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Stability of pathogen-derived *Potato virus Y* resistance in potato under field conditions and some aspects of their ecological impact

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Abstract

The results of three years of field experiments with transgenic potato clones resistant to *Potato virus Y* (PVY) are presented. The plants were transformed either with a truncated NIb gene of PVY^N fused to the Enhanced Blue Fluorescent Protein gene or the coat-protein gene of a PVY^N strain. It was demonstrated that their resistance to PVY can be overcome by several isolates of this virus. The spectrum of PVY strains infecting transgenic plants was different from that of control plants. On resistant clones the virulent strain PVY^NW prevailed while on control plants as well as on susceptible transgenic plants PVY^N was the dominating strain. Susceptibility or resistance of the transgenic plants to several other viruses was altered too. Some of the clones were more attractive to aphids though the reproduction rate of the aphids on transgenic plants was not altered. Recombination was not observed between transgenic and viral RNAs but between RNAs of invading PVY isolates. We cannot rule out the possibility that the recombination rate between viral RNAs is enhanced in transgenic plants. The paper discusses why so far little use has been made of transgenic approaches for the induction of virus resistance.
Keywords: *Potato virus Y*; potato; transgenic; aphids; pathogen-derived resistance

Introduction

From the beginning of agriculture farmers have been faced with the problem of crop diseases. It is only since the last 200 years that we have known that diseases are mainly caused by pathogens, and the history of plant viruses is even younger. In 1892 D.J. Iwanowski, working on a disease of tobacco, described the first plant virus, *Tobacco mosaic virus* (TMV). From about half way through the last century plant virology developed rapidly. Cloning and sequencing techniques introduced in the early 70s enabled the understanding of the molecular organization of viruses and founded the basis for the genetic improvement of crops.

The history of the potato as an important staple food in many parts of the world is also relatively short. It originated from the Andes of South America and the lowlands of Southern Chile and was introduced into Europe at the end of the 16th century, at

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that time being a short-day crop. Later forms were selected which were adopted for the long 16-18-hour day. They were named *Solanum tuberosum* subsp. *tuberosum* (Bradshaw and Mackay 1994; Ross 1986). Potatoes suffer from a large range of diseases, with viruses playing an important role. In Europe, first reports of epidemics caused by viruses date from 1775. At that time the disease was called 'degeneration' or 'senility' of potato (Schmelzer 1974). Nowadays, natural resistance alleles are known against most of the potato viruses, which confer a high level of resistance (Solomon-Blackburn and Barker 2001; Ross 1986). Consequently, the question arises why we are still faced with the virus problem in potato. In a modern cultivar more than 50 traits have to be combined. Most of them are inherited by polygenes and only a small number of pedigrees of a crossing experiment express the desired features. The fact that the potato is a tetraploid crop increases the difficulty of combining specific traits. Therefore, any new variety is always a compromise between traits that are on a desired level and those which are, unfortunately, not. As quality characteristics have been considered to be more important than resistance to viruses, the compromise looks such that breeding clones with better quality traits but lower resistance levels are preferred to those with a high level of virus resistance but unsatisfactory quality, as viruses can be controlled by eliminating their vectors. The situation may change with the appearance of new more aggressive and harmful isolates of *Potato virus Y* (PVY).

In 1986, the first report appeared on induction of resistance to a virus by means of genetic methods (Beachy, Harrison and Wilson 1999): introducing the coat-protein (CP) gene of TMV into tobacco rendered the plants resistant to infection by the virus. This principle of induction of resistance was named pathogen-derived resistance (PDR). Meanwhile, it was demonstrated for almost any of the important crops and harmful viruses infecting them, that expression of a viral gene or part of it may confer resistance to the corresponding virus. Later on it was demonstrated that expression of the mRNA is often sufficient to induce resistance. There are different models explaining mechanisms underlying the process of resistance induction. Nowadays, in most cases it is explained by post-transcriptional gene silencing (PTGS). In this case only low levels of transgenic RNA are detected in the host plant. Upon infection with a virus, which contains a RNA molecule that is highly homologous to the transgenic sequence (homology-dependent resistance), a mechanism is activated in the host leading to the degradation of both the transcript of the transgene and the viral RNA.

Though nearly 20 years have passed since the first report of PDR, the principle is not yet widely used in practical agriculture (Kawchuk and Prufer 1999). There are several reasons for this. One of the most important is a broad public concern on – never demonstrated – adverse environmental effects of the transgenes. Another one is the complicated patent situation, which makes it almost impossible for small breeding companies to market corresponding cultivars. Approved cultivars with PDR originate mainly from the USA, the world leader in growing genetically improved crops. These are the papaya cultivars 'Rainbow' and 'SunUp' with CP-gene-based resistance to *Papaya ringspot virus* (Gonsalves 1998), the squash cultivars Liberator III, Destiny III and Prelude II with combined resistance to *Zucchini yellow mosaic virus*, *Watermelon mosaic virus 2* and *Cucumber mosaic virus* (CMV) (Tricoli et al. 1995; Lin et al. 2003) and the potato cultivars NewLeaf®, Shepody and Russet Burbank with either *Potato leafroll virus* (PLRV) or PVY resistance (Lawson et al. 2001; Duncan et al. 2002). Except for the NewLeaf® potato cultivars it was demonstrated that some virus isolates exist which can overcome transgenic resistance (Flasinski et al. 2002). Unfortunately, in most publications dealing with PDR to viruses, resistance was tested

only with a small number of virus isolates; this does not reflect the natural conditions where the transgenic plants are faced with a large number of different virus isolates. Data on long-term stability of PDR are usually missing. The fact that only limited data exist on the ecological impact of transgenic plants with virus resistance is also an unsatisfactory situation.
Using transgenic potato plants engineered for resistance to PVY we tried to get some answers to open questions such as the stability of resistance, the influence of transgenic plants on aphids settling on them and changes in virus populations occurring in plots with transgenic potatoes. This paper reports the results of field experiments from 2000 until 2002.

