Silencing P25, HC-Pro and Brp1 of Potato Virus (Viroid) Using Artificial microRNA Confers Resistance to PVX, PVY and PSTVd in Transgenic Potato

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Abstract
Virus infection is the key constraint to potato cultivation worldwide. Especially, coinfection by multiple viruses could exacerbate the yield loss. Transgenic plants expressing artificial microRNAs (amiRNAs) have been shown to confer specific resistance to viruses. In this study, three amiRNAs containing Arabidopsis miR159 as a backbone, expressing genes targeting P25, HC-Pro and Brp1 of potato virus X (PVX), potato virus Y (PVY) and potato spindle tuber viroid (PSTVd), were constructed. amiR-159P25, amiR-159HCPro and amiR-159Brp1 were cloned into the plant expression vector pCAMBIA1301 with a CaMV35S promoter, producing the p1301-pre-amiRP25−HCPro−Brp1 vector. Twenty-three transgenic plants (Solanum tuberosum cv. ‘Youjin’) were obtained by Agrobacterium tumefaciens–mediated transformation, and ten PCR-positive transplants were chosen for further analysis. Quantitative real-time PCR results indicated that 10 transgenic plants could express amiRNAs successfully. Southern blotting hybridization proved that amiR-159P25−HCPro−Brp1 had integrated into potato genome in transgenic lines. Viral (viroid) challenge assays revealed that these transgenic plants demonstrated resistance against PVX, PVY and PSTVd coinfection simultaneously, whereas the untransformed controls developed severe symptoms. This study demonstrates a novel amiRNA-based mechanism that may have the potential to develop multiple viral resistance strategies in potato.

Keywords Artificial microRNAs · Multiple virus resistances · Potato virus · Transgenic plants

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Introduction

Potato (Solanum tuberosum L.) is ranked fourth amongst all food crops in total production, following rice, wheat and corn worldwide (Rozentsvet et al. 2022). Potato continues to be recommended as a key food for human consumption, especially when faced with the current population growth, leading to problems with food supply, nutrition and food security and climate change challenges globally (Beh et al. 2022). In 2020, 5,596,000 ha land was used for potato cultivation and approximately 122.94 million tons of potatoes was produced in China (Luo et al. 2021). Unfortunately, potato is vulnerable to around 40 different virus species and two viroids that are endemic throughout the potato-growing areas of the world and significantly reduce crop productivity (Jeffries et al. 2006; Kreuze et al. 2020).

Potato virus X (PVX; genus Potexvirus) causes great economic losses to crop production due to its extensive host range (Yu et al. 2010). Potato virus Y (PVY; genus Potyvirus) is the most detrimental virus for potato, causing 10–80% yield losses and it could be spread within potato fields as well as from field to field (Hosseini et al. 2011; Petrov et al. 2015; Dupuis et al. 2019). Potato spindle tuber viroid (PSTVd; family Pospiviroidae) is a small RNA pathogen that causes severe pandemic diseases in potatoes. In the case of infecting PSTVd, the tubers of potato are reduced in size with an elongated or spindle-shaped morphology and have prominent eyes (Bao et al. 2019).

The simultaneous coinfection of multiple viruses could result in pronounced disease phenotypes and lead to crop failure because of the synergistic or antagonistic interactions amongst viruses (Tollenaere et al. 2016; Hameed et al. 2017; Sajid et al. 2020). For example, in mixed infections involving PVX with PVY, losses can reach up to 55%, and the symptoms of viral infection become worse in mixed infections with PVY and PSTVd (Vance 1991; Qiu et al. 2014).

Artificial microRNAs (amiRNAs) are a class of artificial small RNAs engineered to silence specific transcripts in plants. A single 21-nucleotide amiRNA was produced in plants by expressing a functional miRNA and it could only recognise a target sequence with less than five mismatches (Ossowski et al. 2008). In multiple models and crop species, amiRNAs are artificial small RNAs engineered to silence endogenous transcripts as well as viral RNAs in plants (Carbonell & Daròs 2017; Carbonell et al. 2019). amiRNA technology has been successfully applied to obtain virus-resistant species such as tobacco, tomato, rice and strawberry. The amiRNA approach can also create the constructs of targeting multiple sites within different viral RNAs (Li et al. 2013). Therefore, multiple amiRNAs for multiplex gene silencing could be produced to enhance plant antiviral resistance.

