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An antisense coat protein gene confers immunity to potato leafroll virus in a genetically engineered potato

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Abstract

The Bzura commercial potato cultivar was transformed by sense or antisense constructs which included the coat protein gene of potato leafroll virus RNA. In the sense construct, the coat protein gene was preceded by a leader sequence shorter than that in the subgenomic RNA formed in infected cells. The antisense construct consisted of a sequence complementary to the first 2020 nucleotides of the subgenomic RNA. Selected transformants expressing viral RNA were resistant to virus challenge by viruliferous aphids. In one line, expression of the antisense RNA prevented virus infection even after grafting with scions from infected plants and therefore this transformant might be regarded as virus immune.

Introduction

Potato leafroll virus (PLRV), a member of the genus *Luteovirus*, causes losses world-wide in potato production. PLRV infection spreads easily in potato stocks and is associated with significant reduction of tuber mass. PLRV is transmitted by aphids in the circulative, non propagative manner and in infected tissue is limited mainly to phloem tissue (van den Heuvel et al., 1995). Luteoviruses have isometric particles which contain a single stranded messenger-sense RNA genome (Mayo et al., 1989). The 5′ end of PLRV RNA contains a genome-linked protein (VPg) and lacks a polyadenylate sequence at the 3′ end. The nucleotide sequences of the genomic RNA of a few PLRV isolates have been determined (Keese et al., 1990; van der Wilk et al., 1989; Mayo et al., 1989); the RNA codes for six open reading frames arranged in two gene clusters which are separated by short intergenic region. The nucleotide sequence of the Polish isolate (PLRV-P) (Pałucha et al., 1994) follows the genome organisation of other isolates.

Genetically engineered coat protein-mediated resistance (CP-MR) has been shown to be a promising approach for the control of a number of plant virus diseases (Beachy et al., 1990; Hackland et al., 1994). Expression of the virus coat protein gene in transgenic plants often induces a substantial delay in the development of symptoms following infection by the virus. In some aspects, CP-MR is similar to the phenomenon of 'cross-protection' in which prior infection of a plant with a mild virus strain reduces the effects of infection with a virulent one (McKinney, 1929). However, the mechanism of CP-MR is not well understood. Theoretically, the virus life cycle may be affected by different transgene products including the coat protein per se and/or its transcript. Transgene orientation may be important, and indeed negative sense RNA expression was reported to affect virus accumulation (Huntley and Hall, 1993a; 1993b). The lack of a unified CP-MR mechanism leads us to test different transgene versions when hunting for efficient engineered resistance. Until now several papers report the construction of transgenic potatoes resistant to PLRV infection (Kawchuk et al., 1990; Kawchuk et al., 1991; van der Wilk et al., 1991; Barker et al., 1992).

We decided to create potato transformants expressing either (+) or (-) transcripts covering the PLRV-P

genome regions encoding the coat protein. Here we report the engineering of resistance to PLRV in the cv. Bzura (Bp) potato widely used in Polish agriculture which carries natural resistance to potato virus X and potato virus Y (PVX and PVY). In this study, potato plants were transformed with several constructs carrying the coat protein gene, with flanking sequences of different lengths, in both sense and antisense orientations. The resulting transgenic plants were tested for the presence of viral cDNA, its expression and virus resistance. The potato plants were challenged with PLRV by aphid inoculation and by grafting. The resistance trait observed in two lines was transmitted to the progeny grown from transformant tubers.