PVY is a highly variable virus. Depending on its biological features one can distinguish several strains (Valkonen 1994; Kerlan et al. 1999). PVY^{O} and PVY^{N} have been the most common strains for Europe. The first, also known as the ordinary strain, is characterized by causing heavy leaf symptoms on potatoes while the second, the necrotic strain, does not. Necrotic means that it causes vein necrosis on *Nicotiana tabacum* L. Both strains can also be differentiated by monoclonal antibodies (MAb). In the early 80s of the last century two new strains appeared: PVY^{NTN} (Beczner et al. 1984) and $PVY^{N}W$ (Chrzanowska 1987). The first is the so-called tuber necrosis strain, the second the Wilga strain. $PVY^{N}W$ is a highly virulent biological N-, but serological O-strain. This means that it will react with O-strain specific MAb and cause vein necrosis on *N. tabacum*.

Material and methods

Transgenic potato plants – generation and characteristics

We have reported on the generation of transgenic potato plants with PVY resistance used for field experiments elsewhere (Schubert et al. 2000). Two types of constructs were used for *Agrobacterium tumefaciens*-mediated transformation. In one case the CP gene of strain PVY^{N} CH605 (P. Gugerli, Switzerland), including the 3'-NTR, driven by the CaMV 35S-promoter and terminated by the CaMV 35S-polyadenylation signal, was used (plants provided by G. Barchend, BAZ, Aschersleben). In the other case a truncated NIb gene (lacking at the 3'-end 400 nt) fused in frame to the N-terminus of the Enhanced Blue Fluorescent Protein gene (EBFP, Clontech) was used. The fused genes were also regulated by the CaMV 35S-promoter/terminator sequences. In both cases the cassettes were cloned into a binary plasmid (for NIb-EBFP: pGPTV-Kan, (Becker et al. 1992); for CP: the same plasmid but lacking the NPTII gene). While the CP gene was transferred separately from the plant selection marker NPTII, using a double transformation method including pBIN19 as donor of the NPTII gene (Bevan 1984), in case of NIb-EBFP the selection marker was transferred together with the viral gene on the same binary plasmid. For transformation the variety ‘Linda’ as well as the dihaploid line DH59 (BAZ, Groß Lüsewitz) were used. Plants were tested for virus resistance by manual inoculation with strain PVY^{NTN}-Hessen (DSMZ, Braunschweig, NIb construct) or PVY^{N}-CH605 (CP construct). Among more than two hundred tested clones 5 revealed a high level of resistance: plants did not get infected systemically when tested 35 days after infection (dai) by DAS-ELISA.

Selected resistant genotypes revealed different mechanisms of resistance when tested with the isolates PVY^{N}-CH605 as well as PVY^{NTN}-Hessen. Linda Nb58 showed extreme resistance to infection: no virus was detected in inoculated and following leaves. Lines DH59 Nb93, Nb146 and Nb156 as well as CP102 revealed recovery

from infection: the virus was detected in the inoculated leaves but did not spread. Clones DH59 CP39 and CP41 showed partial resistance (most plants remained free of virus after inoculation). Clones DH59 Nb36, Nb51, Nb80 and Nb88 did not reveal a markedly improved resistance to PNY^{NTN}-Hessen. They were used as susceptible transgenic controls. Linda Nb58 was also extremely resistant to *Potato virus A* (PVA) isolate B11 (BAZ, Aschersleben).

No correlation was found between level of resistance and number of gene copies of the transgene nor their expression level. Apparently, the mechanism of resistance was not based on PTGS as the level of transgenic RNA was equal in resistant and susceptible transgenic plants and did not change after inoculation with PVY (Schubert et al. 2004).

Field experiments for investigation of stability of PVY resistance and changes in virus population in transgenic plants

Field experiments were performed at a location near Aschersleben, Saxony-Anhalt, Germany. This location is characterized by a high incidence of aphid-borne potato viruses.

