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Generation of transgenic potato plants highly resistant to potato virus Y (PVY) through RNA silencing

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Abstract

In this study we applied RNA silencing to engineer potato plants that are resistant to potato virus Y (PVY). We expressed double-stranded (ds) RNA derived from the 3′ terminal part of the coat protein gene of PVY, which is highly conserved in sequence amongst different PVY isolates, in transgenic potatoes of the commercial variety 'Spunta'. Transgenic plants were analyzed for generation of transgene-derived short interfering RNAs (siRNAs) prior to virus inoculation. Twelve of fifteen transgenic lines produced siRNAs and were highly resistant to three strains of PVY, each belonging to three different subtypes of the virus (PVY^N, PVY^O and PVY^{NTN}). Infection of transgenic plants with Potato virus X (PVX) simultaneously or prior to the challenge with PVY did not interfere with PVY-resistance.

Introduction

According to the International Potato Center (CIP), potato is considered the World's fourth most important crop and has been the subject of many breeding efforts. Viruses are very widespread in this crop and cause severe yield losses. Potatoes are mainly vegetatively propagated and this makes viral infections even more destructive: not only viruses persist in the tubers, but the tuber-borne secondary infections are more severe than primary infections. Potato virus Y (PVY) is one of the most damaging viruses of potato and in some parts of Europe, including Greece, the most widespread. PVY can be transmitted mechanically, but the usual transmission route in the field is through vector insects (aphids). It is a single-stranded (ss) plus RNA virus and the type strain of the Potyviruses, which replicate, like all plant RNA viruses, via double-stranded (ds) RNA intermediates. Different potato PVY isolates occur that can be categorized in 4 groups on the basis of virulence and host response: PVY^O, PVY^N, PVY^{NTN}, PVY^NW (Glais et al. 2002). Disease symptoms vary depending on viral strains and potato varieties and can be influenced also by temperature and other environmental conditions. Typical are mild mosaics in the leaves, as well as necrosis of veins and tubers.

Control measures that can be taken to reduce losses caused by viral infections are both limited and expensive. The generation of resistant cultivars is considered the most economic and environmentally acceptable way of controlling viral diseases of potato (Solomon-Blackburn and Barker, 2001). However, attempts, thus far, to engineer PVY-resistance in potato plants (Farinelli et al. 1992; Hassairi et al. 1998; Kaniewski et al. 1990; Lawson et al. 1990; Malnoe et

al. 1994; Okamoto et al. 1996; Pehu et al. 1995; Smith et al. 1995) have been met with mixed success. Resistance to PVY was achieved via the ectopic expression of several viral proteins: the coat protein (CP), the RNA-dependent RNA polymerase (Nib), the nuclear inclusion proteins gene (Nia) and P1 proteinase gene in sense and antisense orientation as well as with a heterologous sequence of lettuce mosaic virus. Resistance was not always very strong (Kaniewski et al. 1990; Lawson et al. 1990), often strain-specific (Farinelli et al. 1992; Maki-Valkama et al. 2000a; Maki-Valkama et al. 2000b; Pehu et al. 1995) and protection appeared almost always in only a few of the transgenic lines generated. In retrospect, attempts at expressing virus-derived sense or antisense sequences can be interpreted such that they actually initiated posttranscriptional gene silencing (PTGS) via accidental formation of dsRNA (Dougherty et al. 1994; Dougherty and Parks 1995; Vaucheret et al. 2001; Waterhouse et al. 1998). In one case, the presence of the protein (p17, movement protein) was reported necessary for resistance (Tacke et al. 1996) and therefore other mechanisms, e.g. an inhibition of normal viral protein function, cannot be completely ruled out.

PTGS, or simply RNA silencing, is a sequence-specific mRNA degradation mechanism in plants that does not affect transcription, at least not initially. It is believed that this mechanism evolved to protect plants against viruses and transposons (Voinnet 2001). A mechanism that shares large similarities with PTGS has been described in many other organisms including *Caenorhabditis elegans*, *Drosophila melanogaster* and mammals and has been termed 'RNA interference' (RNAi) (Fire et al. 1998). A central role in PTGS/RNAi is played by dsRNA (Hutvagner and Zamore 2002; Vaucheret and Fagard 2001). DsRNA molecules are not normal constituents of the eucaryotic cell and it has become clear that they are the key trigger for the process which leads to degradation of homologous RNAs (Vaucheret and Fagard 2001; Voinnet et al. 1999). The next important step of the silencing process is the cleavage of dsRNA into short dsRNA fragments, by a ds-specific ribonuclease called Dicer (Bernstein et al. 2001), which occurs in plants as a family of four enzymes with different functions (Schauer et. al. 2002) and Carpel factory in *Arabidopsis thaliana* (Jacobsen et al. 1999). In all cells where PTGS is active, these small sequence-specific sense and antisense RNAs of 21-25 nts become detectable (Hamilton and Baulcombe 1999; Waterhouse et al. 2001a). The short dsRNAs are also known as "short interfering RNAs (siRNAs)" (Elbashir et al. 2001), as they represent a reaction intermediate that can induce the silencing process, just like its dsRNA precursor. The siRNAs are incorporated into the RNA-induced silencing complex (RISC) (Hammond et al. 2000), which is the actual sequence-specific endoribonuclease that cleaves single-stranded target RNA. Since dsRNA is the key trigger, any RNA virus that replicates via a dsRNA intermediate will elicit a PTGS response in plants. However, specific virus-encoded suppressor proteins impair the antiviral host response to allow, in some virus/host combinations, a systemic infection. The ectopic expression of virus-specific dsRNA mimics a viral infection and likewise activates the systemic virus-directed PTGS response of the host plant. The activated host response can be monitored by the occurrence of siRNA derived from the expressed dsRNA sequences. Unlike an actual viral infection, this response is not impaired by the viral suppressor. If then a viral RNA, penetrating such a plant with activated PTGS, it will be under immediate attack by RISC. Therefore the expression of dsRNA is much more reliable in confering viral resistance than previous strategies of expressing pathogen-derived sense or antisense sequences.

