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A coat protein transgene from a Scottish isolate of potato mop-top virus mediates strong resistance against Scandinavian isolates which have similar coat protein genes

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Abstract

Resistance tests were made on seedlings of transformed lines of *Nicotiana benthamiana* which contain a transgene encoding the coat protein (CP) gene of a Scottish isolate of potato mop-top virus (PMTV). This transgene has been reported to confer strong resistance to the PMTV isolate from which the transgene sequence was derived and also to a second Scottish isolate. Plants of lines of the transgenic *N. benthamiana* were as resistant to two Swedish and two Danish PMTV isolates as to a Scottish isolate, and of five lines tested, greater than 93.5% of transgenic plants were immune. The coat protein gene sequences of these four Scandinavian isolates were very similar to those of the two Scottish isolates. The greatest divergence between the isolates was three amino acid changes and there was less than 2% change in CP gene nucleotide sequence. It is concluded that the PMTV CP transgene used in these experiments could confer resistance against isolates from different geographical areas because it is becoming apparent that the CP genes of PMTV isolates are highly conserved.

Introduction

Potato mop-top virus (PMTV) is a member of the genus *Furovirus* in the family *Togaviridae*. The virus is responsible for economic losses in potato crops grown in areas with cool climates and has been identified in Northern Europe, Canada, China, Japan and the Andean region of South America. PMTV is transmitted by the motile zoospores of the plasmodiophoromycete fungus *Spongospora subterranea* (Wallr.) Lagerh. (Jones and Harrison, 1969; Arif et al., 1995) which causes powdery scab on tubers. Infection with the virus can cause some yield loss but, more importantly, qualitative damage known as 'spraing' which can occur as brown arcs and circles in the flesh of tubers of susceptible cultivars (Harrison and Jones, 1971). Some potato cultivars are particularly sensitive and PMTV-infected plants produce tubers with severe spraing symptoms. For example, cv. Saturna, which is widely used in the Scandinavian potato processing industry, is a particularly sensitive cultivar. In Saturna and two other cultivars used for crisp production in Sweden, incidences of 25% spraing are commonly found (Sandgren, 1995). In Denmark, Nielsen and Engsbro (1992) have often recorded 30–50% of Saturna tubers affected by spraing. Effective and environmentally acceptable chemical control of the fungal vector is not commercially available, and there are no sources of resistance or tolerance to PMTV that have been deliberately used in breeding programs.

The advantages of transgenic resistance to plant viruses using pathogen-derived sequences are well established (Lomonossoff, 1995) and there are many examples where this approach has been successfully applied against potato viruses (reviewed by Acosta et al., 1995). The most common pathogen-derived transgene used for resistance is that encoding the coat protein from which is derived coat protein-mediated resistance (CP-MR) (reviewed by Lomonossoff, 1995). Transgenic lines of *Nicotiana benthamiana* transformed to express a translatable version of the coat protein gene from a Scottish isolate of PMTV exhibit very

strong resistance to infection with that isolate and a second Scottish isolate of the virus. This resistance was effective following manual, graft, or fungal inoculation and three of five lines tested were immune to infection with PMTV (Reavy et al., 1995). Lomonossoff (1995) suggested that CP-MR generally tends to be fairly specific for the virus from which the transgene was isolated, although there are instances where resistance is quite wide. Examination of the CP gene coding similarities between a range of virus isolates, and assessment of the effectiveness of CP-MR against isolates from different geographical locations is essential if transgenes are to be deployed in commercial crops. In this paper we describe a comparison of the CP gene sequences of four Scandinavian PMTV isolates and the two Scottish isolates mentioned above, and the effectiveness of the existing CP transgene derived from a Scottish isolate against inoculation with the Scandinavian isolates.

Materials and methods

ELISA

Accumulation of PMTV antigen in inoculated plants was assessed by triple antibody sandwich ELISA. This used a polyclonal antibody (1 μg/ml) to coat microtitre plates and monoclonal antibody SCR69 (1 μg/ml) to detect trapped antigen followed by alkaline phosphatase conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Maryland, USA) and 4-nitrophenyl phosphate (Boehringer Mannheim) at 1 mg/ml as substrate, essentially as described by Torrance et al. (1993).

