PVX-tolerant potato development using a nucleic acid-hydrolyzing recombinant antibody

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Summary. – 3D8 scFv, a catalytic recombinant antibody developed in the MRL mouse, exhibits nucleic acid-hydrolyzing activity. Previous studies have demonstrated that tobacco plants harboring 3D8 scFv antibodies showed broad-spectrum resistance to infection by both DNA and RNA viruses. In this study, potatoes were transformed with the 3D8 scFv gene and screened by potato virus X (PVX) challenge. Starting with the T0 and T1 potato lines, PVX-tolerant T1 potatoes were identified in the field and characterized by ELISA and RT-PCR analysis. T2 potatoes were propagated for T3 generation and additional virus challenges in the field, and 44% of the 3D8 scFv T3 transgenic potatoes grown in GMO fields were found to be tolerant to PVX infection. Tubers from PVX-tolerant T3 lines were 60% bigger and 24% heavier, compared with tubers from PVX-susceptible transgenic lines and wild-type potatoes. Three-step virus challenge experiments and molecular characterization techniques were used for plants grown in growth chambers or fields to identify 3D8 scFv-transgenic, PVX-tolerant potatoes. These studies also revealed that the viral tolerance enabled by 3D8 scFv persisted during asexual propagation.

Keywords: potato virus X; 3D8 scFv; agrobacterium-mediated transformation; transgenic potato; PVX tolerance

Introduction

Potatoes (*Solanum tuberosum* L.) are the world's fourth-largest agronomical crop after maize, rice, and wheat. Approximately 330 million tons of potatoes are grown per annum, in more than 130 countries (Rakosy-Tican *et al.*, 2007). Potato plants are easily infected by viruses, such as potato leaf roll virus (PLRV), potato virus X (PVX), and potato virus

Y (PVY) (Hull, 1984; Verchot-Lubicz *et al.*, 2007), causing severe yield losses every year. Furthermore, the vegetative propagation of potatoes makes viral infections even more destructive: viruses persist in infected tubers and are then transmitted to propagated tuber clones, thus causing more severe secondary infections over several generations. Due to the lack of available virucides, approaches to control viral damage in potato plants have thus been limited to two main strategies: 1) the production of pathogen-free stocks in tissue cultures, and 2) the development of virus-resistant potato plants through genetic manipulation (Solomon-Blackburn and Barker, 2001a,b).

PVX is a plant pathogenic virus of the family *Alphaflexiviridae* and the order *Tymovirales*. It is the prototype species of the genus *Potexvirus*. PVX is found mainly in potatoes and is only transmitted mechanically. PVX causes mild or

no symptoms in most potato varieties, but when plants are co-infected with PVX and PVY, synergy between these two viruses causes severe symptoms in potatoes. To protect potatoes against PVX infection, several genetic manipulation approaches with different genes have been implemented (Hemenway *et al.*, 1988,1989).

Genetic manipulation strategies for the development of virus-resistant crops rely mainly on resistance genes derived from either the host plants or pathogens (Beachy, 1997; Goldbach *et al.*, 2003; Runo, 2011)**.** Pathogen-derived resistance consists of the transformation of plants with resistance genes derived from viral genomes, and involves either protein-mediated or RNA-mediated mechanisms (Prins *et al.*, 2008).

The coat protein (CP) genes of PVX and PVY were introduced into a major commercial cultivar of potato, Russet Burbank, and transgenic plants that expressed both CP genes were found to be resistant to infection by PVX and PVY by mechanical inoculation. This engineered resistance experiment indicated that CP protection was effective against mixed infection by two different viruses (Lawson *et al.*, 1990). In another study, transgenic tobacco plants harboring either the sense coat protein transcript (CP+sense) of PVX or the antisense coat protein transcript (CP-antisense) were protected against PVX infection. The extent of protection observed in CP+ plants depended primarily upon the level of expression of the coat protein. Plants expressing antisense RNA were protected only at low inoculum concentrations (Hemenway *et al.*, 1988). When mutations were introduced into the sequence encoding the GDD motif of the putative replicase component of PVX, all mutations tested rendered the viral genome completely noninfectious when inoculated into tobacco plants. These results suggest that viral resistance to a plant virus may be engineered by expression of dominant negative mutant forms of viral genes in transformed cells (Longstaff *et al.*, 1993).

In addition to virus-mediated resistance, a host gene-mediated resistance for PVX was reported. Resistance to PVX is characterized by the gene product of a host resistance gene and a viral determinant. PVX strains showed typical `gene-for-gene` type resistance interactions in respect of the host Nx, Nb and Rx genes (Baulcombe *et al.*, 1995). The viral coat protein of PVX is known for the determinants of resistance mediated by the host Nx gene while the movement protein is the determinant of Nb-mediated resistance. Virulence or avirulence by the interactions between the determinants of PVX and host factors was dependent on the different groups of PVX strains (Malcuit *et al.*, 2000). Specially Rx mediated resistance against PVX infection was dependent on RanGAP2, which interacts with the Rx CC domain by disrupting either the intramolecular interaction or the interaction with RanGAP2 (Sacco *et al.*, 2007; Tameling and Baulcombe, 2007). Based on the structural and functional analysis, a new model of CC-NB-LRR function was reported as the LRR and CC domains could co-regulate the signaling activity of the NB domain in a recognition-specific manner (Rairdan *et al.*, 2008).

