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Designing an Effective RNA Interference Construct for Generating PVY-Resistant Transgenic Potato Plants

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RNA interference (RNAi) is a powerful gene silencing technique that blocks specific gene expression. RNAi technology has shown great promise in controlling viral diseases in plants, particularly in potatoes, where viruses cause significant losses in quality and production. The ability of transgenic plants to resist insect viral infection by expressing double-stranded RNAs (dsRNAs) targeting viral genes has been documented. However, selecting target genes is a cornerstone for the success of RNAi in controlling viral diseases. The potato virus Y (PVY) is considered the most economically damaging for potato crops worldwide. The overall aim of the present study is the potential use of RNAi technology to create a virus-resistant potato crop, improving global food security. In the current study, two distinct RNA interference (RNAi) constructs were developed. The first construct is designed to target the *P1* gene, which is responsible for encoding the initial protein of the potato virus Y (PVY). Meanwhile, the second construct targets *the nuclear inclusion-Pro* gene and serves the purpose of developing a transgenic potato variety that is resistant to PVY.

Keywords: Gene silencing, Potato virus Y (PVY), RNA interference, (RNAi), Viral diseases.

Introduction

RNA interference (RNAi) is a biological mechanism that causes the suppression of gene expression after transcription. This process is initiated by double-stranded RNA (dsRNA) molecules, which serve as triggers to prevent the expression of particular genes (Goel & Ploski, 2022; Zabala et al., 2022; Lyu et al., 2023). RNAi is a mechanism in eukaryotic cells that occurs in the cytoplasm. It selectively breaks down mRNA after it is transcribed using specific nucleotide sequences (Routhu et al., 2023).

RNAi shows effectiveness against different types of viral diseases, including RNA and DNA viruses, with the potential for improved crop production and increased food security worldwide. RNAi-mediated virus resistance has been applied in plants by developing genetically modified crops that show low viral titers of infected plants compared to non-transgenic crops resulting in improved plant growth and increased crop yield (Meister & Tuschl, 2004; Younis et al., 2014; Petrov et al., 2019; Mitra et al., 2023).

The defence mechanism of RNAi occurs against double-stranded RNA (dsRNA) by targeting mRNA translation and hence, down regulating gene expression. The dsRNA expressed by transformed DNA insert within the plant genome. This dsRNA gives rise to small interfering RNA (siRNA) that induces viral transcripts to be targeted based on homology. This process leads to a pre-programmed immunity in transgenic plants, adequately safeguarding them against viral infections (Hameed et al., 2017; Jahromi et al., 2022).

It can target both cellular and viral mRNAs, with microRNA (miRNA) and small interfering RNA (siRNA) as the cleavage products of dsRNA, forming small non-coding RNAs. The dsRNA cleavage is digested by a ribonuclease

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named DICER or a Dicer-like enzyme. According to studies, a complex called the RNA-induced silencing complex (RISC), along with effector proteins such as Argonaute (AGO), collaborates to initiate the RNA interference (RNAi) phenomenon (Saurabh et al., 2014; Akbar et al., 2022; Sundaresha et al., 2022).

Many key factors influence the successful application of RNAi technology, including the mode of RNAi delivery, whether the genes being targeted are lethal to the pest after silencing, whether they are safe to non-targets such as natural enemies and humans, and the type of tissue and dose of the dsRNA molecule. Many biological factors, such as variation in the fundamental RNAi machinery, cellular absorption, propagation of silencing signals, dsRNA degrading enzymes, and other genetic variances, all affected the outcome of RNAi tests in different species (Scott et al., 2013; Mamta & Rajam, 2017).

Studies on transgenic plants transformed with hairpin RNA (hpRNA) constructs that are designed with sense and anti-sense arms ranging from 98 to 853 nucleotides and introns in between the two arms have significantly improved their effectiveness in viral suppression. Independent transgenic plants with intron-containing constructs (ihpRNA) exhibited effective silencing at a rate of 90% to 100% (Wesley et al., 2001; Kumar et al., 2022).

RNAi is a favored approach for crop management, addressing pests, pathogens, and abiotic stresses. It regulates gene expression naturally, offering improved resistance to biotic and abiotic stress in crops, despite limitations in gene candidate selection and trigger molecule stability (Bharathi et al., 2022).

The RNAi technique using dsRNA-inducing gene silencing has shown exceptional promise in modulating the expression of target genes and suppressing viral RNA production (Wytinck et al., 2020; Šečić & Kogel, 2021). Two approaches to inducing RNAi in crop pests have recently gained traction: host-induced gene silencing (HIGS) using RNAi cultivars and spray-induced gene silencing (SIGS) using spray-able dsRNA (Willow et al., 2021).

