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ORIGINAL PAPER

Field testing, gene flow assessment and pre-commercial studies on transgenic *Solanum tuberosum* spp. *tuberosum* (cv. Spunta) selected for PVY resistance in Argentina

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Abstract *Solanum tuberosum* ssp. *tuberosum* (cv. Spunta) was transformed with a chimeric transgene containing the Potato virus Y (PVY) coat protein (CP) sequence. Screening for PVY resistance under greenhouse conditions yielded over 100 independent candidate lines. Successive field testing of selected lines allowed the identification of two genetically stable PVY-resistant lines, SY230 and SY233, which were further evaluated in field trials at different potato-producing regions in Argentina. In total, more than 2,000 individuals from each line were tested along a 6-year period. While no or negligible PVY infection was observed in the transgenic lines, infection rates of control plants were consistently high and reached levels of up to 70–80%. Parallel field studies were performed in virus-free environments to assess the agronomical performance of the selected lines. Tubers collected from these assays exhibited agronomical traits and biochemical compositions indistinguishable from those of the non-transformed Spunta cultivar. In addition, an interspecific out-crossing trial to determine the magnitude of possible natural gene flow between transgenic line SY233 and its wild relative *Solanum chacoense* was performed. This trial yielded negative results, suggesting an extremely low probability for such an event to occur.

Keywords Field evaluation · Potato · Spunta · PVY · Coat protein · Transgenic · Resistance · Gene flow

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Introduction

Potato is the fourth most important food crop in the world, with an annual production close to 300 million tons, more than one-third of which are produced in developing countries. In Argentina potato is an important staple crop reaching an annual production of 2–2.5 million tons which are mostly sold in the domestic market. Spunta is the most important potato

cultivar by far and accounts for almost 60% of the national production for human consumption (Rodriguez Quijano 1989). This variety, originally developed in Holland, produces large, pale-color tubers of light-yellow flesh and is widely grown in subtropical regions of Africa and South America.

Viral infections produce important diseases in potato. Potato virus Y (PVY), Potato virus X, and Potato leaf roll virus are present in all major production areas, infecting most cultivars and causing significant yield and economical losses (Rich 1983). PVY, the type member of the Potyvirus group, is naturally transmitted by aphids in a non-persistent manner. Though PVY affects other important solanaceous crops, such as tomato and pepper, it is of particular concern in potato due to the severity of its infections and its persistence in subsequent rounds of seed tubers. Disease symptoms vary widely with virus strains and potato varieties, ranging from barely perceptible damage to extensive necrosis and plant death. In susceptible cultivars, infections can be devastating and losses can reach up to 80% in tuber yield. According to their biological, serological and molecular features, PVY isolates are classified into four groups. The more relevant isolates for potato production are PVY^{N} and PVY^{O} and variants thereof, such as PVY^{NW} (also described as $PVY^{N:O}$) and PVY^{NTN}. Data obtained from PVY detection/characterization studies showed that the different PVY strains have evolved by mutations (acquisition of necrotic abilities) and recombination events (Lorenzen et al. 2006; Ogawa et al. 2008).

Introduction of virus resistance into susceptible potato cultivars by conventional breeding has been hampered by the complex genetics of this crop and the limited number of resistance genes that are currently available. On the other hand, the advent of plant genetic engineering has opened up the possibility of producing resistant crops by transferring viral and non-viral resistance genes (Baulcombe 1994; Beachy et al. 1990; Wilson 1993). Expression of viral CP sequences has been repeatedly used to generate PVY resistant plants (Farinelli et al. 1992; Hassairi et al. 1998; Kaniewski et al. 1990; Mälnoe et al. 1994; Okamoto et al. 1996; Smith et al. 1995) and a number of potato varieties transformed with these constructs have been evaluated under field conditions (Hoekema et al. 1989; Jongedijk et al. 1992; Kaniewski and Thomas 1993; Kaniewski et al. 1990; Lawson et al. 1990; Mälnoe et al. 1994). Satisfactory levels of virus resistance were obtained in most cases but, under certain conditions, protection was overcome by strains differing from that used to obtain the transgenic sequence (Farinelli et al. 1992; Kaniewski et al. 1990; Lawson et al. 1990; Pehu et al. 1995). Post-transcripcional gene silencing (PTGS), a cell-autonomous mechanism implicated in protection to viruses and transposons, has been key to engineer virus-resistant plants for more than ten years (Dougherty et al. 1994; Dougherty and Parks 1995; Voinnet 2001). A growing understanding on PTGS components and mechanisms has allowed the implementation of increasingly efficient strategies to trigger viral RNA degradation (Waterhouse et al. 1998) and, consequently, to generate high levels of resistance. These improvements have also been applied to the development of PVY resistant potatoes (Missiou et al. 2004; Smith et al. 2000). More recently, tobacco transformation with artificial microRNA sequences has been validated as an additional method to induce antiviral protection in plants (Qu et al. 2007).

Prior to its commercial release, transgenic crops intended for human consumption must be exhaustively examined regarding their potential impacts on agricultural environments and food safety. Among other issues, the evaluation process includes assessment of potential gene transfer, impact on non-target organisms and human health, biochemical and phenotypical analyses and allergenicity and toxicity tests of transgenic edible organs. One major concern in environmental analysis is the potential gene flow to sexually compatible and sympatric species that could evolve into new weeds or affect natural biodiversity (Fuchs et al. 2004). Wild potato species present in the commercial potato ecosystems of Argentina, such as *S. commersonii, S. chacoense* and *S. kurtzianum*, could eventually cross with the cultivated potato. The success of these crosses will depend on how the pre- and post-cygotic barriers are overcome (Camadro et al. 2004; Camadro and Peloquin 1981; Johnston et al. 1980; Masuelli and Camadro 1997; Pandey 1968). Viable offspring for such crosses have been reported for *S. chacoense* (Jackson and Hanneman 1999). However, reports estimating the frequency of transgene flow between *S. tuberosum* and *S. chacoense* in natural conditions are still lacking.

In this report, we summarize our studies pointing to the commercial release of two Spunta-derived lines (SY230 and SY233) highly resistant to PVY, including the greenhouse screenings and field trials leading to their selection, their phenotypic and biochemical

evaluation and the outcome of a natural pollen dissemination assay to *S. chacoense*.

Materials and methods

Plant material and virus strains

Virus free minitubers of *S. tuberosum* cv. Spunta were used immediately after harvest or kept at 4°C for periods of 1–6 months before transformation assays. Transgenic and non-transformed potato plants were propagated in vitro on MS medium (Murashige and Skoog 1962) supplemented with 3% sucrose (MS-3) and 0.8% agar to obtain virus-free material for infection assays. Explants were grown at 22°C under a 16/8 h light/dark photoperiod. In vitro grown plants were transferred to soil and grown under greenhouse conditions for virus testing and/or tuber production. Tubers were harvested and kept in darkness at 4°C for up to 6 months before sown in field trials or used directly for the different biochemical and genetic analysis outlined in this study.