For the current study, we expressed a part of the coat protein (CP) sequence of PVY in the form of an intramolecular dsRNA. It has been shown previously that extremely efficient and specific suppression of transgenes can be achieved in the plant by the expression of hairpin-like RNA containing transgene sequences (Waterhouse et al. 1998). Recently, similar strategies have been successfully implemented for the generation of tobacco lines resistant to PVY (Smith et al. 2000) and cucumber mosaic virus (CMV) (Kalantidis et al. 2002) and for the generation of transgenic barley resistant to barley yellow dwarf virus-PAV (BYDV-PAV) (Wang et al. 2000). We have generated 35, and analyzed 15, transgenic potato lines of the variety 'Spunta' a popular variety around the Mediterranean, which is susceptible to all relevant isolates of PVY. We found that the great majority of the lines analyzed were resistant to the virus. The resistance was very strong, not strain-specific and could not be overcome by simultaneous or prior infection with PVX. The presence of PVY-specific siRNAs in the majority of the lines before PVY inoculation correlated with the resistance phenotype showing that the resistance was acquired through RNA silencing.

Materials and methods

Description of the panhandle construct

A fragment 605-bp of the 3′ coding sequence of PVY coat protein (from a local strain of PVY) starting at PVY nucleotide 9100 (numbering according to PVY Acc. No. D00441) was cloned by RT-PCR in the pT3T7-lac vector (formerly Boehringer Mannheim, now Roche Diagnostics/CH), using the DNA oligonucleotides (Y9100) 5′ CTGGATCCTGTCTCCTGATTGAAGTTTACAGTC 3′ and (YREV1) 5′ TTGAATTCAAAGGAACCATATATGCCACGATAT 3′. A 1255 bp *Bam*HI/*Sal*I fragment derived from bacteriophage λ (nucleotide 33244 to 34499) separated the 9100CP sense and antisense orientation, to ensure the stability of the construct. The cassette (pvyPH9100) was subcloned in the pART7/27 binary vector system under the control of a single CaMV 35S promoter, yielding pART27HP9100, which was then transferred to *Agrobacterium tumefaciens* strain LBA4404, which was used for plant transformation via triparental mating (Ditta et al. 1980).

Plant material

Potato tubers of the variety Spunta were kindly provided by Vitro Hellas AE, Alexandria, Greece and potato plants were grown in the greenhouse for all experiments. Plant material for molecular analyses was initially isolated from the T_0 plants that came directly from transformation experiments. Experiments were repeated with plant material isolated from plants grown from the T_0 tubers. Although these plants should be genetically identical to the T_0 originals, we named these T_{01} plants to differentiate them from the original plants.

Plant transformation and regeneration

Originally, four potato transformation protocols (Beaujean et al. 1998; Esna-Ashari and Villiers 1998; Hassairi et al. 1998; Rook and Lindsey 1998) were tested for their efficiency in the transformation of the ‘Spunta’ variety. However, efficient transformation and regeneration of plants was only achieved when transformations were performed according to Beaujean et al. (1998). This protocol was then used for all potato transformation experiments with only minor modifications: sliced internodal explants that were used for transformation, were excised from young potato plants, cv. Spunta, grown in the greenhouse. Explants were co-cultivated with Agrobacteria (*A. tumefaciens, LBA 4404,* harbouring plasmid pART27PH9100) for 24h and then placed on callus-inducing MS media, containing hormones (zeatin, 0.8 μg ml^{-1}, and 2,4-D, 2.0 μg ml^{-1}) and antibiotics (kanamycin, 125 μg ml^{-1}, and cefotaxime, 250 μg ml^{-1}) Then calli were transferred in shoot inducing MS media, containing hormones (zeatin, 0.8 μg ml^{-1}, and GA_3, 2 μg ml^{-1}) and antibiotics (kanamycin, 125 μg ml^{-1} and cefotaxime, 300 μg ml^{-1}). Root regeneration was carried out on MS (Murashige and Skoog 1962) containing IAA, 0.1 μg ml^{-1} and 80 μg ml^{-1}, 200 μg ml^{-1} of kanamycin and cefotaxime respectively. All tissue culture chemicals were purchased from Ducheva / NL. Explants and plants were grown at 25 °C day and 18 °C night in the growth chamber with a 16 hour photoperiod provided by cool white fluorescent tube lights to give 90 μmol $m^{-2}s^{-1}$ PAR. About 5-6 months later, regenerated plantlets were transferred to the greenhouse for further analysis.

Southern blot analysis

Total DNA was isolated from 0.5-1.0 mg of fresh plant material according to Dellaporta et al. (1983). 10 to 15 μg of restricted DNA was separated in a 0.7% agarose gel and transferred to a nylon membrane (Nytran N. Schleicher and Schuell Dassel, Germany). Antisense ^{32}P-labeled riboprobes, *in vitro* transcribed (Sambrook et al. 1989) from the 605 bp fragment of PVY-CP DNA, were used for the detection of transgenes.

Northern blot analysis

Northern analyses for both transcript and siRNA detection were performed according to previously published methods (Kalantidis et al. 2002; Papaefthimiou et al. 2001), except that total RNA was used for both standard mRNA and siRNA analysis. For the analysis of PVY infection an additional proteinase K (0.1 mg/ml, Sigma) treatment was included in the extraction procedure just before the phenol extraction. Hybridization probes were prepared as for Southern hybridizations. Hybridization to U1 RNA (a 156 base RNA of the splicosomal snRNP complexes) using a potato U1 antisense probe was used as an internal standard to control RNA loading in “short RNA” northern hybridizations.