Genetically engineered resistance established using resistance genes can breakdown upon recombination between virus strains or mutations in resistant determinants of the viral genome. Two clones of PVX strains, PVX (UK3) and PVX (HB), have been constructed in plasmid vectors as infectious clones and the PVX (HB) clone was used to systemically infect tobacco and susceptible potato cultivars. However, the infectious clone of PVX (UK3) failed to infect the *Rx* plants or show a hypersensitive response of the *Nx* gene. Hybrid viral genomes between PVX (UK3) and PVX (HB) were constructed to elucidate the resistance breaking determinants of PVX (HB). Transcripts of hybrids were infectious on potato cultivars with either the *Rx* or *Nx* resistance genes. These results indicated that hybrid viruses generated with closely related strains or mutations in viral genomes may overcome the host resistance gene (Kavanagh *et al.*, 1992). Similar experiments have also been performed with another PVX strain (DX), and showed different symptoms based on different host resistant genes, such as systemic top necrosis in presence of the potato hypersensitivity gene *Nx* and mosaics with gene *Nb* (Jones, 1982).

Each approach has its own advantages and disadvantages; however, the virus-resistant potatoes that have been engineered thus far have in general shown only narrow-spectrum resistance to their target viruses (Prins *et al.*, 1996; van den Boogaart *et al.*, 2001). To overcome these limitations, a novel approach was developed as an alternative to pathogen-derived resistance strategies for plant systems. This approach includes the expression of antibodies against viral proteins in the plant body (Boonrod *et al.*, 2004; Tavladoraki *et al.*, 1993). Although the antibody-mediated immune response is known to be unique to animals, the high affinity of some antibodies to their target antigens is sufficient to inhibit viral infection in plants. The essential goal of this technology is to generate a recombinant antibody in a host plant ("plantibody"); specifically, a single-chain variable fragment (scFv) consisting of the variable regions of the heavy (V_H) and light (V_L) chains of an immunoglobulin molecule, connected by a short linker peptide (Bird *et al.*, 1988). The first successful demonstration of plantibody-mediated resistance was performed in tobacco plants (*Nicotiana benthamiana*) transformed with a gene encoding a functional scFv raised against the coat protein of artichoke mottled crinkle virus (Tavladoraki *et al.*, 1993). Subsequently, varieties of transgenic plants with antibody-engineered resistances to citrus tristeza virus (Cervera *et al.*, 2010), tomato yellow leaf curl virus (TYLCV) (Safarnejad *et al.*, 2009), and PLRV (Nickel *et al.*, 2008) were also developed.

The "abzyme" originates from the terms "antibody" and "enzyme" (Tramontano *et al.*, 1986). The catalytic antibodies called abzymes combined the characteristics of antibodies and enzymes with therapeutic effects. Over the 30 years, catalytic antibodies which hydrolyze DNA and RNA have been observed in the sera of patients with various autoimmune diseases such as systemic lupus erythematosus (SLE), multiple sclerosis, primary Sjogren's syndrome, Hashimoto thyroiditis, myasthenia gravis and rheumatoid arthritis (Dubrovskaya *et al.*, 2003; Krasnorutskii *et al.*, 2008). 3D8 scFv immunoglobulin molecules were first isolated from spleen cells of the autoimmune MRL-*lpr/lpr* mouse. These immunoglobulins enable the development of viral resistance via nucleic acid binding/hydrolyzing, without any sequence specificity for single-stranded or double-stranded DNA (Kwon *et al.*, 2002). Moreover, 3D8 scFv molecules can indiscriminately inhibit infection by both animal and plant viruses, since their target is only recognized as a nucleic acid. HeLa cells expressing 3D8 scFv molecules exhibit strong antiviral activity against classical swine fever virus infection (Jun *et al.*, 2010). Recently, these nucleic acid-hydrolyzing recombinant antibodies were also demonstrated to elicit virus resistance in tobacco plants (Lee *et al.*, 2013a,b). Interestingly, we also obtained evidence that resistance to infection by a different virus species, geminivirus, can be induced in 3D8 scFv-transgenic tobacco plants (*Nicotiana benthamiana*).

In the current study, the 3D8 scFv gene was introduced into the potato genome for the first time, and the antiviral effects of 3D8 scFv on a potato virus were investigated in clonal progeny propagated through the tubers of potato transformants, with PVX challenges over three consecutive transgenic generations (T0 to T3). Our results show that PVX tolerance can be stably maintained through multiple 3D8 scFv-transgenic potato generations.

Materials and Methods

Plant material and growth conditions. Solanum tuberosum cv. Superior potatoes were used in this study. For transformations, plants were grown *in vitro* and maintained under a 16/8 h light/dark regimen at 50% humidity on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), supplemented with 2% (w/v) sucrose and agar. Transgenic lines selected for biochemical analysis and virus challenge experiments were micropropagated from seed tubers in a growth chamber under a long-day conditions (16 h) at 25 ± 2°C. The mass propagation and maintenance of transgenic potatoes was performed at the Highland Agriculture Research Center, National Institute of Crop Science, Korea.

