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Generation of double-virus-resistant marker-free transgenic potato plants

Yunfeng Bai, Zhihua Guo, Xiaoqi Wang, Dongmei Bai, Weifeng Zhang *

Institute of Crop Genetics, Shanxi Academy of Agricultural Sciences, Nongke Beilu No. 16, Taiyuan 030031, China

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Abstract

Viruses are very widespread in nature, and they cause severe diseases and yield losses in potato production. The transfer of the dsRNA-producing gene could confer a high level virus resistance by specific targeting of cognate viral RNA. In this study, we constructed a marker-free expression vector of a chimeric gene derived from the coat protein sequence of *Potato virus X* (PVX) and the nuclear inclusion protein sequence of *Potato virus Y* (PVY) in the form of an intramolecular dsRNA. Then this chimeric gene was introduced into potato cv. Zihuabai, a popular variety in China, via *Agrobacterium tumefaciens*-mediated transformation. Marker gene-free transgenic plants resistant to both PVX and PVY were obtained and confirmed by RT-PCR and DAS-ELISA detection. Northern blot analysis showed that transgene-derived mRNA was cleaved into short interfering RNAs (siRNAs), and that the virus resistance was mediated by RNA silencing. One important aspect of the study is that the transgenic viral sequence is not translated and the actual RNA transcript is cleaved, which possibly limit the environmental risks, such as transcapsidation and recombination of the transgene with an incoming virus. In addition, the biosafety risk resulting from marker genes can be avoided because of the absence of marker genes in transgenic plants.
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Keywords: *Solanum tuberosum*; Marker-free; RNA silencing; Double-virus resistance; Transgene

1. Introduction

Tetraploid cultivated potato (*Solanum tuberosum*) is one of the most important food crops in the world in terms of its total production and area cultivated. Viruses are very widespread in nature, and they cause severe yield losses in potato production, not only because of the effects caused by primary infection, but also because the crop is mainly vegetatively propagated and this makes viral infections even more destructive. Viruses persist in the tubers and the tuber-borne secondary infections are more severe than primary infections. Losses attributable to viruses can take the form of reduced yield, downgrading of seed crops, and/or tuber blemishes. *In vitro* virus-free propagation by shoot-tip culture is a major control measure, but it has a high financial cost. The generation of resistant cultivars is considered the most economic and environmentally acceptable way of controlling viral diseases, but virus-resistant germplasm sources available are limited in the conventional breeding program of the potato.

In 1985, Sanford and Johnstone raised the concept of pathogen-derived resistance (PDR) [1]. In 1988, Hemenway transformed potato with the coat protein gene (*cp*) of the *Potato virus X* (PVX) [2], which was one of the first attempts to obtain pathogen-derived resistance to major potato virus. After this, numerous other examples of pathogen-derived resistance to potato viruses followed. Transgenes included the complete or partial sequences of *cp* [3], movement protein gene [4], and nuclear inclusion pro-

* Corresponding author. Tel: +86 351 7123625; fax: +86 351 7136763.
E-mail address: zhwfenok@126.com (W. Zhang).

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tein gene (*NIb*) [5]. Moreover, multiple genes derived from different viruses have been successfully introduced into potato simultaneously for multi-virus resistance [6–9]. However, in a number of cases, resistance conferred by various virus-derived transgenes was not always very strong, and protection appeared almost always in only a few of the transgenic lines. Furthermore, the transgenic viral sequence was transcribed and translated, which possibly caused environmental risks, such as recombination [10], transcapsidation [11] and synergism [12] between a viral RNA produced from a transgene and an infecting plant RNA virus. In addition, consumers and environmental protection groups have expressed concern about the use of selectable marker genes, such as antibiotic- and herbicide-resistance genes, from an ecological and food safety perspective.