Tubers from the PVY-resistant as well as susceptible transgenic potato clones were multiplied up under insect-proof glasshouse conditions starting from *in vitro* material. Samples from them were checked for any contamination with PVY, *Potato virus S* (PVS), *Potato virus M* (PVM), PVA and PLRV by DAS-ELISA. Non-transformed tubers of clone DH59 and cvs. Linda, Bettina, Arosa and/or Ute were used as controls. Planting was done at the end of May to ensure successful infection by naturally occurring aphids. Plots consisted of 15 tubers in three replicates with a randomized block design. In the first two years each plot was flanked on two opposite sides by one row of plants of cv. Hansa infected either with PVY^{N}-CH605 (year 2000) or PVY^{NTN}-Hessen (year 2001), which served as additional sources of infection. Two fields of the same design but with different controls were planted. In case of design B the medium resistant clones DH59 CP39 and CP41 were included while they were omitted in plots with design A. Insecticides were not applied other than *Bacillus thuringiensis* preparations against Colorado potato beetle (Novodor FC, Agrinova). Tubers were harvested at the end of August/beginning of September. Three medium-sized tubers per plant were collected and stored in a cold room at 4°C for testing for secondary virus infection.

Field experiments on the influence of transgenic plants on aphid populations

The cultivars/clones Bettina, Ute, Linda, Hansa, DH59 Nb146, DH59 Nb156, Linda Nb58 and DH59 CP102 were used for this investigation. Plots were each planted with 15 tubers in three replicates with a randomised block design.

Aphids were collected in the year 2001 twice and in 2002 once per week from 5 randomly chosen leaves of each plant of the test field. The ratio adult/larvae was determined. It served as an indicator for the reproduction rate of aphids.

Detection of virus infection

Plants from the field were tested in duplicates for primary infection with PVY, PVA, PVS, PVM and PLRV by means of DAS-ELISA (PVY: MAb cocktail, Bioreba; PVA, PVS, PVM and PLRV: polyclonal IgG/alkaline-phosphatase conjugate; PVY^{N}: specific monoclonal antibody; all provided by F. Rabenstein, BAZ, Aschersleben) at the end of July, beginning of August. For this purpose mixed samples from top leaves of each plant were collected and analysed in duplicates.

Plants were scored as infected if OD_{405nm} values exceeded the mean value for healthy control plants plus three times the corresponding standard deviation (threshold value). Standard deviation was calculated from 10 healthy leaf samples. Sprout testing of tubers was carried out in a glass house from January to March. Two tubers per plant were analysed independently. Mixed samples from top leaves of the sprouts were collected and tested in duplicates.

For serotyping of PVY isolates a MAb-based ELISA kit was used (Adgen). For analysis of the presence of $PVY^{N}W$ leaf-sap extracts from plants negatively reacting with the PVY^{N} specific MAbs were obtained and used to inoculate *N. tabacum* plants. Symptoms were scored 21 dai. Veinal necrosis and mosaic symptoms were indicative for the presence of $PVY^{N}W$ as isolates of PVY^{O} induce only mosaic symptoms.

Investigation of recombination events between viral and transgenic RNAs

Leaf samples were analysed for recombinants from sprouts originating from different PVY-infected tubers. When starting the experiments it was not clear whether some resistance-breaking PVY isolates would appear. Thus, in contrast to the experiments described above, for these investigations the susceptible transgenic potato clones DH59 Nb36, Nb51, Nb80 and Nb88 had been included, which revealed the expression of recombinant viral RNA providing the possibility of recombination. Approximately 25 PVY-infected plants were analysed from each transgenic line per year. Immunocapture reverse-transcription PCR (IC-RT-PCR) was used to ensure that viral and not plant recombinant RNA was evaluated. Two hundred µl of goat PVY antiserum (IgG-fraction, 100 ng protein) was used to coat a PCR tube. The same volume of PBS-extracted leaf sap was added to the tube and incubated overnight at 4°C. After washing the tube with PBS and distilled water, a reverse transcription reaction was performed in a final volume of 25 µl (Superscript II, Gibco) with an appropriate 3'-end primer. For the subsequent PCR reaction (Triple master polymerase, Eppendorf), 5 µl of the RT-mix was used in 50 µl final volume with appropriate 5'- and 3'-end primers:

CP: 3'-end primer - 5'-TACAGCCACTGCTATGACAGAATC-3',
5'-end primer - 5'-GCCAACTGTGATGAATGGGCTTATG-3'.
NIb: 3'-end primer - 5'-CCAATTYTCAGGTARACGCCGAAGC-3',
5'-end primer - 5'-TTCTTCAGGCCTTTGATGGATGC-3'.

Supposing that recombination between transgenic and viral RNA is a rare event under the lack of a strong selection pressure it was necessary to pre-select different sequence variants of viral RNAs to reduce the number of clones that had to be sequenced. For pre-selection the Decode Universal Mutation Detection System (BioRad) with an 8% polyacrylamide gel was used at 58°C to do a Constant Denaturing Gel Electrophoresis (CDGE). Five hundred ng of each PCR product, including probes from genomic DNA, were loaded per lane and bands that represent sequence variants of the viral RNA were visualized with ethidium bromide. DNA with altered melting properties and hence different electrophoretic mobility was isolated from the gel matrix, reamplified by PCR, ligated into pGEM-T (Promega), transformed into *Escherichia coli* XL-1 cells (Stratagene) and sequenced. Sequence alignments were performed with DNASIS software (Hitachi).