Plasmid construction. The 3D8 scFv gene was derived from a hybridoma cell line, originating from an autoimmune-prone MRL-*lpr/lpr* mouse, which produces MAb 3D8 IgG (Kwon *et al.*, 2002). 3D8 scFv consists of the VH (NCBI accession number AAF79128) and VL (NCBI accession number AAF79129) genes with a $(Gly_4Ser)_3$ flexible linker inserted between the V_H and V_L sequences (VH-$(Gly_4Ser)_3$-VL). This gene was subcloned into pGEM®-T Easy (Promega, USA), as described in previous studies (Kim *et al.*, 2006). From this construct, a DNA fragment encoding the 3D8 scFv molecule was amplified by PCR using primers 5'-TCTAGAATGGAGGTCCAGCTGCAGCA-3' and 5'-GAGCTCTTATATTTCCAGCTTGGTCCCCG-3'. The resultant PCR products were subcloned into pGEM®-T Easy, thereby generating pGEM-3D8 scFv. This construct was digested with *Sac*I and *Spe*I, and the resultant fragment was subcloned into the binary vector pBI121 (BD Biosciences Clontech, USA, NCBI accession number AF485783), thereby generating pBI121-3D8scFv. This vector was then transformed into *Agrobacterium tumefaciens* LBA4404 (Fig. 1a).

Potato transformation. Transgenic plants were constructed using an Agrobacterium-mediated transformation method modified from the previously reported protocol (Beaujean *et al.*, 1998). Potato leaf discs were prepared by cutting leaves from 4 weeks old potato plants in length of approximately 4–6 cm. The *A. tumefaciens* strain LBA4404 harboring the plasmid pBI121-3D8scFv was incubated for 16 h with shaking at 28°C in 50 ml YEP broth medium containing antibiotics (100 µg/l rifampicin and 50 µg/l kanamycin). The culture medium was centrifuged and resuspended in two volumes of MS liquid medium (MS salts and vitamins MS, 3% sucrose, pH 5.7). MS media containing *Agrobacterium* cells was poured onto Petri dishes containing potato leaf discs. After two to three days of primary culture on MS medium *in vitro*, sampled leaf explants were co-cultured with *A. tumefaciens* for the next three days. Cultures were maintained on selection medium containing 250 mg/l cefotaxime and 100 mg/l kanamycin. One month after culture initiation, the shoots had regenerated from calli and were transferred to hormone-free medium for rooting (Fig. 1b left). Rooted potato plantlets were subjected to continuous selection and acclimatization for two months to obtain healthy transformants (Fig. 1b right).

Transcript analysis of transgenic plants by RT-PCR. Total RNA was isolated from transgenic plants, as previously described by Schwach *et al.* (2005). cDNA was synthesized from total RNA using a oligo(dT)$_{18}$ primer and M-MLV reverse transcriptase (Rexgene Biotech, Korea) and used as a template for PCR. Potato actin was used as an internal control. Amplifications were carried out in triplicates. Thermocycling conditions were as follows: 94°C for 30 s, 35 cycles of 60°C for 30 s and 72°C for 1 min, followed by a final extension at 72°C for 5 min. The PVX diagnostic primer sets (forward and reverse) were: actin, 5'-GCTTCCCGATGGTCAAGTCA-3' and 5'-GGATTCCAGCTGCTTCCATTC-3'; PVX coat protein, 5'-CAGTCCACCTGCTAACTGG-3' and 5'-TRACAGCTGCATCTAGGCT-3'; and 3D8 scFv, 5'-CTGCAAGGCTTCTGGATACAC-3' and 5'-GTTTAGGAGACTGCCCTGGTT-3'. PCR products were visualized on a 1% agarose gel stained with 50 µg/ml ethidium bromide.

PVX challenge. PVX inoculation was performed on 4 weeks old transgenic potato plants. Homogenates of PVX-infected po-
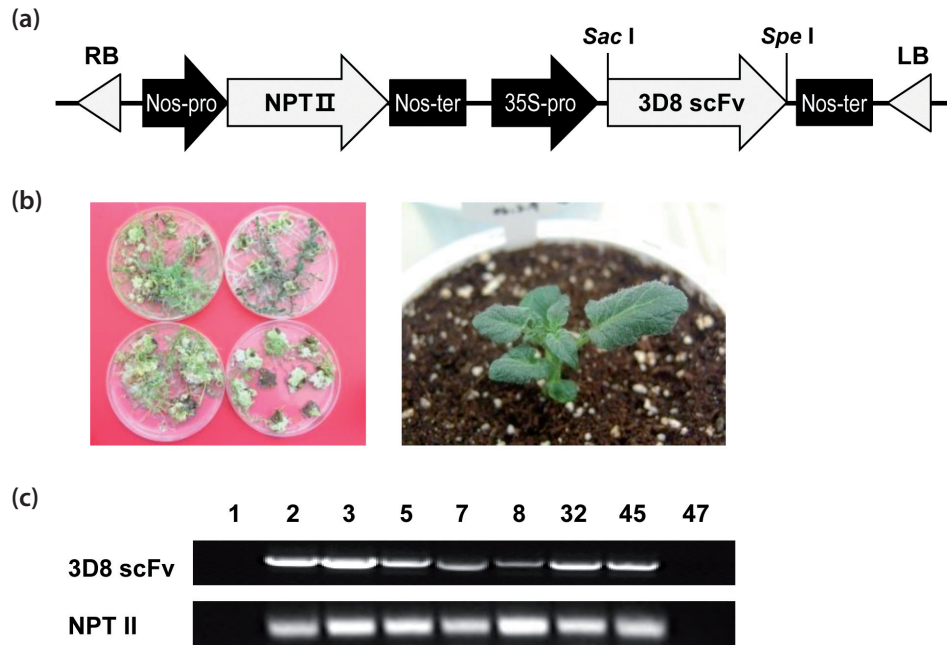
(a)