In this study, the amiRNA approach was adopted for expression against essential genes of PVX, PVY and PSTVd using an endogenous precursor, miR159a. Transgenic potato plants expressing amiRNA to silence two viral genes and one viroid gene were developed, and the resistance against viruses (viroid) of transformants was examined.
Materials and Methods

Cloning of P25, HC-Pro and Brp1 genes

The sequences for P25, HC-Pro and Brp1 genes were obtained using the BLASTn and BLASTX search tools available at the NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Gene-specific primers were designed to amplify P25, HC-Pro and Brp1 genes from potatoes. RNA was isolated from virus (viroid)-infected potato plants using TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa, Dalian, China), and cDNA was synthesised from RNA using PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa) according to the manufacturer’s instructions.

Using gene-specific primers (Table 1), P25, HC-Pro and Brp1 genes were amplified from cDNA. The polymerase chain reaction (PCR)–amplified products were cloned into the pMD18-T vector. The insertion of the amplicon was confirmed by Tsingke Biological Technology (China).

Construction of amiRNA-159P25, amiRNA-159HCPro and amiRNA-159Brp1

The sequences of P25, HC-Pro and Brp1 genes were analysed to predict the target viral (viroid) amiRNA by Web MicroRNA Designer (WMD, http://wmd3.weigelworld.org/cgi-bin/webapp.cgi). According to the criteria for selection of amiRNA (Schwab et al. 2005), the potential sequence without off-targets was 5′-TAGACA CATGGTTGAACCTCA-3′ for P25 amiRNA, 5′-TCGACAGATCTGC GCCATCA -3′ for HC-Pro amiRNA and 5′-TAACCTAAACCATTGCACACGT-3′ for Brp1 amiRNA. The 21-nucleotide was precisely chosen from target genes to minimise the off-target effects. The 228 bp backbone of pre-miRNA159 of Arabidopsis was used to generate artificial pre-miRNA159 for the three genes. Arabidopsis cDNA as a template with appropriate pairs of primers was used to generate the three DNA fragments. The primers for the construction of amiRNA-159P25, amiRNA-159HCPro and amiRNA-159Brp1 are shown in Table 2.

The whole amiRNA cassette (amiRNA-159P25), (amiRNA-159HCPro) and (amiRNA-159Brp1) was designated as amiR-159P25–HCPro–Brp1. The multiple amiRNA cassette was commercially synthesised (Tsingke, China). The amiRNA fragments of 729 bp were ligated into the binary vector pMD-18 T using T4 DNA ligase (TaKaRa) at EcoRI and PstI sites and transferred into the binary vector.

Table 1 Names, sequences and brief description of primers

| Primer name | Sequence from 5’ to 3’ | Amplification |
|-------------|-----------------------|--------------|
| P25-F       | TGTCACCGACGTTGGGTAG   | P25          |
| P25-R       | TGACCGAGGCGGTCAGTCT   |              |
| HC-Pro-F    | GCTGGCCGACTCAGACATTAT | HC-Pro       |
| HC-Pro-R    | TGGCATTAGAACCACCAAG   |              |
| Brp1-F      | CGGAACTAAAACTCGTGTTT   | Brp1         |
| Brp1-R      | TGGAAACCCGAGTTGTTTCT   |              |

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pCAMBIA1301. The amiRNA precursors (pre-amiR-159P25−HCPro−Brp1) containing vectors were transformed into *Agrobacterium tumefaciens* strain LBA4404.