Experiments on stability of resistance to isolates of different strains of PVY

When it became obvious that resistance of transgenic plants can be overcome by certain PVY isolates, experiments were set up in a growth chamber (22°C, 16h/8h

light/dark) to test whether there is a correlation between PVY strains and their ability to overcome resistance. They were done twice. In each experiment three plants of the clones DH59 Nb93, DH59 Nb146, DH59 Nb156 and Linda Nb58 were included. Isolates PVY^{O}-Adgen, PVY^{C}-Adgen and PVY^{N}-Adgen were supplied by Adgen Inc., PVY^{O}-BBA by the DSMZ (Braunschweig, Germany) and PVY^{O}-CZ by P. Dedič (IPB, Havličkov Brod, Czech Republic). PVY isolates 2 and 5 belonging to the strain $PVY^{N}W$ were isolated in 2000 at Aschersleben from primarily infected transgenic plants of clone Linda Nb58. Samples from inoculated leaves were tested 10 dai and from uninoculated newly expanding leaves 28 dai.

In another experiment plants of clone Linda Nb58 were inoculated with different isolates of PVY^{NTN} under the same conditions: isolates Hessen, Igor, Lukava (both from P. Dedič), 12/94, Ditta, Gru99 (M. Chrzanowska, PBAI Mlochow, Poland) and -Langenweddingen1 (BAZ, Aschersleben). Infection was tested by DAS-ELISA 7 dai on inoculated and 35 dai on uninoculated leaves.

Results

Stability of resistance to PVY under field conditions

Results of testing for primary and secondary infection with PVY are given in Table 1. Plants with recovery type of resistance became heavily infected in summer time but recovered from virus infection after storage of tubers. In case of lower infection pressure, as in the years 2001 and 2002, these plants revealed some basal level of resistance as only a part of them became infected Extreme resistance of Linda Nb58 was overcome in each year and the plants did not recover from infection.
In all three years an interesting phenomenon was detected. In the case of DH59 Nb146, Nb156 and CP102 single plants were identified that did not recover from the infection. All were grown in the field with design B, which differed from design A only in that there clones DH59 CP39 and CP41 with partial resistance were grown. In 2003 the same was true for clone Linda Nb58.

Appearance of other potato viruses on transgenic plants

According to di Serio et al. (2002) viruses with known suppressers of gene silencing can block transgenic resistance. On the other hand some biochemical processes may have occurred in the transgenic plants rendering them susceptible to, or more resistant against, other viruses. For this reason we investigated whether the incidence of aphid-transmitted viruses was changed on transgenic plants. Results are given in Table 2. It is notable that the appearance of viruses revealed extreme variations during the three investigated years. Thus it is only possible to recognize tendencies. DH59 CP41 appears to be more susceptible to PLRV and more resistant against PVS. DH59 Nb146 seems also to be more resistant against PVS while Linda Nb58 is more susceptible.

An influence of other viruses on the appearance of PVY, acting as suppressers of the resistance reaction to PVY, was not noticed. For instance, in the case of DH59 Nb146 and Nb156, in 2001 most plants were infected with PVM but remained free from PVY. To verify field data we tested the influence of PVS, PVM and PVA on resistance of Linda Nb58 against PVY in a climate-chamber experiment (Schubert et al. 2004). No influence, either on type or level of resistance, was observed thus supporting field data.

Table 1. Incidence (%) of PVY in transgenic potato plants in three field experiments (design A)

a) 2000 growing season

Clone/cultivar	Primary infection with PVY	Secondary infection with PVY
DH59 Nb93	100#	0
DH59 Nb146	100	0
DH59 Nb156	100	0
Linda Nb58	13	42
Controls (DH59, Linda)	100	100

b) 2001 growing season

Clone/cultivar	Primary infection with PVY	Secondary infection with PVY	Secondary infection with PVY (B)
DH59 Nb93	82	7*	n.t.
DH59 Nb146	58	0	27
DH59 Nb156	27	0	7
Linda Nb58	0	30	15
Controls (DH59, Linda)	95	92	

c) 2002 growing season

Clone/cultivar	Primary infection with PVY	Secondary infection with PVY	Secondary infection with PVY (B)
DH59 Nb93	71	5	n.t.
DH59 Nb146	31	0	0
DH59 Nb156	18	0	0
Linda Nb58	2	0	33
DH59 CP102	4	0	7
Controls (DH59/ Linda)[§]	80/82	18/100	

For each clone 45 plants were tested.
*Only slightly above threshold value.
(B): Design B. In this experimental field, resistant transgenic plants were grown mixed with partially resistant transgenic plants (CP39 and CP41). n.t.: not tested
[§] In 2002 pronounced differences existed in secondary infection between both controls.

Table 2. Incidence (%) of four other viruses in transgenic potato clones in field experiments

Clone/cultivar	PVS	PLRV	PVM	PVA
DH59 CP39	-: -: 2*	-: -:13	-: -: 4	-: -: 8
DH59 CP41	-: 0: 7	57:21:16	-:64: 0	2: 0: 0
DH59 CP102	-: -:11	-: -: 4	-: -: 0	-: -: 0
DH59 Nb146	0: 2: 2	14:13: 4	33:53: 0	7: 0: 0
DH59 Nb156	20: 7:16	27: 2:18	23:62:13	14: 2: 9
Linda Nb58	58: 3:60	44: 0: 7	51:64: 2	0:18:18
Ute	60:20: -	9:20: -	40:91: -	4: 2: -
Bettina	13:18:33	11: 9: 4	29:75: 0	11:11:24
Hansa	45:13:77	33: 4: 2	58:62: 3	4:22:42
Linda	4: 5:64	4: 7: 7	52:82: 4	4: 0:62
DH59	-: -: 7	-: -: 0	-: -: 0	-: -: 0
Arosa	-: -:21	-: -: 2	-: -: 0	-: -:33

* In year 2000 (left figure), year 2001 (middle figure) and 2002 (right figure).
-: not tested. Shaded fields: data mentioned in the text.