(b)

(c)

Fig. 1

Development of 3D8 scFv-transgenic potato plants

(a) Binary construct containing the T-DNA region and 3D8 scFv. The 3D8 scFv gene was subcloned into the binary vector, pBI121, with *Sac*I/*Spe*I and subsequently introduced into *Agrobacterium* LBA4404. The region between the triangles (RB/LB: right/left border) represents the T-DNA fragment in the binary construct. Nos-pro, plant-recognizable promoter sequence derived from the nopaline synthase promoter of *A. tumefaciens*; NPT II, neomycin phosphotransferase II gene, whose product confers resistance to the antibiotic neomycin; Nos-ter, plant-recognizable terminator sequence derived from the nopaline synthase gene; 35S-pro, cauliflower mosaic virus-derived plant-recognizable promoter sequence; 3D8 scFv, 3D8 scFv DNA fragment. **(b)** Regeneration of 3D8 scFv-transformed potato leaf tissue *in vitro*. After cocultivation with *A. tumefaciens*, leaf explants were cultured on selection medium containing 250 mg/l cefotaxime and 100 mg/l kanamycin (left panel). Regenerated shoots were transferred to hormone-free MS medium for rooting. Finally, healthy potato plantlets were selected and grown in a pot (right panel). **(c)** Establishment of the T0 generation for 3D8 scFv potato transformants. A total of nine putative transgenic lines were obtained after acclimatization and analyzed to confirm the presence of the 3D8 scFv gene by RT-PCR. The NPT II gene was used as an internal control. Seven lines were selected as genuine transformants and designated as the T0 generation (lanes 2, 3, 5, 7, 8, 32, and 45). Lane 1 and 47 are negative controls – non-transformants.

tato leaves were used as the inoculum. Young leaves of the potato plants were lightly dusted with carborundum, and virus inoculum (50 μl) was applied to the scratched leaves with a cotton swab. The inoculated plants were incubated at 22°C–25°C and were typically observed for 30 to 45 days (Sonoda *et al.*, 2000). Symptom severity was scored using the external visible symptoms of PVX, based on developed leaves or shoot stunting. ELISAs were performed using shoots that had emerged from the inoculated potatoes. Three quantitative traits (length of major/minor axes, weight, and number) were evaluated for the tubers of PVX-inoculated transgenic and wild-type plants. All measurements were analyzed by Student's *t*-test using GraphPad Prism 5 (USA). A *P*-value of ≤ 0.05 was considered statistically significant.

ELISA. Potato total protein extracts were prepared as previously described by Hemenway *et al.* (1988). A double-antibody sandwich ELISA kit (Agdia, USA) was used to detect the PVX coat protein (CP) in the extracts. A 1:200 dilution was generally used, both for coating plates with polyclonal PVX CP rabbit antiserum and for detecting PVX coat protein with alkaline phosphatase-conjugated antibody. One-way ANOVA was used to test for significant differences between the quantified amounts of PVX protein.

Results

Genetic transformation

A total of 205 calli were induced from the 542 leaf explants used for genetic transformation, and 45 plantlets with roots were regenerated from the 84 callus-derived shoots in the subsequent shooting and rooting steps. The average transformation efficiency was approximately 8.3%, and a total of nine putative transgenic lines were produced after acclimatization. Of these, 7 transgenic lines were confirmed by RT-PCR analyzis to show the 3D8 scFv and NPT II genes and were designated as T0 generation (Fig. 1c).
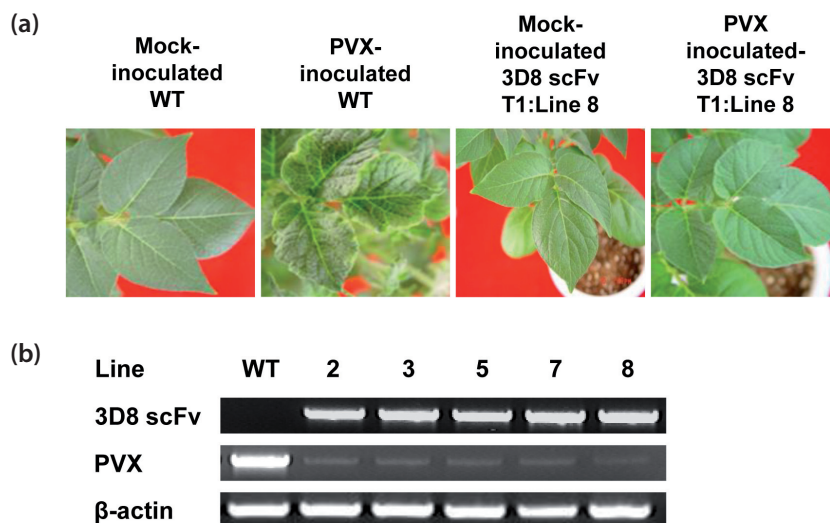
Fig. 2