Potato (*Solanum tuberosum* L.) is a vital crop that has the ability to feed the world's rising population. (Mystkowska et al., 2023; Sharma et al., 2023; Sofy et al., 2013). However, viruses cause significant losses in both quality and quantity of potato production worldwide, with viral infections up to 47% of total crop loss (Mushtaq et al., 2020; Sofy et al., 2022). Out of 128 known Potyvirus species, the *potato virus Y* (PVY) is considered the most economically damaging one for potato crop production (Singh et al., 2008; Al-Mokadem et al., 2022).

The primary objective of the current study is to construct a plasmid containing a designed dsRNA cassette that can be inserted later into a plant transformation vector. The purpose is to induce RNA interference (RNAi) within the transgenic potato cultivar, leading to resistance against PVY viral infection. By leveraging this technology, the study demonstrates a promising alternative to traditional methods such as crop rotation and pesticide use. The focus is on highlighting the potential of RNAi for reducing the need for conventional practices and effectively managing viral diseases in potato crops.

Materials and Methods

Inoculum source

The strain of Potato Virus Y (PVY) originating from Egypt was acquired from the Virology Lab at Al-Azhar University, Cairo, Egypt. To validate the authenticity of this viral strain, serological tests were conducted using ELISA kits generously provided by the Agriculture Research center, Giza, Egypt, along with a double-antibody sandwich enzyme-linked immunoassay test (DAS-ELISA) following the methodology described by Clark & Adams (1977).

Total RNA extraction

In accordance with the manufacturer's recommendations, total RNA was extracted from leaves of Datura metel plants that show PVY infection using the TRIZOL reagent (Invitrogen, USA).

cDNA synthesis

The PVY total RNA was used to synthesize cDNAs. As directed by the manufacturer (Invitrogen), the Superscript II cDNA synthesis kit was used for preparing first-strand cDNA.

Cloning of dsRNA cassette in the pBlueScript SK cloning vector

A) Cassette targeting first protein (P1)

The dsRNA cassette was designed to target the first protein (*P1)* transcript. The cloning strategy basically depends on assembling the dsRNA fragment into pBluescript SK+ (pBSK). The dsRNA cassette was assembled by dual cloning of 352bp fragment of first protein gene, in face-to-face direction, to produce sense and antisense transcript, and sub-cloning a 134bp of potato pyruvate kinase (Accession number Z11964) intron links them and works as a buffer zone between the two copies. The cassette design is shown in Fig. 1.

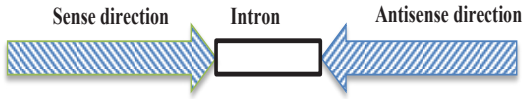

Fig. 1. dsRNA cassette designed as two copies of PVYP1 as face-to-face orientation and intron fragment in between them

B) Cassette targeting the nuclear inclusion-Pro gene (NI-Pro gene)

Another dsRNA cassette was targeted towards *the nuclear inclusion-Pro gene*. The main objective of the cloning approach was the assembly of the dsRNA fragment into pBlueScript SK+ (pBSK). Dual cloning of a 416 bp nuclear inclusion-Pro gene fragment was used to construct the dsRNA cassette to produce sense and antisense transcript., and sub-cloning a134 of the intron of potato pyruvate kinase (Accession number Z11964), which links the two copies, acts as a buffer zone in between them. The cassette design is shown in Fig. 2.

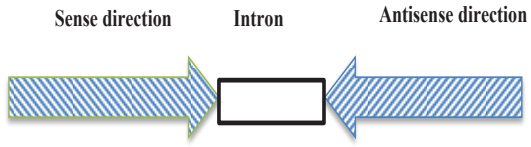

Fig. 2. dsRNA cassette designed as two copies of NI-PRO gene as face-to-face orientation and intron fragment in between them

The cloning process for two cassettes was as follows

PCR amplification

The most conserved genomic sequence of PVY *P1* gene and *nuclear inclusion-Pro* gene, published at the GenBank database (Acc. # AF522296.1), was selected to design two primer sets for *P1* gene (P1SenseFWD-1/P1SenseRVS-1 and P1AntisenseFWD-2/P1AntisenseRVS-2). The nucleotide sequences of primer sets are shown in Table 1. Two primer sets were used to amplify the 352bp DNA fragment of the PVY *P1* gene between nts (418-769) on the PVY genome.

On the other side, another two primer sets for the *NI-PRO gene* are (NI-PRO SenseFWD-1/ NI-PRO SenseRVS-1 and NI-PRO AntisenseFWD-2/ NI-PRO AntisenseRVS-2). The nucleotide sequences of primer sets are shown in Table 2. Two primer sets were used to amplify the 416bp DNA fragment of the PVY *NI-PRO* gene between nts (6139-6554) on the PVY genome.

The two sets were identical in nucleotide sequence except for the restriction sites overhang of each set, which was designed to orient the two copies to face direction during cloning.

The synthesized cDNA prepared from the previous step was used as a template for PCR reaction. The reaction was performed in a total volume of 25 µl. Those primers were used to amplify the target regions in a standard PCR reaction.

A sample of the amplified DNA fragment was visualized on 1% agarose gel supplemented with ethidium bromide. The rest of the PCR product was used for the cloning process.