RNA silencing is a post-transcriptional gene-silencing phenomenon induced by double-stranded RNA (dsRNA), which is not a normal constituent of eukaryotic cells and does not usually occur naturally. When dsRNA occurs in the eukaryotic cell, Dicer, an RNase III-like ribonuclease, specifically cleaves dsRNA into small interfering RNAs of 21–25 nucleotides. siRNAs act as a guide to recognize complementary RNAs for their degradation to suppress gene expression. RNA silencing was described as RNA interference [13]. RNA silencing is an intrinsic plant defense mechanism, and it plays important biological roles in protecting the organism's genome against foreign nucleic acids. dsRNA can be delivered by stably transforming plants with transgenes that express a self-complementary RNA. The resulting transcript hybridizes with itself to form a double-stranded structure that undergoes an efficient RNA silencing, which is a new and agriculturally sustainable strategy to obtain virus-resistant plants [14]. The ectopic expression of virus-specific dsRNA activates the RNA silencing mechanism and recognizes cognate RNAs of invasive viruses for their degradation and transgenic plants obtain virus resistance.

PVX and *Potato virus Y* (PVY) are two of the most prevalent viruses which cause severe diseases in potato cultivars. PVX and PVY are positive sense ssRNA viruses. They belong to the family-genus of Flexiviridae-potexvirus and Flexiviridea-potyvirus, respectively. Under field conditions, mixed PVX–PVY infections are not uncommon, which frequently show synergistic effects, i.e. stronger disease symptoms. Coat protein (CP) is a viral structural protein and NIb is a putative RNA-dependent RNA polymerase. Both CP and NIb have critical functions in the infection and replication process of viral genomic RNAs. In the current study, we used the RNA silencing strategy and the marker-free transgenic method [15] simultaneously to produce double-virus-resistant potato cv. Zihuabai through a cDNA construct of the inverted-repeat sequence of a chimeric gene derived from a *cp* fragment of PVX (PVX-*cp*) and a *NIb* fragment of PVY (PVY-*NIb*). Our strategy provided a reliable and efficient tool for generating high-biosafe and double-virus-resistant transgenic potato plants.

2. Materials and methods

2.1. Gene cloning and vector construction

Total RNA was extracted from the leaves of naturally infected potato plants in the field. The PVX-*cp* sequence was obtained by reverse transcription-PCR (RT-PCR) with a pair of primers (the forward primer: 5′-ATG TCA GCA CCA GCT AGC AC-3′, and the reverse primer: 5′-TTA TGG TGG TGG TAG AGT GA-3′) designed according to the PVX-*cp* sequence (GenBank Accession No. NC_001455). The PVY-*NIb* sequence was also amplified by RT-PCR with a pair of primers (the forward primer: 5′-GCT AAG CAT TCT GCA TGG ATG-3′, and the reverse primer: 5′-GCA TCA ATT GTG TCA TTT GC-3′) designed according to the PVY-*NIb* sequence (GenBank Accession No. U09509). A 520 bp fragment of the amplified PVX-*cp* sequence and a 540 bp fragment of the PVY-*Nib* sequence were fused, and this chimeric gene was ligated to a 742 bp spacer cDNA fragment of the *pdk* intron sequence in the vector pKANNIBALI. A second copy of the chimeric gene was ligated in an inverted orientation to the other side of the intron sequence, resulting in a vector containing the inverted-repeat sequence. For construction of the marker-free expression vector containing the inverted-repeat sequence, the vector pCAMBIA-3301 was used, in which the *gus* gene was substituted by the construct we developed and its *ppt* resistance gene expression cassette was deleted.

2.2. Plant transformation and selection of transformants

The marker-free plant expression vector harboring the inverted-repeat sequence of the chimeric gene was transferred to *Agrobacterium tumefaciens* strain LBA4404. Leaf discs from young plants of potato cv. Zihuaba were transformed by using a marker-free transgenic method [15] via the *A. tumefaciens* route. The upper leaves or stem materials of the regenerated plantlets were harvested for isolating genomic DNA. PCR analysis was performed using the oligonucleotide primers P1 5′-CATGAAGGTGCCCACA GAA-3′ (annealing to the PVX-*cp* sequence) and P2 5′-CGGATTCACAGCAATCAGC-3′ (annealing to the PVY-*NIb* sequence) to check for the presence of transformants. These primers amplified a 930 nt fragment from the chimeric gene. The PCR cycles used for detection of the transgene were 5 min at 95 °C, 35 cycles of 45 s at 94 °C, 45 s at 57 °C, 45 s at 72 °C, and a final extension for 6 min at 72 °C. PCR products were fractionated by electrophoresis in 1% agarose gels.

