (1987). Specific synthesis of DNA *in vitro* via a polymerase catalyzed chain reaction. *Methods in Enzymology* **155**: 335-350.

Mühlegger, K., Huber, E., von der Eltz, H., Rüger, R., and Kessler, C. (1990). Non-radioactive labeling and detection of nucleic acids. *Biological Chemistry Hoppe-Seyler* **371**: 953-965.

Owens, R.A., and Diener, T.O. (1981). Sensitive and rapid diagnosis of potato spindle tuber viroid disease by nucleic acid hybridization. *Science* **213**: 670-672.

Palukaitis, P., Rakowski, A.G., Alexander, D.M., and Symons, R.H. (1981). Rapid indexing of the sunblotch disease of avocados using a complementary DNA probe to avocado sunblotch viroid. *Annals of Applied Biology* **98**: 439-449.

Renz, M., and Kurz, C. (1984). A colorimetric method for DNA hybridization. *Nucleic Acids Research* **12**: 3435-3444.

Roy, B.P., AbouHaidar, M.G., and Alexander, A. (1989). Biotinylated RNA probes for the detection of potato spindle tuber viroid (PSTV) in plants. *Journal of Virological Methods* **23**: 149-156.

Roy, B.P., AbouHaidar, M.G., Sit, T.L., and Alexander, A. (1988). Construction and use of cloned cDNA biotin and ^{32}P-labeled probes for the detection of papaya mosaic potexvirus RNA in plants. *Phytopathology* **78**: 1425-1429.

Rybicki, E.P., and Hughes, F.L. (1990). Detection and typing of maize streak virus and other distantly related geminiviruses of grasses by polymerase chain reaction amplification of a conserved viral sequence. *Journal of General Virology* **71**: 2519-2526.

Saiki, R.K., Scharf, S.J., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., and Arnheim, N. (1985). Enzymatic amplification of ß-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**: 1350-1354.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**: 487-491.

Salazar, L.F. (1989). Potato Spindle Tuber. In Kahn R.P. (ed) *Plant Protection and Quarantine*, vol. II, pp. 155-167. CRC Press, Boca Raton, Florida.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Tchen, P., Fuchs, R.P.P., Sage, E., and Leng, M. (1984). Chemically modified nucleic acids as immunodetectable probes in hybridization experiments. *Proceedings of the National Academy of Science USA* **81**: 3466-3470.

Theissen, G., Richter, A., and Lukacs, N. (1989). Degree of biotinylation in nucleic acids estimated by a gel retardation assay. *Analytical Biochemistry* **179**: 98-105.

Van Brunt, J., and Klausner, A. (1987). Pushing probes to market. *Bio/Technology* **5**: 211-221.
Wilchek, M., and Bayer, E.A. (1988). The avidin-biotin complex in bioanalytical applications. *Analytical Biochemistry* **171**: 1-32.

Chapter 4

Detection of Andean potato virus X isolates by radioactive and nonradioactive nucleic acid spot hybridization tests

SUMMARY

A cDNA probe, pX61, prepared from the Andean potato virus X (PVX) cp strain was tested against a broad spectrum of Andean PVX isolates grouped in two serotypes: 1) the PVX^A Andean serotype detected only in Peru and Bolivia, which includes the cp and HB strains; and 2) the PVX^O common serotype, which contains isolates serologically similar to those occurring elsewhere in the world. In radioactive nucleic acid spot hybridization tests (R-NASH) of virus in crude sap using the ^{32}P-labeled RNA probe pX61, the PVX^A isolates showed stronger hybridization signals and a detectability usually from two to six threefold dilution steps higher than those shown for PVX^O isolates. The difference in the detectability of isolates from PVX^A and PVX^O serotypes was similar to that in nonradioactive nucleic acid spot hybridization tests (NR-NASH) with biotinylated DNA probe pX61. However, detectability in NR-NASH was lower than in R-NASH. When biotinylated DNA probes of pX61 (PVX^A-specific) and pPVX19 (PVX^O-specific), prepared from a British PVX isolate of the common European strain-group 3, were compared in NR-NASH, pPVX19 hybridized much more strongly to most isolates from the PVX^O serotype than to those from the PVX^A serotype. Several PVX^O isolates, mostly from Bolivia, reacted as weakly with pPVX19 as the PVX^A isolates. For R-NASH and NR-NASH tests, virus

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concentration in crude sap was checked by the double-antibody sandwich form of enzyme-linked immunosorbent assay, confirming that the differences obtained were not because of differences in virus concentration. These results were reconfirmed in R-NASH tests with DNA probes pX61 and pPVX19 using known concentrations of purified RNA from selected isolates. The differences shown between PVX^{A} and PVX^{O} isolates stress the importance of using the appropriate probes to detect PVX in breeding programs for resistance and quarantine purposes.

INTRODUCTION

The nucleic acid spot hybridization (NASH) technique (also referred to as dot blot hybridization) has been reported for the detection of potato X (PVX), potato Y (PVY), and potato leafroll (PLRV) viruses using radioactively labeled complementary DNA (cDNA) probes (Owens and Diener, 1981; Salazar *et al.*, 1983; Baulcombe *et al.*, 1984a,b), as well as for potato spindle tuber viroid (PSTVd) in potato, using radioactively labeled RNA probes (Salazar *et al.*, 1988). Nonradioactive, biotinylated RNA and DNA probes for the detection of PVX and potato virus S (PVS) in crude potato extracts have also been reported (Hopp *et al.*, 1988; Eweida *et al.*, 1989). Other nonradioactive systems of nucleic acid labeling and detection, including luminescent DNA probes and luminography, were compared in the detection of purified PVX, PVY, and PVS (Audy *et al.*, 1991). They were also compared in the detection of purified PSTVd and PSTVd in tomato leaf extracts (Kanematsu *et al.*, 1991). Baulcombe and Fernandez-Northcote (1988) evaluated the ability of cDNA probes, prepared from a British PVX isolate of the common European strain (strain-group 3) and from a British common strain of PVY^{O}, to detect distinct PVX strains and a broad spectrum of PVY isolates, respectively. The two PVY probes used in that work did not detect differences among the PVY isolates from the three groups of strains (PVY^{O}, PVY^{N} and PVY^{C}). However, PVX probes showed a stronger reaction with the non-Andean strains than with the Andean strains cp and HB.

In the present work, a cDNA probe (pX61), synthesized from the cp strain, was tested as a probe for detection of a broad spectrum of Andean PVX isolates selected from two serotypes (Fernandez-Northcote and Lizárraga, 1991): 1) the PVX^{A} Andean serotype, detected only in Peru and Bolivia, which includes the cp and HB strains; and 2) the PVX^{O}

serotype, which groups isolates that are serologically similar with those occurring elsewhere in the world. The pX61 was evaluated and compared with Baulcombe's pPVX19 in both radioactive (R) and nonradioactive (NR) NASH tests. The cDNA inserts of both probes were sequenced.

MATERIALS AND METHODS

Isolates and spotting.

Isolates and strains of PVX used in the experiments are listed in Tables 4.1 and 4.2. Strains cp and HB (Moreira *et al.*, 1980) were kindly donated by C. Fribourg (Universidad Nacional Agraria La Molina, Lima, Peru) and maintained at the International Potato Center (CIP). The cp and HB strains were originally isolated from the high Andes of central-southern Peru and southern Bolivia, respectively. All isolates and strains were maintained in *Nicotiana glutinosa* L. grown in a greenhouse at 18-24°C. Crude leaf sap from healthy and infected plants was extracted using a roller press and microfuged at 9,880 *g* x 5 min. Threefold dilutions of the supernatant were prepared in 1.5 M NaCl, 0.15 M trisodium citrate, pH 7.0 (10x SSC). Three microliters of each crude sample or known concentrations of purified viral RNA in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA ($T_{10}E_1$ buffer) were applied to 0.45-μm nitrocellulose filters (Schleicher and Schuell) previously wetted for 5 min in distilled water and then equilibrated in 10x SSC for 5 min. The filters were then air-dried and baked under vacuum at 80°C for 2 h.

Estimation of PVX titer by enzyme-linked immunosorbent assay (ELISA).

An aliquot from the same crude sap samples used for NASH was taken for use in ELISA. Threefold dilutions were prepared in phosphate-buffered saline (PBS: 0.02 M sodium phosphate, pH 7.4; 0.15 M NaCl) containing 0.05% Tween-20, 2% polyvinylpyrrolidone (M_r 44,000), and 1% egg albumin. The direct double-antibody sandwich form of ELISA (DAS-ELISA) was used, as described by Clark and Adams (1977), and 200 μl of each diluted sample was added to each well of the ELISA plate. The coating immunoglobulin G (IgG) was prepared from the PVX_{cp} isolate, and the detecting IgG, conjugated to alkaline phosphatase, was prepared from the PVX_{HB} isolate.

Virus purification and viral RNA extraction.

The Andean PVX_{cp} isolate, used for the preparation of probe pX61, and the other PVX isolates used in this work were purified from infected *N. glutinosa* leaves. Frozen leaves were homogenized in 0.1 M phosphate buffer, pH 8.0, containing 0.2% 2-mercaptoethanol and 10% ethanol. The homogenate was treated with 1% Triton X-100 at 4°C for 1 h, precipitated with 0.2 M NaCl, 4% polyethylene glycol 6,000-8,000, for 1 h at room temperature, and centrifuged at 7,840 *g*. The pellets were resuspended in 0.05 M phosphate buffer, pH 8.0, and subjected to centrifugation through a 30% sucrose cushion (6 ml per tube) at 69,231 *g* x 150 min, followed by centrifugation through a linear sucrose density gradient (10-40%) in 0.05 M phosphate buffer, pH 7.2. Fractions containing virions were further concentrated by ultracentrifugation and resuspended in $T_{10}E_1$ buffer.

For RNA extraction (Hammond and Lawson, 1988), 0.2-0.5 mg/ml of purified virus in $T_{10}E_1$ buffer was incubated for 30 min at 37°C with 50 μg/ml of proteinase K in the presence of 0.1 % sodium dodecyl sulphate (SDS). The mixture was then extracted once with $T_{10}E_1$ buffer-saturated phenol (1:1 w/v), extracted twice with phenol/chloroform (1:1), and extracted once with chloroform. The RNA was concentrated by ethanol precipitation and resuspended in sterile diethyl pyrocarbonate-treated distilled water (Maniatis *et al.*, 1982).

cDNA synthesis and cloning.

PVX_{cp} RNA was used for cDNA preparation. First-strand synthesis was obtained using M-MLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD, [BRL]) by priming the RNA with either oligo dT_{12-18} (Hellmann *et al.*, 1983) or a random hexanucleotide mixture (pdN_6) (both from Pharmacia). After RNA hydrolysis, second-strand cDNA synthesis was carried out using the Klenow fragment of DNA Polymerase I (BRL) by standard procedures (Maniatis *et al.*, 1982).

Double-stranded cDNA was analyzed by gel electrophoresis, and aliquots were digested with three different restriction enzymes (*Bam*HI, *Eco*RI, *Hind*III). The fragments were ligated into pSP64 plasmid that was separately digested with the same restriction enzymes. Recombinant plasmids introduced into *Escherichia coli* JM83 were selected by colony hybridization using ^{32}P-labeled cDNA (Maniatis *et al.*, 1982). The presence and size of inserts was determined by plasmid isolation (Maniatis *et al.*, 1982) followed by restriction enzyme digestion and agarose gel electrophoresis with DNA size markers.

The pX61 clone selected for this work contains a 1,100 bp *Hind*III cDNA fragment inserted into the *Hind*III site of plasmid pSP64.

Probe pPVX19.

Probe pPVX19 (Baulcombe and Fernandez-Northcote, 1988), prepared from a British PVX isolate of the common European strain-group 3, was donated by D. C. Baulcombe. The probe was a 937 bp cDNA inserted in plasmid pBR322 (Baulcombe *et al.*, 1984b).

DNA sequencing.

The nucleotide sequences of cDNA clones pX61 and pPVX19 were obtained by the dideoxy chain termination method (Sanger *et al.*, 1977) using the Taq Track sequencing system from Promega (Madison, WI). The genome coordinates of the two probes were determined by comparison with the sequences reported by Huisman *et al.* (1988) for PVX strain X3, and Orman *et al.* (1990) for PVX strain cp.

R-NASH.

^{32}P-labeled RNA probe was prepared essentially according to the procedure described by Melton *et al.* (1984) using a commercial kit (Promega). Plasmid pSP64:pX61 was linearized with *Eco*RI, extracted twice with phenol/chloroform (1:1), ethanol precipitated, and resuspended in $T_{10}E_1$ buffer (0.5 μg/μl) for use as a riboprobe template. The transcription mixture, containing 1 μg linearized plasmid template, 0.5 mM each of unlabeled nucleoside triphosphates, and 70 μCi of 10 mCi/ml α^{32}P-UTP in the presence of 1 unit per microliter of ribonuclease inhibitor, was incubated with 20 units of SP6 RNA polymerase for 90 min at 38°C. The mixture was then incubated at 37°C for 15 min with RNase-free deoxyribonuclease I to remove the DNA template. Phenol/chloroform-extracted RNA was ethanol precipitated and resuspended in $T_{10}E_1$ buffer, pH 7.5, containing 1% 2-mercaptoethanol. Hybridization, without a prehybridization step, was carried out overnight at 45°C in heat-sealable plastic bags containing 0.05-0.1 ml of the hybridization solution per square centimeter of membrane, using $4x10^5$ cpm of RNA probe per milliliter of hybridization solution (40% formamide; 0.18 M NaCl; 10 mM sodium cacodylate, pH 7.0; 1 mM EDTA; 0.1% SDS; 10% dextran sulphate; and 300 μg/ml calf-thymus DNA). After hybridization, membranes were washed as previously described (Salazar and Querci, 1992) and hybridization was visualized by autoradiography. DNA probes were obtained by

labeling pSP64:pX61 and pBR322:pPVX19 plasmids with α^{32}P-dCTP using a nick-translation kit (BRL). Membranes were prehybridized for 2 h at 45°C in a solution of 50% formamide, 5x SSC, 5x Denhardt's reagent (Maniatis *et al.*, 1982), 50 mM sodium phosphate, pH 6.5, 2.5 mM EDTA, 0.6% SDS, 5% dextran sulphate and 100 μg/ml of denatured herring sperm DNA. Labeled probe (10^6 cpm/ml) was added directly to the prehybridization solution for hybridization and incubated overnight at 45°C. Membranes were washed twice in 2x SSC, 0.1% SDS for 15 min at room temperature; once in 0.2x SSC, 0.2% SDS for 30 min at 37°C; once in 0.1x SSC, 0.2% SDS for 30 min at 55°C; then briefly rinsed in 0.1x SSC and air-dried prior to autoradiography.

NR-NASH.

Plasmids pSP64:pX61 and pBR322:pPVX19 were biotinylated by nick-translation in the presence of 50 μM bio-14-dATP (BRL) using a nick-translation kit (BRL) with a DNase concentration of 4 pg/μl; incubation was carried out at 15°C for 90 min. Under these conditions, the biotinylated nucleotides substituted about 25% of the A residues. Unincorporated nucleotides were then removed by two ethanol precipitations in the presence of 2.5 M ammonium acetate. Biotinylated DNA was resuspended in 1x SSC, and used at a concentration of 0.1-0.3 μg/ml.

Incubations were performed in heat-sealable plastic bags containing 0.05-0.1 ml of the hybridization solution per square centimeter of membrane. Membranes were prehybridized a minimum of 2 h at 42°C in a solution of 50% formamide, 5x SSC, 5x Denhardt's reagent, 25 mM sodium phosphate (pH 6.5), and 500 μg/ml of denatured herring sperm DNA. Hybridization was carried out overnight at 42°C in a solution of 45% formamide, 5x SSC, 1x Denhardt's reagent, 20 mM sodium phosphate (pH 6.5), 5% dextran sulphate, 200 μg/ml of denatured herring sperm DNA, and 0.1-0.3 μg/ml of biotinylated cDNA probe. After hybridization, membranes were washed as previously described (Salazar and Querci, 1992); they were incubated for 20 min in 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl containing streptavidin-alkaline phosphatase conjugate (BRL) diluted 1:2,000, then they were washed in 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 0.05 M $MgCl_2$. Results were visualized by incubation in the dark in the same buffer containing 0.2 mg/ml nitro blue tetrazolium chloride and 0.1 mg/ml 5-bromo-4-chloro-3-indolylphosphate.

RESULTS

Comparison between the nucleotide sequence of pX61 and the corresponding region of PVX_{cp}, reported by Orman *et al.* (1990), showed 100% homology. The probe pX61 corresponds to the region between nucleotides 3008 and 4107 (Fig. 4.1). The pX61 sequence shows 78.5 and 78.8% homology to the corresponding sequences of PVX_{X3} (Huisman *et al.*, 1988) and PVX_S (Skryabin *et al.*, 1988) respectively (Fig. 4.2).

Probe pPVX19 corresponds to the region between nucleotides 2909 and 3845 of PVX RNA (Fig. 4.1). In that region, the nucleotide sequence showed 95.9 and 97.3% homology to the corresponding PVX sequences reported for PVX_{X3} and PVX_S, respectively, and 78.4% homology to PVX_{cp} (Fig. 4.2). The percentage of nucleotide changes in the nonoverlapping regions of the probes 2909-3007 and 3846-4107 were 16 and 20% for the PVX_{cp} and the European isolates, respectively.

These results indicate that both probes, pX61 and pPVX19, derive from the central region of the PVX RNA genome and that they overlap in 838 nucleotides. The overlaps represent 76% of the pX61 towards the 3' end and 89.5% of the pPVX19 towards the 5' end.

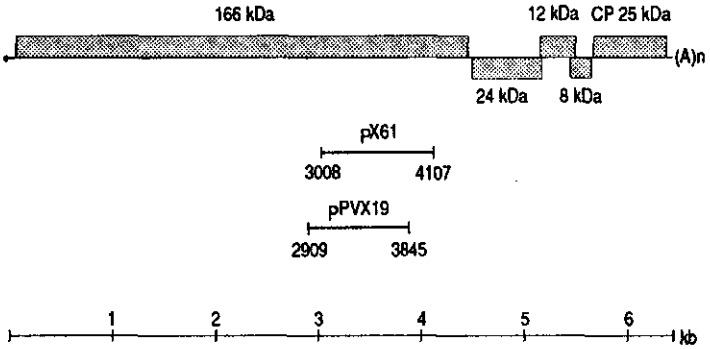
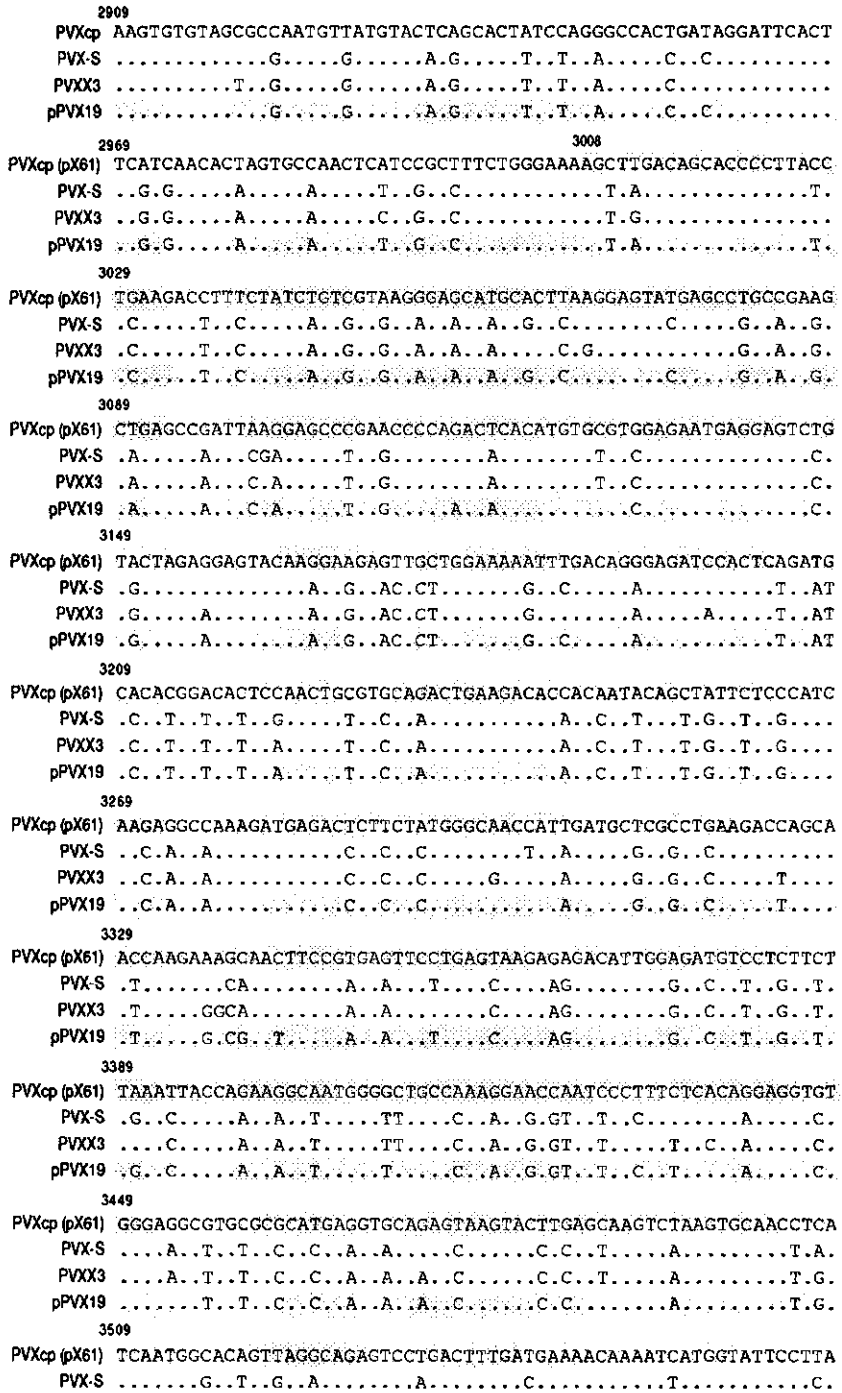


Fig. 4.1. Genomic organization of the potato virus X RNA and coordinates of the cDNA probes pX61 and pPVX19. Shaded boxes are open reading frames as reported by Orman *et al.* (1990). Arrowhead at the 5' end is m^7GpppG cap structure and a poly (A) tail (A)n is at the 3' end. CP = coat protein.


2909
PVXcp AAGTGTGTAGCGCCAATGTTATGTACTCAGCACTATCCAGGGCCACTGATAGGATTCACT
PVX-S .....G.....G.....A.G.....T..T..A.....C..C.....
PVXX3 .....T..G.....G.....A.G.....T..T..A.....C.....
pPVX19 .....G.....G.....A.G.....T..T..A.....C..C.....
2969
3008
PVXcp (pX61) TCATCAACACTAGTGCCAACTCATCCGCTTTCTGGGAAAAGCTTGACAGCACCCCTTACC
PVX-S ..G.G.....A.....A.....T..G..C.....T.A.....T.
PVXX3 ..G.G.....A.....A.....C..G..C.....T.G.....
pPVX19 ..G.G.....A.....A.....T..G..C.....T.A.....T.
3029
PVXcp (pX61) TGAAGACCTTTCTATCTGTCGTAAGGGAGCATGCACTTAAGGAGTATGAGCCTGCCGAAG
PVX-S .C.....T..C.....A..G..G..A..A..A..G..C.....C.....G..A..G.
PVXX3 .C.....T..C.....A..G..G..A..A..A.....C.G.....G..A..G.
pPVX19 .C.....T..C.....A..G..G..A..A..A..G..C.....C.....G..A..G.
3089
PVXcp (pX61) CTGAGCCGATTAAGGAGCCCGAACCCCAGACTCACATGTGCGTGGAGAATGAGGAGTCTG
PVX-S .A.....A...CGA.....T..G.....A.....T..C.....C.
PVXX3 .A.....A...C.A.....T..G.....A.....T..C.....C.
pPVX19 .A.....A...C.A.....T..G.....A..A.....C.....C.
3149
PVXcp (pX61) TACTAGAGGAGTACAAGGAAGAGTTGCTGGAAAAATTTGACAGGGAGATCCACTCAGATG
PVX-S .G.....A..G..AC.CT.....G..C.....A.....T..AT
PVXX3 .G.....A.....A..G..AC.CT.....G.....A.....A.....T..AT
pPVX19 .G.....A.....A..G..AC.CT.....G..C.....A.....T..AT
3209
PVXcp (pX61) CACACGGACACTCCAACTGCGTGCAGACTGAAGACACCACAATACAGCTATTCTCCCATC
PVX-S .C..T..T..T..G.....T..C..A.....A..C..T...T.G..T..G....
PVXX3 .C..T..T..T..A.....T..C..A.....A..C..T...T.G..T..G....
pPVX19 .C..T..T..T..A.....T..C..A.....A..C..T...T.G..T..G....
3269
PVXcp (pX61) AAGAGGCCAAAGATGAGACTCTTCTATGGGCAACCATTGATGCTCGCCTGAAGACCAGCA
PVX-S ..C.A..A.....C..C..C.....T..A.....G..G..C.....
PVXX3 ..C.A..A.....C..C..C.....G.....A.....G..G..C.....T....
pPVX19 ..C.A..A.....C..C..C.....A.....G..G..C.....T....
3329
PVXcp (pX61) ACCAAGAAAGCAACTTCCGTGAGTTCCTGAGTAAGAGAGACATTGGAGATGTCCTCTTCT
PVX-S .T.....CA.....A..A...T....C....AG.....G..C..T..G..T.
PVXX3 .T.....GGCA.....A..A.....C....AG.....G..C..T..G..T.
pPVX19 .T.....G.CG..T.....A..A...T....C....AG.....G..C..T..G..T.
3389
PVXcp (pX61) TAAATTACCAGAAGGCAATGGGGCTGCCAAAGGAACCAATCCCTTTCTCACAGGAGGTGT
PVX-S .G..C.....A..A..T.....TT....C..A..G.GT..T..C.....A.....C.
PVXX3 ....C.....A..A..T.....TT....C..A..G.GT..T.....T..C..A.....C.
pPVX19 .G..C.....A..A..T.....T.....C..A..G.GT..T..C..T.....A.....C.
3449
PVXcp (pX61) GGGAGGCGTGCGCGCATGAGGTGCAGAGTAAGTACTTGAGCAAGTCTAAGTGCAACCTCA
PVX-S ....A..T..T..C..C..A..A.....C.....C.C..T.....A.....T.A.
PVXX3 ....A..T..T..C..C..A..A..A..C.....C.C..T.....A.....T.G.
pPVX19 .....T..T..C..C..A..A..A..C.....C.C.....A.....T.G.
3509
PVXcp (pX61) TCAATGGCACAGTTAGGCAGAGTCCTGACTTTGATGAAAACAAAATCATGGTATTCCTTA
PVX-S .....G..T..G..A.....A.....C.....T.....C.
PVXX3 .....G..T..G..A.....C..A.....C.....G..T.....C.
pPVX19 .....G..T..G..A.....C..A.....T.....C.

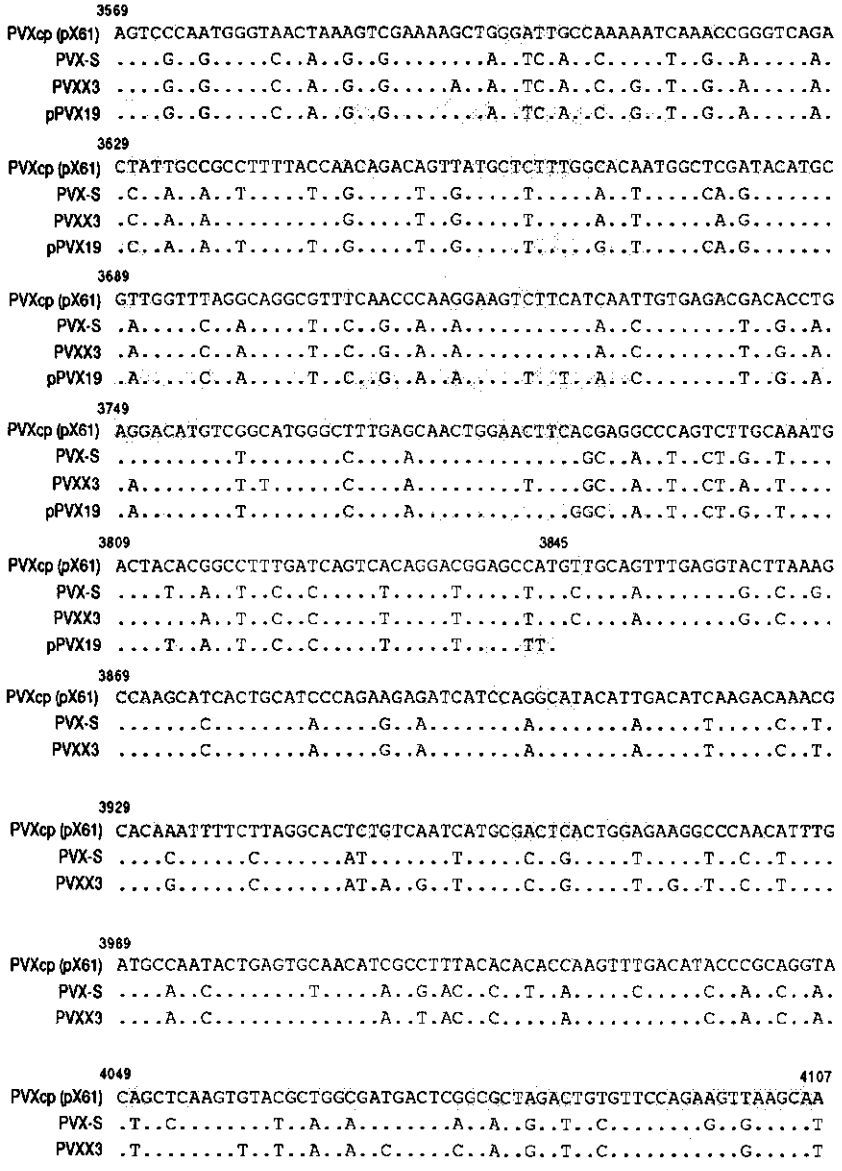


Fig. 4.2. Nucleotide sequence of the potato virus X (PVX) RNA genome covered by probes pX61 and pPVX19 (nucleotides 2909-4107). Upper line: nucleotide sequence of PVXcp according to Orman *et al.* (1990) and corresponding nucleotide sequence (3008-4107) for pX61 (dashed). Lower and botton lines show only the differences in the sequences reported for PVX_S and PVX_{X3}, by Skryabin *et al.* (1988) and Huisman *et al.* (1988), respectively, and in the corresponding nucleotide sequence (2909-3845) determined for pPVX19 (dashed).

