Molecular Analysis of Potato Virus X Isolates in Relation to the Potato Hypersensitivity Gene *Nx*

Simon Santa Cruz and David C. Baulcombe

The Sainsbury Laboratory, Norwich Research Park, Colney, Norwich NR4 7UH, U.K.
Received 7 May 1993. Accepted 9 July 1993.

The coat protein gene of potato virus X is known to affect the outcome of interactions between different strains of the virus and potato plants carrying the *Nx* resistance gene. To analyze the role of the coat protein in interactions with *Nx* hosts, we used the potato virus X strain PVX_{DX}, which induces a hypersensitive response on potato cultivars carrying the *Nx* resistance gene and the strain PVX_{DX4}, which was originally derived from PVX_{DX} and which overcomes *Nx*-mediated resistance. Sequencing of cloned coat protein genes representing the strains PVX_{DX} and PVX_{DX4} showed that they differed at a single nucleotide. This change results in the substitution of glutamine-78 in the PVX_{DX} coat protein for proline in PVX_{DX4}. We constructed hybrid viral genomes by replacing the coat protein gene of a full-length clone of isolate PVX_{UK3} with the corresponding sequence from either PVX_{DX} or PVX_{DX4}. Progeny virus, derived from *in vitro* transcripts of these hybrid clones, showed that the single nucleotide difference between the coat protein genes of isolates PVX_{DX} and PVX_{DX4} was sufficient to alter the outcome of the interaction between the virus and potato plants carrying the resistance gene *Nx*. Additional coat protein mutants generated *in planta* from transcript-derived inocula induced an intermediate host response on *Nx* potato cultivars which is influenced by the presence of a second, PVX-specific, resistance gene in the host plant genome.

A number of dominant genes have been identified in *Solanum* species that determine strain-specific resistance to potato virus X (PVX). The genes *Nx* and *Nb*, which confer a hypersensitive response (HR) in incompatible interactions with PVX, have been used to classify PVX isolates into four resistance groups (Cockerham 1955). Group 1 strains induce a necrotic response on potato cultivars carrying either *Nb* or *Nx*, group 2 strains only induce *Nb*-mediated necrosis, group 3 strains only induce necrosis on *Nx* cultivars, and group 4 strains overcome both resistance genes.

Resistance to PVX in potato cultivars carrying either *Nx* or *Nb* is accompanied by a hypersensitive response (HR) and a reduction in virus accumulation (Adams *et al.* 1986). The HR is expressed either by the development of necrotic lesions on the inoculated leaf or as systemic necrosis. The systemic necrotic response, termed apical necrosis, results in death of the apical tissue and consequently the death of the plant. Whether the local lesion or apical necrotic response is induced in incompatible interactions between PVX and *Nx* potato cultivars depends on both inoculum concentration and environmental conditions. Graft inoculations of incompatible strains routinely induce the systemic necrotic response (Cadman 1942). The development of systemic necrosis rather than the local necrotic reaction may reflect a differential effect of experimental conditions on the components of the resistance response affecting necrosis and virus accumulation. Systemic necrotic responses to plant virus infections are seen in a number of host-virus systems in which the plant response is determined by a single dominant or semi-dominant gene (Holmes 1937, 1938).

A further class of resistance genes found in *Solanum* species, the *Rx* genes from *S. andigena* and *S. acaule* (Cockerham 1970), confer extreme resistance to all strains of PVX with the exception of the group 4 strain PVX_{HB} (Moreira *et al.* 1980). Hybrid viruses based on the strain PVX_{UK3}, which is avirulent on potato cultivars carrying either *Nx* or *Rx*, and the resistance-breaking strain PVX_{HB} have been used to demonstrate that the ability to overcome both *Nx*- and *Rx*-mediated resistance is determined by the viral coat protein gene (Kavanagh *et al.* 1992). Further analysis of PVX-*Rx* interactions has indicated that the coat protein of incompatible strains acts as a dominant avirulence determinant that induces *Rx*-mediated resistance (Köhm *et al.* 1993).

The PVX_{UK3} and PVX_{HB} coat protein genes differ at 20% of their nucleotide positions and 10% of their amino acid residues (Kavanagh *et al.* 1992). However the minimum difference necessary to effect the transition from an avirulent to a virulent phenotype on *Nx* potato cultivars was not established. Strains of PVX belonging to resistance groups 1 and 3 can be differentiated from the majority of strains belonging to resistance groups 2 and 4 on the basis of their interactions with a panel of monoclonal antibodies (Torrance *et al.* 1986). However, the group 4 strain PVX_{DX4} is serologically indistinguishable from the group 3 strain PVX_{DX}, suggesting a close relationship between these strains. Strain PVX_{DX4} was isolated from plants grown from tubers of an *Nx* potato cultivar that had been infected with PVX_{DX}, indicating that PVX_{DX4} may represent a mutant derivative of PVX_{DX}. The *Nx* resistance-breaking property of PVX_{DX4} appeared to be unstable in the absence of selection by *Nx*, since serial passage of PVX_{DX4} through *Nicotiana glutinosa* resulted in the recovery of a group 3 revertant strain (Jones 1982).

MPMI Vol. 6, No. 6, 1993, pp. 707-714

This report describes the construction and analysis of viral genomes in which the coat protein genes of PVX_{DX} and PVX_{DX4} were used to replace the corresponding sequence from a cDNA clone of strain PVX_{UK3}. Additional mutants, generated *in planta* from transcript-derived viruses, have also been analyzed to assess the effects of coat protein amino acid substitutions on interactions between PVX and potato cultivars carrying the *Nx* gene.

RESULTS

Strain PVX_{DX4} is an *Nx* resistance-breaking derivative of strain PVX_{DX}. We have shown previously that for the naturally occurring *Nx* resistance-breaking strain, PVX_{HB}, the ability to overcome *Nx*-mediated resistance is determined by the viral coat protein gene (Kavanagh *et al.* 1992). To establish whether the coat protein of PVX_{DX4} has a similar role in conferring an *Nx* resistance-breaking phenotype, the sequences of the PVX_{DX} and PVX_{DX4} coat protein genes were compared and substituted for the homologous sequence from the PVX_{UK3} transcription clone pTXN.