Materials and methods

Construction of transgenic plants

Two clones pVCP-2 and pVCP-5 carrying viral cDNA (corresponding to the sequence between nucleotides 2263-5466 and 3510-5466 respectively) were as previously constructed (Pałucha et al., 1994). From the pVCP-2 clone, a DNA fragment, extending 428 nucleotides upstream of the AUG initiation codon of the coat protein gene, which covers the leader sequence of sgRNA and 967 nucleotides downstream the amber codon of the coat protein gene which is the 5′ end of ORF5, was excised using *Eco*RI, blunt end ligated into the *Sma*I site of the binary vector pROK2 in antisense orientation (pRA7-a, Figure1. A). From the pVCP-5 clone, a DNA fragment extending 132 nucleotides upstream of the the AUG start codon and 4 nucleotides downstream the amber codon of the PLRV coat protein gene was excised using *Eco*RI and *Acc*I. This fragment was gel purified and inserted into the *Sma*I site of the binary vector pBI121 in sense orientation (pBCP1-s Figure1. B). Both plasmids, pRA7-a and pBCP1-s, were introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation. Leaf discs of *Solanum tuberosum* cultivar Bzura were used for Agrobacterium-mediated transformation experiments as described (Nadolska-Orczyk et al., 1994). Potato cells carrying integrated copies of the introduced T-DNA cassette were selected by growth of calli and the subsequent shoots in the presence of 100 μg/ml kanamycin. Shoots 4 to 7 mm long were excised and rooted on Murashige and Skoog media lacking kanamycin.

Analysis of transgenic plants

Plants propagated *in vitro* were screened for transgene DNA and RNA. From 200 mg of grounded in a 2ml Eppendorf tube leaf material the DNA was extracted using a procedure described by Doyle and Doyle (1989). For RNA isolation, 200 mg of leaf material was powdered in liquid nitrogen and RNA was extracted according the method described by Verwoerd et al. (1989). Southern hybridisation analysis was performed on *Hin*dIII or *Eco*RI and *Hin*dIII digested genomic plant DNA that was transferred to Hybond N membranes (Amersham) and probed as described (Maniatis et al., 1990). RNA for Northern analysis was run after glyoxal denaturation on 1% agarose gels, transferred to positively charged Hybond membranes and probed according to the manufacturer's instruction. Randomly primed ^{32}P-labelled DNA probes were prepared from a gel-purified *Eco*RI restriction fragment of pVCP-2. These probes, which are specific for the coat protein cistron, were used for both Southern and Northern analysis.

Challenge of transgenic plants with PLRV

The resistance of transgenic plants to PLRV was tested by inoculation with aphids or grafting. PLRV was maintained in cv. Osa potato plants infected with the isolate L7 (Syller, 1985). Transgenic potato lines were propagated under sterile conditions and small plantlets were transplanted to pots with soil and grown in the greenhouse. After two weeks, eight plants of each line were inoculated with PLRV by exposure for 4 days to 10 viruliferous *Myzus persicae* that had been reared on PLRV-infected cv. Osa potato. Aphids were placed on plants using a brush, and after this inoculation period the aphids were killed. Two other plants of these lines were inoculated with scions carrying PLRV. Virus concentration in these plants was high, with ELISA A_{405} values over 0.8.

Serological tests were performed on inoculated plants 6 weeks post inoculation. Two to four tubers were collected from each plant and progenies of these tubers were tested for viral antigen. Tests were performed on the fourth leaf from the top of the plant in the sixth week after planting of presprouted tubers. For virus detection, leaf sap samples were extracted from the whole leaves with a Pollähne roller press and a 200μl sample from each leaf was diluted with 300 μl of extraction buffer. Coating globulins and globulins conjugated with alkaline phosphatase, produced