Six experiments were conducted using leaf sap and the cp or HB PVX^A isolates as positive controls. The PVX^O isolates included in the tests (Tables 4.1 and 4.2) varied according to the experiment, but most were tested two to four times. All isolates were positive in the ELISA and indicated sufficient virus concentration in the leaf sap for the R-NASH and NR-NASH tests.

In R-NASH, the PVX^A isolates reacted more strongly than the PVX^O isolates with RNA transcribed from pX61. The cp isolate (homologous isolate) reacted more strongly than the HB (Fig. 4.3). The difference in detectability (detection limit) between them was not higher than two threefold dilution steps (TFDS). However, the difference between the HB isolate and the PVX^O isolates was at least one but usually 2-6 TFDS (Table 4.2). No difference was observed among serogroups I/II and IV from the PVX^O serotype. In addition to the differences observed in the detectability between isolates from PVX^A and PVX^O serotypes, another, more striking, difference was observed in the strength of the hybridization signal at lower dilutions, which was stronger for PVX^A isolates than for PVX^O isolates (Fig. 4.3).

Table 4.1. Identity of isolates used in this work other than those cited in Table 4.2.

Isolate	Geographic origin	Serotype (Serogroup)
73	Huanuco, Peru	X^O(I/II)
D-CP[a]	Peru	X^O(IV)
Luc 3-1	Huancayo, Peru	X^O(IV)
Luc 5-2	Huancayo, Peru	X^O(IV)
Luc 6-1	Huancayo, Peru	X^O(IV)
Luc 9	Huancayo, Peru	X^O(IV)
Luc 10	Huancayo, Peru	X^O(IV)
65	Huanuco, Peru	X^O(IV)
69	Huanuco, Peru	X^O(IV)
57CH	Chile	X^O(IV)
85CH	Chile	X^O(IV)
119CH	Chile	X^O(IV)
GUA2[b]	Guatemala	X^O(IV)

[a] Provided by C. Fribourg (Universidad Nacional Agraria, La Molina, Lima, Peru). [b] Provided by J. Abad (International Potato Center, Lima, Peru).

Table 4.2. Reaction of selected potato virus X (PVX) isolates in DAS-ELISA, and in NASH tests using RNA probe pX61 and DNA probes pPVX19 and pX61 prepared from a PVX^{O} and a PVX^{A} isolate.

Isolate	Geographic origin	Serotype (Serogroup)	DAS-ELISA[a]	R-NASH[b] pX61	NR-NASH[c] pPVX19	NR-NASH[c] pX61
Experiment A						
2	Cuzco, Peru	X^{O} (I/II)	> 10[d]	5	6	4
3	Cuzco, Peru	X^{O} (IV)	> 10	6	6	4
8	Cuzco, Peru	X^{O} (IV)	9	7	6	4
2Bo	Boqueron, Bolivia	X^{O} (IV)	> 10	6	3	4
3Bo	Boqueron, Bolivia	X^{O} (IV)	6	6	5	5
HB	Bolivia	X^{A}(III)	7	8	2	6
cp	Peru	X^{A}(III)	> 10	> 10	2	8
Healthy			0	0	0	0
Experiment B						
20	Cuzco, Peru	X^{O}(I/II)	8	5	1	3
46Bo	Huatajata, Bolivia	X^{O}(I/II)	10	4	1	4
73Bo	Cochabamba, Bol.	X^{O}(I/II)	9	5	1	4
14	Cuzco, Peru	X^{O} (IV)	8	4	4	3
59	Huanuco, Peru	X^{O} (IV)	6	4	4	2
38Bo	Chirapaca, Bol.	X^{O} (IV)	5	5	3	3
HB	Bolivia	X^{A} (III)	9	9	1	7
cp	Peru	X^{A} (III)	9	> 10	1	8
Healthy			0	0	0	0
Experiment C						
2	Cuzco, Peru	X^{O} (I/II)	8	9	8	0
27Bo	Chulchulcani,Bol	X^{O} (I/II)	> 10	5	1	1
20Bo	Montepunto, Bol.	X^{O} (I/II)	> 10	8	1	3
73Bo	Cochabamba, Bol.	X^{O} (I/II)	9	8	1	2
8	Cuzco, Peru	X^{O} (IV)	9	4	5	0
38Bo	Chirapaca, Bol.	X^{O} (IV)	> 10	9	4	1
HB	Bolivia	X^{A}(III)	> 10	10	1	3
cp	Peru	X^{A}(III)	> 10	> 10	1	9
Healthy			0	0	0	0

[a]Double-antibody sandwich form of ELISA. [b]Radioactive nucleic acid spot hybridization technique. [c]Nonradioactive nucleic acid spot hybridization technique. [d]Numbers assigned to reciprocal (x 1,000) of highest dilutions of sap extracted from *Nicotiana glutinosa* leaves healthy or infected with PVX isolates, at which a reaction was noted after visual observation: 1=0.08, 2=0.24, 3=0.73, 4=2.19, 5=6.56, 6=19.7, 7=59.1, 8=177, 9=531, 10=1,594.

Differences in the detectability of isolates from PVX^A and PVX^O serotypes were similar in NR-NASH using the DNA probe pX61 and in R-NASH using RNA pX61. However, the detectability of the homologous cp isolate and the PVX^O isolates in NR-NASH were approximately 1-3 and 1-9 TFDS lower, respectively, than in R-NASH (Fig. 4.3, Table 4.2). In reciprocal NR-NASH tests with DNA probe pPVX19, most PVX^O isolates (serogroups I/II and IV) reacted much more strongly than PVX^A isolates (Fig. 4.3, Table 4.2). Several PVX^O isolates, mostly from Bolivia, reacted as weakly as the PVX^A isolates (Table 4.2). These results were reconfirmed in R-NASH tests with DNA probe pPVX19 (Table 4.3).

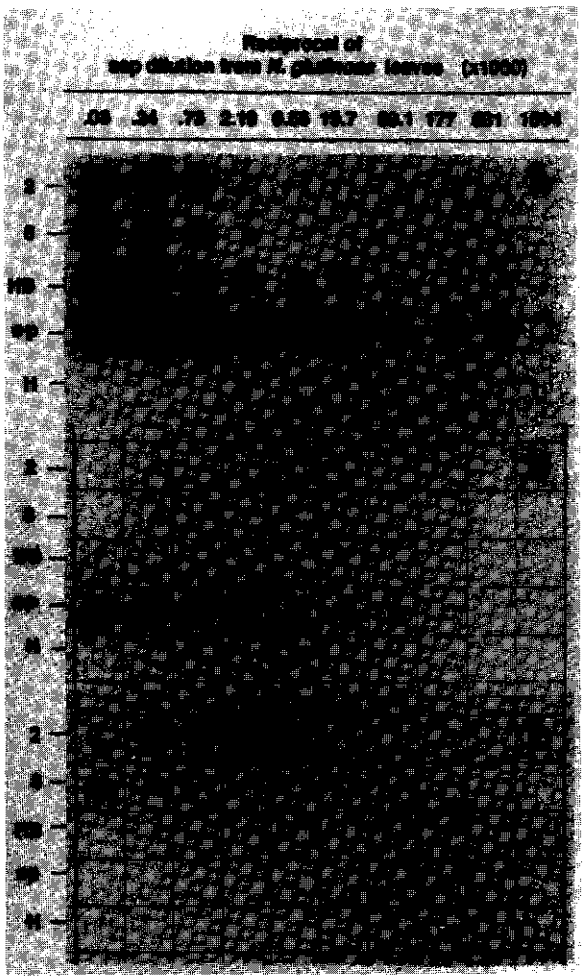


Fig. 4.3. Differential detection of potato virus X (PVX) serotypes by **A**, radioactive nucleic acid spot hybridization using RNA probe pX61, **B**, nonradioactive nucleic acid spot hybridization using DNA probes pX61, or **C**, pPVX19. Isolates 2 and 8 are PVX^O serotype (serogroups I/II and IV, respectively), and HB and cp are PVX^A serotype (serogroup III). H is healthy sap.

Table 4.3. Reaction of selected PVX isolates in radioactive nucleic acid spot hybridization tests using DNA probe pPVX19 and RNA probe pX61 prepared from a PVX^O and a PVX^A isolate, respectively.

Isolate	Serotype (Serogroup)	pPVX19	pX61
GUA2	X^O(IV)	9[a]	4
Luc 10	X^O(IV)	9	5
8	X^O(IV)	8	4
3	X^O(IV)	8	5
Luc 5-2	X^O(IV)	8	6
Luc 3-1	X^O(IV)	8	7
Luc 6-1	X^O(IV)	8	7
59	X^O(IV)	6	4
2	X^O (I/II)	7	5
3Bo	X^O (IV)	6	6
2Bo	X^O (IV)	3	6
29Bo	X^O (II)	3	7
27Bo	X^O (I/II)	1	6
46Bo	X^O (I/II)	1	5
73Bo	X^O (I/II)	1	8
20	X^O (II)	1	7
HB	X^A(III)	1	>9
cp	X^A(III)	1	>9
Healthy		0	0

[a]Numbers assigned to reciprocal (x 1,000) of highest dilution of sap extracted from *Nicotiana glutinosa* leaves healthy or infected with PVX isolates, at which a reaction was noted after visual observation: 1=0.08, 2=0.24, 3=0.73, 4= 2.19, 5=6.56, 6=19.7, 7=59.1, 8=177, 9=531.

Differences observed in the detectability of isolates from PVX^A and PVX^O serotypes were reconfirmed when equal concentrations of purified PVX RNA from selected isolates were tested in R-NASH with the DNA probes pX61 and pPVX19 (Fig. 4.4).

The RNA probe pX61 did not react in R-NASH with: 1) four representative isolates from the three groups of strains of PVY (PVY^O, PVY^N and PVY^C); 2) three isolates of Peru tomato virus-potato strain (potato virus V); 3) two isolates of potato virus A; 4) one isolate each of potato virus S, potato leafroll virus, potato virus M, Andean potato mottle

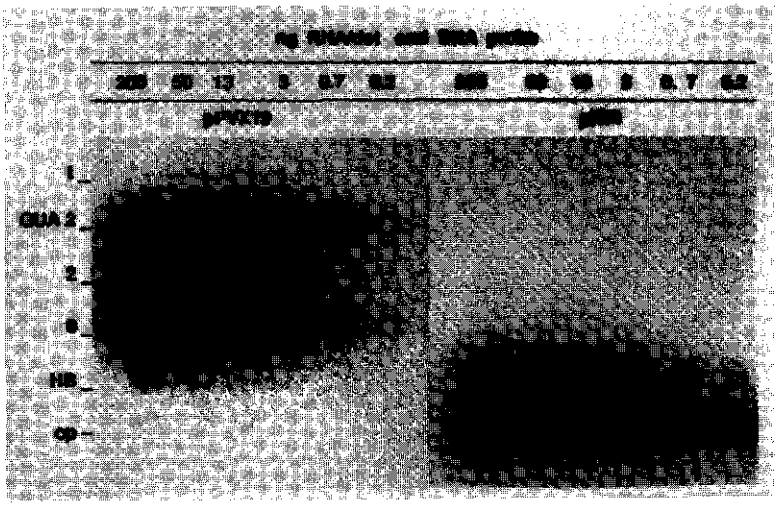


Fig. 4.4. Differential detection of potato virus X (PVX) serotypes PVX^{O} (isolates GUA2, 2, and 8) and PVX^{A} (isolates HB and cp) by radioactive nucleic acid spot hybridization tests using DNA probes pPVX19 and pX61. Samples are dilutions of purified RNA of PVX isolates. t= purified transfer RNA as control.

virus, alfalfa mosaic virus, SB-22, tobacco ringspot virus, cucumber mosaic virus, tobacco etch virus; and 5) three isolates of Andean potato latent virus (data not shown).

DISCUSSION

Results from DAS-ELISA indicated that there was sufficient virus present in leaf sap to detect differences among PVX^{A} and PVX^{O} isolates using each of the NASH variations. Because experiments were conducted at different times, some variability in virus concentration, and therefore in the reaction of a particular isolate, among experiments was expected.

The greater sensitivity of DAS-ELISA for detecting heterologous isolates may be due to an appropriate combination of the coating IgG (cp) and the detecting IgG (HB) enzyme conjugate. The combination of a cp coating IgG and a homologous cp conjugate does not properly detect a broad spectrum of PVX^{O} isolates by direct DAS-ELISA (Fernandez-Northcote and Lizárraga, 1991). Thus, the combination selected ensured high sensitivity to detect the broad spectrum of isolates used in this work.

When probe pX61 prepared from the PVX_{cp} strain was used, it was shown that cp and HB isolates from the Andean serotype (PVX^{A}) differ from other isolates grouped in the common serotype PVX^{O} from the Andean region and from elsewhere. This evidence

supports previous results obtained by using monoclonal antibodies (Torrance *et al.*, 1986; Lizárraga and Fernandez-Northcote, 1989; Fernandez-Northcote and Lizárraga, 1991) and DNA probes prepared from a British isolate (Baulcombe and Fernandez-Northcote, 1988). In this work, for R-NASH with the RNA probe pX61, the difference in detectability between cp and HB isolates (PVX^A), and isolates from PVX^O serotypes, was usually 2-6 TFDS, suggesting that low virus concentrations might not be detected in plants infected with Andean isolates from the PVX^O serotype. For routine detection of PVX, the chances of escaping detection would be greater using NR-NASH. Results in this work and elsewhere (Hopp *et al.*, 1988) show that this technique does not yet seem to be as sensitive as R-NASH. This lower sensitivity affected the reproducibility of results with NR-NASH in this work when, in some experiments, some isolate concentrations were not as high as in other experiments. Although improvements in labeling and signal detection for NR-NASH, such as the chemiluminescence system (Audy *et al.*, 1991; Kanematsu *et al.*, 1991), are under way, the utility of nonradioactive labeled probes for detecting PVX in leaf extracts remains to be determined.

Results of simultaneous tests with DNA probe pPVX19 were opposite to those obtained with DNA or RNA probe pX61 using the same samples. Most PVX^O isolates (from serogroups I/II and IV) reacted much more strongly in R-NASH or NR-NASH than the PVX^A isolates. The lower sensitivity of NR-NASH further lessens efficient detection. Several PVX^O isolates (serogroups I/II and IV), mostly from Bolivia, reacted as weakly as the PVX^A isolates. Probe pPVX19 cannot detect all the PVX^O isolates from the Andean region tested so far, indicating that there are divergent sequences in isolates within the PVX^O serotype. Probe pX61 detected these PVX^O isolates better than pPVX19, despite their differences in the coat protein gene from the PVX^A isolates. Therefore, the PVX^O isolates which have a closer geographic origin to PVX^A, have other areas in the nucleotide sequence that are more homologous with the PVX^A isolates than other PVX^O isolates not geographically related.

Although no European isolates from the PVX^O serotype were tested with the RNA probe pX61, it seems to detect isolates from PVX^O serotype better than the DNA probe pPVX19 detects isolates in the PVX^A serotype (Baulcombe and Fernandez-Northcote, 1988). Therefore, the RNA probe pX61 seems to detect a broader spectrum of PVX isolates.

Orman *et al.* (1990) demonstrated that the PVX_{cp} complete genomic sequence differs significantly from the ones reported for two European isolates, the X3 strain (Huisman *et*

al., 1988) and the Russian strain PVX_S (Skryabin *et al.*, 1988). Although the two European strains showed 97.1% nucleotide homology between them, they showed only about 77% overall homology with PVX_{cp}. From recent studies, it is believed that these two European isolates belong to the PVX^O serotype (Fernandez-Northcote and Lizárraga, 1991). The two probes used detected differences between PVX^O and PVX^A serotypes in the nucleotide sequence of the central part of the genomic RNA of PVX, towards the 3' end of the open reading frame (ORF) 1 that encodes for 166 kDa protein apparently involved in viral replication (Huisman *et al.*, 1988). The differences detected are not in areas of significant divergence between the nucleotide sequence of PVX_{cp} and the two European isolates. Those differences (Orman *et al.*, 1990) are clustered between nucleotides 1490 and 1928 in ORF 1; between 5650 and 5920 in ORF 5, which encodes the coat protein; and in the entire ORF 4, which encodes an 8 kDa polypeptide that might fulfill a function in viral spread (Huisman *et al.*, 1988). Probe pX61 and probe pPVX19, especially, detect differences in nucleotide sequences between PVX^O and PVX^A in a region of low divergence. The two probes hybridize within a region that is relatively conserved but does show 22% nucleotide changes. pX61 hybridizes in the putative region of the polymerase sequence (3968-4073), which is expected to be a conserved region. In comparison, pX61 covers a larger region (262 nucleotides) of lower divergence than pPVX19 (99 nucleotides), probably allowing hybridization with isolates of the heterologous serotype. This explains the ability of the pX61 to detect a broader spectrum of PVX isolates than the pPVX19. It is expected that the divergence among some PVX^O isolates from the Andean region and PVX^A is still lower than among PVX^O isolates from elsewhere and PVX^A.

In our R-NASH tests, the hybridization signal of the heterologous serotype with the DNA probes using purified RNA was weaker than with the RNA probes using leaf sap. This enhanced the differences between PVX^A and PVX^O isolates. Several factors could be involved: higher concentration of viral RNA, coat protein binding of PVX RNA to the filter due to using leaf sap, degradation of PVX RNA during the purification process, or better reactivity of the RNA probe for the heterologous serotype.

Hopp *et al.* (1988) prepared a 500 bp DNA probe (pX1) from the PVX_{cp} isolate. Apparently, this probe was not tested against a broad spectrum of PVX isolates, but it could be more specific than pX61 because of its smaller size. They also found R-NASH to be more sensitive than the NR-NASH, but reported similar sensitivities for NR-NASH and ELISA. In our work, the sensitivities of DAS-ELISA, R-NASH and NR-NASH were

similar when using the homologous PVX_{cp} isolate, but the sensitivity of ELISA was higher with heterologous isolates from the Andean region.

The differences among PVX^{A} and PVX^{O} isolates confirmed in this report stress the importance of using the appropriate antibodies or probes in detection techniques, especially for breeding programs for resistance and quarantine purposes. Probe pX61 will be very valuable for the quarantine detection of the PVX_{HB} pathotype (Fernandez-Northcote, 1990) from the PVX^{A} serotype that breaks the immunity to PVX in potato (Moreira *et al.*, 1980). This pathotype is found only in Bolivia at the present time (Fernandez-Northcote and Lizárraga, 1991).

RESUMEN

El probador de ADNc pX61 preparado usando el strain PVX_{cp}, se utilizó contra un espectro amplio de aislamientos andinos de PVX agrupados en dos serotipos, el andino PVX^{A} detectado únicamente en el Perú y Bolivia y que incluye a los strains cp y HB y el común PVX^{O} que incluye aislamientos serológicamente similares a los que ocurren en otras partes del mundo. En pruebas de hibridación local de ácidos nucleicos modalidad radioactiva (R-NASH) con virus en savia de hojas y con el probador de ARN pX61 marcado con ^{32}P, se obtuvo una señal de hibridación más fuerte y una detectabilidad generalmente de dos a seis pasos más altos en diluciones de la savia 1:3, en el caso de los aislamientos PVX^{A} que en el de los aislamientos PVX^{O}. La diferencia en la detectabilidad entre los serotipos PVX^{A} y PVX^{O}, fue similar en pruebas de hibridación local de ácidos nucleicos modalidad no-radioactiva (NR-NASH) usando el probador de ADN pX61 biotinilado, sin embargo la sensibilidad fue menor en NR-NASH que en R-NASH. Cuando se comparó en NR-NASH probadores de ADN biotinilados de pX61, y de pPVX19 preparado usando un aislamiento británico de PVX del grupo de strains-3 común en Europa, la mayoría de los aislamientos del serotipo PVX^{O} reaccionaron mas fuertemente que los del serotipo PVX^{A} con el probador pPVX19. Varios aislamientos PVX^{O} generalmente provenientes de Bolivia también reaccionaron débilmente con el probador pPVX19. La concentración apropiada de virus para las pruebas de R-NASH y NR-NASH con savia de hojas se chequeó por DAS-ELISA confirmando que las diferencias obtenidas no dependian de diferencias en la concentración de virus. Estos resultados se confirmaron

en R-NASH con los probadores de ADN pX61 y pPVX19 utilizando concentraciones definidas de ARN purificado de aislamientos selectos.

Las diferencias encontradas entre los aislamientos PVX^A y PVX^O enfatizan la importancia del uso de probadores apropiados para la detección de PVX en programas de mejoramiento para resistencia y propósitos cuarentenarios.

REFERENCES

Audy, P., Parent, J-G., and Asselin, A. (1991). A note on four nonradioactive labeling systems for dot hybridization detection of potato viruses. *Phytoprotection* **72**: 81-86.

Baulcombe, D.C., Boulton, R.E., Flavell, R.B., and Jellis, G.J. (1984a). Recombinant DNA probes for detection of viruses in plants. *Proceedings of the 1984 British Crop Protection Conference*, pp. 207-213. British Crop Protection Council, Thornton Heath, Surrey.

Baulcombe, D.C., and Fernandez-Northcote, E.N. (1988). Detection of strains of potato virus X and of a broad spectrum of potato virus Y isolates by nucleic acid spot hybridization (NASH). *Plant Disease* **72**: 307-309.

Baulcome, D.C., Flavell, R.B., Boulton, R.E., and Jellis, G.J. (1984b). The sensitivity and specificity of a rapid nucleic acid hybridization method for the detection of potato virus X in crude sap samples. *Plant Pathology* **33**: 361-370.

Clark, M.F., and Adams, A.N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* **34**: 475-483.

Eweida, M.; Sit, T.L., and AbouHaidar, M.G. (1989). Molecular cloning of the genome of the carlavirus potato virus S: biotinylated RNA transcripts for virus detection in crude potato extracts. *Annals of Applied Biology* **115**: 253-261.

Fernandez-Northcote, E.N. (1990). Variability of PVX and PVY and its relationship to genetic resistance. In *Control of virus and virus-like diseases of potato and sweet potato. Report of the III planning conference*, pp. 131-139. International Potato Center, Lima, 1989.

Fernandez-Northcote, E.N., and Lizárraga, C. (1991). Distribución geográfica de serotipos del virus X de la papa. *Fitopatología* **26**: 13-18.

Hammond, J., and Lawson, R.H. (1988). An improved purification procedure for preparing potyviruses and cytoplasmic inclusions from the same tissue. *Journal of Virological Methods* **20**: 203-217.

Hellmann, G.M., Shahabuddin, M., Shaw, J.G., and Rhoads, R.E. (1983). Molecular cloning of DNA complementary to tobacco vein mottling virus RNA. *Virology* **128**: 210-220.

Hopp, H.E., Giavedoni, L., Mandel, M.A., Arese, A., Orman, B., Bravo Almonacid, F., Torres, H.N., and Mentaberry, A.N. (1988). Biotinylated nucleic acid hybridization probes for potato virus detection. *Archives of Virology* **103**: 231-241.

Huisman, M.J., Linthorst, H.J.M., Bol, J.F., and Cornelissen, B.J.C. (1988). The complete nucleotide sequence of potato virus X and its homologies at the amino acid level with various plus-stranded RNA viruses. *Journal of General Virology* **69**: 1789-1798.

Kanematsu, S., Hibi, T., Hashimoto, J., and Tsuchizaki, T. (1991). Comparison of nonradioactive cDNA probes for detection of potato spindle tuber viroid by dot-blot hybridization assay. *Journal of Virological Methods* **35**: 189-197.

Lizárraga, C., and Fernandez-Northcote, E.N. (1989). Detection of potato viruses X and Y in sap extracts by a modified indirect enzyme-linked immunosorbent assay on nitrocellulose membranes (NCM-ELISA). *Plant Disease* **73**: 11-14.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 545 pp.

Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K., and Green, M.R. (1984). Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Research* **12**: 7035-7056.

Moreira, A., Jones, R.A.C., and Fribourg, C.E. (1980). Properties of a resistance-breaking strain of potato virus X. *Annals of Applied Biology* **95**: 93-103.

Orman, B.E., Celnik, R.M., Mandel, A.M., Torres, H.N., and Mentaberry, A.N. (1990). Complete cDNA sequence of a South American isolate of potato virus X. *Virus Research* **16**: 293-306.

Owens, R.A., and Diener, T.O. (1981). Sensitive and rapid diagnosis of potato spindle tuber viroid disease by nucleic acid hybridization. *Science* **213**: 670-672.

Salazar, L.F., Balbo, I., and Owens, R.A. (1988). Comparison of four radioactive probes for the diagnosis of potato spindle tuber viroid by nucleic acid spot hybridization. *Potato Research* **31**: 431-442.

Salazar, L.F., Owens, R.A., Smith, D.R., and Diener, T.O. (1983). Detection of potato spindle tuber viroid by nucleic acid spot hybridization: evaluation with tuber sprouts and true potato seed. *American Potato Journal* **60**: 587-597.

Salazar, L.F., and Querci, M. (1992). Detection of viroids and viruses by nucleic acid probes. In Duncan J.M. and Torrance L. (eds) *Techniques for the rapid detection of plant pathogens*, pp. 129-144. Blackwell Scientific Publications, Cambridge.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Science USA* **74**: 5463-5467.

Skryabin, K.G., Kraev, A.S., Morozov, S.Yu., Rozanov, M.N., Chernov, B.K., Lukasheva, L.I., and Atabekov, J.G. (1988). The nucleotide sequence of potato virus X RNA. *Nucleic Acids Research* **16**: 10929-10930.

Torrance, L., Larkins, A.P., and Butcher, G.W. (1986). Characterization of monoclonal antibodies against potato virus X and comparison of serotypes with resistance groups. *Journal of General Virology* **67**: 57-67.

Chapter 5

Detection of PVX^{A} and PVX^{O} serotypes by nucleic acid spot hybridization using a broad spectrum recombinant probe

SUMMARY

A broad spectrum of PVX isolates grouped into two serotypes, the Andean PVX^{A} and the common PVX^{O}, was evaluated by nucleic acid spot hybridization. Two cDNA probes, pX61 prepared from PVX$_{cp}$ (serotype PVX^{A}), and pPVX19 from a British PVX isolate of the common European strain-group 3 (serotype PVX^{O}), showed high specificity in detecting isolates belonging to the serotype from which they were prepared and some closely related isolates. A new probe, pX6119, obtained by coupling both cDNAs, allowed the detection of isolates belonging to both serotypes PVX^{A} and PVX^{O}. This probe should be of particular interest for the detection of PVX in breeding programs, for resistance and quarantine purposes.

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INTRODUCTION

The importance of appropriate selection of the technique, antibodies and probes for the detection of viruses, in breeding programs for resistance to viruses, seed production and quarantine purposes, has been demonstrated in previous work for the detection of several strains and isolates of potato virus X (Querci *et al.*, 1993).

Potato virus X (PVX) is one of the most common viruses infecting potato (Schultz and Bonde, 1944) occurring worldwide in potato-growing areas, and therefore a component of potato breeding and quarantine programs. Foliage symptoms on potato are non-reliable indicators of infection by PVX, as they may vary considerably with the cultivar, virus strain, and climate conditions. As a consequence, infected cultivars may be symptomless or develop a mild mottling of the leaves, interveinal or mild mosaic. Some cultivars develop a top necrosis and usually die following infection with PVX (Rich, 1977).

Isolates of PVX have been characterized in groups of strains on the basis of serological cross-absorption tests (Matthews, 1949), symptoms induced on tobacco plants (Ladeburg *et al.*, 1950), thermal inactivation points (Köhler, 1962), and on the basis of their infectivity on different potato genotypes (Cockerham, 1955). The existence of great serological variability between strains (Torrance *et al*, 1986) and isolates (Fernandez-Northcote and Lizárraga, 1991) of PVX has been previously reported.

Nucleic acid spot hybridization (NASH), detecting the viral genome, may be considered a useful alternative to serological methods based on the immunological detection of the viral coat protein. The technique has the possible advantage of higher sensitivity, with a degree of specificity depending on the hybridization conditions and on the selection of the sequence used as a probe. For example, in the case of quarantine or eradication programs, it is possible to design a broad spectrum probe, while for epidemiologic studies a narrow spectrum probe could be developed (Boulton *et al.*, 1986). Several procedures have already been reported to detect plant viruses by molecular probes (Baulcombe *et al.*, 1984a,b; Baulcombe and Fernandez-Northcote, 1988; Eweida *et al.*, 1990; Querci *et al.*, 1993), and the possibility of the simultaneous detection of different viruses using a mixture of recombinant probes has been investigated (Hopp *et al.*, 1991; Querci *et al.*, unpublished results).