Each PCR-positive plantlet was cultivated for vegetative propagation. About 3–4 weeks later, the plants (three plants for each construct tested) were transplanted to the greenhouse for further analysis.

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2.3. Plant virus inoculation

PVX and three PVY strains, PVY^O, PVY^C and PVY^N, were propagated in *Nicotiana benthamiana*. Inocula consisting of PVX and PVY^O, PVY^C, PVY^N were prepared by mixing equal aliquots of the diluted crude sap from plants infected with each of these viruses. Thirty days after transplantation, the two apical leaves of PCR-positive plants (two plants for each construct) were inoculated by rubbing leaf extracts onto carborundum-dusted leaves, and then by rinsing the leaves with water. Wild-type control plants were inoculated at the same time as the control. To avoid escapes of viruses, a secondary inoculation of young emerging leaves was practiced in one week. The plants were then monitored for symptom development.

2.4. Immunological assays

Detection of PVX and PVY in inoculated plants was performed by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). The DAS-ELISA kit was purchased from Agdia, and ELISAs were performed according to the manufacturers' instructions. Optical density of the reaction products was measured at 405 nm.

2.5. Semi-quantitative RT-PCR analysis

Total RNA from inoculated plants was extracted using the RNA plant Reagent (Tiangen, China) and was treated with RNase-free Dnase I. Semi-quantitative RT-PCR was performed according to the manual of the RT-PCR kit (TakaRa). For detection of PVY, amplification was carried out with both PVY-specific primers (P1: 5′-GAA GCC TTG ACA GGA AAT-3′ and P2: 5′-TCT TGC TCG TCA GTG ACA-3′) and *tubulin*-specific primers (P1: 5′-GAC AGT CTG GTG CTG GGA ATA-3′ and P2: 5′-CAG GGA ATC TCA AAC AGC AAG-3′). The *tubulin* was used as an internal standard. For detection of PVX, amplification was carried out with both PVX-specific primers (P1: 5′- ATA GTA GCC AGC AAT GCC G-3′ and P2: 5′-TTA TGG TGG TGG TAG AGT GA-3′) and *tubulin* primers that were the same as those used for detection of PVY. The PVY-specific primers amplified a 330 bp fragment; and the PVX-specific primers amplified a 590 bp fragment. PCR cycles consisted of an initial heating at 95 °C for 5 min; 30 cycles of 94 °C denaturation for 1 min, 53 °C annealing for 1 min, and 72 °C elongation for 1 min; and a final extension of 8 min at 72 °C. The amplified products were analyzed on agarose gels after electrophoresis and ethidium bromide staining.

2.6. Southern blot analysis and detection of siRNA

Total DNA was isolated from fresh plant material, and 10–15 μg was digested with Hind III to determine the number of transgene copies inserted into the genome. The digested DNA was fractioned on 0.7% agarose gels and transferred to a Hybond™-N^+ nylon membrane. Hybridization was conducted at 38 °C using a Dig-labeled 930 bp chimeric gene fragment for the detection of transgene according to the manual of DIG High Prime DNA Labeling and Detection Starter Kit (Roche).

The total RNA was extracted from the leaf tissue of non-inoculated transgenic plants 6 weeks after transplantation using the RNAplant Reagent (Tiangen, China) and was separated on 15% polyacrylamide/7 M urea/TBE gels. Then the RNA was transferred onto a membrane by electrophoretic transfer using a Trans-Blot semi dry apparatus (Junyi) and was UV-crosslinked (120 mJ in a UV cross-linker, SIM). Antisense-specific Dig-labeled riboprobes corresponding to the 930 bp fragment of the chimeric gene transcribed *in vitro* were prepared according to the manual of DIG RNA Labeling Kit (Roche). Hybridization was done as for Southern hybridization.