From gDNA isolated from potato (Spunta), a 134-bp fragment encoding an intron of the *Solanum tuberosum* gene for cytosolic pyruvate kinase was amplified. Using the IntFWD/IntRVS primer sets, the intron that connects them and acts as a buffer zone between the two copies was amplified (Table 1).

Cloning of PVY-P1 fragment and PVY-NI Pro fragment in sense orientation

The PCR fragment of PVY-P1 was digested using *Xho*I and *Pst*I restriction enzymes. The digestion reaction was performed using five units of Fast Digest Restriction Enzyme, according to the manufacturer's instruction (Promega, USA).

The pBSK cloning vector was digested using *Xho*I and *Pst*I enzymes and the same reaction conditions.

TABLE 1. Nucleotide sequences of primer sets used for the amplification of PVYP1fragment and the expected sizes

Primer	Sequence 5'-3'	Expected fragment size (bp)
Cloning primers		
P1SenseFWD-1	CCGGCTCGAGCATATGTGCGCATTCAGAAGAAACTG	352
P1SenseRVS-1	CCGGCTGCAGGGATCCTCTAGACCCATCATATGTGCAGTTCG	
P1AntisenseFWD-2	CCGGCCGCGGGTCGACTGCGCATTCAGAAGAAACTG	352
P1AntisenseRVS-2	CCGGGCGGCCGCGGATCCTCTAGACCCATCATATGTGCAGTTCG	
IntFWD	GGCCCTGCAGCACAGCACTGTGAAAAGAACATC	134
IntRVS	CCGGGCGGCCGCTTTGGAGGATTCGACAACCC	

TABLE 2. Nucleotide sequences of primer sets used for the amplification of NI-PRO gene fragment and the expected sizes

Primer	Sequence 5'-3'	Expected fragment size (bp)
Cloning primers		
NI-PRO SenseFWD-1	CCGGCTCGAGCATATGGGATCCTGCCACATAACCCACTCAAA	416
NI-PRO SenseRVS-1	CCGGCTGCAGAGGGAAATCTTTCGGCATTT	
NI-PRO AntisenseFWD-2	CCGGCCGCGGGTCGACGGATCCTGCCACATAACCCACTCAAA	416
NI-PRO AntisenseRVS-2	CCGGGCGGCCGCAGGGAAATCTTTCGGCATTT	
IntFWD	GGCCCTGCAGCACAGCACTGTGAAAAGAACATC	134
IntRVS	CCGGGCGGCCGCTTTGGAGGATTCGACAACCC	

The *Xho*I / *Pst*I digested fragment was cloned into the corresponding sites linearized pBSK vector by ligation reaction to form the "pBSF" plasmid, according to the manufacturer's instruction (New England Biolabs, UK).

Two µl of the ligation reaction was transformed into 50 µl of DH10B-competent cells. The cells were incubated for 20 minutes on ice and then subjected to heat shock at 42°C for 45–50sec immediately. The reaction was incubated on ice for 2min. One ml of LB broth medium was added to the reaction, and the cells were allowed to grow in a shaking incubator for 1 hour at 37°C. One hundred µl of the transformation culture was selected on a 100µg/ml Ampicillin plate that was supplemented with X- Gal/IPTG. The plate was incubated overnight at 37°C. The white colonies were selected from the plates, and the plasmid DNA was extracted using Wizard® Plus SV Minipreps DNA Purification System (Promega). The plasmid DNA was screened using PCR analysis according to the manufacturer's instructions.

The cloned fragment was subjected to sequence analysis using the Big TriDye sequencing kit (ABI Applied Biosystems) by the facility of Macrogen, Korea (Seoul, Republic of Korea). The sequencing result was blasted against published PVY *P1*gene using the Genbank database of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

Regarding the cloning of the NI Pro fragment in sense orientation, it was carried out as follows: *XhoI* and *PstI* restriction enzymes were used to digest the PCR fragment and the same conditions were used to digest the pBSK cloning vector as they used to digest the PCR fragment. The "pBSF" plasmid was created by ligating the *XhoI / PstI* digested segment into the corresponding sites of the linearized pBSK

vector. As previously mentioned, the processes of ligation, digestion, and transformation were carried out using a similar technique to that of the cloning of the PVY-P1 fragment. At Macrogen, Korea (Seoul, Republic of Korea), the cloned fragment underwent a sequence analysis using the Big TriDye sequencing kit (ABI Applied Biosystems).

Utilizing the Genbank database (www.ncbi.nlm.nih.gov), the sequencing results were compared to the gene PVY *NI Pro* that had been published.