Sequence comparison of the PVX_{DX} and PVX_{DX4} coat protein genes.

Sequence analysis of pKDX and pKDX4 showed only one nucleotide difference between the coat protein genes of PVX_{DX} and PVX_{DX4}. This unique difference was confirmed in additional clones of both pKDX and pKDX4, each derived from separate RNA preparations, indicating that the variant nucleotide had not arisen during the cloning process. The predicted amino acid sequence of the coat protein of strain PVX_{DX} is identical to the previously reported sequence of strain PVX-3 (Huisman *et al.* 1988) and differs from strain PVX_{UK3} at two adjacent residues (positions 10 and 11) in the amino-terminal domain of the protein. Like PVX_{DX}, both PVX-3 and PVX_{UK3} induce the *Nx*-mediated HR. The only observed variation between pKDX and pKDX4 is an A-to-C substitution at nucleotide 5882, which results in substitution of the glutamine

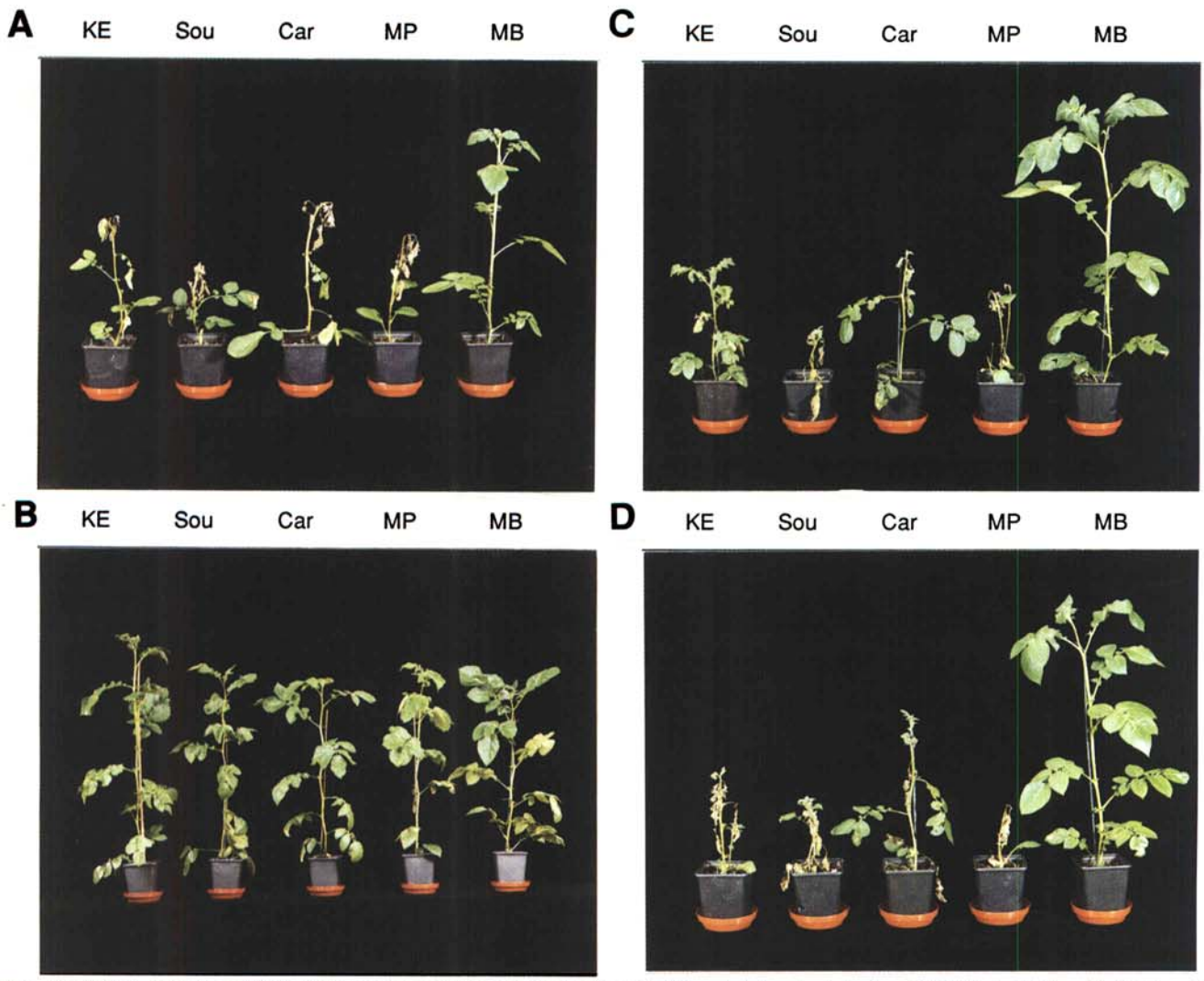

Fig. 1. A selection of potato cultivars showing symptoms induced by infection with different strains of potato virus X (PVX). **A,** PVX_{KD}, **B,** PVX_{KD4}, **C,** PVX_{KD76}, and **D,** $PVX_{KD4\text{-}62}$. KE, King Edward (*Nxnb*), Sou, Southesk (*Nnxb*); Car, Cardinal (*nnxb*); MP, Maris Piper (*Nnxb*); MB, Maris Bard (*nxnb*). the photographs were taken 21 days postinoculation, and all plant tested positive for PVX antigen by ELISA.

residue at position 78 in the PVX_{DX} coat protein by a proline in the PVX_{DX4} protein.

Functional analysis of the PVX_{DX} and PVX_{DX4} coat protein genes.