Resistance to different isolates of PVY

As field experiments revealed that some PVY isolates can overcome resistance of transgenic clones we tested whether this effect is strain-dependent. Data are given in Table 3. They clearly indicate that no correlation exists between the strain used for inoculation and its ability to overcome resistance. The ability to overcome resistance does not depend on differences in the sequences between transgene and invading virus (Schubert et al. 2004).

After storage of tubers harvested in 2001, ring-necrosis symptoms were observed in ca 8% of the tubers of cv. Linda. Tuber ring necrosis was never noticed on transgenic Linda Nb58. The question arose whether Nb58 is resistant against different PVY^{NTN} isolates. Manual inoculation of plants of clone Linda Nb58 with 6 PVY^{NTN} isolates of different geographical origin revealed that this clone was immune to these isolates too. PVY was detected neither on inoculated nor on non inoculated leaves (Table 3).

Table 3. Resistance reactions in four transgenic clones manually inoculated with diverse PVY isolates

PVY isolates	Transgenic clones			
	DH59 Nb93	DH59 Nb146	DH59 Nb156	Linda Nb58
PVY^{N}-CH605	S	S	R	R
PVY^{N}-Adgen	S	RE	RE	S
PVY^{NTN}-Hessen	RE	RE	RE	R
PVY^{NTN}-Igor	-	-	-	R
PVY^{NTN}-Lukava	-	-	-	R
PVY^{NTN}-12/94	-	-	-	R
PVY^{NTN}-Ditta	-	-	-	R
PVY^{NTN}-Gru99	-	-	-	R
PVY^{NTN}-LW1	-	-	-	R
PVY^{O}-Adgen	RE	RE	RE	S
PVY^{O}-BBA	R	S	R	R
PVY^{O}-CZ	S	S	R	S
PVY^{C}-Adgen	S	RE	RE	RE
PVY^{N}W-isolate 2	S	S	R	S
PVY^{N}W-isolate 5	S	R	R	S

S: susceptible (inoculated and uninoculated leaves infected), R: resistant (no virus detected in inoculated or uninoculated leaves), RE: recovery resistance (inoculated leaves infected, no virus detected in uninoculated leaves). Leaves tested 10 and 28 dai by DAS-ELISA.
LW1: isolate Langenweddingen 1; -: not tested.

In 2002 we also compared changes of the strain spectrum of PVY on transgenic and non-transgenic plants. Results are presented in Figure 1. Isolates found on control plants as well as on the resistant clone DH59 Nb93 belonged to PVY^{N}. The same strain prevailed on the susceptible clones DH59 Nb36 and Nb88. In contrast, on the susceptible clones DH59 Nb51 and Nb80 as well as on the other resistant clones mainly isolates of PVY^{O}/PVY^{N}W were detected. All isolates found on DH59 Nb146 and Nb156 belonged to PVY^{N}W too.