Antiviral effects against PVX in the first 3D8 scFv-transgenic generation (T1)

(a) Progression of disease symptoms in a wild-type and a 3D8 scFv-T1 potato plant after PVX inoculation. T1 plants were grown from T0 generation tubers. To test the susceptibility to PVX, five 3D8 scFv-T1 lines and a wild-type line were inoculated with PVX. A T1 plant (line 8) was selected as a representative plant from the T1 generations tested. Mock-inoculated wild-type and transgenic plants were included as internal controls for PVX inoculation. WT, wild-type; 3D8 scFv T1:line 8, T1 clonal progeny of 3D8 scFv-transgenic potato line 8. (b) Transcript analysis of PVX-inoculated 3D8 scFv-T1 potato plants. PVX transcript detection by RT-PCR was used as a molecular indicator of virus susceptibility of the T1 plants tested in Fig. 2a at 4 weeks post PVX inoculation. Actin was used as an internal control.

Viral resistance testing in the 3D8 scFv-T1 generation

Tubers were harvested from each of the seven confirmed transgenic potato plants and grown in a growth chamber (28°C, relative humidity 68%) for 5–6 weeks, thereby producing a T1 generation. Leaf tissues were mechanically inoculated with PVX to evaluate the accumulation of virus (infectivity) and the development of disease (symptom severity) 4 weeks post inoculation.

Strong symptoms of infection were observed in the PVX-inoculated wild-type plants. These symptoms consisted of leaf deformities with dwarfism and curling, which are known to be typical of PVX infection (Fig. 2a). Five transgenic T1 lines were challenged with PVX. Four of the T1 lines tested (3, 5, 7, and 8) showed no symptoms; transgenic line 8 is shown as a representative sample (Fig. 2a). For internal controls, a mock-inoculated wild-type line and a transgenic line (3D8 scFv T1:line 8) (Fig. 2a) were evaluated; these lines did not exhibit any symptoms of PVX infection. Interestingly, however, the T1:line 2 plant developed strong symptoms similar to those of the inoculated wild-type plant (data not shown). The viral infectivities and the development of disease symptoms in the first generation (T1) of 3D8 scFv-transgenic potato plants after PVX inoculation are shown in Table 1.

PVX infectivity was determined by detection of a PVX transcript by RT-PCR in selected T1 lines. This analysis revealed, based on the presence of the PVX transcript, that all five T1 lines (2, 3, 5, 7, and 8) were infected by PVX (Fig. 2b). Collectively, these results strongly suggest that PVX tolerance was acquired in the first generation of four of the 3D8 scFv-transgenic potato lines (T2 lines 3, 5, 7, and 8).

Viral resistance testing in the 3D8 scFv-T2 generation

Tubers of the five T1 lines were planted in fields to obtain a second generation (T2) of 3D8 scFv-transgenic potato plants. The overall patterns of plant growth were similar in all T2 clonal progeny tested, although a slight difference in the vegetative growth phase was observed between T2 lines 3, 5, 7, and 8 compared with line 2.

Three quantitative traits (size, weight, and number of tubers) of the T2 clonal progeny were also evaluated. The average dimensions of the tubers from the wild-type plants were 6.8 cm and 4.2 cm on the major and minor axes, respectively, whereas the tubers from T2 potato plants had average dimensions of 5.95 cm and 4.49 cm, respectively. The average tuber weights were 80.74 g and 88.30 g for the wild-type and T2 potato plants, respectively. Only a slight difference in the average tuber production was observed between the wild-type and T2 potato plants (8 tubers for the wild-type and 7 for the T2 plants) (Table 2).

Table 1. Viral infectivity and manifestation of disease symptoms in the first generation (T1) of 3D8 scFv-transgenic potato plants after PVX inoculation

		T1 potatoes	
		Infectivity (%)	Symptom severity
WT		10/10 (100%)	+
3D8 scFv line	T1-2	1/1 (100%)	+
	T1-3	0/1 (0%)	–
	T1-5	0/1 (0%)	–
	T1-7	0/1 (0%)	–
	T1-8	0/2 (0%)	–

Percentage of infectivity was calculated as the number of plants exhibiting symptoms of disease out of the total number of inoculated plants 2 weeks post PVX inoculation. Symptom severity indicates the extent to which symptoms of PVX infection developed in the infected plants. (+) = symptoms were observed;(–) = no symptoms were observed.

Collectively, these results indicate that the growth of T1 and T2 3D8 scFv-transgenic potato plants was not affected by the transgene, at least in the transgenic lines selected for this test. The above-ground growth and tuber formation rates of the T2 line 8-derived clonal progeny (8–33) represents the T2 generation.