Plant transformation

Transformation of *Agrobacterium* strains was carried out by the "freeze and thaw" method described by Holsters et al. (1978). Plant transformation was performed as described by Hoekema et al. (1989) with minor modifications. Minitubers were washed in tap water, peeled off, briefly treated with an ionic detergent and then sterilized in 70% ethanol and 2% NaClO. After washing in sterile water, tuber tissue was cut in 1 cm^2, 1 mm thin slices and transferred to Petri dishes containing MS-3 medium. For transformation, batches of 50 discs from *S. tuberosum* cv Spunta were co-cultivated in an over-night culture with *A. tumefaciens* (strain LBA4404), blotted dry on sterile filter paper and transferred to solid MS-3 medium supplemented with 100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine chlorohydrate, 2.0 mg/L glycine and 5 μM of indoleacetic acid (IAA) and 5 μM zeatin riboside (ZR). Explants were incubated at 22°C for 48 h under a 16/8 h light/dark photoperiod, and then transferred to a selection medium containing 100 μg/mL kanamycin and 200 μg/mL cefotaxime. After 2–3 weeks, shoots were excised from explants and subcultured in the same growth medium for root development. Rooted plants were micropropagated, grown in vitro for another 2 weeks, and then transferred to soil and grown in the greenhouse.

Genetic constructs

The CP gene from PVY strain O was previously cloned and sequenced (Bravo-Almonacid and Mentaberry 1989). A sequence comprising the last 17 codons of the viral replicase (*NIb*), the CP gene, and the complete 3′ non-coding region of the viral gRNA was fused in a continuous reading frame to a cDNA fragment comprising positions 113–201 of the PVY gRNA (Robaglia et al. 1989) including part of the gRNA 5′ non-coding region, the AUG codon of the viral polyprotein and the first 5 codons of the P1 protein (*CPPVY* sequence). The recombinant polypeptide encoded by this sequence contains the cleavage site between Nib and CP recognized by the NIa viral protease (Riechmann et al. 1992). The *CPPVY* sequence was cloned into the binary expression vector pBI121 (Jefferson 1987) under the control of the 35S promoter from the Cauliflower mosaic virus and the final construct was named pPBI-CPPVY. This plasmid also carries the *nptII* gene which allows for plant selection in media supplemented with kanamycin (Supplementary Figure S1). Putative regenerated transformants were assayed for the presence of *CPPVY* sequence by PCR amplification and for the accumulation of NptII protein by western blot analysis.

Plant inoculations

Greenhouse-grown potato plants of approximately 15 cm in height were manually inoculated with a PVY^N strain in 4-plant batches for each transgenic line. Inoculation was carried out at the fourth leaf from the top using diluted PVY-infected *N. tabacum* leaf extracts. Plants were then grown in the greenhouse under strict containment and primary virus infection was assessed in non-inoculated leaves 40 days post-inoculation (d.p.i) using a commercial DAS–ELISA test (Agdia Inc.). Transgenic lines were ranked as susceptible if any leaf of the 4 plants tested became infected. Tubers from putative resistant lines were propagated in the greenhouse for future field testing. Depending on the transformed line, 5–15 tubers were sown to evaluate their performance to PVY infection

under field infection pressure. The presence of virus in leaves of transformed and non-transformed plants was measured by DAS-ELISA. The same procedure was repeated in nine successive trials over a period of 3 years, discarding at each assay the lines that were infected by PVY. Testing and tuber propagation were conducted under controlled conditions to prevent accidental release of transgenic material.

PCR amplification of the CPPVY sequence

Plant genomic DNA was isolated from leaves of in vitro plantlets as described by Dellaporta et al. (1983). *CPPVY* PCR amplification was carried out in a IHB2024 Hybaid thermocycler using primers 5′-ACT CTGCTTGGAGGCAAAAA-3′ and 5′-ATCACAA CATCACCCAAGCA-3′ located at the *CPPVY* 3′ sequence. Amplification products were separated by electrophoresis in a 1.2% agarose gel and visualized by ethidium bromide staining. PCRs were carried out in 50 µL volume. Cycling conditions were 95°C for 3 min followed by 40 cycles of 95°C for 40 s, 57°C for 40 s, 72°C for 40 s and a final 7-min extension at 72°C.

Southern and northern blot

For Southern analysis, 15 µg aliquots of genomic DNA were treated with the restriction enzymes *EcoRV* or *XbaI* and then separated by electrophoresis in 0.8% agarose gels. For northern assays, 10 µg samples of leaf, stem or root total RNA were electrophoresed in 1.5% denaturing agarose gels. In both cases, plant nucleic acids were transferred to Hybond N^+ Nylon membranes (Amersham Biosciences, Uppsala, Sweden) and hybridized with ^{32}P-labelled probes specific for the *CPPVY* or the *nptII* sequences. Radioactive labeling was performed using a commercial kit (Prime-a-Gene; Promega, Madison, WI, USA). After overnight hybridization, membranes were washed and exposed to a Phosphor Image screen (Molecular Dynamics, USA) and scanned on a Storm 840 Phosphorimaging System (Amersham Biosciences, Uppsala, Sweden).

Immunological assays

Leaf samples for western immunoblots were homogenized in 100 mM Tris–HCl, pH 6.8, 10% glycerol, 5% β-mercaptoethanol, 0.1% SDS, boiled for 3 min, centrifuged for 10 min at 16,000*g* and assayed for soluble protein content according to Bradford (1976). Aliquots of 40 µg of soluble proteins were separated by 12.5% SDS-PAGE and transferred to nitrocellulose membranes. CPPVY protein was detected by incubation with a rabbit anti-PVY antibody raised against purified virions followed by a second incubation with alkaline phosphatase-linked goat anti-rabbit antibody (Jackson Immuno Research Inc.). Visualization was carried out using a commercial BCIP/NBT phosphatase substrate kit (Sigma Chemical Co). For NptII detection, a rabbit anti-NptII antibody (US Biological Inc.) was used (1/250 dilution). Equivalent protein loading of gel lanes was assessed by membrane staining (0.1% Ponceau Red) before incubation with the respective antibodies.

Field trial evaluations

Putative resistant plants were subjected to several stages of field testing in order to identify genetically stable resistance to PVY. The number of transgenic lines included in these trials changed from test to test and fluctuated between 10 and 72. As a rule, not less than 4 individuals from each transformation event were included in a single assay. Wild-type, PVY-infected Spunta plots were distributed across the trial to provide a source of inoculum. Evaluation of infection was carried out using a DAS-ELISA kit specific for PVY (Agdia Inc.). Before crop maturity, the first fully expanded leaves were sampled and kept cool for less than 24 h until freezing at −80°C. Frozen samples were ground using a pellet pestle, suspended in extraction buffer and cleared by centrifugation before pipetting into the ELISA plate. Virus-free samples were used as negative controls. Samples displaying ELISA values above the average of negative controls plus 5 standard deviations were considered as infected. Tubers from transgenic events not displaying infection were advanced to the following stage.