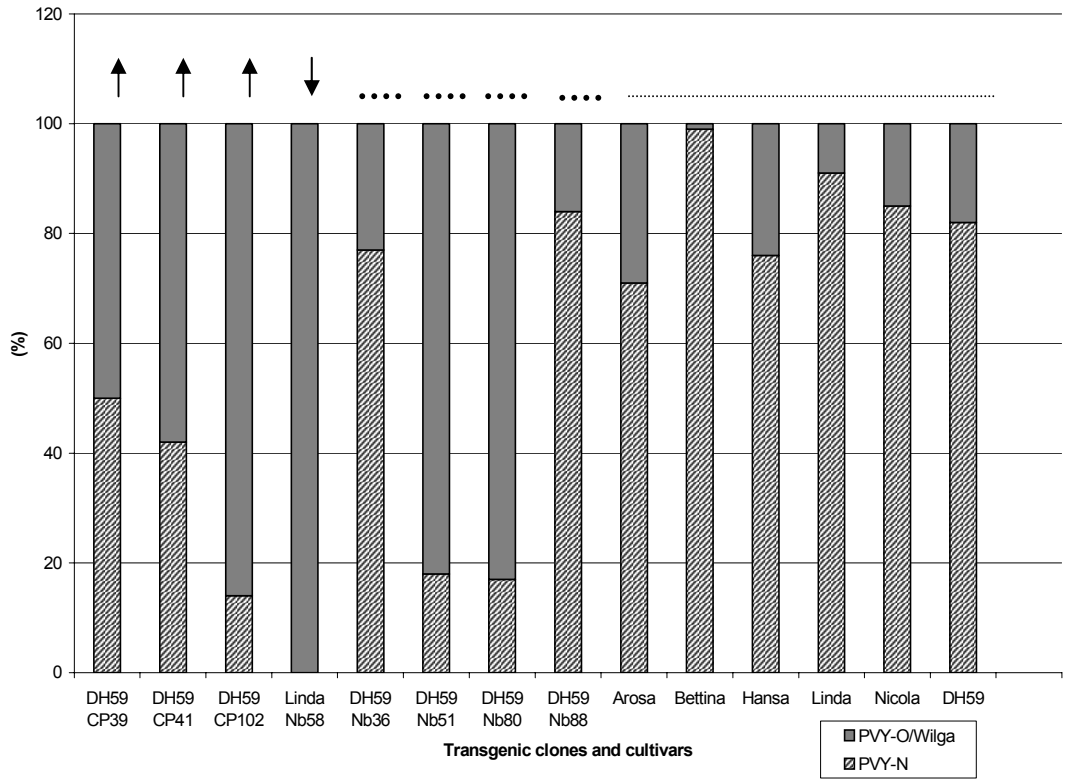

Figure 1. Incidence (%) of PVY^N and PVY^O/PVY^NW^* strains on transgenic potato plants grown in the year 2002.
Data for DH59 Nb93, Nb146 and Nb156 have been omitted as no or only single plants have been infected. ↑: clones with partial resistance and recovery type of resistance; ↓: clone with extreme resistance; ••••: susceptible clones; ….: non-transformed, susceptible cultivars/line.
In case of Arosa and Bettina the dealer provided us with false cultivars

Recombination on RNA level

For transformation we used the cDNA of a PVY^N strain. In 2000, the year in which we started the field experiments, most of the PVY isolates identified belonged to PVY^N strains according to their nucleic-acid sequences. Thus, it was complicated to identify recombination between transgenic and viral RNA as differences in sequences have been only marginal. Investigating several hundred pre-selected cDNA clones no hint of a recombination was obtained. On the other hand some mixed infections took place. In this case it was not possible to recognize whether a recombination between an N- and an O-strain was the result of a recombination between viral RNAs or viral and transgenic RNAs. For this reason potatoes were planted in 2002 in a screen house and infected manually with PVY^NW isolate 5. Investigations of the RNA are in progress.

Among investigated clones that had been pre-selected by CDGE three recombinants were identified, two in experiments of the year 2000 and one in the year 2001. They represent a recombination between O- and N-strains and appeared solely on transgenic plants. The most interesting was the mutant detected in 2001 that originated from a plant of clone DH59 CP102. It was the dominating isolate in this plant. The recombination was rather complex. It was based on a partial duplication in the 3'-non-coding region as well as an exchange between sequences of a PVY^N and PVY^O strain (Figure 2). Whether or not it is competitive enough to survive under conditions possibly favouring non-recombinant isolates will be investigated.

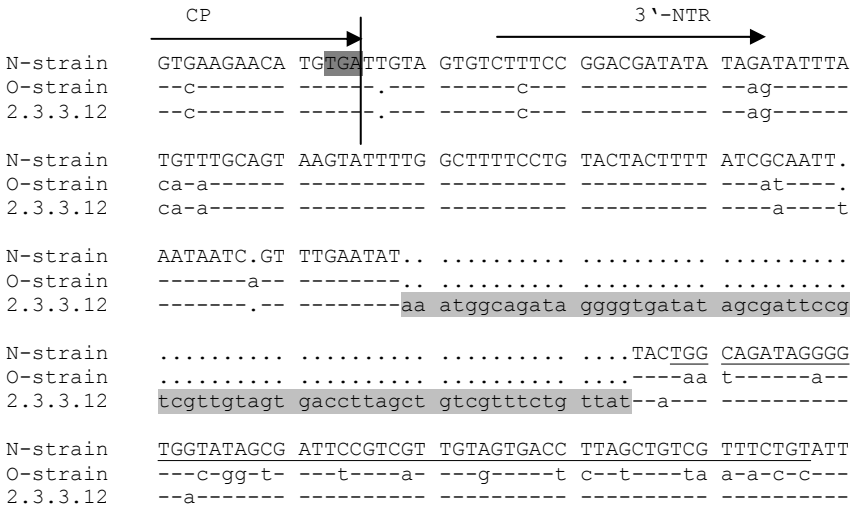

Figure 2. Recombination on RNA level (partial duplication) as found for PVY isolate 2.3.3.12 (infecting clone DH59 CP102).
Dark grey shaded: stop codon of CP gene; grey shaded: duplicated region; underlined: origin of duplicated sequence; ---: identical nucleotide; …: missing nucleotide

Aphid populations

In 2001 we could not notice any pronounced differences in colonization with aphids of transgenic and non transgenic plants.

In 2002 the aphid population was evenly distributed over the experimental field. While in 2001 the overall number of aphids counted was ca 3,000, this number being more than 36,000 individuals in 2002. In this year differences in population density (adults plus larvae) on different clones and varieties were obvious. Data are given in Table 4.