Transcripts synthesized from plasmids pKD and pKD4 were infectious when inoculated to *N. clevelandii*, producing systemic symptoms indistinguishable from those induced by infection with PVX_{UK3}. Progeny virus from *N. clevelandii* plants infected with PVX_{KD} or PVX_{KD4} (derived from pKD and pKD4, respectively) was passaged to potato cultivars carrying different combinations of the resistance genes *Nx* and *Nb*. Infections with PVX_{KD} gave rise to systemic chlorotic symptoms in the fully susceptible cultivar Maris Bard (*nxnb*) and the *nxNb* cultivar Catriona (not shown) but induced an apical necrosis on all of the *Nx*-carrying cultivars tested (Fig. 1A). This contrasts with PVX_{KD4}, which gave rise to systemic chlorotic symptoms on Maris Bard and Catriona as well as on the *Nxnb* cultivars King Edward, Southesk, and Cardinal (Fig. 1B). On the *NxNb* cultivars Maris Piper, Pentland Dell, Kirsty, and Skirza, PVX_{KD4} induced some necrosis in lower leaves but only chlorotic mosaic symptoms in upper leaves. Continued growth of PVX_{KD4}-infected *NxNb* cultivars resulted in the appearance of chlorotic symptoms in newly developed leaves and no further progression of necrotic symptoms. The symptom types seen for each virus-potato cultivar combination described above were consistently observed in several replicate experiments.

Accumulation of PVX_{KD} and PVX_{KD4} RNA in infected plants.

Previous analyses have shown that, in addition to the induction of necrosis, resistance mediated by *Nx* is associated with reduced virus accumulation (Adams *et al.* 1986). To establish whether the same is true for the recombinant isolates described above, total RNA was prepared from *N. clevelandii* and potato plants systemically infected with the transcript-derived viruses PVX_{KD} and PVX_{KD4}. Slot-blot analysis of viral RNA indicated that PVX_{KD} and PVX_{KD4} accumulated to similar levels in *N. clevelandii* by 10 days postinoculation (dpi). On the fully susceptible potato cultivar Maris Bard, the levels of both PVX_{KD} and PVX_{KD4} RNA are also equivalent. However, on the *NxNb* cultivar Maris Piper, the level of PVX_{KD} RNA was reduced compared with the accumulation of PVX_{KD4} (Fig. 2). Comparison of viral RNA present in compatible and incompatible interactions with dilution standards indicated that, in the incompatible interaction between PVX_{KD} and Maris Piper, viral accumulation was less than 10% of that detected in compatible interactions (data not shown). The small variation in the level of viral RNA between samples taken from different compatible interactions was no greater than the variation between replicates of the same virus-host combinations. The probe used to detect viral RNA is complementary to both the viral genomic and subgenomic RNAs. However, at the time when samples were collected, Northern analysis showed that very little subgenomic RNA could be detected (not shown), suggesting that variation in the relative levels of genomic and subgenomic RNAs does not account for the differential accumulation of PVX_{KD} and PVX_{KD4} RNA in incompatible interactions.

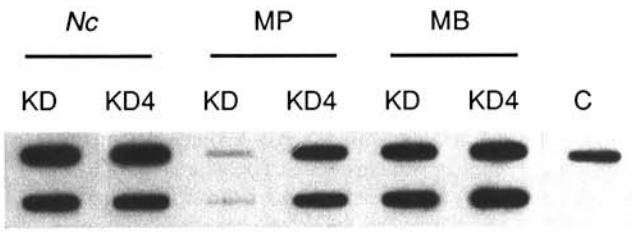

Fig. 2. Slot bot of total RNA prepared from *Nicotiana clevelandii* (*Nc*) and the potato cultivars Maris Piper (MP) and Maris Bard (MB) infected with either PVX_{KD} (KD) and PVX_{KD4} (KD4). One microgram of total RNA from infected plants was blotted to a nylon membrane and the blot probed with a riboprobe complementary to the 3′ 1.5 kb of the viral genome. Controls (C), lower panel 1 μg of total RNA from a healthy *N. clevelandii* plant; upper panel, 5 ng of virion purified PVX_{KD} RNA.

Confirmation of progeny virus genome structure.

Polymerase chain reaction (PCR)-amplified viral cDNA, prepared from infected potato plants, was digested with *Hpa*II. Due to the nucleotide substitution at position 5882, pKD4 has an additional *Hpa*II recognition site that is not present in pKD. Loss of the proline residue encoded at position 78 of the PVX_{KD4} coat protein would result in the loss of the ad-

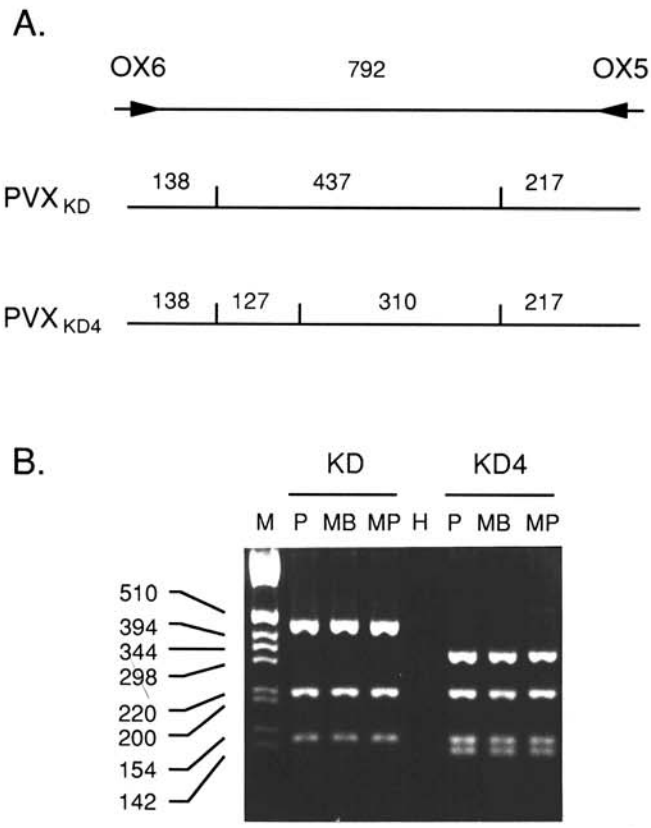

Fig. 3. Restriction fragment length polymorphism detected by digestion of viral cDNA with *Hpa*II. **A,** Sizes of predicted *Hpa*II restriction products generated by digestion of the 792-bp cDNA amplified with the primers OX6 and OX5. **B,** Agarose/TBE gel of digested PCR cDNA fragments. The templates used for PCR amplifications were the transcription plasmids (P) or first-strand cDNA prepared from total RNA of virus-infected Maris Bard (MB) or Maris Piper (MP) potatoes. Total RNA prepared from a healthy Maris Bard plant was usedas a control for cDNA synthesis and amplification (H). Molecular size markers (M) are shown with fragment lengths indicated to the left.

ditional *Hpa*II site. Figure 3 shows digested PCR products resolved on an agarose-TBE gel and indicates that PVX_{KD4} recovered from systemically infected Maris Bard and Maris Piper potatoes retained the proline residue at position 78 of the coat protein. Thus, a single nucleotide difference between the virus strains PVX_{KD} and PVX_{KD4} was sufficient to alter the outcome of interactions between the virus and potato cultivars carrying the *Nx* gene.