Plant inoculations

Four isolates of PVY, were used in this study. Isolate, PVY^{N-8} (Acc. No., AJ609240, donated by Dr. N, Katis, Plant Pathology Laboratory Aristotle University/ Thessaloniki, Greece) was used for RT-PCR, plasmid constructs and most infections. PVY^{O-4} (Acc. No., AJ609242), PVY^{N-14} (Acc. No., AJ609241), PVY^{NTN-7} (Acc. No., AJ609243), donated by Dr. Ch. Varveri of the Benakio Plant Pathology Institute in Athens, Greece) were used for inoculations to test resistance to different strains. All PVY isolates, derived from potato and tobacco, were mechanically inoculated to transgenic potato plants. Inoculi were prepared by grinding PVY-infected wild type potato or tobacco leaves (1: 10 w v-1) in a buffer containing 10 mM sodium phosphate and 0.4% sodium sulphite, pH 7.5. The PVX strain was kindly donated by Dr. D. Baulcombe (John Innes Center, Norwich, UK), and was mechanically inoculated to young leaves in phosphate buffer (pH 8).

Immunological assays

Detection of PVY in plants was performed by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). PVY- and PVX-specific antibodies and PVY- and PVX-specific antibodies conjugated with alkaline phosphatase was purchased from BIOREBA AG, Reinach, Switzerland. ELISA assays were performed according to manufacturers' instructions. Optical density of the reaction products with p-nitrophenylphosphate (Sigma) as substrate was measured at 405 nm. Two measurements were performed for each sample.

Tissue prints

Leaves for tissue prints, 21 dpi from wild type and transgenic potato plants were blotted onto nylon membrane (Nytran N. Schleicher and Schuell Dassel, Germany). Leaves from potato plants were cut and immediately placed in liquid nitrogen. Frozen leaves were then placed on nylon membranes and pressed under filter paper. RNA was fixed on the membrane by UV cross-linking (120 kJoule in a 'Stratalinker', Stratagene La Jolla, CA, USA) and was hybridized with ^{32}P-labeled CP antisense RNA probe.

Results

Construction of 'hairpin plasmid' and potato transformation

We intended to express a PVY-specific dsRNA to engineer virus-resistant potatoes. To ensure a broad resistance range against different isolates of PVY we performed a BLAST search (Altschul et al. 1997) of known PVY sequences. This revealed that the 3′-terminal part of the PVY CP gene is most highly conserved in sequence. We selected a 605 base fragment starting at PVY nucleotide 9100 (numbering according to PVY Acc. No. D00441). The sequence identity of this fragment was 77-99% between different strains, however, the great majority of the strains show similarity of over 95% in this region (data not shown). The corresponding cDNA fragment was cloned by RT-PCR from a local Greek PVY strain. The cDNA fragment was connected to a 1250 bp spacer cDNA fragment derived from bacteriophage λ. A second copy of the cDNA fragment was fused in inverted orientation to the other side of the spacer fragment. The size of the spacer was chosen on the basis of convenience and stability, although as we have since found out, it is possible to use much shorter spacers without greatly destabilizing the construct (Kalantidis, unpublished results). The whole cassette (pvyPH9100) was cloned in the pART7/27 binary vectors system (Gleave 1992) under the control of a single CaMV *35S* promoter (Figure 1), yielding pART27HP9100.

Potatoes of the variety 'Spunta' were transformed using the plasmid described above via the *Agrobacterium tumefaciens* route. Four potato transformation protocols (Beaujean et al. 1998; Esna-Ashari and Villiers 1998; Hassairi et al. 1998; Rook and Lindsey 1998) were tested for their efficiency in the transformation. Of the methods tested only the protocol developed by Beaujean et al. (1998) gave efficient transformation and was used further. The transformation efficiency of the protocol reached 30% (i.e., 30 regenerated for every 100 initial explants) and the whole process from stem segments to rooted plantlets in soil lasted about 5-6 months. No phenotypic abnormalities were observed in the mature plantlets, although somaclonal variability was not analyzed in any other way. We generated 35 transgenic potato lines of which 15 were analyzed further.

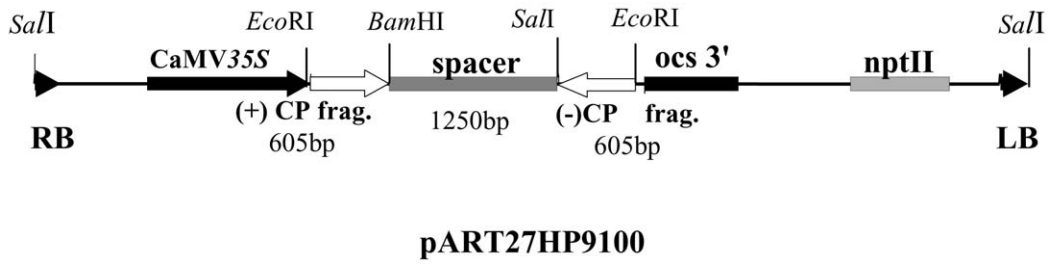

Figure 1. Map of T-DNA fragment of pHP9100 carrying the *PVY* inverted repeat. *CP*: *Coat Protein* sequence, RB, LB: right and left border T-DNA sequences respectively. CaMV*35S*: CaMV promotor, *OCS 3'*: *Octopine synthase*-derived terminator sequence, *nptII*: kanamycin resistance gene.