### Plant Materials and Transformation

Potato (*S. tuberosum* L.) tetraploid cultivar ‘Youjin’ was used in this study, which is widely grown in northern China. ‘Youjin’ is moderately resistant to PVY; however, in mixed infections of PVY and PVX, systemic dwarfing and leaf curling can occur, and yield losses can reach up to 30%. Plants were propagated in vitro using single stem nodes on MS medium (Murashige & Skoog. 1962) supplemented with 3% sucrose and 0.6% agar at 22–24 °C, 16 h light/8 h dark photoperiod and light intensity 100 µmol/m²s; MS Liquid medium contained 8% sucrose and 5 mg/l 6-BA (6-Benzylaminopurine) was added (30 mL per tube) after 21 days’ cultivating, 20 °C and 8 h light/16 h dark photoperiod and microtubers were harvested after 12 weeks.

The plant expression vector pMD-18 T (TaKaRa) was introduced into *A. tumefaciens* strain LBA4404 via the freeze–thaw method and used as a control in the transient transformation experiment. A single colony from the bacterial strain was inoculated into liquid yeast extract beef (YEB) medium containing 50 mg/L kanamycin and 100 mg/L rifampicin and grown overnight at 28 °C with shaking (200 rpm). The bacterial fluid (1 mL) was added into 50 mL liquid YEB medium and shaken to mid-log phase (OD₆₀₀ ≈ 0.5). Microtubers that were harvested in vitro were peeled and cut into 5 mm × 5 mm × 1 mm slices. The explants of tuber slices were transferred into the above bacterial liquid for 5 min with gentle shaking every minute. Subsequently, the tuber slices were blotted on sterile filter papers to remove most of the liquid medium and cocultivated on differentiation medium containing solid MS medium supplemented with 4.0 mg/L ZT (zeatin), 1 mg/L IAA (indole acetic acid) and 0.25 mg/L GA₃ (gibberellin) for 2 days in the dark. The explants were rinsed in the liquid MS medium and then plated on the regeneration medium containing 500 mg/L cefotaxime and 50 mg/L kanamycin. The kanamycin-resistant ratoon buds were sprouted after 3 weeks. The regenerated plants (0.5–1.0 cm) were

| Primer name | Sequence from 5′ to 3′ | Amplification |
|-------------|-------------------------|---------------|
| 159P-F      | CGATGGAAAGTGGATTTCAAGCCGCCTACATCATGAGTTGAGCAGGGTA | amiR-159P25   |
| 159P-R      | TCTAGATCTATGTTAGGAAAGTCATGATAGGAGTAAAGAGCCCA     | amiR-159HC−Pro|
| 159H-F      | TCTAGATCTATGGACCTGAGGTGACATTAGGAGTAAAGAGGGAGA   | amiR-159Brp1  |
| 159H-R      | ACTAG ATGTTCTACCCGATGATGATAGAGGTAAAAAGCCA        |               |
| 159B-F      | ACTACGAGTTGAAGGTTCTTTACTCAATTAGGCGAGCATGTCCA    |               |
| 159B-R      | CTGCAGATGTGGCTCTTTACAAAGGGGCGAGAGAGGGAAGAAGGAAAGCCA |               |

Table 2 Sequence primers of pre-amiRNA-159P25, pre-amiR-159Hc−Pro, pre-amiR-159Brp1
transferred to an MS medium supplemented with 50 mg/L kanamycin and cultured in a growth room at 22–24 °C, 16 h light/8 h dark photoperiod and a light intensity of 100 µmol/m²s.

**PCR for the Identification of Transgenic Plants**

Total DNA of primary transformants (T₀) and nontransgenic control plants was isolated using the CTAB method described by Clarke (2009) and screened by PCR using 159P-F and 159B-R primers for the presence of the transgene. Reactions were performed in a final volume of 20 µL, and the components were individual dNTPs (200 µM), each forward and reverse primers (0.2 µM), 50–100 ng total DNA as the template and Taq polymerase (0.05 U/µL; TaKaRa). The reaction mixture was initially denatured for 5 min at 95 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 45 s. The complete PCR product (20 µL) was resolved by electrophoresis on 1.0% agarose.