Genome stability of strains PVX_{KD} and PVX_{KD4}.

The original investigation of strain PVX_{DX} and its derivative PVX_{DX4} indicated that mutants able to avoid the *Nx*-mediated HR could be isolated with relative ease from a culture of the avirulent strain PVX_{DX}. However, the resistance-breaking trait was found to be genetically unstable and a revertant, avirulent, strain was isolated from a culture of PVX_{DX4} after 10 serial subcultures in the absence of selection for the resistance-breaking trait (Jones 1982). To determine whether the transcript-derived strains PVX_{KD} and PVX_{KD4} could be used to obtain further strain group transition mutants, the experimental approaches used to obtain PVX_{DX4} from PVX_{DX} and group 3 revertants from PVX_{DX4} were repeated using PVX_{KD} and PVX_{KD4}.

Recovery of virus from tubers of PVX_{KD} -infected Maris Piper.

Attempts to obtain *Nx* resistance-breaking mutants from the avirulent strain PVX_{KD} involved the recovery of virus from the tuber progenies of Maris Piper (*NxNb*) potatoes, which had been infected with PVX_{KD}. Of 36 tubers collected from 12 Maris Piper plants, 24 tubers gave rise to healthy progeny plants as determined by the absence of symptoms and the failure to detect viral antigen by enzyme-linked immunosorbent assay (ELISA). Each of the remaining 12 tubers produced both healthy and infected shoots. The timing of symptom appearance varied between tuber progenies, but all progeny plants that tested positive for PVX antigen by ELISA eventually developed lethal apical necrosis. Although there was no evidence for mutants unable to induce the HR, it was thought that the variation in the timing of symptom appearance could have been caused by mutation of the virus. To investigate this possibility, total RNA was isolated from six independent, PVX_{KD}-infected, progeny plants and used to synthesize viral coat protein cDNAs. Sequencing of cloned coat protein genes indicated one of the six clones harbored a coding mutation. The mutant clone carried a nucleotide substitution, G to A at position 5877, which resulted in replacement of a methionine residue by isoleucine at position 76 of the coat protein. The coat protein gene from the plasmid harboring this mutation was used to replace the corresponding sequence of the full-length clone pTXN to give pKD76.

Transcripts synthesized from pKD76 were infectious on *N. clevelandii* plants but accumulated to a lower level than PVX_{KD} in systemically infected leaves (data not shown). Inoculation of the potato cultivars Maris Bard (*nxnb*) and Catriona (*nxNb*) with PVX_{KD76} resulted in systemic infections and no necrotic symptoms (Fig. 1C). On all of the *Nx* potato cultivars tested, infection with PVX_{KD76} induced severe systemic necrosis, although the extent of symptom development varied between different potato cultivars. The *NxNb* cultivars Maris Piper and Pentland Dell responded to infection with PVXKD76 with the development of apical necrosis. Infections of the Nxnb cultivars with PVXKD76 also induced a systemic necrotic response, but the apical leaves remained free of necrotic symptoms (Fig. 1C). Infected plants continued to grow, but as new leaves expanded they developed necrotic symptoms and died. We conclude from the phenotype of PVX_{KD76} that the mutation at position 76 of the coat protein inhibited the ability of PVX_{KD76} to elicit *Nx*-mediated apical necrosis on *Nxnb* potatoes but that on cultivars carrying both *Nx* and *Nb* this mutant behaved like the progenitor strain PVX_{KD}.

Serial culture of PVX_{KD4} in *N. clevelandii*.

The stability of the *Nx* resistance-breaking phenotype of PVX_{KD4} was assessed by repeated subculturing of the virus in *N. clevelandii*. Systemically infected tissue was harvested from transcript-inoculated *N. clevelandii*, and a serial dilution of sap was prepared in 50 mM sodium borate buffer (pH 8.2). Each dilution of sap was inoculated to two plants, and the plant infected with the lowest concentration of virus was used as the source of inoculum for the next passage. All subsequent subcultures were also initiated with a limiting dilution of sap. After each passage, undiluted sap from the plant infected with the lowest concentration of inoculum was retained for inoculation of potatoes. The progeny virus obtained after each subculture was inoculated to the potato cultivars Catriona (*nxNb*) and King Edward (*Nxnb*) to determine the strain group of the recovered virus. Progeny virus, from each of 20 serial subcultures on *N. clevelandii*, produced systemic infections of both Catriona and King Edward potato cultivars without inducing necrosis (data not shown). Cloned coat protein cDNA, prepared from RNA of a Catriona plant infected with a culture of KD4 that had been passaged 19 times through *N. clevelandii*, was identical in nucleotide sequence to the progenitor plasmid pKD4. Thus, under the conditions used for this experiment, the mutation identified in PVX_{KD4} was genetically stable in the absence of selection for the *Nx* resistance-breaking trait.

Isolation of a necrosis-inducing derivative of PVX_{KD4}.