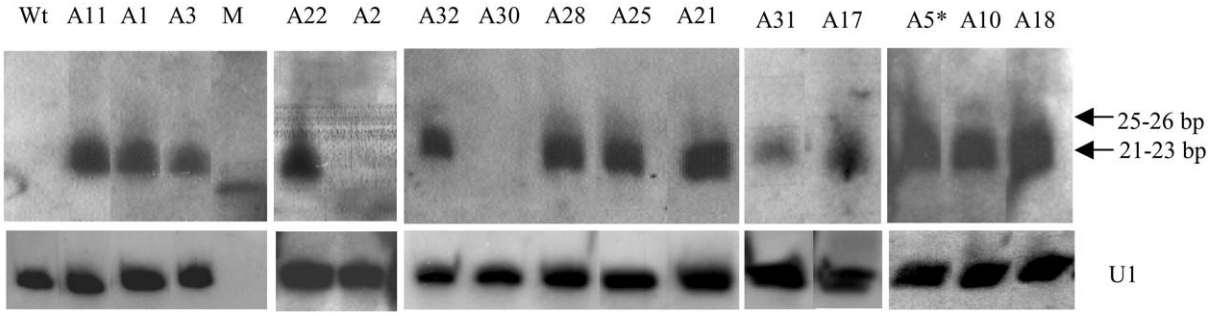

Figure 2. Detection of PVY-CP-specific siRNA from non-inoculated transgenic potato lines (top row). Samples were taken approximately 2 months after the plants were transferred to the greenhouse. Individual blocks represent individual hybridizations. *Line A5 samples were taken from a four-month old plant. In all cases membranes were hybridized with antisense CP RNA-probe. M: 22nt radiolabelled DNA sequences. Lower row: to ensure equal loading, the filters were stripped and re-hybridized with an U1-specific antisense RNA probe (bottom).

Detection of siRNAs

We have previously expressed dsRNA of the CP of CMV (Kalantidis et al. 2002). We could demonstrate, in the case of CMV, that the detection of siRNAs derived from the ectopically expressed dsRNA could be used as a prognostic tool to predict resistance to the virus. Thus, we decided to analyze the transgenic potato lines for the presence of PVY CP-specific siRNAs derived from the introduced transgene approximately two months after their transfer to the greenhouse. To exclude a potential latent infection with PVY, which might result in virus-derived siRNAs that are not of transgene origin, we subjected the starting material to a PVY-specific DAS-ELISA analysis. As expected, no PVY-signal was detected in any of the lines studied (data not shown). In the subsequent northern analysis we could detect siRNAs in 13 lines (A1,A3, A5, A10, A11, A17, A18, A21, A22, A25, A28, A31, A32), while they were undetectable in 2 lines (A2, A30) (Figure 3). CMV-dsRNA-expressing tobacco plants contain greatly varying amounts of siRNA (Kalantidis et al. 2002). However, in the potato lines analyzed here, no significant differences could be detected with regard to quantity or quality of the small RNAs. The siRNAs detected were mainly of the smaller class of RNAs typical for RNA silencing (21-23 bp) (Hamilton et al. 2002) although in a few lines (lines A10 and A18) the slightly larger (~25 bp) class of RNAs could be seen as well (Figure 2). Small differences in quantity of siRNAs can be attributed to variation between individual experiments (Figure 2).

Effects of developmental stage on the generation of siRNAs

Since the detection of transgene-derived siRNA is an important molecular marker to predict virus-resistance, we monitored the timing of siRNA formation in transgenic potato two months after transfer to the greenhouse. When RNA was isolated from transgenic shoots grown in tissue culture, no PVY-CP-specific siRNAs could be detected in any transgenic line. This included lines, which at a later stage of development generated high levels of siRNAs in mature plants

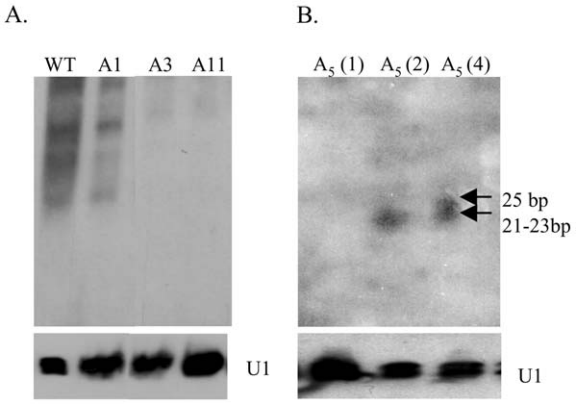

Figure 3. Detection of PVY-CP-specific siRNA in early developmental stages. (A) Samples were taken from transgenic plantlets still in tissue culture. (B) Samples were taken for A5 transgenic plants that were at different developmental stage, 1, 2 and 4 months after transfer to the greenhouse. Membranes were hybridized with a minus CP-RNA probe. Lower lanes in A and B, U1-RNA hybridizations.

(lines A1, A3 and A11) (Figure 3A). One of the lines (A5) was analyzed for the presence of siRNAs at three different time points after transfer to the greenhouse: one, two and four months after the transfer of the T_0 plantlets. No siRNAs were visible one month after transfer, but after two months siRNAs could be detected. The concentration increased further four months post transfer (Figure 3B). Also, the composition of siRNAs changed between two and four months post transfer since the latter sample also contained a slightly larger RNA species (~25 bp) (Figure 3B). Thus, it seems that the generation of transgene-specific siRNA cannot always be expected at very early times of growth.

Transgene copy number and transcript levels

In order to determine whether there is a correlation between transgene copy number and siRNA generation, we analyzed our transgenic potato lines by Southern hybridization. Genomic DNA of each line was digested with *Hin*dIII that cuts once in the hairpin construct (and in the genomic DNA outside of the PVY-CP hairpin cDNA). The number and the size of detectable DNA fragments allow discrimination between individual transformation events. Transgenic lines A5, A11, A17 and possibly A2 carry two transgene copies, whereas all other lines are single-copy transgenic lines (Figure 4A). This shows that in most cases a single transgene is sufficient to confer PVY-resistance. Therefore, no general conclusions can be made as to whether a second copy of the transgene would increase the likelihood of virus-resistance. For two lines (A1 and A21) transgene copy number was not determined (Figure 4D). Next, we determined the accumulation of transgene-derived RNA transcripts. Due to their ds nature, engineered PVY-specific RNA molecules are targeted for degradation so that only small steady-state amounts of the actual hairpin transcripts can be expected in the transgenic lines, especially in lines where siRNAs are detectable. Our northern hybridization analysis confirmed that only very little or no hairpin transcript could be detected (Figure 5). However, it should be added that detection of RNA with perfect self-complementarity is generally difficult, since the probe has to compete with intramolecular base pairing.