Subcloning of intron fragment in two "pBSF" plasmids

The intron fragment and "pBSF" were digested by the restriction enzymes *PstI* and *NotI*. The digested fragments were purified and ligated using T4DNA ligase. The PCR-amplified intron fragment was subcloned into the two linearized "pBSF" plasmids at the corresponding sites, creating the two "pBSFI" plasmids. The cloning procedures were mainly similar to those previously described. The cytosolic pyruvate kinase gene from *Solanum tuberosum* was amplified as a 134-bp fragment. The intron fragment was subcloned into two "pBSF" plasmids in parallel (The first "pBSF," which was previously cloned in sense orientation using the PVY-P1 fragment, and the second "pBSF," which was previously cloned in sense orientation using the PVY-NI Pro fragment).

Subcloning of PVY-P1 fragment into "pBSFI" in antisense orientation

A) Subcloning of PVY-P1 fragment into "pBSFI" in antisense orientation: A 352bp amplified fragment encoding PVY first protein was subcloned in the opposite direction into the first "pBSFI" plasmid (PVY-P1fragment in sense orientation+ intron). Both the fragment and "pBSFI" plasmid were digested using NotI/SacII restriction enzymes and ligated to each other, forming the "pBSFIA" plasmid. The cloning procedures were essentially similar to those described above.

B) Subcloning of PVY-NI Pro fragment into "pBSFI" in antisense orientation: A 416bp amplified fragment encoding PVY, the nuclear inclusion-Pro gene was subcloned opposite into the second "pBSFI" plasmid (PVY-NI Pro fragment in sense orientation+ intron). Both the fragment and "pBSFI" plasmid were digested using NotI/SacII restriction enzymes and ligated to each other, forming the "pBSFIA" plasmid. The cloning procedures were essentially the same as described above.

Results and Discussion

Potato virus Y (PVY) is a member of the Potyvirus genus that belongs to the Potyviridae family. Due to high genomic similarity among the Potyvirus genus, protein activities of PVY have mostly been deduced by comparison with those of other members of the same genus. P1, which is called the first protein, is the most variable protein among potyviruses. It is encoded by the 5' section of the PVY genome. It has a protease domain in the carboxy (C)-terminal region and separates from the nearby helper component protease (HC-Pro). It has been demonstrated to bind both single- and double-stranded RNA and participates in genome amplification. Therefore, it can help to decrease RNA-based plant defenses more effectively (Quenouille et al., 2013). The NIa protease is the major protease responsible for the viral polyprotein's cleavage into functional proteins and acts as a nuclear inclusion body A. The cleavage of polyproteins is allowed by the NIa protein (Gargouri-Bouzid et al., 2006). In addition to its protease activity, NIa-Pro has other important functions during the viral infection cycle. It is involved in viral replication, viral movement, and suppression of host defense mechanisms. Specifically, NIa-Pro is required for the amplification of viral RNA and the assembly of viral particles. Additionally, NIa-Pro interacts with host factors and suppresses host RNA silencing, which is a defense mechanism used by plants to counteract viral infections.

Overall, the multifunctional nature of NIa-Pro makes it a crucial component of the potyviral infection cycle, and it is a promising target for developing antiviral strategies against potyviruses (Gong et al., 2020).

In the present study the virus was propagated by in infecting DATORA plants in a field experiment at field facility of Faculty of science. The morphological symptoms of plant leaves infection by PVY strains were confirmed as shown in Fig. 3. Various symptoms of virus infection,

such as yellow, light green, and dark green mosaic patterns, leaf drop, and necrotic line patterns on veins or shoots were all reviewed within infected DATORA plants. Ultimately, the propagated PVY was used for subsequent experiments. The morphological symptoms of PVY infection in Datora leaves were examined according to standard characteristics of viral infection.

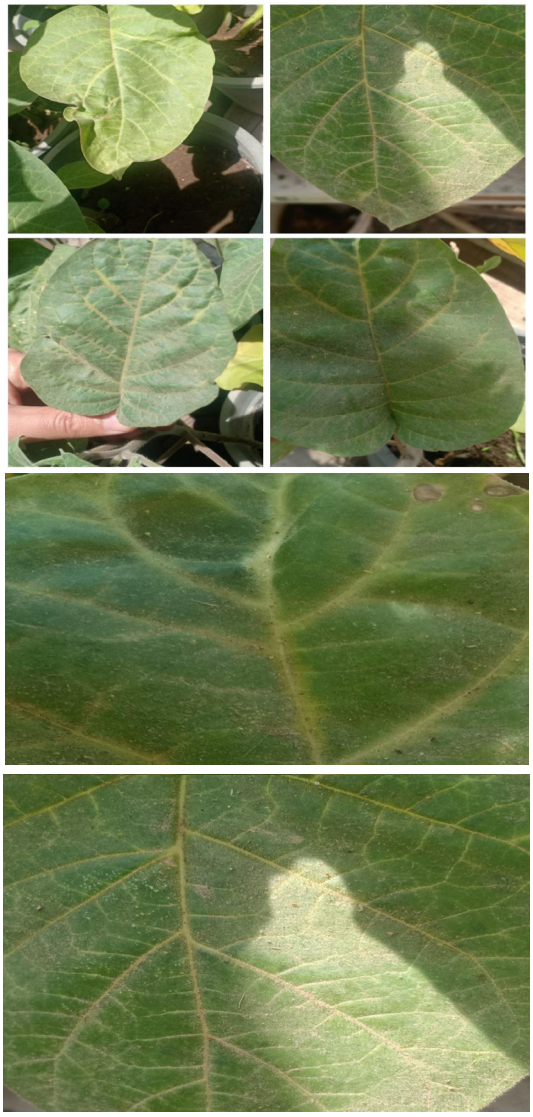

Fig. 3. PVY infection symptoms on *Datora metel leaves* such as yellow, light green, and dark green mosaic patterns, leaf drop, and necrotic line patterns on veins