For the viral resistance test, seven potato plants from four T2 lines (3, 5, 7, and 8) were inoculated with PVX, and RT-PCR was used to detect viral RNA 4 weeks post inoculation. PVX transcripts were detected in all transgenic plants tested, indicating that all T2 clonal progeny had been infected with PVX (Fig. 3). However, the infected potato plants showed no disease

symptoms. Furthermore, no differences were observed between these infected plants and the non-inoculated wild-type plants in terms of above-ground growth and tuber formation (data not shown). The T2:line 2 clonal progeny (2–15, 18) showed the same response to PVX challenge as the T1 generation of this line; that is, severe symptoms of viral infection developed and the PVX transcript was readily detected after PVX inoculation.

Viral resistance testing in the 3D8 scFv-T3 generation

T3 clonal progeny were generated from field-grown tubers from four T2 lines (2, 3, 5, and 8) selected from among the five potato lines tested in the second transgenic generation, and another set of PVX inoculations was carried out on these clonal progeny to monitor PVX tolerance in the third transgenic generation.

Disease symptoms were not consistently observed in the PVX-infected T3 clonal progeny derived from transgenic lines 3, 5, and 8. In other words, virus tolerance was consistently present when these transgenic lines advanced to the T3 generation (data not shown). As a representative example, a PVX-infected line 8-derived T3 clonal plant (T3:8-33) had healthy leaves, similar to those of an uninfected wild-type plant (data not shown) and more expended leaves to those of an uninfected wild-type plant, and the tubers of the T3:8-33 plant were approximately 60% bigger and 24% heavier than those of the wild-type plant (Fig. 4a). On the other hand, two of the line 2-derived clones (2–15-3 and 2–18-3) were highly infected and manifested strong symptoms in the T3 generation (data not shown).

RT-PCR analysis showed that the PVX gene was detectable in all T3 clonal progeny of the tested lines (2, 3, 5, and

Table 2. Evaluation of three quantitative traits (size, weight, and number) in T2 clonal progeny tubers of 3D8 scFv-transgenic potato plants

		Total No. of harvested potato plants	Average No. of tubers/plant	Average length (cm) of tubers/plant		Average weight (g) of tubers/plant
				Major axis	Minor axis	
WT	Total	20	8.00±1.50	6.80±4.10	4.20±2.30	80.74±2.00
3D8 scFv	Total line	190	6.62±0.97	5.95±1.14	4.49±0.92	88.30±5.52
	2-15	23	5.9	3.8	3.0	79
	2-18	21	7.8	7.3	5.9	89
	3-20	16	6.7	4.9	3.7	81
	5-35	22	5.3	6.4	4	93
	5-45	13	7.3	4.9	3.9	85
	7-36	17	7.5	6.3	5.1	89
	7-43	20	5.1	7.2	5.5	92
	8-21	17	6.1	5.5	3.9	86
	8-33	22	7.5	6.3	4.6	93

Data were analyzed with Student's *t*-test, using GraphPad Prism 5. A *P*-value of ≤0.05 was considered statistically significant.
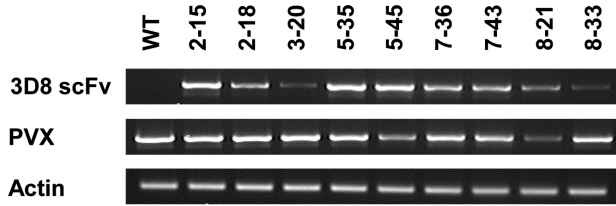
Fig. 3

Antiviral effects against PVX in the second 3D8 scFv-transgenic generation (T2)

Transcript analysis of PVX-inoculated 3D8 scFv-T2 potato plants. RT-PCR was used to detect the presence of the PVX transcript in nine subclonal potato plants from five transgenic lines in the T2 generation (2, 3, 5, 7, and 8) 4 weeks post-PVX inoculation. Actin was used as an internal control. Subclones are designated as follows: first number, transgenic T1 line; second number, T2 subclones from the transgenic T1 line.

8). Morphological observations and molecular data indicated that the PVX tolerance developed in the T2 generation was maintained in T3 clonal progeny of lines 3 and 5 of 3D8 scFv-transgenic potatoes (Fig. 4b).

It was particularly interesting that the line 8–21-derived T3 clonal progeny exhibited interclonal variation in their acquired PVX tolerance; the PVX gene was detected in two transgenic 8–21-derived T3 clonal progeny (8–21-1 and 8–21-5) even though 3D8 scFv was relatively highly expressed in both lines. For T3 lines 2–15-3 and 2–18-3, the results were consistent with those of the 8–21 transgenic lines, in that PVX transcripts were clearly detected in the plants regardless the different degree of 3D8 scFv transcription level.