Resistance to PVY of the original isolate

Plants from all 15 lines under study were mechanically inoculated in T_{01} plants at the 4-5-leaf stage by the same PVY isolate that had been used for RT-PCR-based cloning (PVY^{N-8}). The presence of the virus was evaluated in plant samples collected 21 dpi by DAS-ELISA. PVY infection was detected in the inoculated wt controls and in all plants of lines A2 and A30, which are devoid of any PVY-specific siRNAs (Figure 6 and Table 1). PVY infection was also detected in a portion of plants from lines A17 and A18 (2/4 and 1/4, respectively; Table 1). Plants from all the other lines could not be infected. Infection was also tested by tissue print hybridization using leaves of the plants under study. The nylon membranes produced were then hybridized with a PVY probe. For the 6 lines tested by this method (A1, A3, A17, A21, A28, A30), the ELISA results were confirmed. A virus signal could be detected only in infected wt and A30 samples, but not in any of the other lines or in the non-infected control leaf (Figure 7). Phenotypic data were also in accordance with the ELISA readings since plants that developed PVY symptoms showed high ELISA readings (data not shown). In order to ensure that the other plants were genuinely virus-free, additional sensitive and antibody-independent methods were utilized. The presence of the CP RNA was analyzed by Northern hybridization in plants from 9 lines found to be resistant based on the ELISA results (Figure 8). In addition, RT-PCR for the CP gene was performed on RNA extracts from the same lines (data not shown). Both experiments con-

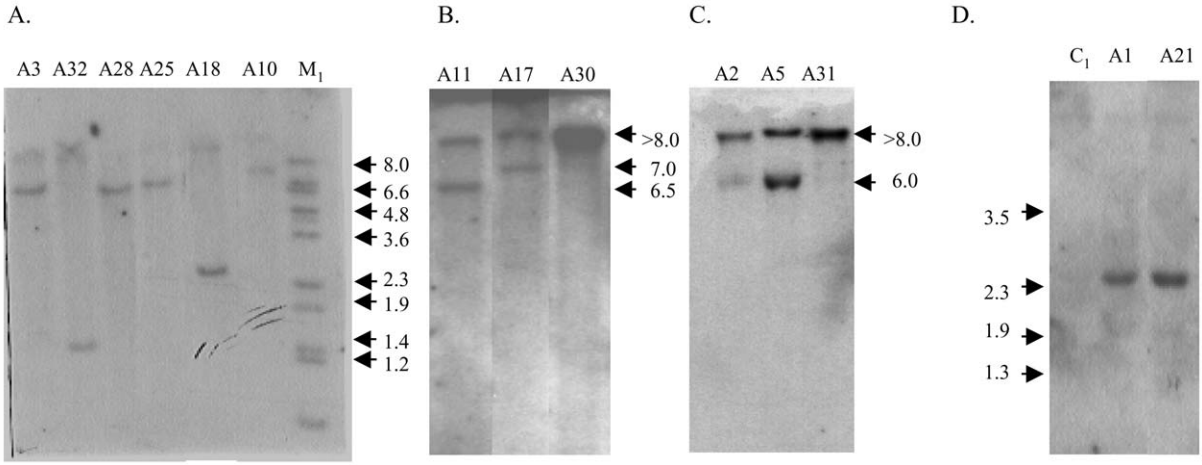

Figure 4. Southern blot analysis of total DNA. (A), (B), (C) *Hin*dIII-digested DNA from different transgenic lines. H*in*dIII cuts once in the transgene outside of the CP probe used and therefore results in fragments unique for each insertion event. M_1: λ DNA Bs*t*EII radiolabeled. Membranes were hybridized with an antisense CP-RNA-probe. Lines A11, A17, A5 and possibly A2 seem to carry two transgene copies whereas all other transgenic lines studied carry a single transgenic insertion. (D) *Eco*R*I*-digested DNA from transgenic lines A1 and A21. *Eco*R*I* gives a 2.5Kb fragment containing the whole hairpin DNA (see map in Figure 1). The expected signal is detected in the two transgenic lines confirming transformation. C_1: E*coR*I-digested DNA from wild-type "Spunta" plant. Sizes in kb.

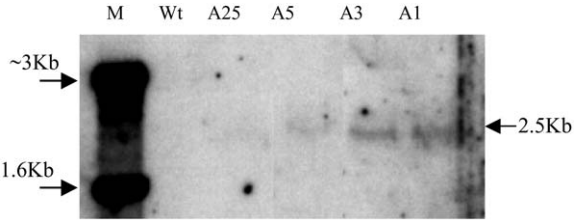

Figure 5. Northern blot analysis for the detection of transgene transcript levels. As expected, the steady-state transcript levels are low due to the specific degradation of the transgene product to siRNA soon after production. The membrane was hybridized with a DNA PVY-CP probe. M: pBR322 *Hin*fI *l*abeled marker (also uncut plasmid, 3kb band visible).

firmed the ELISA readings indicating complete resistance in these plants. The results of the infections together with molecular analyses are summarized in Table 1.

Resistance to other PVY strains

Next, we wanted to test whether resistance would withstand challenge by different PVY strains. Two plants, each of line A21, were inoculated with three different viral inoculi, each containing virus belonging to one of the groups PVY^N, PVY^O and PVY^{NTN}, respectively. In parallel, one wt control plant was also inoculated as well with these three strains. The nucleotide identity between the sequence used for the hairpin and the three isolates used for infections ranges from 93% (with PVY^O) to 98% (with PVY^N). The presence of PVY was evaluated in samples collected 20 dpi, by DAS-ELISA. The results show that only wt potato plants were infected with any of the PVY isolates tested. The PVY^O-infected control plant had lower ELISA readings compared to the plants infected with the other two strains (OD 0.65 compared to OD 2.7 and OD 3.3). Since a mixture of monoclonal antibodies raised against various PVY strains was used, this difference most likely represents lower viral concentration levels rather than different reactivity of antibody. However, both plants of line A21 proved to be resistant to challenge with any of the three PVY isolates, demonstrating a wide PVY-resistance range (Figure 9A).