In this work we report on the evaluation by nucleic acid spot hybridization (NASH) of a broad selection of PVX isolates from South America grouped into the two serotypes, the

Andean PVX^{A}, which includes the cp and HB strains, and the common PVX^{O}, which groups isolates serologically similar to those occurring elsewhere in the world. Two cDNA probes, pX61 prepared from PVX_{cp} (serotype PVX^{A}), and pPVX19 from a British isolate of the common European strain-group 3 (serotype PVX^{O}) were used.
Since in previous work (Querci *et al.*, 1993) both probes showed high specificity in detecting isolates belonging to the serotype from which they were prepared and some closely related isolates, a chimaeric recombinant probe pX6119 containing both pX61 and pPVX19 cDNA sequences was constructed as to attempt to overcome such specificity.

MATERIALS AND METHODS

Probes pX61 and pPVX19

The construction of probe pX61, prepared from the South American strain PVX_{cp} (Fribourg, 1975; Orman *et al.*, 1990) has been described previously (Querci *et al.*, 1993). Clone pX61 is a 1100 bp cDNA insert corresponding to the region between nucleotides 3008 and 4107 of the genomic PVX_{cp} RNA, inserted into the *Hind*III site of plasmid pSP64. Probe pPVX19 (Baulcombe and Fernandez-Northcote, 1988), prepared from a British PVX isolate of the common European strain-group 3, was kindly donated by D.C. Baulcombe; the cDNA is a 937 bp fragment (nucleotides 2909-3845) inserted into the *Pst*I site of plasmid pBR322 (Querci *et al.*, 1993).

Preparation of probe pX6119

Plasmid pPVX19 was digested with restriction endonuclease *Pst*I using conditions recommended by the supplier (BRL), then was run in 1% low melting point agarose gel in 1x TAE (Tris-acetate/EDTA electrophoresis buffer). The pPVX19 cDNA insert was purified from the gel band containing the DNA of interest and by several extractions with equal volumes of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA ($T_{10}E_{1}$) saturated phenol/ chloroform (1:1), and finally with an equal volume of chloroform. The cDNA insert present in the supernatant was then precipitated in 0.3 M sodium acetate pH 5.2 and two volumes cold ethanol. The pPVX19 cDNA resuspended in sterile distilled water was subcloned (Sambrook *et al.*, 1989) into pX61 plasmid previoulsy linearized with *Pst*I.

Chimaeric recombinant plasmids were introduced into *Escherichia coli* strain JM83, furtherly screened by ampicillin resistance. Small scale plasmid purifications were performed following standard procedure (Sambrook *et al.*, 1989), the recombinant plasmids were analyzed by agarose gel electrophoresis and screened according to size. The presence of the two cDNA inserts in the same plasmid was confirmed by double restriction endonuclease digestion using *Hind*III and *Pst*I, followed by gel electrophoresis and Southern hybridization (Sambrook *et al.*, 1989).

Crude sap sample preparation

All the isolates and strains of PVX used in the experiments (Table 5.1) were mantained in *Nicotiana glutinosa* L. and mechanically inoculated onto young potato plants three to four weeks before performing the test. Crude leaf sap from healthy and infected potato plants was extracted and microfuged at 11,000 rpm x 5 min. About 0.3 g of fresh leaves were used for each sample. Ten-fold dilutions of the supernatant were prepared in 10x SSC (1.5 M NaCl, 0.15 M trisodium citrate, pH 7.0). Three μl of each sample were spotted onto nitrocellulose membranes previously equilibrated in 20x SSC and air dried. Spotted membranes were then baked under vacuum at 80°C for 2 h.

Virus purification and viral RNA extraction

Frozen *N. glutinosa* infected leaves were homogenized in 0.1 M phosphate buffer pH 8.0 containing 0.2% 2-mercaptoethanol and 10% ethanol. The homogenate was treated with 1% Triton X-100 at 4°C for 1 h, precipitated with 0.2 M NaCl, 4% PEG 6,000-8,000, for 1 h at room temperature, and centrifuged at 7840 *g*. The pellets were resuspended in 0.05 M phosphate buffer, pH 8.0, and subjected to centrifugation through a 30% sucrose cushion (6ml/tube) at 69,231 *g* x 150 min, followed by centrifugation in linear sucrose density gradient (10-40%). Fractions containing virions were further concentrated by ultracentrifugation and finally resuspended in $T_{10}E_1$ buffer. Viral RNA was extracted by incubating 0.2-0.5 mg/ml of purified virus in $T_{10}E_1$ for 30 min at 37°C with 50 ug/ml proteinase K in the presence of 0.1% sodium dodecyl sulphate (SDS) and by following extraction with an equal volume of $T_{10}E_1$ buffer-saturated phenol, then again with an equal volume of phenol/chloroform (1:1) and once with chloroform (Hammond and Lawson, 1988). The RNA was then precipitated in 0.3 M sodium acetate, pH 5.2, and 2.5 volumes cold ethanol, collected by centrifugation, and resuspended in sterile $T_{10}E_1$. The RNA

concentration was determined spectrophotometrically, RNA was diluted in $T_{10}E_1$ to obtain the final concentrations used in the experiments, as indicated in Fig. 5.3.

Labeling of ^{32}P-DNA probes

^{32}P-labeled DNA probes were obtained by nick-translation using α^{32}P-dCTP (650 Ci/mmol) and a commercial nick-translation kit (BRL), according to the procedure indicated by the supplier.

Hybridization conditions

Hybridization was carried out overnight at 45°C (unless otherwise indicated) using about 1x 10^6 cpm/ml of labeled probe in hybridization solution (50% deionized formamide, 5x SSC, 50 mM sodium phosphate, pH 6.5, 2.5 mM EDTA, 0.6% SDS, 5x Denhardt's reagent, 120 μg/ml herring sperm DNA and 5% dextran sulphate), according to Salazar and Querci (1992). Membranes were washed as previously described (Salazar and Querci, 1992) and air dried. Results were visualized by autoradiography performed overnight at -70°C using Kodak X-OMAT AR films with intensifying screen.

RESULTS AND DISCUSSION

The possibility of simultaneous detection of different viruses using a mixture of recombinant probes has been previously investigated for PVX/PVY, PVX/PLRV, and PVX/PSTVd (Hopp *et al.*, 1991; Querci *et al.*, unpublished results), and the results revealed no interference in the detection. The advantage of handling a single chimaeric plasmid containing the cDNAs of the viruses to be detected was also investigated. Comparison between hybridizations carried out by using the chimaeric recombinant probes and by mixing the single, individually labeled probes, was performed using the same amount of incorporated radioactivity (1x 10^6 cpm/ml) in each experiment. The sensitivity obtained with the chimaeric recombinant probe (cDNAs of the two viruses in the same plasmid) was higher than the one obtained by mixing the specific probes after individual labeling. With the mixture of specific probes, a similar sensitivity was obtained only by increasing the total amount of radioactivity or by extending the time of autoradiography. Indeed, the presence of two cDNA sequences in the same plasmid increases the ratio

between viral and plasmid sequences allowing the use in hybridization of a higher amount of virus specific labeled sequences.

In this work twenty six PVX isolates and strains from South America (Table 5.1) and belonging to the PVX^A and PVX^O serotypes, were tested by nucleic acid spot hybridization (NASH). Dilutions of leaf sap from potato plants infected with each PVX isolate were spotted on nitrocellulose membranes and hybridized with the chimaeric probe pX6119 (Fig 5.1) and with probes pX61 and pPVX19 separately (Fig. 5.2). For routine detection of PVX a dilution of less than 10^{-1} is generally used. In the experiments presented in this work, as shown in Figs. 5.1 and 5.2, a sap dilution of 10^{-2} and 10^{-3} was used. Indeed, more striking differences in the strength of the hybridization signal are observed with lower virus concentration.

The chimaeric recombinant probe pX6119, containing both the pX61 and the pPVX19 cDNA sequences, allowed the detection by NASH of all isolates and strains tested (Fig. 5.1). Results obtained with the individual probes confirmed a previous report (Querci *et al.*, 1993) in which PVX^A and PVX^O were specifically detected by pX61 and pPVX19, respectively. However, as shown in Fig 5.2, several PVX^O isolates, mostly from Bolivia, reacted with probe pPVX19 as weakly as the PVX^A isolates do and were more readily detected by probe pX61.

Several experiments confirmed, even at low stringency conditions, the strong specificity of the two probes used, pX61 and pPVX19. In order to ensure elimination of variability in the detection due to possible different virus concentration in sap from plants inoculated with the different isolates, different ratios of encapsidated/unencapsidated RNA, or due to different ability of the virus isolates to adhere to the nitrocellulose membrane, hybridization experiments were performed using predetermined amounts of purified viral RNA (Fig. 5.3). The results confirmed previous reports (Baulcombe and Fernandez-Northcote, 1988; Querci *et al.*, 1993) of a high specificity of the individual probes. Such specificity depended only on the choice of the sequences used as probes, i.e. probes able to detect isolates belonging to the serotype from which they were prepared or isolates closely related.

Probe pX61 detects strain cp (homologous strain, PVX^A) and HB (also PVX^A serotype) with a strong signal, being higher the reaction with PVXcp, but only very poorly isolates 8, 2 and GUA 2 (belonging to serotype PVX^O). On the contrary, probe pPVX19 detects the isolates belonging to serotype PVX^O strongly, and poorly cp and HB. The lower sensitivity

Table 5.1. Origin, serotype and serogroup of the isolates in Figs. 5.1 and 5.2. Serogroups are according to Torrance *et al.* (1986), and serotypes by NCM-ELISA with monoclonal antibodies MA 58, MA 59 and MA 67 (Fernandez-Northcote and Lizárraga, 1991).

Isolate	Geographic origin	Serotype	Serogroup
14	Andahuaylilla, Cusco, Peru	X^0	IV
8	Urcos, Cusco, Peru	X^0	IV
Luc 6-1	Huancayo, Peru	X^0	IV
3	Oropesa, Cusco, Peru	X^0	IV
73Bo	Morochata, Cochabamba, Bolivia	X^0	I/II
46Bo	Huatajata, L. Titicaca Plateau, Bolivia	X^0	I/II
35Bo	Chirapaca, L. Titicaca Plateau, Bolivia	X^0	IV
27Bo	Chullchuncani, Bolivia	X^0	I/II
20	Oropesa, Cusco, Peru	X^0	I/II
2	Cusco, Peru	X^0	I/II
119CH	Temuco, Chile	X^0	IV
85CH	Chiloe, Chile	X^0	IV
57CH	Riofrio, Osorno, Chile	X^0	IV
49CH	Chile	X^0	IV
3Bo	Boqueron Kasa, Toralapa, Bolivia	X^0	IV
Luc 9	Huancayo, Peru	X^0	IV
D-CF[a]	Peru	X^0	IV
69	Moyobamba, Huanuco, Peru	X^0	IV
65	Moyobamba, Huanuco, Peru	X^0	IV
59	Moyobamba, Huanuco, Peru	X^0	IV
cp[a]	South Central Andes, Peru	X^A	III
HB[a]	Puna, Potosi, Bolivia	X^A	III
GUA 2[b]	Guatemala	X^0	IV
1-U	Uruguay	X^0	IV
2Co	Colombia	X^0	IV
1Co	Colombia	X^0	IV

[a,b] Kindly donated by [a] C. Fribourg (Universidad Nacional Agraria La Molina, Lima, Peru), and [b] J. Abad (International Potato Center, Lima, Peru).

showed by pX61 in this work, contrasts with a higher sensitivity in the detection of isolates 8, 2, and GUA 2 shown in a previous report (Querci *et al.*, 1993), this is probably due to the use in this work of a pX61 DNA probe instead of the pX61 RNA probe used previously.

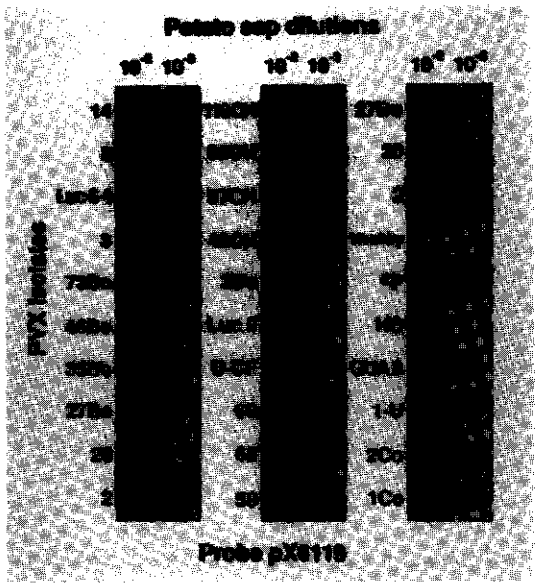

Fig. 5.1. Detection of a broad spectrum of potato virus X isolates by NASH using the chimaeric recombinant probe pX6119.

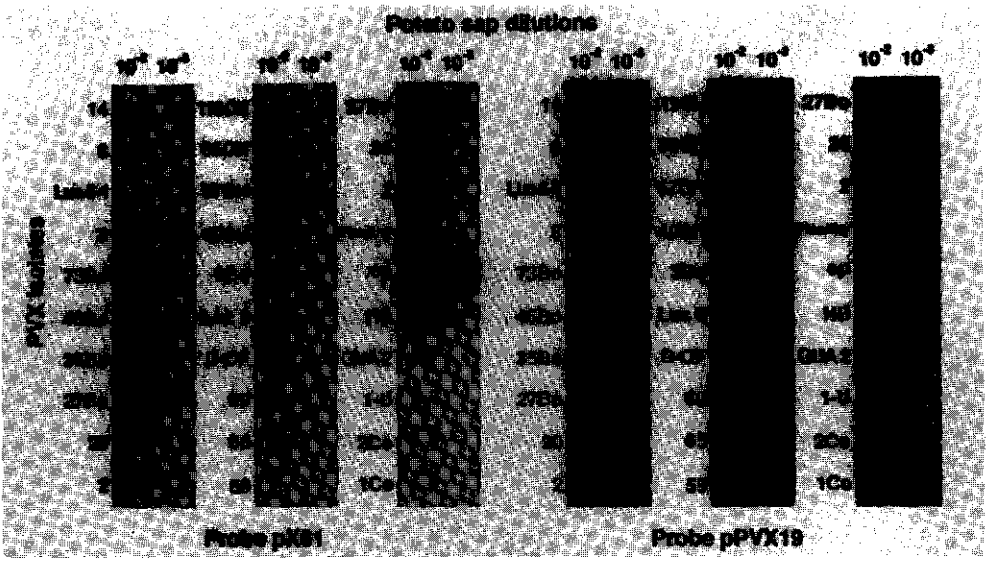

Fig. 5.2. Specific detection of potato virus X isolates by NASH using probes pX61 (from PVX_{cp}, serotype PVX^{A}) and pPVX19 (from a British PVX isolate of the common European strain-group 3, serotype PVX^{O}). Specificity of the probes is shown in the ability of each probe to detect PVX isolates belonging to the serotype group of the strain used for their preparation, and closely related isolates.

NASH may be considered a useful alternative to the serological methods for virus detection. The technique shows the possibility of higher sensitivity and a degree of specificity which depends on the choice of the sequence used as a probe and on the experimental conditions. As shown above and in Fig. 5.4, the hybridization temperature or the stringency conditions in the washing steps affect sensitivity, and therefore enhance or decrease specificity.

Orman *et al.* (1990) reported that the RNA sequence of PVX_{cp} differs significantly from the ones obtained for two European strains, PVX_{X3} (Huisman *et al.*, 1988), and PVX_S (Skryabin *et al.*, 1988). Although the two European strains showed 97.1% nucleotide homology between them, they showed only about 77% overall homology with PVX_{cp}. Specifically, main divergences between the PVX_{cp} isolate and the two European strains, are clustered between nucleotides 1490 and 1928 in the open reading frame 1 (ORF 1); between 5650 and 5920 in ORF 5, which encodes the coat protein; and in the entire ORF 4, which encodes an 8 kDa polypeptide that might fulfill a function in viral spread (Huisman *et al.*, 1988).

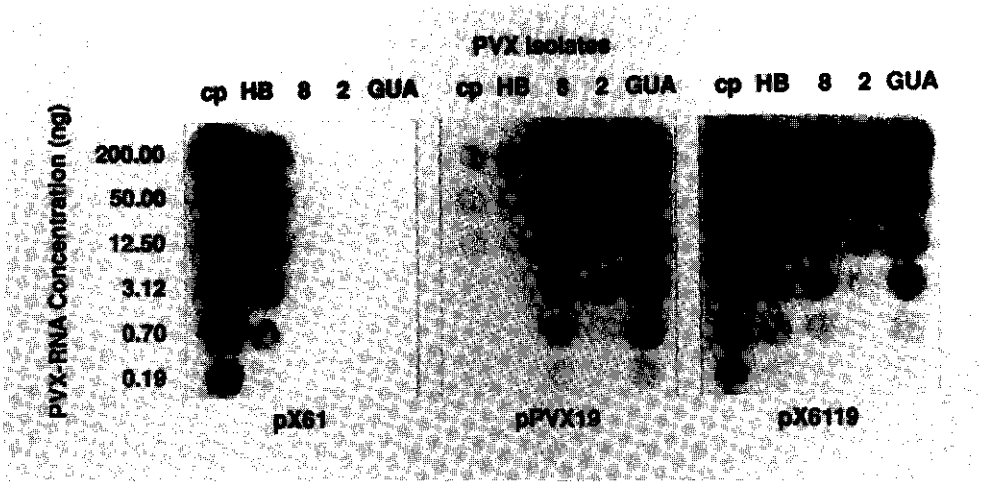

Fig. 5.3. Specific detection of PVX isolates cp and HB from the PVX^A serotype and isolates 8, 2 and GUA (previously called also GUA 2) from the PVX^O serotype in spots containing purified viral RNA at different known concentrations.

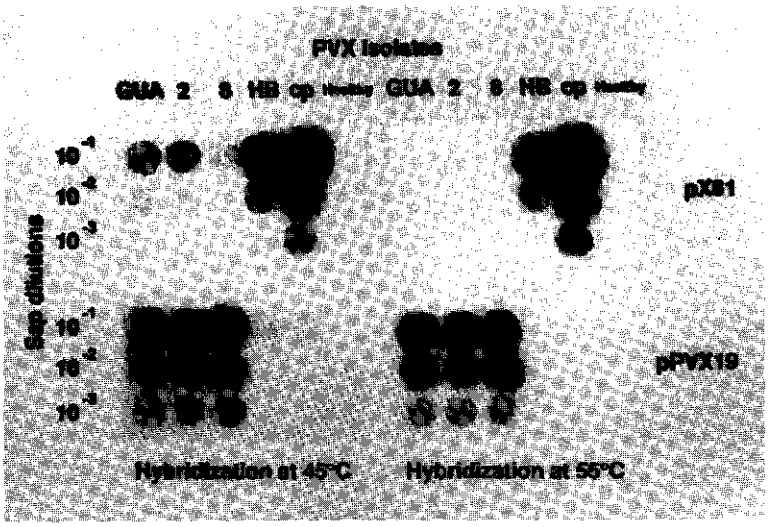

Fig. 5.4. Enhancing or decreasing specific detection by NASH of PVX isolates cp and HB from the PVX^{A} serotype, and isolates 8, 2, and GUA (previously called GUA 2) from the PVX^{O} serotype, in potato leaf sap by hybridization at 45°C or 55°C.

Probe pX61 was reported (Querci *et al.*, 1993) to correspond to the region between nucleotides 3008 and 4107 (1100 bp) of the genomic PVX_{cp} RNA. Probe pPVX19 corresponds to the region between nucleotides 2909 and 3845 in the PVX genome. As shown, the two probes contain a large portion of overlap. Probe pPVX19 overlaps with 76% of pX61 and probe pX61 with 89% of pPVX19 (Fig. 5.5). More importantly, both probes are derived from a highly conserved region in the 3'end of the PVX ORF1, and share a overall homology of about 78%.

In spite of having used two probes from a highly conserved region, a high specificity in the detection was observed, as shown in the ability of pX61 and pPVX19 to detect isolates belonging to the serotype from which they were prepared or closely related isolates. The results suggest the existence of variability in the nucleotide sequence among the different PVX isolates or strains tested, even belonging to the same serotype and/or serogroup.

The potato originated in the Andean region of South America and was later introduced to Europe and other countries; hence the idea that potato viruses, like PVX, were also spread from this region to other areas of potato production and that the divergences observed among the different virus strains and isolates could reflect adaptation to different *Solanum* varieties.

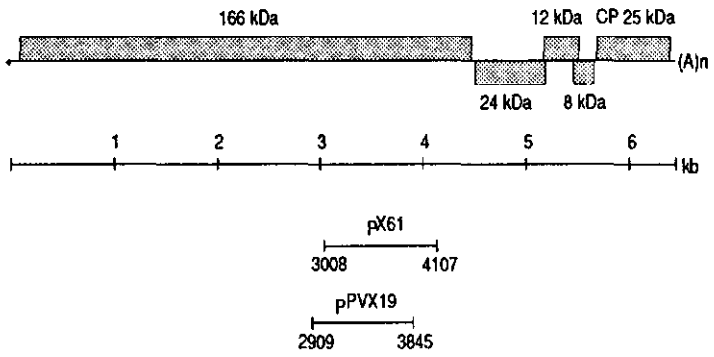

Fig. 5.5. Genomic organization of the PVX RNA and coordinates of the cDNA probes pX61 and pPVX19 (Querci *et al.*, 1993). Shaded boxes represent Open Reading Frames (ORF). Both probes originate from the highly conserved 3' end region of ORF1.

In the case of PVX several studies have been conducted on variability and distribution of the different strains and consequent ability of the techniques currenly used to detect all variants. Cockerham (1955) classified PVX strains into four groups on the basis of their reaction with the dominant resistance genes Nx, Nb and Rx. Torrance *et al.* (1986) found a large degree of serological variation among PVX strains (which were therefore divided into 4 main serological groups or "serogroups") and concluded that serological reactions do not always correlate with the specific classification based on resistance genes. In addition, only a very limited amount of the information contained in the viral genome is involved in the antigenicity of its coat protein (Hull, 1986). Further, differences found in the coat protein region (which determines serological variability or similarity between strains) do not necessary correspond to similar divergences in other regions of the viral genome.

The differences observed among PVX^{A} and PVX^{O} isolates stress the importance of using the appropriate technique for detection, especially for breeding programs for resistance, and quarantine purposes. Since CIP's regional activities require the routine detection of viruses from different countries, sensitive, standardized and highly reproducible procedures are essential; the studies presented in this work are a further step to improve the detection of PVX.

RESUMEN

Un espectro amplio de aislamientos de PVX agrupados en dos serotipos, el andino, PVX^A, y el común, PVX^O, han sido evaluados por hibridación de ácidos nucleicos.

Las sondas de ADNc, pX61 preparada del strain PVX_{cp} (serotipo PVX^A), y la pPVX19 (serotipo PVX^O) son altamente específicas para la detección de aislamientos pertenecientes al serotipo del cual fueron preparadas y algunos aislamientos cercanamente relaccionados. Una nueva sonda, pX6119, formada por el ligamento de los dos ADNc anteriores, permitió detectar aislamientos de ambos serotipos PVX^A y PVX^O.

Esta sonda permitira la detección apropriada de PVX en programas de mejoramiento para resistencia y para propósitos cuarentenarios.

REFERENCES

Baulcombe, D.C., Boulton, R.E., Flavell, R.B., and Jellis, G.J. (1984a). Recombinant DNA probes for detection of viruses in plants. *Proceedings of the 1984 British Crop Protection Conference*, pp. 207-213. British Crop Protection Council, Thornton Heath, Surrey.

Baulcombe, D.C., Flavell, R.B., Boulton, R.E., and Jellis, G.J. (1984b). The sensitivity and specificity of a rapid nucleic acid hybridization method for the detection of potato virus X in crude sap samples. *Plant Pathology* **33**: 361-370.

Baulcombe, D.C., and Fernandez-Northcote, E.N. (1988). Detection of strains of potato virus X and of a broad spectrum of potato virus Y isolates by nucleic acid spot hybridization (NASH). *Plant Disease* **72**: 307-309.

Boulton, R.E., Jellis, G.J., Baulcombe, D.C., and Squire, A.M. (1986). The application of complementary DNA probes to routine virus detection with particular reference to potato viruses. In Jones R.A.C. and Torrance L. (eds) *Developments and Applications in Virus Testing*, pp. 41-53. Association of Applied Biologists, Wellesbourne.

Cockerham, G. (1955). Strains of potato virus X. *Proceedings of the Second Conference on Potato Virus Diseases*, pp.89-92. Lisse-Wageningen, 1954.

Eweida, M., Xu, H., Singh, R.P., and AbouHaidar, M.G. (1990). Comparison between ELISA and biotin-labelled probes from cloned cDNA of potato virus X for the detection of virus in crude tuber extracts. *Plant Pathology* **39**: 623-628.

Fernandez-Northcote, E.N., and Lizárraga, C. (1991). Distribución geográfica de serotipos del virus X de la papa. *Fitopatologia* **26**: 13-18.

Fribourg, C.E. (1975). Studies on potato virus X strains isolated from Peruvian potatoes. *Potato Research* **18**: 216-226.

Hammond, J., and Lawson, R.H. (1988). An improved purification procedure for preparing potyviruses and cytoplasmic inclusions from the same tissue. *Journal of Virological Methods* **20**: 203-217.

Hopp, H.E., Hain, L., Bravo Almonacid, F., Tozzini, A.C., Orman, B., Arese, A.I., Ceriani, M.F., Saladrigas, M.V., Celnik, R., del Vas, M., and Mentaberry, A.N. (1991). Development and application of a nonradioactive nucleic acid hybridization system for simultaneous detection of four potato pathogens. *Journal of Virological Methods* **31**: 11-30.

Huisman, M.J., Linthorst, H.J.M., Bol, J.F., and Cornelissen, B.J.C. (1988). The complete nucleotide sequence of potato virus X and its homologies at the amino acid level with various plus-stranded RNA viruses. *Journal of General Virology* **69**: 1789-1798.

Hull, R. (1986). The potential for using dot-blot hybridisation in the detection of plant viruses. In Jones R.A.C. and Torrance L. (eds) *Developments and Applications in Virus Testing*, pp. 3-12. Association of Applied Biologists, Wellesbourne.

Köhler, E. (1962). Über die unterschiedlichen Temperaturempfindlichkeit von Typen und Stämmen der Kartoffel-X-virus. *Phytopathologisches Zeitschrift* **44**: 189-199.

Ladeburg, R.C., Larson, R.H., and Walker, J.C. (1950). Origin, interrelation and properties of ringspot strains of virus X in American potato varieties. *Wisconsin Agricultural Experiment Station Research Bulletin* **165**, 47 pp.

Matthews, R.E.F. (1949). Studies of potato virus X. *Annals of Applied Biology* **36**: 449-474.

Orman, B.E., Celnik, R.M., Mandel, A.M., Torres, H.N., and Mentaberry, A.N. (1990). Complete cDNA sequence of a South American isolate of potato virus X. *Virus Research* **16**: 293-306.

Querci, M., Salazar, L.F., and Fernandez-Northcote, E.N. (1993). Detection of Andean potato virus X isolates by radioactive and nonradioactive nucleic acid spot hybridization tests. *Phytopathology* **83**: 171-176.

Rich, A.E. (1977). Potato diseases. In Smith O. (ed) *Potatoes: Production, Storing, Processing*, 2nd edn., pp. 506-549. Avi Publication, Westport, Connecticut.

Salazar, L.F., and Querci, M. (1992). Detection of viroids and viruses by nucleic acid probes. In Duncan J.M. and Torrance L. (eds) *Techniques for the rapid detection of plant pathogens*, pp. 129-144. Blackwell Scientific Publications, Cambridge.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schultz, E.S., and Bonde, R. (1944). The effect of latent mosaic (virus X) on yield of potato in Maine. *The American Potato Journal* **21**: 278-283.

Skryabin, K.G., Kraev, A.S., Morozov, S.Yu., Rozanov, M.N., Chernov, B.K., Lukasheva, L.I., and Atabekov, J.G. (1988). The nucleotide sequence of potato virus X RNA. *Nucleic Acids Research* **16**: 10929-10930.

Torrance, L., Larkins, A.P., and Butcher, G.W. (1986). Characterization of monoclonal antibodies against potato virus X and comparison of serotypes with resistance groups. *Journal of General Virology* **67**: 57-67.

Chapter 6

RNA sequence of potato virus X strain HB

SUMMARY

The genomic RNA of the potato virus X strain HB (PVX_{HB}), isolated in Bolivia and able to overcome all known PVX resistance genes, has been cloned and sequenced. The PVX_{HB} RNA sequence is 6432 nucleotides long and contains, similarly to the RNAs of other PVX strains, five open reading frames encoding proteins of 165.1 kilodaltons (kDa), 24.5 kDa, 12.4 kDa, 7.6 kDa and 25.1 kDa (coat protein), respectively.

Multiple amino acid sequence alignment of the coat proteins of four PVX strains identified eight amino acid residues unique for PVX_{HB}. Structural prediction comparisons of the coat proteins of PVX_{HB} and of the other strains suggest a general structural similarity. However, two of the eight amino acid residues unique for strain HB gave rise to a change in the predicted coat protein structure, suggesting a possible involvement in the resistance-breaking activity of PVX_{HB}.

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INTRODUCTION

Potato virus X (PVX), the type member of the potexvirus group, is one of the most common and widespread viruses infecting potato. Strains of PVX have been classified into four groups according to their reactions with the dominant resistance genes Nb and Nx which, in cultivated varieties of potato, determine a hypersensitive response, and with the extreme resistance gene Rx (Cockerham, 1955, 1970).

Group 1 strains cause a hypersensitive response in the presence of Nb or Nx, group 2 only with Nb, group 3 only with Nx and group 4 with neither, but fails to infect plants carrying an Rx gene. A further strain, designated as PVX_{HB}, occurring in 7% of the Bolivian clones from *Solanum tuberosum* ssp. *andigena* and so far found only in South America, overcomes the effects of all known genes conferring resistance to PVX (Nb, Nx, Rx, Rx_{acl} and Rx_{adj}; Moreira *et al.*, 1980; Jones, 1985). This strain is unique in that it causes typical PVX symptoms in most common indicator species but does not produce local lesions in inoculated leaves of *Gomphrena globosa* L., which is the main diagnostic indicator host for PVX (Bercks, 1970). This symptomless infection in inoculated leaves of *G. globosa* distinguishes PVX_{HB} from all previously described strains of PVX.