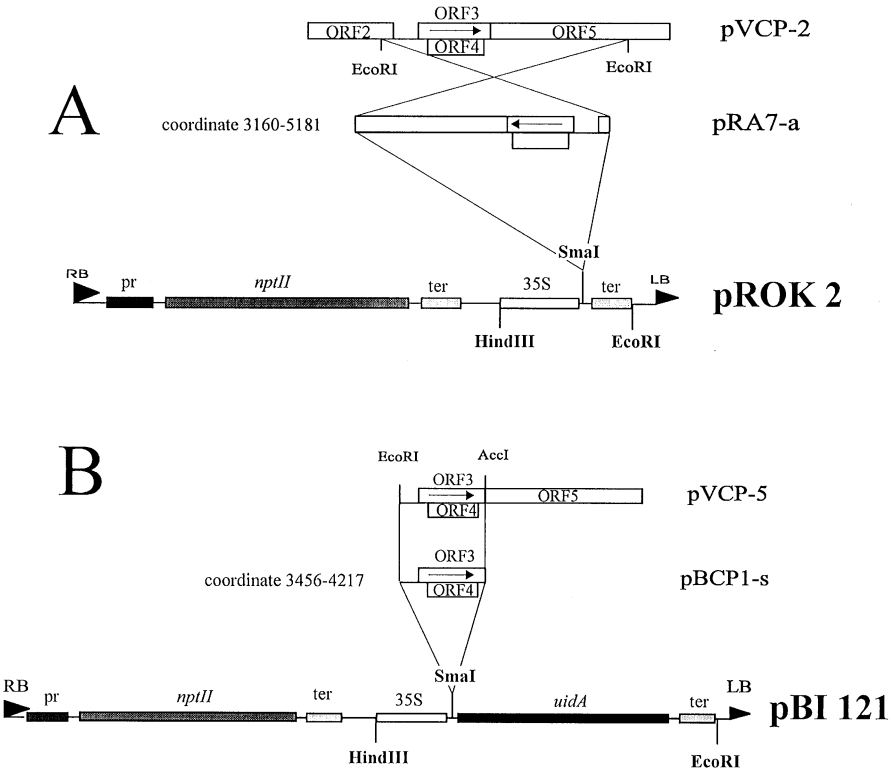

Figure 1. Schematic representation of the construction of the plant expression plasmids pRA7-a and pBCP1-s used to express of the PLRV genome in transgenic potato plants; ORF3 - CP gene.
RB- right border; LB- left border; *uidA*- β-glucuronidase gene; pr- nopaline synthase promoter; ter- nopaline synthase termination signals; *nptII* - neomycine phosphotransferase II gene;.35S- CaMV 35S RNA promoter.

in the Gdañsk laboratory of the Potato Research Institute, were used in the ELISA tests. Coating globulins were used at a concentration 2 μl/ml, and conjugated globulins in a dilution of 1:1000 for testing leaf extracts. Incubation with substrate (PNPP-Sigma catalogue number N104-0) was conducted at room temperature for 2 h. Absorbance at 405nm was recorded.

Challenge of transgenic plants with PVY^N and PVX

Three plants of each line tested were mechanically inoculated with PVY^N. Four weeks after inoculation, well-grown leaves were chosen to prepare samples for ELISA with polyclonal antiserum against PVY.

Transgenic line RA7a-2 was inoculated with PVX. Four and six weeks after inoculation the agglutination test with anti-PVX serum was performed and *Gomphrena globosa* test plants were inoculated with crude sap from tested potato plants.

Results

Transformation of potato plants

From 59 cv. Bzura potato leaf disks infected with *A. tumefaciens* containing the pBCP1-s or the pRA7-a plasmid, 3 and 4 shoots respectively were isolated from independent calli. All isolated potato shoots rooted on medium containing 100μg/ml kanamycin and were further characterised before being selected for virus inoculation. With the exception of line RA7a-2, which was phenotypically different from parental plants being slightly bushy and stunted, whereas other transgenic potato plants were indistinguishable visually from non-transgenic plants. Each transgenic plant was able to produce tubers.

Transgene characterisation by Southern analysis

Plant genomic DNA was digested by either *Hin*dIII or *Hin*dIII plus *Eco*RI. Digestion with *Hin*dIII allows for