Comparative field trials to evaluate the agronomical performance of lines SY230 and SY233 were carried out at four potato-producing regions of Argentina (Balcarce, Córdoba, Malargüe and San Juan) during the period 2005–2006. Geographical locations were chosen considering the different agricultural conditions prevailing in Argentina. Thus, two of the trials

were located in virus-free potato seed-production regions (Malargüe and San Juan) and the other two were located in potato production regions in which PVY infection rates are normally high (Balcarce and Córdoba). A complete randomized block design with four replicates was used to evaluate the two transgenic lines and non-transformed controls. All plots consisted of a single row of 5 m and with a distance of 85 cm between rows. No other plants were grown within 10 m from the trial plot, which was fenced to prevent access of large animals. Plants and tubers of transformed lines were compared to that of non-transformed Spunta according to the description given in the Netherlands Catalogue of Potato Varieties (2007). Biological containment of field trials, assay isolation and disposal of transgenic materials were performed following the guidelines established by the National Commission on Agrobiotechnology (CONABIA, Argentina). A total of nine independent trials were carried out in different locations of Argentina.

Evaluation of tuber characteristics

Total yield (g/plot), number of tubers/stem, average tuber weight (g) and tuber aspect were determined for each plot. Specific gravity (SG) was determined by weighing 5 kg of tubers per plot in air and then in water, according to the formula: SG = tuber weight in air/(tuber weight in air) − (tuber weight in water). Dry matter was assessed gravimetrically by drying mashed tuber samples in an oven at 120°C. Potato chip color was determined by frying 200 g of cut slices (0.7 mm thick), taken from the central region of the tuber, in sunflower oil at 180°C for 5 min. Color was estimated according a 1–9 semi-quantitative scale (1 = dark brown; 9 = golden yellow).

Biochemical measurements

Amino acid concentrations in potato tubers were measured by the Food Technology Laboratory (School of Sciences, University of Buenos Aires), whereas all other biochemical and chemical determinations were performed by the National Institute for Industrial Technology (INTI, Argentina). All measurements were done according to standard methods defined by the Association of Analytical Communities (AOAC) or the American Oil Chemist Society (AOCS).

Glycoalkaloid measurements

A HPLC-based protocol designed according to Hellenäs and Branzell (1997) was developed to measure the α-chaconine and α-solanine contents in potato tubers. At least nine tubers from each transgenic line and from non-transformed Spunta were evaluated regarding their total glycoalkaloid content at each trial site. Ten g of tuber samples were frozen in liquid nitrogen and grinded down to a fine powder in a mortar. The powder was then resuspended in 40 mL of buffer containing 5% (v/v) CH_3COOH and 1% $NaHSO_3$ (v/v). The suspension was vortexed and centrifuged at 20,000*g* for 30 min in a Jovan KR22i centrifuge. The supernatant was frozen at −70°C until examination by HPLC. Chromatographic analyses were performed using an Agilent Serie 1200 Quaternary LC system (Agilent Technologies) equipped with a variable UV wavelength detector and steel columns (250 mm, 4.6 mm, 5 µm). Disposable Waters SepPak Plus C_{18} extraction cartridges (Milford, MA, USA) and 0.45 µm 30 mm PVDF filters were used for sample purification prior to column loading. Chem software (Ezcrom) was used for all data analysis. Analyses were performed injecting 40-µL samples through the HPLC column at a flow rate of 1.5 mL/min. After loading the samples, columns were washed with 15% (v/v) acetonitrile and glycoalkaloids were eluted with 4 mL of 60% (v/v) acetonitrile in a 0.02 M Phosphate buffer, pH 7.6. Glycoalkaloid contents were calculated from the peak areas and plotted against standard calibration curves including concentrations of 5, 10, 25, 50, 100 and 150 µg/mL of either α-solanine or α-chaconine (Sigma Chemical Co.) in 0.1 M KH_2PO_4.

Interspecific transgene flow between SY 233 and *S. chacoense*

A schematic drawing of the field trial performed to evaluate pollen mediated transgene transfer between line SY233 (pollen donor) and *S. chacoense* is shown in Fig. 5. Plants from *S. chacoense* were derived from tubers collected in the previous season in the experimental fields of INTA-Balcarce station. Floral buds from *S. chacoense* were eliminated until flowering was initiated in line SY233. One thousand and seven hundred fruits (1,700) containing 103,000 seeds from the *S. chacoense* population were sampled at distances between 0.20 and 50 m from a central plot planted

with the transgenic line SY233. Forty per cent of the corresponding seeds (41,200), representing all the fruits collected, were in vitro germinated and extracted to obtain total genomic DNA. DNA from 100 seedlings were pooled and tested by a PCR assay designed to detect the *CPPVY* sequence. DNA extracted from a mix of one SY233 and 100 non-transformed seedlings was used as a positive control in the PCR assays.

Statistical analysis

Student's *t*-test was used for statistical analysis of the data. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

Results

Selection of potato transgenic lines resistant to PVY infection

Minituber discs from *S. tuberosum* cv Spunta, were transformed with the genetic construct pPBI-CPPVY (Supplementary Figure S1). Plants growing on media containing kanamycin and showing PCR amplification of the *CPPVY* sequence were selected for further examination. Four hundred transformation events were selected using these criteria. About 30% (141 lines) of all transformed plants were discarded due to poor growth or phenotypic anomalies. The remaining 259 transformants were multiplied and tested for resistance by manual infection with PVY^N-infected potato leaf extracts under greenhouse conditions. As a result, 171 lines were classified as putative resistant to PVY. These lines were subjected to tiered field trials conducted under natural PVY infection at two different geographical locations. This scheme was implemented throughout nine independent field trials carried out during 3 years (1998–2001) and allowed the selection of 22 candidates. A summary of the selection scheme and the results obtained in these trials is shown in Fig. 1 and Table 1. Two of the 22 PVY-resistant lines showing consistent behavior in all field trials (SY230 and SY233) were multiplied and tested in a larger field assay conducted in General Belgrano in October 2000. In this assay, 154 out of 154 SY230 plants were found to be virus free, and only one out of 441 SY233 plants was found to contain detectable PVY levels. In the same trial, 560 out of