During the initial analysis of PVX_{KD4}, one King Edward plant inoculated with transcript progeny developed isolated necrotic sectors in several systemic leaves. Necrotic tissue from this plant was used as a source of inoculum and passaged to further King Edward plants, which responded to infection with the development of a severe systemic necrosis. As PVX_{KD4} usually induced only chlorotic symptoms on *Nxnb* cultivars, it was thought likely that a mutant form of PVX_{KD4} had been obtained. Viral RNA, prepared from a King Edward plant infected with the necrosis-inducing derivative of PVX_{KD4}, was used as template for cDNA synthesis. Amplified viral cDNA was cloned and sequenced. This clone retained the proline residue encoded at position 78 of the PVX_{KD4} coat protein. An additional point mutation, from A to G at position 5833, resulted in the substitution of an isoleucine residue for valine at amino acid 62 of the coat protein. This mutant coat protein was used to replace the corresponding sequence in pTXN to give pKD4-62. Transcripts synthesized from pKD4-62 were infectious on *N. clevelandii*, although the level of viral RNA detected in transcript-inoculated plants 10 dpi was reduced compared with the level of viral RNA ac-

cumulation in plants inoculated with pKD4 transcripts (data not shown). Infections of potato cultivars with PVX_{KD4-62} resulted in very similar infection types to those produced by PVX_{KD76}. The *nx* cultivars Maris Bard and Catriona both developed systemic chlorotic symptoms, the *NxNb* cultivars Maris Piper and Pentland Dell developed lethal apical necrosis, and the *Nxnb* cultivars all developed severe systemic necrosis without the death of apical tissue (Fig. 1D). Thus, the second-site mutation, at amino acid position 62 of the PVX_{KD4-62} coat protein, abolished the resistance-breaking trait of the progenitor strain PVX_{KD4}.

DISCUSSION

Features of PVX affecting the outcome of interactions with *Nx* potatoes.

Analysis of PVX_{KD} and PVX_{KD4} indicated that a single nucleotide substitution within the coat protein gene was sufficient to convert an avirulent strain to virulence on *Nx* potato cultivars. The mutation identified in PVX_{DX4} results in the substitution of a glutamine residue at position 78 of the coat protein for a proline residue in the resistance-breaking mutant. The mutant PVX_{KD4-62} encodes the proline residue at position 78 and an additional mutation from isoleucine to valine at position 62. This double mutant shows an almost complete reversion to the avirulence phenotype of PVX_{KD}. The mutant PVX_{KD76}, in which the methionine residue encoded at position 76 of the coat protein is replaced by isoleucine, only differs from the progenitor strain PVX_{KD} in showing a slightly reduced level of accumulation in *N. clevelandii* and in its failure to induce the lethal necrotic response on *Nxnb* potato cultivars.

The fact that mutations in the coat protein gene that alter the phenotype of interactions on *Nx* potato cultivars alter the coding capacity of the gene provides indirect evidence that the *Nx* response is determined by an interaction involving the coat protein rather than the viral RNA. Other virus-plant systems for which the molecular determinants of resistance gene interactions have been investigated have also shown that coding changes distinguish avirulent from virulent viral strains (Knorr and Dawson 1988; Meshi *et al.* 1988, 1989).

Proof that the host response is determined by a viral protein has been obtained for interactions between tobacco mosaic virus (TMV) and the *N'* gene of *N. sylvestris* (Saito *et al.* 1989; Culver and Dawson 1989a), and between PVX and the potato *Rx* gene (Köhm *et al.* 1993). In both these systems, the viral coat protein determines the outcome of host interactions, and avirulent strains are rendered virulent by mutations that prevent expression of translatable coat protein mRNA. The simplest explanation for the involvement of viral proteins in the induction of resistance is that the viral protein interacts directly with a host-encoded resistance determinant to trigger the resistance-associated responses. Interactions between plant and virus mediated by the coat protein could involve either monomeric or aggregated forms of the protein. For TMV the ability of mutants to induce the *N'*-mediated HR is associated with mutations located at the surface of the coat protein at positions involved in subunit-subunit interactions (Culver and Dawson 1989b). A correlation between weaker subunit-subunit interactions and the ability to induce the *N'*-mediated HR has been observed, which suggests that the aggregation state of the subunits may influence induction of the HR (Culver *et al.* 1991). Alternatively the strong association between coat protein subunits in virulent strains may mask a universal elicitor domain from the putative *N'* receptor (Dawson *et al.* 1993).

A recently proposed structure for the PVX coat protein, based on physical and predictive approaches to protein structure modeling, indicates that the second-site mutation identified at position 62 in PVX_{KD4-62} may be brought into close proximity with the proline residue at position 78 because of an αα-corner between α-helices spanning positions 56 to 71 and positions 73 to 103 (Baratova *et al.* 1992). It is thus possible that in *Nx* potatoes recognition of avirulent strains is determined by a local structural feature of the coat protein that is modified or masked in the coat proteins of PVX strains virulent on *Nx* potatoes.

Recovery of mutant virus from plants inoculated with the transcript-derived strains PVX_{KD} and PVX_{KD4}.

The single nucleotide difference between an avirulent and a virulent strain of PVX is consistent with the relative ease with which group 4 mutants were recovered from *Nx* potatoes inoculated with the group 3 strain PVX_{DX}. However, attempts to generate mutants showing a complete transition from the resistance group of the progenitor strains PVX_{KD} or PVX_{KD4}, using the approaches employed by Jones (1982) to obtain PVX_{DX4} and group 3 revertants, proved unsuccessful. The original study of PVX_{DX} concluded that *Nx* resistance-breaking mutants were selectively amplified in an *Nx* host and that reversion to an avirulent phenotype reflected a conflicting selection for fitness when the virus was cultured in a susceptible host (Jones 1982). Although no difference in accumulation was seen between PVX_{KD} and PVX_{KD4} on susceptible plants, the fact that group 4 strains are very rarely found infecting European potato crops supports the view that *Nx* resistance-breaking mutants are at a selective disadvantage on susceptible hosts. The discrepancy between results obtained using PVX_{DX} and PVX_{DX4} (Jones 1982) and those obtained in our experiments may, in part, reflect the fact that PVX_{KD} and PVX_{KD4} were transcript-derived and thus initially homogeneous. The rapid appearance of the resistance-breaking mutant PVX_{DX4} may have occurred as a result of amplification of a preexisting component of the inoculum rather than by amplification of a novel mutant.