Resistance to PVY at pre-infection or co-inoculation with PVX

Synergistic effects of PVX and PVY have been well characterized (Vance 1991; Vance et al. 1995), and simultaneous infection with both viruses results in enhancement of disease symptoms. Under field conditions, mixed PVX-PVY infections are not uncommon and thus it was important to test the PVY-resistant transgenic lines for resistance following double PVX-PVY inoculation. The mixed inoculations were done in two variants. First, plants of the resistant line A21 were simultaneously inoculated

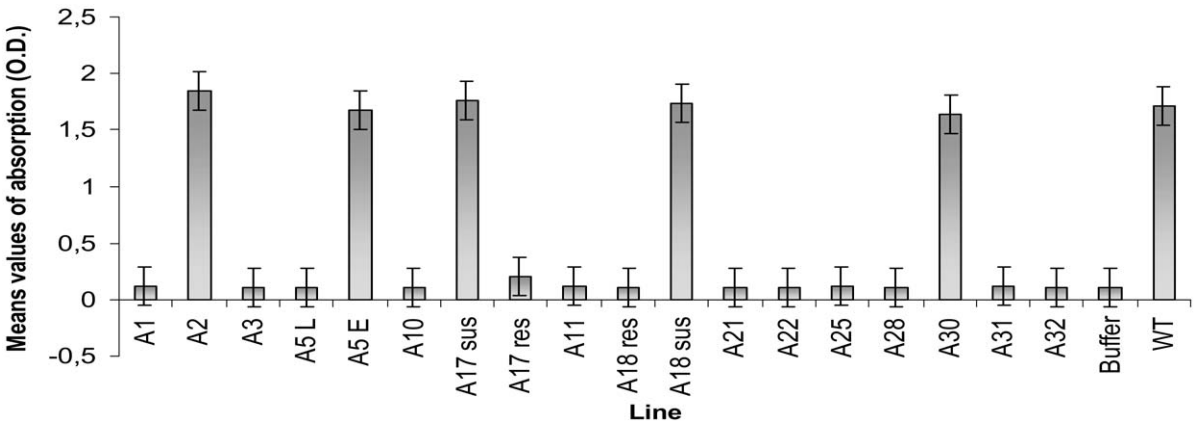

Figure 6. Challenge of transgenic potato lines with PVY^N analyzed by ELISA test for the detection of PVY about 21 days post inoculation (dpi). Virus concentration was expressed as OD units measured photometrically at 405nm. Values shown here represent mean values from all plants studied for each line. In some transgenic lines susceptible (sus) and resistant (res) individual plants were observed. For line A5 measurements for plants inoculated with PVY in an early (E) and in late (L) developmental stage are presented separately.

Table 1. Summary of molecular analysis and virus-resistance of individual transgenic potato lines and wild type controls.

line	copy number	presence of siRNAs	resistance to PVY	number of plants tested	
				R	S
A1	ND	Y	R	12	0
A2	1 or 2	N	S	0	4
A3	1	Y	R	12	0
A5*[1]	2	N	S	0	2
A5*[4]	2	Y	R	4	0
A10	1	Y	R	6	0
A11	2	Y	R	4	0
A17	2	Y	S/R	2	2
A18	1	Y	S/R	3	1
A21	ND	Y	R	7	0
A22	1	Y	R	4	0
A25	1	Y	R	4	0
A28	1	Y	R	4	0
A30	1	N	S	0	4
A31	1	Y	R	4	0
A32	1	Y	R	2	0
WT	0	N	S	0	10

A5 plants showed a delay in the generation of detectable levels of siRNAs and the resistance phenotype. All samples were isolated 1-2 months post-transfer to the greenhouse, except A5, for which samples were selected at 1 (A5[1]) or 4 (A5*[4]) months post-transfer. R: resistant to PVY, S: susceptible to PVY. ND: copy number not determined for these samples.

with PVX and PVY^N. Second, plants of PVY-resistant lines A3 and A21 were initially inoculated with PVX. After one week PVX infection was established as shown by ELISA (data not shown). These plants were then challenged with inoculum containing PVX and PVY^N. In either of the two variations, infections with PVX occurred. However, PVY infection in neither case was found as demonstrated by ELISA (Figure 9B).

Discussion

Engineering resistance to PVY has been approached in the past using a variety of methods (Solomon-Blackburn and Barker 2001). The primary aim of the present work was to efficiently engineer strong PVY-resistance in potato, an economically very important crop that is severely affected by this virus. Moreover, we also set out to explore whether our observations made for CMV-resistant tobacco plants (Kalantidis et