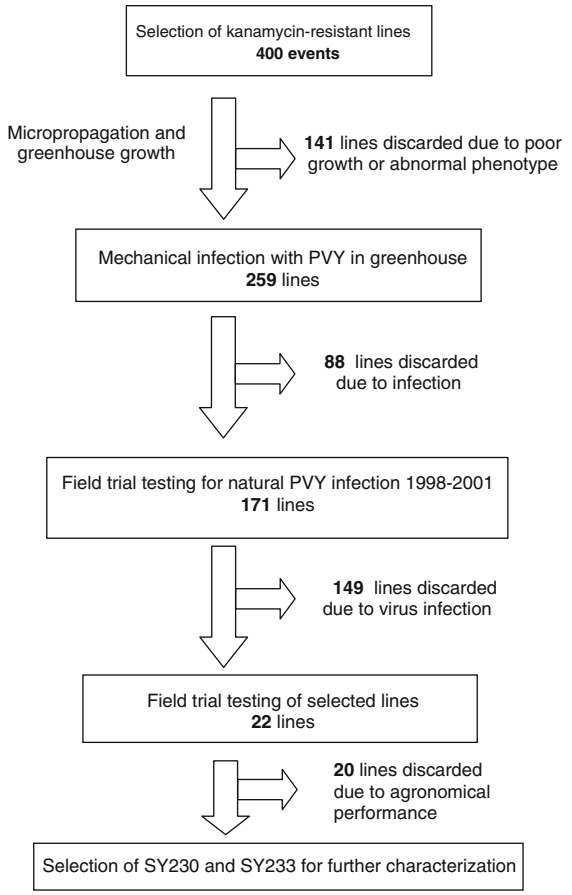

Fig. 1 Screening for selection of PVY-resistant potato plants. Global screening procedure leading to the identification of PVY-resistant lines SY230 and SY233

661 non-transformed control plants were infected with PVY, implying an infection rate of 85%. On this basis, SY230 and SY233 were chosen as candidates for possible commercial release.

Transgene copy number, transcript and protein levels in lines SY230 and SY233

Southern blot analysis showed that SY230 and SY233 are independent transformation events containing multiple transgene insertions. Evidence for the presence of three independent integrations was obtained with a probe recognizing the *nptII* sequence in both lines (Fig. 2, left panel). On the other hand, a probe specific for the *CPPVY* sequence also revealed three bands in line SY230, but only two in the case of SY233 (Fig. 2, right panel). This result could indicate that one

Table 1 Screening for PVY resistance

Screen stage		1st stage	2nd stage	3rd stage
A.				
Events tested		171	93	36
Average plants tested/event		6	11	21
Average % PVY infection across stage		21%	26%	13%
Events discarded		78	57	14
Events passed		93	36	22
Location	Year, season	Screen stages represented	Plants tested	Infection rate across trial (%)
B.				
General Belgrano	1998, Spring	I	49	20
General Belgrano	1999, Fall	I, II	159	14
General Belgrano	1999, Spring	I, II, III	139	8
Colonia Tirolesa	1999, Spring	I, II, III	851	26
General Belgrano	2000, Fall	I, II, III	191	22
Colonia Tirolesa	2000, Fall	I, II, III	294	34
Colonia Tirolesa	2000, Spring	I, II, III	159	6
General Belgrano	2001, Fall	I, II, II	403	14
General Belgrano	2001, Spring	II, III	243	21

A: summary of the tiered field trial screening used to identify PVY-resistant potato lines during 1998–2001. Average infection rates across stages are displayed to illustrate progressive enrichment of the trial populations with resistant genotypes. B: summary of the individual field trials composing the three-stage screening (I, II, III)

of the three *CPPVY* insertions is partially deleted in SY233.

Northern blot assays showed low, but still detectable levels of transgenic *CPPVY* transcripts in leaf, stem and root tissues of both SY230 and SY233 lines after an extended autoradiographic exposure (Fig. 3a, b). A comparison between both lines showed that transcript accumulation is slightly higher in SY230 as compared to SY233. RNA from PVY-infected potato leaves was used as a positive control (Fig. 3a). The NptII transcript was clearly detected in stem, young and old leaves after regular autoradiographic exposure time (Fig. 3d).

Western blot assays conducted with polyclonal antibodies raised against purified *E. coli*-expressed PVY CP failed to detect transgenic CPPVY protein in SY230 or SY233 leaf extracts. In contrast, a band corresponding to the PVY CP could be observed in PVY-infected tobacco leaves in dilutions of up to 5,000 times (Fig. 4a). Similar results were obtained with stem or tuber extracts (data not shown). As expected, the NptII protein was readily detected in leaves and tubers from SY230 and SY233 samples tested with commercial antisera (Fig. 4b). Membrane staining with Ponceau Red before antibody detection confirmed that samples contained equivalent amounts of protein (data not shown).

Agronomical traits and tuber quality

Results from the field trials are presented in Tables 2, 3 and 4. Plant and tuber phenotypical attributes in SY230 and SY233 lines were practically identical to the non-transformed Spunta. The proportion of off-type tubers was found normal considering the environmental variations among trial locations. Scarce flowering was observed in the transgenic lines in all locations. In the Balcarce gene flow trial (see below) low pollen viability (6%) was observed in line SY233. No differential plant (flower color, leaf type, foliage color, anthocyan presence at the stem base, stem wings, plant habit) or tuber (shape, size, flesh color, skin type, eye depth) characteristics were recorded between the two transgenic lines and the non-transformed controls. Similarly, no major differences in total yield, tuber size or virus infection could be