In real-time quantitative Reverse Transcription PCR (qRT-PCR) for the expression analysis, total RNA was isolated from in vitro transgenic plants and nontransgenic control plants. cDNA was synthesised based on the method shown previously. For gene-specific primers used for qRT-PCR analysis, qRT-qPCR was performed in a CFX-96 Instrument (Bio-Rad, USA) using SYBR® Premix ExTaq™ (TaKaRa). The transcript levels of target genes were normalised to those of endogenous StEF-1α (for potato). The primers used for qRT-PCR are listed in Table 3. RT-PCR analysis was carried out in three biological replicates. The quantification results were analysed by the 2−ΔΔCt method (Livak & Schmittgen 2001) for relative gene expression data.

**Southern Blot for the Identification of Transgene Copy Number**

Genomic DNA of potato was isolated from 2 g of leaf tissue from both transgenic and non-transformed control plants by Plant genomic DNA Extraction Kit (TaKaRa), and then 20 µg of genomic DNA was used to perform restriction digestion with HindIII at 37 °C for 16 h. HindIII-digested DNA was separated on a 0.8% agarose gel and the blotted membrane was hybridised with DIG-labelled specific probe, complementary to the sequence of the transgene amiR-159P25−HCPro−Brp1 prepared by PCR DIG Probe Synthesis Kit (Roche). Southern blot was performed according to DIG Application Manual for Filter Hybridisation (Roche).

| Primer name | Sequence from 5’ to 3’ | Amplification |
|-------------|------------------------|---------------|
| **StEF-1α-F** | ATTGGAACGGATATGCTCCA | StEF-1α       |
| **StEF-1α-R** | TCCCTACACGAACCCCTGCA |              |
| **amiR-159F** | ACGATGGAAGTGGTTCAAAAGCC | amiR-159P25−HCPro−Brp1 |
| **amiR-159R** | ATGGTCTCTTACACAAAGGCGAG |              |
**Virus (Viroid) Inoculation and Detection for Transformants**

When the transformants had reached about 8–10 cm height and had a well-developed shoot and root system, the plantlets were taken out from the vials, and the roots were washed under running tap water to remove the medium. The plantlets were transplanted to plastic pots containing vermiculite in the greenhouse at temperatures of 25–28 °C and watered with Hoagland solution (Hoagland & Arnon 1950) once a day. The transplants were mechanically inoculated with 100 ng purified, mixed infection of PVX, PVY and PSTVd (the strain was KX-PVX, PVYN and 351-PSTVd) at the six-leaf stage. For negative control, 0.01 M phosphate buffer (pH 7.0) was used, and wild-type potato plants were inoculated with PVX, PVY and PSTVd mixed infection as a positive control. Symptoms were observed 28 days after virus (viroid) inoculation and virus (viroid) was detected by RT-PCR, the primers used for RT-PCR are listed in Table 4 and the PCR conditions were as follows: 95 °C for 3 min, 40 cycles of 7 s at 95 °C, 10 s at 61 °C and 15 s at 72 °C. The tubers harvested in the T0 generation were planted in the next year and T1 plants were obtained. T1 plants were inoculated with mixed viruses (viroid) at the six-leaf stage, using the same method as that of the T0 generation.

**Results**

**Cloning and Identification of P25, HC-Pro and Brp1 genes**

Primers of P25, HC-Pro and Brp1 were designed according to the sequence provided by the National Center for Biotechnology Information, and the accession numbers were D00344.1, D00441.1 and AJ504728.1, respectively. The sequences of P25, HC-Pro and Brp1 genes were cloned from ‘Youjin’ plants infected with PVX, PVY and PSTVd, respectively. The lengths of their RT-PCR products were 500 bp for P25, 1237 bp for HC-Pro and 1307 bp for Brp1 and showed the same size sequences as the ones published in GenBank (Fig. 1-a).