Table 4. Number of aphids settling on transgenic potato plants in 2002

Clone/cultivar	Adults	Larvae	Overall	Ratio larva/ adults
DH59 Cp39	1,434	4,419	5,853	3.1
DH59 Cp41	1,161	3,256	4,417	2.8
DH59 Cp102	1,123	3,260	4,383	2.9
DH59 Nb146	1,143	2,784	3,927	2.4
DH59 Nb156	826	2,191	3,017	2.7
Linda Nb58	657	1,781	2,438	2.7
			Ø 4,006	**2.8**
DH59	826	2,061	2,887	2.5
Bettina	617	1,293	1,910	2.1
Linda	714	2,055	2,769	2.9
Hansa	854	1,784	2,638	2.1
Arosa	511	1,752	2,263	3.4
			Ø 2,493	**2.6**
			∑ **36,502**	

On all DH59 CP clones and on the clone DH59 Nb146 the density of aphids was significantly increased as compared to DH59 Nb156, Linda Nb58 and the control plants.

In 2001 the aphid species were determined too. The prevailing species was *Macrosiphum euphorbiae* making up approximately 99% of the adult population. No differences in appearance of aphid species were found on the different transgenic clones and controls.

Discussion

During three growing seasons we investigated two main aspects of cultivating transgenic potato plants resistant to PVY, viz., stability of the resistance and biosafety aspects.

It is known that transgenic virus resistance of solanaceous hosts can be rather stable, provided that the sequence of the corresponding viral RNA is invariable. This was shown, for example, for *N. benthamiana* L. transformed with the CP gene of *Potato mop-top virus* (Reavy et al. 1997). For most other transgenic plants with PDR it is known that resistance can be overcome by some isolates of the virus if resistance is homology-dependent and based on PTGS. Ability to overcome resistance of our transgenic lines was not associated with differences in the sequences of the transgene and the invading virus. Probably, some of the virus's genes can block the resistance reaction of the host. Similar results were obtained by Maki-Valkama et al. (2000) using transgenic potato plants with the P1 gene of PVY^{O}. In this case resistance was overcome by a PVY^{N} strain. Sequence differences in the corresponding gene were not observed. Lin et al. (2003) investigated squash plants with CP-mediated resistance to CMV. When grown under field conditions plants were infected by several isolates. Again, it was demonstrated that there was no correlation between ability to overcome resistance and degree of sequence homology between the transgene and the invading virus. Several viral genes, as the HC-Pro of PVY and 2b of CMV, are responsible for suppression of the host resistance reaction (Brigneti et al. 1998; Mlotshwa et al. 2002). Virus-mediated suppression of the PTGS-based resistance reaction against one virus can influence the resistance reaction to another one. A similar phenomenon was observed for clone Linda Nb58, which became susceptible to PVA when infected with PVY^{N}W-isolate 5, able to overcome resistance. Similar results were shown by Savenkov and Valkonen (2001) for *N. benthamiana* L., resistant against PVA but susceptible to PVY. If infected with PVY the plants became susceptible to PVA. This illustrates that transgenic resistance against both viruses is possibly based on a common mechanism and that suppression of resistance is an active mechanism. In the case of potyviruses the viral proteins 6K2 and VPg play an important role in this process (Rajamaki and Valkonen 1999). One of such mechanisms blocked by the pathogen could be the process of recognition in which the recently described initiation factor 4E (eIF4E) (Ruffel et al. 2002) is involved.

Level and type of resistance seem to be highly influenced by the site of integration of the transgene. Clones containing the same construct revealed different mechanisms of resistance as well as differences in resistance to several isolates of PVY (Table 3).

The resistance of Linda Nb58 was stable against any of the tested PVY^{NTN} isolates. One can speculate that the gene(s) or part of them responsible for suppression of the resistance reaction of this potato clone is (are) lacking in all tested PVY^{NTN} isolates.

The results indicated that in many cases PDR is not stable. For this reason approaches have to be developed for its stabilization. One such possibility would be to

combine different resistance mechanisms or types of resistance. In our case we started to fuse DH lines, which revealed differences in resistance to PVY isolates, e.g. DH59 CP102 with Nb156 or Nb146 with Nb156.

The presented data on isolates capable of overcoming resistance and probably originating from partially resistant plants underline that it is of utmost importance to test stability of resistance before release of a transgenic cultivar with PDR. Otherwise it could be that, due to the high selection pressure, new virus strains or quasispecies evolve in transgenic plants which can overcome the transgenic resistance and also, something that has never been excluded, natural resistance genes. In 2001 and 2002 we identified PVY isolates that were able to overcome resistance genes from *S. stoloniferum* (Ry_{sto}, present in cvs. Bettina and Ute) and *S. demissum* (breeding line from BAZ). These isolates were not competitive with contaminating isolates of PVY, which replaced them when transferred to tobacco. Thus it was not possible to characterize them. One can speculate that this was the result of growing transgenic plants with partial resistance. Of course, we cannot rule out that the identified virulent PVY isolates were already present in the virus population. Growing squash plants with CP-mediated CMV resistance, Lin et al. (2003) also identified several isolates overcoming this type of resistance, but these were already present before growing transgenes.

A possibility to avoid the process of erosion of the transgenic resistance would be a resistance-gene management: in different years cultivars with different resistance mechanisms should be grown. Unfortunately, it was not possible to realize such a concept for common resistance genes and it is highly questionable whether or not this can be done with transgenic plants.