PVX_{HB} is serologically most closely related to PVX_{cp} (Fribourg, 1975; originally named PVX_c), the common PVX strain in Peru from the resistance group 2 (Moreira *et al.*, 1980), but it differs from this and all other strains in its resistance-breaking properties. This property is of obvious importance in potato breeding programs.

Here we report the complete nucleotide sequence of PVX_{HB} RNA and its comparison with the sequences reported for PVX_{cp} (Orman *et al.*, 1990), and for PVX_{X3} (Huisman *et al.*, 1988) and PVX_S (Skryabin *et al.*, 1988) both from resistance group 3.

MATERIALS AND METHODS

Virus purification and isolation of RNA

The PVX_{HB} strain was kindly donated by C. Fribourg (Universidad Nacional Agraria La Molina, Lima, Peru). Strain HB was originally isolated from the high Andes of Southern Bolivia (Moreira *et al.*, 1980) and mantained in *Nicotiana glutinosa* L. in greenhouses at

temperatures in a range of 18 to 23 °C, after its reisolation from a *Solanum tuberosum* ssp. *andigena* immune to PVX, to ensure the purity of the strain.

PVX_{HB} was purified from frozen *N. glutinosa* infected leaves as previoulsy described (Querci *et al.*, 1993), and viral RNA was isolated according to Hammond and Lawson (1988).

cDNA synthesis and cloning

cDNA synthesis was performed by using the RiboClone cDNA Synthesis System (Promega) following the procedure indicated by the supplier, but SuperScript Reverse Transcriptase (BRL) was used for first strand synthesis upon priming the reaction with either oligo $dT_{12\text{-}18}$ or a random hexa-nucleotide mixture (pdN_6). A specific 14-mer primer complementary to the 5' end of PVX RNA (5'-GAAAACTAAACCAT-3'), named PVX-01, was also used in one cloning experiment, for priming the second strand synthesis. Recovered double-stranded cDNAs were inserted by blunt-end ligation into *Sma*I-digested pGEM7Zf(+) vectors. Ligation mixtures were used to transform *Escherichia coli* JM103 cells and colonies carrying recombinant plasmids were selected from agar plates containing 50 μg/ml ampicillin, 0.25 mM IPTG and 0.005 % Xgal (w:v). The presence and size of cDNA inserts was determined by plasmid isolation (Sambrook *et al.*, 1989) followed by restriction enzyme digestion upstream and downstream the *Sma*I site, and 1 % agarose gel electrophoresis with DNA size markers. Identity and orientation of the inserts were confirmed by ^{32}P-RNA transcription and hybridization against purified PVX_{HB} RNA.

Nucleotide sequence determination

The nucleotide sequence of PVX_{HB} was determined by the dideoxy-nucleotide chain termination method (Sanger *et al.*, 1977) using the TaqTrack sequencing system (Promega), from either full-length cDNA or subclones obtained by restriction enzymes digestion and Bal 31-generated nested deletions (Sambrook *et al.*, 1989). Using overlapping clones each position was sequenced at least three times. Computer analysis of sequence data was done using the LKB Pharmacia DNASIS program and the University of Wisconsin GCG program package version 7.1 (Devereux *et al.*, 1984).

RESULTS AND DISCUSSION

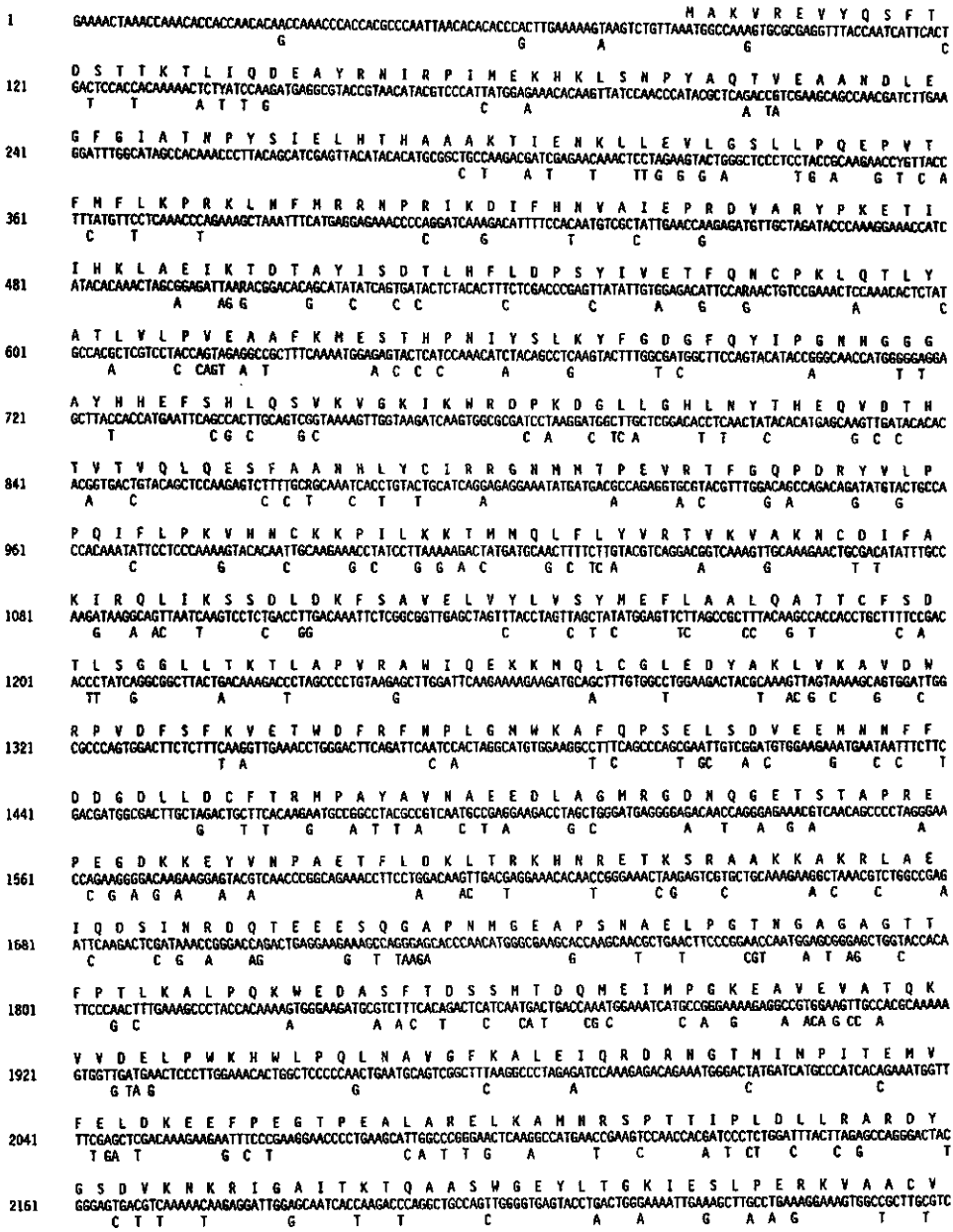
The complete genomic sequence of PVX_{HB}, shown in Fig. 6.1, contains 6432 nucleotides, exactly the length reported for PVX_{cp} (Orman *et al.*, 1990), whereas the RNAs of two European PVX strains, PVX_{X3} (Huisman *et al.*, 1988) and PVX_S (Skryabin *et al.*, 1988), both from the resistance group 3, are 6435 nucleotides long.

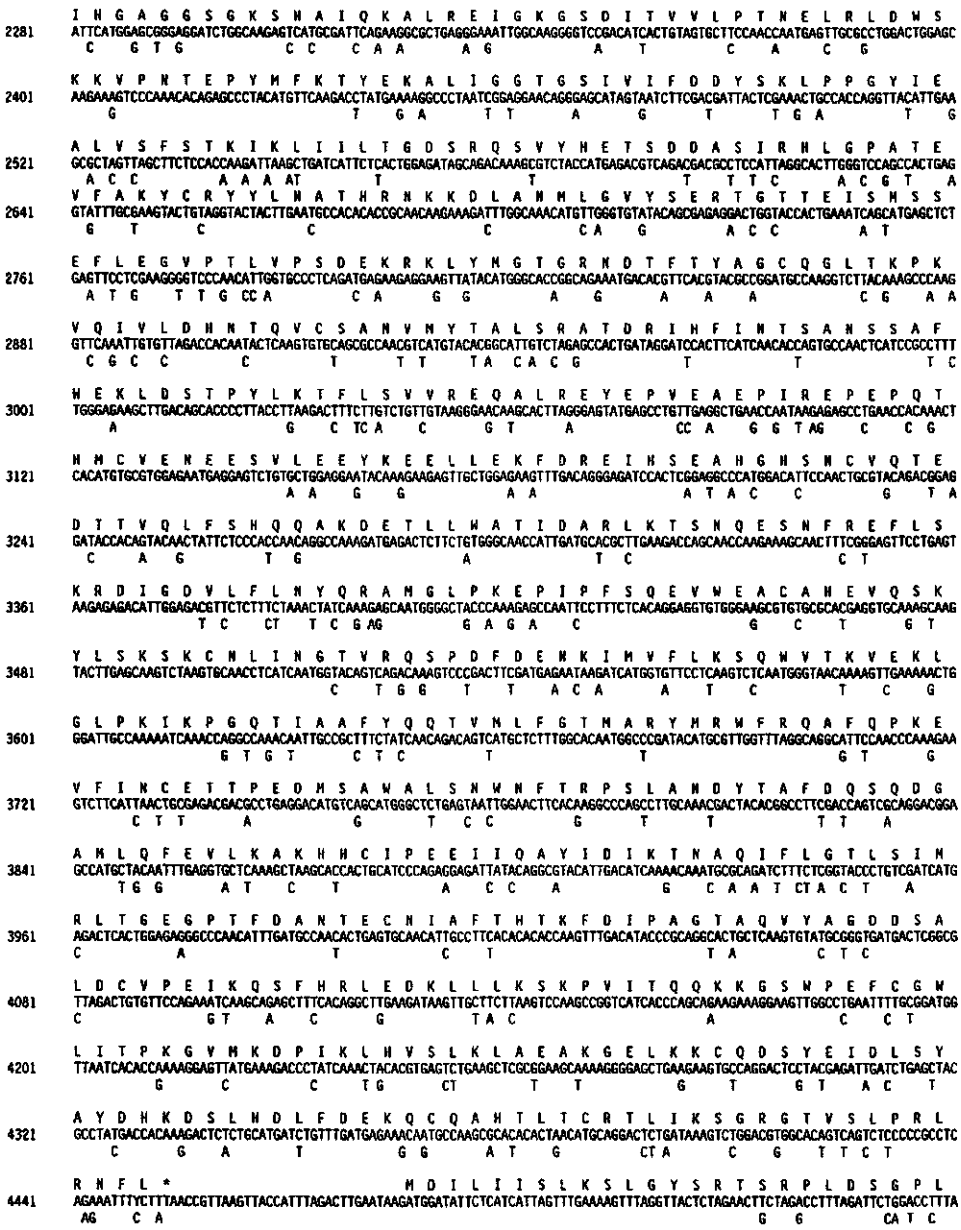
Computer analysis and translation of the sequence confirmed that the general structure was conserved among all PVX strains. The sequence contains five open reading frames (ORFs): ORF1 extends from base 85 until the UAA stop codon at base 4453 and encodes a polypeptide of 165.1 kDa; ORF2, in phase with ORF1, starts at position 4486, ends at position 5163 and encodes a 24.5 kDa protein; partially overlapping with ORF2 and in a different reading frame, ORF3 (from nucleotide 5147 to 5491) encodes a 12.4 kDa protein. ORF3 also overlaps partially with ORF4 which starts in a different reading frame at position 5427 and terminates at position 5639, coding for a protein of 7.6 kDa. Finally, ORF5 encodes the coat protein (CP) of 25.1 kDa and extends from position 5650 to position 6357 in the same reading frame as ORFs 1 and 2. The UAA stop codon at position 6358 is followed by an untranslated region of 84 nucleotides and a poly(A) tract.

In the nucleotide sequence of PVX_{HB}, seven points of variability have been found in which a purine (either A or G; in Fig. 6.1 marked R) or a pyrimidine (either C or T; marked Y) occur. The possibility cannot be excluded that these nucleotide variabilities resulted from the method used for sequence determination. In all cases the variability found did not cause changes in the encoded amino acid sequence.

PILEUP, PRETTY and DISTANCES programs of the GCG program package (Devereux *et al.*, 1984) were used for sequence analysis. Levels of calculated sequence similarities among all potential open reading frames of four PVX strains are summarized in Table 6.1. Sequence similarities varied between 83 and 99%, most sequence divergence occurring in the ORF4 product. From the comparisons in Table 6.1 it can be concluded that PVX_{HB} is most closely related to PVX_{cp}.

The difference in genome length between PVX_{HB} and PVX_{cp} on the one hand (6432 nucleotides), and PVX_{X3} and PVX_S (6435 nucleotides) on the other, is due to a three nucleotide deletion in the CP at positions 5732 to 5734, determining the replacement of threonine (T) and alanine (A) in PVX_{X3} and PVX_S with asparagine (N) in PVX_{HB} and PVX_{cp}. The coat protein sequences of the two South American strains, PVX_{HB} and PVX_{cp},





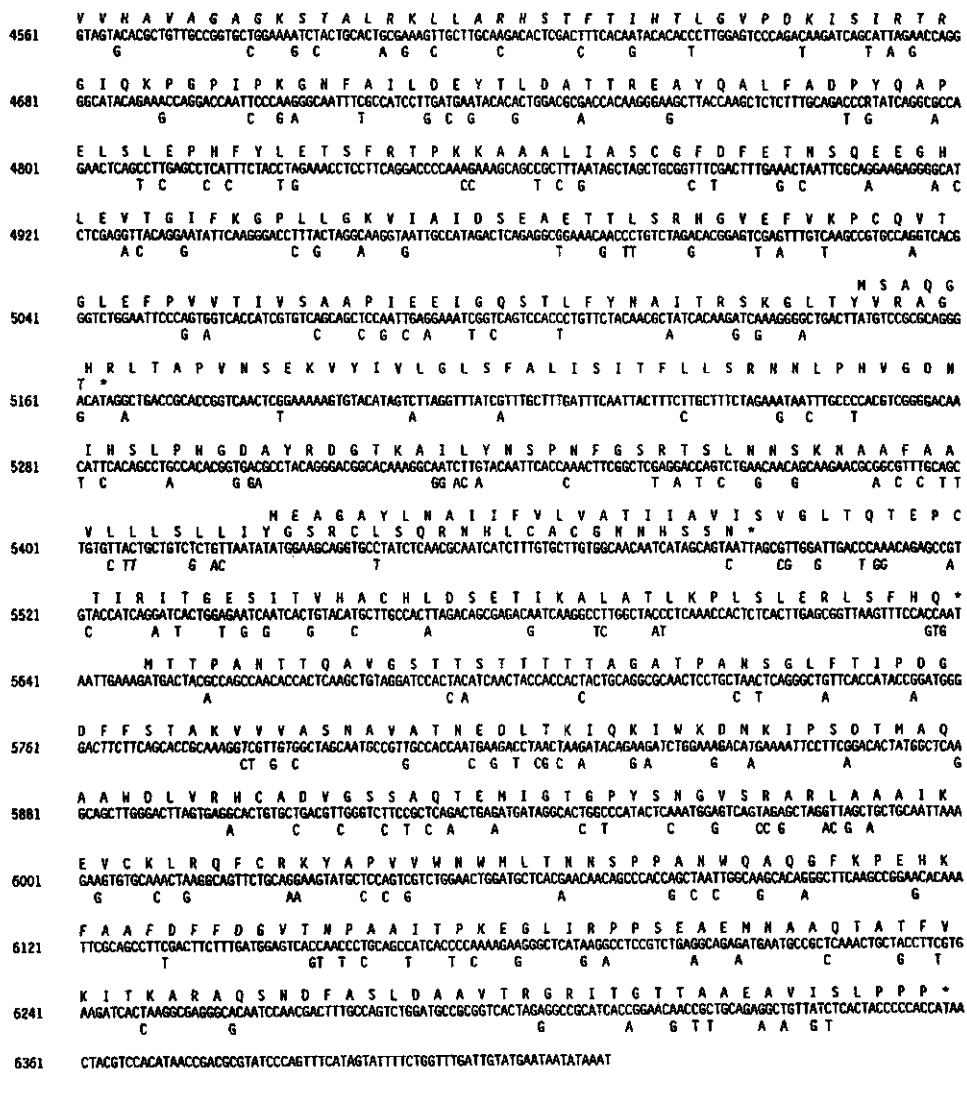


Fig. 6.1. Complete nucleotide sequence of PVX_{HB} and the amino acid sequences of the open reading frames (ORFs) 1 to 5. Nucleotide sequence of PVX_{HB} RNA is shown in the DNA form with nucleotide numbers to the left. Purines (A or G) are indicated by R and pyrimidines (C or T) by Y. The one letter amino acid code is on the first base of each codon within the ORFs in the upper line. The asterisk identifies the first nucleotide of the stop codon terminating an ORF. Nucleotide differences from the sequence reported for PVX_{cp} (Orman *et al.*, 1990) are indicated in the lower line. The nucleotide sequence data reported in this paper have been assigned the accession number X72214 in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases .

Table 6.1. Sequence similarity between the amino acid sequences of ORFs 1 to 5 of four PVX strains: PVX_{HB}, PVX_{cp} (Orman *et al.*, 1990), PVX_{X3} (Huisman *et al.*, 1988) and PVX_S (Skryabin *et al.*, 1988).

Strains	PVX_{HB}	PVX_{cp}	PVX_{X3}	PVX_S	
PVX_{HB}	1.0000	0.9773	0.9348	0.9430	ORF 1
PVX_{cp}		1.0000	0.9286	0.9334	
PVX_{X3}			1.0000	0.9876	
PVX_S				1.0000	
PVX_{HB}	1.0000	0.9823	0.9336	0.9336	ORF 2
PVX_{cp}		1.0000	0.9248	0.9292	
PVX_{X3}			1.0000	0.9912	
PVX_S				1.0000	
PVX_{HB}	1.0000	0.9652	0.9478	0.9478	ORF 3
PVX_{cp}		1.0000	0.9391	0.9391	
PVX_{X3}			1.0000	1.0000	
PVX_S				1.0000	
PVX_{HB}	1.0000	0.9571	0.8286	0.8429	ORF 4
PVX_{cp}		1.0000	0.8571	0.8714	
PVX_{X3}			1.0000	0.9857	
PVX_S				1.0000	
PVX_{HB}	1.0000	0.9619	0.9280	0.9237	ORF 5 (CP)
PVX_{cp}		1.0000	0.9364	0.9322	
PVX_{X3}			1.0000	0.9958	
PVX_S				1.0000	

contain 236 amino acids and are one residue shorter than those of PVX_{X3} and PVX_S (237 amino acids). The one amino acid gap introduced for the alignment is located at position 28. In addition, the polypeptide encoded by ORF4 in PVX_{HB} is one amino acid longer than in all the other strains of PVX; in fact ORF4 terminates at nucleotide position 5636 in PVX_{cp}, PVX_{X3} and PVX_S, whereas in PVX_{HB} it ends at position 5639.

Although the mechanism of Rx resistance-breaking by PVX_{HB} has remained unknown, studies with hybrid PVX genomes (Santa Cruz *et al.*, 1989), showed that the virulence determinants for the Rx gene are located in the 3' portion of the viral genome, outside the replicase gene. Further studies with hybrid PVX genomes, constructed by joining the 5' part of the genome of a group 3 non-breaking strain of PVX (PVX_{UK3}) and the 3' part of the PVX_{HB} genome, mapped the breaking properties of PVX_{HB} in either the coat protein

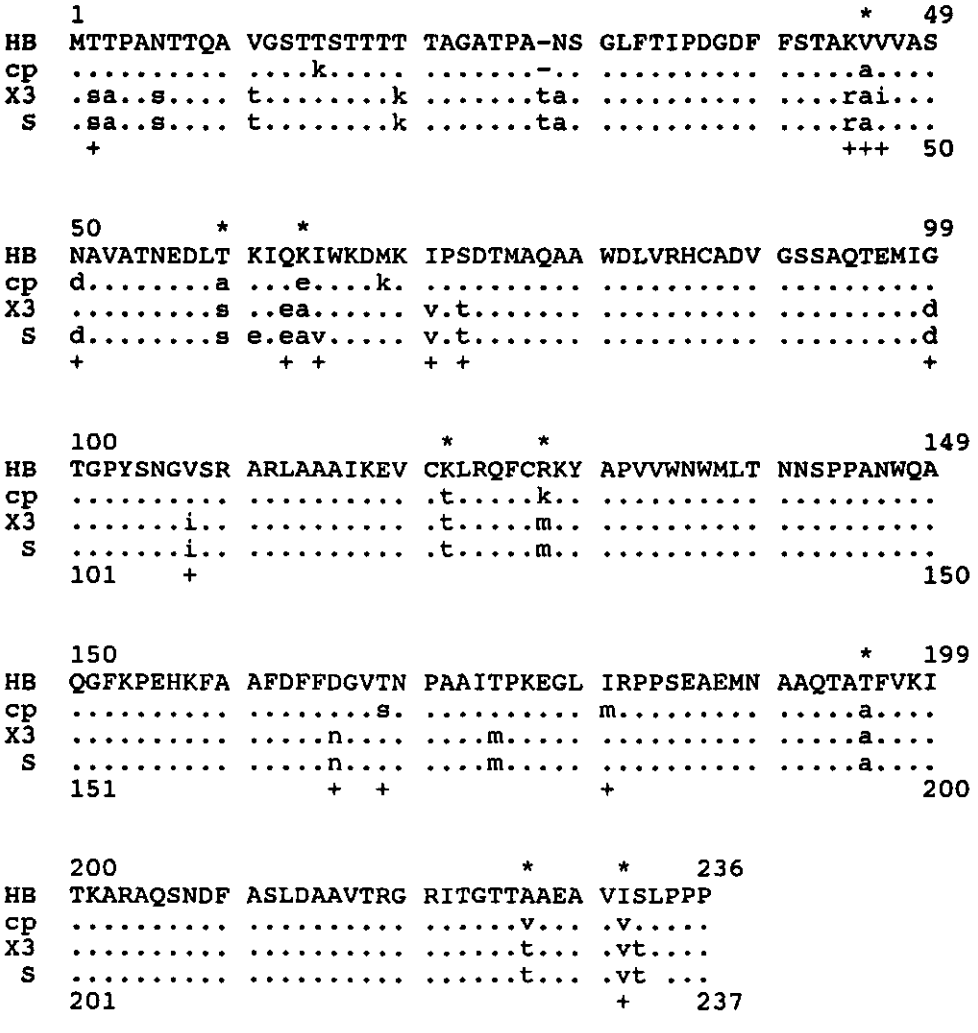


Fig. 6.2. Multiple alignment of the coat protein sequences of four PVX strains, HB, cp, X3 and S (for sources see text). Amino acids identical to HB are indicated by dots (.); residues differing from HB are indicated by lower case lettering. Gaps made to align the sequences are indicated by dashes (-). (+) indicates conserved amino acids changes within the following groups (Kamer and Argos, 1984): acidic and polar (D,E,N,Q), basic (K,R), hydrophobic (A,C,F,H,I,L,M,V,W,Y), polar (S,T) or strong turn formers (D,G,N,P). Amino acid residues unique for HB are indicated by an asterisk (*).

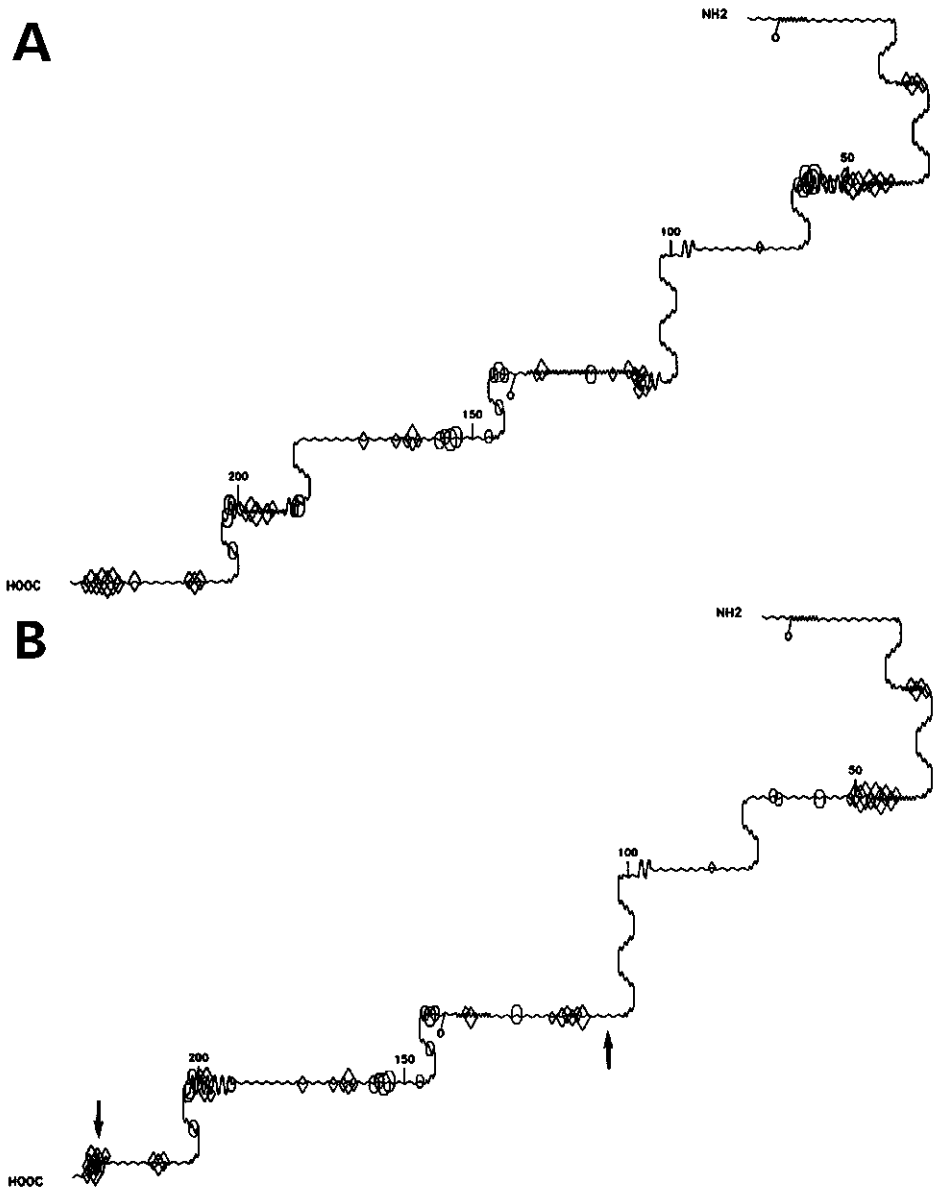
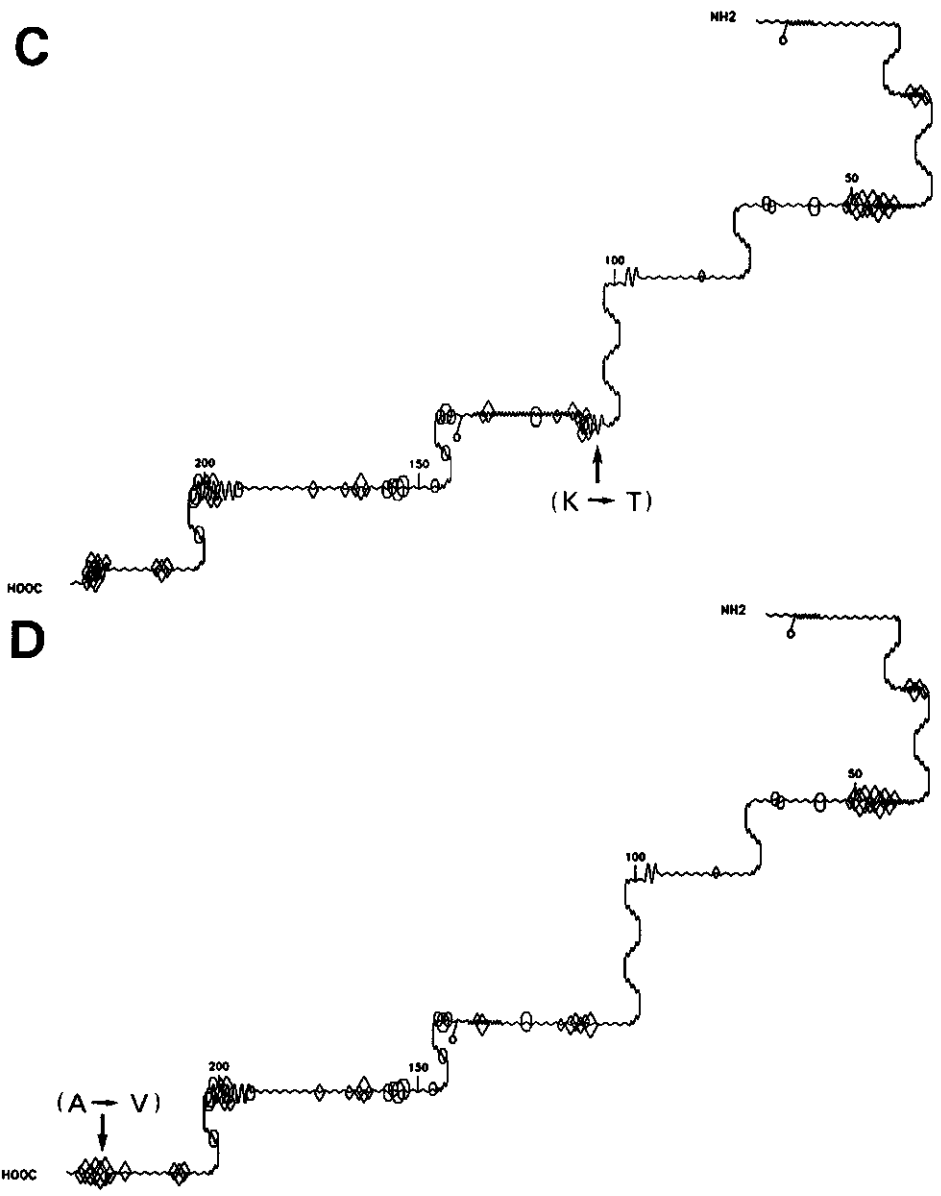


Fig. 6.3. Prediction of protein secondary structure, presented as "PLOTSTRUCTURES" for the PVX_{cp} **(A)** and PVX_{HB} **(B)** coat proteins compared with the structures obtained after single amino acid substitutions (HB to cp) in PVX_{HB} coat protein, at positions 121 (C) and 226 (D). The two-dimentional plot helices are shown as sine waves, ß-sheets with sharp sawtooth waves, 180° turns and coils with dull sawtooth waves.