| Primer name | Sequence from 5' to 3' | Amplification | Length (bp) |
|-------------|------------------------|---------------|-------------|
| PVXF        | ACAGGCCACAGGGTCAACTAC  | PVX           | 711         |
| PVXR        | CATCTAGGCTGGCAAAGTCTG  |               |             |
| PVYF        | GAGCAACTCAATCAGTTGATAC | PVY           | 447         |
| PVYR        | TGTTCTGTCACTGAACTAG    |               |             |
| PSTVdF      | GCCGCAGGGAATTCGAT      | PSTVd         | 356         |
| PSTVdR      | TCCAACCGTGGGAGC        |               |             |
Construction of amiRNAs Targeting P25, HC-Pro and Brp1 Genes

In the pre-amiRNA159a backbone, the original 21-nucleotide miR159a sequence was replaced by amiRNA sequences. Three amiRNA fragments targeting P25, HC-Pro and Brp1 were obtained by PCR amplification, and the sizes of amiRNAs were consistent with the predicted ones (Fig. 1-b). These amiRNA sequences were named pre-amiR-159P25, pre-amiR-159HC−Pro and pre-amiR-159Brp1, respectively.

Cloning into the Binary Vector

Overlapping PCR techniques were applied to generate multiple amiRNA cassettes with the endonuclease recognition sites EcoRI and PstI. The whole amiRNA cassettes (amiR-159P25, (amiR-159HC−Pro) and (amiR-159Brp1) were designated as amiR-159P25–HCPro–Brp1 (Fig. 1-c). Pre-amiR-159P25–HCPro–Brp1 can produce three different amiRNAs (amiR-159P25, amiR-159HC−Pro and amiR-159Brp1) directed target to the mRNA of PVX (P25), PVY (HC-Pro) and PSTVd (Brp1).

pCAMBIA1301 (P1301), a binary vector used for plant genetic transformation, was digested with EcoRI and PstI to connect the multiple amiRNAs, and EcoRI and PstI could maintain the correct orientation of the amiRNAs according to the
orientation of the CaMV promoter and the OCS terminator, which are located in the new expression vector named p1301-pre-amiR-159P25−HCPro−Brp1 (Fig. 1-d).

The presence of the amiRNAs in the p1301 vector was confirmed by PCR detection and restriction enzyme digestion (Fig. 1-e).

**Generation of Transgenic Potato Plants Expressing Pre-amiR-159P25−HCPro−Brp1**

The virus-free minitubers of potato cultivar ‘Youjin’ were transformed with p1301-pre-amiR- 159P25−HCPro−Brp1. The colour of mini-tuber slices gradually became light green when cultured in differentiation medium after 14 days, and the colour turned dark after 21 days of culture. Callus tissue was regenerated after about 28 days of culture, and new shoots regenerated from the callus tissue. The shoots (1–1.5-cm long) were transferred to an MS medium supplemented with 50 mg/L kanamycin, and transgenic microplants were obtained (Fig. 2-a). The regenerated shoot from one explant was considered an independent transgenic line.

**Fig. 2** Generation of transgenic potato plants and identification analysis of transgenic plant. **a** Schematic representation of in vitro plant transformation, (1) Micro-tuber slice infected with Agrobacterium for 2 days; (2) micro-tuber slice infected with Agrobacterium for 21 days; (3) the regenerated plants were transferred to MS medium; (4) propagation of regenerated plants. **b** PCR verification of transgenic plants. (M) DNA ladder, (C) non-transformed plants control, (P) expression vector control, (lane 1–23) transgenic lines. Lines without the transgene are marked with an “X”. **c** The relative expression levels of amiRNAs in transgenic potato lines. Bars with the same letter represent no significant difference at $P \leq 0.05$ according to Duncan’s Multiple Range Test. **d** Expression analysis of amiR-159P25−HCPro−Brp1 in transgenic lines by Southern blot, (P) expression vector control, (C) non-transformed plants control, (lane 1–5) transgenic lines.
Selection of Putative Transgenics

PCR amplification was performed on total DNA isolated from kanamycin-resistant lines in T₀ plants. Amongst the 23 plants analysed, 17 showed DNA amplification of the expected size of 729 bp (Fig. 2-b), yielding a 73.9% positive rate.