3. Results

3.1. Construction and analysis of the marker-free expression vector

A PVX-*cp* gene fragment and a PVY-*NIb* gene fragment were fused for construction of a chimeric gene. Then an inverted-repeat sequence containing sense and antisense chimeric genes flanking an intron sequence derived from the *pdk* gene was constructed. The intron-containing inverted-repeat sequence substituted for the *gus* gene of the selection marker gene-free pCAMBIA-3301 binary vector yielded a marker-free inverted-repeat sequence expression vector (Fig. 1a). The intron sequence provides stability to the pre-mRNA [16,17] and enhances the RNA silencing efficiency [18], but it is spliced out during pre-mRNA processing. It was predicted that the self-complementary structure (Fig. 1b) and intermolecular-complementary structure (Fig. 1c) of RNA transcribed from the inverted-repeat transgene could be produced.

3.2. Potato transformation and direct selection of transformants

The leaf explants of potato variety Zihuabai were transformed using the marker-free expression vector carrying the chimeric inverted-repeat sequence via the *A. tumefaciens* route. A total of 272 plantlets were generated, of which 14 had an amplification of the expected 930 bp fragment (Fig. 2), confirming the presence of the transgene in the genome of these plantlets.

3.3. Screening of transgenic plants resistant to both PVX and PVY

The putative transgenic plants were mechanically inoculated 30 days after transplantation (dpi) by rubbing mixed extracts of PVX-infected tobacco leaves and PVY^O-,

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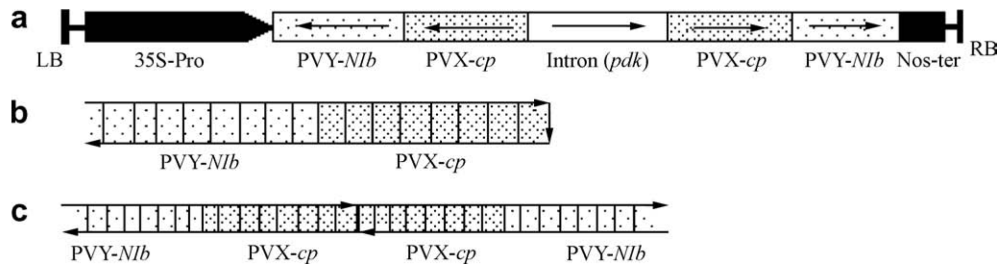

Fig. 1. Construction of the marker-free expression vectors carrying the inverted-repeat sequence of the chimeric gene (a) and the putative self-complementary structure (b) and intermolecular-complementary structure (c) of RNA transcribed from the sequence.

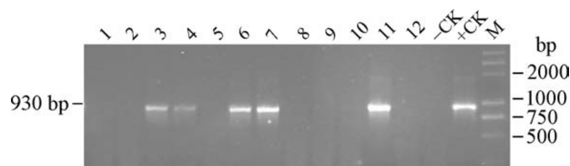

Fig. 2. PCR assay of a portion of generated plantlets. M, DNA molecular marker; −CK and +CK, the expression vector-transferred plants and wild-type plants, respectively. Lanes 1–12 refer to regenerated plantlets.

PVY^{C}-, and PVY^{N}-infected tobacco leaves, respectively. Susceptible phenotype and resistant phenotype were clearly observed at 30 dpi. Eighteen plants were found to be virus symptomless, three plants developed slight virus symptoms and another three plants developed strong virus symptoms as inoculated wild-type control plants (Fig. 3).

Under the field conditions, six tuber-borne transgenic lines derived from six different resistant transgenic plants and wild-type control plants were mechanically inoculated by rubbing mixed extracts of PVX- and PVY^{O}-, PVY^{C}-, and PVY^{N}-infected tobacco leaves. The susceptible phenotype and resistant phenotype were clearly observed at 30 dpi. All plants of the two transgenic lines (L1 and L2) were resistant as their parent plants (Fig. 4); some plants of the other four transgenic lines developed virus infection symptoms, but these were weaker than those that all the wild-type plants developed.

Semi-quantitative RT-PCR amplifications were conducted for evaluation of the presence of the viruses. The *tubulin* was used as an internal standard gene. In all the plants from transgenic lines L1 and L2, both the predicted

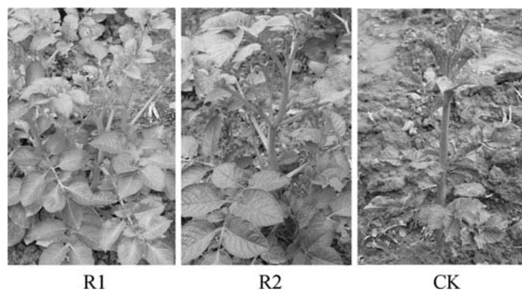

Fig. 3. Reaction of transgenic potato plants to mixed infection of PVX and PVY. Transgenic plants R1 and R2 were virus symptomless, whereas the wild-type plant (CK) developed strong virus symptoms.