In contrast, with the results obtained using PVX_{DX4}, strain PVX_{KD4} did not lose the ability to overcome *Nx*-mediated resistance after serial culture in *N. clevelandii*. Apart from the different *Nicotiana* host species used for this analysis, the PVX_{KD4} inoculum was subjected to a genetic "bottleneck" at each passage resulting from the use of limiting dilutions of inoculum to initiate each subculture. If the selective advantage of revertants was low, then the use of limiting dilutions of viral inoculum may have eliminated rare variant forms before they could accumulate. The use of high inoculum concentrations at each passage would be expected to favor the appearance of novel mutants if the rate of mutation were low.

Effect of the *Nb* gene on the *Nx*-mediated response to the PVX coat protein.

The strains PVX_{KD4}, PVX_{KD76}, and PVX_{KD4-62} each induced symptoms on *NxNb* potato cultivars that differed from the symptoms induced on *Nxnb* potatoes; the correlation between

host genotype and the symptoms induced by infections with the different viral inocula are summarized in Table 1. Although PVX_{DX4} was classified as a group 4 strain on the basis of its ability to accumulate to high levels in *Nx* potato cultivars without inducing apical necrosis, some systemic necrotic symptoms were observed on infected *NxNb* cultivars (Jones 1982). When PVX_{KD4} was analyzed on a range of different potato cultivars, it was found that the appearance of necrotic symptoms was specifically associated with *NxNb* potato genotypes, because none of the *Nxnb* cultivars tested gave a necrotic reaction to PVX_{KD4}. The different response of *Nxnb* cultivars compared with *NxNb* cultivars was also evident in the analysis of the mutants PVX_{KD76} and PVX_{KD4-62}. Both of these mutants induce lethal apical necrosis on *NxNb* cultivars. However, on *Nxnb* cultivars, despite the induction of a severe systemic necrosis, the apical tissues showed no necrotic symptoms. This correlation between genotype and response was consistent in two *NxNb* cultivars and three *Nxnb* cultivars. A mutual, and reciprocal, enhancement of resistance was reported previously for the hypersensitivity genes *Nx* and *Nb*. This conclusion was based on the observation that accumulation of group 2 and group 3 strains of PVX is reduced in *NxNb* cultivars compared with cultivars carrying only the "effective" resistance gene (Adams *et al.* 1986).

The effect of *Nb* on expression of the *Nx*-mediated response may reflect the similarity in the mechanisms of *Nx*- and *Nb*-mediated resistance. This similarity is not at the level of virus recognition, because induction of the *Nb*-mediated response is determined by viral sequence outside the coat protein gene (A. Forsyth, personal communication), suggesting that the *Nb* gene product may affect expression of the *Nx*-mediated response downstream of the initial recognition event.

The results from studies of TMV/*N'* (Culver and Dawson 1991) and PVX/*Rx* (Köhm *et al.* 1993) interactions are consistent with the gene-for-gene model of resistance gene action in which induction of the host response is triggered by the product of the corresponding pathogen avirulence gene. In both the TMV/*N'* and PVX/*Rx* systems, the viral coat protein acts as an avirulence determinant, which triggers the host response in resistant plants. Current research is directed at establishing whether a similar role for the coat protein of PVX as an avirulence determinant can be demonstrated for the PVX/*Nx* interaction.

MATERIALS AND METHODS

Preparation and cloning of viral cDNA.

Total RNA was prepared from PVX_{DX} and PVX_{DX4}-inected *N. tabacum* 'Samsun NN' by the method of Baulcombe and Buffard (1983). One microgram of RNA was used as template for the synthesis of first-strand cDNA using Superscript reverse transcriptase (Bethesda Research Laboratories, Inc., Gaithersburg, MD) and an oligo(dT) linker-primer (dT/Kpn; Table 2) according to the manufacturer's instructions. First-strand cDNA synthesis was terminated by phenol/chloroform extraction, and residual primer was removed by spin dialysis through Sepharose CL6B (Pharmacia, Uppsala, Sweden) equilibrated in water (Murphy and Kavanagh 1988). One tenth of the first-strand cDNA reaction was used as template in a 20-cycle PCR amplification using the primers 5/Xba, which corresponds to PVX nucleotides 5110–5126, and L/Kpn, which allows amplification of cDNAs primed using dT/Kpn (Table 2). Amplifications were performed using *Taq* DNA polymerase (Cetus) at a concentration of 0.2 U/μl according to the manufacturer's instructions. Amplified products were concentrated by precipitation with ethanol and treated with T4 DNA polymerase to generate blunt-ended cDNA molecules. The treated products were ligated to *Eco*RV-digested RK8.2/pKR (Waye *et al.* 1985), and plasmid clones carrying PVX_{DX}- and PVX_{DX4}-specific cDNAs were designated pKDX and pKDX4, respectively. For both PVX_{DX} and PVX_{DX4}, cDNA clones were obtained from two independent RNA preparations. Additional coat protein cDNAs, prepared from the progeny of transcript-derived viruses, were synthesized using total RNA from infected potato plants and cloned in the vector RK8.2/pKR as described above. The sources of the virus preparations from which additional cDNA clones were prepared are detailed in results. Cloned coat protein cDNAs were sequenced across both strands using M13 universal primers and the PVX-specific oligonucleotides listed in Table 2. Sequencing was performed using Sequenase (USB) following the manufacturer's instructions. Manipulations of plasmids and cDNAs were performed using standard techniques (Sambrook *et al.* 1989). Primers used for cDNA synthesis, sequencing, and PCR amplifications (listed in Table 2) were synthesized on an Applied Biosystems DNA synthesizer (model 391). The genome coordinates used to identify oligonucleotide binding sites and nucleotide substitutions are based on the PVX sequence of strain PVX-3 (Huisman *et al.* 1988).

Construction of hybrid virus genomes.