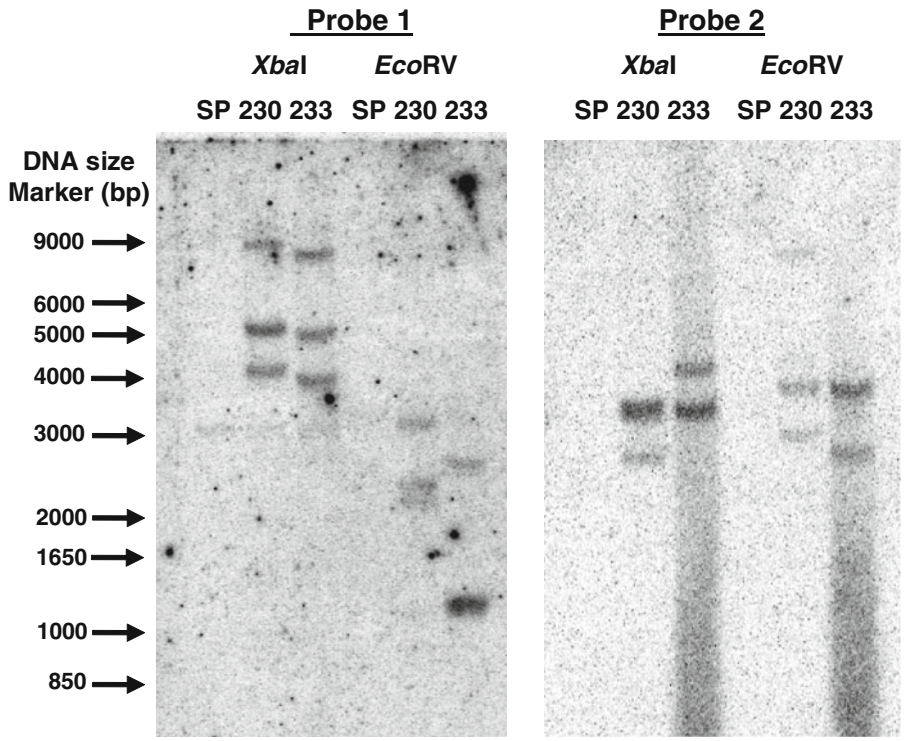

Fig. 2 Southern blot analysis. Purified DNA from SY230 (230), SY233 (233) and non-transformed Spunta (SP) leaves was digested either by restriction enzymes *Xba*I or *Eco*RV. Digestion products were separated in 0.8% agarose gels and transferred to nylon membranes. Detection of restriction bands was performed using DNA probes complementary to either the *nptII* (*probe 1*; *left panel*) or the *CPPVY* (*probe 2*; *right panel*) sequences. *Arrows* shown to the left indicate the position of DNA size markers

detected between transgenic and non-transgenic plants grown in virus-free areas.

Total protein and amino acid content

Tubers collected in the four regional trials for agronomical performance (Malargüe, San Juan, Córdoba and Balcarce) were evaluated for their biochemical composition. Potato tubers are considered a poor protein source and their total protein content usually varies between 2–3 g for 150 g of tubers. Measurements of total protein in tubers harvested at the regional trials (Table 4) showed no significant differences between line SY230 (2.41 g $\pm$ 0.44), SY233 (2.31 g $\pm$ 0.42) and Spunta controls (2.67 g $\pm$ 0.28). Similarly, contents of 18 essential aminoacids were no significantly different in transgenic lines and control plants (data not shown). In addition, several parameters of interest for the potato industry, such as tuber specific gravity, starch content, dry matter and frying color were analyzed. Results from these studies are summarized in Tables 3 and 4. As it was the case for the protein and aminoacid contents, no significant differences were found between the two transgenic lines and Spunta controls.

Glycoalkaloid content

Glycoalkaloids are the most important toxic compounds produced by potato. Two of these compounds, α-chaconine and α-solanine, account for more than 95% of total tuber alkaloids and are the source of major concerns. Concentrations of these glycoalkaloids in the range of 1–15 mg/100 g FW are regarded as acceptably low, while values above 20 mg/100 g FW produce a bitter taste and can induce strong gastroenteric symptoms, coma, and even death (Gregory 1984; Morris and Lee 1984). Consequently, contents higher

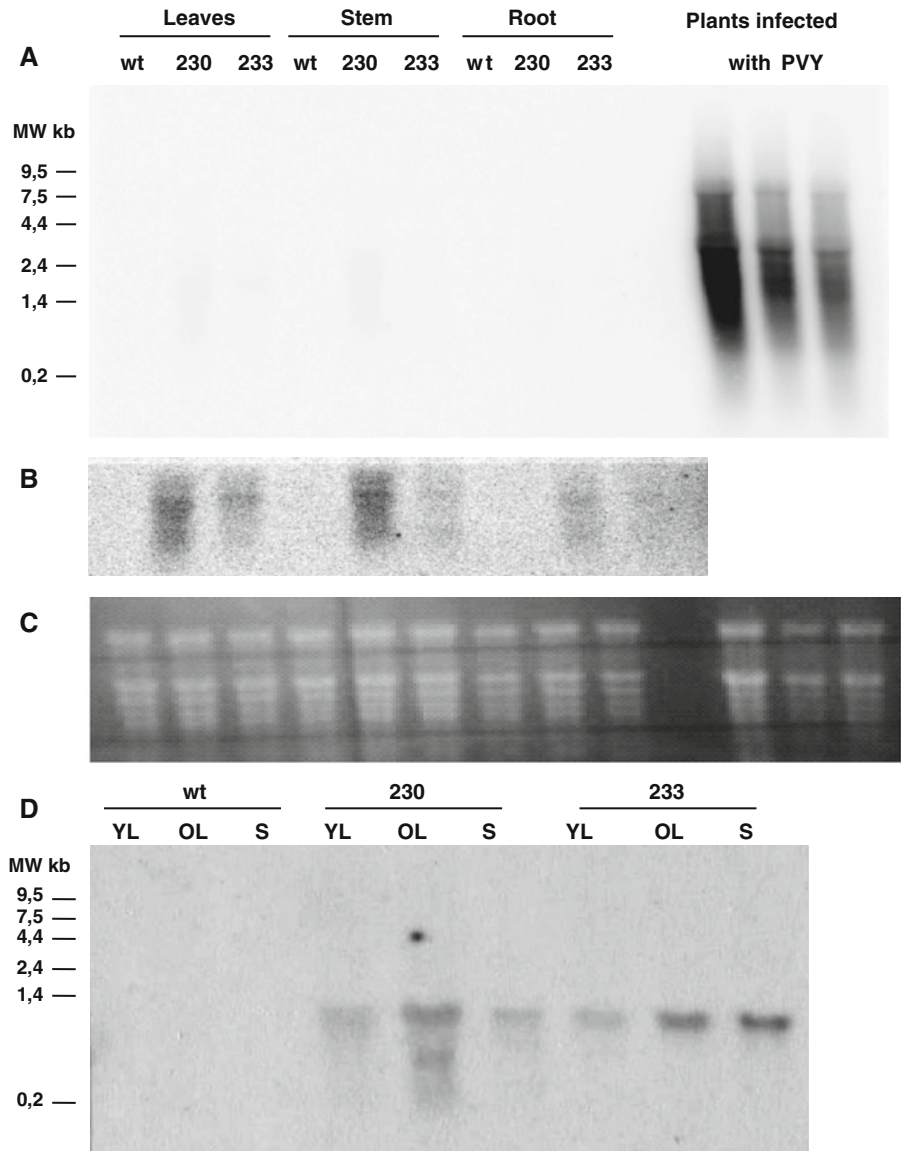

Fig. 3 *CPPVY* transcript accumulation in transgenic lines SY230 and SY233. Total RNA from leaf, stem and root of SY230 (230), SY233 (233) and non-transformed Spunta (wt) was separated in a 1.5% denaturing agarose gel. RNA was transferred to nylon membranes and transgenic RNA was detected using a DNA probe corresponding to the *CPPVY* sequence. RNA purified from three PVY-infected potato leaves was included as a positive control (PVY-infected *lines 1–3*). **a** Autoradiogram corresponding to a 12 h-exposure. **b** Autoradiogram corresponding to a 48 h-exposure. Positive controls were excluded in **b** in order to avoid interference with the signal corresponding to the other lanes. **c** Ethidium bromide staining of the agarose gel. **d** Total RNA from young leaves, old leaves and stems (YL, OL, S, respectively) revealed with an *nptII* probe. Autoradiogram corresponding to a 12 h-exposure. MW: RNA molecular weight markers. NptII: position corresponding to the NptII transcript

than 20 mg/100 g FW exceed food safety recommendations and these tubers should be withdrawn from sale (Percival et al. 1994). No major differences in total glycoalkaloid concentration were found between SY230, SY233 and non-transformed Spunta controls (Table 3) and all values were well bellow the recommended food safety limit of 20 mg/100 g FW (Bushway et al. 1986; Carman et al. 1986).