Fig. 4. Response of transgenic lines to the mixed infection of PVX and PVY in the field. Transgenic lines L1 and L2 were virus symptomless, whereas the wild-type plants (CK) developed severe symptoms.

PVX-specific 590 bp fragment and PVY-specific 330 bp fragment were not amplified, whereas in wild-type plants (CK), the two fragments were amplified. In all the tested plants, a predicted internal standard gene, *tubulin*-specific 450 bp fragment, was amplified (Fig. 5). The results of DAS-ELISA for the detection of PVX and PVY were also in accordance with RT-PCR amplification and phenotypic data (Table 1).

3.4. Southern blot analysis and detection of siRNAs

Two resistant lines, L1 and L2, were further analyzed by Southern hybridization. Genomic DNA was digested with Hind III that cuts once in the plant expression vector harboring the inverted-repeat sequence of the chimeric gene. Southern analysis indicated that the two transgenic lines carried a single transgene copy or a few transgene copies (Fig. 6a).

To confirm that RNA silencing was indeed the underlying mechanism of the observed virus resistance, the transgenic potato plants were analyzed for the presence of both PVY-*NIb*-specific and PVX-*cp*-specific siRNAs derived from the introduced transgene. To exclude a potential latent infection with PVY and PVX, which might result in virus-derived siRNAs that are not of transgene origin, we isolated the RNA from virus-free plants of L1 and L2 and wild-type plants. In the subsequent Northern blot analysis, we could detect siRNAs in L1 and L2, whereas no siRNAs were detected in wild-type plants (Fig. 6b). The results of siRNA detection indicated that the trans-

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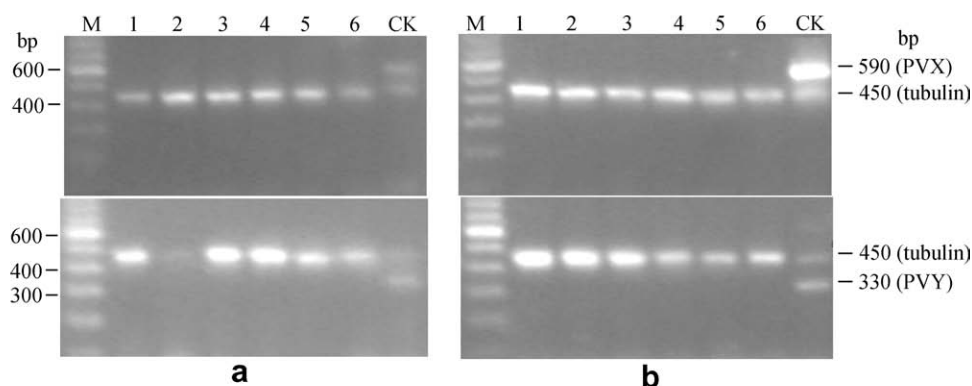

Fig. 5. RT-PCR detection for both PVX and PVY in transgenic lines L1 (a) and L2 (b).

Table 1
DAS-ELISA for the detection of PVX and PVY.

Plant No.	Transgenic line L1		Transgenic line L2	
	PVX	PVY	PVX	PVY
1	0.020 ± 0.009	0.039 ± 0.008	0.032 ± 0.006	0.029 ± 0.010
2	0.038 ± 0.006	0.028 ± 0.010	0.037 ± 0.003	0.036 ± 0.009
3	0.038 ± 0.004	0.026 ± 0.011	0.040 ± 0.003	0.038 ± 0.007
4	0.027 ± 0.002	0.040 ± 0.005	0.029 ± 0.010	0.042 ± 0.004
5	0.024 ± 0.003	0.039 ± 0.006	0.039 ± 0.009	0.032 ± 0.012
6	0.032 ± 0.005	0.041 ± 0.008	0.042 ± 0.004	0.040 ± 0.006
+CK	0.268 ± 0.008	0.520 ± 0.010	0.301 ± 0.009	0.601 ± 0.019
−CK	0.036 ± 0.003	0.037 ± 0.008	0.041 ± 0.004	0.038 ± 0.009

Note: values shown here represent mean absorbance values from three measurements. 1–6 refer to six plants of every transgenic line; +CK refers to wild-type plants inoculated with the virus; −CK refers to virus-free plants.