Transgenic plants

Transgenic lines of *N. benthamiana* expressing the CP gene were those described by Reavy et al. (1995). The transgene contained cDNA encoding the CP gene of PMTV (T isolate from Scotland) in a translatable context under the transcriptional regulation of a cauliflower mosaic virus 35S promotor. All experiments were made with plants grown from seed (T_1 generation) arising from self-fertilisation of the original transformants (Reavy et al., 1995). Seedlings of transgenic test plants were grown on compost without prior kanamycin selection. However, before testing for resistance, DNA extracts of young plants were screened by polymerase chain reaction (PCR). Total leaf DNA extracts were prepared by grinding samples frozen in liquid nitrogen, followed by addition of DNA extraction buffer (50 mM Tris HCl, pH 8.0, containing 0.7 M NaCl, 10 mM EDTA, 1% CTAB and 1% 2-mercaptoethanol; 2 ml/g tissue). Samples were homogenised, an equal volume of Tris stabilized phenol (Bio/Gene) added and homogenised again. Extracts were heated at 56 °C for 10 min with occasional mixing, then a half volume of chloroform : isoamylalcohol (24:1) was added. The mixture was then centrifuged for 5 min at 10,000 g and DNA in the aqueous phase from this final extraction precipitated by addition of 0.1 vol of 3 M sodium acetate and 2 volumes of ice-cold ethanol. Precipitated DNA was washed with 70% ethanol, dried and resuspended in 300 μl of 10 mM Tris HCl, pH 8.0, containing 1 mM EDTA. Between 1 and 5 μl of resuspended DNA were used for PCR amplification essentially as described by Arif et al. (1994); using an oligonucleotide primer complementary to a sequence in the nopaline synthase terminator (*nos*) gene and the PMTV A839 primer. The sequence (5′-GCTCTAGACCGGCAACAGGATTC-3′) of the primer in the *nos* gene corresponded to the complement of nucleotides 615–637 (Bevan et al., 1983) and had mutations (underlined) incorporated to produce an *Xba*I restriction enzyme recognition site. PCR of transgenic plants gave a DNA product of approximately 650 bp. Seedlings which gave DNA extracts from which the specific transgene sequence could not be amplified (non-transgenic segregants) were discarded.

Virus isolates and propagation

The PMTV cultures were originally isolated from field soil samples by the bait-plant method described by Jones and Harrison (1969). The isolates PMTV:174 and PMTV:31 were obtained from two farms in the south west of Sweden (Halland county). Two Danish isolates (PMTV:47 and PMTV:DK) were obtained from two farms used for contract cultivation of cv. Saturna in Jylland. Scottish isolate (PMTV-S) was obtained from a farm at Braco, near Auchterarder, Perthshire. Cultures of PMTV isolates were maintained in *N. benthamiana*. Virus was transmitted by manual inoculation in which extracts of freshly macerated leaves from infected *N. benthamiana* plants (1 g of leaf per 5 ml of water) were rubbed onto Carborundum-dusted leaves of test plants.

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          1                                                  50
T         MAEIRGERKA AVENRYDSWD HEQAMKAAVR KFISYDQFSA QLRNWREARL
S         ...N....R. .......... .......... .......... ..........
47        ...N....R. .......... .......... .......... ..........
DK        ...N....R. .......... .......... .......... ..........
174       ...N....R. .......A.. .......... .......... .......R..
31        ...N....R. .......A.. .......... ...F...... ..........

          51                                                 100
T         NIIEHATSVL SQVSNLGRTH FYSGTERFGG SSLVGDKLYV CLNETRMKTA
S         .......... .......... ...R...... .......... ..........
47        .......... .......... ...R...... .......... ..........
DK        .......... .......... ...R...... .......... ..........
174       .......... .......... ...R...... .......... ..........
31        .......... .......... ...R...... .......... ..........