Expression of amiRNAs in Potato

The transcription levels of amiR-159P25−HCP−Brp1 was analysed by RT-PCR using the 10 putative transgenic lines (Fig. 2-c). As expected, pre-amiR-159P25−HCP−Brp1 was expressed in the 10 transgenic lines. The expression level of the amiRNA in a nontransformed plant was set at 1. All tested transgenic plants demonstrated that the expression levels of amiRNAs increased dramatically. The level of amiRNA expression in transgenic plants relative to nontransformed plants varied from 7.9 in line L6 to 28.4 in line L5. These results suggested that amiRNAs could be transcribed.

Verification of Transgene Integration Using Southern Blot Analysis

A total of five putative T₀ transgenic lines were selected for testing of Southern blot. Southern blot analysis revealed that L1, L3 and L4 have a single copy of amiR-159P25−HCP−Brp1, whereas L2 line has double copies (Fig. 2-d), whilst L5 did not show any copy.

Resistance of amiRNA Transgenic Plants to PVX, PVY and PSTVd Infection

To examine the coinfection resistance conferred by amiRNAs in transgenic potato plants, the same batch of transgenic and nontransgenic plants was inoculated with an equal amount of PVX, PVY and PSTVd. Typical PVX and PVY symptoms were observed in wild-type potato plants after 28 days of inoculation, such as severe leaf curling, shortened internodes and stunted plant growth. The negative control and transgenic plants exhibited no coinfection symptoms with flatted leaves and normal plant height (Fig. 3-A). Twenty-eight days later, wild-type plants displayed obvious leaf developmental defects with upward-curved and mottled leaves and shorter petioles compared to transgenic plants. When the tubers were harvested, their number and diameter of tubers of wild-type plants were significantly lower than those of transgenic plants. The symptoms of viral (viroid) infections were noticed for nontransgenic plants of T₁ generation around the flowering stage, whilst the transgenic plants of T₁ generation grew vigorously.

Specific primers of PVX, PVY and PSTVd sequences were used for further detection. RT-PCR results indicated that PVX, PVY and PSTVd sequences had not been detected in all transgenic lines. In comparison, 562 bp fragment for PVX,
amiRNAs have been widely used to induce resistance against many DNA and RNA viruses (Carbonell et al. 2016). This study attempted to regulate PVX, PVY and PSTVd infection in potato plants using the amiRNA strategy. Three amiRNAs were established targeting PVX, PVY and PSTVd, respectively, and effectively transferred into potatoes to enhance resistance against virus (viroid) infection.

Many Arabidopsis miRNA precursors are used to produce amiRNAs, such as miR159, miR165, miR166, miR167, miR169, miR171 and miR319 (Cisneros & Carbonell 2020; Liu et al. 2010; Jelly et al. 2012; Ai et al. 2012; Tong et al. 2017; Li et al. 2019). miRNA395 of wheat (Fahim et al. 2012) and miRNA528 of rice (Li et al. 2014) as backbones for amiRNAs were also reported; however, no reports were published about potatoes yet. Niu et al. (2006) showed that the complementary strand in pre-miR159 could be easily replaced with any artificial sequences targeting any viral RNAs by PCR. Ai et al. (2012) proposed that the stem loop of pre-amiRNA159a might more efficiently generate the miRNA-miRNA* duplex in the nucleus. Therefore, in this study, the miR159a of Arabidopsis was chosen as a backbone for producing amiRNAs.

Fig. 3 Resistance of amiRNA transgenic plants to PVX, PVY and PSTVd infection. a Analysis of resistance to PVX, PVY and PSTVd infection in transgenic potato plants. b PCR detection of PVX, PVY and PSTVd sequence in transgenic lines. (M) DNA ladder; (C) nontransformed plant controls; (lane 1–10) transgenic lines.