Superimposed over the wave forms are diamonds representing hydrophobic residues and hexagons representing hydrophilic residues. The size of symbols represent the extent of hydrophobicity or hydrophilicity. Arrows indicate positions of the two amino acids unique for PVX_{HB}, causing changes in the coat protein structure.

cistron or 3' non-coding sequence (Kavanagh *et al.*, 1992). Since the 3' non-coding sequences of PVX_{HB} and PVX_{cp} are identical, the coat protein gene could thus be identified as the resistance-breaking factor of PVX_{HB}.

In an attempt to establish more precisely the exact location of the determinants in the PVX coat protein sequence responsible for the ability of strain HB to overcome immunity, the nucleotide and amino acid sequences of four PVX strains were compared. The coat protein sequences of PVX_{HB} and of the other three PVX strains were aligned (Fig. 6.2) and the levels of sequence similarities calculated using the GCG DISTANCES program (Devereux *et al.*, 1984). Within the coat protein, highest variability is located between amino acids 1 and 90. In this region strains X3 and S show a similarity of 99.5% between each other (three substitutions), and only about 92% and 91% with strains HB and cp respectively.

Comparison of PVX_{HB} with all non-breaking strains (PVX_{cp}, PVX_{X3} and PVX_{S}) suggests a high similarity in the general coat protein structure, the differences being limited to a small number of residues. Eight possibly relevant substitutions in the PVX_{HB} coat protein amino acid sequence were found, i.e. residues unique for PVX_{HB} and different from those present in all non-breaking strains of PVX (Fig. 6.2, positions indicated with *). Computer-directed mutational analysis, performed by substitution of each of the eight "unique" amino acids in the PVX_{HB} coat protein sequence with the corresponding ones from the PVX_{cp} sequence, followed by secondary structure predictions using the GCG program PEPTIDESTRUCTURE (Devereux *et al.*, 1984), revealed that within the eight amino acid residues considered, only two determine variations in the protein structure, i.e. K_{121} and A_{226}. As an example of these analyses, Fig. 6.3 shows the prediction of protein secondary structures, presented as "PLOTSTRUCTURE" profiles, of the PVX_{HB} coat protein obtained after single amino acid substitutions (HB to cp) at positions 121 and 226. Fig. 6.3 incorporates α-helix, ß-sheet, hydrophilicity and hydrophobicity predictions based on the method described by Chou and Fasman (1978).

When the lysine (K, basic) at position 121 in the PVX_{HB} coat protein sequence is substituted with a (cp-specific) threonine (T, polar) the α-helix at this position is converted into a ß-sheet structure as found in the coat protein structure of PVX_{cp}. The substitution at position 226 (alanine to valine) also determines a slight alteration of the predicted structure, although the modification is located in a highly hydrophobic region and might therefore be masked. Both amino acid substitutions at positions 121 and 226 lead to a reversion of the

predicted HB-type coat protein structure (Fig. 6.3 B) to the cp-type coat protein structure (Fig. 6.3 A).

It is tempting to assume that one, or both, of these amino acid substitutions is responsible for the biological difference between PVX_{HB} (Rx-breaking) and the other strains (non-breaking). The elucidation of the whole genomic sequence of PVX_{HB}, as presented in this paper, has allowed comparison of this Rx resistance-breaking isolate with non-breaking isolates on the molecular level. The sequence comparisons, compiled in Table 6.1, demonstrate that the Rx resistance-breaking capacity of HB is not accompanied by a high rate of divergence in the (CP) gene involved. Remarkably, whereas most of the other ORF's in the PVX_{HB} genomic sequence show a similar rate of divergence, ORF4, which is part of the "triple gene block" and not involved in resistance-breaking, is less conserved that the coat protein sequence.

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REFERENCES

Bercks, R. (1970). Potato virus X. *C.M.I./A.A.B. Descriptions of Plant Viruses.* No. 4.

Chou, P.Y., and Fasman, G.D. (1978). Empirical predictions of protein conformation. *Annual Review of Biochemistry* 47: 251-276.

Cockerham, G. (1955). Strains of potato virus X. *Proceedings of the Second Conference on Potato Virus Diseases*, pp. 89-92. Lisse-Wageningen, 1954.

Cockerham, G. (1970). Genetical studies on resistance to potato viruses X and Y. *Heredity* 25: 309-348.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Research* 12: 387-397.

Fribourg, C.E. (1975). Studies on potato virus X strains isolated from Peruvian potatoes. *Potato Research* 18: 216-226.

Hammond, J., and Lawson, R.H. (1988). An improved purification procedure for preparing potyviruses and cytoplasmic inclusions from the same tissue. *Journal of Virological Methods* 20: 203-217.

Huisman, M.J., Linthorst, H.J.M., Bol, J.F., and Cornelissen, B.J.C. (1988). The complete nucleotide sequence of potato virus X and its homologies at the amino acid level with various plus-stranded RNA viruses. *Journal of General Virology* 69: 1789-1798.

Jones, R.A.C. (1985). Further studies on resistance-breaking strains of potato virus X. *Plant Pathology* **34**: 182-189.

Kamer, G., and Argos, P. (1984). Primary structural comparisons of RNA-dependent polymerases from plant, animal and bacterial viruses. *Nucleic Acids Research* **9**: 7269-7282.

Kavanagh, T., Goulden, M., Santa Cruz, S., Chapman, S., Barker, I., and Baulcombe, D. (1992). Molecular analysis of a resistance-breaking strain of potato virus X. *Virology* **189**: 609-617.

Moreira, A., Jones, R.A.C., and Fribourg, C.E. (1980). Properties of a resistance-breaking strain of potato virus X. *Annals of Applied Biology* **95**: 93-103.

Orman, B.E., Celnik, R.M., Mandel, A.M., Torres, H.N., and Mentaberry, A.N. (1990). Complete cDNA sequence of a South American isolate of potato virus X. *Virus Research* **16**: 293-306.

Querci, M., Salazar, L.F., and Fernandez-Northcote, E.N. (1993). Detection of Andean potato virus X isolates by radioactive and nonradioactive nucleic acid spot hybridization tests. *Phytopathology* **83**: 171-176.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences USA* **74**: 5463-5467.

Santa Cruz, S., Baulcombe, D.C., and Kavanagh, T. (1989). Virulence and virulence determinants in potato virus X. *Annual Report IPSR/JII/SL*. pp. 43-44.

Skryabin, K.G., Kraev, A.S., Morozov, S.Yu., Rozanov, M.N., Chernov, B.K., Lukasheva, L.I., and Atabekov, J.G. (1988). The nucleotide sequence of potato virus X RNA. *Nucleic Acids Research* **16**: 10929-10930.

Chapter 7

Analysis of the resistance-breaking determinant of potato virus X strain HB on different potato genotypes expressing extreme resistance to PVX

SUMMARY

Different potato genotypes expressing extreme resistance to potato virus X were inoculated with potato virus X strains cp, CP4, and HB and with three *in vitro* constructed PVX mutants. Using these viral strains and mutants the involvement of the coat protein, and specifically of the single amino acid residue at position 121 within this protein, in the resistance-breaking capacity of strain HB was confirmed for all resistance genes tested. It is concluded that the extreme resistance expressed in *Solanum* x *chaucha*, *S.* x *curtilobum*, *S.* x *juzepczukii*, and *S. vernei* are all carried by Rx genes (Rx_{cha}, Rx_{cur}, Rx_{juz}, and Rx_{vrn}, respectively) which employ the same mechanism of interaction with the PVX coat protein as Rx, Rx_{acl}, and Rx_{adg}. The *S. sucrense* accession OCH 11926, immune to both common PVX strains and PVX_{HB}, however, was susceptible to the mutant isolates. It is hypothesized that at least two different viral determinants, one of which being not located in the coat protein, are involved in the interaction with the *S. sucrense* resistance gene.

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INTRODUCTION

The first case of extreme resistance to potato virus X (PVX) was reported by Schultz and Raleigh (1933) and Schultz *et al.* (1934). This resistance, based on a single dominant gene reported to as Rx (Mills, 1965; Cockerham, 1970), was obtained from the Chilean cultivar Villaroela (ssp. *tuberosum*). This gene was transferred to the USDA seedling 41956 and next incorporated into the North American potato variety Atlantic (Webb *et al.*, 1978), Saco (Akeley *et al.*, 1955) and others (Ross, 1986). A further gene, Rx_{adg}, was originally detected by Wiersema (1961) in a progeny from ssp. *andigena* CPC 1673 resistant to the pathotypes Ro-1 and Ro-4 of *Globodera rostochiensis*. Extreme resistance to PVX was also found by Ross (1954) in the wild species *Solanum acaule*. This gene, Rx_{acl}, considered by Cockerham (1970) to be distinct from Rx_{adg}, is also inherited as monogenic dominant character. Rx_{acl} was introduced into several European varieties (Ross, 1986), among them the variety Bzura, and into the Argentine variety Serrana INTA via the back-cross hybrid MPI 44.1016/10. An additional unidentified gene for extreme resistance to PVX was found in *S. vernei* cultivars resistant to the nematode *Globodera pallida* and introduced in some Dutch varieties (Ross, 1986).

Rx, Rx_{adg} and Rx_{acl}, as well as most of the other genes involved in extreme resistance to plant viruses, are not strain specific (Cockerham, 1970; Ross, 1983) and, in contrast to single-gene resistance against fungal pathogens, in general they do not break down at the population level (Cockerham, 1970; Ross, 1986).

Strains of PVX have been traditionally classified into four groups (Cockerham, 1955) according to their reactions with the genes for localized hypersensitivity (Nb, Nx) and extreme resistance (Rx). The strain PVX_{HB}, found in 7% of Bolivian clones of ssp. *andigena* (Moreira *et al.*, 1980; Jones, 1985), resembles the normal group 4 strains in overcoming genes Nb and Nx, but differs from these in being able to overcome also the effects of Rx genes conferring extreme resistance to PVX. However, C. Chuquillanqui and L.F. Salazar (personal communication) found extreme resistance against PVX_{HB} in the wild *S. sucrense* accession OCH 11926 and Brown *et al.* (1984) suggested the implication of a single dominant gene.

With few exceptions, the way in which the various types of resistance work at the biochemical or physiological levels is far from being understood. Several efforts have been devoted in recent years to gain knowledge of the genetic basis of resistance and of the

genetic system controlling the behaviour of pathogens. Particular interest invested the phenomenon called "virulence", the ability of a particular strain of a virus to multiply or cause disease on individuals of the host species containing genes for resistance to that virus. Virulent isolates able to overcome single-gene resistance under normal conditions have been found for several viruses (Moreira *et al.*, 1980; Adams *et al.*, 1984; Ross, 1986).

The specific case of PVX_{HB} has been extensively studied and several experiments have been conducted in order to localize, within the viral genome sequence, the virulence determinants. Santa Cruz *et al.* (1989) showed that the virulence determinants for the Rx gene are located in the 3' portion of the viral genome, outside the replicase gene. Further studies, with hybrid PVX genomes obtained by joining the 5' part of the genome of a British group 3 non-breaking isolate of PVX (PVX_{UK3}) and the 3' part of the PVX_{HB} genome, allowed to reduce the area involved in the resistance-breaking activity of HB to the viral coat protein gene (Kavanagh *et al.*, 1992).

Querci *et al.* (1993b) compared the nucleotide and amino acid sequences of PVX_{HB} and other non-breaking strains of PVX, PVX_{X3} (Huisman *et al.*, 1988), PVX_{S} (Skryabin *et al.*, 1988), and PVX_{cp} (Orman *et al.*, 1990), and highlighted the presence, within the coat protein amino acid sequence, of eight residues "unique" for strain HB. Computer-directed mutational analysis, performed by substitution of each of these eight amino acids in the PVX_{HB} coat protein sequence with the corresponding ones from the PVX_{cp} sequence, followed by secondary structure predictions, revealed that only two (121 and 226) of the eight residues considered, determine variation in the predicted protein structure. These observations suggested the involvement of one (or both) of these amino acid residues in the resistance breaking activity of the strain HB. At the same time, Goulden *et al.* (1993) analyzed a series of hybrid and mutant isolates of PVX_{HB} and PVX_{CP4}, a group 4 isolate (Jones, 1985), and concluded that extreme resistance expressed in the var. Cara (Rx) is affected by amino acids 121 and 127 of the viral coat protein, codon 121 representing the major determinant.

Wild tuber-bearing *Solanum* species possess a broad spectrum of resistance genes, but only few of them have been analyzed and incorporated in the genome of commercial varieties. Resistance actually used in breeding programs is still often limited to only few proveniences. In addition even the relationship between the widely used genes Rx, Rx_{acl} and Rx_{adg} is essentially unknown. However, Ritter *et al.* (1991) mapped genes Rx1 and Rx2, conferring resistance to PVX and present in potato clones originated from clones CPC 1673

and MPI 44.1016/10, at two different positions on the RFLP map of potato. These results confirmed the independence of Rx1 (Rx_{adg}) and Rx2 (Rx_{acl}) genes suggested by Cockerham (1970).

Having been demonstrated that the coat protein, and specifically, only a single amino acid position (121) in the coat protein, affects extreme resistance in Rx genotypes, our interest was devoted to the analysis of a wider pool of Rx genes available. In this paper we report the results obtained after inoculation of several varieties and native potato cultivars carrying Rx genes from different *Solanum* species, with the strains PVX_{HB}, PVX_{cp} and PVX_{CP4}, and with selected mutant isolates of PVX containing specific, virulence-affecting mutations in the coat protein gene.

MATERIALS AND METHODS

Virus strains and mutant isolates

PVX strains and mutant isolates used in this work are listed in Table 7.1. Strains PVX_{cp} and PVX_{HB} were kindly donated by C. Fribourg (Universidad Nacional Agraria La Molina, Lima, Peru) and maintained at the International Potato Center (CIP, Lima, Peru). PVX_{cp}, from strain group 2, was originally isolated from the high Andes of central-southern Peru (Fribourg, 1975; called PVX_C). PVX_{HB} is the resistance-breaking strain isolated in Bolivia by Moreira *et al.* (1980). PVX_{CP4} is a strain group 4 isolate described by Jones (1985).
The construction of the mutant isolates CP4-KR and HB-TK has been previously described by Goulden *et al.* (1993). CP4-KR is a mutant derivative of PVX_{CP4} in which two point mutations have been introduced, such that amino acids 121 and 127 of the viral coat protein are lysine (K) and arginine (R), respectively, typical for strain HB coat protein, instead of threonine (T) and lysine (K), as normally found in PVX_{CP4}. HB-TK is a similarly produced mutant derivative of PVX_{HB} in which mutations have been introduced so that amino acids 121 and 127 of the viral coat protein are threonine (T) and lysine (K), as found in CP4, instead of lysine (K) and arginine (R), respectively.
KH2, finally, is a hybrid PVX genome (K, PVX_{UK3}; H, PVX_{HB}) obtained by replacement of the coat protein gene and 3' non-coding sequence in the PVX_{UK3} genome with the homologous sequence from PVX_{HB} (Kavanagh *et al.*, 1992) (Fig. 7.1).

Plant materials

The potato varieties, native cultivars and clones listed in Table 7.2 and used in experiment 1, have been selected from the germplasm collection of the International Potato Center and propagated *in vitro* conditions. *In vitro* plantlets were cut into single nodes and after removal of large leaves transferred to sterile flasks containing 25 ml of liquid MS medium (Murashige and Skoog, 1962) with 0.1 ppm gibberellic acid and 2.5% sucrose. After growing for 3 weeks at 18-22°C with a 16 hours photoperiod under 3000 lux light intensity, the plantlets were next transferred to pots containing compost mixture and maintained with high relative humidity for few days (Espinoza *et al.*, 1989), and finally transferred to normal greenhouse conditions for optimal leaf development prior to inoculations.

Wild *S. acaule* accessions listed in Table 7.2, kindly supplied by C. Ochoa, and used in experiment 2, were raised from botanical seeds. Plants of the wild *S. sucrense* accession OCH 11926 were grown from tubers which originated from the previously selected seedling number 4 (OCH 11926.4) kindly supplied by Charlotte Lizárraga. Indicator plants listed in Table 7.4 were obtained from commercially available seeds.

Table 7.1. Characteristics of the potato virus X strains and mutant isolates used in this work. * Resistance groups according to Cockerham (1955). RB= resistance-breaking.

Isolate	Resistance group*	Origin	Reference
PVX_{cp}	2	Peru	Fribourg, 1975
PVX_{CP4}	4	UK	Jones, 1985
PVX_{HB}	RB	Bolivia	Moreira *et al.*, 1980
PVX_{KH2}	UK3 + (5650-6432) HB = RB		Kavanagh *et al.*, 1992
$PVX_{CP4\text{-}KR}$	RB		Goulden *et al.*, 1993
$PVX_{HB\text{-}TK}$	not RB		Goulden *et al.*, 1993

Plant inoculations

All experiments were conducted in growth chambers at 18-22°C, with constant relative humidity (80%), and 12 hours photoperiod, under 1500 lux light intensity. Wild strains and isolates of PVX (cp, CP4, and HB) were maintained in *Nicotiana glutinosa* L. The mutant and hybrid viral cDNAs (CP4-KR, HB-TK, and KH2) were transcribed into infectious RNAs (Chapman *et al.*, 1992) and inoculated to *N. glutinosa*. and *N. clevelandii* as previously described (Goulden *et al.*, 1993; Köhm *et al.*, 1993).

Potato and indicator plants were manually inoculated with infective sap extracts. Non-inoculated leaves of infected *N. glutinosa* plants were homogenized in 10 volumes of 50 mM sodium phosphate buffer (pH 7.0) using pestle and mortar. The inoculum was rubbed onto four leaves per plant previously dusted with 600 mesh carborundum. Inoculated plants were then mantained in a growth chamber at the conditions previously indicated and tested for PVX infection three and four weeks after inoculation.

ELISA and NASH tests

Infection of plants was monitored by the enzyme-linked immunosorbent assay on nitrocellulose membranes (NCM-ELISA) and by nucleic acid spot hybridization (NASH). NCM-ELISA was performed as described (Lizárraga and Fernandez-Northcote, 1989) but samples were macerated in TBS buffer (0.02 M Tris-HCl, 0.5 M NaCl, 0.01% NaN_3) containing 0.01 M DIECA and 0.01 M EDTA (International Potato Center, 1993). NASH test was performed according to Querci *et al.* (1993a) using the pX61 negative-sense ^{32}P-labeled RNA probe specific to PVX_{cp} and able to detect all PVX strains and isolates used in the experiments.

RESULTS

To test whether different potato genotypes, which express extreme resistance to PVX, all react in a similar way with the virus, these plants were inoculated with the PVX strains cp, CP4 and HB, and with the mutant constructs presented in Fig. 7.1. Plant materials used in the experiments are listed in Table 7.2. For experiment 1 four varieties were selected: Atlantic (Webb *et al.*, 1978) is a North American variety carrying the gene (Rx) that originated from the Chilean cultivar Villaroela (ssp. *tuberosum*) and has been transferred

Table 7.2. List of potato varieties, native cultivars, clones and wild species accessions used in the experiments, origin and resistance genes sources.

	CIP number	Origin		Gene	Resistance gene source
Atlantic	800827	USA	variety	Rx	*S. tuberosum* ssp. *tuberosum* (USDA 41956)
Bzura	800953	Poland	variety	Rx_{acl}	*S. acaule*
Carnera	703482	Peru	native cultivar	Rx_{adg}	*S. tuberosum* ssp. *andigena*
DTO-33	800174	CIP	clone	rx	
Huayro	703304	Peru	native cultivar	Rx_{chc}	*S.* x *chaucha* (natural hybrid)
LT-8	379706.27	CIP	clone	Rx_{adg}	*S. tuberosum* ssp. *andigena*
Maria Huanca	279142.12	Peru	variety	$Rx_{vrn?}$	*S. vernei* ?
Ugro Shiri	702937	Peru	native cultivar	Rx_{cur}	*S.* x *curtilobum* (nat. hybrid)
Yagana INIA	720139	Chile	variety	Rx_{vrn}	*S. vernei*
Yurac Kaipi	702078	Peru	native cultivar	Rx_{juz}	*S.* x *juzepczukii* (nat. hybrid)
OCH 11818	761027	Bolivia	wild species	Rx_{acl}	*S. acaule*
OCH 11823	761286	Bolivia	wild species	Rx_{acl}	*S. acaule*
OCH 11825	761288	Bolivia	wild species	Rx_{acl}	*S. acaule*
OCH 11889	761330	Peru	wild species	Rx_{acl}	*S. acaule*
OCH 11890	761331	Peru	wild species	Rx_{acl}	*S. acaule*
OCH 11912	761347	Bolivia	wild species	Rx_{acl}	*S. acaule*
OCH 11983	761379	Bolivia	wild species	Rx_{acl}	*S. acaule*
OCH 14391	762032	Peru	wild species	rx	
OCH 14392	762032	Peru	wild species	rx	
OCH 11926	761351	Bolivia	wild species	?	*S. sucrense*

to the USDA seedling 41956 prior to incorporation into this and others important varieties. Bzura is a Polish variety carrying the Rx_{acl} gene from *S. acaule*. The variety Yagana INIA, from Chile, as well as possibly the Peruvian Maria Huanca, carry the Rx_{vm} gene from *S. vernei*. The Rx_{adg} gene (from ssp. *andigena*) is present in the clone LT-8 (International Potato Center) as well as in the native cultivar Carnera (ssp. *andigena* f. *Carnera*). Three additional native cultivars of Peruvian origin, and considered also immune to PVX (E.N. Fernandez-Northcote, personal communication), were selected: Huayro (*S.* x *chaucha*), Ugro Shiri (*S.* x *curtilobum*) and Yurac Kaipi (*S.* x *juzepczukii*). The clone DTO-33 (International Potato Center) was used as susceptible (rx) control.

Five repetitions of each genotype were used for inoculation with each of the potato virus X strains or mutants listed in Table 7.1 and Fig. 7.1. The results from experiment 1, as summarized in Table 7.3, were all confirmed in a second experiment conducted in a similar way. Isolates cp and CP4 behaved similarly on all Rx genotypes and on most indicator plants used in this work. Both isolates were used in all experiments but for the sake of simplicity only the results with the CP4 isolate are shown. Similarly, two HB isolates have been tested: HB-CIP (Querci *et al.*, 1993b), which has been maintained at the International Potato Center (CIP, Lima, Peru), and a second isolate maintained at the Sainsbury Laboratory (Norwich, UK). Results reported for the HB strain mean similar results obtained with both isolates, differences in behaviour or symptoms production, found between the two HB isolates, will be specifically indicated.

As expected cp and CP4 isolates gave systemic infection with mild mosaic only on the clone DTO-33 used as susceptible (rx) control (Table 7.3). Equally expected was the ability of strain HB to infect all Rx potato genotypes tested. As indicated in Table 7.3 the recombinant clone KH2, originating from the group 3 PVX isolate UK3, but carrying the PVX_{HB} coat protein gene (Kavanagh *et al.*, 1992), infected all genotypes tested. Goulden *et al.* (1993) tested several mutant derivatives of PVX_{HB} and PVX_{CP4} and showed that a single feature in the coat protein affected virulence of HB on var. Cara (Rx genotype). These results indicated that only amino acid 121 of the coat protein is directly involved in the Rx-breaking activity of HB, while mutation at position 127 provides stability needed for normal replication of the virus (Goulden *et al.*, 1993). Accordingly, the mutant CP4-KR (mutated to have lysine (K) and arginine (R) in positions 121 and 127 of the viral coat protein respectively instead of threonine (T) and lysine (K), as normally found in PVX_{CP4}) behaves on var. Cara as HB does. Reversely, the mutant derivative of HB, HB-TK, in

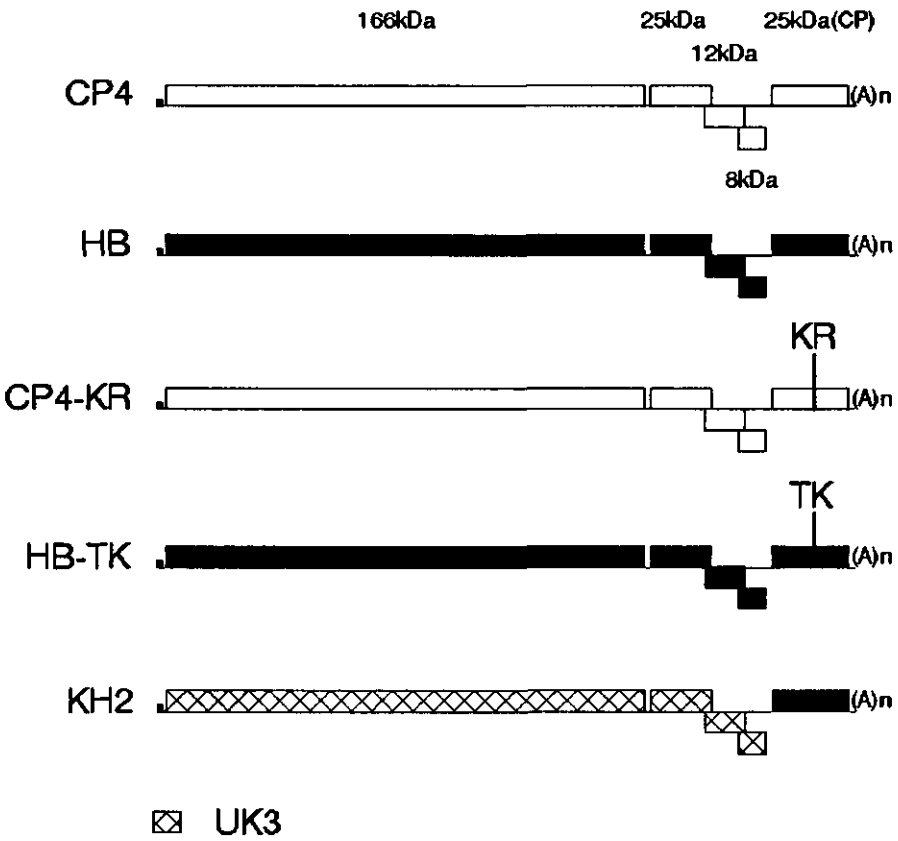

Fig. 7.1. Gene organization in potato virus X indicating approximate size of the five open reading frames (ORFs), and schematic representation of the recombinant and mutant isolates used. CP4 = wild type PVX_{CP4} isolate from resistance group 4. HB = wild type resistance-breaking strain PVX_{HB}. CP4-KR = mutant isolate obtained in PVX_{CP4} cDNA by replacement within the coat protein of amino acids 121 and 127 with the corresponding ones from PVX_{HB}. HB-TK = mutant isolate of PVX_{HB} in which amino acids 121 and 127 within the coat protein have been replaced with the corresponding ones from PVX_{CP4}. KH2 = recombinant isolate from PVX_{UK3} in which the entire coat protein (CP) gene and 3' non-coding sequence have been substituted with the corresponding region from PVX_{HB}. For references see Table 7.1.

which amino acids 121 and 127 of the viral coat protein are threonine (T) and lysine (K) respectively instead of lysine (K) and arginine (R), behave similarly to PVX_{CP4}. As shown in Table 7.3 and summarized in Fig. 7.2 the behaviour of these two mutant isolates, previously tested on var. Cara (Rx) was confirmed on all genotypes tested carrying resistance genes from different *Solanum* species. While PVX_{HB} infection was symptomless in all genotypes tested, the mutant isolate CP4-KR induced a very mild mosaic in most genotypes, best visible on vars. Atlantic and Bzura. Genotypes infected with the recombinant clone KH2 developed a more defined and readily visible mosaic.

A selection of wild *S. acaule* accessions was included in a further experiment (Table 7.3, Exp. 2). Accessions OCH 11818, OCH 11823, OCH 11825, OCH 11912, and OCH 11983, collected in different locations in Bolivia, as well as the Peruvian accessions OCH 11889 and OCH 11890, previously tested, were considered immune to PVX (L.F. Salazar, unpublished results). The Peruvian accessions OCH 14391 and OCH 14392 were included as susceptible controls. Again, systemic infection was obtained in all accessions inoculated with strain HB as well as with the recombinant clone KH2 and the mutant isolate CP4-KR.