In addition, ELISA was also conducted to confirm the presence of infection on the protein level in selected T3
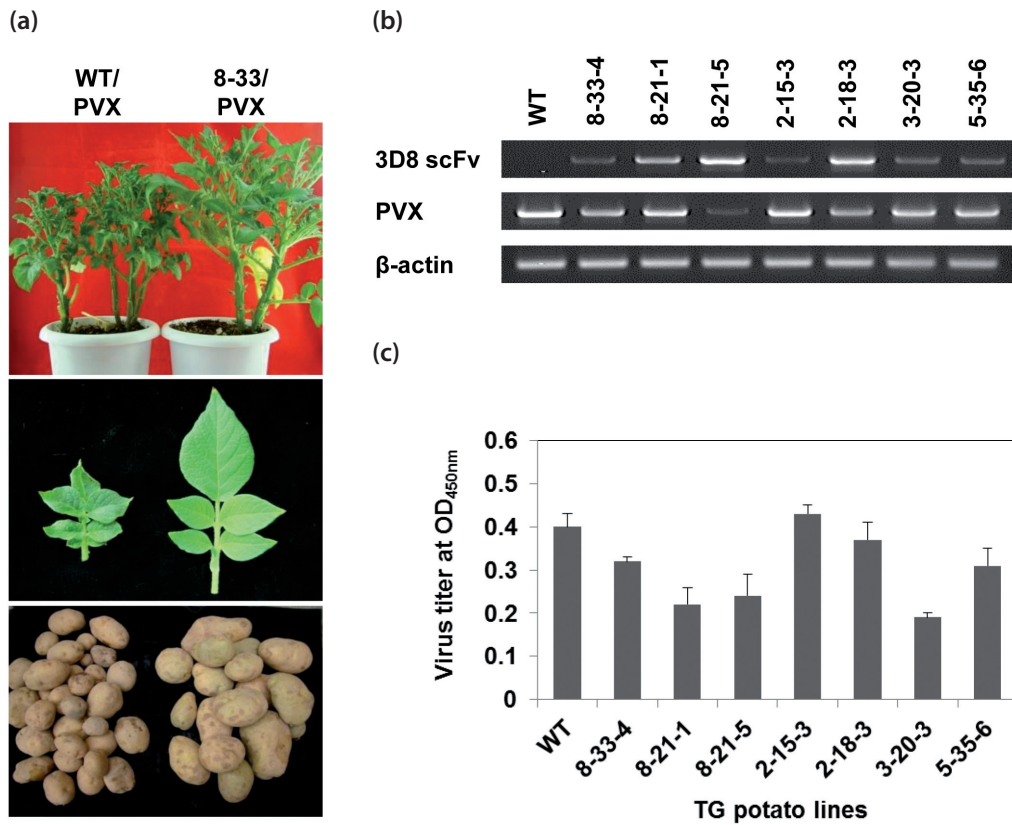

Fig. 4

Antiviral effects against PVX in the third 3D8 scFv-transgenic generation (T3)

(a) Growth of 3D8 scFv-T3 potato plants after PVX inoculation. T2 tuber-derived potato plants (T3 generation) were evaluated based on the severity of their disease symptoms and tuber formation to evaluate yield performance after PVX inoculation. A transgenic line 8-derived T2 subclone (T2:8-33) and a wild-type (WT) plant are shown as representative samples. **(b)** Transcript analysis of PVX-inoculated 3D8 scFv-T2 potato plants. The presence of PVX RNA was analyzed by RT-PCR in eight subclonal potato plants from the T3 clonal progeny of four transgenic lines (2, 3, 5, and 8) at 4 weeks post-PVX inoculation. Actin was used as an internal control. Subclones are designated as follows: first number, transgenic T1 line; middle number, T2 subclone from the transgenic T1 line; last number, T3 clonal progeny from subclones tested in the T2 generation. **(c)** Detection of PVX accumulation by ELISA. The extent of PVX infection after PVX inoculation was assessed by ELISA for the selected T3 clonal progeny. The ELISA detected the PVX coat protein (CP) in total transgenic potato extracts. X-axis, 3D8 scFv-transgenic potato lines tested; Y-axis, virus concentration (virus titer) at OD 450 nm. A *P*-value <0.05, indicating a statistically significant difference, is marked with an asterisk.

clonal progeny after PVX inoculation. In general, viral protein was detected in all clonal progeny tested. In particular, the amount of viral protein detected, was nearly equal to or higher than that in the infected wild-type plant in the line 2-derived clones (2–15-3 and 2–18-3) (Fig. 4c).

Discussion

In agriculture, plant breeding has proven to be indispensable for the development of new cultivars with valuable genetic traits. However, classical breeding is based upon laborious and time-consuming genetic crosses. This process is even more intricate if the target crop species has complex genetics and reproductive organs. Furthermore, each step involves the mass migration and recombination of chromosomes during meiosis, resulting in the acquisition of novel desired traits in addition to unwanted ones, which entails an inevitable loss of desirable features. These major drawbacks of the classical breeding process can potentially be overcome through the use of genetic transformation techniques. However, successful transformation-mediated plant breeding requires stable transgene transmission over generations of the transformed plant species.

In this study, we aimed to devise a strategy for protecting crops against severe PVX infection. To do so, we introduced the 3D8 scFv gene into the potato genome to develop a PVX-resistant potato cultivar and monitored the stability of the transgene in tuber-propagated transgenic potato generations from T0 to T3. Transgene genetics were simplified in this study by the clonal propagation of transformed potato plants via tuber formation. Direct transmission of the 3D8 scFv gene, with no genetic recombination or other genetic complexities, was assumed in all of the transgenic clonal progeny.