          101                                                150
T         LNNIIVALQT VNGEGRARRL GPREASANTG GEDSALNVAH QLAEVDDLLT
S         .......... .......... .......... .......... ..........
47        .......... .......... .......... .......... ..........
DK        .......... .......... .......... .......... ..........
174       .......... .......... .......... .......... ..........
31        .......... .......... .......... .......... ..........

          151                      176
T         DESFLREAVF TQDKYELVNG LRWAGA
S         .......... .......... ......
47        .......... .......... ......
DK        .......... .......... ......
174       .......... .......... ......
31        .......... .......... ......
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Figure 1. Comparison of the amino acid sequences of the coat proteins of the PMTV-S Scottish isolate (S) and four Scandinavian isolates (47, DK, 174 and 31) with PMTV-T (T) isolate from which the CP transgene was obtained.

Virus resistance tests

For each experiment, test plants of the T_1 generation of the transgenic lines and of a control non-transgenic line were manually inoculated. Extracts of the challenge-inoculated plants were tested approximately 18 days post inoculation by a very sensitive assay for infectious virus as described by Reavy et al. (1995). Infectivity assay was performed by manually inoculating indicator plants of *N. benthamiana* with macerated leaf tissue obtained from test plants. Infection of indicator plants was assessed by symptomatology and ELISA approximately 3 wks after inoculation.

Sequencing of coat protein genes

Total RNA was extracted from *N. benthamiana* leaves infected with PMTV as described by Verwoerd et al. (1989). Reverse transcription was performed using oligonucleotide 671 (5′ CTGGATCCCCTGAGCGGTTAATTGC 3′) which is complementary to PMTV RNA 3 nucleotides 819-835 (Kashiwazaki et al., 1995) with additional nucleotides (underlined) to create a *Bam*HI site. PCR was performed using oligonucleotides 671 and A839 (5′-TCGGATCCTCTCGGATACCACCCTT-3′) which is the same as the 5′ untranslated region upstream of the CP gene with additional nucleotides (underlined) incorporated to create a *Bam*H1 site. PCR products were cloned into pGEMT (Promega) or pT7 Blue (Novagen) and sequenced using M13 forward and reverse sequencing primers with an Applied Biosystems Dye-Terminator sequencing kit and a 373A automated sequencing machine. A minimum of two independent clones were sequenced for each isolate to detect mutations possibly caused by Taq DNA polymerase during the PCR reactions. Sequences were analysed and compared using the UWGCG computer software package (Devereaux et al., 1984).

Results

Coat protein gene sequences

The CP gene amino acid sequences of the four Scandinavian PMTV isolates were very similar to those of the Scottish isolates PMTV-T (Kashiwazaki et al., 1995) and PMTV-S (Mayo et al., 1996) (Figure 1). All the

Table 1. Resistance of five transgenic lines of *Nicotiana benthamiana* expressing the PMTV-T CP gene to manual inoculation with one Scottish (S) and three Scandinavian (174, 47 and 31) PMTV isolates

Line	Resistance[a] to manual inoculation with PMTV isolates			
	S	174	47	31
W1	NT	NT	1/20	1/17
W2	1/26	13/93	NT	NT
W5	NT	0/33	3/23	1/24
W8	0/33	1/32	NT	NT
W9	NT	NT	0/16	1/20
WT control	48/48	51/64	24/24	24/24

[a] Resistance given as no. of infected transgenic plants/no. of transgenic plants inoculated.
NT = not tested.

Scandinavian isolates and PMTV-S, when compared to PMTV-T, had the following amino acid changes: I4 to N, K8 to R, G74 to R. The Swedish isolates had additional changes: S18 to A in both PMTV-174 and PMTV-31; A48 to R in PMTV-174; S34 to F in PMTV-31. In addition there were < 2% non-coding changes in the nucleotide sequences of the other isolates when compared to PMTV-T.