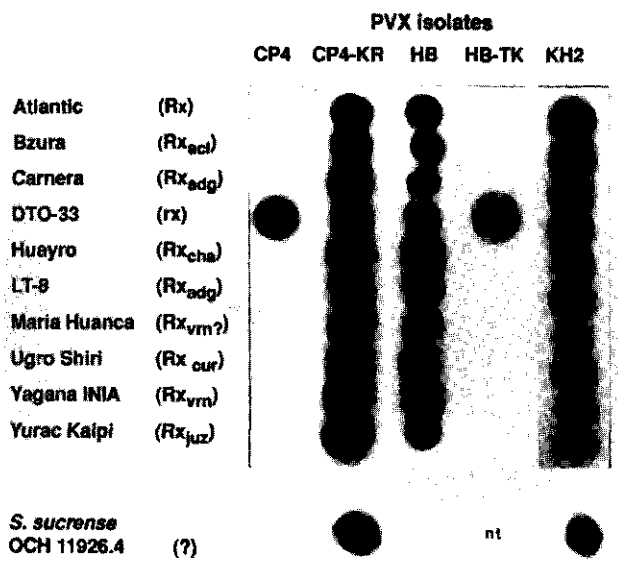

Fig. 7.2. Infectivity of wild and mutant potato virus X isolates on potato genotypes expressing extreme resistance to PVX. CP4 = wild type PVX_{CP4} isolate from resistance group 4. HB = wild type resistance-breaking strain PVX_{HB}. CP4-KR = mutant isolate obtained in PVX_{CP4} cDNA by replacement within the coat protein of amino acids 121 and 127 with the corresponding ones from PVX_{HB}. HB-TK = mutant isolate of PVX_{HB} in which amino acids 121 and 127 within the coat protein have been replaced with the corresponding ones from PVX_{CP4}. KH2 = recombinant isolate from PVX_{UK3} in which the entire coat protein gene and 3' non coding region have been substituted with the corresponding sequence from PVX_{HB}. For references see Table 7.1.

Table 7.3. Infectivity of wild strains and mutant derivatives of potato virus X tested on potato varieties, native cultivars and wild species carrying extreme resistance genes. PVX infection was determined by NCM-ELISA and NASH tests 3 and 4 weeks post inoculation. Results are indicated as systemically infected plants per inoculated plants; nt means not tested. Tendency of results here reported have been confirmed in an additional experiment similarly conducted. For detailed informations on plant materials used see Table 7.2. Detailed characteristics of viral isolates are given in the Materials and Methods section, for reference see Table 7.1.

Exp. 1	Gene	CP4	HB	CP4-KR	HB-TK	KH2
Atlantic	Rx	0/5	5/5	5/5	0/5	5/5
Bzura	Rx_{acl}	0/4	3/3	4/4	0/5	3/3
Carnera	Rx_{adg}	0/5	5/5	5/5	0/5	5/5
DTO-33	rx	5/5	5/5	5/5	5/5	5/5
Huayro	Rx_{chn}	0/5	5/5	5/5	0/5	5/5
LT-8	Rx_{adg}	0/5	5/5	5/5	0/5	5/5
Maria Huanca	$Rx_{vrn?}$	0/5	5/5	5/5	0/5	5/5
Ugro Shiri	Rx_{cur}	0/5	5/5	5/5	0/5	5/5
Yagana INIA	Rx_{vrn}	0/5	5/5	5/5	0/5	5/5
Yurac Kaipi	Rx_{jur}	0/5	5/5	5/5	0/5	5/5
Exp. 2						
OCH 11818	Rx_{acl}	0/4	4/4	4/4	0/4	4/4
OCH 11823	Rx_{acl}	0/4	4/4	4/4	0/4	4/4
OCH 11825	Rx_{acl}	0/4	4/4	4/4	0/4	4/4
OCH 11889	Rx_{acl}	0/4	4/4	4/4	0/4	4/4
OCH 11890	Rx_{acl}	0/4	4/4	4/4	0/4	4/4
OCH 11912	Rx_{acl}	0/4	4/4	4/4	0/4	4/4
OCH 11983	Rx_{acl}	0/4	3/4	4/4	0/4	3/4
OCH 14391	rx	3/3	1/3	1/2	2/3	3/3
OCH 14392	rx	nt	2/2	nt	nt	2/2
Exp. 3						
OCH 11926.4	?	0/7	0/7	4/7	nt	7/7

Isolates cp and CP4 infected only the susceptible accessions OCH 14391 and OCH 14392 and induced a mild mosaic in both accessions. The mutant isolate HB-TK also infected only the accessions susceptible for CP4, but infection was symptomless. Immune accessions, systemically infected with the two isolates of the HB strain, remained symptomless. The mutant isolate CP4-KR induced on susceptible and immune systemically infected accessions, a mild mosaic similar to the effects of CP4 on susceptible accessions. The hybrid clone KH2, which produces symptoms similar to PVX_{UK3} rather than PVX_{HB} on both tobacco and potato (Kavanagh *et al.*, 1992), produced a more severe mosaic in all infected accessions. These results indicate that the extreme resistance carried by all the different genotypes tested interact in the same way with the virus.

In addition, the wild *S. sucrense* accession OCH 11926, reported to be immune to PVX_{HB} (C. Chuquillanqui and L.F. Salazar, personal communication; Brown *et al.*, 1984) was included in a further experiment. In this case plants were grown from tubers which originated from the same plant (OCH 11926.4) and therefore were genotypically identical. For testing each viral strain or isolate seven plants were mechanically inoculated and maintained as indicated in the Materials and Methods section. Inoculated plants were assayed by NASH and NCM-ELISA tests 3 and 4 weeks post inoculation. As expected no infection of *S. sucrense* was obtained with strains cp, CP4 and HB. Surprisingly, however, the mutant isolate CP4-KR and the recombinant clone KH2 produced systemic infection in 4/7 and 7/7 inoculated plants, respectively (Table 7.3, Exp. 3). The presence of PVX particles in the non-inoculated leaves of these plants was also confirmed by electron microscopy. Furthermore, plants inoculated with the recombinant clone KH2 developed a necrotic response. Necrotic spots appeared 10 days after inoculation first on inoculated leaves (Fig. 7.3). Necrosis spread to other parts of the plants and general necrosis led to death of all the plants in approximately 4 weeks after inoculation. Two weeks after inoculation no visible symptoms were present on the CP4-KR infected plants but later they also developed general necrosis resulting in death of plants. However, two of the four plants infected, in which PVX was present at lower concentration, could recover and developed a mild mosaic in the young leaves produced.

The symptom expression of the PVX strains and mutants was analyzed on a broad range of indicator plants. Mechanically inoculated indicator plants (Table 7.4) were visually analyzed daily for a total period of one month and thereafter tested by NASH for systemic presence of each PVX isolate. The results obtained are summarized in Table 7.4. Isolate

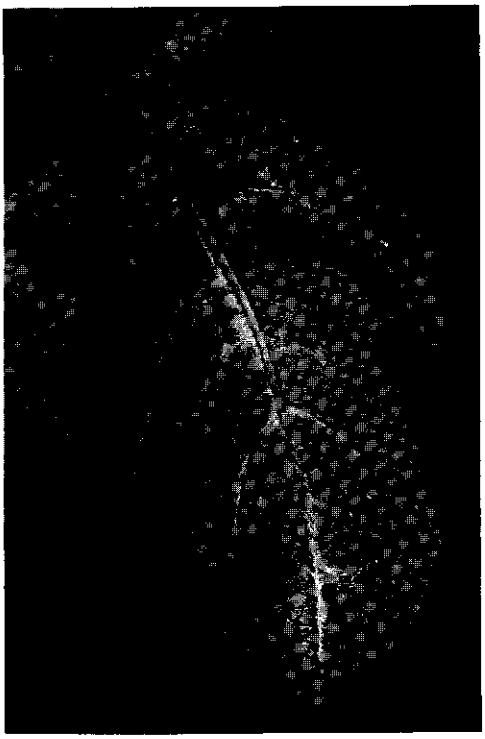

Fig. 7.3. Symptoms induced by the PVX recombinant isolate KH2 (Kavanagh *et al.*, 1992) on the *S. sucrense* accession OCH 11926.4. Necrotic spots appeared first on inoculated leaves about 10 days after inoculation. Necrosis spread then to other parts and led to death of the plant.

CP4 determined in general visible and quite severe symptoms in most indicator plants tested, including local necrotic spots with well defined red rings on inoculated leaves of *Gomphrena globosa* L. (Fig. 7.4). On the contrary, both HB isolates induced a very mild reaction, the symptoms produced by the CIP isolate being more defined and best visible (Fig. 7.5 and Table 7.4). As previously reported (Moreira *et al.*, 1980) and repeatedly confirmed, the HB strain does not produce local lesions on *G. globosa* leaves (Fig. 7.4). Analysis on symptoms production of the two mutant isolates CP4-KR and HB-TK showed that, with exception of *G. globosa*, all indicator plants tested developed symptoms which were indistinguishable from the original parental strains from which the mutants were derived (Figs. 7.5 and 7.6). Indeed symptom production determinants for almost all indicator plants were not affected by the mutations. On the contrary *G. globosa*, which is the only indicator plant available to clearly distinguish PVX_{HB} from all other PVX strains, seems to be susceptible to the mutations induced in the coat protein gene. Indeed, while isolate CP4 developed well defined necrotic spots 5-6 days post inoculation, the mutant CP4-KR did not. Similarly, mutant HB-TK behaved on this host as a common PVX strain,

Table 7.4. Symptoms induced on indicator plants by PVX strains CP4, HB and mutant isolates CP4-KR, HB-TK, and KH2.

Indicator plant	CP4	CP4-KR	HB	HB (CIP)	HB-TK	KH2
Amaranthus caudatus	LCS	LCS	-	-	-	LCS
A. edulis	-	LCS	-	-	-	LCS
Gomphrena globosa	LNS+red rings	late LNS no red rings, SI	-	-	LNS+ red rings	late LNS no red rings
Chenopodium amaranticolor	LCS, few SCS	LCS, SCS	mild LCS	LCS, SCS	mild LCS, few SCS	LCS, SCS
C. murale	LNS	LNS	-	-	-	LNS
C. quinoa	LCS, SCS	LCS, SCS	LCS	LCS	LCS, SCS	LCS, SCS
Helianthus annuus	MM, LCR	LCR	-	-	-	-
Cucumis sativus	-	-	-	-	-	-
Datura metel	SM, SNS, ND	SM, SNS, ND	very MM, LNS, LNS, SVC	MM, LNS, SVC	very MM, LNS	SM, SNS, ND
D. stramonium	SM, SNS, SNR, Df	SM, SNS, SNR, Df	MM	MM	MM	SM, SNS, Df
Lycopersicon esculentum cv. Rutgers	SM, SVC	SM, SVC	-	MM	MM	SM
Nicandra physaloides	SM, SNR	SM, SNR	very MM	very MM	very MM	SM, few SNS
Nicotiana benthamiana	SM, AN, SNS, Df	SM, AN, SNS, Df	MM, SCS	MM, SCS	MM, SCS	SM, AN, SNS, Df
N. clevelandii	SM, SVN	SM, SVN	MM	MM	MM	SM
N. debneyi	SCR	SM, SCR, SVN	MM	MM, SVN	MM	SM, SVN
N. glutinosa	SM, SVC, SNS	SM, SVC, SNS	very MM	MM	very MM	SM, SNS
N. occidentalis	SM, SVN	SM, SVN	SCR	MM	MM	SM, SVN
N. rustica	SCR	SCR	mild SCR	mild SCR	mild SCR	SM, SCR
N. tabacum cv. Samsun	SNS, SNR, SCS	SNS, SNR, SCS	SS	mild SCR	SS	SNS, SNR
Physalis floridana	SM, SVC, SNR	SM, SVC, SNS	SS	very MM	SS	SM, SVC, SNS
Coriandrum sativum	SS	SS	-	-	SS	-
Phaseolus vulgaris	-	-	-	-	-	-

LCS, local chlorotic spots; LCR, local chlorotic rings or ringspots; LNS, local necrotic spots; LNR, local necrotic rings or ringspots; MM, mild mosaic; SM, severe mosaic; SCS, systemic chlorotic spotting; SNS, systemic necrotic spots; SNR, systemic necrotic rings or ringspots; SVC, systemic vein clearing; SS, symptomless systemic infection; SI, systemic infection; Df, systemic leaf deformation; ND, necrotic defoliation; SVN, systemic vein necrosis; AN, apical necrosis; -, no symptoms, no systemic infection.

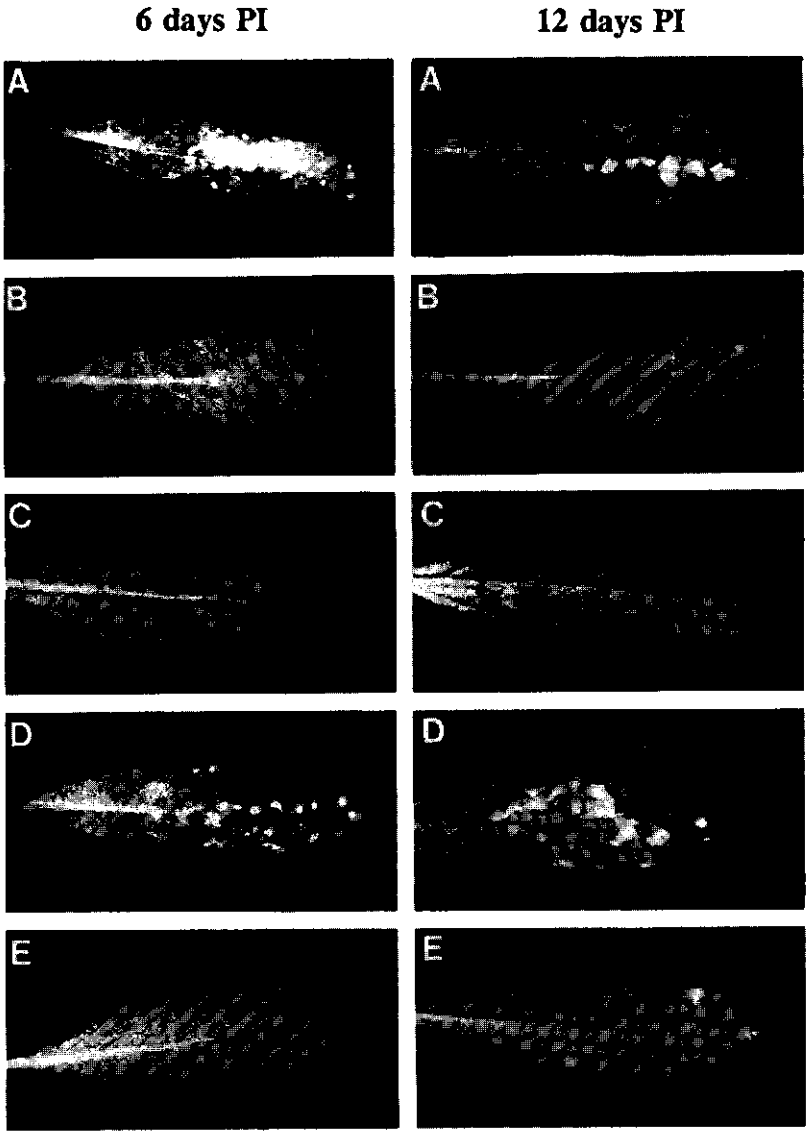

Fig. 7.4. Symptoms induced on inoculated leaves of *Gomphrena globosa* by wild and mutant isolates of PVX, 6 and 12 days post inoculation (PI). A: PVX_{CP4}; B: $PVX_{CP4\text{-}KR}$; C: PVX_{HB}; D: $PVX_{HB\text{-}TK}$; E: PVX_{KH2}.

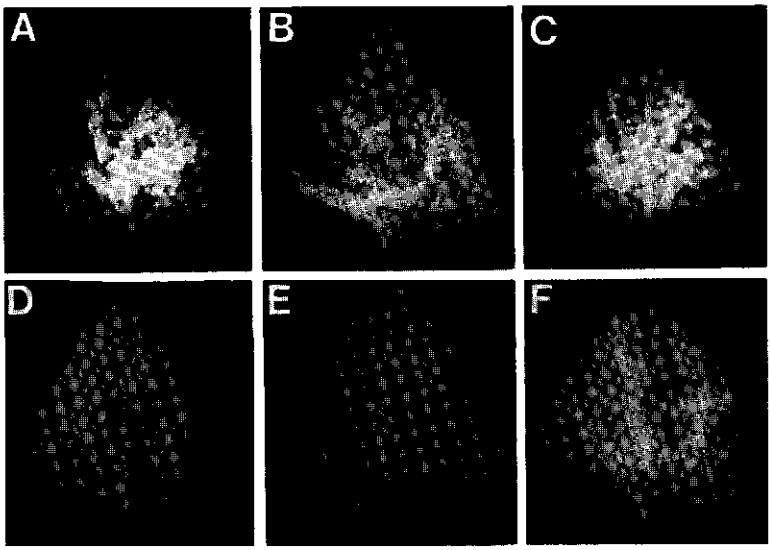

Fig. 7.5. *Chenopodium amaranticolor* leaves inoculated with PVX wild and mutant isolates showing local chlorotic spots. A: PVX_{CP4}; B: $PVX_{CP4\text{-}KR}$; C: PVX_{KH2}; D: PVX_{HB}; E: $PVX_{HB\text{-}TK}$; F: $PVX_{HB(CIP)}$.

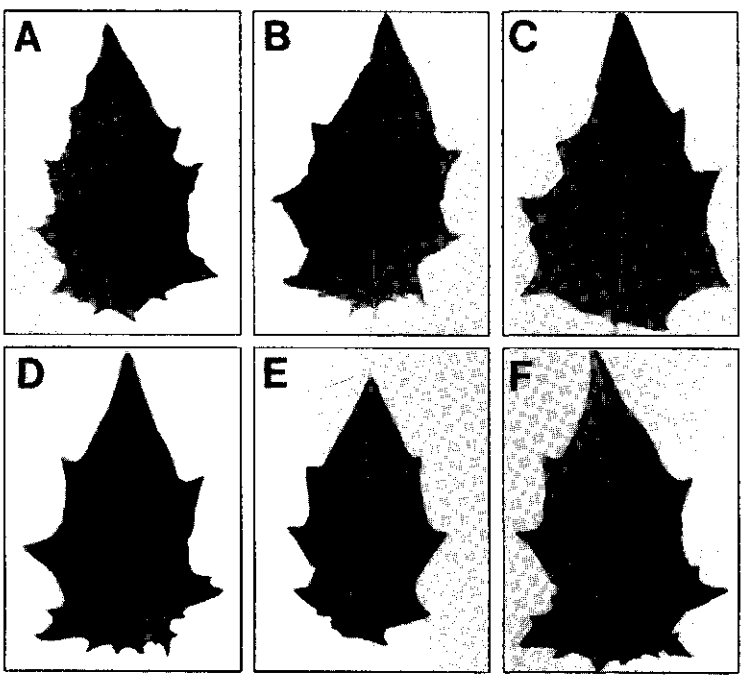

Fig. 7.6. Non inoculated leaves of *Datura stramonium* plants infected with PVX wild and mutant isolates. A: PVX_{CP4}; B: $PVX_{CP4\text{-}KR}$; C: PVX_{KH2}; D: PVX_{HB}; E: $PVX_{HB\text{-}TK}$; F: $PVX_{HB(CIP)}$.

producing local necrotic spots with well defined red rings, while the wild strain HB did not produce any symptoms.

The involvement of non-coat protein determinants in symptom development on most indicator plants and of the coat protein in the case of *G. globosa*, has also been confirmed with KH2. This recombinant clone developed in all indicator plants tested severe symptoms similar to CP4, but behaved on *G. globosa* as HB, in that it did not produce local lesions. However, some necrotic spots, not well defined and without typical red rings, appeared approximately 2 weeks after inoculation on *G. globosa* leaves inoculated with the mutant isolates CP4-KR and KH2 (Fig. 7.4).

DISCUSSION

After penetration into a host plant, viruses may activate resistance genes which initiate different types of resistance reactions. Despite the considerable attention in recent years, the primary products of resistance genes, and the way in which these products interact with viruses, have remained essentially unknown.

The importance of the viral coat protein in resistance and symptoms expression has been shown for several virus-plant combinations (Culver and Dawson, 1989; Petty and Jackson, 1990; Neeleman *et al.*, 1991; Shintaku *et al.*, 1992). Kavanagh *et al.* (1992) demonstrated that also in the case of PVX the coat protein gene plays an important role in viral pathogenicity and, analyzing different hybrid PVX genomes including elements of PVX_{UK3} and PVX_{HB}, located the resistance-breaking properties of PVX_{HB} in the coat protein gene. In an attempt to establish more precisely the exact location of the deteminants in the PVX coat protein sequence responsible for the ability fo HB to overcome immunity, Querci *et al.* (1993b) compared the nucleotide and amino acid sequence of PVX_{HB} with those reported for three non resistance-breaking strains of PVX (PVX_{X3}, Huisman *et al.*, 1988; PVX_{S}, Skryabin *et al.*, 1988; PVX_{cp}, Orman *et al.*, 1990). This comparison highlighted a high similarity in the coat protein sequences with only few differences that were limited to a small number of residues. Out of the eight amino acid residues unique for the HB coat protein sequence, only two determined variation in the coat protein structure, i.e. K_{121} and A_{226}. In that work it was assumed that one, or both, of these amino acid residues was responsible for the biological differences between PVX_{HB} (Rx-breaking) and the other

strains (non-breaking). Using a series of hybrid and mutant isolates of PVX_{HB} and PVX_{CP4}, Goulden *et al.* (1993) determined that elicitation of the resistance was affected only by amino acids 121 and 127 of the viral coat protein. PVX_{HB} and hybrid or mutant isolates with lysine and arginine at positions 121 and 127 were able to overcome resistance expressed on var. Cara, whereas those with threonine and arginine were resistance sensitive. In that work, and in accordance with the prediction of Querci *et al.* (1993b), it was also pointed out that position 121 is the major determinant in the resistance-breaking activity, while the importance of residue 127 is suggested to be linked to stability of the mutants. In this work we analyzed the recombinant clone KH2 (Kavanagh *et al.*, 1992) and two mutant isolates, CP4-KR and HB-TK (Goulden *et al.*, 1993), and tested their ability to overcome the effects of Rx genes originating from different *Solanum* species. From the results obtained in this work it can be concluded that the resistance-breaking capacity determined by the two amino acid residues in positions 121 and 127 within the HB coat protein gene are also active on all Rx genes tested (Rx, Rx_{acl}, Rx_{adg}, Rx_{cha}, Rx_{cur}, Rx_{juz}, and Rx_{vrn}). Hence, the mechanism of recognition (of the PVX coat protein) is the same for all these genes considered (Table 7.3; Fig. 7.2).

The various primitive cultivated species and wild species represent diverse gene-pools and possess great variability for many traits, moreover they are and have been indispensable sources for resistance to pathogens (Ross, 1986). Genes from several wild species have been incorporated in the genome of many cultivars even if at present only a small portion of the valuable genes found (or present) in wild species has been used. Most of this breeding for resistance has been done mostly at an empirical level, without detailed knowledge of the genetic basis. In the specific case of PVX, the exact relationship between the different Rx genes incorporated in various potato varieties was not known.

Here we can identify extreme resistance found in *S.* x *chaucha*, *S.* x *curtilobum*, *S.* x *juzepczukii*, and *S. vernei* as carried by Rx genes (Rx_{cha}, Rx_{cur}, Rx_{juz}, and Rx_{vrn}, respectively) which interact with the PVX coat protein in the same manner as Rx (USDA 41956), Rx_{acl}, and Rx_{adg}, do.

Symptoms produced on *N. glutinosa* and *N. clevelandii* by the recombinant clone KH2 and the mutant isolates CP4-KR and HB-TK resembled symptoms produced by the PVX strain from which they were prepared (UK3, CP4, and HB, respectively). The substitutions or alterations induced within the coat protein gene did not affect symptom production on these hosts. For that reason, we analyzed a broad range of indicator plants and compared

the symptoms produced by the mutant isolates and by the wild type PVX strains (Table 7.4). In all cases, except for *G. globosa*, it was confirmed that alterations produced in the coat protein gene did not affect or alterate symptoms production (Figs.7.5 and 7.6). *G. globosa* is the only indicator plant available at present, to clearly distinguish PVX_{HB} from all other strains of PVX. As indicated by Goulden *et al.* (1993) the coat protein and specifically residues 121 and 127 of the coat protein, are involved also in this characteristic of HB. Indeed the ability to overcome resistance is associated to a lack of local lesion production on inoculated leaves of *G. globosa* (Fig. 7.4).

All PVX strains and mutant isolates KH2, and CP4-KR were tested on the *S. sucrense* accession OCH 11926 (genotype OCH 11926.4) reported to be immune to PVX_{HB}. In addition to the results obtained and summarized in Table 7.3 and Fig. 7.2, in an additional experiment, conducted to confirm the results, also the mutant isolate HB-TK was included. All previous results were confirmed, while in addition HB-TK was also found to infect OCH 11926.4 plants (data not shown). HB-TK infected plants produced very small necrotic lesions on inoculated leaves and again decay of plant fitness and early death of plants. The findings that both the recombinant clone KH2 and the mutant isolates CP4-KR and HB-TK, but not the parental isolates, are able to systemically infect this accession, opens up a new question on what kind of resistance mechanism is involved. As shown in Table 7.5, homologous combinations of coat protein genes and upstream sequences result in resistance. However, when the coat protein gene of HB is placed in a CP4/UK3 context, the resistance is broken. Reversely, a CP4 coat protein gene placed in a HB context, also lead to resistance breakage. Although at this time we cannot exactly define the mechanism of action of the resistance gene found in the *S. sucrense* accession OCH 11926.4, a first interpretation of the results suggest that in this case resistance or susceptibility is not determined by an interaction with a single determinant in the coat protein solely, but also by a second determinant, which maps outside the coat protein and 3' non-coding region.

It is also important to stress the high value of the resistance carried by this accession. OCH 11926 carries, in addition to immunity to PVX, other useful characteristics of resistance to several important viruses infecting potato. In inoculation experiments conducted (L.F. Salazar, unpublished results) this *S. sucrense* accession showed also resistance to potato leafroll virus (PLRV), hypersensitivity to potato virus Y (PVY), resistance to potato virus T (PVT) and potato virus S (PVS), and immunity to Andean potato mottle virus (APMV). Recently Tozzini *et al.* (1993) reported an additional source

Table 7.5. Reaction of *S. sucrense* OCH 11926.4 with wild strains and mutant isolates of PVX. R = resistance; s = susceptibility; nt = not tested.

PVX strain/mutant	5' region characteristics	coat protein origin	coat protein aa. 121	reaction of *S. sucrense* OCH 11926.4
cp	cp	cp	T	R
CP4	CP4	CP4	T	R
HB	HB	HB	K	R
UK3	UK3	UK3	T	nt
CP4-KR	CP4	CP4	K	s
KH2	UK3	HB	K	s
HB-TK	HB	HB	T	s

of resistance to PVX_{HB} found in *S. commersonii* and tentatively defined this new type of resistance as IRx_{com}. At present it is not known if resistance induced by this IRx gene acts similarly to that found in the *S. sucrense* accession OCH 11926. Knowledge of the genetic basis of this resistance, and of the complementary genetic system controlling behaviour of viral pathogens, and of their interaction, could provide clues to understand the mechanism of operation. Further studies are needed to specifically characterize the novel *S. sucrense* gene. The use of specific mutant isolates and approach followed in this work could help in the further understanding of the mechanism of resistance involved.

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REFERENCES

Adams, S.E., Jones, R.A.C., and Coutts, R.H.A. (1984). Occurrence of resistance-breaking strains of potato virus X in potato stocks in England and Wales. *Plant Pathology* **33**: 435-437.

Akeley, R.V., Stevenson, F.J., Schultz, E.S., Bonde, R., Nielsen, K.F., and Hawkings, A. (1955). Saco: A new late-maturing variety of potato, immune from common race of the late blight fungus, highly resistant to if not immune from net necrosis, and immune from mild and latent mosaic. *American Potato Journal* **32**: 41-48.

Brown, C.R., Salazar, L.F., Ochoa, C., and Chuquillanqui, C. (1984). Strain-specific immunity to PVX_{HB} is controlled by a single dominant gene. *9th Triennial Conference of the European Association for Potato Research*, pp. 249-250, Interlaken.

Chapman, S., Hills, G., Watts., J., and Baulcombe, D. (1992). Mutational analysis of the coat protein gene of potato virus X: Effects on virion morphology and viral pathogenicity. *Virology* **191**: 223-230.

Cockerham, G. (1955). Strains of potato virus X. *Proceedings of the Second Conference on Potato Virus Diseases*, pp. 89-92. Lisse-Wageningen, 1954.

Cockerham, G. (1970). Genetical studies on resistance to potato viruses X and Y. *Heredity* **25**: 309-348.

Culver, J.N., and Dawson, W.O. (1989). Point mutations in the coat protein gene of tobacco mosaic virus induce hypersensitivity in *Nicotiana silvestris*. *Molecular Plant-Microbe Interactions* **2**: 209-213.

Espinoza, N., Lizárraga, R., Silva-Rodriguez, D., Buitrón, F., Bryan, J., and Dodds, J.H. (1989). Tissue culture micropropagation, conservation, and export of potato germplasm. *CIP Research Guide 1*. International Potato Center, Lima, Peru. 22 pp.

Fribourg, C.E. (1975). Studies on potato virus X strains isolated from Peruvian potatoes. *Potato Research* **18**: 216-226.

Goulden, M.G., Köhm, B.A., Santa Cruz, S., Kavanagh, T.A., and Baulcombe, D.C. (1993). A feature of the coat protein of potato virus X affects both induced virus resistance in potato and viral fitness. *Virology* **197**: 293-302.

Huisman, M.J., Linthorst, H.J.M., Bol, J.F., and Cornelissen, B.J.C. (1988). The complete nucleotide sequence of potato virus X and its homologies at the amino acid level with various plus-stranded RNA viruses. *Journal of General Virology* **69**: 1789-1798.