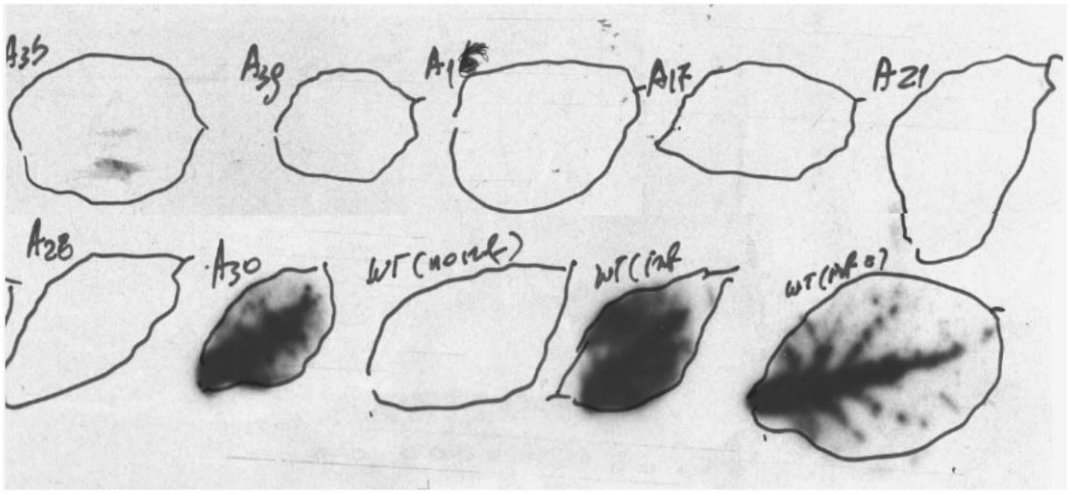

Figure 7. Tissue print for the detection of PVY in inoculated transgenic lines. About 20 days post inoculation one (upper) leaf of each potato plant was printed on nylon membrane. The membrane was hybridized with an antisense CP RNA-probe. Top row : transgenic lines A3 (plant B), A3 (plant G), A1, A17, A21. Lower row: transgenic lines A28, A30 and leaves of WT (non-infected control and two WT infected plants).

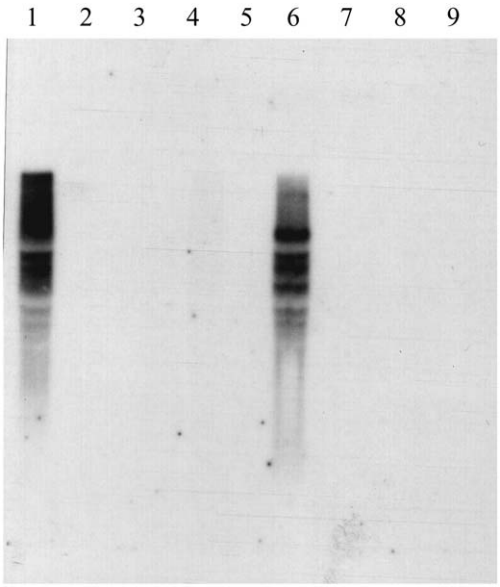

Figure 8. Northern hybridization of transgenic potato plants for the detection of viral infection. DNA from the PVY-Nib gene was used to make a probe that was hybridized to RNA from potato plants inoculated with PVY. Lanes 1: PVY-infected control plant; lanes 2-9, transgenic lines: A32, A28, A25, A22, A19, A11, A3, A1

al. 2002) could be extended to the combination of PVY and potato. In particular we were interested to see whether the generation of virus-specific siRNAs from ectopically expressed virus-specific dsRNA is also indicative of resistance in this combination of virus and host plant. Additionally, we wished to determine whether resistance to different virus strains could be achieved, as that is of practical importance for this virus/host combination. Furthermore, we wanted to explore whether infection of a second commonly occurring potato virus – PVX – would interfere with the PVY-resistance. Such an interference might be expected since PVX expresses a suppressor gene for RNA silencing.

The method of choice to confer resistance to PVY in potato was the induction of RNA silencing by an ectopically expressed dsRNA that was conserved between different PVY strains. Engineering virus-resistant transgenic crops through RNA silencing takes advantage of the natural defense mechanism of plants against viruses (Baulcombe 2001; Vance and Vaucheret 2001; Waterhouse et al. 2001b). The same mechanism is active in wild type plants. However, in this case two factors limit the host defense. First, the silencing process is activated after the virus has entered the plant. Second, most – if not all – plant RNA viruses express proteins, known as suppressors, that strongly impair the efficiency of the silencing response. In the case of PVY this is the Hc-Pro protein and its mechanism has been studied previously in transgenic plants (Anandalakshmi et al. 1998; Mallory et al. 2001). Different RNA viruses encode various viral suppressors of silencing that act at different stages of the process. The expression of a suppressor protein seems necessary for the establishment

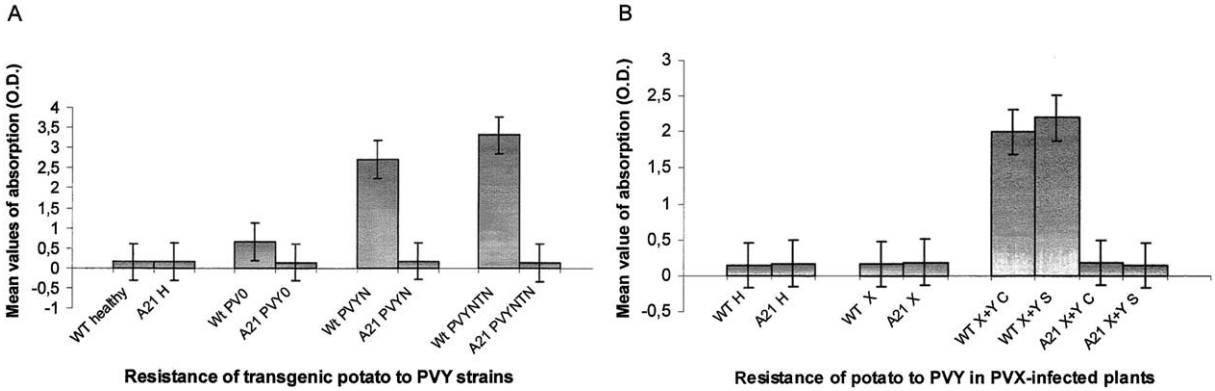

Figure 9. Resistance of a transgenic line to PVY is broad and is not influenced by PVX infection.(A) Resistance of transgenic line A21 to different strains of PVY. Infection by the diferrent viral strains was evaluated by ELISA, optical density absorbance measured at 405nm. (B) Resistance of A21 transgenic plants to PVY after simultaneous (A21X + YS) or consecutive (A21X + YC) infections with PVX. Plants showed susceptibility to PVX inoculation (data not shown). The presence of PVX in plants did not change resistance to PVY. WT: wild type H: Healthy plants. X: plants only infected with PVX. ELISA was conducted with PVY specific antibodies.