Plasmid pTXN carries a full-length cDNA of strain PVX_{UK3} downstream of a T7 RNA polymerase promoter. This plasmid is identical to pTXS (Kavanagh *et al.* 1992) except that the *Spe*I restriction site used to linearize the template prior to synthesis of *in vitro* run-off transcripts is replaced by a unique *Nsi*I restriction site. Plasmid pTXN linearized with *Nsi*I is predicted to give rise to transcripts with a poly(A) tail of 25

Table 1. Interactions between strains of PVX and potato cultivars carrying different combinations of the genes *Nx* and *Nb*

	Potato genotype and cultivar								
	nxnb	*nxNb*	*Nxnb*			*NxNb*			
Inoculum	Maris Bard	Catriona	Southesk	Cardinal	King Edward	Maris Piper	Pentland Dell	Kirsty	Skirza
KD	SM[a]	SM	AN	AN	AN	AN	AN	AN	AN
KD76	SM	SM	SN	SN	SN	AN	AN	NT	NT
KD4-62	SM	SM	SN	SN	SN	AN	AN	NT	NT
KD4	SM	SM	SM	SM	SM	MN	MN	MN	MN

[a] Symptom types: SM, systemic mosaic; AN, apical necrosis; SN, systemic necrosis; MN, mild necrosis; NT, not tested.

residues with no nonviral sequences at either end of the genome. The full-length clones pKD and pKD4 were constructed by replacing coat protein gene-coding sequences between unique *Nhe*I and *Xho*I restriction sites of pTXN (positions 5663 to 6303; Fig. 4) with the equivalent fragments from pKDX and pKDX4. Two further full-length clones carrying mutations in the coat protein gene, pKD76 and pKD4-62, were prepared by replacement of the pTXN *Nhe*I/*Xho*I fragment with cloned viral cDNAs recovered from potatoes infected with PVX_{KD} and PVX_{KD4}, respectively. Virus recovered from transcript-inoculated plants is referred to using the name of the transcription vector from which it was derived as a subscript suffix to PVX.

Plant inoculations with transcripts and progeny virus.

Potato and *N. clevelandii* plants were maintained and propagated as described in Kavanagh *et al.* (1992). Capped transcripts were synthesized using 2 μg of *Nsi*I linearized plasmid in a total volume of 20 μl as described in Chapman *et al.* (1992). Transcription reactions were terminated by phenol/chloroform extraction, and the reaction products were precipitated with ethanol. Transcripts were resuspended in 70 μl of water containing 5 mg/ml of bentonite, and 15 μl of the diluted transcription reaction was inoculated to two leaves of two *N. clevelandii* plants. Progeny virus from systemically infected tissue of transcript inoculated *N. clevelandii* plants was used to inoculate potato cultivars (*Solanum tuberosum* subsp. *tuberosum*) both with and without the *Nx* gene. Sap preparation and potato inoculation were as described in Kavanagh *et al.* (1992). Experiments to determine the resistance group phenotype of the transcript-derived virus preparations were performed using four replicates of each potato cultivar, inoculating small plants. These experiments were repeated at least twice. For the recovery of virus from tubers of infected plants, mature Maris Piper plants were inoculated with PVX_{KD}, and tubers were harvested 2 wk after the onset of necrotic symptoms.

Table 2. Oligonucleotides used for cDNA priming, PCR amplification, and sequencing

Primer	Sequence	Sense[a]	Position[b]
5/Xba	TTCTACAACGCTATCAC	+VE	5110-5126
OX6	TTGAGCGGTTAAGTTTCCA	+VE	5617-5635
OX8/3	CAACAAATGACGACCTCAGC	+VE	5809-5828
5/Hpa	AACTGGATGTTAACAAACAACAG	+VE	6055-6077
3/N3	CTAAAGAAATCCCCATC	−VE	5758-5774
3/Hpa	TGTTTGTTAACATCCAGTTCCA	−VE	6052-6073
OX9	AAGGCAGCAGTTTGGGC	−VE	6223-6239
OX5	GAAAATACTATCAAACTGGG	−VE	6389-6408
dT/Kpn	$ATCCGCGGGCCCATGGTACC(T)_{17}$		
L/Kpn	ATCCGCGGGCCCATGGTA		

[a] Orientation of primer with respect to virus genome.
[b] The binding sites of PVX-specific oligonucleotides are shown using the nucleotide numbering of Huisman *et al.* (1988).

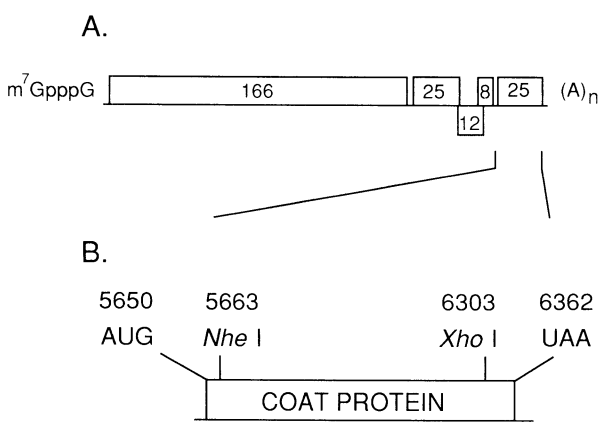

Fig. 4. A, Schematic representation of the potato virus X (PVX) genome. Boxes represent open reading frames with the sizes of the predicted translation products in kilodaltons. **B,** The coat protein gene indicating the position of the initiation and termination codons and the restriction sites in viral genomes. Numbering is based on that of strain PVX-3 (Huisman *et al.* 1988).

Confirmation of infection by ELISA.

Two weeks after inoculation, samples were collected from the systemic leaves of inoculated potato plants. Symptomatic tissue was sampled in preference to asymptomatic tissue, and where necrotic symptoms had developed, samples were taken from tissues adjacent to necrotic zones. Samples were collected using the lid of a 1.5-ml microcentrifuge tube to pinch out a disk of material into the tube. Samples (about 0.02 g) were ground in 1 ml of sample buffer (20 g/L PVP-40 in phosphate-buffered saline, pH 7.4) using a Pöllahne leaf press. Indirect ELISA (Clarke and Bar-Joseph 1984) was as described previously (Kavanagh *et al.* 1992).

Slot-blot analysis of PVX_{KD} and PVX_{KD4} RNA accumulation.