As demonstrated in our previous work, 3D8 scFv exhibits nucleic acid-hydrolyzing activity (Kim *et al.*, 2006), and can digest any nucleic acid from either plant or animal viruses in a sequence-nonspecific manner (Jun *et al.*, 2010). These characteristics may have practical applications in economically important crop plants. Among the many strategies for genetically-induced resistance against virus infections, 3D8 scFv is potentially more advantageous to crops in terms of viral resistance compared with other viral resistance approaches.

In our previous researches, 3D8 scFv was shown to exhibit antiviral effects against various plant (beet curly top virus, beet severe curly top virus, pepper mild mottle virus, tobacco mild green mosaic virus, tomato mosaic virus, tobacco mosaic virus and cucumber mosaic virus) and animal (pseudorabies virus, herpes simplex virus, classical swine fever virus, murine norovirus, influenza virus H1N1, H9N2) viruses (Cho *et al.*, 2015; Hoang *et al.*, 2015; Jun *et al.*, 2010;

Kim *et al.*, 2012; Lee *et al.*, 2013a,b; 2014) at low 3D8 scFv protein concentrations. In the transgenic cells or mouse system, 3D8 scFv could only be detected by ELISA, but not by Western blot analysis of protein lysates (Jun *et al.*, 2010; Lee *et al.*, 2014). In previous study, the stability of cytosolicly expressed 3D8 scFv on plant development was confirmed by intrinsic nuclease activity of 3D8 scFv proteins (Lee *et al.*, 2013a). When the stable transformants were established, they did not show significant difference observed in wt plants vs. transgenic plants. 3D8 scFv expression in fully developed transgenic plants could be hardly detected by western blot analysis even though they showed the complete resistance against virus infection. This was also observed on transgenic animal cell lines and mouse system (Lee *et al.*, 2014). Taken together, we hypothesized that expression of low levels of 3D8 scFv could be good enough to protect against virus infection with no harmful effects on potato development.

The 3D8 scFv transgene was shown to be constitutively expressed in the selected T0 potato plants. However, among these transformants, transgenic line 2 was of particular interest due to its transgene effect. This plant, which was validated as transgenic, showed symptoms of infection and accumulated a level of virus similar to that in the PVX-infected wild-type plant after PVX inoculation, even though this transgenic plant harbored the 3D8 scFv gene. Moreover, this observed transgene abnormality in line 2 persisted in its clonal progeny throughout all generations examined (T1 to T3). The mechanism of this effect remains unclear. However, we consider the most likely explanation to be that the 3D8 scFv gene product had unidentified problems at the level of translation. It is quite possible that the final protein product of the 3D8 scFv gene underwent abnormal post-translational modification, or adopted a misfolded conformation. In such cases, an active nuclease would not have been formed by the defective 3D8 scFv protein, even though the gene was successfully expressed in the transgenic plant.

Another interesting observation was that viral tolerance was established as the generations advanced. Pathological and molecular data indicated that PVX tolerance was acquired in the T1 generation and persisted over in next generations of the transgenic clonal progeny. It would be informative to determine which factor(s) induced viral tolerance in later transgenic generations. The factor(s) involved may be either intrinsic (genetic) or extrinsic (environment-related) and may function to ensure optimal stability for plant survival. The most plausible explanation for the viral tolerance observed in this study may be viral-directed silencing suppression, as has been shown for TYLCV. The silencing of two partial TYLCV (C2 and C4) genes has been shown to interfere with the ability of the virus to counteract suppression of the virus by the host plant genes, thus engendering resistance or tolerance in tomato plants (Peretz *et al.*, 2011). It is likely that PVX genes act through mechanisms similar

to those of the TYLCV genes to generate viral tolerance to the 3D8 scFv gene, even though the precise factor(s)/mechanism(s) involved in this process have not yet been identified in potato plants. If this is the case, the detection of the PVX gene in PVX-inoculated 3D8 scFv-transgenic potato clonal progeny strongly indicates that PVX gene suppression may occur at the post-transcriptional gene silencing level.

Couple of transgenic lines showed the correlation between tolerance against PVX infection and 3D8 scFv expression levels but in other lines, there was no correlation between them. In our previous works, (Lee *et al.*, 2013a,b), we also observed similar observation. This might be caused by the catalytic activity of 3D8 scFv. The intrinsic catalytic activity of 3D8 scFv in transgenic lines may affect the difference between the transcriptional level of 3D8 scFv mRNA and the translational level of 3D8 scFv. In this case, we now understand that RT-PCR data are not good enough to explain this correlation between resistance/tolerance against virus infection and 3D8 scFv expression levels. To prove this correlation, we need to have highly sensitive and specific anti-3D8 scFv antibody for quantifying 3D8 scFv proteins in each line.

Two of the T3 clones from transgenic line 8–21 (8–21-1, 5) showed variations in viral tolerance. The precise cause of this variation is unknown; however, clonal uniformity may be difficult to maintain in a fluctuating environmental milieu. One possibility is that unidentified environmental stressors may have caused genomic stress, thereby affecting the genomes of the transgenic potatoes in the field. This fine-tuning process may randomly affect 3D8 scFv transgene regulation in field-grown T3 clonal progeny, thus resulting in interclonal variation in PVX tolerance.

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