The PVY symptoms, such as on *Datura metel* plant leaves, as shown in Fig. 3, were confirmed serologically by specific PVY polyclonal antibodies by DAS-ELISA.

The ultimate aims of the present study are to establish an RNAi-mediated defense mechanism of potato cultivars against PVY and develop PVY-resistant potato cultivars by knocking down the essential genes in Potato Virus Y using dsRNA-mediated RNAi. Thus, the current study focuses on designing a dsRNA construct that specifically targets the P1 transcript of the PVY genome, and we have chosen another important viral protein was chosen as a target, namely the NIa protein gene of the PVY genome, to generate more stable virus resistance. Such construct is an intermediate step that will be used later to build up the dsRNA-plant transformation vector. Finally, potato plants will be genetically modified to induce RNAi conferring a high level of PVY resistance.

To achieve this goal, a 352bp fragment between nts 417 and 769 of the PVY genome representing the P1 gene was amplified and cloned into a pBSK cloning vector using *Xho*I/ *Pst*I enzymes. The positive clones were screened using the P1SenseFWD/ P1SenseRVSprimer set. The expected size band of 352bp is shown in Fig. 4 and Fig. 10.

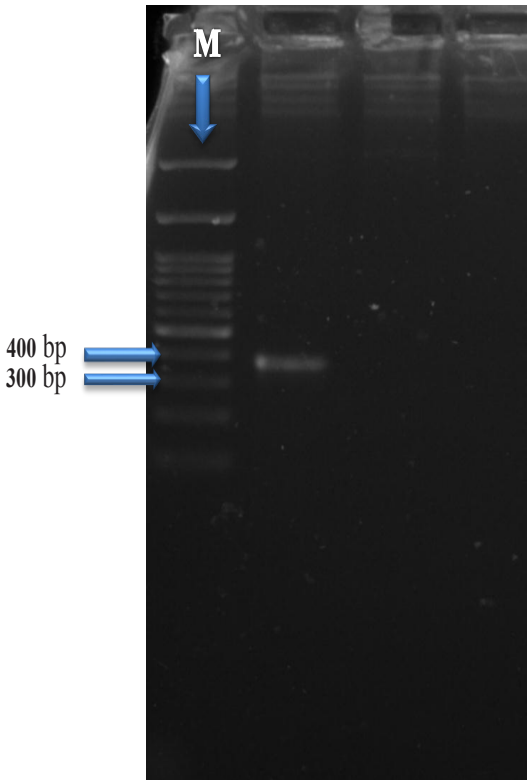

Fig.4. PCR product of 352bp fragment of sense PVYP1 and M is 100bp molecular DNA ladder

A 134bp fragment of the intron shown in Fig. 11 was cloned next to the P1 sense fragment using *Pst*I/*Not*I enzymes. The positive clone was confirmed by dual PCR reaction using a combination of P1SenseFWD-1/IntRVS and IntFWD/P1SenseRVS-1 primer sets as shown in Fig. 5 and Fig. 12.

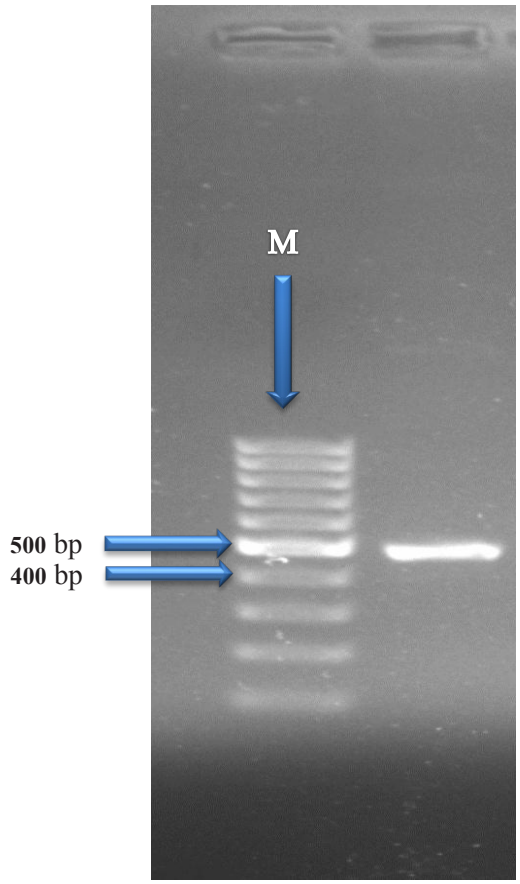

Fig. 5. PCR product of positive pBSFI of PVYP1clones showing an expected band size at 486bp and M is 100bp molecular DNA ladder

Finally, the same 352bp PVYP1 fragment was cloned in an opposite position flanked to intron using *Not*I/*Sac*II enzymes. The positive clones were confirmed by PCR. The produced positive clones are 838bp long-sized fragments, as shown in Fig. 6.