Total RNA, prepared as described above, was isolated from systemic tissue of *N. clevelandii* and potato plants infected with the transcript-derived strains PVX_{KD} and PVX_{KD4}. Samples from *N. clevelandii* plants were taken 10 dpi; potato RNA samples were taken 20 dpi. One microgram of total RNA was transferred to nylon membranes (Hybond N, Amersham) using a slot-blot apparatus as described in Goulden *et al.* (1993). A ^{32}P-labeled RNA probe, complementary to positive-strand PVX RNA between positions 4950 and 6435, was synthesized from *Apa*I digested pTXS template using T3 RNA polymerase. Probe preparation and hybridization were as described in Chapman *et al.* (1992).

Virus detection and analysis by PCR amplification of first-strand cDNAs.

Progeny virus RNA from potatoes infected with PVX_{KD} or PVX_{KD4} was analyzed by restriction enzyme digestion of PCR-amplified cDNA. Samples were taken from apical tissue of infected potato plants. RNA extraction, first-strand cDNA synthesis, and PCR amplification were as described above except that the primers used for PCR amplification were OX6 and OX5 (Table 2). Amplified cDNA products were digested with the restriction enzyme *Hpa*II. Digestion products were resolved on 3% agarose/TBE gels (Nusieve; FMC Corp., Philadelphia, PA) and compared with *Hpa*II-digested fragments amplified from the transcription plasmids pKD and pKD4.

ACKNOWLEDGMENTS

We thank Gill Brewer for supplying the strains PVX_{DX} and PVX_{DX4} used in this study, David Morton for maintaining regular supplies of potato plants, and Fred Boccard, Sean Chapman, Chris Davies, and Matthew

Goulden for helpful discussion. Genetic manipulation of PVX was carried out under the MAFF licence PHF 1185A/8C(40) in accordance with the Plant Pest (Great Britain) Order, 1980. The Sainsbury Laboratory is supported by the Gatsby Charitable Foundation.

LITERATURE CITED

Adams, S. E., Jones, R. A. C., and Coutts, R. H. A. 1986. Effect of temperature on potato virus X infection in potato cultivars carrying different combinations of hypersensitivity genes. Plant Pathol. 35:517-526.

Baratova, L. A., Grebenshchikov, N. I., Dobrov, E. N., Gedrovich, A.V., Kasharin, I. A., Shishkov, A.V., Efimov, A.V., Järvekulg, L., Radavsky, Y. L., and Saarma, M. 1992. The organization of potato virus X coat proteins in virus particles studied by tritium planigraphy and model building. Virology 188:175-180.

Baulcombe, D. C., and Buffard, D. 1983. Gibberellic acid regulated expression of α-amylase and six other genes in wheat aleurone layers. Planta 157:493-501.

Cadman, C. 1942. Autotetraploid inheritance in the potato: Some new evidence. J. Genet. 44:33-53.

Chapman, S., Kavanagh, T., and Baulcombe, D. 1992. Potato virus X as a vector for gene expression in plants. Plant J. 2:549-557.

Clarke, M. F., and Bar-Joseph, M. 1984. Enzyme immunosorbent assays in plant virology. Pages 51-85 in: Methods in Virology, K. Maramorsoch and H. Koprowski, eds. Academic Press, San Diego.

Cockerham, G. 1955. Strains of potato virus X. Proc. Conf. Potato Virus Dis., 2nd. 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. 1989a. Tobacco mosaic virus coat protein: An elicitor of the hypersensitive reaction but not required for the development of mosaic symptoms in *Nicotiana sylvestris*. Virology 173:755-758.

Culver, J. N., and Dawson, W. O. 1989b. Point mutations in the coat protein gene of tobacco mosaic virus induced hypersensitivity in *Nicotiana sylvestris*. Mol. Plant-Microbe Interact. 2:209-213.

Culver, J. N., and Dawson, W. O. 1991. Tobacco mosaic virus elicitor coat protein genes produce a hypersensitive phenotype in transgenic *Nicotiana sylvestris* plants. Mol. Plant-Microbe Interact. 4:458-463.

Culver, J. N., Lindbeck, A.G. C., and Dawson, W. O. 1991. Virus-host interactions: Induction of chlorotic and necrotic responses in plants by tobamoviruses. Annu. Rev. Phytopathol. 29:193-217.

Dawson, W. O., Lindbeck, A. G. C., Lewandowski, D. J., Culver, J. N., Hilf, M. E., and Stubbs, G. 1993. Precise interactions between tobamoviruses and plants. In: Biotechnology and Plant Protection: Viral Pathogenesis and Disease, D.D. Bills, ed. World Scientific Publishing,Teaneck, NJ.

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.).

Holmes, F. O. 1937. Inheritance of resistance to tobacco-mosaic disease in the pepper. Phytopathology 27:637-642.

Holmes, F. O. 1938. Inheritance of resistance to tobacco mosaic virus in tobacco. Phytopathology 28:553-561.

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. J. Gen. Virol. 69:1789-1798.

Jones, R. A. C. 1982. Breakdown of potato virus X resistance gene Nx: Selection of a group four strain from strain group three. Plant Pathol. 31:325-331.

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, non-specific resistance in protoplasts. Plant Cell (In press.)

Knorr, D. A., and Dawson, W. O. 1988. A point mutation in the tobacco mosaic virus capsid protein gene induces hypersensitivity in *Nicotiana sylvestris*. Proc. Natl. Acad. Sci.USA 85:170-174.

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*. EMBO J. 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. Plant Cell 1:515-522.

Moreira, A., Jones, R. A. C., and Fribourg, C. E. 1980. Properties of a resistance-breaking strain of potato virus X. Ann. Appl. Biol. 95:93-103.

Murphy, G., and Kavanagh, T. 1988. Speeding up the sequencing of double-stranded DNA. Nucleic Acids Res. 16:5198.

Saito, T., Yamanaka, K., Watanabe, Y., Takamatsu, N., Meshi, T., and Okada, Y. 1989. Mutational analysis of the coat protein gene of tobacco mosaic virus in relation to hypersensitive response in tobacco plants with the N′ gene. Virology 173:11-20.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

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. J. Gen. Virol. 67:57-67.

Waye, M. M. Y., Verhoeyen, M. E., Jones, P. T., and Winter, G. 1985. EcoK selection vectors for shotgun cloning into M13 and deletion mutagenesis. Nucleic Acids Res. 13:8561-8571.