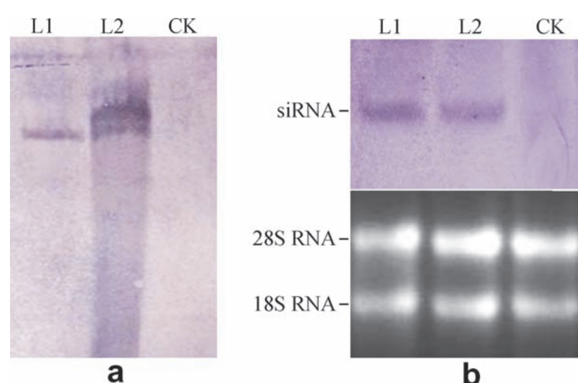

Fig. 6. Southern blot analysis (a) and detection of siRNA in transgenic lines (b). L1 and L2, two resistant transgenic lines; CK, wild-type plants; rRNA, shown as loading controls.

gene-derived mRNA was degraded, and that the double-virus resistance was mediated by RNA silencing, a mechanism trigged prior to virus inoculation.

4. Discussion

Double-stranded RNA is the key trigger of RNA silencing [13]. Recently, it has been shown that the expression of virus-derived dsRNA from transgenes can fully suppress viral infection through RNA silencing. The strategies have been successfully implemented for the generation of tobacco lines resistant to PVY [19], barley lines resistant to barley yellow dwarf virus-PAV (BYDV-PAV) [20], maize lines resistant to sugarcane mosaic virus [21] and potato lines resistant to PVY [14]. Under the field conditions, potato is frequently infected by multiple viruses. Mixed PVX-PVY infections frequently show synergistic effects, i.e. stronger crinkle mosaic disease symptoms and more severe yield losses. For the current study, the expression of virus-derived dsRNA from PVX-*cp* and PVY-*NIb* chimeric inverted-repeat transgenes construct can fully suppress the infection of both PVX and PVY through RNA silencing, which further confirms that double-virus-resistant potato can be obtained through RNA silencing, and that the double resistance can be stably inherited through vegetable propagation and be expressed well under the field conditions. The results of siRNA detection by Northern blot showed that RNA silencing mechanism was trigged prior to virus inoculation, indicating that a viral RNA will be under immediate attack by the RNA-induced silencing complex (RISC) if it penetrates such a plant with activated RNA silencing. Therefore, resistance is very strong. Further studies are needed to know whether the RNA silencing efficiency of the construct that we developed is consistent or not.

Artificial inoculation of both PVX and PVY was easily practiced, and mixed PVX-PVY infections frequently show synergistic effects and develop identifiable disease symptoms. The aim of the current study was to select resistant transgenic lines, hence only double-resistant transgenic lines without significantly different agronomic performance from their non-transgenic counterparts were further analyzed by Southern blot and Northern blot, which greatly reduced the workload for performing the experiments. But it is obvious that more transgenic information would be obtained by analyzing all transformants through Southern blot and Northern blot [14].

During recent years environmentalists and consumers have expressed concern about the transgenic biosafety [22]. RNA silencing techniques more easily fulfil the cur-

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rent high demands with respect to biosafety because the transgenic viral sequence is not translated and the actual RNA transcript is almost undetectable, most likely, for it gets cleaved quickly to small fragments [14]. The small non-translatable segments minimize the environmental risks of recombination, transcapsidation, synergism or complementation between an infecting plant RNA virus and a viral RNA produced from a transgene. For the current study, the RNA silencing technology and the marker-free transgenic method [15] were simultaneously used and the biosafety risk of both gene of interest and marker gene could be avoided, which would certainly contribute to the public acceptance of transgenic potato.

Acknowledgements

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