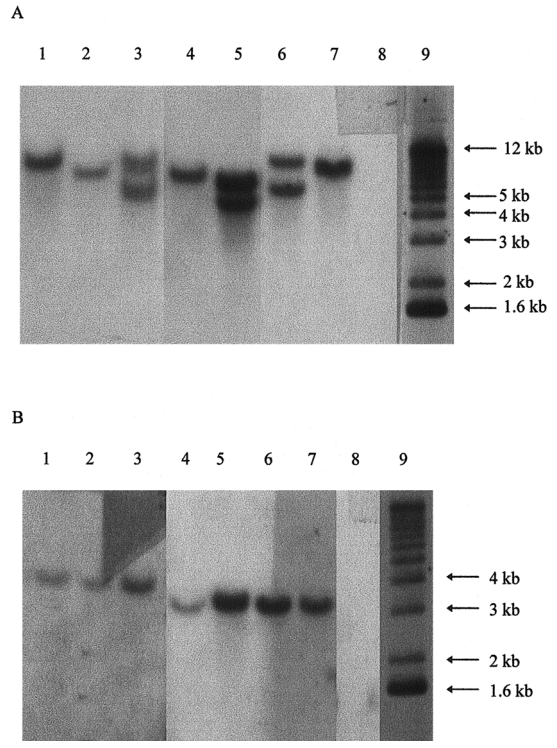

Figure 2. Southern blot analysis of transgenic potato plants transformed with the potato leafroll virus (PLRV) cDNA. Ten μg of DNA purified from transgenic leaf material and digested with *Hin*dIII (A) or with *Eco*RI and *Hin*dIII (B) were loaded onto a 1% agarose gel. Lanes 1-3, DNA from transgenic plants BCP1s-1 BCP1s-2 and BCP1s-4 respectively. Lanes 4-7, DNA from transgenic plants RA7a-1 RA7a-2 RA7a-4 and RA7a-5 respectively. Lane 8, DNA from an untransformed potato plant. Lane 9, DIG labelled 1kb LADDER (Gibco-BRL).

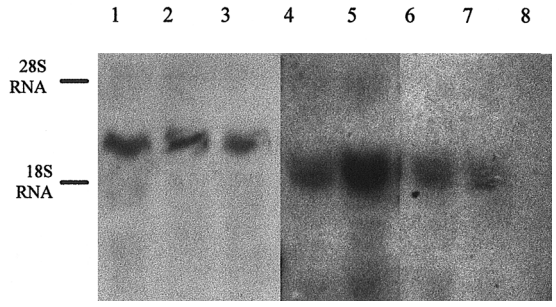

Figure 3. Northern analysis of PLRV cDNA in transgenic potato plants. Twenty μg of total plant RNA purified from transgenic leaf material were loaded onto a 1% agarose gel. Lanes 1-3, RNA from transgenic plants BCP1s-1 BCP1s-2 and BCP1s-4 respectively. Lanes 4-7, RNA from transgenic plants RA7a-1 RA7a-2 RA7a-4 and RA7a-5 respectively. Lane 8, RNA from an untransformed potato plant.

the analysis of the number of genetic loci where viral cDNA integrated, since one restriction site is located in plant DNA whereas the second site is located in the expression cassette. The presence of a unique restriction fragment after hybridisation with the probe indicates that in lines BCP1s-1, BCP1s-2, RA7a-1 and RA7a-5, the integration occurred in a single locus (Figure 2, A, lanes 1, 2, 4, and 7). Judging from the presence of additional restriction fragments (Figure 2, A, lanes 3, 5 and 6) integration of viral cDNA fragments in lines BCP1s-4 RA7a-2 and RA7a-4 occurred in multiple loci. The integrity of the introduced expression cassettes was assessed by double digestion with *Hin*dIII and *Eco*RI. These enzymes excised the entire 3100 nucleotides of integrated expression cassette from lines RA7-a, which contain the 2020 nucleotides of inserted PLRV cDNA covering the sgRNA leader sequence, the CP gene, and part of the ORF5 (Figure 2 B, lanes 5-8). In BCP1-s plant lines, the excised 3700 nucleotide restriction fragment, which contains the CP gene with part of the leader sequence was identified (Figure 2 B, lanes 1-4). The remainder of the restriction fragments originated from the pROK2 or pBI121 plasmid and polylinker. This analysis confirmed that all transformants contain the appropriate DNA insert.

Transcript analysis

Southern analysis suggested that intact transgenes were present in transformants. To assess transgene expression, Northern analysis was performed. All pBCP1-s derived transformants gave unique positive signals with the coat protein specific probe confirming the presence of viral cDNA transcripts of 3000 nucleotides (CP+GUS; Figure 3, lanes 1-3). pRA7-a derived transformants also synthesised unique, but shorter transcripts migrating in the region of 2300 nucleotides (Figure 3, lanes 4-7). As expected, the length of transcripts was determined by the length of the integrated cDNA fragment and an additional 250-300 nucleotides provided by the terminator sequence and a poly A tail. Northern analysis confirms the integrity and transcriptional activity of integrated genes.