Transgenic resistance to Scandinavian PMTV isolates

PCR analysis of progeny plants indicated that the transgenic lines of *N. benthamiana* used for resistance tests contained either 1 or 2 T-DNA insertion events (Reavy et al., 1995; H. Barker, unpublished results), although we cannot discount the possibility that some insertion events contained several copies of the transgene in tandem arrays. For resistance tests reported in Table 1, DNA extracts of seedlings were screened by PCR in order to discard non-transgenic segregants. Seedlings of non-transgenic control *N. benthamiana* were also inoculated in each resistance test; a mean of 92% became infected whereas a mean of 6.5% of transgenic plants became infected as indicated by the infectivity assay to test plants. These results indicate that infectious virus could not be detected in the majority of inoculated transgenic plants. However, most transgenic plants in which virus was detected did not develop symptoms, whereas symptoms developed in non-transgenic control plants. In other tests, unscreened seedlings of several lines were inoculated with isolate DK, and these were also resistant to PMTV. Thus, 24 plants from each of lines W8, W16 and W20 were inoculated with isolate DK, and the numbers of plants which became infected were 0, 4 and 1, respectively. These seedlings were not selected or tested by PCR, and thus some of the infected plants may have been non-transgenic segregants which are expected to be susceptible. These results are consistent with others (Reavy et al., 1995; H. Barker, unpublished results) which indicate that in some tests infectious PMTV can be recovered from a few transgenic plants, although the majority are immune.

Discussion

We show here that a number of European isolates of PMTV are highly conserved in the amino acid sequence of the CP gene. The Scottish isolate PMTV-S and the Danish isolates have identical coat protein amino acid sequences. The Swedish isolates diverge from these three by two amino acid changes and PMTV-T has three amino acid changes compared to the Danish isolates. Mayo et al. (1996) have reported that there is remarkably little sequence diversity between the coat protein sequences of eight Andean PMTV isolates and three Scottish isolates. Indeed, the CP sequences of three Andean isolates of PMTV are identical to that of PMTV-S and the Danish isolates described here. Thus, the coat protein gene of PMTV isolates is highly conserved with isolates appearing to be more like minor variants. So far, the biological significance of any sequence variation in the coat protein of PMTV isolates has not been determined.

Some forms of CP-MR have been found to be effective against closely related virus strains. Thus, Barker (1995) showed that a PLRV CP transgene sequence obtained from a Scottish isolate, was effective against a Peruvian isolate whose amino acid sequence was

97.6% identical. Nejidat and Beachy (1990) obtained CP-MR with a range of tobamoviruses when their coat proteins were greater than 60% homologous in amino acid sequence. Similarly, transformation of plants with the soybean mosaic virus (SMV) CP gene sequence, protects plants against infection with potato virus Y and tobacco etch virus whose coat proteins are about 60% identical with SMV CP (Stark and Beachy, 1989). However, there are examples in which CP-MR is not expressed to related CP sequences. Thus, tobacco plants transgenic for the coat protein of the U_1 strain of tobacco mosaic virus (TMV) are most resistant to that strain of TMV and its close relatives and show less resistance to more distantly related tobamoviruses (Anderson et al., 1989). Similarly, transgenic expression of CP from one strain of tobacco rattle virus did not protect plants against infection with another strain where the CPs are 39% identical (Van Dun & Bol, 1981). More significantly, single amino acid changes in the coat protein transgene sequence of alfalfa mosaic virus abolishes resistance to an otherwise homologous virus (Turner et al., 1991). However, this seems to be the only such example in which protection breaks down to virus isolates with small changes in amino acid sequences.

The strong form of CP-MR to Scottish isolates T and S of PMTV is conferred by transgenic expression of the PMTV (isolate T) CP gene in *N. benthamiana*, is effective against PMTV infection following inoculation by mechanical means, grafting or by the fungal vector and prevents viral replication and symptom development (Reavy et al., 1995). There is a great degree of CP homology between the Scottish and Scandinivian isolates we have examined and we have now shown that such CP-MR using a transgene from a Scottish isolate is as effective against four Scandinavian PMTV isolates as it is against the Scottish isolates. Given the high degree of coat protein sequence conservation between PMTV isolates shown here and by Mayo et al. (1996), we expect this transgene to be effective against other isolates including those from the Andes.

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