The *P1* and *NIa* genes have been chosen as two important PVY genes to be silenced utilizing RNA technology. A viral replica is encoded by the *NIa* gene, whereas the *P1* gene produces a protein that is involved in viral replication. The severity of PVY infection can be lessened by silencing these genes, which can stop viral replication.

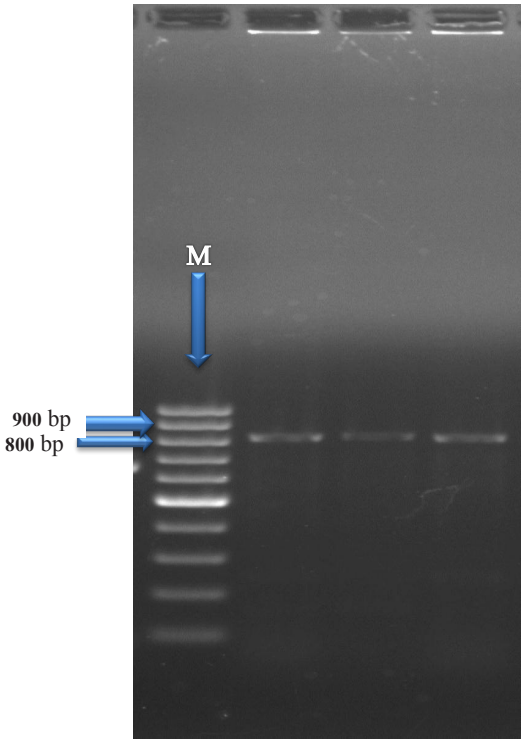

Fig. 6. PCR product of positive pBSFIA of PVYP1 clones showing an expected band size at 838bp and M is 100bp molecular DNA ladder

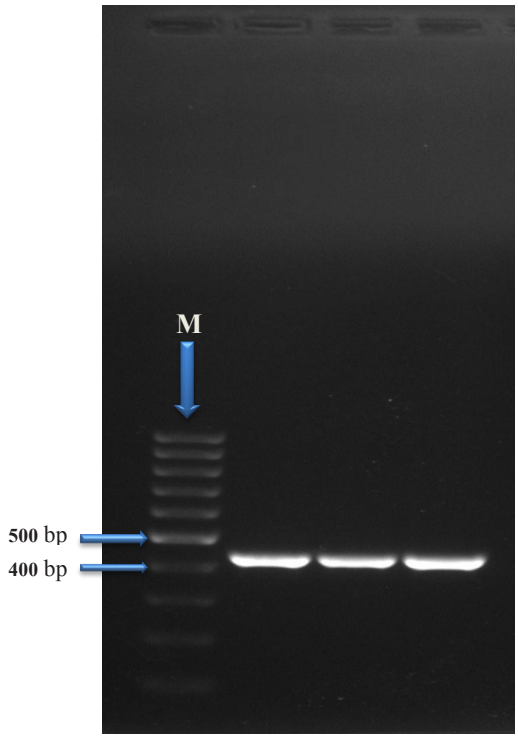

Fig. 7. PCR product of 416bp fragment of sense NI-PRO gene, and M is 100bp molecular DNA ladder

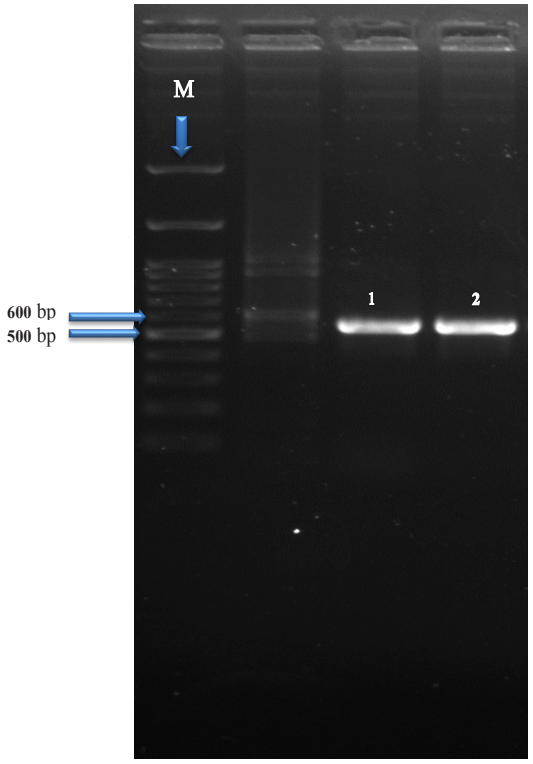

Fig. 8. PCR product of positive pBSFI of NI-PRO gene clones showing an expected band size at 550bp and M is 100bp molecular DNA ladder