and propagation of the respective virus. It has been shown in an increasing number of studies that the expression of virus-derived dsRNA from transgenes can fully suppress viral infection through RNA silencing, thus overcoming viral suppressors (Kalantidis et al. 2002; Smith et al. 2000; Wang et al. 2000). Resistance engineered through RNA silencing is very attractive for several reasons. Resistance is very strong, in most cases reaching immunity (Kalantidis et al. 2002; Smith et al. 2000; Wang et al. 2000). Although there is some degree of variability amongst transgenic lines, a relatively high proportion of them are highly resistant or immune. This feature makes the method attractive also for crops where transformation is difficult. Here, we confirmed that transgenic potato lines, expected to be highly resistant can be selected relatively early in development by the presence of specific siRNAs, an observation that we made previously for the combination CMV/tobacco (Kalantidis et al. 2002). By using strains from three different groups of PVY, we could demonstrate that resistance range may be broad.

No phenotypic abnormalities were observed in the transgenic plants and the large majority of the transgenic lines carried a single copy of the transgene. Of the 15 transgenic lines analyzed only two did not produce detectable levels of siRNAs while 13 produced comparable levels of siRNA. This is a much higher proportion than we had previously observed in CMV-resistant transgenic tobacco plants through RNA silencing (Kalantidis et al. 2002). The difference in the transgenic lines producing siRNAs must lie either in the transformation procedure, which results in insertion in different positions, or some as yet unknown factors. In addition, differences in the plant species (allotetraploidy versus tetraploidy) and the reduced growth rate of potato compared with tobacco may also play a role. At variance with our previous analysis is the low degree of variability in the quantities of siRNAs generated by individual lines. This may reflect the different transformation behavior of potato as we obtained primarily single copy transgenes.

As outlined above, transgenic lines that did not generate detectable levels of PVY-specific siRNAs were susceptible to PVY infection, whereas transgenic lines in which siRNAs were detected, were predominantly resistant. Notable exceptions were lines A17 and A18. In these lines both resistant and susceptible individuals were found. The possible reason for this variability in these lines is not clear. Although unlikely, it is possible that not all plants were homogenous in their transgenic content, and therefore may not represent a single “line”. Alternatively, the resistance in this line may be in a fragile balance and may sometimes be overcome by the virus due to small variations in the inoculum or the portion of virus that is actually penetrating the plant.

Resistance was found to have the form of immunity, since no viral particles could be detected in the resistant transgenic lines as shown by ELISA measurements, northern hybridizations and RT-PCR. In addition, no viral RNA could be detected in the resistant lines tested, as shown in the tissue print hy-

bridizations. Resistance was observed against all three PVY strains tested here, which belong to three different PVY groups. Sequence analysis showed that the strains used for infections in our experiments share over 93% identity with the original sequence used in transformations. Our plants remain resistant, even after infection with the most distant of the three PVY strains used, PVY^{O}. Since the majority of PVY strains found in the database have sequence identity greater than 95% within the part of the PVY sequence used in our experiments, the range of PVY-resistance of the plants presented here is likely to be very broad. Resistance to other potyviruses has not been tested. Under field conditions is not uncommon that multiple viral infections occur simultaneously, which frequently show synergistic effects, i.e. stronger disease symptoms. This is common for PVX and PVY. Synergistic effects of viruses can be at least in part explained by the simultaneous presence of more than one viral suppressor of silencing. For example, PVX and PVY carry the silencing suppressors p25 and HC-Pro, respectively (Brigneti et al. 1998; Davies et al. 1993; Voinnet et al. 2000). We found in our transgenic potatoes that PVX infection was unable to suppress PVY-resistance. Of particular interest is the experiment, where PVX infection had already been established. First, this indicates that the Dicer/RISC system of RNA silencing is not easy to saturate from the presence of PVX-specific siRNAs, which are expected to be generated, following the infection of the virus (Hamilton and Baulcombe 1999). This is unlike what has been observed in similar situations in mammalian tissue culture systems (Holen et al. 2002). Second, PVY-resistance could not be overcome, despite the presence of the PVX-derived p25, which is a suppressor of the systemic silencing signal (Voinnet et al. 2000). Since PVY-specific dsRNA is produced in each cell of the transgenic plant, there is no need for a systemic spread of the signal. Therefore, it is not surprising that p25, even if present before the PVY infection, has no influence on PYV-resistance.

As far as we are aware, there is only limited information regarding the developmental stages at which the plant is capable to perform RNA silencing. The study of Mitsuhara et al. (2002) is based mainly on protein data and showed that in strongly proliferating tissues of silenced plants gene expression is re-established. Similarly, they did not observe silencing in very young progeny of a post-transcriptionally silenced line (Mitsuhara et al. 2002). It should be noted that the authors studied luciferase overexpressing transgenic tobacco plants with no evidence of hairpin formation. In general agreement with the slow accumulation of siRNA reported earlier (Kalantidis et al. 2002), we were able to detect transgene-derived siRNAs in potato in plants not earlier than one month after their transfer to the greenhouse, although detailed analysis of siRNAs at different developmental stages was not conducted.

One important aspect of this strategy to engineer virus-resistant plants is the fact that the transgenic viral sequence is not translated. Moreover, the actual RNA transcript is almost undetectable, most likely, because it gets cleaved quickly in small fragments. These two features possibly limit the environmental risks of this strategy, such as transencapsidation or recombination of the transgene with an incoming virus. The transgenic lines generated have not been yet tested under field conditions, which would be necessary for further use of these lines.

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