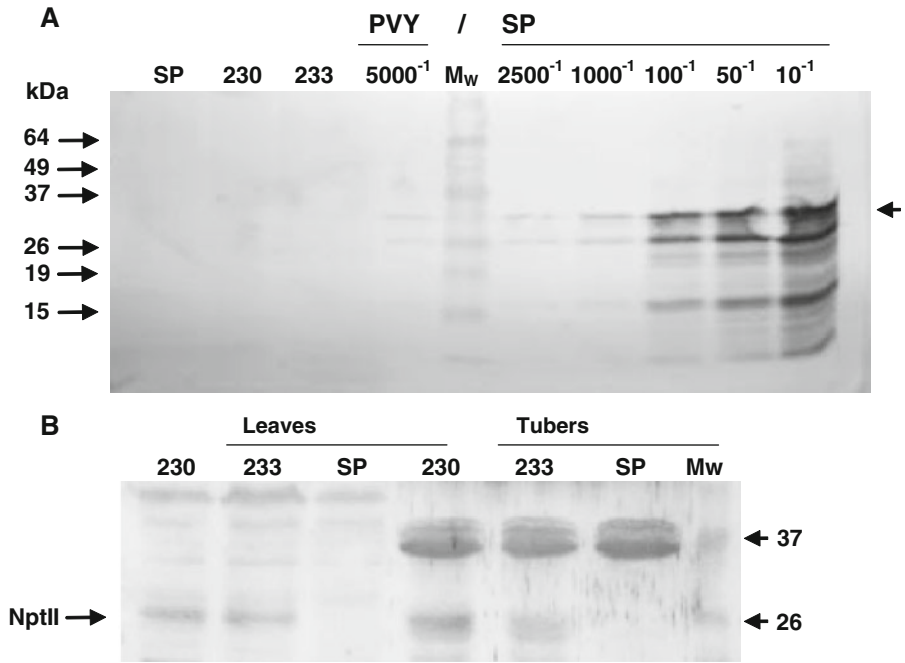

Fig. 4 Western blot analysis of lines SY230 and SY233. **a** Total soluble proteins were isolated from SY230 (230), SY233 (233), non-transformed Spunta (SP) and PVY-infected Spunta (PVY/SP) leaf extracts, separated by PAGE-SDS, transferred to a nitrocellulose membranes and revealed with anti-PVY antibodies. Numbers (10^{-1}–5.000^{-1}) indicate the dilutions of PVY-infected potato leaf extracts loaded in each lane. **b** Total soluble proteins from leaf or tuber extracts revealed with anti-NptII antibodies. *Arrows* indicate the position of molecular weight markers (BenchMark Pre-Stained Protein Ladder, Invitrogen), the PVY CP (CP) or the NptII protein (NtpII)

Evaluation of possible inter-species crossings between *S. tuberosum* spp. *tuberosum* (cv. Spunta) and *S. chacoense* under natural conditions

In order to determine if natural crossings could occur between the transgenic varieties and wild *Solanum* species, a field trial using SY233 as pollen donor and *S. chacoense* as pollen acceptor was performed in an experimental field located in a major potato producing area (Balcarce). A scheme of the experimental design used in this assay is shown in Fig. 5. About 103,000 seeds from the *S. chacoense* plants were collected at different distances of the central plot planted with line SY233. About 40% of these seeds (41,200) were randomly pooled in 100-seed groups and germinated. DNA representing each seed pool was then analyzed by PCR for presence of the *CPPVY* sequence. No DNA amplification was obtained in any of these assays, indicating that no natural interspecific crossing had taken place at the test site and strongly suggesting that an extremely low probability for such an event to occur might be expected in the case of a commercial release. A typical result from the PCR amplification study is shown in Supplementary Figure S2.

DISCUSSION

Since the first report on CP-mediated virus resistance two decades ago (CP-MR) (Beachy 1990), this approach has been used to confer plant virus resistance to many different crops (reviewed in Dasgupta et al. 2003; Prins 2003; Prins et al. 2008). The mechanism underlying CP-MR involves the presence of viral protein as direct or indirect effector, and induces interference with virus assembly, virus replication, long-distance and cell-to-cell virus movement (Beachy 1999; Bendahmane et al. 1997; Lu et al. 1998). Though this mechanism operates in some specific cases, it was soon realized that overexpression of CP viral sequences could trigger viral RNA degradation by a pathway independent of protein accumulation (Lindbo and Dougherty 1992). The discovery of this mechanism—today named as post-transcriptional

Table 2 Comparative tuber yield studies

Geographical location	Plant line	Total tuber yield (g/plot)
Balcarce		
Assay 1	SY 230	3,426 ± 899
Assay 1	Spunta	2,565 ± 557
Assay 2	SY 233	4,225 ± 376
Assay 2	Spunta	4,265 ± 1,497
Córdoba		
	SY 230	4,025 ± 1,281
	SY 233	4,010 ± 1,536
	Spunta	3,040 ± 1,627
Malargüe		
	SY 230	2,450 ± 490
	SY 233	2,100 ± 540
	Spunta	2,900 ± 1,000
San Juan		
	SY 230	8,271 ± 2,985
	SY 233	8,780 ± 3,088
	Spunta	8,275 ± 2,323

Field trials to compare tuber yields between lines SY230, SY233 and non-transformed Spunta were performed at Balcarce, Córdoba, Malargüe and San Juan during the growth season 2005/2006. Total tuber yield values for each location are presented as the mean-values resulting from 4 independent plots and their respective standard deviations

gene silencing (PTGS)—opened up new avenues of research and represented an important milestone in contemporary biology (Vaucheret and Fagard 2001; Voinnet 2001; Wassenegger and Pélissier 1998; Waterhouse et al. 2001). Constitutive induction of PTGS, both by the CP or other viral sequences, leads to degradation of homologous viral RNA and is now widely employed to prevent virus infections.

At the beginning of this work, it was decided to implement a strategy involving classical CP-MR. Earlier reports on PVY resistance engineered by CP-MR have indeed shown that resistance can be overcome by strains other than that providing the transgenic sequence (Farinelli et al. 1992, Mäki-Valkama et al. 2000; Pehu et al. 1995). For this reason, our main concern was how to select transgenic lines showing genetic stability and broad-strain resistance to PVY under field conditions. To increase the probability of finding a line resistant to most common PVY strains, we expressed a chimeric gene construct containing de PVY^O CP sequence and conducted all manual screenings infecting with a PVY^N strain. With the same objective, field tests were designed to cover a broad period of time and different geographic locations.