480 bp fragment for PVY and 408 bp fragment for PSTVd were amplified in non-transgenic plants (Fig. 3-B).

Discussion
P25 is a viral movement protein (25 kDa) in plants, and the PVX-encoded P25 protein could prevent systemic posttranscriptional gene silencing (Voinnet et al. 2000). HC-Pro (helper component proteinase) is a major factor in viral pathogenicity and encoded by plant viruses of the genus *Potyvirus*. HC-Pro could counteract antiviral defence in plants by decreasing their endonuclease activity (Plisson et al. 2003). Virp1 is a bromodomain-containing protein that contains a nuclear localization signal and a RNA-binding domain. Virp1 could affect viroid replication and the systemic spread of PSTVd in the host, and Brp1 is bromodomain-containing RNA-binding protein for Virp1 (Maniataki et al. 2003; Kalantidis et al. 2008). In this study, *P25*, *HC-Pro* and *Brp1* genes were selected to develop an amiRNA construct for resistance against PVX, PVY and PSTVd, respectively.

Optimal amiRNA target sites were crucial for the silencing activity of the generated amiRNAs (Kis et al. 2016). Three amiRNAs targeting the *P25*, *HC-Pro* and *Brp1* genes were chosen from the top of the WMD output list for candidate target sites. As complete base pairing of amiRNA could enhance the effect of gene-silencing effect, the Spud DB (http://solanaceae.plantbiology.msu.edu/index.shtml) was used as a reference database when selecting amiRNA sites to reduce the potential off-target effects. The results demonstrated that the transgenic potato plants have a powerful capacity against virus (viroid).

Multiple virus resistance could be engineered by expressing two or more amiRNAs in tandem repeats as a single transcript with one promoter (Carbonell et al. 2019; Liang et al. 2012; Zhang et al. 2018; Fetchthai et al. 2018). In this study, three amiRNAs were designed to silence *P25*, *HC-Pro* and *Brp1* genes simultaneously and effectively transferred into potatoes coinfected with PVX, PVY and PSTVd.

In this study, RT-PCR results showed that the expression level of the target gene varies in individual transgenic lines. No viral (viroid) symptoms were detected in these lines upon infection with three viruses and viroid. Southern blotting hybridization proved that amiR-159*P25−HCPro−Brp1* had integrated into potato genome in four putative transgenic lines. One important limitation of the amiRNA approach is a breakdown of specific viral resistance in some transgenic plants (Niu et al. 2006). When devising strategies for expressing amiRNAs in plants, it may be difficult to stably suppress a target virus gene because of the rapid mutation rate of viruses (Jiang et al. 2022). Fetchthai et al. (2018) reported that the amiRNA transgenic lines targeting Cymbidium mosaic virus (CymMV) and Odontoglossum ringspot virus (ORSV) could confer high percentage resistance to CymMV but weak resistance to ORSV in the T3 generation and no resistance in T4. The reason for this phenomenon could be attributed to the rapid replication of the nontargeted virus, and the silencing suppressor of nontargeted virus would block cleavage of the targeted viral RNA. However, in this study, the breakdown of resistance to PVX, PVY and PSTVd of T1 generation was not significant as compared to T0 generation. Since potatoes are reproduced asexually, the viral (viroid) resistance of transgenic plants in potatoes could be more stably heritable. Furthermore, higher generations of transgenic plants (T3–T5) need to be tested for viral (viroid) resistance in future research.
Conclusions

In this study, the miRNA159 backbone precursor from A. thaliana was modified to express the amiRNA construct targeting P25, HC-Pro and Brp1 of PVX, PVY and PSTVd. The amiRNA construct was stably transformed in potatoes. The transformed plants could express amiRNAs successfully and demonstrated resistance against PVX, PVY and PSTVd coinfection simultaneously. Overall, the outcome of our study establishes a practical amiRNA strategy for generating stable antiviral immunity in potatoes. Theoretically, this antiviral strategy would be suitable for targeting different viruses in potato.

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Declarations

Conflict of Interest The authors declare no competing interests.

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