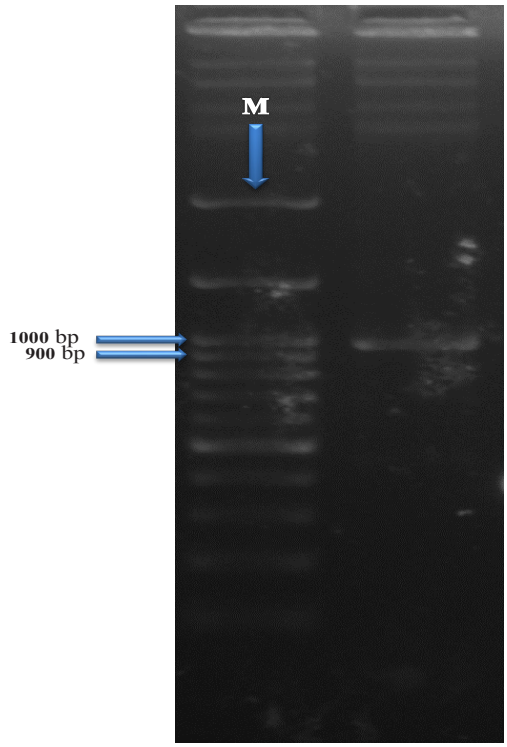

Fig. 9. PCR product of positive pBSFIA of NI-PRO gene clones showing an expected band size at 966bp and M is 100bp molecular DNA ladder

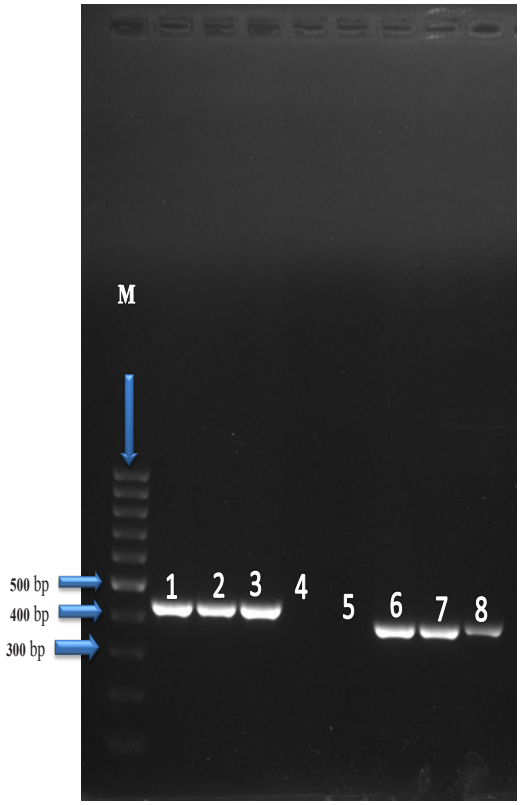

Fig. 10. PCR product of positive pBSF of NI-PRO gene and p1 gene, M is 100bp molecular DNA ladder, lane 1-3 PCR product of positive pBSF of NI-PRO gene clones showing an expected band size at 416bp, lane 4 negative control for them, lane 5 negative control for p1 sample, and lane 6-8 PCR product of positive pBSF of p1 gene clones showing an expected band size at 352bp

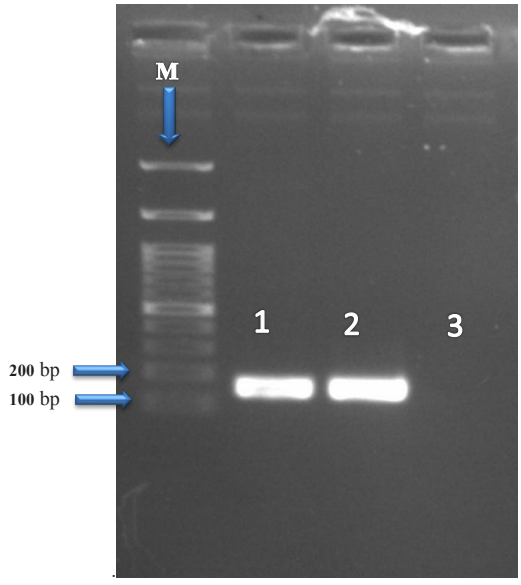

Fig. 11. PCR product of intron samples; lanes 1–2 show an expected band size of 134 bp, lane