Testing under field conditions is difficult because environmental stresses change frequently and it is impossible to ensure an optimal condition with all

Table 3 Tuber analysis

Location	Variety	Specific gravity	Starch contents (%)	Dry matter (%)	Chip color	Glycoalkaloids (mg/100 g fresh weight)		
						Solanine	Chaconine	Total
Balcarce	SY230	1.066	11.0	18.0	4.5	2.40 ± 0.18	6.47 ± 0.37	8.87
	SY233	1.068	11.3	18.6	7.5	3.18 ± 0.48	8.94 ± 1.03	12.12
	Spunta	1.069	11.6	18.8	6	1.98 ± 0.27	6.05 ± 0.50	8.03
Córdoba	SY230	1.083	14.6	20.7	8	3.10 ± 0.43	7.10 ± 0.70	10.20
	SY233	1.081	14.0	20.5	8	3.26 ± 0.67	7.63 ± 1.37	10.89
	Spunta	1.086	15.1	21.5	6.5	3.43 ± 0.09	9.17 ± 1.34	12.60
Malargüe	SY230	1.057	9.1	16.5	3.5	1.63 ± 0.44	4.68 ± 1.68	6.31
	SY233	1.064	10.6	17.5	3	1.71 ± 0.21	5.51 ± 0.77	7.22
	Spunta	1.063	10.3	17.4	3	2.79 ± 1.79	5.93 ± 2.70	8.72
San Juan	SY230	1.080	10.7	22.8	6	1.32 ± 0.03	5.07 ± 0.19	6.39
	SY233	1.081	11.1	22.2	6	2.73 ± 0.57	8.27 ± 3.26	11.00
	Spunta	–	–	–	–	2.60 ± 0.25	8.44 ± 3.16	11.04

Biochemical and culinary parameters of potato tubers were measured in SY230, SY233 and non-transformed Spunta samples obtained from the comparative assays referred in Table 2. Values for each location are presented as mean-values resulting from 4 independent plots and their respective standard deviations

Table 4 Tuber analysis

Location	Variety	Total protein	Soluble proteins	Fat	Crude fiber	Ashes	Total carbohydrates
San Juan	SY230	2.25 ± 0.17	68.5 ± 6.8	0.45 ± 0.06	0.60 ± 0.00	1.22 ± 0.07	19.6 ± 0.13
	SY233	1.85 ± 0.06	70.4 ± 5.1	0.45 ± 0.13	0.55 ± 0.06	1.12 ± 0.08	21.1 ± 1.09
	Spunta	2.42 ± 0.61	68.3 ± 7.6	0.48 ± 0.10	0.45 ± 0.06	1.23 ± 0.23	20.8 ± 2.37
Balcarce	SY230	2.32 ± 0.10	64.3 ± 6.9	0.42 ± 0.10	0.65 ± 0.17	1.09 ± 0.10	16.6 ± 2.23
	SY233	2.18 ± 0.05	61.5 ± 11.3	0.35 ± 0.06	0.62 ± 0.10	1.15 ± 0.05	16.8 ± 2.38
	Spunta	2.25 ± 0.19	62.1 ± 20.1	0.45 ± 0.06	0.62 ± 0.05	1.11 ± 0.17	16.6 ± 3.75
Malargüe	SY230	2.25 ± 0.37	65.8 ± 3.9	0.40 ± 0.00	0.58 ± 0.05	1.11 ± 0.08	13.8 ± 1.05
	SY233	2.45 ± 0.06	70.0 ± 7.4	0.48 ± 0.05	0.65 ± 0.17	1.05 ± 0.13	17.4 ± 1.18
	Spunta	2.50 ± 0.14	78.1 ± 10.0	0.42 ± 0.10	0.48 ± 0.05	1.08 ± 0.03	15.6 ± 0.63

Biochemical parameters of potato tubers were measured in SY230, SY233 and non-transformed Spunta samples obtained from the comparative assays referred in Table 2. Values for each location are presented as mean-values resulting from 4 samples taken from 2 independent field trials at consecutive growth seasons. Values are expressed as g/100 g dried weight

required factors for the stress to be tested. This was indeed the case for several of our field trials, in which low levels of viral inoculum and/or insect vectors produced low infection rates. As shown in Table 1, infection rates varied considerably from one testing site to another, ranging from 32% (Colonia Tirolesa, Fall, 2000) to only 5% (General Belgrano, Spring, 2000). The low infection rates found in several trial sites considerably delayed our selection time schedule. Nevertheless, in one particular trial (General Belgrano, October 2000) in which infection levels of non-transformed control plants reached 85%, it was possible to identify lines SY230 and SY233 as highly resistant to PVY. In subsequent testing at different Argentine agro-ecosystems, these lines consistently displayed immunity to PVY during several planting cycles. Taken together, all these results suggest that the antiviral trait has been stably integrated in the genome of both lines and confers a range of resistance encompassing most common PVY strains. In addition, except for the trait conferring PVY resistance, no phenotypic changes could be detected in these lines as compared with the non-transformed Spunta controls.

Though the initial goal was to introduce viral resistance by a protein-based mechanism (i.e., classical CP-MR), it soon became apparent that the mechanism acting on lines SY230 and SY233 could involve the presence of PTGS. The first evidence pointing to this direction was the low accumulation of transgenic RNA transcripts detected in both lines. The 35S CaMV promoter -used to drive CPPVY expression- ranks among the most efficient constitutive promoters and normally yields high transcription levels that can be easily detected in short autoradiographic exposures. Moreover, plants transformed with multiple insertions -as it is the case of SY230 and SY233- normally exhibit increased transcript and protein accumulation. In contrast with this assumption, *CPPVY* transcripts could only be detected after very long exposure times in both transgenic lines. The amount of *CPPVY* transcripts in the transgenic lines could not be directly measured but it appeared extremely low as compared with PVY RNA accumulation in infected leaves. Consistent with this, analysis of samples from lines SY230 and SY233 in western immunoblots failed to demonstrate accumulation of CPPVY protein over a detection limit of about 100 pg. Although this result does not exclude the presence of lower amounts of recombinant protein, it strongly suggests that the high level of resistance observed in these lines is not protein-mediated. Together with the very low levels of transcript accumulation, all these results agree with a predominant role for PTGS in both SY230 and SY233.