Virus resistance in transgenic plants

The evaluation of resistance to PLRV was done with 7 transgenic lines (Table 1). Two transgenic lines, RA7a-2 and BCP1s-4, were resistant to PLRV after inoculation by aphids (*Myzus persicae*). After 6 weeks post inoculation, the virus concentration was monitored by

Table 1. Values of ELISA (average of A_{405}) of tested transgenic potato plants. The values of the resistant lines (BCP1s-4 and RA7a-2) are in bold letters

Plant line	Inoculated plants			Progeny grown from:					
	Six weeks post inoculation by aphids			i) aphid inoculated plants			ii) plants inoculated by grafting		
	Number of tested plants	Average A_{405} value	SE	Number of tested plants	Average A_{405} value	SE	Number of tested plants	Average A_{405} value	SE
Bp not inoculated	4	0.056	0.011	4	0.040	0.009	2	0.056	0.012
Bp inoculated	7	0.168	0.065	7	0.232	0.147	3	0.696	0.322
Bp-GUS	7	0.143	0.070	8	0.316	0.330	5	0.900	0.500
BCP1s-1	6	0.427	0.498	8	0.262	0.227	4	0.667	0.368
BCP1s-2	7	0.453	0.253	9	0.256	0.240	3	0.789	0.453
BCP1s-4	7	**0.033**	0.008	7	**0.030**	0.017	4	**0.410**	0.080
RA7a-1	6	0.768	0.360	12	0.529	0.362	4	0.646	0.428
RA7a-2	7	**0.036**	0.012	7	**0.027**	0.007	3	**0.069**	0.070
RA7a-4	5	0.246	0.265	12	0.166	0.200	4	0.711	0.410
RA7a-5	4	0.318	0289	10	0.257	0.218	2	0.698	0.114

SE- standard error.

ELISA. Three types of control plants were used in this assay: 1. *In vitro* propagated non transgenic plants (Bp-*wild type*); 2. transgenic plants carrying the reporter gene from pBI121 (Bp-GUS); 3. transgenic plants RA-7a-2 not infected (healthy control). The plants were challenged with viruliferous aphids (see Methods) and virus accumulation was evaluated. Wild type regenerated plants (line Bp-*wild type*, Figure 4) as well as control transformants carrying the GUS gene (line Bp-GUS, Table 1) showed high virus accumulation. In contrast, PLRV antigen did not accumulate in lines RA7a-2 and BCP1s-4 (Table 1). Challenged plants were further cultured until tuber production, and tuber progeny were tested for the PLRV antigen (Table 1). A serological test showed that progeny of the tubers from seven transgenic plants of BCP1s-4 and seven of RA7a-2 were healthy, whereas 10 out 14 of control plants were infected with the PLRV.

Infection by grafting results in a continuous supply of virus directly to the phloem tissue. Under such heavy PLRV load, control plants showed average ELISA A_{405} values around 0.7. The antigen accumulation was at least twice as high compared to aphid inoculated controls (line Bp-GUS and Bp-*wild type*, Table 1). Under this heavy challenge, line BCP1s-4 carrying the coat protein gene in sense orientation showed resistance, with an average ELISA A_{405}value being only 0.41. Therefore, even under such virus pressure, the line BCP1s-4 showed enhanced resistance to PLRV. Line RA7a-2, expressing the antisense PLRV RNA, showed very high resistance because the A_{405} values were on the same level as uninoculated control with the exception of one plant in which A_{405}=0.15. We conclude that both transformant lines, RA7a-2 and BCP1s-4, are highly resistant to PLRV. Progeny plants of both lines (BCP1s-4 and RA7a-2) were virus free after aphid inoculation, whereas after inoculation by grafting only progeny of line RA7a-2 did not show any virus accumulation. Indeed, RA7a-2 line therefore may be considered as virus immune.

Both transformants were independently challenged with PVY and PVX (see materials and methods). Six weeks post PVY inoculation, average ELISA A_{405} values with anti PVY polyclonal antiserum in transformants reached 0.085 (SD=0.016). In mock inoculated control plants, the ELISA A_{405} values were 0.091 (SD=0.016). In transgenic line RA7a-2 inoculated with PVX the virus was not detected unlike in the control potato and tobacco plants. Therefore natural resistance to PVY and PVX present in parental Bzura cultivar was fully preserved in transformants carrying engineered PLRV resistance.