International Potato Center (CIP) (1993). Enzyme-linked inmunosorbent assay on nitrocellulose membranes. In Jayasinghe U. and Salazar L.F. (eds) *Basic techniques in plant virology*. CIP, Lima, Peru. Fasc. **3.3.7**. 3 p.

Jones, R.A.C. (1985). Further studies on resistance-breaking strains of potato virus X. *Plant Pathology* **34**: 182-189.

Kavanagh, T., Goulden, M., Santa Cruz, S., Chapman, S., Barker, I., and Baulcombe, D. (1992). Molecular analysis of a resistance-breaking strain of potato virus X. *Virology* **189**: 609-617.

Köhm, B.A., Goulden, M.G., Gilbert, J.E., Kavanagh, T.A., and Baulcombe, D.C. (1993). A potato virus X resistance gene mediates an induced, nonspecific resistance in protoplasts. *Plant Cell* **5**: 913-920.

Lizárraga, C., and Fernandez-Northcote, E.N. (1989). Detection of potato viruses X and Y in sap extracts by a modified indirect enzyme-linked immunosorbent assay on nitrocellulose membranes (NCM-ELISA). *Plant Disease* **73**: 11-14.

Mills, W.R. (1965). Inheritance of immunity to potato virus X. *American Potato Journal* **42**: 294-295.

Moreira, A., Jones, R.A.C., and Fribourg, C.E. (1980). Properties of a resistance-breaking strain of potato virus X. *Annals of Applied Biology* **95**: 93-103.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiological Plantarum* **15**: 473-497.

Neeleman, L., Van der Kuyl, A.C., and Bol, J.F. (1991). Role of alfalfa mosaic virus coat protein gene in symptom expression. *Virology* **181**: 687-693.

Orman, B.E., Celnik, R.M., Mandel, A.M., Torres, H.N., and Mentaberry, A.N. (1990). Complete cDNA sequence of a South American isolate of potato virus X. *Virus Research* **16**: 293-306.

Petty, I.T.D., and Jackson, A.O. (1990). Mutational analysis of barley stripe mosaic virus RNA ß. *Virology* **179**: 712-718.

Querci, M., Salazar, L.F., and Fernandez-Northcote, E.N. (1993a). Detection of Andean potato virus X isolates by radioactive and nonradioactive nucleic acid spot hybridization tests. *Phytopathology* **83**: 171-176.

Querci, M., van der Vlugt, R., Goldbach, R., and Salazar, L.F. (1993b). RNA sequence of potato virus X strain HB. *Journal of General Virology* **74**: 2251-2255.

Ritter, E., Debener, T., Barone, A., Salamini, F., and Gebhardt, C. (1991). RFLP mapping on potato chromosomes of two genes controlling extreme resistance to potato virus X (PVX). *Molecular and General Genetics* **227**: 81-85.

Ross, H. (1954). Die vererbung der "Immunität" gegen das X-virus in tetraploidem *Solanum acaule*. *Caryologia* suppl. **6**: 1128-1132. *Proceedings of the 9th International Congress of Genetics*, Bellagio, 1953.

Ross, H. (1983). Major and minor genes in breeding virus resistant varieties. In Hooker W.J. (ed) *Research for the potato in the year 2000*, pp.165-166. International Potato Center, Lima.

Ross, H. (1986). Potato breeding - Problems and perspectives. In Horn W. and Robbelen G. (eds) *Advances in plant breeding, vol.13*. Verlag Paul Parey, Berlin. 132 pp.

Santa Cruz, S., Baulcombe, D.C., and Kavanagh, T. (1989). Virulence and virulence determinants in potato virus X. *Annual Report IPSR/JII/SL*. pp. 43-44.

Schultz, E.S., Clark, C.F., Bonde, R., Raleigh, W.P., and Stevenson, F.J. (1934). Resistance of potato to mosaic and other virus diseases. *Phytopathology* **24**: 116-132.

Schultz, E.S., and Raleigh, W.P. (1933). Resistance of potato to latent mosaic. *Phytopathology* **23**: 22.

Shintaku, M.H., Zhang, L., and Palukaitis, P. (1992). A single amino acid substitution in the coat protein of cucumber mosaic virus induces chlorosis in tobacco. *The Plant Cell* 4: 751-757.

Skryabin, K.G., Kraev, A.S., Morozov, S.Yu., Rozanov, M.N., Chernov, B.K., Lukasheva, L.I., and Atabekov, J.G. (1988). The nucleotide sequence of potato virus X RNA. *Nucleic Acids Research* **16**: 10929-10930.

Tozzini, A., Cramer, P., Ceriani, H.F., and Hopp, H.E. (1993). Interaction between potato virus X virulent strains and resistance genes. *Abstracts Conference papers of the 6th International Congress of Plant Pathology*, pp. 306, Montreal, 1993.

Webb, R.E., Wilson, D.R., Shumaker, J.R., Graves, B., Henninger, M.R., Watts, J., Frank, J.A., and Murphy, H.G. (1978). Atlantic: A new potato variety with high solids, good processing quality, and resistance to pests. *American Potato Journal* **55**: 141-145.

Wiersema. H.T. (1961). Methods and means used in breeding potatoes with extreme resistance to virus X and Y. *Proceedings of the 4th conference on potato virus diseases*, pp. 30-36. Braunschweig, 1960.

Chapter 8

Summary and concluding remarks

One of the principal threats to potato production is the high susceptibility of this food crop to diseases, the causal agents including bacteria, fungi, mycoplasmas, nematodes, viruses and viroids. In particular, close to 30 different viruses, and one viroid, are known to infect potato worldwide (Salazar, 1990). Most, if not all, potato viruses are tuber-perpetuated through clonal propagation, while only few of them (including the viroid PSTVd), are known to be transmitted also through true potato seeds.

Potatoes are usually propagated by planting potato seed pieces or sets, or whole tubers. This method of propagation lends itself to the introduction of pathogens from one area to another and to the overwintering of pathogens in the tubers stored for propagative purposes. In particular, in many tropical and sub-tropical countries, yields of potatoes are very low as a result of several factors, but poor quality of the seeds used is one of the main limiting factors. For that reason, sanitation, systems of seed certification, and quarantine programs invest a great importance in attempt to control the spread of potato viruses. The use of virus-free seed potatoes may control at least initially the spread of the viruses, however, it seems to be difficult, if not impossible, to maintain stocks completely free of viruses, especially those viruses which are easily transmitted. Potato virus X (PVX), for example, has a tendency to build up rapidly in seed stocks if not checked regularly (Manzer *et al.*, 1975).

For inspection and certification, rapid and reliable tests for potato viruses are essential. In the case of some crops, such as potatoes, and in the analysis of seed samples, these procedures should allow daily assays of a large number of samples. To apply the techniques in viral seed health evaluation in developing countries it is important to stress the needs of

poorly-equipped laboratories for tests which are cheap, simple and labour-saving yet reliable. Further, the sensitivity should be high enough to reliably detect low levels of the disease agent as well as the various strains that may occur in the field.

The current interest in virus and viroid detection techniques and the enormous scope for their application both as research tools and in practical large-scale routine testing led to the development of several procedures and methods now widely used. It is essential to point out the importance of a reliable detection of a specific virus (independently from the strain) for the institutions which include in their mandate the distribution of kits for/and the testing of viruses in different areas in the world.

The more specific diagnostic methods fall into two classes: the immunological approaches rely on the use of antibodies, usually prepared against the viral coat protein, the second involves the use of hybridization analysis. In the latter case complementary DNA prepared from the purified viral or viroid nucleic acid, or a recombinant DNA clone of that nucleic acid, is incubated with plant extracts to test for the presence of viral or viroid nucleic acid by the formation of a highly specific cDNA:viral nucleic acid hybrid (Chapter 2).

Up to now most nucleic acid probes have been prepared by radioactive labeling which has obvious disadvantages for routine use. However, an increasing number of nonradioactive labeling systems have been developed and the most relevant ones are discussed in Chapter 3. Most of these involve enzymes to give color reactions. A limitation of nonradioactive approaches for large-scale practical application is still the interference by components of plant sap. The sensitivity of detection, however, is often similar to that of the most commonly used serological methods. As well as for detection of viruses in plant samples, nucleic acid hybridization can also be used to assess relationships between viruses.

Potato virus X (PVX), is the type member of the genus *Potexvirus*. PVX is known also as potato latent virus (Miller and Polland, 1977), potato mottle virus, potato virus B, healthy potato virus, and potato mild mosaic virus (Harrison, 1971; Smith, 1972). PVX is of worldwide distribution and it is considered as the most common virus infecting potato (Schultz and Bonde, 1944). Since the potato originated in the Andean region of South America and was later introduced to Europe and other continents, it has been proposed that potato viruses, like PVX, were also spread from this region into other areas of potato production and that the divergences observed among the different virus strains and isolates could reflect adaptation to different *Solanum* varieties. In the case of PVX several studies

have been conducted on variability and distribution of the different strains and on the consequent (in)ability of the techniques currently used to detect all variants. Torrance *et al.* (1986) found a large degree of serological variation among PVX strains (which could be divided into 4 main serological groups or "serogroups"), they also concluded that serological reactions do not always correlate with the specific classification (Cockerham, 1955) based on resistance genes. Strains of PVX have also been distinguished as PVX^{A} serotype (found only in South America), and the PVX^{O} or common serotype, found elsewhere (Fernandez-Northcote and Lizárraga, 1991). It has been shown that differences found in the coat protein gene (which determines serological variability or similarity between strains) do not necessary correspond to similar divergences in other regions of the viral genome (Chapter 4).

In Chapter 4 two selected probes, pX61, derived from PVX strain cp (serotype PVX^{A}) and probe pPVX19, derived from an European strain-group 3 isolate (serotype PVX^{O}) were used and their specificity tested on a broad range of PVX isolates and strains. Probe pX61 corresponded to the region between nucleotides 3008 and 4107 of the genomic PVX_{cp} RNA, probe pPVX19 corresponded to the region between nucleotides 2909 and 3845 in the PVX genome, thus comprising a large portion of overlap. Both probes were derived from a highly conserved region in the 3' end of the PVX ORF1, sharing an overall homology of about 78%. In general, probe pX61 detected strain cp (homologous strain, PVX^{A}) and HB (also PVX^{A} serotype) with a strong signal, but poorly isolates belonging to serotype PVX^{O}. On the contrary, probe pPVX19 detected the isolates belonging to serotype PVX^{O} readily, and cp and HB poorly. However, several PVX^{O} isolates, mostly from Bolivia, reacted with this probe as weakly as PVX^{A} isolates did and were more readily detected by probe pX61. Despite the use of two probes derived from the highly conserved region in the 3' end of the PVX ORF1, a high specificity in the detection was observed, as demonstrated by the ability of pX61 and pPVX19 to detect isolates belonging to the serotype from which they were prepared or closely related isolates. The results suggest the existence of variability in the nucleotide sequence among the different PVX strains and isolates tested, even belonging to the same serotype and/or serogroup. The differences observed among PVX^{A} and PVX^{O} isolates stress the importance of using the appropriate technique for detection, especially for breeding programs for resistance, and quarantine purposes. As a next step towards the development of a universal probe for broad spectrum detection of PVX isolates, a chimaeric recombinant probe was prepared, consisting of sequences from both original probes pX61

and pPVX19. This recombinant probe (pX6119) gave a strong reaction (and therefore reliable detection) with all isolates tested so far (Chapter 5). This result indicates that recombinant probes, containing cDNA fragments from different strains of a virus (as shown here) or from different viruses, may become powerful tools which may find wide application.

The following part of my work, presented here in Chapters 6 and 7, was focussed on the characterization of a virulent strain of PVX. Different genes have been found in *Solanum* sp. conferring resistance to PVX. Hence, Cockerham (1955) classified known PVX strains into four groups on the basis of their interactions with the dominant resistance genes Nx and Nb, which, in cultivated varieties of potato, determine a hypersensitive response, and with the extreme resistance gene Rx. Group 1 strains are able to infect only susceptible plants. Several PVX strains, classified in groups 2 and 3, are able to overcome genes Nx and Nb, respectively. Finally, group 4 strains overcome both Nx and Nb but fail to infect plants carrying gene Rx. Only one strain, PVX_{HB}, isolated in Bolivia, has been reported to be able to overcome all Nx, Nb and Rx resistance genes (Moreira *et al.*, 1980). This strain is unique in that it causes typical PVX symptoms in most common indicator species but it does not produce local lesions in inoculated leaves of *Gomphrena globosa* which is the main indicator host for PVX.

PVX_{HB} is serologically very closely related to PVX_{cp} (Fribourg, 1975), the common Peruvian strain from the resistance group 2. Using polyclonal antibodies in diagnostic tests such as direct ELISA, latex agglutination or electron microscope serology it is not possible to differentiate between these two strains. Indeed, Torrance *et al.* (1986) could distinguish PVX_{HB} from other PVX isolates only using selected monoclonal antibodies. However, PVX_{HB} behaves on potato genotypes differently from this and all other known strains of PVX.

In Chapter 6 the genomic RNA of the strain HB was cloned, entirely sequenced, and analyzed. Sequence comparison of the two South American strains (PVX_{cp} and PVX_{HB}) at the nucleotide level revealed a high rate of homology in all ORFs, ranging between 95 and 98%.

The aim of the work was to localize, within the viral genomic sequence, the viral determinant(s) responsible of the ability of strain HB to overcome the resistance provided by the gene Rx, and to understand the mechanism involved. Some information on the involvement of the coat protein in resistance (breaking) and symptoms expression was

already available. Santa Cruz *et al.* (1989) showed with experimental data that the virulence determinants for the Rx gene are located in the 3' portion of the viral genome, outside the replicase gene. Kavanagh *et al.* (1992) demonstrated that in the case of PVX the coat protein gene plays an important role in viral pathogenicity and, analyzing different hybrid PVX genomes obtained by joining elements of PVX_{UK3} (a British group 3 non-breaking isolate) and PVX_{HB}, located the resistance-breaking properties of PVX_{HB} in the coat protein gene. Therefore, as shown in Chapter 6, the nucleotide and amino acid sequences of four PVX strains were compared, but particular attention was given to the coat protein gene. It was supposed that difference in behaviour between HB and the non resistance-breaking strains of PVX could be determined by differences in the secondary structure of a particular region of the coat protein. Coat protein sequence comparisons between HB and three non resistance-breaking strains of PVX, i.e. strains cp (Orman *et al.*, 1990), X3 (Huisman *et al.*, 1988), and S (Skryabin *et al.*, 1988) indicated the presence of eight amino acid residues "unique" for PVX_{HB}. Computer-directed mutational analysis and secondary structure predictions of the viral coat protein allowed the analysis of the possible effect of single amino acids changes in the predicted secondary structure of the coat protein. Only two positions (K_{121} and A_{226}) were found to determine variation in the protein structure and were therefore considered as being potentially involved in the resistance-breaking capacity of strain HB. When in the PVX_{HB} coat protein sequence the lysine (K, basic) at position 121 is substituted with a (cp-specific) threonine (T, polar) the alpha-helix at this position is converted into a beta-sheet structure as found in the coat protein structure of PVX_{cp}. The substitution at position 226 (alanine to valine) also determines a slight alteration of the prediction of the coat protein structure, although the modification is located in a highly hydrophobic region and might therefore be masked. Both amino acid substitutions at positions 121 and 226 lead to a reversion of the predicted HB-type coat protein structure to the cp-type coat protein structure.

In the meantime, Goulden *et al.* (1993) analyzed a series of hybrid and mutant isolates of PVX_{HB} and PVX_{CP4}, a group 4 isolate (Jones, 1985) and concluded that elicitation of extreme resistance expressed in the var. Cara (Rx) is affected by amino acids 121 and 127 of the viral coat protein. PVX_{HB} and hybrid or mutant isolates with lysine and arginine at positions 121 and 127 were able to overcome resistance expressed on var. Cara, whereas those with threonine and arginine were not. In that work, and in accordance with the prediction in Chapter 6, it was also pointed out that position 121 is the major determinant

in the resistance-breaking activity, while the importance of residue 127 is proposed to be linked to stability of the mutants.

Having been demonstrated that the coat protein, and specifically, only a single amino acid position (121) in the coat protein, affects extreme resistance in Rx genotypes, the interest was devoted to the analysis of a wider pool of Rx genes available. Indeed, wild tuber-bearing *Solanum* species possess great variability for many traits, having been indispensable sources for resistance to pathogens (Ross, 1986), but only a small portion of the valuable genes found in those species has been incorporated in the genome of commercial varieties. Resistance actually used in breeding programs is still often limited to only few proveniences. In addition, in the specific case of resistance to PVX, even the relationship between the different Rx genes incorporated in various potato varieties is essentially unknown.

To gain insight in this matter, in Chapter 7 a recombinant clone KH2 (Kavanagh *et al.*, 1992) and two mutant isolates, CP4-KR and HB-TK (Goulden *et al.*, 1993), were analyzed and tested for their ability to overcome the effects of various extreme resistance genes originating from different *Solanum* species. The experiments confirmed in all cases the involvement of amino acid 121 of the PVX coat protein in resistance breaking. Hence, the extreme resistance found in *S.* x *chaucha*, *S.* x *curtilobum*, *S.* x *juzepczukii*, and *S. vernei* as carried by Rx genes (Rx_{cha}, Rx_{cur}, Rx_{juz}, and Rx_{vrn}, respectively) interacts with the PVX coat protein in the same manner as Rx (USDA 41956), Rx_{acl}, and Rx_{adg}.

However, when the *S. sucrense* accession OCH 11926 (genotype used OCH 11926.4) reported to be immune to PVX_{HB} (C. Chuquillanqui and L.F. Salazar, personal communication) was inoculated with the same strains and mutant clones, it was found that the recombinant clone KH2 and the mutant isolates CP4-KR and HB-TK were able to systemically infect this genotype and induced a lethal necrosis. The finding that both the recombinant clone KH2 and the mutant isolates CP4-KR and HB-TK, but not the parental isolates, are able to systemically infect this accession, opens up a new question on what kind of resistance mechanism is involved. Although at this time is not possible to exactly define the mechanism of action of this gene, a first interpretation of the results suggests that in this case resistance or susceptibility is not determined only by an interaction with a specific determinant in the viral coat protein, but also by a second determinant, located outside the coat protein and 3' non-coding region.

Even though the involvement of the coat protein gene, in resistance (breaking) and symptom expression, has been confirmed in the case of various other plant-virus interactions (Culver and Dawson, 1989; Meshi *et al.*, 1989; Neeleman *et al.*, 1991; Shintaku *et al.*, 1992), it could also be supposed that other viral components are the recognition entity or play an important role in the recognition with the plant gene product(s).

Indeed, plant species and viruses of plants interact with each other in various ways which range from non-pathogenesis through to symptoms formation and virus multiplication. The interactions controlled by single genes are likely to involve discrete recognition events between appropriate gene products in the host and factors in the virus. Because of the small size of most viral genomes, and of their limited coding capacity, it is unlikely that any viral gene would be involved solely in the determination of virulence/avirulence. Therefore, a determinant is likely to be located in a gene for some other primary function, such as viral replication, assembling, movement, or transmission, but it could be also possible that the viral nucleic acid (and not a viral protein) could be the recognition entity. Fraser (1986) suggested that in different virus groups, virulence against the resistance genes of various hosts maps to various genomic segments, probably specifying a number of different functions. Examples of known cases in which changes in a viral gene, other than the coat protein gene, determine whether the virus exhibits virulence or avirulence against a host resistance gene, are found in the works of Meshi *et al.* (1988) and Yamafuji *et al.* (1991) in which it has been proposed that the product of the *Tm1* gene of tomato confers resistance to tobacco mosaic virus (TMV) by interacting with the virus-encoded replicase component, and in the work of Padgett and Beachy (1993) in which the induction of the N gene-mediated hypersensitive response by tomato mosaic virus (ToMV-Ob) is also supposed to be associated to the viral replicase.

In the case of the resistance expressed in the *S. sucrense* accession OCH 11926, further studies are needed to specifically characterize the gene and the mechanism involved. The approach followed in Chapter 6 and the use of additional mutants could lead to insight in the genetic basis of this resistance, and in the complementary genetic system of PVX and could provide clues to understand the mechanism of operation.

REFERENCES

Cockerham, G. (1955). Strains of potato virus X. *Proceedings of the Second Conference on Potato Virus Diseases*, pp. 89-92. Lisse-Wageningen, 1954.

Culver, J.N., and Dawson, W.O. (1989). Point mutations in the coat protein gene of tobacco mosaic virus induce hypersensitivity in *Nicotiana sylvestris*. *Molecular Plant-Microbe Interactions* **2**: 209-213.

Fernandez-Northcote, E.N., and Lizárraga, C. (1991). Distribución geográfica de serotipos del virus X de la papa. *Fitopatologia* **26**: 13-18.

Fraser, R.S.S. (1986). Genes for resistance to plant viruses. *CRC Critical Reviews in Plant Sciences* **3**: 257-294.

Fribourg, C.E. (1975). Studies on potato virus X strains isolated from Peruvian potatoes. *Potato Research* **18**: 216-226.

Goulden, M.G., Köhm, B.A., Santa Cruz, S., Kavanagh, T.A., and Baulcombe, D.C. (1993). A feature of the coat protein of potato virus X affects both induced virus resistance in potato and viral fitness. *Virology*. (*in press*).

Harrison, B.D. (1971). Potato viruses in Britain. In J.H. Western (ed) *Diseases of crop plants*, pp.123-159. Wiley, New York.

Huisman, M.J., Linthorst, H.J.M., Bol, J.F., and Cornelissen, B.J.C. (1988). The complete nucleotide sequence of potato virus X and its homologies at the amino acid level with various plus-stranded RNA viruses. *Journal of General Virology* **69**: 1789-1798.

Jones, R.A.C. (1985). Further studies on resistance-breaking strains of potato virus X. *Plant Pathology* **34**: 182-189.

Kavanagh, T., Goulden, M., Santa Cruz, S., Chapman, S., Barker, I., and Baulcombe, D. (1992). Molecular analysis of a resistance-breaking strain of potato virus X. *Virology* **189**: 609-617.

Manzer, F.E., McCrum, R.C., and Merriam, D.C. (1975). Testing Maine potato seed stocks in Florida for presence of virus X. *American Potato Journal* **52**: 137-141.

Meshi, T., Motoyoshi, F., Adachi, A., Watanabe, Y., Takamatsu, N., and Okada, Y. (1988). Two concomitant base substitutions in the putative replicase genes of tobacco mosaic virus confer the ability to overcome the effects of a tomato resistance gene, *Tm-1*. *The EMBO Journal* **7**: 1575-1581.

Meshi, T., Motoyoshi, F., Maeda, T., Yoshiwoka, S., Watanabe, H., and Okada, Y. (1989). Mutations in the tobacco mosaic virus 30-kD protein gene overcome *Tm-2* resistance in tomato. *The Plant Cell* **1**: 515-522.

Miller, P.R., and Polland H.L. (1977). *Multilingual compendium of plant diseases*. Vol. II. Phytopathol. Soc. St. Paul, Minnesota.

Moreira, A., Jones, R.A.C., and Fribourg, C.E. (1980). Properties of a resistance-breaking strain of potato virus X. *Annals of Applied Biology* **95**: 93-103.

Neeleman, L., Van der Kuyl, A.C., and Bol, J.F. (1991). Role of alfalfa mosaic virus coat protein gene in symptom expression. *Virology* **181**: 687-693.

Orman, B.E., Celnik, R.M., Mandel, A.M., Torres, H.N., and Mentaberry, A.N. (1990). Complete cDNA sequence of a South American isolate of potato virus X. *Virus Research* **16**: 293-306.

Padgett, H.S., and Beachy, R.N. (1993). Analysis of a tobacco mosaic virus strain capable of overcoming *N* gene-mediated resistance. *The Plant Cell* **5**: 577-586.

Ross, H. (1986). Potato Breeding - Problems and Perspectives. In Horn W. and Robbelen G. (eds) *Advances in plant breeding vol.13*. Verlag Paul Parey, Berlin. 132 pp.

Salazar, L.F. (1990). Main virus diseases of potato. In *Control of virus and virus-like diseases of potato and sweet potato. Report of the III planning conference*, pp. 9-12. International Potato Center, Lima, 1989.

Santa Cruz, S., Baulcombe, D.C., and Kavanagh, T. (1989). Virulence and virulence determinants in potato virus X. *Annual Report IPSR/JII/SL*. pp. 43-44.

Schultz, E.S., and Bonde, R. (1944). The effect of latent mosaic (virus X) on yield of potato in Maine. *The American Potato Journal* **21**: 278-283.

Shintaku, M.H., Zhang, L., and Palukaitis, P. (1992). A single amino acid substitution in the coat protein of cucumber mosaic virus induces chlorosis in tobacco. *The Plant Cell* **4**: 751-757.

Skryabin, K.G., Kraev, A.S., Morozov, S.Yu., Rozanov, M.N., Chernov, B.K., Lukasheva, L.I., and Atabekov, J.G. (1988). The nucleotide sequence of potato virus X RNA. *Nucleic Acids Research* **16**: 10929-10930.

Smith, K.M. (1972). *A textbook of plant virus diseases*, 3rd edn. Academic Press, New York.

Torrance, L., Larkins, A.P., and Butcher, G.W. (1986). Characterization of monoclonal antibodies against potato virus X and comparison of serotypes with resistance groups. *Journal of General Virology* **67**: 57-67.

Yamafuji, R., Watanabe, Y., Meshi, T., and Okada, Y. (1991). Replication of TMV-L and Lta1 RNAs and their recombinants in TMV-resistant *Tm-1* tomato protoplasts. *Virology* **183**: 99-105.

Samenvatting en conclusies

Ziekteverwekkers, waaronder bacteriën, schimmels, mycoplasma's, nematoden, virussen en viroïden, vormen een grote bedreiging voor de produktie van aardappelen. Aardappelplanten worden doorgaans vegetatief vermeerderd, hetgeen grote risico's met zich meebrengt m.b.t. de introductie, verspreiding en accumulatie van pathogenen in dit gewas. Tot op heden zijn bijna 30 verschillende virussen en één viroïde geïdentificeerd in aardappel, die wereldwijd belangrijke oogstverliezen veroorzaken.

Voor controle en certificering van pootaardappelen en aardappelzaad is het essentieel om over snelle en betrouwbare toetsen voor het aantonen van virussen te beschikken. Deze toetsen dienen geschikt te zijn voor het verwerken van vele monsters per dag. Voor ontwikkelingslanden komt daar nog bij dat deze toetsen goedkoop moeten zijn en simpel uit te voeren onder suboptimale (laboratorium)condities, zonder dat de betrouwbaarheid daaronder lijdt. Met de toetsen moeten alle stammen kunnen worden aangetoond die in het veld voorkomen. De gevoeligheid moet verder zo groot zijn dat ook lage concentraties van de ziekteverwekkers kunnen worden opgespoord.

De huidige interesse in detectietechnieken en in de toepassingsmogelijkheden ervan in grootschalige routine-testen en in het wetenschappelijk onderzoek heeft geleid tot de ontwikkeling van diverse technieken en methoden, die thans algemeen gebruikt worden. Hierbij kan onderscheid gemaakt worden in serologische technieken en moleculaire hybridisatietechnieken. De eerste categorie maakt gebruik van antilichamen, meestal opgewekt tegen het virale manteleiwit, de tweede categorie gaat uit van het principe dat viraal (en viroïd) nucleïnezuur detecteerbaar is door hybridisatie met een gemerkte, complementaire streng van dit nucleïnezuur. Zoals besproken in Hoofdstuk 2 wordt hiertoe meestal een (gekloneerd) complementair DNA-molekuul ("cDNA probe") gebruikt. Tot op heden worden deze cDNA probes in de meeste gevallen radioactief gemerkt, met alle

nadelen van dien voor routinematig gebruik. Gedurende de laatste jaren zijn echter een aantal niet-radioactieve detectiesystemen ontwikkeld, waarvan de meest relevante in Hoofdstuk 3 beschreven zijn. Meestal wordt hierbij gebruik gemaakt van enzymen die een (meetbare) kleurreactie teweegbrengen. De gevoeligheden die hierbij bereikt kunnen worden zijn vergelijkbaar met die van serologische technieken. Een beperking van zulke niet-radioactieve technieken voor grootschalige toepassing in de praktijk vormt nog steeds de interferentie door andere componenten in het te testen plante-extract.

In het experimentele werk dat in dit proefschrift beschreven is, staat het aardappelvirus X (PVX) centraal. Dit virus, dat het type-virus is van het geslacht *Potexvirus*, staat internationaal ook bekend onder de namen potato latent virus (Miller and Polland, 1977), potato mottle virus, potato virus B, healthy potato virus, en potato mild mosaic virus (Harrison, 1971; Smith, 1972). PVX heeft een wereldwijde verspreiding en wordt beschouwd als het meest voorkomende virus in aardappel (Shultz and Bonde, 1944). Omdat de aardappel oorspronkelijk afkomstig is uit het Andesgebied van Zuid-Amerika en later geïntroduceerd werd in Europa en andere werelddelen, zou men zich kunnen voorstellen dat aardappelvirussen, zoals PVX, eveneens vanuit dit gebied verspreid zijn naar andere regio's waar aardappels geteeld worden, en dat de variatie die tussen de verschillende PVX-stammen wordt waargenomen een gevolg is van aanpassing aan verschillende *Solanum*-varianten. Voor PVX zijn diverse studies gedaan naar de variabiliteit en verspreiding van verschillende stammen, en naar de daaruit voortvloeiende (on)mogelijkheid om met de huidige technieken alle varianten aan te tonen. Torrance *et al.* (1986) toonde een grote serologische variatie tussen PVX stammen aan (welke ingedeeld konden worden in 4 "serogroepen") en concludeerde dat deze serologische variatie niet samenvalt met de specifieke indeling van dit virus gebaseerd op interactie met resistentiegenen in de aardappel (Cockerham, 1955). PVX stammen zijn ook ingedeeld in PVX^{A} serotype (uitsluitend aangetroffen in Zuid-Amerika) en PVX^{O} serotype, elders voorkomend (Fernandez-Northcote and Lizárrage, 1991). Vastgesteld is bovendien, dat de mate van verschil in manteleiwit (het eiwit dat de serologische verschillen tussen stammen bepaalt) niet noodzakelijkerwijs correspondeert met de mate waarin de andere delen van het virale genoom verschillen (Hoofdstuk 4).