Southern blot analysis showed that lines SY230 and SY233 possess three and two independent transgene integrations, respectively. It was not possible to establish whether virus resistance observed in these lines is associated to a particular integration of the transgene or to more of one of them. Implementation of straightforward outcrossing assays is very difficult to accomplish in this case, due to the tetraploid nature of potato and the low fertility level of cultivar Spunta (González et al. 2002). The analysis of the integration of the transgene sequences in the potato genome should give a better understanding on the mechanisms involved in virus protection. The availability of a draft genome for potato

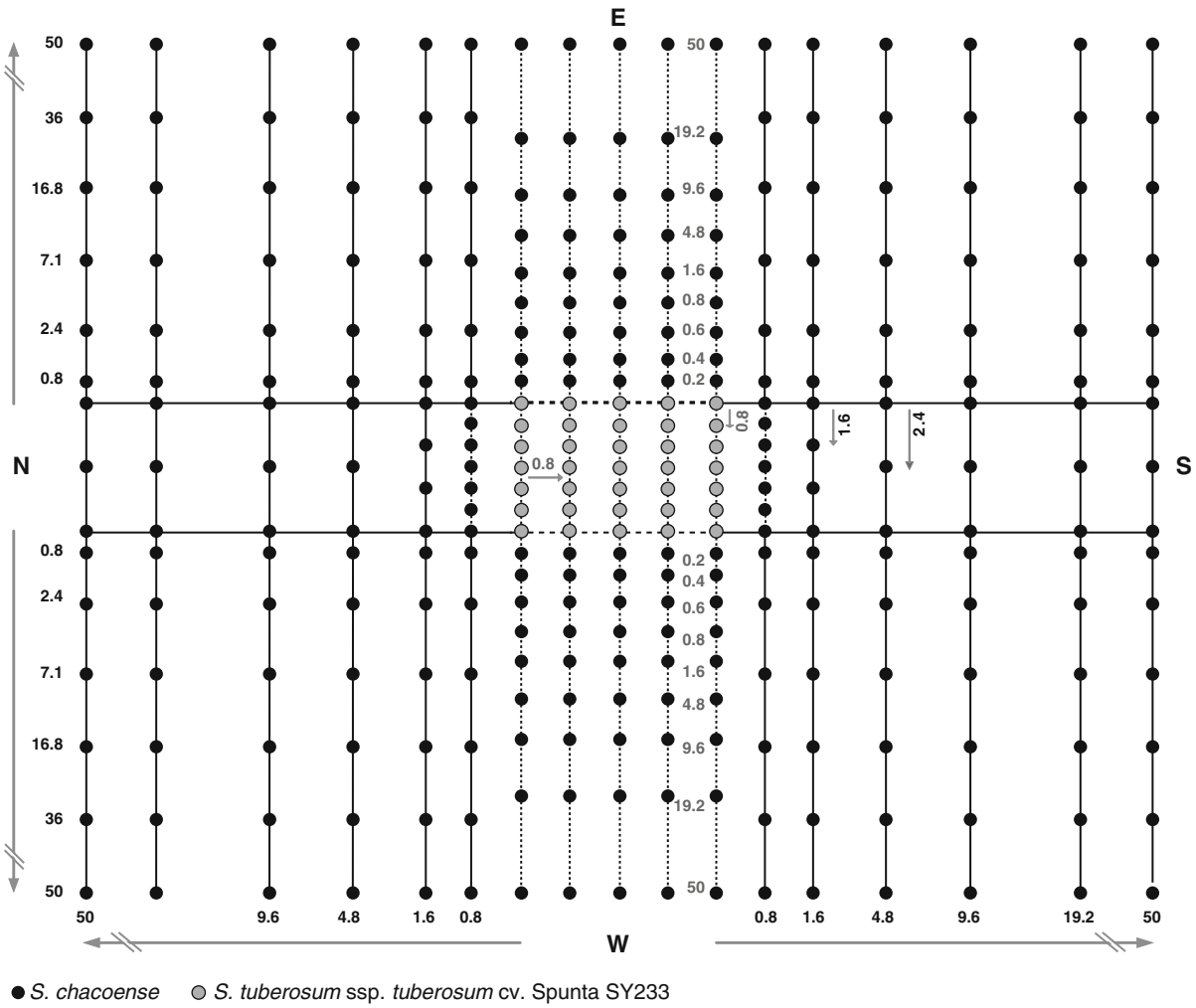

Fig. 5 Field trial design to assess pollen-mediated gene transfer from *S. tuberosum* (cv Spunta) to *S. chacoense*. Each *dot* corresponds to an individual plant. *Black dots*: *S chacoense* plants. *Grey dots*: *S. tuberosum* plants. Distance between plants *rows* are indicated in meters

(The Potato Genome Sequencing Consortium 2011) will be very useful to facilitate such analysis by using next generation sequencing techniques.

Multiple transgene insertions are usually not desirable in commercial crops because presence of repeated sequences could promote intra- or inter-chromosomal rearrangements potentially affecting their agronomic performance. Nevertheless, more than 6 years of continuous regeneration and testing confirmed that both the SY230 and SY233 lines showed a remarkable stability with regards to their transgene copy number and agronomical phenotype.

Several biochemical and physico-chemical parameters were measured to evaluate possible changes between the transgenic lines and the original cultivar. Values obtained for protein and aminoacid contents in different testing sites did not significantly differ between the SY230 and SY233 lines and cultivar Spunta or from the reference levels established for potato tuber composition. Similarly, glycoalkaloid content, an important parameter regarding the nutritional value of potato, was comparable to that of non-transformed controls. Other parameters of industrial interest, such as specific gravity, starch content, dry matter and frying color were also indistinguishable between transgenic and control plants. As a whole, these results indicate that the biochemical and physical characteristics of SY230 and SY233 tubers are

essentially equivalent to those of non-transformed Spunta.

The possible occurrence of gene flow from transgenic varieties to wild species is the most important issue concerning biosafety and biodiversity preservation. Natural crossings between cultivated potato and wild relatives is commonly restricted by reproductive barriers but have been reported in some cases (Hawkes and Hjerting 1969). Crossing studies between cultivated potatoes and wild *Solanum* accessions showed that only one of the species present in Argentina (*S. chacoense*) could possibly cross with *S. tuberosum*. Nevertheless, germinative seeds from this crossing could be only obtained using *S. chacoense* as male donor, and the resulting plants were infertile (Jackson and Hanneman 1999). Crossings using Spunta as male donor were extremely difficult because of the very low rate of pollen production of this cultivar. In line with this precedent, our cross-pollination trial between *S. tuberosum* and *S. chacoense*, failed to detect any event of transgene transfer under natural field conditions. The result of this study, which included the analysis of 1,700 fruits and 41,200 seeds from *S. chacoense*, indicated that the probability for such a crossing—should it occur in nature—is extremely low.

Based on these results, it was concluded that, except for the presence of the specific trait conferring PVY resistance, the agronomical behavior and the nutritional features of these two lines are substantially equivalent to those of the original non-transformed Spunta and represent an extremely low risk for gene flow.

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