3 is the negative control, and M is the 100 bp molecular DNA ladder

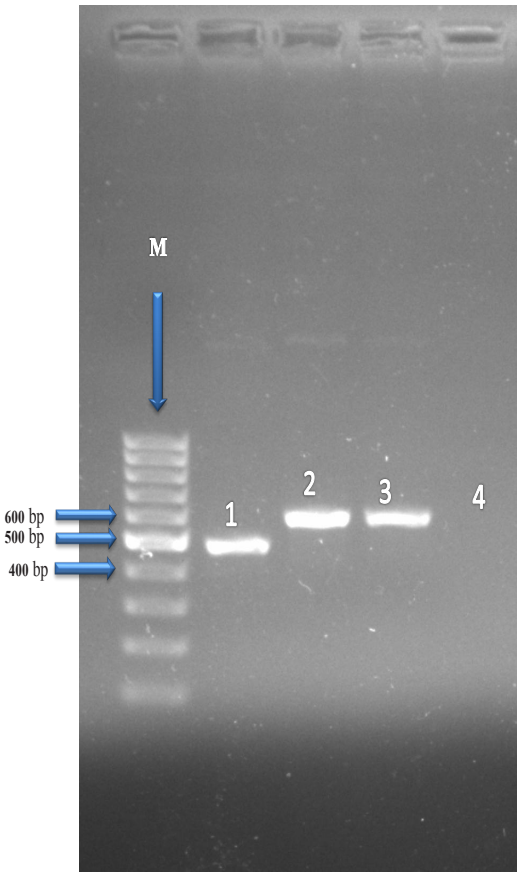

Fig. 12. PCR product of positive pBSFI of the NI-PRO gene and p1 gene, M is the 100-bp molecular DNA ladder, lane 1 PCR product of positive pBSFI of P1 gene clones showing an expected band size of 486 bp, lane 2–3 PCR product of positive pBSFI of the NI-PRO gene showing an expected band size of 550 bp, and lane 4 negative control

The targeting of two important genes (*P1* and *NIa*) was performed to confirm the occurrence of gene silencing for the virus using RNA technology. Both of these genes play a crucial role in the viral replication process. The use of RNA technology resulted in the reduction of virus accumulation, indicating the successful occurrence of gene silencing for the virus.

Conclusion

In conclusion, the present study highlights the potential of RNA interference (RNAi) technology in the pursuit of virus-resistant potato cultivation, primarily focused on mitigating the menace posed by potato virus Y (PVY). The key takeaway from this research lies in the efficient use of RNAi to specifically target essential PVY genes, notably P1 and NIa. This strategic gene silencing approach represents a significant stride towards developing transgenic potato varieties resilient to PVY, ultimately bolstering global food security by safeguarding potato crops from this economically damaging virus.

Looking ahead, it is imperative to further investigate and optimize the application of RNAi in creating disease-resistant crops. Additionally, research should continue to refine the delivery and stability of RNAi trigger molecules while ensuring the safety of non-target organisms. Further research is needed to assess the long-term effects of RNAi constructs on crops and to optimize their efficiency. Further research is required to evaluate the enduring impacts of RNAi constructs on crops and enhance their effectiveness.

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Competing interests The authors report no conflicts of interest regarding this work.

*Authors' contributions:*All authors contributed to the research with the same degree of participation. The final manuscript was read and approved by all the authors.

Ethics approval: Not applicable

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تصميم بناء فعال لتطبيق تقنية (RNAi) كوسيلة لإنتاج نباتات بطاطس مقاومة لفيروس PVY

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تداخل الحمض النووي الريبي (RNAi) هو تقنية قوية لإسكات الجينات، وتستخدم لتمنع جين معين من التعبير. لقد أظهرت تقنية RNAi إنجازا كبيرا في السيطرة على الأمراض الفيروسية في النباتات، وخاصة في البطاطس، حيث تتسبب الفيروسات في خسائر كبيرة في الجودة والإنتاج. وأظهرت قدرة النباتات المعدلة وراثيا على مقاومة العدوى الفيروسية من خلال التعبير عن السبل المزدوجة (dsRNAs) وتم توثيق الحمض النووي الريبي الذي يستهدف الجينات الفيروسية. ومع ذلك، فإن اختيار الجينات المستهدفة هو حجر الزاوية لنجاح الحمض النووي الريبي في السيطرة على الأمراض الفيروسية. وفيروس البطاطس (PVY) يعتبر من الأكثر ضررا اقتصاديا لمحاصيل البطاطس في جميع أنحاء العالم فكان الهدف العام من الدراسة الحالية هو الاستخدام المحتمل لتقنية RNAiلإنشاء بطاطس مقاومة للفيروس، وتحسين الأمن الغذائي العالمي. وفي الدراسة الحالية، هناك تداخلان متميزان في الحمض النووي الريبي (RNAi) تم تطوير البناء. تم تصميم البناء الأول لاستهداف جينP1، والذي مسؤول عن تشفير البروتين الأولي لفيروس البطاطس(PVY)، يستهدف البناء الثاني جينا Nuclear inclusion- Pro gene ويخدم الغرض من تطويرتنوع البطاطس المعدلة وراثيا المقاوم لPVY