In Hoofdstuk 4 staan experimenten beschreven waarin twee verschillende cDNA probes, probe pX61 (afgeleid van PVX stam cp; serotype PVX^{A}) en probe pPVX19 (afgeleid van

een Europese "strain-group" 3 isolate; serotype PVX^{O}), werden getest op hun vermogen om een breed scala aan PVX-isolaten en -stammen aan te tonen.

Probe pX61 bevat een DNA-fragment corresponderend met de PVX_{cp}-RNA-regio tussen de nucleotiden 3008 en 4107, probe pPVX19 bevat een DNA-fragment corresponderend met de regio tussen nucleotiden 2909 en 3845 in het PVX genoom. Beide, in nucleotidenvolgorde overlappende probes, representeren het sterk geconserveerde gebied in het 3'-deel van het eerste open leesraam (ORF1) en vertonen een onderlinge homologie van 78%. Met probe pX61 kon zowel de homologe stam (cp, PVX^{A}) als stam HB (eveneens van serotype PVX^{A}) uitstekend aangetoond worden, maar isolaten van het serotype PVX^{O} reageerden slechts met een zwak signaal. Anderzijds kon met probe PVX19 verschillende isolaten van serotype PVX^{O} gevoelig aangetoond worden, maar de stammen cp en HB veel minder goed. Een aantal isolaten, met name isolaten afkomstig uit Bolivia, reageerde ook zeer slecht met deze probe, hoewel ze toch tot serotype PVX^{O} behoorden. Deze resultaten wijzen erop dat er nogal wat variabiliteit in nucleotidenvolgorde is, zelfs tussen isolaten en stammen die tot hetzelfde serotype behoren. De gevonden verschillen in aantoonbaarheid tussen de verschillende PVX^{A} en PVX^{O} isolaten onderstrepen het belang van toepassing van de juiste detectietechniek t.b.v. bijvoorbeeld veredelingsprogramma's en quarantaine-maatregelen.

Als een volgende stap naar de ontwikkeling van een universele probe, geschikt voor detectie van een breed spectrum van PVX-isolaten, werd een chimaere recombinant-probe gemaakt bestaande uit sequenties van zowel pX61 als van pPVX19. Deze recombinant probe (pX6119) bleek een sterk signaal (en derhalve een betrouwbare detectie) te geven met alle geteste isolaten (Hoofdstuk 5). Dit resultaat laat zien dat probes, waarin cDNA-fragmenten, afkomstig van het genoom van verschillende stammen van een virus of zelfs van verschillende virussen, een krachtig hulpmiddel kunnen zijn met een brede toepasbaarheid.

Na het ontwikkelen en testen van een aantal cDNA detectieprobes werd aandacht besteed aan de karakterisering van een agressieve stam van PVX, stam HB (Hoofdstukken 6 en 7). Er zijn verschillende genen bekend die resistentie tegen PVX veroorzaken. Cockerham (1955) heeft de PVX-stammen in vier categorieën ingedeeld op basis van hun interactie met de dominante resistentiegenen Nx en Nb, die in aardappelcultivars een overgevoeligheidsreactie teweegbrengen, en het dominante resistentiegen Rx, dat een extreme vorm van resistentie geeft. Groep 1-stammen kunnen alleen vatbare (nx, nb, rx) planten infecteren,

groep 2- en groep 3-stammen kunnen respectievelijk Nx en Nb doorbreken maar zijn niet in staat Rx planten te infecteren. Slechts één stam, PVX_{HB}, is bekend die, naast Nx en Nb, ook het Rx resistentiegen kan doorbreken. Deze stam, afkomstig uit Bolivia, induceert typische PVX-symptomen op de meeste indicator-soorten maar veroorzaakt geen lokale lesies op geïnoculeerde bladeren van *Gomphrena globosa*, de belangrijkste indicatorplant voor PVX. In serologisch opzicht is PVX_{HB} sterk verwant met PVX_{cp} (Fribourg, 1975), een Peruaanse stam behorend tot groep 2.

In Hoofdstuk 6 staan de experimenten beschreven die geleid hebben tot de opheldering van de complete nucleotidenvolgorde van het RNA genoom van stam HB. Vergelijking van deze volgorde met die van het PVX_{cp}-genoom, liet een hoge mate van overeenkomst zien: 95 tot 98% sequentie-homologie voor alle open leesramen.

De beschikbaarheid van de nucleotidenvolgorde van het PVX_{HB} RNA maakte het mogelijk om de virale determinant(en), verantwoordelijk voor doorbreking van het Rx-gen, te lokaliseren. Dankzij het onderzoek van Santa Cruz *et al.* (1989) en Kavanagh *et al.* (1992) stond inmiddels vast dat het manteleiwitgen hierbij betrokken was. Derhalve werd een vergelijking gemaakt tussen de aminozuurvolgorde van het manteleiwit van stam HB en die van drie niet-doorbrekende stammen, nl. de stammen cp (Orman *et al.*, 1990), X3 (Huisman *et al.*, 1988), en S (Skryabin *et al.*, 1988). Aldus werden acht aminozuurresiduen geïdentificeerd die uniek zijn voor stam HB. Op basis van de veronderstelde secundaire structuur en gesimuleerde mutatie-analyse kon vervolgens vastgesteld worden dat voor slechts twee van de acht posities (posities 121 en 226) het verschil in aminozuur aanleiding gaf tot een duidelijke verandering in eiwitvouwing. Derhalve zouden de aminozuren op positie 121 (lysine) en 226 (alanine) betrokken kunnen zijn bij resistentiedoorbreking. Omdat het effect van de vervanging op positie 226 minder duidelijk was en wellicht zelfs gemaskeerd door het hydrofobe karakter van de omgeving, werd verondersteld dat het aminozuur op positie 121 het meest belangrijk is voor de resistentiedoorbreking. Deze veronderstelling werd, ongeveer tegelijkertijd, experimenteel bevestigd door Goulden *et al.* (1993), die aan de hand van een aantal mutante en hybride virussen, afgeleid van PVX_{HB} en PVX_{CP4} (een groep 4-isolaat; Jones, 1985), konden laten zien dat het lysine-residu op positie 121 en het arginine-residu op positie 127 betrokken zijn bij de doorbreking van het Rx-gen. In overeenstemming met de resultaten van Hoofdstuk 6 werd daarbij vastgesteld dat het aminozuur op positie 121 de belangrijkste determinant voor resistentiedoorbreking

was, terwijl het belang van positie 127 meer leek samen te hangen met de stabiliteit van de geteste mutanten.

Nadat vastgesteld was dat het manteleiwit van stam HB, en met name een enkele aminozuurpositie (121) hierin, betrokken is bij de doorbreking van resistentie in een Rx-genotype, drong de vraag zich op of ook andere door stam HB doorbroken Rx-genen, afkomstig van verschillende *Solanum* soorten, op dezelfde wijze een interactie aangaan met het PVX-manteleiwit. Om hier inzicht in te krijgen zijn een recombinant virus (KH2, Kavanagh *et al.*, 1992) en twee mutant isolaten, P4-KR en HB-TK (Goulden *et al.*, 1993), getest op hun vermogen om een reeks Rx-genen al dan niet te doorbreken (Hoofdstuk 7). Aangetoond kon worden dat de extreme resistenties in *S.* x *chaucha*, *S.* x *curtilogu*, *S.* x *juzepczukii*, en *S. vernei*, respectievelijk gebaseerd op enkelvoudige, dominante resistentiegenen Rx_{cha}, Rx_{cur}, Rx_{juz} en Rx_{vrn}, op eenzelfde wijze het PVX-manteleiwit herkennen als de Rx-genen zoals die reeds in commerciële cultivars geïntroduceerd zijn (Rx-USDA 41956, Rx_{acl}, en Rx_{adg}). Echter, de *S. sucrense* lijn OCH 11926 (met genotype OCH 11926.4), die onvatbaar is voor stam HB (C. Chuquillanqui en L.F. Salazar, pers. meded.), bleek vatbaar voor zowel het recombinante virus KH2 als voor de mutant-isolaten CP4-KR en HB-TK, waarbij geïnfecteerde planten systemische necrose vertoonden. Dit resultaat laat zien dat de resistentie die door dit sucrense-gen bewerkstelligd wordt, op een ander mechanisme berust dan dat van eerder genoemde Rx-genen. Een eerste interpretatie van de resultaten suggereert dat bij dit gen (a)virulentie niet alleen bepaald wordt door een determinant in het manteleiwit maar daarnaast door tenminste een tweede, virale determinant die zich buiten het manteleiwit-gen en het 3' niet-vertaalde gebied van het PVX genoom bevindt.

Vanwege de beperkte afmetingen van de meeste virale genomen, is het onwaarschijnlijk dat er sprake is van een viraal genprodukt dat louter betrokken is bij de bepaling van virulentie en avirulentie. Het is waarschijnlijker dat (a)virulentie bepaald wordt door één of meer virale genprodukten, die primair betrokken zijn bij een voor het virus essentieel proces zoals genoomreplicatie, assemblage, intercellulair transport of overdracht. Daarnaast is het ook mogelijk dat niet een viraal genprodukt (eiwit) maar het virale RNA zelf als determinant van (a)virulentie herkend wordt. Fraser (1986) heeft gesuggereerd dat bij verschillende virusgroepen (a)virulentie bepaald wordt door verschillende segmenten van het virale genoom, die waarschijnlijk corresponderen met verschillende functies. Er zijn enkele voorbeelden voorhanden, waar aangetoond is dat niet het manteleiwit maar een ander

viraal genprodukt de (a)virulentie bepaald. Zo is door het werk van Meshi *et al.* (1988) en Yamafuji *et al.* (1991) aannemelijk gemaakt dat het produkt van het Tm1-gen, dat tomaat resistent maakt tegen het tabaksmozaïekvirus, een viraal gecodeerde replicase-subeenheid herkent, terwijl ook bij de inductie van de overgevoeligheidsresistentie in NN tabaksplanten door het tomate-mozaïekvirus (ToMV-Ob) herkenning van het virale replicase centraal staat.

Met betrekking tot het PVX-resistentiegen in *S. sucrense* is vervolgonderzoek nodig om het werkingsmechanisme van dit gen vast te stellen. Hierbij kan, indien gebruik gemaakt wordt van additionele mutanten, de benadering zoals die in Hoofdstuk 6 beschreven staat, van grote betekenis zijn.

Voor Referenties zie pagina 116.

Resumen y conclusiones

Uno de los principales problemas en la producción da papa es la alta susceptibilidad de este cultivo a enfermedades. Los agentes causantes de enfermedades incluyen a bacterias, hongos, micoplasmas, nematodes, virus y viroides. Cerca de 30 virus diferentes y un viroide infectan a la papa en el mundo (Salazar, 1990). La mayoría o casi todos los virus de papa son perpetuados a través de la propagación clonal mientras que algunos de ellos (incluyendo el viroide PSTVd) se sabe que son transmitidos también a través de la semilla botanica de la papa.

La papa es usualmente propagada plantando piezas de tubérculos o semillas. Este método de propagación se presta a la introducción de patógenos de una área a otra y a la permanencia de los patógenos en los tubérculos almacenados con propósitos de propagación. En muchos países tropicales y subtropicales, el rendimiento de la papa es muy bajo como resultado de varios factores pero la calidad de la semilla usada es uno de los principales factores limitantes. Por esta razón los sistemas de certificación de semilla, y los programas de cuarentena le dan gran importancia al control de la diseminación de virus de papa. El uso de semilla de papa libre de virus puede controlar al menos inicialmente la diseminación de los virus, sin embargo, es dificil mantener stocks o núcleos de semilla completamente libres de estos patógenos, especialmente de aquellos que son fácilmente transmitidos. El virus X de la papa (PVX), por ejemplo, tiene una tendencia a incrementarse rápidamente en los núcleos de semilla sino se elimina el material infectado regularmente (Manzer *et al.*, 1975).

Para inspección y certificación se requieren pruebas rápidas y confiables. En el caso de algunos cultivos como la papa, y en el análisis de muestras de semillas, estos procedimientos deben permitir la prueba diaria de un gran número de muestras. Para aplicar

las técnicas de evaluación de semilla para virus en países en desarrollo debe resaltarse la importancia y la necesidad de procedimentos simples, que sean de bajo costo y confiables. Además, la sensibilidad de los procedimientos debe ser lo suficientemente alta para detectar en forma confiable niveles bajos de agentes de enfermedad asi como de las diferentes variantes que puedan ocurrir en el campo.

El interés en la detección de virus y viroides tanto para su aplicación como instrumento de investigación como en la detección práctica rutinaria en gran escala ha llevado al desarrollo de varios procedimientos y métodos que son largamente usados en la actualidad. Es esencial enfatizar la importancia de un método confiable de detección para aquellas instituciones que incluyen en su mandato la distribución de equipos ("kits") de detección de virus y que tienen la necesidad de diagnosticar virus en diferentes lugares del mundo.

Los métodos más específicos de diagnóstico caen en dos categorias: el procedimiento inmunológico se basa en el uso de anticuerpos, usualmente preparados contra la cubierta proteica viral. El segundo método hace uso del ensayo de hibridación de ácidos nucléicos. En este último caso, ADN complementario (cADN) preparado del ácido nucléico del virus o viroide, o un clon de ácido nucléico recombinante convenientemente marcado, se incuba con extractos de plantas a probar los cuales son detectados por la formación de híbridos altamente específicos entre el cADN y el ácido nucléico viral (Capítulo 2).

Hasta el presente la mayoría de sondas de ácidos nucléicos han sido preparadas por marcaje radioactivo lo qual tiene desventajas obvias para aplicación rutinaria en países en desarrollo. Sin embargo, un número grande de sistemas de marcaje no radioactivo han sido desarrollados y los más importantes son discutidos en el Capítulo 3. La mayoría de estos hacen uso de enzimas que permiten la obtención de una reacción coloreada. Una limitación del método no radioactivo para la aplicación en gran escala es aún la interferencia con la reacción de algunos componentes de la savia de la planta. La sensibilidad de la detección, sin embargo, es a menudo similar a la de los métodos serológicos mayormente usados. La hibridación de los ácidos nucléicos puede también ser usada para medir el grado de relacion entre virus.

El virus X de la papa (PVX), es el miembro tipo del género *Potexvirus*. PVX tiene distribución mundial y es considerado como el virus más común que infecta a la papa (Schultz y Bonde, 1944). Desde que la papa se originó el la región andina de Sudamérica desde donde fue posteriormente introducida a Europa y otros países, se ha propuesto que los virus de papa como el PVX, se dispersaron también desde esta región a otras áreas de

producción de papa y que las divergencias observadas entre los diferentes strains o variantes del virus puede indicar adaptación a diferentes especies de *Solanum*. En el caso de PVX varios estudios han sido realizados sobre la variabilidad y distribución de los diferentes strains o variantes y sobre la consecuente habilidad de la técnica usada al momento para detectar todas las variantes. Torrance *et al.* (1986) encontraron un alto grado de variación serológica entre las variantes de PVX las cuales podían ser divididas en cuatro grupos serológicos principales, o serogrupos. Ellos también concluyeron que las reacciones serológicas no siempre se correlacionan a la clasificación basada en genes de resistencia (Cockerham, 1955). Los strains de PVX también han sido distinguidos como PVX^{A} (encontrado solamente en Sudamérica), y el PVX^{O}, o serotipo común, encontrado en otros lugares del mundo (Fernandez-Northcote y Lizárraga, 1991). Ha sido demonstrado que las diferencias halladas en el gen la cubierta proteica (lo cual determina la variabilidad serológica entre strains o variantes) no necesariamente corresponde a divergencias similares en otras regiones del genome viral (Capítulo 4).

En el Capítulo 4 se discute la especificidad para detectar un rango amplio de aislamientos de strains de PVX con dos sondas seleccionadas. Estas sondas son la siguientes: pX61, derivada del strain PVX_{cp} (serotipo PVX^{A}) y la sonda pPVX19, derivada de un strain europeo, aislamiento del grupo 3 (serotipo PVX^{O}). La sonda pX61 corresponde a la región entre los nucleotidos 3008 y 4107 del ARN genómico de PVX_{cp}, mientras que la sonda pPVX19 corresponde a la región entre los nucleotidos 2909 y 3845 en el genome de PVX. De esta manera ambos tienen una gran porción de superposición en su secuencia. Estas sondas fueron derivadas de una región altamente conservada del terminal 3' del ORF1 de PVX, y comparten una homología general de cerca de 78%. En general la sonda pX61 detectó al strain cp (strain homólogo) y HB (también del serotipo PVX^{A}) con una señal fuerte pero detectó muy pobremente a los aislamientos que pertenecen al serogrupo PVX^{O}. Al contrario, la sonda pPVX19 detectó muy fácilmente los aislamientos que pertenecen al serotipo PVX^{O} y muy pobremente a los aislamientos cp y HB. Sin embargo, varios aislamientos de PVX^{O} mayormente de Bolivia reaccionaron con esta sonda tan débilmente como los aislamientos PVX^{A} y fueron más fácilmente detectados por la sonda pX61. A pesar de haber usado sondas derivadas de una región altamente conservada en el terminal 3' del ORF1, se observó una alta especificidad en la detección. Estos resultados sugieren la existencia de variabilidad en la secuencia de nucleotidos entre los diferentes strains de PVX de los aislamientos probados aún cuando pertenecen al mismo serotipo y/o serogrupo.

Las diferencias observadas entre los aislamientos PVX^A y PVX^O enfatizan la importancia de usar la tecnica apropiada para detección especialmente para programas de mejoramiento para resistencia y con propósitos de cuarentena. Como un próximo paso al desarrollo de una sonda universal para la detección amplia de aislamientos de PVX una sonda quimérica recombinante fue preparada, la cual consistía de la unión de las dos sondas originales (pX61 y pPVX19). Esta sonda recombinante (pX6119) dió una reacción fuerte (y por ello detección confiable) con todos los aislamientos hasta ahora probados (Capítulo 5). Este resultado indica que sondas recombinantes que contienen fragmentos de cADN de diferentes strains de un virus o de diferentes virus, pueden llegar a ser un instrumento poderoso en la detección de virus y pudiesen tener una amplia aplicación.

La parte siguiente de mi trabajo presentada en los Capítulos 6 y 7, fue dirigida a la caracterización de un strain virulento de PVX. Genes diferentes se han encontrado en *Solanum* sp. que confieren resistencia a PVX. Cockerham (1955) clasificó a los strains de PVX conocidos en cuatro grupos en base a sus interacciones con los genes dominantes de resistencia Nx y Nb, los cuales en variedades cultivables de papa determinan una respuesta de hipersensibilidad, y con el gene de resistencia extrema Rx. Los strains del grupo 1 son capaces de infectar solo plantas susceptibles. Varios strains de PVX, clasificados en grupos 2 y 3 son capaces de romper los genes de resistencia Nx y Nb, respectivamente. Finalmente, los strains del grupo 4 rompen la resistencia de los genes Nx y Nb y no pueden infectar plantas que lleven el gen Rx. Solamente un strain, PVX_{HB}, aislado en Bolivia ha sido reporado como capaz de romper los genes de resistencia Nx, Nb y Rx (Moreira *et al.*, 1980). Este strain es único por causar síntomas típicos de PVX en la mayoría de las especies indicadoras comunes. No produce lesiones locales en hojas inoculadas de *Gomphrena globosa* la cual es el indicador principal para PVX.

PVX_{HB} es serológicamente muy estrechamente relaccionado a PVX_{cp} (Fribourg, 1975), el strain de Perú del grupo de resistencia 2. Usando anticuerpos policlonales en prueba de diagnóstico, tales como ELISA directo, aglutinación de látex, y por serología en el microscopio electrónico no es posible diferenciar ambos strains. Torrance *et al.* (1986) pudieron distinguir PVX_{HB} de otros aislamientos de PVX solamente con el uso de anticuerpos monoclonales. Sin embargo, PVX_{HB} se comporta en genotipos de papa en forma diferente a este y todos los otros strains conocidos de PVX.

En el Capítulo 6 el ARN genómico del strain HB fue clonado, secuenciado completamente y analizado. La comparación de la secuencia de nucleótidos de los dos

strains Sudamericanos (PVX_{cp} y PVX_{HB}) confirmaron un alto grado de homología en todos los ORFs, la cual varia entre 95 y 98%.

El objetivo del trabajo fue localizar dentro de la secuencia genómica viral, los determinantes virales responsables de la habilidad de los strains HB de romper la resistencia provista por el gen Rx, y de entender el mecanismo involucrado. Alguna información sobre el comportamiento de la cubierta proteica en la resistencia y la expresión de sintomas ya existia. Santa Cruz *et al.* (1989) demonstraron con datos experimentales que los determinantes de virulencia para el gen Rx estan localizados en el terminal 3' del genome viral, fuera del gen de replicasa. Kavanagh *et al.* (1992) demonstraron que en el caso de PVX el gen de la cubierta proteica juega un rol importante en la patogenicidad viral y, analizando diferentes genomes híbridos de PVX obtenidos de la unión de elementos de PVX_{UK3} (un strain británico del grupo 3 que no rompe la resistencia) y PVX_{HB}, localizaron las propriedades de romper la resistencia de PVX_{HB} en el gen de la cubierta proteica. Por ello, como se muestra en el Capítulo 6, las secuencias de nucleótidos y aminoácidos de 4 strains de PVX fueron comparadas, pero la atención particular se dio al gen de la cubierta proteica. Se supone que la diferencia en comportamiento entre HB y los strains de PVX que no rompen la resistencia, tiene que estar determinada entre las diferencias en la estructura secundaria de una region particular de la cubierta proteica. Comparaciones de la secuencia de la cubierta proteica entre HB y tres strains que no rompen la resistencia, por ejemplo strains cp (Orman *et al.*, 1990), X3 (Huisman *et al.*, 1988) y S (Skryabin *et al.*, 1988) indicaron la presencia de ocho residuos de aminoácidos únicos para PVX_{HB}. Análisis mutacional dirigido por computadora y predicciones de la estructura secundaria de la cubierta proteica del virus permitieron el análisis del posible efecto de cambios simples de aminoácidos en la estructura secundaria prevista de la cubierta proteica. Solo dos posiciones (K_{121} y A_{226}) se hallaron como determinantes de la variación en la estructura proteica y por ello fueron considerados de estar involucrados potencialmente en la capacidad de romper la resistencia del strain HB. Cuando en la secuencia de la cubierta proteica PVX_{HB}, lysina (K, básica) en la posición 121 es sustituida con una treonina (T, polar) específica de cp, la hélice alfa en esta posición es convertida en una estructura de lámina beta tal como se encontró en la estructura de la cubierta proteica de PVX_{cp}. La sustitución en la posición 226 (alanina a valina) también determina una ligera alteración en la predicción de la estructura de la cubierta proteica aunque la modificación es localizada en una región altamente hidrofóbica y podria por ello estar enmascarada. Ambas sustituciones de aminoácidos en

las posiciones 121 y 226 llevan a una reversión de la estructura de la cubierta proteica tipo HB al tipo cp.

Mientras tanto, Goulden *et al.* (1993) analizaron una serie de híbridos y aislamientos mutantes de PVX_{HB} y PVX_{CP4}, un aislamiento del grupo 4 (Jones, 1985), y concluyeron que la iniciación de resistencia extrema expresada en la variedad Cara es afectada por los aminoácidos 121 y 127 de la cubierta proteica viral. PVX_{HB} y los aislamientos híbridos o mutantes con lysina y arginina en las posiciones 121 y 127, fueron capaces de romper la resistencia expresada en la variedad Cara, mientras que aquellos con treonina y arginina no lo hicieron. En este trabajo y de acuerdo con la predicción del Capítulo 6 se enfatizó que la posición 121 es el mayor determiante en la actividad de ruptura de la resistencia mientras que la importancia del residuo 127 se propone que esta relaccionada a la estabilidad de los mutantes.

Habiendose demonstrado que la cubierta proteica y específicatamente solo un aminoácido en la posición 121 en la cubierta proteica afecta la resistencia extrema en genotipos Rx el interés fue dedicado al análisis de estos descubrimientos en un grupo más amplio de genes Rx. Las especies de *Solanum* que producen tubérculos poseen gran variabilidad en muchas de sus características y han sido fuentes indispensables de resistencia a patógenos (Ross, 1986), pero sólo una porción pequeña de los genes valiosos encontrados en estas especies han sido incorporados en el genome de variedades comerciales. La resistencia actualmente usada en los programas de mejoramiento está aún limitada a algunas fuentes de genes. Además en el caso específico de resistencia a PVX, la relación entre los diferentes genes Rx incorporados en varias variedades de papa es prácticamente desconocida. Con el fin de profundizar en esta materia en el Capítulo 7 el clon recombinante KH2 (Kavanagh *et al.*, 1992) y dos aislamientos mutantes, CP4-KR y HB-TK (Goulden *et al.*, 1993), fueron analizados y probados por su habilidad de romper los efectos de varios genes de resistencia extrema originados de diversas especies de *Solanum*. Los esperimentos confirmaron en todos los casos la implicación del aminoácido 121 de la cubierta proteica de PVX en la ruptura de la resistencia. Por ello la resistencia extrema encontrada en *S.* x *chaucha*, *S.* x *curtilobum*, *S.* x *juzepczukii*, y *S. vernei* condicionada por genes Rx (Rx_{cha}, Rx_{cur}, Rx_{juz}, y Rx_{vrn}, respectivamente) interacionan con la cubierta proteica de PVX como el Rx (USDA 41956), Rx_{acl}, y Rx_{adg}.

Sin embargo, cuando la accesión de *S. sucrense* OCH 11926 (genotipo usado OCH 11926.4) reportado como inmune a PVX_{HB} (C. Chuquillanqui y L.F. Salazar, comunicacion

personal) fue inoculado con los mismos strains y clones mutantes, se halló que el clon recombinante KH2 y los aislamientos mutantes CP4-KR y HB-TK fueron capaces de infectar sistémicamente este genotipo e indujeron una necrosis letal. Los descubrimientos de que ambos clones recombinantes y los aislamientos mutantes, pero no los aislamientos parentales, son capaces de infectar sistémicamente a esta accesión, abren una nueva pregunta acerca del tipo de mecanismo de resistencia envuelto. Aunque en este momento no es posible definir exactamente el mecanismo de acción de este gen, una primera interpretación de los resultados sugiere que en este caso la resistencia o susceptibilidad no esta determinada sólo por una interacción con un determinante específico de la cubierta proteica viral sino también por un segundo determinante localizado fuera de la cubierta proteica y la región no codificadora de 3'.

Aún si el gen de la cubierta proteica a sido implicado en la capacidad de romper la resistencia y de modificar la expresión de sintomas, lo cual ha sido también confirmado en el caso de otras interacciones entre virus y plantas (Culver y Dawson, 1989; Meshi *et al.*, 1989; Neeleman *et al.*, 1991; Shintaku *et al.*, 1992), podría suponerse que otros componentes virales podrian actuar como entidades de reconocimiento y jugar un rol importante en el reconocimiento de los productos de los genes de las plantas.

Ciertamente, las especies de plantas y los virus interaccionan en varias formas que varian de no-patogenesis hasta formación de sintomas, y multiplicación viral. Las interacciones controladas por genes simples probablemente envuelven eventos de reconocimiento discretos entre productos de los genes apropiados en el huésped y factores en el virus. Debido al tamaño pequeño de los genomes virales y su limitada capacidad de codificación es improbable que cualquier gen viral podría estar involucrado solamente en la determinación de virulencia/avirulancia. Por ello un determinante es probablemente localizado en un gen para otras funciones primarias tales como replicación viral, movimiento o transmisión pero también podría ser posible que el ácido nucléico viral (y no una proteina viral) pudiese ser una entidad de reconocimiento. Fraser (1986) sugirió que en diferentes grupos de virus virulencia contra los genes de resistencia de varios huéspedes estan localizados en varios segmentos genómicos, probablemente especificando un número diferente de funciones.

Hay algunos ejemplos de casos conocidos en los cuales cambios en el gen viral diferente al de la proteína de la cubierta, determinan si el virus exhibe virulencia o avirulencia contra un gen de resistencia de huésped. En los trabajos de Meshi *et al.* (1988) y Yamafuji *et al.*

(1991) se ha propuesto que el producto del gen *Tm1* de tomate confiere resistencia a mosaico del tabaco (TMV) al desorganizar la función del componente de la replicasa codificado por el virus, y en el trabajo de Padgett y Beachy (1993) la inducción de la respuesta hipersensitiva mediada por el gen N por el virus del mosaico del tomate strain Ob (ToMV-Ob) se supone que esta asociado a la replicasa viral.

En el caso de la resistencia expresada en *S. sucrense* accesión OCH 11926, se necesitan mayores estudios para caracterizar especificamente el gen y el mecanismo implicado. El camino usado en el Capítulo 6 y el uso de mutantes adicionales podrian llevar al conocimiento de las bases genéticas de estas resistencia y del sistema genetico complementario de PVX y podría dar indicaciones para entender el mecanismo de operación.

Para referencias ver pag. 116.

Curriculum vitae

Maddalena Querci was born on 24 June 1962 in Varese, Italy. She obtained her doctoral degree in Biological Sciences at the Università degli Studi di Milano, Milano, Italy, in February 1987.

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The work presented in this thesis has been conducted within this Department, while finalization of the Ph.D. study took place at the Department of Virology in Wageningen, The Netherlands, supported by a sandwich Ph.D. grant from the Wageningen Agricultural University.