Recombination between transgenic and viral RNAs was not detected. On the other hand several recombinants between O- and N-strains of PVY were identified solely on transgenic plants. One can speculate that replication of viruses is influenced in transgenic plants resulting in an enhanced level of recombination between viral RNAs. An indication that recombination of PVY in transgenic tobacco was greatly enhanced was also obtained by Jakab et al. (2002). In this case isolates appeared as a result of recombination between N- and O-strains that could overcome resistance. Results presented in Table 1 can be interpreted as an indication that incompletely resistant plants fulfil a bridge function for PVY between susceptible and resistant plants in that they exert a selection pressure on the virus to adapt to PDR. Once mutated and able to overcome an incomplete resistance a second mutation could enable the virus to infect plants with a stronger resistance. Probability for such double mutations, necessary to overcome strong resistance, might be too low for it to happen in highly resistant clones.

Although other authors have described how plant viruses with known suppressers of PTGS have enabled the target virus to infect transgenic plants, this phenomenon was not observed in the case of our transgenic lines. One reason could be that transgenic resistance of our lines is not based on PTGS. For practical use of plants with PDR these results imply that clones revealing protein-based resistance should be preferred for practical use as their resistance is more stable against strains differing in their sequence and this type of resistance will not be suppressed by unrelated viruses.

Another issue of the experiments was the investigation of the influence of transgenic resistance on the appearance of other than the target virus PVY as well as on its strain spectrum. Data in Table 2 illustrate that such influence exists. Unfortunately, the data are not fully conclusive, because there are sometimes large differences between the data of different years. Nevertheless, some tendencies can be

recognized. While Linda Nb58 is more susceptible to PVS the DH59 clones CP41 and Nb146 seem to be more resistant against PVS. Though more resistant against PVS, DH59 CP41 is more susceptible to PLRV. These data underline that transgenic virus-resistant plants must be tested for susceptibility to other unrelated viruses before being released.

Besides influence on non-target viruses, some influence of the transgenic plants was detected on strains of PVY (Figure 1). As a rule, control plants were spontaneously infected by PVY^N. The same is true for the susceptible clones DH59 Nb36 and Nb88. In contrast to this the resistant transgenic plants were infected mainly by PVY^NW. One explanation for this may be that only more aggressive strains – and actually PVY^NW is the most aggressive among them – can infect transgenic plants. Changes in the spectrum of PVY strains had to be expected as this is a common process when growing plants with resistance genes. The question of whether the isolates infecting the (normally) resistant transgenic clones are more virulent on common potato cultivars and, if so, if this is the result of some unusual recombination remains to be answered.

In the literature no data are available describing the influence of transgenic plants on aphids. Using the ubiquitous active CaMV 35S promoter one has to expect high expression levels of the transgenic proteins in phloem tissue too. This could render transgenic plants more attractive for aphids as it possibly improves the nutritional value of the plants due to an enhanced level of proteins in the phloem. On the other hand, a reduced multiplication rate of aphids on transgenic plants would be an indicator for some intrinsic toxic compounds of these plants. Such toxic effects were not observed, as the multiplication rate remained unchanged. In 2001 the density of the aphid population was low. In that year the non-transgenic control cvs. Bettina and Ute were covered slightly more densely with aphids than other control plants and transgenic clones. Probably, data for 2002 are more reliable. In this year we detected a statistically significant denser population on all transgenic CP clones and on clone DH59 Nb146 (Figure 3). Possibly, these plants produce some compounds which make them more attractive for aphids, or the nutritional value of such plants is really enhanced despite the fact that their reproduction rate is not influenced at all. The epidemiological consequence of this feature would be that plants that are more attractive to aphids should be treated with insecticides. In addition, these plants should have high levels of resistance to other aphid-transmitted viruses because the infection pressure would grow with an increased number of aphids attracted by these plants. Consequently, before releasing a new cultivar with transgenic virus resistance, or any other transgenic feature, it is necessary to test whether this feature is influencing attractiveness of the plants to insects. This might also hold true for the Colorado potato beetle.

Summarizing the presented data we conclude that no obvious risk is expected from growing transgenic plants with PDR against viruses. However, it is necessary to investigate whether the suspected enhanced recombination frequency among virus isolates exists on transgenic plants.

The principle of PDR against plant viruses has been known for nearly 20 years. The question arises why only a limited number of commercially available cultivars with this type of resistance exist. One of the answers is provided by the presented results – the probable instability of this type of resistance in the case of highly variable viruses. Plant-breeding companies have to look at this question mainly from the viewpoint of economics: which development costs are necessary and what benefits they can expect. At least five to six years are necessary to develop a transgenic

cultivar with virus resistance. The cost of license fees that would have to be paid is an unknown variable, especially, as the patent situation is confusing at the moment. Additional expenditure is necessary to solve this problem. Further investments have to be made in promotion of such cultivars. At a time of low acceptance expenditures for this must be expected to be high. In addition, the reputation of a breeding firm could suffer from announcing that it is involved in the production of transgenic plants, at least in Europe at the current time. On the other hand, the expected benefits are – under European conditions – marginal. In most cases virus vectors can be controlled by insecticides. Consequently, taking into account the comparably small sizes of European breeding companies one should not expect the appearance of crops with PDR against viruses in the near future. This situation may change rapidly, if methods are found to stabilize the resistance, problems with virus diseases appear that cannot be controlled by conventional methods, public acceptance of GMOs is improved and the patent situation becomes clearer.

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