Detection of coat protein in transgenic plants was not performed. Attempts to detect of coat protein in transgenic potato were not successful (van der Wilk et al., 1991; Kawchuk et al., 1991).

Discussion

Transformation with the constructs described in this paper, resulted in resistance to PLRV in transgenic

potato, expressing the coat protein gene transcripts in either the negative or positive sense orientation. Several transgenic potato lines were obtained, and two tested showed resistance to PLRV (see Table 1). These transformants were resistant not only to PLRV infection with viruliferous aphids but also by grafting with scions from PLRV-infected plants (Table 1). Such challenge is recognised as the most potent method of virus infection and was used by other authors (Derrick and Barker, 1992). This result strongly suggests that RA7a-2 line plants are indeed highly resistant or even immune to virus under control conditions (growth at 25 °C). It is likely that under field conditions they will not become infected even at a high infection pressure. Moreover, cv. Bzura RA7a -2 and BCP1s-4 transgenic plants remained resistant to PVY and PVX and thus these lines are resistant to three important potato viruses.

So far, attempts to develop transgenic plant resistant to viral infections using antisense RNA (AS RNA) of CP gene have usually ended with limited success (Cuozzo et al., 1988; Rezaian et al., 1988; Powell et al., 1989; Fang and Grumet, 1993; Farinelli and Malnoe, 1993: Hammond and Kamo, 1995). However, other authors (van der Wilk et al., 1991; Kawchuk et al., 1991) working with PLRV reported to reach the same level of resistance with AS RNA constructs as with the CP gene.

The AS strategy seems to be more effective when the engineered transcript is complementary to the intercistronic or non-coding region of the viral genome. Transgenic *Nicotiana tabacum* cv. Xanthi plants expressing the AS transcripts of a 5′ nucleotide fragment of TMV leader sequence were highly resistant to TMV infections. Systemic accumulation of TMV RNA and progeny virus was diminished 15 to 30 fold in these plants (Nelson et al., 1993). Another author (Huntley and Hall, 1993a) showed that AS RNA to the brome mosaic virus RNA3 intercistronic region significantly reduced virus replication when co-electroporated into protoplasts with viral RNA transcripts. AS RNA targeted to the 3′ non-coding regions carrying the minus strands promoters of BMV genomic RNAs significantly reduced replication in protoplasts co-electroporated with viral RNA (Huntley and Hall, 1993b). Recently, construction of transgenic *Nicotiana benthamiana* lines carrying the carboxy-terminal portion of the coat protein gene, the complete 3′ non-coding sequence and a short poly(A) tract of the bean yellow mosaic potyvirus genome (BYMV) in antisense orientation has been reported [Hammond and Kamo, 1995). One transgenic line out of 10 examined was extremely resistant to infection by mechanical inoculation of BYMV.

It should be stressed that the resistant Bzura RA7a-2 plant expresses the AS RNA covering the leader sequence of subgenomic RNA, the coat protein gene and 967 adjacent nucleotides from the ORF5. The substantial homology between the 5′ end of the genomic RNA and sgRNA (ACAAAAGAA) has been previously observed (Miller and Mayo, 1991). The (-) strand sequence complementary to the ACAAAAGAA domain may act as a promoter for viral replicase. AS RNA complementary to this region might interfere with amplification of viral genomic and sgRNA. Interestingly, one can observe a correlation between high AS RNA accumulation and high resistance of RA7a-2 line (Figure 3).

Effective antisense expression-based resistance observed in RA7a-2 line could be of interest for breeders for several reasons. As the antisense RNA does not code for viral polypeptides, the resistant plant could be regarded as virus antigen-free. Antisense RNA of coat protein gene does not express the coat polypeptide. Thus, transgenic plants could not be a source for hetero-encapsidation, a phenomenon which may propagate unexpected versions of pathogens (Zoeten, 1991). Chimeric plants, expressing viral positive strand RNA, infected with related virus are potential sources of recombinants (Greene and Allison, 1994).

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